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ORIGINAL ARTICLE

HDAC1 and HDAC2 integrate the expression of p53 mutants in pancreatic cancer

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Mutation of p53 is a frequent genetic lesion in pancreatic cancer being an unmet clinical challenge. Mutants of p53 have lost the tumour-suppressive functions of wild type p53. In addition, p53 mutants exert tumour-promoting functions, qualifying them as important therapeutic targets. Here, we show that the class I histone deacetylases HDAC1 and HDAC2 contribute to maintain the expression of p53 mutants in human and genetically defined murine pancreatic cancer cells. Our data reveal that the inhibition of these HDACs with small molecule HDAC inhibitors (HDACi), as well as the specific genetic elimination of HDAC1 and HDAC2, reduce the expression of mutant *p53* mRNA and protein levels. We further show that HDAC1, HDAC2 and MYC directly bind to the *TP53* gene and that MYC recruitment drops upon HDAC inhibitor treatment. Therefore, our results illustrate a previously unrecognized class I HDAC-dependent control of the *TP53* gene and provide evidence for a contribution of MYC. A combined approach targeting HDAC1/HDAC2 and MYC may present a novel and molecularly defined strategy to target mutant p53 in pancreatic cancer.

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INTRODUCTION

After 2020 pancreatic ductal adenocarcinoma (PDAC) will be the second leading cause of cancer-related deaths in the United States.[1] In contrast to other cancers, the prognosis, reflected by a 5-year survival rate of only 8%, remains dismal. Since clinical studies have failed to improve the survival of patients suffering from PDAC significantly, there is an urgent need to better understand the biology of the disease and to define therapies.

Mutations of the tumour suppressor p53 are frequently found in PDAC.[2] These mutations disturb its tumour-suppressive functions. Mutants of p53 are expressed at high levels in cancer cells and, in addition to the loss of tumour-suppressive properties, p53 mutants can actively contribute to tumour development by gain-of-function properties.[3–6] Such gain-of-functions of mutant p53 are observed in mice genetically engineered to express p53 mutants. Compared to p53 null animals, these mice develop different tumour spectra with increased aggressiveness and metastatic potential.[7–9] Importantly, recent *in vivo* data demonstrate that tumours depend on mutant p53. This finding identifies p53 mutants as *bona fide* therapeutic targets.[10]

In PDAC, gain-of-functions of p53 mutants promote therapeutic resistance.[11–13] In genetically defined murine PDAC models, the NFκB member p65 and $p53^{R172H}$ contribute to NFκB-dependent survival signalling,[11] which may add to the drug resistance phenotype of p53 mutant PDACs. Cross-signalling of p65 with $p53^{R273H}$ upon a pro-inflammatory stimulus was also described in Panc1 cells and this cross-signalling occurs at the promoters of NFκB target genes *in cis*.[14] Furthermore, p53 mutants promote the metastatic cascade in PDAC.[15,16] Mechanistically, a pro-metastatic function of p53 mutants was connected to platelet-derived growth factor receptor beta signalling.[16] In addition, the murine $p53^{R172H}$ mutant promotes activity of the small GTPase RhoA and thereby invasiveness.[17] These current data point to important roles of p53 mutants in drug resistance and metastasis formation in PDAC. Hence, drugs targeting the expression of p53 mutants are of particular interest.

HDACs deacetylate the ε-amino group of lysine residues of histones and non-histone substrates.[18] HDACs are grouped into class I–IV enzymes. Class I contains HDAC1, 2, 3 and 8, class II HDAC4, 5, 6, 7, 9 and 10, and class IV HDAC11.[18] The class III contains the SIRT deacetylases. In PDAC, overexpression of the class I HDACs 1, 2 and 3[19–21] and the class II HDAC7[22] was demonstrated. In PDAC, links of HDACs towards processes contributing to metastasis[23,24] and therapeutic resistance[19,25–28] are evident. Thus, the described HDAC functions in PDAC overlap with programmes assigned to mutant p53.[11,13,15,16]

Previous reports show that HDAC8 controls the *TP53* gene through the transcription factors HoxA5[29] or Yin Yang 1 (YY1)[30] and that HDAC6 regulates the stability of the p53 protein through the deacetylation of the heat shock protein 90 (HSP90).[31] We found that the benzamide HDAC inhibitor (HDACi) MS275, an inhibitor with restricted activity towards HDAC1, HDAC2 and HDAC3 (Table 1), significantly reduces the expression of p53 mutants. Therefore, we analysed whether HDAC1, HDAC2 or

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HDAC3 modulate the expression of mutant p53. Here, we provide genetic and pharmacological evidence that HDAC1/HDAC2 and MYC are connected and contribute to maintain the expression of p53 mutants.

RESULTS

HDACi control the expression of mutant p53

Due to the described gain-of-functions of p53 mutants, we sought to investigate whether and how HDACs control the expression. To clarify which HDACs might be involved, we used the hydroxamic acid SAHA and the benzamide MS275 to block these enzymes. SAHA targets mainly HDAC1, 2, 3, 6, and to some extent HDAC8[32–35] (Table 1). MS275 specifically blocks HDAC1, 2 and 3, but not other HDACs (Table 1)[33,34] and is hence suited to identify class I HDAC-specific functions.

Table 1. Target profile of SAHA and MS275

Reference	*Value*	*HDAC1*	*HDAC2*	*HDAC3*	*HDAC8*	*HDAC6*
MS275						
[35]	IC_{50} (μM)	0.24	0.45	0.25	>10	>10
[34]	K_D^{app} (μM)	6.8	7.9	3.9	206	>600
[32]	K_i (μM)	0.02	0.07	0.36	—	—
SAHA						
[35]	IC_{50} (μM)	0.06	0.25	0.02	0.83	0.009
[34]	K_D^{app} (μM)	0.28	0.35	0.29	>10	0.13
[32]	K_i (μM)	0.001	0.002	0.005	0.48	0.002

Abbreviations: IC_{50}: Inhibitor concentration 50%; K_D: apparent dissociation constant; K_i: inhibitory constant.

We tested the murine $Kras^{G12D}$-driven PDAC cell line PPT-5436, engineered to express mutant $p53^{R172H}$ from the endogenous promoter, as well as the human cell lines PaTu8988t ($p53^{R282W}$) and Panc1 ($p53^{R273H}$). Western blots of SAHA- and MS275-treated PPT-5436 (Figure 1a), Panc1 (Figure 1b) and PaTu8988t (Figure 1c) cell lysates demonstrated that HDACi reduced the expression of mutant p53. Consistently, p53 immunocytochemistry showed that SAHA and MS275 decreased the nuclear expression of mutant p53 in PaTu8988t cells (Figure 1d). Thus, the ability of HDACi to control expression of p53 mutants is not strictly confined to a specific *p53* mutant.

Roles of MDM2 and the proteasome

The E3 ligases MDM2 and CHIP were reported to accelerate proteasomal degradation of mutant p53 in HDACi-treated cells.[31] To test whether MDM2 contributes to the regulation of mutated p53 by HDACi, we used the MDM2 inhibitor Nutlin3. In Panc1 cells, SAHA (Figure 2a) and MS275 (Figure 2b) decreased the expression of mutant p53 over time, an effect mildly rescued by Nutlin3. Likewise, no effect of the MDM2 inhibitor on mutant p53 was detected in HDACi-treated PaTu8988t (Figure 2c) and PPT-5436 (Figure 2d) cells. Expression of the p53 mutants was not changed by Nutlin3 in PaTu8988t (Figure 2c), PPT-5436 (Figure 2d) and Panc1 cells (data not shown). These results correspond with data showing that mutant p53 is not regulated upon MDM2 inhibition in PDAC.[36–38] In other models, it is clear that MDM2 can interact with and control mutant p53.[39–41] Up-regulation of the $p53^{R172H}$ mutant in murine PDAC cells upon MDM2 inhibition was observed.[42] Therefore, the influence of MDM2 to the regulation of mutant p53 in PDAC cells is heterogeneous and context-dependent. Irrespective thereof, the HDACi-induced down-regulation of p53 mutants was not or incompletely rescued by Nutlin3. Therefore, we focused on MDM2-independent mechanisms.

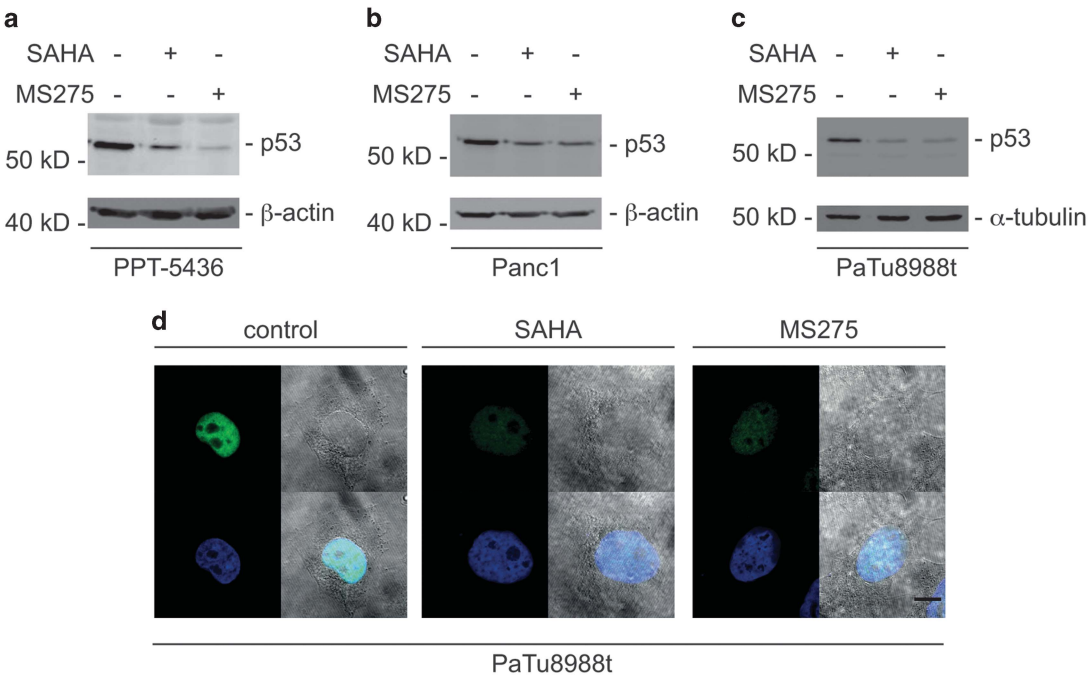

Figure 1. HDAC inhibitors control protein expression of p53 mutants in PDAC. (**a**–**c**) PPT-5436, Panc1, and PaTu8988t cells were treated with SAHA (4 μM, 48 h) or MS275 (4 μM, 48 h) or were left as a vehicle treated control. Western blot of p53 and β-actin or α-tubulin (loading control) expression ($n = 3$). (**d**) p53 immunocytochemistry of vehicle control, SAHA (4 μM, 48 h) or MS275 (4 μM, 48 h) treated PaTu8988t cells. Shown is the fluorescence microscopy of p53 (green), the nuclear DAPI staining (blue), a differential interference contrast of the cells and a merged picture. Scale bar: 10 μm.

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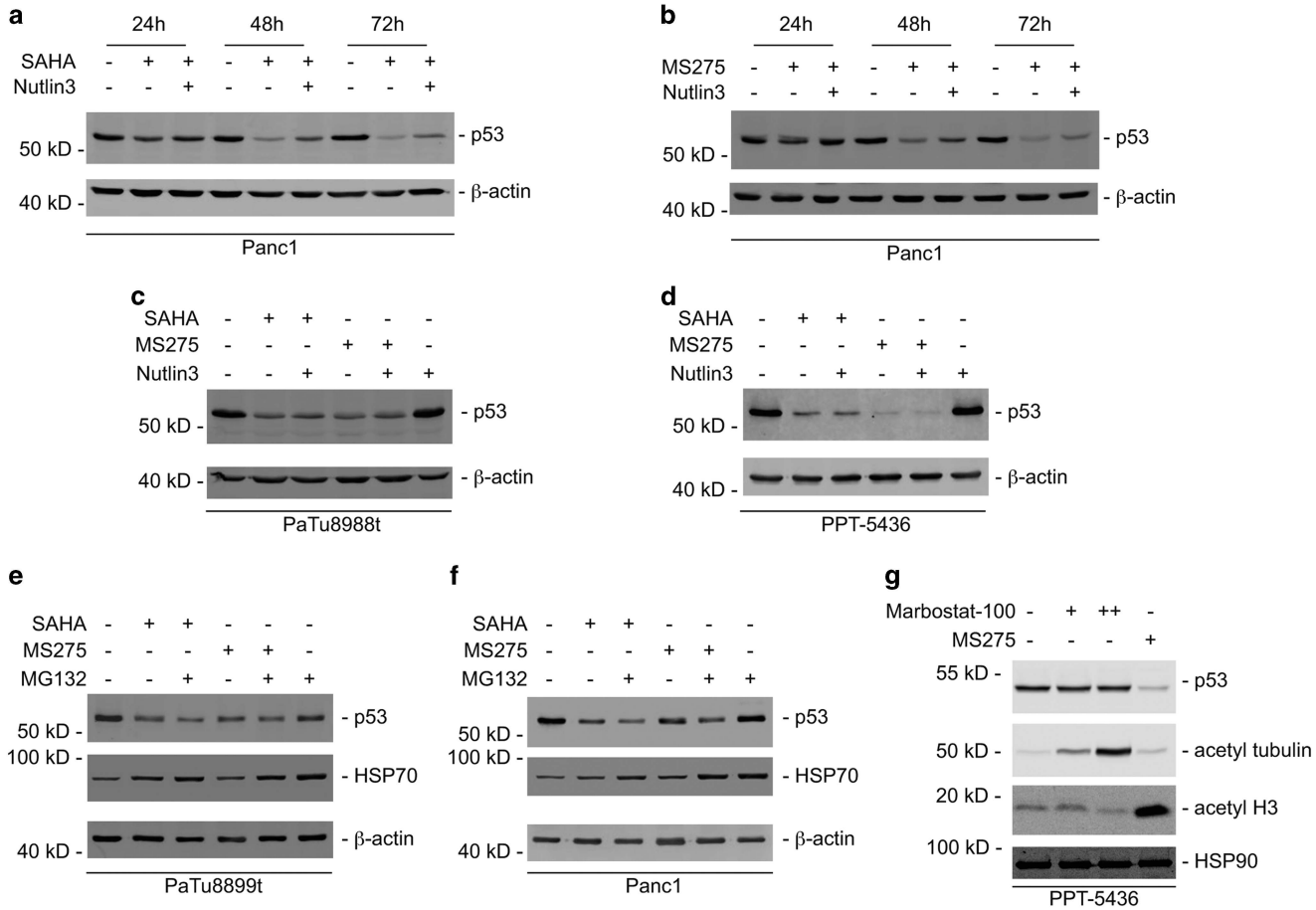

Figure 2. Role of MDM2 and the proteasome in the HDAC inhibitor (HDACi) controlled p53 expression. (**a** and **b**) Panc1 cells were treated with (**a**) SAHA (4 μM) and (**b**) MS275 (4 μM) or the combination of the HDACi and Nutlin3 (10 μM) over time as indicated. Vehicle treated cells were used as controls. Western blot of p53 and β-actin (loading control) expression ($n = 3$). (**c**) PaTu8988t and (**d**) PPT-5436 cells were treated with SAHA (4 μM), MS275 (4 μM), Nutlin3 (10 μM) or the combination of the HDACi and Nutlin3 for 48 h. Vehicle treated cells were used as controls. Western blot of p53 and β-actin (loading control) expression ($n = 2$). (**e**) PaTu8988t and (**f**) Panc1 cells were pretreated with SAHA (4 μM) or MS275 (4 μM) for 24 h. Afterwards cells were again treated with the HDACi or the combination of the HDACi and MG132 (1 μM) for additional 6 h. Vehicle treated cells and cells treated for 6 h with MG132 were used as controls. Western blot of p53, HSP70 and β-actin (loading control) expression ($n = 2$). (**g**) PPT-5436 cells were treated with the HDAC6-specific inhibitor Marbostat-100 (+: 200 nM, ++: 500 nM, 48 h), MS275 (5 μM, 48 h) or were left as an untreated control. Western blot of p53, acetylated tubulin and acetylated histone H3 expression. Same extracts were blotted to a different membrane to detect HSP90 expression (loading control) ($n = 2$).

To further test for proteasomal degradation pathways, we used the proteasome inhibitor MG132. Due to the potent cytotoxic action of HDACi and MG132 combinations, we were only able to investigate a limited time window of 6 h. We pre-treated PaTu8988t and Panc1 cells for 24 h with HDACi. Afterwards, HDACi treatment was continued for 6 h with or without MG132. This proteasome inhibitor did not affect the SAHA- or MS275-induced down-regulation of mutant p53 in both cell lines (Figures 2e and f). HSP70 expression was induced by MG132, arguing that proteasomes were efficiently targeted in this assay (Figures 2e and f).

Since SAHA promoted the hyperacetylation of α-tubulin being a *bona fide* HDAC6 target, it was concluded that HDAC6 and an effect on HSP90 acetylation were responsible for a loss of p53 expression.[31] To test for such a direct contribution of HDAC6, we utilized a novel and highly specific HDAC6 inhibitor, Marbostat-100.[43] Whereas Marbostat-100 increased acetylation of the HDAC6 target tubulin in PPT-5436 cells, this inhibitor did not alter expression of mutant $p53^{R172H}$ (Figure 2g). As expected for an HDAC6-specific inhibitor, no acetylation of histone H3 was detected in Marbostat-100-treated cells (Figure 2g). In contrast, a parallel treatment of PPT-5436 cells with the class I-specific HDACi MS275 increased the acetylation of histone H3 and decreased expression of the p53 mutant. No effect of the MS275 treatment towards acetylation of tubulin was detected, demonstrating its specificity for class I HDACs (Figure 2g). In summary, these results suggest that alternative pathways beyond proteasomal degradation and HDAC6 activity contribute to HDACi-induced regulation of mutant p53.

HDACi reduce p53 mRNA expression

To identify the molecular mechanism(s) that decreased the expression of mutant p53 in response to HDACi, we measured *p53* mRNA levels. We found that SAHA and MS275 treatment decreased the expression of *p53* mRNA in Panc1 (Figure 3a) and PPT-5436 cells (Figure 3b) over time. Reduced *p53* mRNA expression levels were first detected 9 h after the treatment of Panc1 cells with MS275 (Figure 3c). Interestingly, the kinetics of *p53* mRNA regulation after the treatment with MS275 follows the histone H3 acetylation kinetics (Figure 3d). We detected only slight acetylation of histone H3 within the first 6 h (Figure 3c). A clear acetylation of histone H3 was observed after 8 h and further increased after 10 h (Figure 3d). These observations are in

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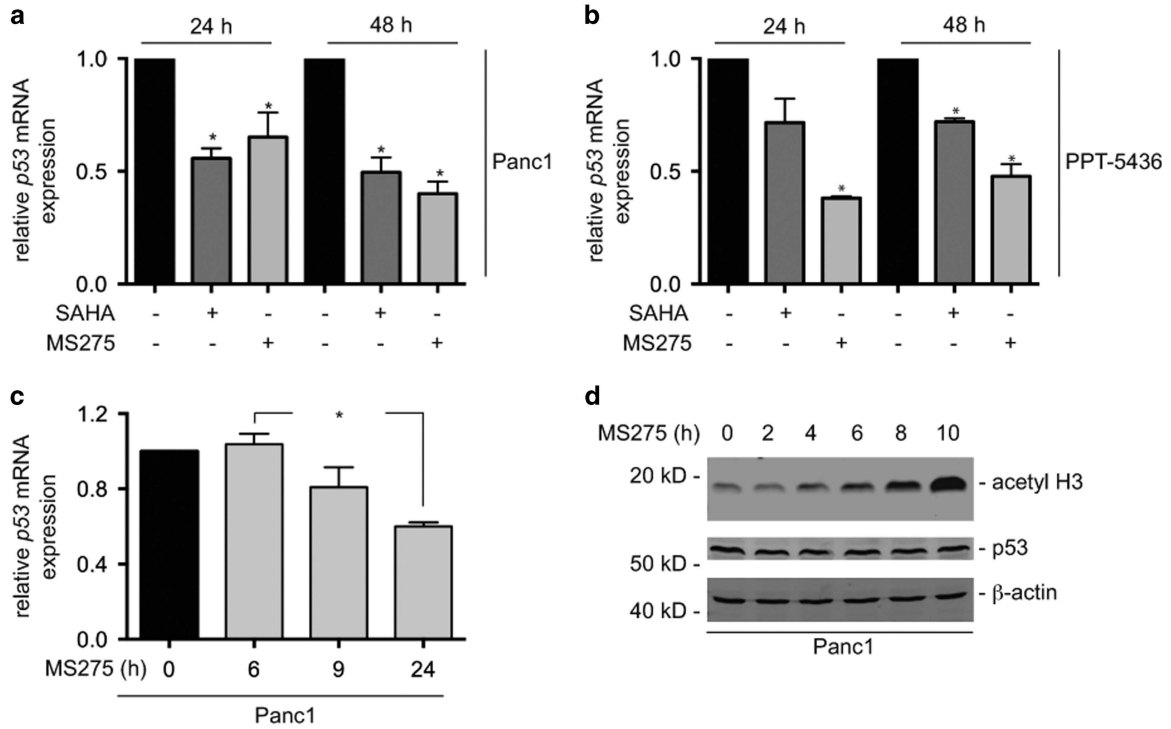

Figure 3. HDACs maintain expression of p53 mRNA. (**a**) Panc1 and (**b**) PPT-5436 cells were treated SAHA (4 μM) or MS275 (4 μM) over time as indicated or were left as a vehicle treated control. Relative *p53* mRNA expression was determined by qPCR using cyclophilin A mRNA as reference (A:$n = 6$;B:$n = 4$). *paired two-tailed Student's *t*-test $P < 0.05$. (**c**) Panc1 cells were treated with MS275 (4 μM) over time as indicated or were left as a vehicle treated control. Relative p53 mRNA expression was determined by qPCR using cyclophilin A mRNA as reference ($n = 3$). *one-way ANOVA $P < 0.05$. (**d**) Panc1 cells were treated with MS275 (4 μM) over time as indicated or were left as a vehicle treated control. Western blot of acetylated histone H3, p53 and β-actin (loading control) expression ($n = 3$).

agreement with the slow target binding kinetics of MS275.[35] No decreased expression of the p53 mutant was detected in the first 10 h after the treatment with MS275 (Figure 3d). Therefore, reduction of the *p53* mRNA preceded the decline of the protein.

To cross-tumour validate our findings, we accessed RNA-sequencing profiles of HDACi treated U-266 myeloma cells,[44,45] which express a $p53^{T161A}$ mutant. Also here, *p53* mRNA expression was attenuated by the HDACi (Supplementary Figure S1) proposing a common mode of regulation. Together, these results suggest that reduced *p53* mRNA levels likely account for the loss of the protein.

Contribution of HDAC1 and HDAC2

MS275 specifically inhibits HDAC1-3 (Table 1) and decreases mutant p53 expression. Hence, one or more of these HDACs is likely critical for the expression of p53. To address such isoenzyme-specific functions, we genetically engineered $Kras^{G12D}$-driven murine PDAC cells that express $p53^{R172H}$ and lack HDAC1 or HDAC2 (Supplementary Table 1). *Ex vivo* cultures were collected from these mice and all of these new models express the p53 mutant from the endogenous promoter. Thus, the *TP53* gene is faithfully controlled by endogenous transcription factors. When we analysed such cell lines, we noted that HDAC2-deficient cells still expressed $p53^{R172H}$ (Figure 4a). Furthermore, SAHA and MS275 reduced p53 expression potently in HDAC2-proficient and -deficient cells (Figure 4a), indicating that additional class I HDAC(s) contribute(s).

In order to investigate if HDAC3 maintains expression of mutant p53, we transfected HDAC2-deficient and -proficient cells with HDAC3 siRNAs. No clear regulation of the p53 mutant was detected upon the transfection of HDAC3 siRNAs (Figure 4b). In consequence, we can exclude a significant regulation of mutant p53 expression by HDAC3.

To determine the impact of HDAC1, we engineered HDAC1-deficient PDAC cells that express $p53^{R172H}$. All HDAC1-deficient cells express the p53 mutant and MS275 reduced distinctly the expression of $p53^{R172H}$ (Figure 4c). Acetylation of histone H3 was measured to demonstrate efficient HDAC inhibition (Figure 4c).

We next attempted to generate conventional HDAC1 and HDAC2 double-deficient PDAC cells. However, deleting *Hdac1* and *Hdac2* genes simultaneously in the *Pdx1* lineage in the pancreas (using a *Pdx1-Cre* driver line, which is known to mediate recombination in the developing mouse pancreas) resulted in the replacement of most of the acinar cells with adipose tissue (Figure 4d). Due to the described mosaic expression of the pancreatic *Pdx1-Cre* driver line,[46] only some small areas of not recombined, normal appearing pancreas were present. Therefore, systematic generation of HDAC1 and HDAC2 double-deficient PDAC cell lines is not possible using this model *in vivo*.

To analyse the contribution of HDAC1 and HDAC2 alternatively, we isolated cells from different parts of the primary tumour or metastases from the same animal carrying floxed *Hdac1* and/or *Hdac2* alleles. This strategy allowed us to analyse the contribution of HDAC1 and HDAC2 in a similar genetic background. We observed a trend of higher HDAC1 protein levels in HDAC2-deficient cells (Figure 5a). Furthermore, the HDAC1 expression levels in the HDAC2-deficient models correlated with the protein expression of the $p53^{R172H}$ mutant (Figure 5a). Similar effects for HDAC2 were detected in HDAC1-deficient PDAC cells (Figure 5b). Note that the same identifier number given to the PDAC cell line in Figures 5a and b indicates cell lines from the same animal. In trend, HDAC2 protein expression levels correlated with the expression of the $p53^{R172H}$ mutant (Figures 5b and 4c), which argues that HDAC1 and HDAC2 cooperatively maintain the expression of the p53 mutant.

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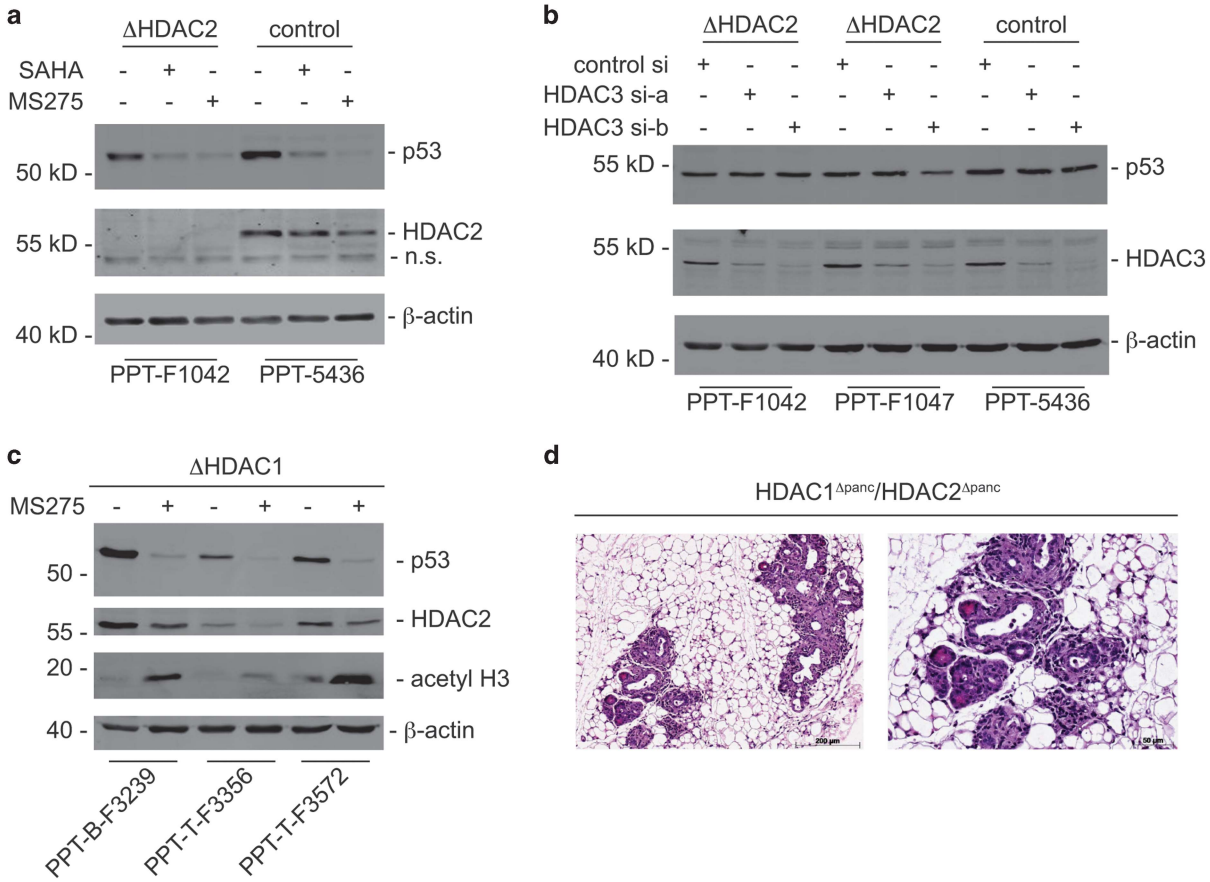

Figure 4. p53 is regulated by HDAC inhibitors in HDAC1- and HDAC2-deficient PDAC cells. (**a**) The HDAC2-deficient (PPT-F1042) and -proficient (PPT-5436) murine PDAC cell lines were treated with SAHA (4 μM) or MS275 (4 μM) for 48 h. Vehicle treated cells were used as controls. Western blot of HDAC2, p53 and β-actin (loading control) expression ($n = 3$). n.s.: non-specific band. (**b**) The HDAC2-deficient (PPT-F1042 and PPT-F1047) and -proficient (PPT-5436) murine PDAC cell lines were transfected with two HDAC3-specific siRNAs or a control siRNA. Western blot was used to determine the expression of HDAC3, p53 and β-actin (loading control) ($n = 2$). (**c**) The HDAC1-deficient pancreatic cancer cell lines were treated with MS275 (4 μM; 48 h). Vehicle treated cells were used as controls. Western blot of p53, HDAC2, acetyl-histone H3 and β-actin (loading control) expression ($n = 3$). (**d**) H&E staining of the pancreas of *Pdx1-Cre;HDAC1$^{lox/lox}$;HDAC2$^{lox/lox}$* mice. The scale bar is depicted. Left: 200 μm, right: 50 μm.

To test whether HDAC1 and HDAC2 maintain mRNA expression of mutant p53 we analysed *p53* mRNA levels in HDAC1/HDAC2-proficient and HDAC1- as well as HDAC2-deficient PDAC cell lines. As shown in Figure 5c, there is a lower expression of *p53* mRNA in HDAC1-deficient and HDAC2-deficient cell lines, respectively. A significance level of $P < 0.05$ was not reached, which suggests for compensatory mechanisms and that both HDACs are needed to maintain the expression of mutant p53 (Figures 1–3).

Of note, we isolated HDAC1- or HDAC2-deficient PDAC cells also from animals where three of the four *Hdac1* and *Hdac2* alleles were deleted, respectively. In contrast to the ductal or mixed ductal/undifferentiated PDACs we observed in *Pdx1-Cre;LSL-Kras$^{G12D/+}$;LSL-p53$^{R172H/+}$* mice, we detected mixed ductal/acinar differentiated tumours in such mice (data not shown). These data suggest that an HDAC1/HDAC2 activity threshold determines tumour type development/differentiation.

To cope with the above-mentioned shortcomings and to elaborate that HDAC1 and HDAC2 contribute to maintain the expression of p53 mutants in PDAC, we took advantage of a dual-recombinase system (Supplementary Figure S2). This system allows the time-specific manipulation of genes.[47,48] We isolated murine PDAC cell lines from *Pdx1-Flp;FSF-Kras$^{G12D/+}$;FSF-R26$^{CAG-CreERT2/+}$;LSL-p53$^{R172H/+}$;Hdac1$^{lox/lox}$;Hdac2$^{lox/lox}$* mice. In such cells, treatment with 4-Hydroxtamoxifen (4-OHT) activates the expression of the p53 mutant. Simultaneously, floxed *Hdac1* and *Hdac2* alleles become deleted (Supplementary Figure S2C). We isolated two cell lines from the same animal (tumour in the head and tail of the pancreas). When we treated PDAC cells isolated from the head (PPT-H-F5382) with 4-OHT, HDAC1 and HDAC2 expression became reduced (Figure 6a). Consistent with our hypothesis that these HDACs contribute to the regulation of p53 mutants, expression of p53^{R172H} is not detected (Figure 6a). HDAC1 expression is reduced in the cell line isolated from the tumour in the pancreas tail (PPT-T-F5382), but this line escapes from HDAC2 deletion (Figure 6a). The fact that such a remaining HDAC2 expression maintains expression of p53^{R172H} (Figure 6a) supports our conclusion that both HDAC1 and HDAC2 are necessary to sustain the levels of mutant p53 (Figures 4c and 5). Genotyping PCR indicates loss of the STOP cassette in the *Lox-Stop-Lox* (*LSL*)*-p53^{R172H}* allele of PPT-H-F5382 cells upon treatment with 4-OHT (Figure 6b), demonstrating that the loss of mutant p53 expression cannot be explained by insufficient recombination. Since our hypothesis is a regulation of p53^{R172H} at the level of its mRNA, we determined its expression. Whereas 4-OHT induced expression *p53* mRNA to levels comparable to conventional p53^{R172H} expressing cells in PPT-T-F5382 cells, *p53* mRNA is not induced in PPT-H-F5382 cells (Figure 6c). mRNA expression of *Hdac1* (Figure 6d) and *Hdac2* (Figure 6e) is reduced in PPT-H-F5382 cells upon 4-OHT treatment. Consistent with protein

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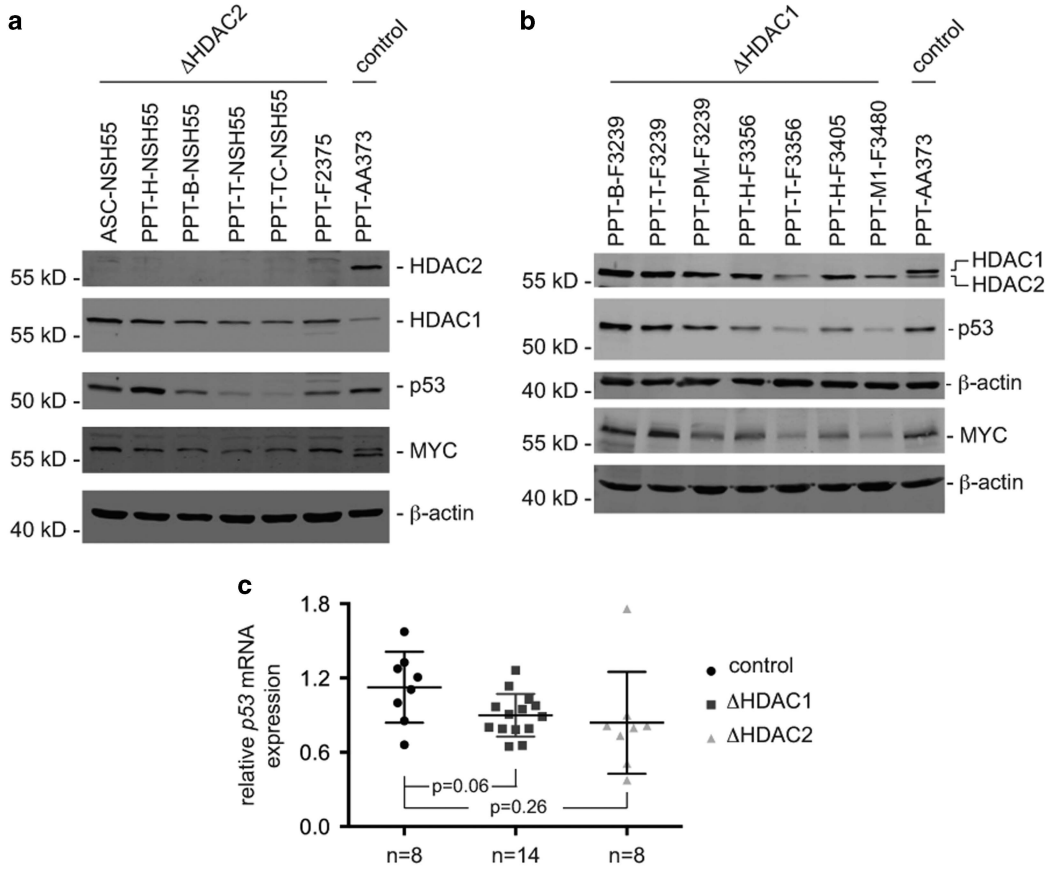

Figure 5. HDAC1 and HDAC2 maintain expression of $p53^{R172H}$. (**a**) The indicated HDAC2-deficient and -proficient murine pancreatic cancer cell lines were analysed for the expression of HDAC1, HDAC2, p53, MYC and β-actin (loading control) by western blot ($n = 2$). Cell lines numbered NSH55 were from the same animal. ASC: ascites; H: head of the pancreas; B: body of the pancreas; T: tail of the pancreas; TC: cyst in the tail of the pancreas. (**b**) The indicated HDAC1-deficient and -proficient murine pancreatic cancer cell lines were analysed for the expression of HDAC1, HDAC2, p53, MYC and β-actin (loading control) by western blot ($n = 2$). B: body of the pancreas; T: tail of the pancreas; PM: peritoneal metastasis; H: head of the pancreas; M1: pancreatic tumour infiltrating stomach. Same extracts were blotted to different membranes to detect HDAC1, HDAC2, and p53 or MYC expression. (**c**) *p53* mRNA expression of eight HDAC1- and HDAC2-proficient (PPT-434, PPT-1048, PPT-C-3040, PPT-5193, PPT-5486, PPT-4917, PPT-6051, PPT-AA373), fourteen HDAC1-deficient (PPT-B-F3239, PPT-T-F3239, PPT-PM-F3239, PPT-H-F3356, PPT-T-F3356, PPT-H-F3405, PPT-M1-F3480, PPT-M2-F3480, PPT-T1-F3546, PPT-T2-F3546, PPT-T-F3572, PPT-B-F3572, PPT-B1-F3632, PPT-B2-F3632) and eight HDAC2-deficient (ASC-NSH55, PPT-H-NSH55, PPT-B-NSH55, PPT-T-NSH55, PPT-TC-NSH55, PPT-F-2375, PPT-H-F3124, PPT-F3549) pancreatic cancer cell lines were analysed by qPCR using cyclophilin A mRNA as reference. Shown is the mean *p53* mRNA expression per cell line of three independent mRNA preparations. A detailed description of the genotype is presented in Supplementary Table 1. *P*-values of a two tailed unpaired Student's *t*-test are indicated.

expression, no reduction of the *Hdac2* mRNA was detected in PPT-T-F5382 cells (Figure 6e).

Together, these genetic experiments demonstrate that HDAC1 and HDAC2 act together to maintain $p53^{R172H}$ mRNA and protein expression. Of note, data in PPT-H-F5382 cells were collected with cells in very early passages. However, even without 4-OHT treatment, we observed a clonal outgrowth of cells after serial passaging. As shown in Figure 6f, PPT-H-F5382 cells in higher passages escaped complete *Hdac2* deletion. In the shown experiment, the extent of HDAC2 reduction (Figure 6f) is sufficient to lower expression of the p53 mutant. Nonetheless, PPT-H-F5382 cells completely escape *Hdac2* deletion and levels of $p53^{R172H}$ remain stable upon further passaging of the cells.

To investigate a direct binding of HDAC1/HDAC2 to the *TP53* gene, we used quantitative chromatin immunoprecipitations (qChIP) and analysed Panc1 cells. The *TP53* promoter is controlled by numerous transcription factors.[49] In promoter-scanning quantitative qChIP with primers covering the promoter, exon 2 and a 3´ region of the *TP53* gene, clear recruitment of HDAC1 was observed to the *TP53* promoter and the gene body (Figure 6g). HDAC1 binding peaks at the transcriptional start site (TSS) of the gene. No distinct change in the HDAC1 binding behaviour was detected upon the treatment with MS275 (Figure 6g). Low recruitment of HDAC2 was detected at the *TP53* promoter and no significant recruitment to the *TP53* gene body was observed (Figure 6h). There is a HDAC2 peak at the TSS of the gene, which becomes reduced upon the MS275 treatment (Figure 6h).

Furthermore, we analysed epigenetic marks at the *TP53* promoter. Acetylation of histone H3 and H4 at the *TP53* promoter is reduced upon MS275 treatment in Panc1 cells, whereas histone acetylation is increased in the *TP53* gene body (Supplementary Figure S3). In addition, a slight reduction of trimethylated lysine 4 of histone H3 was detected at the TSS upon MS275 treatment (Supplementary Figure S3D). Direct binding of HDAC1 and HDAC2 together with altered acetylation histone marks at the *TP53* gene upon HDACi treatment support the notion that both HDACs maintain transcription of this gene.

MYC binds to the proximal *TP53* gene promoter

Next, we aimed to identify transcription factors that bind to the *TP53* gene promoter and that are regulated by HDACs. In our

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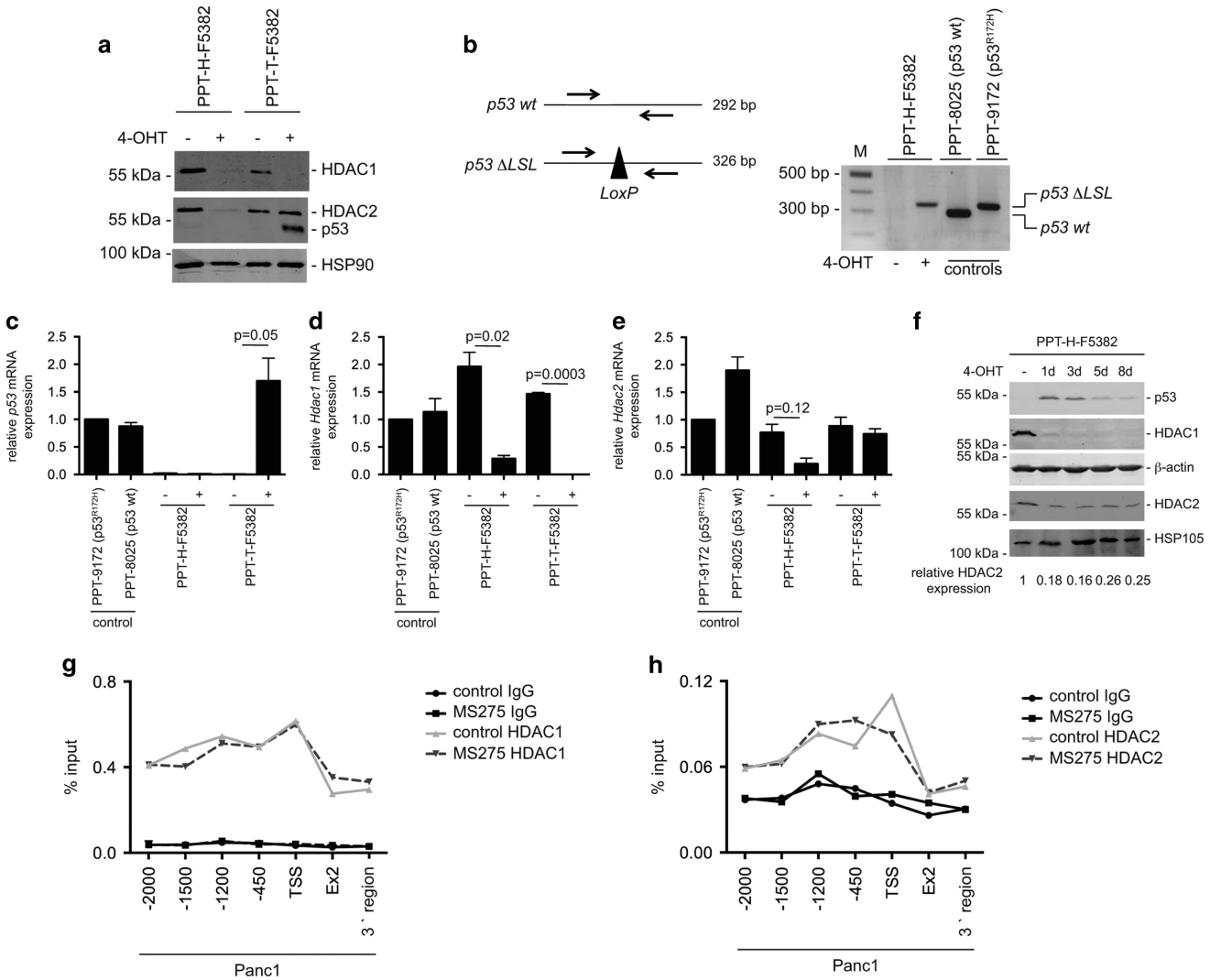

Figure 6. Simultaneous deletion of HDAC1 and HDAC2 reduced expression of p53^{R172H}. (**a**) Murine PDAC cells were isolated from a head (PPT-H-F5382) or tail (PPT-T-F5382) PDAC of the same *Pdx1-Flp;FSF-Kras$^{G12D/+}$;FSF-R26$^{CAG-CreERT2/+}$;LSL-p53$^{R172H/+}$;Hdac1$^{lox/lox}$;Hdac2$^{lox/lox}$* mouse. Freshly isolated cells (passage <4) were treated with 500 nM 4-Hydroxytamoxifen (4-OHT) or vehicle control for 5 days as indicated. Expression of HDAC1, HDAC2, p53, and HSP90 (loading control) was analysed by western blot ($n=2$). (**b**) PPT-H-F5382 PDAC cells were treated with 4-OHT or vehicle control over 5 days. Untreated PPT-8025 (p53 wild type) and conventional PPT-9172 (p53^{R172H}) cells served as controls. Genotyping PCR, according to the depicted strategy was performed and analysed by an agarose gel electrophoresis. M: marker. (**c–e**) PPT-H-F5382 and PPT-T-F5382 PDAC cells were treated with 4-OHT or vehicle control over 12 days as indicated. Untreated PPT-8025 (p53 wild type) and conventional PPT-9172 (p53^{R172H}) cells served as controls. mRNA expression in PPT-9172 cells was arbitrary set to 1. Relative (**c**) *p53*, (**d**) *Hdac1*, (**e**) *Hdac2* mRNA expression was determined by qPCR using *β-actin* mRNA as reference ($n=3$). *P*-values of a paired two-tailed Student's *t*-test are depicted. (**f**) PPT-H-F5382 cells were treated with 4-OHT (500 nM) for 1, 3, 5 and 8 days. Expression of HDAC1, HDAC2, p53 and β-actin or HSP105 (loading controls) by western blot ($n=1$). Same protein lysates were blotted on two membranes to detect p53 and HDAC1 or HDAC2 expression. The ratio of HDAC2 to HSP105 was measured and set to 1 in vehicle treated controls. The relative HDAC2 expression is depicted. (**g**) HDAC1 and (**h**) HDAC2 qChIP. Panc1 cells were treated with MS275 (4 μM, 24 h) or were left as vehicle treated control. Promoter qChIP of HDAC1 and HDAC2 represented as percentage of input and qChIP with negative control IgG antibody is shown. Primers covering the promoter, in Exon2 and a 3` region of the gene were used. Each point in the qChIP represents the mean from at least three independent immunoprecipitations. *P*-value of a paired two tailed Student's *t*-test at the TSS comparing HDAC2 recruitment in controls versus MS275 treated precipitations: $P=0.36$.

HDAC1-deficient models, c-MYC (afterward MYC) protein expression correlated with the expression of mutant p53 (Figure 5b). As comparison, Figure 5a showed MYC expression in HDAC2-deficient models. The human and murine *TP53* promoters harbour an E-box in their proximal regions. Furthermore, gene set enrichment analysis (GSEA) of microarray data from MS275 treated HCT116 cells, pancreatic endocrine alpha and beta (αTC1 or βTC3) cells,[50] and embryonic stem (ES) cells engineered to allow the genetic deletion of HDAC1 and HDAC2,[51] showed a significant negative enrichment of several MYC signatures. The normalized enrichment score of one of these MYC signatures[52] is exemplified in Figure 7a. These data illustrate that class I HDAC inhibition restrains MYC-directed networks. Notably, the combined inhibition of HDAC1 and HDAC2 by genetic means in ES cells is sufficient to impair MYC-dependent gene expression patterns, mimicking the MS275 treatment. In the investigated transcriptome profiles, class I HDACs are properly inhibited or HDAC1 and HDAC2 expression is relevantly reduced, since HDACi signatures were positive enriched.

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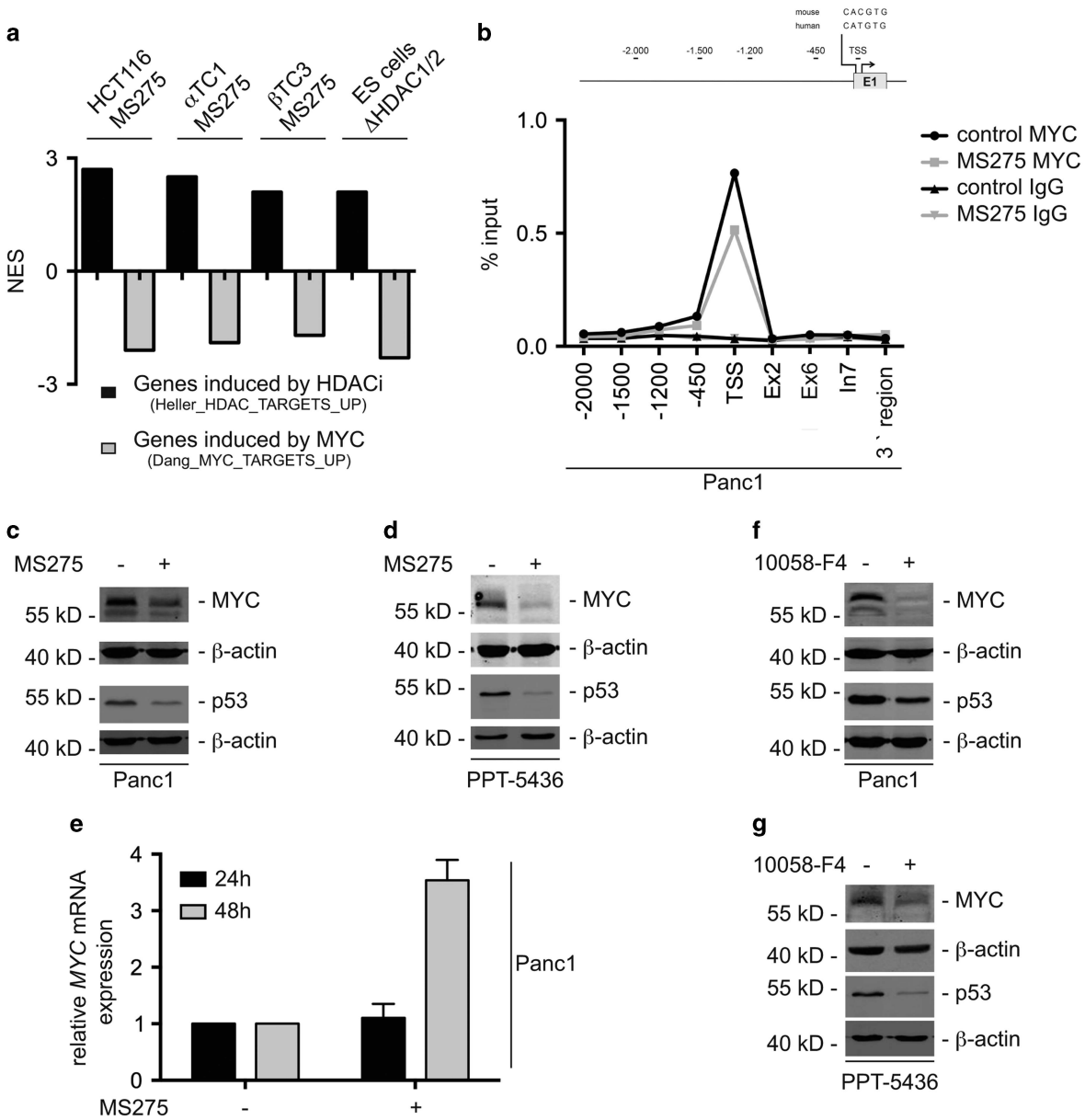

Figure 7. MYC expression and its binding to the *TP53* gene are controlled by class I HDACs. (**a**) Transcriptome profiles of HCT116, αTC1 and βTC3 cells treated for 24 h with MS275 as well as of embryonic stem (ES) cells with genetically deleted HDAC1 and HDAC2 alleles were analysed by GSEA. Shown is the normalized enrichment score of the indicated gene signatures. All signatures reveal a FDR q-value < 0.05 and a nominal *P*-value < 0.05. (**b**) Schematic illustration of *TP53* promoter with the location of the primers in the promoter used in qChIP. In addition primers in Exon2, Exon6, Intron7 and a 3´ region were used for the qChIP. Panc1 cells were treated with MS275 (4 μM, 24 h) or were left as vehicle treated control. Promoter qChIP of MYC represented as percentage of input and qChIP with negative control IgG antibody is shown. Each point in the qChIP represents the mean from at least three independent immunoprecipitations. *P*-value of a paired two tailed Student's *t*-test at the TSS comparing MYC recruitment in controls versus MS275 treated precipitations: $P = 0.18$. (**c**) Panc1 and (**d**) PPT-5436 cells were treated with MS275 (4 μM, 48 h) or were left as vehicle treated controls. Western blot of MYC, p53 and β-actin (loading control) expression ($n = 2$). (**e**) Panc1 cells were treated with MS275 (4 μM) as indicated. Vehicle treated cells were used as controls. Relative MYC mRNA expression was determined by qPCR using cyclophilin A mRNA as reference ($n = 3$). (**f**) Panc1 and (**g**) PPT-5436 cells were treated with 10058-F4 (Panc1: 100 μM, PPT-5436: 80 μM, 48 h) or were left as vehicle treated control. Western blot of MYC, p53 and β-actin (loading control) expression (**f**) $n = 2$; (**g**) $n = 3$). Same extracts were blotted to different membranes to detect MYC and p53 expression in (**c, d, f** and **g**).

The normalized enrichment score of one such HDACi signature[53] is shown (Figure 7a). Because these data suggest that class I HDACs, especially HDAC1 and HDAC2, are connected to the MYC network in different models, we tested the recruitment of MYC to the *TP53* promoter. The E-box is in close proximity to the TSS of the *TP53* gene (Figure 7b). In qChIPs, we found a distinct MYC peak at the TSS of the *TP53* gene in Panc1 cells (Figure 7b). This peak declined 24 h after the treatment with MS275 (Figure 7b). Moreover, MYC protein expression was reduced in Panc1 (Figure 7c) and PPT-5436 (Figure 7d) cells treated with MS275 and this finding was correlated with reduced expression of the p53 mutants. In contrast to the MYC protein, *MYC* mRNA

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expression was induced by MS275 in Panc1 cells (Figure 7e). Therefore, we conclude that class I HDACs regulate MYC protein expression at the post-transcriptional level.

To test the functional relevance of MYC for the expression of mutant p53, we used the MYC/MAX dimerization inhibitor 10058-F4.[54] Consistent with the binding of MYC to the *TP53* promoter, we observed reduced protein expression of p53 and MYC in 10058-F4 treated Panc1 (Figure 7f) and PPT-5436 (Figure 7g) cells. Since MYC can activate HDAC2 expression in PDAC models,[21] the 10058-F4 treatment reduced the HDAC2 expression over time in Panc1 and PPT-5436 cells (Supplementary Figure S4). Furthermore, 10058-F4 slightly attenuated HDAC1 expression (Supplementary Figure S4). Together, these results provide evidence that MYC contributes to the expression of mutant p53 and connect class I HDACs to oncogenic functions of MYC. Considering that a transcriptional mechanism is engaged by HDACi treatment, the *TP53* gene should be regulated irrespective of the mutational status. Consistent with this note, *p53* mRNA is reduced in microarrays of class I HDACi-treated wild type p53 expressing HCT116 cells, pancreatic endocrine alpha and beta cells or ES cells with reduced HDAC1/2 expression (Supplementary Figure S5). Pancreatic endocrine alpha and beta cells (αTC1 or βTC3) and ES cells presumably express wild type p53. Consistently, decreased wild type *p53* mRNA upon HDACi treatment was detected in several cancer models.[55–58] The regulation of the *p53* mRNA irrespective of the mutational status might further assist the note of a transcriptional mechanism engaged in response to HDACi.

DISCUSSION

Here, we show that HDACi target the expression of mutated p53 in PDAC. We provide evidence that HDAC1/HDAC2 and MYC contribute to the expression of p53 mutants across species.

Due to the pleiotropic actions of HDACs, it is likely that HDACi impinge onto the expression of mutated p53 at multiple levels. HDAC6 blockade by SAHA was shown to allow the degradation of mutant p53 by its E3 ubiquitin ligases MDM2 and CHIP.[31] However, inhibitors of MDM2 and a broad acting proteasomal inhibitor cannot completely rescue the HDACi-induced depletion of the p53 mutant in our models. Furthermore, consistent with the idea of an HDAC6-independent pathway, we detected no regulation of mutant p53 by a new and specific HDAC6 inhibitor. Such findings suggest that other HDACs are required to maintain p53 expression in PDAC cells. HDAC8 controls the expression of the transcription factor HoxA5,[29] a known activator of the *TP53* gene.[49] In addition, HDAC8 controls YY1 to maintain transcription of the mutant *TP53* gene in an HDAC6-independent manner in triple-negative breast cancer cells.[30] However, our data now provide evidence for an HDAC8-independent mechanism in PDAC models. MS275 has no activity against HDAC6 and HDAC8 at the doses we used,[32–35] but this agent efficiently depletes p53. In genetically defined HDAC1- or HDAC2-deficient conventional murine PDAC cells, the protein levels of mutated p53 correlates with expression levels of the remaining HDAC. Compared to proficient cells, there is a trend that conventional HDAC1- or HDAC2-deficient cell lines have reduced *p53* mRNA expression. Thus, both HDACs regulate p53 expression positively. This interpretation is corroborated in a new model, which we generated by a dual-recombinase system allowing genetic manipulation of PDAC cells.[47] Here, the $p53^{R172H}$ protein and mRNA is not expressed if *Hdac1* and *Hdac2* are simultaneously deleted. Importantly, we detected that specifically *Hdac2*, but not *Hdac1*, escaped genetic deletion in PDAC cell lines that we generated from the dual-recombinase system. In these escaper cells the p53 mutant remains expressed. This selection could indicate a selective pressure to express HDAC2. Although these observations are coherent with an important iso-enzyme specific function of HDAC2 in PDAC, the HDAC2 controlled oncogenic programme needs further clarification.

Despite their initial characterization as co-repressors, our data add further credit to the notion that HDAC1 and HDAC2 can actively promote transcription.[59–61] In a genome wide mapping of HDAC1 and HDAC2 binding sites, recruitment of HDAC1 and HDAC2 to the transcriptional start of active genes was demonstrated and correlated with binding of RNA polymerase II and acetylated histones.[59] It was suggested that HDACs participate in transcription cycles to reset histone modifications after the activities of histone acetyltransferases and RNA polymerase II.[59] These findings are in agreement with the observed binding of HDAC1 and HDAC2 to the *TP53* gene in our model. Consistent with a positive and activating role of HDACs in transcription, HDACi tuned down transcription of an oncogenic driver-programme of highly expressed genes in breast cancer cells by blocking transcriptional elongation.[62] Importantly, recent genome wide analyses of HDACi-treated cells also illustrate that HDACs promote transcriptional elongation for actively transcribed genes.[63] Such results are consistent with the control of the *TP53* gene that we demonstrate in MS275-treated cells. (I) Increased recruitment of HDAC1 to the bodies of genes repressed by HDACi treatment was described;[63] consistently, we detected a significant recruitment of HDAC1 to the *TP53* gene body. (II) Acetylation of histones at the proximal promoter of HDACi-repressed genes was reduced upon HDACi treatment, whereas HDACi increased histone acetylation at the gene bodies.[63] Again, we demonstrate a similar modulation of histone acetylation marks occurs at the *TP53* gene upon MS275 treatment in our model. Although these data provide further evidence that particularly HDAC1 and HDAC2 maintain transcription of the mutant *TP53* gene, additional layers of control may operate.

Decreased expression of the wild type *p53* mRNA in response to HDACi treatment is observed by us and others.[55–58] It is important to consider that HDACi treatment increases acetylation of wild type p53, contributing to its stabilization/activation.[64,65] Therefore, the net outcome of HDACi on wild type p53 protein expression and activity varies with context.

We show that HDACi reduces protein expression and genetic programmes of the oncogenic transcription factor MYC. MYC is an essential factor in PDAC.[48,66–71] Down-regulation of MYC expression in tumours upon inhibition of HDACs is commonly detected.[72–76] We observed increased *MYC* mRNA expression and decreased MYC protein expression upon MS275 treatment. Apparently, regulation of MYC expression upon HDACi treatment occurs at the post-transcriptional level. Of interest, our GSEA analysis argues that MYC and class I HDACs, especially HDAC1/HDAC2, are more generally connected. Accordingly, the control of MYC activity by HDACs was recently corroborated in MYC-driven medulloblastoma.[77]

Consistent with the reduced expression of MYC, we detected less MYC recruitment to an E-box near the TSS of the *TP53* gene upon the inhibition of class I HDACs. Congruent with these observations, MYC binding to the proximal *TP53* promoter is described[78–80] and mutated p53 has been suggested to be one important oncogenic MYC target.[80] Binding of MYC to the $p53^{R172H}$ gene in murine PDAC cells was described.[67] Moreover, treatment with SAHA causes a functionally relevant, reduced recruitment of MYC to its target gene $p21^{CIP1}$,[81] arguing that HDACi target MYC at additional genes. Although our data show that MYC maintains the expression of mutant p53, additional HDACi-triggered mechanisms may also operate on mutant p53. For example, MS275 was shown to induce acetylation of non-histone proteins especially in the nuclear compartment, including many proteins assuring accurate function of the transcriptional machinery.[82] Therefore, it is likely that such mechanisms additionally play a role.

In sum, our data show that important tumour drivers, like mutant p53 and MYC, are targeted by class I-specific HDACi in

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PDAC models. Certain HDACi-based combination therapies demonstrate exciting efficacy in PDAC *in vivo* models.[68] Therefore, HDACi should be further characterized and developed to increase therapeutic opportunities for PDAC.

MATERIALS AND METHODS

Materials

Please see Supplementary material and methods (SM&M).

PDAC cell lines, mouse lines, transfection, siRNAs

Panc1 and PaTu8988t cells were propagated as described.[19,83] Human PDAC cell lines were authenticated by Single Nucleotide Polymorphism-profiling conducted by Multiplexion (Multiplexion, Heidelberg, Germany). Cell lines were tested for Mycoplasma contamination by a PCR-based method.[84] For murine PDAC cell lines see SM&M and Supplementary Table 1. Animal studies were conducted in compliance with European guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees (IACUC) of the Technische Universität München and Regierung von Oberbayern. See SM&M for a description of mouse lines. siRNAs, purchased from Eurofins (Ebersberg, München, Germany), were transfected with polyethylenimine (Sigma-Aldrich, Taufkirchen, Germany) (final concentration: 100 nM) as described.[85] Sequences can be found in SM&M.

Cell lysis and western blot

Please see SM&M.

Histochemistry and immunocytochemistry

Please see SM&M.

Quantitative reverse-transcriptase PCR

Please see SM&M.

Quantitative chromatin immunoprecipitation

Chromatin immunoprecipitations were performed using SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, #9003) as described.[86] For detailed description see SM&M.

Microarray, RNA-Seq, GSEA and statistical methods

Generation and analysis of gene expression profiles was performed as described[86] and briefly mentioned in SM&M. We used the following gene expression data sets GSE52134,[51] GSE3637,[50] GSE56623,[44,45] which we have accessed via NCBI GEO. Profiles of MS275 treated HCT116 cells can be accessed via ArrayExpress (No. E-MTAB-4303). For analysis of microarrays, RNA-Seq, GSEA and statistical methods see SM&M.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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