Article

Dynamically regulated sumoylation of HDAC2 controls p53 deacetylation and restricts apoptosis following genotoxic stress

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Histone deacetylase 2 (HDAC2) is relevant for homeostasis and plays a critical role in gastrointestinal cancers. Here, we report that post-translational modification of endogenous HDAC2 with small ubiquitin-related modifier 1 (SUMO1) is a new regulatory switch for the tumor suppressor p53. Sumoylation of HDAC2 at lysine 462 allows binding of HDAC2 to p53. Moreover, sumoylated HDAC2 is a previously not recognized biologically relevant site-specific deacetylase for p53. Deacetylation of p53 at lysine 320 by sumoylated HDAC2 blocks recruitment of p53 into promoter-associated complexes and p53-dependent expression of genes for cell cycle control and apoptosis. Thereby, catalytically active sumoylated HDAC2 restricts p53 functions and attenuates DNA damage-induced apoptosis. Genotoxic stress evokes desumoylation of HDAC2, enabling p53-dependent gene expression. Our data show a new molecular mechanism involving a dynamically controlled HDAC2-sumoylation/p53-acetylation switch that regulates cell fate decisions following genotoxic stress.

Keywords: acetylation, apoptosis, HDAC2, p53, SUMO

Introduction

Histone deacetylases (HDACs) are crucial regulators of gene expression, signaling, and homeostasis. These enzymes remove acetyl moieties from ε-N-acetylated lysine residues of histones and non-histone proteins. In higher eukaryotes, deacetylases are grouped into four classes. Class I deacetylases (HDAC1, 2, 3, and 8) are ubiquitously expressed and often show deregulated expression patterns in transformed cells. Class IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10) enzymes are larger and their expression is restricted to certain cell types. HDAC11 belongs to class IV. Sirtuins (SIRT1-7), mammalian orthologs of yeast Sir2, comprise class III (Yang and Seto, 2008).

The functions of HDACs are modulated by post-translational modifications (PTMs) (Brandl et al., 2009). Recent findings reveal unique and isoenzyme-specific roles for HDAC2 that are controlled via PTMs (Krämer, 2009). HDAC2 is subject to phosphorylation, ubiquitinylation, nitration, and nitrosylation.

Sumoylation is the covalent attachment of SUMO (small ubiquitin-related modifier) to lysine residues of proteins. Four SUMO proteins (SUMO1-4) have been identified in humans. Sumoylation occurs by an enzymatic cascade involving the E1-activating enzyme SAE1/2, the E2 SUMO-conjugase UBC9, and E3 SUMO-ligases enhancing sumoylation (Geiss-Friedlander and Melchior, 2007; Gareau and Lima, 2010). SUMO-specific proteases (SEPNs) deconjugate SUMO rendering sumoylation highly dynamic (Kim and Baek, 2009). A link between sumoylation and the deacetylation machinery has been noticed (Ouyang and Gill, 2009) and the NAD⁺-dependent SIRT1 as well as HDAC1, 4, 6, and 9 can be sumoylated (Yang et al., 2007; Brandl et al., 2009). Although SUMO modification of the three first mentioned enzymes was found necessary for full catalytic activity, others measured similar catalytic activities of sumoylated and non-sumoylated HDAC1 (Brandl et al., 2009). It has also not been resolved whether sumoylation of class I HDACs can specifically affect their association with transcription factors.

HDAC inhibitors (HDACi) block the catalytic activity of HDACs. Treatment with HDACi attenuates proliferation and evokes...
tumor cell apoptosis. Interestingly, HDACi affect the functions of the tumor suppressor protein p53 by increasing its acetylation (Terui et al., 2003; Zhao et al., 2006; Palani et al., 2012). This and other modifications alter the capability of p53 to associate with chromatin and to induce the expression of its target genes that encode factors controlling cell cycle arrest and apoptosis. Cell cycle control genes are usually induced early and possess high-affinity p53 binding sites (e.g. p21). Pro-apoptotic gene expression is regulated by p53 via lower affinity p53-binding sites (e.g. BAX) and is induced upon persistent or strong stress (Vouuden and Prives, 2009; Beckerman and Prives, 2010; Schneider and Kramer, 2011).

It is unclear whether sumoylation of HDACs affects endogenous gene expression and how this may modulate p53 during the DNA damage response (DDR). Moreover, although the epigenetic regulator HDAC2 and the tumor suppressor p53 are discussed to have antagonistic functions with physiological relevance, the molecular mechanisms orchestrating their interplay still need to be defined. In this study we show that catalytically active endogenous HDAC2 can be modified by SUMO1 and that cell fate decisions depend on HDAC2 tagged with SUMO. We reveal that only sumoylated HDAC2 binds to and selectively deacetylates p53 at lysine 320 (K320) in genetically defined reconstituted cells. Consequently, this interferes with the DNA binding of p53 and alters the expression of endogenous p53 target genes. These processes ultimately impair the apoptotic response and increase the robustness of cells exposed to genotoxic stress. Our results identify dynamically controlled sumoylation of HDAC2 as a previously unrecognized regulatory switch for the tumor suppressor p53.

Results

Lysine 462 is essential for sumoylation of HDAC2 in vitro and in vivo

Analyzing whole cell extracts from various cell lines with different anti-HDAC2 antibodies revealed a prominent HDAC2-reactive band at the expected size of 55–60 kDa and a slower migrating species (Figure 1A and Supplementary Figure S1A). The 15–20 kDa molecular weight shift of this band pointed to a PTM with ubiquitin-like factors, e.g. SUMO. To exclude that we detected a splice variant of HDAC2, we expressed HA-tagged HDAC2 in HEK293T cells. We again observed a higher molecular weight fraction with anti-HA antibodies (Figure 1B), suggesting that this slower migrating form indeed represents post-translationally modified HDAC2.

To test whether HDAC2 can be conjugated to SUMO, we employed an in vitro SUMO modification assay (Werner et al., 2009). In vitro translated HDAC2 was incubated with recombinant SUMO1, SUMO-E1, and SUMO-E2 enzymes. In the presence of ATP, a single higher migrating HDAC2 band emerged. This protein fraction corresponded in size to SUMO1-modified HDAC2 (Figure 1C). Subsequently, we used immunoprecipitation (IP) with SUMO1-specific antibodies to probe for endogenously sumoylated HDAC2. Indeed, SUMO1-modified HDAC2 was detected in anti-SUMO1 precipitates from lysates of HCT116 human colon cancer cells (Figure 1D) and HEK293T human embryonic kidney cells (Supplementary Figure S1B). In addition, SUMO1-HDAC2 was enriched in IPs compared with the input.

Consistent with our in vitro data, one SUMO1-modified version of HDAC2 was observed. These findings suggest that endogenous HDAC2 is a bona fide substrate for modification with SUMO1.

Sumoylation usually occurs at lysine residues within the consensus sequence ΨKXE, where Ψ represents a large hydrophobic amino acid, K is the modified lysine, x can be any amino acid, and E is glutamic acid (Geiss-Friedlander and Melchior, 2007). Lysine 462 (K462) in the structurally flexible C-terminus is the only residue of HDAC2 within a ΨKXE motif. Comparison of HDAC2 from Pan troglodytes, Mus musculus, Rattus norvegicus, and Xenopus laevis revealed interspecies conservation of this sumoylation consensus sequence (Figure 2A). To test the relevance of K462 to HDAC2 sumoylation, we mutated K462 into a non-sumoylatable but charge-conserving arginine (HDAC2K462R) and generated a C-terminal deletion mutant lacking the last 28 amino acids including K462 (HDAC2Δ1–460). In vitro translated HDAC2, HDAC2K462R, and HDAC2Δ1–460 were subjected to in vitro sumoylation. Notably, wild-type HDAC2 was modified with SUMO1, but HDAC2K462R and HDAC2Δ1–460 remained unmodified (Supplementary Figure S1C). To assess whether K462 is critical for sumoylation in vivo, HEK293T cells were transfected with V5-tagged HDAC2 or HDAC2K462R. Anti-V5 IPs contained a higher migrating form of HDAC2 which was absent in HDAC2K462R precipitates (Figure 2B). Coexpression of SENP1, which recognizes and
deconjugates SUMO from proteins (Kim and Baek, 2009), led to disappearance of the slower migrating HDAC2 band. This effect was not observed when catalytically inactive SENP1 was cotransfected (Figure 2B). Also, SENP1 specifically coprecipitated only with wild-type HDAC2 but not with HDAC2K462R (Figure 2C). Finally, we performed anti-SUMO1 precipitates from lysates of HEK293T cells expressing V5-tagged HDAC2 or HDAC2K462R. Probing for HDAC2 showed sumoylation of the wild type but not of the K462R protein (Figure 2D). Taken together, these experiments show that K462 is strictly required for modification of HDAC2 with SUMO1. Next, we tested whether sumoylation of HDAC2 controls its general functions or whether this PTM has an effect on specific HDAC2 functions. Cell lines with defined genetic backgrounds lacking endogenous HDAC2 provide the opportunity to clearly discriminate between biological functions of HDAC2 and HDAC2K462R. RKO colon cancer cells lack HDAC2 (Ropero et al., 2008). Screening for an additional cellular model revealed that HCT116 p53−/− cells (Bunz et al., 1998) are also HDAC2-deficient (Supplementary Figure S2A). We investigated whether HDAC2 sumoylation determines its intracellular targeting with immunofluorescence microscopy. In RKO cells, wild-type HDAC2 and sumoylation-deficient HDAC2K462R and HDAC21460 were strictly nuclear (Supplementary Figure S2B). Thus, sumoylation of HDAC2 unlikely controls its nuclear localization. Next, we determined if the transcriptional repression capacities of HDAC2 are sumoylation dependent. A GAL4-driven

Figure 2 Lysine 462 is essential for sumoylation of HDAC2. (A) Schematic representation of HDAC2 with its catalytic domain shaded in gray, and K462 at the C-terminus within a SUMO modification consensus motif (YKxE) conserved among vertebrates. (B) HEK293T cells were transfected with plasmids encoding HDAC2 or SENP1. HDAC2 was immunoprecipitated and detected by the V5 tag (upper panel). Asterisk indicates SUMO-modified HDAC2. The expression of the overexpressed proteins is shown in the input (lower panels). (C) The indicated proteins were expressed in HEK293T cells and HDAC2-V5 proteins were immunoprecipitated. The interaction of SENP1 with HDAC2 was analyzed by anti-FLAG immunoblotting. Anti-V5 reprobes show equal IP efficiencies. The lower panels show immunoblots of 5% input. (D) HEK293T cells were transfected with plasmids coding for the indicated V5-tagged HDAC2 proteins. IPs with anti-SUMO1 antibodies or mouse preimmunserum were analyzed by immunoblotting using anti-V5 antibodies (upper panel in the right) and anti-SUMO1 antibodies (lower panel in the right). Left panel shows anti-V5 immunoblot (5% input); NSB, non-specific band. (E) HEK293T cells (left panel, n = 13) and HDAC2-negative HCT116 p53−/− cells (right panel, n = 3) were transfected with a GAL4-driven luciferase reporter (2xUAS-TK-Luc) together with pCMX-GAL4-HDAC2 or pCMX-GAL4-HDAC2K462R. Fold repression was determined relative to the luciferase activity obtained by transfecting pCMX-GAL4. The error bars represent standard errors (SEM).
HDAC2 sumoylation controls p53 activity

Luciferase reporter was employed with GAL4-HDAC2 or GAL4-HDAC2 K462R in HEK293T cells and HDAC2-negative HCT116 p53−/− cells. The latter was chosen to exclude an influence of endogenous HDAC2. Both HDAC2 variants suppressed reporter gene expression to a similar extent in both cell lines (Figure 2E). These data indicate equivalent repression by HDAC2 and HDAC2 K462R when artificially tethered to a promoter.

Contrary to other SUMO-modified deacetylases (Yang et al., 2007; Brandl et al., 2009), yet in agreement with our reporter gene assays, immunoprecipitated wild-type and sumoylation-deficient HDAC2 were similarly active in an in vitro deacetylase assay (Supplementary Figure S2C). This finding agrees with sumoylation of HDAC2 at the far C-terminus, away from the central catalytic domain of the protein. Also, binding to the corepressor mSin3a did not differ between HDAC2 and HDAC2 K462R (Supplementary Figure S2D). A requirement of sumoylation for HDAC2 enzymatic capacity in vitro is therefore not supported by our data. Still, sumoylation of HDAC2 might have an effect towards specific in vivo targets by allowing or disrupting protein complex with these targets. Sumoylation though seems not to influence HDAC2 stability. IPs from HEK293T cells expressing HDAC2-V5 or HDAC2 K462R-V5 revealed equal interaction of HDAC2 with UBC8 and RLIM and both HDAC2 variants displayed similar degradation kinetics (Supplementary Figure S2D and E).

HDAC2 sumoylation modulates gene expression controlled by p53

What could be the relevance of HDAC2 sumoylation? To address this question, we stably reconstituted RKO cells with wild-type HDAC2-V5 or HDAC2 K462R-V5 to achieve a situation resembling endogenous HDAC2. Compensatory regulation of HDAC1/HDAC3 was not observed (Figure 3A). As in other cells, wild-type HDAC2-V5 underwent sumoylation while HDAC2 K462R-V5 did not (Supplementary Figure S3A). Since HDAC2 can affect p53 functions (Harms and Chen, 2007; Ropero et al., 2008; Krämer, 2009; Li et al., 2010), we investigated putative effects of HDAC2 sumoylation on p53 target gene expression. The cell cycle regulator CDKN1A (p21cip1/waf1), the pro-apoptotic factors Pig3 and Bax, as well as p53’s major ubiquitin-E3 ligase HDM2 (human Mdm2) are positively regulated p53 target genes (Vouwen and Prives, 2009). Remarkably, we noticed drastically reduced levels of these proteins in RKO cells expressing wild-type HDAC2 compared with those with non-sumoylatable HDAC2 K462R (Figure 3A). Cytoprotective Survivin (Birc5c) on the other hand is directly repressed by p53 (Schneider and Krämer, 2011; Xia et al., 2011). Concordantly, Survivin levels were elevated in HDAC2 RKO cells compared with RKO cells harboring HDAC2 K462R (Figure 3A). Quantitative real-time PCR-based mRNA expression analyses showed that downregulation of HDM2 and p21 in HDAC2 RKO cells corresponds to reduced mRNA levels (Figure 3B). Consistent with the lower expression of HDM2, p53 levels are elevated in cells carrying wild-type HDAC2 (Figure 3A and Supplementary Figure S3B). While mutations of p53 can cause its accumulation, RKO cells harbor wild-type p53 (Xia et al., 2011), suggesting that reduced expression of HDM2 leads to increased wild-type p53 levels in RKO cells with wild-type HDAC2. Comparing the original mixed cell populations of stable RKO cells with single clones revealed similar expression profiles for the p53-inducible proteins mentioned above (Supplementary Figure S3B). Therefore, our results are not limited to single clones. In sum, these results argue that functionally impaired p53 accumulates in cells with wild-type HDAC2. These results strongly indicate that sumoylated HDAC2 blocks p53-dependent target gene expression and that HDAC2 sumoylation interferes with positive and negative transcriptional regulation by p53.

To corroborate these findings, we transiently expressed HDAC2 and HDAC2 K462R in HCT116 cells. Consistent with our hypothesis of a sumoylation-dependent functionality of HDAC2 towards p53, only wild-type HDAC2 suppressed the p53-responsive reporter pRGC-Luc (Figure 3C, left). This effect was p53-dependent as it was not present in HCT116 p53−/− cells (Figure 3C, right), in which reexpression of p53 activated pRGC-Luc. This was again antagonized by HDAC2 but not by sumoylation-deficient HDAC2 K462R (Figure 3C, right). To exclude that a modification at K462 other than sumoylation caused this repression, we mutated additional positions of the ψKXE sumoylation consensus. HDAC2 E463A with the variable x position mutated still repressed pRGC-Luc. Changing the critical ψ and E sites in HDAC2 (HDAC2 V461A and HDAC2 W464A) though resulted in complete loss of repression capacity, similar as for HDAC2 K462R (Supplementary Figure S3C). Thus, repression of p53-dependent transcription is mediated specifically by sumoylation-competent HDAC2.

Next, we tested whether p53 levels per se or HDAC activity cause the inert state of p53 in HDAC2-positive RKO. Incubation with Nutlin-3a, a specific inhibitor of HDM2 (Schneider and Krämer, 2011), stabilized p53 and resulted in comparable p53 protein levels in RKO cells harboring wild-type HDAC2 or HDAC2 K462R (Figure 3D). This treatment promoted the expression of p21 and HDM2 in control and HDAC2 K462R RKO, but not in cells expressing wild-type HDAC2 (Figure 3D, left). Furthermore, increasing p53 levels by such treatment resulted in stronger repression of Survivin in HDAC2 K462R cells, while it was ineffective in wild-type HDAC2 cells. Thus, sumoylated HDAC2 seems to inactivate p53 regardless of its expression levels.

To test whether the catalytic activity of HDAC2 locks p53 in an inert state, we used the HDACi VPA which targets HDAC2 (Brandl et al., 2009). In contrast to Nutlin-3a, VPA induced p21 expression in all stable RKO cells and decreased Survivin expression in cells carrying wild-type HDAC2 (Figure 3D, right). These data show that VPA restored p53 functionality regarding induction of p21 and repression of Survivin. The finding that VPA reduced HDM2 expression (Figure 3D, right) reflects the described suppression of this promoter upon HDACi-induced hyperacetylation (Ropero et al., 2008; Ku et al., 2009). Taken together, these experiments indicate that sumoylatable catalytically active HDAC2 antagonizes p53 functions. Whereas increasing the levels of p53 with Nutlin-3a cannot compensate for this effect, blocking the catalytic activity of sumoylatable HDAC2 with VPA restores p53-dependent transcription.

HDAC2 is a previously not recognized sumoylation-dependent deacetylase for p53

Acetylation of several C-terminal lysine residues can influence p53-dependent apoptosis by increasing p53’s DNA binding capacity, target gene selectivity and transcription-activating properties (Liu et al., 1999; Luo et al., 2004; Zhao et al., 2006; Yang
PCAF-triggered acetylation of p53 strongly effects of wild-type HDAC2

Knights et al., 2006

HDAC2-positive RKO cells compared with those harboring sumoylation-deficient HDAC2K462R (Figure 4B). These data cannot be explained by different PCAF levels (Figure 3A). Importantly, wild-type HDAC2 deacetylated p53K320, whereas non-sumoylatable HDAC2K462R was not able to deacetylate p53 at K320 (Figure 4C). We further examined the interaction of p53 with wild-type HDAC2 and HDAC2K462R. Western blot analyses of anti-p53 immunoprecipitates revealed that endogenous p53 readily interacts with HDAC2, but not with sumoylation-deficient HDAC2 (Figure 4D). These data argue for sumoylation as the molecular trigger directing HDAC2 against acetylation of p53K320.

HDAC2 sumoylation regulates stress-induced apoptosis by impeding acetylation and DNA binding of p53

Although the above data implicate sumoylated HDAC2 in the control of p53, they cannot prove that deacetylation of p53 by wild-type HDAC2 inactivates p53. To elucidate the functional consequences of HDAC2 on p53, avidin–biotin complex–DNA (ABCD) binding assays were used to test if the lower relative p53K320 acetylation level in wild-type HDAC2 RKO cells prevents in vitro DNA binding of p53. HDAC2-RKO and HDAC2K462R-RKO cells were treated with Nutlin-3a to achieve equal levels of p53.
Two independent p53-binding site oligonucleotides captured less p53 from RKO cells expressing HDAC2 compared with RKO cells with HDAC2K462R (Figure 5A). We further performed chromatin-IP (ChIP) analyses to evaluate the role of sumoylated HDAC2 for p53 promoter-specific DNA binding in vivo. Recruitment of p53 to the p21 and HDM2 promoters in wild-type HDAC2 cells was strongly reduced when compared with HDAC2K462R cells (Figure 5B). These experiments suggest that HDAC2 deacetylates p53 to inhibit its recruitment to different promoters encoding physiologically relevant genes.

Both, HDAC2 and p53 regulate the proliferation and survival of normal and cancer cells under genotoxic stress (Krämer, 2009; Vousten and Prives, 2009). We tested whether sumoylation of HDAC2 determines apoptosis in the context of DNA damage caused by doxorubicin. This drug induces double-strand DNA breaks and causes p53-dependent apoptosis (Schneider and Krämer, 2011; Xia et al., 2011). Of note, flow cytometric analyses disclosed that RKO cells expressing the wild-type HDAC2 were significantly less susceptible to doxorubicin-induced apoptosis compared with RKO cells reconstituted with sumoylation-deficient HDAC2K462R (Figure 5C). Dead cells with a DNA content below 2N (sub-G1) were ~20% for RKO cells with the wild-type HDAC2 and ~70% for RKO cells with HDAC2K462R (P < 0.001). Additionally, cotreatment of HDAC2K462R/V5 RKO cells with a lower dose of doxorubicin and Nutlin-3a led to markedly enhanced apoptosis (Supplementary Figure S3E) pointing out that enhanced p53 levels in the presence of genotoxic stress greatly enhance apoptosis in cells reconstituted with HDAC2K462R. In perfect agreement with our hypothesis that sumoylated HDAC2 blocks p53-dependent gene expression (Figure 3D), wild-type HDAC2-positive cells are less sensitive to this drug combination (Supplementary Figure S3E). These data demonstrate that sumoylated wild-type HDAC2 attenuates p53-dependent apoptosis in cells exposed to DNA damage.

Nonetheless, HDAC2-positive cells undergo apoptosis in response to doxorubicin, and we also see a significantly lower but discernable effect of doxorubicin on HDAC2-positive cells (Figure 5C). Based on these findings and on the fact that stress alters sumoylation patterns within cells (Gareau and Lima, 2010), we hypothesized that HDAC2 sumoylation is a dynamically controlled upstream regulator of p53. Remarkably, HDAC2 became desumoylated upon genotoxic exposure of cells (Figure 5D). Treatment of RKO-HDAC2-V5 cells results in a drastic increase in p53 and p21 expression (Figure 5E), hinting that HDAC2 desumoylation after genotoxic stress allows transcriptional competence of p53.

Taken together, our data suggest that sumoylation of HDAC2 is a dynamic regulator for p53-dependent gene expression and cell fate decisions.

Discussion

In this article, we show that HDAC2 is covalently modified with SUMO1. We demonstrate that an intact ΨKxK sumoylation consensus motif around lysine 462 of HDAC2 is essential for this...
modification. The fact that this consensus motif is evolutionarily conserved from *Xenopus* to humans points to a biologically important role of HDAC2 sumoylation. We reveal that sumoylation-deficient HDAC2 does not interact with p53, whereas sumoylation-competent HDAC2 does. Our new finding that modification with SUMO controls the HDAC2 interactome is a major conceptual advance. Upon analyzing the biological consequences of HDAC2 sumoylation, we noticed that disrupting this site-specific PTM has a strong impact on p53-dependent gene expression in transformed human cells. We found that HDAC2 can inhibit p53-dependent induction of p21, PIG3, BAX, and HDM2, as well as p53-dependent suppression of *Survivin*. In search for the molecular relay underlying the control of p53 by HDAC2, we discovered that HDAC2 is a p53 deacetylase. PCAF-triggered p53<sup>K320</sup> acetylation promotes binding of p53 to its consensus sites *in vitro* and to the p21 promoter *in vivo* (Liu et al., 1999; Knights et al., 2006; Xenaki et al., 2008). We reveal here that HDAC2 binds to p53 and reverses its acetylation at K320. Both processes strictly depend on sumoylation of HDAC2. In line with this, we detect impaired acetylation of p53<sup>K320</sup> together with strongly diminished binding of p53 to the p21 and HDM2 promoters in cells positive for wild-type HDAC2. Of note, this is not the case in cells carrying sumoylation-deficient HDAC2. Reduced amounts of HDM2, the major ubiquitin ligase for p53, might contribute to the accumulation of p53 we noticed. It is noteworthy that p53 in the wild-type HDAC2 reconstituted cells is hypoacetylated and consequently not able to modulate steady-state levels of p53 target genes. Hypoacetylated p53 could even cause dominant-negative effects as tetramer formation is required for the functionality of p53 as a transcription factor (Chan et al., 2004). Therefore, we speculate that reduced acetylation of p53<sup>K320</sup>, located near the DNA-binding domain and the tetramerization domain of p53 (Beckerman and Prives, 2010), generates inactive p53 tetramers which may account for the pronounced DNA-binding deficiency of p53 in cells expressing wild-type HDAC2 (Figure 6).

Albeit the amount of sumylated species represents only a fraction of total HDAC2, our data reveal that this pool has unique and privileged biological functions. Similar results were collected for the majority of sumoylation targets. Furthermore, it is well known that sumoylation engages permissive states for complex formation and protein functions (Hay, 2005; Geiss-Friedlander and Melchior, 2007). Studies on HDAC sumoylation often rely on reporter assay systems and cells carrying the endogenous protein. This strategy can be problematic as sumoylation affects on reporter assay systems and cells carrying the endogenous protein. However, we here demonstrate that sumoylation also affects regulation of p53 as a transcription factor (Chan et al., 2004). Therefore, we speculate that reduced acetylation of p53<sup>K320</sup>, located near the DNA-binding domain and the tetramerization domain of p53 (Beckerman and Prives, 2010), generates inactive p53 tetramers which may account for the pronounced DNA-binding deficiency of p53 in cells expressing wild-type HDAC2 (Figure 6).

Figure 5 HDAC2 regulates stress-induced apoptosis by preventing DNA binding of p53. (A) ABCD assays with p53 binding oligonucleotides. Stably transfected RKO cells (HDAC2-V5, HDAC2<sup>k462R-V5</sup>) were treated with Nutlin-3a for 24 h to equalize p53 protein levels. Cell extracts were incubated with oligonucleotides containing a p53 consensus binding site (p53-site consensus), a p53 responsive element from the CDKN1A promoter (p21), or a mutated p53 consensus binding site (-CTR). The amount of bound p53 compared with % input was determined by western blotting. (B) ChIP analysis revealing the binding of p53 to the p21 (left panel) and HDM2 promoter (right panel) in RKO cells stably expressing HDAC2-V5 or HDAC2<sup>k462R-V5</sup> (n = 3; ± SEM). Quantities of chromatin fragments precipitated with anti-p53 antibodies and control IgG are shown relative to the amount of IP input. Amplification of GAPDH after p53 served as an internal negative control. (C) FACS analysis of RKO cells stably expressing HDAC2-V5 or HDAC2<sup>k462R-V5</sup> (n = 7; ± SEM). Cells remained untreated (upper panel) or were treated with 1 µM doxorubicin for 24 h (lower panel). Treated and untreated cells were always from one batch and cultured side-by-side. ***p<0.001. (D) RKO-HDAC2-V5 cells were treated with 1 µM doxorubicin for the indicated time points. Sumoylation levels of HDAC2 were assessed with IP. (E) RKO-HDAC2-V5 cells were treated as in C. The expression of p53 and p21 was analyzed by immunoblot.
leaves to decreased p53^K320 acetylation in our cell system. Data supporting a crucial role of HDAC2 for p53-dependent gene expression were also collected in murine fibroblasts lacking the ubiquitin ligase Mule which degrades HDAC2 (Zhang et al., 2011). Moreover, knocking down HDAC2 induces the p21 and HDM2 genes due to enhanced p53 promoter binding (Harms and Chen, 2007). Our work links HDAC2- and SUMO-dependent mechanism to reduced endogenous p53-dependent gene expression and apoptosis.

Overall, our results are consistent with studies revealing negative effects of the epigenetic regulator HDAC2 on p53 (Harms and Chen, 2007; LeBoeuf et al., 2010; Li et al., 2010; Wilting et al., 2010; Zhang et al., 2011). We extend these studies as we delineate a previously unrecognized molecular mechanism that connects p53 with HDAC2. It should also be considered that p53 can be dynamically regulated by methylation, phosphorylation, and other PTMs (Knights et al., 2006; Huang et al., 2007; Beckerman and Prives, 2010). For example the methyltransferase Set7/9 promotes an increase in p53 acetylation levels in cells exposed to genotoxic stress (Liu et al., 2011). It will be interesting to find out whether this mechanism is associated with HDAC sumoylation. Furthermore, it is possible that HDAC2 deacetylates p53 at additional lysine residues in a cell-type and stimulus-dependent fashion. Such complex regulatory circuits should be investigated in future studies.

Expression of the pro-apoptotic proteins PIG3 and BAX is repressed by wild-type HDAC2, which deacetylates p53^K320. Accordingly, cells carrying a p53^K320R mutant are resistant to apoptosis (Terui et al., 2003). However, p53^K317R knock-in mice (murine K317 corresponds to human K320) can transactivate pro-apoptotic target genes (Chao et al., 2006). Independent of cell-type/context-dependent effects, human cells carrying sumoylated HDAC2 have low p21 levels that could facilitate tumor proliferation. While BAX and PIG mediate p53-dependent apoptosis, HDM2 provides a negative feedback on p53 stability to permit the survival of stressed cells (Brooks and Gu, 2006). Suppression of HDM2 in HDAC2 wild-type cells allows p53 accumulation, which is potentially dangerous for cells. Deacetylation by HDAC2 compensates this effect by impairing the transcription factor p53 and the expression of its tumor suppressive target genes.

Dysregulation of HDAC2 and p53 in numerous human malignancies indeed implies that the balanced activity of both factors is required for cellular homeostasis (Olsson et al., 2007; Krämer, 2009). Numerous cancers, including colon tumors, over-express HDAC2 which promotes their survival and proliferation (Fritsche et al., 2009; Krämer, 2009; Weichert, 2009; Fakhry et al., 2010). Moreover, HDAC2 is an independent prognostic marker in colorectal carcinomas and oral cancers (Huang et al., 2005; Weichert et al., 2008; Chang et al., 2009). The present data suggest that a selection for high HDAC2 levels in some cancer types bypasses the potent tumor suppressor p53 without the need for inactivating p53 mutations. We show that colon cancer cells expressing sumoylation-deficient HDAC2, i.e. HDAC2 unable to deacetylate p53, are much more sensitive to DNA damage-induced apoptosis than cells expressing wild-type

Figure 6 HDAC2 sumoylation regulates p53 activities. Catalytically active sumoylatable HDAC2 locks the tumor suppressor p53 in an inactive state. The mechanism by which HDAC2 negatively controls p53-dependent transcription relies on the ability of HDAC2 to become sumoylated at K462. Consequently, HDAC2 binds to and deacetylates p53 at K320 which strongly attenuates its DNA binding. This process suppresses the expression of the pro-apoptotic factors BAX and PIG3, of the cell cycle regulator p21 and of the E3 ubiquitin-ligase HDM2. It also blocks the p53-mediated transcriptional repression of the anti-apoptotic protein Survivin (Surv). This ultimately modulates the apoptotic response to genotoxic stress.
HDAC2. Thus, sumoylation of HDAC2 and not its mere presence appears as relevant marker for the control of p53 and the (patho-) physiological role of HDAC2.

Chemotherapy-induced genotoxic stress reduces HDAC2 sumoylation and allows p53-dependent target gene expression. However, cells with sumoylated HDAC2 have already less basal p53-dependent gene expression and are accordingly more resistant against DDR-induced apoptosis. We speculate that inactivation of p53 by aberrantly expressed HDAC2 is perhaps an early necessary step for cell transformation. The importance of HDAC activity in the control of p53 is stressed by studies showing increased p53 acetylation and p53-dependent gene expression after treatment of cells with HDACi (Terui et al., 2003; Roy et al., 2005; Zhao et al., 2006; Lin et al., 2008; Palani et al., 2012). We provide a mechanistic explanation for these observations and extend the knowledge on PTMs and the crosstalk of the epigenetic and transcriptional regulators HDAC2 and p53.

Materials and methods

Cell culture, transfections, cell extracts, IP and western blotting

Cells were maintained in Dulbecco’s modified Eagle’s medium containing 2% l-glutamine (PAAs, now GE Healthcare) supplemented with 10% fetal calf serum (Sigma-Aldrich). Experimental details on western blotting and IPs and buffer composition, drugs, chemicals, plasmids and antibodies can be found in Supplementary material.

Luciferase assays

Details for this method are listed in Supplementary material.

In vitro sumoylation assays

V5-tagged HDAC2, HDAC2 K462R, and HDAC2 1-460 were expressed in vitro using the TnT® T7 Coupled Reticulocyte Lysate System (Promega). Sumoylation assays using 1 μl TNT-lysate per reaction were performed as described (Werner et al., 2009).

Generation of stable RKO cell lines

HDAC2-negative RKO cells were transfected with plasmids encoding GFP, V5-tagged wild-type HDAC2, and HDAC2 K462R, respectively, on two successive days. Cells were treated with 800 μg/ml G418 (PAa) to select transfectants.

ABCD assays

Experimental details on ABCD assays can be found in Supplementary material.

Quantitative real-time PCR

Details for this method can be found in Supplementary material.

Flow cytometry

Cells were treated with doxorubicin 1 day after seeding. Cells were detached with accutase (PAa), washed with PBS (PAa) and fixed (70% ETOH, −20°C; at least 1 h). DNA was stained with 10 mM propidium iodide, 38 mM sodium citrate, and 100 μg/ml RNaseA (Roth) for 30 min. Measurements were performed with an FACSCanto Flow Cytometer and FACSDiVa software (BD).

Chromatin IP

Chromatin IP (ChiP) was performed as stated (Baus et al., 2009). For p53-ChIPs, mouse α-p53 (DO-1; Santa Cruz) was used. Determination of the amount of precipitated DNA was done by RT-qPCR in principle as described above. Primer sequences are given in Supplementary Table S1.

Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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References


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