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Oliver H. Krämer *Editor*

HDAC/HAT Function Assessment and Inhibitor Development

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Preface

Reversible protein acetylation has emerged as a critical regulator of protein functions and ultimately of cellular fate. This book covers how the biological functions of HDACs can be detected in various experimental settings, in vivo and in vitro. The book also covers the generation and specificity of deacetylase inhibitors and how such agents can be used to test experimental hypotheses.

Mainz, Germany *Oliver H. Krämer*

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Part I

Analysis of Cellular Processes That Are Regulated by HDACs

Chapter 1

Detection of Autophagy Induction After HDAC Inhibitor Treatment in Leukemic Cells

Anja Göder, Nisintha Mahendrarajah, and Oliver H. Krämer

Abstract

Autophagy is a lysosome-dependent, intracellular pathway for the recycling of cellular components. It plays a pivotal role in both cancer development and the response to chemotherapy. Here we describe how autophagy can be monitored in living cells by flow cytometry using the cationic amphiphilic tracer dye Cyto-ID® Green. The detection of autophagy induction in the human leukemia cell line K562 after the treatment with the HDAC class I inhibitor MS-275 serves as an example for this approach.

Key words Autophagy, Leukemia, HDACi, Flow cytometry

1 Introduction

The term autophagy describes an evolutionarily conserved, catabolic process that serves to degrade cytoplasmic components and organelles by delivering them to lysosomes [1]. One of the central steps during macroautophagy (usually termed autophagy) is the formation of double-membrane-bound autophagosomes that engulf a portion of the cytoplasm [2]. This process can be performed either in a selective way, using adaptor proteins [1], or in an unselective manner [3]. The presence of autophagosomes and the accumulation/processing of proteins associated with cellular membranes, like the LC3-II protein, can be used to measure autophagy induction [4].

All mammalian cells exhibit a basal level of autophagy to eliminate misfolded proteins and damaged organelles [5]. Under environmental stress autophagy is upregulated as a survival mechanism to maintain cellular homeostasis and to protect the cell from harmful components [5].

The role of autophagy in cancer is controversial as there is evidence for tumor-suppressive [6–8] as well as tumor-promoting properties [9–11]. While it has been shown that autophagy

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contributes to genomic integrity under stress conditions [12] (therefore impeding tumor initiation and progression), it can also protect cancer cells from chemotherapy [13]. Moreover, several chemotherapeutics induce autophagy and this also applies for epigenetic drugs belonging to the group of histone deacetylase inhibitors (HDAC) [14, 15].

The targets of these drugs, histone deacetylases (HDACs), are a class of enzymes that catalyze the removal of acetyl groups from histones and also from nonhistone targets [16]. 18 human HDACs have been identified. These can be divided into two distinct families, the NAD^+-dependent sirtuins (class III) and the Zn^{2+}-dependent HDACs (class I, II, IV), referred to as "classical HDACs" [17]. Since HDACs have been shown to be deregulated in cancer cells, their inhibition appears as a promising strategy for cancer therapy [18].

Up to date, a large variety of HDACi have been generated. Some of them, like the pan-HDACi suberoylanilide hydroxamic acid (SAHA) [19] and LBH589 as well as the class I-specific inhibitors MS-275 and valproic acid (VPA), have already progressed into clinical trials or have been approved for treatment [18, 20]. The influence of HDACi treatment on autophagy has been shown in different tumor types [14, 21–24] including leukemia [15, 25, 26]. Depending on the leukemia cell line, HDAC inhibition either blocked autophagy leading to increased cell death [25, 27] or induced pro-survival autophagy and the resistance against HDACi [15, 28].

A highly appreciated method to detect autophagy is transmission electron microscopy (TEM), which requires very specific equipment and technical expertise [4]. More common is the use of

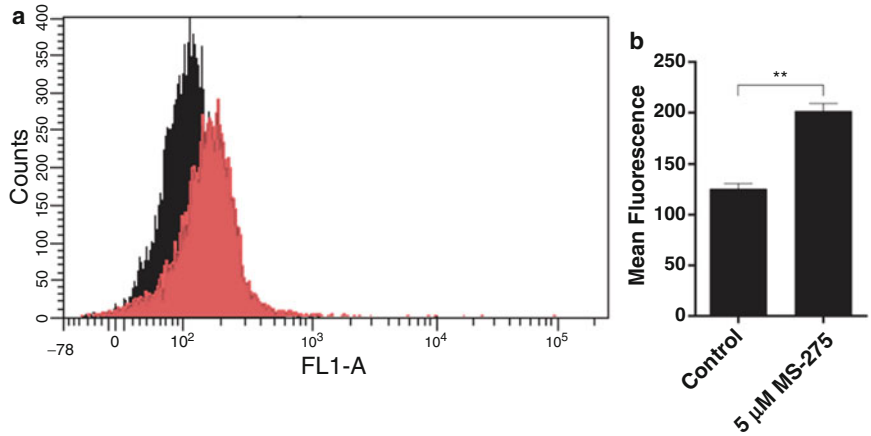

Fig. 1 Detection of autophagy induction after MS-275 treatment in K562 cells. K562 were treated with the HDACi MS-275 (5 μM) for 48 h. Autophagy induction was measured by flow cytometry using the Cyto-ID® Autophagy detection kit. (**a**) Comparison of Cyto-ID® fluorescence signals of untreated control (*black*) and MS-275-treated K562 cells (*red*). A representative overlay histogram is shown. (**b**) The Cyto-ID® fluorescence signals are displayed as mean ± SD ($n = 3$); $^{**}p \leq 0.01$, calculated with the Student's *t*-test

western blot analysis or immunohistochemistry to determine the protein levels of autophagy marker proteins like LC3-II or p62 [4]. Using the example of autophagy induction after MS-275 treatment of K562 cells (Fig. 1), this chapter describes a flow cytometry-based method to monitor autophagy using the cationic amphiphilic tracer dye Cyto-ID® Green.

2 Materials

Materials and kits used for detection of autophagy induction are listed below. Equipment from other providers should be useful as well.

1. Suspension cell line: K562 (human chronic myelogenous leukemia), propagated in RPMI-1640 medium with l-glutamine containing 10% fetal bovine serum (FBS) with 100 U/100 µg/mL penicillin/streptomycin.
2. Cyto-ID® Autophagy Detection Kit (Enzo Life Sciences). Store at −20 °C for short-term storage. Store at −80 °C for long-term storage. Protect from light.
3. HDAC inhibitor: MS-275 (Selleckchem). Dissolved in DMSO. Store at −80 °C.
4. Autophagy inhibitor: Chloroquine (Enzo Life Sciences). Dissolved in deionized water. Store at −20 °C for short-term storage. Store at −80 °C for long-term storage.
5. 37 °C, 5% CO_2 humidified incubator (Thermo Scientific).
6. RPMI medium with no phenol red (Gibco Life Technologies) containing 5% FBS.
7. Dulbecco's phosphate-buffered saline (PBS) (Biochrom).
8. Flow cytometry tubes (Sarstedt).
9. FACS Canto II flow cytometer (BD Biosciences).

3 Methods

3.1 Quantitative Analysis of Autophagy Induction by Flow Cytometry

The Cyto-ID® Autophagy Detection Kit is used to monitor the autophagic flux in living cells. The Cyto-ID® Green Reagent is a cationic amphiphilic tracer dye which selectively labels pre-autophagosomes, autophagosomes, and autophagolysosomes. Upon autophagy induction (e.g., after rapamycin treatment, starvation) an accumulation of autophagic vesicles can be detected by flow cytometry.

Flow cytometry measures multiple characteristics of single cells as they pass in a stream through a cytometer. As the particles pass through one or more lasers, the system detects the angle and direction of scattered light providing information on differences in cell

size and texture. Light scattered in the forward direction (FSC) measures the size of the cells, whereas the side-scattered light (SSC) can be used to obtain data about the granularity of cells. Thus, particular cell populations can be resolved.

Further biological information can be provided by staining cells with one or more fluorescent dyes. A suitable laser excites these fluorochromes while several detectors measure their emission. It is possible to use multiple fluorochromes to analyze several cell properties simultaneously. These fluorochromes should have similar excitation wavelengths but different emission wavelengths.

Here we show how the induction of autophagy can be measured with the Cyto-ID® Autophagy Detection Kit in response to HDACi treatment in K562 cells (Fig. 1a). Furthermore, we demonstrate the use of chloroquine as a positive control for this method (Fig. 2).

3.2 Harvesting

1. Seed human leukemic suspension cells on a 12-well plate at a density of 2×10^5 cells/well in fresh RPMI-1640 medium containing 10% fetal bovine serum with 100 U/mL penicillin and 100 µg/mL streptomycin (*see* **Notes 1** and **2**).
2. Stimulate cells with an HDACi (e.g., MS-275) after an adaptation time of approximately 2 h following seeding (*see* **Note 3**).
3. Harvest cells 48 h after stimulation by transferring the cell suspensions into 15 mL tubes and centrifuge them at $250 \times g$ for 5 min (*see* **Note 1**). To achieve a higher yield of cells wash the dishes with 1 mL PBS.
4. Aspirate the supernatants completely.
5. Resuspend cell pellets in 2 mL PBS and centrifuge at $250 \times g$ for 5 min to remove all remaining growth medium.

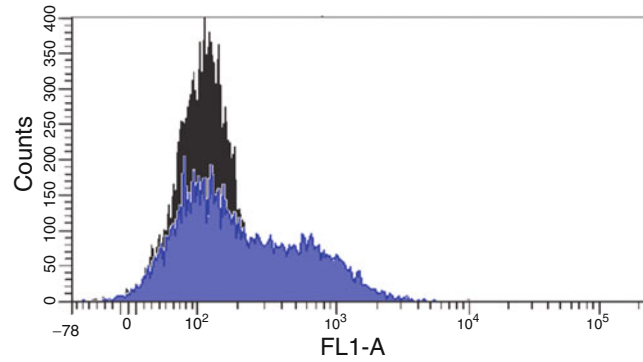

Fig. 2 Chloroquine treatment as positive control for autophagy induction in K562 cells. To provide a positive control for Cyto-ID® staining, K562 were treated with the autophagy inhibitor chloroquine (10 µM) for 48 h. The presence of autophagic vesicles was measured by flow cytometry using the Cyto-ID® Autophagy detection kit. A representative overlay histogram of Cyto-ID® fluorescence signals is shown (untreated control: *black*; chloroquine: *blue*)

6. Aspirate the supernatants completely.
7. Prepare working solution of the Cyto-ID® Green Reagent: Mix 1 µl Cyto-ID® Dye with 1 mL phenol red-free RPMI medium (*see* **Notes 4** and **5**) containing 5% FBS. Protect the solution from light.
8. Prepare 1× assay buffer: Mix 1 mL 10× assay buffer with 9 mL ddH_2O (*see* **Note 6**).

3.3 Cyto-ID Staining

1. Resuspend cell pellets in 0.25 mL phenol red-free RPMI medium containing 5% FBS.
2. All the following steps are performed in the dark (*see* **Note 4**).
3. Add 0.25 mL diluted Cyto-ID® working solution to each tube and mix well (*see* **Note 7**).
4. Incubate in a cell incubator for 30 min at 37 °C and 5% CO_2.
5. Centrifuge at 250×*g* for 5 min.
6. Aspirate the supernatants completely and resuspend the cell pellets in 1× assay buffer to remove excess Cyto-ID® dye (*see* **Note 6**).
7. Centrifuge at 250×*g* for 5 min.
8. Aspirate the supernatants completely. Resuspend cell pellets in 0.5 mL 1× assay buffer and transfer cell suspensions to flow cytometry tubes (*see* **Note 6**).
9. Keep the samples on ice and in the dark.
10. Measure samples in FL1 or FL2 channel of FACSCanto II flow cytometer using a 488 nm laser source (Figs. 1a and 2) and analyze the data using the FACSDiva software (BD Biosciences) (*see* **Notes 8**–**10**).

4 Notes

1. Different human cell lines, including adherent cells such as HCT116, may be used for this analysis. Adherent cells are to be cultivated in DMEM medium containing 10% FBS and 100 U/mL penicillin, 100 µg/mL streptomycin, or other appropriate media. Harvest adherent cells by trypsinization. Then transfer the supernatant and cell suspension into 15 mL tubes followed by a centrifugation step at 250×*g* for 5 min. However, in our hands the Cyto-ID® staining did not work with mouse embryonic fibroblasts.
2. Please note that at the time of harvesting, cells should be below 80% confluency. This avoids nutrition limitation and the presence of toxic metabolites that increase the basal level of autophagy.

3. It is suggested to include a positive control. Positive controls are autophagy inducers like the mTOR inhibitor rapamycin or starvation. It is also advisory to use autophagy inhibitors like chloroquine (CQ) or ammonium chloride (NH_4Cl). These compounds are lysosomotropic and therefore block autophagolysosomal degradation, resulting in an accumulation of autophagosomes. Additionally, the use of autophagy inhibitors in combination with your substance provides information about whether a positive Cyto-ID® signal results from an inhibition of the autophagic flux or the induction of autophagy. The concentrations of these compounds should be established for each cell line.
4. It is important to perform the staining steps in the dark to prevent the decay of the Cyto-ID® Dye. Upon staining keep the samples always on ice and in the dark until the time of analysis.
5. Phenol red-free medium should be used to avoid interference of the indicator dye with the Cyto-ID® Dye.
6. Instead of 1× assay buffer, 1× PBS can be used as well.
7. Suggested cell density for the staining and measurement of K562 is 10^5 to 10^6 cells/mL.
8. Untreated cells should be used to determine basal autophagy levels.
9. To distinguish live autophagic cells from apoptotic/necrotic cells, they can be co-stained with a nuclear dye (Hoechst 33342), which is also included in the Cyto-ID® Autophagy Detection Kit.
10. Cyto-ID® fluorescence signal should be measured using a histogram (cell count vs. FL1-A or FL2-A). Autophagy induction can be displayed as increase in mean fluorescence intensity.

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Chapter 2

Assessment of HDACi-Induced Protein Cleavage by Caspases

Fabian Treude, Tobias Gladbach, Jacqueline Plaster, and Jörg Hartkamp

Abstract

Aberrant histone deacetylase (HDAC) activity often correlates with neoplastic transformation and inhibition of HDACs by small molecules has emerged as a promising strategy to treat hematological malignancies in particular. Treatment with HDAC inhibitors (HDACis) often prompts tumor cells to undergo apoptosis, thereby causing a caspase-dependent cleavage of target proteins. An unexpectedly large number of proteins are in vivo caspase substrates and defining caspase-mediated substrate specificity is a major challenge. In this chapter we demonstrate that the hematopoietic transcription factor PU.1 becomes cleaved after treatment of acute myeloid leukemia (AML) cells with the HDACis LBH589 (panobinostat) or MS-275 (entinostat). To define caspase specificity for PU.1, an in vitro caspase assay including caspases 1–10 with in vitro-translated PU.1 is described in detail.

Key words Caspase-8, PU.1, HDACi, LBH589, MS-275, In vitro translation

1 Introduction

Reversible acetylation regulated by histone acetyl transferases (HATs) and histone deacetylases (HDACs) plays a key role in the epigenetic regulation of gene expression [1]. Abnormal epigenetic changes are frequent and early events in tumorigenesis, and deregulated acetylation in particular, has been associated to tumor progression [2]. HDACs regulate acetylation by deacetylating lysine residues of nuclear histones as well as a large number of non-histone proteins that are involved in tumor progression, cell cycle control, and apoptosis [2–4]. 18 HDAC family members are divided into Zn^{2+}-dependent class I (HDAC 1, 2, 3, 8), class II (HDAC 4, 5, 6, 7, 9, 10), class IV (HDAC 11), and the NAD^{+}-dependent class III HDACs, which are termed sirtuins (SIRT 1–7) [4, 5].

Most HDAC inhibitors (HDACis) developed so far target the Zn^{2+}-dependent class I, II, and IV enzymes and many of these have entered clinical trials [4, 6]. In cell-based studies HDACis are powerful antiproliferative agents that can promote a broad

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spectrum of cellular changes, including apoptosis in cancer cells [1]. Furthermore, therapeutic efficacy and HDACi-induced tumor cell death have been shown to be directly connected in preclinical model systems [6]. Transformed cells are up to ten times more sensitive to HDACi treatment than their normal cellular counterparts, but why tumor cells are more sensitive to HDACi-induced apoptosis is not entirely understood [6].

Most morphological changes triggered by the induction of apoptosis are associated to the activation of cysteine-dependent aspartate-specific proteases (caspases) [7]. Human caspases are typically divided into inflammatory caspases (caspases 1, 2, 4, 5), apoptotic effector caspases (caspases 3, 6, 7), and apoptotic initiator caspases (caspases 8, 9, 10) [8]. Catalysis of caspases is executed by a conserved cysteine side chain in the catalytic domain and a highly stringent selectivity for cleaving substrate proteins or peptides on the C-terminal side of aspartate residues [7, 9]. Their biochemical properties are tightly regulated to ensure that the irreversible proteolysis catalyzed by caspases occurs specifically and efficiently. Caspase substrate recognition depends on primary, secondary, tertiary, and quaternary structure effects, posttranslational modifications, as well as spatial and temporal localization [7]. Recent advances in the field of proteome-scale studies of proteases and their corresponding substrates have led to the identification of almost 1000 human proteins that are cleaved by caspases [7]. In order to analyze caspase substrate specificity under certain physiological conditions, two different types of experiments, which complement each other, can be employed. Initially, cell culture or in vivo experiments can reveal which proteins are cleaved during a biologically relevant process. Following the discovery of a potential caspase substrate in vitro experiments can illustrate which caspase is capable of cleaving the identified protein. After the initial examination of a functionally relevant cleavage event the specificity of the caspase substrate interaction should be validated. In some cases, a specific caspase may cleave a target protein in vitro but may not have access to it in the cell. Therefore, overexpression of the identified caspase and substrate protein in cells should verify direct cleavage of the target protein. Furthermore, the same caspase should be active under the cell culture conditions under which the protein of interest was identified as a caspase target in the first place.

Here, we analyzed how the clinically tested HDACis MS-275 (inhibits HDAC 1–3) [10] and LBH589 (inhibits HDAC 1–11) [11] affect the transcription factor purine-rich box1 (PU.1) in the promyelocytic leukemia cell line HL-60. PU.1 belongs to the ETS transcription factor family and plays a key role in the development of most hematopoietic cell lineages [12, 13]. Downregulation of PU.1 expression is observed in human acute myeloid leukemia (AML) patients [13] and a reduction of PU.1 expression leads to AML in mouse models [14, 15], suggesting that PU.1 functions as

a tumor suppressor. PU.1 contains a C-terminal DNA-binding domain and an N-terminal activation domain followed by a domain enriched in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) (Fig. 1a). Treatment of HL-60 cells with LBH589 and MS-275 leads to cleavage of the caspase-3 substrate PARP-1 in a dose-dependent manner, indicating that both HDACis induced apoptosis under these conditions (Fig. 1b). Under apoptotic conditions triggered by HDAC inhibition a cleaved band at ~24 kDa could be detected by anti-PU.1 antibody (detects C-terminal part of PU.1) while full-length PU.1 disappears under these conditions (Fig. 1b). Interestingly, Zhao and coworkers could demonstrate before that PU.1 is cleaved into two C-terminal fragments of ~24 and ~16 kDa under apoptotic conditions in leukemia cell lines [16]. The cleavage sites were mapped at $HVLD_{97}\downarrow T$ and $GEAD_{151}\downarrow G$ and this cleavage process is specifically catalyzed by caspase-3 (Fig. 1a) [16].

To validate and confirm the specificity of caspase-3 for PU.1 in vitro-translated PU.1 was subjected to a caspase cleavage assay

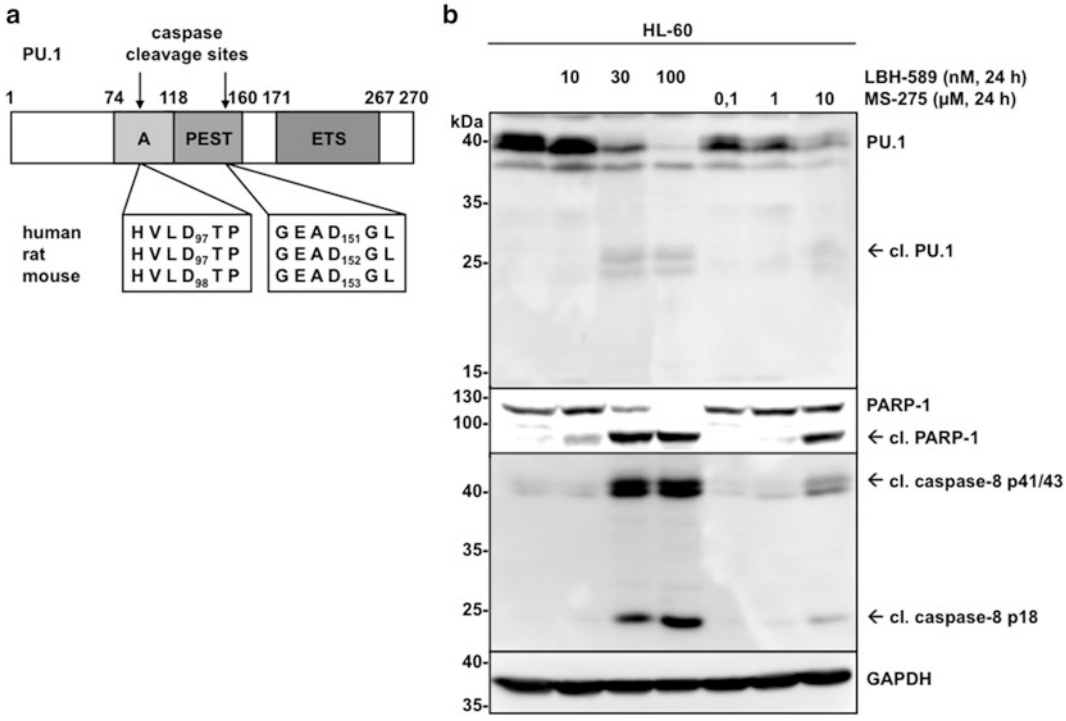

Fig. 1 PU.1 is cleaved upon HDACi treatment in HL-60 cells. (**a**) Schematic overview of human PU.1 protein: The caspase cleavage sites at positions D97 and D151; activation domain (amino acids 74–118); PEST domain (domain enriched in proline (P), glutamic acid (E), serine (S), and threonine (T), amino acids 118–160); ETS-domain (amino acids 171–267). (**b**) HL-60 cells were incubated with 10, 30, and 100 nM LBH589, or with 0.1, 1, and 10 μM MS-275 for 24 h. Cell lysates were analyzed by immunoblotting using antibodies against PU.1, PARP-1 as a marker for apoptosis, cleaved-caspase-8 and GAPDH as loading control

with recombinant caspases 1–10 (Fig. 2). Alternatively, the caspase assay can be performed with purified recombinant proteins or with immunoprecipitated proteins [17, 18]. Figure 2 demonstrates that only caspase-1 and caspase-8 were able to hydrolyze full-length PU.1, whereas caspase-3 failed to do so. Caspase-8 processing of PU.1 resulted in the appearance of a single ~16 kDa fragment, whereas caspase-1-mediated PU.1 cleavage resulted in the generation of an ~24 kDa and a main ~16 kDa fragment detected with the C-terminal PU.1 antibody (Fig. 2). These data are in agreement with the two reported caspase cleavage sites at $HVLD_{97}\downarrow T$ and $GEAD_{151}\downarrow G$ [16]. Caspase-1 and -8 were able to cleave PU.1 to various degrees in vitro with caspase-8 being the most efficient to hydrolyze PU.1 as judged by the complete disappearance of full-length PU.1 and the absence of the ~24 kDa cleavage fragment. The use of antibodies has got its limitations especially when more than one cleavage event occurs. In this case the synthesis of an in vitro [^{35}S] methionine-labeled target protein provides a useful alternative to analyze cleavage events if no suitable antibody is available [19].

Our in vitro data suggest that caspase-8 might cleave PU.1 in HL-60 cells that are exposed to apoptosis-inducing concentrations of HDACis. To confirm caspase-8 activation under conditions where the HDACi treatment results in PU.1 cleavage, we made use of an antibody that specifically recognizes the activated and cleaved form of caspase-8. Figure 1b demonstrates that caspase-8 activation correlates with PU.1 cleavage, suggesting that HDACi-induced PU.1 cleavage is caspase-8 dependent. While caspase-8-mediated PU.1 cleavage in vitro results in the generation of a C-terminal ~16 kDa PU.1 fragment, HDACi-induced apoptosis in vivo mainly generates a C-terminal ~24 kDa PU.1 fragment, indicating that under these conditions only one PU.1 cleavage site is targeted by caspase-8.

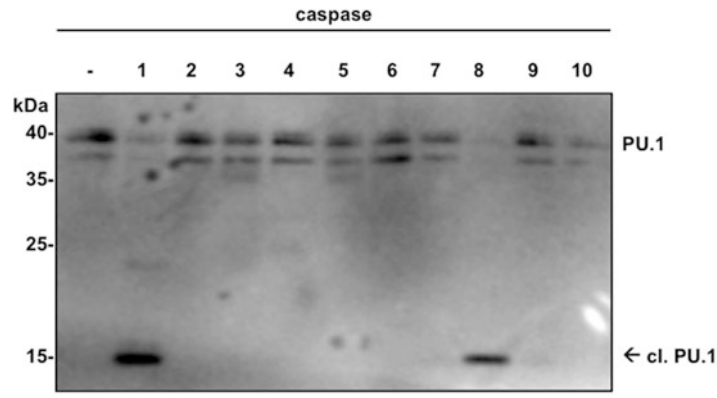

Fig. 2 PU.1 is a substrate of caspase-1 and caspase-8. Caspase assay using in vitro-translated PU.1 and recombinant human caspases 1–10. The reaction was stopped after 30 min by adding sample buffer and proteins were analyzed by immunoblotting using PU.1 antibodies

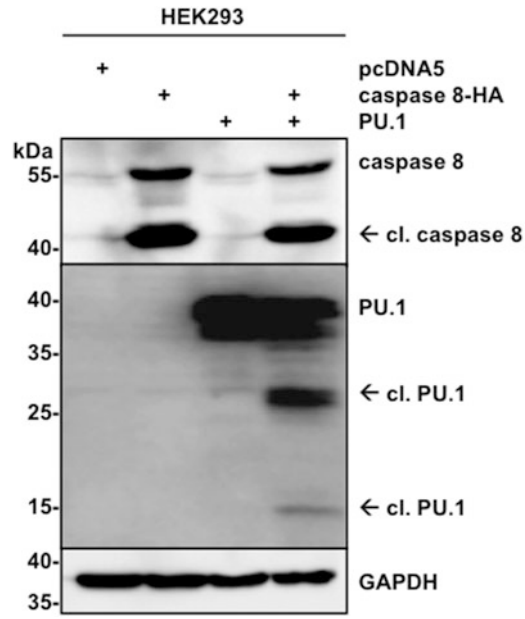

Fig. 3 PU.1 is cleaved by caspase-8 in cells. HEK293 cells were transfected with 1 μg of the indicated plasmids for 16 h using the calcium phosphate transfection method. Cells were lysed and subjected to immunoblotting using antibodies recognizing caspase-8, PU.1, and GAPDH

On the other hand, coexpression of PU.1 and caspase-8 in HEK293 cells results in the generation of both PU.1 cleavage products, indicating that caspase-8 has access to PU.1 in the cell and is capable of inducing PU.1 cleavage at both sites (Fig. 3).

Caspase-mediated cleavage of a substrate protein can lead to a gain, loss, or change of a protein's functions [7]. To analyze the contribution of PU.1 hydrolysis towards HDACi-induced apoptosis in HL-60 cells the creation of a cleavage-resistant PU.1 mutant should help to investigate the importance of caspase-8-mediated PU.1 cleavage. In addition, the overexpression of PU.1 cleavage fragments should complement these studies to address the biological effect of the HDACi-mediated PU.1 cleavage.

2 Materials

Materials and antibodies listed below are routinely used in our laboratory. However, equipment and reagents from other providers should be equally suitable.

2.1 Preparation of Whole-Cell Extracts

1. TLB lysis buffer: 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (v/v), 1 mM DTT. Freshly add 0.5 mM NaF, 1 mM $NaVO_3$, and a protease inhibitor cocktail.

2. Phosphate-buffered saline (PBS).
3. SDS-PAGE loading buffer (2×): 20 mM Tris–HCl pH 6.8, 20% glycerol (v/v), 4% SDS (w/v), spatula tip bromophenol blue. Freshly add 200 mM DTT.
4. Bio-Rad Protein Assay Dye Reagent Concentrate (Bio Rad).

2.2 SDS-Polyacrylamide Gel Electrophoresis

1. Separating gel buffer: 1.5 M Tris-HCl pH 8.8.
2. Stacking gel buffer: 2 M Tris–HCl pH 6.8.
3. 20% SDS: 20% (w/v) aqueous solution.
4. 20% Ammonium persulfate (APS): 20% (w/v) aqueous solution.
5. Tetramethylethylenediamine (TEMED).
6. 30% Acrylamide/bisacrylamide 29/1.
7. Methanol.
8. Mini PROTEAN 3 system-casting stand with corresponding casting frames, combs, and glass plates (Bio-Rad).
9. SDS-running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS (*see* **Note 1**).
10. Protein ladder.

2.3 Protein Transfer: Western Blot

1. Polyvinylidene difluoride (PVDF) membrane.
2. Whatman paper (3 M).
3. Methanol.
4. Transfer buffer: 25 mM Tris, 250 mM glycine, 10% methanol.
5. Semidry transfer blotter.

2.4 Antibody Detection

1. Antibodies, *see* Subheading 2.8.
2. Nonfat dry milk.
3. BSA.
4. PBS-T: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween 20.
5. Enhanced chemiluminescence (ECL): 100 mM Tris–HCl pH 8.8, 2.5 mM Luminol, 0.2 mM poly-coumaric acid.
6. 30% H_2O_2 (v/v).

2.5 In Vitro Translation

1. TNT® Quick Coupled Transcription/Translation Systems (Promega; # L4610).
2. PEQ176P2-PU.1 was subcloned into the EcoRI/HindIII sites of pCDNA3.1 (+).

2.6 Caspase Assay

1. Recombinant human caspases (1–10) (PromoKine; PK-RP577-K233).
2. Caspase assay buffer: 50 mM Hepes pH 7.2, 50 mM NaCl, 0.1% Chaps (v/v), 10 mM EDTA, 5% Glycerol (v/v). Freshly add 10 mM DTT.

2.7 Calcium Phosphate Transfection

1. Hepes-buffered saline (HBS): 21 mM Hepes pH 6.95, 136.9 mM NaCl, 5 mM KCl, 0.89 mM Na_2HPO_4.
2. 2.5 M $CaCl_2$.

2.8 Antibodies and HDAC Inhibitors

1. PU.1 (Santa Cruz; sc-352 [rabbit]).
2. GAPDH (Santa Cruz; sc-32233 [mouse]).
3. Caspase-8 (Cell Signaling; #9746 [mouse]).
4. PARP-1 (Cell Signaling; #9542 [rabbit]).
5. Cleaved caspase-8 (Asp 391) (Cell Signaling; #9496 [rabbit]).
6. LBH589 (Selleckchem).
7. MS-275 (Selleckchem).

3 Methods

3.1 Preparation of Whole-Cell Extracts

1. Human HL-60 cells are cultured in 60 mm dishes and stimulated with increasing concentrations of the HDACis LBH589 (10 nM, 30 nM, 100 nM) or MS-275 (0.1 μM, 1 μM, 10 μM) for 24 h.
2. The following steps are performed on ice. Cells are centrifuged at 200 × *g* for 5 min at 4 °C and the supernatant is carefully aspirated. Before cell lysis, cells are washed with ice-cold PBS and centrifuged at 200 × *g* for 5 min at 4 °C and the supernatant is aspirated.
3. Cells are lysed immediately using 200 μl ice-cold complete TLB lysis buffer. Vortex and keep on ice for 20 min.
4. A centrifugation step at 20,000 × *g* for 20 min at 4 °C is performed to remove cell debris. 3 μl of the supernatant is used for protein concentration.
5. Protein concentration is measured using Bio-Rad Protein Assay Dye Reagent Concentrate at OD 595.
6. Mix 150 μl protein lysate with 150 μl 2× SDS-PAGE loading buffer and heat at 95 °C for 5 min. Spin down the protein samples and perform SDS-PAGE with equal protein amounts.

3.2 SDS-Polyacrylamide Gel Electrophoresis

1. Cast the 12.5% acrylamide (v/v) separating gel using 4.9 ml H_2O, 6.0 ml acrylamide, 3.8 ml 1.5 M Tris-HCl (pH: 8.8), and 75 μl 20% SDS (w/v). 15 μl TEMED (catalyst) and 40 μl 20% APS (w/v) (radical former) should be added last. After vortexing the mixture is immediately poured into the assembled gel plate to ¾ of the volume. Cover the surface of the gel with 500 μl isopropanol to ensure proper polymerization of the gel.
2. After the separating gel is polymerized (approx. 10 min) the isopropanol is carefully removed using filter paper. Cast the stacking gel using 4 ml H_2O, 635 μl acrylamide, 313 μl 2 M

Tris (pH: 6.8), 25 µl 20% SDS (w/v), 5 µl TEMED, and 40 µl 20% APS (w/v) and quickly insert the comb.

3. After polymerization of the stacking gel (approx. 10 min) the comb should be removed carefully and the slots carefully rinsed with ddH_2O.
4. Assemble the gel in an electrophoresis chamber (Bio-Rad) and fill it up with SDS-running buffer (*see* **Note 2**).
5. Use a Hamilton syringe to load equal amounts of protein samples (*see* Subheading 3.1), and load a protein ladder as size standard.

3.3 Protein Transfer: Western Blot

Western blotting involves the transfer of proteins separated on SDS-polyacrylamide gel electrophoresis to a solid matrix such as a PVDF membrane. Western blotting can be performed by either complete immersion of a gel-membrane sandwich in a transfer buffer (wet transfer) or placing the gel-membrane sandwich between absorbent Whatman paper soaked in transfer buffer (semidry transfer). Semidry transfer is generally more rapid and efficient than wet transfer and is in general better suited to low-molecular-weight protein (smaller 150 kDa).

1. A PVDF membrane (usually 6×9 cm) is equilibrated in methanol for 20 s. Afterwards the PVDF membrane is washed in ddH_2O for 2 min and incubated in transfer buffer for 5 min for equilibration (*see* **Note 3**).
2. Take the polyacrylamide gel and remove the stacking gel. The protein gel is also incubated in transfer buffer for 5 min for equilibration (*see* **Note 3**).
3. In the meantime soak four Whatman papers (same size as PVDF membrane) in transfer buffer.
4. Carefully arrange Whatman papers, polyacrylamide gel, and PVDF membrane on the semidry blotter in the following order: (anode +), 2× Whatman paper, PVDF membrane, polyacrylamide gel, 2× Whatman paper (cathode –). Avoid air bubbles between layers as they result in insufficient protein transfer.
5. The transfer should run for 1 h at 50 mA per polyacrylamide gel.
6. Disassemble the semidry blotter, transfer the PVDF membrane to a tray with PBS-T, and incubate the membrane on a rocking platform for 5 min.

3.4 Antibody Detection

1. Incubate the PVDF membrane in PBS-T with 5% (w/v) nonfat dry milk for 1 h on a rocking platform to block unspecific binding sites on the membrane.
2. Wash the PVDF membrane three times for 5 min in PBS-T. Add the membrane to a tray with PBS-T 5% (w/v) BSA and PARP-1 antibody (e.g., 9542) diluted 1:2000 (*see* **Note 4**). Incubate overnight at 4 °C on a rocking platform.

3. Wash the PVDF membrane three times for 5 min in PBS-T on a rocking platform to wash away unbound antibodies.
4. Incubate the PVDF membrane for 1 h at room temperature with the secondary antibody diluted 1:8000 in PBS-T with 5% nonfat dry milk.
5. Afterwards the membrane is washed three times for 5 min with PBS-T to wash away unbound secondary antibodies.
6. The signal is detected by autoradiography using ECL. In brief, 10 ml ECL buffer is mixed with 10 µl 30% H_2O_2 (v/v). Incubate the PVDF membrane for 1 min in ECL solution, wipe off excessive solution, and develop the PVDF membrane.

3.5 In Vitro Translation

For the in vitro synthesis of proteins cell-free systems derived from cells engaged in a high rate of protein synthesis have been developed. The most frequently used cell-free translation systems consist of extracts from rabbit reticulocytes, wheat germ, or *Escherichia coli*. They contain all macromolecular components (tRNAs, aminoacyl-tRNA synthetases, ribosomes, initiation, elongation and termination factors, etc.) for efficient translation of exogenous RNA. Whereas standard translation systems (reticulocyte lysates or wheat germ extracts) use RNA as template, coupled systems start with DNA templates, which are transcribed into RNA and subsequently translated. Such systems typically combine a prokaryotic phage RNA polymerase and promoter (T7, T3, or SP6) with eukaryotic or prokaryotic extracts to synthesize proteins from DNA templates. The TNT® Quick Coupled Transcription/Translation System (Promega) is a coupled transcription/translation kit for eukaryotic in vitro translation purposes and is available for transcription/translation of genes cloned downstream of the T7 or SP6 RNA polymerase promoters. It simplifies in vitro reactions by combining RNA polymerase, nucleotides, salts, and recombinant RNasin ribonuclease inhibitor with the reticulocyte lysate in a single master mix (*see* **Note 5**). Starting with circular plasmid DNA in vitro transcription/translation results may be generated in 3–4 h and can be used to analyze posttranslational modifications subsequently (*see* Subheading 3.6).

1. Rapidly thaw the TNT® Quick Master Mix by hand-warming and store it on ice.
2. Pipet 40 µl TNT® Quick Master Mix, 1 µl 1 mM methionine (*see* **Note 6**), and 2 µl pCDNA3-PU.1 (0.5 µg/µl) and fill up to a final volume of 50 µl with nuclease-free H_2O.
3. Incubate the reaction at 30 °C for 60–90 min.
4. Analyze the results by Western blotting (*see* Subheadings 3.2–3.4).

3.6 Caspase Assay

Before performing the caspase assay the efficacy of the in vitro translation should be tested and analyzed by Western blotting.

1. 2 µl in vitro-translated PU.1, 0.1 unit/enzyme recombinant human caspases 1–10 (PromoKine), and caspase assay buffer to a final volume of 20 µl are incubated at 37 °C for 30 min (*see* **Note** 7).
2. The caspase reaction is stopped, by adding equal amounts of 2× SDS loading dye.
3. The caspase assay is analyzed by Western blotting (*see* Subheadings 3.2–3.4).

3.7 Calcium Phosphate Transfection

Transient transfection of plasmid DNA into cells is an indispensable tool in molecular biology. Though plenty of lipid-based transfection reagents are commercially available, a quick, efficient, and inexpensive method is to transfect eukaryotic cells via the calcium phosphate co-precipitation method. Calcium phosphate transfection typically results in transient expression of the delivered plasmid DNA and the efficacy can be close to 100% depending on the cell lines used.

1. For transient transfections HEK293 cells are seeded in 6-well plates 1 day prior to transfections.
2. 1 µg of plasmid DNA is diluted in 160 µl HBS buffer and afterwards 8.5 µl 2.5 mM $CaCl_2$ is added drop-wise.
3. The transfection mixture is carefully vortexed and incubated for 25 min at room temperature.
4. Finally the transfection mixture is added drop-wise to the cells (50% confluency is desired) and the cells are incubated for 16–24 h.
5. The transfected cells are lysed and analyzed by Western blotting (*see* Subheadings 3.1–3.4).

4 Notes

1. A solution of a tenfold concentrated SDS running buffer can be used as a stock.
2. SDS-PAGE polyacrylamide gels can be stored up to 2 weeks wrapped in wet paper and sealed in a plastic bag to prevent drying out at 4 °C. Longer storage is not recommended as it results in structurally unstable gels.
3. The equilibration step in Western blotting refers to the soaking of your PVDF membrane and your polyacrylamide gel in transfer buffer for at least 5 min. The equilibration step ensures that any contaminating electrophoresis buffer salts are removed, the protein gel is allowed to shrink to its final size before the

transfer, and it prevents the protein gel or membrane to dry out if you cannot do the transfer immediately.

4. After the protein transfer from the gel it is important to block the PVDF membrane to prevent nonspecific binding of the antibodies during subsequent steps. The suitable blocking buffer (nonfat dry milk, BSA, etc.) should improve the sensitivity of the assay by reducing background. For most antibodies a recommendation for the blocking buffer is provided. In other cases this has to be tested empirically.
5. To reduce RNase contamination Recombinant RNasin® Ribonuclease inhibitor is included in the TNT® Quick Master Mix (Promega).
6. If no suitable antibody is available coupled in vitro reactions from plasmid DNA can be used to synthesize [^{35}S] methionine-labeled proteins by incorporating 2 μl [^{35}S] methionine (1000 Ci/mmol at 10 mCi/ml) in the transcription/translation reaction.
7. The amount of time for the caspase/substrate assay can vary considerably and should be titrated/adjusted.

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Chapter 3

Assessment of HDACi-Induced Cytotoxicity

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Abstract

The chromatin contains the genetic and the epigenetic information of a eukaryotic organism. Posttranslational modifications of histones, such as acetylation and methylation, regulate their structure and control gene expression. Histone acetyltransferases (HATs) acetylate lysine residues in histones while histone deacetylases (HDACs) remove this modification. HDAC inhibitors (HDACi) can alter gene expression patterns and induce cytotoxicity in cancer cells. Here we provide an overview of methods to determine the cytotoxic effects of HDACi treatment. Our chapter describes colorimetric methods, like trypan blue exclusion test, crystal violet staining, lactate dehydrogenase assay, MTT and Alamar Blue assays, as well as fluorogenic methods like TUNEL staining and the caspase-3/7 activity assay. Moreover, we summarize flow cytometric analysis of propidium iodide uptake, annexin V staining, cell cycle status, ROS levels, and mitochondrial membrane potential as well as detection of apoptosis by Western blot.

Key words Cell death, Viability, Vitality, Colorimetric assay, Fluorogenic assay, Flow cytometry, Immunoblotting, Histone deacetylase inhibitor, HDACi, Toxicity

1 Introduction

The blockage of histone deacetylases (HDACs) by HDAC inhibitors (HDACi) has multiple cell type-specific effects in vitro and in vivo, such as growth arrest, cell differentiation, induction of reactive oxygen species (ROS), and apoptosis in malignant cells [1, 2]. Since many aspects of HDACs and HDACi are still not fully understood, further in-depth investigations into the effects of HDACi, their targets, and mechanisms of action are needed. This will be critical for the advancement of these compounds, especially to facilitate the rational design of HDACi that are effective as antineoplastic agents.

There are different possibilities to determine HDACi-induced cellular cytotoxic effects, such as colorimetric or fluorogenic assays that assess cell viability and vitality. Viability is defined as the percentage of living cells in the whole population [3]. Although cell death is the ultimate consequence of cytotoxicity, alterations that compromise the cellular ability to divide, without necessarily

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leading to cell death, may also operate. This aspect represents the term cell vitality and is defined as the physiological capability of cells. Yet, a single toxicity assay cannot stand alone in determining the extent of cell damage or death [4]. Therefore, a panel of different methods is required to elucidate the cytotoxic effects induced by treatment with HDACi.

A very quick method to assess cell viability microscopically is to stain cells and distinguish between dead and live cells on the basis of membrane integrity and the associated ability to absorb a dye, such as trypan blue. The trypan blue dye exclusion assay is based on the fact that living cells possess intact cell membranes whereas dead cells do not. Thus, intact cell membranes exclude the dye, while dead cells appear blue due to trypan blue uptake [5]. This technique is feasible without the need of elaborated equipment, but it is not appropriate for high-throughput analyses.

Tetrazolium reduction assays, including the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Alamar Blue assay, are common methods for determining the extent of cytotoxicity by measuring cell vitality and proliferation [3]. These methods are realizable in 96- or 384-well formats and are indispensable for analyzing a greater number of samples.

Tetrazolium derivatives are soluble salts that are readily taken up by living cells. They are converted by mitochondrial succinate dehydrogenase and cytosolic nicotinamide adenine dinucleotide as well as nicotinamide adenine dinucleotide phosphate (NADH/NADPH) [6] into colored insoluble compounds. In case of MTT, a purple formazan product is formed [7]. These end products can be easily quantified by measuring their absorbance using a UV-Vis spectrophotometer. Besides MTT, there are other, closely related tetrazolium dyes such as 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), and the water-soluble tetrazolium salts (WSTs).

A disadvantage of these methods is that a number of other factors may inhibit mitochondrial reductases and change cytosolic NADH/NADPH levels [6], implying that the conversion of tetrazolium derivatives per se cannot provide unequivocal information on cell viability [4]. MTT-based assays, thus, are highly susceptible to metabolic interference and thereby may generate false results. Additionally, reducing agents and respiratory chain inhibitors as well as a number of other parameters, such as medium pH, D-glucose concentration in the growth medium, and cellular concentration of pyrimidine nucleotides, can significantly affect formazan formation. Thus, although metabolism-orientated tests are very useful to obtain preliminary information, their results need to be validated in secondary screens [8].

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide), the Alamar Blue dye, is a nontoxic derivative of the tetrazolium salt. It becomes pink and fluorescent upon reduction to resorufin. This change in absorbance parallels with an increase in fluorescence and can be measured at distinct wavelengths. Thereby, the amount of fluorescence or absorbance is proportional to the cells' metabolic activity. Both readouts can be used to obtain the production of resorufin and are a measure for cell growth and vitality [9].

Crystal violet staining (gentian violet) is a quick and easy way to determine cell proliferation colorimetrically. Crystal violet permeates into cells and intercalates with DNA. It has the disadvantage compared to the previously described assays that all adherent cells, including dead cells, are stained and regarded as viable cells.

Another possibility to determine cytotoxic effects is to measure cell vitality using the lactate dehydrogenase (LDH) assay. LDH is an enzyme that physiologically catalyzes the oxidation of lactate to pyruvate, thereby regenerating NADH. It is released out of cells after severe cell damage or plasma membrane ruptures. Therefore, the oxidation of lactate can be assessed by measuring the production of NADH within the supernatant of cultured cells. The LDH assay is relatively inexpensive and can easily be realized in a 96- or 384-well format [4]. A major drawback of this high-throughput screening is that the activity of LDH may be affected by physicochemical parameters such as changes in the pH of the culture medium or side effects of pharmacological inhibitors. Additionally, enzyme activity may decay with time in the extracellular milieu [4].

To discriminate cell death from cytostatic events, flow cytometric analyses have to be considered. Fluorogenic assays are suitable to assess cell viability as well as vitality [3]. Fluorescence-activated cell sorting (FACS) is a technique for the analysis of several parameters within cell suspensions. By doing so, suspensions are streamed through a fluid sheath pipe intersected by a laser. The laser beam is scattered and dissected by the cell surface. Thereby, the forward light scatter directly correlates with the cell size, whereas the side light scatter corresponds directly to the granularity of the cells [10]. The fluorescence intensities of coupled fluorophores are also detectable by FACS. There are different possibilities to label cells and thereby gather simultaneously information on various cellular processes like expression of surface markers, intracellular cytokines and signaling proteins, cell death, DNA fragmentation, ROS levels, cell cycle status, and mitochondrial membrane integrity of a cell [11]. Propidium iodide (PI) and annexin V are compounds used for cell viability measurements, whereas 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) is suitable for cell vitality determination by measuring the loss of the mitochondrial membrane potential ($\Delta\psi_m$) [12]. ROS can be easily detected by 2′,7′-dichlorofluorescein diacetate (DCFDA) or its derivates like CM-H_2DCFDA and carboxy-DCFDA [13].

PI, which is a fluorescent nuclear and chromosome counterstain, can cross the plasma membrane only after loss of its integrity [8]. Since PI is extruded by living cells, it is used to detect dead cells in a population. Another staining method to discriminate dead from living cells is 7-aminoactinomycin D (7-AAD).

An important marker for apoptotic cells is the occurrence of internucleosomal DNA fragmentation [14]. This causes a reduction of the cellular DNA content in apoptotic cells compared to non-apoptotic ones. Cellular DNA levels can be determined by the application of the DNA-intercalating dye PI in fixed cells, whose fluorescence is stoichiometrically equal to the cellular DNA content. Since the cellular DNA level depends on the present cell cycle phase, each phase has its corresponding fluorescence intensity. Cells that are in G_1 phase have a diploid set of chromosomes and therefore offer a high fluorescence. The fraction of apoptotic cells with fragmented DNA exhibits a weaker fluorescence and is termed sub-G_1 cells.

Annexin V staining is used to detect cells undergoing apoptosis. In viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. The exposure of PS on the outer leaflet of the plasma membrane constitutes an early event in apoptosis. Externalized PS can be readily detected by fluorescent or streptavidin-labeled annexin V. To distinguish apoptotic cells that are annexin V positive but still retain intact plasma membranes from non-apoptotic or late-apoptotic cells, annexin V staining is usually combined with cell-impermeable dyes, such as PI [4].

$DiOC_6(3)$ is a positively charged molecule that permeates through the plasma membrane. At low concentrations, it accumulates in mitochondria due to their negative membrane potential and is retained inside them. If the mitochondrial membrane is disrupted, mitochondria can no longer retain $DiOC_6(3)$, resulting in a decay of fluorescence. Besides $DiOC_6(3)$ and related oxa-carbocyanine derivates, other probes for measuring the membrane potential, like 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) or 3,3′-dimethyl-α-naphthoxacarbocyanine iodide (JC-9), exist [12]. Loss of the mitochondrial membrane potential is, like annexin V staining, a marker for early apoptosis [8].

Several HDACi induce ROS and ROS-mediated apoptosis [15]. The cell-permeant non-fluorescent dye DCFDA is oxidized by hydrogen peroxide resulting in the fluorescent product 2′,7′-dichlorofluorescein (DCF). Whereas DCFDA and its derivatives as well as Amplex Red® are dyes specific for cytosolic hydrogen peroxide, other dyes have been developed to react with different cellular ROS. MitoSOX™ and dihydroethidium react with mitochondrial superoxide [16], and 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate reacts with nitrogen peroxide [17]. Hence, by the use of these dyes, it is possible

to specifically delineate the source and species of ROS after HDACi treatment.

An advantage of FACS analyses is, in case of multicolor flow cytometric analyses, the possibility to use different dyes in parallel. This gives the opportunity to collect several data from single samples. Consequently, not only cell death can be detected, but also the mode of cell death. However, analyses that employ more fluorophores are subject to signal spillover, rendering data analyses more sophisticated [11].

Since the activation of cysteine-dependent aspartate-directed proteases (caspases) is an important step in the apoptotic process, assessing caspase activation is another possibility to determine cytotoxic effects [4]. Caspase-3/7 activity can be measured using the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-amido-4-methylcoumarin (Ac-DEVD-AMC). Caspases-3 and -7 are members of the cysteine protease family and belong to the group II caspases, the so-called effector caspases. Both are activated and cleaved during apoptosis [18]. They are involved in the proteolysis of poly-(ADP)-ribose-polymerase 1 (PARP-1). PARP-1 is a nuclear enzyme activated by DNA damage, which, in contrast to its role as a survival factor in the presence of basal levels of DNA damage, can induce cell death in the presence of extensive DNA damage [19]. Since PARP-1 is a NAD^+-dependent enzyme, it is normally cleaved and therefore inactivated by caspase-3/7 or -8 during apoptosis to prevent an enhanced NAD^+ consumption and therefore an ATP depletion that may lead to necrotic cell death [19]. Ac-DEVD-AMC contains the amino acid sequence of the PARP-1 cleavage site and is therefore useful for the identification and quantification of caspase-3/7 activity in apoptotic cells. The tetrapeptide is cleaved by caspase-3/7 between AMC and the aspartic acid residue leading to the release of the fluorogenic AMC, which can be detected by spectrofluorometry.

Since there are other, caspase-independent cell death mechanisms that manifest with apoptotic morphology and as it was found that initiator and executioner caspases exert nonlethal functions as intracellular signaling molecules, caspase activation alone is not a universal marker of apoptosis [4]. Therefore, co-application of the pan-caspase inhibitor z-VAD-fmk is useful to inhibit caspase-dependent apoptotic events. Its potentially protective effect can be assessed by flow cytometric analyses of cell death and mitochondrial depolarization using PI and $DiOC_6(3)$, respectively. However, the supposedly specific caspase inhibitors, such as the putative-specific caspase-9 inhibitor z-LEHD-fmk, should be used with caution. They do not guarantee a specific inhibition of individual caspases [20, 21].

The use of z-VAD-fmk in cell cycle analysis can also be helpful for the detection of cell cycle arrest. HDACi treatment can elicit both cell death and cell cycle arrest [2]. However, in the presence of

a fully active caspase machinery, cell cycle arrest may be concealed by cell death, but may become visible when cell death is blocked through the inhibition of caspases. Examples of this phenomenon have been reported in, e.g., studies investigating the effects of HDACi in lung, prostate, and colon cancer cells [22, 23].

The activation of caspases is commonly accompanied by cleavage of the caspase zymogens into distinct fragments [18]. Thus, the involvement of specific caspases can be elucidated by assessing caspase cleavage through Western blot analysis. This method enables the identification of specific proteins from a complex mixture of proteins extracted from cells and allows quantifying the protein levels. Therefore, three main elements are necessary to accomplish this task: (1) separation by size, (2) transfer to a solid support, and (3) marking target protein using a proper primary and secondary antibody for visualization [24]. An example of caspase-8 cleavage detection complementing its activity measurements can be found in [25].

As mentioned above, the cleavage of PARP-1, which is catalyzed mainly by caspases-3, -7, or -8, is another typical marker for cells undergoing apoptosis [18]. These proteins and their cleavage products can be detected by immunoblotting. Signs for the activation of the mitochondrial (intrinsic) pathway of apoptosis are the localization of pro-apoptotic BCL-2 family proteins, such as BAX, BID, and BIM, or monomeric p53 at mitochondria. The interaction of different BCL-2 family proteins leads to the permeabilization of the outer mitochondrial membrane and to the release of pro-apoptotic proteins, like cytochrome *c*, SMAC/DIABLO, or apoptosis-inducing factor (AIF), into the cytosol. Following, cytochrome *c* forms together with APAF-1 the so-called apoptosome and activates effector caspases to induce apoptosis [26]. The cleavage of the BCL-2 family member BID to truncated BID (tBID) connects the death receptor-mediated (extrinsic) pathway of apoptosis with the intrinsic one [27]. Hence, the detection of tBID on Western blots can be useful to disclose whether HDACi facilitate the engagement of the intrinsic pathway in death receptor-mediated apoptosis; *see* e.g. [28].

A useful marker for apoptosis or at least severe cytotoxic effects is DNA fragmentation. Detection of DNA fragments in situ can be realized by using the terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP)-digoxigenin nick end labeling (TUNEL) assay [4, 29]. This assay is based on internucleosomal DNA cleavage, a characteristic biochemical hallmark of the apoptotic mode of cell death. However, DNA fragmentation is common in different kinds of cell death and therefore its detection should not be considered as a specific marker of apoptosis [4, 8].

HDACi may also induce immunogenic cell death [30, 31]. Yet a discussion of the methods pertinent to immunogenic cell death is beyond the scope of this chapter and has recently been published [32].

Apparently, every method has its advantages and disadvantages. This suggests the necessity of using these assays not as standalone methods but rather as parts of a panel of methods using different markers for cytotoxicity to evaluate HDACi-induced cytotoxic effects.

2 Materials

All gels and buffers should be prepared with double-distilled water (ddH_2O), whereas solutions for tissue culture use should be sterile filtered or autoclaved before usage. The concentration information given for all buffers and solutions refers to the final concentration, unless noted otherwise. All reagents should be prepared and stored at room temperature (RT) unless indicated otherwise. Materials and antibodies listed here are routinely used by our lab and many other groups. However, equipment from other providers should be equally useful [33].

2.1 Staining Assays and Colorimetric Analyses

2.1.1 Trypan Blue Exclusion Test

1. Hemocytometer (Assistant Germany).
2. Dulbecco's phosphate-buffered saline (PBS) 1× (PAA Laboratories).
3. Trypan blue 0.5% (w/v) in PBS 1× (PAA Laboratories).
4. Trypsin/ethylenediaminetetraacetic acid (EDTA) 1× (PAA Laboratories).

2.1.2 Tetrazolium Reduction Assays

3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

1. FLUOstar Omega multi-mode microplate reader (BMG LABTECH GmbH).
2. 0.5% (w/v) MTT solution (5 mg/ml MTT in H_2O, Alfa Aesar GmbH & Co KG).
3. 2-Propanol (Carl Roth).

Alamar Blue® Assay

1. FLUOstar Omega multi-mode microplate reader (BMG LABTECH GmbH).
2. Resazurin solution (PromoKine).

2.1.3 Crystal Violet Staining (Gentian Violet) Assay

1. FLUOstar Omega multi-mode microplate reader (BMG LABTECH GmbH).
2. 10% Acetic acid (in H_2O; Carl Roth).
3. 0.1% Crystal violet (in H_2O; Sigma).
4. 3.7% Formaldehyde (Carl Roth) in PBS 1× (PAA Laboratories).

2.1.4 Lactate Dehydrogenase Assay

1. Dounce tissue grinder (Sigma).
2. FLUOstar Omega multi-mode microplate reader (BMG LABTECH GmbH).

3. Dulbecco's PBS 1× (PAA Laboratories).
4. Lactate dehydrogenase assay kit (colorimetric) (Abcam, ab102526).

2.2 Fluorogenic Assays

1. BD Cytometer Setup & Tracking Beads™ (BD Bioscience).
2. BD FACSCANTO™ II flow cytometer (BD Bioscience).

2.2.1 Flow Cytometric Analysis

3. Dulbecco's PBS 1× (PAA Laboratories).
4. Trypsin/EDTA 1× (PAA Laboratories).

Propidium Iodide (PI) Uptake

1. Resuspension buffer: 15 µg/ml PI (Sigma) in PBS 1× (PAA Laboratories).

Annexin V Staining

1. Annexin V-APC (Immuno Tools GmbH).
2. Binding buffer: 0.1 M 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid (HEPES) (Sigma) pH 7.4 (adjust with HCl/NaOH), 1.4 M NaCl (Carl Roth), 25 mM $CaCl_2$ (Carl Roth).

Cell Cycle Analysis

1. Ethanol.
2. Resuspension buffer: PBS 1× (PAA Laboratories), 1% glucose (PAA Laboratories), 50 µg/ml RNase A (Roche), 50 µg/ml PI (Sigma).

Accumulation of 3,3′-Dihexyloxacarbocyanine Iodide ($DiOC_6(3)$)

1. 50 µM $DiOC_6(3)$ (Molecular Probes) in DMSO.

ROS Detection with 2′,7′-Dichlorofluorescein Diacetate (DCFDA)

1. 20 mM DCFDA (Sigma) in DMSO.

2.2.2 Caspase-3/7 Activity

1. FLUOstar Omega multi-mode microplate reader (BMG LABTECH GmbH).
2. Dulbecco's PBS 1× (PAA Laboratories).
3. Activity buffer (pH 7.5, adjust with HCl/NaOH): 20 mM HEPES (Sigma), 10% glycerol (Merck), add freshly: 2 mM dithiothreitol (DTT) (Sigma), 25 µg/ml caspase-3/7 substrate (Ac-DEVD-AMC) (Bachem).
4. Lysis buffer (pH 7.5, adjust with HCl/NaOH): 10 mM NaH_2PO_4 (Carl Roth), 10 mM Tris–HCl (Carl Roth), 10 mM Na_2HPO_4 (Carl Roth), 130 mM NaCl (Carl Roth), 10 mM $Na_4P_2O_7$ (Sigma), 1% Triton-X 100 (Sigma).
5. Trypsin/EDTA 1× (PAA Laboratories).

2.2.3 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

1. FLUOstar Omega multi-mode microplate reader (BMG LABTECH GmbH).
2. Fluorescence microscope (Nikon).
3. Blocking buffer: 1% Bovine serum albumin (BSA) (Carl Roth) in PBS 1× (PAA Laboratories).
4. DAPI-containing mounting medium (Fluoroshield™ with DAPI, Sigma).
5. Dulbecco's PBS 1× (PAA Laboratories).
6. 3.7% Formaldehyde (Carl Roth) in PBS 1× (PAA Laboratories).
7. Terminal transferase solution: 200 µl Reaction buffer 5× and 250 units terminal deoxynucleotidyl transferase (final conc.: 0.25 units/µl) (Thermo Scientific), 6.66 µM Biotin-16-dUTP (Roche), add H_2O to a final volume of 1 ml.
8. Antibody solution: Streptavidin-Cy3 (Sigma), 1:500 in PBS 1× (PAA Laboratories) with 1% BSA (Carl Roth).

2.3 Western Blot Analysis

2.3.1 Preparation of Whole-Cell Extracts

1. Sonifier® WD-250 (Branson).
2. Dulbecco's PBS 1× (PAA Laboratories).
3. Protease inhibitor cocktail (PIC): 10,000 Units/ml aprotinin (Carl Roth), 100 mg/ml benzamidine, 2 mg/ml antipain (Carl Roth), 1 mg/ml leupeptin (Carl Roth).
4. Radioactive immunoprecipitation assay (RIPA) buffer: 150 mM NaCl (Carl Roth), 50 mM Tris–HCl (Carl Roth) pH 8.0 (adjust with HCl/NaOH), 1% NP-40 (Sigma) or Triton-X 100 (Carl Roth), 1% sodium deoxycholate (Carl Roth), add freshly: 0.5 mM phenylmethyl sulfonyl fluoride (PMSF) (Merck), 0.2% PIC (*see* **Note 1**).
5. Trypsin/EDTA 1× (PAA Laboratories).

2.3.2 Determination of Protein Concentrations

1. FLUOstar Omega multi-mode microplate reader (BMG LABTECH GmbH).
2. BSA (Carl Roth).
3. Coomassie Brilliant Blue G-250 (Roti® Nanoquant) (Carl Roth) or Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific) (*see* **Note 2**).

2.3.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. Mini-PROTEAN® 3 System (Bio-Rad).
2. Laemmli buffer (6×): 35% β-Mercaptoethanol (SERVA), 350 mM Tris–HCl (Carl Roth) pH 6.8 (adjust with HCl/NaOH), 30% glycerol (Merck), 10% SDS (AppliChem), 0.025% bromophenol blue (SERVA).
3. Page Ruler™ Plus Prestained Protein Ladder 10–250kDa (Thermo Scientific).

Table 1
Gel mixtures for different SDS gel concentrations

	Stacking gel	Resolving gel			
Final acrylamide concentration	4%	7.5%	10%	12.5%	15%
Rotiphorese® Gel30	0.66 ml	2.5 ml	3.33 ml	4.1 ml	5 ml
1 M Tris–HCl pH 6.8	1.25 ml	–	–	–	–
1 M Tris–HCl pH 8.8	–	1.9 ml	1.9 ml	1.9 ml	1.9 ml
H_2O	3.1 ml	5.6 ml	4.8 ml	4 ml	3.1 ml
20% SDS (w/v)	25 µl	50 µl	50 µl	50 µl	50 µl
20% APS (w/v)	30 µl	40 µl	40 µl	40 µl	40 µl
TEMED	2 µl	4 µl	4 µl	4 µl	4 µl

4. Resolving and stacking gel: Acrylamide (Rotiphorese® Gel30; Carl Roth), Tris–HCl (Carl Roth), SDS (AppliChem), ammonium persulfate (APS; AppliChem), N,N,N′,N′-tetramethylethane-1,2-diamine (TEMED) (Carl Roth) (Table 1).
5. SDS running buffer (10×): 2.5 M Glycine (Carl Roth), 250 mM Tris–HCl (Carl Roth), 1% SDS (AppliChem).

2.3.4 *Western Blotting*

1. Blotting paper Whatman®, 3 mm (Sigma).
2. ECL film processor Protec® (OptiMax).
3. Enhanced chemiluminescence (ECL) solution (Pierce, Thermo Scientific).
4. Mini-PROTEAN® 3 blot chamber (Bio-Rad).
5. Polyvinylidene difluoride membrane (Roti-PVDF) (Carl Roth).
6. Roll and mix device (RM-5 Ingenieurbüro CAT).
7. X-ray films Fuji Super RX (Fujifilm).
8. Blocking buffer: PBS 1× containing 5% fat-free milk powder (Carl Roth), 0.05% Tween-20 (Carl Roth).
9. Ethanol, methyl ethyl ketone (MEK) denatured (Carl Roth).
10. PBS (10×): 1370 mM NaCl (Carl Roth), 100 mM Na_2HPO_4 (Carl Roth), 27 mM KCl (Carl Roth), 18 mM KH_2PO_4 (FLUKA), pH 7.25 (adjust with HCl/NaOH).
11. PBS-T (1×): 1× PBS containing 0.05% Tween-20 (Carl Roth).
12. Primary antibody solution: Appropriate amount of antibody in PBS-T with 5% blocking buffer, 0.02% sodium azide (primary

antibody: anti-PARP-1 cleaved and anti-PARP-1 full length, mouse monoclonal IgG1 (BD PharMingen, 552597 and 556494, respectively), 1:5000; anti-GAPDH, rabbit monoclonal IgG1 (Abcam, ab128915), 1:50,000, anti-caspase-8, mouse monoclonal IgG1 (Cell Signaling, 9746), 1:1000).

13. Secondary antibody solution: Appropriate amount of antibody in PBS-T with 50% blocking buffer (secondary antibody: goat anti-mouse IgG H&L (HRP) or goat anti-rabbit IgG H&L (HRP) (Abcam, ab97023 and ab97051, respectively), 1:10,000).
14. Transfer buffer (10×): 250 mM Glycine (Carl Roth), 25 mM Tris–HCl (Carl Roth), 20% ethanol MEK denatured (Carl Roth), 0.1% SDS (AppliChem).

3 Methods

All cell culture preparations are performed under sterile conditions using a sterile working bench and are designed for adherent cells. All steps are performed at room temperature (RT) unless otherwise indicated.

3.1 Staining Assays and Colorimetric Analyses

Cell viability can be assessed by trypan blue dye exclusion, a test that is used to identify the number of viable cells present in a cell suspension.

3.1.1 Trypan Blue Dye Exclusion Test

1. Culture cells in 6-well plates to approximately 90% confluence and stimulate them with the HDACi of choice.
2. For harvesting cells, collect the medium, wash cells with 500 µl PBS, and detach them afterwards by incubation with 250 µl trypsin/EDTA at 37 °C.
3. Add 500 µl PBS and resuspend cells within.
4. Centrifuge the cell suspension and the collected medium together for 5 min at 1800×*g* and discard the supernatant afterwards (*see* **Note 3**).
5. Resuspend the cell pellet in fresh medium and mix 20 µl of this cell suspension with 20 µl trypan blue.
6. Transfer this solution between a slide and cover glass of a hemocytometer, and determine cell viability by counting trypan blue-positive and -negative cells.

3.1.2 Tetrazolium Reduction Assays

MTT and tetrazolium salt-based assays measure the metabolic activity of mitochondria by the reduction of tetrazolium salts to formazan via mitochondrial reductases, thereby assessing cell vitality.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay

1. Culture cells in 48-well plates to approximately 70% confluence and stimulate them using an appropriate HDACi.
2. Add 50 µl of a 0.5% (w/v) MTT solution to 500 µl culture medium and incubate the plate for 2 h at 37 °C.
3. Afterwards, remove the supernatant containing the remaining MTT thiazole and resolve the violet, water-insoluble formazan in 300 µl 2-propanol on a shaker for 20 min (*see* **Note 4**).
4. Analyze the staining photometrically by measuring the absorbance of the formazan at 596 nm in a fresh 48- or 96-well plate (*see* **Note 5**).

Alamar Blue® Assay

A widely used alternative to the MTT assay is the Alamar Blue assay®, which is easier to carry out.

1. Culture cells in 48-well plates to approximately 70% confluence and stimulate them using an appropriate HDACi.
2. Add 50 µl of the resazurin solution to 500 µl culture medium and incubate the plates at 37 °C for 3 h.
3. Afterwards, directly measure the fluorescence of the produced resorufin using an excitation/emission wavelength of 560/590 nm (*see* **Note 6**).
4. Alternatively, measure the absorbance of the produced resorufin at 570 nm in a fresh 48- or 96-well plate (*see* **Note 6**).

3.1.3 Crystal Violet Staining (Gentian Violet) Assay

Another assay to determine cytotoxicity is the crystal violet staining. It is based on the growth rate reduction reflected by the colorimetric determination of the stained cells.

1. Culture cells in 48-well plates to approximately 70% confluence and stimulate them using an appropriate HDACi.
2. Remove the supernatant medium and fix cells within 250 µl of a 3.7% formaldehyde solution for 15 min (*see* **Note 7**).
3. Discard the supernatant, add 250 µl of a 0.1% crystal violet solution (in H_2O), and incubate the plate for 30 min at 37 °C.
4. Wash cells two times with H_2O (*see* **Note 7**).
5. Afterwards, dissolve the dye in 300 µl 10% acetic acid and measure the absorption at 570 nm in a fresh 48- or 96-well plate [9, 10].

3.1.4 Lactate Dehydrogenase Assay

Plasma membrane ruptures can also be biochemically quantified by measuring the release of intracellular proteins, such as LDH, into cell culture supernatants. In the LDH Assay Kit (Abcam), LDH reduces NAD^+ to NADH, which then interacts with a specific probe to produce a color that can be measured at 450 nm.

1. Culture cells in 96-well plates to approximately 90% confluence and stimulate them using an appropriate HDACi.

2. Perform all further steps according to the manufacturer's protocols (lactate dehydrogenase assay kit (colorimetric) (Abcam)).

3.2 Fluorogenic Assays

Cell death can be assessed by flow cytometric analyses of cellular PI uptake.

3.2.1 Flow Cytometric Analysis

Propidium Iodide (PI) Uptake

1. Culture cells in 6-well plates to approximately 90% confluence and stimulate them using an appropriate HDACi.
2. For cell harvesting, collect the supernatant medium, wash cells with 500 µl PBS, and detach them afterwards by incubation with 250 µl trypsin/EDTA at 37 °C.
3. Add 500 µl PBS and resuspend cells within. Repeat this step once.
4. Centrifuge the collected medium and the cell suspension together for 5 min at 1800 × *g* (*see* **Note 3**).
5. Perform all the following steps on ice.
6. Discard the supernatant, resuspend the pellet in 1 ml resuspension buffer, and incubate cells for 5 min at 4 °C under light exclusion (*see* **Note 11**).
7. Analyze 10,000 cells per sample and gate data to exclude debris.

Annexin V Staining

Cells undergoing apoptosis are detectable by annexin V staining of externalized phosphatidylserine, which is an early event during apoptosis.

1. Culture cells in 6-well plates to approximately 90% confluence and stimulate them using an appropriate HDACi.
2. For cell harvesting, collect the supernatant medium, wash cells with 500 µl PBS, and detach them afterwards by incubation with 250 µl trypsin/EDTA at 37 °C.
3. Add 500 µl PBS and resuspend cells within. Repeat this step once.
4. Centrifuge the collected medium and the cell suspension together for 5 min at 1800 × *g* (*see* **Note 3**).
5. Perform all the following steps on ice.
6. Discard the supernatant and resuspend the pellet in 500 µl binding buffer.
7. Add 5 µl APC-annexin V, mix carefully, and incubate cells for 15 min under light exclusion (*see* **Note 11**).
8. Analyze 10,000 cells per sample and gate data to exclude debris.

Cell Cycle Analyses

Cytotoxic effects can also be evaluated by measuring the distribution of cells in different phases of the cell cycle and their DNA content.

1. Culture cells in 6-well plates to approximately 90% confluence and stimulate them using an appropriate HDACi.
2. For cell harvesting, collect the supernatant medium, wash cells with 500 µl PBS, and detach them afterwards by incubation with 250 µl trypsin/EDTA at 37 °C.
3. Add 500 µl PBS and resuspend cells within. Repeat this step once.
4. Centrifuge the collected medium and the cell suspension together for 5 min at 1000 × *g*.
5. Perform all the following steps on ice.
6. Discard the supernatant and resuspend the pellet in 500 µl binding buffer.
7. Add 1 ml ice-cold 70% ethanol dropwise while shaking, in order to avoid cell clumping.
8. Incubate and fix cells overnight at 4 °C for 30 min at –20 °C.
9. After fixation, centrifuge cells for 10 min at 1000 × *g* and wash them two times with PBS afterwards.
10. Resuspend cells dropwise while shaking in 500 µl resuspension buffer and incubate them for 30 min at 37 °C in the dark.
11. Analyze 10,000 cells per sample and gate data to exclude debris.
12. Afterwards, calculate the percentage of cells in different phases of the cell cycle as areas under the distribution curve. Assess the percentage of hypo-diploid cells, the cells that are undergoing cell death, by quantitating the sub-G_1 peak (Fig. 1).

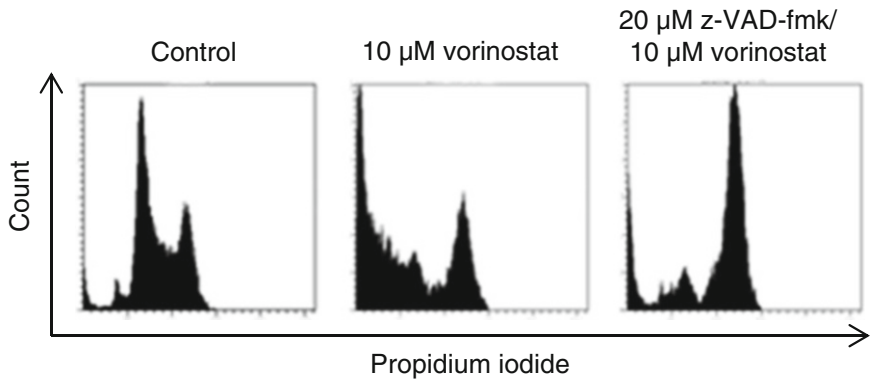

Fig. 1 Cell cycle analysis in HCT-116 $p53^{-/-}$ cells. Cells were exposed to 10 mM vorinostat for 48 h; 20 µM z-VAD-fmk was applied 1 h before treatment with vorinostat. Cell cycle phases were determined by measuring PI incorporation into DNA in ethanol-fixed cells by flow cytometry. Histograms were created with FACSDiva software. Data were gated to exclude debris

Accumulation of 3,3′-Dihexyloxacarbocyanine Iodide ($DiOC_6(3)$)

$\Delta\psi_m$ loss can be determined by assessing the accumulation of the cationic lipophilic fluorochrome $DiOC_6(3)$ in the mitochondrial matrix.

1. Culture cells in 6-well plates to approximately 90% confluence and stimulate them using an appropriate HDACi.
2. Incubate cells with 50 nM $DiOC_6(3)$ at 37 °C for 30 min prior to harvesting under light exclusion (*see* **Note 11**).
3. For cell harvesting, collect the supernatant medium, wash cells with 500 µl PBS, and detach them afterwards by incubation with 250 µl trypsin/EDTA at 37 °C.
4. Add 500 µl PBS and resuspend cells within. Repeat this step once.
5. Centrifuge the collected medium and the cell suspension together for 5 min at 1800 × *g* (*see* **Note 3**).
6. Discard the supernatant and resuspend the pellet in 500 µl binding buffer.
7. Wash cells at least once with PBS.
8. Analyze 10,000 cells per sample and gate data to exclude debris.

Detection of Cytosolic Reactive Oxygen Species with 2′,7′-Dichlorofluorescein Diacetate (DCFDA)

1. Culture cells in 6-well plates to approximately 90% confluence and stimulate them using an appropriate HDACi.
2. Incubate cells with 20 µM DCFDA at 37 °C for 30 min prior to harvesting under light exclusion (*see* **Note 11**).
3. For cell harvesting, collect the supernatant medium, wash cells with 500 µl PBS, and detach them afterwards by incubation with 250 µl trypsin/EDTA at 37 °C.
4. Add 500 µl PBS and resuspend cells within. Repeat this step once.
5. Centrifuge the collected medium and the cell suspension together for 5 min at 1000 × *g*.
6. Discard the supernatant and resuspend the pellet in 500 µl binding buffer.
7. Wash cells at least once with PBS.
8. Analyze 10,000 cells per sample and gate data to exclude debris. Afterwards, quantify the levels of ROS as the ratio of treated to untreated cells (Fig. 2).

3.2.2 Caspase-3/7 Activity

Caspase-3/7 activity can be determined using the fluorogenic substrate Ac-DEVD-AMC.

1. Culture cells in 6-well plates to approximately 90% confluence and stimulate them using an appropriate HDACi.
2. For cell harvesting, collect the supernatant medium, wash cells with 1 ml PBS, and detach them afterwards by incubation with 250 µl trypsin/EDTA at 37 °C.

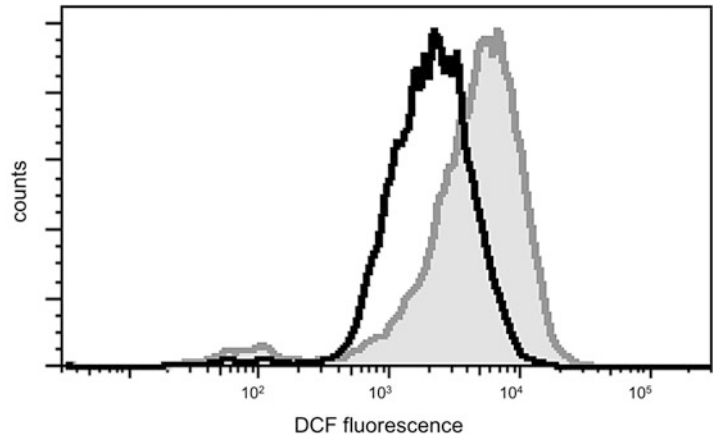

Fig. 2 Tenovin-1-triggered alterations in cellular ROS level in HCT-116 cells. Cells were exposed to 10 μM tenovin-1 for 48 h (*gray*). Control cells were treated with PBS (*black*). ROS levels were determined by flow cytometric analysis of the available amount of DCF within cells using the FITC channel. Histograms were edited using the FlowJo™ software. Data were gated to exclude debris

3. Add 500 μl PBS and resuspend cells within. Repeat this step once.
4. Centrifuge the collected medium and the cell suspension together for 5 min at 1000 × *g*.
5. Perform all the following steps on ice.
6. Discard the supernatant, resuspend the pellets in 100 μl lysing buffer, and incubate them for 15 min.
7. Add 110 μl caspase-3/7 activity buffer to each sample, mix well, and transfer the whole volume to a sterile 96-well plate.
8. Incubate this plate for 2 h at 37 °C under light exclusion (*see* **Note 12**).
9. Determine the release of AMC using an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a spectrofluorometer.
10. Calculate the relative caspase-3/7 activity as the ratio of emission of treated to untreated cells.

3.2.3 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling Assay

To investigate apoptosis, the detection of DNA fragments using TUNEL assay, which is based on internucleosomal DNA cleavage in apoptotic cells, can be applied.

1. Seed cells at a density of 50,000 cells per well on cover slips in 6-well plates.
2. After attachment overnight, treat cells with an appropriate HDACi and fix them afterwards with 500 μl 3.7% formaldehyde for 10 min.

3. Then, wash cells three times with 500 µl PBS for 5 min each (*see* **Note 13**).
4. Add 50 µl terminal transferase solution per cover slip and incubate them for 1 h at 37 °C in a humidified chamber.
5. Afterwards, wash cells again three times with 500 µl PBS for 5 min each.
6. Block unspecific binding sites by incubating the samples for 1 h in blocking buffer.
7. Probe each cover slip with the antibody solution for 2 h in a humidified chamber.
8. Following, wash cells three times with 500 µl PBS for 5 min each and mount the cover slips with DAPI containing mounting medium onto slides.
9. Analyze the cells by immunofluorescence microscopy. Quantify both DAPI and Cy3 fluorescence and calculate the percentage of TUNEL-positive cells per DAPI staining afterwards.

3.3 Western Blot Analysis

3.3.1 Preparation of Whole-Cell Extracts

1. Culture cells in 6-well plates to approximately 90% confluence and stimulate them using an appropriate HDACi.
2. Perform all the following steps on ice to keep protease activities at a minimum.
3. For harvesting cells, collect supernatant medium, wash cells with 1 ml PBS, and detach them afterwards by incubation with 250 µl trypsin/EDTA at 37 °C or by scraping.
4. If trypsinized, add 1 ml PBS and resuspend the cells within. Repeat this step once.
5. Centrifuge the collected medium and the cell suspension together for 5 min at 1000 × *g*. Discard the supernatant and repeat this step once.
6. After harvesting, resuspend cells in 100 µl RIPA buffer (*see* **Note 14**) and lyse them by sonication (10% amplitude, 10 × 0.5 s pulse, 0.5 s pause).
7. Remove cell debris for 10 min at 20,000 × *g* and 4 °C.
8. Transfer the supernatant cell lysate into a fresh tube and either use it immediately or store it at −80 °C.

3.3.2 Determination of Protein Concentrations

To determine the protein concentration within the lysates, Bradford or BCA assays can be performed [2].

The Bradford assay involves the binding of Coomassie Brilliant Blue G-250 to cationic, nonpolar, and hydrophobic amino acid residues of proteins, which causes a shift in the absorption maximum of this dye from 365 to 595 nm. The increase in absorption at 595 nm can be measured using UV-Vis spectrophotometer.

The BCA assay is based on the Biuret reaction, the reduction of Cu^{2+} to Cu^{1+} by proteins. Two molecules of BCA bind to one molecule CU^{1+} forming a purple color with a strong absorbance at 562 nm. In both cases, the amount of protein within the solution correlates directly with the observed absorption.

1. Determine the absorption of BSA (0, 10, 20, 30, 40, 60 µg/ml), mixed with Bradford solution or BCA, as protein standard and use it for the calculation of a calibration curve.

Bradford Assay

2. Dilute the Bradford reagent 1:5 with ddH_2O, and mix 200 µl of this solution with 2 µl sample in a 96-well cell culture plate.
3. Measure the absorption at 595 nm.
4. Finally, calculate the protein concentration of the samples with the help of the calibration curve, using the linear regression function in Microsoft Excel.

BCA Assay

1. Mix reagents A and B 50:1 following the manufacturer's protocol (Pierce™ BCA Protein Assay Kit (Thermo Scientific)) and mix 200 µl of this solution with 2 µl sample in a 96-well cell culture plate.
2. Incubate the plate for 30 min at 37 °C and measure the absorbance at 562 nm afterwards.
3. Finally, calculate the protein concentration of the samples with the help of the calibration curve, using the linear regression function in Microsoft Excel.

3.3.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to their molecular weight using a denaturing, discontinuous gel electrophoresis in accord with the Laemmli gel method.

1. Prior to gel loading, mix protein samples or whole-cell extracts 6:1 with 6× Laemmli buffer and heat them for 5–10 min at 95 °C to denaturize the proteins (*see* **Note 15**).
2. Load 5 µl of Page Ruler™ Plus Prestained Protein Ladder into one gel pocket, to enable estimation of protein size.
3. Load and separate at least 10–20 µg of protein per lane by SDS-PAGE in 1× SDS running buffer at constant 130 V (*see* **Note 16**).

3.3.4 Western Blotting

After separation, proteins are electrophoretically transferred to a PVDF membrane using wet transfer. Although semidry transfer is faster, wet blotting is less prone to errors. During blotting, negatively charged proteins travel towards the positively charged electrode and bind to the PVDF membrane surface, providing access to immunodetection reagents.

1. Prior to transfer, activate the membrane by a short incubation in ethanol.
2. Afterwards, equilibrate the membrane and the filter papers in transfer buffer.
3. Sandwich gel and membrane between the filter papers.
4. Place the whole stack between transfer buffer-soaked sponges and clamp them tightly together to avoid air bubbles (*see* **Note 17**).
5. Subsequently, submerge the sandwich in a transfer buffer-filled tank apparatus and apply an electrical field at 150 mA per membrane for 2 h. Cool the blotting apparatus during the run with a thermal pack.
6. After blotting, block unspecific binding sites by incubating the membrane for 1 h in blocking buffer (*see* **Note 18**).
7. Probe the membrane with the primary antibody overnight at 4 °C while constant shaking (*see* **Notes 19** and **20**).
8. On the following day, wash the membrane three times for 10 min in PBS supplemented with 0.05% (v/v) Tween-20 (PBS-T), incubate it for 1 h in a secondary horseradish peroxidase (HRP)-coupled antibody solution, and wash it again three times for 10 min in PBS-T (*see* **Note 21**).
9. For protein-antibody complex detection, treat the membrane with an enhanced chemiluminescence (ECL) solution.
10. By doing so, HRP catalyzes the oxidation of luminol in the presence of hydrogen peroxide leading to light emission. Expose the membranes to X-ray films to detect the specific signals.
11. Exposure time depends on the intensity of the signal (*see* **Note 22**).
12. After exposure, develop the film using an automatic film processor and finally digitalize it (Fig. 3).

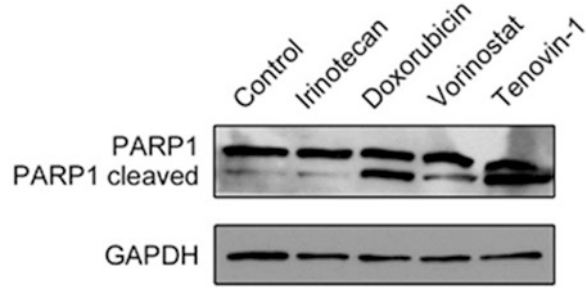

Fig. 3 Effects of different chemotherapeutic compounds and HDACi on PARP-1 cleavage in HCT-116 cells. Cells were exposed to 10 μM irinotecan, 1 μM doxorubicin, 10 μM tenovin-1, and 2 μM vorinostat for 24 h. Control cells were treated with PBS. Protein expression levels of whole-cell lysates were determined by Western blotting using a mixture of anti-PARP-1 cleaved and anti-PARP-1 full-length mouse monoclonal IgG1 antibodies. GAPDH was used to detect equal protein loading

4 Notes

1. It is optional to add EDTA: EDTA is a chelating compound and thereby sequesters metal ions, which are necessary in some physiologically occurring enzymatic reaction. Sequestering metal ions by EDTA provokes a diminished enzymatic activity, including HDAC activity, and thereby makes lysates more stable. It is optional to add SDS to achieve harsher lysis conditions. Further inhibitors can be added to block phosphatases and/or HDACs within the lysates to analyze posttranslational protein modifications afterwards.
2. We recommend using the BCA Protein Assay Kit if you are working with SDS-containing lysis buffers.
3. This unusually high rotation speed is used to ensure that the dead cells are not lost during centrifugation.
4. The appropriate amount of 2-propanol can be higher or lower, depending on the format you seeded your cells in, the density of your culture, and the MTT turnover rate of your cells; maximum absorbance should be < 1; you can dilute the samples with further 2-propanol if the absorbance is higher; keep in mind that you have to adjust the volume of all samples.
5. MTT assay can be performed in suspension cell culture: add 1/10 volume of a 0.5 % (w/v) MTT solution to the cell suspension and incubate them for 2 h at 37 °C; afterwards spin down the cells (5 min at 1000 × *g*), dissolve the pellet in an appropriate amount of 2-propanol with a pipette, and leave them on a shaker for 20 min at RT; repeat the centrifugation step and analyze the supernatant photometrically, by measuring the absorbance of the formazan at 596 nm in a 96-well plate.
6. The absorption and fluorescence of resorufin are pH dependent and decrease strongly at pH values lower than 7.5. Additionally the maximum absorbance is shifted from 570 nm to 485 nm. Similar to the absorbance, the fluorescence is decreased at pH values lower than 7.5 and is nearly diminished at pH values lower than 4, whereas the excitation and emission wavelengths do not change between different pH values. Absorbance and fluorescence are stable between pH 7.5 and 12 [34].
7. If formaldehyde does not work well or is not available, the same volume of ice-cold methanol may be used. Nevertheless, this step is not necessary at all but can help to fix not very well adherent cells.
8. If you want to store the cells you can dry them after the washing step. To ensure that the cells are dry, it is better to dry them overnight.

9. Like for the MTT assay, the appropriate amount of acetic acid can be higher or lower depending on the preconditions you are using; maximum absorbance should be <1. Crystal violet is also solvable using 100 µl 1% SDS per well.
10. The maximal absorption is pH dependent. It varies from 590 nm at pH 7.0 to 420 nm at pH 1.0.
11. It is important to protect cells from light after staining.
12. The fluorogenic substrate Ac-DEVD-AMC is very labile and light sensitive. An opened batch should not be used for more than a few weeks.
13. It is also possible to use 500 µl 100% ice-cold methanol for fixation.
14. Unusual or unexpected bands at unexpected positions can be due to protease degradation caused by an inappropriate lysis buffer or incomplete cooling. In this case it is advisable to use a fresh sample that had been kept on ice and new buffer to ensure protease inhibition [24].
15. Insufficient heating can lead to an incomplete breakage of the quaternary protein structure, which may further lead to too high positions of detected bands. Therefore, reheat the samples to ensure protein denaturation. Too extensive heating can remove posttranslational modifications from your protein of interest. Thus, you should not heat your samples for more than 10 min.
16. Too low concentrations of antigens can cause a weak signal. Therefore it is sometimes necessary to increase the amount of protein loaded to the SDS gel.
17. Patchy and uneven spots on the blot are usually caused by an improper transfer due to air bubbles between the gel and the membrane [24].
18. Maybe fat-free milk powder masks the antigen leading to undetectable signal. Therefore use BSA as blocking agent or decrease the amount of fat-free milk powder.
19. Patchy and uneven spots can also be caused by an unequal distribution during the incubation. To avoid this, use a shaker for all incubation steps.
20. The concentration of the antibody should be appropriate. If the concentration is too low, the signal may not be visible. Therefore you can increase the exposure time. But an excess antibody concentration often causes background.
21. Prolonging the washing steps can help to decrease background. But too long washing may decrease the signal.
22. A too long exposure time can lead to high background or overexposed signals and mask differences between the applied samples.

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Chapter 4

Analysis of HDACi-Induced Changes in Chromosomal Passenger Complex Localization

Britta Unruhe-Knauf and Shirley K. Knauer

Abstract

The chromosomal passenger complex (CPC) is a key regulator of cell division. Its proper localization during the different phases of mitosis and cytokinesis is crucial for the exertion of its various functions. HDACi treatment has been demonstrated to disturb the centromeric localization of the CPC in tumor cells, thus leading to severe mitotic defects often followed by apoptosis. In this chapter, we describe how HDACi-induced changes of the CPC localization can be analyzed by indirect immunofluorescence using CPC-specific primary and fluorophore-coupled secondary antibodies followed by confocal microscopy.

Key words HDACi, CPC, Aurora B, Survivin, Borealin, INCENP, Mitosis, Immunofluorescence

1 Introduction

In addition to genetic alterations, epigenetic aberrations, including changes in DNA methylation, histone modifications, chromatin remodeling, and regulation by noncoding RNAs, have emerged as important contributors to tumor development. Posttranslational modifications (PTMs) of specific residues of core histone tails, such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deamination, and proline isomerization, regulate the accessibility of chromatin for DNA-binding proteins [1].

Histone acetylation is generally associated with enhanced transcriptional activity as the transfer of an acetyl moiety to lysine residues of histone tails neutralizes their positive charge leading to a decreased affinity of acetylated histones to the negatively charged DNA backbone. Thus, chromatin packaging is loosened, phenotypically manifesting in a heterochromatin to euchromatin transition, and proteins of the transcription machinery are able to access promotor regions and to induce gene expression. In contrast, hypoacetylated histones are found in regions of tightly packed chromatin (heterochromatin) where transcription is repressed [2].

Oliver H. Krämer (ed.), *HDAC/HAT Function Assessment and Inhibitor Development*: *Methods and Protocols,* Methods in Molecular Biology, vol. 1510, DOI 10.1007/978-1-4939-6527-4_4,

However, recent studies have revealed that also histone hyperacetylation can lead to the repression of gene transcription [3]. The second function of acetylation and other PTMs occurring at histone tails is the recruitment of nonhistone proteins, which are not only involved in gene transcription, but also in further processes, for example in mitosis [4].

Acetylation of lysine residues in histones as well as in nonhistone proteins is regulated by two counteracting classes of enzymes: histone acetyltransferases (HATs) transferring the acetyl moiety to the ε-amino group of the lysine side chain, and histone deacetylases (HDACs) removing the acetyl group. As imbalances can have severe effects on various physiological processes like gene transcription, proliferation, or differentiation [5, 6], the activity of HATs and HDACs, and thereby the level of substrate acetylation and deacetylation, has to be tightly controlled [7]. Hence, it is not surprising that the expression of HATs and HDACs is deregulated in many tumor types [8, 9]. Overexpression of HDAC2, for example, has been detected in colon and colorectal, gastric, as well as pancreatic cancer and is often correlated with an unfavorable outcome [10]. Due to these findings, HDACs have emerged as attractive targets for anticancer therapy. Eighteen mammalian HDAC enzymes have been identified so far differing in their structure, subcellular localization, and expression patterns. According to their homology to yeast proteins, they are divided into four classes. Class I (HDAC1, 2, 3, and 8), class IIa (HDAC4, 5, 7 and 9), class IIb (HDAC 6 and 10), and class IV (HDAC11) HDACs belong to the classical family of Zn^{2+}-dependent enzymes, whereas class III HDACs, also called sirtuins, require the cofactor NAD^{+} for their activity [2, 11].

Notably, the discovery that the antifungal antibiotic trichostatin A (TSA) induces differentiation of tumor cells through acting as an HDAC inhibitor (HDACi) [12, 13] set the stage for the development of HDACi as chemotherapeutics. Today, a variety of HDACi are available, differing in their chemical properties and HDAC class specificity.

Based on their structure, HDACi are classified into four groups: hydroxamates including the pan-specific HDACi TSA and SAHA targeting class I, II, and IV HDACs; the cyclic peptide FK228 which targets class I HDACs; benzamides, e.g., MS-275 which inhibit HDAC1, 2, and 3; and fatty acids like valproic acid (VPA) and butyrate which are active against class I and IIa HDACs [2, 11].

HDACi induce various cellular responses including cell cycle arrest, differentiation, apoptosis, or autophagy. Apart from the cell cycle block, these effects have proven to be mostly restricted to tumor cells, while normal cells remain relatively unaffected [2, 7]. Low HDACi concentrations induce G1 arrest, which is mainly attributed to the transcriptional activation of genes coding for

cyclin-dependent kinase (CDK) inhibitors like *CDKN1A*. The protein product of *CDKN1A*, p21, binds to and inhibits the cyclin/CDK complexes cyclin E/CDK2 and cyclin A/CDK2, the major drivers of G1/S progression, thus inducing cell cycle arrest and differentiation [14].

In addition, HDACi treatment can also cause a halt of the cell cycle in G2 phase when applied in high concentrations. However, this arrest only occurs when high doses of HDACi are applied during S phase and mostly affects normal cells while many tumor cell lines remain unaffected [7, 15]. Even though the underlying mechanisms are not completely understood, recent data hint towards an involvement of different regulatory factors. This includes upregulation of p21, reduction of cyclin A and B1 protein levels, as well as downregulation of the mitotic kinase polo-like kinase 1 (PLK1) [7]. In contrast, tumor cells fail to pause their cell cycle and instead undergo aberrant mitosis and cytokinesis, which is often followed by apoptosis [15, 16]. The HDACi-induced phenotype is characterized by the formation of multipolar spindles as well as erroneous chromosome alignment and segregation [17–19]. However, these cells do not arrest in prometaphase for a prolonged period of time, but after a relatively short halt progress to cytokinesis and separate their chromosomes unequally to multiple daughter cells. This indicates that HDACi treatment does not only overcome G2 arrest, but also disrupt the spindle assembly checkpoint (SAC) in tumor cells, leading to a premature exit from mitosis [17]. As one of the most important cellular signaling networks, the SAC ensures proper chromosome segregation to daughter cells by sensing the attachment of spindle microtubules to the kinetochores of mitotic chromosomes. It delays sister chromatid separation and progression of mitosis until all kinetochores obtain bipolar spindle attachments. This is achieved by blocking the activation of the anaphase-promoting complex (APC/C), an E3 ubiquitin ligase that tags specific mitotic proteins like cyclin B for proteasomal degradation, thereby inducing anaphase onset [20]. The SAC is regulated by the kinase aurora B, which is a member of the chromosomal passenger complex (CPC) (Fig. 1).

The CPC is a key regulator of cell division, exerting crucial functions during mitosis and cytokinesis. It consists of the four proteins aurora B kinase, the inner centromere protein (INCENP), borealin, and survivin. While aurora B, together with the C-terminus of INCENP, provides the enzymatic activity of the complex, the localization module comprising INCENP's N-terminus, borealin, and survivin ensures its dynamic localization [21–23] (Fig. 2). In prophase, the CPC can be detected at chromosome arms and centromeres and is assumed to be involved in the regulation of chromosome condensation [4, 24, 25]. During prometaphase and metaphase, the CPC further accumulates at centromeres, where it activates the SAC and corrects erroneous

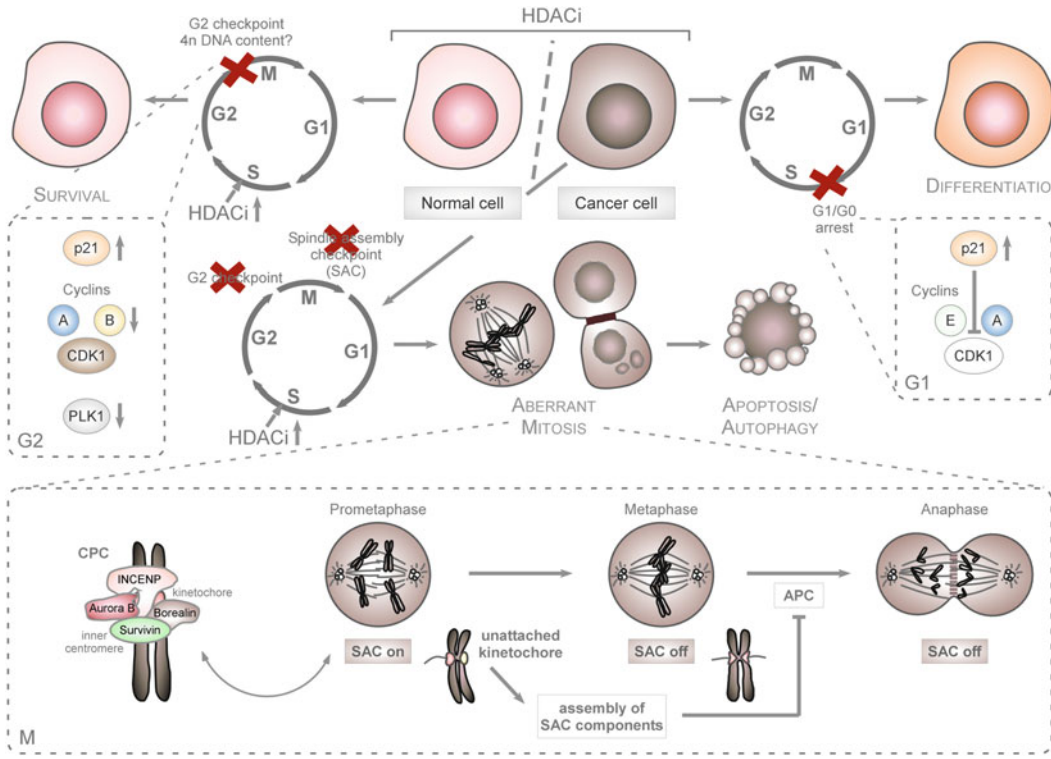

Fig. 1 Effects of HDACi treatment on the cell cycle of normal and tumor cells. When challenged with HDACi, normal cells undergo G2 arrest and survive. This involves upregulation of the CDK inhibitor p21 as well as downregulation of cyclins A and B1 and PLK1. Tumor cells, however, are affected by HDACi in two ways: on the one hand, they are susceptible to cell cycle arrest in G1 phase when treated with low inhibitor concentrations. This is caused by the upregulation of CDK inhibitors, targeting cyclin/CDK complexes that drive G1/S progression. Arrest of cells in G1 inhibits apoptosis and instead leads to differentiation, thereby interfering with cancer cell proliferation. On the other hand, treatment with high inhibitor concentrations induces tumor cell death during mitosis. In contrast to normal cells, malignant cells fail to arrest in G2 phase and instead undergo aberrant mitosis and cytokinesis, which is often followed by apoptosis *(upperv part)*. The ability to proceed through mitosis in the presence of malformed spindles and erroneous kinetochore-spindle attachments implies that tumor cells overcome the SAC, which delays anaphase onset until all kinetochores obtain bipolar spindle attachments. This is regulated by the CPC, which recruits SAC components to kinetochores and corrects defective kinetochore-microtubule attachments. HDACi treatment impairs proper CPC function by disrupting its localization at centromeres *(lower part)*. *HDACi* histone deacetylase inhibitors, *CDK1* cyclin-dependent kinase 1, *PLK1* polo-like kinase 1, *SAC* spindle assembly checkpoint, *CPC* chromosomal passenger complex, *APC* anaphase-promoting complex

kinetochore-microtubule attachments [4, 26–28]. In anaphase, the CPC relocalizes from centromeres to the spindle midzone, where it facilitates the formation of the central spindle. A population of the complex moves to the equatorial cortex, the region of the cell membrane where the cytokinetic machinery is assembled [4, 29]. In telophase and cytokinesis, an accumulation of the CPC at the midbody, a remnant of the central spindle, is observed. Here,

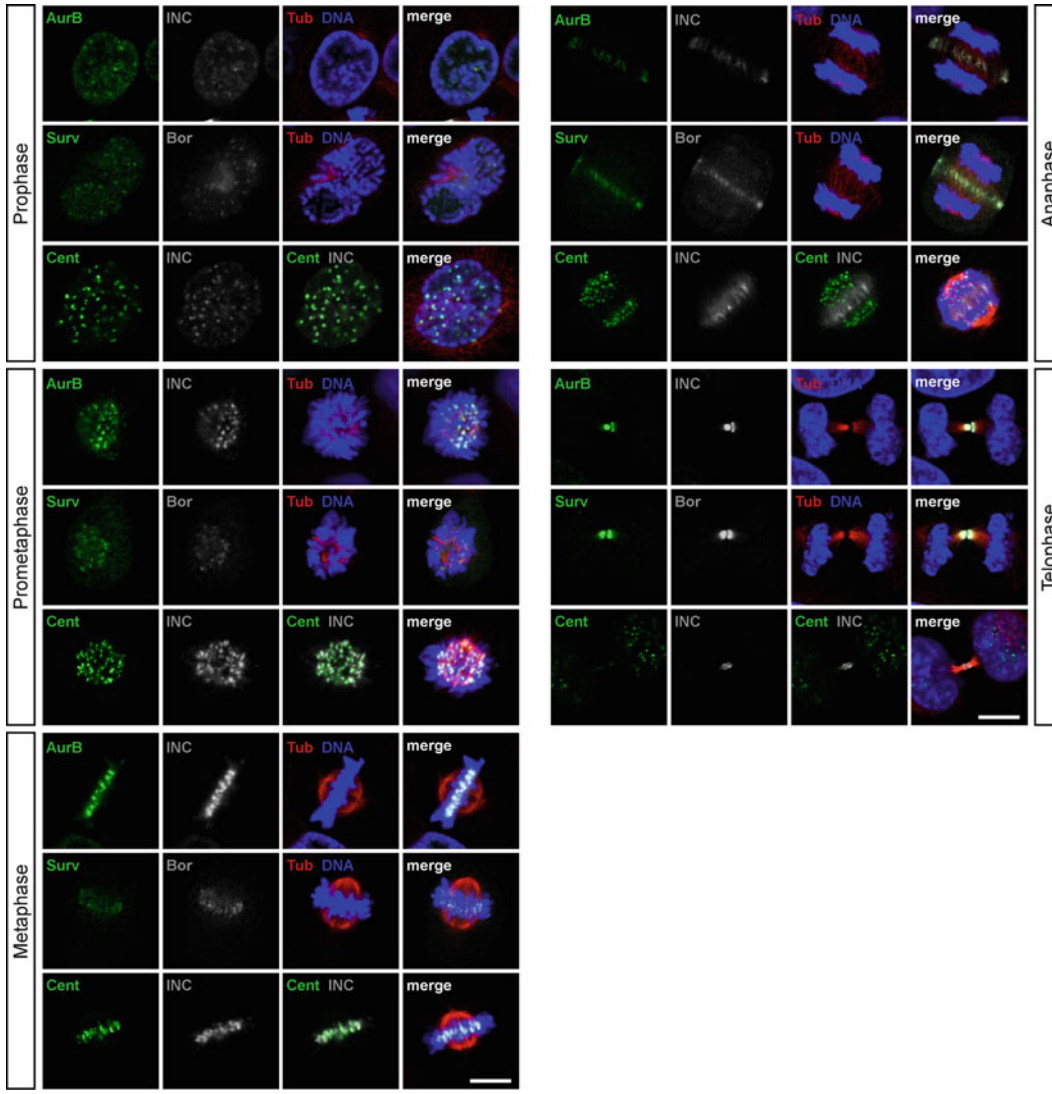

Fig. 2 Characteristic localization of the CPC in mitosis detected by indirect immunofluorescence staining. Mitotic HeLa Kyoto cells were fixed, permeabilized, and immunostained. Primary antibodies were specific for aurora B (AurB), INCENP (INC), survivin (Surv), borealin (Bor), alpha-tubulin (Tub), and centromere protein (Cent), and subsequently detected with fluorophore-conjugated secondary antibodies. DNA was stained with Hoechst 33342. Scale bar, 10 μm

it contributes to the maturation and constriction of the contractile ring that drives the abscission of the daughter cells [4, 29, 30].

The HDACi-induced mitotic defects as well as the disruption of the SAC are at least partly attributed to a reduction of the centromeric localization of the CPC, which is also observed after HDACi treatment [17, 19, 31] (Fig. 3). Centromeric targeting of the CPC in prophase is mediated via an interaction of survivin's baculovirus IAP repeat (BIR) domain with histone 3 phosphorylated at

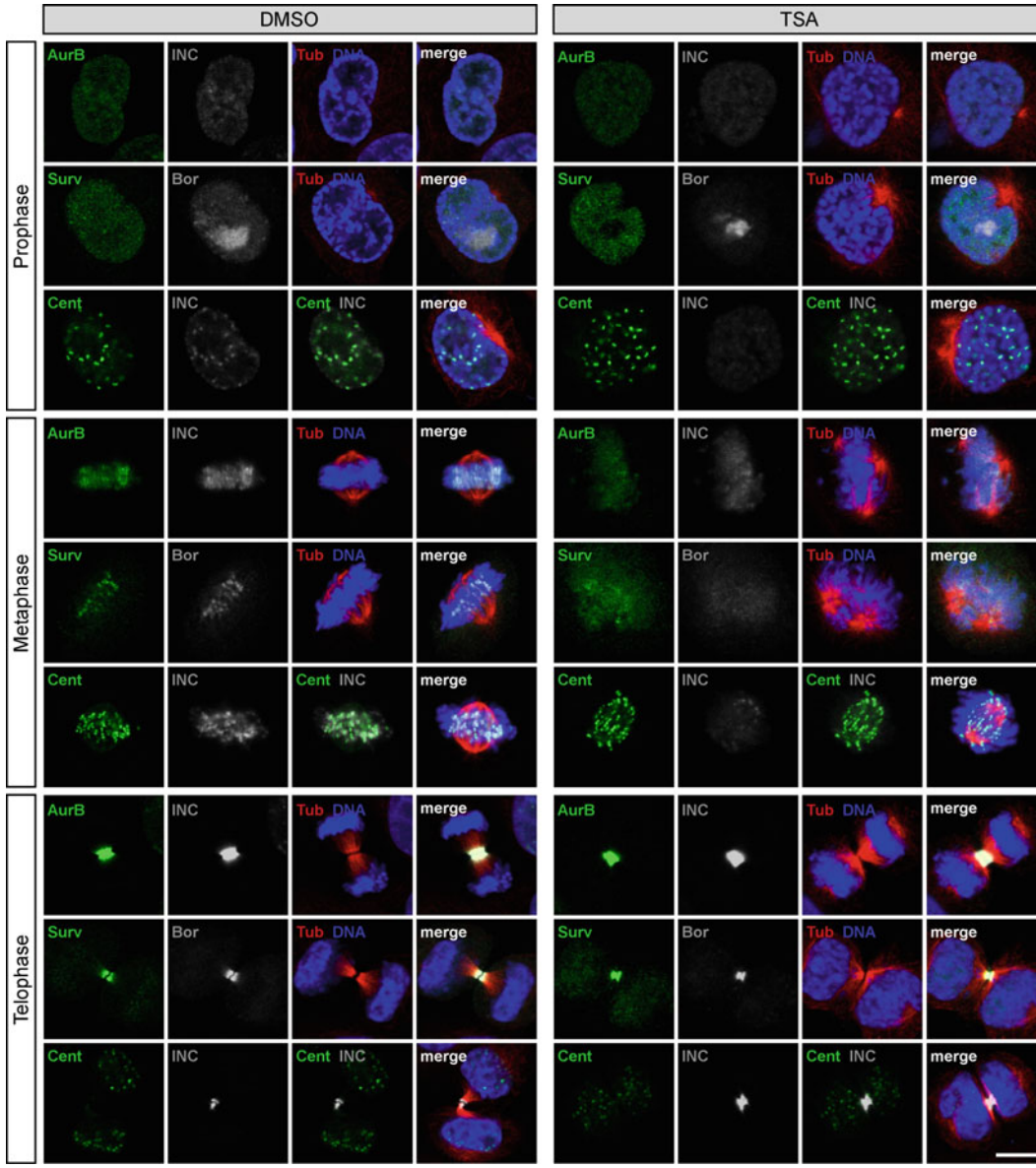

Fig. 3 Localization of the CPC in mitosis after HDACi treatment detected by indirect immunofluorescence staining. HeLa Kyoto cells arrested in S phase were treated with 0.02 % DMSO as control or 1 μM TSA. Cells were fixed in different phases of mitosis, permeabilized, and immunostained. Primary antibodies were specific for aurora B (AurB), INCENP (INC), survivin (Surv), borealin (Bor), alpha-tubulin (Tub), or centromere protein (Cent), and subsequently detected with fluorophore-conjugated secondary antibodies. DNA was stained with Hoechst 33342. Scale bar, 10 μm

threonine 3 (H3T3ph) by haspin kinase [32]. HDACi might disrupt this interaction by an increase in H3K9 acetylation (H3K9ac) detected in response to drug treatment, finally leading to the recruitment of the H3K4 methyltransferase MLL. This in turn elevates the levels of trimethylated H3K4 (H3K4me3) which

Fig. 4 Experimental procedure for analyzing HDACi-induced changes of CPC localization. Tumor cells are arrested in G1/S phase using hydroxyurea. The block is released, and cells are immediately treated with HDACi to induce aberrant mitosis. Mitotic cells are fixed, and CPC proteins are detected by indirect immunofluorescence staining utilizing specific antibodies and subsequent confocal microscopy

inhibits the binding of survivin to the adjacent H3T3ph, thus preventing the CPC from localizing to centromeres [7]. However, accumulation of the CPC at the midbody in telophase is not affected by HDACi [17].

Mitotic defects induced by HDACi treatment of tumor cells, such as spindle malformation or misaligned chromosomes, can be easily analyzed by indirect immunofluorescence using commercially available antibodies. For this purpose, tumor cells are first treated with, for example, hydroxyurea in order to arrest their cell cycle in G1/S phase [33, 34]. Subsequently, the cell cycle blockade is released and the synchronized cells are immediately treated with HDACi until they enter mitosis. Cells are fixed, and the proteins of interest are detected using specific primary as well as fluorophore-conjugated secondary antibodies, which allows to investigate the protein localization using a fluorescence microscope (Fig. 4). This chapter provides a simple and commonly used protocol for analyzing the effects of HDACi treatment on cell division of tumor cells applying immunofluorescence staining followed by confocal microscopy. It exemplarily describes this method for the detection of the four CPC members, aurora B, INCENP, survivin, and borealin.

2 Materials

Materials listed here are routinely used in our and other labs. However, they should be interchangeable by products from other providers.

2.1 Seeding of Cells

1. Dulbecco's phosphate-buffered saline (DPBS).
2. TrypLE Express Enzyme (Life Technologies) or trypsin.

3. Cell culture medium (CCM): Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 1× antibiotic-antimycotic (Life Technologies).
4. Human tumor cell line, e.g., HeLa Kyoto.
5. Neubauer chamber.
6. Microscopy dishes (35 mm cell culture dishes with 10 mm glass bottom).

2.2 Cell Cycle Arrest in G1/S Phase

1. 0.5 M Hydroxyurea (HU) in water (*see* **Note 1**).
2. CCM.

2.3 HDACi Treatment

1. CCM.
2. Dimethyl sulfoxide (DMSO).
3. Trichostatin A (TSA) stock solution: 5 mM TSA in DMSO, stored at –20 °C.
4. DPBS.

2.4 Fixation of Cells

1. 4% Formaldehyde in DPBS.
2. DPBS.

2.5 Immunostaining

1. DPBS.
2. Blocking buffer: 5% (v/v) normal goat serum (*see* **Note 2**), 0.3% (v/v) Triton X-100 in DPBS.
3. Antibody dilution buffer (ADB): 1% (w/v) bovine serum albumin, 0.3% (v/v) Triton X-100 in DPBS.
4. Primary antibodies: Alpha-tubulin (AbD Serotec; MCA77G [rat]), aurora B (Sigma; A5102 [rabbit]), INCENP (Life Technologies; 39-2800 [mouse]), survivin (Novus Biologicals; NB500-201 [rabbit]), borealin (MBL; M147-3 [mouse]), centromere protein/antiserum from CREST patients (Antibodies Incorporated; 15-234 [human]).
5. Fluorophore-conjugated secondary antibodies: Anti-rabbit-AlexaFluor (AF) 488 (Life Technologies; A-11008 [goat]), anti-human-AF488 (Life Technologies; A-11013 [goat]), anti-rat-AF568 (Life Technologies; A-11077 [goat]), anti-mouse-AF633 (Life Technologies; A-21052 [goat]).
6. DNA staining dye: 0.5 mg/mL Hoechst 33342 in water.
7. 0.1% (w/v) sodium azide in DPBS.
8. Humidity chamber.

2.6 Imaging

1. Confocal microscope featuring a 63× water objective and 405/488/568/633 nm laser lines, e.g., Leica SP5 equipped with a HCX PL Apo CS 63.0×1.20 water UV objective.
2. Image-processing software.

3 Methods

3.1 Seeding of Cells

1. HeLa Kyoto cells are trypsinized and counted using a Neubauer chamber.
2. 1.2×10^5 cells are seeded in CCM per microscopy dish and cultured for approx. 20 h. Three microscopy dishes are required for every treatment condition.

3.2 Cell Cycle Arrest in G1/S Phase

An accumulation of cells in G1/S phase is necessary as HDACi-induced mitotic defects only occur when HDACi are applied during S phase [7, 17]. A reversible arrest can be obtained by chemical agents interfering with different steps of DNA replication, for example HU, which inhibits ribonucleotide reductase [34].

1. 2 mL of a 2 mM dilution of HU in CCM is prepared per microscopy dish and added to the cells.
2. Cells are incubated in HU-containing medium for 24 h (see **Note 3**).

3.3 HDACi Treatment

TSA is a pan-specific HDAC inhibitor of the hydroxamate group targeting class I, II, and IV HDACs [2].

1. 6 mL of the following dilutions are prepared per treatment condition:
   (a) 0.02% (v/v) DMSO in CCM.
   (b) 1 μM TSA in CCM (*see* **Note 4**).
2. The HU-containing medium is aspirated and cells are rinsed three times with 2 mL DPBS (*see* **Note 5**).
3. The HDACi-containing medium is added and cells are monitored for mitosis onset microscopically (see Note 6).

3.4 Fixation of Cells

Mitotic cells are only loosely attached to the bottom of the cell culture dish. Thus, pipetting and aspirating have to be carried out very carefully. Prevent direct pipetting to or aspirating from the center of the glass insert of the dish.

1. The HDACi-containing medium is aspirated and cells are fixed with 1 mL 4% formaldehyde for 15 min at room temperature (RT) (*see* **Note 7**).
2. The fixative is aspirated and cells are rinsed three times with 1 mL DPBS for 5 min each.

3.5 Immunostaining

Indirect immunofluorescence is a two-step technique. It utilizes an unlabeled primary antibody which binds to a specific antigen, and subsequently, a fluorophore-labeled secondary antibody directed against the Fc region of the primary antibody. This technique is more time consuming than direct immunofluorescence, but it is more sensitive as more than one secondary antibody can bind to

each primary antibody, leading to an amplification of the fluorescence signal [35].

1. Samples are blocked in 1 mL blocking buffer for 1 h at RT.
2. During blocking, 200 µL of the following primary antibody dilutions are prepared in ADB. Each dilution is used for the pairwise staining of one DMSO- and TSA-treated sample, respectively (*see* **Note 8**):
   (a) Alpha-tubulin (1:1000 dilution); aurora B (1:2000 dilution; *see* **Note 9**); INCENP (1:150 dilution).
   (b) Alpha-tubulin (1:1000 dilution); survivin (1:300 dilution); borealin (1:200 dilution).
   (c) Alpha-tubulin (1:1000 dilution); INCENP (1:150 dilution); centromere protein (1:400 dilution).
3. Blocking buffer is aspirated and 80 µL of the corresponding antibody dilution is pipetted to the edge of the glass insert of each dish.
4. Samples are incubated in a humidity chamber at 4 °C overnight.
5. Samples are rinsed three times with 1 mL DPBS for 5 min each.
6. During washing, the following secondary antibody dilutions are prepared in ADB (*see* **Note 10**):
   (a) Anti-rat-AF568 (1:1000 dilution); anti-rabbit-AF488 (1:1000 dilution); anti-mouse-AF633 (1:1000 dilution); 0.5 µg/mL Hoechst 33342 in a total volume of 400 µL (for staining the samples previously incubated with primary antibody dilutions a and b).
   (b) Anti-rat-AF568 (1:1000 dilution); anti-human-AF488 (1:1000 dilution); anti-mouse-AF633 (1:1000 dilution); 0.5 µg/mL Hoechst 33342 in a total volume of 200 µL (for staining the samples previously incubated with primary antibody dilution c).
7. 80 µL of the corresponding antibody dilution is pipetted to the edge of the glass insert of each dish. From now on, all incubation and washing steps have to be carried out protected from light in order to prevent bleaching of fluorophores.
8. Samples are incubated for 1 h at RT.
9. Samples are rinsed three times with 1 mL DPBS for 5 min each. DPBS is aspirated and 2 mL 0.1 % (w/v) sodium azide is added (*see* **Note 11**).

3.6 Imaging

1. Samples are imaged with a confocal microscope equipped with a 63× water objective and a sensitive hybrid detector. Fluorophores are excited with 405/488/568/633 nm laser lines performing a sequential scan beginning with the longest wavelength. Images are recorded with a resolution of 1024 × 1024 pixels at a scan speed of 200 Hz.
2. Image processing is performed using appropriate software.

4 Notes

1. HU is soluble in water to at least 50 mg/mL (0.657 M); thus, the preparation of a 0.5 M solution is useful. As HU is unstable in water, aqueous HU solutions should be prepared freshly. However, an excess of the 0.5 M solution can be aliquoted and stored at −20 °C for up to 1 month.
2. Normal serum is a suitable blocking reagent as it contains antibodies that bind to reactive sites and prevent the nonspecific binding of secondary antibodies. It is important, however, to use serum from the host species the secondary antibody was generated in. Serum from the primary antibody species would also bind to reactive sites, but the secondary antibody would recognize those nonspecifically bound antibodies along with the primary antibody bound to the target antigen, leading to a high background.
3. In order to achieve the maximum number of cells arrested in G1/S phase, it is recommended to incubate cells with HU approx. for the duration of one cell cycle (approx. 24 h in Hela Kyoto). However, as other cell lines might be more sensitive to the treatment, the optimal incubation time has to be tested to avoid unwanted toxicity.
4. HDACi-induced mitotic defects only occur, when cells arrested in S phase are treated with high inhibitor concentrations. As the IC_{50} of TSA lies in the low nanomolar range [36, 37], a concentration of 1 μM is sufficient.
5. The synchronized cells have to be thoroughly rinsed with PBS to remove the remaining HU. Less washing steps will result in an incomplete release from G1/S block and a reduced number of mitotic cells.
6. The duration of the G2 phase differs depending on the cell line used. In HeLa Kyoto cells, the first cells enter mitosis after approx. 9–10 h. Mitotic cells can be identified by their spherical shape as they partly detach from the bottom of the cell culture dish.
7. As formaldehyde is toxic, perform fixation in a fume hood and collect waste separately.
8. When using any primary or secondary antibody for the first time, titrate the antibody to determine with which dilution the strongest specific signal and the weakest background can be obtained.
9. Prepare 1:10 predilutions of the alpha-tubulin and aurora B antibodies in order to prevent pipetting of very small volumes.
10. Before applying all secondary antibodies together in one step, make sure that none of the antibodies cross-reacts with additional species.

11. Sodium azide (toxic) is added to the samples in order to prevent the growth of microbial organisms. Samples can be stored in a lighttight humidity chamber for approx. 1 week at 4 °C.

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Chapter 5

Analysis of Histone Deacetylase-Dependent Effects on Cell Migration Using the Stripe Assay

Sonja Mertsch and Solon Thanos

Abstract

For normal embryonic development/morphogenesis, cell migration and homing are well-orchestrated and important events requiring specific cellular mechanisms. In diseases such as cancer deregulated cell migration represents a major problem. Therefore, numerous efforts are under way to understand the molecular mechanisms of tumor cell migration and to generate more efficient tumor therapies. Cell migration assays are one of the most commonly used functional assays. The wound-healing assay or the Boyden chamber assay are variations of these assays. Nearly all of them are two-dimensional assays and the cells can only migrate on one substrate at a time. This is in contrast to the in vivo situation where the cells are faced simultaneously with different surfaces and interact with different cell types. To approach this in vivo situation we used a modified version of the stripe assay designed by Bonhoeffer and colleagues to examine mechanisms of axonal guidance. The design of this assay allows cells to decide between two different substrates offered at the same time. Utilizing alternating neuronal substrates for migration analyses we can partially mimic the complex in vivo situation for brain tumor cells. Here we describe the detailed protocol to perform a modified version of the stripe assay in order to observe substrate-dependent migration effects in vitro, to analyze the effect of Rho-dependent kinases (ROCKS), of histone deacetylases (HDACs) and of other molecules on glioma cells.

Key words Stripe assay, Glioblastoma, HDAC, Substrate-dependent cell migration, Migration preference

1 Introduction

Cell migration is a complex and dynamic process, which is orchestrated by a large number of different chemical messengers and signaling pathways. Directed cell migration is important for normal development and tissue functions. However, dysregulation of cell migration can increase the pathological metastasis of cancer. Therefore, analyzing cell migration is an important tool in studying cancer progression as cancer cell migration, invasion, and metastasis represent one of the major problems in the treatment of cancer. In the last decades a various number of different in vitro migration assays have been established [1]. The new generation of

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migration assays often uses 3D systems such as brain slices to mimic the in vivo situation, but these assays are complicated to handle and measurement of cell migration/behavior is physically limited [2, 3]. Therefore, analyzing cell migration is mainly based on in vitro single-substrate migration assays because of the simple method and of the possibility of live cell imaging during the experiments. The main approaches of migration assays used in the literature are two-dimensional migration assays where cells are removed from the substrate by using chemical, thermal, electrical, or mechanical means or cells are excluded by mechanical barriers. Both types of assays lack the possibility to confront the cells with different migration substrates at the same time.

To circumvent the limitations of these single-substrate assays, we adapted the so-called stripe assay as a migration assay for the use of cell lines [4, 5]. The original assay was developed to scrutinize navigation of axonal growth cones, and since then several variants of this assay have been developed for several different applications [6]. The first advantage over one substrate assays is the possibility to test the influence of different compounds on the substrate-dependent cell migration. As the cells are confronted simultaneously with two different substrates at the physical border of two substrates of choice, a binary decision is enforced, seen as preferential migration on either substrate. The second advantage of our stripe assay over slice assays or 3D assays is the high-resolution monitoring of cell behavior using video time-lapse microscopy. Substrate preferences as well as repulsive effects are visualized and directed migration can be identified. The third advantage is that pharmacological and/or genetic interventions in the migration behavior of the cells can be evaluated. In recent studies we showed the practicability of the stripe assay to examine the importance of the different migratory substrates encountered by glioblastoma cells [7, 8]. As an example, using a ROCK inhibitor in commonly used migration assays, we prove a significant impact on cell migration [9]. Expanding the studies with the stripe assay we could show that the influence of Rho kinases is not limited to an inhibition of cell migration but it leads to a switch of substrate preference in the cells. Untreated glioblastoma cells show a clear preference for extracellular matrix (ECM, biomatrix) compared to myelin or other migration substrates [7]. Inhibition of Rho kinase leads to a significant switch of this preference during migration and not to an inhibition in migration. On this ground, concerning the development of new therapeutic agents, not only cell migration but also substrate-dependent cell migration should be considered. Although the importance of the cell-substrate adhesion in cell migration is well known [10, 11], the preferential migration on different substrates seems of crucial importance for it points to molecular mechanisms based on the balance of Rho kinases or other mediators of these preferences.

Besides the complex events within the cytoskeleton and the cellular protrusions, additional events within the cell nuclei may crucially influence cell migration. HDACs are known to be involved in chromatin remodeling. These enzymes regulate DNA expression through protein acetylation and deacetylation [12]. Furthermore they play an important role in cancer development and progression [13–15]. HDACs influence some of the main characteristics of different malignant tumor cells, like uncontrolled proliferation, invasion, and resistance to apoptosis [16, 17]. Currently, different clinical trials using HDAC inhibitors are being pursued in different cancer types [18, 19]. HDAC inhibitors also play a role in cell migration as shown in colon carcinoma [20, 21].

The prognosis of patients with glioblastoma still remains poor, mainly because of the rapid invasion of single tumor cells into the surrounding tissue and the resistance of these cells to conventional therapeutic agents [22, 23]. Therefore our aim is to analyze the impact of different HDAC inhibitors on substrate-dependent cell migration using the stripe assay. Here we describe the stripe assay as done recently [7, 8] and suggest the addition of HDAC inhibitors.

2 Materials

2.1 Stripe Assay Equipment

1. Vacuum pump with manometer.
2. Suction plate (glass frit).
3. Silicon tubes.
4. Plastic clips.
5. Sidearm flask (Büchner flask).
6. Bored rubber bung.
7. Check valve.
8. 15 ml Centrifuge tube.
9. Silicon matrices (distributor: Dr. M. Bastmeyer, Karlsruher Institut für Technologie (KIT), Institut für Zoologie I, University Karlsruhe, Germany): Matrix 1 with a channel system (white) and matrix 2 with a mesh-like structure (blue).
10. Polycarbonate Nucleopore Track-Etches membranes 0.1 µm (Whatman) cut into rectangles of approximate 1.5 × 1.2 cm, facing the shiny side upwards, the upper left corner should be cut out as a marking point.
11. Small plastic tub.

2.2 Additional Equipment

1. 2 × 1000 µl Pipettes (one for washing and one for the stripe solutions) and 100 µl pipette.
2. 60 mm Bacterial dishes.
3. Centrifuge (for 2 and 15 ml tubes).

4. Spectrophotometer.
5. Cuvette.
6. 2 ml Reaction tube.
7. 15 ml centrifuge tubes.
8. Scalpels (No. 11).
9. Vortexer.
10. Heating plate with stirrer.
11. Forceps (No. 5, A. Dumont).
12. Curved forceps (No. 7, A. Dumont).

2.3 Stripe Material

1. Matrigel (e.g., BD Matrigel™ Basement Membrane Matrix growth factor reduced).
2. Chicken embryos (*Gallus gallus domesticus*) white leghorn strain E6–E10.
3. Postnatal rats (*Rattus norvegicus*) Sprague-Dawley strain postnatal day 10.

All animal experiments must be permitted by the national guidelines for animal testing.

2.4 Reagents

1. Homogenization buffer (HB):
   10 mM Tris–HCl, pH 7.4, 1 mM spermidine × 3HCl, 1.5 mM $CaCl_2$.
   Dissolve 1.2114 g Tris in 800 ml dH_2O, mix, and adjust pH to 7.4 with the appropriate volume of concentrated HCl. Add 0.25463 g spermidine × 3HCl to the solution and mix gently. Finally add 0.16647 g $CaCl_2$ to the solution, mix, and fill up to 1 L with dH_2O. Aliquot the solution and store at −20 °C.
2. Sucrose gradient:
   For 50% (w/w) sucrose solution add 50 g of sucrose in 50 g HB, sterilized by filtration (0.4 μm filter), and store at −20 °C. For 5% (v/v) sucrose solution dilute 50% sucrose solution 1:10 in HB.
3. 2% (w/v) SDS solution in H_2O_{dest}.
4. Protease Inhibitor Cocktail (e.g., cOmplete™, Mini, EDTA-free).
5. Sterile PBS+ (PBS without calcium chloride and magnesium chloride with protease inhibitors).
6. Cell culture media (for glioma cells, DMEM with 4.5 g glucose/L plus 10% FCS and 1% pen-strep).
7. Trypsin-EDTA solution.
8. Fluorescent microspheres 0.5 μm in two different colors (e.g., Invitrogen, Molecular Probes F8813 (yellow) and F8812 (red)).
9. Sucrose.
10. Betaisodona solution.

2.5 Cell Lines

1. Human glioma cell lines (U87MG, U343MG, 86HG39, U373MG, D54MG, H4, T98G, and A172): All used cell lines were cultured using standard cell culture conditions in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO_2.

3 Methods

All experiments should be performed under a laminar flow hood at room temperature (unless otherwise specified).

3.1 Preparation of Stripe Assay Material

Different materials are used to mimic the CNS environment. For non-myelinated neuronal membrane fraction (to represent grey matter), we used rat retina (RR), CNS myelin was prepared from the myelinated brain areas of rats (white matter, pons) (RM), and chick embryo retina (CR) was used to mimic white matter.

3.1.1 Removal of Brain and Eyes from the Animals

1. For rat tissue: Decapitate the rats using surgical scissors and enucleate eyes. To remove the brain, use a scalpel (No. 11) to cut along the sagittal suture and then cut temporally along the left and right coronal suture. Remove the parietal bones carefully with forceps. The exposed brain can now be lifted out of the cranial cavity after removing the spinal cord. Eyes and brains should be stored separately in ice-cold phosphate-buffered saline (PBS) until further processing.
2. For chicken tissue: Before opening the eggs, they should be sprayed with 70% (v/v) ethanol (EtOH) for anesthesia of the embryos. Open the eggs using a scalpel in a petri dish (10 cm diameter) and decapitate the embryo immediately using scissors. Remove the eyeballs using curved forceps (e.g., No. 7 from A. Dumont) and store in ice-cold PBS until required.

3.1.2 Preparation of Retina

1. Rinse eyeballs in ice-cold PBS containing penicillin (100 U/ml) and streptomycin (100 µg/ml) followed by a 5-min incubation in iodine solution and an additional washing step with PBS.
2. Perform the following steps in a half-filled petri dish with ice-cold PBS under a dissecting microscope under sterile conditions.
3. Remove the anterior segment (including cornea and lens) of the eyeball by circumferential incision immediately below the cornea. To remove the vitreous body, use two pairs of forceps to grab repeatedly in the optic cup until you are able to carefully pull out the complete vitreous body (Fig. 1).
4. Make four incisions into the eyeball to flatten out the cup (Fig. 2).

Fig. 1 Graphical depiction of the removal of the vitreous body using two forceps

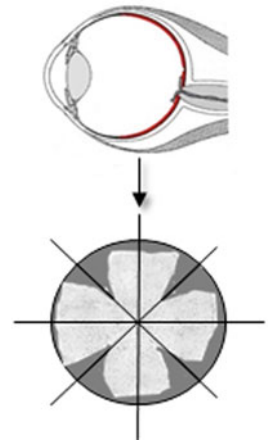

Fig. 2 Flatten eye cup for removal of the retina

5. Use the tips of a closed pair of forceps to carefully peel the retina away from the pigment epithelium and cut off the optic nerve head to sever the retina from the optic nerve.
6. In the chick embryo eye, the optical fissure (the exit of the optic nerve in avian eye) also needs to be excised before the retina can be completely removed from the pigment epithelium and the eye cup.
7. Collect the retina into 600 µl precooled homogenization buffer containing the protease inhibitor cocktail (HB+).
8. Homogenize the tissue carefully first using a 1000 µl pipette, followed by a 100 µl pipette, and lastly with a 1 ml syringe and 27G needle. Try to avoid air bubbles and store the tissue on ice between each step.

3.1.3 Preparation of Myelin from Different Brain Areas

1. Remove the brains after euthanasia.
2. Gently prise apart the two hemispheres of the rat brain ensuring that the corpus callosum is not disrupted. This allows easy access to the corpus callosum.

3. Dissect the myelin-containing regions such as the fornix/corpus callosum and parts of the white matter. Also dissect parts of the cortex but ensure that meninges are removed from the cortex first.
4. Collect the tissue fragments together in 600 µl ice-cold HB+.
5. Homogenize the tissue as described above.

3.1.4 Gradient Centrifugation

In order to observe the surface properties of the different tissues, cells are separated into their components, and only cell membranes and associated surface proteins are used for generating the different stripes. To separate cell components, a sucrose gradient centrifugation is used.

1. Pipette 350 µl of 50% sucrose solution into a 15 ml Falcon tube.
2. Add 150 µl of 5% sucrose solution on top of the first layer; let the solution slowly run down the side of the tube.
3. Add no more than 700 µl of the homogenized tissue in the same manner on top of the sucrose layers.
4. Centrifuge at 52,000 × *g* (or maximum of your centrifuge) for 10 min at 4 °C.
5. In the meantime, prepare 15 ml tubes with 1 ml ice-cold PBS+.
6. After centrifugation, carefully remove the tubes from the centrifuge. A white layer of crude membrane extract should now be present between the two layers of sucrose.
7. Transfer this layer carefully into the prepared tubes containing 1 ml PBS+; be sure to transfer as much material as possible while avoiding sucrose layers.
8. Mix gently with a pipette.
9. Centrifuge again at 13,000 × *g* for 7 min at 4 °C.
10. Discard the supernatant and resuspend the pellet in 500–1000 µl (depending on the size of the pellet) ice-cold PBS+. Use a 1 ml syringe with a 27G needle if the pellet does not dissolve.
11. This material can be stored at –80 °C after freezing with liquid nitrogen or directly in liquid nitrogen for several months. To avoid freeze/thaw cycles, aliquot the material. The concentration of the material should be measured immediately before use (the concentrations may be affected by freezing and the duration of storage). Since the membrane fractions lose some activity upon long-term storage, it is recommended to use fresh material whenever possible.

3.1.5 Measurement of Stripe Material

1. All stripes should be prepared at a density of 70 µg/ml protein.
2. For measurement use 1:50 dilution of the probes (and of PBS+ as reference) in 2% (w/v) SDS (add 20 µl of each sample to 980 µl of 2% (w/v) SDS solution). Mix well and prepare at least duplicates for each sample.

3. Measure OD at 220 nm in a disposable UV/Vis cuvette.
4. Walter et al. showed that a 1:15 dilution of the material results in an OD_{220nm} of 0.1 at a substrate concentration of 70 µg/ml [4]. In order to save material, use a 1:50 dilution. To convert results to the 1:15 dilution, use a factor of 3.33 to get a final OD_{220nm} of 0.1.
5. Calculation formula: $0.1 \times \text{volume (total)}/OD_{220} \times 3.33 = \text{volume (substrate)} + \text{PBS+ (filled to total volume)}$ [6].
6. Total volumes required per membrane are 150 µl for the first stripe and 300 µl for the second stripe.
7. Dilute the different fractions appropriately with ice-cold PBS+ and store on ice.
8. Label the different stripe solutions using fluorescence beads in two different colors. Do not just label one stripe as the labeling can lead to chemoattractive effects in some cell lines.
9. Use a 1:50 dilution in PBS+ of fluorescence beads as a pre-dilution. For 150 µl of membrane solution, add 7.5 µl of the pre-diluted beads (0.05 µl beads per µl membrane solution). Keep the labeled membrane solution in the dark (use aluminum foil to cover the tube) on ice until use (*see* **Notes 1** and **2**).

3.2 Preparation of Membranes

1. Before starting, cut the membranes into rectangles of approximately 1.5×1.2 cm. To protect the membrane, lay the intermediate (blue) sheet on top of the membrane and cut both sheet and membrane. To mark the shiny side (which should be facing up during the preparation of the stripes), cut the upper left corner as a marking point.
2. Do not touch the membrane surface with your fingers. Boil the membranes in sterile H_2O for approximately 5 min to sterilize, and let them cool down slowly in the H_2O. Store the membranes in the water until use.

3.3 Preparation of the Stripes

Prepare 10–20 membranes at a time (*see* **Notes 3–5**).

1. Set up the vacuum device and sterilize the suction plate using 70% EtOH.
2. Rinse with PBS+.
3. Two different kinds of vacuum are needed: low vacuum (−200 to −400 mbar), where the bored rubber bung is not sealed (Fig. 3a), and high vacuum (−800 to −1000 mbar), where a 15 ml tube is used to seal the bore hole of the rubber bung (Fig. 3b).
4. Prepare 35 mm dishes with a drop of PBS+ for storage of the membranes after preparation of the first stripe (you can store two membranes in one 35 mm dish).

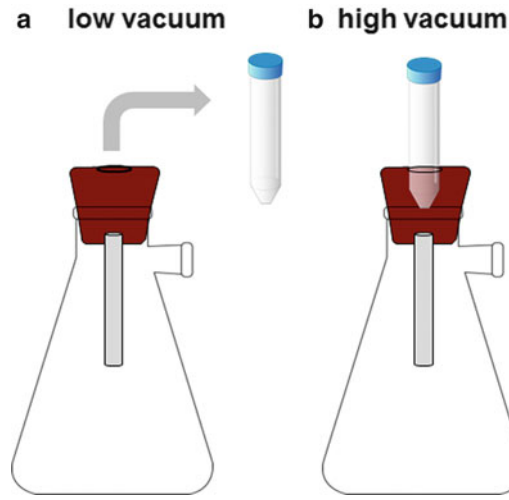

Fig. 3 Presenting the structure of the different vacuum states. (**a**) Low vacuum (−200 to −400 mbar), where the bored rubber bung is not sealed, and (**b**) high vacuum (−800 to −1000 mbar), where a 15 ml tube is used to seal the bore hole of the rubber bung

3.3.1 *First Stripes*

1. Close the clip (so that no vacuum is present) (Figs. 4a and 5a).
2. Place a drop of PBS+ onto the suction plate and place the first (transparent) matrix onto the drop avoiding air bubbles (*see* **Note 6**). Open and close the clip briefly to suck the matrix onto the plate. The red lines of the matrix should face upwards (Fig. 5b, c).
3. Rinse the matrix a few times with PBS+ under high vacuum.
4. Close the clip (no vacuum) and place a small drop of PBS+ onto the matrix (Fig. 5d).
5. Now place the trimmed membrane (shiny side up, marked edge on the upper left side) onto the matrix (*see* **Notes 7–10**).
6. Open the clip briefly again to suck the membrane onto the matrix (high vacuum).
7. Apply 150 µl of the first stripe solution to the membrane (*see* **Note 11**, Fig. 5e, f).
8. Open the clip to apply high vacuum for exactly 90 s (starting the countdown after the vacuum reaches −600 mbar) (*see* **Note 12**).
9. Stop the high vacuum by removing the 15 ml tube (now only low vacuum is applied to the membrane).
10. Carefully remove the excess volume on the membrane.
11. Wash the membrane gently by applying 1000 µl PBS+ to the membrane while it is held at a slight angle with the suction plate (*see* **Note 13**).

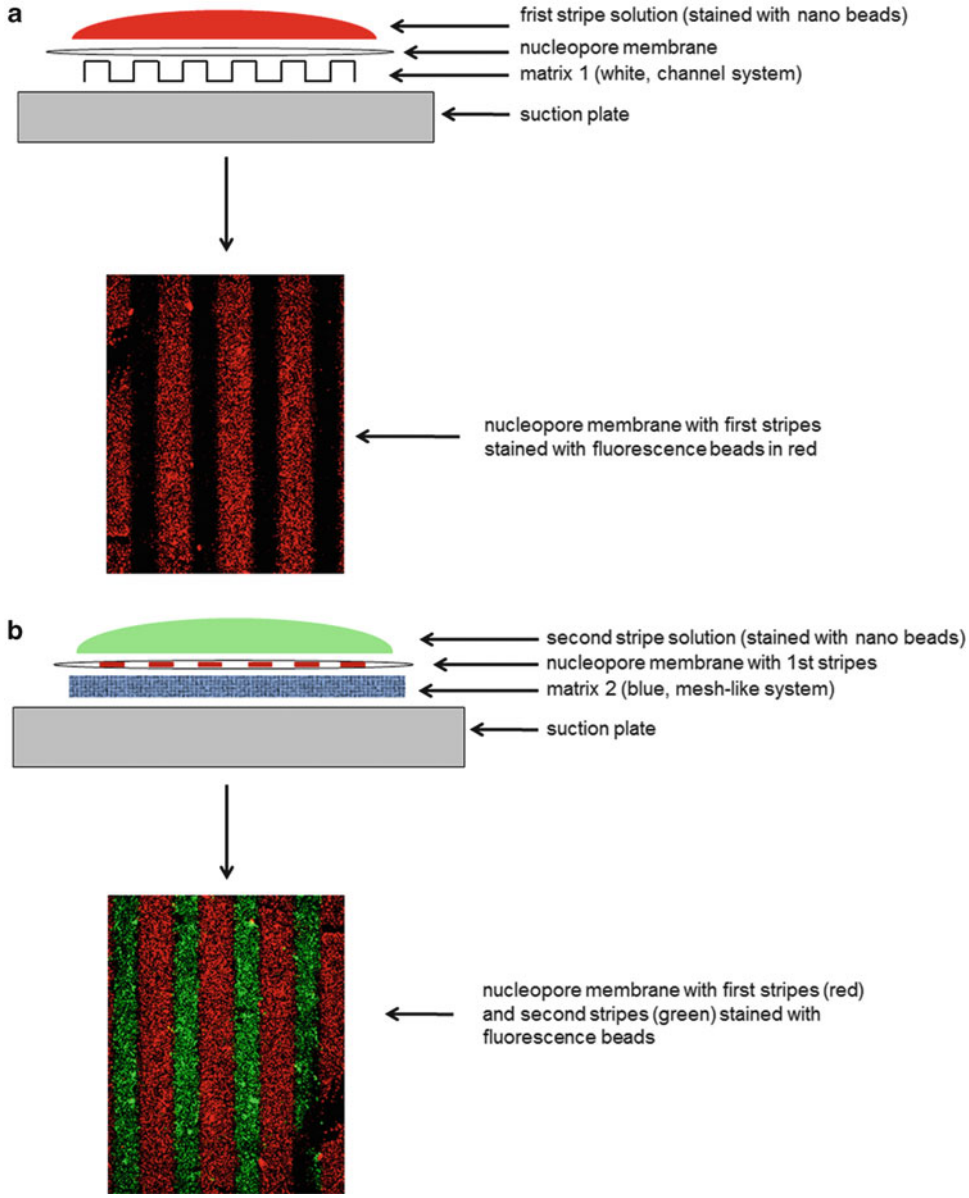

Fig. 4 Graphical depiction of the composition of the assembly of the matrix, membrane, and suction plate for generating the first stripe (**a**) and the second stripe (**b**)

12. Leave the membrane under low vacuum until the first stripes appear at the margin.
13. Close the clip (no vacuum) and transfer the membrane to the petri dish with PBS+.
14. Carefully place a drop of PBS+ on top of the membrane in the petri dish to prevent dehydration.
15. Store the membrane in the dark (cover using a black cloth or aluminum foil).

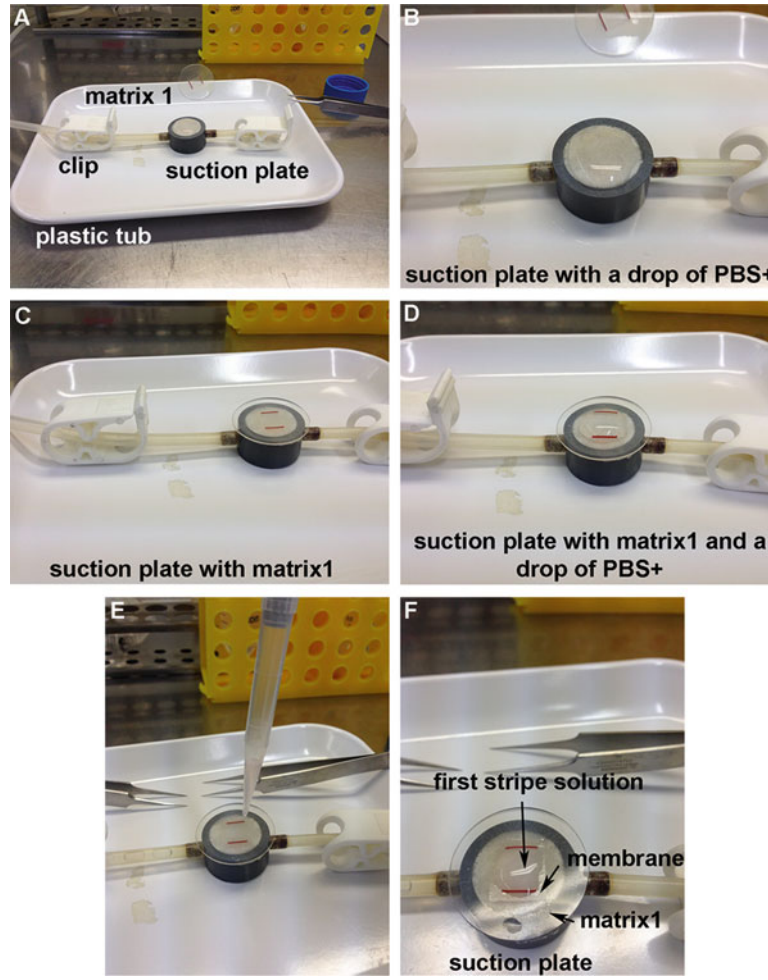

Fig. 5 Procedure of generating the first stripe. All steps are performed in the plastic tub (**a**). Before placing the matrix on the suction plate, place a drop of PBS+ onto the suction plate (**b**) followed by the matrix (**c**) and shortly open the clip to apply high vacuum. Before placing the membrane on the matrix, again put a drop of PBS+ on the matrix to prevent air bubbles underneath the membrane (**d**, **e**). Place the first stripe solution in the center of the membrane and open the clip for full vacuum (**f**)

16. Wash the matrix before preparing the next membranes by rinsing the matrix with PBS+ under high vacuum (*see* **Note 14**).
17. Prepare all first stripes before starting with the second stripes because you have to use a different matrix for the second stripes.

3.3.2 *Second Stripes*

1. Wash the suction plate at least three times with PBS+ under high vacuum (Fig. 4b).
2. Close the clip and apply a drop of PBS+ to the suction plate.
3. Gently place the matrix 2 (blue) onto the drop avoiding air bubbles (*see* **Note 7**).

4. Open the clip briefly to suck the matrix to the suction plate.
5. Transfer the membrane with the first stripe onto the matrix and make sure that there are no air bubbles underneath the membrane (*see* **Notes 8–10**).
6. Place 300 µl of the second stripe solution onto the membrane and apply high vacuum by opening the clip (*see* **Note 11**).
7. Apply high vacuum for exactly 90 s (from as soon as pressure reached −600 mbar) (*see* **Note 12**).
8. After 90 s, apply low vacuum and wash the membrane carefully three times using PBS+ (*see* **Note 13**).
9. Transfer the membrane into a 35 mm dish prepared with a drop of PBS+ and apply a drop of PBS+ on top of the membrane for short-term storage.
10. Remember to store the membranes in the dark after the stripes are applied.
11. You can store the membranes at 37 °C in the incubator until the cells are ready for use but ensure that the membranes do not dry out.

3.4 Preparation of Cells and Drug Treatment

1. Culture the cells of interest under normal conditions (in our case they are cultured in DMEM with 10% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO_2).
2. After preparation of all membranes, trypsinize the cells and count them using a hemocytometer.
3. Prepare 1×10^5 cells/ml media (the cell number has to be optimized for each cell line depending on their growth rate).
4. Remove the PBS+ carefully from the membranes and carefully apply the cell suspension to the membranes.
5. Before adding the cell suspension, but after removing of the PBS+, place small glass weights (broken pieces of glass slides) on each edge of the membrane to prevent them from floating (*see* **Notes 1** and **15**).
6. Two membranes may be stored in one dish using one glass weight per side for each membrane.
7. Add the inhibitor to the media after the cells are attached (3–4 h after seeding) (*see* **Note 16**).
8. Incubate the membranes with the cells for 24–48 h in an incubator using the standard media for your cells.

3.5 Evaluation of Cell Distribution

1. To stop the assay, remove the media and gently wash the membrane with sterile PBS (suitable for cell culture) (*see* **Note 17**).
2. Fix the membrane in 4% PFA for 10 min and remove the glass weights (*see* **Note 18**).
3. Wash three times for 5 min with PBS.

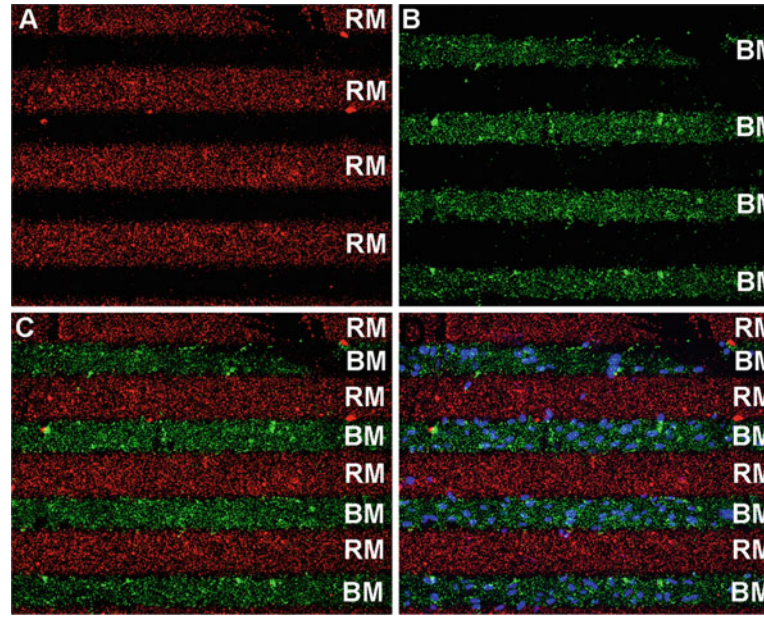

Fig. 6 Examples of a membrane with rat myelin (RM) as the first stripe (**a**, *stained red*) and biomatrix (BM) as the second stripe (**b**, *stained green*). The merge shows the carpet of both labeled solutions (**c**) and the fourth picture shows the membrane seeded with human glioblastoma cells (nuclei stained with Hoechst) after incubation of 24 h

4. Transfer the membranes to a glass slide with a drop of mounting media (suitable for fluorescence staining, such as MOWIOL) onto the slide.
5. Put a drop of mounting media containing DAPI or Hoechst on top of the membrane and cover with a cover slip avoiding air bubbles under or on top of the membrane.
6. Take at least 20 photos of each membrane from different areas and count the cells on the different types of stripes (Fig. 6).

4 Notes

1. Change the labeling of the stripes to blind the analysis (e.g., do not use the same color for biomatrix all the time).
2. Always stain both stripes to reduce the influence of the fluorescence beads to the cellular behavior.
3. Be sure that the membrane did not dry out at any time of the procedure.
4. Perform all experiments vice versa, means reverse the order of the solutions so that you use for example the combination biomatrix (first stripe) versus rat myelin (second stripe and vice versa rat myelin (first stripe) versus biomatrix (second stripe).

5. Also prepare a control only using the same solution for first and second stripes to obtain a random cell distribution.
6. If the stripe pattern is weak, make sure that there are no air bubbles between matrix, suction plate, and membrane and matrix.
7. Check if the matrices have adhered to the suction plate evenly and firmly.
8. Make sure that the membrane overlaps the channel area of the matrix 1 and the mesh-like areas of matrix 2. If this is not the case, cut the membranes in a different size than suggested.
9. It is important that there are no air bubbles underneath the membrane.
10. Check if the right side of the membrane is faced up. The use of the wrong side leads to poor results in the stripe pattern.
11. Do not let the solution fall over the edge of the membrane; otherwise the solution is sucked under the membrane and not on the membrane.
12. Check if you use the right vacuum pressure during the procedure.
13. Do not wash the membranes too intensely but also not too little.
14. Clean the matrices to be sure that the channels and the mesh are not blocked by old substrate.
15. Be sure that the membranes do not float; check the glass weights.
16. When the cells do not adhere to the membrane: Do not add the inhibitor directly to the cells after seeding. Cells have to attach first to the membrane before using the different inhibitors.
17. If you have used fluorescent microspheres to stain the stripes, you do not have to perform additional staining steps.
18. You can reuse the glass weights, by cleaning with ethanol, rinsing with distilled water, and autoclaving.

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Chapter 6

Using Histone Deacetylase Inhibitors to Analyze the Relevance of HDACs for Translation

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Abstract

Gene expression is regulated in part through the reversible acetylation of histones, by the action of histone acetyltransferases (HAT) and histone deacetylases (HDAC). HAT activity results in the addition of acetyl groups on the lysine residues of histone tails leading to decondensation of the chromatin, and increased gene transcription in general, whereas HDACs remove these acetyl groups, thus leading to an overall suppression of gene transcription. Recent evidence has elucidated that histones are not the only components of the proteome that are targeted by HATs and HDACs. A large number of nonhistone proteins undergo posttranslational acetylation. They include proteins involved in mRNA stability, protein localization and degradation, as well as protein–protein and protein–DNA interactions. In recent years, numerous studies have discovered increased HDAC expression and/or activity in numerous disease states, including cancer, where the upregulation of HDAC family members leads to dysregulation of genes and proteins involved in cell proliferation, cell cycle regulation, and apoptosis. These observations have pushed HDAC inhibitors (HDACi) to the forefront of therapeutic development of oncological conditions. HDACi, such as Vorinostat (Suberoylanilide hydroxamic acid (SAHA)), affect cancer cells in part by suppressing the translation of key proteins linked to tumorigenesis, such as cyclin D1 and hypoxia inducible factor 1 alpha (HIF-1α). Herein we describe methodologies to analyze the impact of the HDACi Vorinostat on HIF-1α translational regulation and downstream effectors.

Key words HDAC, HDACi, HIF-1α, Translation, Vorinostat

1 Introduction

Histone modifications, such as methylation, phosphorylation, and acetylation, can affect the structural conformation of chromatin, leading to altered expression of genes [1]. The reversible acetylation of histones by histone acetyltransferases (HATs) leads to a more relaxed chromatin structure, resulting in a general increase in gene transcription, whereas the removal of the acetyl group on

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lysine residues through the action of histone deacetylases (HDACs) results in a more condensed chromatin structure, thus promoting a general repression of gene transcription. It is now well established that more than 1750 nonhistone proteins, such as transcription factors, steroid hormone receptors, and cytoplasmic proteins, such as chaperones and cytoskeletal proteins, are also regulated by reversible acetylation [2].

In humans, 18 HDACs are classified into four classes based on their homology with yeast proteins. Class I includes HDAC1, 2, 3, and 8, which are related to the yeast RPD3 deacetylase. Class II, which is related to the yeast HDA1, is further subdivided into IIa (HDAC 4, 7, and 9) and IIb (HDAC6 and 10), based on the nature of N- and C-terminal protein-interacting motifs. These two classes differ in their subcellular localization, with class I being primarily localized into the nucleus [3] and class II shuttling between the nucleus and the cytoplasm, suggesting that class II family members could be involved in the deacetylation of nonhistone proteins. Class IV has only one member, HDAC11, which shares a catalytic domain with class I and II HDACs. Classes I, II, and IV are zinc-dependent enzymes and belong to the Zn^{2+} superfamily, whereas class III HDACs, also named sirtuins (Sir 1–7), are related to the yeast Sir2 family and have a NAD^{+}-dependent enzymatic activity.

One of the characteristic features of cancer is the deregulation of DNA methylation and histone acetylation, which contributes to abnormal transcription profiles in cancerous cells. Cancer cells are "addicted" to HDACs, where their abnormal expression pattern and/or function is associated with key events in carcinogenesis, including the repression of tumor-suppressor genes, such as *CDKN1A*, *p53*, and *BRCA1* [4–7]. Increased expression of HDACs has been observed in several types of cancer [8, 9] including HDAC1 in prostate, breast, and colon carcinomas [10–12], HDAC2 in cervical, lung, and gastric cancer [13–15], and HDAC 3 and 5 in hepatocarcinoma [16–19], and upregulation of HDAC9 is associated with poor prognosis in acute lymphoblastic leukemia and medulloblastoma [20, 21]. Collectively, these data support the development of HDACi for therapeutic interventions in cancer [22, 23]. However, even though many new HDACi are currently in preclinical development or clinical trials, to date only three HDACi, Vorinostat, Panobinostat/LBH-589, and Romidepsin, are FDA approved for the treatment of specific cancers.

Vorinostat is a general class I/II/IV HDAC inhibitor, which has been shown to induce apoptosis [24], cell cycle arrest [25, 26], autophagic cell death [27], and senescence [28] in cancer cells, as well as to prevent angiogenesis [29–32]. These effects are in part through blocking the translation of key cancer-related proteins [32–34]. Kawamata et al. have shown that Vorinostat drastically decreased cyclin D1 protein levels in mantle cell lymphoma [33]. Their results show that Vorinostat did not affect the mRNA levels of *cyclin D1*

nor its protein stability, but rather it inhibited the translation of *cyclin D1* by regulating the activity of PI3K and other proteins modulating the Akt/mTOR/eIF4E-BP pathway [33]. Recently, Emmrich et al. have shown that the treatment of acute myeloid leukemia cells with Vorinostat leads to increased expression of microRNA-139-5p through acetylation of histone 3 at the miR-139 promoter [34]. This microRNA can disrupt the translation initiation complex by targeting eIF4G2, which alters the rate of global protein synthesis and thereby decreases cell viability [34]. Previously, we have shown that Vorinostat drastically decreases hypoxia inducible factor 1 alpha (HIF-1α) expression in hepatocellular carcinoma cells without altering its transcription or proteolysis [32], reminiscent of the effect on cyclin D1 in mantle cell lymphoma [33]. We provided evidence that Vorinostat regulates members of the eukaryotic translation initiation (eIF) machinery through inhibition of HDAC9 [32]. This observation shows a new way to target the eIF machinery and this could open novel avenues for therapeutic development in cancer by looking for small-molecule inhibitors of the eIF complex machinery. Herein, we provide the methodology used to investigate and pharmacologically inhibit HIF-1α expression with HDACi in hepatocellular carcinoma cells.

2 Materials

2.1 Cell Line

1. Human *HuH7* hepatocellular carcinoma-derived cell line and cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS).

2.2 Chemicals

1. HDACi: Suberoylanilide hydroxamic acid (Vorinostat/SAHA).
2. Desferrioxamine (DFO).
3. Dimethyloxallyl glycine (DMOG).
4. MG132 (Z-leu-leu-leu-al).
5. Acrylamide–Bis-acrylamide 30:1.
6. Sodium dodecyl sulfate (SDS).
7. Uptilight US WBlot Chemiluminescent Substrate.

2.3 Lysis Buffer Components

1. 1 M Tris-HCl solution, pH 7.4: Dissolve 121.1 g of Tris base in 900 ml of distilled water. Mix and adjust the pH with HCl. Make up to 1 L with distilled water.
2. 20% Triton X-100 solution: Mix 10 ml of Triton X-100 in 40 ml of distilled water in a 50 ml conical tube. Mix at room temperature overnight. Store at room temperature.

3. 5 M NaCl: Dissolve 146.1 g of NaCl in 400 ml of distilled water and complete volume to 500 ml. Store at room temperature.
4. Lysis buffer—50 mM Tris-HCl solution, pH 7.4; 150 mM NaCl; 1% Triton X100: Mix 50 µl of 1 M Tris-HCl pH 7.4, 30 µl of 5 M NaCl, and 50 µl of 20% Triton X100 in 870 µl of distilled water per ml of lysis buffer.

2.4 Antibodies

1. Mouse anti-human GAPDH (Genetex, Irvine, CA, USA; Catalog number GTX627408).
2. Mouse anti-human HIF-1α antibody (BD Biosciences, Franklin Lakes, NJ, USA; Catalog number 610958).
3. Mouse anti-human p53 (Calbiochem, Merck Bioscience, Darmstadt, Germany; Catalog number OP43L).
4. Rabbit anti-human H3 acetylated (Millipore, Billerica, MA, USA; Catalog number OP43L 06-599).
5. Secondary antibodies: Anti-Mouse HRP (Dako, Santa Clara, CA, USA; Catalog number P0447) or anti-Rabbit HRP (Dako; Catalog number P0448).

2.5 SDS-PAGE Gel Components

1. Resolving gel buffer—1.5 M Tris-HCl solution, pH 8.8: Dissolve 181.7 g of Tris base in 900 ml distilled water. Adjust pH with HCl and complete volume to 1 L with distilled water. Filter sterilize using 0.4 µm filter and store at room temperature.
2. Stacking gel buffer—0.5 M Tris-HCl solution, pH 6.8: Dissolve 60.6 g of Tris base in 900 ml of distilled water. Adjust pH with HCl and complete to 1 L with distilled water. Filter sterilize using 0.4 µm filter and store at room temperature.
3. 10% (w/v) SDS solution: Dissolve 100 g of SDS in 900 ml of distilled water. Complete volume to 1 L and store at room temperature.
4. 10% (w/v) Ammonium persulfate solution: Dissolve 1 g of $(NH_4)_2S_2O_8$ in 10 ml of distilled water and store at 4 °C.
5. 30% Acrylamide–Bis solution (29.2:0.8 acrylamide–Bis). Store at 4 °C.
6. *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED). Store at 4 °C.
7. Running buffer, 1× solution: Dissolve 3.02 g of Tris base, 1.0 g of SDS, and 14.4 g of glycine in 900 ml of distilled water. Complete volume to 1 L with distilled water and store at room temperature.
8. Sample buffer (6× solution): Mix 3 ml of glycerol with 2.4 ml of 0.5 M Tris-HCl pH 6.8 (120 mM final concentration (*see* Subheading 2.5, **item 2**)). Dissolve 0.6% (W:V) of bromophenol blue and 0.6% (W:V) of SDS in glycerol/Tris-HCl solution.

Complete to 10 ml with distilled water. Aliquot in 1.5 ml microtubes placed at −20 °C for long-term storage. Add 2-mercaptoethanol to 15% final concentration (V:V) or dithiothreitol (DTT) to 35 mM just before use.

2.6 Immunoblotting Components

1. Nitrocellulose Blotting membrane (Amersham, GE Healthcare).
2. Western blot transfer buffer: Dissolve 58.2 g of Tris base and 29.3 g of glycine in 900 ml of distilled water. Complete volume to 1 L with distilled water and store at 4 °C.
3. Phosphate-buffered saline (PBS 10×): Dissolve 80 g of NaCl, 2 g of KCl, 2.4 g of KH_2PO_4, and 21.6 g of $Na_2HPO_4 \cdot 7H_2O$ in 800 ml of distilled water. Adjust pH to 7.4 and complete volume to 1 L with distilled water.
4. PBS-Tween 20: Dissolve 0.5 ml of Tween 20 in 1 L of 1× PBS.
5. Blocking solution: 5% Milk (W:V) in PBS-Tween 20. Store at 4 °C.
6. Antibody solution/diluent solution: 1% Milk in PBS-Tween. Store at 4 °C. Dilution for each antibodies: GAPDH 1/3000; HIF-1α 1/1000; p53 1/1000; H3 acetylated 1/1000 (*see* Subheading 2.4).
7. Mini PROTEAN 3 System glass plates; medium binder clips (11/4 in.); plastic container.
8. Whatman™ 3MM Chr Chromatography Blotting Paper sheet, relatively thick (0.34 mm).

2.7 PCR Primers, siRNAs, and siRNA Components

2.7.1 qPCR Primers

1. qPCR HIF-1α: 5′-GAACAAAACACACAGCGAAG-3′ and 5′-ACAAATCAGCACCAAGCAG-3′.
2. qPCR p53: 5′-TCTCCACTTCTTGTTCCCC-3′ and 5′-CTCCCCACAACAAAACACC-3′.
3. qPCR GAPDH: 5′-AAGGTGAAGGTCGGAGTCAA-3′ and 5′-CATGGGTGGAATCATATTGG-3′.

2.7.2 siRNAs

1. siRNA GL2: 5′-CGUACGCGGAAUACUUCGA-3′ (*see* **Note 3**).
2. siRNA eIF3H, siRNA ID s16509 (Ambio/Applied Biosystem/Thermo Scientific).

2.7.3 siRNA Components

1. Opti-Mem® Reduced Serum Medium.
2. Lipofectamine® RNAIMAX transfection reagent.

2.8 mRNA Extraction and qRT-PCR

2.8.1 mRNA Extraction

1. TRIzol® Reagent.
2. Chloroform.
3. Isopropanol.
4. Ethanol 70%.

2.8.2 Reverse Transcription

RT² First Strand Kit (Qiagen; Catalog number: 330401; RT First Strand Kit from other providers may equally work); 0.5 μg total RNA, RNase-free water, Genomic Elimination buffer, control P2, RE3 Reverse Transcriptase Mix, 5× buffer BC3.

2.8.3 qPCR Amplification

RT² SYBR Green qPCR Mastermix (Qiagen; Catalog number: 330500; SYBR Green qPCR Mastermix from other providers may equally work); RNase-free water, Hot start DNA Taq Polymerase, PCR buffer, dNTP Mix, SYBR Green dye, cDNA synthesis reaction.

2.8.4 qPCR Array

qPCR 96-well plate; Catalog number: PAHS-032YD-2—RT² Profiler™ PCR Array Human Hypoxia Signaling Pathway Plus (Qiagen; Catalog number 330231).

2.8.5 qPCR Machine

CFX connect real-time PCR detection Biorad system. qPCR machine from other providers may equally work.

3 Methods

3.1 Hypoxia Mimics and HDACi Treatments

1. HuH7 cells are cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS), plated in 12-well tissue culture dishes, and grown to 90–95% confluency.

3.1.1 DMOG Treatment

2. Treat cells with 0.01% DMSO, 5 μM Vorinostat, 0.01% DMSO + 500 mM dimethyloxallyl glycine (DMOG) or 5 μM Vorinostat + 500 mM DMOG for 24 h (Fig. 1).

3.1.2 DFO Treatment

3. Treat cells with 0.01% DMSO and 5 μM Vorinostat for 24 h. After 4 h of treatment with DMSO and Vorinostat, add 100 mM of desferrioxamine (DFO) for remaining 18 h (Fig. 1) (*see* **Note 3**).

3.1.3 MG132 Treatment

4. Treat cells with 0.01% DMSO and 5 μM Vorinostat for 24 h. After 20 h of treatment with DMSO and Vorinostat, add 50 mM MG132 for remaining 4 h (Fig. 1).

3.2 eIF3 Silencing

3.2.1 siRNA Treatment

1. HuH7 cells were plated in 12-well tissue culture dishes and grown to 40% confluency (day 0) (Fig. 4).
2. Wash cells once with 1× PBS and add 800 μl of Opti-MEM. For each well to be transfected, dilute 1 μl of siRNA in 100 μl of opti-MEM (*see* Subheading 2.7.3) in a sterile tube. In a second sterile tube dilute 2 μl of lipofectamine RNAiMax (*see* Subheading 2.7.3) in 100 μl of Opti-MEM. Incubate these tubes for 5 min at room temperature. Combine equal parts of diluted siRNA and lipofectamine RNAiMax, vortex briefly, and incubate for 20 min at room temperature. Add 200 μl of the appropriate transfection complex to a well of a 12-well dish containing 800 μl of Opti-MEM. Mix by gentle lateral shaking and incubate overnight at 37 °C/5% CO_2 (day 1) (Fig. 4).

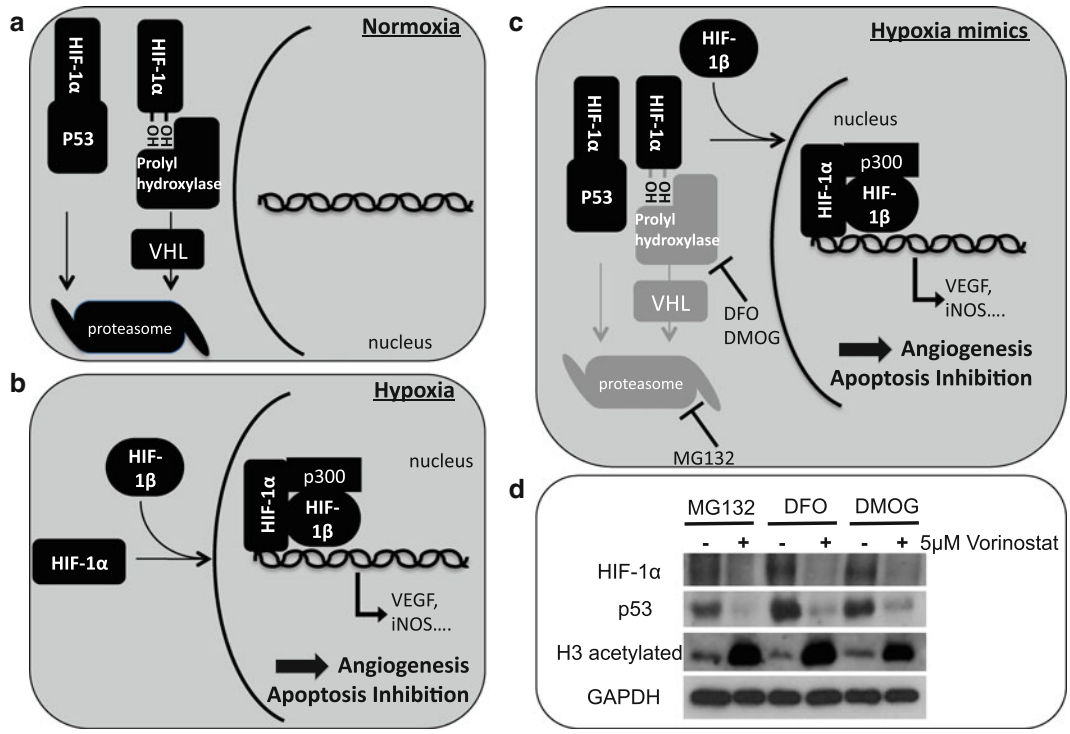

Fig. 1 Analysis of Vorinostat-mediated HIFα protein regulation. Schematic representation of HIF-1α regulation during normoxia, hypoxia, and hypoxia mimicking. HIF-1 is a heterodimeric complex composed of the HIF-1α and HIF-1β subunits. Oxygen levels strictly regulate HIF-1α. (**a**) Under normoxic conditions, proline residues in HIF-1α are hydroxylated by prolyl hydroxylase domain (PHD) containing oxygenases, which serve as a recognition signal for the E3 ubiquitin ligase, von Hippel-Lindau (VHL) complex, which targets HIF-1α for degradation by the proteasome. HIF-1α levels are also regulated by p53, which promotes MDM2-mediated ubiquitination [32]. (**b**) Under hypoxic conditions, the oxygen-dependent PHD-containing oxygenases have reduced activity, resulting in stabilization of HIF-1α, which dimerizes with HIF-1β to activate the transcription of genes that affect angiogenesis and apoptosis [32]. (**c**) Hypoxia can be mimicked by treating cells with prolyl hydroxylase inhibitors, such as desferrioxamine (DFO) or dimethyloxallyl glycine (DMOG) or with a proteasome inhibitor, such as MG132, resulting in stabilization of HIF-1α, and consequently transcription activation of angiogenesis and apoptosis-related genes. (**d**) HuH7 cells were treated with DFO (100 mM), DMOG (500 mM), or MG132 (50 mM), without or with addition of 5 μM Vorinostat, lysed and protein samples were resolved by SDS-PAGE, following transfer onto nitrocellulose membranes. Immunoblots for HIF-1α, p53, acetylated H3, and GAPDH (used as loading control) show the effect of compounds on HIF-1α, p53, and histone H3 expression (*see* Subheading 2.6) (*see* **Notes 1–3**). Vorinostat drastically decreases HIF-1α expression in HuH7 cell line by a pathway independent of p53- or prolyl-hydroxylases/VHL-mediated proteasomal degradation

3. Next day aspirate Opti-MEM containing transfection complexes and wash wells with 1 ml of 1× PBS. Replace PBS with complete culture medium (day 2) (Fig. 4).
4. Repeat the siRNA treatment as described in **step 2** on day 3.
5. Repeat washing and feeding of cells described in **step 3** on day 4.
6. Prepare cell lysates as described in Subheading 3.3 (day 5).

3.2.2 Synergistic Effect of Vorinostat and eIF3H Silencing

1. On day 4 of transfection protocol (*see* Subheading 3.2.1) replace complete culture media with complete culture media containing (Fig. 4) (*see* **Note 3**):
   (a) 0.05% DMSO, 5 μM Vorinostat, 0.05% DMSO+500 mM DMOG or 5 μM Vorinostat+500 mM DMOG for 24 h (Fig. 4).
   (b) 0.05% DMSO, 5 μM Vorinostat+100 mM DFO for 18 h.
   (c) 0.05% DMSO or 5 μM Vorinostat+50 mM MG132 for 4 h.
2. Prepare cell lysates as described in Subheading 3.3 (day 5).

3.3 SDS-PAGE Analysis

1. Prepare the 10% resolving gel by mixing 4.0 ml of water, 3.3 ml of 30% acrylamide mixture, and 2.5 ml of 1.5 M Tris-HCl pH 8.8 (*see* Subheading 2.5) in a 50 ml conical flask. Add 100 μl of SDS, 100 μl of ammonium persulfate, and 4 μl of TEMED (*see* Subheading 2.5), and cast gel within a 7.25 cm×10 cm×1.5 mm gel cassette (*see* Subheading 2.6). Allow space for stacking the gel and gently overlay with ethanol.
2. Prepare the stacking gel by mixing 4.1 ml water, 1.0 ml of acrylamide mixture, and 0.75 ml 0.5 M Tris pH 6.8 (*see* Subheading 2.5) in a 15 ml conical flask. Add 60 μl of SDS, 60 μl of ammonium persulfate, and 6 μl of TEMED. Remove the ethanol from resolving gel in **step 1**, rinse with distilled water, and overlay with the stacking gel mixture. Insert a 15-well gel comb immediately avoiding the introduction of air bubbles.
3. Sample preparation and electrophoresis: Wash cells in 1× PBS at the end of respective treatments (*see* Subheadings 3.1 and 3.2) and lyse them in 50 μl of lysis buffer with protease inhibitors (*see* Subheading 2.3). Determine protein concentrations by Bradford protein assay. Add 10 μl of 6× sample buffer (*see* Subheading 2.5) to 50 μl cell lysate. Heat at 95 °C for 5 min, chill samples on ice for 1 min, and centrifuge at 2000×*g* for 15 s to bring down the condensate. Load 15 μg of each sample and 10 μl of protein standard in the appropriate well of 10% SDS-PAGE gel. Electrophoresis is performed at 30 mA per gel (*see* **Note 1**).

3.4 Immunoblot

1. Resolve samples by SDS-PAGE and transfer onto nitrocellulose membranes using liquid transfer for 80 min at 100 V in transfer buffer (25 mM Tris-HCl, 192 mM glycine pH 8.8) (*see* Subheading 2.6).
2. Wash membranes with distilled water and incubate with Ponceau S (0.1% (W:V) Ponceau S in 1% (V:V) acetic acid) for 5 min prior to extensive washing with distilled water. Block membranes with 1× PBS, 0.05% Tween 20, and 5% (w/v) milk for 1 h at room temperature (*see* Subheading 2.6).
3. Dilute primary antibodies with 1× PBS, 0.05% Tween 20 (V:V), and 1% (W:V) milk at the appropriate dilution (*see* Subheadings 2.4 and 2.6) and incubate with the membrane overnight at 4 °C (*see* **Note 2**).

4. Wash membranes 4× 10 min with 1× PBS and 0.05 % Tween 20 (V:V) prior to incubating with HRP-conjugated secondary antibodies (*see* Subheadings 2.4 and 2.6) for 1 h at room temperature.
5. Wash membranes 3× 10 min with 1× PBS and 0.05 % Tween 20 (V:V).
6. Incubate membranes with chemiluminescent reagent (*see* Subheading 2.2) as recommended by the manufacturer and expose to X-ray films (Figs. 1, 3, 4). Quantify bands with ImageJ software (NIH).

3.5 mRNA Extraction and qRT-PCR

3.5.1 mRNA Extraction

1. The cells are treated as described in Subheading 3.1. At the end of those different treatments, remove the medium from the wells and wash cells 2× with 1× PBS.
2. Add 1 ml of TRIzol® Reagent in each well. Scrape the cells with a cell scraper directly in the wells and harvest the cell lysate by pipetting.
3. Transfer each extract in a clean 1.5 ml tube and add 200 µl of chloroform.
4. Mix thoroughly by inverting the tube ten times and incubate for 3 min at room temperature.
5. Centrifuge the samples at 12,000 × *g* for 15 min at 4 °C. Pipette 500 µl of the aqueous phase of the sample onto a new clean 1.5 ml tube. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.
6. Add 500 µl of isopropanol to the aqueous phase. Incubate at room temperature for 10 min.
7. Centrifuge at 12,000 × *g* for 10 min at 4 °C. Carefully discard the supernatant making sure not to disrupt the RNA pellet.
8. Wash the pellet, with 1 ml of 70 % ethanol (V:V), and then centrifuge the tube at 8000 × *g* for 5 min at 4 °C. Remove the 70 % ethanol (V:V) wash.
9. Air-dry the RNA pellet for 5–10 min (*see* **Note 5**).
10. Resuspend the RNA pellet in 26 µl of RNase-free water and incubate on ice for 20 min.
11. After homogenization, determine the RNA concentration at 260 nm making sure to assess the 260/230 and 260/280 ratios (*see* **Note 5**).

3.5.2 Reverse Transcription

1. Start with the genomic DNA elimination mix. In a clean 200 µl tube, use 0.5 µg of RNA as template for each reaction, and then add the following reaction components (manufacturer protocol, Qiagen): 2 µl buffer GE and complete with RNase-free water to 10 µl.
2. Incubate the mix for 5 min at 42 °C and then incubate on ice for at least 1 min.

3. During this incubation prepare the reverse transcription mix: 4 µl 5× buffer BC3, 1 µl control P2, 2 µl RE3 reverse transcriptase, and complete with RNase-free water 3 µl.
4. Add 10 µl of the reverse transcription mix to each tube containing 10 µl of genomic DNA elimination mix. Vortex and centrifuge the sample briefly.
5. Incubate for 15 min at 42 °C and stop the reaction by heating at 95 °C for 5 min.
6. Add 91 µl of RNase-free water, vortex, and centrifuge the sample briefly. Store at −20 °C.

3.5.3 qPCR Array

qPCR reaction should be performed in a DNA-free environment. Use dedicated pipettes and tips.

1. In a clean 5 ml tube, use 102 µl from the reverse transcription reaction as template, and then add the following reaction components (manufacturer protocol, Qiagen): 1350 µl of 2× RT² SYBR Green Mastermix and 1248 µl RNase-free water. The validated qPCR primers for the genes of interest are already dispensed in the wells.
2. Add 25 µl of the qPCR mix to each well of the RT² profiler PCR Array (manufacturer protocol, Qiagen).
3. Centrifuge the 96-well plate briefly to remove bubbles.
4. qPCR program: Start at 95 °C for 10 min to activate the Hot Start DNA Taq Polymerase, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C and perform fluorescence data collection. Finally, run a melting curve program (to verify PCR specificity) using the following program: 95 °C for 1 min; 65 °C for 2 min; 65 °C to 95 °C at 2 °C/min. A single peak should appear per well.
5. Quantification of ΔC_T was performed by normalization of each gene to the average C_T value of all six housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*) included in RT² Profiler™ PCR Array (ΔC_T = target gene C_T − housekeeping gene C_T) (Fig. 2) (*see* **Note 6**). Three different and independent biological replicates were used for each condition and relative quantification of gene expression was performed by calculation of $\Delta\Delta C_T$ referent to the control condition ($\Delta\Delta C_T = \Delta C_T$ of target sample − ΔC_T of control sample).

4 Notes

1. SDS-PAGE gels should be run at 30 mA per gel to ensure a perfect resolution of bands. The transfer should be run at 100 V.
2. For the detection of HIF-1α, it is better to dilute the primary HIF-1α antibody with a higher concentration of milk

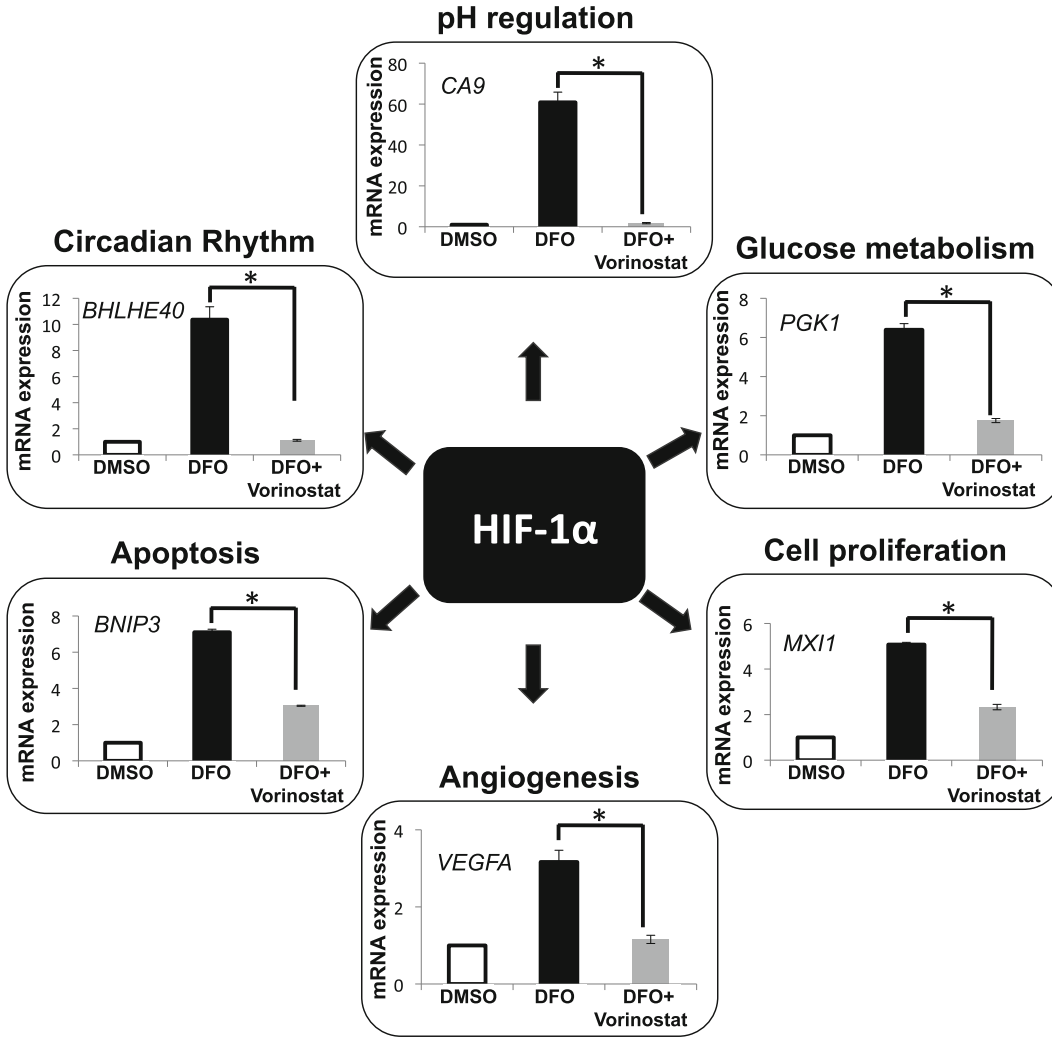

Fig. 2 Analysis of HIF-1α downstream signaling. HIF-1α is a central regulator of angiogenesis (VEGF-A), pH regulation (CA9), glucose metabolism (PGK1), apoptosis (BNIP3), cell proliferation (MXI1), and circadian rhythm (BHLHE40). HuH7 cells treated with DFO (100 mM) or DFO + Vorinostat (5 μM) were lysed and mRNA extracted. HIF-1α transcriptional regulation analysis was monitored by qRT-PCR analysis (RT^2 profiler PCR Array, Qiagen) (*see* Subheading 2.8.4). Three different and independent biological replicates were used for each condition and gene expression of target genes was normalized to the average C_T value of six housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*). Data are shown as the fold change of the ratio of target mRNA relative to that seen for vehicle control DMSO. Asterisks indicates $p < 0.05$ as determined by two-tailed *t*-test using DMSO (*white bar*) as the reference. As expected, Vorinostat drastically decreases HIF-1α downstream targets and signaling in HuH7 cell line

(5% milk (W:V) diluted in 1× PBS + 0.05% Tween 20 (V:V)) to decrease background (Figs. 1, 3, 4).

3. Treatments to mimic hypoxia: (1) Treatment with DFO at 100 mM must be performed for 18 h (Fig. 1). We observed that the effect of DFO on HIF-1α stabilization is less efficient

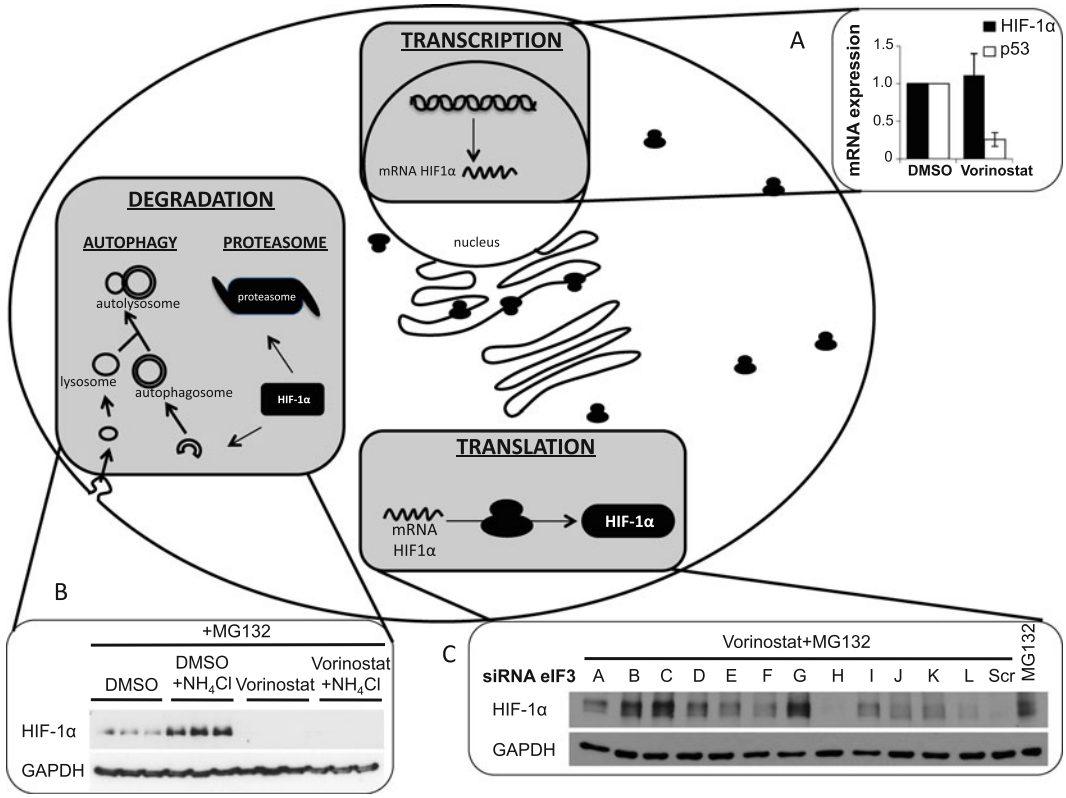

Fig. 3 Representation of the effect of Vorinostat on HIF-1α translation. Schematic representation of HIF-1α regulation by Vorinostat. (**a**) Analysis of *HIF-1α* and *p53* transcriptional regulation in HuH7 treated with DMSO or Vorinostat (5 μM). Cells were lysed and mRNA extracted to analyze the gene expression of *HIF-1α* and *p53* by qRT-PCR (*see* **Note 4**). (**b**) Analysis of the impact of inhibition of autophagy (10 mM ammonium chloride (NH_4Cl) for 8 h) and proteasomal degradation (MG132) on HIF-1α regulation. HuH7 cells were treated with DMSO + MG132, DMSO + NH_4Cl + MG132, Vorinostat + MG132 (5 μM), or Vorinostat + NH_4Cl + MG132 and subsequently lysed for protein analysis by SDS-PAGE. Following transfer onto nitrocellulose membranes, HIF-1α and GAPDH (loading control) were analyzed by immunoblot with corresponding antibodies. (**c**) Analysis of the impact of silencing components of the translation initiation eIF3 complex on HIF-1α. HuH7 cells were treated with eIF3 siRNAs and 5 μM Vorinostat + 50 mM MG132 and subsequently lysed for protein analysis by SDS-PAGE, following transfer onto nitrocellulose membranes. HIF-1α and GAPDH (loading control) were visualized by immunoblot with corresponding antibodies. Vorinostat decreases HIF-1α expression without altering its transcription or degradation. Vorinostat regulates HIF-1α translation in HuH7 cell line in a mechanism dependent on the eukaryotic translation initiation machinery. Figures (**b**) and (**c**) are adapted from our original paper Hutt DM et al. deacetylase inhibitor, Vorinostat, represses hypoxia inducible factor 1 alpha expression through translational inhibition. Plos one 2014 Aug 28;9(8):e106224. doi: 10.1371/journal.pone.0106224) [32]

for treatments longer than 18 h. (2) We obtained the same Vorinostat-mediated HIF-1α translation repression when using other hypoxia mimetics such as cobalt chloride (treatment at 150 μM for 24 h) and also other human hepatocellular carcinoma cell lines (Hep3B) [32]. The results for the synergistic effect of sieIF3H + Vorinostat on HIF-1α protein expression (Fig. 4) were also observed using other hypoxia

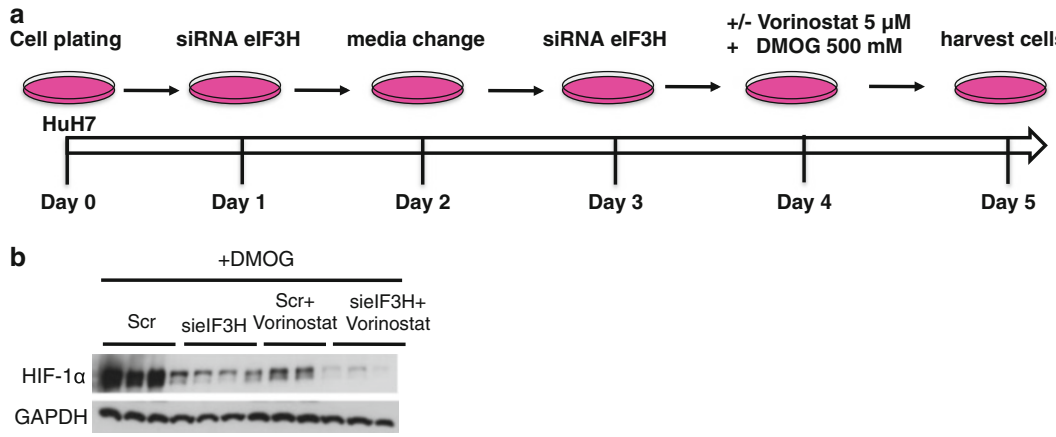

Fig. 4 Schematic representation of the experimental approach for combining eIF3H silencing and Vorinostat treatment in HuH7 cells. (**a**) Schematic representation of the protocol for the combined treatments of eIF3H siRNA and Vorinostat. (**b**) Silencing of eIF3H in combination with Vorinostat further decreased HIF-1α protein levels. HuH7 cells treated with eIF3H siRNA + 500 mM DMOG with or without 5 μM Vorinostat were lysed and protein samples were resolved by SDS-PAGE, following transfer onto nitrocellulose membranes. HIF-1α and GAPDH (loading control) are visualized by immunoblot with corresponding antibodies. Combining eIF3H silencing with Vorinostat treatment had an additive effect on HIF-1α repression

mimics as DFO (100 mM) or MG132 (50 mM) [32]. The Scramble siRNA (Scr) is siGL2 (*see* Subheading 2.7.2) and is used as a non-targeting siRNA control.

4. qPCR quality control: If you intend to study transcriptional regulation mediated by Vorinostat, use *p53* mRNA as positive control. We and others [6, 32, 35] have observed that p53 (wild type or mutated) is regulated at a transcriptional level by HDACi such as Vorinostat (*see* Subheading 2.7.1 for primers) (Fig. 3).
5. mRNA extraction quality control: Residual ethanol might inhibit downstream analyses. Ensure that all residual ethanol is evaporated from the sample prior to resuspension of the RNA pellet. The RNA pellet is white following alcohol precipitation and becomes translucent when all the ethanol has evaporated. The RNA absorbance A260:A230 ratio should be greater than 1.7 and A260:A280 ratio should be between 1.8 and 2.0.
6. Housekeeping genes: The expression levels of the housekeeping genes for the normalization must be the same in each experimental condition. If no housekeeping genes have been previously identified, test different housekeeping genes and choose housekeeping genes with the smallest ΔC_T across the condition. More than one housekeeping gene may be chosen for analysis of gene expression. In our experiments, no variation was observed in the C_T values of all the six housekeeping genes included in the RT^2 Profiler™ PCR Array (Qiagen) for each condition; therefore we used their C_T value average.

Acknowledgements

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Chapter 7

Effects of HDACi on Immunological Functions

René Winkler and Christian Kosan

Abstract

Histone deacetylase inhibitors (HDACi) are used as therapeutics for several B cell-derived malignancies. Furthermore, they have been shown to modulate the response of the immune system, like the B cell function. HDACi treatment affects differentiation, proliferation, and survival of B cells. Here we describe how to investigate the effects of HDACi treatment on naïve B cells regarding class-switch recombination (CSR) in vitro using flow cytometry.

Key words Class switch recombination, Naïve B lymphocytes, HDAC inhibition, Magnetic separation, Flow cytometry

1 Introduction

Histone deacetylase inhibitors (HDACi) are small molecules that bind to the catalytic domain of histone deacetylases (HDACs) and this leads to a reduction or complete inhibition of their enzymatic activity. HDACi regulate on the one hand gene expression by modulating histone acetylation. On the other hand they also modify the acetylation status of nonhistone proteins. HDACi are nowadays widely used as therapeutic treatments; in particular, different T and B lymphocyte-derived diseases are treated with HDACi [1]. For example, B cell lymphomas and multiple myelomas respond to pan-HDACi treatment in mouse models [2, 3]. Furthermore, several findings from in vitro and in vivo studies demonstrate that other tumors of the hematopoietic system also respond to HDACi treatment.

Interestingly, HDACi have been shown to enhance antitumor immunity and to modulate physiological innate and adaptive immune responses. Important cellular subsets of the adaptive immune system like T cells, Tregs, and B lymphocytes are modified by HDACi treatment [4].

B cells undergo several developmental and maturation steps that are sensitive to epigenetic alterations like acetylation. The

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production of high-affinity antibody responses is critical for the host defense against foreign pathogens. Therefore, activated B cells undergo class switch recombination (CSR) and somatic hypermutation (SHM) [5]. Both CSR and SHM require an open chromatin structure and are characterized by hyperacetylation of several histone residues [6]. Therefore, HDACs might be important regulators of CSR or SHM and this process appears to be regulated by HDACi. For instance, valproic acid (VPA) dampens class switching and hypermutation of B lymphocytes in a T cell-dependent and T cell-independent manner by upregulation of microRNAs that silence AID and Blimp-1 expression, key regulators for B cell maturation [7]. Furthermore, H3 and H4 histone acetylation events in the switch (S) regions are associated with CSR and required to recruit the DNA repair machinery to the respective S region [8, 9]. In line with this, a recent study showed that HDACi treatment impairs the primary antibody response in vitro and in vivo [10].

To study the effect on CSR in vitro we provide a protocol on how the impact of HDACi on primary B cell function can be assessed. For this, splenic B cells have to be isolated and purified by magnetic separation. Purified naïve B cells are treated with various HDACi and subsequently stimulated with different cytokine combinations to induce CSR towards the different immunoglobulins (Igs) IgG, IgG2a, IgG2b, or IgG3. To determine the effect of HDACi treatment on stimulated B cells we recommend to analyze B cell cultures for proliferation, apoptosis, and class switching by flow cytometry.

2 Materials

2.1 Mice and Cell Isolation

1. Sterile scissors and forceps.
2. 6-Well plates.
3. Sterile 1×PBS without $MgCl_2$ and $CaCl_2$.
4. 70% EtOH for disinfection.

2.2 Cell Culture

1. 12-Well plates.
2. Sterile 1×PBS without $MgCl_2$ and $CaCl_2$.
3. Sterile cell strainer 100 μM.
4. Sterile syringe.
5. 15 mL/50 mL Round-bottom tubes.
6. 5 mL FACS tubes (with caps).

7. Red cell lysis (RCL) buffer: 8.3 g/L Ammonium chloride in 0.01 M Tris–HCl pH 7.5.
8. 5 mL Magnet like MagniSort™ Magnet/Magneto (eBioscience; we want to state throughout that materials from other providers may work as well).
9. Negative selection antibody cocktail and magnetic beads like MagniSort™ Mouse B cell enrichment kit (catalog number 8804-6827-74) (eBioscience).
10. B cell culture medium: RPMI 1640, 10% fetal calf serum (FCS), 50 μM β-mercaptoethanol, 10 mM HEPES, 0.5% gentamicin.
11. Anti-B220 (CD45R) rat APC antibody; clone RA3-6B2 (catalog number 17-0452-81) (eBioscience).
12. Anti-CD3e hamster PE antibody; clone 145-2C11 (catalog number 12-0031-91) (eBioscience).

2.3 CSR Stimulation

1. HDACi of interest; solve as recommended by the supplier. For example, MS-275/Entinostat in dimethyl sulfoxide (DMSO), LBH-589/Panobinostat in DMSO, EX-527/Selisistat in DMSO, VPA in 1×PBS.
2. Cytokines: CD40L, IFN-γ, IL-4, IL-5, TGF-β; prepare as recommended by the supplier or purchase as ready-to-use solutions.
3. LPS from *E. coli* 0111:B4 (L4391) (Sigma-Aldrich; LPS from other strains and providers might work as well) solved in 1×PBS.

2.4 Functional Analysis

1. Antibodies against specific murine immunoglobulins: Anti-IgG1 biotin (clone A85-1) (BD Biosciences), anti-IgG2a biotin (clone R19-15) (BD Biosciences), anti-IgG2b biotin (clone R12-3) (BD Biosciences), anti-IgG3 biotin (clone R40-82) (BD Biosciences), anti-IgA biotin (clone C10-1) (BD Biosciences).
2. Streptavidin PE antibody (catalog number 12-4317-87) (eBioscience).
3. Annexin V Apoptosis Detection Kit FITC (catalog number 88-8005-74) (eBioscience) including Annexin-binding buffer (10×), anti-Annexin V FITC antibody, and propidium iodide staining solution.
4. Cold 70% EtOH in 1×PBS.
5. RNase A (10 mg/mL).
6. Propidium iodide (PI) solution (2.5 mg/L).

3 Methods

3.1 Isolation and Purification of Naïve B Cell Populations from Murine Spleens

All steps after **step 1** should be performed under a cell culture hood to avoid contamination.

1. Sacrifice a mouse (8- to 16-week-old C57BL/6), disinfect fur with 70% EtOH, and isolate the complete spleen by using sterile scissors and forceps. Transfer spleen to a 6-well plate containing 3 mL of sterile 1×PBS. Animal handling should be conducted according to local guidelines approved by an authority.
2. Generate a single-cell suspension by passing the spleen through a 100 μm cell strainer. Use the plunger from a syringe to mash the spleen through the cell strainer into a 50 mL round-bottom tube. Rinse cell strainer with 5 mL 1×PBS (*see* **Note 1**).
3. Centrifuge cells at 700 ×*g* for 5 min and remove supernatant.
4. Resuspend cell pellet in 1 mL RCL buffer. Incubate splenocytes for 10 min at room temperature to lyse red blood cells. Wash with 5 mL 1×PBS, centrifuge cells (700 ×*g* for 5 min), remove supernatant, and resuspend splenic cells in 10 ml 1×PBS (*see* **Note 2**).
5. Count cells and prepare a single-cell suspension in a 5 mL FACS tube at a concentration of 1×10^7 cells/100 μL (1×10^8/mL) in 1×PBS (*see* **Note 3**).
6. Add 20 μL of MagniSort™ Enrichment Antibody Cocktail for B cell isolation per 100 μL of the 1×10^7 cell suspension (*see* **Note 4**). Mix well by pulse vortexing five times (*see* **Note 5**). Incubate at room temperature for 10 min.
7. Wash cells by bringing the volume up to a final volume of 4 mL with 1×PBS and centrifuge at 700 ×*g* for 5 min.
8. Discard supernatant and thoroughly resuspend cells to their original volume from **step 5** with 1×PBS.
9. Add 20 μL of resuspended MagniSort™ Negative Selection Beads per 100 μL of the 1×10^7 cell suspension (*see* **Note 6**). Mix well by pulse vortexing five times. Incubate at room temperature for 5 min.
10. Bring the volume up to 2.5 mL with 1×PBS. Mix gently by pipetting up and down three times. Avoid vortexing.
11. Insert the 5 mL FACS tube into Magneto. Incubate at room temperature for 5 min.
12. Pick up the magnet containing the 5 mL FACS tube and pour the supernatant into a 15 mL collection tube with an uninterrupted move. Hold inverted tube for approximately 1 s and return it to the original position (*see* **Note 7**).
13. Discard the 5 mL tube containing bound cells. The purity of the isolated B cell population can be analyzed by flow cytometry

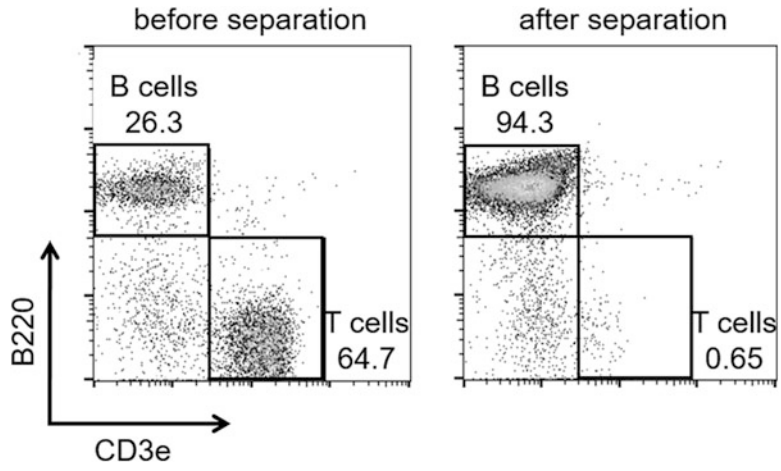

Fig. 1 Purity of B cell population after magnetic separation. B220-positive B cells were isolated by magnetic separation from a splenic cell suspension containing T cells (CD3-positive) among others

using antibodies for B cells (anti-B220 APC) and T cells (anti-CD3e FITC) (*see* **Note 2** and Fig. 1). Obtained cells can now be stimulated with HDACi before induction of CSR.

3.2 Treatment and Stimulation of Splenic B Cells with HDACi

1. Seed 0.5×10^6 purified B cells in 1 mL medium per well into 12-well plates (*see* **Note 8**).
2. Treat B cells with HDACi: for example, MS-275/Entinostat (0.2 μM), LBH-589/Panobinostat (10 nM), EX-527/Selisistat (10 μM), VPA (1.5 mM), or other HDACi for 2 h before stimulation with cytokines (*see* **Note 9**). Total time of HDACi treatment is approximately 74 or 98 h. Use appropriate controls (*see* **Note 10**).
3. Induce proliferation and class switch recombination by adding combinations of CD40L (1 μg/mL), IFN-γ (50 ng/mL), IL-4 (5 ng/mL), IL-5 (1.5 ng/mL), TGFβ-1 (1 ng/mL), or LPS (10 μg/mL) for 72 or 96 h (*see* Table 1) (*see* **Note 11**).
4. Perform functional analysis (*see* Subheadings 3.3–3.5 and **Note 12**).

3.3 Immunoglobulin Staining

1. Transfer around 0.5×10^6 cells into 5 mL FACS tubes, add 2 mL 1×PBS, and centrifuge at $700 \times g$ for 5 min.
2. Discard supernatant by carefully inverting the tube and repeat washing from **step 1**.
3. Discard supernatant and resuspend cell pellet in 100 μL 1×PBS.
4. Add 0.5 μL anti-mouse anti-Ig antibody (1:200) conjugated to biotin (e.g., anti-IgG1 biotin). Incubate for 10 min on ice in the dark (*see* **Note 13**).

Table 1
Cytokine combinations to stimulate specific immunoglobulin subclasses (*see* ref. 13)

Cytokines	Immunoglobulin subclass
LPS	IgG2b, IgG3
LPS and IL-4	IgG1, IgE
CD40L and IL-4	IgG1, IgE
LPS and IFN-γ	IgG2a
LPS, TGFβ-1, and IL-5	IgA

5. Add 2 ml of 1×PBS and centrifuge as in **step 1**. Discard supernatant and resuspend stained cells in 100 µl 1×PBS.
6. Add 0.5 µL fluorophore-conjugated streptavidin antibody (e.g., streptavidin PE). Incubate for 10 min on ice in the dark.
7. Add 2 ml of 1×PBS and centrifuge as in **step 1**. Discard supernatant and resuspend stained cells in 100 µl 1×PBS. Perform flow cytometry analysis to determine CSR (*see* Fig. 2).

3.4 Annexin V and PI Staining

1. Transfer around 0.5×10^6 cells into 5 mL FACS tubes, add 2 mL 1×PBS, and centrifuge at 700 × *g* for 5 min.
2. Prepare 1×Annexin-binding buffer. Discard supernatant by carefully inverting the tube and add 2 mL 1×Annexin-binding buffer.
3. Discard supernatant and resuspend cell pellet in 100 µL 1×Annexin-binding buffer.
4. Add 2 µL anti-Annexin V FITC antibody. Incubate for 15 min on ice in the dark (*see* **Note 13**).
5. Add 0.5 mL 1×Annexin-binding buffer and centrifuge as in **step 1**. Discard supernatant and resuspend stained cells in 200 µL 1×Annexin-binding buffer.
6. Add 3 µL PI staining solution provided in the kit (*see* **Note 14**).

3.5 Cell Cycle Analysis

1. Transfer around 0.5×10^6 cells into 5 mL FACS tubes, add 2 mL 1×PBS, and centrifuge at 700 × *g* for 5 min.
2. Discard supernatant by carefully inverting the tube.
3. Add slowly 1 mL cold 70% EtOH by dropping EtOH while vortexing the tube containing cells.
4. Store samples overnight or at least for 4 h at –20 °C to fix cells.
5. Wash cells by adding 2 mL 1×PBS and centrifuge at 700 × *g* for 5 min.

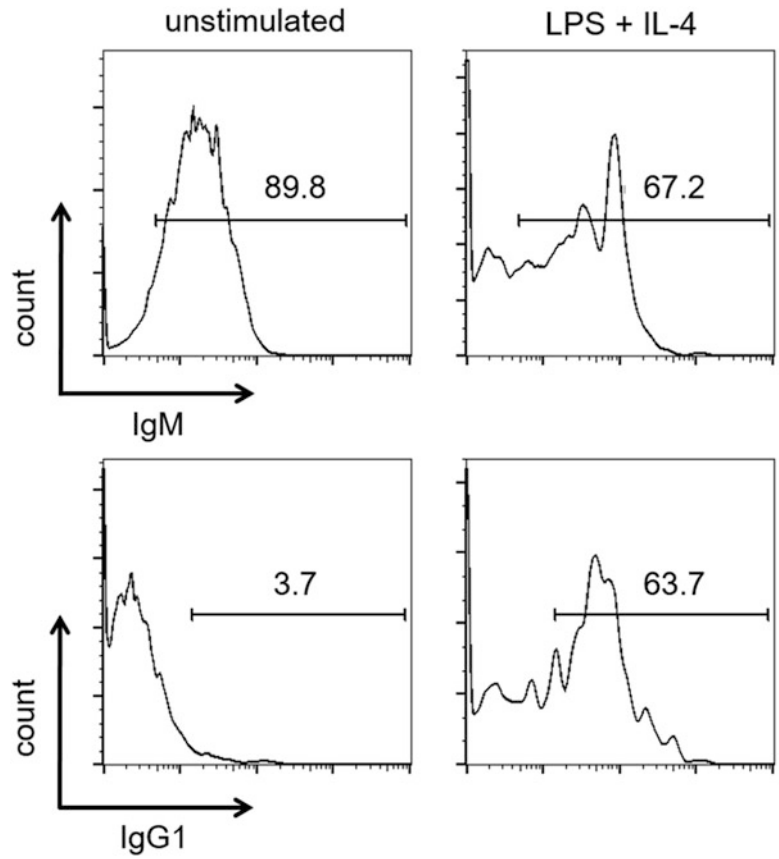

Fig. 2 Induction of CSR changes surface immunoglobulin subtypes. B cells undergoing CSR after stimulation with LPS and IL-4 downregulate IgM and show increased IgG1 surface expression

6. Discard supernatant carefully and stain cells with a mixture containing 390 µL 1×PBS, 5 µL RNase A, and 5 µL PI (2.5 mg/mL) (*see* **Note 13**). Incubate for 20 min at 37 °C in the dark. Perform flow cytometry analysis (*see* Fig. 3).

4 Notes

1. An alternative method is described in the following: Use two sterile microscopy slides with frosted ends to grind the spleen directly in the PBS-containing 6-well plate. Prepare a 50 mL round-bottom tube by inserting a sterile glass pipette that is stuffed with a small piece of cotton and transfer cell suspension into the glass pipette. Let the suspension pass through the glass pipette and add 5 mL 1×PBS into the 50 mL round-bottom tube. Invert tube one time.
2. To evaluate if purification was successful, a sample of cells should be taken before and after the separation. Cells can be

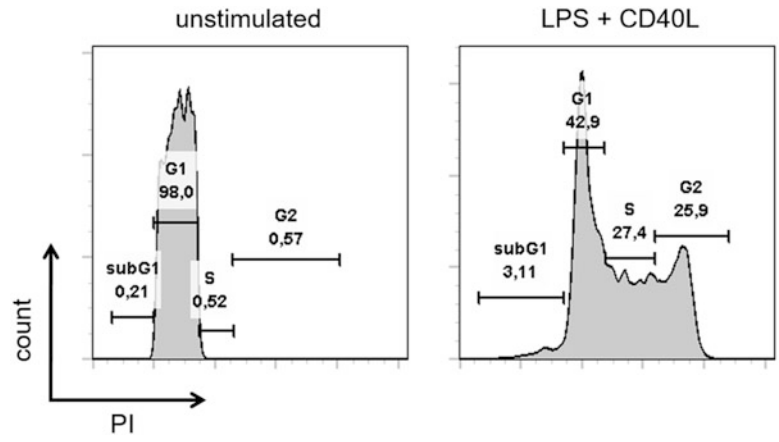

Fig. 3 Cell cycle profiles of unstimulated and stimulated B cells. While unstimulated cells are mainly resting in G1 phase, stimulated B cells show increased S phase indicating cell proliferation

stained with anti-CD3e PE antibody (1:200) and anti-B220 APC antibody (1:200) as described (*see* Subheading 3.3, **steps 1–5**, and *see* Fig. 1).

3. It is not recommended to use more than 2×10^8 cells.
4. Besides negative selection via magnetic separation, a positive selection method is possible (e.g., MagniSort™ Mouse CD19 positive selection kit) (eBioscience). However, positive selection may lead to a pre-activation of B cells through antibody contact.
5. Use FACS tube caps when leaving cell culture hood to avoid contamination.
6. Magnetic beads should be resuspended properly before use, for example by pipetting up and down five times with a P1000 pipette.
7. Do not shake the inverted tube as this may reduce the purity of unbound cells.
8. Besides using primary B cells, CSR experiments can be performed with the murine lymphoma cell line CH12F3. This cell line switches exclusively to IgA after exposure to CD40L, IL-4, and TGF-β. Seed CH12F3 cells in a density of 50,000 cells/mL in 12-well plates.
9. Always titrate your best working concentrations of HDACi, in particular when working with new HDACi. Avoid too high concentrations of HDACi to prevent excessive cell death.
10. Be careful when using controls with DMSO because DMSO can act as a HDACi as well [11]. An additional control without DMSO can indicate unwanted effects.
11. Titrate your best working concentrations of cytokines as they may vary between manufacturers and batches. Try different

combinations of concentrations to achieve the highest CSR without affecting cell survival. Always thaw reagents on ice because cytokines are highly sensitive to temperature changes.

12. It is recommended to check functional HDAC inhibition by western blotting with antibodies such as anti-acetyl-H3 and anti-acetyl-tubulin. Use pan-HDACi in lysis buffer for maintaining current acetylation status in the cell [12].
13. Always prepare an unstained control that is treated in the same and measure it before stained samples by flow cytometry. This helps to distinguish stained cell populations better.
14. Too high concentrations of PI can induce cell death. Add PI right before measurement and do not keep cells with PI for a longer time span.

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Chapter 8

DNA Fiber Spreading Assay to Test HDACi Effects on DNA and Its Replication

Teodora Nikolova, Anja Göder, Ann Parplys, and Kerstin Borgmann

Abstract

DNA fiber spreading assay is an invaluable technique to visualize and follow the spatial and temporal progress of individual DNA replication forks. It provides information on the DNA replication progress and its regulation under normal conditions as well as on replication stress induced by environmental genotoxic agents or cancer drugs. The method relies on the detection of incorporated thymidine analogues during DNA synthesis in the S phase of the cell cycle by indirect immunofluorescence. Here, we describe the procedure established in our laboratories for sequential pulse labeling of human cells with 5-chloro-2′-deoxyuridine (CldU) and 5-iodo-2′-deoxyuridine (IdU), cell lysis, and DNA fiber spreading on slides and sequential immunodetection of the incorporated thymidine analogues by primary antibodies recognizing specifically CldU or IdU alone. We describe also the laser scanning imaging, classification, and measurement of the detected DNA fiber tracks. The obtained quantitative data can be evaluated statistically to reveal the immediate or long-term effects of DNA-damaging agents, DNA repair inhibitors, and epigenetic modulators like HDAC inhibitors on DNA replication in normal and tumor cells.

Key words Thymidine analogue labeling, DNA fiber spreading, Anti-BrdU antibody, Immunodetection, LSM microscopy

1 Introduction

Replication forks can be stalled during the S phase by DNA lesions or due to transcription events at highly expressed genes [1]. Stalling DNA replication lesions are also induced by genotoxic agents and the features of the stalling (sequence, duration) and the consequences thereof depend on the nature of the induced primary and secondary DNA lesions [2]. The outcome may be fork restart, activation of new origins of firing, or fork collapse.

Whereas prokaryotic organisms have developed efficient fork repair mechanisms to restart stalled forks, eukaryotic cells seem to have evolved a different strategy to deal with replication stress and to prevent genomic instability during S phase, which is based on origin redundancy [1]. It is considered that specific

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origins or groups of origins which function at determined time points of S phase can be spatially and temporally regulated [3]. Only about 10% of the available potential replicons in human cells are involved in DNA synthesis at any time during the S phase and replicon clusters exhibit sequential activation. In the absence of DNA damage, the cell cycle checkpoint pathway Chk1-Cdc25A-CDK2 regulates origin activation throughout the S phase, but how the program of origin activation is developed and ordered spatially remains to be elucidated [4]. When compared to normal cells, most cancer cells exhibit a similar speed of replication fork movement from one origin to the next one [5], still some temporal differences in the formation of origin clustering were observed for glioma cells in comparison with normal human fibroblasts [6].

In the prevailing number of contemporary studies on DNA replication, the DNA fiber spreading assay is applied to accumulate important scientific data. The DNA fiber spreading, or alternatively DNA fiber combing, techniques are an invaluable tool to gain information on both DNA replication progress and regulation under normal conditions or under replication stress induced by environmental genotoxic agents or cancer drugs. DNA fiber spreading assay is a single-molecule assay developed to monitor DNA replication at the level of individual chromosomes [1]. Using the strategy for DNA fiber labeling with thymidine analogues, which are incorporated into DNA during replication, DNA synthesis during the S phase of the cell cycle can be assessed on the level of single replication forks [7]. The most widely used labeling agents are 5-iodo-2′-deoxyuridine (IdU) and 5-chloro-2′-deoxyuridine (CldU). The method is based on the immunodetection of the above-indicated thymidine analogues by specific antibodies. The finding that two commercially available antibodies bind specifically to IdU alone but not to CldU (namely a mouse anti-BrdU antibody) or vice versa to CldU alone (a rat anti-BrdU antibody) [8] allows to visualize and follow the spatial and temporal progress of the replication forks. This approach enables the analysis of replication dynamics throughout the genome, including immediate and long-term effects after DNA-damaging treatments [7]. The obtained data is quantitative and can be evaluated statistically [7].

Of note, this approach showed that the HDAC inhibitor SAHA (Vorinostat) slowed down DNA replication fork velocity, and caused activation of dormant origins and DNA damage in cancer cells [9]. HDAC3 inhibition by Romidepsin and other novel synthetic HDAC3 inhibitors also reduced replication speed and induced DNA damage and apoptosis [10]. In ongoing studies we apply this technique to reveal the effects of the HDAC inhibitor MS-275/Entinostat [11] on DNA replication in colon cancer cells.

2 Materials

Prepare all solutions using autoclaved deionized water and analytical grade reagents. Prepare and store all reagents at the indicated temperatures. Before use, thaw or take the reagents from the fridge and equilibrate them at room temperature or at 37 °C in the incubator as indicated. Follow waste disposal regulations when disposing waste materials (e.g., collect and dispose separately organic solvents like methanol and acetic acid waste). We do not need to add sodium azide to the reagents as preservative because we prepare them shortly before and use them up during each experiment.

2.1 Labeling and DNA Spreading

1. Stock solution 2.5 mM IdU (5-iodo-2′-deoxyuridine, TCI Europe IO258): Add 40 mg IdU substance to 45 ml pre-warmed (37 °C) serum-free DMEM culture medium. Vortex the solution and incubate for 30–60 min in a water bath at 70 °C (soluble to up to 2 mg/ml at 25 °C in water). Check carefully if the substance has dissolved completely. Aliquot as 2 ml samples, freeze, and store at −20 °C until required.
2. Stock 2.5 mM CldU (5-chloro-2′-deoxyuridine, Sigma C6891-100MG). Add 6.57 mg IdU substance to 10 ml pre-warmed at 37 °C serum-free DMEM culture medium. Vortex the solution and incubate for 30–60 min in a water bath at 37 °C. Check carefully if the substance has dissolved completely. Aliquot in 200–250 µl samples, freeze, and store at −20 °C until required.
3. Spreading buffer (200 mM Tris–HCl pH7.4, 50 mM EDTA, 0.5% SDS): Adjust pH adding 37% HCl drop by drop while mixing on the electromagnetic mixer, aliquot in 0.5 ml samples in vials, freeze, and store at −20 °C until required.
4. Superfrost microscope slides with a white, writable area and dimensions of 26 × 76 mm.
5. Fixative: Methanol/acetic acid (3:1, v/v). Wear a mask and gloves, and protect your eyes with lab goggles. Work under the fume hood. Example: Mix 60 ml methanol with 20 ml acetic acid and divide portions of 13–14 ml in several plastic carrier buckets with tamper evident closure for four slides each (*see* **Note 1**).

2.2 Washing and Blocking

1. Wash buffer I: Mix 9.55 instant PBS powder in 1 L dH_2O or dilute 1 part 10×PBS with 9 parts dH_2O.
2. Wash buffer II: Add 1% BSA and 1% Tween-20 to PBS. Leave at RT and mix now and then until completely dissolved, and then store at 4 °C until used (*see* **Note 2**).
3. Blocking solution: 5% Goat serum in BS. Prepare shortly before use.

4. Post-fixative 4% paraformaldehyde in PBS (PFA): To avoid exposure to paraformaldehyde fumes, work under the fume hood. Incubate 4 g in 100 ml PBS at 60 °C in the water bath (or on the magnet stirrer) in a heat-resistant flask covered with aluminum foil and placed under the hood. Stir now and then (or continuously if using the stirrer) until completely dissolved, then aliquot in 14 ml samples, and freeze at −20 °C until required (*see* **Note 3**).

2.3 DNA Denaturation

1. 2.5 mol/L Hydrochloric acid (HCl): Wear a mask, protective goggles, and gloves. To avoid exposing co-workers to HCl work under a fume hood. Add slowly 26 ml 37% HCl to 99 ml cooled at 4 °C ddH_2O under the fume hood. Mix well and equilibrate at room temperature for 15–30 min (*see* **Note 4**).

2.4 Antibodies

1. Rat monoclonal anti-BrdU, clone BU1/75 (ICR1) from AbD Serotec (catalogue number 0BT0030G). Keep at 4 °C.
2. Goat anti-rat Cy3 F(ab′)2 IgG (H + L) (Jackson Immuno Research, catalogue number 112-166-062): Dilute in ddH_2O, aliquot in 5–10 μl samples, and freeze at −80 °C until required. Optional: Use goat anti-rat AlexaFluor555 conjugate IgG (H + L) (Molecular Probes, catalogue number A-21434). Keep at 4 °C in the dark.
3. Mouse monoclonal anti-BrdU, clone B44 from BD Biosciences. Keep at 4 °C.
4. Goat anti-mouse AlexaFluor488 F(ab′)2 IgG (H + L) (Molecular Probes, catalogue number A-11017). Keep at 4 °C in the dark.

2.5 Materials for Microscopy

1. Vectashield mounting medium (catalogue number H-1000, Vector Laboratories).
2. Immersion oil for laser scanning microscopy Immersol 518F (ISO 8036, Carl Zeiss).

3 Methods

3.1 Labeling

1. Seed 150,000–300,000 cells in 5–6 ml culture medium in 50 ml sterile culture flasks (Greiner). Incubate the cells at 37 °C in 5–7% CO_2 for 1–2 days. Treat the cells with the chemical of choice (pulse or continuous treatment).
2. Several hours before labeling, thaw IdU- and CldU-containing aliquots at 37 °C. Mix CldU with culture medium, prewarmed at 37 °C, at a concentration of 1:100 (to obtain a 25 μM working concentration). Incubate at 37 °C in the dark until used, but at least for 1 h before labeling.

3. Incubate IdU-containing aliquot at 70 °C in a heating block or in a water bath for 30–60 min until it is completely dissolved. Then mix with pre-warmed culture medium (37 °C) 1:10 (i.e., 250 μM working concentration) and incubate at 37 °C in the dark until used (at least for 1 h before the labeling).
4. Label exponentially growing cells with 25 μM CldU for 20–40 min (you must estimate the optimal labeling time in preliminary experiments). Stick to the pulse treatment time precisely to the second in further experiments (*see* **Note 5**).
5. Remove the medium with CldU and pulse label with medium containing 250 μM IdU for another 20–60 min (*see* **Note 6**). Stick to the labeling time estimated in preliminary experiments very precisely (*see* **Note 7**).
6. During labeling, prepare Superfrost slides (at least 4–5 per sample, signed with a pencil). Thaw a sample of the spreading buffer (*see* Subheading 2), vortex until transparent, and equilibrate at room temperature for 20–30 min.
7. Wash the cells twice with ice-cold PBS and scrape each sample in a small volume of ice-cold PBS using individual cell scrapers for each variant. Transfer the cell suspension to 1.5 ml cooled on ice microcentrifuge vials. Count the cells using hemocytometer or cell counter. Adjust the cell number to 5×10^6 cells/ml in ice-cold PBS. Keep further the cell samples on ice during slide preparation.

3.2 Spreading

1. Spread each sample separately. Vortex the vial and drop 2 μl in the middle upper part of each horizontally lying slide (~1.5–2 cm under the writable area). Let it air-dry for 2.5–3 min until the droplet border becomes "sticky" but not dry.
2. Vortex again the spreading buffer, add 7–8 μl spreading buffer to the droplets, mix by gentle stirring with the pipet tip, and wait for 2 min. Then tilt the slides at an angle of ~15° and let them run down slowly along the slide leaving a wide track (~1 cm). It should take the drops 2–3 min to reach the bottom edge of the slide (*see* **Note 8**).
3. Air-dry the tracks; meanwhile prepare the methanol/acetic acid fixative (*see* "Needed materials"). Fix the slides in staining cuvettes for 10 min in the dark, air-dry, and store in the fridge in a black box at 4 °C (better on a tray filled with ice).

3.3 DNA Denaturation

1. Wash the slides in dH_2O in plastic staining cuvettes two times 5 min each (*see* **Note 9**).
2. Wash once in 2.5 mol/L HCl for 5 min.
3. Denature for 75 min in 2.5 mol/L HCl.
4. Rinse twice with PBS. Lay the slides in a black StainTray (catalogue number 631-1923, VWR International GmbH).

3.4 Immunodetection

1. Wash twice 5 min each with 2 ml BS (*see* "Subheading 2").
2. Add 1 ml 5% goat serum in BS and incubate for 1 h to block unspecific binding of the antibodies used for immunodetection. Take frozen PFA aliquots out of the freezer and leave them at RT to thaw and equilibrate.
3. Prepare rat anti-BrdU primary antibody (*see* item 4 in Subheading 2) in BS at a concentration 1:1000 (calculate the quantity by multiplying the number of stained slides to 120–125 μl/slide). The antibody is known to recognize CldU, but not IdU [8]. Cut Parafilm stripes wide and long enough to cover all side-by-side laying slides. Remove the blocking solution, drain the slides shortly on paper tissue, lay the slides back into the tray, add 115 μl onto each slide, and cover with the Parafilm stripes. Incubate for 1 h in the dark at RT.
4. Remove the stripes, rinse three times with PBS, and then fix for 10 min in the thawed and equilibrated 4% PFA solution. Rinse again three times in PBS and wash two times with BS 5 min each. Meanwhile dilute Cy3-coupled anti-rat antibody in BS (1:500, ~120 μl/slide). Drain the slides onto paper tissue from BS, lay them back into the tray, pipet 115 μl Cy3-coupled antibody solution onto each slide, and then cover with parafilm stripes. Incubate for 2 h in the dark at RT.
5. Remove the stripes, rinse twice with PBS, and then wash three times 5 min each with BS. Meanwhile prepare the dilution of mouse anti-BrdU antibody in BS (1:1500, ~155–160 μl/slide). Drain the slides onto paper, return them back to the black tray, pipet 150 μl/slide from the solution, cover with Parafilm stripes, and lay the tray at 4 °C overnight.
6. On the next day remove the stripes, rinse twice in PBS, and wash three times 5 min each in BS. Meanwhile prepare AlexaFluor488-coupled anti-mouse antibody solution in BS (1:500, ~120 μl/slide), drain the slides as described above, pipet 115 μl of the antibody solution, cover with stripes, and incubate for 2 h in the dark at RT.
7. Rinse twice in PBS and wash twice 5 min each in BS. During the washing, drop onto 22 × 50 mm cover slips a drop of the Vectashield anti-fade medium. Rinse again twice in PBS, rinse twice in dH_2O, drain the slides onto paper, and turn the slides upside down (with the DNA fibers turned to the anti-fade medium) onto the cover slips. Turn them, so that the cover slip is on the upper side of the slide, allow the drop to spread and drive away the air, and then glue the cover slip to the slide with nail polish. Store the slides in a black box (light protected) on ice at 4 °C until microscopy (at least 15–20 min for the nail polish to harden).

3.5 Laser Scanning Microscopy (LSM) and DNA Fiber Analysis

The images are captured using LSM 710 (Carl Zeiss) equipped with the ZEN2009 software. The applied settings for acquisition are 63× objective (oil), 16 bit, average of four scans, sequential scans for Cy3 and Alexa488 tracks (Best signal mode in the Smart Setup Function in the Menu), and max pinhole (non-confocal mode).

3.5.1 DNA Fiber Track Determination

Export the images captured by LSM in JPEG format. We identify the following five types of DNA tracks according to [12, 13] (Fig. 1).

The exported images can be opened in the free software ImageJ (Wayne Rasband, NIH, USA).

1. Use the function *File open* in the menu, and *then Plugins*; in *Plugins* choose *Analyse*, and in *Analyse* choose *Cell counter*.
2. Remove counters to leave only 5 (which will correspond to the five track types). Initialize, choose the desired type of counter (e.g., type 1 = first pulse origin) by a click with the mouse left button, and then click with the left button onto the tracks in the image which you classify as type 1 track. They are all marked by small figures (Fig. 2).
3. Identify sequentially all other available track types, and then choose in the *Cell counter* window *Result*. The results will appear in a small window as a table, which can be copied to and further processed in Excel.
4. Calculate the percentage of each type of DNA track in Excel.
5. Close the image and open another one until you have scored at least 100 DNA tracks for the investigated treatment variant in a single experiment. Note: Repeat the experiment at least thrice and score at least 300 tracks per treatment for statistics.

Fig. 1 *Red tracks* represent the first pulse labeling with CldU detected by a specific primary antibody and a Cy3-coupled secondary antibody. *Green tracks* represent the second pulse labeling of IdU detected by a specific primary antibody and an Alexa488-coupled secondary antibody. Type 1 are replication forks, where the replication started at a certain origin and went on in both directions during the first pulse labeling with CldU and continued during the second pulse with IdU. Type 2 represents one-directional ongoing replication forks during both pulses. Type 3 are the stalled forks which incorporated CldU but the replication stopped before the second pulse with IdU. Type 4 are the sites where forks starting at neighboring origins of replication converge and the DNA synthesis in this section of the genome is completed. Type 5 may represent new origins of firing because no incorporation of CldU is observed; however, incorporation (DNA replication) begins during the second (IdU) pulse

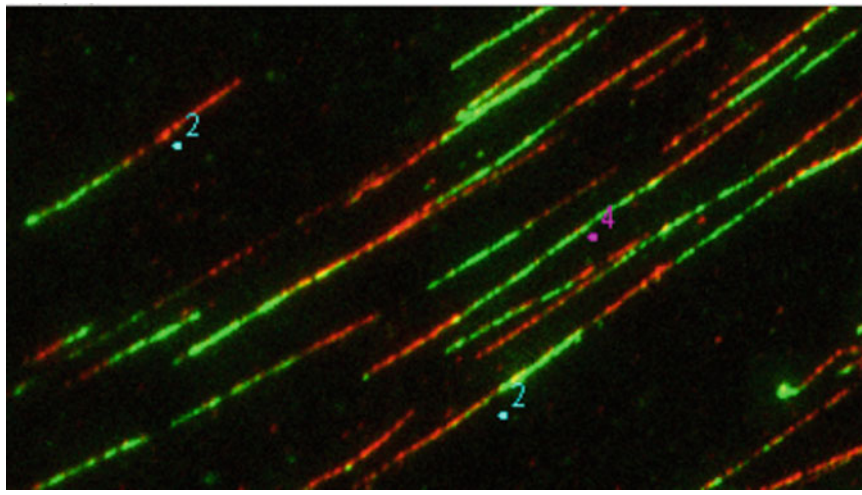

Fig. 2 HCT116 cells treated with the HDAC inhibitor 2 μM MS-275 for 24 h were incubated successively with 25 μM CldU and 250 μM IdU for 30 min. DNA fiber preparations were fixed and stained successively with a primary rat anti-BrdU antibody which recognized CldU and was detected by Cy3 anti-rat antibody *(red tracks)* and mouse anti-BrdU which recognized JdU and was detected by Alexa 488 anti-mouse antibody *(green tracks)*. The tracks were imaged by ZEN 2009 software of LSM 710. Replication tracks were classified according to the schemes in Fig. 1 as different types of replication forks using the Cell Counter function of the ImageJ software on exported in JPEG-format images

3.5.2 Measurement of the DNA Tracks

Measurement of the DNA replication tracks was performed as previously described [13, 14].

1. Use the original images in LSM format captured by the ZEN2009:
2. Open the images with the LSM Image Browser software which can be loaded from the Carl-Zeiss website.
3. Activate the *Overlay* function in the menu.
4. Activate in the expanded *Overlay* menu the *Line Drawing Mode* function by a left-button click.
5. Choose a track and drag a line with the mouse from the beginning till the end of the track.
6. Activate the *Measure* function by a left-button click.
7. A figure appears next to the line to show the length in μm (Fig. 3).
8. Repeat the measurement for the green part of the track (IdU pulse) and then subtract it from the first measurement to calculate the length of the red track (CldU pulse) in μm.
9. Insert the calculated lengths in a new Excel table.
10. *Note*: For quantification, measure at least 50 tracks per sample in a single experiment (i.e., at least 150 tracks in three experiments). If the expected effects are low, increase the number of measured tracks to 100 or more for a single experiment.

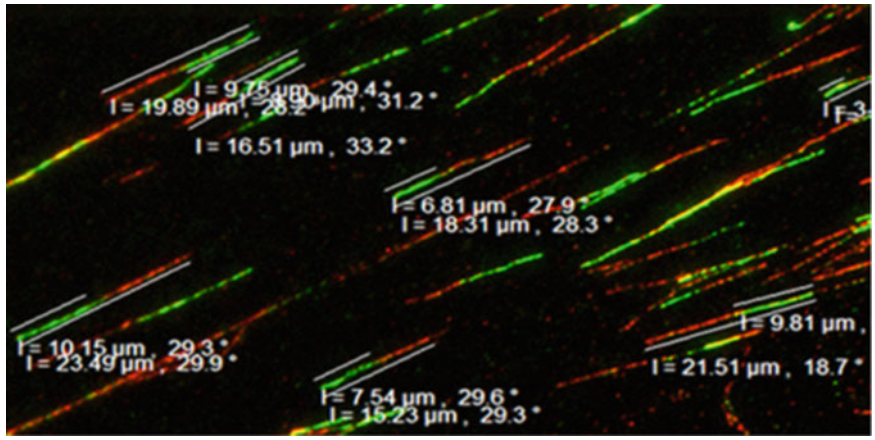

Fig. 3 *Green* and *red tracks* were measured on the original LSM images of the slides described in Fig. 2. The tracks were measured in µm by the overlay function of the LSM Image Browser software and the length was calculated in kb pairs/min (2.59 kb pairs = 1 µm)

11. Multiply the length of the tracks by a factor of 2.59 to calculate the length in kilobases (kb). Divide the results for the red and green track by the duration of the corresponding CldU, resp. IdU pulse in min (e.g., by 20 min). The quotients show the speed of the nucleotide incorporation during the corresponding pulse labeling in kb per min.
12. Compare the lengths of the green and red tracks in untreated cells and cells treated with a stalling agent from at least three experiments using a suitable statistical test.

4 Notes

1. Prepare methanol:acetic acid fixative each time fresh shortly before use.
2. Use BSA- and Tween-containing solutions within 2–3 days; they can be easily contaminated at RT.
3. Equilibrate thawed or stored at 4 °C buffers for 30–60 min at RT before use.
4. Prepare 2.5 mmol/L HCl fresh each time for optimal DNA denaturation.
5. Depending on the investigated issue, CldU can be added to the treatment medium containing the investigated agent (e.g., hydroxyurea).
6. Short-time treatments can be performed simultaneously with the IdU labelling (e.g., methyl methanesulfonate treatment for 40–60 min).

7. Protect the cells during and after the labeling with CldU and IdU from direct light (substituted with thymidine analogues DNA can undergo photolysis).
8. Work under dimmed light when spreading DNA onto slides (we use yellow light in our lab). If the drop cell suspension mixed with lysing buffer runs too slowly along the tilted slide or cannot start increase the angle.
9. Perform the washing steps in plastic cuvettes during denaturation and immunodetection in the StainTray under dimmed (yellow) light. Expose the slides as short as possible to the dimmed light. During the incubation with the antibodies, the slides must be strictly protected from light in the black StainTray.

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Chapter 9

Assessing the Effect of Class I Histone Deacetylase Activity on DNA Double-Strand Break Repair by Homologous Recombination

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Abstract

Here we describe the method used in our laboratory for determining the activity of homologous recombination repair of DNA double-strand breaks in cell lines. This plasmid-based method, first published by Pierce et al. 1999 from Maria Jasin's laboratory, is used along with flow cytometry for demonstrating the positive regulation of class I histone deacetylases on the repair of DNA double-strand breaks by homologous recombination.

Key words Class I histone deacetylases, Homologous recombination, Valproic acid

1 Introduction

In recent years, the importance of histone deacetylases (HDACs) on the regulation of DNA repair and the maintenance of genomic stability has come to light. Thus, it has been shown that inhibition of HDACs with the broad-spectrum phenyl hydroxamic acid HDAC inhibitor PCI-24781 decreases RAD51 expression [1]. RAD51 is involved in the homology search and strand invasion step of homologous recombination (HR) repair of DNA double-strand breaks (DSBs) [2] and, therefore, suppression of RAD51 expression by PCI-24781 leads to decreased HR repair and sensitization of cells to genotoxic insults [1, 3]. Furthermore, by using a siRNA approach, it was shown that the class II HDACs HDAC9 and HDAC10 are required for HR [4]. The class III HDAC SIRT1 has been ascribed a role in a sub-pathway of HR, namely the single-strand annealing pathway that functions in a RAD51-independent manner. SIRT1 stimulates single-strand annealing in a Werner helicase (WRN)-dependent manner [5]. As HR is required for maintaining the stability of the genome [6] and HDACs are deregulated in cancer [7], the influence of HDACs on HR that might occur during cancer transformation deserves further examination.

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Here we describe the method, first published by Pierce et al. [8], we use for qualitatively quantifying the error-free homologous-directed repair of DSBs by HR and the influence that class I HDACs have on this process. This method has been successfully employed in many studies dealing with the repair of DSBs by HR. It was used to show that ATM can be activated by a DSB in the absence of the MRN complex [9], that p53 prevents hyper-recombination by modulating BRCA1 function [10] and that the HDACs 9 and 10 are required for HR [4]. The method by Pierce et al. makes use of a two-plasmid based system. The first plasmid, pDRGFP, contains two modified nonfunctional green fluorescent protein (GFP) genes: one with the 18-bp 5′-TAGGGATAACAGGGTAAT-3′ recognition site of the rare cutting endonuclease I-SceI, the other truncated. A DSB induced by I-SceI in this DNA sequence can be repaired by HR using the truncated GFP gene. Upon successful repair by HR, GFP is expressed [8]. Therefore, pDRGFP serves as a target for DSB induction by I-SceI, a substrate for HR repair and as a reporter for HR activity. In order for this substrate to be used by the cell, stable integration of the pDRGFP plasmid into the host cell's DNA (chromatin) has to occur.

Therefore, the first part of the protocol describes how this cell line is generated (Subheading 3.1). The second plasmid, pCBASceI described in [11], contains the cDNA of the rare cutting endonuclease I-SceI. Transient transfection of the cell line containing the chromatin integrated pDRGFP substrate with pCBASceI, induces a DSB at the I-SceI recognition site, which can be repaired by HR. Upon successful repair by HR, GFP is expressed. The expression of GFP is then detected by flow cytometry (Subheading 3.2). Lastly, in Subheading 3.3 we describe how the experiment was performed for determining the contribution of class I HDACs on HR repair activity in the melanoma cell line D05.

2 Materials

The authors realize that most of the materials described in this section can be obtained from any provider. The inclusion of the provider in the text is specified where the preparation method we used is dependent on the composition of the manufacturer's product.

1. Puromycin stock solution: We prepare a 10 mg/ml puromycin stock solution in double distilled H_2O (ddH_2O). Dissolve 25 mg puromycin in 2.5 ml ddH_2O. Sterile filter the puromycin solution using a syringe filter with pore size 0.22 μm and aliquot 250 μl of the stock solution into sterile 1.5 ml Eppendorf tubes. Store at −20 °C until use.
2. Effectene® transfection reagent (Qiagen): We use the Effectene® transfection reagent because, in our hands, it always gives a high transfection efficiency.

3. The HR repair substrate and reporter are contained in the Addgene plasmid 26475 pDRGFP [8].
4. The expression vector containing cDNA of I-SceI is the Addgene plasmid 26477 pCBASceI [11].
5. Phosphate-buffered saline (PBS): We use PBS Dulbecco powder without Ca^{2+} or Ma^{2+} from Biochrom GmbH. Weigh 9.55 g powder and dissolve in 1 l ddH_2O. Autoclave to sterilize.
6. Valproic acid (VPA) stock solution: We prepare a 100 mM stock solution of valproic acid sodium salt by dissolving 1 g powder in 60.2 ml ddH_2O. Sterile filter the VPA stock solution using a syringe filter with pore size 0.22 µm, aliquot 1 ml into sterile 1.5 ml Eppendorf tubes and store at –20 °C until use.
7. Fixative (optional): Add 100 ml methanol and 100 ml acetic acid to 800 ml ddH_2O. Mix well and store at room temperature in a well-sealed bottle.
8. Staining solution (Optional): Add 10.5 ml Giemsa stock solution (Fluka Chemika) and 1.25 g crystal violet to 800 ml ddH_2O. Cover container with cling wrap (also known as plastic wrap, cling film or food wrap) place on magnetic stirrer and stir overnight. Add ddH_2O to a final volume of 1 L. Store at room temperature and protect from light.

3 Methods

The determination of HR repair activity by this method relies on generating a cell line containing a genome-integrated repair substrate and reporter. In this case the repair substrate is provided by the pDRGFP and the reporter is the HR repair-mediated activation of GFP expression. The methods section will therefore be broken into three sections: the first pertaining to the generation of the stable transfectants, the second pertaining to the flow cytometric analysis and the last where the influence of class I HDAC inhibition on HR is demonstrated.

3.1 Culture and Transfection

1. As pDRGFP contains the puromycin resistance (*pac*) gene, we first have to determine the puromycin concentration that is able to kill untransfected cells. Prepare ten 6 cm Petri dishes each containing 100,000 cells of your chosen cell line (we used the melanoma cell line D05) in 5 ml growth medium. Place them in an incubator overnight under normal growth conditions (generally 37 °C and 5 % CO_2).
2. The next day, add puromycin (10 mg/ml stock) to each Petri dish in order to obtain the following concentrations: 0 µg/ml, 0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml, 2.5 µg/ml, 3 µg/

ml, 3.5 μg/ml, 4 μg/ml, and 4.5 μg/ml. Place the Petri dishes back in the incubator and wait for colony formation. This can take up to 2 weeks, depending on the cell line (*see* **Note 1**). If the cells do not die, the concentration of puromycin can be increased up to 10 μg/ml.

3. Identify the dish where no colonies are present (*see* **Note 2**). This is the concentration of puromycin you will use in the transfection experiment.
4. On the day before transfection, plate out sufficient cells on a 6 cm Petri dish so that the next day they will be between 60 and 80% confluent. Place Petri dish back in incubator overnight.
5. Transfect 1 μg pDRGFP into cells using the Effectene® transfection reagent (Qiagen), according to the manufacturer's instructions (*see* **Note 3**). Place back in incubator for 8 h. Ensure that the Petri dishes are not moved during this time.
6. Following the 8-h incubation, the growth medium is replaced with fresh medium (**Note 4**). Place Petri dishes back in incubator for 48 h (*see* **Note 5**).
7. Re-passage (1:10) the transfected cells onto ten 6 cm Petri dishes, allow cells to settle, attach, and start cycling. Add puromycin (concentration determined in steps 1–3) and place back in incubator for 1–2 weeks. This gives puromycin time to kill the cells without the genome-integrated plasmid and provides sufficient time for the survivors to form colonies.
8. Pick up the surviving colonies and transfer them to a 24-well culture plate containing 1 ml culture medium per well and puromycin for selection (*see* **Note 6**). Remember to number the wells of the 24-well plate in order to keep track of which colony is in which well. Transfer the cells from a single colony into a single well. Be mindful not to mix the different colonies. Place the 24-well plates into incubator.
9. Those cells that survived the picking up process should now be given sufficient time to reach confluence in the 24-well plates. Once they are confluent, clones are serially expanded by transferring cells from each well to a 6 cm Petri dish. When the 6 cm Petri dishes are full, they are transferred to 10 cm Petri dishes, then to two 10 cm Petri dishes. Mark the Petri dishes with the corresponding numbers found on the 24-well plate. Keep on expanding the colonies until there are sufficient cells that can be tested for plasmid integration and for cryopreservation (*see* **Note 7**).
10. In order to test for the integration of the HR repair substrate (pDRGFP) into the genome of your cell line, you now have to induce a DNA double-strand break in this substrate and test for the activation of the GFP that occurs due to successful repair by HR. The repair substrate (pDRGFP) contains a cleavage site for the endonuclease I-SceI. We therefore transiently

transfect the clones with a plasmid containing the cDNA of this endonuclease, namely pCBASceI.

11. Plate out enough cells of the clone in a 6 cm Petri dish so that the next day they will be 50–60% confluent. Place clone in incubator overnight.
12. Using Effectene® transfection reagent (Qiagen) (*see* **Note 3**), we transfect 1 μg pCBASceI into the clone according to the manufacturer's instructions. Place Petri dish in incubator for 8 h (*see* **Note 4**), and then replace growth medium.
13. Incubate pCBASceI-transfected clones for 48–72 h (*see* **Note 8**). Analyze using flow cytometry.

3.2 Flow Cytometry

1. Live cells will be used for the analysis. After the 48- or 72-h incubation, cells are harvested, suspended in 500 μl phosphate-buffered saline (PBS), and transferred to a flow cytometry tube. Keep samples on ice till analysis.
2. The flow cytometer is set up to acquire the following parameters: forward scatter channel (FSC), side scatter channel (SSC), and the fluorescence channels FL-1 (excitation wavelength 488 nm and emissions wavelength 515–545 nm, green) for detecting the GFP signal and FL-3 (excitation wavelength 488 nm and emissions wavelength 670–735 nm, red) for detecting any change in auto-fluorescence (*see* **Note 9**).
3. We acquire at least 50,000 events for each sample (*see* **Note 10**).
4. In Fig. 1, results are presented for a positive clone. Results are presented as dot plots for FL-1 (GFP-A) on the *x*-axis and FL-3 (auto-fluorescence) on the *y*-axis. In the left panel, clone 25 pDRGFP was mock transfected (transfection that omits the pCBASceI plasmid). No GFP-fluorescence signal is detected in gate P1. In the right panel, clone 25 pDRGFP was transiently transfected with pCBASceI. Here a clear GFP-fluorescence signal was detected in gate P1. The reader will also observe an increase in auto-fluorescence caused by either the transfection or by the response of the cell to the induced DSB. The results show that this clone contains the repair substrate and that I-SceI induced a DSB that was repaired by HR.

3.3 Influence of Class I HDACs on Homologous Recombination

Results are presented (Fig. 2) showing the effect of class I HDACs, namely HDAC1, HDAC2, HDAC3 and HDAC8, on HR. Here the class I HDAC inhibitor VPA [12] was used. The melanoma cell line D05 clone 25 pDRGFP, generated in Subheading 3.1 and tested in Subheading 3.2, was treated with 1 mM VPA for 168 h. Medium was changed every 48 h and replaced with fresh medium containing VPA (1 mM). Following 144-h treatment, the cells were passaged for transfection in medium containing VPA (1 mM). On the day of transfection, VPA was removed by medium change

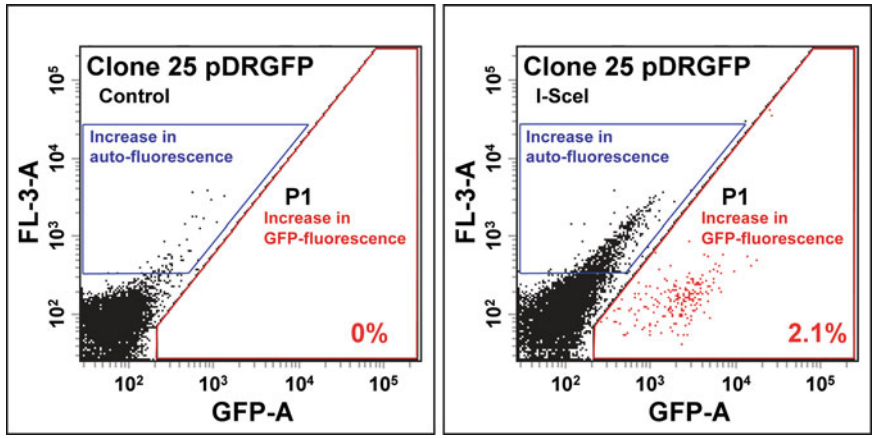

Fig. 1 The testing of cells for stable integration of pDRGFP into genomic DNA. Melanoma cells (D05) were transfected with pDRGFP and selected for stable plasmid integration with puromycin. Following clonal expansion, clone 25 was transiently transfected with pCBAScel to induce a DSB in the substrate provided by pDRGFP. Following a 72-h incubation, which allowed for sufficient time for DNA repair by HR, samples were prepared for flow cytometry analysis

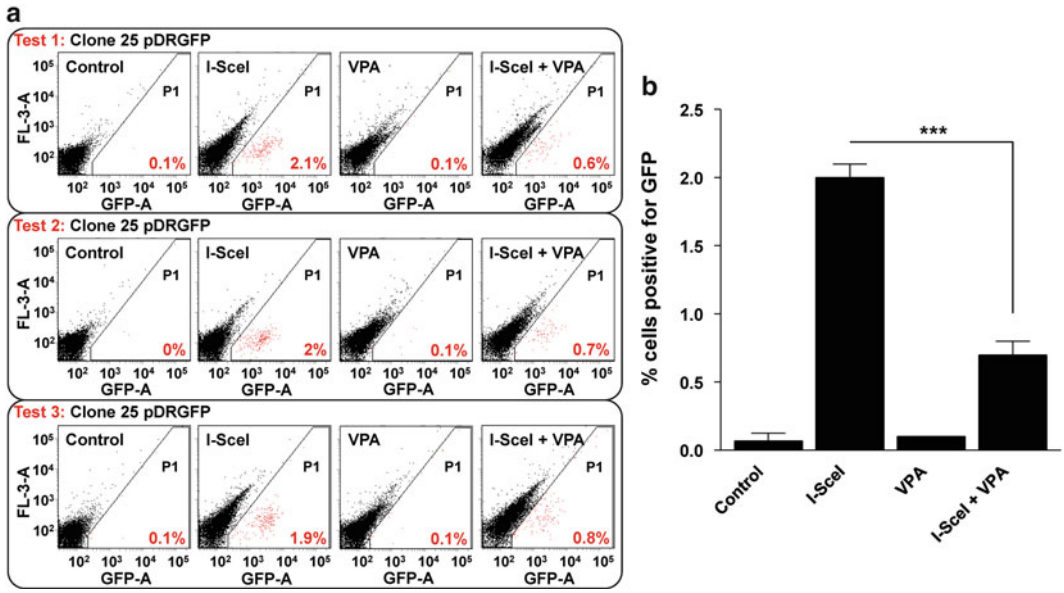

Fig. 2 Effect of HDAC inhibition on HR repair activity. (**a**) Three independent experiments, namely test 1, test 2, and test 3, show the effect of HDAC inhibition by VPA in the melanoma cell line D05 (D05 clone 25 pDRGFP). Cells were pretreated with 1 mM VPA for 168 h. Following pretreatment, VPA was removed by medium change and the cells were transiently transfected with pCBAScel. 72 h later, the cells were prepared for flow cytometry analysis. "Control" was mock transfected, "I-SceI" was transiently transfected with pCBAScel, "VPA" was subjected to the 168-h pretreatment, and "I-SceI + VPA" was subjected to the 168-h VPA pretreatment and then transiently transfected with pCBAScel. **(b)** Quantification and statistical analysis of results obtained in **(a);** $^{***}p < 0.001$

and the cells were transfected with pCBASceI. The cells were harvested 72 h after transfection and analysed by flow cytometry. Results from three independent experiments are shown (Fig. 2a). The GFP signal in the Control (Test 1: 0.1%, Test 2: 0% and Test 3: 0.1%) and VPA samples (Test 1: 0.1%, Test 2: 0.1% and Test 3: 0.1%) did not increase significantly above basal levels, showing that the DSB induced by I-SceI is required for the activation of GFP expression. The results obtained in the pCBASceI transfected samples (Test 1: 2.1%, Test 2: 2%, and Test 3: 1.9%) only varied slightly from each other, showing that this method provides reproducible results. The pCBASceI and VPA treated (Test 1: 0.6%, Test 2: 0.7%, and Test 3: 0.8%) cells showed a significant decrease in the repair of the I-SceI induced DSB by HR repair compared to control pCBASceI-transfected samples (Fig. 2a, b). From these results we can draw the conclusion that class I HDAC activity in the melanoma cell line D05 contributes to the DSB repair activity of HR.

4 Notes

1. Puromycin is known to kill cells within 2 days of exposure. However, from our experience this can take longer depending on the cell line. It is also important to note that the efficacy of antibiotics such as puromycin can differ greatly between providers and lot numbers. It is therefore good laboratory practice to retest your cell line as to its antibiotic susceptibility when new puromycin enters the lab. In this step we are also testing the capacity of the cell line to form colonies.
2. Identification of colonies can be accomplished by microscope or by carefully holding the Petri dish up to a light source and looking for colonies. If you are still not able to differentiate between dishes that contain surviving cells and those that do not, you can fix and stain the cells. To do this, remove medium and add 2 ml fixative to the dish. Fix for 30 min at room temperature, remove fixative, wait for dishes to dry completely, and add staining solution. Stain for 30 min, remove stain, and carefully rinse with water.
3. It is important to use a transfection method that has a high transfection efficiency while showing low transfection toxicity. In our hands we obtain the best results with Effectene®, although other groups have also had success with electroporation [8] or Lipofectamine2000 (Invitrogen) [13] as a transfection method.
4. We find that an 8-h transfection, followed by medium change, greatly reduces toxicity in transfected cells.

5. Integration of the foreign (plasmid) DNA into the genome of the cell occurs mostly in the S-phase of the cell cycle. 48 h is sufficient time for the cell to recover from the stress responses triggered by transfection, for the cell to start cycling, for plasmid integration to occur and for transcription and translation of the puromycin resistance gene.
6. We find that it makes the picking up of colonies easy if we first remove the dead cells by medium change. Following medium change, the colony positions are marked on the bottom of the Petri dish using a felt tip pen. Now, very carefully, the cells in the colony are scraped off using the tip of a pipet (200 μl) while simultaneously sucking up the scraped off cells into the tip. Use a new tip for every colony.
7. At this stage you can cryopreserve the clones in order to make the testing procedure more manageable.
8. Time may vary greatly depending on the growth rate of the cell line. Sufficient time should be allowed for I-SceI transcription and translation, cleavage of the substrate, repair by HR and transcription and translation of GFP. Please keep in mind that HR occurs predominantly in the S-phase of the cell cycle due to the regulation of this repair process by the S-phase cyclin-dependent kinase [14] and E2F1 transcription factor [15]. Therefore, for this assay to function, the DNA double-strand break should be present during the S-phase of the cell cycle.
9. Make sure that your manipulation of cells, whether it is manipulation by transfection, drug exposure or differential buffer use, does not cause a false positive in the signal you want to acquire with flow cytometry. False positives are often caused by changes in the cell leading to changes in auto-fluorescence. Changes in auto-fluorescence can normally be detected in all fluorescence channels. For this reason, we include the channel FL-3. For all intents and purposes, GFP (emission maximum 509 nm) does not give a signal in this channel (FL-3, emission wavelength 670–735 nm) and thus this channel only registers changes in auto-fluorescence.
10. The more events you acquire, the better your statistical analysis will be, i.e., the reproducibility becomes better with increasing events.

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Part II

Primary Cell Systems and In Vivo Models to Assess HDAC Functions

Chapter 10

Establishment and Characterization of Long-Term Cultures Derived from Primary Acute Myeloid Leukemia Cells for HDAC Inhibitor Research

Annette Romanski and Gesine Bug

Abstract

Histone deacetylase (HDAC) inhibitors are promising drugs. These agents lead to growth inhibition, cell cycle arrest, premature senescence, and apoptosis of malignant cells. Aim of our studies was to determine the efficacy of HDAC inhibitors on the clinically most relevant population of human leukemic progenitor cells in vitro. We here present stroma-free long-term cultures (LTC) of primary acute myeloid leukemia (AML) cells as a useful system for drug sensitivity testing in functional assays. AML-LTC are established by isolating mononuclear cells from peripheral blood samples of AML patients followed by selection of $CD34^+$ progenitor cells. AML-LTC cells can be maintained in liquid culture supplemented with cytokines and utilized for in vitro analyses to assess proliferation, apoptosis, expression of surface proteins or intracellular proteins and signal transduction, respectively.

Key words AML long-term culture, HDAC inhibitor, $CD34^+$ progenitor cells, Leukemia

1 Introduction

AML is an aggressive malignant disease which remains incurable in the majority of patients. As a clonal stem cell disorder, it is maintained by a hierarchy of leukemic cells that differ in their phenotypic characteristics and proliferation potential. Similar to normal hematopoietic stem cells, leukemic stem cells from all subtypes of AML reside mostly, but not exclusively, in the $CD34^+38^-$ population [1]. They are characterized by an infinite self-renewal potential and give rise to a population of extensively proliferating progenitor cells which produce the vast pool of incompletely differentiated blasts. Thus, the efficacy of any molecular therapy will ultimately depend on the treatment's ability to eradicate the leukemic stem and progenitor cell compartment. The most primitive AML progenitor cells detectable in vitro can give rise to colony-forming cells after several weeks of cytokine-dependent proliferation in suspension culture [2].

Oliver H. Krämer (ed.), *HDAC/HAT Function Assessment and Inhibitor Development*: *Methods and Protocols,* Methods in Molecular Biology, vol. 1510, DOI 10.1007/978-1-4939-6527-4_10,

Modifying the epigenome by inhibition of HDACs represents a novel approach in cancer therapy. There is increasing evidence that HDAC inhibitors are effective therapeutic agents in the treatment of a variety of tumors including malignant lymphoma and leukemia. Recently, the cinnamon acid derivatives vorinostat and panobinostat have been approved for clinical use in hematologic malignancies, i.e. cutaneous T-cell lymphoma and multiple myeloma, respectively [3, 4]. Both drugs induce the hyperacetylation of histones and are thus involved in chromatin remodeling, but they also affect transcription factors and other non-histone proteins, such as p53 or tubulin-α [5]. In AML, an aberrant recruitment of HDAC leads to the repression of genes that are critical for myeloid differentiation, apoptosis, and cell cycle regulation. Accordingly, HDAC inhibitors have been shown to reverse these leukemic phenotypes independently of the underlying genetic alteration [6]. However, most of these in vitro results have been obtained in leukemic cell lines which, due to extensive culture periods, tend to gain complex genetic aberrations and may no longer represent the clinical situation. In addition, leukemic cell lines mirroring unique genetically defined AML subtypes may not be available at all. To overcome these confinements and to allow for further analysis of the rare AML progenitor cells, we have established and characterized stroma-free LTC of genetically heterogeneous AML subgroups. We show that AML samples contain primitive leukemic progenitor cells that are capable of initiating stroma-free LTC in nearly half of the cases with extensive proliferative potential. These LTC differ in their susceptibility to the HDAC inhibitors vorinostat, panobinostat, dacinostat, and valproic acid. Thus, LTC are suitable in vitro models to comprehensively evaluate the effects of novel, HDAC inhibitor-based targeted therapies in primary AML cells.

2 Materials

2.1 Establishment of AML-LTC

1. Ficoll. Store at 4 °C (*see* **Note 2**).
2. PBS (sterile) (Thermo Fisher Scientific).
3. $CD34^+$ selection Kit (indirect CD34 MicroBead kit—Miltenyi Biotech: #130-046-701) containing: CD34-Hapten antibody, anti-Hapten microbeads, and FcR blocking reagent (please note: Here and elsewhere we name the provider, but we do not want to imply that material from other providers may equally be useful).
4. MACS cell separation columns for isolation (Miltenyi Biotech).
5. Turks solution (Merck).
6. Trypan blue solution (Thermo Fisher Scientific).
7. MACS buffer (sterile): PBS containing 0.5% BSA and 2 mM EDTA.

2.2 Culturing of AML-LTC

1. Wash medium (sterile): X-Vivo 10 medium containing 10% FCS Hyclone.
2. hTPO stock solution (sterile): 25 ng/μL in PBS. Please note for all cytokine stock solutions: Store aliquoted stocks at –20 °C. After thawing store aliquots at 4 °C for a maximum of 2 weeks.
3. hSCF stock solution (sterile): 50 ng/μL in PBS.
4. hFlt3-ligand stock solution (sterile): 50 ng/μL in PBS.
5. hIL3 stock solution (sterile): 20 ng/μL in PBS.
6. Culture medium for 1×10^6 AML cells (sterile, prepare always fresh): Take 4.5 mL of X-Vivo 10 (Lonza) and add 0.5 mL FBS Hyclone (final conc.: 10%), 50 μL L-Glutamine (final conc.: 1%), 5 μL hTPO (final conc.: 25 ng/mL), 5 μL hSCF (final conc.: 50 ng/mL), 5 μL hFlt3-ligand (final conc.: 50 ng/mL), 5 μL hIL3 (final conc.: 20 ng/mL).

2.3 Characterization of AML-LTC: Analysis of Phenotypically Different Leukemic Subpopulations

1. FACS buffer: PBS containing 1% FCS and 0.1% NaN_3.
2. Antibodies: CD45-FITC (BD Bioscience: #345808), CD11b-FITC (BD Bioscience: #562793), CD13-PE (BD Bioscience: #347406), CD34-APC (BD Bioscience: #345804), CD34-PE-Cy7 (BD Bioscience: #348811), CD34-PerCP-Cy5.5 (BD Bioscience: #347222), CD33-PE (BD Bioscience: #345799), CD33-APC (BD Bioscience: #345800), CD33-PE-Cy7 (BD Bioscience: #333952), CD33-PerCP-Cy5.5 (BD Bioscience: #333146), CD38-FITC (BD Bioscience: #340909), CD7-PE (BD Bioscience: #332774), CD7-APC (BD Bioscience: #653311), CD14-Pacific blue (BD Bioscience: #653311), CD15-FITC (BD Bioscience: #332778), CD65-FITC (eBioscience: #11-0659-42), HLA-DR-Pacific blue (Biolegend: #307624).
3. 7-Amino-Actinomycin (7AAD) (BD Bioscience: #559925).
4. FACS fixation buffer : PBS containing 1% paraformaldehyde.

2.4 Cell Sorting of $CD34^+$ AML-LTC (Re-enrichment of $CD34^+$ Cells from AML-LTC)

1. FACS buffer (sterile): PBS containing 1% FCS and 0.1% NaN_3.
2. FACS sorting solution (sterile): PBS containing 2 mM EDTA.
3. Wash medium (sterile): X-Vivo 10 containing 10% FCS Hyclone.
4. CD34-APC (BD Bioscience: #345804), CD38-FITC (BD Bioscience: #340909).

2.5 Effect of HDAC Inhibitors on AML Progenitor Cells

2.5.1 HDAC Inhibitors

1. VPA: 150 μg/mL in PBS (Orfiril®, Desitin Pharma). Store at –20 °C.
2. Dacinostat stock solution: 20 μM in DMSO (Novartis Pharmaceuticals). Store at –20 °C.
3. Panobinostat stock solution: 20–50 μM in DMSO (Selleckchem). Store at –20 °C.
4. Vorinostat stock solution: 2 mM in DMSO (Biozol). Store at –20 °C.

2.5.2 Western Blotting

1. Antibodies: anti-β-actin (Cell Signalling Technology: #4970), anti-acetyl-Histone-3 (Cell Signalling Technology: #9674), goat anti-rabbit secondary antibody (Jackson Immuno Research: #111-035-003).
2. 10×TBS: 1.5 M NaCl, 1 M Tris–HCl, pH 8.0.
3. TBS-T: 1×TBS containing 0.1% Tween 20.
4. Blocking solution: 5% low fat dry milk or 5% BSA in TBS-T.

2.5.3 Apoptosis: Annexin V Staining

1. Apoptosis detection kit (BD Bioscience): containing Annexin V binding buffer (10×) (BD Bioscience: #556454), Annexin V-APC (BD Bioscience: #550474) or Annexin V-FITC (BD Bioscience: #556419) and 7AAD (BD Bioscience: #559925). Take 500 µL 10× Annexin V binding buffer dissolved in 4.5 mL H_2O for 20 tubes (*see* **Note 17**).
2. Optional: CD34-APC (BD Bioscience: #345804).

2.5.4 Survivin Staining

1. FACS buffer: PBS containing 1% FCS and 0.1% NaN_3.
2. FACS fixation for intracellular survivin staining: Take PBS and add formaldehyde to obtain a final concentration of 3–4% (1 mL ad 10 mL).
3. 90% methanol (ice-cold). Store at –20 °C.
4. Incubation buffer (for intracellular staining): PBS containing 0.5% BSA.
5. Anti-CD34-PE (BD Bioscience: # 345802), anti-Survivin Alexa-Fluor 488 (Cell Signalling Technologies: #2810).

2.5.5 Detection of pSTAT3 in $CD34^+$ Cells

1. Medium for starvation: Take X-Vivo10 and add 1% L-Glutamine w/o FCS.
2. IFN alpha stock solution: 6×10^6 U/mL.
3. Reagents for stimulation of 2.5 mL cell suspension: 0.25 mL FCS Hyclone (final conc.: 10%) 2.5 µL hTPO (*see* Subheading 2.2, **item 2**) (final conc.: 25 ng/mL), 2.5 µL hSCF (*see* Subheading 2.2, **item 3**) (final conc.: 50 ng/mL), 2.5 µL hFlt3-ligand (*see* Subheading 2.2, **item 4**) (final conc.: 50 ng/mL), 2.5 µL hIL3 (*see* Subheading 2.2, **item 5**) (final conc.: 20 ng/mL), 4.15 µL IFN alpha (final conc.: 10,000 U/mL).
4. PBS (ice-cold). Store at 4 °C.
5. Paraformaldehyde (3.7%): Take 730 µL PBS ice-cold and add 270 µL Paraformaldehyde.
6. 90% methanol (ice-cold): Store at –20 °C.
7. Incubation buffer (for intracellular staining): PBS containing 0.5% BSA.
8. Antibody: anti-pSTAT3 (Beckman Coulter) (*see* **Note 26**).

2.5.6 CFU Assay, Replating Assay

1. Methylcellulose (Methocult® GF H4434—Stem Cell Technologies).
2. Medium: IMDM containing 10% FCS-Hyclone.
3. PBS (sterile).

3 Methods

3.1 Establishment of AML-LTC

3.1.1 Routine Clinical Evaluation of AML Samples

To set up AML-LTC for further functional assays (flow cytometry, Western blot, colony assays, qPCR, *see* Fig. 1), peripheral blood samples were obtained from AML patients at diagnosis after informed consent and with the approval of the ethics committee of the Goethe-University of Frankfurt am Main. Diagnosis and classification of the AML were based on the criteria of the French-American-British (FAB) group and WHO classification after routine evaluation of baseline morphology, cell surface antigen expression and cytogenetic as well as molecular genetic aberrations of the leukemic cell population in the bone marrow [7].

To give an example, we fully characterized the cells of patient FFM05. These cells have continuously proliferated and they have generated AML colony-forming cells in suspension culture for more than 3 years. To ensure that all of the proliferating progenitor cells are part of the malignant clone, the cytogenetic analysis was repeated after 65 weeks of LTC. We observed clonal evolution, but the initially described loss of 5q and chromosome 7 were still obvious. At the outset, the karyotype of FFM05 was given as:

43-45,XY,t(1;3)(q42;q27),del(5)(q14q33),-7,del(16)(q23),+Mar1,+Mar2;

and at week 65:

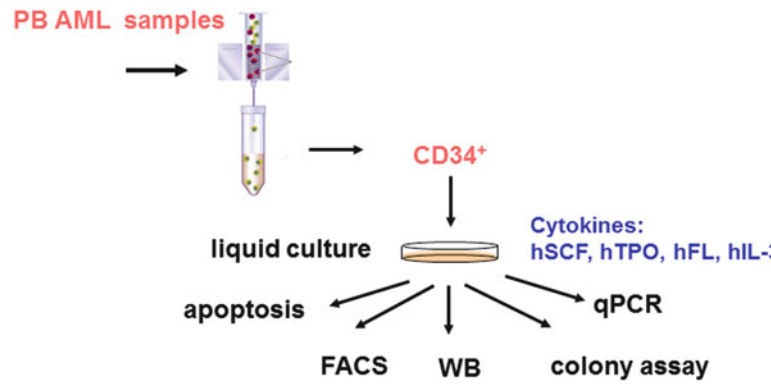

Fig. 1 Schematic overview of cell separation and functional analysis of patient-derived AML cells

44,XY,del(5)(q22q34),der(6)t(6;6)(p25;?)del(6)(q15q21),-7,i(8)(q10),der(12)t(7;12)(p15;p12)del(12) (q11q21), del(15)(q21q24),-16;

In addition, AML cells from patient FFM12 with the karyotype 45,XX,-7(12)/46,XX(8) were used for some analyses.

3.1.2 Isolation of Peripheral Blood Mononuclear Cells from AML Samples

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood samples by a density gradient centrifugation using Ficoll.

1. Mix blood samples collected in heparinized vials (*see* **Note 1**) with PBS in a 50 mL Falcon tube by gently inverting the tube several times.
2. Transfer 15 mL of Ficoll into a 50 mL centrifuge tube (*see* **Note 2**).
3. Gently layer the blood-PBS mixture on the top of Ficoll. The layering should be done very slowly so that blood and Ficoll remain in two different phases.
4. Centrifuge the tubes without any delay for 30 min at 250 × *g* at room temperature in a swing-out bucket rotor without brake (*see* **Note 3**). Fixed angle rotors can also be used but require more caution when separating cells in interphase.
5. After centrifugation aspirate the white layer of PBMCs in the interphase between Ficoll and medium without delay with 5-mL serological pipette (*see* **Note 3**) and transfer to a new 50 mL Falcon tube.
6. Wash the cells twice with 20 mL of sterile PBS and centrifuge with 250 × *g* for 10 min with brake.
7. Determine total cell number using Turks solution (*see* **Note 4**). For that dilute 10 µL of the cell suspension with 90 µL Turks solution and count the cells using a Neubauer chamber.

3.1.3 Enrichment of $CD34^+$ Stem and Progenitor Cells from PBMCs of AML Samples

1. Transfer up to 2×10^8 cells to a 50 mL Falcon tube, wash the cells once again with 20 mL sterile PBS and aspirate the supernatant completely.
2. Resuspend the cells in a final volume of 300 µL of MACS buffer (*see* Subheading 2.1) for up to 2×10^8 cells and mix by gently pipetting (*see* **Note 5**).
3. Perform magnetic labeling by adding 100 µL of FcR blocking reagent (component of Microbead kit) for up to 2×10^8 total cells and 100 µL of CD34-Hapten antibody (component of indirect CD34 Microbead kit). Mix the suspension well and incubate for 15–30 min at 4 °C.
4. Wash the cells by adding 20 mL of MACS buffer and centrifuge at 300 × *g* for 10 min.

5. Discard supernatant completely and resuspend cells in 100 µL anti-Hapten microbeads (for up to 2×10^8 cells). Again mix the suspension well, incubate for 15–30 min at 4 °C and wash the cells with 20 mL of MACS buffer with centrifugation at $300 \times g$ for 10 min.
6. Aspirate the supernatant completely and add 500 µL MACS buffer for up to 2×10^8 cells.
7. Pass the cells through a 30 µm nylon mesh to obtain a single-cell suspension and to remove cell clumps which may clog the column before magnetic labeling. For that moisten the filter with MACS buffer before use and rinse afterward with 1 mL MACS buffer (2–3 times).
8. Proceeding with magnetic cell separation: Choose an appropriate MACS column and MACS separator according to the number of total cells and the number of $CD34^+$ cells (*see* **Note 5**). Place the column in the magnetic field of a suitable MACS separator and prepare it by rinsing with 2 mL MACS buffer. Discard the flow-through of equilibration.
9. Apply labeled cell suspension onto the column (steps with 2 mL). Collect the flow-through containing unlabelled cells ($CD34^-$).
10. Wash the column 3 times with 2 mL MACS buffer and combine collected unlabelled cells that passed through with the flow-through ($CD34^-$ fraction).
11. Remove the column from the separator and place it on a suitable collection tube (capped FACS tube or 15 mL Falcon tube). Add 3 mL MACS buffer onto the column and immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column ($CD34^+$ fraction).
12. Take 10 µL of $CD34^+$ and $CD34^-$ cell suspension and count $CD34^+$ cells using trypan blue and $CD34^-$ cells using Turks solution (*see* **Note 4**).
13. The purity of the isolated hematopoietic progenitor cells can be evaluated by flow cytometry (*see* Subheading 3.3). Accomplish analysis of 2.5×10^5 $CD34^+$ cells by direct immunofluorescent staining using CD34-APC antibody recognizing an epitope different from that recognized by the CD34 monoclonal antibody of the CD34 Microbead kit and CD38-FITC.
14. Culture 1×10^6 cells as described in Subheading 3.2.
15. Freeze super numerous cells in N_2 with $2–4 \times 10^6$ cells per vial (*see* **Note 6**).

3.2 Culturing of AML-LTC

$CD34^+$ cells were maintained in liquid culture supplemented with interleukin-3 (20 ng/mL), thrombopoietin (25 ng/mL), stem cell factor and Flt-3 ligand (50 ng/mL each) for 7 days.

1. Transfer AML cells after CD34+ selection or cells for culturing in a 15 mL Falcon tube (up to 1×10^6 cells) and wash with 10 mL X-Vivo10/10% FCS.
2. Centrifuge ($300 \times g$, 10 min, room temperature) and prepare fresh culture medium (*see* Subheading 2.2).
3. Discard supernatant completely and resuspend AML-LTC cells in 5 mL fresh culture medium by gently mixing with a 5-mL serological pipette.
4. Transfer cell suspension to a 6-well plate and culture for 1 week (*see* **Notes 7** and **8**). Cells that were not used for culturing or in vitro analysis could be frozen in cryovials ($2–4 \times 10^6$ cells) for storage and further usage (*see* **Note 6**).

It is also possible to set-up AML-LTC from cryopreserved material (*see* **Note 9**).

To analyze the cytokine dependency of AML-LTC they have been cultured with different conditions under cytokine deprivation (*see* **Note 10**). We tested the dependency of AML-LTC on each of the cytokines hTPO, hSCF, hFlt3-ligand, and hIL3 separately and observed a slightly reduced proliferation and viability of FFM05 cells lacking hTPO, hSCF, and/or hFlt3-ligand. Nonetheless, the AML-LTC could be subcultured without these cytokines. In contrary, hIL-3 proved to be mandatory for the proliferation and viability of the AML-LTC, hIL-3 removal resulted in a complete abrogation of cell growth (data not shown).

For some AML-LTC it is possible to culture them directly after Ficoll separation without CD34+ selection (*see* **Note 11**), but as AML-LTC are not like cell lines, only low-scale expansion is possible (*see* **Note 12**).

3.3 Characterization of AML-LTC: Analysis of Phenotypically Different Leukemic Subpopulations

Similar to its normal counterpart, the malignant hematopoiesis is known to be hierarchically organized [8]. Primary AML samples consist of a small population of proliferating AML progenitor cells giving rise to the bulk of more or less differentiated blasts. We thus aimed to study if the hierarchical composition was retained by our AML-LTC and performed FACS analysis to determine different leukemic subpopulations.

1. For FACS analysis harvest 5×10^5 cells and wash with 2 mL FACS buffer (*see* Subheading 2.3).
2. Add 5 µL of the indicated antibody or isotype control (*see* Subheading 2.3) and incubate for 30–60 min at room temperature in the dark.
3. Wash the cells with 3 mL FACS buffer, discard supernatant and resuspend cells in 0.5 mL FACS fixation buffer to analyze them by flow cytometry (*see* **Note 13**).

The FFM05 was initiated with an 85% pure CD34+ population without coexpression of CD38. After 100 weeks of culture, four distinct subpopulations were detected (Fig. 2).

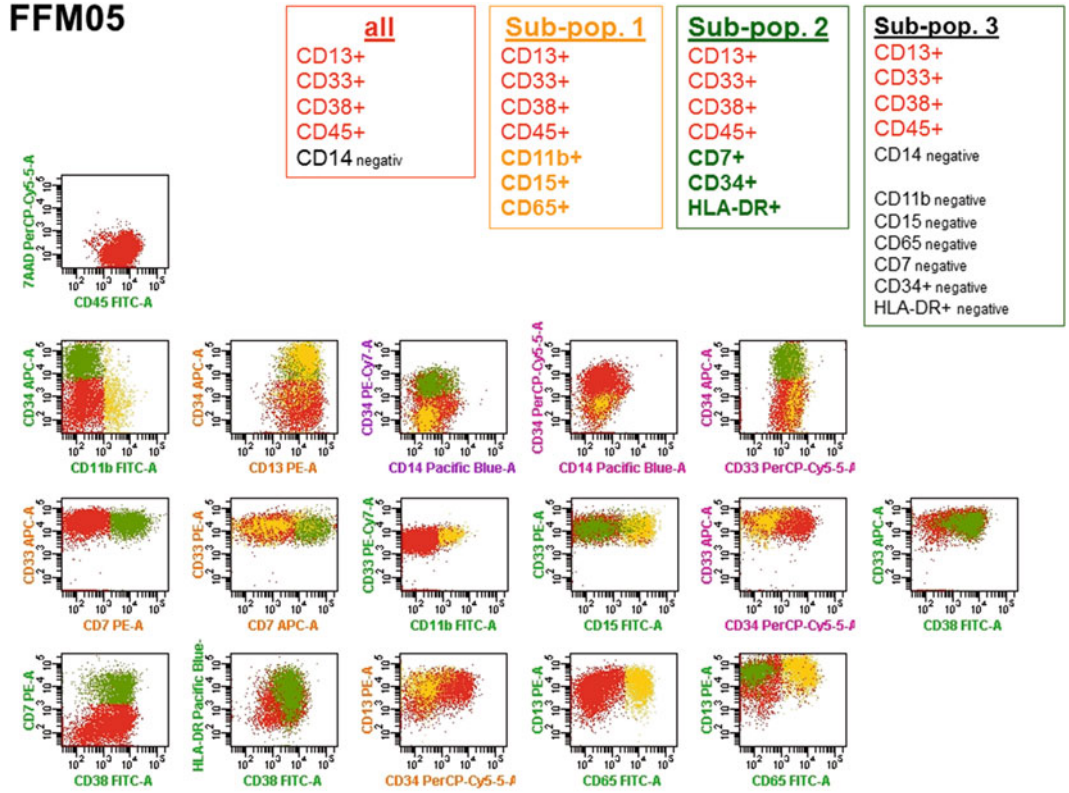

Fig. 2 Surface marker characterization of AML-LTC FFM05 is exemplarily shown. FFM05 cells were labeled with the indicated surface markers and analyzed by flow cytometry. The myeloid lineage markers CD33 and CD13 were detected on the surface of all progeny, and the majority of cells demonstrated additional expression of CD38 antigen. Subpopulation 1 (10–15 %) additionally expressed CD15 which is typically found on neutrophils as well as the myeloid and monocytic antigens CD11b and CD65. By acquiring additional granulocytic antigens, subpopulation 1 displayed a more mature phenotype suggestive of an intrinsic differentiation potential of FFM05. The most immature $CD34^+$ subpopulation 2 (35–40 %) aberrantly carries the T-cell marker CD7. Subpopulation 3 (30–35 %) can be distinguished from subpopulation 4 (10–15 %) via either positive visualization or lack of CD38 antigen expression, while both compartments (3 and 4) are consistently negative for the stem cell marker CD34 and the T-cell antigen CD7

3.4 Re-enrichment of $CD34^+$ Progenitor Cells from AML-LTC

In the course of AML-LTC, the $CD34^+$ and $CD34^-$ cells form an equilibrium after $CD34^+$ selection which seemed to be distinct and stable for each LTC, e.g. 60 % $CD34^+$ cells for FFM12 and approximately 80 % for FFM05. Thus, we also performed FACS sorting of primitive leukemic progenitor cells of AML-LTC. The total number of cells required depends upon the sample. For samples in which the desired population is rare, a higher number of starting cells and a longer sorting time is needed than for samples in which the desired population is relatively abundant.

1. Start with 2×10^6 AML-LTC cells in a capped tube and wash with sterile FACS buffer (*see* Subheading 2.4).
2. Add 5 µL CD34-APC or IgG-APC and incubate for 30 min at room temperature in the dark.
3. For washing use 2 mL sterile FACS buffer and centrifuge (10 min, $300 \times g$).
4. Discard supernatant and resuspend the cells in 1 mL FACS sorting solution (*see* Subheading 2.4 + **Note 14**).
5. Pass cells through a cell strainer to remove cell clumps for optimal performance to obtain a single-cell suspension before sorting. Humidify the filter with FACS sorting solution before use and rinse afterward with 0.5 mL FACS sorting solution.
6. To ensure that viable cells are analyzed or collected, a viability dye can be used. For example 7-AAD may be added to cells just prior to sorting.
7. Before collecting $CD34^+$ cells, use AML cells stained with IgG control to adjust the settings of FACS Aria (*see* **Note 15**).
8. Collect $CD34^+$ and $CD34^-$ cells of AML-LTC directly in X-Vivo 10 (*see* **Note 15**).
9. Wash the sorted cells with X-Vivo10/10% FCS and culture as described in Subheading 3.2.
10. Determine CD34 expression once a week using flow cytometry (*see* Subheading 3.3).

Established AML-LTC FFM12 contains approximately 60% of $CD34^+$ cells. With sorting, $CD34^+$ cells of FFM12 were enriched to 99% in the CD34-positive fraction and not detectable in the CD34-negative fraction (as detected directly after sorting by flow cytometry). Within 14 days FFM12 reached again the 60% of $CD34^+$ expression (Fig. 3).

3.5 Effect of HDAC Inhibitors on AML Progenitor Cells

HDAC inhibitor-induced hyperacetylation of histone H3 was examined by Western blot analysis.

3.5.1 Acetylation of Histone Proteins

Western blotting was done according to widely used protocols [9] with the indicated antibodies (*see* Subheading 2.5.2) [10, 11]. All antibodies were diluted in 5% low fat dry milk or 5% BSA. Blocking was performed in 5% low fat dry milk; washing was performed in TBS-T (*see* Subheading 2.5.2).

Figure 4 shows the effect of VPA, dacinostat, and vorinostat on cellular signalling of FFM05 cells after 24 h of treatment (Fig. 4). HDAC inhibitor treatment induced a dose-dependent hyperacetylation of histone H3 as proof of HDAC inhibitor activity.

3.5.2 Proliferation

AML-LTC (mostly FFM05 and FFM12) were treated with different HDAC inhibitors: valproic acid (VPA), dacinostat, vorinostat, or panobinostat (*see* Subheading 2.5.1). Trypan blue exclusion was used to determine proliferation and cell viability after HDAC inhibitor treatment at different time points (*see* **Note 16**).

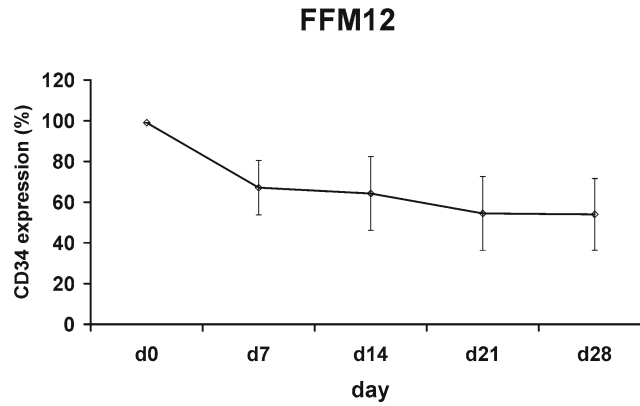

Fig. 3 CD34⁺ sorting with FACS-Aria and subculture of the CD34⁺ fraction of FFM12. CD34 expression of FFM12 was analyzed using flow cytometry once a week. After sorting, CD34 expressing cells decreased over time until a CD34⁺ cell content distinctive for each AML-LTC has been achieved, indicating that AML-LTC equilibrate the percentage of CD34⁺ and CD34⁻ cells. The content of CD34 positive cells of FFM12 proved to be about 60 %

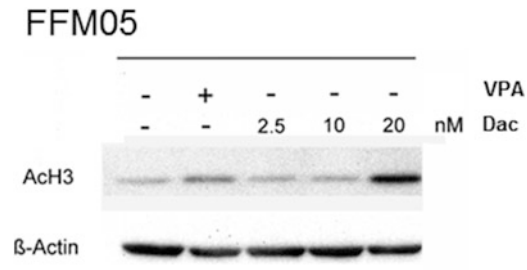

Fig. 4 Effect of HDAC inhibitors on cellular signalling of FFM05 cells. FFM05 cells were cultured ± valproic acid (VPA, 150 μg/mL) or dacinostat (Dac, in increasing concentrations as indicated) for 24 h and protein lysates were prepared. HDAC inhibitor treatment induced a dose-dependent hyperacetylation of histone H3 as proof of HDAC inhibitor activity. β-actin was used as a loading control

Figure 5 shows the proliferation kinetic of FFM05 approximating one log over 7 days. A dose-dependent inhibition of cell growth was observed in presence of panobinostat resulting in complete inhibition of proliferation at concentrations of 20 and 50 nM (Fig. 5).

3.5.3 Apoptosis: Annexin V Staining

We have previously shown that HDAC inhibitors induce apoptosis in leukemia cells [12]. Therefore, we analyzed the induction of apoptosis in AML-LTC after HDAC inhibitor treatment. One tool for identifying cells that are undergoing apoptosis is Annexin V. Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes [13, 14]. Therefore, staining with Annexin V is typically used in conjunction with a vital dye

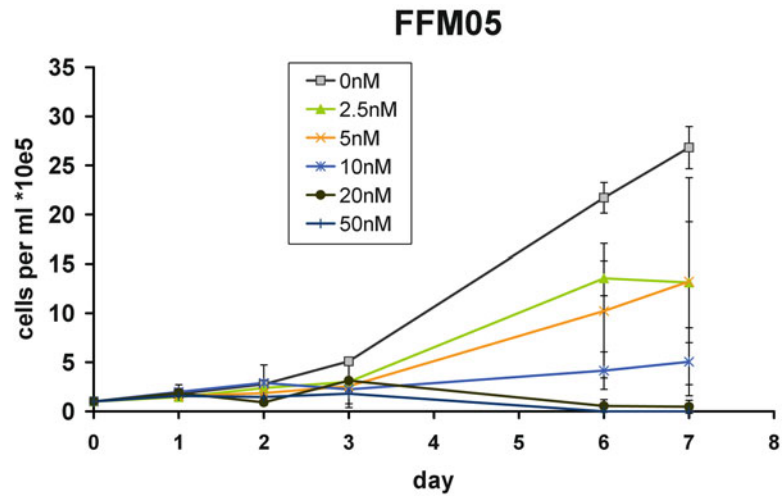

Fig. 5 Effect of HDAC inhibitor treatment on cell growth of FFM05. FFM05 cells were treated with the indicated panobinostat concentrations and proliferation was determined by trypan blue dye exclusion assay. A dose-dependent inhibition of proliferation was observed, with nearly complete inhibition using 20 and 50 nM of panobinostat

such as 7-AAD to allow the investigator to identify early apoptotic cells (7-AAD negative, Annexin V positive). Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD [13, 14].

Apoptosis of AML-LTC was quantified 48 h after HDAC inhibitor treatment using flow cytometry.

1. Transfer 1 × 105 cells to a FACS tube and wash twice with cold PBS (centrifugation: 300 × *g*, 10 min).
2. Prepare 1× Annexin binding buffer during centrifugation (*see* Subheading 2.5.3 + **Note 17**).
3. Resuspend the cells in 100 µL 1× Annexin binding buffer and add 2.5 µL of Annexin V and 2.5 µL of 7-AAD to each tube.
4. Following gently vortexing incubate for 15 min at room temperature (25 °C) in the dark.
5. Add additional 400 µL of 1× Annexin binding buffer to each tube and analyze the cells by flow cytometry within 1 h (*see* **Note 18**).

In addition, apoptosis can be determined in $CD34^+$ population (*see* **Note 19**). Differential effects of VPA and dacinostat on $CD34^+$ and $CD34^-$ AML progenitor cells could be observed [10]. VPA showed no effect on apoptosis in AML $CD34^+$ and $CD34^-$ cells, whereas dacinostat induced apoptosis about 1.5- and 1.8-fold in $CD34^+$ and $CD34^-$ cells [10].

3.5.4 Survivin Staining

Survivin, an inhibitor of apoptosis protein (IAP), is expressed in the G2/M phase of the cell cycle and tightly regulated during cell cycle [15, 16]. This suggests that survivin may counteract default induction of apoptosis. As survivin is localized intracellular, the cells have to be permeabilized before staining for survivin.

1. To determine survivin expression after HDAC inhibitor treatment harvest the cells (5×10^5) 48 h after treatment and wash with PBS.
2. Add 10 µL CD34-PE or isotype control and incubate for 30 min at room temperature in the dark.
3. Wash the cells with 5 mL FACS buffer and discard supernatant.
4. Resuspend cells in 0.5–1 mL FACS fixation for intracellular staining (*see* Subheading 2.5.4), centrifuge (300 × *g*, 10 min) and incubate for 10 min at 37 °C.
5. Chill tubes on ice for 1 min.
6. Remove fixation solution prior to permeabilization by centrifugation (300 × *g*, 10 min).
7. Resuspend cells by slowly adding of ice-cold 90% methanol to pre-chilled cells, while gently vortexing and incubate for 30 min on ice (*see* **Note 20**).
8. Immunostaining with survivin can be done directly or cells can be stored at −20 °C in 90% methanol for up to 1 week for further analysis.
9. For immunostaining wash the cells twice with 2 mL incubation buffer (*see* Subheading 2.5.4) and aspirate supernatant.
10. Resuspend cells in 100 µL incubation buffer and incubate for 10 min at room temperature.
11. Add 10 µL survivin antibody or IgG control and incubate for 30–60 min at room temperature in the dark.
12. Wash the cells with 2–3 mL incubation buffer and centrifuge (300 × *g*, 10 min).
13. Resuspend in 0.4 mL PBS and analyze by flow cytometer within 1 h.
14. Gate first on $CD34^+$ population to analyze survivin expression.

As shown in Fig. 6, dacinostat treatment resulted in a reduction of survivin in FFM05 cells whereas VPA did not alter the survivin level (Fig. 6).

3.5.5 Intracellular Signalling: pSTAT3 in $CD34^+$ Cells

For hepatocellular carcinoma, it was shown that pSTAT3 is downregulated by panobinostat treatment [17]. As STAT3 is a major downstream target of Janus kinase (JAK) in response to cytokines, growth factors, or hormone receptor signalling [18], the analysis of pSTAT3 was performed after HDAC inhibitor treatment of AML-LTC.

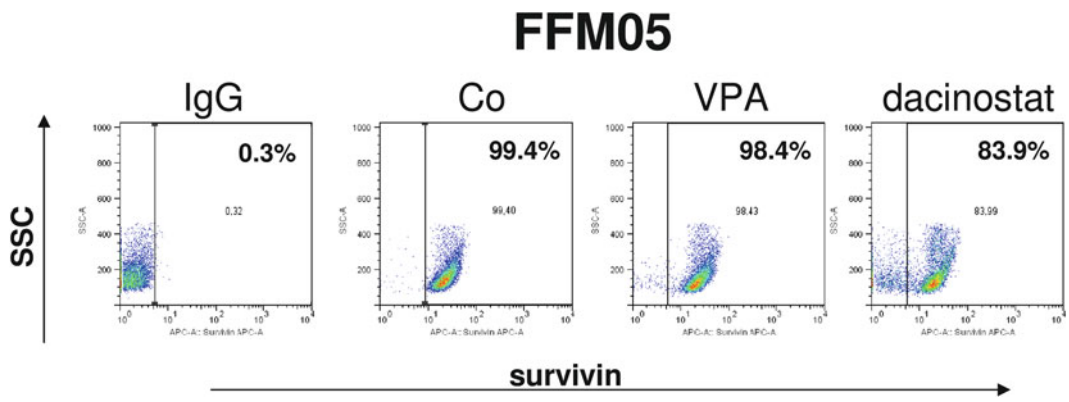

Fig. 6 Effect of HDAC inhibitor treatment on survivin expression in FFM05. FFM05 cells were treated with valproic acid (VPA, 150 μg/mL) or dacinostat (20 nM) for 48 h, and then intracellular staining of survivin was performed. While VPA had no effect, dacinostat treatment resulted in a reduction of survivin

1. To prepare the cells for the analysis of pSTAT3 activity transfer at a time twice 5×10^5 AML-LTC cells to 15 mL Falcon tubes.
2. Wash each tube with 5 mL PBS, centrifuge (300 × *g*, 10 min) and discard supernatant completely.
3. Resuspend 5×10^5 cells in 2.5 mL medium for starvation (*see* Subheading 2.5.5 + **Note 21**) and seed cells in a 24-well plate.
4. Treat with the indicated HDAC inhibitors and incubate for 24 h at 37 °C/5% CO_2.
5. For stimulation add FCS and cytokines to each well (*see* Subheading 2.5.5) and incubate for another 30 min at 37 °C/5% CO_2.
6. Transfer the cells to capped FACS tubes and centrifuge for 10 min at 300 × *g*.
7. Aspirate supernatant completely and resuspend the cell pellet in 730 μL ice-cold PBS.
8. For fixation add 270 μL 3.7% paraformaldehyde and incubate for 10 min at 37 °C.
9. Chill tubes on ice for 1 min.
10. Remove fixation solution prior to permeabilization by centrifugation (10 min at 300 × *g*).
11. Resuspend cells by slowly adding ice-cold 90% methanol to pre-chilled cells, while gently vortexing (*see* **Note 20**).
12. Incubate samples for 30 min on ice.
13. Immunostaining of pSTAT3 can be done directly or cells can be stored at −20 °C in 90% methanol for up to 1 week for further analysis.
14. Wash cells twice with 2 mL incubation buffer, centrifuge (300 × *g*, 10 min) and aspirate supernatant.

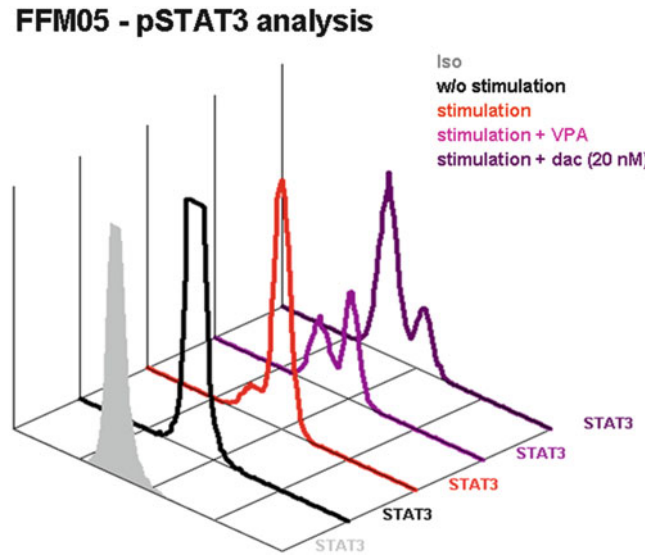

Fig. 7 Effect of HDAC inhibitor treatment on pSTAT3 regulation in FFM05. pSTAT3 is downregulated after valproic acid (VPA, 150 μg/mL) or dacinostat (Dac, 20 nM) treatment. The effect was obvious with VPA but stronger with dacinostat

15. Resuspend the cells in 100 μL incubation buffer and incubate for 10 min at room temperature.
16. Add 10 μL pSTAT3 antibody or IgG control and incubate for 30–60 min at room temperature in the dark.
17. Wash by centrifugation (300 × *g*, 10 min) with 2–3 mL incubation buffer and resuspend in 0.4 mL PBS. Analyze immediately by flow cytometry.

Dacinostat (20 nM) and VPA were added to monitor the signaling of pSTAT3 of FFM05 after HDAC inhibitor treatment. Both VPA as well as dacinostat lead to a reduction of pSTAT3 in FFM05 cells (Fig. 7) indicating the involvement of pSTAT3 in HDAC inhibitor-mediated signalling in AML cells.

3.5.6 Functional CFU and Replating Assay

To analyze the effect of HDAC inhibitors on stem and progenitor cells replating assays with semisolid medium can be used. For that cells should be harvested from suspension culture and plated in methylcellulose (*see* Subheading 2.5.6) treated with HDAC inhibitors.

1. Transfer 1 mL Methocult® GF H4434 slowly to a 15 mL Falcon tube by a 5 mL pipette (*see* **Note 22**). If necessary, add HDAC inhibitors (dacinostat, VPA, vorinostat, panobinostat) too.
2. Collect 0.1×10^5 AML cells by centrifugation (300 × *g*, 10 min) and aspirate supernatant completely.

3. Resuspend the cells in 100 µL IMDM/10% FCS and add cell suspension directly to the top of methylcellulose in a prepared 15 mL Falcon.
4. After mixing well by vortexing incubate at room temperature for 30–60 min to remove air bubbles (*see* **Note 23**).
5. Take the whole 1 mL Methocult with cells using 1-mL serological pipettes and transfer 3 times 300 µL in 3 wells (for one condition) of a 24-well plate. Slowly working allows avoiding air bubble formation (*see* **Note 23**).
6. To distribute the methylcellulose completely in the wells swing round the 24-well plate after transfer.
7. Add 1 mL H_2O in each of the surrounding wells to avoid drying-out of methylcellulose (*see* **Note 24**).
8. Culture 24-well plates for 10–14 days at 37 °C, 5% CO_2.
9. Count colonies (>20 cells) by microscopy after 12–14 days and replate, if possible.

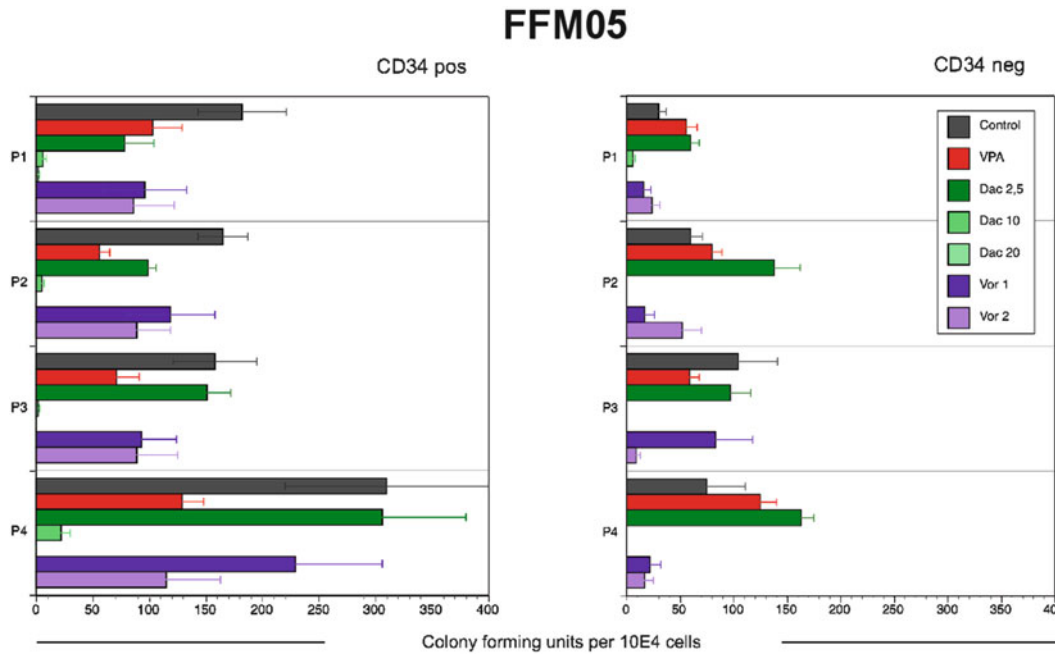

Fig. 8 Self-renewal of FFM05 is differentially influenced by HDAC inhibitors. Dose-dependent inhibition of colony formation by VPA, dacinostat (Dac), and vorinostat (Vor) is shown in $CD34^+$ and $CD34^-$ populations of FFM05. Both, $CD34^+$ and $CD34^-$ population, contain colony-forming cells and allow serial replating, but the number of colony-forming cells at each plating was significantly higher in the $CD34^+$ fractions compared to their $CD34^-$ counterparts. $CD34^+$ and $CD34^-$ FFM05 cells were separated using FACS cell sorting and plated in methylcellulose medium treated with the respective HDAC inhibitor (VPA (150 µg/mL), dacinostat (2.5, 10, 20 nM), vorinostat (1, 2 µM)) to assess colony formation. The colony number was counted after 12–14 days of culture. Cells were then harvested and serially replated. One representative experiment of three with similar results is shown

10. For replating rinse methylcellulose of one condition (3 wells) with 10 mL PBS and transfer to a 15 mL Falcon tube.
11. Centrifuge tubes for 10 min with 250 × *g* and aspirate supernatant with 10-mL pipettes.
12. Resuspend the cells in 1 mL sterile PBS and count cells using 20 μL cell suspension and 20 μL trypan blue.
13. For replating take 0.1×10^5 cells as described before using fresh methylcellulose treated with HDAC inhibitor if necessary (*see* **step 2** in Subheading 3.5.6). If no colonies are visible replate once again with all cells to determine if there are no cells/colonies at all. Replating efficiency has to be determined by serial plating and counting (*see* **Note 25**).

We asked, if differences in the CD34 phenotype are reflected in functionality and sorted the FFM05 according to their CD34 expression. Importantly, both populations ($CD34^+$, $CD34^-$) contain colony-forming cells and allow serial replating, an evidence for in vitro self-renewal. The frequency of colony-forming cells at each plating was calculated to about 1 in 100 cells with a significantly higher number in the $CD34^+$ fraction compared to their $CD34^-$ counterparts.

The impact of increasing concentrations of dacinostat and vorinostat has been studied in colony assays. A significant suppression of colony growth with 20 nM dacinostat and 0.5–2 μM vorinostat was shown. Among the $CD34^+$ cells, colony formation was significantly inhibited by 10 and 20 nM dacinostat, but not by vorinostat or VPA (except for the second plating, where VPA significantly reduced CFU, $p = 0.000$). In the $CD34^-$ fraction, very similar results with dacinostat and vorinostat were obtained. However, a significantly enhanced colony formation was obtained if $CD34^-$ cells treated with the lowest dacinostat dose of 2.5 nM during the first and second plating (30 ± 7 vs. 60 ± 8, $p = 0.008$ and 60 ± 11 vs. 138 ± 24, $p = 0.024$). Serial replating experiments were performed in presence of HDAC inhibitor supplemented to the methylcellulose (Fig. 8).

4 Notes

1. It is important to take blood samples in heparinized vials. Other anticoagulants such as EDTA or citrate proved to be not suitable for the collection of blood samples intended for the establishment of AML-LTC.
2. Ficoll is stored at 4 °C. Before use Ficoll needs to be kept at room temperature for 1–2 h to avoid aggregation of PBMCs when layered on pre-chilled Ficoll.
3. Ficoll separation should be centrifuged without brake to not disturb the PBMC layer in the interphase otherwise you would lose the cells. In addition, it is necessary to proceed directly

after centrifugation because PBMCs from the interphase will get disturbed and start settling down if the tubes are kept standing for more than 10 min.

4. Turks solution allows distinguishing nucleated and non-nucleated cells. Nucleated cells are stained with Turk and appear as dark cells, whereas erythrocytes are lysed in this solution. Trypan blue is a vital stain used to tint dead cells blue. Viable cells do not absorb trypan blue, thus, living cells with intact cell membranes are not dyed, whereas dead cells appear in blue color under a microscope. Mix 20 µL trypan blue with 20 µL cell suspension and analyze cell number by microscope.
5. It is important to use 300 µL MACS buffer for up to 2×10^8 cells, 100 µL blocking solution and 100 µL CD34 hapten antibody. For higher cell numbers, increase the volume accordingly. The various MACS cell separation columns are configured for specific cell numbers, i.e. $\leq 1 \times 10^7$ cells with MS column, $>1 \times 10^7$ to $\leq 1 \times 10^8$ cells with LS column, $>1 \times 10^8$ cells use more than one LS column—Miltenyi Biotech. Use the appropriate size for your isolation.
6. Freeze $2–4 \times 10^6$ cells per cryovial. After centrifugation (10 min, $300 \times g$) discard supernatant completely and resuspend $2–4 \times 10^6$ in 500 µL RPMI containing 20% FCS. Afterward add 500 µL RPMI containing 20% DMSO and transfer to a cryovial. Put cryovials immediately to a Mr. Frosty box and transfer the box to −80 °C for 1 day. At day 2 transfer cryovials to liquid N_2 for long-term storage.
7. In our hands, AML-LTC did not grow well in culture flasks. Thus, we prefer 12- or 6-well plates for long-term culture. For expansion it is better to seed cells in more wells in a 6-well plate than to transfer them to culture flasks.
8. It is not possible to establish AML-LTC of all primary AMLs. In our hands nearly 40% of primary AML samples generated AML-LTC which are defined by a culture period of at least 6 weeks.
9. Take out the cryopreserved vial of liquid N_2 at day 1. Transport on ice and thaw cells at room temperature. Prepare wash solution with 7 mL X-Vivo 10 medium with 1 mL FCS HyClone in a 15 mL Falcon tube. As far as the cells are thawed transfer those to the tube containing X-Vivo/10% FCS. Centrifuge ($300 \times g$, 10 min, room temperature) and prepare the culture medium (*see* Subheading 2.2). Aspirate wash solution completely and resuspend AML cells by gently mixing with 5 mL fresh culture medium. Transfer cell suspension to a 6-well plate and culture for 2 days. At day 3, count the cells using trypan blue staining (*see* **Note 4**). If viability of the cells is less than 50%, a density gradient centrifugation using Ficoll has to be performed (3 mL Ficoll + 10 cell suspension in 15 mL Falcon tubes, *see* Subheading 3.1.2, **steps 2–6**). If the viability is >50%, cells can be cultured as described in Subheading 3.2.

10. For the analysis of cytokine dependency, harvest and collect the cells by centrifugation (300×*g*, 10 min). Aspirate supernatant and resuspend AML-LTC cells in 5 mL X-Vivo10/10% FCS-Hyclone/1%L-glutamine without cytokines. Add only three out of the four cytokines hTPO, hSCF, hIL-3, and hFlt3-ligand (*see* Subheading 2.2) to determine the effect of cytokine withdrawal and culture the cells for 7 days. Determine proliferation and viability by counting the cells using trypan blue at day 2, day 4, and day 7. For further analysis of cytokine dependence leave out 2 or 3 cytokines and analyze the cells in the same way.
11. For some of the AML patients it was possible to establish AML-LTC from MNCs without $CD34^+$ selection, i.e. when CD34 expression was very low (<1%) or very high (nearly 100%). Without $CD34^+$ selection we observed some adherent cells in the beginning of the culture period, but we did not subculture these cells (as trypsin was not used) and after a few weeks the LTC did not contain adherent cells anymore.
12. AML-LTC are not like cell lines, only low-scale expansion is possible, if cells are available directly start the experiments for in vitro analysis.
13. For the analysis of surface markers using flow cytometry it is important to add FACS fixation after antibody incubation, because adding fixation before antibody will increase the unspecific binding of antibodies. But this is different for intracellular staining.
14. For cell sorting, the cells should be prepared in PBS containing 2 mM EDTA. For optimal performance, it is important to obtain a single-cell suspension before sorting and cells have to be passed through a cell strainer to remove cell clumps. The filter has to be humidified with buffer before use and rinsed afterward with 0.5 mL PBS/2 mM EDTA.
15. In addition, it would be the best to have unstained and IgG stained cells as a control for the adjustment of the sorting.

    For better recovery in culture after cell sorting fill the collection tubes with X-Vivo10-medium.
16. Proliferation and viability of cells can be determined using trypan blue solution (*see* **Note 4**). It is calculated as the number of viable cells divided by the total number of cells within the grids in the Neubauer chamber. If cells take up trypan blue, they are considered nonviable. To calculate the number of viable cells per mL of culture, the formula below was used and corrected by the dilution factor.

$$\%\ \text{viability} = \text{number of viable cells}\,(\text{unstained}) / \text{number of total cells}\,(\text{unstained} + \text{blue}) \times 100$$

17. For stable apoptosis measurements, it is required to prepare the binding buffer solution and staining solution fresh per working day during the wash of the cells. Do not use PBS for dilution of the binding buffer solution, but the recommended H_2O.
18. To avoid false-positive detection of apoptotic and necrotic cells, it is necessary to analyze the cells with flow cytometry within 1 h after application of 7AAD, because 7AAD is toxic to the cells and leads to false-positive results.

    Cells without HDAC inhibitor treatment were used to define the basal level of apoptotic and dead cells. The percentage of cells that have been induced to undergo apoptosis was determined by subtracting the percentage of apoptotic cells in the untreated population from the percentage of apoptotic cells in the treated population.
19. To quantify the number of apoptotic $CD34^+$ cells, 10^6 cells/mL were first labeled with APC-conjugated anti-CD34 antibody (*see* Subheading 3.3, 20 min incubation). After washing with PBS proceed with Subheading 3.5.3.
20. For permeabilization it is important to vortex the cells during dropwise addition of ice-cold 90% methanol, because this avoids clumps and allows good intracellular staining conditions.
21. To determine a stronger pSTAT3 signal, it is crucial to do the pre-incubation of the cells without FCS.
22. For replating assays, it is important to pipette the methylcellulose very slowly to transfer most of the material to the tube or well of the plate and not to lose half of the sample.
23. In addition, it is necessary to wait 30–60 min after adding of cells to remove the air bubbles. To remove the air bubbles faster, it is also possible to centrifuge the tube for 1 min at $150 \times g$. But for transfer of the methylcellulose containing the cells slowly working is essential otherwise you will add air bubbles again. These air bubbles would be handicaps for counting of the colonies using the microscope.
24. It is crucial to add H_2O or PBS in each of the surrounding wells in the 24-well plate to avoid drying-out of methylcellulose during 10–14 days of incubation.
25. In case no colonies could be determined replating should be repeated once more with all cells from methylcellulose to finish this series of experiments and to determine if colony-forming potential has completely ceased.
26. The pSTAT3 antibody we used is not available anymore at Beckman Coulter. So a different p-STAT3 has to be used for current analysis.

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Chapter 11

Assessing the Histone Deacetylase Activity of SIRT6 in Primary Murine Hepatocytes via Proximity Ligation Assay

Katharina Wolf and Susanne Strand

Abstract

Generation of primary cell culture of hepatocytes by mouse liver perfusion (MLP) combines the advantages of in vivo and in vitro models. It provides hepatocytes that grow under physiological conditions in mice, with the genotype of the whole organism or a specific gene knockout. In contrast to immortalized cell cultures, primary murine hepatocytes (pmHep) are non-cancerous cells with a limited lifespan but still amenable to classical in vitro methods such as treatment with drugs, small molecule inhibitors, and agonistic/antagonistic antibodies of surface receptors as well as transfection. One technique, which has gained popularity recently, is the analysis of protein–protein interactions by the proximity ligation assay (PLA). Here, we describe a liver perfusion protocol and the detection of the histone deacetylase function of Sirtuin 6 (SIRT6) using PLA in pmHep.

Key words Sirtuin 6, Histone deacetylase, Mouse liver perfusion, Primary murine hepatocytes, Proximity ligation assay, Protein–protein interactions

1 Introduction

The understanding of cellular processes and their modulation is required for the decoding of pathways involved in the development of diseases. Most of the cellular processes are controlled by protein–protein interactions or protein modifications, so new therapeutic approaches could arise by studying those under different conditions. Recently, a new method called the proximity ligation assay offers the possibility to detect proteins and their interaction partners or modifications based on immunofluorescent imaging techniques. Here, we describe this method and its advantages in detail using murine hepatocytes lacking the histone deacetylase SIRT6 as example. Therefore, pmHep gained by MLP [1] were seeded in an appropriate chamber slide and proteins of interest were detected by antibodies from different host species. These were recognized by secondary antibodies linked to oligonucleotides. If the proteins are

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in close *proximity* (<40 nm), bridging oligonucleotides build a DNA circle by hybridization and *ligation*. After rolling circle amplification (RCA) and labeling with fluorescent probes, the samples can be *assay*ed by fluorescent microscopy [2, 3]. An alternative route to study protein–protein interactions is, for instance, co-immunoprecipitation, in which a protein of interest and its interaction partners are affinity purified using appropriate antibodies and agarose or magnetic beads for capturing the antibodies followed by SDS-PAGE and Western blot analysis. An advantage of using PLA instead of immunoprecipitation is the detection of the subcellular localization of the interacting proteins as well as the possibility to quantify the interactions in a particular cellular compartment.

Sirtuin 6 is a NAD^+-dependent deacetylase, that also shows deacylase and ribosyltransferase activity [4–7]. It is expressed ubiquitously with a molecular weight of 37 kDa in mice and 42 kDa in humans. Its deletion induces a severe degenerative phenotype in mammals [8, 9]. The embryogenesis of SIRT6-deficient mice seems to be normal, but they start to develop a different outward appearance after 1 week: they look smaller and are less active. Three weeks after birth they exhibit kyphosis, osteopenia, colitis, hypoglycemia, hepatic steatosis, lower subcutaneous fat levels, lymphopenia, and they die by the age of 28 days [10]. This strong phenotype is thought to be related with the various functions of SIRT6: it shows all three enzymatic activities of the Sirtuin family and thus influences cell metabolism, DNA repair and telomere maintenance, inflammation, and cancer development [11]. Biomolecular analyses revealed increased acetylation of proteins, especially of lysine residues of histone H3 in absence of SIRT6—a strong indication for its epigenetic activity [12, 13]. We could show that epigenetic changes due to loss of SIRT6 may be relevant to chronic liver disease and development of hepatocellular carcinoma [14]. Likewise, the wide range of enzymatic activities of SIRT6 implies multiple protein–protein interactions with regulatory factors and histones [11]. A PLA of pmHep from SIRT6-deficient and their control mice could elucidate further targets of SIRT6 and thus expose new pathways in which SIRT6 could be involved. In our example, the differential acetylation of the lysine residues of the known SIRT6-target protein histone H3 was assessed (Fig. 1).

2 Materials

All solutions are prepared using ultra-pure water and analytical-grade reagents. In addition, reagents used for mouse liver perfusion and cell culture must be sterile. Store all buffers and solutions at 4 °C or follow the manufacturer's instructions.

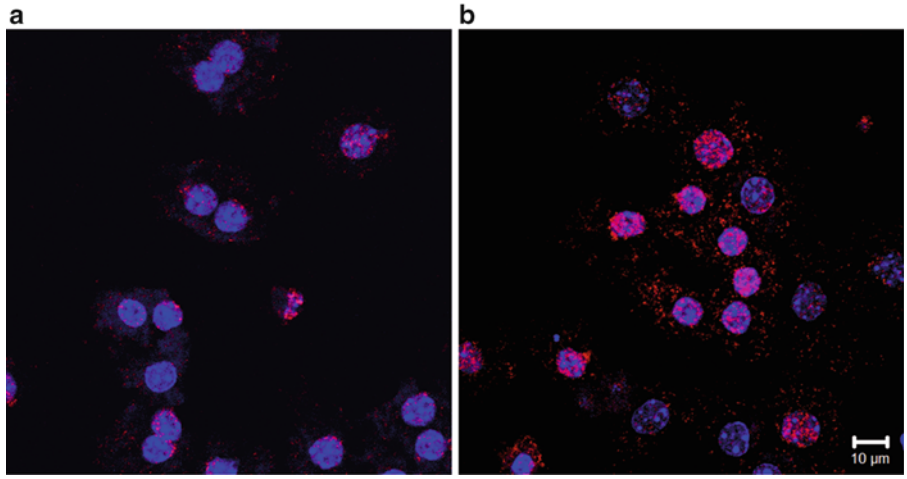

Fig. 1 Proximity ligation assay of acetylated histone H3 in pmHep of wild-type (**a**) and Sirt6-deficient (**b**) mice. Confocal laser scanning images of primary mouse hepatocytes on which a proximity ligation assay (*red*) was performed with a combination of antibodies to histone H3 and acetyl-lysine. DNA was stained with Hoechst33342 shown in *blue*. Images were captured using a Zeiss LSM710-NLO microscope equipped with a Plan-Apochromat 63×/1.4 oil objective. Samples were illuminated by a 405 nm Diode laser and the 543 laser line of the HeNe laser with a pinhole opened to 1.5 Airy units. Scale bar is 10 µm

2.1 Mouse Liver Perfusion

1. Collagen coating buffer: Dilute Collagen R Solution 0.2% (e.g., SERVA Electrophoresis GmbH, Heidelberg, Germany) 1:10 in water to cover the bottom of the tissue culture plate or slides in our case. The volume depends on the size and number of the tissue culture slides needed.
2. MLP washing buffer: 140 mM NaCl, 7 mM KCl, 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7.4. Weigh 8.3 g NaCl, 0.5 g KCl, and 2.4 g HEPES. Add 900 mL water and mix thoroughly. Adjust the pH to 7.4 with 5 M HCl and make up to 1 L with water. Filter the MLP washing buffer with a 0.2 µm filter.
3. MLP collagenase buffer: 67 mM NaCl, 7 mM KCl, 5 mM $CaCl_2$, 100 mM HEPES, 0.05% collagenase (e.g., Collagenase NB 4G Proved Grade from *Clostridium histolyticum*, SERVA Electrophoresis GmbH, Heidelberg, Germany) pH 7.6. Weigh 3.9 g NaCl, 0.5 g KCl, 0.7 g $CaCl_2 \times 2H_2O$ (*see* **Note 1**) and 24 g HEPES. Prepare buffer and adjust pH as in previous step. Shortly before starting the MLP add 50 mL of the MLP collagenase buffer to 25 mg of collagenase (0.5 mg/mL) for each mouse.
4. MLP dissolving buffer (*see* **Note 2**): 140 mM NaCl, 7 mM KCl, 1 mM $CaCl_2$, 10 mM HEPES pH 7.4. Weigh 8.3 g NaCl, 0.5 g KCl, 0.18 g $CaCl_2 \times 2H_2O$ and 2.4 g HEPES. Prepare buffer and adjust pH as in **item 2**.
5. Primary hepatocyte medium: DMEM with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1%l-glutamine, 20 mM HEPES, 1% sodium pyruvate.

6. Anesthetics: Mix Xylazin 1:5 with Ketamin. To inject *intraperitoneal* in mice, dilute the anesthetics 1:1 with a sterile, isotonic NaCl solution.

Assemble the mouse preparation setup, including a preparation pad (*see* **Note 3**) and a set of instruments (Fig. 2). Furthermore, a butterfly cannula, a non-slip, oblong object to fix the butterfly cannula (e.g., a rubber) (*see* **Note 4**), and thread (alternatively histoacryl tissue adhesive, *see* **Note 5**), will be needed for MLP. Place everything in a waterbath at 46 °C to prewarm the buffers, so that 37 °C is maintained at the site of MLP (*see* **Note 6**). A peristaltic pump is needed to flush the buffers through the liver. Therefore, we recommend using a platinum-cured silicone tube (wrapped around a glass beaker and laid into the waterbath, *see* **Note 7**) with the head of a sterile plastic pipette (2 mL) on the one end and the head of a single use syringe (1 mL, without the plunger) on the other end. Thus, the pipet end facilitates the suction of buffer out of a 50 mL tube and the single use syringe end can be inserted into

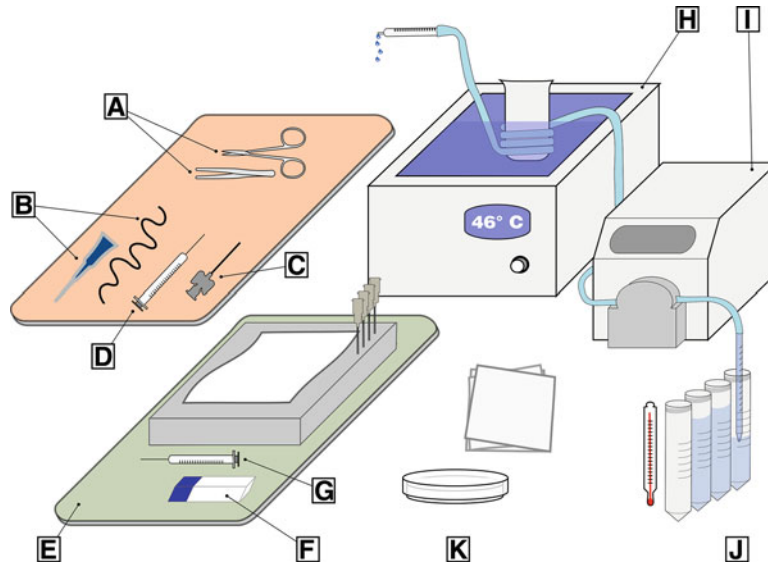

Fig. 2 Experimental setup for MLP. (**a**) Set of instruments (sanitized), e.g., forceps and scissors, (**b**) Thread (sanitized), about 10 cm and/or histoacryl tissue adhesive, (**c**) Butterfly cannula 22G, (**d**) Single use fine dosage syringe (1 mL) with integrated needle filled with the appropriate amount of anesthetics, (**e**) Preparation pad on an absorbent cover tissue with a paper tissue on *top*, (**f**) Non-slip oblong object to fix the butterfly cannula (e.g., a standard rubber eraser), (**g**) Single use syringe (1 mL) filled with MLP washing buffer, (**h**) Waterbath at 46 °C with a rack for 50 mL tubes to prewarm the buffers and a glass beaker with the tube wrapped around, (**i**) Peristaltic pump with the appropriate platinum-cured silicone tubes, (**j**) Rack with 50 mL tubes with water, ethanol, and the MLP buffers. Use a 15 mL tube as a mount for a thermometer, (**k**) Sterile 10 cm culture dish and gauze compresses for the dissolving of the pmHep

a butterfly cannula used for perfusion of the portal vein. Additionally, have a rack with 50 mL tubes of water, ethanol, and MLP buffers as well as a thermometer, sterile 10 cm dishes and wound fleece available. Standard cell culture equipment and medium are required to seed the pmHeps after MLP.

2.2 Proximity Ligation Assay (PLA)

1. Chamber slide (*see* **Note 8**) with pmHep (e.g., SIRT6-deficient and wild-type cells) at about 60–80% confluence.
2. Fixation and permeabilization reagents: 4% paraformaldehyde and 0.2% saponin in phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4. Thus weigh 8 g NaCl, 0.2 g KCl, 1.78 g $Na_2HPO_4 \times 2H_2O$, 0.27 g KH_2PO_4 and adjust to 1 L with dH_2O. Verify with a pH meter that the pH is neutral (7.4).
3. Primary antibodies validated for IF/ICC from different host species.
4. Duolink® Kit (OLINK Biosciences/Sigma-Aldrich): Make sure that the kit contains the appropriate PLA probes (e.g., Duolink® In Situ PLA® Probe Anti-Rabbit PLUS, Affinity purified Donkey anti-Rabbit IgG (H + L) and Duolink® In Situ PLA® Probe Anti-Mouse MINUS, Affinity purified Donkey anti-Mouse IgG (H + L)), the Duolink® In Situ detection reagents, the Duolink® In Situ mounting medium with DAPI, and the Duolink® In Situ wash buffers (*see* **Note 9**).

3 Methods

Carry out all procedures at room temperature unless otherwise specified (*see* **Note 10**).

3.1 Mouse Liver Perfusion

1. Prepare the collagen coating buffer and cover the bottom of the culture dishes. Shake them gently overnight at room temperature or incubate them at 37 °C for 2–4 h. Wash them twice with water, let them dry and sterilize them with UV light for at least 30 min (*see* **Note 11**).
2. Meanwhile, ensure that the experimental setup is ready-to-use and all MLP buffers are pre-warmed to 37 °C. Recheck the pH values of the respective buffers. Sterilize the MLP devices by circulation of ethanol for 5 min, followed by circulation of water and MLP washing buffer. Therefore, put the pipet and the syringe end in the same filled 50 mL tube.
3. Inject the anesthetics *intraperitoneal* depending on the size of the mouse (*see* **Note 12**). Fix the anesthetized mouse on the preparation pad and open the abdomen by cutting ventral from the groin to the chest in the form of the letter "V". Fix the abdominal opening and carefully move the bowel aside to

uncover the portal vein (*vena portae*). Take the thread with forceps and thread it below the portal vein. Insert the butterfly cannula in the portal vein without further disruption of the blood vessels (*see* **Note 13**). Then, tense the portal vein by *slightly* pulling on the lower end with a forceps. Fix the butterfly cannula, e.g., by laying the piece of rubber underneath. Now carefully wrap the thread around the part of the portal vein where the butterfly cannula is inserted and tie knot to fix it in the vein and create a seal.

4. Flush the butterfly cannula with a syringe filled with MLP washing buffer to eliminate air bubbles in the MLP system and put the syringe end of the tube into the butterfly cannula while leaving the pipet end in MLP washing buffer. Immediately, cut the *vena cava inferior* to create an efflux for the buffers.
5. Now, let 50 mL MLP washing buffer run through the mouse liver followed by 50 mL MLP collagenase buffer (*see* **Note 14**). The flow rate should be 20 mL/min for a tube of 16 mm diameter. No air bubbles should pass the butterfly cannula—if there are air bubbles in the tube, shortly pull the syringe end out of the cannula, when the air bubbles reach it, let them escape and reinsert the syringe.
6. After digestion, excise the liver and shake it in a 10 cm culture dish filled with MLP dissolving buffer by using tweezers. This allows the cells to detach from the remaining connective tissue. Resuspend the pmHep cells by pipetting up and down and filter the suspension through gauze into a sterile 50 mL tube.
7. Centrifuge the cells at 150 × *g* and 4 °C for 5 min. Suspend cells in an appropriate amount of fresh medium and plate them (*see* **Note 16**).

3.2 Proximity Ligation Assay

1. Plate 20,000–30,000 pmHep in each well of a collagen-coated 8-well chamber slide. Let them adhere ideally overnight.
2. Wash the cells carefully with 200–300 µl PBS (*see* **Note 14**), add 100 µl paraformaldehyde (4%) to each well, and incubate for 15 min. Wash the cells twice with PBS. The cells are now fixed by extensive cross-linking and should retain conformation through subsequent steps.
3. Add 100 µl Saponin (0.2% in PBS) to each well and incubate for 20 min to permeabilize the cell membrane.
4. Unspecific antibody binding can be avoided by usage of a blocking solution. Add one droplet of the blocking solution (*see* **Note 15**), which is part of the Duolink® In Situ PLA® Probe Kit, to each well. Put the lid on the chamber slide and wrap a flexible sealing film (e.g., Parafilm M®, Bemis NA, Neenah, WI, United States) around to seal it and prevent evaporation. Incubate the slide for 30 min at 37 °C.

5. Dilute the primary antibodies in antibody diluent (also part of the Duolink® In Situ PLA® Probe Kit) in a *user-defined* concentration (*see* **Note 16**). Prepare 100 µl for each well and apply it to the cells (*see* **Note 17**). Seal the chamber slide again and incubate it at 4 °C over night.
6. Pre-warm the humidity chamber (*see* **Note 18**) to 37 °C.
7. Wash the cells with buffer A of the Duolink® In Situ Wash buffers one time for 5 min. Now, remove the chamber from the slide following manufacturer's instructions. Encircle the single wells each with a PAP liquid blocker pen. Wash the slide again in buffer A for 5 min under gentle orbital agitation using a small jar.
8. Prepare 30 µl of the PLA probes 1:5 in antibody diluent for each well. Thoroughly eliminate the wash buffer. Place the slide in a pre-warmed humidity chamber and add the probes carefully to the sample. Seal the chamber and incubate it for 60 min at 37 °C.
9. Prepare the ligation mix by diluting the ligation stock 1:5 in ultra-pure water. Wash the slide two times with buffer A for 5 min. Add ligase at a 1:40 dilution to the ligation mix and add 30 µl to each well (*see* **Note 19**). Seal the chamber and incubate it at 37 °C for 30 min.
10. Prepare the amplification mix by diluting the amplification stock 1:5 in ultra-pure water. Wash the slide two times with buffer A for 2 min. Add polymerase at a 1:40 dilution to the amplification mix and add 30 µl to each well. Seal the chamber again and incubate it at 37 °C for 100 min in the dark.
11. If not using the Duolink® In Situ mounting medium with DAPI, stain the cells with Hoechst 33342 diluted 1:5000 in PBS for 15 min in the dark.
12. Wash the slide two times with buffer B for 10 min. Afterward, wash again with a 1:100 aqueous dilution of buffer B for 1 min. Aspirate the buffer and carefully dry the slide with a paper tissue without touching the cell layer. Let it air-dry in the dark for 15 min. Cover the cells with mounting medium and a coverslip (*see* **Note 20**).
13. Analyze the proximity ligation assay by laser scanning microscopy (*see* **Note 21** and **22**) (Fig. 1).

4 Notes

1. Be aware that there is also calcium chloride anhydrous, monohydrate, tetrahydrate, and hexahydrate with divergent molecular masses available. We used calcium chloride dihydrate to prepare the MLP collagenase/dissolving buffer or adjust the mass accordingly.

2. MLP dissolving buffer can be replaced by primary hepatocyte medium if needed.
3. We use s rectangular piece of styrofoam (e.g., the lid of a transport box) covered by aluminum foil with a paper tissue on top and four needles to fix the mouse on the pad.
4. Any non-slip object with similar proportions (±5 cm × 2.5 cm × 1 cm) can be used.
5. If the mouse is extremely small, it can be difficult to tie the butterfly cannula in the portal vein with thread. Instead, one can use a histoacryl tissue adhesive but one has to pay attention to not block the vein.
6. In our experience, the buffer reaching the mouse liver through the tube has body temperature if the water bath is warmed to 46 °C. Use a thermometer to verify the final temperature.
7. It is important that the buffer does not run too shortly through the warm water bath, so making a spiral of the tubing will help. If there is no appropriate commercial device available, we suggest a glass beaker with the tube wrapped around it. Make sure that the tube is not blocked at any time.
8. A broad range of sample types can be used for PLA, e.g., adherent tissue culture cells, cytospin preparations, and tissue sections. For adherent cells (e.g., pmHep), we recommend the use of eight chamber tissue culture slides from BD Falcon or 18-well slides from ibidi.
9. It is absolutely essential for the proximity ligation assay that the primary antibodies are from different host species and that one of the equivalent PLA probes is MINUS, the other one PLUS. The Duolink® In Situ Mounting Medium with DAPI can be replaced by usual DNA staining and other available mounting media.
10. Vortex all solutions slightly before you use them. Let the anesthetics and all buffers adapt to room temperature and check for precipitations. If precipitations (of, e.g., Duolink® In Situ Wash buffer A) persist after adapting to room temperature, filter the Wash buffer. For the proximity ligation assay (Duolink® from OLINK Biosciences sold by Sigma-Aldrich) consider their instructions additionally.
11. Storage of Collagen R covered tissue culture plates is possible. Place them back into the original covering material and store them at room temperature.
12. It is recommended to test the correct amount of anesthetics for the particular mouse strains used in advance, so that the mouse is in deep anesthesia as long as needed for the complete perfusion. It is important to keep the mouse alive to ensure blood flow. Start with a minimum of 20 μl of diluted anesthetics per gram body weight.

13. The vascular system of the perfused mouse has to be completely intact to enable MLP. Disruption of blood vessels or blockage by blood clots must be avoided.
14. A good indicator for a successful MLP and the integrity of the vascular system is the change in color and volume of the liver: During the washing procedure, the mouse liver should swell and become pale (as blood is washed out). When the collagenase buffer is digesting the liver, the liver should become smaller and mucilaginous.
15. PmHep cells are very sensitive cells, especially after the stressful perfusion. Avoid rough vortexing or fast centrifugation. During PLA washing procedures, aspirate and replenish the wells setting the pipet tip in the edge of the well and pipette slowly, do not directly splash the buffer on the cell layer.
16. The blocking solution and antibody diluent are part of the Duolink® In Situ PLA® Probe Kit, but also self-made solutions can be used. The agents used for blocking should also be contained in the antibody and PLA probe diluent.
17. We strongly recommend testing the primary antibodies before the PLA in immunofluorescence staining. Test different antibody concentrations starting with 1:100–1:200 and incubation temperatures and times. Also consider manufacturer's instructions. Do not forget to incorporate controls during PLA (e.g., a positive control of proteins already known to interact as well as negative controls with only one of the primary antibodies or using cells overexpressing or not expressing one protein of interest).
18. The samples should not dry out during the staining process.
19. We use a humidity chamber with several compartments, putting wet paper tissue in the unused compartments.
20. Set the ligase and the polymerase in a freezing block when removing them from the freezer.
21. We recommend sealing the edge of the coverslip with clear nail polish, especially if the slide should be analyzed directly after the mounting. Avoid or diminish air bubbles as they will disturb the imaging.
22. When performing microscopy look at several different areas or positions of the sample as we have occasionally observed an uneven signal distribution over the slide.

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Chapter 12

Purification and Analysis of Male Germ Cells from Adult Mouse Testis

Thierry Buchou, Minjia Tan, Sophie Barral, Anne-Laure Vitte, Sophie Rousseaux, Juan Arechaga, and Saadi Khochbin

Abstract

Isolation of pools of spermatogenic cells at specific developmental stages is essential for the investigations of molecular events controlling critical transitions during spermatogenesis. Large-scale cell purification techniques allow for combined proteomics, genomics, and transcriptomics studies. Herein, we describe a procedure for the purification of meiotic and post-meiotic male germ cells from adult mouse testes. We also describe how the fractionated cell populations could be used for further studies. In our laboratory, these protocols are routinely used to specifically investigate the molecular basis of histone acetylation/acylation-driven epigenetic programming.

Key words Mouse testis, Male germ cell, Haploid post-meiotic spermatid

1 Introduction

One of the most dramatic genomic reorganizations and chromatin remodeling processes takes place after the commitment of progenitor spermatogenic cells, spermatogonia, into meiotic and post-meiotic differentiation [1]. This transition is characterized by chromosome-wide and genome-scale exchange of histones by specific variants and massive histone posttranslational modifications [2, 3]. At post-meiotic stages haploid cells undergo a general transcriptional activity shutdown associated with histone hyperacetylation and a genome-wide histone replacement by non-histone basic proteins [4]. These dramatic events are driven by factors encoded by genes that are parts of male-specific gene expression programs that are activated in a stage-specific manner [5]. Histone acetylation as well as other lysine acylation events appears to be not only determinant elements in controlling these gene expression programs but also essential in mediating the final metamorphosis of a nucleosome-based genome organization into a new type of genome packaging based on non-histone proteins [2, 6].

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Although these genome reorganizations are essential for the life cycle and procreation, almost nothing is known on the underlying molecular basis [7]. The fact that testis is formed of a variety of very different cell types including somatic type cells and the absence of satisfactory in vitro or ex vivo systems make these studies particularly challenging.

In our laboratory we are using the sedimentation based spermatogenic cell fractionation procedure and have adapted chromatin, RNA, protein and histone extraction protocols to these isolated cell populations to characterize at large-scale all the molecular events that drive critical genome organization transitions and programing. They include the mapping of histone posttranslational modifications (PTMs) and histone variants on chromatin, as well as analyzing gene expression and protein complexes.

Detailed protocols are provided here that aim at enabling interested investigators to fractionate dissociated spermatogenic cells from adult mouse testes and to proceed with the molecular analyses.

2 Materials

2.1 BSA Gradient

1. Fluorinert-FC77 (3M, ZV-0170-0734-5).
2. Na Butyrate 2 M: 4 ml n-butyric acid, 8 ml NaOH 5 N, 10 ml 1× PBS, keep at 4 °C.
3. BSA 4%: 8 g/200 ml of DMEM/F12 medium, 2%: 4 g/200 ml of DMEM/F12 medium, and 0.5%: 1 g/200 ml of DMEM/F12 medium; dissolve at room temperature using magnetic stirring and keep in cold room.
4. Collagenase solution: 10 mg of collagenase in 10 ml 1× PBS, final concentration 1 mg/ml.

2.2 Chromatin Immunoprecipitation (ChIP)

1. Lysis buffer: Tris–HCl pH 7.4 15 mM, KCl 60 mM, NaCl 15 mM, sucrose 0.34 M, EDTA 2 mM, EGTA 0.5 mM, spermidine 0.65 mM, dithiothreitol 1 mM, Triton X-100 0.03%, glycerol 1%, "cOmplete, EDTA-free" Protease Cocktail Inhibitor according to manufacturer (Roche, 05056489001).
2. Wash buffer: Tris–HCl pH 7.4 15 mM, KCl 60 mM, NaCl 15 mM, sucrose 0.34 M, spermidine 0.65 mM, dithiothreitol 1 mM, Protease Cocktail Inhibitor.
3. Micrococcal nuclease (MNAse) buffer: Tris–HCl pH 7.5 10 mM, KCl 10 mM, $CaCl_2$ 2 mM.
4. Micrococcal nuclease (S7 nuclease Roche; 10107921001; 15,000 U dissolved in 3 ml Tris–HCl pH 7.5, NaCl 100 mM, glycerol 50%; keep at −20 °C).
5. LSDB500 buffer: Hepes 50 mM pH 7, $MgCl_2$ 3 mM, KCl 500 mM, glycerol 20%, Protease Cocktail Inhibitor.

6. Dynabeads protein G (Invitrogen, 10003D).
7. LSDB350 buffer: Hepes 50 mM pH 7, $MgCl_2$ 3 mM, KCl 350 mM, glycerol 20%, Protease Cocktail Inhibitor.
8. Elution buffer: Tris–HCl 10 mM pH 8.5, EDTA 1 mM.

2.3 RNA Purification

1. TRIzol Reagent (Ambion/Invitrogen, 15596026).
2. RNeasy Mini Kit (Qiagen, 74104).

2.4 Histone Extraction

1. Extraction buffer: HEPES 10 mM pH 7, KCl 10 mM, $MgCl_2$ 1.5 mM, sucrose 0.34 M, Nonidet P-40 0.5%, Protease Cocktail Inhibitor, Na Butyrate 2 mM, and nicotinamide 5 mM.
2. Wash buffer: HEPES 10 mM pH 7.9, KCl 10 mM, $MgCl_2$ 1.5 mM, sucrose 0.34 M, without Nonidet P-40, Protease Cocktail Inhibitor, Na Butyrate 2 mM, and nicotinamide 5 mM.

2.5 Pull Down

1. LSBD250 buffer: Hepes 50 mM pH 7, $MgCl_2$ 3 mM, KCl 250 mM, glycerol 20%, Protease Cocktail Inhibitor.
2. LSBD0 buffer: Hepes 50 mM pH 7, $MgCl_2$ 3 mM, glycerol 20%, Protease Cocktail Inhibitor.

3 Methods

3.1 Fractionation of Male Germ Cells by Sedimentation on a BSA Gradient

3.1.1 Preparation of Apparatus/Solutions

1. In order to ivnhibit Histone DeAcCetylase (HDAC) activities, add Na Butyrate at a final 10 mM concentration in all solutions.
2. In the cold room, fill up the bottles with Fluorinert-FC77, up to a volume greater than 800 ml each. Assemble the chambers, tighten the screw diagonally on the lid with the bottom chamber (not too tight), install the soft tube with stopper on the top, and the hard tube at the bottom. Place the chambers in the cold room horizontally. Place the stirrer, two graduated cylinders connected with the tubes, and a syringe in the cold room. Place the second cylinder on the stirrer. Make sure the stirrer bar works, fix the position with tape. Place the first cylinder on a platform to adjust the height. Close the gate of the tube on first cylinder. Cover the top of cylinders with Parafilm. Take the tube from the bottom of the chamber into a bottle of FC77. Make sure that the tip is reaching to the bottom of the bottle. Fix the position of the tube with tape. Cover the top of the bottle with Parafilm. Label (1–36) 36×15 ml *Falcon* tubes. Remove the cap and keep them.
3. Prepare BSA 4%, 2%, 0.5%, and 1 liter 1× PBS. These solutions are kept at 4 °C.
4. Weight Collagenase in 50 ml *Falcon* tube and keep at –20 °C.
5. Prepare 36 slides aligned (twice as many if two chambers are used).

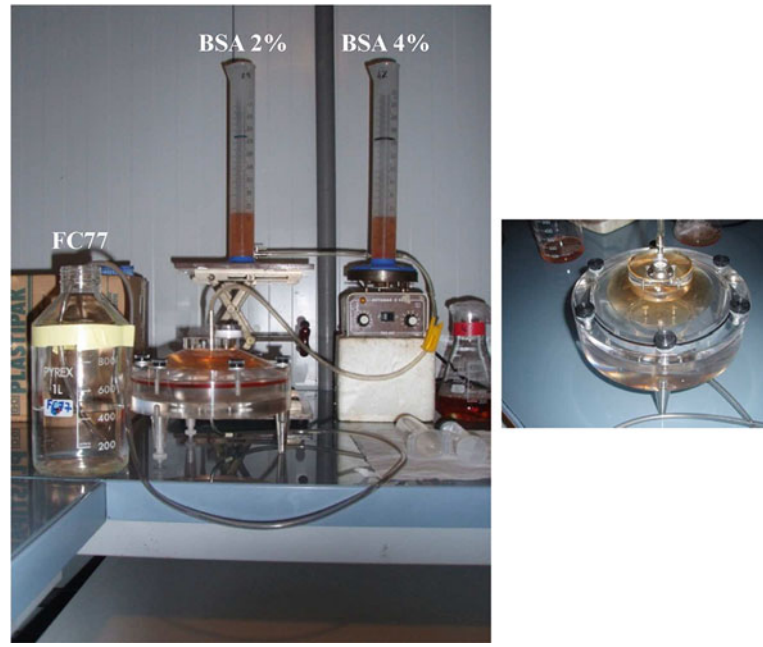

Fig. 1 BSA gradient in chamber. The chamber is filled up with the BSA 2–4 % gradient in the cold room

3.1.2 Prepare Gradient in Chamber

1. In the cold room, fill up the sedimentation chamber with Fluorinert-FC77, prime using syringe (*see* **Notes 1** and **2**).
2. Fill up the two graduated cylinders respectively with BSA 4 % (right) and BSA 2 % (left), 180 ml each. To allow the BSA gradient to develop, fill up the chamber (approximately 1 ml/10 s; Figs. 1 and 2).
3. When this process is over, the sample of total germ cells suspension (*see* below) can be added. Add 90 ml of cold 1× PBS to "center" the gradient into the homogenous part of the chamber.

3.1.3 Preparation of Total Germ Cells (TGCs) Suspension and Sedimentation

1. Turn on centrifuge to cool it down, and water bath at 35 °C. Euthanize two male mice and recover the four testes. Remove albuginae and transfer seminiferous tubules in 50 ml tube containing a fresh collagenase/1× PBS solution. Incubate during 10–15 min at 35 °C and vortex regularly (follow digestion: the seminiferous tubules should unravel in the collagenase solution). Centrifuge at 200 × *g* for 10 min at 4 °C. Resuspend the pellet in 10 ml BSA 0.5 %. Dissociate the aggregates by rapid pipetting for 10 min on ice with transfer pipets (graduated 3 ml large bulb). Centrifuge at 200 × *g* for 10 min at 4 °C. Resuspend the pellet in 5 ml BSA 0.5 %. Filter the suspension on 15 ml tube with a 100 μm filter. Rinse the tube with 1 ml BSA 0.5 % and filter the suspension again using the same filter. Filter the

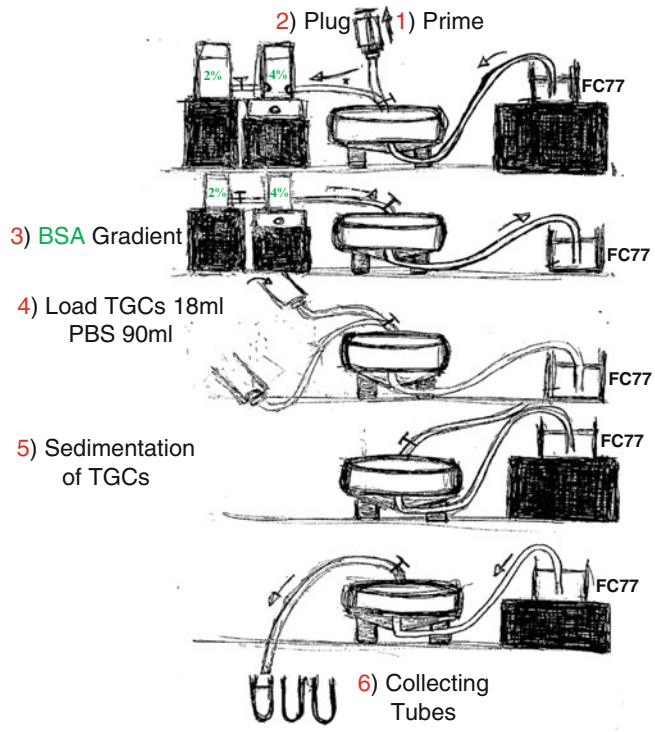

Fig. 2 Purification of male germ cells by sedimentation. Fill up the bottles with Fluorinert-FC77 (1). Connect the tube with the stopper on the top of the chamber with the cylinder containing BSA 4 % (2). Fill up the chamber with the BSA gradient (3). Gently add the TGCs suspension on the top of the BSA gradient (4). Leave cells to sediment for 70–75 min (5). Collect the cell fractions (6)

suspension on a new 15 ml tube with a second 100 μm filter. Rinse the tube with 1 ml BSA 0.5% and filter using the same filter. Add BSA 0.5 % up to a total volume of 18 ml.

2. Gently add the TGCs suspension on the top of the BSA gradient in the cold room. Add 90 ml of cold 1× PBS to "center" the gradient as described above. Leave cells to sediment for 70–75 min at 4 °C (Fig. 2).
3. Remove 1× PBS (approximately 90 ml) and start collecting the cell fractions in the 15 ml labeled tubes (10 ml/ tube, Fig. 2).
4. Centrifuge each of the collected fractions at 200 × *g* for 10 min at 4 °C and remove the supernatant (approximately 9 ml); resuspend cells in 1 ml of the remaining fluid. Use a small aliquot of each fraction (on a slide with coverslip) for examination with a phase contrast microscope. Pool the fractions with the same cell types (Spermatocytes, Round Spermatids, Elongating/Condensing Spermatids; Fig. 3) and wash them in 10 ml cold 1× PBS by centrifugation at 200 × *g* for 10 min at 4 °C (*See* **Note 3**).

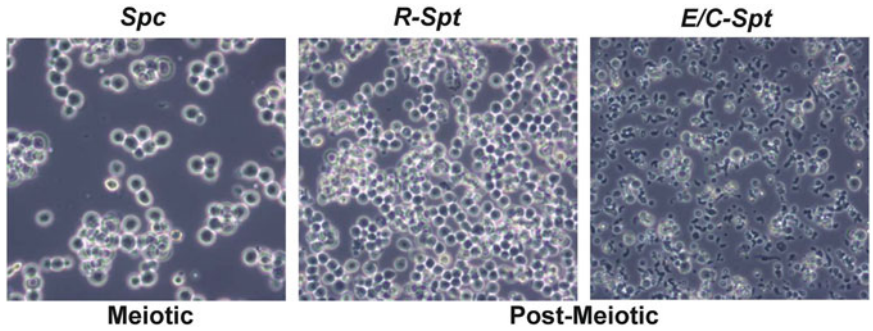

Fig. 3 Purified meiotic and post-meiotic male germ cells. Examination of collected cell fractions with a phase contrast microscope. *Spc* pachytene spermatocytes, *R-Spt* early post-meiotic Round Spermatids, *E/C-Spt* elongated/condensed spermatids

3.2 Chromatin Preparation and ChIP

1. Purified pool fractions of germ cell nuclei from ten testes (2× sedimentation procedures) are prepared by resuspension of the PBS-washed cell pellets with 1.5 ml lysis buffer with Na Butyrate 10 mM in a 1.5 ml tube at 4 °C followed by centrifugation for 15 min at 200 × *g* at 4 °C. Nuclei (pellet) are resuspended in 1.5 ml wash buffer with Na Butyrate 10 mM and centrifuged again. Nuclei can be stored at −20 °C in 500 µl wash buffer with Na Butyrate 10 mM/Glycerol 50% (mix 1/1 v/v, wash buffer with Na Butyrate 10 mM and Glycerol 100% carefully) or are processed directly. Resuspend nuclei in 200 µl of MNAse buffer with Na Butyrate 10 mM. 5 µl of nuclei solution are treated with 5 µl micrococcal nuclease S7 (5 U/µl) in 100 µl MNAse buffer for 5 min at 37 °C. 900 µl Urea 8 M is added to the reaction and DNA content is measured by OD at 260 nm using a NanoDrop apparatus (1 unit OD260 corresponds to 50 µg DNA/ml). Nuclei (200 µg) are incubated in 200 µl MNAse buffer (final concentration 1 µg/µl) with Na Butyrate 10 mM in the presence of 2 µl micrococcal nuclease (5 U/µl; 20 µg/U) at 37 °C for the desired time, to obtain mononucleosomes. The digestion reaction is stopped by adding EDTA at 5 mM final concentration. The nucleosome fraction is isolated by centrifugation at 600 × *g* for 15 min at 4 °C. In order to quantify the mononucleosomes in the supernatant, 100 µl of SDS 0.2% is added to 1 µl of the fraction and the DNA content is measured at OD260 nm. The mononucleosome fraction can be tested: 10 µl of the supernatant is added to 40 µl H_2O and mixed with 50 µl phenol–chloroform–isoamyl Alcohol. After strong mixing by vortex and centrifugation (9400 × *g* for 5 min at 4 °C) the aqueous phase (40 µl) is mixed with 5 µl loading buffer and analyzed after migration on a regular agarose gel 2%. The mononucleosome DNA signal is around 150 bp. Save chromatin input (10 µl).

2. Chromatin Immuno Precipitations (ChIPs) are carried with 5 µg of antibodies (*see* **Note 4**) and 100 µg (100 µl) of mononucleosomes diluted in 200 µl LSDB500 buffer with Na Butyrate 10 mM for 16 h at 4 °C. Antibodies have to be previously coupled to magnetic beads (1/10th of the final volume, 30 µl) according to the manufacturer's manual (Dynabeads protein G) for 6–16 h in 1× PBS, BSA 5 µg/µl (note that the final KCl concentration is therefore 350 mM). Beads were washed three times with LSDB350 with Na Butyrate 10 mM at 4 °C (tenfold volume of beads) and once with elution buffer with Na Butyrate 10 mM before nucleosome elution with 150 µl elution buffer containing, SDS 1%.
3. DNA purification: Process the input in parallel: 140 µl of elution buffer containing SDS 1% is added to 10 µl of the conserved fraction before ChIP. Elution and input samples are incubated for 20 min at 65 °C (mixed every 5 min). Isolate the flow-through and add 250 µl elution buffer. Add 4 µl of RNAseA 20 mg/ml and incubate for 1 h at 37 °C. Add 8 µl of proteinase K 10 mg/ml and incubate for 1 h at 55 °C. Add 400 µl phenol–chloroform–isoamyl alcohol and mix gently (no vortex). Centrifuge at 18,500 × *g* for 10 min. Transfer the aqueous layer and add 300 µl chloroform (mix gently, no vortex). Centrifuge at 18,500 × *g* for 10 min. Transfer the aqueous layer and add NaCl (200 mM, final concentration), Glycogen 40 µg, and 2× volume cold ethanol 100%. Mix briefly and incubate at −80 °C for 30 min. Centrifuge at 18,500 × *g* for 15 min at 4 °C. Wash the pellet with 1 ml cold ethanol 70% and centrifuge at 18,500 × *g* for 5 min at 4 °C. Let the pellet dry and resuspend into 20 µl of pure water. The samples can be kept at −20 °C.

   (*See* **Note 5**).

3.3 RNA Purification

1. Add in a 1.5 ml tube 1 ml of Trizol reagent onto the PBS-washed germ cell pellet and resuspended robustly. This can be kept at −80 °C.
2. Add chloroform (0.2 ml), shake 30 s and incubate for 2–3 min at room temperature. Centrifuge at 12,000 × *g* for 10 min at 4 °C. The aqueous phase is transferred into a new tube and isopropanol 0.5 ml is added, mixed, and incubated for 10 min at room temperature. Centrifuge at 12,000 × *g* for 15 min at 4 °C. The pellet is washed with ethanol 75%. Centrifuge at 7500 × *g* for 5 min at 4 °C. Air-dry the pellet and dissolves in 50 µl RNase-free water. Incubate at 55 °C for 10 min. Quantify using a NanoDrop apparatus. The RNA samples can be kept at −20 °C and/or tested by RT-qPCR.
3. For transcriptomic analysis, RNAs are further purified using the RNeasy Minit Kit (Qiagen). Adjust to 100 µl RNA

samples with RNase-free water. Add 250 µl ethanol 100%. Load onto an RNeasy Mini spin column and centrifuge at 10,000 × *g* for 30 s at room temperature. Wash with 350 µl of the RW1 solution and centrifuge at 10,000 × *g* for 15 s at room temperature. Add onto the membrane of the column a mix of 10 µl RNase-free DNaseI (Qiagen) with 70 µl RDD buffer and let incubate for 15 min at room temperature. Add 350 µl RW1 and centrifuge at 10,000 × *g* for 15 s at room temperature. Wash twice with 500 µl RPE buffer and centrifuge: the first washing step at 10,000 × *g* for 15 s and the second at 10,000 × *g* for 3 min, both at room temperature, in order to dry the membrane. The elution step is processed twice into a new collection tube with 20 µl of RNase-free water after centrifugation at 10,000 × *g* for 1 min at room temperature. Quantify using a NanoDrop apparatus (1 unit OD260 corresponds to 40 µg RNA/ml). The RNA samples can be kept at −20 °C.

(*See* **Note 6**).

3.4 Acidic Histone Extraction

1. Add 3× PBS-washed germ cell pellet volumes of ice-cold extraction buffer. Gently resuspend the cells with a pipette, and incubate on ice for 20 min. Check under the microscope to ensure that the plasma membrane is disrupted but not the nuclear membrane. Centrifuge at 1000 × *g* for 5 min at 4 °C, discard the supernatant with pipette, save the pellet. Wash the pellets in ice-cold wash buffer twice by resuspending the pellets completely. Spin the pellets in cooled centrifuge, 1000 × *g* for 5 min and completely remove the supernatant.
2. Add 10× pellet volumes of H_2SO_4 0.2 M into pellets, vortex the solution until the pellets are dissolved, and incubate the mixture for 16 h on rotator at 4 °C. Centrifuge at 20,000 × *g* for 15 min and save the supernatant. Add TCA drop by drop with pipette into the supernatant with a final concentration of Trichloroacetic acid 20% (TCA: supernatant, v/v). Place the tubes on ice for at least 30 min and centrifuge at 16,000 × *g* for 10 min to obtain the histone pellets. Wash samples with cold acetone with HCl 0.1% (v/v), which is followed by washing with cold acetone 100% twice. Allow precipitates to dry completely at room temperature.

(*See* **Notes 7** and **8**).

3.5 GST-Fusion Protein Pull-Down

1. Prepare Trichostatin A (TSA) 1 mg/ml in DMSO and keep at −20 °C. Dilute before use to 25 µg/ml into H_2O (250×). In order to inhibit Histone DeACetylase (HDAC) activities, add TSA at a final 100 ng/ml concentration.

2. Incubate PBS-washed germ cell pellets from four testes in 2 ml lysis buffer (LSBD500 with Nonidet P-40 0.1 %, dithiothreitol 1 mM, and TSA 100 ng/ml) for 15–20 min at 4 °C. Centrifuge at 18,000 × *g* for 10 min at 4 °C. Transfer the supernatant (1.8 ml) and separate equally (0.9 ml) to 250 µl of packed pre-bound Glutathione Sepharose beads, which have been pre-incubated with 130 µg GST (control) or with 130 µg GST-fusion protein of interest according to the manufacturer's protocol in the equilibration buffer (LSBD250 with Nonidet P-40 0.1 %, dithiothreitol 1 mM, and TSA 100 ng/ml) buffer. Immediately, add an equal volume (0.9 ml) of LSBD0 buffer with Nonidet P-40 0.1 %, dithiothreitol 1 mM, and TSA 100 ng/ml and incubate for 16 h with rotation at 4 °C. Wash beads twice with 1.5 ml LSBD250 buffer with Nonidet P-40 0.1 %, dithiothreitol 1 mM, and TSA 100 ng/ml and twice with 1.5 ml 1× PBS with TSA 100 ng/ml.
3. The cDNA of interest corresponding to the GST-fusion protein is cloned into a pGEX4T2 vector. This strategy allows the elution of the protein of interest after thrombin treatment. Therefore, 1× PBS with TSA 100 ng/ml equilibrated beads are incubated for 16 h with rotation at 4 °C with 350 µl 1× PBS with TSA 100 ng/ml containing 50 units of thrombin. Centrifuge at 2400 × *g* for 5 min at 4 °C in order to remove the beads and the GST only bound fraction. Supernatant aliquots of 50 µl can be kept at −20 °C for further proteomic analysis.

4 Notes

1. The sides of the chambers are available upon request.
2. Fluorinert-FC77 is a high density liquid that maintains a stable BSA gradient.
3. An original protocol has been described previously [8].
4. Antibodies should be selected as ChIP-grade quality according to manufacturer.
5. This protocol has been validated in our laboratory (see refs. 3, 5, 9, 10).
6. This purification has been tested in our team (see refs. 3, 5).
7. In Early Round spermatid extract the testis-specific TH2B histone variant [3] replaces most of the H2B histone specie detected in HeLa cell somatic extract (see Fig. 4).
8. This extraction protocol is routinely used in different laboratories (*see* refs. 3, 9).

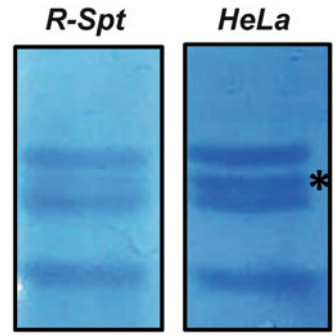

Fig. 4 Acidic histone extracts analysis by SDS-PAGE and Coomassie staining. *R-Spt* early post-meiotic Round Spermatid extract, *HeLa* HeLa cell extract. *Asterisk* H2B histone variant signal in HeLa cell extract almost not present in early Round Spermatid extract

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Chapter 13

Generation of Tissue-Specific Mouse Models to Analyze HDAC Functions

Astrid Hagelkruys, Mirjam A. Moser, and Christian Seiser

Abstract

Histone deacetylases (HDACs) play crucial roles during mammalian development and for cellular homeostasis. In addition, these enzymes are promising targets for small molecule inhibitors in the treatment of cancer and neurological diseases. Conditional HDAC knock-out mice are excellent tools for defining the functions of individual HDACs in vivo and for identifying the molecular targets of HDAC inhibitors in disease. Here, we describe the generation of tissue-specific HDAC knock-out mice and delineate a strategy for the generation of conditional HDAC knock-in mice.

Key words Transgenic mice, Knock-out mice, Knock-in mice, Loss-of-function, Gain-of-function, Histone deacetylases, Chromatin modifications, Epigenetics

1 Introduction

The HDAC subfamily of mammalian histone deacetylases encompasses 11 members named HDAC1-HDAC11. These enzymes have important functions in the control of cellular proliferation and differentiation and dysfunctions of some of these enzymes have been implicated in different human diseases. Strikingly, mice with a full knock-out of individual class I and class II HDACs show distinct phenotypes, some of them affecting embryonic development [1]. Pharmacological inhibition of HDACs is a promising strategy for therapeutic intervention to reverse aberrant epigenetic changes in cancer and other disorders (reviewed in refs. 2–5). Therefore, one important aspect of HDAC biology is the impact of catalytic and noncatalytic functions of mammalian deacetylases on gene regulation and cellular homeostasis. Some class II HDACs such as HDAC4, HDAC5, and HDAC7 have been reported to be catalytically inactive [6] and to require interaction with HDAC3 for their deacetylase function [7]. On the other hand, the class I enzymes HDAC1, HDAC2, and HDAC3 are able to dimerize and constitute the catalytic components of multiprotein corepressor complexes [6, 8, 9]

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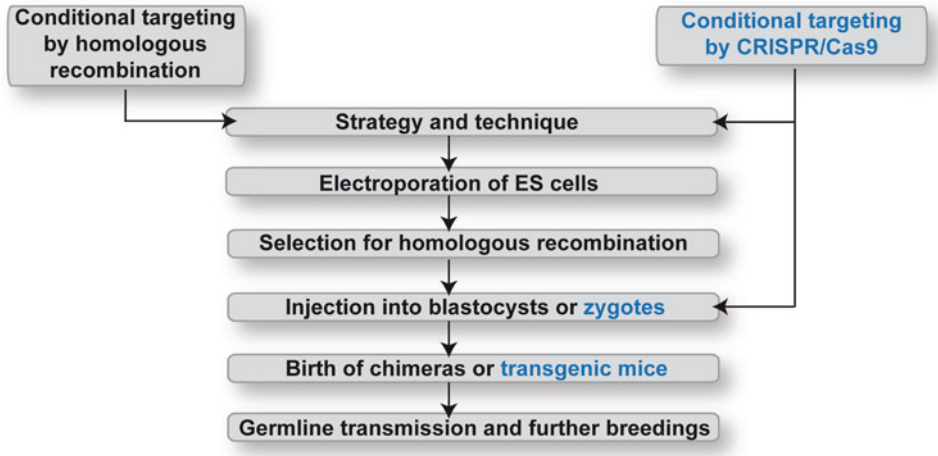

Fig. 1 Schematic overview of two approaches for the generation of mice with floxed *Hdac* alleles

and seem to have also scaffolding functions [10–12]. The class II enzyme HDAC6 is a tubulin deacetylase and has two catalytic domains [13]. Recently, by using a knock-in mouse model catalytically inactive HDAC2 has been shown to have a dominant negative function during mouse development [14].

Hence, conditional HDAC knock-out mice and conditional knock-in mice expressing catalytically inactive HDACs are valuable tools for the analysis of cell type- and tissue-specific functions of these deacetylases during development and in disease models.

Here, we describe the individual steps for the generation and validation of conditional HDAC knock-out mice, as depicted in the scheme shown in Fig. 1. This includes the construction of the targeting vector, electroporation of ES cells, selection and characterization of cells with floxed alleles, generation of chimeric mice, and the validation of the conditional knock-out mice. An alternative approach by using CRISPR/Cas9 for the generation of floxed alleles is also described. Furthermore, we outline a strategy for creating conditional HDAC knock-in mice.

2 Materials

2.1 Cloning and Preparation of Targeting Vectors

1. C57BL/6J BAC clones, for instance, from BACPAC resources center.
2. Pfu proof reading DNA polymerase.
3. Site-Directed Mutagenesis System.
4. MidiPrep Kit.
5. Restriction enzymes and buffers.
6. Ligation kit.
7. Competent *E. coli* cells.

8. Miniprep DNA Purification System.
9. Phenol-Chloroform-Isoamyl alcohol (PCI, 25:24:1) stored at 4 °C.
10. 3 M sodium acetate pH 5.2: for 1 L solution dissolve 246.1 g sodium acetate in water and adjust the pH with acetic acid to 5.2.
11. Isopropanol.
12. 70% ethanol.
13. Safe lock tubes.
14. Thermoshaker.
15. Vortexer.
16. Cooling centrifuge.
17. Nanodrop spectrophotometer.

2.2 ES Cell Cultivation, Electroporation, and Selection

1. DR4 Feeder Cell Medium: 450 ml high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1× Glutamine, and 1× Penicillin/Streptomycin.
2. Standard medium for ES cells: 450 ml DMEM high glucose supplemented with 10% FBS, 1× Glutamine, 1× Penicillin/Streptomycin, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 100 μM β-mercaptoethanol (diluted in dH_2O), 50 μl ESGRO LIF.
3. 100× G418: 34.5 mg/ml G418 sulfate powder dissolved in ES cell medium.
4. 1× (0.05%) and 2× (0.1%) Trypsin/ETDA prepared from 0.5% Trypsin/EDTA.
5. Phosphate Buffered Saline (PBS): 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4. Adjust pH with HCl or NaOH to 7.4.
6. ES Cell Proteinase K digestion buffer: 0.1% SDS, 100 mM Tris–HCl, pH 8.0, 5 mM EDTA, 200 mM sodium chloride, Proteinase K (1 mg/ml).
7. TAE buffer (10×): 400 mM Tris–Acetate, 20 mM EDTA pH 8.3.
8. 96-well, 24-well, 6-well, 10 cm tissue culture plates.
9. CO_2 incubator.
10. 0.1% gelatine for coating.
11. 2× Freezing Medium: 80% FBS, 20% DMSO.
12. 50 ml Falcon tubes.
13. Electroporation cuvettes.
14. Sterile microscope.
15. Centrifuge with a 96-well plate adaptor.
16. Gene Pulser.
17. PCR block for 96-well plates.

2.3 Southern Blot Analysis

1. Southern blot digestion buffer: 0.1% SDS, 50 mM Tris–HCl, pH 8.0, 20 mM EDTA, 100 mM sodium chloride.
2. Proteinase K (20 mg/ml in dH_2O).
3. 20% SDS in dH_2O.
4. 5 M sodium chloride: dissolve 292 g of NaCl in 800 ml of dH_2O. Adjust the volume to 1 L with dH_2O.
5. Centrifuge for 15 ml falcon tubes.
6. Phenol-Chloroform-Isoamyl alcohol (PCI, 25:24:1) stored at 4 °C.
7. Chloroform, stored in the dark.
8. 96% ethanol.
9. 70% ethanol.
10. Tris–HCl/EDTA (pH 8.0).
11. DNA ladder (GeneRuler 1 kb Plus DNA Ladder or Lambda DNA/HindIII marker).
12. Restriction enzyme.
13. Centrifugal evaporator.
14. Agarose gel + TAE buffer (10×: 400 mM Tris–Acetate, 20 mM EDTA pH 8.3).
15. Orange G loading dye: 40% w/v sucrose in dH_2O 0.5% w/v Orange G.
16. Depurination solution: 0.25 M HCl.
17. Denaturation solution: 0.5 M sodium hydroxide, 1.5 M sodium chloride.
18. Neutralization solution: 0.5 M Tris–HCl, pH 7.4, 0.5 M sodium chloride.
19. 10× SSC (transfer buffer): 150 mM sodium citrate, pH 7.0, containing 1.5 M sodium chloride.
20. Southern transfer device (as shown in Fig. 4).
21. Nylon membrane (for instance Genescreen, *PerkinElmer*).
22. Blotting paper (for instance Whatman 3MM).
23. Soaking paper towels.
24. UV crosslinker.
25. Methylene blue solution: 0.5 M sodium acetate, 0.04% Methylene blue.
26. Random Primer Labeling Kit.
27. (alpha-32P) deoxycytidine 5′-triphosphate (dCTP; 10 mCi/ml).
28. Quick Spin Columns.
29. 100× Denhardt solution: 2% Ficoll, 2% Polyvinylpyrrolidone, 2% BSA.

30. 20× SSPE pH 7.4: 3 M sodium chloride, 0.2 M NaH_2PO_4, 0.02 M EDTA.
31. Hybridization/blocking solution: 5× SSPE, 0.1 M sodium phosphate, 50% formamide, 5× Denhardt, 10 mM EDTA, 1% N-Laurylsarcosidate, 200 μg/ml freshly added tRNA from baker's yeast. Alternatively, a commercially available hybridization mix such as PerfectHyb™ Plus Hybridization Buffer (*Sigma*) can be used. In the latter case, hybridization, temperature, and wash conditions are different (all at 65 °C).
32. Wash solution I: 6× SSC with 0.1% SDS.
33. Wash solution II: 2× SSC with 0.1% SDS.
34. Wash solution III: 0.2× SSC with 0.1% SDS.
35. Stripping solution: 0.4 N sodium hydroxide.
36. Strip-neutralization solution, pH 7.4: 0.1× SSC, 0.1% SDS, 0.2 M Tris–HCl.
37. Hybridization oven.
38. Saran wrap.
39. PhosphorImager.

2.4 Generation of Floxed Alleles with CRISPR/ Cas9

1. DNA template for in vitro transcription (for instance, Double stranded gBlocks® fragments (*IDT*)).
2. Short RNA in vitro transcription kit (for instance, MEGAshortscript kit from *Thermo Scientific*).
3. Clean-up kit for in vitro transcripts (for instance, MEGAclear transcription clean-up kit, *Thermo Scientific*).
4. Two single stranded repair oligodeoxynucleotides.
5. Cas9 mRNA.
6. NHEJ inhibitor Scr7.

2.5 Identification and Characterization of Transgenic Mice

1. Biopsy lysis buffer: 100 mM Tris–HCl pH 8.0, 5 mM EDTA, 200 mM sodium chloride, 0.1% SDS.
2. Proteinase K (20 mg/ml in dH_2O).
3. PCR Mastermix.
4. Thermoshaker.
5. PCR machine.
6. RNA isolation reagent (for instance, Trizol (*Invitrogen*)).
7. 10× MOPS/EDTA, pH 7.0: 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA.
8. 6× RNA sample buffer: 15 ml formamide (deionized), 3 ml 10× MOPS/EDTA, 4.8 ml 37% formaldehyde, 2 ml dH_2O, 1.7 ml 100% glycerol, 1.6 ml 10% bromophenol blue.
9. MOPS/EDTA gel: 1.2 g agarose in 100 ml 1× MOPS and 5.2 ml 37% formaldehyde added after heating.

10. RNAse-free gel electrophoresis apparatus.
11. Nanodrop spectrophotometer.
12. cDNA synthesis kit.
13. qPCR reagent (for instance, qPCR MasterMix; *Peqlab*).
14. Real-time PCR machine, for instance iCycler IQ system (*BioRad*).
15. Protease—Deacetylase—Phosphatase inhibitor mix: 1× complete protease inhibitor cocktail, 100 μM PMSF, 10 mM sodium butyrate, 10 mM sodium fluoride, 10 mM β-glycerophosphate, 100 μM orthovanadate (activated 5 min at 95 °C), 10 μM sodium molybdate.
16. Bradford protein assay (*BioRad*): diluted 1:5 in dH_2O.
17. Protein gel apparatus.
18. Immunoblot apparatus.
19. Enhanced Chemo Luminescence kit.
20. X-ray films.

3 Methods

We describe here in detail the classical method for generation of floxed *Hdac* alleles via homologous recombination. However, the recently established CRISPR/Cas9 methodology provides a valuable alternative. Therefore, we additionally briefly describe the generation of mice carrying conditional alleles by CRISPR/Cas-mediated genome engineering in Subheading 3.4.

Before you start with your experiments contact the ethics and animal care committee of your country to clarify the guidelines for transgenic mouse lines and to submit a corresponding proposal.

3.1 Generation of Targeting Vectors for Conditional HDAC Knock-Out and Knock-In

3.1.1 Generation of Targeting Vectors for Conditional HDAC Knock-Out

A typical knock-out targeting vector contains the following sequences [15], as depicted in Fig. 2a. These are the long homology arm and the short homology arm, which encompass genomic sequences adjacent to the targeted exon(s) and two *loxP* sites, which flank the targeted exon(s) and thereby mediate removal of the exon by Cre recombinase. In addition, the vector contains a positive selection marker, usually a neo gene preferentially flanked by *frt* sites that allow removing the neo gene with the FlpE recombinase after selection (*see* Subheading 3.5). A negative selection marker, usually diphteria toxin gene (DTA), is lost upon homologous recombination and therefore prevents unfavorable integration outside the locus (*see* **Note 1**).

1. Choose the optimal exon(s) for targeting. Removal of the exon(s) should result in out-of-frame splicing and a frameshift and the resulting mRNA can become a target for nonsense-mediated mRNA decay. The choice of the exons also depends

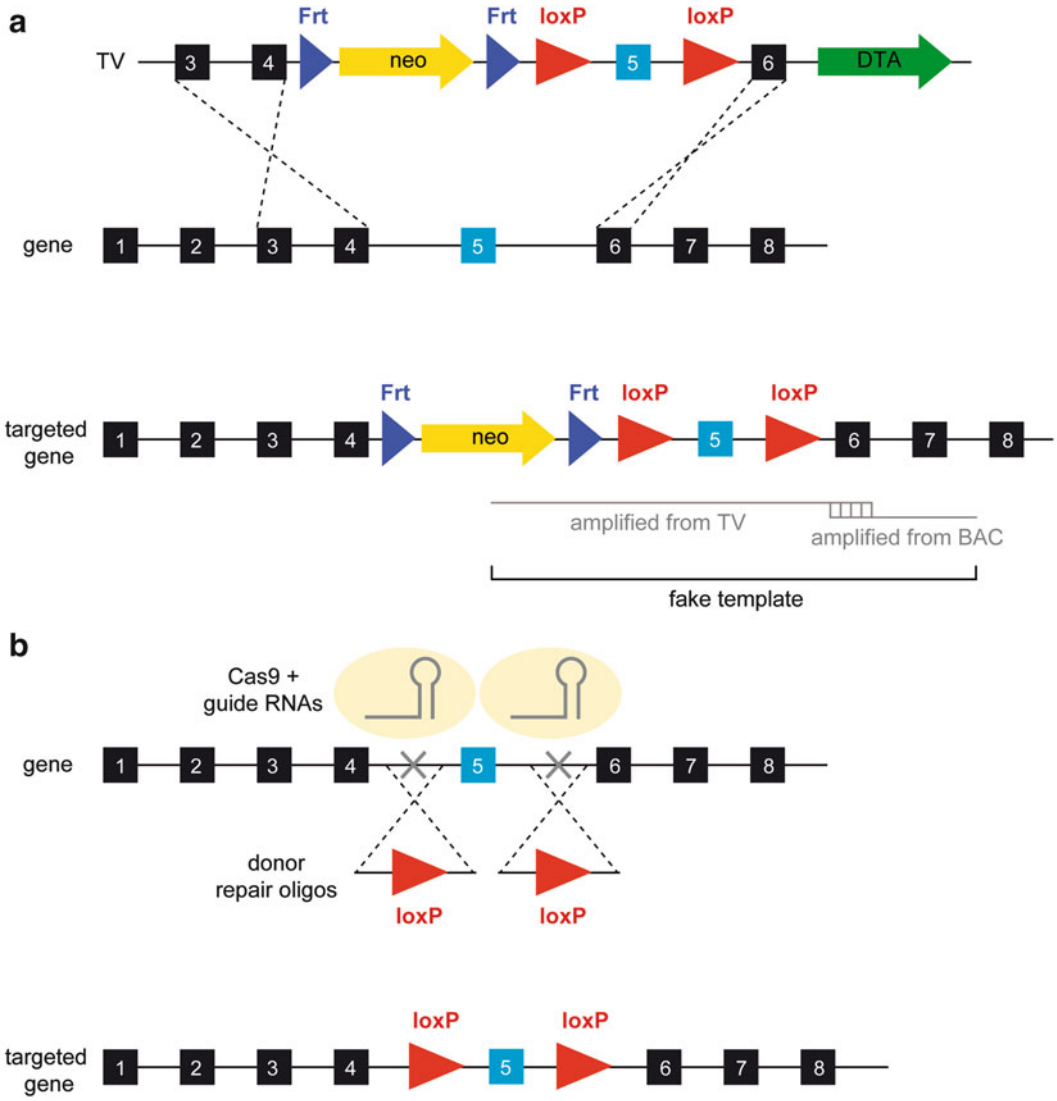

Fig. 2 Generation of floxed alleles by homologous recombination (a) or CRISPR/Cas9-mediated genome engineering (b). Exons are depicted as *black rectangles* carrying the exon number and the exon (exon 5) to be flanked with *loxP* sites (*red triangles*) is shown in *blue*. (a) The homology arms (exon 3–4 and exon 6) allow homologous recombination (visualized by *dashed lines*) in ES cells resulting in the insertion of the targeting cassette (containing *loxP* sites flanking exon 5) into the genome. The *neomycin phosphotransferase* gene (neo, *yellow*) and the *Diphtheria toxin A fragment* gene (DTA, *green*) for positive and negative selection permit selection of successfully targeted ES cells. To screen potentially targeted ES cells, an optimized PCR screen and a positive control are necessary. Therefore, a fake template containing parts of the targeting cassette (amplified from TV) and sequences extending 3′ to the genomic region (amplified from BAC) with an overlap of approximately 200 base pairs has to be generated. (b) Two sgRNAs recognizing sequences before and after the exon of choice (blue exon 5) in a complex together with Cas9 (*yellow*) result in two double strand breaks, which can be repaired by homology directed repair (*dashed lines*) with two donor repair oligonucleotides containing a *loxP* site and short homology sequences. The targeted gene contains the desired exon with flanking *loxP* sites without any unnecessary cassette for selection

on the specific properties of the HDAC. In several cases exons that encode parts of the catalytic domain have been targeted. Avoid that the expression of a remaining truncated HDAC protein affects the function or activity of another HDAC, for example, via its dimerization domain.

2. Define appropriate long and short homology arms. Typically, the total length of homology is 6–14 kb, with a short arm of about 1–2 kb and a long arm of 4–6 kb [15]. Order the corresponding BAC clone and generate the short arm, the long arm, and the targeted exon plus adjacent intron sequences by PCR and insert them into the targeting vector (TV) (Fig. 2a). The genomic BAC clone should be isogenic to the targeted ES cell line. This is important because it increases the efficiency of homologous recombination [15].
3. Generate a fake template that contains parts of the targeting cassette and sequences extending 3′ to the genomic region (Fig. 2a). This sequence mimics part of the targeted gene and will be used for optimizing the nested PCR reactions for rapid detection of ES cell clones with a correctly targeted locus (Subheading 3.2.3) (*see* **Note 2**).
4. Verify the correct amplification and insertion by sequencing (*see* **Note 3**).

3.1.2 Generation of Targeting Vectors for Conditional HDAC Knock-In

Cre-lox-mediated generation of conditional point mutation into *Hdac* genes can be achieved as described by Zhang and Lutz [16] (Fig. 3). In addition to a long homology and a short homology arm, the targeting vector contains a central sequence (MA), which comprises the targeted exon and an inverted version of the same exon containing the desired mutation. This sequence is flanked by two mutant *loxP* sites (lox66 and lox71) oriented in a head-to-head position. In the absence of Cre recombinase, the wildtype exon is positioned to be transcribed and spliced correctly. When Cre recombinase is present, the DNA flanked by the two mutant *loxP* sites will be inverted, forming one *loxP* and one double mutated *loxP* site. As the double mutated *loxP* site shows low affinity for Cre recombinase, the favorable reaction leads to a product where the mutated exon 5* is placed into the position to be correctly transcribed and spliced (*see* **Note 4**).

A typical knock-in targeting vector contains the following sequences (Fig. 3):

- A long homology arm and a short homology arm, which encompass genomic sequences adjacent to the targeted exon.
- A middle arm containing the targeted exon and the inverted mutated exon flanked by two mutant *loxP* sites.
- A positive selection marker, usually a neo gene preferentially flanked by *frt* sites that allow removing the neo gene with FlpE recombinase after selection (*see* Subheading 3.5).

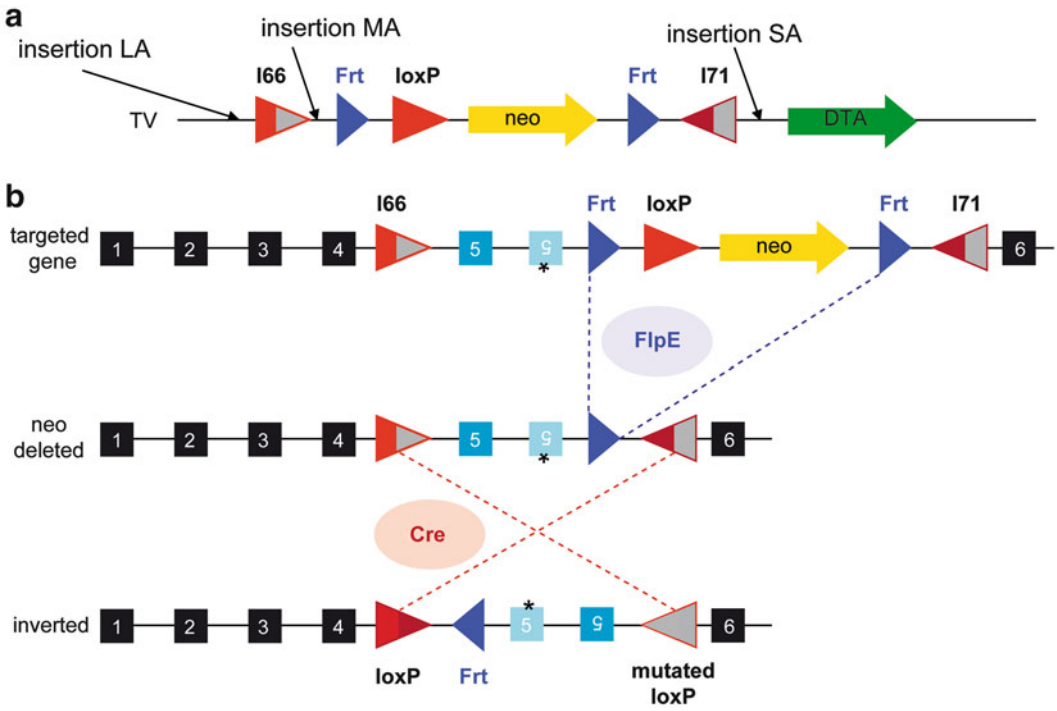

Fig. 3 Generation of targeting vector (TV) for a conditional knock-in and further recombination of the targeted allele to express an inactive HDAC mutant. (a) The middle arm (MA) and the two arms of homology LA/SA (long arm = LA, short arm = SA) are introduced at the marked positions to construct the final targeting vector. Containing a *neomycin phosphotransferase* gene (neo, *yellow*) and a *Diphtheria toxin A fragment* gene (DTA, *green*) for positive and negative selection, the pFLNeo-DTA-lox66-lox71 vector permits selection of successfully targeted ES cells. (b) Before Cre-mediated recombination, one additional unmutated *loxP* site has to be removed together with the neo cassette by FlpE recombinase, which detects the flanking *frt* sites (*dark blue*). Upon Cre recombinase-mediated recombination, the two half-mutated and head-to-head oriented *loxP* sites lox66 and lox71 (*red*/*gray*) lead to inversion of the interjacent sequence. The introduced flipping cassette contains the wildtype version of *Hdac1* exon 5 (*blue*) in sense direction to be expressed properly. The point-mutated version of *Hdac1* exon 5* (*light blue*) is positioned in inverse orientation and only expressed after Cre recombinase-mediated inversion of the flipping cassette. Exons are depicted as *black rectangles* carrying the exon number. FlpE- and Cre-mediated recombination is illustrated by *dashed lines*

- A negative selection marker, usually *diphteria toxin* gene (DTA), that is lost upon homologous recombination and therefore prevents unfavorable integration outside the locus.

1. Order the corresponding BAC clone and generate the short arm, the long arm and insert them into the targeting vector (TV) (Fig. 3).
2. Generate a PCR fragment encompassing the targeted exon plus adjacent intron sequences.

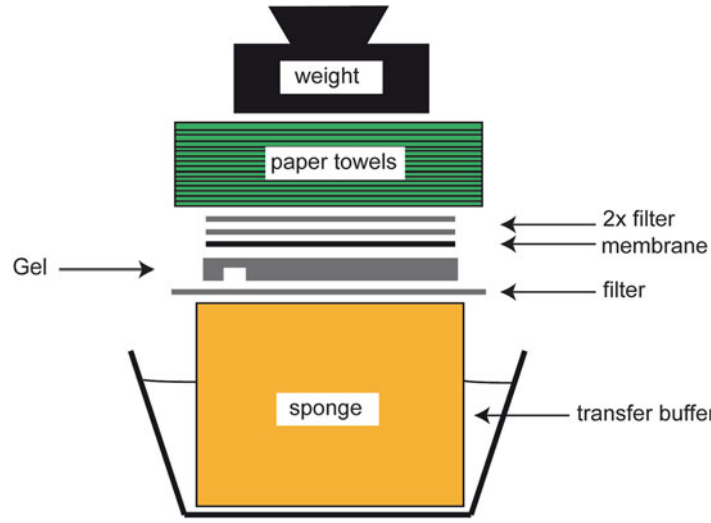

Fig. 4 Scheme of Southern blot device assembly

3. Generate a mutated version of this sequence by inserting the desired mutation into the exon with a DNA mutagenesis kit.
4. Verify the mutation by sequencing.
5. Clone the wildtype exon and the inverted mutated exon into the TV (see Note 5).
6. Verify the correct amplification and insertion by sequencing.

3.2 Targeting of ES Cells by Electroporation

For the whole procedure use cut tips and safe-lock tubes. Perform all centrifugation steps at 4 °C.

3.2.1 Targeting Vector Preparation

1. Isolate the final targeting vector with a Midi Prep kit (see Note 6).
2. Linearize and purify the targeting vector (TV) plasmid before the electroporation into ES cells.
3. If doing a single electroporation experiment digest 30–60 µg of the TV in a volume of 100–200 µl with an appropriate restriction enzyme. Incubate overnight at 37 °C on a thermoshaker.
4. On the next day add dH_2O to a total volume of 500 µl and add one volume Phenol-Chloroform-Isoamyl alcohol (PCI, 25:24:1). Vortex vigorously for 1 min and centrifuge the sample at 14,000 × *g* for 5 min at 4 °C.
5. Use cut tips to transfer the aqueous upper phase containing the DNA into a fresh safe-lock tube. Purify the DNA once again with one volume PCI and then wash it twice with chloroform.
6. After the second chloroform-washing step, transfer the upper phase to a fresh safe-lock tube. Fill up with dH_2O to 500 µl.

7. For precipitation, add 10% 3 M sodium acetate (50 µl) and 0.7 volumes isopropanol (385 µl) and mix by inverting until the DNA becomes visible as white thread. Centrifuge for 15 min at 14,000 × *g* and let the DNA form a tight pellet.
8. Discard the supernatant and wash the pellet with 500 µl 70% ethanol.
9. After removal of ethanol air-dry the pellet and then resuspend it in 40 µl dH_2O. Use 1 µl for measuring the concentration with the Nanodrop spectrophotometer, 1 µl to check for complete linearization on an agarose gel and use the rest for electroporation into ES cells (15 µg per electroporation). Store the DNA at −20 °C.

3.2.2 Electroporation and Selection

Germline-competent ES cells (for instance, B6129F1 background) are used for the generation of transgenic mice due to their germ line competence and the convenient color detection of chimeric mice when injected into B6-albino mice blastocysts (*see* **Note 7**).

1. Thaw appropriate ES cells. Split them at least two times before the electroporation to let them recover from thawing (15 cm dish).
2. Prepare feeder-coated 10 cm dishes at least 3 h before use for the cultivation of ES cells (*see* **Note 8**).
3. Harvest ES cells from a 15 cm dish (approximately 10^7 cells) by using trypsin. Dissociate the cells carefully by pipetting up and down. Pellet the cells.
4. Resuspend the cells gently in 800 µl PBS and transfer them into a tube containing 15 µg of the linearized and purified targeting vector.
5. Mix it and transfer the suspension to an electroporation cuvette. Perform electroporation with 0.24 kV and 500 F with a resulting pulse of approximately 6 ms using a GenePulser.
6. Incubate the ES cell suspension for 10 min at room temperature. Then transfer the cells to a 50 ml Falcon tube, dilute them with ES cell medium. Distribute it to the four prepared 10 cm dishes with feeder cells.
7. Let the ES cells regenerate for 36 h after the electroporation. Then select with G418-containing medium for 6–7 days. Replace media each day. After this time period resistant colonies become visible.

3.2.3 Picking of ES Colonies and Nested PCR Screening for Homologous Recombinants

After 6–7 days of selection most nontargeted cells (lacking the *neomycin phosphotransferase* gene) die. Pick targeted, G418-resistant clones as soon as the colonies appear as round balls.

1. Therefore, wash the resistant ES cells twice with PBS. Add 5 ml PBS for picking. Use a sterile microscope and detach single ES cell clones carefully in a volume of 20 µl from the feeder cells using a yellow pipette tip. Transfer each clone separately into a well of a 96-well PCR plate (*see* **Note 9**).
2. After isolation of 96 clones (or after 30 min picking) add 20 µl 2× (0.1 %) Trypsin/ETDA to the cells in 20 µl PBS and trypsinize them for 4 min at 37 °C. Pipet up and down very carefully to obtain single cell suspensions.
3. Transfer half of each clone (20 µl) to a feeder-coated 96-well culture plate containing 200 µl ES cell medium. Add 200 µl PBS to the remaining half of picked cells in the 96-well PCR plate.
4. Pellet the cells by centrifugation at 350 × g for 5 min. Shake off the supernatant and resuspend the cells in 10 µl ES Cell Proteinase K digestion buffer. Incubate the plate for 1 h at 55 °C on a PCR block.
5. Centrifuge briefly at 350 × g and add 50 µl dH2O per well. Incubate at 95 °C for 10 min to inactivate proteinase K.
6. To check successful targeting, take 1.5 µl of the solution containing the ES cell DNA and perform the first nested PCR reaction. Use one well per plate for the positive control (see Note 10).
7. Perform the second PCR with 1.5 µl of the first PCR reaction. Load the final PCR products on a 0.8 % agarose gel next to the marker and the positive control.
8. After 2 days the majority of the clones in the 96-well culture plate usually reach 80–90 % confluency. Expand the positive PCR-tested clones and split them every 2–3 days (96-well → 24-well → 6-well → 10 cm) according to the procedure given in Note 7.
9. Take aliquots while splitting from the 24-well and from the 6-well plate for later blastocyst injection according to the freezing procedure given in **Note 7**. In the last splitting step, grow ES cells on gelatin-coated 10 cm dishes for Southern blot analysis to confirm full-length integration of the desired construct.

3.2.4 Southern Blot Analysis of Positive ES Clones

Southern blot analysis is the method of choice to confirm the full-length integration of the targeting construct by homologous recombination and to differentiate between single and multiple integration events.

1. Grow the clones tested positive for homologous recombination in the nested PCR assay on gelatin-coated 10 cm plates for several days for DNA extraction and Southern blot analysis.

2. Wash the cells in PBS, harvest them by using cell scrapers, and pellet the cells in a 15 ml Falcon tube.
3. Remove the supernatant and resuspend the pellet in 4.5 ml Southern blot digestion buffer and add 300 µl Proteinase K (20 mg/ml) to reach a final concentration of 1 mg/ml.
4. Mix the samples and then quickly add 250 µl 20% SDS (final concentration of 1% SDS) to lyse the cells during an overnight incubation in a water bath at 55 °C.
5. On the next day, add 200 µl 5 M sodium chloride, mix and further add one volume (5 ml) PCI.
6. Mix the solutions by vortexing for 1 min. Separate the aqueous and the organic phases by centrifuging at 3200 × *g* for 5 min at 4 °C (Beckman GS-6KR centrifuge).
7. Transfer the aqueous phase to a new tube and repeat the last step once with 5 ml PCI and once with chloroform only.
8. After the last washing step, fill up to 5 ml with dH_2O. Add 3 volumes of 96% ethanol to precipitate the DNA. Invert several times until the DNA becomes visible as white thread.
9. Use a Pasteur pipette or a similar "hook" to fish the DNA. Wash the DNA in 70% ethanol, shortly dry and dissolve it in 400 µl prewarmed Tris/EDTA (pH 8.0) (*see* **Note 11**).
10. Let the DNA fully dissolve and then measure the concentration with a Nanodrop spectrophotometer.
11. Store genomic DNA for Southern blotting at 4 °C, but do not freeze to avoid nicking and fragmentation.
12. Digest genomic DNA overnight with 50–100 units of the appropriate restriction enzyme in a total reaction volume of 80 µl.
13. On the next day, reduce the reaction volume to approximately 40 µl by concentrating the samples in a centrifugal evaporator.
14. Add Orange G DNA loading dye to the samples before loading them on an agarose gel next to the DNA ladder.
15. Run the gel at up to 70 V for 5–15 h depending on the size of the expected bands.
16. Take a gel photo under UV light and then rinse the gel in dH_2O.
17. Shake the gel in depurination solution on a rocker shaker for 15 min.
18. Rinse the gel with water and incubate the gel in denaturation solution for 15 min.
19. Then put the gel into the neutralization solution for 15 min, rinse it again in dH2O.

20. Equilibrate the gel in transfer buffer (10× SSC). Equilibrate as well the sponge, Whatman 3MM filter papers, and the nylon membrane in transfer buffer before the blotting assembly (shown in Fig. 4).
21. Fill a big container with transfer buffer and put in the sponge and soak it.
22. Assemble the blotting sandwich on the top of the sponge as follows: one sheet of Whatman 3MM paper, the gel upside down to the Whatman paper, the equilibrated membrane, two sheets of Whatman 3MM paper (see Note 12).
23. Then put a stack of soaking paper towels covered with a glass plate, onto which a balanced weight can be put to enhance the overnight transfer.
24. On the next day rinse the membrane in transfer buffer and crosslink the DNA under UV light using the autocrosslink mode.
25. To check successful blotting, stain the membrane with methylene blue and destain with dH2O. Mark the marker bands and lanes with a pen.
26. The next step is the hybridization procedure. For prehybridization put the membrane between two sheets of Whatman paper soaked with the hybridization solution supplemented with tRNA. Seal the sandwich in a plastic bag and put it into a hybridization oven at 42 °C for 2 h. Alternatively, the membrane can be hybridized in hybridization flasks.
27. Perform the probe labeling with a Random Primer Labeling Kit, as described in the protocol provided by the manufacturer. 25 ng of purified DNA probe is radioactively labeled with (alpha-32P) deoxycytidine 5′-triphosphate. Clean the probe by using Quick Spin Columns for radiolabeled DNA purification according to the instruction of the manufacturer. Measure the specific activity of the probe with a Liquid Scintillation analyzer (Packard).
28. Denature the purified and labeled DNA probe at 95 °C for 5 min, mix it with hybridization/blocking solution (2 × 106 million cpm probe/ml blocking solution) and put it onto the hybridization sandwich as described before for the prehybridization and incubate it at 42 °C overnight. Alternatively, the membrane can be hybridized in hybridization flasks.
29. On the next day wash the blot twice in 6× SSC with 0.1 % SDS on a rocker shaker at room temperature. Then wash it once in 2× SSC with 0.1 % SDS and finally once in 0.2× SSC with 0.1 % SDS each for 15 min in a 55 °C shaking water bath or in hybridization flasks.

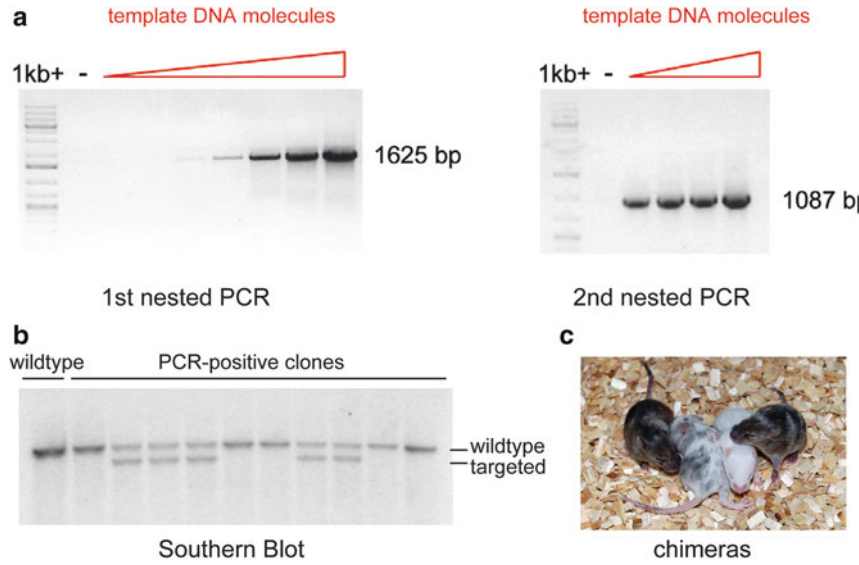

Fig. 5 Identification of correctly targeted ES cells by a highly sensitive nested PCR screen and Southern blot analysis. **(a)** Establishment and optimization of the highly sensitive nested PCR screen using the "fake template." After adjusting annealing temperature and cycle number, a dilution series of "fake template" was used for the first and second rounds of nested PCR. Taking account of the "fake template" concentration, the "fake template" length, the average molar mass of 650 g per base pair and the Avogadro constant of 6.022×10^{23} molecules per mole, concentrations of "fake template" from 10 to 10^7 molecules were generated and used as template DNA. Increasing concentrations of "fake template" ranging from 10^1 to 10^7 molecules used as template DNA and yielded a 1625 bp product (*left panel*). These 1st nested PCR products (10^1 to 10^4 molecules) used as template DNA for the 2nd nested PCR generating a 1087 bp product (*right panel*). **(b)** Southern blot analysis of nested PCR positive ES cell clones. Genomic DNAs of one wildtype negative control and several nested PCR positive ES cell clones used for a Southern blot to discriminate between the wildtype and the targeted allele. **(c)** Picture of chimeric mice after blastocyst injection of Southern blot positive ES cell clones

30. After washing wrap the blot in plastic foil and expose it to a PhosphorImager developer cassette at room temperature for an appropriate period of time. Scan the blot by using Typhoon 8600 PhosphorImager and perform data analysis using ImageQuant software.
31. To check the size of bands, expose the radioactively labeled blot additionally to an X-ray film at −80 °C and develop. A typical result of a Southern blot based screen for targeted ES cells is shown in Fig. 5b.
32. For stripping of the blot keep it in stripping solution for 30 min in a 55 °C shaking water bath. Then incubate it with strip-neutralization solution also for 30 min in a 55 °C shaking water bath. To store the membrane, dry it and seal it in a plastic foil and store it at −20 °C.

3.3 Blastocyst Injection

ES cell injection should be performed by a trained person, for instance a member of the animal facility. Typically, three successfully targeted ES cell clones are injected into 3.5 dpc blastocysts. The blastocysts are isolated on day E3 from uteri of fertilized female B6(Cg)-*Tyrc-2J/*J mice. B6(Cg)-*Tyrc-2J/*J (B6-albino mice) are C57BL/6J mice that carry a mutation in the *tyrosinase* gene. Pigment is completely absent from skin, hair, and eyes in these mice homozygous for *Tyrc-2J* (Jackson Laboratory homepage). The white fur of these mice makes these mice ideal for creation of novel strains with targeted mutations. If the targeted ES cells are derived from the B6129F1 or similar background (brown fur) and are injected into blastocysts derived from B6-albino mice (white fur), chimerism of the born offspring can be estimated as the percentage of brown/agouti versus white fur color.

Up to ten ES cells are injected into one blastocyst, which are then implanted into the uteri of pseudopregnant foster mothers. The inner cell mass of these manipulated blastocysts displays a mosaic of wildtype and transgenic ES cells giving rise to chimeras. Correctly targeted ES cells are thawed and split at least two times before blastocyst injection to let them recover. They are trypsinized, centrifuged, and seeded on a 10 cm dish for 30 min to let the feeder cells settle down. The supernatant containing enriched ES cells is centrifuged, resuspended gently in 1–2 ml medium, and stored on ice until injection.

3.4 Generation of Floxed Alleles with CRISPR/ Cas9

An attractive alternative to the conventional gene targeting approach by homologous recombination is the introduction of *loxP* sites by CRISPR/Cas9 [17]. The major advantage of this method is the generation of mice with a floxed allele in one step. There are several online tools (e.g., http://crispr.mit.edu) for finding CRISPR target sites (20 nucleotides followed by the protospacer adjacent motif (PAM) "NGG" near the region of interest. Recent findings [15] show that the CRISPR/Cas nuclease specificity can be improved by truncating guide RNAs to 18 nucleotides. To insert two *loxP* sites into the same allele of the desired gene, (1) two guide RNAs targeting the introns flanking the desired exon and (2) the corresponding two repair oligonucleotides containing the *loxP* site need to be generated (Fig. 2b).

1. Generate two guide RNAs for the exon of choice. Double stranded gBlocks® fragments (*IDT*) can be used as template for in vitro transcription using the MEGAshortscript kit (*Thermo Scientific*) followed by purification using the MEGAclear transcription clean-up kit (*Thermo Scientific*). gBlocks® fragments can be designed in the following way with a 6 bp filler, followed by the T7 promoter and the chimeric sgRNA containing the 18 nucleotide target recognition sequence (selected CRISPR target without PAM sequence, N_{18}) and a gRNA scaffold:

ccgctgTTAATACGACTCACTATAGGGN$_{18}$gttttagagc-taGAAAtagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcacc-gagtcggtgcTTTT.

2. Design the two single stranded repair oligodeoxynucleotides containing a *loxP* site and 60 bp homology to sequences on each side surrounding each sgRNA-mediated double strand break according to [17]. To facilitate detection of correct insertions, the repair oligodeoxynucleotides should include different restriction sites in addition to the *loxP* sequence. Moreover, the repair template has to be designed in a way that it is not recognized and thereby cleaved by the Cas9-gRNA complex.
3. For intracytoplasmic injection of fertilized zygotes prepare a mixture of the following CRISPR components and centrifuge at full speed for 1 h at 4 °C: 50 ng/μl sgRNAs, 100 ng/μl of the repair oligonucleotides, and 100 ng/μl Cas9 mRNA (*Sigma*). The Cas9-mediated double strand break can be repaired either by the error-prone nonhomologous end joining (NHEJ) pathway or the less efficient but high-fidelity homology directed repair (HDR) pathway. The efficiency of precise HDR-mediated genome editing was reported to be increased by the NHEJ inhibitor Scr7, which can be coinjected into zygotes with the CRISPR components at a final concentration of 1 mM [18].
4. The zygote injection should be performed by a trained person, preferentially a member of the animal facility.
5. Test for successful targeting by genotyping as described in Subheading 3.5.2 (*see* **Note 13**).

3.5 Identification and Characterization of Transgenic Mice

3.5.1 Breeding to Obtain Conditional HDAC Knockout Mice

When injecting targeted ES cell clones (ES cells of B6129F1 background; *brown* fur) into blastocysts (B6(Cg)-Tyrc-2J/J (C57BL/6 albino mice)) carrying a mutation in the *tyrosinase* gene; *white* fur) and implanting them into a foster mother, the obtained offspring can be chimeric for the knock-out. Further breeding steps are needed in order to obtain mice that are homozygous for the targeted allele.

1. Analyze the chimerism by estimating the percentage of brown versus white fur color (Fig. 5c).
2. Select mice (preferentially male) with highest chimerism: the higher the chimerism, the higher the chance that these mice transmit the targeted allele via the germline.
3. Backcross these mice to a C57BL/6 strain and genotype the offspring to screen for heterozygous fl/+ alleles of the gene of interest. At this point, the selection cassette (e.g., neomycin) can be removed by backcrossing the chimera to a FlpE recombinase-expressing C57BL/6 strain.

4. By intercrossing fl/+ heterozygous mice, individuals homozygous for the floxed allele can be obtained with a 25% chance.
5. As soon as fl/+ heterozygous mice are available, also start breeding them to (tissue-specific) cre-recombinase expressing mice (*see* **Notes 14** and **15**).
6. To obtain fl/fl cre+ mice in the final breeding step, breed fl/fl cre− mice with fl/fl cre+ mice (*see* **Note 16**).

3.5.2 Identification of Conditional Hdac Knock-Out Mice

At any of the breeding steps it is necessary to determine the genotype of the obtained mice. Therefore, genotyping strategies to test for presence of the floxed allele (wt, hetero- or homozygous), effective deletion of the allele, as well as successful expression of the cre recombinase are needed. Transgenic mice are usually genotyped by PCR analysis of DNA from biopsy samples as described in detail by Bonaparte et al. [19].

Genomic DNA isolation and genotype analysis of murine biopsy samples

1. Digest biopsy sample in 100 µl biopsy lysis buffer and Proteinase K at a final concentration of 1 mg/ml.
2. Incubate the samples on a thermoshaker at 55 °C overnight.
3. In order to inactivate Proteinase K incubate samples for 5 min at 95 °C.
4. Add 200–400 µl of autoclaved dH_2O and store at 4 °C.
5. For genotyping PCR centrifuge the samples for 10 min at 4 °C (full speed) and use 0.5–2 µl of the supernatant as template for the PCR.
6. Perform genotyping with tested primers and PCR conditions.
7. Always include a positive and a negative control for the genotyping PCR. A representative example for a genotype analysis of conditional *Hdac* knock-out mice is shown in Fig. 6a.

3.5.3 Validation of the Conditional HDAC Knock-Out

To determine if cre-mediated deletion also leads to the loss of the respective mRNA and protein in the specific cell type or tissue, different approaches can be performed:

mRNA expression:

- qRT-PCR
- In situ hybridization

Protein expression:

- immunoblot
- immunohistochemistry

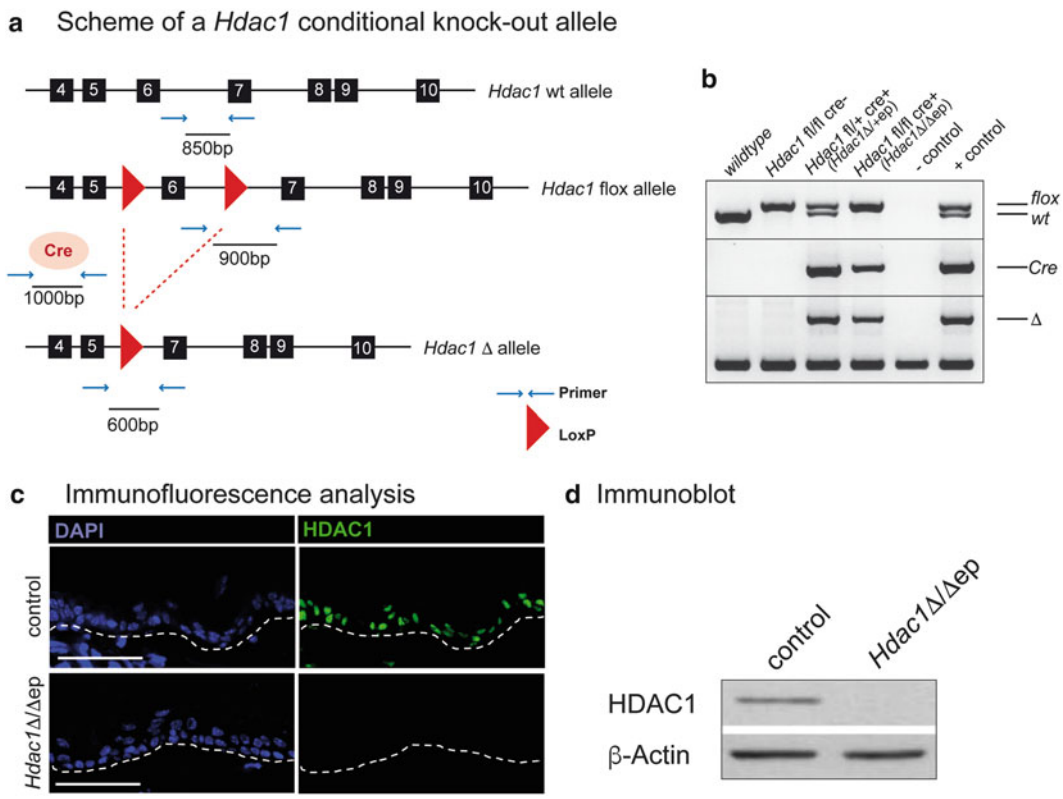

Fig. 6 Verification of a *Hdac1* conditional knock-out allele. **(a)** Two *loxP* sites (*red triangles*) flanking exon 6 of the *Hdac1* gene (*upper panel*) are introduced in ES cells, creating an *Hdac1* flox allele (*middle panel*). The *Hdac1* flox ES cells are used to create chimeras by injection into blastocysts flowed by implantation into foster mice. After crossing of *Hdac1* flox mice to animals carrying a Cre transgene, exon 6 of the *Hdac1* gene is deleted, resulting in an *Hdac1* Δ allele (*lower panel*). Exons are depicted as *black rectangles* carrying the exon number. Genotyping primers (*blue arrows*) and resulting PCR product sizes are indicated for each allele. In the wildtype situation, the genotyping primers give an 850 bp band for the *Hdac1* allele. In mice with *loxP* sites flanking exon 6 of the *Hdac1* allele, a 900 bp PCR product is generated and in the heterozygous mice both bands are obtained. The primers for the existence of the Cre recombinase in the genome yield a 1000 bp band if Cre is present. Primers detecting the *Hdac1* Δ allele only show a 600 bp band if the part between the *loxP* sites is deleted by Cre recombinase. **(b)** Picture of an agarose gel loaded with genotyping PCR samples. Lane 1 (wildtype): shows an 850 bp band (wildtype band), no band for the cre recombinase and no band for the Δ PCR. Lane 2 (*Hdac1* fl/fl cre–) with an *Hdac1* allele carrying *loxP* sites (flox) shows homozygous flox alleles (900 bp band), but no Cre recombinase PCR band and no Δ PCR band. Lane 3 (*Hdac1* fl/+ cre+) shows the wildtype and the flox band (heterozygous for the flox allele), the Cre PCR band, as well as the Δ PCR for the deleted allele. Lane 4 (*Hdac1* fl/fl cre+) shows homozygous flox alleles (900 bp), the presence of the cre recombinase gene, and the deletion of the floxed *Hdac1* allele (600 bp, Δ PCR band). **(c)** Verification of the epidermis-specific knock-out of HDAC1 by immunofluorescence staining of skin sections. The epidermis is the outer part of the skin, which is the region above the *dashed line*. Scale bar: 50 μm. **(d)** Verification of the knock-out of HDAC1 in the epidermis by using an immunoblot. Epidermal extracts from control and *Hdac1* knock-out mice (*Hdac1*Δ/Δep) were isolated and loaded onto a SDS PAGE and HDAC1 expression was detected with an antibody specific for HDAC1. β-Actin served as loading control

Hdac mRNA Expression (*See* Note 17)

1. Total RNA is extracted using Trizol reagent. It might be necessary to adapt the RNA isolation protocol for the specific cell type or tissue.
2. Resuspend cells or tissue in 1 ml Trizol reagent, incubate for 5 min at room temperature and store at −80 °C. After thawing, centrifuge samples for 10 min at 14,000 × *g* and transfer the supernatant into a new tube.
3. Add 200 µl of chloroform and shake the tubes vigorously for 15 s. For phase separation, incubate samples at room temperature for 3 min and centrifuge at 14,000 × *g* for 15 min. Transfer the upper, aqueous phase into a fresh tube and precipitate at room temperature with 500 µl of isopropanol for 10 min.
4. Pellet the precipitated RNA by centrifuging at 14,000 × *g* for 10 min. Remove the supernatant, wash the RNA pellet with 1 ml 75% ethanol, and centrifuge again at 14,000 × *g* for 5 min. Let the RNA pellet briefly dry and dissolve it in 200 µl dH_2O.
5. Precipitate the RNA a second time by adding 20 µl 3 M sodium acetate and 500 µl 96% ethanol.
6. After overnight incubation at −20 °C centrifuge the samples at 14,000 × *g* for 30 min, and wash the resulting pellet with 75% ethanol.
7. After air-drying, dissolve the pellet in 30–100 µl dH_2O for 10 min at 55 °C on a thermoshaker and measure the RNA concentration by using a Nanodrop spectrophotometer.
8. Check the integrity of the RNA on a MOPS/EDTA gel: incubate 1 µg of RNA with RNA sample buffer containing ethidium bromide at 65 °C for 5 min and load onto a MOPS/EDTA gel running at 70 V.
9. Reversely transcribe the RNA using the iScript cDNA synthesis kit. Prior to cDNA synthesis, dilute RNA samples to an appropriate concentration and use 0.5–1 µg RNA for the following reaction mix:

1 µg RNA

4 µl 5× iScript Reaction buffer

1 µl iScript Reverse Transcriptase

dH_2O to 20 µl

Mix samples gently, centrifuge briefly and reversely transcribe using the following program:

25 °C	5 min
42 °C	30 min
85 °C	5 min

10. Select appropriate exon-spanning primers for the targeted exon and a neighboring exon and perform quantitative real-time PCR analysis (*see* **Note 18**).

HDAC Protein Expression

To verify the loss of the protein either immunoblot analysis or immunohistochemical analysis of the affected tissue are the methods of choice. Examples for both methods are shown for the epidermis-specific deletion of HDAC1 in Fig. 6b, c. The specific method for the analysis of HDAC proteins in tissues using immunohistochemistry entirely depends on the given tissue and will not be described here.

HDAC Immunoblot Analysis

1. Extract proteins with the appropriate buffer. It might be necessary to adapt the protein isolation protocol for the specific cell type or tissue. Mouse tissues are homogenized in a suitable amount of extraction buffer containing appropriate inhibitors (protease and phosphatase inhibitors).
2. Measure the protein concentration using the Bradford Protein Assay.
3. Analyze HDAC expression in equal amounts (20 μg) of protein extracts by conventional immunoblot analysis (*see* **Note 19**).

4 Notes

1. A large number of vectors and conditional targeted alleles have been produced in a genome-wide approach [17]. In addition, several companies offer commercial DNA cloning and vector construction services: GenScript Corporation, inGenious, InvivoGen, OzGene, SeqWright, Vega Biolab, Cyagen.
2. As alternative to conventional cloning, the targeting vector can be generated by recombineering (recombinogenic engineering). This method is based on heat shock-induced recombination in bacteria [18] instead of cutting/ligation of DNA fragments and is less time-consuming.
3. To reduce errors in the amplified sequences, use a proofreading polymerase such as Pfu polymerase for the PCR reactions.
4. We have generated conditional knock-in alleles for *Hdac1* and *Hdac2* using this strategy. In both cases turning of the cassette was induced by Cre recombinase resulting in the correct sense orientation of the mutated exon. However, expression of the targeted allele in the wildtype configuration was strongly impaired for both HDAC1 and HDAC2, most probably by the insertion of the additional sequences into introns 4 and 5 of the respective genes.

5. During the construction of the TV for conditional knock-in we have experienced serious cloning problems, when we attempted to clone the middle arm containing wildtype exon und the inverted mutated exon. This might be due to the fact that *E. coli* cells do not tolerate these large palindromic sequences. One way to overcome these problems would be to avoid complete symmetry of the middle arm.
6. If the eluate is too diluted, evaporate the final TV obtained by Midi Prep on a Speed-vac to get a proper concentration.
7. ES cells are always grown on feeder-coated plates, except for ES cells used for DNA isolation (Southern blot), which are grown on 0.1% gelatin-coated plates. Medium is changed every day. ES cells are passaged usually every second day (apart from colonies under selection) after they are about 80% confluent. For freezing, ES cells or feeders are trypsinized and pelleted as for passaging, but resuspended in medium and slowly diluted 1:1 with 2× Freezing medium. This is done according to their splitting ratio and 1 ml aliquots are transferred to 1.5 ml freezing vials. The vials are wrapped in paper and placed in the −80 °C freezer to cool them down slowly. A few days later the cells are transferred to the liquid nitrogen tank for long-term storage.
8. DR4 primary mouse embryonic fibroblasts with resistance against neomycin, hygromycin, puromycin, and 6-thioguanin are used as feeder cells. For nonirradiated feeder cells medium is changed every second day. In case of irradiated feeder cells, medium is changed every third to fourth day. Feeder cells are split 1:3 and latest after five passages are γ-irradiated (11Gy) for 3:30 min to mitotically inactivate them. If feeder cells are used directly for ES cell cultivation, they have to be seeded at least 3 h prior to ES cells to allow attachment to the surface.
9. Since ES cells should not be longer than half an hour without medium, stop picking after 30 min.
10. A fake template that mimics the successful homologous recombination has to be constructed in order to establish the nested PCR. You need to set up the nested PCR procedure *before* you start with the electroporation of the ES cells. The nested PCR is very sensitive and detects as few as ten molecules of the fake template (Fig. 5a).
11. Bend the Pasteur pipette into a shape of a hook with a Bunsen burner.
12. Assemble the Southern Blot carefully to avoid air bubbles. In order to prevent short-circuit of blotting, cover the area surrounding the gel with parafilm.

13. Potential off-target sites are predicted by the CRISPR/guides selection server at crispr.mit.edu (http://crispr.mit.edu) and can be tested by PCR as described [17].
14. To choose the appropriate cre strain for tissue or cell type specific HDAC deletion, consult http://www.informatics.jax.org/recombinase.shtml and search either by tissue of interest (Recombinase activity in) or by the recombinase itself (Recombinase driven by). If the suitable promoter for your Cre recombinase was found, you can find a direct link to the homepage of International Mouse Strain Resource (IMSR) http://www.findmice.org, where you can order the mice from the repository of *The Jackson Laboratory* or you order directly at their webpage: http://www.homeimprovement.com/mouse-order.jax.org/.
15. For some cre-expressing mice the cre recombinase has to be passed through the male germline to circumvent general recombination when introduced maternally, for instance [20].
16. If homozygous fl/fl cre+ mice cannot be bred due to lethality or infertility, perform the final breeding by crossing heterozygous fl/+ cre+ mice with fl/fl cre− mice.
17. To avoid RNA degradation treat all tools and working surfaces with 1 M sodium hydroxide and perform centrifuging steps at 4 °C.
18. In addition, it is recommended to examine expression of transcripts containing sequences 5′ and 3′ to the deleted exon(s) by using appropriate primers. Some but not all mutated transcripts are recognized by the nonsense-mediated mRNA decay pathway. The remaining transcript might encode functional parts of the protein.
19. To detect truncated HDAC polypeptides expressed from the conditional knock-out allele, it might be helpful to perform immunoblot analyses with both N-terminus specific and C-terminus specific antibodies.

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Chapter 14

Engineering of Conditional Class I *Hdac* Knockout Mice and Generation of a Time-Spatial Knockout by a Dual Recombination System

Sieglinde Bayer and Matthias Wirth

Abstract

The protein sequences of class I HDACs in mice and humans are 96–99% identical. These highly conserved proteins have crucial roles in biological processes, such as proliferation and development, which is reflected in the lethality that occurs in conventional whole body knockout mice. Therefore, conditional knockouts are inevitable to investigate the functions of class I HDACs in mice. Here, we describe the generation of conditional class I *Hdac* knockout mice, using *Hdac1* as an example. We explain a relatively quick procedure to generate the necessary target vectors by recombination-mediated genetic engineering and gateway techniques. Furthermore, we show how to culture, target, and screen for positively recombined ES cells. Additionally, we present a dual recombination system, which allows the deletion of class I *Hdacs* at any time by a tamoxifen inducible Cre.

Key words HDAC, GEMM, Knockout, Mouse, Transgenic, Cre/lox, Recombination, Flp/FRT, Dual recombination, Tamoxifen

1 Introduction

For in vivo loss-of-function studies, two common techniques are used. (1) The conventional knockout, in which the gene is irreversibly deleted in the embryonic stem cell (ES cell) and (2) the conditional knockout. In the case of the conditional knockout, the target region is flanked by recognition sites for site-specific recombinases, such as the Cre recombinase, which recognizes *lox*P sites [1]. Due to many disadvantages of the conventional knockout, conditional knockouts are currently the state of the art. In principle the genetic modification, which is part of a targeting vector that contains homologous regions 5′ and 3′ of the modified DNA, is introduced into ES cells. By homologous recombination, the modified DNA is introduced into ES cells. Selection markers allow survival of the ES cells with a positive insertion of the targeted region [2].

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The four members of the Class I HDAC family are located on different chromosomes: *Hdac1* on chromosome 4, *Hdac2* on chromosome 10, *Hdac3* on chromosome 18, and *Hdac8* that is located on the X chromosome. None of the conventional knockouts of class I *Hdac*s are viable. *Hdac1* and *Hdac3* whole body knockout mice die both prenatal at embryonic days 10.5 and 9.5, respectively, due to proliferation or gastrulation defects [3–6]. The conventional knockouts of Hdac2 and Hdac8 die perinatal due to cardiac malfunction or skull instability [3, 7, 8].

Therefore, to investigate the function of class I HDACs in mice in vivo, an organ-specific deletion of the *Hdac*s is necessary. Conditional mouse models are an adequate alternative to conventional knockouts and inevitable to study gene functions of lethal genes, such as class I *Hdac*s. Mechanistically, the dimer of two identical *lox*P sites is recognized by the Cre recombinase, which then cuts out the inter-*lox*P-site region of interest [1, 9]. The Cre-*lox* site-specific recombination system has its origin in the procaryotic coliphage P1 [10]. The phage codes for a short asymmetric 34 bp-long DNA sequence (*lox*P site) that consists of two 13 bp-long inverted repeats and is separated by an asymmetric 8 bp-long core sequence [10]. The Cre protein (encoded by the *P1* gene), which may be fused to a nuclear localization sequence (NLS), enters the nucleus and recognizes the *lox*P sites [11]. Synapsis of the DNA at the two *lox*P sites is followed by the break and a rejoin of the DNA. The recombination can occur (1) inter- or intramolecularly, (2) on supercoiled or linear DNA, and (3) regardless of the relative orientation of the *lox*P sites within the DNA molecule. To achieve the deletion of a DNA segment, this region needs to be flanked by identical *lox*P sites. Upon recombination, this sequence will be removed [10, 12]. Many Cre driver mouse lines with a tissue-restricted expression of Cre are available (www.jax.org) to gain insights into gene functions.

The knockout of possible therapeutic targets is a common method in cancer research, so there is a trend to use conditional knockouts to investigate tumor maintenance and tumor resistance. The dual-recombinase system is very useful for investigation of such questions. The Flp/*FRT* system is analogous to the Cre/*lox*P system. The Flippase recombinase (Flp) binds to two identical *FRT* sites (*F*lippase *R*ecognition *T*arget sites), which results in cleavage and recombination of the sequence in between the *FRT* sites [13]. In this system the active Flp recognizes *FRT* sites that could be located at any place in the genome. These *FRT* sites are, for instance, part of a *FRT*-STOP-*FRT* (FSF) cassette, which are located in front of a mutated oncogene, such as $Kras^{G12D}$ and additionally in front of an inducible Cre [14, 15]. As a result of the active Flp the *FRT* sites are recognized, leading to the cleavage of the stop codon. Upon recombination, the oncogene (e.g., $Kras^{G12D}$) and the inducible Cre protein will be expressed locally. There are multiple ways to obtain inducible genes or proteins, for

instance by TetOn/Off systems or light-sensitive proteins (optogenetics). To induce a Cre-mediated recombination, the protein needs to be localized in the nucleus. It is possible to fuse the Cre protein to a mutated ligand binding domain of the human estrogen receptor, called CreER^T [16]. Due to binding to the chaperone HSP90, CreER^T is not able to shuttle into the nucleus. Only in the presence of the antiestrogen, tamoxifen, CreER^T dissociates from HSP90 and can be shuttled into the nucleus (Fig. 1). There it recognizes and cuts *lox*P sites, which flank certain target regions, leading for instance to a knockout of a gene. By combining the Flp/*FRT* and the Cre/*lox*P recombination technologies in one mouse model at any time, a Cre-mediated recombination of *lox*P flanked regions is possible [14, 15].

2 Materials

Comparable compounds, kits, enzymes, etc., from other suppliers may also work well.

2.1 Buffer and Media

1. ES Cell Lysis Buffer: 100 mM Tris-HCl, pH 8.5; 5 mM EDTA; 0.8 mM HCl; 0.2% SDS; 200 mM NaCl; 0.1 mg/ml Proteinase K (add prior to use, may be degraded if not stored properly).
2. 6× Loading buffer: 60% Glycerol; 60 mM EDTA; 0.24% Orange G.

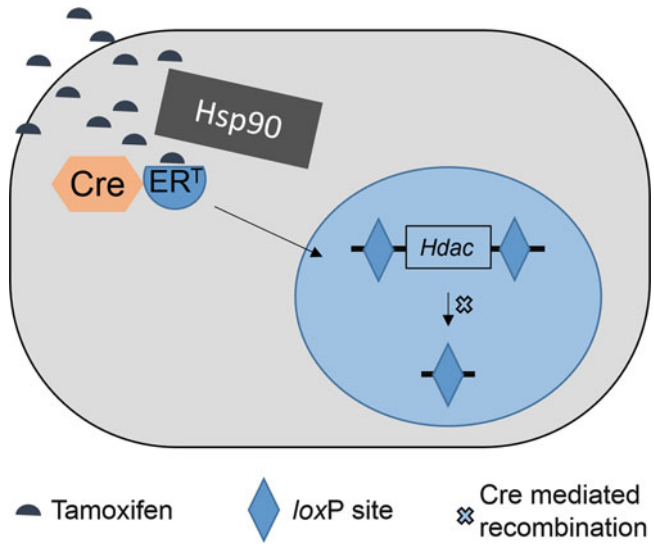

Fig. 1 Mode of action of the inducible CreER^T. Under physiological conditions the CreER^T (Cre protein, which is fused to a modified human estrogen receptor) is sequestered in the cytosol by the Hsp90 chaperone. Upon treatment with antiestrogen (for instance tamoxifen) Hsp90 dissociates and the CreER^T fusion protein enters the nucleus and recombines *lox*P site flanked target genes (e.g., *Hdacs*)

3. PCR lysis buffer (Soriano buffer): 0.5% Triton®X-100; 1% 2-Mercaptoethanol; 1× Gitschier's buffer; 400 µg/ml Proteinase K (add prior to use).
4. 10× Gitschier's buffer: 670 mM Tris-HCl, pH 8.8; 166 mM $(NH_4)_2SO_4$; 67 mM $MgCl_2$.
5. IP-buffer, pH 7.9: 50 mM HEPES; 150 mM NaCl; 1 mM EDTA; 0.5% NP-40; 10% Glycerol.
6. 50× Tris acetate EDTA (TAE) buffer, pH 8.5: 2 M Tris; 50 mM EDTA; 5.71% acetic acid.
7. 5× KCM buffer: 500 mM KCl; 150 mM $CaCl_2$; 250 mM $MgCl_2$.
8. TE-buffer, pH 8: 10 mM Tris; 1 mM EDTA.
9. TSB-buffer: 10% PEG4000; 5% DMSO; 10 mM $MgCl_2$; 10 mM $MgSO_4$; LB-Broth pH 6.1 and filter through 0.45 µM membrane (store at 4 °C).
10. Luria-Bertani (LB) medium: 10 g tryptone; 5 g yeast extract and 10 g NaCl in 950 ml deionized water. Adjust the pH to 7.0 and autoclave at 121 °C for 15 min. To obtain LB-Agar just add 15 g/L agar before autoclaving.
11. Super optimal broth medium with catabolite repression (S.O.C.): 2% tryptone; 0.5% yeast extract; 20 mM glucose; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$.
12. Embryonic stem cell medium D-MEM without glutamine: 15% FCS; 1% Penicillin-streptomycin; 1% L-Glutamine; 1% sodium pyruvate MEM; 1% MEM nonessential amino acids; 0.1% 0.1 M 2-Mercaptoethanol; 1000 U/ml LIF.
13. MEF medium D-MEM: 10% FCS; 1% Penicillin-streptomycin; 1% L-Glutamine.
14. Freezing medium 70% D-MEM: 70% D-MEM; 20% FCS; 10% DMSO.

2.2 Consumables

Phosphate buffered saline (PBS), fetal calf serum (FCS), Penicillin/Streptomycin (PenStrep), 4-Hydroxytamoxifen (4-OHT), ethanol (absolute), Tris, EDTA, HCl, NaCl, Orange G, Glycerol, Triton® X-100, beta-Mercaptoethanol, ammonium sulfate, $MgCl_2$, HEPES, Nondiet P-40, acetic acid, KCl, $CaCl_2$, L-Glutamine, sodium pyruvate, ethidium bromide, Dulbeccos Modified Eagle Medium (D-MEM), dimethyl sulfoxide (DMSO), gelatin, agarose, 2-Propanol, SSC-buffer concentrate (Sigma Aldrich), BBXF agarose gel loading dye, Hybond-N+ membrane, Amersham Rapid-hyb™ buffer, and Kodak® BioMax™ MS film.

2.3 Kits

Topo® TA cloning® kit Invitrogen GmbH.

Zero Blunt® TOPO® PCR cloning kit Invitrogen GmbH.

GeneJET PCR Purification Kit, Thermo Scientific.

NucleoSpin Plasmid, Macherey-Nagel.

QIAamp DNA Mini Kit.

Amersham Rediprime™ II DNA labeling system.

Amersham micro columns Illustra ProbeQuant™ G-50.

2.4 Antibodies

α-HDAC1 antibody	1:1000	Merck Millipore: #05-100
α-HDAC2 antibody	1:500	Santa Cruz: sc-7899
α-HDAC3 antibody	1:1000	Santa Cruz: sc-11417
β-actin antibody	1:2000	Sigma-Aldrich: A5316

(*see* **Note 1**).

2.5 Enzymes

Taq-polymerase, Pfu-polymerase, T4 ligase, specific restriction enzymes, RNase A, Collagenase Typ II, Proteinase K, Clonase (from TOPO® Cloning Kit, Invitrogen).

3 Methods

3.1 Generation of the Targeting Construct

To generate conditional knockouts of each of these *Hdacs* first the respective target vector has to be designed. Instead of using word processing applications, it is more efficient to use plasmid mapping tools, such as ApE or Snapgene (http://biologylabs.utah.edu/jorgensen/wayned/ape/; http://www.snapgene.com/). To obtain the sequence information of the respective gene locus, databases such as ensembl.org or ncbi.nlm.nih.gov are recommendable. Ensembl.org enables visualizing BAC (bacterial artificial chromosomes) clones (Fig. 2) [17]. These BAC clones can be obtained by several sources, e.g., http://dna.brc.riken.jp/en/NBRPB6Nbacen.html. By a method, called recombineering, the final targeting vector can be created (Fig. 3).

1. Design oligonucleotides with ~50 bp of homology to the BAC clone + 20 bp of the vector containing l phage attachment sites for Gateway® cloning (e.g., pENTR/D-TOPO) to finally obtain two oligonucleotides with a total length of 70 bp. The targeting construct should contain an origin of replication (ori), a selection marker (e.g., Kanamycin), and attL1/attL2 sites (Invitrogen) (*see* **Note 2**).
2. The previously designed oligos are used to amplify the plasmid DNA by using a proofreading polymerase (e.g., Pfu), if TOPO® cloning is not used. At least 150 ng/μl of the resulting PCR product has to be used for the following steps (Table 1). To

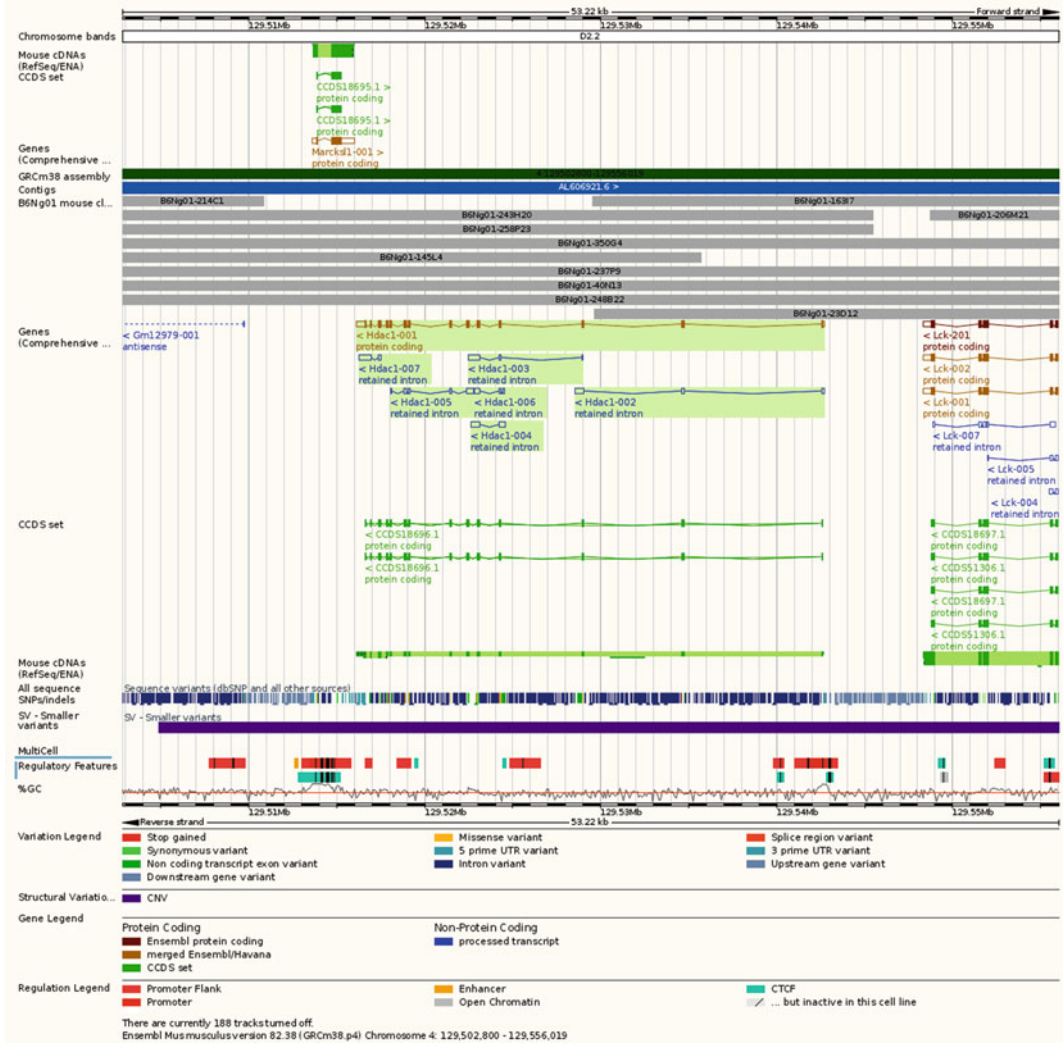

Fig. 2 Screenshot of the murine *Hdac1* locus with BAC clones (ensembl.org). The location of *Hdac1* on chromosome 4 is displayed. The track legend is displayed on the left-hand side and the bottom, respectively. The bacterial artificial chromosome (BAC) track needs to be added to the default setting by click on 'region in detail' and subsequently by clicking on 'configure this page.' In this screenshot, the BAC clones (B6Ng01) for the C57Bl/6 mouse strain are depicted [17, 18]

reach this amount, five to ten reactions (20 μl each) have to be produced. Check the amplified PCR products by using gel electrophoresis.

3. The PCR product now needs to be purified (e.g., with GeneJET PCR Purification Kit, Thermo Scientific) to obtain clean DNA, which can be used for subsequent transformation in Red-competent bacteria (Table 2). Red-competent bacteria can also be self-made by previous transformation of a Red recombinase expressing plasmid (e.g., pKD46) [19]. Before

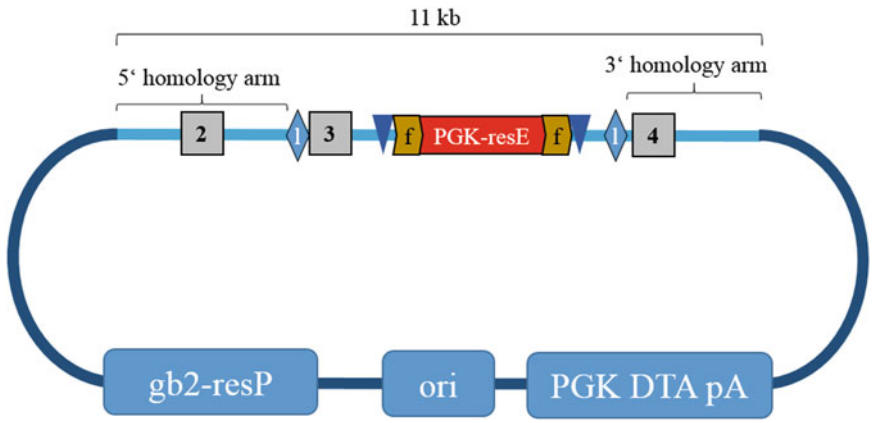

Fig. 3 Scheme of a potential targeting construct. Light blue = homologous regions (derived from a BAC clone, *see* Fig. 1); dark blue = vector backbone; 2, 3, 4 = putative exons; l = *loxP* site; f = *FRT* site; triangles = restriction enzyme recognition site; *gb2-reP* = prokaryotic *gb2* promoter with a prokaryotic resistance marker; *PGK-resE* = eukaryotic *PGK* promoter with an eukaryotic resistance marker; *PGK DTA pA* = *PGK* promoter with Diphteria toxin and a polyadenylation site as a negative selection marker; *ori* = origin of replication

introducing the PCR product (pENTR + Oligo-Plasmid DNA) the BAC clone, which contains the right targeting regions of the respective HDACs, needs to be transformed into the respective Red competent bacteria (Table 2).

4. By Red recombination the genomic fragment from the chosen HDAC containing BAC clone is inserted into the Gateway® pENTR + Oligo vector. Since there is a possibility for pENTR self-recombined containing colonies, at least 20–30 colonies have to be picked and transferred into 5 ml LB-medium (+ antibiotics) for a subsequent pDNA isolation (e.g., NucleoSpin Plasmid, Macherey-Nagel).
5. The validation of successful recombined plasmid DNA is now performed by PCR (Table 1—replace Pfu with a Taq polymerase), restriction analysis (Table 3), and sequencing. The correct plasmid now is called pEntrBAC-Hdac1.
6. The entire plasmid (pEntrBAC-Hd1) now contains genomic DNA with the region of interest; for instance, intronic regions between exon 2 and exon 3 (as the 5′ homologous region ~5 kb), the complete exon 3 (target region), and exon 4 (as the 3′ homologous region ~5 kb) of the *Hdac1* gene. The target region (exon 3) now needs to be flanked by *lox*P sites. Additionally, a mammalian resistance marker or reporter has to be introduced.

   Gene cassettes with flanked resistance marker regions can be amplified from plasmids such as pTC201B [20] or they can be obtained by certain suppliers (e.g., b-Bridge offers a FRT-PGK-gb2-neo-FRT template with a neomycin and kanamycin resistance in combination with a prokaryotic (gb2) and

Table 1
PCR conditions

Single reaction	Conditions
2 µl 10× Pfu PCR buffer	94 °C 2 min (initial denaturation)
2 µl dNTP mix, 10 mM each	
2 µl Forward primer (10 µM)	*35 cycles*:
2 µl Reverse primer (10 µM)	94 °C 30 s
1 µl proof reading polymerase (e.g., Pfu)	60 °C 30 s
1 µl Plasmid (50 ng/µl)	72 °C 1 min 30 s
ad 20 µl H_2O	72 °C 10 min (final elongation)

Table 2
Transformation protocol [21]

Generation and transformation of chemical competent bacteria
• Bacteria (e.g., StBl3) are streaked on agar plates
• An isolated colony is grown at 37 °C in 5 ml LB medium for 16 h (overnight)
• 4 ml of this culture is used for inoculation of 250 ml LB medium
• Bacteria are grown until the early log phase is reached OD_{600} 0.3–0.6 (duration approximately 1–2 h) and subsequently the bacteria will be placed on ice
• Cells are centrifuged for 10 min at 1000 × *g* (4 °C)
• Resuspension in 25 ml ice cold TSB buffer with subsequent incubation on ice for 10 min
• 100 µl aliquots are subsequently snap-frozen in liquid nitrogen and stored at −80 °C until further use (transformation efficiency > 1×10^8)
• Freshly transformed cells are thawed on ice
• 20 µl 5× KCM buffer, 10–200 ng of the DNA and dH_2O (final volume 100 µl) are mixed and added to 100 µl of the competent bacteria
• Bacteria are kept at 4 °C for 20 min plus 10 min at room temperature
• 1 ml S.O.C. medium is added and the bacteria are shaken for 2 h at 25 °C
• Bacteria are now streaked in various amounts on agar plates (with respective antibiotics) and incubated at 25 °C for 24 h

an eukaryotic (PGK) promoter). One of such gene cassettes now can be amplified with specific oligonucleotides, which harbor specific and unique recognition sites for one restriction enzyme (E2). If the dual resistance marker cassette will be used, two 50 bp flanking regions of homology to the targeting sites have to be added to the respective oligonucleotides (*see* **Note 3**).

Table 3
Restriction/ligation

Restriction digestion	
Restriction enzyme	10 units
DNA	1 µg
10× Reaction buffer	2 µl (1×)
Reaction volume	20 µl
Incubation time	1–16 h
Incubation temperature	37 °C (may differ, depends on enzyme)
Ligation:	
(a) Dephosphorylation of vector DNA by alkaline phosphatase	
(b) Pipette for one reaction:	
10× T4 DNA ligase buffer	2 µl
Vector DNA	0.020 pmol
Insert DNA	0.060 pmol
T4 DNA ligase	1 µl
H_2O	ad 20 µl
(c) Mix the reaction gently	
(d) Incubate at 16 °C overnight	
(e) Heat inactivate at 65 °C for 10 min	
(f) Chill on ice and transform 1–5 µl of the reaction into 50 µl competent cells (Table 2)	

7. To integrate the first *lox*P site into the region of interest—in this example between exon 2 and exon 3—a prokaryotic selection marker is needed. The gene cassette, which will be cloned into the vector, is composed of (from 5′ -> 3′):
   (a) A 50 bp of homology to the target region (BAC clone).
   (b) One *lox*P site.
   (c) One recognition site for a unique restriction enzyme (E1).
   (d) The selection marker (R).
   (e) Again the same restriction enzyme recognition site (E1) and
   (f) A 50 bp homology region of the target region.

   To get this cassette, just two oligonucleotides have to be designed; each of this oligo contains the 50 bp homology region, the E1

site and additionally 20 bp as a primer for the resistance marker. One of these oligonucleotides (forward or reverse) also needs to harbor the 34 bp *lox*P site. By using a proofread polymerase this cassette now can be amplified (PCR conditions: Table 1) and transferred into Red competent bacteria containing the pEntr-BAC-Hd1 construct. After transformation (Table 2) 10–30 colonies can be picked and grown in 5 ml of LB medium + respective antibiotics, the containing plasmid DNA subsequently should be isolated (e.g., NucleoSpin Plasmid, Macherey-Nagel) and further be analyzed by PCR, restriction analysis, and sequencing (*see* **Note 4**).

8. By restriction of the E1 sites (Table 3), which are flanking the selection marker, only the *lox*P site will be left. The resulting plasmid now can be ligated by using T4 or T7 ligases. By the previously mentioned screening methods (PCR, restriction analysis, and sequencing), a positive restriction and successful ligation can be proved.
9. To obtain the second *lox*P site 3′ from exon 3 of Hdac1 the previously amplified "50 bp homo-FRT-eukaryotic + prokaryotic selection marker-*FRT-loxP*-50 bp homo" cassette now will be introduced into the cloning vector by Red recombination (transformation: *see* Table 2). If only single selection marker cassettes (prokaryotic or eukaryotic) will be used, a further cloning step is needed. Here almost the same gene cassette can be used, but another restriction enzyme recognition site (E2) should be used (as selection marker, the same as in **step 7** may be used). As the dual selection cassette, also the single selection cassette, now may be transformed into Red competent bacteria, which are selected for the pEntr-BAC-Hd1-loxP-5′ plasmid (generated in **step 8**). After successful transformation (*see* Table 2; confirmation: screening by PCR, restriction analysis, sequencing) the prokaryotic selection marker can be easily replaced by E2 restriction of the plasmid and the E2-FRT-selection marker-FRT-E2 gene cassette with a subsequent ligation step (*see* Table 3).
10. The final cloning vector, which contains two flanking att sites (attL1 and attL2), now may be used for a gateway recombination into a destination vector, containing a negative selection marker (e.g., Diphteria toxin, DTA).

3.2 ES Cell Culture

There are several BAC clones for specific mouse strains available. In Fig. 2, BAC clones for the C57BL/6 mouse strain are depicted. Besides C57BL/6 embryonic cells, ES cells from the W4/129S6 strain may be used as well.

1. Embryonic stem (ES) cells need to be grown on a monolayer of mitotically inactivated mouse embryonic fibroblasts (feeder cells). By irradiation (34 gray) the mouse embryonic fibro-

blasts are mitotically inactivated. One day before seeding the ES cells MEFs need to be cultured on plates, which are coated with 0.1% gelatin. To keep the ES cells in an undifferentiated status, they should not become confluent but passaged by trypsinization.

2. For the genetic transfer into the ES cells the electroporation method was used [22, 23]. Prior to transfection the targeting vector needs to be linearized (Table 3) by a single cutter restriction enzyme (e.g., load the full sequence into the web-based tool NEBcutter and choose single cutter only). To check for a successful linearization 100 ng of digested and 100 ng of the original targeting vector can be analyzed by gel electrophoresis (0.7–1% agarose). Uncut DNA yields more than one band: coiled, super-coiled, nicked, linear, covalently bonded, and circular single stranded DNA can be observed, whereas a complete digestion should show only one band for the linearized DNA.
3. 25 µg of the linearized targeting vector now can be transfected into the previously prepared ES cells. Together with 1×10^7 ES cells the DNA should be resuspended in 750 µl PBS (4 °C) and subsequently transferred into a precooled electroporation cuvette. Two pulses at 250 V and 500 µF (e.g., Gene Pulser® II) should be enough for transfection of the DNA into the ES cells. Immediately after electroporation, 50 ml of prewarmed ES medium has to be added and pipetted onto 6-well plates covered with four to eight feeder cells. As a control for successful selection nonelectroporated ES cells may be seeded onto another prepared 6-well plate.
4. 24 h after transfection, the selection for successful transfected ES cells can start. For selection of the ES cells 250 µg/ml Geneticin® has to be added to the normal ES medium. For the period of 1 week, the medium has to be changed every day. Using filter tips, single clones can be picked and transferred onto feeder cell-covered 24-well plates for further culturing. Growing clones can be further subdivided on prepared 96-well plates for screening of the ES cell clones.

3.3 ES Cell Screening

1. Before screening of ES cell clones DNA needs to be isolated. Out of the 96-well plate the ES cell clones are collected in 300 µl ES cell lysis buffer and incubated at 55 °C for 4–8 h. The lysed cells are subsequently centrifuged at $24{,}000 \times g$ for 10 min. 200 µl of the supernatant is mixed with 200 µl of 2-propanol and again centrifuged at $24{,}000 \times g$ for 10–15 min. The supernatant now will be discarded and the pellet will be dissolved in 300 µl ice cold 70% Ethanol. After centrifugation (10 min at $24{,}000 \times g$) the supernatant will be discarded. The previously dried pellet now may be dissolved in 100 µl

TE-buffer (pH 8.0). Alternatively the DNA may be isolated, using silica spin columns (e.g., QIAamp DNA Mini Kit).

2. The purified DNA now can be used for a PCR-mediated screening of a successfully integration of the modified DNA in the targeting region (e.g., *Hdac1*). It is important to use a bunch of primers and the respective controls. Positive clones should further be confirmed by Southern Blot.
3. Purified DNA (**step 16**) from ES cell clones, showing the correct results in the screening PCR, as well as DNA from wild type ES cells, should be digested by a specific restriction enzyme (Table 3) and RNaseA (12 μg/ml).
   (a) The digested DNA is loaded on a 1% agarose gel with BBXF agarose gel loading dye (together with a DNA standard). Gel electrophoresis should run overnight (14–16 h) at 40 V. Under UV light the ethidium bromide stained gel now may be photographed.
   (b) The DNA now should be transferred onto a Hybond-N+ membrane (by vacuum blotting). By 60–75 min incubation in 0.25 M HCl and a subsequent 0.4 M NaOH incubation the gel is denaturized. The DNA now is transferred to the membrane by blotting for 4 h at 55 mbar. To fix the DNA, the membrane has to be washed in SSC buffer first and is subsequently incubated for 2 h at 80 °C.
   (c) The membrane now should be prehybridized for 2 h at 65 °C in 0.1 ml Amersham Rapid-hyb™ buffer per 1 cm^2 membrane.
   (d) Using the Amersham Rediprime™ II DNA labeling system, 25 ng of the sample is labeled with deoxycytidine 5′-triphosphate, [alpha-32P] during the prehybridization step.
   (e) The labeled sample needs to be purified (e.g., by Amersham micro columns Illustra ProbeQuant™ G-50). Incorporation can be measured by a liquid scintillation counter.
   (f) Before mixing with the prewarmed Amersham Rapid-hyb™ buffer the labeled samples should be denaturized for 5 min at 95 °C and cooled for 5 min on ice. At 65 °C the samples are hybridized overnight.
   (g) After hybridization the membrane needs to be washed in 2× SSC-buffer (one time, 20 min), in 1× SSC-buffer (two times, 1 h), and in 0.1× SSC-buffer (one time, 1 h).
   (h) Finally, the membrane can be exposed to a film (e.g., Kodak® BioMax™ MS film).

3.4 Blastocyst Injection

By microinjection the ES cells are transferred into host blastocysts to generate germline chimeras [24].

3.5 Genotyping of the Genetic Engineered Mouse Models Hdac1, Hdac2 and Hdac3

For genotyping of the mice usually a tail biopsy (2–3 mm) is taken and lysed with 50 µl Soriano buffer + appropriate amount of Proteinase K to release the DNA. Short centrifugation makes sure that all tail biopsies are covered with Soriano buffer. The following PCR conditions should be used for lysis: 55 °C for 90 min, 95 °C for 15 min, pause at 4 °C. Centrifuge the samples at 12,000 × *g* for 10 min and transfer 45 µl of the supernatant, which contains the DNA, to fresh reaction tubes and store them at −20 °C until further use. The DNA can be used for genotyping with respective primers for the gene of interest according to Table 1 (here a Taq polymerase instead of a proofreading polymerase may be used).

3.6 Generation of a HDAC Knockout Cell Line Using Inducible CreER^T

When the mice with the inducible dual-recombination system develop cancer, the isolation of these cancer cells can be started.

A piece of the tumor tissue has to be separated from the primary tumor, put into sterile PBS, and cut into small pieces. For isolation of the tumor cells the tissue pieces have to be transferred to D-MEM medium with 10% FCS, 1% Pen/Strep, and 0.1% Collagenase type II, followed by incubation in a water bath at 37.5 °C overnight. The next step is the centrifugation of the suspension at 250 × *g*, 5 min, and room temperature, the supernatant has to be discarded. Afterward the cells have to be transferred to a cell culture dish with fresh medium. Cultivation of cancer cells should be done at 37.5 °C and 5% CO_2 and near 100% humidity.

After a few days, when the cells are approximately 80% confluent, the tamoxifen treatment to knock out the floxed gene sequences can be started.

To activate the shuttling of the cytosolic ER^T-fusion protein to the nucleus, the cells have to be treated with 0.5–1 µM tamoxifen [25]. After seeding the cells in cell culture dishes, add medium dosed with tamoxifen or, as a control, with the vector (ethanol absolute). Replace this media daily. Whether the knock out was successful or not, should be checked on the DNA, mRNA, or protein level (*see* Subheading 3.7) after 3, 5, and 8 days.

3.7 Validation of HDAC Deficiency by Western Blot

After tamoxifen/ethanol treatment the medium has to be removed and the cells need to be washed with cold PBS. For harvesting the proteins 200 µl IP buffer including phosphatase- and protease inhibitors will be added to the cells. The cells are collected with a

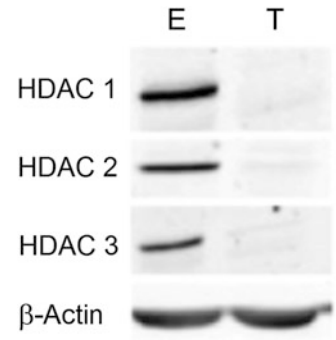

Fig. 4 Western blot analysis of cell lines, derived from genetic engineered *Hdac1–Hdac3* mouse models. *Hdac1*$^{lox/lox}$, *Hdac2*$^{lox/lox}$, *Hdac3*$^{lox/lox}$ cell lines (each with additional alles: *Pdx1-Flp*, *FSF-KrasG12D*$^{/+}$, *R26FSF CreERT*$^{/+}$) treated for 8 days with 600 nM Tamoxifen (T) or Ethanol (E) as a control. Western Blot analysis performed with primary antibody: α-HDAC1 antibody (Merck Millipore: #05-100), α-HDAC2 antibody (Santa Cruz: sc-7899), α-HDAC3 antibody (Santa Cruz: sc-11417). Loading control: β-actin (Sigma-Aldrich: A5316)

cell scraper and transferred to a reaction tube, followed by shock freezing the cells in liquid nitrogen. To test whether the knock out was successful a Western Blot analysis may now be performed [25] (Fig. 4).

3.8 Major Problems with the Technique Used

3.8.1 Time Consuming: Available Quick GEMM

If multiple alleles are desired (e.g., eight alleles such as *Hdac1*$^{lox/lox}$, *R26-*$^{CreERT/lacZ}$, *FSF-Kras*$^{G12D/+}$, *Pdx1-Flp*, *Trp53*$^{frt/frt}$), breading of those mice is quite time consuming and takes up to several months. A faster method is an embryonic stem cell approach (ESC-GEMM), where necessary alleles are already targeted and the desired gene of interest can be targeted (or mutant variants introduced) additionally, for example, by an introduction of a specific shRNA or Crispr/Cas9 using the recombinase-cassette mediated exchange (RCME) method [26–29].

3.8.2 Cre Toxicity

A range of publications show that Cre expression comes along with genotoxicity and/or antiproliferative effects [30, 31].

Hameyer et al. [32] compared different ligand-dependent Cre recombinases concerning genotoxicity and recombination efficiency by use of fusion proteins that consist of Cre and a mutated human estrogen receptor (ER^T). During this study, they showed that the Cre with the deleted D domain of the human estrogen receptor shows the best recombination efficiency and also antiproliferative effects after adding the ligand tamoxifen. Other Cre recombinases with high or low affinity to tamoxifen had a lower

recombination efficiency, but no effect in proliferation could be detected [32].

Nevertheless, Cre recombinases are a widely used tool for inducing genomic recombination, but it is mandatory to exclude any possible side effects. One option to check whether Cre toxicity is affecting the experiment is to determine an appropriate control, e.g., a Cre expressing cell line without *lox*P sites.

3.9 Alternative Recombination Systems

The optimal temperature of the Flp/*FRT* system is near 30 °C, compared to the Cre-*lox*P system, which has an optimal temperature at 37 °C. Due to the optimum at 30 °C the Flp recombinase is less efficient than the Cre recombinase in mammalian cells, which usually grow at 37 °C [33].

3.9.1 Disadvantage of the Flp/FRT Recombination System

3.9.2 Dre/rox Recombination System

Analog to Cre/*lox*P and Flp/*FRT*, there is another recombination system, which has efficiencies similar to the Cre/*lox*P-system. Here, a Dre recombinase recognizes so-called *rox* sites for recombination. In phage D6 Sauer and McDermott found this site specific DNA recombinase (Dre), which is not catalyzing recombination at *lox*P sites [34]. Anastassiadis and colleagues demonstrated that the Dre/*rox* system is highly effective in mammalian cells and in mice [35].

4 Notes

1. Antibodies are dissolved in 5 % skim milk, 0.1 % TBS-Tween
2. When using the TOPO® cloning the PCR product needs to have the following sequence (TOPO® cloning manual, Invitrogen):

   5′-CACCATG NNNNNN ---- NNN -3′

   3′-GTGGTAC NNNNNN ---- NNN -5′

3. If a recombination cassette with selection markers for both prokaryotic and eukaryotic species is used, these recognition sites do not necessarily have to be used.
4. It is necessary to use different selection markers to get a positive selection for the different constructs (e.g., Ampicilin, Kanamycin, Chloramphenicol, Blasticidin).

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Chapter 15

Xenografting of Cancer Cell Lines for In Vivo Screening of the Therapeutic Potential of HDAC Inhibitors

Daniel Nettersheim, Sina Jostes, and Hubert Schorle

Abstract

Histone deacetylase inhibitor application is lethal to many cancer types. To screen for the therapeutic potential of HDIs it is necessary to analyze their ability to target and kill cancer cells in vivo. Here, we describe the xenografting of (germ cell) cancer cell lines into the flank of nude mice and the subsequent intravenous application of HDIs.

Key words Xenografting, Cancer cell lines, HDAC inhibitors, In vivo vein injection, Therapy

1 Introduction

Histone deacetylase inhibitors (HDIs) cause global hyperacetylation of histones leading to a relaxed chromatin state termed euchromatin, which can be associated mainly with hypertranscription. Furthermore, HDIs induce cellular stress, cell cycle arrest, and apoptosis in a broad range of cancer cell types, highlighting HDIs as a therapeutic option in cancer treatment [1–3]. Besides in vitro screens on cell lines, demonstration of the in vivo potential of HDIs to specifically and efficiently target tumor cells has to be demonstrated. To address this question, cancer cell lines can be xenografted into the flank of nude mice. Subsequently, grown tumors can be treated in vivo by intravenous injection of HDIs. Here, we describe in detail the xenografting of an adherent growing embryonal carcinoma cell line (2102EP, [4]) and the in vivo treatment of transplanted animals with a HDI. The technique can simply be adapted to other cell lines.

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2 Materials

2.1 Components for Preparation of Cells for Xenografting

Our suppliers are given in brackets behind each indicated material, but the described techniques should work well with any supplier of choice.

1. Standard culture medium (SCM): RPMI/DMEM supplemented with 10% fetal calf serum (PAA, Pasching, Austria), 1% penicilin/streptomycin (PAN, Aidenbach, Germany), and 200 mM l-Gutamine (PAN, Aidenbach, Germany).
2. DPBS, pH 7–7.3 (Gibco, via Life Technologies, Darmstadt, Germany).
3. 0.05% Trypsin/1× EDTA (PAN, Vienna, Austria).
4. Neubauer counting chamber.
5. Light microscope.
6. Centrifuge.
7. Corning Matrigel Matrix (Corning, Kaiserslautern, Germany).

2.2 Components for Xenografting of Cells

1. CD1 nude mice (Crl:CD1-*Foxn1*^{nu}; Charles River, Erkrath, Germany) or other immunodeficient strain.
2. 1 ml syringe + needle (28G) (BD, Heidelberg, Germany).

2.3 Components for Intravenous Application of HDIs

1. 1 ml syringe + needle (28G) (BD, Heidelberg, Germany).
2. Heating plate.
3. Mouse fixator.

3 Methods

3.1 Preparation of Cells for Xenografting

The following protocol describes preparation of cells for the xenografting of one mouse.

1. In a T75 cell culture flask 2102EP cells are grown to 90% confluency at 37 °C and 7.5% CO_2 in SCM.
2. On the day of injection, remove cells from incubator, aspirate SCM and wash cells once with 15 ml DPBS.
3. Remove PBS and add 1 ml 0.05% Trypsin/1× EDTA solution. Incubate cells for 5 min at 37 °C, 7.5% CO_2. Check continuously for detachment of the cells under the microscope.
4. Once cells have fully detached, add 3 ml fresh SCM.
5. Prepare a 1:10 dilution of the cell suspension in DPBS (e.g., 50 µl cell suspension + 450 µl DPBS) and determine the cell number under the microscope by utilizing a Neubauer counting chamber.
6. Transfer cell suspension containing 1×10^7 cells into a 15 ml Falcon tube and centrifuge for 5 min at 800 × *g*, ideally in a swing-out rotor centrifuge.

7. Remove SCM supernatant and carefully resuspend cell pellet in 500 µl Matrigel (*see* **Notes 1** and **2**). Keep sample on ice until injection.

3.2 Xenografting of Cells into the Flank of Nude Mice

Xenografting is best performed by two persons (right-handed). The following protocol describes an injection with both persons sitting side by side (person 1 sitting on the left side and person 2 on the right side). For left-handed persons switch positions.

1. Take mouse out of its cage. Researcher 1 places the mouse on the wires of the top of the cage. With the right hand, hold on the tail, the left hand grasps the skin of the neck. Fix mouse but make sure that the mouse is able to grasp and breathe. In the meantime, Researcher 2 fills the syringe with 500 µl cell/Matrigel suspension.
2. Researcher 1 grabs skin at right flank of mice and pulls it up softly. Afterward, skin is penetrated with the needle and the cell/Matrigel suspension is injected carefully. During injection, slightly loosen grip on the skin to make sure that Matrigel has enough room to distribute.
3. Slowly remove needle, while grabbing the skin around the injection site and pressing it carefully and shortly together to prevent Matrigel from leaking out. Procedure was successful, when a roundish pea-size structure is visible under the skin (*see* **Note 3**) (Fig. 1a, black arrow).
4. Place mouse back in cage and let tumors grow for 2 weeks or until reaching size similar to a pea.

3.3 In Vivo Application of HDI Intravenously

1. Take a mouse out of its cage and put it on a 37 °C warm plate (Fig. 1b). Prewarming of the mouse allows for a more easy injection procedure due to enlarging of blood vessels and a better visualization of the tail vein.
2. Load 100 µl HDI (in appropriate solvent) in a sterile syringe (*see* **Note 4**).
3. After a few minutes, when the mouse starts cleaning itself (Fig. 1b) the injection procedure can be started. Fix mouse in a fixator (*see* **Note 5**) (Fig. 1c, inlay). Grab the tail with left hand and place it between forefinger and thumb and carefully bend it not more than 90° (Fig. 1c). Locate the tail vein and carefully insert injection needle parallel to the vein (Fig. 1c). Slowly inject the 100 µl HDI solution. Injection was successful when the vein blanches (*see* **Note 6**). Afterward, remove needle and apply pressure to injection site to ensure hemostasis.
4. Place mouse back in cage and check the health and condition of the mouse frequently (*see* **Note 7**).

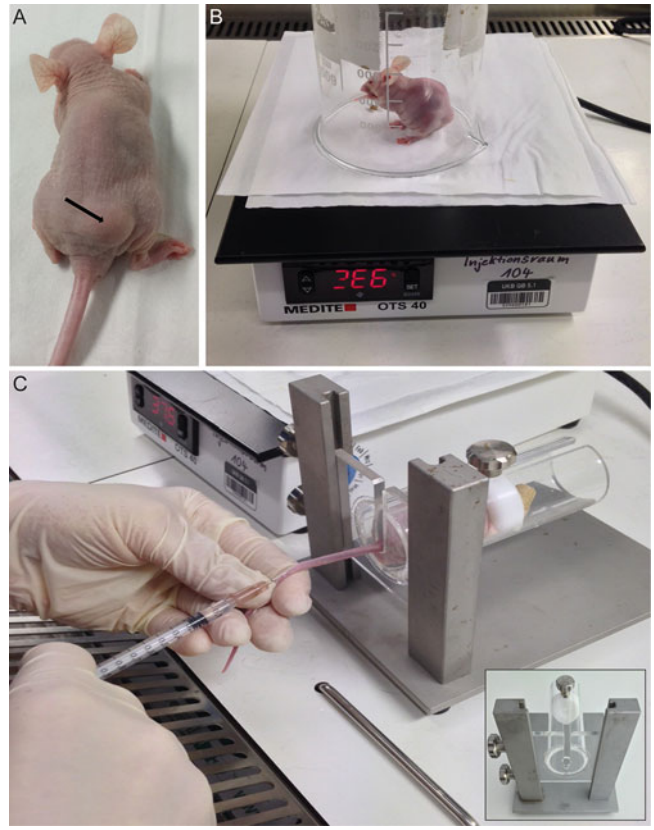

Fig. 1 (**a**) CD1 nude mouse with a tumor (*arrow*) grown from xenografted 2120EP cells. (**b**) Prewarming of a mouse on a 37 °C warm plate prior to the i.v.-injection process. (**c**) I.v. injection of a 100 μl HDI solution into the tail vein of a nude mouse fixed in fixator (inlay)

4 Notes

1. Matrigel is very viscous and sticks to reaction tube walls. Thus, we recommend to prepare cell/Matrigel solution for one or even two more mice than needed (e.g., if six mice should be xenografted, prepare cell/Matrigel solution for 7/8 mice). Additionally, Matrigel is solid at <0 °C, becomes liquid around 4 °C, but adapts a gel-like structure at higher temperatures. Therefore, make sure Matrigel is liquid (i.e., above 4 °C) before use, but does not get too warm.
2. Before resuspending the cell pellet make sure that the reaction tube used is large enough to insert the syringe and needle to fully soak up cell/Matrigel suspension.
3. Matrigel becomes resorbed 3–14 days after injection. So, size of the injected cell/Matrigel mass will first decrease, but increases again, when tumor cells start to grow.

4. Concentrations of applied HDI have to be determined by the user. Make sure to include control mice, which will be treated with the solvent.
5. Alternatively, mice can be anesthetized by intraperitoneal injection of a Ketamin/Xylazine mix (0.5 ml Ketanest (100 mg/ml, Parke-Davis via Pfizer, Berlin, Germany) + 1 ml Rompun (20 mg/ml, Bayer, Leverkusen, Germany) + 9 ml isotonic NaCl solution; 250 µl/mouse i.p.)
6. Formation of a bubble at injection site indicates an unsuccessful vein injection procedure.
7. Please make sure that the experimental setup has been approved by your local ethics committee.

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Chapter 16

Generation of Xenotransplants from Human Cancer Biopsies to Assess Anti-cancer Activities of HDACi

Christina Susanne Mullins, Stephanie Bock, Mathias Krohn, and Michael Linnebacher

Abstract

Human tumor in vivo cancer models raised in immunodeficient mice, the so-called patient-derived xenografts, are increasingly in use in preclinical development and evaluation of novel drug candidates including HDAC inhibitors. Here, we describe the techniques needed to generate novel patient-derived xenografts. The focus lies on vitally frozen tumor biopsies as starting material. First, the preparative steps on the animals, followed by the engraftment procedure itself, the tumor growth surveillance, the explantation procedure, and finally the handling of obtained xenograft tissues are described step by step. This technical description is completed by numerous tips and alternatives designed to allow for an easy adaptation and transfer to other laboratories.

Key words Xenografts, Xenopatients, PDX, Xenotransplantation, Preclinical tumor models, Tumor cryopreservation

1 Introduction

For decades, the development of novel antitumoral drugs was based on preclinical in vitro efficiency testing on a panel of standard (highly passaged) cancer cell lines, i.e., the NCI60 panel for the most part. The best drug candidates were subsequently validated in vivo—typically using tumors generated by heterotopic engraftment of the very same cell lines into immunodeficient animals. Thus, it did not come as a big surprise that the majority of novel drug candidates later failed in clinical phase II and/or III studies [1–3].

One of the consequences is a shift in the design of clinical studies [3] but also of the preclinical and early clinical drug testing procedure [3, 4]. The backbone of early development remains cell line panels, but primary, patient-derived cells, ideally with meticulously selected molecular features, are increasingly recognized as better predictors for later clinical outcomes [5]. In addition to

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panels of patient-derived xenograft (PDX) models, a significantly increased predictive power of the preclinical drug delivery pipelines can be expected [4, 6]. Support comes from the fact that in clinical trials, rigid selection of patients most likely to benefit from a given treatment scheme, has increased success rates significantly [2, 3]. Panels of PDX models offer the very same opportunities: preselection of cases based on histological, clinical, molecular, and further criteria of the original cancers/patients [6].

Cryopreservation of clinical cases collected in the context of state-of-the-art biobanking [7] adds additional value to this strategy. Choosing suitable cases from hundreds of vitally frozen tumor tissues in appropriate biobanks allows for the targeted generation of cell line and PDX models fulfilling the respective selection criteria [8, 9].

Recently, we described a simple yet effective method for cryopreservation of freshly operated human colorectal cancer biopsies before engraftment into immunodeficient animals [10]. Since this cryo-step simplifies the complex logistics of xenograft model generation and subsequent studies, the description of the xenografting procedure in this article bases on such cryopreserved human tumor tissues. Of course, using fresh biopsy material is also possible and explicitly not excluded from this technical description.

Finding the best treatment option for the individual cancer patient is the ultimate goal of personalized tumor therapy. Preclinical oncological research is primarily devoted to this objective and histone-deacetylase inhibitors (HDACi) have repeatedly been suggested as promising drug candidates for clinical management of colorectal and other cancers [11]. For the most part they are tested in combinatorial treatment schemes but also as single agents [12, 13]. In the following, we describe the technical procedures for the generation of novel PDX models from vitally frozen tumor biopsies. Subsequent to the general technical prerequisites, the implantation procedure is outlined followed by a description of growth measurement, the explantation procedure, and finally the ex-vivo handling steps of the explanted PDX tissues. This is completed by many tips and hints on frequent pitfalls, alternatives, and options.

This procedure is especially suited for the generation of colorectal cancer PDX where success rates of 60% or higher have been obtained when following this protocol [11]. However, similar results can be expected for many other tumor entities including lung, pancreatic, gastric, and liver carcinoma.

2 Materials

All materials and equipment listed are used in routine in our lab. Equivalent products from other providers should be exchangeable and equally suitable.

Bold numbers in parenthesis (e.g., **#1**) correspond to material depicted in Figs. 1 and 6.

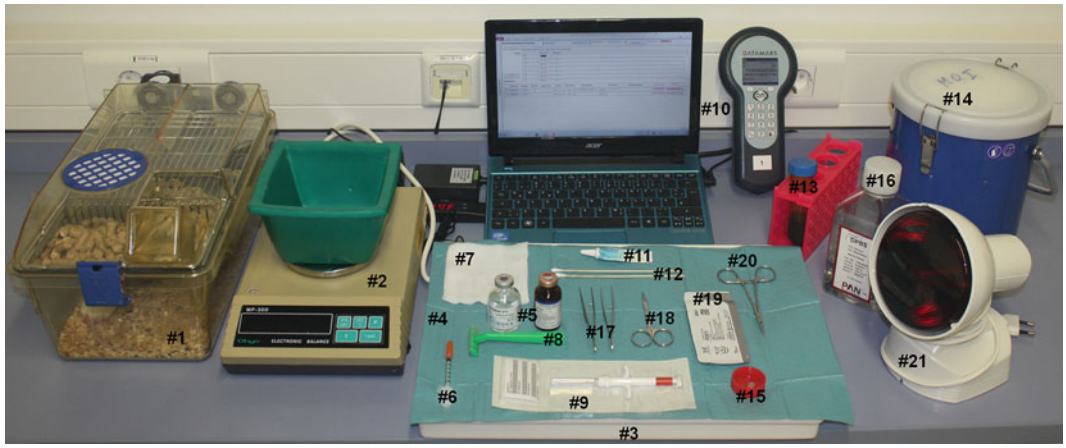

Fig. 1 Overview of equipment and reagents used for tumor engraftment into immunodeficient mice. #1: individually ventilated cage with filter, #2: precision scale and bowl for weight determination, #3: heating plate for animals, #4: sterile surgical utility drape, #5: narcotics (xylazin and ketamine), #6: microfine syringes (0.3 mL insulin syringe), #7: gauze bandage, #8: disposable razor, #9: RFID transponder, #10: RFID chip reader and portable computer, #11: eye ointment, #12: cotton buds, #13: 7.5% povidone-iodine solution, #14: liquid nitrogen container, #15: tumor tissue cube (3 × 3 × 3 mm), #16: sterile PBS without Ca and Mg, #17: forceps (one hemostat and one tissue), #18: iris straight scissor, #19: surgical suture, #20: hemostatic clamp, #21: infrared lamp

2.1 Equipment

1. Bowl for mice weight determination (**#2**).
2. Caliper square.
3. Cotton bud (**#12**).
4. Cryogenic vials for cell culture and biobanking (optimal: 2 mL; **#23**).
5. Disposable razor (**#8**).
6. Forceps (one hemostat and one tissue; **#17**).
7. Freezing container (**#30**).
8. Gauze bandage (**#7**).
9. Heating plate for animals (**#3**).
10. Hemostat/hemostatic clamp (**#20**).
11. Individually ventilated cages with filter (**#1**).
12. Infrared lamp (**#21**).
13. Iris straight scissor (**#18**).
14. Liquid nitrogen container (**#14**).
15. Microfine syringes (e.g., 0.3 mL insulin syringe; **#6**).
16. Electronic pipetting aid (**#29**).
17. Precision scale (**#2**).
18. RFID chip reader (e.g., GES3S Reader by Datamars, Bedano, Switzerland, **#10**).
19. Scalpel blades (**#24**).

20. Scalpel blade holders (**#26**).
21. Scalpel blade remover (**#25**).
22. Sterile petri dish (**#27**).
23. Sterile serological pipette (**#28**).
24. Sterile surgical utility drape (**#4**).
25. Sterile working bench.
26. 15 mL sterile plastic tubes.
27. −80 °C ultra-low freezer.

    optional: portable computer (**#10**).

2.2 Reagents

1. Clinical grade NaCl solution.
2. Disinfectant.
3. Drinking water with antibiotics: 480 mg Cotrimoxazol + 100 mL 0.9% NaCl (w/v) + 333 mL tab water.
4. Eye ointment (**#11**).
5. Freezing medium (fetal calf serum with 10% DMSO, **#22**).
6. Immunodeficient mice (e.g., NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ), NMRI$^{nu/nu}$ (NMRI-Foxn1^{nu}), or NOD SCID (NOD.CB17-Prkdc^{scid}/NcrCrl)).
7. Narcotics (2% (w/v) xylazin(hydrochlorid) and ketamine (10% (w/v), **#5**).
8. RFID transponder (e.g., PICO-ID ISO Transponder from UNO, Zevenaar, The Netherlands, **#9**).
9. Sterile PBS without Ca and Mg (**#16**).
10. Surgical suture (size 5.0, 6.0, or 7.0, **#19**).
11. Tumor tissue cubes (3 × 3 × 3 mm; fresh or vitally frozen, **#15**).
12. 7.5% povidone-iodine solution (**#13**).

3 Methods

All processes described here are for vitally frozen tumor tissue pieces. In case of implantation of fresh tumor tissue (either directly receiving patient material fresh from surgery or regrafting of previously outgrown xenograft tumors) proceed as described neglecting unthawing steps.

Important: It is mandatory to fulfill and obey all local regulatory requirements applying for the work with laboratory animals **before** starting any experiments as described in the following.

3.1 Preparation

1. Liberally disinfect area of operation procedure.
2. Prepare operation place: plug in heating plate for animals and cover with sterile surgical utility drape (Fig. 1).

3. Prepare fresh cage for operated mice (Fig. 1).
4. Weigh mice to calculate anesthesia dose: 1 μL narcotics (xylazin:ketamine (1:3)) per 1 g body weight for NSG and NOD SCID mice, 1.3 μL narcotics (xylazin:ketamine (1:1)) per 1 g body weight for NMRI$^{nu/nu}$.
5. Administer narcotics dose (using microfine syringe) to maximum of two mice at the same time, continue surgical procedures after about 3 min (Fig. 2).
6. During the 3 min waiting period (anesthesia) unthaw vitally frozen tissue pieces and rinse properly with PBS.
7. Test proper onset of anesthesia by lightly pinching mouse in hind leg with the tissue forceps. The kicking response must be absent before continuing with the next steps.
8. Inject RFID transponder into nuchal fold of mouse (*see* **Notes 1** and **2**) (Fig. 2).
9. Apply eye ointment to avoid dry eyes.
10. For mice strains with fur (like NOD SCID, and NSG), remove fur on flanks using a disposable razor.
11. Apply povidone-iodine solution to the (shaved) skin liberally (using a cotton bud) (Fig. 3).

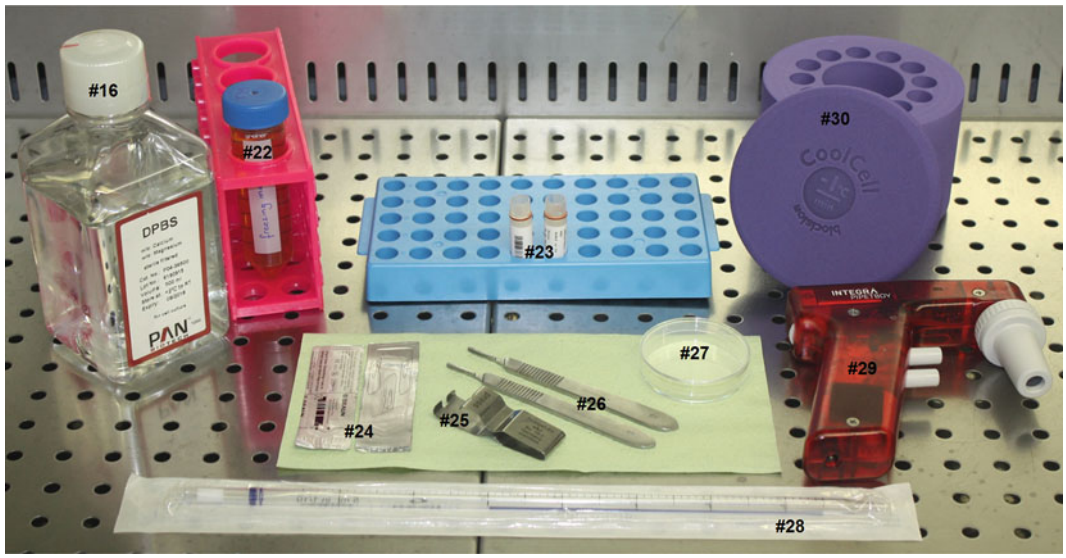

Fig. 6 Overview of equipment and reagents used for xenograft preparation and conservation. #16: sterile PBS without Ca and Mg, #22: freezing medium, #23: cryogenic vials, #24: scalpel blades, #25: scalpel blade remover, #26: scalpel blade holders, #27: sterile petri dish, #28: sterile serological pipette, #29: electronic pipetting aid, #30: freezing container

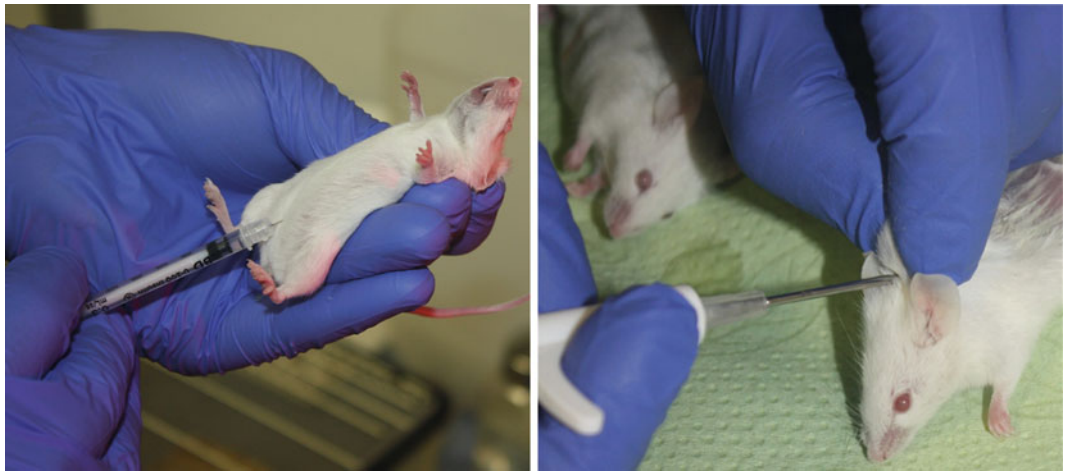

Fig. 2 Administration of anesthesia and implantation of RFID transponder. First narcotics (xylazin-ketamine mixture) are administered intraperitoneal (*left image*), after onset of anesthesia, mice are injected with a RFID transponder into the nuchal fold (*right image*)

3.2 Implantation

1. Use an iris straight scissor to make an incision (about 0.5 cm long) on one or both sides of the mouse flank(s) (*see* **Note 3**) (Fig. 3).
2. Hold open the incision with one forceps and insert tumor piece using the other forceps (Fig. 3).
3. Immediately close incision with two surgical knots (the hemostatic clamp may be used to hold and better navigate the suture) (Fig. 3).
4. Apply povidone-iodine solution to the wound.
5. Place mouse in a new cage and warm with infrared lamp.
6. After awakening of all mice in a given cage (up to six mice, *see* **Note 4**), place the cage in the cage ventilation system (*see* **Note 5**).

Optional: use gauze bandage to remove spills (e.g., excess povidone-iodine solution) that may occur during the operation procedure.

3.3 Tumor Outgrowth Surveillance

1. Supplement drinking water of mice for a period of 6 weeks with antibiotics to exclude outgrowth of potential bacterial contaminations present in the xenografted tumor tissues (*see* **Note 6**).
2. Check mice (starting 15–30 days postimplantation) twice a week for tumor outgrowth (*see* **Note 7**).
3. Measure outgrowing xenografts at least once (better twice) per week using a caliper square (*see* **Notes 8** and **9**) (Fig. 4). Volumes of outgrowing tumors are calculated using the following formula: $\text{width}^2 \times \text{length} \times 0.52$.

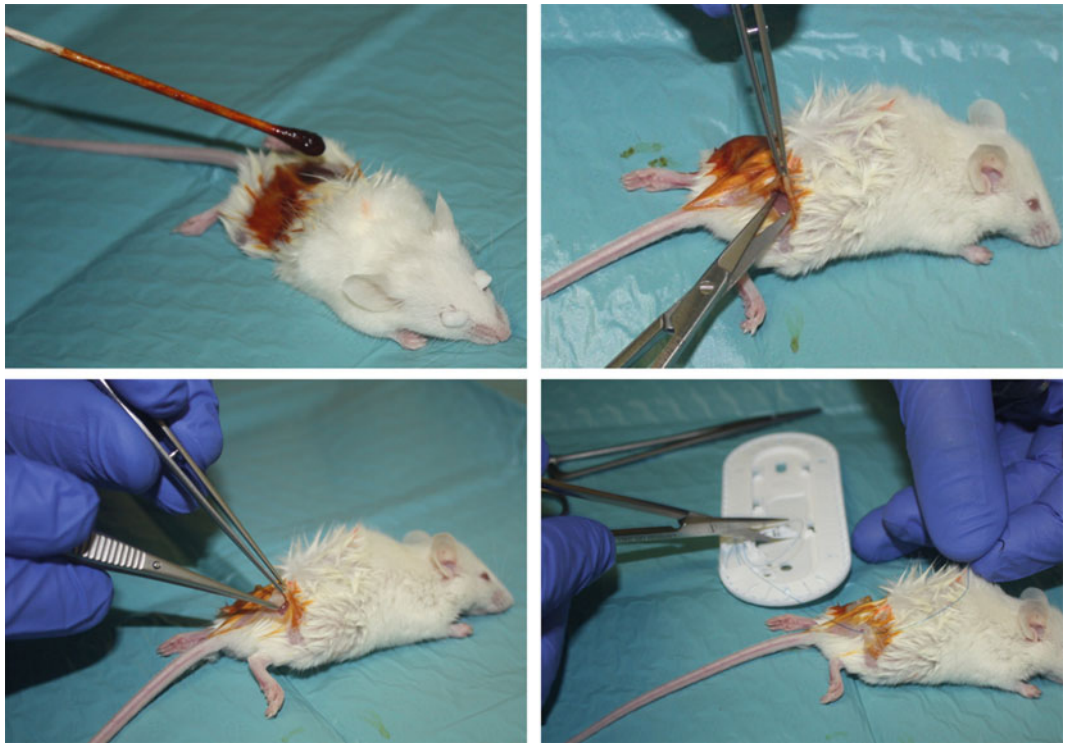

Fig. 3 Tumor implantation. First povidone-iodine solution (after shaving in case of mice with fur) is applied to site(s) of tumor implantation (*upper left image*), then subcutaneous pocket(s) generation performed (*upper right image*), followed by insertion of tumor piece(s) into the pocket(s) (*lower left image*), and finalized by closing the incision site(s) with two surgical knots (*lower right image*)

3.4 Explantation

1. Sacrifice mice when tumors have reached a volume of 500–1000 mm^3 (*see* **Note 10**).
2. Make an incision on the edge of tumor bulk and explant the tumor by a blunt dissection. For this, hold the incision open with a forceps, insert a scissor (closed) directly into the incision (remaining just barely subcutaneous at all times), open the scissor to expand the skin above the tumor, and accomplish detaching the tumor from mouse skin. When the tumor is solely linked to the subcutis, seize the tumor with a forceps and separate the tumor from the remaining skin (Fig. 5). Immediately submerge the tumor in PBS (for washing and short-term storage use 5–15 mL PBS in a 15 mL sterile plastic tube).

3.5 Xenograft Preparation and Conservation

1. Transfer plastic tube containing the explanted xenograft tissue under a sterile working bench, disinfect and arrange material needed (Fig. 6).
2. Transfer the tumor into a sterile petri dish and cut/dice it using crossed scalpels (Fig. 7). Depending on the requirements (future applications), process the tumor as described in the following two steps.

Fig. 4 Mouse identification and tumor formation surveillance. Mice are identified by placing the hand-held RFID chip reader just above the animal and by pressing the ON button (*left image*), before the xenograft size of tumor bearing mice is determined using a caliper square (*right image*)

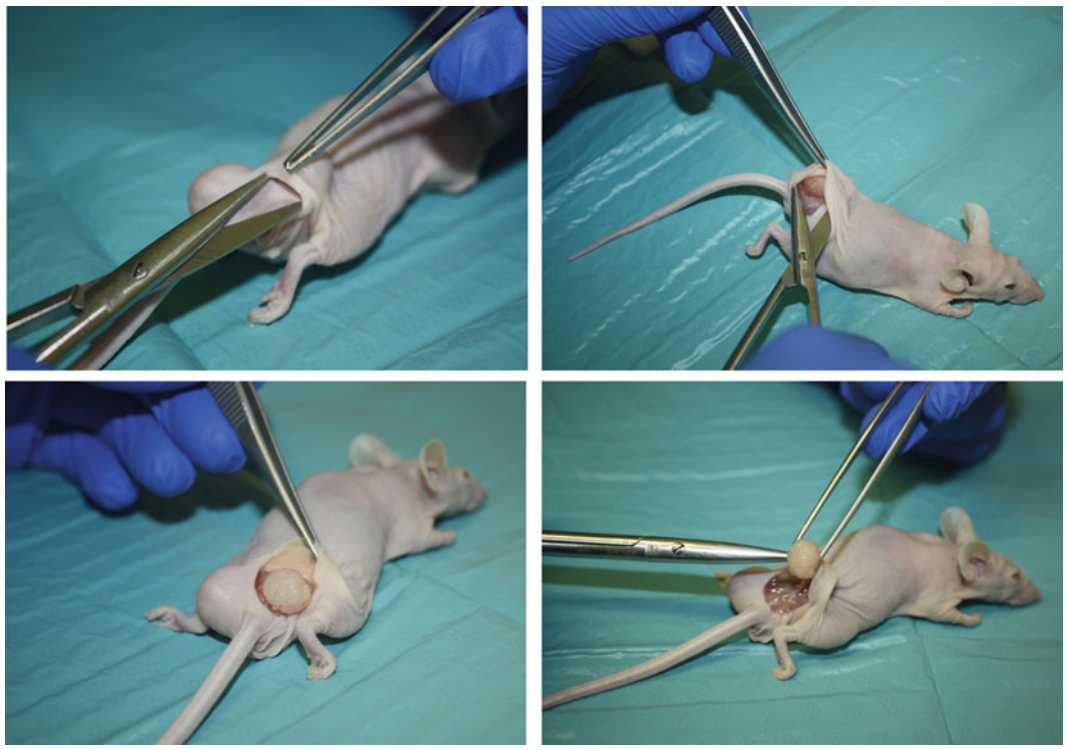

Fig. 5 Tumor explantation. First, an incision about the size of the xenograft length is made just beneath the tumor and the scissor inserted (*upper left image*), then the xenograft is detached from the mouse skin by blunt dissection (*upper right image*) until its sole attachment is to the mouse subcutis (*lower left image*), and finally the xenograft is completely separated from the mouse skin by seizing the xenograft tumor piece with a forceps and cropping the subcutis with the scissor (*lower right image*)

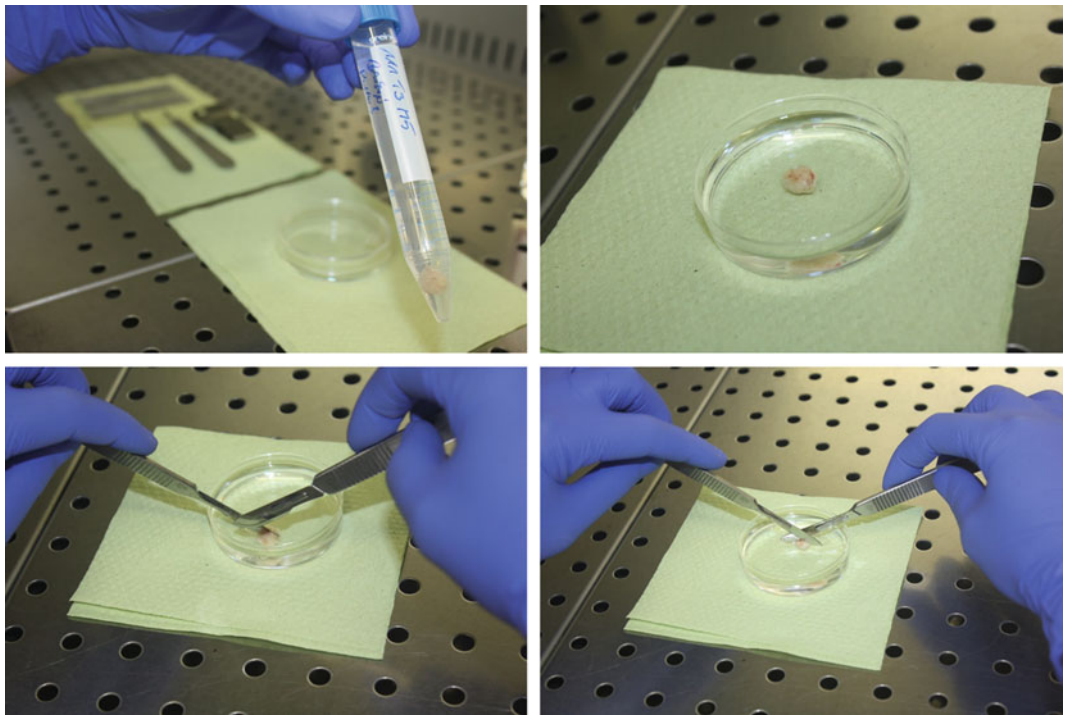

Fig. 7 Xenograft dissection. Bring 15 mL tube containing xenograft tumor under a sterile working bench (*upper left image*), transfer xenograft tumor to sterile petri dish containing 3–4 mL sterile PBS (*upper right image*), and dice xenograft by crossed scalpels into pieces (*lower images*)

3. For conservation and engraftments at later time points, cut xenografts into 3 × 3 × 3 mm pieces and insert pieces each in a 2 mL cryogenic vial containing 1.5 mL freezing medium (*see* **Note 11**). Seal tubes, place them immediately into a suitable freezing container, and transfer them to −80 °C.
4. For subsequent molecular analyses cut the xenograft material into pieces of 10–50 mm^3. Transfer the pieces in 2 mL cryogenic vials, screw on the lids carefully, and immediately snap freeze them in liquid nitrogen (*see* **Note 11**) (Fig. 8).
5. To further expand a given tumor model, directly reimplant 3 × 3 × 3 mm tumor pieces into immunodeficient mice (as described before). Alternatively, vitally frozen pieces can be reimplanted at a later time point (see the vital freezing procedure above).

4 Notes

1. Different strategies for mouse identification are in use. We recommend the RFID transponders since this best avoids misidentification of animals and allows tracking also over longer

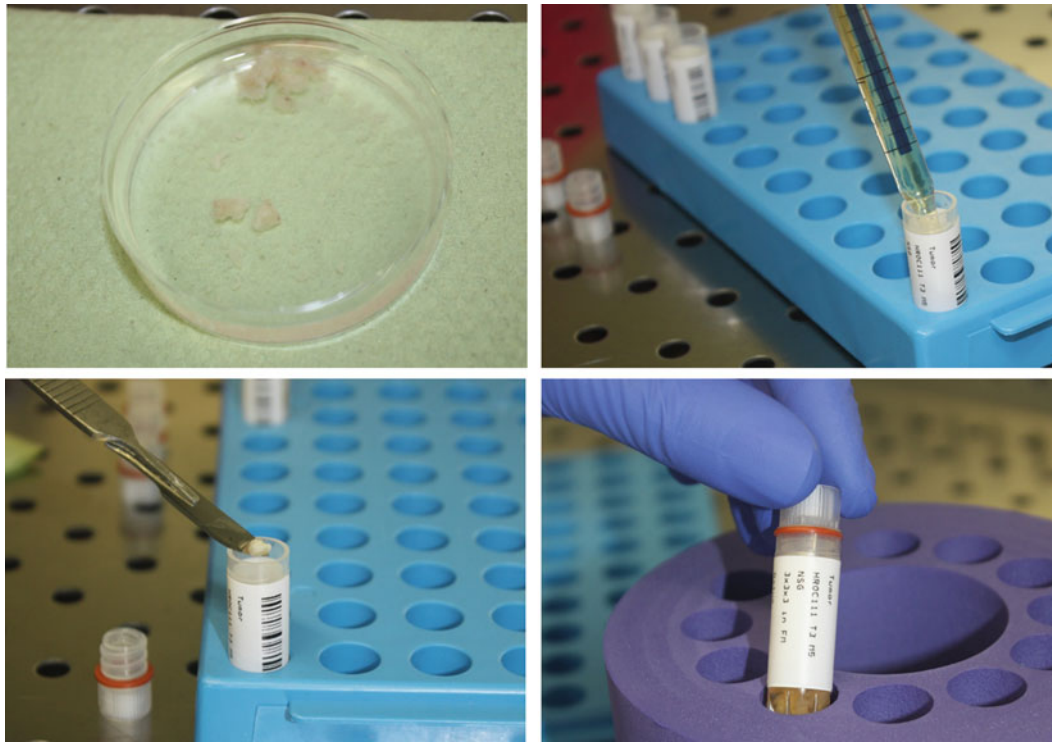

Fig. 8 Xenograft freezing. Xenograft cubes of 3 × 3 × 3 mm (*upper left image*) are inserted into cryogenic vials containing 1.5 mL freezing medium (*upper right image*) using the scalpel's blade (*lower left image*), closed and immediately placed into a freezing container (*lower right image*)

time periods. Direct data transfer from the handheld RFID reading device into a database via Bluetooth is an additional advantage. However, alternatives such as simple coloring, piercing, or tattooing are suitable as well.

2. On rare occasions, a RFID chipped animal cannot be identified because the chip is not readable. Typically, these chips simply (were) moved deeper into the animals' body and are usually readable again after explantation and cleaning. However, it might happen that a chip is permanently lost. If this starts to be a frequent problem, the RFID chip insertion site should be routinely closed with a stitch.
3. In case both sides are used for engraftment, perform two incisions one on the left and one on the right flank in **step 1** and implant two tumor pieces by repeating **steps 2** and **3** for both flanks before continuing with **step 4**.
4. Specific pathogen free (SPF) conditions are implemented for animal care facilities breeding and keeping immune deficient animals to ensure an optimal specific pathogen-free environment. This is achieved by airlocks, powerful room ventilation systems, and specific racks allowing ventilation for each individual cage.

5. The number of mice in a single cage should be at least two to avoid isolation, but not more than six. Females better accept more animals per cage and especially animals from different litters. Check acceptance of cage mates when mixing animals from different cages carefully to avoid stress for and potential loss of animals.
6. Bacterial contaminations may be an intrinsic property of some tumor tissues obtained fresh from surgery or pathology. Additionally, they might occur in the implantation process itself or during other handling steps of the tumor tissues. If there is any doubt that the implanted tissue is absolutely sterile, it is recommended to supply antibiotics in the drinking water to exclude damage to the animal(s) as well as a potential loss of the xenograft(s).
7. Freshly engrafted tumor pieces very frequently seem to disappear approximately 7–10 days after implantation. The time point of measurable tumor outgrowth after implantation is highly variable and additionally depends on the passage number of a xenograft, amount and quality (e.g., necrosis) of tissue implanted, and to some extent also the individual mouse. Generally, engraftment is better in younger mice, the take rate is typically higher in the NOD SCID and NSG strains than in the NMRI$^{nu/nu}$, and finally, the fitness of an individual mouse also is a factor that should be carefully surveilled.
8. For a given patient tumor, one might wish to "force" the successful generation of a PDX model. To augment the likelihood of success, multiple tumor pieces can be implanted into multiple mice. The size of the engrafted tissue piece can also slightly be increased up to approximately 125 mm^3 when using fresh tissue. The former will not work for frozen tissue cubes due to the limitations of freezing medium diffusion. Also, presoaking of tumor cubes with Matrigel (BD Biosciences) might increase outgrowth rates further.
9. Another possibility of optimizing success rates is the careful selection of the most suitable mouse strains. As a general rule, one can assume that the higher the immunosuppression level of the strain, the better the overall take rate. For the three strains used in our laboratory, the order is (listed decreasing): NSG—NOD SCID—NMRI$^{nu/nu}$. However, one has to be aware of the following risks: In NSG, the spontaneous outgrowth of human lymphoid-like tumors at the site of xenografting has been described [14]. It seems that due to the very high level of immunosuppression, EBV-positive B lymphocytes present in an implanted human tumor piece are the major risk, but occasionally, EBV-negative T cells might also start proliferating. Similarly, lymphoid-like tumors will also spontaneously arise in NOD SCID mice [15]. But according to our experience, those are contrary to the NSG lymphoid-like tumors of

mouse origin. Additionally, some human tumors seem to be associated with a very high likelihood of mouse lymphoid-like tumor occurrence when implanted into NOD SCID. Of note, NOD SCID was originally described as a model of spontaneous thymoma [16].

10. When an individual tumor has successfully engrafted for the first time we recommend explanting it at an approximate size of 500–750 mm^3. Larger tumor sizes are possible before explantation but have to be tested individually. The larger a tumor is, the greater the risk of central necrosis. In some cases, one may even receive more viable tumor tissue from smaller tumors. Thus, establishing optimal parameters for each individual tumor model is optimal. Moreover, for NSG mice or other strains with fur, the real size of a given tumor is smaller than the measured size (in comparison to a nude mouse). Here, sizes of up to 1500 mm^3 might be optimal.
11. The amount of tumor pieces to be put into a single cryogenic vial may of course vary. We would recommend four pieces for the $3 \times 3 \times 3$ mm cubes in freezing medium but one or two in case of the snap frozen pieces for molecular analyses.

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Part III

Assessment of HDAC-Dependent Effects on Signaling Cascades in Primary and Permanent Cells

Chapter 17

Phospho-Flow Analysis of Primary Mouse Cells After HDAC Inhibitor Treatment

Dagmar Hildebrand and Katharina F. Kubatzky

Abstract

Flow cytometric techniques allow fast, sensitive, and multiparametric analyses at the single cell level. This makes it possible to distinguish subsets of cells within heterogeneous samples. Moreover, flow cytometry has become a frequently used method for the evaluation of therapeutic effects. Here, we describe the analysis of the phosphorylation status of signal transducer and activator of transcription-1 (STAT-1) in primary mouse cells after treatment with histone deacetylase inhibitors (HDACi) that are currently considered anticancer agents. We provide detailed protocols for the preparation of murine bone marrow cells and the staining of HDACi-treated cells, as well as an insight into the concepts of flow cytometry analysis.

Key words Fluorescence cytometry, Phospho-flow analysis, Intracellular staining, HDACi, Bone marrow-derived cells

1 Introduction

Flow cytometry is a technology that enables the simultaneous detection and analysis of multiple physical characteristics of single cells. Single cells are generated through separation in a fluidic stream (sheath stream). When these cells then pass through a light beam, the degree of light scattering gives information on the relative size, granularity, and internal complexity, as well as the relative fluorescence intensity (Fig. 1) [1]. These capacities are determined by an optical-to-electronic coupling system that registers how the cell scatters incident laser light and emits fluorescence [2–5].

Flow cytometry can be performed using a variety of tissues, such as peripheral blood, bone marrow, and tissue culture cell lines, if the sample is processed to a cellular suspension. In the case of a mixed cell population such as bone marrow cells, subpopulations of interest can be identified through the use of antibodies that only bind to a specific subset of cells. These marker proteins can be surface proteins, for example transmembrane proteins called cluster of differentiation (CD) or intracellular markers such as specifically

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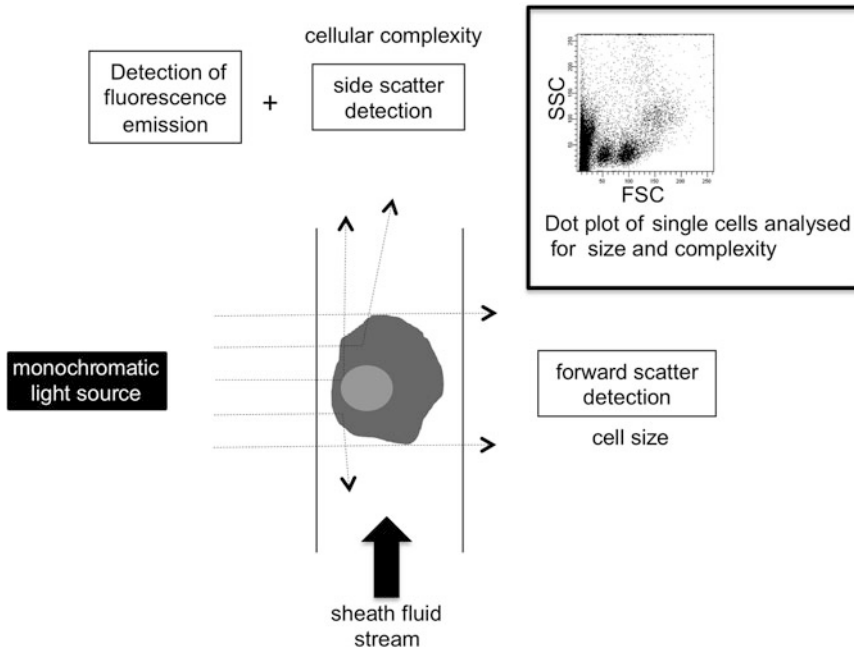

Fig. 1 Light-scattering: particles or cells deflect light according to their size and internal complexity or granularity. This phenomenon is referred to as light scattering and is affected by the membrane, nucleus, and any granular material inside the cell which contributes to the cellular complexity. In addition, cell shape as well as surface composition also affect light scattering. Light scattering that is detected by a photodiode parallel to the axis of the laser beam gives information about the size of the cell and is called the forward scatter (FCS). Light scattered perpendicular to the axis of the laser beam (sideward scatter, SSC) consists of refracted and deflected light and gives information about the cellular granularity or internal complexity. In addition, the emission of fluorescence is detected perpendicular to the laser beam. FSC and SSC are usually displayed together in a dot plot to define cellular populations

expressed transcription factors [6, 7]. The antibody-coupled fluorophore (fluorescent chemical compound) has to be excited at a specific wavelength with a laser beam that is part of the flow cytometer. The argon laser emits at 488 nm and some cytometers also have an additional red diode laser that emits at 635 nm, or a violet laser that emits at 407 nm. Depending on the filters used, a variety of different fluorophores can be excited at their specific excitation wavelengths. If cells have the selected marker, the fluorophore absorbs the light and subsequently emits light at a specific wavelength that is detected by the optical system [8]. It is possible to simultaneously use three (argon laser only), up to eight (blue and red lasers) or even ten (violet, blue and red laser) different fluorophores to monitor several proteins at the same time (polychromatic flow cytometry) and to quantify the number of positive cells. As excitation or emission spectra of fluorochromes can overlap, a specific detector may also record signals from a different fluorochrome. However, this overlap can be corrected using a mathematical algorithm in a process called compensation [9]. The compensation may even be performed after the measurement.

For analysis, specialized software graphically illustrates the distribution of the cell populations in linear scatter plots, where the forward scatter (FSC, *x*-axis) shows the relative size of the cells, and the sideward scatter (SSC, *y*-axis) gives information about the granularity. The analysis of fluorescence can be presented in logarithmic one-dimensional histograms or in dot plots, where the fluorescence intensity of the fluorophore is depicted versus the forward scattering light or against a second fluorophore, respectively [8].

For the staining of proteins for flow cytometry it is important to consider their cellular localisation. Thus, not only the discrimination between surface and intracellular but also between cytoplasmic and nuclear localization can be of interest or even necessary. For example, phosphorylated signalling proteins are detected with a protocol different from that to monitor secreted cytokines or nuclear proteins [10, 11].

Here, we describe the analysis of the phosphorylation status of signal transduction molecules (phospho-flow analysis) in primary mouse cells after HDACi treatment [10]. In transformed cells, HDACi trigger acetylation that eventually causes the dephosphorylation and thereby inactivation of signal transducer and activator of transcription-1 molecules (STAT-1) through T cell protein tyrosine phosphatase (TCP45) [12]. The staining method presented here was established to check the influence of HDACi on interferon (IFN)-γ-induced phosphorylation of STAT-1 in murine bone marrow-derived cells. The technique allows the simultaneous measurement of phospho-protein levels and total protein expression within one cell, thus enabling the discrimination between modulated activation status and general protein expression.

2 Materials

The materials, antibodies, and reagents listed here are used by our lab. Obviously, equipment from other providers should be equally useful; however, it might be necessary to adjust the concentrations of the respective antibodies.

2.1 Cell Preparation

1. Styrofoam surface.
2. Needles and sterile dissection instruments (10 min incubated in boiling water).
3. Petri dishes.
4. Cell culture 12-well plates.
5. 15 mL tubes, 10 mL syringes with 27G × 3/4 needles, cell strainer 100 μm.
6. 70% ethanol, RPMI-1640 medium (Biochrom AG), foetal bovine serum, and penicillin/streptomycin (both PAA laboratories).

2.2 Cell Stimulation

IFN-γ (Immunotools Ref. 12343534), HDAC inhibitor valproic acid (VPA, Cayman Chemicals Ref. 13033), HDACi MS-275 (Cayman Chemicals, Ref. 13284).

2.3 Staining

1. FACS polystyrene round-bottom 12×75 mm Falcon tubes, pipettes, centrifuge, gloves, vortexer.
2. PBS (9.0 g NaCl; 1.62 g $Na_2HPO_4 \cdot H_2O$; 0.37 g KH_2PO_4/L H_2O), Paraformaldehyde, Triton X-100.
3. Anti-p (Y701) STAT-1-PE (BD Biosciences cat. 612564), anti-IgG 2a Isotype-PE (cat. 565363, clone MOPC-173), anti-STAT-1 p84/p91 (Santa Cruz Biotechnology sc-346, clone E-23), anti-rabbit IgG-FITC (Santa Cruz Biotechnology sc-2012).
4. Optional: Annexin V-FITC Apoptosis Detection Kit (Abcam Ref. ab14085).

2.4 Flow Cytometry

1. Flow cytometer and associated software. For example: FACSCanto (BD Biosciences) and FACSDiva software (BD Biosciences), pipettes, vortexer.
2. FACSFlow (BD Ref. 342003), FACSClean (BD Ref. 340345).

3 Methods

3.1 Preparation of Bone Marrow Cells and Subsequent Stimulation

1. Euthanize a C57BL/6 mouse (either male or female, 20–25 g, 8–10 weeks old) by CO_2 asphyxiation, or another approved method of euthanasia, such as cervical dislocation [13] according to national laws on animal welfare.
2. Fix the mouse with needles through the paws on its back onto a styrofoam surface. Spray the whole area thoroughly with 70% ethanol for disinfection and as protection against contamination with fur or other particles.
3. Cut into each hind leg using scissors that were sterilized for 10 min in boiling water. Grab the skin with tweezers and gently pull downward to expose the muscles.
4. Cut the hind leg above the hip joint.
5. Transfer the hind leg to RPMI-1640 medium in a sterile petri dish.
6. From this point onward you should work under the laminar flow (*see* **Note 1**).
7. Cut the hind leg below the knee-joint to separate tibia from femur and remove excess tissue using sterile forceps and scissors.
8. Trim the ends of the bones carefully.
9. Flush the contents of the bone with RPMI (10 mL for the bones of one mouse) using a 10 mL syringe with a 27G×3/4 needle. Collect the marrow into a sterile 15 mL plastic tube.

10. Fill the tube with RPMI and centrifuge the cell suspension at 300 × *g* for 5 min.
11. Remove the supernatant and resuspend the cell pellet in 15 mL of RPMI and filter through a 100 μm cell strainer. Repeat the washing step once.
12. Resuspend the cell pellet in culture medium (RPMI-1640 containing 10% FBS and 1% penicillin/streptomycin) to achieve a final cell density of 1×10^6 cells/mL and plate them in a 12-well plate.
13. Add 2 mM of valproic acid (VPA) and 5 μM MS-275, respectively, to one well and leave another two wells unstimulated to be used as negative and positive control samples.
14. After 24 h add 100 ng/mL IFN-γ to the HDACi-treated and one untreated well for an additional 30 min incubation time (*see* **Note 2**).

3.2 Staining of Cells

1. Harvest cells (*see* **Note 3**) and pipet 500 μL (500,000 cells) in each FACS tube. Six tubes per condition (unstained, anti-STAT-1+ anti-rabbit-FITC, anti-rabbit-FITC, anti-p-STAT-1-PE, IgG2a Isotype-PE, anti-STAT-1/anti-rabbit-FITC + anti-p-STAT-1-PE). Add 500 μL of PBS and centrifuge at 300 × *g* for 5 min.
2. Gently discard the supernatant by decanting the tube once. Resuspend cell pellet (pellet might be hard to see) in 1 mL PBS and centrifuge again (300 × *g*, 5 min) (*see* **Note 4**).
3. An additional surface stain could be done at this point (1 h, at 4 °C in the dark) (*see* **Note 5**).
4. To fix the cells, resuspend the cell pellet in 500 μL 4% paraformaldehyde/PBS and incubate for 20 min at room temperature (wear gloves at this step—paraformaldehyde is a suspected carcinogen!).
5. Wash twice with PBS (500 μL, 300 × *g*, 5 min). Discard the supernatant. By doing so, around 100 μL of liquid will flow back. Keep this in mind for further calculations.
6. For permeabilization resuspend cells in 0.1% Triton X-100/PBS and incubate for 5 min at room temperature (*see* **Note 6**).
7. After washing the cells three times as described before, resuspend the cells in 100 μL PBS/10% foetal bovine serum (FBS, heat-inactivated) to block unspecific antibody binding. Do not add FBS before that step, as this would result in disturbed fixation and permeabilization (*see* **Note 7**).
8. Add the first antibody to the cell suspension after a 15 min blocking step.

   Tube 1: 1:5 anti-p (Y701) STAT-1-PE (*see* **Note 8**).

Tube 2: 1:50 IgG isotype-PE (see Note 9).

Tube 3: 1:50 anti-STAT-1 purified (anti-rabbit IgG-FITC will be incubated later).

Tube 4: 1:5 anti-p (Y701) STAT-1-PE + 1:50 anti-STAT-1 purified (anti-rabbit IgG-FITC will be incubated later).

Tube 5: PBS (anti-rabbit IgG-FITC will be incubated later).

Tube 6: PBS (unstained control).

9. The incubation of 1 h at 4 °C in the dark (*see* **Note 10**) is followed by a washing step with PBS/10% FCS (3 × 300 × *g*, 5 min).
10. Add the secondary antibody (anti-rabbit IgG-FITC) to tubes 3 and 5 and incubate for 30 min at 4 °C. Wash 3× with PBS/10% FCS.
11. For the analysis on the flow cytometer, the cells should be resuspended in around 300 µL of PBS. Because the cells were fixed with paraformaldehyde, they can be stored for a couple of days at 4 °C if direct analysis is not possible.

3.3 Flow Analysis

For measurement and analysis of flow cytometry data a FACSCanto (BD Biosciences) with FACSDiva software (BD Biosciences) was used. For detailed instructions read the respective manuals ("BD FACSCanto II Instructions For Use" and "Getting Started with BD FACSDiva Software").

1. Create your worksheet: FSC vs SSC plot (illustrates the distribution of the cells in linear scatter plots according to their size and granularity) (Fig. 2a).
2. Create a PE histogram (illustrating pSTAT-1-PE signal) and a FITC histogram (illustrating STAT-1/anti-rabbit FITC signal) (Fig. 2b).
3. In case of an additional surface stain for the identification of a specific subpopulation, create a dot plot fluorescence vs FSC.
4. Create a PE vs FITC dot plot (illustrates single-positive and double-positive cells) (Fig. 2c).
5. In case of an additional Annexin V-FITC stain, create a FITC vs pSTAT-1-PE dot plot, so that the Annexin V-FITC-positive, apoptotic cells can be excluded.
6. Adjust the voltage settings for your experiment while measuring unstained cells at a low rate, so that the cells or the population of interest is displayed in the center of the FSC vs SSC plot. The histograms of the fluorescence (Fl) signal should be in the left lower region of the scale. If possible count 10,000 cells per condition.
7. Draw a gate (P1) around the living cells in the FSC vs SSC plot. Dead cells and cell debris appear in the left lower part of the diagram. Adjust the settings of the further histograms and dot plots so that only "P1" cells are displayed (Fig. 2a).

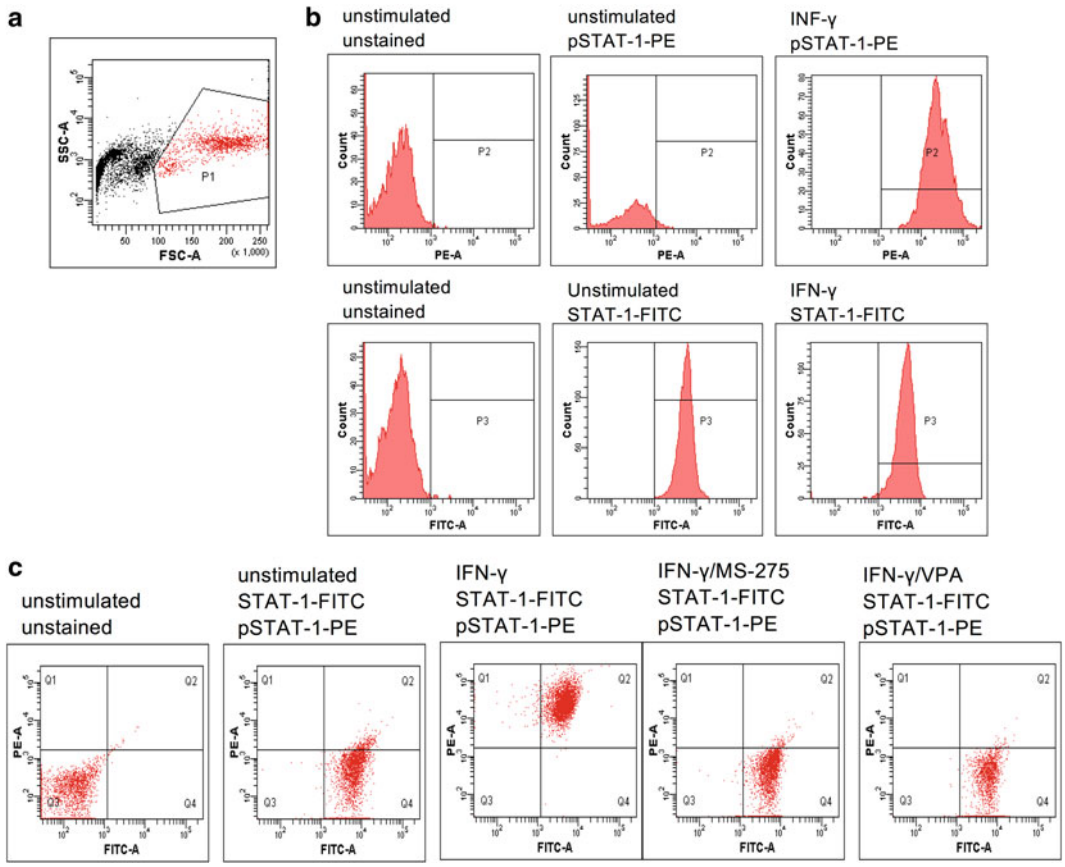

Fig. 2 Analysis of bone marrow cells with BD FACSDiva. (**a**) Graphical display of the size and the internal complexity of bone marrow cells in a FSC/SSC dot plot. The gate P1 is drawn around living cells. (**b**) FITC and PE histograms of the cells gated in P1. Bone marrow cells were stimulated with IFN-γ (100 ng/mL, 30 min) and intracellularly stained with anti-STAT-1 (1:50)/anti-rabbit IgG-FITC (1:100) or anti-p (Y701) STAT-1-PE (1:5). PE and FITC signals of cells in the P1 gate are displayed as histograms. (**c**) FITC vs PE dot plots of cells gated in P1. Bone marrow cells were pretreated with 2 mM of valproic acid (VPA) or 5 μM MS-275 or were left untreated. After 24 h, IFN-γ (100 ng/mL) was added for 30 min. Shown are unstained cells and intracellularly stained cells (1:5 anti-p (Y701) STAT-1-PE + 1:50 anti-STAT-1 purified/1:100 anti-rabbit IgG-FITC that were either unstimulated or treated with IFN-γ. Q1: FITC-positive, PE-negative; Q2: double-positive cells; Q3: double-negative cells; Q4 PE-positive, FITC-negative cells

8. Draw a quadrant gate in the PE vs FITC plot so that it divides the diagram into four quadrants (Q1: FITC-positive, PE-negative; Q2: double-positive cells; Q3: double-negative cells; Q4: PE-positive, FITC-negative cells). In this way, you can analyze the level of total STAT-1 in each cell and additionally determine the phosphorylation status.
9. Quantification and compensation: Create a "statistics view" table showing the number of "events," the "mean fluorescence," and the "percentage of parent cells" for each window and gate.

10. Light detectors may record fluorescence from multiple fluorophores as their emission spectra can overlap causing unspecific signals. This overlap (the amount of light spilling over to different detectors) must be compensated before data analysis of double-stained cells. For this compensation process you will need cells that have been stained only with one of the antibodies to determine how much of its fluorescence is detected in the second, unspecific channel. Further information about the procedure can be found in "An Introduction to Compensation for Multicolor Assays on Digital Flow Cytometers" (BD Biosciences) [14].
11. Data presentation.
    It is possible to show original flow cytometry data such as the dot plot of STAT-1 vs pSTAT-1 with the percentage of positive cells calculated for each quadrant. Alternatively, you can export the FSC files and analyze and display the data with freely available software such as WEASEL flow cytometry software or flowJo (30 days trial versions), or Flowing Software (free software) that for example allow to overlay the histograms of the various conditions tested (Fig. 3).

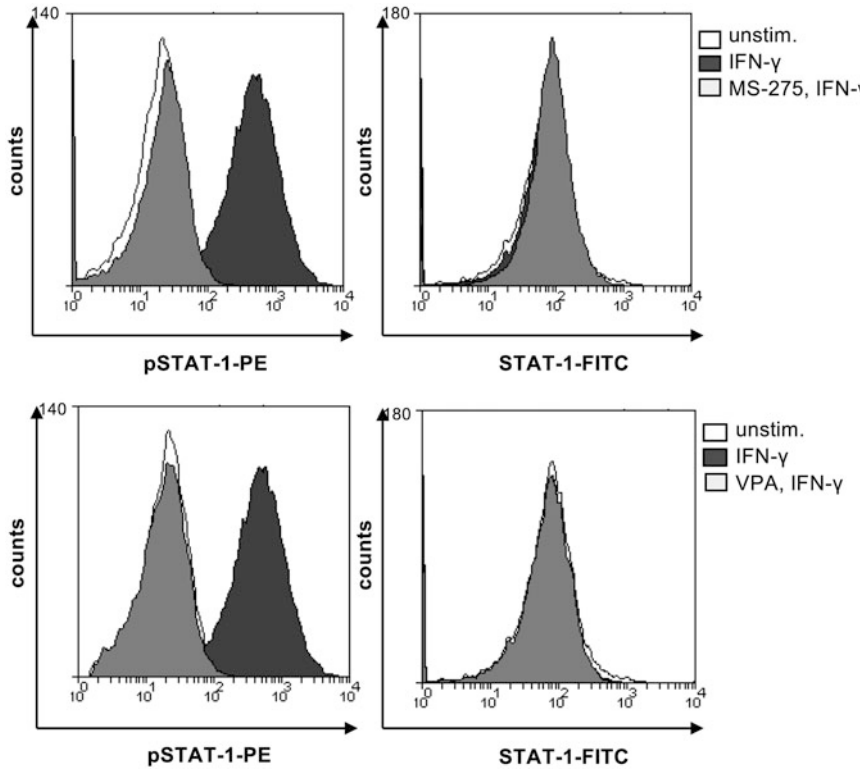

Fig. 3 Histogram overlay of bone marrow-derived dendritic cells. Dendritic cells (DCs) were generated by culturing BMCs in GM-CSF-containing growth medium [15]. DCs were treated with the HDACi MS-275 or VPA (5 μM and 2 mM, respectively) and IFN-γ (100 ng/mL, 30 min) as indicated and stained with anti-STAT-1 (1:50)/anti-rabbit IgG-FITC (1:100) and anti-p (Y701) STAT-1-PE (1:5) antibodies. Overlay histograms were made by importing the original FCS files into WEASEL flow cytometry analysis software for analysis

Good advice for analyzing and presenting data can be found here: interpreting flow cytometry data: a guide for the perplexed [16].

4 Notes

1. If you have access to a laminar flow that is exclusively used for the work with mice, you can start right from the beginning under sterile conditions. From our experience it is fine to switch to sterile conditions when you start to open the bone.
2. If you culture your bone marrow-derived primary cells for more than 1 day it is advisable to transfer the cells into a new plate as fibroblasts adhere to the bottom of the plate and potentially disturb the culture.
3. Harvest the cells for staining using a cell scraper if necessary (some cells such as macrophages can tightly stick to the plastic surface). Check afterwards in the microscope that no cells are left in the petri dish.
4. Alternatively, you can perform the staining in 1.5 mL Eppendorf tubes. Centrifuge the cells for 30 s at 8400×*g*. Aspirate the supernatant and be careful not to lose the cell pellet. This method is much faster, but requires higher speed for centrifugation.
5. Optional: we recommend an additional stain with Annexin V-FITC to exclude apoptotic cells, which tend to nonspecifically bind to antibodies. Annexin V binds to phosphatidylserine residues that are hidden within the plasma membrane in living cells but accessible in apoptotic cells. Cells must be incubated with Annexin V-FITC before fixation since any cell membrane disruption will cause binding of Annexin. You can use for example the Annexin V-FITC Apoptosis Detection Kit from abcam (ab14085). Add 5 µL Annexin V-FITC to each sample (app. 500,000 cells) in 100 µL binding buffer. As this staining procedure is very efficient, 5 min incubation time at room temperature should be sufficient.
6. Detergents such as Triton X-100 and Tween-20 are nonselective and may extract proteins along with the lipids.

   Alternatively, Saponin (0.1–1 %) can be used for permeabilization, which is less harsh than Triton X-100 or Tween-20. This substance from soap nuts interacts with membrane cholesterol, selectively removing it and leaving holes in the membrane.
   If you cannot detect phosphorylation after permeabilization with Triton X-100 or Saponin you should try cold (–20 °C) methanol as it can increase the reactivity of antibodies to certain antigens. Incubate for 30 min at 4 °C. A great disadvantage, however, is its high toxicity.

7. Alternative blocking: in 2% bovine serum albumin (BSA) or normal serum. Cave: selection of the type of normal serum is important to prevent interactions with the primary or secondary antibodies. For example, goat serum would not be advised as a blocking reagent for use with a goat-derived primary antibody. Instead, a serum identical to the host animal of the secondary antibody or from an unrelated species is recommended.
8. A 1:5 dilution (20 µL) is suggested by BD Biosciences. It is definitively possible to use lower amounts of antibody, but this should be titrated carefully.
9. The isotype antibody is used to ensure that the fluorescence signal detected is due to a specific interaction between antibody and antigen. The isotype control must be the same type of antibody as your specific antibody (for example here the mouse IgG 2a istoype), labeled with the same fluorophore. However, this control antibody is not directed against any specific epitope. If the isotype control generates a signal, unspecific binding occurred. This can often be reduced by a longer preincubation of the cells with up to 2% BSA in PBS or by increasing the number of washing steps after staining the cells. The isotype control antibody should be from the same company as the controlled antibody, as different manufacturers label the antibodies with variable amounts of fluorophores. In addition, the concentrations of the isotope and the antibody it controls have to be the same and predilutions (as for the pSTAT1 antibody used here) need to be taken into account.
10. The incubation time of 1 h at 4 °C can be reduced to 15–20 min at room temperature (in the dark). Incubation at room temperature can increase unspecific binding of the antibodies and may allow receptor internalization. In our hands this did not make any difference.

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Chapter 18

Evaluation of Innate Immune Gene Expression Following HDAC Inhibitor Treatment by High Throughput qPCR and PhosFlow Cytometry

David Olagnier, Cindy Chiang, and John Hiscott

Abstract

The dynamics of chromatin structure contribute to the regulation of gene transcription and in part, the changes in chromatin structure associated with gene activation/repression are a function of the state of histone acetylation. Histone deacetylases (HDACs) deacetylate histone tails leading to a more compact structure of chromatin that in turn represses gene transcription. Given the rapid activation and/or repression of gene networks following microbial infection, the role of HDACs in the epigenetic regulation of genes involved in the innate and adaptive immune responses has become an area of extensive research. In relation to the immune-modulatory properties of HDAC inhibitors, we provide in the following methodological article an extended description of two techniques—a high throughput qPCR assay combined with PhosFlow cytometry—to evaluate the modulation of antiviral and inflammatory signaling cascades following HDAC inhibitor treatment. The high-throughput qPCR assay is based on the nanofluidic Fluidigm BioMark system that permits the analysis of up to 9216 qPCR reactions at once in a self-design open array chip. Together with the more refined analysis provided with the Phosflow technique, these two strategies offer invaluable tools to measure modulation of innate immune gene networks.

Key words Immune response, Antiviral response, High throughput qPCR, Phosflow, Cytometry, HDAC, HDAC inhibitor, Gene transcription

1 Introduction

Modulation of gene transcription is a critical and often rapid response of eukaryotic cells to changes in the microenvironment that ultimately enhances cellular versatility and adaptability through the modulation of cellular signaling pathways and subsequent protein expression. The dynamics of chromatin structure is critically involved in the regulation of gene transcription and is dramatically altered by the epigenetic modification of histones [1]. Two groups of enzymes, the histone acetyltransferases (HAT) and the histone deacetylases

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(HDAC), regulate this process by either facilitating or restricting the accessibility and binding of transcription factors and regulatory proteins to DNA [2, 3]. It is now widely recognized, for example, that activated immune-related transcription factors such as NF-kB, a master regulator of inflammatory responses, bind to promoter regions leading to the recruitment of HATs such as CREB binding protein (CBP) and adenoviral protein E1A (p300) [4]. These proteins form a transcriptional initiation complex, leading to HAT-mediated acetylation of the histone tails, thus conferring a low-density packing of DNA that facilitates gene transcription [3]. Conversely, the removal of acetylated residues by HDACs reverses this process and leads to a high-density chromatin structure and transcriptional repression. Furthermore, dysregulation of HAT and HDAC activities is associated with aberrant gene transcription, leading to increased cell proliferation or excessive inflammatory conditions [1].

The role of HDACs in the epigenetic regulation of innate and adaptive immune responses is equally critical to transcriptional control [5]. Indeed, HDAC activity has been previously shown to be involved in the control of inflammatory response by regulating the acetylation status of NF-kB RelA/p65 [6]. HDAC activity also inhibits dendritic cell function by repressing acetylation of STAT3, which negatively regulates adaptive immunity [7]. Finally, HDAC enzymes can also regulate Th1 and Th2 differentiation by modulating the IFN-γ promoter activity [8]. Since HDAC activity influences the establishment of immune responses, small molecules repressing HDAC activity offer a promising approach to the treatment of immuno-inflammatory diseases [5]. However, most studies using HDAC inhibitors (HDIs) have focused on their antiproliferative and anticancer properties [9]. Our previous studies have shown that HDIs can also act as repressors of the transcriptional activation of select antiviral genes following type I interferon (IFN) stimulation or virus infection [10]. Because of their effect on antiviral gene suppression, pretreatment of tumors with HDIs enhances the oncolytic activity of viruses such as vesicular stomatitis virus (VSV) [10]. More recently, we demonstrated that HDIs not only block IFN responses but also induce a subset of NF-kB-regulated genes that enhance VSV oncolytic activity, in part via stimulation of NF-kB-mediated autophagy [11].

In a different series of experiments, we evaluated the expression of immune gene networks that mediate antiviral protection and linked innate and adaptive immune responses in different cell types and infectious contexts using a Fluidigm BioMark™ assay [12–15]. This high-throughput nanofluidic qPCR system allows detection of 96 genes and 96 samples with less than 100 cells and can be optimized for single-cell analysis. BioMark is an “open array” chip and a flexible and highly sensitive analytical tool that can be easily modified to study different combinations of genes from various signaling pathways. The choice of genes to be

analyzed is selected depending on the project (for example, IFN stimulated genes, chemokines, inflammation markers, cell death related genes), and thus eliminates the need to evaluate large amounts of data from genome-wide transcriptional profiling.

The analysis of antiviral and immune gene expression can be complemented with the measurement of the phosphorylation state of the STAT1 transcription factor or other immune-related transcription factors by flow cytometry [12, 13]. Monitoring the phosphorylation of specific residues on transcription factors is critical to confirm the transient activation of signaling cascades during immune modulation. Flow cytometry is a rapid, sensitive, and powerful technique that can measure simultaneously multiple characteristics of a single cell including phosphorylated epitopes or surface markers. Based on our studies on HDIs in the modulation of antiviral and inflammatory responses [10, 11], we describe step-by-step protocols to thoroughly evaluate the modulation of signaling cascades in either immune or nonimmune HDIs-stimulated cells using two rapid, flexible, and sensitive methods—nanofluidic Fluidigm BioMark analysis and PhosFlow cytometry.

2 Materials

2.1 PhosFlow Cytometry

1. Fixation buffer: use the Fix Buffer I from BD (BD Phosflow, Cat 557870) or make a home-made 4% formaldehyde solution in PBS. Dilute approximately 2.1 mL of a 37% formaldehyde solution in 17.9 mL of water. The 4% formaldehyde solution can be kept at room temperature for future experiments.
2. Permeabilization buffer: use the PERM III buffer from BD (III) (BDPhosflow, Cat. 558050) or make a 90% methanol solution starting from absolute methanol. Dilute 9 mL of 100% methanol in 1 mL of PBS. The 90% methanol solution can be kept at room temperature for future experiments.
3. Saturation buffer: PBS with 5% Fetal Bovine Serum (FBS) solution. To make 40 mL of this solution, dilute 2 mL of FBS in 38 mL of sterile PBS. Keep at 4 °C once diluted.
4. Staining buffer: use the BD Stain Buffer (FBS) (BD, Cat. 554656) or PBS containing 2% FBS. To make 40 mL of this solution, dilute 800 μL of FBS in 39.2 mL of sterile PBS. Keep at 4 °C once diluted.
5. Although we have only used antibodies from BD Phosflow to detect phosphorylated forms—anti-STAT1 (pY701) Pacific blue (Cat. 560310) and anti-NF-kB p65 (pS529) Alexa Fluor 488 (Cat. 558421)—other phosphospecific antibodies are likely to detect phosphorylated STAT1 and NF-kB p65 as well. A BD LSRII Flow Cytometer was used in these analyses.
6. For representation of the data, we recommend the use of FlowJo Software to display overlays of phosphorylated epitope stainings.

2.2 High Throughput qPCR

1. RNA extraction: RNeasy mini kit (Qiagen #74004), 100% ethanol.
2. cDNA synthesis: superscript VILO cDNA synthesis kit (Invitrogen #11754-250).
3. Primer plate: primers (Integrated DNA Technologies).
4. Preamplification: TaqManPreAmp Master Mix (Invitrogen #4391128).
5. Assay plate: assay loading reagent (Fluidigm #85000736), Universal Probe Library (Roche).
6. Exonuclease treatment: Exonuclease I (*E. coli*) (NEB #M0293S).
7. Sample plate: 2× FastStartTaqMan Probe Master (ROX) (Roche #4673450001), GE Sample Loading Reagent (Fluidigm #85000735), TAQ polymerase (Invitrogen #18038-042).
8. BioMark chip prime, run, and read: 48.48 Chip (Fluidigm #BMK-M-48.48), control line fluid syringes (Fluidigm #89000020). Use the BioMark HD and IFC Controller hardware for the run of the BioMark chips.
9. For analysis of the data we recommend Fluidigm Real-Time PCR Analysis software.

3 Methods

3.1 PhosFlow Cytometry

The protocol presented below has been optimized for detection of phosphorylated forms of STAT1 and NF-kB in IFN-α and TNF-α-stimulated monocyte-derived dendritic cells (Mo-DCs). The staining protocol has also been optimized for a procedure in a 96-well plate format. Essentially, the same procedure can be used to detect phosphorylated forms of STAT1 and NF-kB in other cell types and in any other conditions where the phosphorylation levels of STAT1 and NF-kB are altered. Tips will be given throughout the notes section to troubleshoot and optimize the procedure (*see* **Note 1**).

1. Prior to beginning the experiment, plan to have extra wells available in your experiment for both positive and negative controls. Stimulate cells with IFN-α (10 ng/mL), TNF-α (10 ng/mL) for 1 h, or leave untreated as control (*see* **Note 2**).
2. Rinse cells in prewarmed PBS (37 °C).
3. If starting from adherent cells, remove PBS and lift off the cells using a 0.25% trypsin solution and transfer cells into a 96-well plate or into FACS tubes. In the case of Mo-DC or any other cell type in suspension, simply harvest the cells and transfer in a 96-well plate or in a FACS tube.
4. Centrifuge cells by spinning them down at 13.4×*g* for 3 min.

5. Flick out supernatant.
6. Resuspend cells in 100 µL PBS.
7. Fix cells using 100 µL of prewarmed (37 °C) Fix Buffer I or 4% formaldehyde (*see* **Note 3**).
8. Incubate plate at 37 °C for 10 min.
9. Centrifuge plate at 1500 rpm for 3 min.
10. Flick out supernatant.
11. Resuspend cells with 200 µL ice cold PERM buffer III or 90% methanol (*see* **Notes 4** and **5**).
12. Incubate plate for 20 min on ice.
13. Precool centrifuge at 4 °C during this step.
14. Centrifuge plate at 1500 rpm for 3 min at 4 °C.
15. Wash three times with 200 µL of saturation buffer.
16. Resuspend cells in 200 µL of staining buffer.
17. Incubate plate for 30 min on ice.
18. Centrifuge plate at 1500 rpm for 3 min.
19. Resuspend cells in 90 µL of staining buffer.
20. Add 10 µL of antibodies per well (*see* **Note 6**).
21. Mix well by pipetting up and down using a multichannel pipette.
22. Incubate plate for 30 min at room temperature and protect from light (*see* **Note 7**).
23. Wash twice with 200 µL of staining buffer.
24. Resuspend in 150 µL BD stain buffer or PBS/2% FBS.
25. Create compensations and analyze by flow cytometry (*see* **Note 8**).
26. Use the FlowJo Software to display overlays of phosphorylated epitope staining.

An excellent example of such an analysis of phosphorylated STAT is provided by BD Biosciences: http://www.bdbiosciences.com/br/research/phosflow_old/features/optimizedreagents.jsp.

3.2 High Throughput qPCR

This protocol has been tailored for the 48.48 Fluidigm BioMark chip. The same principles apply for the 96.96 chip; however, it is necessary to modify volumes of reagents and programs used.

3.2.1 RNA Extraction Using the Qiagen RNeasy Mini Kit

1. Harvest cells. If starting from adherent cells, remove PBS and lift off the cells using a 0.25% trypsin solution and transfer cells into a 96-well plate. In the case of Mo-DC or any other cell type in suspension, simply harvest the cells and transfer in a 96-well plate.
2. Centrifuge cells by spinning them down at 1500 rpm for 3 min.

3. Flick out supernatant.
4. Resuspend cells in 100 µL PBS.
5. Repeat **steps 3** and **4**.
6. Resuspend cells in 350 µL Buffer RLT.
7. Add 350 µL 70% ethanol and mix well by pipetting.
8. Transfer the sample to an RNeasy Min Elute spin column in a 2 mL collection tube.
9. Centrifuge for 30 s at 9000 × *g*; discard the flowthrough.
10. Add 700 µL Buffer RW1 to the spin column. Centrifuge for 30 s at 9000 × *g*; discard the flowthrough.
11. Add 500 µL Buffer RPE to the spin column. Centrifuge for 30 s at 9000 × *g*; discard the flowthrough.
12. Add 500 µL Buffer RPE to the spin column. Centrifuge for 2 min at 9000 × *g*; discard the flowthrough.
13. Place the spin column in a new collection tube. Centrifuge for 1 min at maximum speed.
14. Place the spin column in a new 1.5 mL Eppendorf tube. Add 35 µL RNase-free water directly onto the spin column membrane. Centrifuge for 1 min at 9000 × *g* to elute.
15. Measure RNA concentration by Nanodrop.

3.2.2 cDNA Synthesis

1. Using the Superscript VILO cDNA synthesis kit reagents, assemble reactions consisting of 100 ng RNA, 2 µL 5× VILO reaction mix, 1 µL SuperScript enzyme mix, and PCR-grade water to make up the volume to 10 µL in a 96-well PCR plate.
2. Seal, vortex, and briefly spin the plate.
3. Place plate in thermocycler and run program at 25 °C for 10 min, 42 °C for 60 min, and 85 °C for 5 min (optional holding stage of 4 °C).
4. Dilute cDNA by adding 90 µL PCR-grade water to each sample (final concentration of 1 ng/µL).
5. Plate can be stored at −20 °C at this step.

3.2.3 Assembling Primer Plate and PCR Preamplification

1. Aliquot 7uL of 20uM (forward & reverse) primers into a 96-well plate, as seen in Fig. 1, utilizing rows A-H columns 1–8 (*see* **Note 9**).
2. From the primer plate (**step 1**), make a primer pool for the pre-amp reaction (2 µL of each primer × 48 primer pairs = 96 µL, +104 µL of PCR-grade water.
3. For the preamplification step (Specific Target Amplification—STA), assemble a master mix of pre-amp reagent using TaqMan PreAmp Master Mix. For one reaction, use 5 µL of 2× TaqMan pre-amp mix and 2.5 µL primer pool.

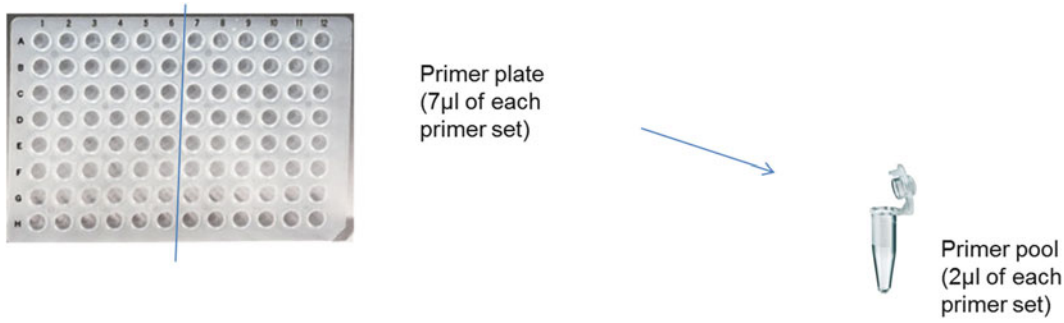

Fig. 1 Setup of primer plate and primer pool. For 48.48 chips, primers are aliquoted in wells rows A-H, columns 1–6. After aliquoting each desired forward and reverse primer set, a small aliquot of each is transferred to an Eppendorf tube for the subsequent preamplification step

4. In a new 96-well plate add 7.5 µL pre-amp mix from **step 1** to each well, again utilizing rows A-H, columns 1–8.
5. Add 2.5 µL cDNA sample to 7.5 µL pre-amp mix.
6. Seal, vortex, and briefly spin the plate.
7. Place plate in thermocycler and run program at 95 °C for 10 min; denature: 95 °C for 15 s, anneal and extend: 60 °C for 4 min repeat denature and anneal steps 14 cycles (optional holding stage of 4 °C forever) (*see* **Note 10**).
8. Plate can be stored at −20 °C at this step.
9. Next, assemble the assay mix using Fluidigm assay loading reagent. For a 1.5× reaction use 3.75 µL 2× assay loading reagent, 3 µL primer set (20 µM) from primer plate, and 0.75 µL corresponding Universal Probe Library (10 µM) (*see* **Note 11**).
10. The next step is to treat the preamplification samples with exonuclease to remove excess primers.
11. Assemble a master mix of exonuclease I. For one reaction use 0.8 µL exonuclease I, 1.4 µL 10× exonuclease buffer, and 1.8 µL water.
12. Add 4 µL of exonuclease I mix to each well of the pre-amp plate.
13. Seal, vortex, and briefly spin.
14. Place plate in thermocycler and run program at 37 °C for 30 min and at 80 °C for 15 min (optional holding stage of 4 °C forever).
15. Dilute exonuclease samples by adding 36 µL PCR-grade water to each sample (total volume of 50 µL) and mix carefully.
16. Plate can be stored at −20 °C at this step.

3.2.4 Assemble Sample Plate and Prime for PCR Amplification

1. Assemble a master mix of sample mix using 2× FastStart TaqMan Probe Master Mix, GE Sample Loading Reagent, and TAQ polymerase. For a 1.5× reaction use 3.75 µL 2× FastStartTaqMan Probe Master Mix (Rox), 0.375 µL GE

Sample Loading Reagent, and 0.15 μL TAQ polymerase (*see* **Note 12**).

2. Add 4.275 μL sample mix from **step 1** to a new 96-well plate.
3. Transfer 2.25 μL of diluted exonuclease sample from the pre-amp plate to the sample plate using a multichannel pipette.
4. Seal, vortex, and briefly spin the plate.
5. Take the chip out of the package along with two control line fluid-filled syringes for priming (*see* **Note 13**).
6. Using the black syringe cap, actuate the accumulator by pushing it open.
7. Uncap the syringe, push the black O-ring to the side with the syringe tip to create a gap, and dispense the liquid into the chamber as in Fig. 2 (*see* **Note 14**).
8. Repeat **step 3** using the other syringe in the other chamber.
9. Load the chip into the appropriate dynamic array IFC controller: HX—96.96 or MX—48.48.
10. Select "Prime" then "Run Script" on the touch screen (*see* **Note 15**).
11. To load samples and assays to the chip, add 5 μL of each sample to the BioMark chip using a multichannel pipette using the layout seen in Fig. 3.
12. Add 5 μL of each assay to the BioMark chip using a multichannel pipette using the layout seen in Fig. 3.
13. Scan the wells for any air bubbles with an individual sterile syringe tip. These bubbles can interfere with the fluidics and prevent the assay or sample from being pushed through into the chip.

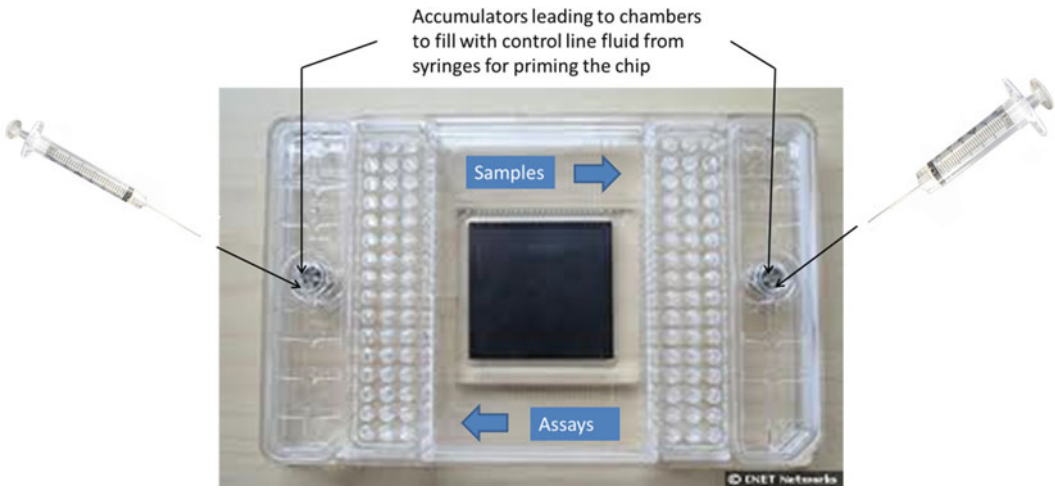

Fig. 2 Priming of BioMark chip with control line fluid. Syringes filled with control line fluid are loaded on either side of the BioMark chip to prepare the chip for priming

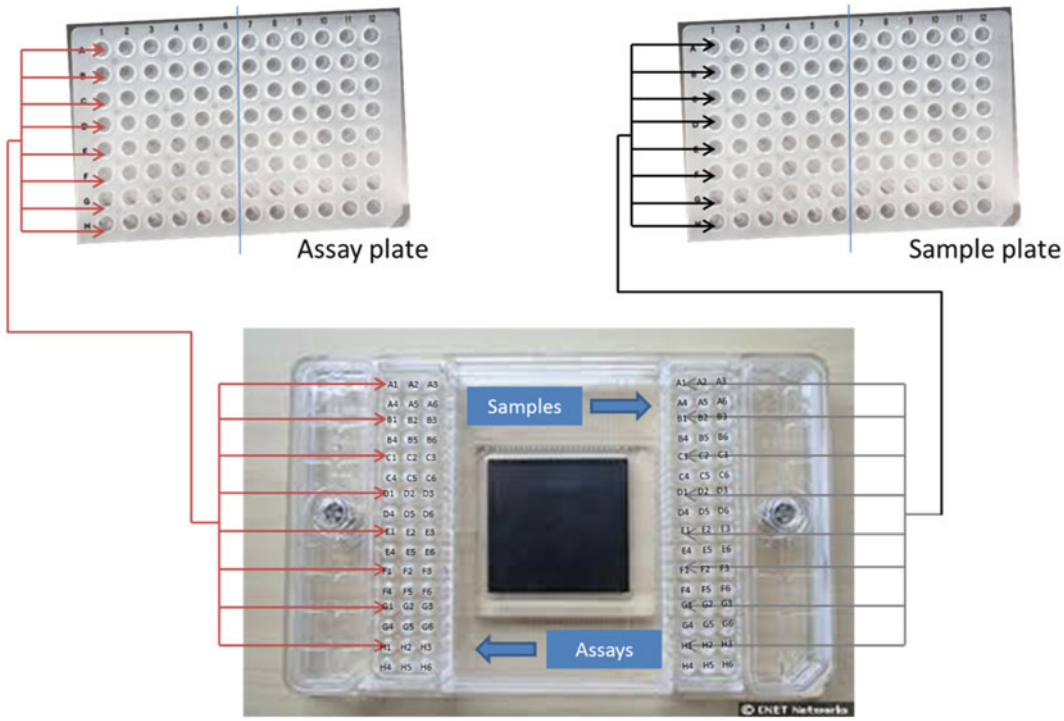

Fig. 3 Arrangement of assays and samples for loading BioMark chip. The orders of assays and samples to be transferred from 96-well assay and sample plates to the BioMark chip are shown. Using a multichannel pipette, assays and samples are loaded to the *left* and *right* of the chip, respectively

14. Return the chip to the controller and select "Load Mix" script on the screen (*see* **Note 16**).
15. Remove the chip from the IFC controller.
16. Use a piece of tape to remove any dust that has collected on the surface of the chip. Gently allow the tape to stick to the surface of the chip without applying pressure.
17. Remove the backing sticker from the chip immediately prior to loading the chip into the BioMark HD machine.
18. Open the BioMark software and follow prompt instructions.
19. Select the following details:

    App: Gene expression

    Pass ref: ROX

    Assay: Single probe: FAM-MGB

    Protocol file: 48.48 UPL
20. Select "Start".
21. After the run, analyze the results using Fluidigm Real-Time PCR Analysis software.

4 Notes

1. A key to success with PhosFlow is perseverance and trouble-shooting; if the experiment fails on first attempt, try it again.
2. Always start with a control system for experimental conditions such as IFN-α or TNF-α treatment to detect p-STAT1 and p-NF-kB, respectively. Make sure that the phosphorylation of STAT1 and NF-kB is clearly observed in your specific samples by western blot. If no difference is observed between stimulated and unstimulated samples by flow cytometry, check the stimulation conditions and if necessary adapt the concentration and/or the stimulation time to your specific cell type, also recognizing that HDACi treatment may modulate phosphorylation levels [11].
3. Resuspension volume in PBS and fixation volume should be the same to optimize the fixing process.
4. Signaling events happening within the cells must be frozen rapidly and efficiently. The percentage as well as the time of formaldehyde fixation can be adjusted to optimize the signal-to-noise-ratio in your specific samples.
5. It is highly recommended to screen each antibody to make sure it works with PERM buffer used in your settings. Different permeabilization buffers may preserve particular epitopes.
6. Antibody must always be titrated before the first use and a range of 1:100–1:5000 is recommended to identify the optimized signal:noise ratio.
7. In most cases, 30 min staining should be adequate but this timing can still be optimized.
8. Pretest the cocktails before real experiments. The concentrations of antibodies within the cocktail may need further adjustment to allow the best compensation and optimal results.
9. If you wish to use the same customized panel of genes for multiple BioMark chips, you may increase the volume of primers transferred to the primer plate and primer pool for future use. Store these primers in a sealed plate at –20 °C.
10. 18 or 20 cycles can be used for experiments where less RNA is used as the starting material.
11. The assay plate can be prepared up to 2 days prior to running the chip. It should be stored at 4 °C, protected from light until the following day.
12. The sample plate must be prepared the same day that the chip is run.
13. Do not start this step until both the assay and sample plates are ready. Do not open the chip package until ready to prime.
14. Do not hold the syringe over the IFC or chip inlets. Remove any extra liquid remaining in the accumulator without apply-

ing pressure and do not break the seal between the O-ring and the chamber from this point on.

15. No more than 1 h should pass before returning the chip to the IFC controller in the next step.
16. Between running and reading the chip, no more than 4 h should pass.

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Chapter 19

Assessing HDAC Function in the Regulation of Signal Transducer and Activator of Transcription 5 (STAT5) Activity Using Chromatin Immunoprecipitation (ChIP)

Sophia Pinz and Anne Rascle

Abstract

Transcriptional activation by STAT5 is repressed by deacetylase inhibitors. Investigating the role of deacetylases (HDACs) in STAT5-mediated transcription implies the analysis of molecular events taking place at the chromatin level. We describe here two alternative methods of chromatin immunoprecipitation that allow the characterization of chromatin modifications ensuing STAT5 activation and its inhibition by deacetylase inhibitors, in particular changes in histone acetylation, in histone occupancy, and in the association/dissociation of transcription factors and other chromatin-associated factors.

Key words STAT5, HDAC, Deacetylase, Brd2, Deacetylase inhibitor, Trichostatin A, TSA, Histone acetylation, Chromatin immunoprecipitation, ChIP, Sonication, Micrococcal nuclease, MNase digestion

1 Introduction

The deacetylation of histone and non-histone proteins by deacetylases (the so-called HDACs, for histone deacetylases) has major impacts on the regulation of protein activity and hence on numerous signaling pathways, both in physiological and pathological conditions [1, 2]. Characterization of HDAC functions has been greatly facilitated by the use of deacetylase inhibitors. This approach allowed our group to demonstrate the implication of HDAC activity in the regulation of the transcription factor signal transducer and activator of transcription 5 (STAT5). In particular, we showed that a variety of pan- and class specific-deacetylase inhibitors (trichostatin A [TSA], sodium butyrate [NaB], suberoylanilide hydroxamic acid [SAHA], valproic acid [VPA], apicidin) inhibit STAT5-mediated transcription by interfering with the function of a chromatin-associated factor normally required for the proper recruitment of the transcriptional machinery [3–5]. Deciphering the role of HDACs in the regulation of chromatin

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function can be tackled employing chromatin immunoprecipitation (ChIP) assays. We describe here two alternative ChIP methods allowing the characterization of histone modifications and occupancy, as well as the detection of DNA- and chromatin-associated factors. The basic chromatin immunoprecipitation procedure was first reported by the group of James Broach in 1993 in their study of the implication of histone acetylation in gene silencing in yeast *Saccharomyces cerevisiae* [6]. Using formaldheyde-cross-linked whole yeast cells and acetylated histone H4-specific antibodies, Braunstein and colleagues demonstrated that gene silencing is associated with hypoacetylated nucleosomes. Since then, the ChIP method has been widely used from both yeast cells and higher eukaryote cells and tissues (reviewed in ref. [7]).

The ChIP method can be divided into five major steps: (1) cross-linking of protein–DNA complexes, usually using formaldehyde, (2) cell lysis and chromatin fragmentation, whether mechanical or enzymatic, (3) immunoprecipitation of DNA-associated proteins using specific antibodies, (4) purification of the co-immunoprecipitated genomic DNA (gDNA), and (5) quantification of isolated gDNA, typically by quantitative PCR. Enrichment of a DNA region of interest (previously bound directly or indirectly to the immunoprecipitated protein) is evaluated by normalizing the amount of ChIPed gDNA to the amount of gDNA present in the input lysate (expressed as percentage of input DNA, thereafter referred to as % input DNA).

The versatility of the method relies on the multiplicity of antibodies that can be used and on their specificity, permitting not only to determine the presence or absence of a particular protein at a specific locus, but also the detection of protein modifications (acetylation, methylation, phosphorylation, etc.) under a given condition. On the other hand, ChIP's main limitation resides in its immunoprecipitation step itself, as not all antibodies function under ChIP experimental conditions, mainly due to inadequate buffer composition or epitope masking upon formaldehyde cross-link. Another limitation is the protein abundance and its cellular distribution. Hence, ChIP of abundant nuclear proteins such as histones or their acetylated version usually yield high % input DNA, while nuclear proteins of low abundance or indirectly bound to DNA evoke low % input DNA. Similarly, protein abundant in both the cytosolic and nuclear fractions, such as the transcription factor STAT5 which is present in a latent form in the cytoplasm and is partially translocated into the nucleus upon activation, might yield low % input DNA when performing ChIP from whole cells due to competition for antibody binding.

For these reasons, we describe here two ChIP protocols, referred to as "standard ChIP protocol" using whole cell extracts, and "alternative ChIP protocol" starting from nuclei preparations. They differ in the cell lysis and chromatin fragmentation step. While whole cell lysis and chromatin shearing is performed in one sonication step in the "standard ChIP protocol" (Fig. 1a), the "alternative ChIP protocol" requires the prior isolation of nuclei, a sonication step and a limited micrococcal nuclease (MNase)

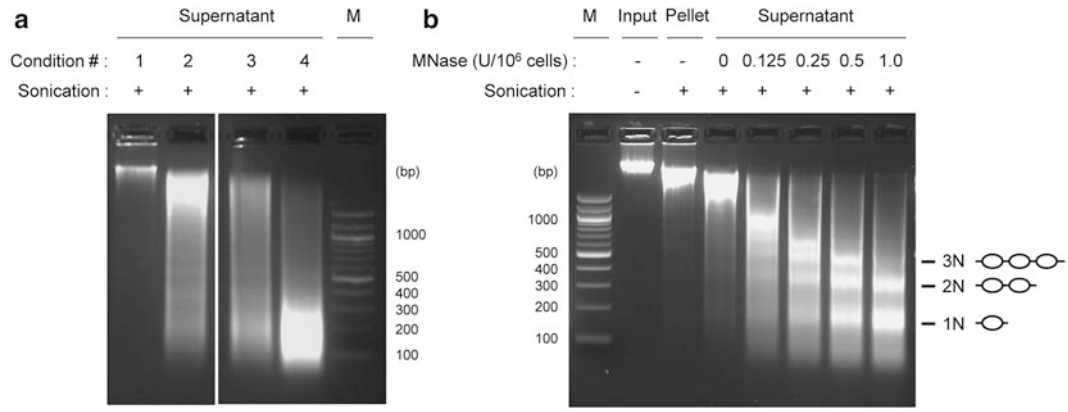

Fig. 1 Agarose gel electrophoresis of fragmented DNA. Shown are results from the standard (a) and alternative (b) cell lysis and chromatin preparation protocols. (**a**) Formaldehyde-cross-linked Ba/F3-derived cells were lysed and sonified using the protocol described in Subheadings 3.3.1 and 3.3.2 using distinct sonication parameters and sonifiers (conditions #1–4). Whole cell sonified lysates (Supernatant; Subheading 3.3.1, step 3) were further processed for agarose gel analysis (Subheading 3.3.1, steps 4–10). The agarose gel image shows that condition #4 (corresponding to the sonication settings described in Subheading 3.3.2, step 2) yields fragments <500 bp in length and is thus appropriate for chromatin immunoprecipitation from whole cell lysates, whereas conditions #1–3 are not. (**b**) Formaldehyde-cross-linked Ba/F3-1*6 cells were lysed and the isolated nuclei were sonified using the protocol described in Subheading 3.3.3. Input is the non-sonified sample (Subheading 3.3.3, step 7). Pellet is the insoluble fraction recovered after sonication (Subheading 3.3.3, step 8). Following sonication, nuclear lysates (Supernatant; Subheading 3.3.3, step 8) were subjected to MNase digestion. The agarose gel image illustrates the effect of increasing amounts of MNase on nucleosomal DNA production. The optimal condition was obtained using 0.25 U MNase/10^6 cells (Subheading 3.3.3, step 9), which yields fragment lengths corresponding mainly to mono-, di-, and tri-nucleosomes (1N, 2N, and 3N, respectively). M is the 100 bp ladder DNA marker

digestion (Fig. 1b). The latter protocol revealed to greatly improve the detection of STAT5 binding to the promoter region of the STAT5 target gene *Cis* (Fig. 2) or the detection of the chromatin-associated factor Brd2 (Fig. 3b and ref. [3]), while the monitoring of histone association and acetylation is readily achievable using the "standard ChIP protocol" (Fig. 3a and refs. [3–5, 8]).

2 Materials

Reagents and materials were obtained from the indicated providers. However, unless indicated otherwise (e.g., antibodies), other providers might be selected.

2.1 Ba/F3 Cell Culture and Treatment

1. RPMI 1640, with l-glutamine and 2 g/l $NaHCO_3$ (PAN Biotech), stored at 4 °C and pre-warmed before use.
2. Heat-inactivated fetal calf serum (FCS) (PAN Biotech), stored at −20 °C and used 10% final in medium.

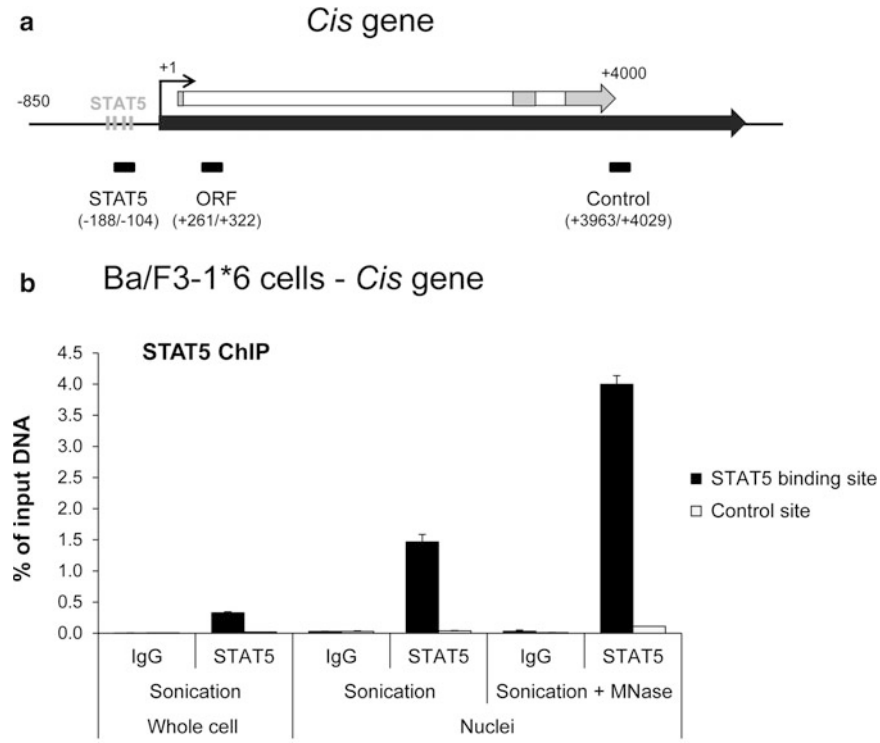

Fig. 2 Comparison of STAT5 ChIP using the standard and alternative ChIP protocols. (**a**) Schematic representation of the model STAT5 target gene *Cis* and of the amplicons analyzed by quantitative PCR in this study. Positions are relative to the transcription start site. The *Cis* gene contains four STAT5 binding sites within its proximal promoter. STAT5, STAT5 binding sites; ORF, open reading frame. (**b**) Formaldehyde-cross-linked Ba/F3-1*6 cells were lysed following the standard (whole cell lysis) or the alternative (nuclear lysis) protocol. The standard protocol was conducted as described in Subheading 3.3.2, steps 1–6. Nuclei isolated following the alternative protocol were resuspended in either SDS^+ (for sonication alone) or HDG150 (for sonication + MNase treatment) buffer, sonified and centrifuged as described in Subheading 3.3.3, steps 7–8. MNase treatment was conducted as in Subheading 3.3.3, steps 9–10 and sample was diluted in IP buffer, as indicated in Subheading 3.3.3, step 12. The sonified untreated sample (in SDS^+ buffer) was adjusted to IP buffer by addition of 0.5 volume of Triton Dilution buffer, as described in Subheading 3.3.2, step 4. ChIP was further performed from all samples as described in Subheadings 3.4 and 3.5, using STAT5-specific antibodies or the same amount of nonspecific IgG (background control). Quantitative PCR (Subheading 3.6) was performed using primers specific for the STAT5 binding sites (−188/−104) or a distal control region (+3963/+4029) of the STAT5 target gene *Cis*, as depicted in panel (a). The figure shows STAT5A-1*6 constitutive binding to the *Cis* promoter [3, 4, 11]. The alternative protocol combining sonication and MNase treatment of nuclei dramatically improves the detection of STAT5 binding to DNA (increased % input DNA and higher fold enrichment relative to the IgG background)

3. Penicillin/streptomycin solution (10,000 U/ml penicillin, 10 mg/ml streptomycin) (PAN Biotech), stored at −20 °C and used 1% final in medium.
4. Recombinant mouse interleukin-3 (IL-3) (Immunotools). IL-3 is reconstituted at 100 µg/ml in RPMI 1640 supplemented with 10% FCS and stored in small aliquots at −20 °C. Before use, IL-3 is diluted 1:10–10 µg/ml in RPMI

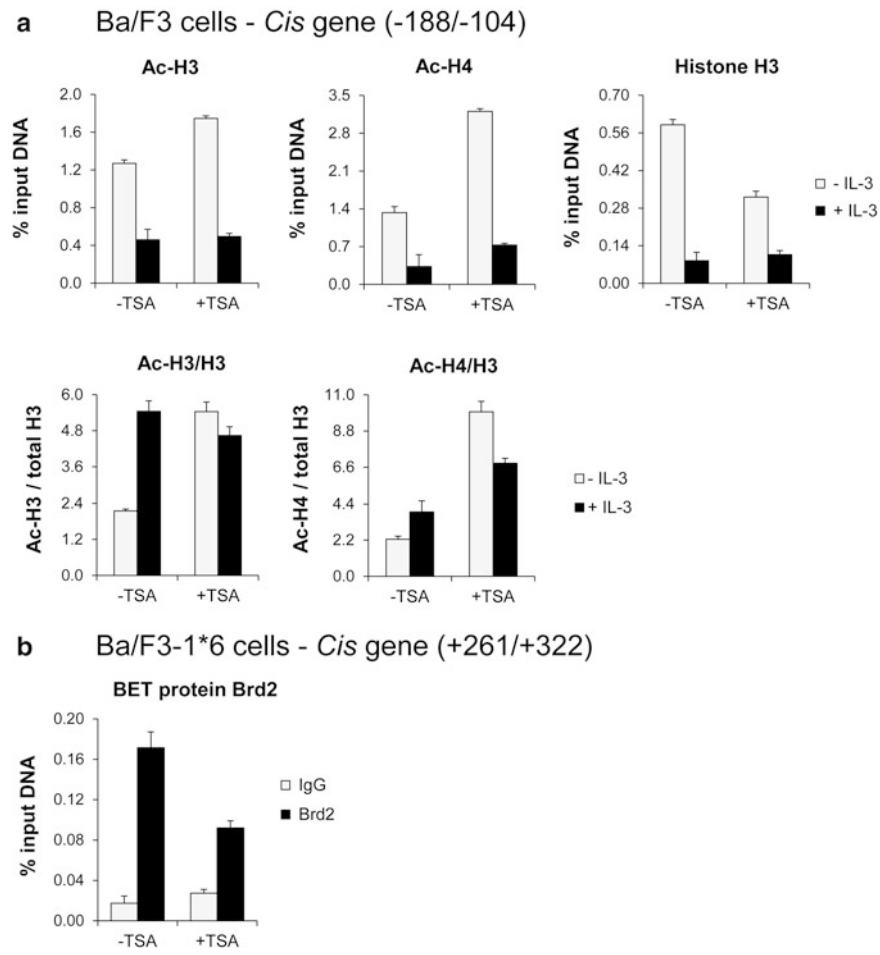

Fig. 3 Investigation of HDAC function in STAT5 regulation using ChIP. (**a**) Effect of the deacetylase inhibitor TSA on histone occupancy and acetylation around the STAT5 binding site of the *Cis* gene (Fig. 2a) upon IL-3-induced STAT5 activation. Rested Ba/F3 cells were pretreated with 200 nM TSA for 30 min and stimulated with IL-3 for an additional 30 min. ChIP using the standard protocol (Subheading 3.3.2) was performed using antibodies specific for histone H3, Ac-H3 and Ac-H4 (Subheading 2.4). Data are expressed as percentage of input DNA (% input DNA). Histone H3 and H4 acetylation was also evaluated upon normalization to total H3 (Ac-H3/H3 and Ac-H4/H3). Histone H3 content and acetylation are altered upon both IL-3 stimulation and TSA treatment (refer to ref. [3]). (**b**) Effect of the deacetylase inhibitor TSA on Brd2 binding at the vicinity of the transcription start site of the *Cis* gene (beginning of ORF; Fig. 2a) in cells expressing constitutively active STAT5. Ba/F3-1*6 cells were treated with 200 nM TSA for 60 min and Brd2 ChIP was performed using the alternative protocol (Subheading 3.3.3). Brd2 association with the transcriptionally active *Cis* gene is reduced upon TSA treatment (refer to ref. [3])

1640 supplemented with 10% FCS, stored at 4 °C and added fresh in medium. IL-3 is used at 2 ng/ml for cell maintenance and at 5 ng/ml for IL-3 stimulation.

5. Chemicals: Dimethyl sulfoxide (DMSO; SIGMA), trichostatin A (TSA; SIGMA). TSA is dissolved in DMSO at a concentration of 1 mM, stored in small aliquots at −80 °C, and used fresh for cell treatment at 200 nM final. DMSO is used as a vehicle control (0.02% final).

6. Cells are cultivated at 37 °C under 5% CO_2 in a humidified incubator.

2.2 Formaldehyde-Induced Cross-Linking

1. Chemical fume hood.
2. 37% formaldehyde solution (SIGMA); stored at room temperature (RT).
3. 2 M glycine in H_2O; stored at RT.
4. Phosphate buffered saline (PBS): 0.137 M NaCl, 2.67 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4; stored at 4 °C.
5. Dewar flask with liquid nitrogen.

2.3 Cell Extract Preparation and Chromatin Fragmentation

2.3.1 Sonication Optimization Protocol

1. 10% NaN_3 in H_2O; stored at room temperature (RT) (*see* **Note 1**).
2. SDS^+ buffer: 100 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5% SDS, 0.02% NaN_3; stored at room temperature. Protease and phosphatase inhibitors are added fresh at the final concentration of 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.5 mM PMSF, 10 mM NaF, and 1 mM Na_3VO_4.
3. Branson 250 sonifier, 3 mm microtip (*see* **Note 2**).
4. Chemical fume hood.
5. Proteinase K (SIGMA): 20 mg/ml in H_2O; stored at –20 °C.
6. Glycogen solution (20 mg/ml; Roche or Affymetrix); stored at –20 °C.
7. Proteinase K digestion buffer, 4×: 0.8 mg/ml proteinase K, 0.48 mg/ml glycogen, TE; prepared fresh.
8. Phenol–chloroform–isoamyl alcohol (24:24:1, pH 8.0) (Fisher Scientific); stored at 4 °C but brought to room temperature before use.
9. 3 M Na acetate in H_2O; stored at RT.
10. Ethanol p.a.; stored at RT; precooled at –20 °C before use.
11. RNase A solution (~30 mg/ml; SIGMA); stored at –20 °C.
12. TE: 10 mM Tris pH 7.6, 1 mM EDTA; autoclaved and stored at RT.
13. RNase A digestion buffer: 1 mg/ml RNase A in TE; prepared fresh.
14. Loading dye, 6×: 40% glycerol, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol in TE; stored at 4 °C.
15. 2% agarose gel containing 0.2 µg/ml ethidium bromide, prepared in 0.5× TAE buffer (before use).
16. TAE buffer, 50×: 2 M Tris, 50 mM EDTA, 1 M acetic acid; autoclaved and stored at RT.
17. 100 bp DNA ladder (New England Biolabs); stored at 4 °C. Load 0.5 µg/lane.

18. Ultraviolet (UV) transilluminator and gel documentation system.

2.3.2 Standard ChIP Protocol (Whole Cell Extraction)

1. Branson 250 sonifier, 5 mm microtip (*see* **Note 2**).
2. SDS^+ buffer (*see* Subheading 2.3.1).
3. Triton Dilution buffer: 100 mM NaCl, 100 mM Tris pH 8.0, 5 mM EDTA, 5% Triton X-100, 0.02% NaN_3; stored at room temperature (RT).
4. IP buffer: 1 volume SDS^+ buffer + 0.5 volume Triton Dilution Buffer (fresh).
5. Protease and phosphatase inhibitors, added fresh to all buffers at the final concentration of 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM PMSF, 10 mM NaF, and 1 mM Na_3VO_4.

2.3.3 Alternative Protocol (Nuclei Extraction)

1. Branson 250 sonifier, 3 mm microtip (*see* **Note 2**).
2. Cell Collection Buffer (CCB): 100 mM Tris pH 9.4, 100 mM DTT; prepared fresh.
3. Buffer MA: 10 mM Hepes pH 6.5, 10 mM EDTA, 0.25% Triton X-100; prepared fresh.
4. Buffer MB: 10 mM Hepes pH 6.5, 1 mM EDTA, 200 mM NaCl; prepared fresh.
5. HDG150 buffer: 20 mM Hepes pH 7.6, 150 mM KCl, 10% (v/v) glycerol, 0.5 mM DTT (added fresh); stored at 4 °C.
6. Protease and phosphatase inhibitors, added fresh to all buffers at the final concentration of 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM PMSF, and 10 mM NaF.
7. MNase (SIGMA): reconstituted at 200 U/ml in 5 mM Tris pH 6.8, 50 mM NaCl, 50% (v/v) glycerol; stored aliquoted at −20 °C.
8. 1 M $CaCl_2$ in H_2O (for Ca^{2+}-dependent MNase activity); stored at RT.
9. 0.5 M EGTA in H_2O; stored at RT.
10. IP buffer (Subheading 2.3.2).

2.4 Immunoprecipitation

1. Protein A Sepharose CL-4B (GE Healthcare Life Sciences); stored at 4 °C.
2. Milli-Q Water.
3. PBS (Subheading 2.2).
4. TE: 10 mM Tris-HCl pH 7.6, 1 mM EDTA; autoclaved and stored at room temperature (RT).
5. BSA, Fatty Acid-free (SIGMA), 25 mg/ml in H_2O; stored at −20 °C.
6. Salmon sperm DNA solution, 10 mg/ml (Thermo Fisher Scientific/Life Technologies); stored at −20 °C.

7. 10% NaN_3 in H_2O; stored at RT.
8. Antibodies: STAT5A (Santa Cruz Biotechnology, sc-1081), STAT5A+B (Santa Cruz Biotechnology, sc-835), histone H3 (Abcam, ab1791), acetylated histone H3 (Ac-H3; Millipore, 06–599), acetylated histone H4 (Ac-H4; Millipore, 06–866), BRD2 (Bethyl, A302–583A) and IgG from rabbit serum as isotype control (SIGMA, I-5006); stored at 4 °C or –20 °C, following the manufacturer's recommendations.
9. 150 mM NaCl Wash Buffer: 150 mM NaCl, 20 mM Tris pH 8.0, 5 mM EDTA, 1% Triton X-100, 0.2% SDS, 0.02% NaN_3; stored at RT.
10. 500 mM NaCl Wash Buffer: 500 mM NaCl, 20 mM Tris pH 8.0, 5 mM EDTA, 1% Triton X-100, 0.2% SDS, 0.02% NaN_3; stored at RT.
11. LiCl Wash Buffer: 250 mM LiCl, 10 mM Tris pH 8.0, 1 mM EDTA, 0.5% (w/v) deoxycholic acid (sodium salt), 0.5% (v/v) NP40, 0.02% NaN_3; stored at RT.

2.5 DNA Isolation

1. Cross-link reversal buffers: 1% SDS, 0.1 M $NaHCO_3$; 5 M NaCl; stored at room temperature (RT).
2. Air incubator (or oven) set at 65, 55 and 37 °C.
3. RNase A solution (30 mg/ml; SIGMA); stored at –20 °C.
4. Proteinase K (SIGMA): 20 mg/ml in H_2O; stored at –20 °C.
5. DNA purification columns: Nucleospin PCR clean-up kit (Macherey-Nagel) with NTB Binding Buffer (Macherey-Nagel); stored at RT.

2.6 DNA Analysis by Quantitative PCR

1. Thermocycler (e.g., Rotorgene Q, Qiagen) using a two-step PCR program (95 °C 15 s, 60 °C 60 s; 40 cycles).
2. SYBR-Green-containing PCR master mix (commercial or home-made using a hot start Taq; recipe provided upon request).
3. Quantitative PCR forward and reverse primers (*see* **Note 3**) specific for the mouse *Cis* gene locus (5′–3′): STAT5 binding sites (–188/–104), GTCCAAAGCACTAGACGCCTG and TTCCCGGAAGCCTCATCTT; beginning of ORF (+261/+322), GGACTTCGAGTGGTGTGCCTA and GGCTCCGTTTCCCTATCCA; end of ORF control (+3963/+4029), TACCCCTTCCAACTCTGACTGAGC and TTCCCTCCAGGATGTGACTGTG; used at 400 nM final.

3 Methods

The following protocols have been optimized for the suspension mouse cell line Ba/F3 (and its derivative Ba/F3-1*6) to study HDAC function in STAT5 signaling [3–5, 8–10]. Ba/F3 is a

IL-3-dependent pro-B cell line widely used to study STAT5 signaling in non-transformed cells. Ba/F3-1*6 cells express and are transformed by the constitutively active STAT5A-1*6 mutant, and consequently grow independently of IL-3. Ba/F3-1*6 constitutes an ideal cellular model to study deregulated STAT5 activity, mimicking the situation found in many cancers [3–5, 8, 10, 11]. Cell lysis and chromatin fragmentation (Subheading 3.3) is cell type-dependent. Optimizations will be required when working with other cell lines (Subheading 3.3.1).

3.1 Ba/F3 Cell Treatment

1. Ba/F3 cells are grown at a maximal density of 10^6 cells/ml in RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin and 2 ng/ml IL-3. The IL-3-independent Ba/F3-1*6 cells, are grown at a maximal density of 10^6 cells/ml in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin.
2. Before cell treatment with the deacetylase inhibitor TSA and IL-3 stimulation, Ba/F3 cells are deprived of IL-3 for several hours (to turn endogenous STAT5 activity off). Cells are transferred in a 50 ml conical tube, harvested by centrifugation (5 min, 300 × *g* at room temperature) and washed twice with 50 ml pre-warmed RPMI 1640. Cells are resuspended at a maximal density of 10^6 cells/ml in RPMI 1640, 10% FCS, 1% penicillin/streptomycin (in cell culture T-flask) and incubated for 9–12 h at 37 °C under 5% CO_2.
3. Rested Ba/F3 cells are divided into two T-flasks and pretreated for 30 min with either 200 nM TSA or 0.02% DMSO (vehicle). Each flask is divided into two new flasks and either stimulated with 5 ng/ml IL-3 or left unstimulated for the desired duration (usually 5–30 min). Growing Ba/F3-1*6 cells are divided into two T-flasks and directly treated with TSA or DMSO, as above.
4. Per lysate preparation, 2×10^7 and 5×10^7 cells are needed for the standard and alternative protocols respectively. Typically, double cell amount is prepared for cross-linking (4×10^7 cells in 40 ml medium and 10^8 cells in 120 ml medium, respectively), allowing two independent cell extractions. Therefore, for a four condition experiment (−/+ TSA, −/+ IL-3), 1.6×10^8 and 4×10^8 Ba/F3 cells are needed for the standard and alternative ChIP protocols respectively (half for IL-3-independent Ba/F3-1*6 cells, for the −/+ TSA conditions).

3.2 Cross-Link of Protein–DNA Complexes

1. Treated cells are transferred into a 50 ml conical tube. For the standard protocol, 4×10^7 cells in 40 ml medium are transferred into one tube. For the alternative protocol, cells (10^8 cells in 120 ml medium) are transferred into three conical tubes (40 ml each).

2. Cells are cross-linked at room temperature under a fume hood.
3. 1.2 ml of a 37% formaldehyde solution is added per tube (1% formaldehyde final). Tubes are closed, mixed per inversion several times, and incubated for 10 min (*see* **Notes 4–6**).
4. Cross-link is stopped by addition of 2.8 ml 2 M glycine (125 mM final). Tubes are mixed by inversion as before and incubated for 5 min at room temperature.
5. Cells are harvested by centrifugation (5 min, 500 × *g* at 4 °C) and washed twice with ice cold-PBS. Cells from the alternative protocol are pooled into one 50 ml conical tube at this stage.
6. Cross-linked cells are resuspended in 2 ml ice cold-PBS, transferred into two 1.5 ml reaction tubes (1 ml each) and harvested by centrifugation (1 min, 2000 × *g* at 4 °C). Dry pellets (2×10^7 and 5×10^7 cells each for the standard and alternative protocol respectively) are snap-frozen in liquid nitrogen and stored at −80 °C until use.

3.3 Cell Extract Preparation and Chromatin Fragmentation

Unless indicated otherwise, all steps are performed on ice.

3.3.1 Sonication Optimization Protocol (Standard Procedure)

Chromatin shearing efficiency is not only dependent on the cell type but also on the sonifier used. Moreover, a same sonifier brand and model will exhibit varying sonication properties in function of its age and of the quality of its microtip (*see* **Note 7**). Therefore, a protocol meant to establish optimal chromatin fragmentation conditions is presented here.

1. Formaldehyde-cross-linked cells (from Subheading 3.2, **step 6**) are resuspended in precooled SDS^+ buffer at a density of 5×10^6 cells/ml, and 1 ml cell suspension is distributed in 1.5 ml microtubes (5×10^6 cells per sonication condition).
2. Sonication parameters to be optimized include: output control amplitude, number and duration of pulses, duty cycle %. Samples are kept on ice during sonication and cooled for at least 30 s between pulses. A non-sonified control is included, which is submitted to a single and brief (5 s) sonication pulse to break the cell membrane.
3. Following sonication, cell debris and insoluble chromatin are eliminated by centrifugation (10 min, 20,000 × *g* at 4 °C) and 300 µl supernatant (whole cell sonified lysate of 1.5×10^6 cells) is transferred to a new 1.5 ml reaction tube. Optional: the pellet (insoluble fraction) can be further analyzed by resuspending in 300 µl SDS^+ buffer and proceeding with **step 4**.
4. To each tube, 100 µl of 4× Proteinase K digestion buffer is added and the samples (now containing 0.2 mg/ml proteinase

K and 0.12 mg/ml glycogen) are incubated in an air incubator for 2–3 h at 55 °C then overnight at 65 °C, to allow protein degradation and cross-link reversal respectively.

5. One volume (400 µl) of phenol–chloroform–isoamyl alcohol is added per tube, samples are vigorously vortexed for 1 min at room temperature, centrifuged (5 min, 20,000 × *g* at RT) and the upper aqueous phase containing the DNA transferred into a new 1.5 ml microtube.
6. To precipitate the gDNA, 0.1 volume of 3 M Na acetate (40 µl) and 2.5 volumes of 100% ethanol (1 ml) are added to the extracted DNA fraction. Samples are mixed by vortexing, cooled at −20 °C for 20 min and DNA recovered by centrifugation (30 min, 20,000 × *g* at 4 °C).
7. Supernatants are carefully removed, DNA pellets are washed with 1 ml ice-cold 70% ethanol, samples are centrifuged once more (10 min, 20,000 × *g* at 4 °C) and the purified gDNA pellets are air-dried at room temperature (*see* **Note 8**).
8. DNA is resuspended in 50 µl of RNase A digestion buffer and incubated at 37 °C for 30 min.
9. 10 µl of 6× loading dye is added per sample and 5 µl of purified sonified gDNA (then corresponding to ~0.1×10^6 cells) is loaded on a 2% agarose gel (*see* **Note 9**).
10. Gel is ran at 100 V for approximately 30 min in 0.5× TAE buffer, DNA is visualized using an UV transilluminator and a gel image is captured using a gel documentation system. The optimal size of sonified DNA fragments is 200–500 bp (Fig. 1a, condition #4).

3.3.2 Standard Protocol for Whole Cell Extraction

1. Cross-linked Ba/F3 cells (2×10^7 cells from Subheading 3.2, **step 6**) are resuspended in 3 ml ice-cold SDS^+ buffer and transferred into a 15 ml conical tube.
2. Samples are placed on ice and sonified using a 5 mm microtip using the following parameters (Branson 250 sonifier): output control 5, duty cycle 60%, six pulses of 60 s, 2 min cooling between pulses (*see* **Note 7**).
3. Optional: an aliquot of the sonified samples (250 µl, corresponding to 1.5×10^6 cells, adjusted to 300 µl with TE) can be processed for the analysis on DNA fragmentation on agarose gel, as described in Subheading 3.3.1, **steps 4–10** (Fig. 1a).
4. Following **step 2**, 0.5 volume of Triton Dilution buffer (1.5 ml) is added to the sonified samples (now 4.5 ml final volume of IP buffer).
5. Cell debris and insoluble chromatin are eliminated by centrifugation (10 min, 3200 × *g* at 4 °C) and the supernatant (sonified whole cell lysate) is carefully transferred into a new 15 ml conical tube.

6. Samples at this stage can be snap-frozen in liquid nitrogen and stored at −80 °C or processed immediately for immunoprecipitation (Subheading 3.4).

3.3.3 Alternative Protocol for Nuclei Preparation

This protocol is adapted from nuclei preparation and MNase digestion procedures described by Métivier et al. [12] and Okada and Fukagawa [13] respectively.

1. Cross-linked Ba/F3 cells (5×10^7 cells from Subheading 3.2, **step 6**) are resuspended in 1 ml ice-cold CCB buffer and vigorously vortexed.
2. Samples are incubated on ice for 15 min, briefly vortexed, and incubated at 30 °C for an additional 15 min (selective lysis of the plasma membrane).
3. Lysates are briefly vortexed and nuclei recovered by centrifugation (1 min, 2000 × *g* at 4 °C).
4. The supernatant (containing cytosolic proteins) is carefully removed (*see* **Note 10**) and the nuclei are washed sequentially with 1 ml buffer MA and 1 ml buffer MB by vigorous vortexing and centrifugation (1 min, 2000 × *g* at 4 °C). Washed nuclei are harvested by centrifugation (1 min, 2000 × *g* at 4 °C) and the washing buffer (supernatant) is carefully and completely eliminated.
5. Washed nuclei (dry pellet) can be snap-frozen in liquid nitrogen and stored at −80 °C or processed further for sonication (**step 6**).
6. Nuclei from **step 5** are resuspended in 1 ml ice-cold HDG150 buffer (*see* **Note 10**).
7. Samples are placed on ice and sonified using a 3 mm microtip using the following parameters (Branson 250 sonifier): output control 3, duty cycle 50%, 6 pulses of 20 s, 1 min cooling between pulses (*see* **Note 7**). For agarose gel electrophoresis analysis (**step 8**), a non-sonified control is included, which is submitted to a single and brief (5 s) sonication pulse to break the nuclear membrane (Fig. 1b).
8. Optional (*see* **Note 11**): for agarose gel electrophoresis of DNA fragmentation following the sonication step, cell debris and insoluble chromatin are eliminated by centrifugation (10 min, 20,000 × *g* at 4 °C) and the supernatant (sonified nuclear lysate) is transferred to a new 1.5 ml reaction tube. An aliquot of the sonified nuclear lysate (30 µl, corresponding to 1.5×10^6 cells, adjusted to 300 µl with TE) can be processed for the analysis on DNA fragmentation on agarose gel, as described in Subheading 3.3.1, **steps 4–10**. Similarly, the pellet from the centrifugation step (insoluble nuclear fraction) can be further analyzed upon resuspension in 300 µl TE and processing for agarose gel

analysis, as described in Subheading 3.3.1, **steps 4–10**, with the following modification: at **step 9** of Subheading 3.3.1, sample is further diluted four-times in 1× loading dye (60 µl sample in 1× loading dye from Subheading 3.3.1, **step 9** + 180 µl 1× loading dye) before loading 5 µl on agarose gel (Fig. 1b).

9. Following sonication (samples from **step 7** or **8**), 3 µl of 1 M $CaCl_2$ (3 mM final) and 62.5 µl of 200 U/ml MNase (0.25 U/10^6 cells) are added to the sonified nuclear lysate.
10. MNase digestion is conducted for 60 min at 4 °C on a rotating wheel and stopped by addition of 11 µl of 0.5 M EGTA (5 mM final).
11. Optional: an aliquot of the sonified MNase-digested samples (30 µl, corresponding to 1.5×10^6 cells, adjusted to 300 µl with TE) can be processed for the analysis of DNA fragmentation on agarose gel, as described in Subheading 3.3.1, **steps 4–10**. The optimal DNA size corresponds to mono- to tri-nucleosomes (Fig. 1b).
12. The sonified MNase-digested nuclear lysate from **step 10** is transferred into a 15 ml conical tube and 3.5 ml of IP buffer is added (final volume of 4.5 ml) to adjust buffer conditions for immunoprecipitation. Samples are incubated for 20 min at 4 °C on a rotating wheel. The SDS present in the IP buffer assures a better chromatin solubilization prior to immunoprecipitation.
13. Remaining insoluble debris are eliminated by centrifugation (10 min, 3200 × *g* at 4 °C) and the supernatant (sheared nuclear lysate) is carefully transferred into a new 15 ml conical tube (*see* **Note 11**).
14. Samples at this stage can be snap-frozen in liquid nitrogen and stored at −80 °C or processed immediately for immunoprecipitation (Subheading 3.4).

3.4 Immunoprecipitation

Unless indicated otherwise, all steps are performed on ice.

3.4.1 Preparation of Protein A Sepharose Beads

1. The required amount of desiccated protein A sepharose is rehydrated in Milli-Q water (30 min at room temperature on a rotating wheel) and recovered by centrifugation (30 s, 500 × *g* at 4 °C). 1.5 g of desiccated protein A sepharose resin yields 7 ml of reconstituted beads.
2. Reconstituted beads are washed twice in PBS, equilibrated by washing twice in TE and recovered by centrifugation (30 s, 500 × *g* at 4 °C).
3. The supernatant (washing buffer) is carefully removed and 1 volume of TE containing 500 µg/ml fatty acid-free BSA, 200 µg/ml salmon sperm DNA and 0.02 % NaN_3 is added to 1 volume of beads (1:1 slurry).

4. Beads are incubated overnight at 4 °C on a rotating wheel, to coat the beads and diminish nonspecific protein and DNA binding.
5. The 1:1 bead slurry is briefly centrifuged (30 s, 500 × *g* at 4 °C) and stored at 4 °C until use (*see* **Note 12**).

3.4.2 Lysate Pre-clearing

1. To reduce background due to possible nonspecific binding of proteins and DNA to protein A sepharose beads, the sonified lysates (4.5 ml from Subheading 3.3.2, **step 6** and/or Subheading 3.3.3, **step 14**) are pre-incubated with 200 µl of protein A sepharose slurry (from Subheading 3.4.1, **step 5**) (*see* **Note 13**).
2. Samples are incubated for 1 h at 4 °C on a rotating wheel, briefly centrifuged (30 s, 500 × *g* at 4 °C), and the supernatant (pre-cleared lysate) transferred into a new 15 ml conical tube.
3. Pre-cleared lysate can be snap-frozen in liquid nitrogen and stored at −80 °C or processed immediately for immunoprecipitation (IP). 4.5 ml pre-cleared lysate is sufficient for 6 IPs (whole cell lysate, standard protocol) and 12 IPs (nuclear lysate, alternative protocol).

3.4.3 Immunoprecipitation

1. Pre-cleared lysate from Subheading 3.4.2, **step 3** is dispensed into 1.5 ml microtubes for the immunoprecipitation (IP) and some lysate is kept as input control for the ChIP quantification. From the pre-cleared whole cell lysate (standard protocol), 750 µl (equivalent to 3.3×10^6 cells) is used per IP and 50 µl for input. From the pre-cleared nuclear lysate (alternative protocol), 375 µl (equivalent to 4.2×10^6 cells) is used per IP and 25 µl is kept as input.
2. Input samples and stored at 4 °C or −20 °C until Subheading 3.5.1, **step 1**.
3. The volume of nuclear lysate is completed to 500 µl by adding 125 µl of IP buffer, to allow sufficient mixing during immunoprecipitation on the rotating wheel.
4. Antibodies are added to the 750 µl whole cell or 500 µl nuclear lysates respectively. Typically, 2.4 and 1.2 µg STAT5 antibodies (a 1:1 mixture of antibodies sc-835 and sc-1081) is used per whole cell and nuclear lysate respectively; 3 µg Brd2 antibody per both whole cell and nuclear lysate; 4 µg histone H3, 3 µg Ac-H3 and 3 µl Ac-H4 antibodies are used per whole cell lysate (*see* **Note 14**).
5. An IgG isotype control should be included (same amount as test antibody; Figures 2b and 3b). If not available, a "no antibody" control (protein A sepharose beads alone) should be included.

6. Samples are incubated for at least 3 h at 4 °C on a rotating wheel.
7. Samples are briefly spun and 30 µl of protein A sepharose slurry (from Subheading 3.4.1, **step 5**) is added (*see* **Notes 12** and **13**).
8. Immunoprecipitations are further conducted for at least another 2 h at 4 °C on a rotating wheel (*see* **Note 15**).
9. Immunocomplexes are recovered by centrifugation (30 s, 500 × *g* at 4 °C). Supernatants are discarded, unless the immunoprecipitating property of the antibody is being tested (*see* **Note 14**).
10. Beads are washed successively with 900 µl of the following buffers: IP buffer (short), 150 mM NaCl buffer (short), 500 mM NaCl buffer (5 min at 4 °C on a rotating wheel), LiCl Wash Buffer (5 min at 4 °C on a rotating wheel), TE (short). Buffer is dispensed into the tubes and samples are mixed by tube inversion, not by pipetting (*see* **Note 16**). Beads are recovered by centrifugation as before (30 s, 500 × *g* at 4 °C) (*see* **Note 17**).

3.5 DNA Isolation

3.5.1 Cross-Link Reversal

1. After complete removal of the last TE wash (Subheading 3.4.3, **step 10**), 120 µl of cross-link reversal buffer (1 % SDS, 0.1 M $NaHCO_3$) is added to the beads. Input samples from Subheading 3.4.3, **step 2** are now processed in parallel to the IP samples, and 70 or 95 µl of cross-link reversal buffer is added to the input samples from the standard or alternative protocol respectively (120 µl final volume) (*see* **Note 16**).
2. 6 µl of 5 M NaCl (250 mM final) is added to all samples to avoid melting of low GC content DNA fragments during cross-link reversal at 65 °C.
3. Bead samples are mixed gently. Beads should remain immersed in buffer (*see* **Note 16**).
4. Samples are incubated overnight at 65 °C to reverse cross-link and to elute proteins from beads.
5. Samples are mixed gently and briefly centrifuged (30 s, 500 × *g* at 4 °C). Supernatants from the IP samples are transferred into a new 1.5 ml tube and the beads discarded. Input samples are left in their tube.

3.5.2 DNA Purification

1. RNAs are eliminated by adding 1 µl of 30 mg/ml RNase A (0.25 mg/ml final) to all samples and incubating 1–2 h at 37 °C.
2. Proteins are degraded by adding 2.5 µl of 20 mg/ml Proteinase K (0.4 mg/ml final) to all samples and incubating 1–2 h at 55 °C.
3. Co-immunoprecipitated genomic DNA fragments are isolated on a DNA purification resin compatible with SDS-containing buffers (*see* **Note 18**). We use Macherey-Nagel's Nucleospin

DNA clean-up kit with NTB Binding Buffer, as follows: 600 µl NTB Binding Buffer is added to the 120 µl IP and input samples from **step 2**. Samples are loaded on the columns, centrifuged and washed as recommended in the kit's manual. After the last wash, columns are thoroughly dried (2 min spin followed by 2–5 min air-drying, lid open) before two rounds of elution with 50 µl each of pre-warmed (70 °C) elution buffer are performed. Columns are incubated 2–5 min at room temperature before proceeding to centrifugation and recovery of eluted DNA into new 1.5 ml collection tubes, as recommended by the manufacturer.

4. The recovered eluates (100 µl) are diluted in Milli-Q water. 200 µl water is added to IP samples and 800 µl water is added to input samples. Samples are stored at −20 °C until analyzed by quantitative PCR.

3.6 DNA Analysis by Quantitative PCR

1. Quantitative PCR reactions are performed in triplicate in 20 µl final volume, using 5 µl template DNA (input and immunoprecipitated) and 400 nM final of each specific primer (*see* **Note 3**).
2. Quantitative PCR data are expressed as percentage of input DNA (% input DNA) using the following formula:
$2^{(CT^{input} - CT^{IP})} \times R \times 100$
where CT is the threshold value from the qPCR analysis and R is the ratio of input to IP sample. In our assay, $R = 0.0222$ (*see* **Note 19**).
3. Following STAT5, Brd2 and histone H3 ChIPs, which assess protein occupancy at a specific locus, data are expressed as % input DNA (Figs. 2b and 3a, b). In the case of histone H3 and H4 acetylation (Ac-H3 and Ac-H4 respectively), the % input DNA values are normalized to histone (H3) occupancy (Ac-H3/H3 and Ac-H4/H3 respectively), to more accurately evaluate changes in histone acetylation levels (Fig. 3a).

4 Notes

1. Sodium azide (NaN_3) is extremely toxic and poisonous when in contact with skin or swallowed, and thus should be handled cautiously. It is recommended to wear gloves during the ChIP procedure.
2. For the sonication of cross-linked cells, a microtip of 3 mm and 5 mm in diameter is recommended for sample volumes ≤1 ml and ≥1 ml respectively.
3. qPCR primer pairs should be designed to generate small amplicons (50–80 bp in length). Primers should be tested for their amplification efficiency (as close to 100% as possible, to allow

comparison of distinct loci) by quantitative PCR, using 20 ng of intact genomic DNA isolated from the same cells as the ones used for ChIP (Subheading 3.6). Moreover, when analyzing a particular locus (for instance STAT5 binding site of the *Cis* gene), a control primer amplifying a remote region along the same gene or/and at a different gene locus should be included in the analysis to confirm specificity (Fig. 2a, b).

4. The conditions of formaldehyde treatment—in particular its duration (10 min) and the temperature at which it is being conducted (room temperature)—are critical for the efficiency and reproducibility of the cross-link, and thus of the chromatin immunoprecipitation outcome.
5. When a kinetic of cell treatment is performed, cells are instead transferred at the desired time points from the same cell culture T-flask into formaldehyde-containing 50 ml conical tubes (prepared under the fume hood).
6. In case adherent cells are used, formaldehyde-induced cross-link is performed directly in the petri dish, at room temperature, under a fume hood and under gentle shaking.
7. Sonication efficiency depends on both the cell type and sonifier used. Optimal shearing of chromatin of Ba/F3 cells on a Branson 450 sonifier (instead of Branson 250 sonifier, as described in this protocol) was achieved using the following parameters: output control 4, duty cycle constant, one pulse of 20 s; 3 mm microtip). On the other hand, sonication of a mouse adherent epithelial cell line using Branson 250 sonifier was performed as follows: output control 3, duty cycle 25%, three pulses of 30 s, 1 min cooling between pulses; 3 mm microtip. In addition, sonication efficiency is influenced by the buffer composition, in particular its SDS content and concentration. It is best illustrated in Fig. 1, where sonication of nuclei in the absence of SDS (HDG150 buffer) does not result in significant chromatin fragmentation (Fig. 1B, lane 4), while sonication of nuclei in SDS^+ buffer using the same sonication parameters (Fig. 2b) efficiently sheared chromatin (not shown).
8. The purified gDNA pellet should not be overdried, for two reasons: (1) it becomes more difficult to resuspend and (2) it might detach from the bottom of the reaction tube and thus possibly get lost upon tube opening.
9. No more than the DNA amount equivalent to $0.1–0.2 \times 10^6$ cells (i.e., 660 ng–1.2 μg, assuming a 100% recovery) should be loaded on the agarose gel. Overloading interferes with proper DNA electrophoretic migration and thus prevents accurate interpretation of the sonication efficiency.
10. The quality of the nuclear fractionation can be verified by Western blot using antibodies specific for the cytosolic and

nuclear proteins α-tubulin and HDAC1 respectively [3]. In that case, the cytosolic fraction (supernatant from **step 4**) is not eliminated but transferred into a new 1.5 ml reaction tube. The protein amount equivalent to 5×10^5 cells, i.e., 10 μl of the cytosolic fraction (**step 4**) and 10 μl of the nucleus suspension (in HDG150 buffer, **step 6**), is separated by electrophoresis on a 10% SDS-polyacrylamide gel (SDS-PAGE), transferred to PVDF membrane and subjected to Western blot analysis [10, 14].

11. The centrifugation in **step 8** can be omitted in case soluble and insoluble chromatin fractions are not analyzed by agarose gel electrophoresis, and instead directly subjected to MNase treatment (**step 9**). In that case, remaining cell debris and insoluble chromatin should be eliminated by centrifugation at **step 13**.
12. Rabbit polyclonal antibodies are preferred to mouse monoclonal antibodies, as epitopes can be masked as a result of formaldehyde cross-link, making monoclonal not as powerful in ChIP assays as polyclonal antibodies. Therefore, protein A sepharose, which binds all rabbit IgG isotypes with a strong affinity, is usually chosen [14]. In case mouse antibodies are used, attention should be drawn to the IgG isotype before opting for protein A sepharose, as mouse IgG1 and IgG3 bind poorly to protein A [14]. In such case, protein G sepharose should be chosen. Some companies also offer mixed protein A/G sepharose beads, which can be used with any type of IgG in ChIP assays. In addition, the amount of protein A sepharose used in ChIP should be determined based on the amount of antibody used. Protein A sepharose has an IgG binding capacity of 20 mg IgG/ml beads. The use of 30 μl protein A slurry for 2.4 μg STAT5 antibody (Subheading 3.4.3, **step 4**) represents a 125-fold excess protein A sepharose in term of binding capacity.
13. To prevent damaging the beads, the protein A sepharose slurry should not be vortexed. To allow homogeneous mixing and pipetting of the protein A sepharose slurry, we recommend to cut the extremity of the 200 μl pipette tips.
14. Unless validated (so called ChIP-grade) antibodies are used, antibody validation and optimization should be conducted prior to the ChIP assay. An antibody that works in conventional immunoprecipitation might not function in ChIP, either due to the high SDS concentration in the IP buffer or/and due to formaldehyde-induced epitope masking. For antibody optimization, sonified lysates from formaldehyde-cross-linked cells are used in an immunoprecipitation assay using increasing amounts of antibody, following the protocol described here (Subheadings 3.1–3.4) until **step 10** of Subheading 3.4.3. In that case, immunoprecipitation supernatants from Subheading 3.4.3, **step 9** are recovered in a new 1.5 ml tube.

Washed beads from Subheading 3.4.3, **step 10** are resuspended in 50 µl 2× Laemmli sample buffer (62.5 mM Tris–HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol), boiled 10 min to reverse cross-link and denature proteins, and 20 µl is analyzed by SDS-PAGE and Western blot [14]. An equivalent amount of input (pre-cleared lysates from Subheading 3.4.2, **step 3**) and IP supernatant (Subheading 3.4.3, **step 9**) is boiled in Laemmli sample buffer and analyzed by Western blot in parallel. The comparison of the Western blot signal in input, IP supernatant and beads will identify the optimal antibody amount (i.e., yielding the weakest signal in IP supernatant and the strongest in bead fraction).

15. When weak signals are expected, for instance when immunoprecipitating factors indirectly bound to DNA (e.g., Brd2), incubation in the presence of antibody and protein A sepharose can be conducted overnight (at 4 °C on a rotating wheel).
16. Protein A sepharose beads should be washed neither by vortexing (*see* **Note 13**) nor by pipetting which might result in beads sticking to the plastic wall of the pipette tips and be lost. Washing steps should be performed by tube inversion (Subheading 3.4.3, **step 10**). However, mixing small volume (cross-link reversal, Subheading 3.5.1) should be achieved by gentle mixing of the tube with the finger tip. Care should be taken that beads do not stick along the plastic tube wall and dry. In case beads are not immersed in liquid and stick to the tube wall, samples should be spun for a few seconds and beads gently resuspended again.
17. Washing buffers should be eliminated as completely as possible between each wash step but without aspirating beads. Alternatively, two washes of 600 µl can be performed per buffer, in which case ~50 µl buffer from the previous step can remain. At the last washing step, TE should be removed as completely as possible.
18. In case no DNA purification kit is available, genomic DNA can be purified and isolated by phenol–chloroform extraction and ethanol precipitation, as follows. Subsequent to the last bead wash (Subheading 3.4.3, **step 10**), 250 µl of cross-link reversal buffer (1% SDS, 0.1 M $NaHCO_3$) and 250 µl of 2× proteinase K digestion buffer (Subheading 2.3.1) are added to the beads and to the input samples (from Subheading 3.4.3, **step 2**). Samples are incubated for 2 h at 55 °C, then overnight at 65 °C. Beads are centrifuged (30 s, 500 × *g* at 4 °C), supernatant is transferred into a new 1.5 ml tube, 50 µl of 4 M LiCl and 500 µl phenol–chloroform–isoamyl alcohol are added and DNA is isolated by phenol–chloroform extraction and ethanol precipitation, as described in Subheading 3.3.1, **steps 5**–7. Input DNA is resuspended in 50 µl TE containing 0.25 mg/ml RNase A and incubated 30 min at 37 °C; final volume is

adjusted to 900 µl with Milli-Q water. Immunoprecipitated DNA is resuspended in 283 µl Milli-Q water + 17 µl TE (300 µl final) to adjust TE concentration in all samples. Samples are stored at −20 °C until analyzed by quantitative PCR.

19. Calculation of ratio (R) of input to immunoprecipitated sample in our assay:
    Whole cell lysate (standard protocol): $R = (5/900 \times 50)/(5/300 \times 750) = 0.0222$
    Nuclear lysate (alternative protocol): $R = (5/900 \times 25)/(5/300 \times 375) = 0.0222$

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Chapter 20

Modulation of STAT1-Driven Transcriptional Activity by Histone Deacetylases

Benjamin Y. Owusu and Lidija Klampfer

Abstract

The luciferase (LUC) reporter assay is commonly used to study gene expression at the transcriptional level. It is convenient, fast, sensitive, inexpensive, and provides quantitative data about small changes in transcription. Signal transducer and activator of transcription 1 (STAT1) is a transcription factor that plays a crucial role in signaling by interferons (IFNs). Here, we describe LUC reporter studies that address the role of histone deacetylase (HDAC) activity in STAT1-dependent gene activation. These experiments include overexpression of HDAC1, HDAC2, HDAC3, and HDAC4 as well as silencing of HDAC1, HDAC2, and HDAC3 through RNA interference in mammalian cancer cells.

Key words STAT1, HDAC, HDAC inhibitors, Interferons, Luciferase gene reporter

1 Introduction

Transcription factors are proteins that display sequence-specific DNA binding activity and thereby regulate the rate of transcription of the target genes. Their DNA binding activity is modulated by alterations in the extracellular environment, such as the levels of cytokines and growth factors. STAT1 is a member of the family of signal transducers and activators of transcription which activates gene expression in response to stimulation of cells with interferons (IFNs) [1, 2]. Binding of IFN gamma (IFNγ) to cell surface receptors initiates signaling through transphosphorylation and activation of the Janus kinases, JAK1/JAK2, leading to tyrosine phosphorylation of STAT1 [1]. The JAK1/JAK2-mediated tyrosine phosphorylation of STAT1 is required for STAT1 dimerization via interactions between the phosphorylated tyrosine residue and the SH2 domain of STAT1, its nuclear translocation, DNA binding, and transcriptional activation [3]. In addition, serine phosphorylation at S727 is required for the full transcriptional activity of STAT1 [4], but much less is known about the kinase

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responsible for serine phosphorylation of STAT1. Recently, cyclin-dependent kinase 8 (CDK8) has been shown to phosphorylate serine 727 in the transactivation domain of STAT1 [5].

In response to IFNγ stimulation, STAT1 forms homodimers or heterodimers with STAT3 that bind to the Interferon-Gamma-Activated Sequence (GAS) promoter element [6]. Treatment of cells with IFNα or IFNβ triggers the formation of STAT1/STAT2 or STAT1/STAT3 heterodimers that recognize the Interferon-Stimulated Response Element (ISRE) promoter element [7–9].

We have reported that pan-HDAC inhibitors, such as butyrate, trichostatin A (TSA), and suberanilohydroxamic acid (SAHA), impede tyrosine and serine phosphorylation of STAT1, and inhibit its nuclear translocation and DNA binding activity [10, 11]. Consistent with our findings, induction of interferon-stimulated genes and antiviral activity of interferon have been shown to require HDAC activity [12–15].

Here, we describe experiments that demonstrate that HDAC activity is essential for STAT1-dependent transcriptional activity.

2 Materials

2.1 Cell Culture

1. HCT116 and HKe-3 colon cancer cells (*see* **Note 1**).
2. Sterile phosphate buffered saline (PBS).
3. Trypsin: 0.5% trypsin in PBS.
4. Minimum Essential Medium (MEM), supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and penicillin (100 I.U./ml), streptomycin (100 μg/ml) and 0.25 mg/ml of Fungizone (antymicotic).
5. Cell culture incubator (5% CO_2, 37 °C, humidified atmosphere).

2.2 Plasmid DNA for Transfection and siRNAs

1. The reporter plasmid 8XGAS-p36LUC contains eight gamma-activated sequence (GAS) STAT1 binding sites cloned upstream of the LUC reporter gene [16].
2. Expression vectors: pBJ5-FLAG-HDAC1 [17], pME18S-FLAG-HDAC2 [18], pCMV-FLAG-HDAC3 [19], pcDNA3-HDAC4 [20]. All plasmids are diluted in DNase/RNase-free water (*see* **Note 2**).
3. siRNAs specific for HDAC1 (# 003493), HDAC2 (#003495), or HDAC3 (#003496) are purchased (Dharmacon).

2.3 Profection Mammalian Transfection System (Promega, Madison, WI)

1. Nuclease-free water.
2. 2 M $CaCl_2$.
3. 2× HEPES-Buffered Saline (HBS): 280 nM NaCl, 1.5 mM Na_2HPO_4, 50 mM Hepes. Adjust pH to 7 with 1 N HCl (*see* **Note 3**).

2.4 Dual Firefly-Renilla Luciferase Reporter Assay Kit (Promega, Madison, WI)

1. 5× Passive Lysis Buffer (PLB).
2. Luciferase Assay Substrate (LAR II) (lyophilized).
3. Luciferase assay buffer II.
4. 50× Stop&Glo Substrate.
5. Stop&Glo buffer (*see* **Note 4**).

3 Methods

3.1 Transfection of Colon Cancer Cells with 8XGAS-36 LUC Reporter

1. Cells are plated a day before transfection and must be seeded at a density that they will reach approximately 50–60% confluence on the day of transfection. Seed 1×10^5 HCT116 or HKe-3 cells per well of a 12-well plate.
2. Replace the medium of cells to be transfected with fresh growth medium 3–4 h prior to transfection.
3. Thaw all transfection reagents and warm them to room temperature under sterile conditions.
4. Prepare two separate sterile tubes for each transfection. To the first tube, add water and DNA (*see* Table 1), mix by pipetting up and down and then gently add the $CaCl_2$ in a dropwise manner and mix again. To the second tube, add the 2× HBS as specified in Table 1 (*see* **Note 5**).
5. Gently bubble air with a pipette through the 2 X HBS solution and slowly add the DNA/$CaCl_2$ solution in a dropwise manner. The solution should appear slightly opaque at this stage due to the formation of DNA-Calcium Phosphate co-precipitate.
6. Incubate the transfection solution at room temperature for about 15–20 min (*see* **Note 6**).
7. Mix or vortex the transfection solution before adding to the cells. Add 100 µl of the solution dropwise to the cells in a

Table 1
Preparation of DNA complexes for transfection in 12-well or 6-well plates

	Per well of a 12-well plate	Per well of a 6-well plate
Tube 1		
8XGAS-LUC TK renilla	1 µg 0.1 µg	3 µg 0.3 µg
2 M $CaCl_2$	6 µl	18 µl
Nuclease-free water	Add to a total volume of 50 µl	Add to a total volume of 150 µl
Tube 2		
2 X HBS	50 µl	150 µl

12-well plate (or 300 µl to cells cultured in 6-well plates) and swirl the plate to evenly distribute the precipitate over the cells. Incubate the plates at 37 °C in the 5% CO_2 incubator. Avoid forceful dispensing of transfection complexes in the wells because this may cause damage to the cell monolayer.

8. After incubation overnight, rinse the plates twice with 1× PBS, add fresh growth medium and return cells to the 5% CO_2 incubator.
9. Upon changing the medium, let cells recover for 4–6 h before treatment with modulators of cell growth, such as interferons and HDAC inhibitors. Measure luciferase activity 24 h after treatment.
10. For co-transfection experiments transfect cells with 8XGAS-LUC (described above) together with expression plasmids for HDAC1, HDAC2, HDAC3, or HDAC4. We tested four different concentrations of expression plasmids (0.05, 0.25, 0.5, and 0.75 µg) (Fig. 1). Adjust the amount of total DNA with an empty pCMV-neo plasmid, so that each tube has a total of 1.85 µg of DNA for a 12-well plate transfection.

3.2 Gene Silencing Using siRNA

1. Plate cells a day before transfection at a density that they will reach approximately 50–60% confluence on the day of transfection. Seed 1×10^5 of HCT116 or Hke-3 colon cancer cells per well of a 12 well plate.

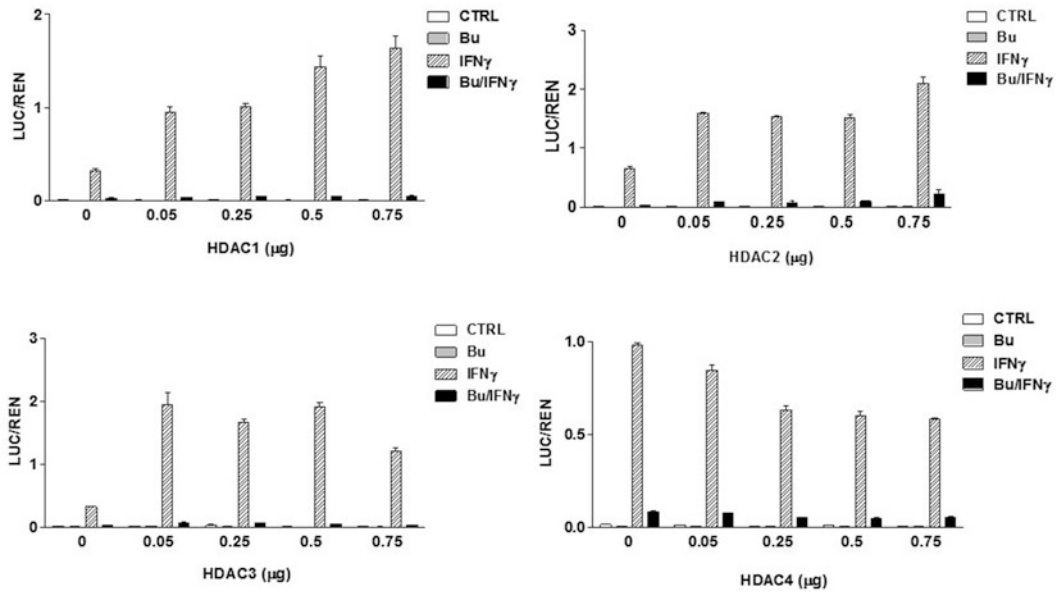

Fig. 1 HDAC1, HDAC2, and HDAC3 support STAT1-dependent transcription. HKe-3 cells were transiently transfected with 8XGAS-LUC reporter gene alone or together with increasing amount of plasmids coding for HDAC1, HDAC2, HDAC3, or HDAC4 as indicated. Cells were left untreated or were treated with interferon gamma (IFNγ) or HDAC inhibitor butyrate (Bu) for 24 h. The results are expressed as the ratio between LUC and Renilla (LUC/REN). Reprinted with permission from The Journal of Biological Chemistry [11]

2. Replace the medium of the cells to be transfected with a fresh growth medium at about 3–4 h prior to transfection.
3. Thaw all transfection reagents and warm to room temperature under sterile conditions.
4. Prepare two separate sterile tubes for each transfection. To the first tube, add the appropriate amount of water and siRNA to reach a final concentration of 50 nM. Mix the solution by pipetting up and down and then gently add the $CaCl_2$ in a dropwise manner and mix again. To the second tube, add the 2 X HBS as specified in Table 2.
5. Mix the transfection solution before adding to the cells. Add 100 µl (per well of 12-well plate) or 300 µl (per well of 6-well plate) of the solution dropwise to the cells and swirl the plate to distribute the precipitate evenly over the cells. Incubate the plates at 37 °C in the 5% CO_2 incubator.
6. After incubation overnight, rinse the plate twice with 1× PBS, add fresh growth medium and return the cultures to the 5% CO_2 incubator.
7. Leave the cells to recover for 3–4 h before treatment with IFNγ. Measure the LUC activity 24 h after treatment (Fig. 2b) (*see* **Note 7**).

3.3 Dual Luciferase Reporter Assay

1. Prepare 1× passive lysis buffer (PLB) by adding 1 volume of 5× PLB to 4 volumes of distilled water.
2. Prepare luciferase assay substrate (LARII) by resuspending the lyophilized luciferase assay substrate in 10 ml of luciferase buffer. Aliquot in 1.5 ml Eppendorf tubes and store at −80 °C.
3. Prepare 1× Stop&Glo reagent by adding 20 µl of 50× Stop&Glo substrate to 980 µl of Stop&Glo buffer and store at −80 °C.

Table 2
Preparation of HDAC-specific siRNAs for transfection

	Per well of a 12-well plate	Per well of a 6-well plate
Tube 1		
siRNA (NSP, HDAC1, HDAC2, HDAC3, HDAC4)	1 µl	3 µl
2 M $CaCl_2$	6 µl	18 µl
Nuclease-free water	Add to a total volume of 50 µl	Add to a total volume of 150 µl
Tube 2		
2 X HBS	50 µl	150 µl

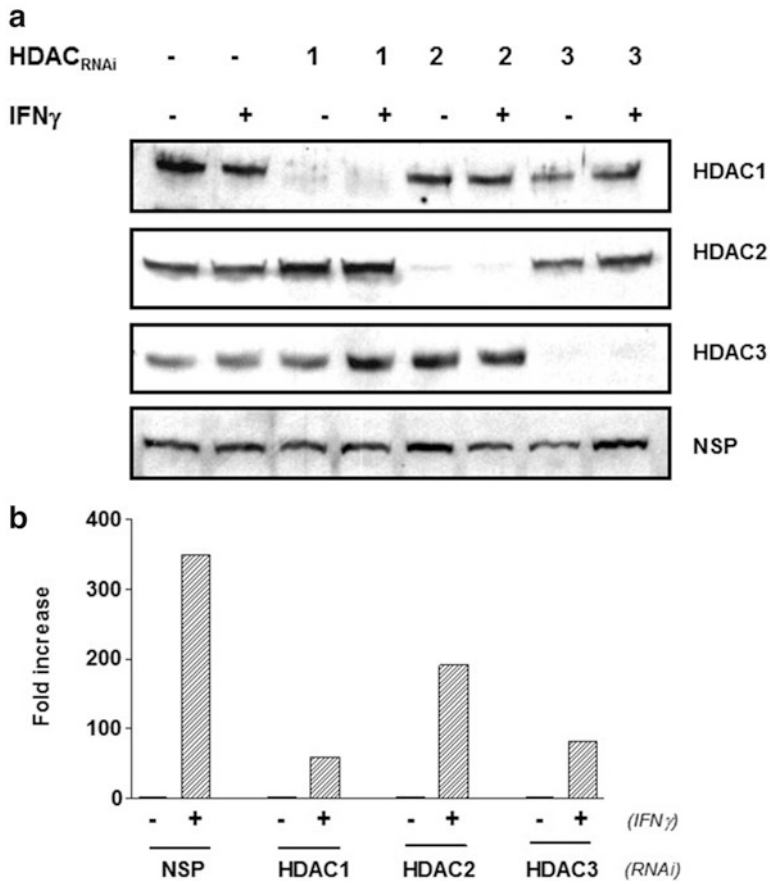

Fig. 2 Silencing of HDAC1, HDAC2, and HDAC3 inhibits IFN-driven STAT1 transcription. (**a**) Hke-3 cells were transfected with nonspecific siRNA (NSP) or siRNAS specific for HDAC1, HDAC2, or HDAC3. Cells were either left untreated or were treated with IFNγ as indicated. (**b**) Hke-3 cells were transfected with 8XGAS-LUC reporter plasmid alone or together with siRNA as indicated. 24 h after transfection, the cells were left untreated (–) or were treated with IFNγ as indicated. Reprinted with permission from The Journal of Biological Chemistry [11]

4. Take out cultured cells from the 5 % CO_2 incubator and remove growth medium from the cells.
5. Rinse cells twice with 1× PBS and remove all PBS solution after rinsing.
6. Add sufficient amount of 1× PLB to cover the cell monolayer. The recommended volumes of 1× PLB per well are shown in Table 3.
7. Gently shake or rock the cell culture plate or dish for 15–30 min at room temperature to achieve complete lysis.
8. Transfer cell lysates into clean tubes, use them immediately or store the lysates at –80 °C. Subjecting cell lysates to more than 2–3 freeze–thaw cycles will result in gradual loss of luciferase reporter enzyme activities.

Table 3
Cell lysis for the dual luciferase reporter assay

	6-Well plate	12-Well plate	24-Well plate	48-Well plate
Volume of 1× PLB (μl)	500	250	100	65

9. Dispense 100 μl of LARII into the appropriate tubes. Use of a 96-well plate is recommended for multiple samples and replicates.
10. Add 20 μl of cell lysate per tube and gently mix by pipetting up and down 2 or 3 times.
11. Place the tube or plate in the luminometer and measure Firefly luciferase (LUC) activity.
12. Take the tube or plate out and add 100 μl of 1× Stop&Glo Reagent.
13. Place the tube or plate in the luminometer and measure Renilla (REN) luciferase activity.
14. The results are expressed as the ratio of Firefly to Renilla luciferase activities (LUC/REN).

4 Notes

1. Use only cells with >90% viability for transfection experiments. The viability can be monitored by staining cells with 0.4% solution of Trypan blue. The test is performed while counting cells with the hemocytometer. The dye exclusion test is based upon the concept that viable cells do not take up Trypan blue, but dead cells are permeable for the dye. Use cells that were not seeded under sparse conditions or were overgrown, as this changes their growth characteristics and transfection efficiency.
2. The plasmid DNA used for transfection should be free of RNA and protein. The purity of the DNA solution can be assessed by determining the ratio of the absorbance at 260–280 nm (A_{260}/A_{280}). A DNA solution with A_{260}/A_{280} ratio of at least 1.8 is required for transfection. Qiagen maxi kit for plasmid isolation yields DNA of good quality that is suitable for transfection.
3. Transfection protocols need to be optimized for each cell line. It is important to choose the right transfection reagent and establish optimal conditions for transfection. For example, the calcium-phosphate transfection protocol described here works very well for a panel of adherent cancer cells and some primary cells, but it is not suitable for the transfection of cells that grow in suspension.

4. The composition of the buffers in the kit has not been disclosed by the company. A similar system that allows for simultaneous detection of Firefly and Renilla luciferases is available at Thermo Scientific Pierce (#16185). Upon receipt, store the Dual-Luciferase Reporter Assay System at –20 °C. Once the Luciferase Assay Substrate has been reconstituted, it should be divided into working aliquots of 1 ml and stored at –20 °C for up to 1 month or at –80 °C for up to 1 year. Stop&Glo reagent should be stored at –80 °C.
5. For a dual luciferase reporter assay, transfection efficiency is normalized by simultaneously transfecting cells with a second reporter plasmid, TK-Renilla, which provides an internal control. This ensures that experimental variability as a result of differences in cell viability or transfection efficiency is minimized. The amount of TK-Renilla added to the transfection mixture in tube 1 is usually about 1/10th of the gene reporter plasmid (Table 1). Firefly and Renilla luciferases have distinct enzyme structures and substrate requirements. Thus, using the dual luciferase reporter assay system, the luminescence from the Firefly luciferase reaction is quenched while simultaneously activating the luminescent reaction of Renilla luciferase.
6. Transfection complexes can be added to cells cultured in media containing antibiotics and serum without impacting cell viability or transfection efficiency.
7. It is important to confirm the efficiency of silencing by monitoring the expression of the target genes at the mRNA or protein levels. We determined the levels of HDAC1, HDAC2, and HDAC3 in our experiments by immunoblotting (Fig. 2a).

Acknowledgments

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Chapter 21

Proximity Ligation Assay to Quantify Foxp3 Acetylation in Regulatory T Cells

Jing Jiao, Rongxiang Han, Wayne W. Hancock, and Ulf H. Beier

Abstract

Determining protein acetylation by immunoprecipitation and immunoblotting can be challenging, especially if the tissue of interest is low in quantity, and when good quality acetylation site-specific antibodies are not available. Proximity ligation assays allow a sensitive and quantitative method to assess Foxp3 acetylation in regulatory T cells, with as little as 1.5×10^5 cells within two days turnaround time. This method is of potential use in other similar scenarios, when post-translational modifications of a protein of interest need to be determined with only a small amount of sample and in the absence of specific antibodies that can assess the post-translational modification in the protein of interest.

Key words Protein acetylation, Immunopreciptation, Immunoblot, Proximity ligation assay

1 Introduction

In order to study post-translational modifications of proteins, either immunoblotting or a combination of immunoprecipitation and immunoblotting are considered as standard experimental procedures. These techniques are common and practical when quality antibodies are available and the protein of interest is abundant. However, in the absence of either, post-translational modifications can be much harder to assess. The discovery that inhibition of several histone/protein deacetylases (HDACs) favors the generation and suppressive function of regulatory T cells (Tregs) by increasing the abundance and acetylation of Forkhead box P3 (Foxp3) led to substantial interest in Foxp3 acetylation [1]. Foxp3 is a key transcription factor of Tregs and essential to their suppressive function [2]. A study by van Loosdregt et al. reported that p300 promotes, and Sirtuin-1 reduces Foxp3 acetylation, utilizing tagged Foxp3 vector-transfected HEK 293 cells [3]. These studies allowed important insights into Foxp3 biology, suggesting that blocking deacetylation by Sirtuin-1 increases Foxp3 acetylation and protein level. To investigate the role of Sirtuin-1 in vivo, we had mated floxed-Sirtuin-1 mice with CD4cre and Foxp3cre mice, observed increase in

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Foxp3 protein expression and improved Treg function [4]. However, measuring Foxp3 acetylation in Tregs isolated from $Sirt1^{fl/fl}CD4^{cre}$ or $Sirt1^{fl/fl}Foxp3^{cre}$ mice proved to be methodologically difficult due to the limited amount of Tregs, especially in the absence of acetylation site-specific antibodies at that time [5]. This prompted a search for alternative methods to assess protein acetylation.

Proximity ligation assay is a technique developed by Landegren et al from the University of Uppsala, Sweden [6]. It allows detection of trace amounts of protein in situ by a localized signal amplification, which triggers if two different types of secondary antibodies are close enough together [6]. The reaction has been used to detect protein bound to DNA [6], protein–protein interactions [7], as well as post-translational modifications such as tyrosine phosphorylation [8]. With regards to Foxp3, van Loosdregt et al used this method to document interactions of Sirtuin-1 and Foxp3 [9]. We have adapted this method to determine Foxp3 acetylation in Tregs isolated from $HDAC6^{KO}$, $HDAC9^{KO}$, and $Sirt1^{fl/fl}CD4^{cre}$ mice [10]. In our approach, we used primary antibodies of different species against acetyl-lysine residues and against Foxp3, and combined them with matching secondary antibodies (Fig. 1a). The species-specific secondary antibodies each carry a DNA stand, which if in close proximity to each other form a joint DNA segment that can be amplified using a polymerase chain reaction. The replicated DNA can be detected by fluorescence-labeled complementary oligonucleotides, which will create fluorescence in the close proximity of Foxp3 and acetyl-lysine antibodies (Fig. 1a). Thus, proximity ligation assay is an alternative procedure that is feasible and sensitive to assess protein acetylation.

2 Materials

2.1 Cell Isolation, Cytospin

1. Spleens and lymph nodes from C57BL/6 and $HDAC6^{KO}$, $HDAC9^{KO}$ and $Sirt1^{fl/fl}CD4^{cre}$ mice.
2. Magnetic beads (catalog #130-091-04, Miltenyi Biotec) to process tissue into single-cell suspensions and to separate conventional T cells ($CD4^+CD25^-$) and Tregs ($CD4^+CD25^-$). Other modalities of purifying Tregs can be used, as well.
3. Shandon™ Double Cytoslides™ (catalog #5991054, Thermo Scientific).
4. Shandon Cytospin 3 centrifuge (Thermo Scientific). Other centrifuge and cytospin slide products with comparable parameters may be used as well.
5. Phosphate buffered saline with 2% fetal bovine serum (pH 7.4).

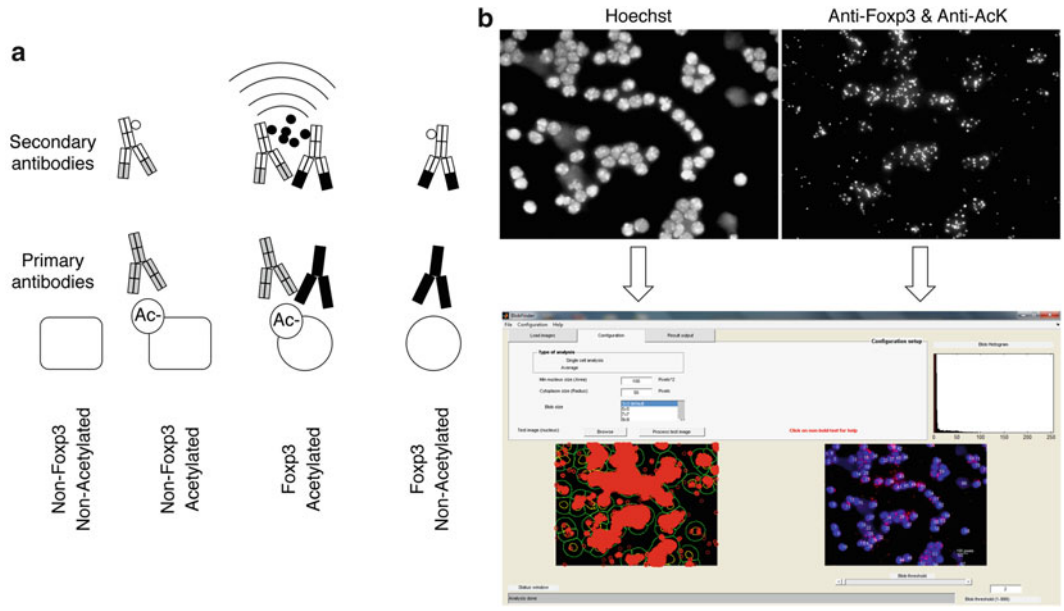

Fig. 1 Proximity ligation assay to measure Foxp3 acetylation in Tregs. (**a**) Experimental design: Regulatory T cells are fixated and permeabilized on a cytospin slide and incubated with primary of different species antibodies against Foxp3 and acetylated lysine. Subsequently, secondary antibodies specific to the species of the primary antibodies are applied. Each of the two different secondary antibodies have a short specific DNA strand attached. If two different secondary antibodies are in close proximity to each other, their DNA oligonucleotides undergo a reaction that forms a circular DNA oligonucleotide, which can then be amplified using a polymerase. Subsequently, the local accumulation of the DNA oligonucleotide can be detected using fluorescent probes, which will be detectible only in the presence of two opposite secondary antibodies. Therefore, fluorescence represents Foxp3 protein that is acetylated. (**b**) Examples of immunofluorescence images from cytospin samples of Treg demonstrating Hoechst DNA staining (*left*) and the DNA oligonucleotide fluorescence reaction (*right*, each at 100× magnification). The DNA staining is used to define nuclei, and overlaid with the proximity ligation assay images derived from Anti-Foxp3 and Anti-Acetyl-lysine (AcK) co-localizing antibodies, and analyzed using BlobFinder software (screenshot). Overlay of DNA staining and the oligonucleotide fluorescence allows assessment of nuclear acetylated Foxp3

2.2 Antibodies and Reagents

1. Antibodies against acetyl-lysine and Foxp3 were chosen to be matching mouse and rabbit-based antibodies (i.e. Foxp3 mouse and acetyl-lysine rabbit). We purchased mouse monoclonal anti-Foxp3 (catalog #14-7979-80, eBioscience) and rabbit anti-acetylated-lysine antibody (catalog #9441, Cell signaling).
2. Double-stranded DNA was visualized with Hoechst 33342 (Invitrogen). We used a Duolink® in situ proximity ligation assay kit "Orange" (Olink Bioscience, Uppsala, Sweden), with corresponding secondary antibodies (PLUS-rabbit, MINUS-mouse), according to the manufacturer's instructions.
3. Normal goat serum to be used as blocking solution.
4. Triton X-100 was used for permeabilization (Sigma Aldrich or other manufactures).
5. Periodate-lysine-paraformaldehyde (PLP) for fixation, which can be purchased or mixed as reported [11].

2.3 Microscope and Image Analysis Software

1. Images were obtained with an Olympus BX51 Brightfield microscope (Olympus) at 100× magnifications.
2. For image analysis and processing, we used ImageJ version 1.45 s, available at the National Institutes of Health, http://rsbweb.nih.gov/ij/ [12] as well as BlobFinder by Allalou and Wählby (Centre for Image Analysis, Uppsala, Sweden, http://www.cb.uu.se/~amin/BlobFinder/) [13]. Both image analysis applications are in the public domain.

3 Methods

3.1 Cytospin

1. Assemble purified regulatory T cells (e.g., $CD4^+CD25^+$ using Miltenyi magnetic columns) and suspend the cells in phosphate buffered saline (PBS) with 2% fetal bovine serum at 1×10^6 cells per ml (*see* **Note 1**).
2. Add the cells to the cytospin slide. For a double cytoslide, we use 150–200 µl of the cell solution per sample.
3. Centrifuge at ~$112.9 \times g \times 5$ min. The g force was derived from converting the centrifuge's rounds per minute value (1000). A similar setting on a different centrifuge is likely to produce comparable results.
4. After centrifugation, allow the cells to dry for ~2 h at room temperature. Slides not to be immediately used can be wrapped in aluminum foil and stored at –80 °C for later use (*see* **Note 2**).

3.2 Blocking and Primary Antibodies

1. To achieve fixation, apply periodate-lysine-paraformaldehyde (PLP) solution and incubate for 5 min at 4 °C.
2. Wash the slides with PBS for 5 min at room temperature.
3. Pre-treat samples with a blocking solution composed of 10% normal goat serum and 0.2% Triton X-100 for 1 h at room temperature.
4. During incubation with the blocking solution, prepare primary antibodies in 1% normal goat serum with 0.2% Triton X-100. Plan to use 80 µl final volume per sample (*see* **Notes 3** and **4**).
5. In addition, add Hoechst 33342 dye to the primary antibodies at a final concentration of 1 µg/ml (*see* **Note 5**).
6. Tap off the pre-treatment solution. Draw a relatively tight circle around the sample using a liquid blocker pen. This aids in orientation and allows preserving antibodies and reagents.
7. After tapping off the pre-treatment solution, apply 80 µl primary antibody solution to each sample. Now cover the samples in a dark humidity chamber, and incubate the samples overnight at 4 °C (*see* **Note 6**).

3.3 Proximity Ligation Assay Probes (Secondary Antibodies)

1. On the next day, mix the two proximity ligation assay probes at 1:5 in 1% normal goat serum with 0.2% Triton X-100, and incubate the solution for 20 min at room temperature.
2. Tap off the primary antibody solution.
3. Wash the cells with 1% normal goat serum with 0.2% Triton X-100. Washing should be performed in a staining jar, with a minimum volume of 70 ml on a shaker with gentle orbital shaking. Bring the wash buffers to room temperature before use.
4. After tapping off the 1% normal goat serum with 0.2% Triton X-100 solution, add the diluted proximity ligation assay probes, and incubate the slides in a pre-heated humidity chamber for 1 h at 37 °C.

3.4 Detection Protocol

1. During incubation, mix ligation stock and purified water. Hold off on adding ligase to the mix, which is to occur just before application. However, account for the volume of ligase to be later added when making a 1:5 dilution of the ligation stock solution, by using the following ratio: 1 μl ligase, 8 μl 5× ligation stock and 31 μl high purity water per sample.
2. Tap off the proximity ligation assay probe solution from the slides.
3. Wash the slides in wash buffer A for 5 min under gentle agitation twice.
4. Add ligase to the ligation solution at above noted ratio (*see* Subheading 3.4, **step 1**). Add the ligation-ligase solution to each sample.
5. Incubate the slides in a pre-heated humidity chamber for 30 min at 37 °C.
6. Mix amplification stock and purified water. Hold off on adding polymerase until immediately before adding the amplification solution to the samples. However, account for the volume of polymerase when making a 1:5 dilution of the amplification stock solution, by using the following ratio: 0.5 μl polymerase, 8 μl 5× amplification stock, and 31.5 μl high purity water per sample.
7. Tap off the ligation-ligase solution from Subheading 3.4, **step 4**.
8. Wash the slides in 1× wash buffer A for 2× 2 min under gentle agitation. Tap off all wash solution after the last washing.
9. Add polymerase to the amplification solution at above noted ratio (*see* Subheading 3.4, **step 6**). Add the amplification-polymerase solution to each sample.
10. Incubate the slides in a pre-heated humidity chamber for 100 min at 37 °C.
11. Tap off the amplification-polymerase solution from Subheading 3.4, **step 9**.
12. Wash the slides in 1× Wash buffer B for 10 min twice.

13. Wash the slides in 0.01× Wash buffer B for 1 min.
14. Let the slides dry at room temperature in the dark.

3.5 Microscopy and Image Processing

1. Mount slides with a coverslip using a minimal volume of Duolink in situ Mounting Medium with DAPI, ensuring no air bubbles get caught under the cover slip. Use nail polish to seal the edges. Wait for approximately 15 min before analyzing in a fluorescence or confocal microscope (*see* **Note 7**).
2. Images can be processed and analyzed using ImageJ analyzed using BlobFinder software by Allalou and Wählby (Centre for Image Analysis, Uppsala, Sweden, http://www.cb.uu.se/~amin/BlobFinder/) [13], allowing quantification of cytoplasmatic and nuclear events per cell and per photograph (Fig. 1b).

4 Notes

1. Adding 2 % fetal bovine serum to the phosphate buffered saline solution reduces clumping of T cells, and helps prevent the cells from stacking in clusters on the microscopy slide.
2. If the cell isolation ends late in the day, it is possible to preserve the slides after the cytospin by allowing the slides to dry at room temperature for 1 h, and then wrapping them in aluminum foil and a closed Ziploc bag to store them at –80 °C, where they can be stored long term, even years. Prior to use, the slides should be allowed to warm to room temperature before being unwrapped from the aluminum foil.
3. The volume of the primary (and secondary) antibody solution applied to the samples depends upon the surface area of the sample. If the surface area is 2 cm^2, this is approximately 80 µl. The general idea is that each sample should be fully covered by a drop, and has an equal amount and exposure to the antibody solution. To that end, prior solutions (e.g., blocking solution) should be completely removed, however, without allowing the sample to dry, which can cause artifacts.
4. Typical dilution of primary antibodies in 1 % normal goat serum with 0.2 % Triton X-100 ranges from 1:100 to 1:1000. For both the Foxp3 and acetyl-lysine antibodies indicated here, we used 1:100. This may be variable based upon the antibodies and samples used.
5. Other modalities of DNA staining can be used, as well, including 4′,6-diamidino-2-phenylindole. The DNA staining can be done with either the primary or the secondary antibody staining.
6. Add wet tissue paper to the chamber to minimize evaporation of the primary antibody solution. Avoid shaking or unnecessary

moving the chamber to prevent spilling of the primary antibody solution outside of the lipid markings drawn by the liquid blocker pen.

7. After the images are obtained, the slides can be stored at –20 °C in the dark for several months.

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Part IV

PTMs Controlling Acetylation Balances and HDAC Functions

Chapter 22

Testing the Effects of SIAH Ubiquitin E3 Ligases on Lysine Acetyl Transferases

Jan Hagenbucher, Hilda Stekman, Alfonso Rodriguez-Gil, Michael Kracht, and M. Lienhard Schmitz

Abstract

The family of seven-in-absentia (SIAH) ubiquitin E3 ligases functions in the control of numerous key signaling pathways. These enzymes belong to the RING (really interesting new gene) group of E3 ligases and mediate the attachment of ubiquitin chains to substrates, which then leads to their proteasomal degradation. Here, we describe a protocol that allows measuring SIAH-mediated ubiquitination and degradation of its client proteins as exemplified by acetyl transferases using simple overexpression experiments. The impact of SIAH expression on the relative amounts of target proteins and their mRNAs can be quantified by Western blotting and quantitative PCR (qPCR) as described here.

Key words Ubiquitin E3 ligases, Proteasome, Protein degradation, Transfection, Western blot, qPCR

1 Introduction

Many posttranslational modifications (PTMs) occur in a hierarchical and timely organized fashion, where acetylation or phosphorylation occurs early in the signal process, while degradative ubiquitination is an irreversible end point of the modification cascade [1]. Modifying enzymes (writers) serve to attach chemical groups to the side chains of amino acids, while erasors antagonize this process and remove the modification [2]. The different enzymes can also cross-regulate each other, as exemplified by the ubiquitin-dependent elimination of lysine acetylases which is in the focus of this chapter.

Ubiquitin is a peptide composed of 76 amino acids that can be attached by an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a lysine in the substrate protein [3]. Ubiquitin itself has seven lysine residues and can also serve as a substrate for further modifications, thus leading to the formation of polyubiquitin chains. While Lys48- or Lys11-linked chains serve

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as a signal for proteasomal degradation, the formation of alternative bondages (e.g., at Lys63) is relevant for nonproteolytic functions [4, 5].

The ubiquitination process is achieved by the concerted action of an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase [6]. The number of E3 ligases encoded in the human genome exceeds that of protein kinases [3], showing that ubiquitination is a highly substrate-specific process. Disturbed protein degradation underlies many pathological processes including muscle dystrophies, inflammation, and cancer [7]. Hence, enzymes affecting protein ubiquitination such as ubiquitin E3 ligases or deubiquitinases are therapeutic targets [8]. Indeed, drugs affecting the ubiquitin/proteasome system such as bortezomib are used to treat multiple myeloma [9] and numerous further drugs acting on enzymes of the ubiquitin/proteasome system are currently evaluated in advanced clinical trials [10].

Ubiquitin E3 ligases fall into several groups including the largest group of RING finger and RING finger-related E3s [11, 12]. The RING family of ubiquitin E3 ligases also comprises the SIAH family of E3 ligases. The human genome encodes SIAH1 and the highly homologous SIAH2 protein. SIAH1 and SIAH2 proteins have a largely divergent N-terminal part, but are highly conserved in the C3HC4 cysteine-rich zinc-binding RING domain and the C-terminal substrate binding domain (SBD) [13–15].

Various forms of cellular stress including hypoxia, glucose supply, or DNA damage lead to increased levels and activities of SIAH1 and SIAH2 [14]. In addition, the activity of SIAH proteins is regulated by phosphorylation [16–18] and its association with the cytosolic adaptor protein DAB1 or the deubiquitinating USP13 protein. SIAH proteins form dimers and have overlapping and distinct substrate-binding abilities, as comprehensively described in a recent review [15]. Among them are the acetyl transferases CBP/p300 (CREB binding protein), TIP60 (Tat-interactive protein 60 kDa), and PCAF (P300/CBP-associated factor), thus allowing a crosstalk between protein ubiquitination and acetylation [17].

As exemplified by the acetyl transferase PCAF we will describe a method that is generally used for the validation of SIAH substrate proteins. This approach relies on the finding that overexpression or knockdown of SIAH proteins results in altered amounts of the SIAH target proteins which can easily be measured by immunoblotting [19–23]. In the experimental design described here, the SIAH target protein PCAF is expressed at low levels, either alone or together with active wildtype forms of SIAH1 or SIAH2, which are sufficient to trigger ubiquitination and degradation of PCAF. As an internal control, point mutated forms of SIAH proteins are expressed in order to ensure that loss of function is attributable to the E3 ligase function and not to indirect effects. In addition, it is relevant to downregulate the expression of the endogenous SIAH proteins with specific shRNAs or siRNAs in

order to ensure regulation of PCAF protein stability without overexpression of components. To reveal the contribution of the proteasome system for PCAF degradation it must be ensured that the degradation of this lysine acetyl transferase is prevented in the presence of a proteasome inhibitor such as MG132. Moreover, a further control ensures that reduced PCAF protein levels are not due to changes in its mRNA abundance. An example for such an experiment is displayed in Fig. 1.

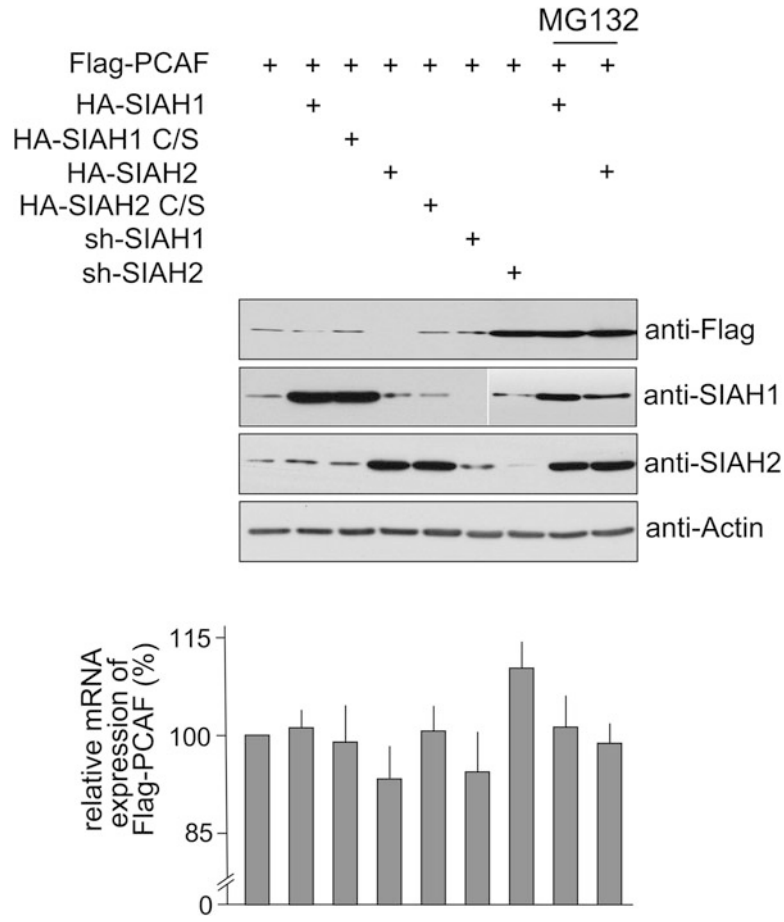

Fig. 1 SIAH-2-mediated degradation of Flag-PCAF. *Upper panel:* cells were transfected with plasmids directing expression of the indicated proteins or shRNAs as shown. Cells were treated with MG132 as described in detail above, followed by cell harvesting 40 h after transfection. One part of the cells was analyzed for the expression of Flag-PCAF, SIAH1, and SIAH2 by immunoblotting as shown. Note that the Flag-PCAF protein is hardly visible in the presence of overexpressed wild-type SIAH2. This downregulation can be antagonized by MG132 or by a SIAH2-specific shRNA, indicating the contribution of SIAH2 for the control of Flag-PCAF protein stability. *Lower panel*: the mRNA amounts of Flag-PCAF were measured by qPCR. The expression of Flag-PCAF alone was arbitrarily set as 100%, error bars show standard errors of the mean. Note the scale on the *y*-axis, indicating that mRNA levels are largely comparable and show no major fluctuations

2 Materials

Materials and antibodies listed here can be obtained by various providers.

2.1 Cell Transfection

1. DMEM (Dulbecco's Modified Eagle's Medium) transfection medium is commercially available DMEM containing 4.5 g/L glucose and 2 mM L-glutamine, but not supplemented with fetal calf serum (FCS) and antibiotics.
2. Complete DMEM medium containing 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin.
3. Linear Polyethylenimine (PEI), MW 25 kDa is prepared by dissolving the PEI powder to a final concentration of 1 mg/mL in preheated H_2O. PEI will completely dissolve at a temperature close to its melting point (72–75 °C). After the PEI has been dissolved, cool down the solution to room temperature and adjust the pH to 7.0 with HCl. The solution is filtrated through a 0.22 μm filter in the cell culture hood to sterilize the solution and to remove small, undissolved PEI particles. Make some aliquots and store them at −20 °C, a working stock can be kept at 4 °C (*see* **Note 1**).
4. Phosphate-buffered saline (PBS): 137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8 mM Na_2HPO_4, pH 7.4.
5. A stock solution of MG132 (10 mg/mL) is prepared in dimethylsulfoxid (DMSO) and should be stored in aliquots at −20 °C.

2.2 Cell Harvest and Protein Extraction

1. NP-40 lysis buffer: 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% (v/v) NP-40. Please add freshly: 10 mM NaF, 0.5 mM Na_3VO_4, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin.
2. 5× SDS (sodium dodecyl sulfate) sample buffer: 0.3 M Tris/HCl, pH 6.8, 50% (v/v) glycerol, 25% (v/v) β-mercaptoethanol, 10% (w/v) SDS, 0.02% (w/v) bromophenol blue.
3. Cooled table top centrifuge.
4. −80 °C deep freezer.

2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 4× Separating gel buffer: 1.5 M Tris/HCl, pH 8.8, 0.4% (w/v) SDS.
2. 4× Stacking gel buffer: 0.5 M Tris/HCl, pH 6.8, 0.4% (w/v) SDS.
3. 10% Ammonium persulfate (APS): 10% (w/v) aqueous solution.
4. Tetramethylethylenediamine (TEMED).
5. 30% Acrylamide/bisacrylamide.
6. Water-saturated Butanol (*see* **Note 2**).

7. Mini Protean 3 system-casting stand with corresponding casting frames, combs, and glass plates with spacers.
8. Hamilton syringe.
9. SDS-Running buffer: 250 mM glycine, 25 mM Tris, 0.1% (w/v) SDS.
10. Prestained protein standards.

2.4 Western Blotting

1. Polyvinylidene difluoride (PVDF) membrane.
2. Whatman paper (3MM).
3. Ethanol for wetting of the PVDF membrane.
4. Transfer buffer: 50 mM Tris, 40 mM glycine, 20% (v/v) methanol, 0.04% (w/v) SDS.
5. Western transfer apparatus.
6. Power supply.
7. TBS-T: 25 mM Tris, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1% (v/v) Tween 20.
8. Nonfat milk powder can be purchased from a supermarket.
9. Antibodies recognizing the Flag epitope (M2), the HA epitope (12CA5), or beta-Actin as a loading control are offered by various companies. Anti-SIAH1 (N-15, Santa Cruz Biotechnology), anti-SIAH2 (N-14, Santa Cruz Biotechnology), and anti-beta Actin (Abcam ab8227-50) were obtained from the indicated suppliers.
10. Sodium azide.
11. Western developing kit.
12. Transparent dossier.
13. Film, film box, and film processor or alternatively an electronic image acquisition device.

2.5 RNA Extraction and cDNA Synthesis

1. RNeasy kit (Qiagen): the contents of the used buffers (RLT buffer containing ß-mercaptoethanol, RDD buffer containing DNAse I, RW1 buffer, RPE buffer) are not disclosed by the manufacturer.
2. Needle and syringe.
3. RNeasy spin columns.
4. 70% (v/v) ethanol.
5. Cooled table top centrifuge.
6. UV spectrometer.
7. RNase inhibitor.

2.6 cDNA Synthesis

1. RNA from the experiment.
2. dNTP (deoxynucleotide triphosphate) mix (10 mM each).

3. Oligo (dT) 12–18 primer.
4. MilliQ water.
5. 5× First strand buffer: 250 mM Tris–HCl, pH 8.3 at room temperature, 375 mM KCl, 15 mM $MgCl_2$.
6. 100 mM 1,4-Dithiothreitol (DTT).
7. Reverse transcriptase.
8. RNase Inhibitor.
9. Thermomixer.

2.7 qPCR

1. 96-well PCR plates.
2. Centrifuge for 96-well PCR plates.
3. Template cDNA.
4. SYBR Green qPCR master mix containing all the components necessary to perform qPCR, with the exception of templates and primers.
5. Forward and reverse primers for plasmid-encoded PCAF and the housekeeping gene.
6. MilliQ water.
7. Real-time PCR cycler.

3 Methods

3.1 Cell Transfection

1. The degradation assay can be performed in any cell type, as the required components leading to ubiquitination and destruction of PCAF are present in all standard cell culture lines. We prefer human embryonic kidney 293T cells as they are easily transfectable with a high efficiency. One day prior to transfection the cells are split and grown on a 10-cm dish in order to reach 1/3rd confluency on the day of transfection. This allows growth of the cells for further 36 h. Cells are transfected with 4 μg of plasmid DNA in total, consisting of 1 μg of a plasmid encoding PCAF, either alone or together with 3 μg of plasmids directing the expression of SIAH proteins, SIAH-specific shRNAs, or empty vector DNA. Fill up with the empty expression vector in order to ensure that each transfection reaction is performed with the same amount of plasmid DNA.
2. Add DMEM transfection medium (lacking FCS and antibiotics) to the Eppendorf tube to reach a volume of 100 μL for your DNA mix.
3. Prepare a premix solution with your transfection reagent such as linear PEI. For each μg of DNA use a 1.5-fold excess of PEI (stock solution 1 mg/mL). This amount is optimal for 293T cells and may be different for other cell types (*see* **Note 3**). Mix

6 μL of PEI with 54 μL of DMEM transfection medium to create a premix solution. Add 60 μL of the premix solution to the DNA mix and pipet the solution up and down 15 times.

4. While PEI condenses DNA into positively charged particles that bind to anionic cell surfaces during 20 min at room temperature, the 293T cells are prepared for the transfection.
5. Aspirate the medium and carefully add 10 mL of warm PBS to the cells (*see* **Note 4**). Aspirate off the PBS and carefully add 3 mL of DMEM containing 10% (v/v) FCS but lacking antibiotics, as they could interfere with the DNA/PEI complexes. Add 160 μL of the PEI/DNA mix that had formed for 20 min and gently agitate the 10-cm plate to allow mixing of the solutions.
6. The cells are now covered with a thin layer of medium and transfection mix and will be incubated for 4 h in a humidified incubator at 37 °C and 5% CO_2 (*see* **Note 5**).
7. After 4 h the transfection medium is aspirated off and 10 mL of complete DMEM containing 10% FCS and 1% (v/v) penicillin/streptomycin is added.
8. As the proteasome inhibitor MG132 is detrimental for cells upon prolonged incubation, this agent is added only 12 h prior to cell lysis at a final concentration of 10 μM. Alternatively, also other proteasome inhibitors such as lactacystin may be used. Cells are harvested 36–40 h after the transfection.

3.2 Cell Harvest and Protein Extraction

1. The DMEM medium is removed by aspiration and cells are washed upon addition of 10 mL cold PBS. After removal of the buffer another 2 mL of cold PBS is added and cells are carefully scraped off until they are fully detached. The 2 mL of PBS containing the detached cells is then split into 2 Eppendorf tubes (1 mL in each tube).
2. The cells are pelleted upon centrifugation for 3 min in a microfuge at 1500 × *g* and 4 °C. The supernatant is carefully removed by aspirating off using a pipette or a pump. Be careful not to remove the pelleted cells. For each sample there are now two cell pellets: one of the cell pellets is snap frozen in liquid nitrogen and is then stored at −80 °C for quantitative analysis of *PCAF* mRNA expression at later time points (see below). The other pellet is immediately used for protein extraction.
3. The cell pellet is resuspended in 150 μL of NP-40 lysis buffer and incubated for 20 min on ice. Gently dissolve the cell pellet by pipetting up and down, strictly avoid foaming to prevent oxidation of proteins. After the incubation step, the lysates are cleared by centrifugation (15,000 × g, 10 min, 4 °C) and the supernatants are transferred into a fresh Eppendorf tube. Lysates are directly mixed with 5× SDS sample buffer and

heated for 4 min at 95 °C. The samples are now ready for denaturing SDS-PAGE or alternatively can be stored at –20 °C and used at a later time point. The PCAF protein is easily extracted in the NP-40 buffer that solubilizes cytosolic and most nuclear proteins. In the case you want to investigate proteins residing in the insoluble fraction, it will be necessary to lyse the cells directly in SDS sample buffer.

3.3 SDS-PAGE

1. A SDS Gel is prepared and the adequate percentage of the separation gel must be chosen in order to allow optimal detection of the protein of interest (*see* Fig. 2). As SIAH1 (31 kDa) and SIAH2 (35 kDa) proteins are relatively small and PCAF migrates at 93 kDa, the E3 ligases are separated on a 10% SDS gel and PCAF on a 8% SDS gel. Cast the separating gels as detailed in Table 1. As the polymerization process of acrylamide is started by APS (radical former) and TEMED (catalyst), both reagents should be added lastly to the mixture. Pour the liquid acrylamide into the assembled gel plate to 3/4 of the volume leaving enough space for the stacking gel. The surface is covered with butanol (water saturated) so that the upper margin of the separating gel stays even.
2. Polymerization proceeds during 20–30 min depending on the temperature. Its completion can be seen upon slight tilting of the gel, which results in the flow of butanol but not of the polymerized acrylamide. Pour away the butanol and remove remaining butanol by washing twice with water. Carefully remove residual water using a pipette. Then prepare the stacking gel as outlined in Table 1 and insert the comb without any air bubbles.

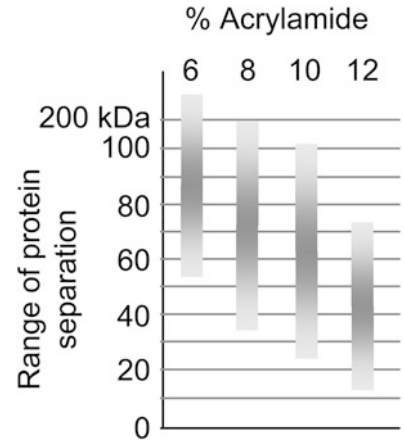

Fig. 2 Schematic display of optimal SDS-PAGE separation ranges. Molecular weights of different proteins and acrylamide concentrations contained in the separation gel are indicated

Table 1
Composition of the SDS gel

	Running		Stacking
Solutions	**8%**	**10%**	**4%**
Acrylamide	2.1 mL	2.7 mL	0.5 mL
H_2O	3.9 mL	3.3 mL	2.5 mL
4× Separating gel buffer	2 mL	2 mL	
4× Stacking gel buffer			1 mL
TEMED	6 µL	6 µL	3 µL
10% (w/v) APS	40 µL	40 µL	20 µL

3. After 20 min of polymerization the comb is pulled out and the wells are rinsed twice with water in order to remove any traces of unpolymerized acrylamide. The gels are then assembled in an electrophoresis chamber that is then filled up with 1× SDS-running buffer.
4. The gel can now be loaded with 30 µL of the samples. If the samples have been frozen before, they should be boiled for 3 min at 95 °C before loading with a Hamilton syringe (*see* **Note 6**). A protein marker is added and empty pockets can be filled with 25 µL of 1× SDS sample buffer to prevent the slightly retarded migration of samples loaded at the margins ("smiling" of the gel).
5. The gel is then run with 80 V for 30 min until the bromphenol blue has entered the separation gel. Subsequently, the electrophoresis is continued with 120 V for further 90 min until the bromphenol blue has left the separation gel.

3.4 Western Blotting

The separated proteins are then transferred by electrical field force to a PVDF membrane. The transfer efficiency decreases with the size of the protein and the transfer time for proteins exceeding 150 kDa is twice as long as for smaller proteins.

1. Cut a PVDF membrane (wearing powder free gloves) to the size of 9×6 cm and also cut three pieces of thick Whatman 3MM blotting paper to the same size.
2. The hydrophobicity of PVDF makes it an ideal support for binding proteins, but also necessitates a previous wetting step in alcohol. The membrane is equilibrated in ethanol for 1 min and then incubated in transfer buffer for 2 min. In parallel, the 3MM blotting papers are soaked in transfer buffer for 1 min until they are completely wet.

3. Pour 4 mL of transfer buffer to the surface of the Western blot device, assemble two pieces of wet 3MM paper, and place the PVDF membrane on the top. Take the acrylamide gel and cut off the stacking gel. The separating gel is carefully placed onto the PVDF membrane and covered with another piece of wet 3MM paper. Any air bubbles will prohibit transfer of proteins, thus a 15 mL tube is used to remove any air bubbles between the layers.
4. Add another 2 mL of transfer buffer to the top 3MM paper and then close the lid and start the transfer at 150 mA per gel. Stop the transfer after 2 h and disassemble the gel. The transfer of the protein marker to the PVDF membrane verifies the successful blotting. The membrane is then washed once for 1 min in TBS-T and then incubated for 60 min in TBS-T containing 5% (w/v) nonfat milk powder for blocking (*see* **Note 7**). In parallel, discard the gel and the 3MM paper and clean the Western blot device carefully with distilled water. Any remaining gel pieces or dried transfer buffer will impede the quality of future blots.
5. After blocking the PVDF membranes are washed for 1 min in 1× TBS-T and further incubated in 10 mL TBS-T containing 1% (w/v) nonfat milk powder and antibodies. The antibodies are incubated between 2 and 14 h at 4°C on a shaker. Incubation for prolonged periods (e.g., over the weekend) is not recommended.
6. Pour the TBS-T solution containing the antibodies into a labeled 15 mL tube and add sodium azide to a final concentration of 0.02% (w/v) as a preservative. Most of the antibodies show a good stability and can be extensively reused. The membrane is then washed five times with 10 mL of TBS-T for 3 min per washing step on a shaker (*see* **Note 8**).
7. Subsequently, the membranes are incubated with the appropriate secondary antibody diluted 1:5000 in TBS-T containing 1% nonfat dry milk for 1–2 h at room temperature or in the cold room overnight.
8. The secondary antibody may be reused within the next days (*see* **Note 9**). The membrane is then again extensively washed five times in 10 mL of TBS-T for 3 min per washing step.
9. The signal is detected by chemiluminescence using a Western developing kit. The system is based on the chemical activation of a substrate, which then turns back to its ground state under emission of light. In the presence of H_2O_2, horseradish peroxidase catalyzes the oxidation of luminol. A transparent dossier is put into a film box. The membrane is slightly dried on a paper towel and placed into the dossier. Mix equal volumes of the two detection solutions and equally distribute over the

membrane with a pipette. Close the dossier and expose to an X-ray film for various periods, followed by insertion into the film processor. The optimal exposure time allows the detection of the protein of interest and changes of its protein levels. Alternatively, the membrane is not exposed to a film and the emitted light is measured and quantified using an electronic image acquisition device.

3.5 RNA Extraction and cDNA Synthesis

In the case that the Western blot reveals differences in SIAH-dependent protein expression, it will be important to ensure that these changes are not due to variations in the levels of mRNA encoding PCAF or the protein of interest. The frozen cell pellets (*see* Subheading 3.2) are then used to isolate the RNA. As RNAses are abundantly active it is important to perform all steps with gloves and autoclaved pipette tips, Eppendorf tubes, and solutions (*see* **Note 10**).

1. Total RNA can be isolated from cells using the RNeasy kit (Qiagen). Cell pellets are directly lysed by adding 600 µL RLT buffer containing β-mercaptoethanol. To avoid RNA degradation, quickly resuspend/homogenize the whole cell pellet. Cell lysates are transferred into a tube and homogenized by passing the lysate through a needle for several times to shear the genomic DNA.
2. After adding 600 µL 70% (v/v) ethanol to the lysate the samples are loaded on RNeasy spin columns. They allow binding of the RNA to the silica gel membrane contained in the column. After loading 700 µL of solution to the column and centrifugation for 15 s at 13,000 rpm the flowthrough is discarded. Load the remaining lysate from **step 1** and repeat the centrifugation step.
3. Wash the columns with 350 µL of washing buffer RWl and spin at 13,000 rpm for 15 s, discard the flowthrough.
4. Add 80 µL of DNAse I-containing RDD buffer on the membrane of the RNA column and incubate for 15 min at room temperature to digest the genomic DNA. This step must be complete in order to avoid artifacts, as incomplete DNA digestion could cause misleading qPCR results.
5. Wash the column with 350 µL of RW1 buffer and once with 500 µL RPE buffer, spin for 15 s at 13,000 rpm.
6. Wash the column with 500 µL RPE buffer, spin for 2 min at 13,000 rpm to remove remaining ethanol.
7. Elute RNA in 30 µL RNase-free water. RNA concentration and purity are determined in a spectrometer and RNA is stored at −80 °C after adding RNase inhibitor.
8. Measure concentration (1 $OD_{260nm} = 40$ µg RNA/mL) and purity ($OD_{260nm}/OD_{280nm} = \sim 2.0$) in a UV spectrometer.

3.6 cDNA Synthesis

This step also critically depends on the integrity of RNAs, so that all precautions for the preservation of RNA apply (*see* also Subheading 3.5). For reverse transcription of RNA into complementary DNA (cDNA) the following reagents and protocol are used.

1. The annealing of the primers is done as shown in Table 2.
2. Incubate for 5 min at 65 °C, put on ice. Then add the following reagents as specified in Table 3.
3. Mix, incubate for 2 min at 42 °C.
4. Add 1 μL of reverse transcriptase and incubate for further 55 min at 42 °C.
5. After inactivation of reverse transcriptase by incubation at 70 °C for 15 min the cDNA is diluted with sterile water to a volume of 100 μL and used as a template for quantitative real-time PCR.

3.7 qPCR

The qPCR method allows the relative quantification of the expression level of specific target genes between different samples. For qPCR, target gene specific oligonucleotides are used which are typically already found in public resources such as primerbank (http://pga.mgh.harvard.edu/primerbank/) or need to be designed using the PRIMER BLAST tool from the NCBI webpage. The size of the amplicons should be kept between

Table 2
Primer annealing for the cDNA synthesis reaction

Reagent	Volume (μL)
1 μg of total RNA	x
dNTP mix (10 mM each)	1
Oligo (dT) 12–18 primer 500 μg/mL	1
MilliQ water	ad 11

Table 3
The cDNA synthesis reaction

Reagent	Volume (μL)
5× First strand buffer	4
DTT (100 mM)	2
RNase inhibitor	1
MilliQ water	1

70 and 250 bp. When possible, the forward and reverse primers should be separated by at least one intron in the genomic sequence, to avoid misquantification of cDNA due to genomic contamination of the RNA samples (*see* **Note 11**). Normalization of gene expression data against housekeeping gene is required for accurate and reliable gene expression analysis. It is important to note that some commonly used housekeeping genes including hypoxanthine phosphoribosyltransferase 1 (HPRT1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) may vary considerably under different experimental conditions. A meta-analysis of data from gene arrays, massive parallel sequencing, and RNA-seq has revealed a list of genes that show extremely low variations in gene expression and are thus recommendable for normalization [24]. In this experiment *PCAF* mRNA expression derived from the expression vector is detected in order to ensure that changes in the protein level are not attributable to variations in transfection efficiency or other parameters. To detect the mRNA encoding Flag-PCAF the upper primer was designed to anneal to the region encompassing the Flag-tag. DNA amplification is detected by SYBR green, a commonly used reporter dye. It intercalates with double-stranded DNA, thus leading to an increased signal intensity during DNA amplification.

1. In a 96-well qPCR plate, pipet 2 μL of cDNA from each sample in three wells per primer pair to test. Pipet also 2 μL of water in three wells per primer pair as a template-free control (*see* **Note 12**).
2. Make one master mix per primer pair. Per well please mix (*see* **Note 13**) 5 μL of 2× SYBR Green qPCR master mix, 0.8 μL of primer pair working stock solution (5 μM) and 2.2 μL of H_2O.
3. Distribute 8 μL of the master mix in the corresponding wells of the qPCR plate. Seal the plate with the appropriate sealing foil and centrifuge briefly at 700 rpm for 30 s in order to remove any bubbles or liquid on the sides of the wells.
4. Run in the qPCR machine using the following settings in the 7300 system SDS software: Assay type: Absolute quantification; Detector type: SYBR green; Thermal profile:

Stage1: 10 min at 95 °C, 1 repetition.

Stage 2: 15 s at 95 °C, 1 min at 60 °C, 40 repetitions.

Stage 3 (dissociation stage, *see* **Note 14**): 15 s at 95 °C, 1 min at 60 °C, 15 s at 95 °C, 15 s at 60 °C, 1 repetition. Use the "Add Dissociation Stage" button to add this stage.

5. Quantification: the Ct (cycle in which fluorescence reaches the threshold) of amplification is determined for each well using the 7300 system SDS software. The average Ct of the three

replicates is then obtained. Data are normalized to the housekeeping gene Glucose-6-phosphate isomerase (GPI) and the resulting ΔCt values are compared to the sample that is chosen as a calibrator (in this experiment cells expressing HA-PCAF). The relative expression level is then calculated according to the following formula: $R = 2^{-\Delta\Delta Ct}$ as described [25].

4 Notes

1. To allow heating of the solution, a glass beaker might be better than preparing the solution in a Falcon tube.
2. Add MilliQ water until the butanol is saturated with water, this material is in the lower phase. The water-saturated butanol does not change the water content of the separating gel.
3. The optimal PEI/plasmid DNA ratio should be defined for each cell line. This can be easily done using a plasmid encoding green fluorescent protein (GFP), which allows the quantification of transfection efficiency by fluorescence microscopy.
4. The PBS should be prewarmed in order to avoid a cold shock reaction of the cells. As 293T cells quickly detach from surfaces, the PBS (and all other solutions) needs to be added carefully to the periphery of the dish.
5. Make sure that the shelf is absolutely horizontal and not inclined, as this might result in incomplete coverage of cells with liquid and subsequent cell damage or incomplete transfection.
6. Make sure to clean the syringe between the individual loading steps by loading the syringe twice with 1× SDS running buffer.
7. We recommend clipping off the upper right corner of the PVDF membrane in order to allow identification of the side containing the transferred proteins. Alternatively, the membrane can be labeled with a pencil.
8. This extensive washing is rewarded by high quality blots with low background.
9. No sodium azide should be added to peroxidase-coupled secondary antibodies as it inhibits the peroxidase. Instead thimerosal (0.01% (w/v) can be used as a preservative.
10. Another dangerous source for RNases is droplets, which are exhaled during talking. So keep your mouth shut while working with RNA.
11. To check the efficiency of the designed primer pairs it is highly recommended to test them prior to further analysis using different serial dilutions of a control cDNA. The resulting curves

of amplification should be separated by approximately 1 Ct ($\log_2 2$) in 1:2 dilutions or approximately 3.3 ($\log_2 10$) Cts in 1:10 dilutions.

12. One control of cDNA synthesized without reverse transcriptase can be added as a negative control for DNA contamination in the RNA extraction. This is especially important when the primers could not be designed to span an intronic region in the corresponding genomic DNA.
13. Calculate the master mix for at least three more wells than the total number of real wells, to avoid running out of it before it can be distributed to all wells. Normally, there are losses of solution during the pipetting due to the viscosity of the SYBR green solution.
14. The dissociation curve should be determined in order to detect possible unspecific amplifications. The results should show one single peak around 85 °C in the melting temperature. Additional peaks indicating melting points at temperatures lower than 80 °C are indicative for the occurrence of primer dimers or other aberrantly short PCR products.

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Chapter 23

Assessment of HDACi-Induced *Acetylation* of Nonhistone Proteins by Mass Spectrometry

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Abstract

Posttranslational acetylation of lysine residues has been discovered as multifaceted regulatory modification for various nuclear, cytoplasmic, and mitochondrial proteins. The implementation of high-resolution and high-throughput mass spectrometry (MS) approaches has led to the identification of a hitherto underappreciated, large number of acetylation sites for a broad spectrum of cellular proteins. In this chapter, we describe a comprehensive protocol for the purification of an in vivo-acetylated, ectopically expressed, FLAG-epitope tagged nonhistone protein through immunoprecipitation (IP). The protocol also covers the sample preparation by SDS-PAGE, proteolytic digestion, and the analysis by LC-ESI MS. The success of this methodology, however, strongly depends on the physico-chemical properties of the respective protein(s) and the quality of selected peptide mass spectra.

Key words Lysine acetylation, Posttranslational modification, Histone deacetylase inhibitor, Immunoprecipitation, FLAG-tag purification, Mass spectrometry analysis, LC ESI tandem MS

1 Introduction

After full translation and folding, proteins can adopt new structural and functional features through posttranslational modifications (PTM) [1–3]. Next to phosphorylation, acetylation of the ε-amino group in lysine residues is considered a most common PTM [4]. In addition to histones, a myriad of nonhistone proteins such as metabolic enzymes [5], key transcription factors [3], epigenetic regulators [6], as well as transport and cytoskeleton proteins [7, 8] undergo acetylation. In many cases, acetylation impacts protein features, such as stability, subcellular localization, transcriptional activation, or binding affinity to DNA or proteins [9].

Lysine acetylation is a reversible modification determined by the opposing enzymatic activities of lysine acetyltransferases (also known as histone acetyltransferases, HATs) and deacetylases (also known as histone deacetylases, HDACs) [3]. Although they are not well evolutionary conserved, HATs are classified into the three major families: CBP/p300, GCN5, and MYST [10, 11]. HATs

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catalyze the transfer of the acetyl-group to lysine residues using acetyl-coenzyme A as a cofactor. The 18 known human HDACs are categorized into four classes according to the sequence homology with their yeast homologues and their domain organization [12]. Class I (HDAC1-3; 8), II (HDAC4-7; 9; 10), and IV (HDAC11) HDACs require Zn^{2+} for the hydrolysis of acetyl-groups. The sirtuin family of deacetylases (Class III HDACs) comprises seven members, which are related to the yeast SIR2 protein [13, 14]. In contrast to other HDACs, class III enzymes use a different catalytic mechanism including NAD^{+} as cofactor. Inhibitors against different HDACs have been used for the identification of acetylated proteins [5, 6, 15].

After the elucidation of their functional impact, some deacetylase inhibitors have been scrutinized as efficient drugs to treat malignancies caused by an aberrant HDAC activation [16–18]. HDAC inhibitors (HDACi) interfere with the equilibrium of the enzymatic acetylation machinery favoring HAT activity and thereby provoke intensive acetylation of their protein substrates. Trichostatin A (TSA), panobinostat (LBH-589), and suberoylanilide hydroxamic acid (SAHA) are potent (pan-) inhibitors of class I, II, and IV HDACs [19, 20], while nicotinamide (NAM) is a noncompetitive inhibitor of sirtuins [21, 22].

The analysis of protein acetylation is often carried out by radiolabeling via [^{3}H] acetate or [1-^{14}C] acetyl-CoA in vivo or in vitro, in combination with a phosphocellulose P81 filter retention and detection by autoradiography [23]. This is an easy to perform assay albeit it is rather insensitive compared to alternative approaches. Alternatively, immunoblotting of isolated proteins using antiacetyl lysine antibodies is suitable for the estimation of the degree of their acetylation. However, it does not provide information about the position of the modified lysine residue. Site-specific antibodies are often not available. As another method, Edman sequencing can be employed to determine the precise positioning of acetylation sites starting from the N-terminus [24]. But due to its restriction to only short peptide sequences (30aa) and the requirement of rather high protein amounts, this method is often not the first choice. Within the last decades the development of sensitive mass spectrometry devices has paved new ways for the identification, mapping, as well as the relative quantification of global protein acetylation events. Different MS instruments and MS methodological approaches have been successfully implemented to measure acetylated-peptides and proteins. PTMs in histones, for instance, have been extensively studied by so-called top-down MS, using intact, purified proteins. In contrast, in most large-scale acetylome analyses bottom-up or "shotgun" proteomics is preferred. It involves the enzymatic digestion of the isolated proteins, separation of the resulting peptides via liquid chromatography, and the determination of the mass and sequence of individual peptides in the mass spectrometer [25].

In general, the coverage of a protein sequence by peptide sequences experimentally measured by mass spectrometry is limited in approaches of bottom-up proteomics. This is the consequence of peptide losses during sample preparation and of the incapability of LCMS setups to detect all possible peptides. The reason for the losses is largely obscure and the incomplete detection in the mass spectrometer is caused by imperfect ionization or by the occurrence of tryptic peptides that either do not bind to the column or are outside the detection range of the mass spectrometer. Although algorithms for the prediction of peptide retention times have been approved in recent years, the prediction of the retention of modified peptides is still difficult and imprecise. The reliability of the detection of acetylated peptides, which, predominantly, are much less abundant than their nonacetylated counterparts, can be significantly improved by techniques that provide the spectra of the modified species. Synthetic peptides containing stable isotopes are frequently used for the determination of the retention time and the quantity of interesting physiologic peptides and proteins. If the existence of various acetylated peptides of a protein is not proven, the synthesis of many candidate peptides can however be impractical or expensive. In such cases, the chemical acetylation (also called hyperacetylation) of a protein of interest can help to determine the retention times of interesting peptides and support the detection of acetylation sites.

Here, we describe the mass spectrometry analysis of an ectopically expressed FLAG-tagged fusion protein in HeLa cells, including the stabilization of protein acetylation in vivo by a combinatorial treatment with TSA and NAM. The cells were lysed and the protein of interest was isolated using immunoprecipitation with FLAG antibodies and one-dimensional SDS-PAGE. The gel was stained with Coomassie Brilliat Blue G-250 (CBB) to visualize the isolated proteins. To confirm the position of the isolated protein in the gel as well as its acetylation after HDACi treatment, immunoblotting was performed with the same samples using acetyl-lysine antibodies and specific antibodies against the protein respectively (Fig. 1). The CBB stained protein spot was excised and digested by proteases in gel. The resulting peptides were subsequently analyzed by electrospray ionization tandem MS (LC-ESI-MS/MS).

As the insights that we obtained for the FLAG-tagged protein are unpublished yet, we decided to name the respective protein as "protein of interest" (POI).

2 Materials

Materials listed here are routinely used in our lab. However, equipment from other suppliers should lead to the same results. Preparation of MS samples requires certain precautions as working under dust-reduced conditions (*see* **Note 1**) or specific plastic ware (*see* **Note 2**).

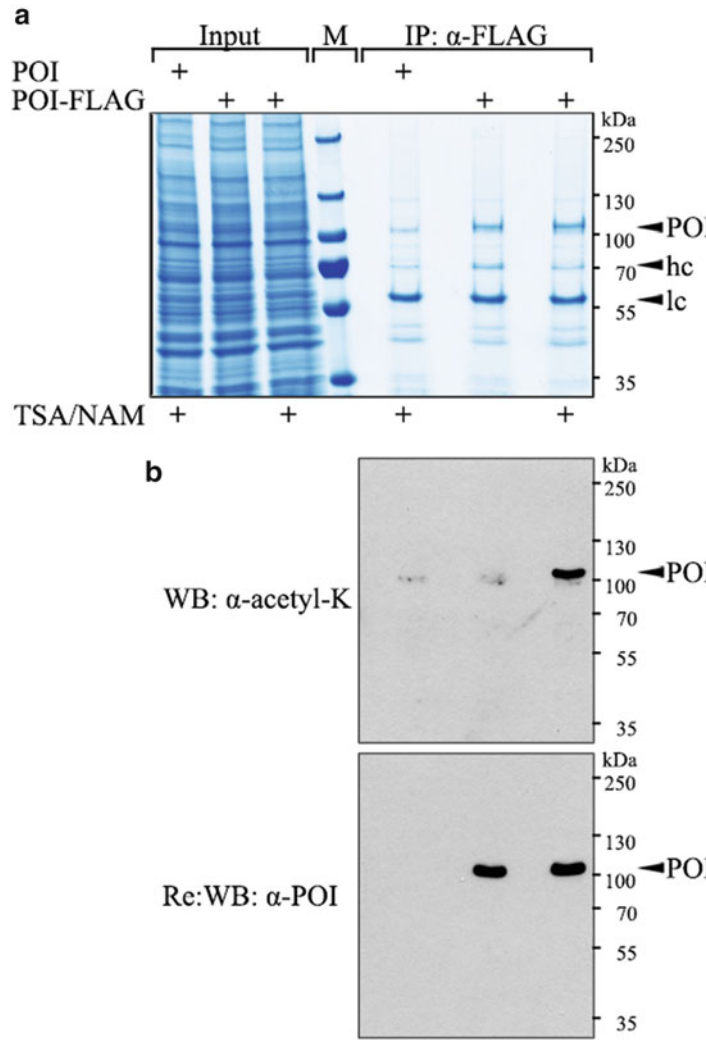

Fig. 1 SDS-PAGE and Western-Blot analysis of an in vivo-acetylated and FLAG-tag purified protein. (**a**) Coomassie G-250 staining of SDS polyacrylamide protein gel after SDS-PAGE. For inputs 35 μg protein was loaded per sample. Proteins isolated by immunoprecipitation (IP) using anti-FLAG agarose (Sigma Aldrich™, Cat. No. #A-2220) were loaded onto the same gel. *Black arrow* heads indicate the anticipated FLAG fusion protein (POI, protein of interest) and the light chain (lc) and heavy chain (hc) of the anti-FLAG antibody. PageRuler™ Plus Prestained Protein Ladder, 10–250 kDa (Thermo Scientific™) was used as a marker (M). (**b**) Control western blot analysis with same samples as used for (**a**). Immunoblotting (WB) with acetylated-lysine antibody (#9441, Cell Signaling Technology®) and reprobe with specific antibodies against the protein was performed to assess position of the FLAG-tagged protein within the gel and to confirm the induction of acetylation following HDACi treatment. The indicated POI (*black arrow head*) was excised from the gel and further processed for MS analysis

2.1 Cell Culture

The human cervical cancer cell line HeLa is maintained in RPMI medium, supplemented with 10% (v/v) fetal calf serum (FCS) and 0.5% (v/v) gentamicin.

2.2 Preparation of Whole Cell Extracts

1. Dulbecco's Phosphate-Buffered Saline (PBS): 1.37 M NaCl, 27 mM KCl, 80 mM $Na_2HPO_4 \times 3 \times H_2O$, 14 mM KH_2PO_4.
2. RIPA lysis buffer: 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 1% (v/v) Nonidet P-40 (NP-40), 1% (v/v) Na-deoxycholate, 0.1% (w/v) SDS. Add freshly: 0.2% (v/v) protein inhibitor cocktail (PIC: 10,000 units/ml aprotinin; 100 mg/ml benzamidin; 2 mg/ml antipain; 1 mg/ml leupeptin), 1 mM PMSF, 10 mM NAM, 100 nM trichostatin A (TSA, Sigma Aldrich).
3. Sonication: Branson Sonifier W250D, 40% amplitude, pulse 0.3 s, pause 0.7 s.

2.3 Immunoprecipitation and SDS-Polyacrylamid Gel Electrophoresis

1. ANTI-FLAG M2 Affinity Gel agarose (Sigma Aldrich, *see* **Note 3**).
2. SDS-Laemmli (2×) loading buffer: 116 mM Tris–HCl pH 6.8, 1.4 M β-mercaptoethanol, 10% glycerol, 3.3% SDS (w/v), spatula tip bromophenol blue.
3. Separating gel buffer: 1 M Tris–HCl pH 8.8.
4. Stacking gel buffer: 1 M Tris–HCl pH 6.8.
5. 10% (w/v) ammonium persulfate (APS).
6. Tetramethylethylenediamine (TEMED).
7. 30% Acrylamide/bisacrylamide (37.5/1).
8. Butanol.
9. Mini Protean 3 system (Bio-Rad), casting stand with corresponding casting frames, combs, and glass plates (spacers included).
10. SDS-running buffer: 250 mM glycine, 25 mM Tris, 0.1% (w/v) SDS.
11. PageRuler Plus Prestained Protein Ladder: 10–250 kDa (Thermo Scientific).
12. Protein staining solution: 0.02% (w/v) Coomassie Brilliant Blue G-250 (CBB), 5% (w/v) aluminiumsulfate-(14–18)-hydrate, 10% (v/v) ethanol, 2% (v/v) orthophosphoric acid (*see* **Note 4**).
13. Gel destaining solution: 10% (v/v) ethanol, 2% (v/v) orthophosphoric acid.

2.4 Sample Preparation and MS Analyses

1 Trypsin NB sequencing grade (Serva).

2 Enodoproteinase Asp-N (Roche Applied Science).

3 Chymotrypsin sequencing grade (Promega).

4 Ammonium bicarbonate, solutions of 25 mM and 0.1 M are prepared in HPLC grade water.

5 Trifluoroacetic acid sequencing grade (Thermo Scientific Pierce).

6 Water LC-MS, acetonitrile LC-MS, formic acid 0.1% in ACN LC-MS, formic acid 0.1% in water LC-MS (J. T. Baker).

7 Trypsin digestion buffer (25 mM ammonium bicarbonate in 10% acetonitrile).

8 Peptide extraction solution 0.1% trifluoroacetic acid in 33% acetonitrile.

9 Measurement of mass spectra with LTQ Orbitrap XL ETD (Thermo Scientific) controlled by Xcalibur 2.1 (Thermo Scientific) coupled online to Eksigent 2D nanoLC (eksigent AB Sciex), controlled by plug-in in Xcalibur.

10 Spectra processing by ProteomeDiscoverer v1.4 (Thermo Scientific). Database searches by Mascot v2.4 (Matrix Science), result visualization by ProteomeDiscoverer v1.4 and Scaffold v2 (Proteome Software).

2.5 Chemical Hyperacetylation

1. 10% Solution of deuterated (D6) acetic anhydride in 10 mM aqueous trimethylamine.
2. 30% Solution of D6 acetic anhydride in 10 mM aqueous trimethylamine.
3. 25 mM ammonium bicarbonate in HPLC grade water.
4. Acetonitrile:H_2O (7:3, v/v).

3 Methods

3.1 Purification of Acetylated Proteins

3.1.1 Preparation of Whole Cell Extracts

During sample preparation it is challenging to preserve the acetylation status of the proteins. Therefore, the cells are lysed and further processed on ice under highly stringent conditions, using a lysis buffer with a high ionic strength. Importantly, washing and lysis buffer should contain inhibitors against all classes of HDACs. Acetylation sometimes occurs only in response to a certain stimulus, such as a ligand binding to a receptor or an environmental condition, which provoke an activation or repression state of the protein. Therefore, it is necessary to culture and treat the cells under specific conditions. Sometimes, the quantities of the acetylated protein of interest (POI) are below the detection threshold of the mass spectrometer. To enhance the signal quality, acetylation of lysine residues can be amplified by overexpression of distinct HATs and pharmacologic inhibition or genetic depletion (e.g., by siRNA knockdown) of HDACs.

1. Grow HeLa cells in 145 mm diameter dishes to 90% confluence.
2. Transfect cells of one dish with 60 μg of plasmid DNA coding for a FLAG-tagged fusion POI (*see* **Note 5**). Use a nontagged

version of the encoded gene as negative control for transfection of another dish.

3. After 24 h, treat the cells with stimuli activating the POI (e.g., ligand) in combination with 100 nM TSA or 10 mM NAM for up to 24 h (*see* **Note 6**).
4. Aspirate the medium and immediately rinse the cells with ice-cold PBS containing 100 nM TSA and 10 mM NAM.
5. Aspirate the PBS and lyse the cells on the dish with 1.5 ml RIPA containing 100 nM TSA, 10 mM NAM and protease inhibitors on ice for 30 min with occasional shaking. Keep carrying out all further steps on ice unless otherwise specified.
6. Scratch off the lysates with a cell scraper and transfer them to a 1.5 ml tube.
7. Sonicate lysates for homogenization 20 times with the protocol indicated above.
8. Centrifuge lysates at 20,000 × *g* for 30 min, 4 °C.
9. Transfer the supernatant to a fresh tube.
10. Perform a protein quantification assay (BCA or Bradford assay) (*see* **Note 7**).

3.1.2 Immunoprecipitation of FLAG-Tagged Proteins

1. Perform immunoprecipitation (IP) with ANTI-FLAG M2 Affinity Gel agarose, which consists of a purified murine monoclonal antibody against FLAG peptide covalently attached to agarose.
2. Store the beads in PBS with 50% (v/v) glycerol at –20 °C.
3. Wash the bead slurry twice with RIPA buffer by centrifugation at 2000 × *g*, for 1 min, 4 °C.
4. For each IP sample use 1500 µg protein lysate with 30 µl washed beads (50% (v/v) settled beads) and a final volume of 1000 µl (filled-up with RIPA buffer incl. protease inhibitors and HDAC inhibitors).
5. Incubate the samples at 4 °C for 4 h or overnight in a rotator shaker.
6. Wash the beads five times at 2000 × *g*, for 1 min, 4 °C with 1 ml RIPA buffer.
7. Aspirate the residual RIPA buffer with an insulin syringe (100 gauge needle).
8. Resuspend the bead pellet in 30 µl 2× Laemmli buffer (*see* **Note 8**), vortex gently, incubate the beads at 95 °C for 5 min, and centrifuge for 30 s at 20,000 × *g*, room temperature (*see* **Note 9**).

3.1.4 SDS-Polyacrylamid Gel Electrophoresis and CBB Staining

1. Use continuous Tris-glycine SDS gels with the appropriate pore size according to the size of your POI. For casting gels please check reference [26].

2. Assemble the gel in an electrophoresis chamber (Bio-Rad) and fill up with SDS-running buffer (*see* **Note 10**).
3. Load the supernatant of the heated samples into the gel slots with a Hamilton syringe without transferring any beads (*see* **Note 11**).
4. Separate the proteins with a constant current of 20 mA until full resolution.
5. Wash the gel once with distillated water for 10 min to reduce SDS background and stain the proteins with colloidal CBB for 2 h at room temperature with gentle shaking.
6. Wash the gel twice with distillated water and incubate it with destaining solution for 1 h (*see* **Note 12**).
7. If the gel is not fully destained, replace staining solution with deionized water and heat it in the microwave at 800 W until boiling (*see* **Note 13**).
8. Repeat **step 7** if necessary.
9. Place the gel onto an illumination desk and excise the spot containing your POI (estimated by size) with a clean scalpel.
10. Cut the spot to pieces of an approximate size of 1 mm^3 and transfer the pieces with forceps into a fresh tube in 100 µl deionized pure water.
11. The gel dices can be stored for several weeks at 4 °C.
12. Often, the protein yield from one gel piece will be sufficient for digestion with one selected protease and subsequent MS analysis.

3.2 Sample Preparation for MS

3.2.1 In-Gel Reduction, Alkylation, and Destaining of Proteins

1. Destain the gel piece with 100 µl of acetonitrile:H_2O (7:3, v/v) for 10 min, remove the supernatant, and incubate the gel piece in 100 µl deionized water for 10 min. Remove the supernatant and repeat the previous steps two more times.
2. To reduce and alkylate cysteine residues in the proteins, incubate the dried sample first with 50 µl of 10 mM DTT for 45 min at 60 °C. Remove the supernatant and add 50 µl of 50 mM iodoacetamide and incubate for 45 min in the dark.
3. Wash the sample three times with acetonitrile:H_2O (7:3, v/v) and with deionized water as done in **step 1**.
4. Air-dry the gel piece and keep it in a closed tube. The dried gel piece is very small and often electrostatically charged and has therefore to be handled carefully.

3.2.2 In Gel Digest

1. Digest your protein samples with trypsin or alternatively with other proteases (*see* **Notes 14** and **15**) such as chymotrypsin, Asp-N, or Glu-C.
2. For trypsin digestion, prepare a solution with 2.5 µg/ml of the enzyme dissolved in 1 mM HCl.

3. Add 30 µl of the trypsin solution to the dried samples and incubate at 0–4 °C for >30 min.
4. Completely remove the left supernatant and add 30 µl of digestion buffer (10% acetonitrile, 25 mM NH_4HCO_3) and digest overnight at 37 °C.
5. Transfer the supernatant to a fresh tube and wash the residual samples in the following order:
   (a) 30 µl 25 mM NH_4HCO_3 for 10 min,
   (b) 70 µl 0.1% trifluoroacetic acid in 33% acetonitrile for 10 min, and
   (c) 150 µl acetonitrile:H_2O (7:3, v/v) for 10 min.

   After each step, collect the supernatant and pool it together with the first supernatant (after proteolysis).
6. Concentrate the samples by vacuum drying to a volume of less than 10 µl.
7. The digestion with chymotrypsin or with a combination of trypsin and chymotrypsin can essentially be performed as described in **steps 1–5** (*see* **Note 16**). The only alteration is the replacement of the 25 mM ammonium bicarbonate buffer by 50 mM Tris–HCl, 5 mM $CaCl_2$ (pH 8.0) in the digestion buffer because chymotrypsin requires the presence of Ca^{2+} ions for its full activity.

3.2.3 MS Analysis

1. Dissolve the dried peptide extracts in 25 µl HPLC-buffer A (5% acetonitrile in 0.1% formic acid) and incubate for 10 min (*see* **Note 15**).
2. The applied nanoLC method permits the use of two columns. The first column (5 µm C18 packing, 100 µm × 3 cm, NanoSeparations) is used with an aqueous buffer (0–5% acetonitrile) as an offline trapping column that binds the peptides but does not bind highly polar components such as salts or urea. After a wash step of 15 min with a flow of 5 µl/min, the trapping column is switched by an appropriate combination of microvalves in-line with a separation column.
3. Peptide separation is achieved using columns filled with C18 packings (either 75 µm × 10 cm, 3 µm, or 75 µm × 20 cm, 5 µm, both NanoSeparations) with a gradient from 5 to 40% acetonitrile in 0.1% formic acid.
4. The nano-HPLC device is coupled to an ESI-mass spectrometer (LTQ Orbitrap XL ETD, Thermo Scientific).
5. The mass spectrometer is set to record a full scan spectrum from 300 to 2000 Da (*see* **Note 16**) followed by the generation of three MS/MS spectra by CID (collision-induced dissociation) of selected precursor ions (Xcalibur triple play). Dynamic exclusion of 30 s of previously fragmented ions is used to reduce the redundancy of abundant precursors.

6. For annotation of peptide sequences to the MS/MS spectrum the software Proteome Discoverer (v1.4, Thermo Scientific) was used in combination with the Mascot (v 2.4, Matrix Science) search engine and the Swiss-Prot database (http://www.uniprot.org/downloads).
7. In setting up the database searches by Mascot the following parameters were used.
   - The accuracy of the precursor masses is set to 10 ppm and the accuracy of the fragment masses in MS/MS experiments to 0.5 Da. In general, the spectra used for the peptide identification and characterization of modification states were within a range of 4 ppm mass deviation.
   - When using single enzyme digests, the number of allowed missed cleavages should be set to the value 2.
   - When using enzyme combinations this value is limited to 1. Alternatively, trypsin cleavage only at one peptide terminus and unspecific cleavage at the other terminus (semitrypsin specificity) was used in combination with 1 allowed missed cleavage in some cases but without significant gain of information.
   - Carbamidomethylation of Cys was set as a static modification.
   - Oxidation of Met, acetylation of the protein N-terminus and of Lys residues, and phosphorylation of Ser and Thr were allowed as dynamic modifications.

3.3 Chemical Hyperacetylation

To verify the findings of the explorative mass spectrometric analyses, purified protein samples can be chemically hyperacetylated in vitro using deuterium (D6) acetic anhydride. Use the deuterated compound to facilitate the simultaneous detection of enzymatic acetylation. Determine the suitable degree of chemical hyperacetylation for each set of samples by experiments with different amounts of alkylation reagent and/or different time scales of the reaction (*see* **Notes 14** and **17**).

1. Prepare the gel pieces as described in Subheading 3.2.1.
2. Add 50 µl of the selected solution (e.g., 10% or 30%) of D6 acetic anhydride in 10 mM trimethylamine to one dried gel piece and incubate for up to 2 h at room temperature. Add the different concentrations of D6 acetic anhydride to the other gel pieces.
3. Remove the supernatant completely and wash the gel pieces with 25 mM ammonium bicarbonate.
4. Remove the supernatant completely and wash the gel pieces with acetonitrile:water = 70:30 (v/v).
5. Repeat **steps 3** and **4** at least four times.

6. Digest the proteins in the gel bands as described in Subheading 3.2.2.
7. Perform the MS analyses as described in Subheading 3.3.
8. Include acetyl and 3D acetyl as a variable modification of lysine residues and exclude any other variable modification except methionine oxidation.
9. Increase the allowed number of missed cleavages stepwise and perform database searches.
10. Exclude hits with assigned acetylation of C-terminal lysine residues from your hit list as they are chemically meaningless and frequently related to low quality spectra.
11. Compare the changes of peptide distribution at different conditions and extract the changes related to the alteration of the extent of acetylation.

With the progression of the chemical acetylation reaction the number of peptides with missed cleavage sites increases. The peptides containing deuterated acetyl groups can be used to determine the retention times of candidate peptides modified enzymatically in vivo. The mass difference of the peptide precursors allows the simultaneous detection of enzymatically and chemically acetylated peptides in the MS1 spectra. The ion series of the fragmented peptides are similar when considering the different isotope composition and support the correct localization of actual sites of modification.

4 Notes

1. Keratin contamination and certain chemical compounds interfere with the signal quality of the sample during MS analysis. Therefore, it is important to wear a lab coat and gloves throughout the protocol. Rinse the gloves occasionally with ethanol as they readily accumulate static charge and attract dust and pieces of hair and wool. Perform all operations in a dust-reduced environment (ideally in a laminar flow hood) and always use cleaned pipettes, filter-tips, tubes (Safe-lock Eppendorf Tubes). Do not use polymeric detergents (e.g., Triton X-100, Tween 20, etc.) for cleaning flasks and glass plates for electrophoresis.
2. Safe-lock Eppendorf tubes consistently yield low chemical background when used for MS sample preparation. Some plastic tubes from certain providers release contaminant ions, such as polymers of polypropylene glycol (PPG) or polyethylene glycol (PEG) and slip agents (e.g., erucamide) to the sample during the preparation process. Such ions can strongly interfere with the peptide spectra of the POI [27]. Of course, we do not want to state that Safe-lock Eppendorf tubes are the only tubes that can be used.

3. ANTI-FLAG M2 affinity gel should be stored in 50% glycerol at −20 °C for maximum stability. After constitution, the resin should be washed and stored in 50% glycerol with PBS containing 0.02% sodium azide to prevent microbial growth. Do not freeze in the absence of glycerol.
4. The staining procedure is carried out with colloidal CBB G-250. When preparing the staining solution the order of adding of each ingredient is crucial. Keep the order as indicated. Shelf life at room temperature is approximately 4 months. This staining solution is highly sensitive (>1 ng protein) and does not contain toxic methanol. Protein staining is visible after 30 min and is normally finished after 4 h. Destaining can often be omitted due to absent background. Do not filter the staining solution. Shake the solution thoroughly before use to homogenize the colloidal particles.
5. The FLAG epitope recognition by the FLAG antibody creates a strong binding that is suitable for protein isolation for biochemical analysis. However, other protein fusion tags as His6, HA, or V5 might also be compatible for this protocol.
6. It is preferable to prepare a stock of 100 mM TSA dissolved in DMSO and a 1 M aqueous stock solution of nicotinamide. Both stocks should be stored at −80 °C. Before starting the experiment, it is recommended to test the activity of the HDACi stocks. This can be done by analyzing global histone acetylation (acetylated-lysine antibody, Cell Signaling Technology). However, the positive result of this test does not ensure the preservation of protein acetylation under IP conditions.
7. Different compounds of the cell lysis buffer considerably affect the accuracy of protein quantification assays. The BCA assay is tolerant up to 5% SDS while the Bradford assay is only reliable for SDS concentrations up to 0.125%.
8. Alternatively, POI can be eluted with 3× FLAG peptide with high efficiency. For this, prepare 3× FLAG elution solution (TBS: 50 mM Tris–HCl, pH 7.4, with 150 mM NaCl). Dissolve 3× FLAG peptide (Sigma Aldrich) in 0.5 M Tris–HCl, pH 7.5, with 1 M NaCl at a concentration of 25 mg/ml. Dilute fivefold with water to prepare a 3× FLAG stock solution containing 5 mg/ml of 3× FLAG peptide. For elution, add 3 ml of 5 mg/ml of 3× FLAG peptide stock solution to 100 ml of TBS (150 ng/ml final concentration). Add 100 µl of 3× FLAG elution solution to each sample and control resin. Incubate the samples and controls with gentle shaking for 30 min at 2–8 °C. Centrifuge the resin for 30 s at 5000–8200 × *g*. Transfer the supernatants to fresh tubes.
9. The Laemmli sample buffer contains high concentrations of SDS. Cooling of the samples at 4 °C or on ice will cause precipitation of SDS. Avoid heavy vortexing of the beads as they will attach to the tube wall.

10. If possible load and run the SDS PAGE under a sterile hood.
11. When loading the IP samples onto the gel leave one slot empty between each lane. This will prevent cross contamination when excising the protein spots. Fill the empty slots with the same amount of 1× Laemmli sample buffer. This will ensure equal and linear migration of the proteins through the gel.
12. This washing step will reduce the background caused by residual, nonincorporated SDS from the gel.
13. Prevent long boiling of the water as this will harm the structure of the gel.
14. Trypsin cleaves the peptide bond in proteins at the C-termini of unmodified arginine and lysine residues at the position P1. Because the side chain acetylation renders the lysine bond resistant to cleavage by trypsin, the result of the acetylation will be an altered cleavage pattern generating longer peptide fragments compared to nonacetylated samples. The change of the pattern as well as the alteration of the detected peptide amounts can be used as an indicator for the localization of the acetylated side chain within the protein chain. In many cases, the tryptic peptides of the acetylated protein molecules may become very large and, therefore, can elude the mass spectrometric analysis. The parallel use of other proteases or of a combination of proteases, such as trypsin and chymotrypsin, generates different or additional peptides, which compared to a sole digestion by trypsin allow a more comprehensive characterization of the protein sequence and conclusions about the occurrence and the position of acetylated lysine residues within the protein of interest. This alternative digestion can be performed with a fresh gel piece (prepared according to Subheading 3.2.1) as described above for the trypsin digestion. In some cases, it might be useful to treat the gel piece already digested with trypsin by another protease or protease combination because very large peptides can resist the extraction procedure. As an additional option, the extracted tryptic peptides can be further digested with chymotrypsin in solution. Depending on the sequence of the POI the application of the additional protease might increase the number of peptides that are detectable by the mass spectrometric analyses. However, make sure that the pH value of the dissolved tryptic peptides is in the appropriate range of about 8.0 and that the right buffer is used.
15. The volumes may differ according to the injection scheme of the applied mass spectrometric analysis template.
16. The given range is useful if the investigated proteins contain lysine/arginine clusters that may yield very small tryptic peptides together with rather long ones. Studying other proteins of interest, the applied mass limits can be set to more appropriate m/z values.

17. The suitable concentration of the alkylation agent and the incubation time are dependent on the individual protein and on the gel used and have to be determined individually. In general, the use of concentration series or time series with different gel pieces from the same gel band increases the chance to find multiple combinations of acetylation sites for the same part of the protein.

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Chapter 24

Assessing the Role of Paralog-Specific Sumoylation of HDAC1

Simona Citro and Susanna Chiocca

Abstract

Attachment of ubiquitin or ubiquitin-like (Ubl) modifiers, such as the small ubiquitin-related modifier SUMO, is a posttranslational modification (PTM) that reversibly regulates the function and the stability of target proteins. The SUMO paralogs SUMO1 and SUMO2/3, although sharing a common conjugation pathway, seem to play different roles in the cell. Many regulatory mechanisms, which contribute to SUMO-paralog-specific modification, have emerged. We have recently found that cell environment affects SUMO-paralog-specific sumoylation of HDAC1, whose conjugation to SUMO1 and not to SUMO2 facilitates its protein turnover. Here, we describe how to identify SUMO-paralog-specific conjugation of HDAC1 and how the different expression of SUMO E3 ligases in the cell plays an important role in this mechanism.

Key words HDAC1, HDAC, SUMO1, SUMO2/3, PTM, PIASy

1 Introduction

Histone deacetylase 1 (HDAC1) is an important epigenetic regulator, which changes nucleosomal conformation in both transformed and nontransformed cells. It belongs to the human class I HDACs together with HDAC2, HDAC3, and HDAC8, which are often overexpressed in various types of cancers, compared to the corresponding normal tissues [1, 2]. The activity and expression of HDAC1 is regulated by PTM, such as phosphorylation, sumoylation, and ubiquitination [3–5].

Ubiquitin (Ub) PTM plays a central role in targeting proteins for proteolytic degradation by the proteasome, but recent findings show that also SUMO, mainly involved in the regulation of nuclear processes [6], can act as a signal for ubiquitination and proteasomal degradation [7]. Covalent conjugation of Ub or ubiquitin-like modifiers, such as SUMO, to a lysine residue of their substrates requires an analogous enzymatic cascade comprising the sequential action of three enzymes [8]: a modifier activating enzyme (E1),

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one modifier carrier enzymes (E2s), and a member of the large group of ligases (E3s). The ubiquitin pathway presents several E2 enzymes, whereas only one, the UBC9 conjugating enzyme, has been identified so far for the sumoylation machinery. Moreover, E3 enzymes, necessary to carry out the final step of the ubiquitination cascade, are dispensable in the conjugation of SUMO to the target protein and they play a role in the SUMO isoform-specific conjugation of particular targets.

Human tissues ubiquitously express three SUMO family members, SUMO1, and the nearly identical paralogs SUMO2 and SUMO3, which can selectively modify different target proteins [9, 10]. HDAC1 is sumoylated on two lysine residues, K444 and K476 and this modification affects the function and the expression of the enzyme [4, 11]. In particular, we have recently described how the different expression of SUMO E3 ligases among cancer (MCF7) and nontumorigenic (MCF10A) mammary epithelial cell lines contributes to paralog-specific HDAC1 sumoylation, affecting HDAC1 protein levels [12]. Specifically, the SUMO E3 ligase PIASy, which is overexpressed in breast cancer cells, selectively promotes the conjugation of HDAC1 to SUMO2. As a result, HDAC1 is more conjugated to SUMO2 in MCF7 cells and more conjugated to SUMO1 in MCF10A cells. Finally, we showed that modification of HDAC1 by SUMO1, and not by SUMO2, facilitates HDAC1 ubiquitination and degradation.

This chapter describes the detection of SUMO-paralog-specific modification of HDAC1 and how this modification can be specifically modulated by the PIASy ligase. Although SUMO modification can have a great effect, it is typically conjugated only to a small fraction of the target protein. Thus, detection of the endogenously modified target protein can be difficult [13]. Here, we show how to detect endogenously modified HDAC1, by immunoprecipitating HDAC1 from the cell lysate and subsequent immunoblotting using anti-SUMO antibodies. We also show how to increase the amount of endogenously modified HDAC1 by overexpressing SUMO and the E2 conjugating enzyme UBC9. Finally, we show how overexpression of the PIASy ligase specifically modulates SUMO2 conjugation to HDAC1. We describe how to prepare lysates from mammalian cells in denaturing lysis buffer to block the action of deconjugating enzymes as well as all the methods and best settings to detect SUMO modification for endogenous or overexpressed HDAC1. This chapter describes the detection of HDAC1 in mammalian cells and the same protocol can be used to detect sumoylation of other target proteins.

2 Materials

2.1 Cell Culture

1. Hela, MCF7, and MCF10A cell lines (American Type Culture Collection).
2. 100 mm tissue culture dishes.
3. Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics, 2 mM L-glutamine and 10% Fetal Bovine Serum (FBS) for culturing Hela and MCF7 cells.
4. F-12 Dulbecco's modified Eagle's medium (1:1) supplemented with horse serum, 20 ng/ml epidermal growth factor, 0.5 μM hydrocortisone, 50 ng/ml cholera toxin, 10 μg/ml insulin, and antibiotics for culturing MCF10A cells.
5. Trypsin-EDTA.
6. Sterile Phosphate Buffered Saline (PBS).

2.2 Transfection

1. DMEM with L-glutamine, without FBS and antibiotics.
2. Fugene® 6 transfection reagent (Promega).
3. Plasmid DNA: pcDNA3 HA-SUMO1 (described in [14]; pcDNA3 HA-SUMO2 (described in [15]); pcDNA3 UBC9 (described in [4]); pCI-neo MYC-HDAC1 and MYC-HDAC1K444/476R (described in [4]); pcDNA3.1 FLAG-PIASy (described in [12]) and pcDNA3 empty vector.

2.3 Cell Lysis

1. SDS lysis buffer: solution I: 5% SDS, 0.15 M Tris–HCl pH 6.8, 30% glycerol, stored at RT; solution II: 25 mM Tris–HCl pH 8.3, 50 mM NaCl, 0.5% NP40, 0.5% deoxycholate, 0.1% SDS, stored at 4 °C.

2.4 Immunoblotting

1. Resolving gel buffer: 1.5 M Tris–HCl, pH 8.8.
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8.
3. 30% Acrylamide/Bis solution.
4. Ammonium persulfate (APS): 10% solution in water (*see* **Note 1**).
5. *N,N,N,N′*-tetramethyl-ethylenediamine (TEMED).
6. SDS-PAGE running buffer: 2 M glycine, 0.25 M Tris–HCl pH 8.3, 0.02 M SDS.
7. Benchmark prestained molecular weight standards.
8. Immobilon® Polyvinylidene difluoride (PVDF) membrane (for example from Millipore).
9. Western blot transfer buffer: 0.025 M Tris, 0.192 M glycine and 20% methanol.
10. Tris buffered saline (TBS; 1×): 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4; TBST is TBS containing 0.1% Tween-20.

11. Blocking solution: 5% low-fat milk in TBST.
12. Enhanced chemiluminescence (ECL) solutions.
13. 5× Loading buffer: 312 mM Tris–HCl pH 6.8, 10% SDS, 40% glycerol, 20% β-mercaptoethanol, spatula tip Bromophenol blue.
14. Antibodies: monoclonal anti-HDAC1 (clone 2E10) and anti-MYC (06-549, Millipore), anti-SUMO1 (S8070) and anti-FLAG M2 (Sigma), anti-GMP1 (Gap Modifying Protein 1, SUMO-1 (33-2400, Thermofisher), anti-SUMO2/3 (Ab22654) and anti-HDAC1 rabbit polyclonal (Ab7028, Abcam), anti-HA mouse monoclonal (clone 16B12, Covance), anti-UBC9 rabbit polyclonal (sc-10759, Santa Cruz).
15. Blot Stripping solution: 62.5 mM Tris–HCl, pH 6.7, 100 mM β-mercaptoethanol and 2% SDS.

2.5 Immunoprecipitation

1. E1A lysis buffer: 50 mM HEPES pH 7, 250 mM NaCl, 0.1% NP-40, stored at RT.
2. Protein-A/sepharose-beads.
3. 2.5× Loading buffer: 156 mM Tris–HCl pH 6.8, 5% SDS, 20% glycerol, 10% β-mercaptoethanol, spatula tip Bromophenol blue.

3 Methods

3.1 Transfection of Human Cell Lines HeLa, MCF7 and MCF10A (see Note 2)

1. Plate cells the day before transfection in 100 mm tissue culture dishes in order to obtain a 60% of confluence the day of transfection.
2. For each transfection sample prepare one tube of transfection reagent: add 24 μl of Fugene® 6 in 600 μl of serum and DMEM without antibiotics and incubate at RT for 5 min. Add 5–10 μg of each DNA to the solution and incubate 20 min at RT to promote the formation of membrane delimited vesicles containing DNA.
3. When waiting for the complex to form, remove old media from cells, wash once with PBS 1× and add 5 ml of serum and DMEM without antibiotics.
4. Add the mixed DNA/Fugene® 6 solution dropwise on the cells.
5. Incubate at 37 °C in a 5% CO_2 incubator for 6 h.
6. Replace with fresh complete media.
7. Incubate at 37 °C in a 5% CO_2 incubator for 24 h.

3.2 Cell Lysis

1. Collect cells by trypsinization and wash them in cold PBS containing SUMO and ubiquitin protease inhibitor: 5 mM *N*-Ethylmaleimide (NEM) (*see* **Note 3**).
2. Lyse cells under denaturing condition using an SDS containing lysis buffer composed of one volume of buffer I and 3 volumes of buffer II. Add protease inhibitors: 100 μg/ml Phenyl-Methyl-Sulfonyl Fluoride (PMSF), 1 μg/ml leupetin, 1 μg/ml aprotinin (*see* **Note 4**), and 5 mM NEM (*see* **Note 5**).
3. Incubate lysates on ice for 15 min.
4. Sonicate each sample on ice using probe sonifier for 20 s (*see* **Note 6**).
5. Centrifuge for 10 min at 10,000 × *g* at 4 °C and transfer supernatants to clean tubes.
6. Use a protein quantitation assay (Bradford or Lowry) to determine the protein concentration of each sample.
7. Dilute sample using equivalent amount of proteins in 5× loading buffer for immunoblotting or use lysates for immunoprecipitation (*see* **Note 7**).

3.3 Immunoprecipitation

1. Dilute SDS cell lysates containing an equivalent amount of proteins 1:4 in E1A lysis buffer, containing protease inhibitors: 100 μg/ml Phenyl-Methyl-Sulfonyl Fluoride (PMSF), 1 μg/ml leupetin, 1 μg/ml aprotinin (*see* **Note 4**), and 5 mM NEM (*see* **Note 5**).
2. Incubate diluted cell lysates with primary antibody overnight at 4 °C on a rotating wheel (*see* **Note 8**).
3. Add 40 μl of protein-A/sepharose-beads (50% slurry) to the samples for 1 h at 4 °C on a rotating wheel (*see* **Note 9**).
4. Abundantly wash beads for five times with E1A lysis buffer, spinning down samples for 1 min at 1000 × *g*.
5. Remove lysis buffer and add 20 μl of 2.5× loading buffer to the beads.

3.4 Immunoblotting

1. Prepare a 8% PolyAcrylamide gel composed by a resolving portion (Acrylamide/Bis solution, Resolving solution, 0.1% APS and TEMED) and a stacking portion (Acrylamide/Bis solution, Stacking solution, 0.1% APS and TEMED) (*see* **Notes 10** and **11**).
2. Heat samples at 95 °C for 5 min.
3. Load sample on the gel together with a prestained molecular weight standard.
4. Run gel in Running buffer at 100 mV until samples have entered the resolving gel and then continue at 180 mV until the dye front has come out from the gel.

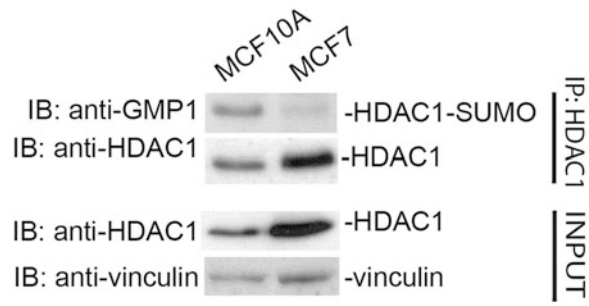

Fig. 1 Detection of endogenously sumoylated HDAC1 in mammary cells. MCF7 and MCF10A cells were lysed in SDS lysis buffer and lysates were subjected to immunoprecipitation (IP) using an anti-HDAC1 polyclonal antibody. Immunoblotting (IB) was then performed: expression of immunoprecipitated HDAC1 was detected using a monoclonal anti-HDAC1 antibody, sumoylated HDAC1 was detected on immunoprecipitated samples using an anti-GMP1/SUMO1 antibody. Whole cell extracts were used as input control

5. Transfer protein from the gel to a PVDF membrane at 250 mA for 1 h in Transfer buffer using a wet transfer apparatus (*see* **Note 12**).
6. Incubate membrane in blocking solution for 1 h at RT.
7. Incubate with primary antibody diluted in 5% milk/TBST overnight at 4 °C.
8. Wash membrane three times for 5 min with TBST.
9. Incubate with secondary antibody diluted in 5% milk/TBST for 1 h at RT.
10. Wash membrane three times with TBST.
11. Develop the membrane using ECL according to manufacturer's instructions (*see* **Note 13**). The results of the immunoblotting analysis are shown in Figs. 1, 2, and 3 (*see* **Note 14**).

4 Notes

1. APS can be dissolved in water and aliquots can be stored at −20 °C.
2. Hela cells can be efficiently transfected using also Calcium Phosphate transfection procedure. MCF7 and MCF10A can be also transfected using Lipofectamine® 2000 (Life Technologies), but we noticed an increased cell death using this reagent compared to Fugene® 6.
3. NEM is a SUMO and ubiquitin protease inhibitor used in the buffers to avoid deconjugation of sumoylated proteins.

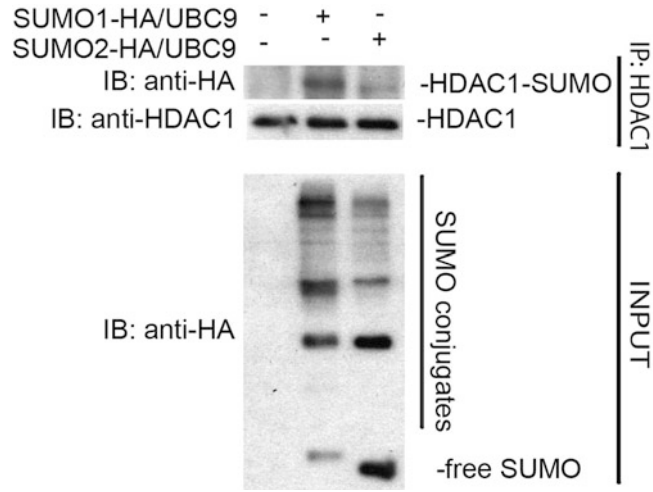

Fig. 2 Paralog-specific sumoylation of HDAC1. Hela cells were cotransfected with HA-SUMO1 or HA-SUMO2 and UBC9. Cells were lysed in SDS lysis buffer and lysates were subjected to immunoprecipitation (IP) using an anti-HDAC1 polyclonal antibody. Immunoblotting (IB) was then performed: expression of immunoprecipitated HDAC1 was checked using a monoclonal anti-HDAC1 antibody, sumoylated HDAC1 was detected on immunoprecipitated samples using an anti-HA antibody. Whole cell extracts were used as input control

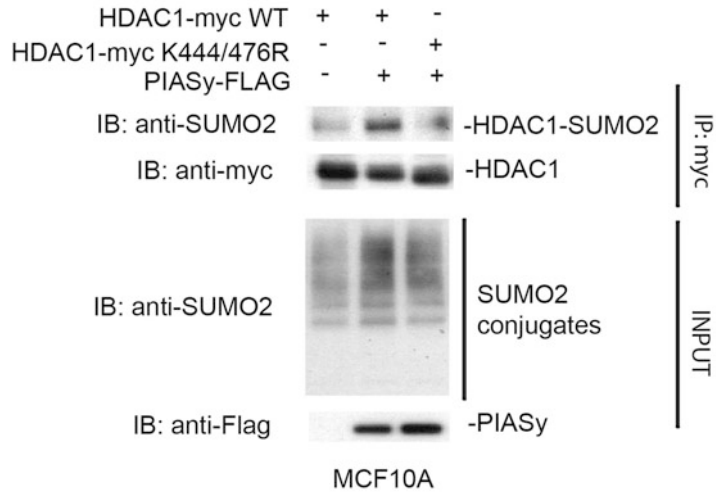

Fig. 3 Effect of PIASy expression on the conjugation of SUMO2 to HDAC1. MCF10A cells were cotransfected with wild-type MYC-tagged HDAC1 or the MYC-HDAC1 K444R/K476R mutant together with FLAG-PIASy. Cells were then lysed in SDS lysis buffer and lysates were subjected to immunoprecipitation (IP) using an anti-MYC polyclonal antibody. Immunoblotting (IB) was then performed: expression of immunoprecipitated HDAC1 was checked using a monoclonal anti-MYC antibody, sumoylated HDAC1 was detected on immunoprecipitated samples using an anti-SUMO2 antibody, and anti-FLAG antibody was used to detect PIASy expression. Whole cell extracts were used as input control

4. The inhibitors are freshly added to the buffers. A commercially available protease inhibitor cocktail for mammalian cell extract can be used instead.
5. 0.5 M NEM is prepared in absolute ethanol, stored at −20 °C, and freshly added to the buffers.
6. Due to the high concentration of SDS in the lysis buffer, lysates appear very viscous.
7. β-Mercaptoethanol can be replaced by dithiothreitol (DTT), used at a final concentration of 0.04 M in 1× loading buffer.
8. To perform immunoprecipitation of endogenous HDAC1 3–5 mg of lysates should be used to pull down enough protein to detect endogenous SUMO modification (Fig. 1); in the case where either SUMO or HDAC1 are overexpressed around 0.5 μg is enough (Figs. 2 and 3 respectively).
9. Protein-A/sepharose-beads usually come packaged in 20% ethanol: wash the desired amount of beads in E1A lysis buffer 3 times, spin down beads for 1 min at 1000 × *g* and resuspend them in an equal amount of lysis buffer.
10. HDAC1's molecular weight is around 60 kDa, the addition of one SUMO protein covalently bound to HDAC1 increases the size of the protein in a range of 15–17 kDa for the single modification and around 30 kDa for the double modification.
11. To detect SUMO conjugating proteins, which have multiple sizes, together with free SUMO a 15% acrylamide gel is necessary (Fig. 2).
12. PVDF membranes are preferred compared to nitrocellulose membranes. PVDF has to be activated by soaking it in pure methanol. After a minute or less of incubation, methanol is removed and the membrane is washed once in TBST. Do not let the membrane dry. It is preferred not to stain the membrane with Ponceau after transfer.
13. Detection can be done by the preferred method.
14. After the incubation and detection with the first antibody (anti-HDAC1), the membrane can be stripped using a blot stripping solution and probed with other antibodies (e.g., anti-FLAG and anti-tubulin, as shown in figures).

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Chapter 25

Analysis of Histone Deacetylases Sumoylation by Immunoprecipitation Techniques

Tobias Wagner, Maren Godmann, and Thorsten Heinzel

Abstract

Histone deacetylases (HDACs) are controlling dynamic protein acetylation by removing acetyl moieties from lysine. Histone deacetylases themselves are regulated on the posttranslational level, including modifications with small ubiquitin-like modifier (SUMO) proteins. Detecting SUMO modifications of deacetylases by immunoblotting is technically challenging due to the typically low ratio of the modified compared to the unmodified species. Here, we describe a set of methods for the detection of endogenous sumoylated HDACs by immunoprecipitation and immunoblotting techniques.

Key words Epigenetics, HDAC, Histone deacetylases, Immunoblotting, Immunoprecipitation, Posttranslational modification, SUMO, Sumoylation

1 Introduction

Acetylation is important for intracellular signaling, epigenetic mechanisms, chromatin accessibility, and the regulation of gene expression [1]. Acetylation of lysine residues is a very dynamic posttranslational modification (PTM) that is regulated by the action of lysine acetyl transferases and lysine deacetylases [2]. Deacetylases are traditionally referred to by their first discovered targets—histones—and hence called histone deacetylases (HDACs). The deacetylase enzymes in mammals are subdivided into four classes based on homology to the yeast enzymes Rpd3, Hda1, and Sir2 [3]. The eleven human enzymes related to Rpd3 and Hda1 are termed the "classical family" of HDACs and are further subdivided into class I (HDAC1-3 and HDAC8), class IIa (HDAC4, 5, 7, and 9), class IIb (HDAC6 and HDAC10), and class IV (HDAC11). The seven human Sir2-related HDACs, which are named Sirtuins (SIRT1-7), use a distinct catalytic mechanism and comprise class III.

The activity of deacetylases can be regulated at the posttranslational level. Accordingly, HDACs are subject to numerous posttranslational modifications [4, 5], including modification with small

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ubiquitin-related modifier (SUMO) proteins at lysine residues. Sumoylation occurs in an enzymatic cascade of three enzymes (E1-3). After an ATP-dependent activation of SUMO by the E1 activating enzyme dimer SAE1/UBA2, the C-terminal carboxyl group of SUMO is coupled to a cysteine residue on the E2 conjugating enzyme UBC9 to form a thioester. SUMO is then directly transferred from UBC9 to the ε-amino group of lysine residues on target proteins [6]. In vivo, E3 ligases provide specificity to direct the E2 enzyme toward its targets and enhance sumoylation efficiency [6]. In vitro, the E3 enzyme might not be required for the transfer of SUMO [7]. By the activity of desumoylating isopeptidase enzymes (sentrin-specific proteases, SENPs), the modification with SUMO is reversible and highly dynamic in vivo [8, 9]. Four SUMO isoforms exist in mammals, with SUMO1-3 being ubiquitously expressed. While mature SUMO2 and SUMO3 are 97% identical in amino acid sequence, SUMO1 shares around 50% homology with them. A distinctive feature of sumoylation is that SUMO2/3 can form intermolecular chains similar to ubiquitin, while in vivo SUMO1 is attached to its target exclusively as a monomer. The majority of SUMO1 in cells is attached to its target proteins, while a substantial pool of unconjugated SUMO2/3 exists. SUMO4 is mostly expressed in kidney, spleen, and lymph nodes and is less characterized compared to the other three isoforms [6, 7].

In the last years, the use of mass spectrometry techniques has greatly facilitated the identification of SUMO modification sites and the analysis of sumoylation dynamics [10]. Mass spectrometry (MS) methods to map sumoylation sites have constantly evolved in the last years [11–14]. The introduction of point mutations in SUMO that generate smaller tryptic fragments in MS together combined with knock-in tags on endogenous SUMO genes enables the purification of sumoylated proteins and global analysis of sumoylation patterns from cell culture and mouse tissues [15–17]. Recently, an elegant proteomic technique for the first time allowed the system-wide identification of the SUMO2-modified proteome without mutated SUMO [18]. When used together with a method to covalently couple SUMO1- or SUMO2/3-specific antibodies during immunoprecipitation, this is a powerful technique to globally analyze endogenous sumoylation patterns from tissue or cell culture material [19].

To date, eight of the 18 deacetylases in mammals have been identified as targets of sumoylation (Table 1). The consequences of sumoylation are diverse for the individual HDACs. For HDAC1, HDAC4, and SIRT1 disruption of sumoylation reduces their catalytic activity. Modification of HDAC1 with either SUMO1 or SUMO2/3 results in paralogous-specific regulation of HDAC1 stability [20]. The adenoviral protein Gam1 reduces HDAC1 sumoylation through interference with E1 enzyme activity [21]. Sumoylation can alter the interactome of its target proteins by

Table 1
List of SUMO modified human histone deacetylases

	Site(s)	SUMO isoform	Reference
HDAC1	*K90*, K444, *K456*, K476	SUMO1, 2/3[a]	[16, 20, 34]
HDAC2	K462, *K481*	SUMO1	[16, 22]
HDAC4	K559	SUMO1, 2/3[a,b]	[35]
HDAC5	*K35, K533*	SUMO1	[16]
HDAC6	nd	nd	[35]
HDAC7	*K160, K382*	SUMO1	[16]
HDAC9	nd (exon 12)	SUMO1, 2/3[a]	[36]
SIRT1	K734	SUMO1	[23]

nd not determined (site/isoform not identified); sites in *italic* have been identified solely with mass spectrometry [16]
[a]SUMO2 was tested
[b]In vitro

blocking surface exposure of binding sites or adding additional interaction surfaces. The sumoylation of HDAC2 and SIRT1 both attenuate the activity of p53 by targeting distinct p53 acetylation sites [22–24]. HDAC2 modification with SUMO further increases NF-kB transcriptional activity [25]. Of note, a deacetylase independent role of HDAC2 and HDAC4 is discussed for enhancing the sumoylation of their interaction partner eIF4 and the androgen receptor, respectively [26, 27]. For HDAC5 and HDAC7 sumoylation sites were identified in a proteome-wide screen after heat stress [16]. The same data also point to additionally sumoylated lysine residues in HDAC1 and HDAC2 after heat stress (Table 1), the functional role of which has yet to be uncovered.

There are several ways to investigate deacetylase sumoylation by immunoblotting. In principle, sumoylation can already be detected in western blots of crude cell lysates. Modification with SUMO increases the apparent molecular weight of its targets in western blots around 15–20 kDa (*see* **Note 1**). Making use of this, mono-sumoylation appears as a single higher migrating species when probing for the target deacetylase (*see* **Note 2**). Multi-mono-sumoylation and poly-sumoylation appear as a ladder of bands that eventually might turn into a smear. However, the interpretation of these bands at higher molecular weight strongly depends on the quality of the antibody, and it might be difficult if the used antibody cross-reacts with other proteins or when unspecific signals occur in the molecular weight range where sumoylated HDACs migrate.

A major disadvantage of the "simple" detection of higher migrating bands in crude lysates is the inability to distinguish between SUMO (isoforms) and other modifications of the

ubiquitin-like protein family, including ubiquitin itself. They all result in a shift toward slower migrating bands. Especially at higher molecular weights, the shift size might not be identified correctly. Furthermore, splice variants with different molecular weights can be misinterpreted as a modified protein species. Thus, combinations of immunoprecipitation (IP) and immunoblotting techniques enable a more specific detection of SUMO-modified proteins [28]. Three different combinations are generally applicable and can be used to detect sumoylation of deacetylases. One option is an IP against SUMO1 or SUMO2/3 with probing for the deacetylase of interest, which shows the fraction of sumoylated deacetylase among all the sumoylated proteins. Alternatively, IP of the deacetylase and probing for a specific SUMO paralogue variant visualizes the SUMO-modified fraction of this specific deacetylase. Lastly, an IP for the deacetylase of interest followed by probing or for the immunoprecipitated deacetylase itself can be useful to visualize higher migrating bands with less background or unspecific bands than in crude lysates. Which IP setup works best has to be determined empirically.

2 Materials

All buffers should be prepared using DEPC-treated and autoclaved deionized H_2O. For RIPA and NETN buffers the inhibitors should be added freshly directly before use, as some have a short half-life in aqueous solutions. If not stated otherwise buffers should be stored at 4 °C for long-term storage. Buffers supplemented with inhibitors should be kept on ice directly before usage. Our lab and other groups routinely use the materials listed here. However, equipment from other providers should be equally suitable when tested accordingly. For the experiments shown here, standard laboratory chemicals, cell culture media, PBS, and plastic consumables were purchased at Carl Roth (Karlsruhe, Germany), Sarstedt (Nümbrecht, Germany), or Sigma Aldrich (Munich, Germany) if not indicated otherwise.

2.1 Cell Lysis Buffers

1. RIPA buffer: 150 mM NaCl, 50 mM Tris/HCl pH 7.4, 1% Nonidet P-40 (NP-40), 1% sodium desoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA); add directly before use: 10–20 mM *N*-Ethylmaleimide (NEM), 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail (PIC; end concentrations, 4 μg/ml antipain, 20 units/ml aprotinin, 0.2 mg/ml benzamidine, 2 μg/ml leupeptine).
2. NETN buffer: 150 mM NaCl, 20 mM Tris/HCl pH 7.4, 0.5% NP-40, 10% glycerol, 1 mM EDTA; add directly before use: 10–25 mM NEM, 1 mM PMSF, PIC.

2.2 Inhibitor Solutions

1. 1000× PIC (protease inhibitor cocktail) in H_2O: 4 mg/ml antipain, 20,000 units/ml aprotinin, 200 mg/ml benzamidine, 2 mg/ml leupeptine. Aliquote and store stock solution at −20 °C. Alternatively, use commercially available PIC according to the manufacturers' instruction.
2. 0.5 M PMSF (phenylmethanesulfonylfluoride) in 96% EtOH: 87 mg PMSF in 1 ml EtOH. Store at −20 °C (*see* **Note 3**).
3. 1 M NEM (*N*-ethylmaleimide) in DMSO or 96% EtOH: 125 mg NEM, 0.5 ml solvent. Add NEM to buffers for final concentrations of 10–25 mM (*see* **Note 4**).

2.3 SDS-PAGE and Blotting Buffers

1. SDS-PAGE running buffer (10×): 2.5 M glycine, 250 mM Tris, 1% SDS. Store at room temperature.
2. Tank Blotting Buffer (10×): 1.92 M glycine, 250 mM Tris. Store at 4 °C.
3. PBS (10×, for PBS-T): 1.37 M NaCl, 80 mM Na_2HPO_4, 27 mM KCl, 14 mM KH_2PO_4, pH 7.25 (adjust with HCl). Store at 4 °C.
4. SDS-PAGE running buffer (1×): 0.25 M glycine, 25 mM Tris, 0.1% SDS. Mix 100 ml 10× SDS-PAGE running buffer (10×) and 900 ml H_2O.
5. Tank Blotting Buffer (1×): 0.192 M glycine, 25 mM Tris, 20% EtOH. Mix 200 ml EtOH, 100 ml Tank Blotting Buffer (10×), 700 ml H_2O, and 200 ml 96% EtOH [29, 30].
6. PBS-T: 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.4 mM KH_2PO_4, pH 7.25, 0.05% (v/v) Tween 20. For 1 l PBS-T, mix 100 ml 10× PBS, 900 ml H_2O, and 0.5 ml Tween 20.
7. 2× Laemmli buffer: 0.125 M Tris/HCl pH 6.8, 10% β-mercaptoethanol (β-ME), 4% SDS, 20% glycerol, 0.004% bromophenol blue [31]. For 50 ml: 6.25 ml of 1 M Tris/HCl pH 6.8, 5 ml β-ME, 10 ml of 20% SDS, 10 ml glycerol, 200 µl of 1% bromophenol blue, fill up with H_2O (18.55 ml) to 50 ml. Keep Laemmli buffer in use at room temperature to avoid precipitation of SDS, long-term storage of aliquots at −20 °C.
8. 6× Laemmli buffer: 0.35 M Tris/HCl pH 6.8, 4.3 M β-ME, 10% SDS, 30% glycerol, 0.02% bromophenol blue. For 10 ml: 3.5 ml of 1 M Tris/HCl pH 6.8, 3 ml β-ME, 1 g of SDS, 3 ml glycerol, 200 µl 1% bromophenol blue, fill up with H_2O (300 µl) to 10 ml. Keep 6× Laemmli buffer in use at room temperature to avoid precipitation of SDS, long-term storage of aliquots at −20 °C.
9. Stripping buffer: 62.5 mM Tris/HCl pH 6.8, 2% (w/v) SDS, add freshly 100 mM β-ME.
10. Blocking solution: 5% (w/v) non-fat dry milk in PBS-T.
11. PVDF (Polyvinylidene difluoride) membrane.

12. Whatman paper (GE).
13. 96% EtOH.
14. Molecular weight marker: PageRuler Plus prestained protein ladder (Thermo).
15. Bio-Rad (Munich, Germany) tank blotting systems. Western blotting details have previously been described [22, 30].

2.4 Antibodies and Immunoprecipitation Materials

1. Bead storage buffer: NETN containing 0.05% (v/v) NP-40, 0.1% (w/v) BSA (Bovine Serum Albumin), and 0.01% (w/v) NaN_3.
2. Protein A and protein G agarose/sepharose beads (Sigma Aldrich, GE).
3. Protein A/G slurry: resuspend beads according to the manufacturer's instruction. Store beads at 4 °C in the same volume of storage buffer as a 50% slurry.
4. Mouse and rabbit normal IgG.
5. Specific antibodies for IP and western blot: (used here: mouse anti-SUMO1 (D-11), rabbit anti-HDAC2 (H-54) (Santa Cruz), and mouse anti-Vinculin (Biozol)).
6. Secondary antibodies (used here: goat antirabbit and goat antimouse (Thermo)).
7. Antibodies solutions: 2% (w/v) nonfat dry milk in PBS-T, 0.01% (w/v) NaN_3 (only for primary antibodies), antibodies diluted 1:1000–1:5000.

2.5 Kits

1. BCA Protein Assay Kit (Thermo).
2. SuperSignal West Pico Chemiluminescent Substrate (Thermo).

3 Methods

3.1 Cell Lysis

Cells of an 80–90% confluent 10 cm dish with adherent cells or an equivalent amount of suspension cells yield sufficient protein amounts for IP. Seed and transfect or treat cells as usual before harvesting (*see* **Note 5**). A typical workflow is depicted in Fig. 1.

Fig. 1 (continued) but without antibodies is used for preclearing of the lysate (**step 14**) by reducing the amount of proteins that bind to beads. The precleared lysate is incubated together with antibodies and protein A/G agarose beads to precipitate the sumoylated HDAC (**step 15–19**). Two options should be considered for IP: precipitation with a specific HDAC antibody and or precipitation with an antibody against a specific SUMO isoform. When immunoprecipitating SUMO, the immunoblot is then probed for the HDAC and vice versa. When precipitating HDACs, also an immunodetection with an HDAC antibody can be used to visualize higher migrating forms of that particular HDAC as an indicator for sumoylation. Diagrams of expected immunoblots are depicted. IgG heavy and light chain (IgG HC/LC) might be detected in the immunoblot. Preimmune serum/normal IgG is used as a control for IP specificity

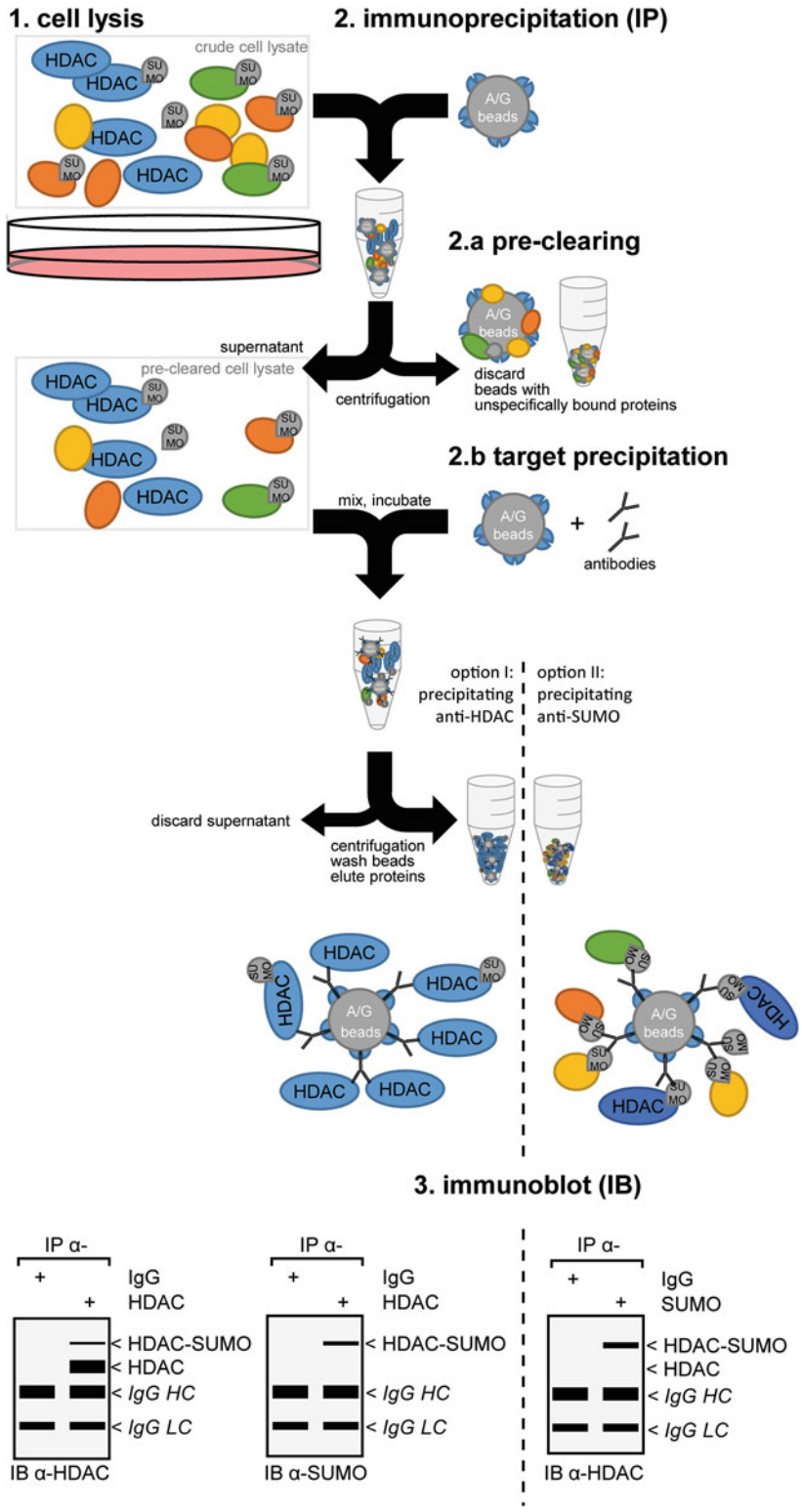

Fig. 1 Workflow for detecting HDAC sumoylation by immunoprecipitation and immunoblot. An exemplary workflow for the detection of sumoylated HDACs from cell culture is depicted. Cells are grown in a 10 cm dish and harvested for cell lysis when being 80–90 % confluent (**steps 1**–**12**). Incubation with Protein A/G agarose beads

Preparations: gather a box of ice, gather/prepare stock solution(s) of inhibitors and other material needed, precool PBS and lysis buffer to 4 °C, precool centrifuge to 4 °C, label tubes.

1. Grow cells to 80–90% confluency in a 10 cm dish according to cell type-specific protocol (*see* **Note 6**).
2. Put cells on ice and aspirate the culturing medium (*see* **Note 7**).
3. Wash cells: add 1 ml ice cold PBS to the dish, scratch of cells with a rubber scraper and transfer them into a 1.5 ml tube.
4. Centrifuge cells at 700 × *g* for 5 min in a precooled centrifuge at 4 °C.
5. In the meantime, prepare an appropriate amount (1 ml per 10 cm dish) of RIPA lysis buffer by adding PIC, PMSF, and NEM.
6. Carefully aspirate the supernatant and put the 1.5 ml tube back on ice.
7. Immediately add RIPA lysis buffer to the cell pellet (900 µl/tube), pipette up and down to homogenize the cell lysate.
8. Incubate on ice for 20 min.
9. Homogenize the cell lysate by sonification (e.g., with a WD-250 Branson sonifier; 70% intensity, 0.3 s pulse/0.6 s pause 10 cycles).
10. Clear the lysate from remaining cell debris by centrifugation: full speed/>20,000 × *g*, 4 °C, 10 min.
11. Transfer the supernatant into a new 1.5 ml tube, be careful to not transfer the pelleted cell debris; place the new tube with supernatant on ice; discard the old tube with the debris.
12. Determine protein concentrations in the lysates by performing an appropriate test, e.g., BCA or Bradford assay.

3.2 Immunoprecipitation

After lysis, proceed with preparing the immunoprecipitations. Freezing and thawing of lysates will decrease the amounts of detectable HDAC sumoylation up to making it undetectable. However, lysates might be stored on ice for at least 1 h if needed.

Preparations: continue with the material from cell lysis, label tubes, and gather material for IP (beads, antibodies) (*see* **Note 8**).

13. For each IP, pipette lysate containing 300–500 µg of total protein into a new 1.5 ml tube (do not forget to hold back lysate for inputs, *see* **Note 9**).
14. Optional: preclearing.
    Incubate the crude lysate with the same amount of beads (*see* **Note 10**) that are used for the actual IP but without antibodies for 2 h on a rotating wheel. Centrifuge for 30 s/700 × *g* at 4 °C and transfer the supernatant into a new 1.5 ml tube. Discard the beads with unspecifically bound proteins (*see* **Note 11**).

15. Add 20 µl of protein A and/or protein G beads (*see* **Note 8**) and 2-3 µg of antibody to the (precleared) cell lysate (*see* **Note 12**).
16. Put on a rotating wheel at 4 °C and incubate for a minimum of 4 h up to overnight.
17. Wash 5× with RIPA buffer:
    Centrifuge at 700 × *g* for 30 s in a precooled centrifuge at 4 °C to sediment beads, aspirate RIPA (be careful not to aspirate beads), and add 0.5 ml of RIPA.
18. After the last washing step aspirate RIPA completely and use an insulin syringe to remove residual buffer.
19. Add 30 µl 2× Laemmli buffer to the beads to release and denature bound antibodies and proteins (*see* **Note 13**).

Proceed with SDS-PAGE and immunoblotting.

3.3 SDS-PAGE, Immunoblotting, Immunodetection

Proceed with SDS-PAGE and immunodetection in accordance with the lab standard procedures. Details for a standard SDS-PAGE setup and immunoblotting workflow can be found elsewhere [30]. Figure 2 shows an exemplary result for IP and immunoblotting of HDAC2.

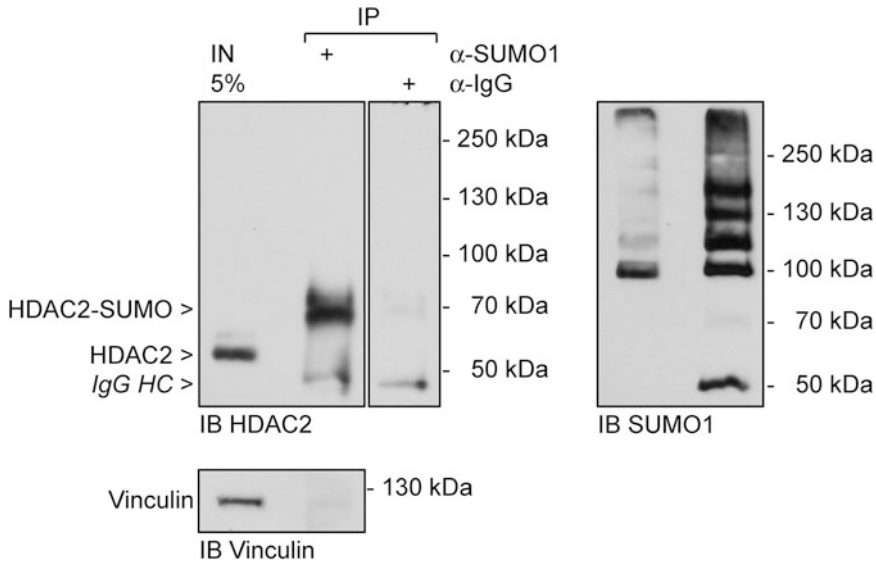

Fig. 2 Detection of SUMO1-modified HDAC2 by immunoprecipitation. HEK293T cells were seeded in a 10 cm dish and grown until 90 % confluence. Cells were harvested and lysed in RIPA buffer containing inhibitors (PIC, PMSF, and NEM). 500 µg of total protein was used for IP anti-SUMO1. IP with normal mouse IgG was used as a control. Immunoblot with HDAC2 antibody shows sumoylated HDAC2 (HDAC2-SUMO) in the IP migrating at circa 70 kDa and the IgG heavy chain at 50 kDa. A reprobe with SUMO1 controls the IP efficiency, Vinculin was tested as a loading control. The membrane was stripped before reprobes

4 Notes

1. The molecular weight of the 96 amino acid (aa) processed version of SUMO1 that is conjugated to target lysines is 11.1 kDa (SUMO2/3: 93/92 aa, 10.6/10.5 kDa). Because the connection of SUMO via an isopeptide bond creates a branched peptide, the migration behavior can change more than a simple addition of the individual molecular weights of the target protein and SUMO. Typical changes are in the range of 10–20 kDa for mono-sumoylation.
2. Not all antibodies might show the expected higher molecular weight bands. If possible, use antibodies that bind an epitope not close to the sumoylation site. Sumoylation is a rather bulky modification and thus can reduce the binding affinity of the antibody to the sumoylated target protein or sumoylated proteins might not be recognized at all.
3. Under those conditions, PMSF forms a saturated solution and crystals precipitate. Dilute the ice-cold PMSF stock 1:500–1:250 when adding to buffers to achieve ca. 1–2 mM end concentration. Heating the stock solution to 37 °C will dissolve the crystals, but PMSF will recrystallize and clog the pipet tips when pipetted into the cold lysis buffer. In aquaeous solutions PMSF is instable and has a pH- and temperature-dependent degradation kinetic [32]. Add directly before usage. No additional PMSF must be added if a commercial PIC including PMSF or a similar serine protease inhibitor (like AEBSF) is used.
4. The addition of *N*-ethylmaleimide (NEM) to inhibit SENPs is critical. The alkene double bond in NEM will react with thiols in a nuclear addition. Thus, cysteine residues in the catalytic center of SENPs (and other cysteine residues) are irreversibly blocked at pH 6.5–7.5 [33]. Usually, NEM is added to lysis buffers in concentration of 10–25 mM. Fresh preparation of a 1 M NEM stock solution in DMSO or 100% EtOH is recommended. However, the stock solution can be stored 24 h at –20 °C.
5. Cells can be lysed in buffers that lead to at least a partial disruption of the native protein structure due to high levels of detergents, or in buffers that leave protein folding largely intact. RIPA buffer is one example for a buffer containing high levels of strong detergents; NETN is a milder buffer containing less detergents. When the levels of sumoylated HDACs are to be analyzed, lysis with RIPA should be preferred. When also interaction with binding partners of the sumoylated deacetylase is investigated, cells should be lysed in NETN to keep protein structure close to native state. Of course, other lysis buffers can be used or the detergent content of the buffer can be adjusted. However, detergent concentration in buffers must not exceed levels that lead to antibody denaturation.

6. The protocol can be adopted for the lysis of 300–500 µg of tissue. Prepare lysates according to the tissue specific protocols and proceed with the immunoprecipitation (**step 13**).
7. Harvesting should be carried out as quickly as possible toward the step where lysis buffer containing NEM is added. As noted above, the enzymatic machinery for sumoylation is ATP-dependent, while the reversal reaction by SENPs is not. The ATP pool is rapidly decreasing when cells/tissue are harvested and thus sumoylation is no longer possible. All steps must be on ice to slow down the enzymatic desumoylation reaction by SENPs.
8. If possible, antibodies coupled covalently to beads are preferable. Covalently coupled antibodies usually show higher binding capacities and coupling will reduce occurrence of IgG heavy (ca. 50 kDa) and light chain bands (ca. 25 kDa) in the immunoblot. This is especially relevant in the case of HDAC1 (482 aa) and HDAC2 (488 aa), which are both similar in size (ca. 55 kDa) to the IgG heavy chain. A protocol for coupling SUMO-specific antibodies to beads was described recently [19]. In addition, the use of light-chain-specific secondary antibodies for western blotting can eliminate detection of the heavy chain efficiently.
9. Hold back 2–5% input of the sample(s) for immunoblotting; add an equal volume of 2× Laemmli buffer to of 2–5% of lysate volume amount used for IP. Inputs can be stored at −20 °C until needed.
10. To best pipette bead slurry, cut off about 1 cm from the front of a 200 µl pipette tip.
11. Preclearing reduces the amount of unspecific protein binding to beads and coprecipitation during the IP and is thus generally recommended. However, for each experimental setup it can be tested if preclearing actually improves the results. When the IP is performed with antibodies covalently coupled to beads, the preclearing can be performed with empty agarose/sepharose beads.
12. Protein A/G have varying affinities for antibodies depending on host species and isotype. Please refer to the information provided by the vendors for affinities.
13. The best results are obtained when proceeding directly with immunoblotting, but it is possible to freeze the beads in 2× Laemmli buffer and to store them for at least 24 h until further usage.

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Part V

Improved, Novel, and Specific HDACi

Chapter 26

How to Distinguish Between the Activity of HDAC1-3 and HDAC6 with Western Blot

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Abstract

Histone deacetylases (HDACs) catalyze the deacetylation of lysine residues in their target proteins. This biochemical modification can have profound effects on the functions of these proteins and a dysregulation of HDAC activity contributes to severe diseases, including neoplastic transformation. In the following chapter, we present a strategy that allows to distinguish between the inhibition of the class I HDACs HDAC1, 2, and 3 and of the class IIb HDAC HDAC6. This method is based on Western blot and relies on the detection of hyperacetylated substrates of class I or class IIb HDACs in lysates from cells that were treated with histone deacetylase inhibitors (HDACi).

Key words Acetylation, HDAC6, HDACs, HDACi, Histone, Tubulin

1 Introduction

Histone deacetylases are enzymes that remove acetyl groups from histones and non-histone proteins [1, 2]. Eighteen HDACs have been identified in mammalian cells [3]. As shown in Table 1, eleven of the known 18 HDACs depend on zinc (Zn^{2+}) for catalysis. The exception are the class III HDACs as their activity is NAD^{+}-dependent [4]. An aberrant overexpression of HDACs has been observed in a variety of diseases and particularly in carcinomas [5–10]. For example, an overexpression of HDAC6 can be found in breast cancer specimens [10]. Furthermore, HDAC3 and other class I HDACs are frequently overexpressed in colon cancer [6]. Thus, the development of selective inhibitors against specific HDACs is of major interest for their clinical application [11, 12]. Moreover, such specific agents will aid in deciphering the divergent mechanisms of action of particular HDACs [3, 4].

Unlike other HDACs, HDAC6 has two active catalytic domains and an ubiquitin binding site [13]. Prominent targets of HDAC6 are HSP90, Tubulin, and Cortactin [14]. However, in contrast to HDAC1-3, HDAC6 does not deacetylate histones in vivo as it is not located in the nucleus [14].

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Table 1
Classification of HDACs; compare [20]

Class of HDACs	HDACs	Cofactor
Class I	HDAC1 HDAC2 HDAC3 HDAC8	Zn^{2+}
Class II a	HDAC4 HDAC5 HDAC7 HDAC9	
Class II b	HDAC6 HDAC10	
Class III	Sirtuins (e.g., SIRT1)	NAD^{+}
Class IV	HDAC11	Zn^{2+}

Table 2
HDACi used in this chapter; compare [15]

Structural subclass	Compound	Inhibited HDACs
Short-chain fatty acid	Valproic acid (VPA)	HDAC1,2,3,8
Hydroxamate	Marbostat-100 LBH589	HDAC6 All Zn^{2+}-dependent HDACs
Benzamide	MS-275	HDAC1,2,3

This disparity in target proteins and an increasing number of specific HDACi provides the possibility to differentiate between the activities of different HDACs and to assess their specific inhibition by Western blot.

To describe this method, we chose HDACi that are either specific for HDAC1-3 (VPA or MS-275; [15, 16]), for HDAC6 (Marbostat-100; Krämer and Mahboobi, manuscript in preparation) or for all Zinc-dependent HDACs (LBH589; [15]). These agents belong to different structural subclasses of HDACi (Table 2).

Treating two cell lines (MV4-11 and Renca cells) with these HDACi promotes the acetylation of distinct target proteins. For example, a treatment of cells with MS-275 or VPA leads to a specific inhibition of the HDACs 1–3 or of all four class I HDACs, respectively. These HDACs catalyze the deacetylation of histones in vivo [15–18]. Accordingly, the hyperacetylation of Histone H3 (H3) can be detected in Western blot analyses (Fig. 1). Marbostat-100 is a novel HDAC6-specific inhibitor

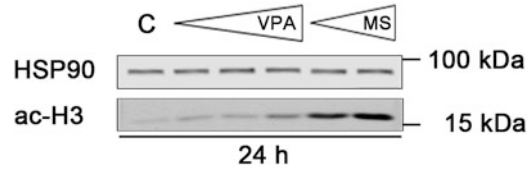

Fig. 1 Detection of HDAC inhibition in adherent Renca cells treated with VPA and MS-275. Renca cells were treated with VPA (0.5, 1.5, and 5 mM) and MS-275 (1.5 and 5 μM) for 24 h. Cells were harvested on ice and whole cell lysates were prepared. The samples were analyzed by SDS-PAGE and Western blot. The figure shows a representative blot ($n = 4$); *C* untreated cells, *ac* acetyl

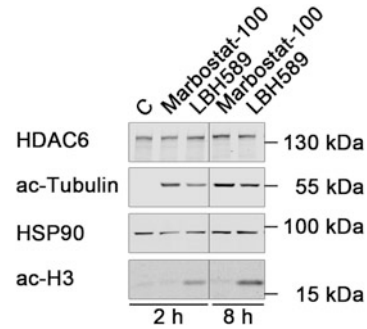

Fig. 2 Detection of HDAC inhibition in MV4-11 suspension cells treated with Marbostat-100 and LBH589. MV4-11 cells were treated with Marbostat-100 (50 nM) or LBH589 (50 nM) for 2 or 8 h. Cells were harvested on ice and whole cell lysates were prepared. The samples were analyzed by SDS-PAGE and Western blot. The figure shows a representative blot ($n = 3$); *C* untreated cells, *ac* acetyl

(Krämer and Mahboobi, manuscript in preparation). Figure 2 clearly shows that cells display a hyperacetylation of Tubulin but not of H3 following the treatment with this inhibitor. LBH589 as pan-HDACi consequently leads to a detectable acetyl-Tubulin signal as well as a clear hyperacetylation of histones (Fig. 2) [17, 19]. Figures 1 and 2 therefore show an easy and practical way to differentiate between the inhibition of HDAC1-3 and HDAC6 using Western blot.

2 Materials

Prepare all solutions using deionized water. Prepare and store all reagents as indicated. We routinely use the listed materials and antibodies in our lab but equipment from other providers may be also suitable.

2.1 Preparation of Whole Cell Extracts

1. NETN lysis buffer: 10 mM Tris–HCl pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% NP-40. Freshly add protease inhibitor (protease inhibitor cocktail tablets) (cOmplete Tablets, Mini, EDTA-free, EASY*pack*, Roche) and phosphatase inhibitor cocktail 2 (Sigma-Aldrich).
2. Sonification: Hielscher Sonifier UP200Ht, amplitude 40% for 10 pulses (0.1 s pulse duration).
3. Dulbecco's Phosphate-Buffered Saline (PBS), e.g. from Biochrom.
4. SDS-Laemmli (6×) loading buffer: 375 mM Tris–HCl pH 6.8, 12% SDS (*w/v*), 30% glycerol, 500 mM dithiothreitol (DTT), spatula tip Bromophenol blue.

2.2 SDS Polyacrylamide Gel Electrophoresis

1. Separating gel buffer: 1.5 M Tris–HCl pH 8.8.
2. Stacking gel buffer: 1 M Tris–HCl pH 6.8.
3. 10% SDS: 10% (*w/v*) aqueous solution.
4. 40% Acrylamide/bisacrylamide 37.5/1 (Roth).
5. 10% Ammonium persulfate (APS): 10% (*w/v*) aqueous solution.
6. Tetramethylethylenediamine (TEMED).
7. Isopropanol.
8. Mini Protean Tetra Cell: casting module, glass plates (spacer included), electrode assembly, buffer tank and lid (Bio-Rad).
9. SDS-Running buffer: 50 mM Tris, 400 mM Glycin, 0.1% SDS (*w/v*).
10. PageRuler™, Prestained Protein Ladder (Thermo Scientific).

2.3 Protein Transfer: Western Blot

1. Nitrocellulose (NC) membrane (Hartenstein).
2. Blotting paper (Whatman paper), grade 703 (VWR).
3. Transfer Buffer: 25 mM Tris, 200 mM Glycin, 20% ethanol.
4. Mini Trans-Blot® Cell system: Mini Trans-Blot central core, foam pad and mini gel holder cassette (Bio-Rad).
5. Tris buffered saline (TBS; 10×): 0.2 M Tris–HCl pH 7.6, 1.4 M NaCl.
6. TBS containing 0.05% Tween-20 (TBST).

2.4 Blocking and Antibodies

1. 5 and 2% non-fat dry milk in TBST.
2. Antibodies for Western Blot.
   (a) HDAC6 (Cell Signaling; #7558 (D2E5) [rabbit]).
   (b) Acetyl-Histone H3 (Millipore; 06-599 [rabbit]).
   (c) Acetyl-Tubulin (Sigma-Aldrich; T7451 [mouse]).
   (d) HSP90 (Enzo; ADI-SPA-830, AC88 [mouse]).
   (e) Goat-anti-mouse 800 CW (LI-COR).
   (f) Goat-anti-rabbit 800 CW (LI-COR).

3 Methods

3.1 Preparation of Whole Cell Lysates

1. Suspension cells are cultured in six-well dishes with a density of 200,000 cells per mL. Before the cells are treated with different HDACi, an adaption time of at atleast 2 h is necessary. Adherent cells are cultured in 60 mm dishes (300,000 cells per dish), after an adaption time of 24 h the cells are stimulated with different HDACi.
2. The following steps are done on ice to slow down the activity of proteases, phosphatases and HDACs. If you use suspension cells, place the six-well dishes on ice and gently resuspend the cells. Transfer the cell suspensions into labeled 15 mL tubes, wash each well with 1 mL ice-cold PBS to collect remaining cells and proceed with **step 3**. If you use adherent cells, place the 60 mm dishes containing the adherent cells on ice, transfer the supernatants into labeled 15 mL tubes and pipette 2 mL ice-cold PBS in every dish (*see* **Note 1**). Take a rubber policeman to scratch the cells from the bottoms of the plate. Transfer the cells into correspondingly labeled 15 mL tubes and wash the dishes each with 1 mL ice-cold PBS to ensure a complete cell yield.
3. Centrifuge the cells at 317 × *g* for 5 min. Aspirate the supernatants and wash the pellets with 1 mL ice-cold PBS. Transfer the cell suspensions into 1.5 mL Eppendorf tubes and perform a second centrifugation step at 17,000 × *g* for 5 min at 4 °C.
4. Aspirate the supernatant and resuspend the pellet in 100–150 μL NETN lysis buffer. Incubate the cells for at least 15 min on ice and sonify to completely rupture the cells (*see* Subheading 2.1).
5. Perform a third centrifugation step at 17,000 × *g* for 25 min at 4 °C to remove cellular debris. Transfer the supernatants into new 1.5 mL tubes. Store the lysates at −80 °C.
6. Determine the protein concentrations of the lysates with an appropriate protein assay (e.g., Bradford assay).
7. For an even protein loading of the gel lanes, ensure an equal protein concentration in every sample (25–50 μg). Adjust samples to the same total volume using ddH_2O.
8. Add sample buffer (6×) to every sample (for example, add 20 μL 6× sample buffer to 100 μL lysate) and heat the lysates for 5 min at 95 °C to denature the proteins (*see* **Note 2**).

3.2 SDS Polyacrylamide Gel Electrophoresis

1. Assemble the gel plates by using a spacer plate and a cover plate and set them into the casting module. Prepare a 10% or a 12.5% separating gel as described in Table 3 and fill up the gel plates to 3/4 of the volume (*see* **Notes 3–6**). Cover the surface with 500 μL isopropanol to receive a straight surface and to prevent oxidation (*see* **Note 7**).

Table 3
Composition of separating and stacking gel

	Separating gel		Stacking gel
	10%	12.5%	
H_2O	5.7 mL	5.1 mL	4.4 mL
Separating gel buffer	3 mL	3 mL	–
Stacking gel buffer	–	–	760 µL
SDS 10% (w/v)	120 µL	120 µL	60 µL
Rotiphorese® Gel 40	3 mL	3.6 mL	760 µL
APS 10% (w/v)	60 µL	60 µL	60 µL
TEMED	6 µL	6 µL	6 µL

2. After approximately 30 min, the gel is polymerized (*see* **Note 8**). Remove the isopropanol using a small piece of filter paper (*see* **Note 9**). Prepare the stacking gel as described in Table 2. Fill up the glass plates with the stacking gel solution and insert the comb quickly.
3. About 15 min later, the stacking gel is polymerized. The gels can be used immediately or stored in wet tissues at 4 °C overnight (*see* **Note 10**).
4. Remove the combs carefully. Rinse the slots with ddH_2O to remove gel residues. Assemble the glass plates with the gel in the buffer tank and add SDS running buffer to the tank (*see* **Note 11**).
5. Apply a protein ladder and samples from Subheading 3.1, **step 8** (*see* **Note 12**).
6. Run the gel at 95–120 V for approximately 1.5 h (*see* **Note 13**). Bromophenol blue from 6× sample buffer marks the running front for orientation. Protein separation is finished when the running front passes the bottom of the glass plates.

3.3 Western Blot

1. Equilibrate nitrocellulose membrane in ddH_2O for approximately 5 min. The preferred size of the membrane is 6 × 9 cm.
2. Prepare a buffer tank with the transfer blotting core and a cool pack and fill up the tank with blotting buffer. Fill a tray with transfer buffer for assembling the blot sandwich. Place a gel holder cassette in the tray and soak a sponge with buffer and lay it on the cassette. Take two Whatman papers with a size of 7 × 10 cm and soak them with buffer. Place the Whatman papers on the sponge. Use for example a falcon tube to roll over the sandwich in order to remove air bubbles between the layers. Place the membrane carefully on the Whatman papers (do not touch the membrane without gloves).

3. Use a spatula to carefully separate the glass plates. Cut of the stacking gel from the separating gel and place the separating gel on the membrane. Lay two Whatman papers soaked with transfer buffer on the gel. Gently role over the sandwich to remove air bubbles. Soak a second sponge with blotting buffer and place it on the sandwich (*see* **Note 14**). Close the gel holder cassette and set it in the blotting core.
4. The protein transfer usually takes 2 h at 150 mA per gel. To avoid warming, change the cool pack after 1 h.
5. Prepare a tray with TBST. Dissemble the blotting apparatus. Transfer the membrane in the tray using a tweezer to avoid touching the membrane (*see* **Note 15**). Incubate nitrocellulose membrane on rocking platform for 5 min.

3.4 Detection via Fluorescence-Coupled Antibodies

1. Remove the TBST and add 5 % non-fat dry milk in TBST to the nitrocellulose membrane (membrane has to be fully covered). Incubate the membrane 1 h on rocking plate to cover unspecific binding sites of the membrane.
2. Wash the membrane three times for 5 min with TBST (*see* **Note 16**). Cut the membrane in different pieces to detect the proteins of choice. Transfer the nitrocellulose membrane pieces into a 50 mL tube containing 5 mL 2 % non-fat dry milk in TBST and the antibody of interest. Use the antibody dilutions shown in Table 4. Incubate the membranes rotating overnight at 4 °C.
3. After incubation wash the membranes three times with TBST for 5 min to reduce unspecific antibody binding.
4. Prepare the secondary antibody in a dilution of 1:10,000 using 2 % non-fat dry milk in TBST. Incubate the membranes in the dark for 2 h by rotating at room temperature.
5. Wash the membranes three times for 5 min in TBS to reduce unspecific antibody binding (*see* **Notes 17** and **18**).
6. Measure the signal of the proteins by using an infrared imager (e.g., Odyssey® from LI-COR) (*see* **Note 19**).

Table 4
Dilution of antibodies for Western blot

Antibody	Dilution
HDAC6	1:1000
Acetyl-Tubulin	1:2000
HSP90	1:1000
Acetyl-Histone H3	1:1000

4 Notes

1. When harvesting adherent cells, discarding the supernatant will lead to a loss of dead cells. This can prevent a faithful detection of markers for dead cells.
2. Do not heat the samples again as this leads to protein loss. Defrost the samples at room temperature.
3. Dissolve adequate amount of Tris in ddH_2O and use HCl to adjust pH value to prepare Tris buffer.
4. Prepare a 10× concentrated SDS solution as working stock. Wear a mask while preparing the solution because SDS powder can cause respiratory damage.
5. Always wear gloves while casting gels. Acrylamide is carcinogenic and can be absorbed over skin. The best way to cast gels is under a fume hood.
6. Bring all components from the separating gel together expect TEMED. Mix the solution thoroughly to prevent uneven polymerization and smears.
7. Carefully pipette the isopropanol on the surface of the separating gel to avoid mixing with the separating gel solution.
8. If the border between the separating gel and isopropanol becomes visible, it indicates that the gel is polymerized. Another way to make sure that the gel is polymerized is to have a look at the remaining residue in the reaction vessel as it should also be polymerized.
9. Be careful when removing isopropanol from the surface of the gel. Do not touch the gel with the filter paper as this leads to an uneven surface of the gel.
10. Store casted gels not longer than 1 week to maintain their structural stability.
11. Preparing a 5× stock solution (250 mM Tris, 1.92 M) simplifies the subsequent preparation of electrophoresis and blotting buffer. To prepare the electrophoresis buffer use 1× stock solution and 0.1% SDS (*w/v*). To prepare blotting buffer use 0.5× stock solution and add 20% ethanol.
12. Do not overload the slots of your gel. It is better to run the gel for some minutes and then reload the rest of the samples.
13. If you start running the gel, choose a voltage around 95 V until the running front reaches the separating gel. This is important to obtain straight protein bands.
14. Many workgroups use methanol as blotting agent. However, ethanol is a good substitute as it allows the same results while having less toxic effects.

15. Always wear gloves when touching the nitrocellulose membrane. Otherwise you can detect your fingerprints on the membrane.
16. The washing steps are very important to reduce unspecific background on the nitrocellulose membrane.
17. Wash the membrane with TBS without Tween-20 after incubation with the secondary antibody, because Tween-20 may cause background during detection.
18. You can store the membrane in a darkened tray in TBS for a week at 4 °C for later detection.
19. After measuring the band intensities, you can laminate the membrane with a small portion of buffer and store it at –20 °C for later use.

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Chapter 27

Evaluating the Effect of HDAC8 Inhibition in Malignant Peripheral Nerve Sheath Tumors

Gonzalo Lopez and Raphael E. Pollock

Abstract

Malignant peripheral nerve sheath tumor (MPNST) is a highly aggressive disease with a dismal prognosis. The disease can occur sporadically or in patients with inherited neurofibromatosis (NF-1). MPNST is typically resistant to therapeutic intervention. Hence, the need for improved therapies is warranted. Several broad spectrum histone deacetylase (HDAC) inhibitors have a high affinity for class I HDAC isoforms. Inhibition of multiple HDAC isoforms often results in undesirable side effects, while inhibiting a single isoform could possibly improve the therapeutic window and limit toxicity. Recently, HDAC8 inhibitors have been developed and in initial preclinical studies, they demonstrate anticancer efficacy. Little is known about the role of HDAC8 in MPNST. We recently revealed an anticancer effect of HDAC8 inhibition in human and murine MPNST models. The goal of our previous study was to determine the potential therapeutic efficacy of HDAC8 inhibition in MPNST. In this chapter, we briefly describe the methods for determining the role of pharmacological HDAC inhibition in MPNST.

Key words HDAC8, Malignant peripheral nerve sheath tumor, MPNST, Histone deacetylase, HDAC, HDAC inhibitor

1 Introduction

Histone deacetylases (HDAC) are a family of specialized proteins that deacetylate lysine tails on histones and non-histone proteins (e.g. Rb, p53, NFkB, E2F proteins, β-catenin). Hyperacetylation of the core histone proteins results in chromatin condensation. Histone acetyltransferases (HAT) acetylate lysine tails and induce the chromatin to "unwind". HDAC and HAT activity play a critical role in epigenetic functions.

To date, eleven HDAC isoforms have been discovered in mammalian systems [1, 2]. The HDAC catalytic domain requires a metal ion and coordinates with the oxygen molecule of the carbonyl group to stimulate an H_2O molecule supported by histidine-aspartic acid for a nucleophilic attack, resulting in the removal of the acetyl group from the protein lysine residue [3].

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HDAC isoforms are ordered into four classes based on their sequence identity and domain organization. Class I HDACs consist of HDAC1, HDAC2, HDAC3, and HDAC8 where HDAC1 and HDAC2 exhibit nuclear subcellular location. HDAC3 and HDAC8 locate to the nucleus and cytoplasm [2]. Class IIa (HDAC4, HDAC5, HDAC7, HDAC9) and Class IIb (HDAC6, HDAC10) HDAC isoforms are expressed and functionally relevant in the nuclear and cytoplasmic compartments of various cells [2, 4]. HDAC11 is the only isoform represented as class IV. It is comprised of conserved residues in the catalytic core region domains that are similarly found in class I and class II HDAC isoforms [5].

HDAC inhibitors chelate the metal ion in the HDAC active site, thus inhibiting the enzymatic mechanism of deacetylation [6]. The role and efficacy of HDAC inhibition in sarcoma is currently limited. HDAC inhibition has also shown superior anticancer effects in simple karyotypic and complex karyotypic soft tissue sarcomas [7–21].

Many HDAC inhibitors that are clinically used today are broad-spectrum drugs, targeting numerous HDAC isoforms and with high affinities to HDAC1, HDAC2, and HDAC3.

Inhibition of many HDAC isoforms results in unwanted side effects where isoforms significant for their tumorigenic contribution can be inhibited, potentially resulting in reduced adverse effects. Park et al. [22] demonstrated HDAC1, HDAC6, and HDAC8 but not HDAC4 inhibition decreased matrix metalloproteinase 9 expression and invasive capacity in breast cancer cells. These data exemplify the significance of individual HDAC isoforms and their role in cancer, creating a basis for the development of isoform-specific HDAC inhibitors.

To date, isoform-specific inhibitors for HDAC6 and HDAC8 have been developed and are currently being utilized in preclinical studies [23]. The structural uniqueness of HDAC8 among other class I isoforms gave way to the development of HDAC8-specific inhibitors [24].

The gene for HDAC8 is found on the Xq13 chromosome and encodes a protein consisting of 377 amino acids. Among the class I HDAC isoforms, HDAC1, HDAC2, and HDAC3 are phosphorylated by casein kinases, while HDAC8 is phosphorylated by cyclic AMP-dependent protein kinase A (PKA) at serine 39 [25]. Analogous to other class I members, HDAC8 contains a nuclear localization sequence in its catalytic domain. While other class I isoforms are ubiquitously expressed and distributed in many tissues, HDAC8 displays variable distribution. HDAC8 expression is higher in the brain and pancreas compared to HDAC1 and HDAC3, yet is expressed at lower levels in the heart, placenta, kidney, and liver compared to other class I members [2, 26]. In various tumors, HDAC8, among the other class I HDACs, is overexpressed [2, 26].

HDAC8 has been identified to play a role in numerous function in a variety of cell types [25, 27]. For example, HDAC8 deacetylates estrogen-related receptor alpha (ERα) to augment its DNA binding affinity and transcriptional regulation [28].

Phospho-HDAC8 cooperates with human ever shorter telomeres 1B (hEST1B) in the recruitment of Hsp70 to inhibit C-terminal heat shock protein interacting protein (CHIP). The phospho-HDAC8-mediated interaction with hEST1B does not require the enzyme's deacetylating function [29]. The role of cytoplasmic HDAC8 has been shown to interact with smooth muscle alpha-actin (α-SMA) in muscle cells undergoing differentiation [30], and used as a potential diagnostic tool in mesenchymal tumors of the uterus [31]. With the emergence and utility of novel HDAC8 inhibitors, the biological function of HDAC8 can be further explored.

Compounds that inhibit HDAC8 have been developed. The HDAC8-specific inhibitor PCI-34051 demonstrates a >200-fold selectivity over other HDAC isoforms. PCI-34051 induced apoptosis in T-cell lymphoma and leukemia cells lines; B-cell or solid tumor cells lines displayed tolerance to this compound. In this study, PCI-34051 did not induce histone or tubulin acetylation in the tested cell lines [32].

HDAC8 expression correlates with an unfavorable outcome in neuroblastoma [33]. HDAC8 inhibition (siRNA and pharmacological inhibition with Compound 2, [34]) was shown to induce neuroblastoma cell line differentiation by inducing a neurite-like structural outgrowth. Inhibition of HDAC8 abrogated neuroblastoma cell growth, however, no apoptosis was observed [33, 35]. Intriguingly, neuroblastoma and malignant peripheral nerve sheath tumors (MPNST) both originate from neural crest cell origins, thus suggesting role for HDAC8 in the progression of these diseases. The materials, methods, and protocols to study the role of HDAC8 in MPNST are described in the following sections.

2 Materials

2.1 Cell Culture and HDAC8 Inhibitors

1. Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS, Lifetech) (*see* **Note 1**).
2. Trypsin-EDTA solution: 0.05% Trypsin-EDTA.
3. Dulbecco's phosphate buffered saline (DPBS 1×, e.g. from Gibco).
4. HDAC8 inhibitors: PCI-34051 and PCI-48012 (Pharmacyclics, Inc.) dissolved in 100% DMSO, aliquoted and stored at −20 °C for short term use; stock solutions were stored −80 °C (*see* **Note 2**).

2.2 Reagent and Antibodies for Western Blots

1. Laemmli Sample Buffer: 31.5 mM Tris–HCl, pH 6.8, 10% glycerol, 1% SDS, 0.005% Bromophenol Blue, 355 mM β-mercaptoethanol. Mix and adjust Tris–HCl solution pH with HCl.
2. 10% and 15% acrylamide SDS-PAGE gels.
3. Gel running apparatus.
4. 1× Running buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Mix and adjust pH with HCl.
5. Protein transfer apparatus.
6. 1× Transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. Mix and adjust pH with HCl.
7. PVDF membrane.
8. Western Lightning Plus-ECL solutions for Western blot chemiluminescence (PerkinElmer).
9. Primary antibodies: Rabbit anti-acetyl-histone H3 (Millipore, 06-599), rabbit anti-acetyl-histone H4 (Millipore, 06-866), mouse anti-acetylated tubulin (Sigma, T7451), rabbit anti-cleaved caspase 3 (Cell Signaling, 9661), mouse anti-β-actin HRP (Santa Cruz, sc-47778HRP).
10. Secondary antibodies: Rabbit anti-mouse HRP (Santa Cruz, sc-358920), mouse ant-rabbit HRP (Santa Cruz, sc-2357).

2.3 In Vitro Cell Growth Assays

1. 96-Well microplates.
2. CellTiter 96 Aqueous One Solution Reagent (Promega).

2.3.1 (MTS) Assay

3. Microplate reader.

2.3.2 Clonogenic Assay

1. 6-Well culture plates.
2. 0.5% Crystal violet solution: 0.5% crystal violet, 20% methanol, 80% ddH_2O.

2.4 Cell Cycle Analysis

1. 10 cm culture dishes.
2. PBS.
3. 70% Ethanol.
4. Propidium iodide solution: 0.0025 g propidium iodide, 0.05 g sodium citrate, 50 μL Triton X-100, 0.5 mg/mL RNAse. Make up to 50 mL with PBS. Avoid light and store at 4 °C.
5. Flow cytometer.

2.5 Annexin V Assay

1. 6-Well culture plates.
2. FITC Annexin V Apoptosis Detection Kit (BD Pharmingen).

2.6 In Vivo Tumor Growth Xenograft

1. 6–7-Week-old female SCID mice.
2. MPNST6IEPVI cell line [36].

3. Syringes with 27G needles.
4. Sterile PBS.
5. Sterile 5% methylcellulose solution (vehicle).
6. Caliper.
7. Digital scale.

2.7 Proteomics and Mass Spectrometry

1. SDS sample buffer: 10% glycerol, 50 mM dithiothreitol, 2.3% SDS, 0.0625 M Tris, pH 6.8. Mix and adjust pH with HCl.
2. 10% acrylamide slab gel (0.75 mm thick).

2.7.1 2D Electrophoresis

3. Buffer "O": 10% glycerol, 50 mM dithiothreitol, 2.3% SDS, 0.0625 M Tris, pH 6.8. Mix and adjust pH with HCl.
4. Transfer buffer: 10 mM Caps, pH 11.0, 10% methanol. Mix and adjust pH with HCl.
5. PVDF membrane.
6. Molecular weight standards (Sigma): myosin, phosphorylase A, catalase, actin, carbonic anhydrase, lysozyme.
7. Coomassie Brilliant Blue R-250.
8. Primary antibody: Rabbit anti-acetylated lysine (Cell Signaling, 9441), secondary antibody: anti-rabbit IgG-HRP (GE Healthcare, NA934).
9. ECL chemiluminescence kit.

2.7.2 Western Blot Spot Analysis

1. Laser densitometer (Molecular Dynamics).
2. Image analysis software: Progenesis Same Spot software (Nonlinear Dynamics), Progenesis PG240 software (Nonlinear Dynamics).

2.7.3 Mass Spectrometry (Protein Chemistry Core Facility, Columbia University)

1. Trypsin.
2. MALDI-MS.

3 Methods

3.1 Acetylated Proteins Western Blots

Western blots are conducted according to previous methods [14].

1. Treat MPNST cell lines with HDAC8 inhibitors.
2. Rinse attached cells with cold PBS prior to harvesting via scrapping and 1× lysis buffer. Place lysate in a tube and set on a rotator at 4° for 30 min. Centrifuge lysate centrifuged at 21130 × *g* (4 °C) for 10 min.
3. Determined protein concentration using the Bradford assay.
4. Dilute equal concentration of protein with Laemmli Sample buffer and heated at 100 °C for 5 min.

5. Separate protein samples on SDS-PAGE gels and transferred (1 h) onto PVDF membranes.
6. Block membranes with 5% nonfat dry milk (in PBST) for 1 h at room temperature.
7. Add primary antibodies against the acetylated proteins (acetyl-H3, acetyl-H4, acetyl-tubulin) at 1:1000 dilution, and incubate overnight at 4 °C.
8. Wash each membrane with PBST on a shaker for 3 × 10 min.
9. Add secondary antibodies at 1:5000 dilution, and incubate for 1 h at room temperature.
10. Use the ECL kit to develop the membranes on X-ray film.

3.2 Determine the Effect of HDAC8 Inhibition on MPNST Cell Growth In Vitro

3.2.1 MTS Assay (Fig. 1)

1. Plate cells in 96-well plates at 1000 cells per well. Allow 24 h for cell attachment to plate.
2. Aspirate media from each well and add control (DMSO) and varying drug concentration in media to the indicated wells at a total volume of 100 µL per well (*see* **Note 3**).
3. Incubate each plate at normal tissue culture conditions for 96 h.
4. Add 20 µL of MTS solution per well and incubate at 37 °C for 2 h.
5. Use a plate reader and measure the plate absorbance at 490 nm, record and analyze the readout data.

3.2.2 Clonogenic Assay

1. Plate cells in 6-well plates at 200 cells per well and allow 24 h for cell attachment.
2. Add control (DMSO) and varying drug concentrations to the desired wells and incubate at normal tissue culture conditions for about 10–15 days. Replace the control and drug for each well every 96 h (*see* **Note 4**).

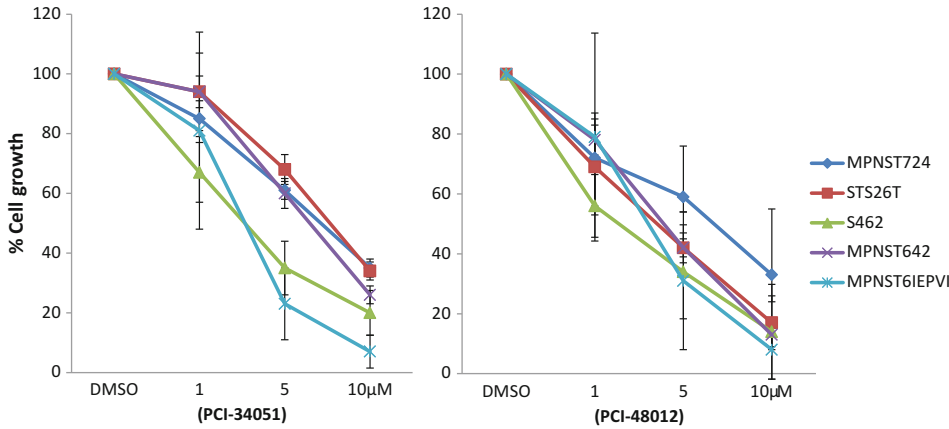

Fig. 1 MTT assay demonstrating the growth inhibitory effect of HDAC8 inhibitors (PCI-34051, PCI-48012) on human and mouse MPNST cell lines

3. On the final day of treatment, remove the media and carefully rinse the cells with PBS. Add 1 mL of 0.5% crystal violet solution to each well and incubate at room temperature for 30 min on a rocker (slow setting).
4. Aspirate the 0.5% crystal violet solution and thoroughly rinse each well with ddH_2O 5–10×. Several rinses may be needed to remove excess crystal violet.
5. Allow the plates to dry and count the colonies.

3.3 Effect of HDAC8 Inhibition on Cell Cycle

1. Treat cells with control (DMSO) and HDAC8 inhibitors for 48 h.
2. Trypsinize the cells into 15 mL conical tubes (neutralize the trypsin with media containing FBS) and centrifuge at 376 × *g* for 5 min.
3. Aspirate the media and resuspend the cells in 1 mL of 70% ethanol for fixation of the cells (*see* **Note 5**).
4. Centrifuge the fixed cells at 587 × *g* for 5 min. Aspirate the ethanol and wash cells in PBS. Centrifuge at 587 × *g* for 5 min and aspirate the PBS.
5. Resuspend the cells in 500 μL of the propidium iodide solution.
6. Analyze the cells on a Flow Cytometer (FACS analysis was conducted at the MD Anderson Flow Cytometry core facility, Houston, TX).

3.4 Effect of HDAC8 Inhibition on Apoptosis

3.4.1 Annexin V Assay

1. Treat cells with control (DMSO) and HDAC8 inhibitors for 48 and 96 h.
2. After treatment, collect media (containing detached cells) from each well into 15 mL tubes. Trypsinize the attached cells and add them to the same tube containing the detached cells.
3. Centrifuge at 304 × *g* for 5 min.
4. Aspirate the media and resuspend the cells in 100 μL of 1× Binding Buffer (provided in FITC Annexin V Apoptosis Detection Kit) and add to 5 mL culture tubes. Add 5 μL of FITC Annexin V and 5 μL PI solution to the cells, and incubate the cells at room temperature (avoid light) for 15 min.
5. Add 400 μL of 1× Binding Buffer to each tube and analyze using a Flow Cytometer within 1 h.

3.4.2 Cleaved Caspase 3 Western Blot

1. When extracting proteins from cell culture, include detached and attached cells.
2. Use Western blot methods as described in Subheading 3.1.
3. Add primary antibodies against cleaved caspase 3 at 1:1000 dilution, and incubate overnight at 4 °C.
4. Wash each membrane with PBST on a shaker for 3 × 10 min.

5. Add appropriate secondary antibodies at 1:5000 dilution, and incubate for 1 h at room temperature.
6. Use the ECL kit to develop the membranes on X-ray film.

3.5 Effect of HDAC8 Inhibition on MPNST Cell Growth In Vivo

1. Grow MPNST6IEPVI cells to 70–80% confluency. Collect viable cells and centrifuge. Wash cells with sterile PBS and resuspend the cells in sterile PBS at 1×10^7 cells per 1 mL.
2. Anesthetize the SCID mice using isoflurane. Inject 1×10^6 (100 μL) of MPNST6IEPVI cells subcutaneously into the right flank of each mouse and monitor daily for tumor take and growth.
3. When tumors reach ~5 mm^3 in size, allocate mice into two treatment arms ($n = 10$ mice/arm): vehicle and PCI-48012 treatment.
4. Inject the vehicle and the compound (dissolved in vehicle at 20 mg/kg BID) intraperitoneal (100 μL) into each mouse of their respective group. Treat each mouse once in the morning and once in the afternoon 5 days per week.
5. Measure the tumors of both groups using a caliper twice a week.
6. Upon completion of the experiment, take final tumor measurements and weigh tumors. Use the 2-tailed Student's *t*-test for statistical analysis.

3.6 2D Gel Proteomics and Mass Spectrometry to Identify Potential HDAC8 Substrates

1. Treat cells with control (DMSO) or HDAC8 inhibitor for 24 h.
2. Send cell pellets on dry ice to Kendrick Labs, Inc. for 2D gel electrophoresis and spot analysis.
3. Mass spectrometry of potential HDAC8 substrates was carried out by Kendrick Labs, Inc. and Columbia University.

4 Notes

1. Providers are examples and other providers may equally have appropriate materials.
2. Stock concentrations of 100 mM were aliquoted into several 1.5 mL tubes.
3. Internal triplicates were conducted per MTS assay. Biological triplicates were conducted for each MTS assays.
4. In our experience with the cell lines used, the doubling time of the cells vary. Some cell lines will yield visible colonies within 10 days, while others will require longer incubation times.
5. Cells can be fixed in 70% ethanol for 1 h on ice or stored at −20 °C. We used cells stored at −20 °C within 1 week.

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Chapter 28

Analyzing the Impact of Pan- and Class-Specific HDACi on Differentiation-Associated Factors

Katrin Noack and Oliver H. Krämer

Abstract

The differentiation of hematopoietic stem cells into mature blood cells is a highly ordered process and dysregulation of this process can lead to leukemogenesis. Agents that are used to cure acute promyelocytic leukemia (APL) can induce differentiation and/or apoptosis. Here, we describe how effects of all-trans retinoic acid (ATRA) and histone deacetylase inhibitors (HDACi) on APL cell differentiation can be evaluated by immunoblotting and by flow cytometry. We show how the levels of differentiation-associated transcription factors of the CCAAT enhancer binding protein (C/EBP) family can be determined by Western blot and we explain how the cell surface expression of the leukocyte surface antigen CD11b can be measured by flow cytometry.

Key words APL, CD11b, C/EBPβ, C/EBPε, HDACi, Flow cytometry, Western blot

1 Introduction

Chemotherapy can rely on the induction of cell death, which aims to eliminate transformed cells, and chemotherapy can act as an inducer of cell differentiation, which aims to restore normal cell physiology [1–3]. The treatment of APL cells with ATRA as an inducer of cell maturation is a paradigm for differentiation therapy. The treatment of APL cells with ATRA accelerates the proteasomal degradation of the leukemogenic fusion protein PML-RARα, which causally contributes to the differentiation and apoptosis block seen in these tumor cells [4, 5].

Histone deacetylase inhibitors (HDACi) can also evoke the differentiation of transformed cells [6]. However, the molecular mechanisms by which these agents promote maturation are largely undefined. It is unexpected that HDACi can decrease the levels of PML-RARα in NB4 APL cells [7], but that they cause at most a very partial differentiation of these cells [8]. Moreover, the induction of granulocytic maturation by ATRA protects NB4 cells from cytotoxic effects of HDACi that specifically inactivate the catalytic

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activity of class I HDACs [8], which comprise HDAC1, -2, -3, and -8 [6, 9]. HDACi that additionally inactivate class II and IV HDACs are though effective inducers of apoptosis in ATRA-treated NB4 cells [8].

Unlike the other HDACs, which use Zn^{2+} for catalysis [6, 9], the class III HDAC family uses the metabolite NAD^{+} to catalyze the removal of the acetyl group from histones and non-histone proteins [10]. The inhibition of these enzymes can also trigger the maturation of NB4 cells [11].

The findings summarized above demonstrate that several enzymes belonging to the HDAC family regulate the maturation process, and that cellular differentiation may determine the success of treatment regimen involving HDACi. Accordingly, it is important to have reliable molecular markers for cell maturation as well as for apoptosis and other types of cell death. The maturation of NB4 cells can be determined by flow cytometry, RNA expression profiling, or by the detection of proteins that are associated with or promote cellular differentiation [8, 12].

C/EBP transcription factors are causally involved in the control of leukemic cell maturation and they are markers for hematopoietic maturation [13]. C/EBPα acts upstream of C/EBPβ and C/EBPε in hematopoietic cells, and C/EBPε particularly directs granulocytic maturation [13–15]. ATRA is a strong inducer of C/EBP proteins in NB4 cells and this illustrates ATRA's strong effect on cell differentiation [8, 16]. The adhesion molecule CD11b is also positively regulated by ATRA [8, 17, 18].

Taking C/EBPβ and C/EBPε as examples, we demonstrate how their expression can be detected by immunoblotting (Fig. 1). In addition, we provide an experimental strategy for the detection of CD11b on the surface of leukemic cells (Fig. 2).

2 Materials

We routinely use these methods and materials, but we do want to state that equipment from other providers is equally useful [19].

2.1 Stimulants and Cell Culture

1. All-trans-retinoic-acid (ATRA) (Sigma-Aldrich). It is recommended to prepare a 5 mM stock-solution by dissolving 1.5 mg ATRA in 1 ml ethanol. Store at −80 °C. Dilute 20 µl of this solution with 80 µl ethanol prior to use to prepare a working solution of 1 mM, store at −20 °C. The solution is light-sensitive and must be protected from light.
2. MS-275 (Selleck Chemicals). Prepare a 5 mM stock solution by dissolving 1.8 mg MS-275 in 1 ml DMSO. Store aliquots at −20 °C.
3. LBH589 (Novartis). Dissolve 10 mg in 286.1 µl DMSO to prepare a 100 mM stock solution. Prepare a 100 µM dilution

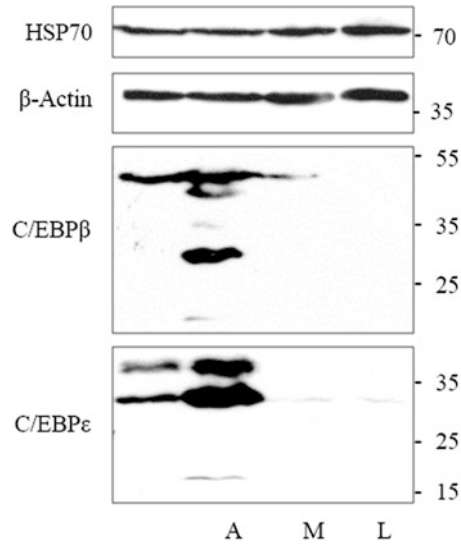

Fig. 1 Expression of C/EBPβ and C/EBPε in NB4 cells after ATRA treatment. NB4 cells were treated for 24 h with ATRA (1 μM) (A) or the HDACi MS-275 (5 μM) (M) or LBH589 (100 nM) (L). Subsequently the cells were lysed and whole cell lysate were analyzed by Western Blot. The proteins were detected with the corresponding antibodies (*see* Subheading 2.5 item 1) on different blots. Molecular weights are shown in kDa; $n = 4$

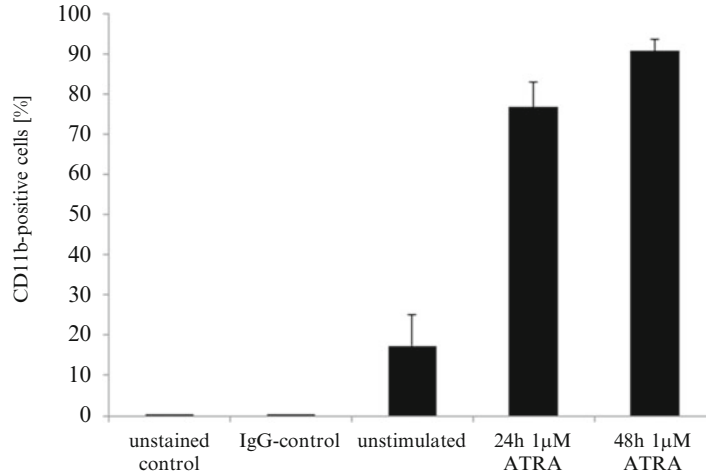

Fig. 2 Expression of CD11b in NB4 cells after ATRA treatment. NB4 cells were treated for 24 h or 48 h with 1 μM ATRA or left untreated. Subsequently, they were stained with PE-coupled antibody either for CD11b or an isotype-specific control (IgG1-PE) and analyzed with FACS. Shown are the percentages of CD11b-positive cells compared to all cells (mean ± SEM, $n = 3$)

by mixing 1 µl of the solution with 999 µl of DMSO. Store aliquots at −80 °C.

4. NB4 cells [7, 8] are kept in Roswell Park Memorial Institute (RPMI) medium containing 10% FCS and 0.5% Gentamicin in a humidified incubator at 37 °C and 5% CO_2. Change the cells to fresh media every 3–4 days.

2.2 Preparation of Whole Cell Lysates and Bradford Analysis for Protein Detection

1. RIPA lysis buffer: 0.1% sodium dodecyl sulfate (SDS) (w/v) (*see* **Note 1**), 1% sodium desoxycholate (w/v), 1% NP-40 (v/v), 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA. Freshly add the protease inhibitor cocktail (PIC) (1:500, *see* **Note 2**), 1 mM NaF (1 M stock, dissolved in water). For the detection of phosphorylated proteins add 1 mM sodium vanadate (*see* **Note 3**), 3 mM phenylmethylsulfonyl fluoride (PMSF) (1.5 M stock, dissolved in ethanol) and 40 mM β-glycerophosphate (0.5 M stock, dissolved in water).
2. Branson Sonifier W250D, amplitude 40% for 3 s ten times.
3. Dulbecco's Phosphate-Buffered Saline (PBS-buffer), e.g., from Sigma-Aldrich.
4. SDS-loading buffer (6×): 62 mM Tris pH 6.8, 5% β-mercaptoethanol (β-ME), 2% SDS, 10% Glycerol, Spatula tip Bromophenole blue.
5. Roti Nanoquant (Roth), 5×-concentrate.
6. Plate-reader (BioTek).

2.3 SDS Polyacrylamide Gel Electrophoresis

1. Separating gel buffer: 1 M Tris–HCl, pH 8.8.
2. Stacking gel buffer: 1 M Tris–HCl, pH 6.8.
3. 20% SDS: 20% (w/v) aqueous solution.
4. 10% Ammonium persulfate (APS): 10% (w/v) aqueous solution.
5. Tetramethylethylenediamine (TEMED) (Sigma).
6. 30% Acrylamide/bisacrylamide 37.5/1 (Roth).
7. 2-Propanol.
8. Mini Protean 3 system, casting stand with corresponding or multiple gel-casting system (Bio-Rad).
9. Casting frames, combs, and glass plates (spacers included) (Bio-Rad).
10. SDS-Running buffer: 250 mM glycine, 25 mM Tris, 0.1% SDS (w/v) (*see* **Note 1** and **Note 4**).
11. Protein ladder (Fermentas).

2.4 Protein Transfer: Western Blot

1. Polyvinylidene difluoride (PVDF) membrane (Millipore).
2. Whatman paper (3 M) (VWR Scientific).

3. Transfer buffer: 25 mM Tris, 192 mM Glycine, pH 8.9 (*see* **Note 5**), 20% ethanol, 250 mM glycine, 25 mM Tris, 0.1% (w/v), 20% ethanol (*see* **Note 6**).
4. Western transfer apparatus (Bio-Rad).

2.5 Detection of Proteins with Western Blot and Stripping of Membranes After Detection

1. Antibodies: HSP70 (Santa Cruz; sc-24 [mouse]); C/EBPβ (Santa Cruz; sc-150 [rabbit]); C/EBPε (Santa Cruz; sc-157 [rabbit]); β-Actin (Sigma; A5316 [mouse]). All antibodies were used as 1:1000 dilutions in 1% (w/v) nonfat dry milk (NFDM) in TBST-buffer, with sodium azide 0.01% (w/v) to avoid microbial contamination. When stored at –20 °C most antibody solutions can be used several times.
2. TBS-T: 150 mM NaCl, 10 mM Tris, pH 7.5, 0.05% Tween-20.
3. Immobilon Western Chemiluminescent HRP Substrate kit (Merck).
4. Stripping buffer: 50 mM Tris–HCl pH 6.8, 2% (w/v) SDS, add freshly 100 mM β-ME.

2.6 FACS-Analysis

1. CD11b-Antibody, PE-coupled (Biozol, R0841); used 1:100 in PBS-buffer.
2. PE-coupled Antibody, adjusted to the isotype of the CD11b-Antibody. In this case: IgG1-PE (EuroBioSciences, C12385P, used 1:100 in PBS-buffer).
3. PBS-Buffer (*see* Subheading 2.2, **item 3**).
4. PBA-Buffer: PBS with 0.2% (w/v) BSA, 0.05% sodium azide.

3 Methods

3.1 Preparation of Cell Lysates

1. For preparation of whole cell lysates needed for further analysis, seed human NB4 suspension cells in 6-well plates with a density of 0.5 mio cells per ml. Usually 3×10^6 cells per well should be sufficient for Western blot analysis. Immediately after seeding, stimulate the cells with ATRA, which remains on the cells until lysis. Pipette gently up and down to make sure that the solution is equally distributed in the well.
2. After 24 h, treat the cells with HDACi. Make sure the inhibitors are equally distributed in the well.
3. Prior to harvest, gently resuspend the cells that have sunk to the ground with a pipette. Transfer the cells to a 15 ml-falcon tube and centrifuge for 5 min at $700 \times g$.
4. All steps afterwards should be done on ice to reduce the protein degradation and the potential loss of posttranslational protein modifications (e.g., of phosphorylation).

5. Wash the cells in 1 ml PBS, then transfer them to a 1.5 ml reaction tube, and centrifuge them again for 5 min at 425 × *g*. Remove the PBS and lyse the cells in approximately fivefold of the volume of the cell pellet. This should be done rapidly to avoid protein degradation.
6. Incubate the cells for 10 min to allow complete lysis. Sonify the lysate to reduce viscosity with a Branson Sonifier W250D at an amplitude of 40% for 3 s and ten times.
7. Remove the cellular debris by a final centrifugation step at 20,000 × *g* for 10 min at 4 °C.
8. Measure the protein concentrations with the Bradford assay-kit from BioRad. Briefly, dilute the Bradford reagent concentrate 1:5 with water and add 200 µl of solution to 2 µl of sample. Measure the resulting color with a plate reader at OD595. A standard curve with known concentrations of BSA enables to calculate the corresponding protein content in the samples.
9. Prepare lysates with the same protein content in each sample for equal loading (60 µl of final volume). Add 12 µl of Laemmli-buffer (6×) and heat the sample for 5 min at 95 °C. This allows the loading of several gels with the same lysates, without having to prepare new samples each time.
10. Load samples either immediately or store at –20 °C or below until further use.

3.2 SDS Polyacrylamide Gel Electrophoresis

1. Prepare a mix of 15% acrylamide (v/v) according to Table 1 for the first part of the separation gel (*see* also **Note 7**). Polyacrylamide polymerization is a radical process with APS and TEMED acting as the radical former and the catalyst.

Table 1
Composition of stacking and separation gel (sufficient for 8 gels. Gels, that are not used immediately, can be stored as described in Subheading 3.2, step 4)

	Stacking gel	Separation gel	
		10%	15%
dH_2O (ml)	11.16	7.03	2.83
Separating gel buffer (ml)	–	9.4	9.4
Stacking gel buffer (ml)	2	–	–
Acrylamide (ml)	2.66	8.3	12.5
SDS (20% w/v) (µl)	80	125	125
APS (20% w/v) (µl)	80	125	125
TEMED (µl)	16	16.5	16.5

Therefore, these two agents should be added last to the mix before casting the gels. Pour the mix into approximately 1/3 of the prepared gel casting chambers and cover immediately after the layer with 500 µl of 2-propanol to ensure proper polymerization.

2. The separating gel should polymerize within about half an hour. Pour away the 2-propanol and carefully wash the gels with water. Do not damage the lining of the gel. Then prepare another separation layer of 10% and cast according to Table 1 and Subheading 3.2, **step 1**.
3. After the gel has polymerized, cast the stacking gel accordingly and immediately insert the comb.
4. 30 min later, the gel should have polymerized and the comb can be removed carefully. The slots should be rinsed with distillated water. The gels can be used immediately but storing the gels in wet paper overnight at 4 °C ensures complete polymerization and results in better resolution of protein separation.
5. Rinse two gels or one gel and a dummy sheet with water and place them in the running chamber. Add running buffer until the chamber is filled and load the samples with a Hamilton syringe. Add 5 µl of marker for later identification of approximate protein sizes. If two lanes at opposite ends of the samples are used for the marker, they can be used later for easier cutting of the membrane.
6. Run the gels at 90 V initially until the samples have fully migrated into the gel, then raise the voltage to 130 V until the running front reaches the far end of the gel.

3.3 Western Blot Transfer

1. Shortly equilibrate the PVDF-membrane in ethanol for 15 s then place it in transfer buffer (*see* **Note 8**). Make sure that the membrane is thoroughly wet, as after the incubation with ethanol it is slightly hydrophobic and should be gently rocked to ensure full soaking. Also, wet Whatman papers and foam sheets with buffer.
2. After protein separation, rinse the gel shortly with distilled water, cut the stacking gel off and remove it. Assemble separating gel in a tray filled with buffer to a sandwich as shown in Fig. 3. Remove air bubbles carefully with a roler.
3. Place the cassette and blocking apparatus inside the Wet Blot running chamber together with an ice pack for cooling the gel during the transfer. Fill the chamber with chilled transfer buffer and close it. Run the transfer with 75 mA per membrane for 135 min, although higher voltages are also possible.
4. After transfer, take the chamber apart. If more than one protein should be detected and they are sufficiently apart in size, the membrane can be cut with a sharp scalpel in several parts (*see also* **Note 9**).

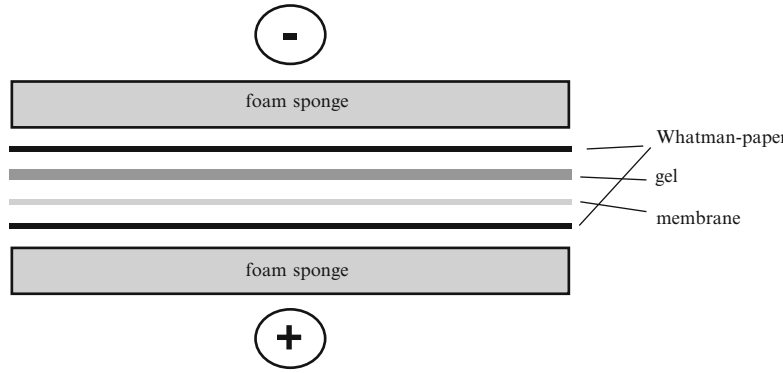

Fig. 3 Basic structure of a Western blot-sandwich for the transfer procedure

5. Wash the membrane shortly in TBST-buffer to remove remaining transfer buffer. During that time the remains of the transfer can be cleared up.

3.4 Protein Detection

1. To block unspecific binding sites on the membrane, block it for 1 h in a solution of 5% (w/v) nonfat milk in TBST-buffer at room temperature.
2. Afterwards place the membrane into the appropriate first antibody solution and incubate it with agitation at 4 °C overnight. We place membranes in 50 ml Falcon tubes, which allows us to use only 5 ml of antibody solution.
3. After incubation, wash the membrane three times for 5 min in 20 ml of TBST to remove remaining antibody and sodium azide.
4. Prepare the secondary antibody freshly in a 1% (w/v)-solution of nonfat milk in TBST (mouse 1:5000 and rabbit 1:10,000) and incubate the membrane for 1 h at room temperature with slight agitation.
5. Wash the membrane three times for 5 min in 20 ml TBST-buffer.
6. For detection, we use Chemiluminescent HRP Substrate from Merck accordingly to the manufacturer's instructions. Prepare a 50:50-solution of the two agents and shortly incubate the membrane with the mix. It is important to soak the whole membrane fully to achieve an even detection. Then whipe off excess solution and place the membrane between thin sheets of plastic in a cassette for detection. Expose it to a film and develop the films with a developing machine (Protec, Germany) or per hand using baths of developer, fixer, and water.
7. For detection of several proteins that have roughly the same sizes, it is necessary to strip the membrane (*see* **Note 10**).

Incubate the membrane for 30 min in 5 ml stripping buffer that has been heated to approximately 50 °C. To remove residual β-ME, wash the membrane three times in distilled water for 10 min. Since stripping not only removes the antibodies that have been bound to the membrane, but also the blocking proteins, it is necessary to perform the blocking step again. Incubate the membrane for 1 h in 5% (w/v) nonfat milk in TBST-buffer at room temperature. Afterwards the membrane can be placed into the primary antibody as described in Subheading 3.4, **step 2**.

3.5 FACS-Analysis for CD11b-Surface Expression

1. Transfer the cells into FACS tubes and centrifuge at 700 × *g* for 5 min at room temperature.
2. Then wash the cells once with 1 ml PBS.
3. Afterwards, resuspend the cells in 300 µl PBA-Buffer and add 3 µl of the appropriate antibody. Do not forget the isotype control, which contains the respective IgG-antibody, coupled with the dye (in this case PE) as well as a non-stained control, to ensure specificity for the detection process.
4. Incubate the cells 30 min on ice in the dark.
5. Afterwards, wash the samples three times with 1 ml PBS-buffer.
6. Again, resuspend the cells in 500 µl PBA-buffer and analyze them with the FACS-machine. For analysis use a dot blot of SSC-A against FSC-A to select the main cell population, then a histogram of count against PE-A for gating. The unstained sample should show the background signal of the antibody and serves to exclude the negative from the positive count. Set a gate for all cells that do not show the fluorescence as measured in the unstained sample. It is also possible to measure the mean of all cell fluorescence and subtract the unstained samples mean from the samples of interest.

4 Notes

1. Since sodium dodecyl sulfate is highly aggressive to the airways, use a dusk mask while weighting. If SDS-pellets are available, prefer them over the powder.
2. The Complete Protease Inhibitor cocktail (PIC) stock consists of: 1 mg/ml Leupeptin, 2 mg/ml Antipain, 100 mg/ml Benzamidin,10,000 U/ml Aprotinin, dissolved in distilled water, stored at −20 °C, and used at 1:500 dilution.
3. For being in the active conformation sodium vanadate should be heated prior to using to 95 °C for 5 min and placed immediately after on ice. Prepare a 100 mM stock in water.

4. A stock of tenfold running-buffer is preferred.
5. A stock of tenfold transfer-buffer is preferred.
6. Methanol is widely used, but ethanol works as well and is less noxious.
7. Always wear gloves while handling acrylamide or gels because acrylamide is carcinogenic and is rapidly absorbed through the skin.
8. Wearing gloves is highly recommended while handling membranes, as proteins from the skin can adhere to the membrane and cause background noise.
9. Gels should not be used after 2 weeks as their quality decreases with time.
10. Since stripping not only removes antibodies and blocking proteins but also the proteins of interest, try to strip as little as possible. Alternatively, another gel might be run, the membrane can be cut, or the species in which the antibodies were generated could be changed.

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Chapter 29

Encapsulation of the HDACi Ex527 into Liposomes and Polymer-Based Particles

Dorle Hennig and Diana Imhof

Abstract

Incorporation of drugs into particles can improve their therapeutic effectiveness. Solubility, half-life time, targeting, and the release of the drug can be modified by the encapsulation into a particle. Histone deacetylase inhibitors have a great potential to be used as therapeutics for many different diseases. In this chapter, we describe the inclusion of the low molar mass HDACi Ex527 into polymer-based particles and liposomes.

Key words Histone deacetylase inhibitor, Ex527, PLGA, Acetalated dextran, Liposomes, Cellular uptake

1 Introduction

Histone deacetylase inhibitors (HDACi) are derived from different classes of chemical substances. Due to their broad spectrum of activity, HDACi have a high potential to be used as drugs in therapy of a great variety of diseases including cancer [1, 2], immune and inflammatory diseases [3, 4], neurodegenerative disorders [5], and epilepsy [6]. To improve the effectiveness of HDACi the respective compound can be encapsulated in particles, which can increase the water solubility of the HDACi [7] and prolong their half-life time in the blood stream [8]. A further, big advantage of an encapsulated drug in comparison to the free drug is the enrichment within the target tissue. This can be mediated by a size-dependent accumulation of particles in solid tumors or by the modification of the particle surface [9]. Targeting of drugs to the cell/tissue of interest improves their effectiveness and reduces side effects. Another very important point is that the release of the drug can be controlled by the type of the particle [10].

This chapter describes the inclusion of Ex527 into acid- or ester-terminated poly(lactic-co-glycolic acid) (PLGA) and acetalated dextran (Ac-Dex) particles using a single emulsion oil/water (o/w) evaporation method. Ex527 is an HDACi which shows a

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relatively short half-life time of 1–3 h in the blood of mice [11, 12]. Ex527 passed phase I and II studies for therapy of Huntington's disease (NCT01485952, NCT01485965, NCT01521585, NCT01521832; data from http://clinicaltrials.gov, retrieved on 22.10.2015). Due to these facts, Ex527 was incorporated into different polymer-based particles and liposomes to investigate the inclusion efficiency and activity of the encapsulated drug. PLGA and dextran are approved by the Food and Drug Administration (FDA) for several applications [13–15]. Both polymers are soluble in organic solvents and therefore suitable for emulsification-solvent evaporation techniques. Besides polymer-based particles, Ex527 was further incorporated into liposomes using the ethanol injection method. For the preparation of the liposomes, the lipid phosphatidylcholine from egg or soybean was used.

Analysis of the Ex527 inclusion efficiency was performed after degradation of the particles followed by a quantification of the compound by HPLC measurements. In addition, the activity of the incorporated drug was examined using a cellular assay followed by Western blot analysis. Finally, the cellular uptake of the polymer-based particles was investigated using confocal microscopy and flow cytometry.

2 Materials

2.1 Polymers

1. Poly(lactic-co-glycolic acid) (PLGA) polymer samples, purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany); all providers are given as examples, material from others may also work well:
   PLGA-0 (MW = 24,000–38,000 g/mol, lactide:glycolide = 50:50, acid terminated, Resomer® RG 503H);
   PLGA-1 (MW = 7000–17,000 g/mol, lactide:glycolide = 50:50, acid terminated, Resomer® RG 502H);
   PLGA-2 (MW = 7000–17,000 g/mol, lactide:glycolide = 50:50, ester terminated, Resomer® RG 502).
2. Acetalated dextran (Ac-Dex) was synthesized according to a modified literature procedure [16]. 2.0 g of dextran (MW = 60,000 g/mol, PharmaGrade, Pharmacosmos, Holbaek, Denmark) was modified with 2-methoxypropene (7.2 ml) and catalytic amounts of pyridinium-p-toluenesulfonate (32 mg) yielding acetalated dextran with a total degree of substitution (DS) of 2.2 (DS(cyclic acetals) = 1.57, DS(acyclic acetals) = 0.63). The degree of substitution was determined by ^{1}H-NMR spectroscopy in DCl/D_2O.
3. Rhodamine B-labeled polymer samples were prepared by reaction of 3 mg rhodamine B-isothiocyanate with 300 mg PLGA-1 or Ac-Dex, respectively, in 3 ml DMSO at 50 °C for

5 h (plus addition of 30 µl Triethylamine for Ac-Dex). The solutions were first precipitated in water, washed, redissolved in DMSO, extensively dialyzed against water and subsequently lyophilized. A closer characterization by NMR spectroscopy or mass spectrometry is not feasible due to the low rhodamine B content.

2.2 Lipids

Liposomes were prepared using egg phosphatidylcholine (EPC) and Lipoid S 100 (phosphatidylcholine from soybean).

2.3 Solvents and Further Substances

Dichloromethane, DMSO and HPLC gradient grade 96% ethanol.

Ex527 (6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1--carboxamide, 98% HPLC pure).

Poly(vinyl alcohol) (PVA, Mw = 67,000 g/mol, 87–89% hydrolyzed).

Triton X-100.

PBS: 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.4 mM KH_2PO_4, adjust with HCl to pH 7.25.

2.4 HPLC-Analysis

Analytical HPLC was performed on a Shimadzu LC-10AT system (Duisburg, Germany) using a Vydac 218TP54 C18 reversed-phase column (5 mm particle size, 300 Å pore size, 4.6 × 25 mm) from Macherey-Nagel (Dueren, Germany).

HPLC gradient grade acetonitrile and water.

Trifluoroacetic acid.

Buffer for Ac-Dex particle degradation: 0.1 M NaH_2PO_4, pH 2.3.

D-tryptophan.

Corning® Costar® Spin-X® centrifuge tube filters were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany).

2.5 Cell Culture

HEK293T cells were cultivated with Dulbecco's modified Eagle's medium supplemented with 2% L-glutamine, 10% fetal calf serum, and 1% penicillin/streptomycin. Dulbecco's modified Eagle's medium, fetal calf serum, and penicillin/streptomycin were purchased from PAA Laboratories GmbH (Coelbe, Germany).

Plastic material needed for cell culture.

2.6 Western Blot Analysis

NETN lysis buffer: 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris–HCl (pH 7.8), 0.5% Nonidet P-40 (NP-40), add freshly 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin, 10 mM nicotinamide and 100 nM Trichostatin A (*see* **Note 1**).

Laemmli buffer (6x): 312.5 mM Tris (pH 6.8), 25% β-mercaptoethanol, 10% sodium dodecyl sulfate, 50% glycerol, 0.005% bromophenol blue.

PVDF-membrane, pore diameter maximum 0.45 µm.

PBS-T: 137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.4 mM KH_2PO_4, adjust with HCl to pH 7.25, 0.05% Tween 20.

Anti-p53 antibody was purchased from Santa Cruz Biotechnology (#sc-126, Heidelberg, Germany), and the acetyl-p53 (Lys382) antibody was from Cell Signaling Technology (#2525, Beverly, MA, USA).

Enhanced Chemoluminescence (ECL) kit.

Stripping buffer: 62.5 mM Tris–HCl pH 6.8, 2% (w/v) SDS, add freshly 100 mM β-mercaptoethanol.

2.7 Microscopy

Alexa Fluor® 488 Phalloidin and Pro Long®Gold antifade reagent with DAPI were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

LSM710 laser scanning confocal microscope from Carl Zeiss GmbH (Jena, Germany) was used.

2.8 FACS Analysis

Accutase.

FACS-tubes.

Fluorescence-activated cell sorter (FACS) Canto II flow cytometer from BD Biosciences (Heidelberg, Germany).

2.9 Machines and Other Devices

Sonicator (VC 505) from Sonics & Materials, Inc. (Newtown, CT 06470-1614, USA).

Ultrasound/sonication bath.

Ultracentrifuge.

Freeze-dryer.

Photon correlation spectroscopy (PCS) or dynamic light scattering (DLS) Zetasizer Nano ZS from Malvern Instruments (Herrenberg, Germany).

Scanning electron microscopy LEO-1450 VP from Leo Elektronenmikroskopie GmbH (Oberkochen, Germany), sputter coating device BAL-TEC SCD005 (60 mA, 80 s) from Balzers (Lichtenstein).

100 nm polycarbonate membrane.

Sterile filter, hydrophilic Durapore, 0.22 μm pore diameter.

3 Methods

3.1 Preparation of Polymer-Based Ex527-Containing Particles

Ex527-loaded particles are prepared using a single emulsion oil/water (o/w) evaporation method.

1. 0.625 mg of Ex527 and 25 mg of the polymer are dissolved in 0.5 ml of dichloromethane.
2. The solution is sonicated with 1 ml 3 wt% PVA solution 10-times for 1 s with each 1 s pause on an ice bath using a probe sonicator with an amplitude of 30%.
3. The resulting single emulsion is immediately poured into 10 ml of a 0.3 wt% PVA solution and stirred for 3 h at room temperature to allow the dichloromethane to evaporate completely.

4. The particles are then isolated by ultracentrifugation at approximately 17,000 × *g* for 15 min (*see* **Note 2**), washed three times with distilled water by resuspension in a sonication bath, and lyophilized to yield an amorphous white powder.
5. Particles are stored at −20 °C until analysis.
6. The particle size is determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS operating with a laser beam at 633 nm and a scattering angle of 173°. The intensity distribution of the particle size is calculated from 15 runs for each 10 s at 25 °C.
7. Scanning electron microscopy images are obtained using a LEO-1450 VP. The sputter coating device BAL-TEC SCD005 is used. The system is operated at 8 kV.

3.2 Preparation of Ex527-Containing Liposomes

Ex527-containing liposomes are prepared by the ethanol injection method.

1. Dissolve lipids (EPC or Lipoid S 100) and Ex527 in 100% ethanol. The concentration of lipids has to be kept constant as 40 mg/ml. The drug concentration can vary from 0.25 to 3 mg/ml.
2. Each 36 µl of ethanolic lipid drug solution is placed in 144 µl of PBS buffer and resuspended three times (pipetting robot, FasTrans, Analytik Jena AG, Jena, Germany).
3. The produced liposome samples are analyzed by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) or DLS for uniform particle sizes. Samples of the same batch are combined.
4. 20-fold extrusion of the liposomes through a 100 nm polycarbonate membrane (if necessary dilution of the original samples to a sample volume of at least 400 µl).
5. Particle size after extrusion is examined by PCS.
6. Then, the free drug molecules are removed by ultracentrifugation for 60 min at 183 960 × *g* in a 500 µl sample tube. Sediments are resuspended in 500 µl of purified water. Repeat the procedure once under the same conditions.
7. Absorption of the liposome sediment is done in a defined amount of purified water, whereby the maximum Ex527 final concentration is adjusted to 252 µg/ml.
8. This is followed by sterile filtration of the sample.
9. Liposomes are stored at 4 °C up to a maximum of 24 h prior to analysis.

3.3 Degradation of Particles

PLGA particles

1. 1–7 mg of particles are suspended with 400 µl of 100% acetonitrile and treated with ultrasound for 30 s.
2. Water is added to result in a 40% acetonitrile/water solution for freezing and subsequent lyophilization.
3. The resulting powder is dissolved in 300 µl 96% ethanol to get Ex527 in solution, while PLGA is still in the solid state.
4. Solutions are filtered using centrifuge tube filters and analyzed by RP-HPLC.

Ac-Dex particles

1. 1–7 mg of particles are degraded using 400 µl of 0.1 M NaH_2PO_4 buffer (pH 2.3).
2. The solution is frozen and lyophilized.
3. The resulting powder is dissolved in 96% ethanol for Ex527 to get Ex527 in solution, while Ac-Dex is still in the solid state.
4. Solutions ware filtered using centrifuge tube filters and analyzed by RP-HPLC.

Liposomes

1. 200 µl of ethanol are added to 50 µl of the liposomal formulation to reach a final concentration of 80% ethanol, which destroys the liposomes.
2. 200 µl water was then added for freezing and lyophilization.
3. The remaining part is resuspended in 200 µl of 96% ethanol.
4. The resulting solutions are filtered using centrifuge tube filters and analyzed by RP-HPLC.

3.4 Quantification of Encapsulated Ex527

An internal standard (D-tryptophan, 1 mg/ml in 50% acetonitrile/water) is used in HPLC to ensure the same conditions and concentrations throughout the analyses. 2.5 µg of D-tryptophan are injected for HPLC analysis.

1. 30 µl of the Ex527-containing sample solution is well mixed with 10 µl of the internal standard.
2. 20 µl of this mixture are injected for HPLC analysis on a C18 reversed phase column.
3. Ex527 is eluted with the gradient 0–50% of eluent B in 50 min at a flow rate of 1 ml/min, where A is 0.1% TFA in H_2O and B 0.1% TFA in acetonitrile. Detection is at 233 nm.
4. The area under the curve is used to calculate the amount of Ex527 according to a standard curve.

Figure 1 shows the Ex527 inclusion efficiency as a percentage of incorporated Ex527 versus the total amount of Ex527 applied for particle preparation.

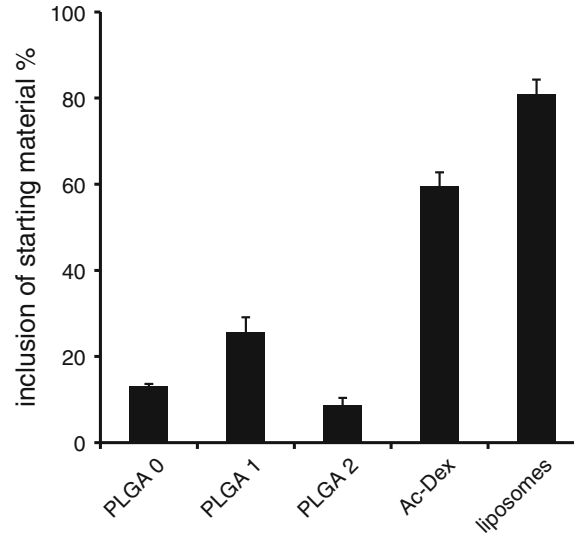

Fig. 1 Inclusion efficiency of Ex527. Inclusion efficiency of Ex527 into different kinds of particles, which were prepared via the described protocols. Particles were degraded and the incorporated Ex527 was quantified. Ex527 inclusion efficiency is expressed as percentage of incorporated versus the total amount of Ex527 applied for particle preparation (starting material)

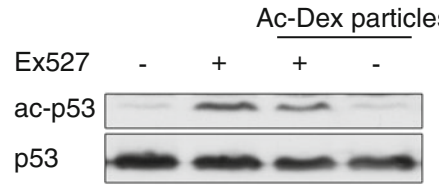

Fig. 2 Biological activity of incorporated Ex527. HEK293T cells were treated with 4 μM Ex527 as free drug or loaded into Ac-Dex-based particles for 6 h, cells were washed, lysed, and the acetylation status of p53 (K382) was analyzed by Western blotting. All lysates were from the same experiment and analyzed on one membrane

3.5 In Vitro Biological Activity of Encapsulated Ex527

SIRT1 is known to be a deacetylase for the lysine residue 382 in the p53 protein [17]. Ex527 is a specific and very potent SIRT1-inhibitor. Acetylation of p53 at lysine 382 was used to analyze the biological activity of the encapsulated Ex527 in a cellular system (Fig. 2).

1. HEK293T cells are maintained in Dulbecco's modified Eagle's medium supplemented with 2% L-glutamine, 10% fetal calf serum, and 1% penicillin/streptomycin. Cells are cultured and grown in cell culture flasks to approximately 80% confluence in a 5% CO_2 incubator at 37 °C.
2. HEK293T cells are seeded in six-well plates 1 day prior to stimulation. Then, cells are stimulated with the same amount of Ex527 (4 μM) in the control (non-encapsulated drug) stimulation.

3. Afterwards, cells are lysed in 80 µl NETN buffer for 20 min at 4 °C.
4. This step is followed by sonification.
5. A centrifugation step at 20,000 × *g* for 5 min at 4 °C is performed to remove cellular debris. It is recommended to directly use the fresh lysate for the SDS-PAGE. Alternatively, it can be stored at −80 °C or lower temperature.
6. Protein lysate is boiled in Laemmli buffer.
7. Samples are separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide (v/v) as separating gel [18].
8. Proteins are blotted to a PVDF-membrane using a tank blot for 2 h at 150 mA (per gel).
9. Western blotting with specific antibodies according to the protocol of Ginter et al. [18].
   (a) Membrane is blocked with PBS-T containing 5% (w/v) non-fat dry milk.
   (b) Then, the membrane is incubated at 4°C over night with acetyl-p53 (Lys382) antibody (1:1000) in PBS-T with 1% (w/v) non-fat dry milk (*see* **Note 3**).
   (c) Wash the membrane three times with PBS-T.
   (d) Subsequently, membrane is incubated with the secondary antibody diluted 1:5000 in PBS-T containing 1% non-fat dry milk for 1 h at room temperature.
   (e) Membrane is then washed three times with PBS-T.
   (f) The signal is detected by autoradiography using an ECL kit as recommended by the manufacturer.
   (g) After detection of acetylation the identification of total p53 protein level is relevant. For that purpose, the membrane is washed quickly in PBS-T and incubated afterwards in stripping buffer for 1 h at room temperature on a shaker.
   (h) The membrane is washed with distilled water until the smell of β-mercaptoethanol has disappeared entirely. The membrane is incubated in PBS-T, and the procedure is repeated with **steps a–f** using p53 antibody (*see* **Note 3**) in a 1:30,000 dilution.

3.6 Microscopy and Flow Cytometry

Microscopy and flow cytometry analysis are used to investigate the cellular uptake of the prepared particles by HEK293T cells.

Microscopy (*see* **Note 4**).

1. HEK293T cells are seeded in six-well plates on 12 mm coverslips 1 day prior to stimulation.
2. Cells are stimulated with rhodamine B-labeled particles for an indicated time period (from 6 to 24 h).

3. Then, medium is sucked off.
4. Wash the cells on the coverslip carefully three times with 2 ml of PBS.
5. Fix the cells with 2% formaldehyde for 10 min at room temperature.
6. Wash the cells carefully two times with 2 ml of PBS.
7. Cells are then permeabilized with 0.15% Triton X-100 for 5 min at room temperature.
8. Wash the cells carefully two times with 2 ml of PBS.
9. Then, cells are stained with 100 µl of AlexaFluor® 488 Phalloidin solution (1:40 in PBS) for 15 min in the dark at room temperature.
10. Wash the cells carefully three times with 2 ml of PBS.
11. A drop of Pro Long® Gold antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI) is added on top of the cells, then carefully place a coverslip avoiding air bubbles on microscope slides and incubate at room temperature overnight in the dark before imaging on a confocal microscope (Fig. 3).
12. For longer storage keep the cells at −20 °C.

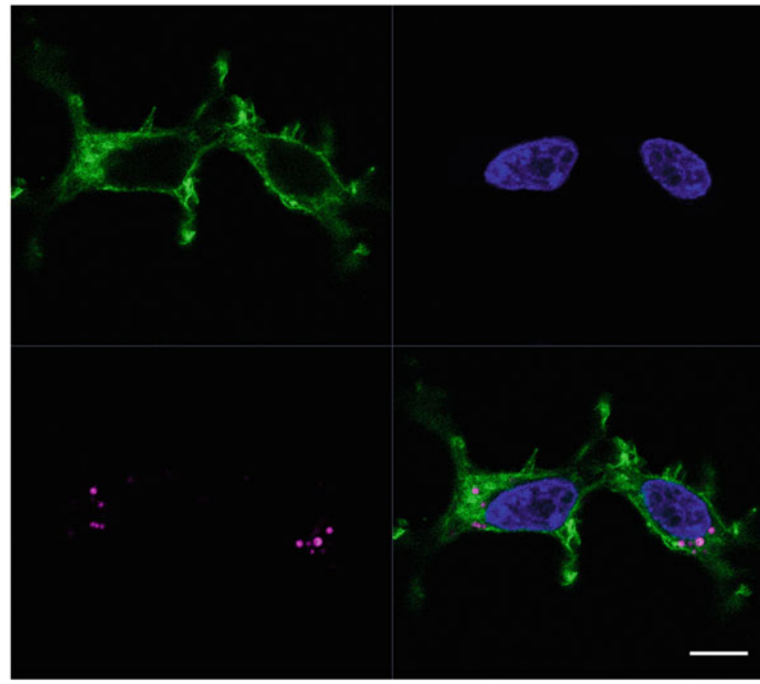

Fig. 3 Confocal microscopy of the cellular uptake of particles. Confocal microscopy of HEK293T cells treated with rhodamine B-labeled Ac-Dex-based particles for 24 h. Cells were treated with Ex527-loaded nanoparticles (*magenta*), washed, fixed, and stained with DAPI (nucleus, *blue*) and Alexa Fluor 488 Phalloidin (actin, *green*). Scale bar equivalent to 10 µm

Flow cytometry

1. HEK293T cells are seeded in six-well plates 1 day prior to stimulation.
2. Stimulate the cells with rhodamine B-labeled particles for an indicated time period (from 6 to 24 h).
3. Wash the cells three times with 2 ml of PBS.
4. Cells are then treated with 300 µl accutase for 5 min to detach them from the six-well plate.
5. 1 ml of PBS is added to the cells, and the resulting cell suspension is transferred to a 5 ml FACS tube.
6. Wash the cells three times with PBS and centrifuge at 300 × *g*.
7. Samples are then analyzed using Canto II, counting cells positive for rhodamine B-labeled particles using the PE-channel (Fig. 4).

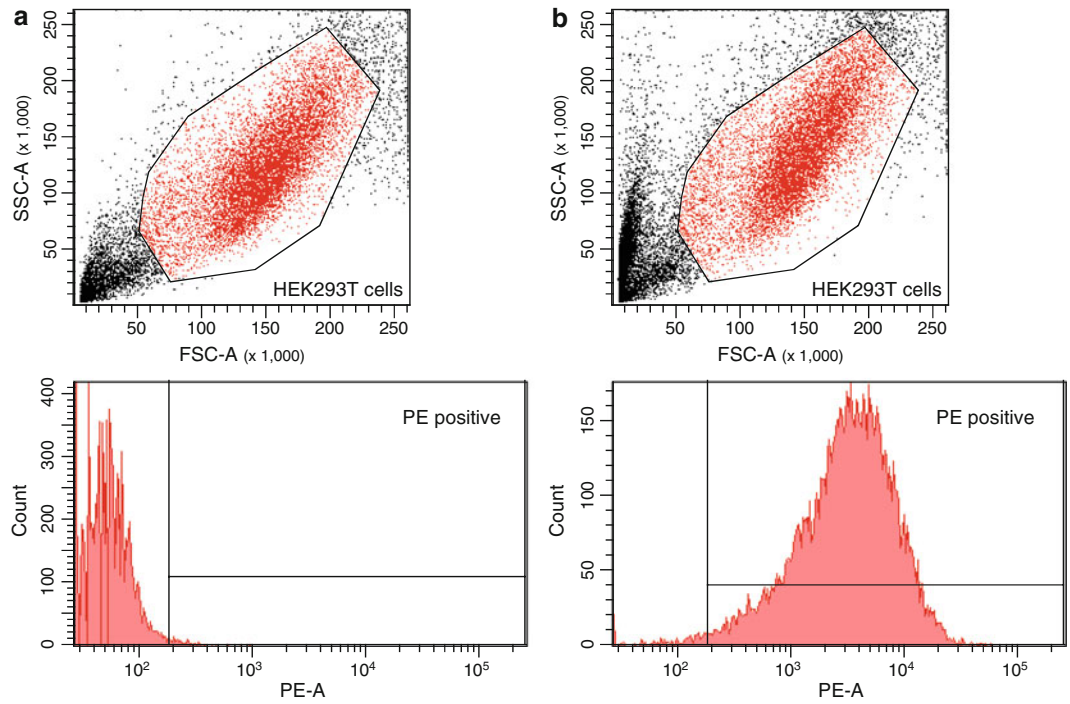

Fig. 4 Flow cytometry analysis of the cellular uptake of particles. FACS analysis of HEK293T cells treated with unlabeled (**a**) and rhodamine B-labeled Ex527-loaded PLGA 1-based particles (**b**) for 6 h. In a first step, whole cells (293T) were gated using forward (FSC) versus side scatter (SSC, density plot). In a second step, the gate for PE-positive cells was determined using cells treated with unlabeled PLGA-based particles (**a**, histogram). Using the gate "PE-positive" the total amount of cells positive for rhodamine B-labeled PLGA 1-based particles was analyzed (**b**, histogram)

4 Notes

1. It is recommended to prepare a TSA stock solution of 100 mM dissolved in DMSO and a 1 M aqueous solution of nicotinamide. Both stock solutions should be stored at −150 °C.
2. Time and strength of centrifugation depends on the size and type of particles.
3. Add 0.01 % (w/v) sodium azide (very toxic) to prevent microbial contamination, store antibody solution at −20 °C and reuse it several times.
4. Handle HEK293T cells very carefully while washing and staining, otherwise they will detach from the coverslip.

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Chapter 30

Preparation of Histone Deacetylase Inhibitor Vorinostat-Loaded Poly D, L-Lactide-co-Glycolide Polymeric Nanoparticles by Nanoprecipitation Method

Vilwanathan Ravikumar and Renu Sankar

Abstract

Nanotechnology is a comparatively new branch of science that includes harnessing the unique properties of particles that are nanometers in scale. Nanoparticles can be tailored in a precise fashion where their size, composition, and surface chemistry can be carefully controlled. The nanoprecipitation is a simple, powerful, and low-energy requiring technique, commonly used for the preparation of defined nanoparticles. Histone deacetylase inhibitor Vorinostat-loaded Poly D, L-lactide-co-glycolide (PLGA) polymeric nanoparticles were prepared by the nanoprecipitation technique. The technique commonly relies on the precipitation of a solvent-dissolved material as nanosize particles after the addition to a non-solvent-containing stabilizer. The particle size and size distribution of the Vorinostat polymeric nanoparticles are significantly influenced by the surfactants used in the fabrication process of the formulation. The surfactants prevent aggregate formation and improve the stability of the nanoparticles. The partitioning and evaporation of organic solvents allowed the formation of Vorinostat-loaded polymeric nanoparticles.

Key words Nanoprecipitation, Vorinostat, Poly D, L-lactide-co-glycolide, Surfactants

1 Introduction

Nanoprecipitation is a commonly used and promising technique for the formulation of both hydrophilic and hydrophobic drugs loaded on cationic biodegradable polymeric nanoparticles [1]. The method is quite flexible and can be mostly used for the preparation of a wide range of hydrophobic biodegradable polymeric nanoparticles [2]. Moreover, the method is simple to perform, powerful, can be certainly scaled up, and it is a one-step procedure that results in a rapid formation of desired nanoparticles [3].

The nanoprecipitation technique needs two miscible solvents: the biodegradable polymer and drug dissolved in a solvent (organic phase), and the stabilizer dissolved in a non-solvent

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(aqueous phase) [4, 5]. The nanosize particles are spontaneously formed by the addition of the organic phase to the aqueous phase under standard magnetic stirring condition, due to the phase separation and solvent diffusion process [6, 7]. The particle size, size distribution, and drug encapsulation efficiency of the nanoparticles are significantly influenced by the surfactants used in the fabrication process of the formulation [8]. The lipophilic surfactant Span 80 aligns at the oil–water interface and prevents the aggregation and improves the stability of the nanoparticles [9]. The separation and evaporation of solvent permitted the formation of drug-loaded polymeric nanoparticles [10, 11]. Here, we describe how to formulate the histone deacetylase inhibitor Vorinostat loaded on Poly D, L-lactide-co-glycolide (PLGA) nanoparticles using the nanoprecipitation technique.

2 Materials

The apparatus used in this technique was thoroughly rinsed with Milli-Q water. For the solution preparation, use Milli-Q water and analytical-grade reagents. The reagent preparation was achieved in a clean chamber and store at 4 °C (unless indicated otherwise).

2.1 Preparation of Organic Phase Solution

2.1.1 Vorinostat Preparation

1. The Vorinostat has to be stored at 4 °C until the start of the experiment (*see* **Note 1**).
2. Accurately weigh 2 mg of Vorinostat in a microcentrifuge tube (*see* **Note 2**).
3. Dissolve Vorinostat with 1 mL of ethanol (*see* **Note 2**).
4. Vortex them and allow to mix well by sonication for 5 min (*see* **Note 3**).

2.1.2 PLGA Polymer Preparation

1. Accurately weigh 10 mg of the PLGA polymer in 150 × 19 mm glass test tube (*see* **Note 4**).
2. Dissolve PLGA polymer with 5 mL of acetone (*see* **Note 2**).
3. Seal the test tube with aluminum foil.
4. Vortex them and allow to mix well by sonication for 15 min (*see* **Note 5**).

2.1.3 Preparation of Aqueous Phase Solution

1. Accurately weigh 20 mg of Tween 80 in the clean, sterile 25 mL glass bottle (*see* **Note 6**).
2. Add 5 mL of Milli-Q water.
3. Keep the bottle under magnetic stirring (220 g) for 30 min at room temperature (*see* **Note 7**).

3 Methods

Freshly prepare an organic and aqueous phase solution while initiating the experiment. The nanoprecipitation technique has to be carried out at room temperature.

3.1 Preparation of Vorinostat Polymeric Nanoparticles by Nanoprecipitation Method (Fig. 1)

1. 2 mg of Vorinostat in ethanol was mixed with 10 mg of the PLGA polymer in acetone solution.
2. Seal the test tube with aluminum foil.
3. Vigorously vortex the mixture for 10 min.
4. For preparation of the organic phase solution, add 50 mg of Span 80 to the above mixture and vortex it for 5 min.

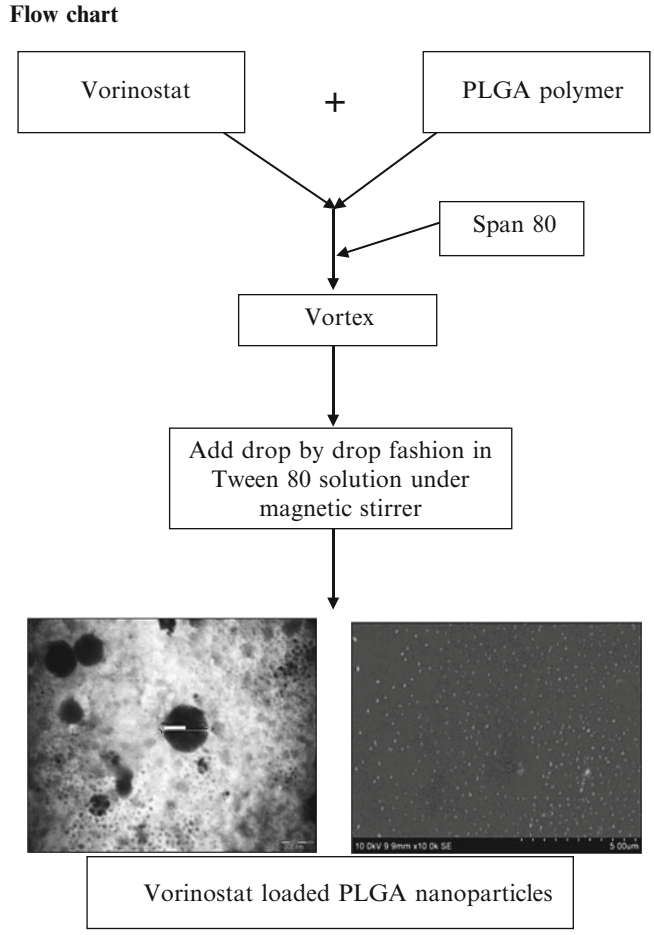

Fig. 1 Schematic illustration of the formulation of histone deacetylase inhibitor Vorinostat-loaded Poly D, L-lactide-co-glycolide polymeric nanoparticles

5. For the formulation of drug-loaded polymeric nanoparticles, organic phase solution was added drop by drop in an aqueous phase solution (Tween 80) using a 12.7 mm syringe dispenser under magnetic stirring (220 g) (*see* **Note 8**).
6. Seal the mixture tightly with aluminum foil and pierce holes using 1 mm needle.
7. Permit the preparation to continuously stir for 12 h.
8. The formulation was centrifuged at 71,000 × *g* for 15 min, freeze dried and stored at 4 °C (*see* **Note 9**).

4 Notes

1. The optimum temperature for Vorinostat is –20 °C. Care should be taken while preparing the drug, use ice bags to maintain the temperature.
2. The organic solvents used in this method evaporate quickly; to avoid the solvent loss use tubes and bottles with cap.
3. To dissolve the drug completely, vortex and then sonify.
4. The procedure should be carried out under 4 °C to maintain the nature of the polymer.
5. Solubility of the PLGA polymer in acetone is poor. To achieve complete solubility do the vigorous vortex and then sonication.
6. Tween 80 is viscous in nature, care should be taken (Pipette handling) while weighing.
7. To achieve the equilibrium, maintain the same g in the stirring process.
8. The PLGA polymer can aggregate and form a thread-like structure when added to the aqueous phase solution. To avoid the aggregate formation, add the organic phase solution drop by drop into the aqueous phase solution.
9. The nanoparticulate was freeze dried using 5% glucose as a cryoprotectant.

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Chapter 31

Generation and Assessment of Fusions Between HDACi and TKi

Siavosh Mahboobi, Bernadette Pilsl, and Andreas Sellmer

Abstract

Chimeric compounds combine the structural features of inhibitors of histone deacetylases (HDACi) and tyrosine kinase inhibitors (TKi), and therefore unite the effects of a dual-targeting strategy in one compound. Here, we describe the generation of such hybrid molecules. Small molecules, known as TKi, are combined with a Zn^{2+} chelating motive, preferentially a hydroxamic acid, in addition. The resulting small molecules also can inhibit histone deacetylases, which are dependent on the catalytically active Zn^{2+}. Moreover, we summarize how the growth-inhibitory effects of these combined compounds can be determined with a simple proliferation assay with a leukemic cell line.

Key words Histone deacetylase inhibitor, Tyrosine kinase inhibitor, Chimeric molecules, Dual targeting strategy, Hybrid structures, Proliferation assay

1 Introduction

The treatment with HDACi causes changes in chromatin and thereby modulates the expression of numerous genes. This includes the re-expression of tumor suppressor genes, whereby cellular differentiation, growth inhibition, and apoptosis induction can be triggered. Different HDACi are currently under development or in preclinical and clinical studies of phase I/II for cancer therapy [1–3]. HDACi have been shown to inhibit growth and induce apoptosis in cancer cells with minimal toxicity. In addition to changes in gene expression, HDACi effect the degradation of various oncoproteins [4]. Moreover, a synergy with other agents, including TKi [5–7] has been shown, which is at least in part based on previously reported mechanisms by which HDAC inhibitors can destabilize chaperones [8], which are responsible for oncogene stability.

This has led to a number of investigations regarding the effect of simultaneous treatment with HDACi and TKi

A dual TKi (EGF/Her2) and HDACi CUDC 101 3 is currently in Phase I clinical trials [2, 9]. Due to the combination of the

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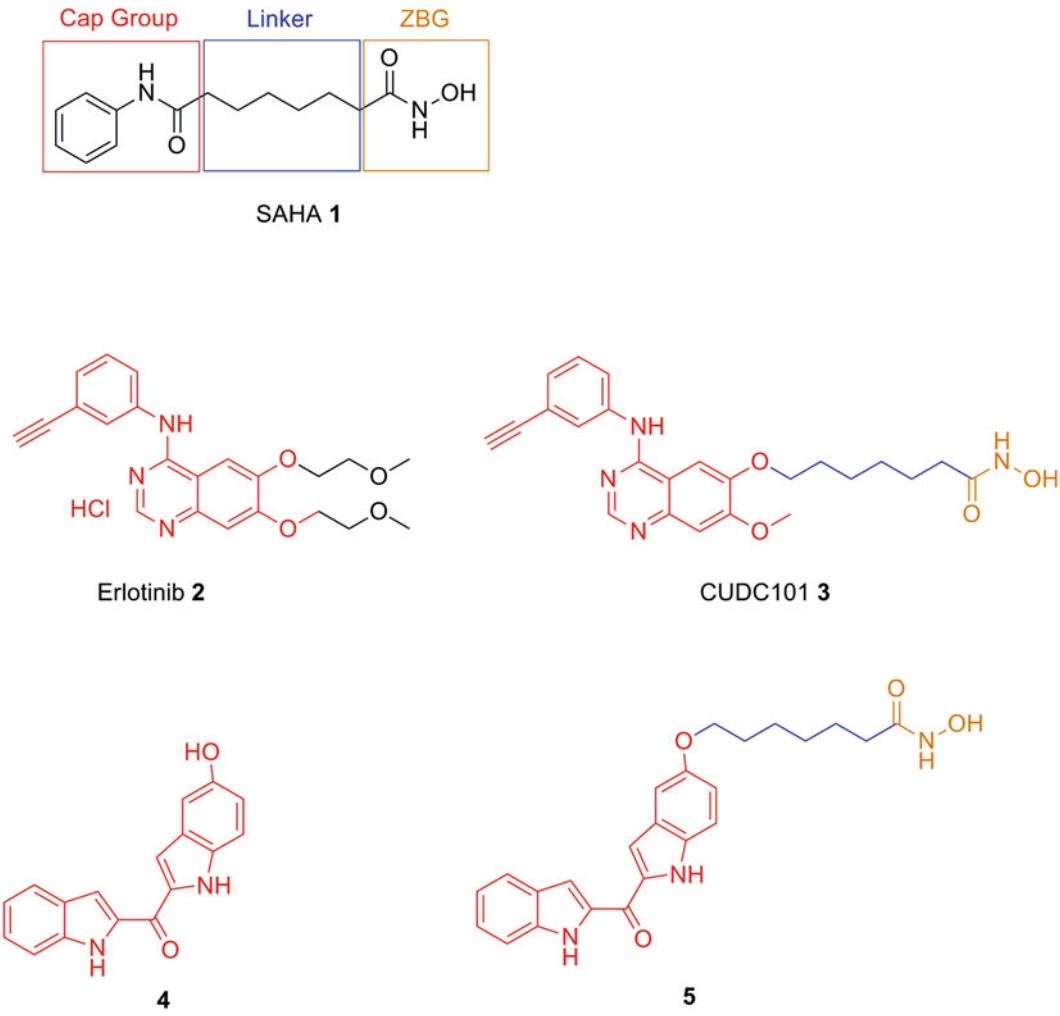


Fig. 1 Structures of SAHA **1**, a pan HDACi, the parental TK inhibitors 2, 4, and the chimeric designed compounds 3, 5. The chimeric compounds combine the structural features of HDACi and TKi and, therefore, unite the biological effects of these different inhibitors

essential pharmacophore of Erlotinib 2 and the HDAC recognition sequence of SAHA [1] succeeded in developing a hybrid (CUDC 101) (Fig. 1). Its inhibitory action in the enzymatic assay is by a factor of 5–10 higher than that of the individual substances. Further, the active chimeric CUDC 101 3 provides significantly improved inhibition of the growth of cancer cell lines in the system as a combined use of the individual substances [10]. Another new generation of hybrids [5] following the same concept was synthesized as potent dual FLT3 and HDACi. These hybrids used the skeletal structure of the bis(1*H*-indol-2-yl)methanone 4 as an FLT3 inhibitor, and structural features of SAHA as a HDACi on the other.

A common model for the description of the pharmacophore for inhibitors of the zinc-dependent HDACs comprising class I

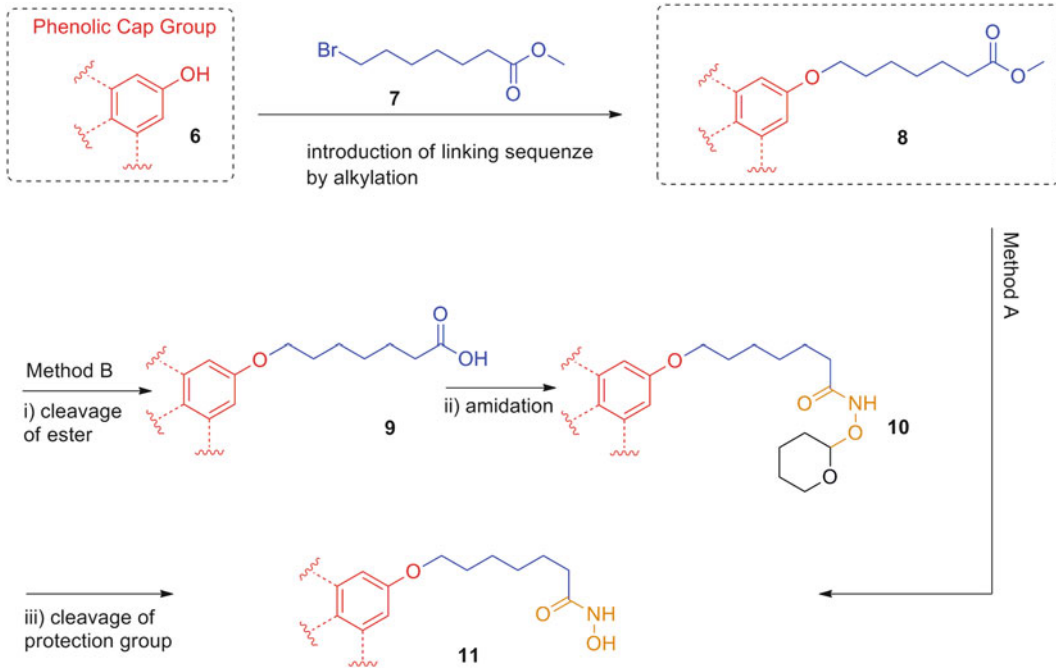


Fig. 2 General reaction sequence for introduction of a linking region and a hydroxamic acid motif to phenolic compounds

and class II HDACs divides the chemical structure in (a) a group that interacts with amino acids of the binding site (cap group), (b) a group that interacts with the catalytic zinc in the active site (ZBG) (chelating head group, warhead), and (c) a linker [11–13] (Fig. 1). In the chimeric designed hybrids, the TK inhibitory increment represents the head group of the HDACi.

Here we describe the generation of such fused dual inhibitors using the phenols, in special of the parental TKi, as an intermediate to obtain substituted esters by alkylation with methyl 7-bromoheptanoate. The hydroxamic acids can be obtained in the following either by treatment with hydroxylamine in one step or by alkaline cleavage of the ester to obtain the carboxylic acid, amidation with an protected hydroxylamine precursor and cleavage of the protecting group (Fig. 2).

By this way, the linking sequence and the hydroxamic acid motif taken from SAHA can be introduced to phenolic compounds with TKi motifs. To conserve TKi activity, the modifications of the parental inhibitor have to be performed at positions sticking outside the receptor binding pocket [14].

MV4-11 cells were used for a proliferation assay and assessment of the experiment. MV4-11 cells expressing endogenously FLT3-ITD are dependent on the signals of mutant FLT3 receptor. Additionally, the active FLT3-ITD receptor can be depleted by HDACi [9, 15].

2 Materials

Anhydrous solvents were purchased from commercial suppliers. These agents were used in all cases, and reactions were carried out under nitrogen atmosphere at room temperature unless stated otherwise.

2.1 Alkylation Components

1. Reaction mixture: Dissolve the phenolic compound 6 (1.0 mmol) in dimethylformamide (6.0 ml). Add powdered potassium carbonate (5.0 mmol; 0.69 g) and methyl 7-bromoheptanoate 7 (1.2 mmol; 0.27 g; 0.21 ml).
2. Diethyl ether.

2.2 Components for Preparation of Hydroxamic Acids from the Corresponding Esters Using Method A

1. Prepare a fresh hydroxylamine solution as follows: Add a solution of potassium hydroxide (11.2 g, 19.6 mmol) in methanol (28 ml) to a stirred solution of hydroxylamine hydrochloride (9.34 g, 134.4 mmol) in methanol (48 ml) at 0 °C. Stir the reaction mixture at 0 °C for 30 min. Remove the resultant precipitate by filtration to obtain the free hydroxylamine solution. Store it in a refrigerator till use.
2. Aqueous HCl (2 N).

2.3 Components for Preparation of the Carboxylic Acid

1. Dissolve the appropriate ester 8 (10.0 mmol) in the necessary amount of methanol and tetrahydrofuran (1:1; 50–100 ml) and add a solution of lithium hydroxide (0.48 g, 20.0 mmol) in water (10 ml).
2. Aqueous HCl (1 N).

2.4 Components for Amidation

1. Dissolve the carboxylic acid 9 (8.0 mmol) in Dimethylformamide (15.0 ml), add (Benzotriazol-1-yloxy) tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (8.2 mmol; 3.62 g), *N*,*N*-diisopropylethylamine (3.62 ml) and O-(Tetrahydro-2*H*-pyran-2-yl)hydroxylamine (NH_2OTHP) (24.0 mmol; equates to 2.80 g).
2. Dichloromethane 300 ml.
3. Sodium sulfate.

2.5 Components for Cleavage of the Tetrahydropyranyl-Group

1. Methanol.
2. Aqueous HCl (0.6 N).

2.6 Chromatography Equipment

1. Aluminum sheets, precoated with silica gel 60 with fluorescence indicator UV_{254}.
2. Geduran® Si 60 Silica gel for column chromatography.
3. Eluents for chromatography: Dichloromethane/ethyl acetate (5:1); dichloromethane/methanol (20/1); ethyl acetate.

2.7 Cell Culture

Cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, GER).

1. Cell media: RPMI-1640 medium supplemented with 20% FBS, 50 U/ml penicillin and streptomycin.

3 Methods

3.1 Alkylation

1. Stir the reaction mixture containing all alkylation components at 40 °C till complete consumption of the reactant (1–12 h) as indicated by thin layer chromatography (TLC) control on silica gel (*see* **Note 1**).
2. Filter the reaction mixture, remove the solvent in vacuo and wash the remaining solid with diethyl ether. If necessary, the obtained product 8 can be purified by column chromatography on silica gel 60 using a mixture of dichloromethane/ethyl acetate (5:1).

3.2 Method A: Preparation of Hydroxamic Acids from the Corresponding Esters

1. Add the appropriate ester 8 (10.0 mmol) to the freshly prepared hydroxylamine solution (30.0 ml) at 0 °C. Warm the reaction mixture to room temperature and stir it at 25 °C till complete consumption of the reactant (1–12 h) as indicated by TLC control on silica gel.
2. Neutralize the mixture with acetic acid whilst stirring, cool the mixture in an ice bath, remove the precipitating product by filtration and wash with a little amount of ice water (*see* **Note 2**). If no crystallization can be obtained, it is necessary to use method B for preparation of the desired product in a gram scale.
3. Dry the crystalline solid in vacuo till it has reached constant weight.

3.3 Method B: Preparation of Hydroxamic Acids from the Corresponding Esters by Cleavage to the Carboxylic Acid, Amidation, and Deprotection

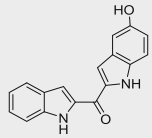
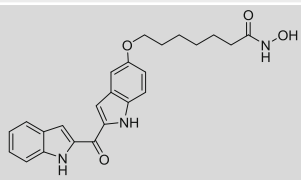
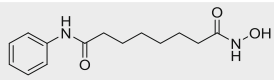
1. To obtain the carboxylic acid from the respective esters, stir the solution containing the ester in a mixture of the given solvents and lithium hydroxide at 40 °C till complete consumption of the reactant (ca. 24 h) as indicated by TLC control on silica gel using ethyl acetate as solvent for development. Add an additional amount of water (10 ml), remove the organic solvents under reduced pressure (*see* **Note 3**).
2. Acidify the solution whilst stirring with HCl till pH = 1.
3. Filter the precipitated product off and dry it in vacuo.
4. For subsequent amidation of the carboxylic acid **9** obtained in **step 3**, stir the mixture containing all components for the amidation reaction given in Subheading 2.4, **item 1** for 18 h at room temperature.

5. Pour the mixture into water, extract the mixture with dichloromethane (3 × 100 ml), dry the combined organic layers by use of sodium sulfate and remove the solvent under reduced pressure.
6. Purify the remaining solid 10 by column chromatography (l = 60 cm, Ø 4 cm) on silica gel 60 using a mixture of dichloromethane/methanol (20:1) and remove the solvent under reduced pressure (bath temperature 40 °C).
7. For preparation of hydroxamic acids 11 dissolve the amide 10 obtained by the procedure described above in the necessary amount of methanol by gentle warming.
8. Add dropwise HCl (0.6 N) whilst intensive stirring till the solution gets slightly opaque. Continue stirring till the product precipitates directly from the methanolic solution (1–12 h) (*see* **Note 4**).
9. Remove the crystalline product by filtration, wash with a small amount of hydrochloric acid and dry it in vacuo till it keeps a constant weight.

3.4 Biological Assessment of the Chimeric Compounds with a Proliferation Assay

1. MV4-11 cells grow in RPMI-1640 medium supplemented with 20% FBS, 50 U/ml penicillin and streptomycin at 37 °C and 5% CO_2. Every third day, the cells have to be splitted 1:3.
2. Seed cells at a density of 2×10^5 cells per well.
3. Treat cells with the indicated concentrations of compound for 72 h (*see* **Note 5**) at 37 °C.
4. Count cells by using trypan blue exclusion test (*see* **Note 6**) with *Vi-CELL* Cell Counter or a *Neubauer Chamber* (*see* **Note 7**).

Table 1
Calculated IC_{50}-values for the investigated compounds

Chemical structure	Cpd.	IC_{50} (nM)
	**4**	1.71 ± 0.412
	**5**	0.18 ± 0.504
	**SAHA 1**	>100

5. Calculate the desired IC_{50}-values (*see* **Note 8**) by using, e.g., GraphPadPrism.
6. Compare the different calculated IC_{50}-values, like in our example (Table 1). In our example, the introduction of an additional HDAC inhibiting sequence taken from SAHA significantly enhances activity of the parental TKI.

4 Notes

1. For TLC control dilute 1 drop of the reaction mixture with dichloromethane (1.0 ml) and dry the TLC sheet after substance application for 5 min at 60 °C before development in the TLC chamber. Otherwise traces of DMF might falsify the result. Use a diluted solution of the phenolic compound in dichloromethane as reference.
2. If no crystallization can be obtained by this way, remove the solvent and purify the title compound by reversed-phase HPLC. A purification on silica gel usually is not possible by instability of the hydroxamic acid.
3. If you obtain no clear solution, add an additional amount of water (10 ml) and warm the mixture and filter the mixture before acidification.
4. If no crystallization appears, cool the mixture to −18 °C. After precipitation add the same amount of hydrochloric acid you have used for dissolving the reactant.
5. Control cells were cultivated just in the presence of medium.
6. Trypan blue exclusion test: The dye "Trypan Blue" can penetrate in the cells when the cell membrane is perforated and stained blue. In the light microscope these cells will appear blue, while the viable cells are colorless.
7. To count the cells via a *Vi-CELL* Cell Counter, you need 500 µl of the cell suspension and the *Vi-CELL* Cell Counter is measuring the cell count by using the trypan blue exclusion test. With "a Neubauer Chamber" you have to calculate your cell count:

   Number of living cells/ml = number of living cells/4 × dilution factor × 10,000.
8. The number of the untreated cells was set as 100 %.

   To obtain IC_{50}-values you have to repeat the experiment thrice and build the mean of the values.

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