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Coordination of PAD4 and HDAC2 in the regulation of p53 target gene expression

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Abstract

Histone Arg methylation and Lys acetylation have been found to cooperatively regulate the expression of p53 target genes. Peptidylarginine deiminase 4 (PAD4) is an enzyme that citrullinates histone arginine and monomethyl-arginine residues thereby regulating histone Arg methylation. We have recently found that PAD4 serves as a p53 corepressor to regulate histone Arg methylation at the p53 target gene p21/WAF1/CIP1 promoter. However, it has not been tested whether histone Arg citrullination coordinates with other histone modifications to repress transcription. Here, we show that HDAC2 and PAD4 interact with p53 via distinct domains and simultaneously associate with the p21 promoter to regulate gene expression. After DNA damage, PAD4 and HDAC2 dissociate from several p53-target gene promoters (e.g., p21, GADD45, and PUMA) with a concomitant increase in histone Lys acetylation and Arg methylation at these promoters. Furthermore, PAD4 promoter association and histone Arg modifications are regulated by p53 and HDAC activity. In contrast, HDAC2 promoter association and histone Lys acetylation are affected by p53 and PAD4 activity at minor degrees. Importantly, PAD4 inhibitor Cl-amidine and HDAC inhibitor SAHA demonstrate additive effects in inducing p21, GADD45, and PUMA expression and inhibiting cancer cell growth in a p53-dependent manner. Our results unveil an important crosstalk between histone deacetylation and citrullination, suggesting that a combination of PAD4 and HDAC2 inhibitors as a potential strategy for cancer treatment.

Keywords

Histone citrullination; Histone deacetylation; PAD4; HDAC2; p53

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Supplementary material, including two figures and figure legends, is available at Oncogene's website.

Introduction

In eukaryotic cells, 146 bp DNA is wrapped around two copies of each histones H3, H2B, H2A and H4 to form a nucleosome – the basic structural unit of chromatin (Kornberg and Lorch, 1999; Luger *et al.*, 1997). Various posttranslational modifications, such as methylation, acetylation, phosphorylation, ubiquitination and citrullination, are believed to play a significant role in chromatin activities, such as transcription (Barski *et al.*, 2007; Berger, 2007; Klose and Zhang, 2007; Li *et al.*, 2007; Shilatifard, 2006). In light of the biological significance of histone modifications in cellular activities, a “histone code” hypothesis was proposed (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000), which suggests that different histone modifications, either working singularly or in combination, fine tune the outcomes of various chromatin-based cellular processes.

Histone modifying enzymes with opposing activities counteract each other's effect via crosstalks (Fischle *et al.*, 2003). For example, histone acetyltransferases (HATs) catalyze histone acetylation, while histone deacetylases (HDACs) remove the pre-existing acetyl groups. Histone Arg methylation is catalyzed by protein arginine methyltransferases (PRMTs) (Bedford and Clarke, 2009). Conversely, peptidylarginine deiminase 4 (PAD4/PADI4/PADV) was identified as the first mechanism mediating histone Arg demethylation (Cuthbert *et al.*, 2004; Wang *et al.*, 2004), which converts monomethyl-Arg to citrulline through a biochemical reaction termed demethyelimination (Wang *et al.*, 2004).

The tumor suppressor protein p53 functions at the center of an extremely complicated signaling network in human cells (Laptenko and Prives, 2006; Vogelstein *et al.*, 2000). p53 is a transcription factor containing an activation domain at its N-terminus, a DNA binding domain in the middle and a regulatory domain at the C-terminus (Kitayner *et al.*, 2006). Multiple cell stress signals and DNA damaging reagents can activate p53 that in turn activates the expression of various target genes, such as p21, GADD45, PUMA (Harris and Levine, 2005; Vogelstein *et al.*, 2000). The protein products of p53-target genes further result in cell growth arrest and apoptosis. Mutations of p53 cause severe cellular defects in coping with genomic stress. The significance of p53 in genome integrity and cancer prevention is manifested by that over 50% of all cancers carry mutant p53 (Levesque and Eastman, 2007). p53 plays multifaceted roles in gene expression, which regulates transcription by recruiting both coactivators and corepressors to its target gene promoter (Ho and Benchimol, 2003; Laptenko and Prives, 2006). Multiple HATs have been found as p53 coactivators (Barlev *et al.*, 2001; Espinosa and Emerson, 2001), while histone deacetylases (HDACs) function as p53 corepressors by counteracting the function of HATs (Dannenberg *et al.*, 2005; Harms and Chen, 2007; Luo *et al.*, 2000). Recently, we have found that PAD4 functions as a p53 corepressor and counteracts Arg methylation at p21 promoter region to repress gene expression (Li *et al.*, 2008). However, whether histone citrullination and deacetylation are coordinated with each other to mediate the p53 target gene expression remains unknown. Here, we report that HDAC2 interacts with PAD4, and both enzymes act on p53 target genes to repress gene expression.

RESULTS

In order to identify PAD4 interacting proteins, we affinity purified endogenous PAD4 and its associated protein using PAD4 antibody conjugated to Sepharose beads from MCF-7 cell nuclear extracts following a previously described method (Baek *et al.*, 2002). Mass spectrometry analyses identified SHARP as a putative PAD4 interacting protein (Data not shown). SHARP is a large transcriptional repressor protein, which was found to interact with HDACs to repress gene expression (Shi *et al.*, 2001). Given that PAD4 (Li *et al.*, 2008) and HDACs (Harms and Chen, 2007) regulate p53-mediated transcription, we postulated that

PAD4 coordinates with HDACs to regulate p53 functions. To test this idea, M2 agarose beads were used for affinity purification of FLAG-PAD4 and its associated proteins from 293T and MCF-7 cells. Western blot analyses found that HDAC2 and p53 were recovered together with FLAG-PAD4 (Figure 1a). By comparing the amount of HDAC2 purified by M2 agarose beads with the amount of input protein, we found that about 1% of HDAC2 was retained by M2 agarose beads together with FLAG-PAD4 (Figure 1b), suggesting the sub-stoichiometry interaction of HDAC2 and PAD4. To further corroborate PAD4 and HDAC2 interaction, GST pull-down experiments were performed. HA-PAD4 was retained by GST-HDAC2 beads but not by control GST beads (Figure 1c). Likewise, HDAC2 was retained by GST-PAD4 beads but not by control GST beads (Figure 1d). To further test if endogenous PAD4 and HDAC2 interact, we performed co-immunoprecipitation experiments and found that HDAC2 was co-immunoprecipitated by a PAD4 rabbit polyclonal antibody but not by the control normal rabbit IgG (Figure 1e).

The interaction of PAD4 and HDAC2 prompted us to test if histone deacetylase and histone citrullination activities associate with each other. First, GST-PAD4 purified from *E.coli* and FLAG-PAD4 purified from FLAG-PAD4/293T cells showed histone citrullination activities when free histone H3 was used as a substrate (Figure 2a). We have previously showed that PAD4 demethylates free histone H3 in *in vitro* biochemical assays (Wang *et al.*, 2004). However, the demethylation activity has not been tested using nucleosomal substrate *in vitro*. Here, we further tested GST-PAD4 activity using oligo nucleosomes purified from HeLa cells as substrates. Concomitant with an increase in histone citrullination, a decrease of histone H3 Arg17 methylation was detected in a GST-PAD4 dose dependent manner (Figure 2b), indicating the demethylation activity of PAD4. Since GST-PAD4 pulled down HDAC2, we further tested if HDAC activity is associated with GST-PAD4. Using a fluorometric HDAC assay method, we found that GST-PAD4 but not GST beads retained HDAC activity (Figure 2c, **column 10**), suggesting that histone citrullination and deacetylation activities can associate with each other via protein-protein interaction.

Neither PAD4 nor HDAC2 has a DNA binding domain, we therefore postulated that these two proteins associate with specific gene promoters by interacting with transcription factors, such as p53. PAD4 has two immunoglobulin-like domains at its N-terminus and a catalytic domain at its C-terminus (Arita *et al.*, 2004). We have previously found that the two N-terminal immunoglobulin-like (IgL) domains of PAD4 interact with the C-terminal regulatory domain of PAD4 (Li *et al.*, 2008). To analyze protein-protein interaction domains between PAD4 and HDAC2, we performed GST-pull down experiments using GST-PAD4 and GST-PAD4-IgL1 (containing PAD4 IgL1, residues 1–125) or GST-PAD4-IgL1&2 (residues 1–300) fusion proteins purified from *E.coli* (Figure 2d). We found that the two Ig-like domains of PAD4 are required for PAD4 and HDAC2 interaction (Figure 2e, lane 8). Moreover, this part of PAD4 was also sufficient to retain the HDAC activity (Figure 2c, **column 9**). To further analyze which part of p53 interacts with HDAC2, we used GST-p53 and its derivatives (Figure 2f) to pull down HDAC2, and found that both the C-terminal regulatory domain of p53 (residues 301–393) and the N-terminal part of p53 (residues 1–300, containing the N-terminal activation domain and the middle DNA binding domain) interact with HDAC2 (Figure 2g), with a slightly stronger interaction of HDAC2 with the p53 C-terminal regulatory domain detected (Figure 2g, compare lane 6 with lane 7). Taken together, protein-protein interaction studies suggest a model of p53, HDAC2, and PAD4 interaction (Figure 2h), in which the N-terminal Ig-like domains of PAD4 interact with the C-terminal regulatory domain of p53 (Li *et al.*, 2008) and HDAC2 (Figure 2e), while p53 and HDAC2 interaction is mediated by both the p53 C-terminal regulatory domain as well as its N-terminal domain.

After DNA damage treatment, p53 is stabilized and binds to its target gene p21 via two defined binding sites, PBS1 and PBS2 (**illustrated in** Figure 3a) to regulate transcription. The stabilization of p53 and the induction of p21 expression were detected at 6 hr after doxorubicin (a DNA damaging drug) treatment in osteosarcoma U2OS cells (Figure 3b, lanes 1–2). To analyze the association of PAD4 and HDAC2 with the p21 promoter during DNA damage response, we performed chromatin immunoprecipitation (ChIP) experiments after treatment of U2OS cells with doxorubicin for 6 hr. The amount of p53 increased at the two p53-binding sites (PBS1 and PBS2) of p21 after DNA damage (Figure 3c). Compared with untreated cells, the amount of PAD4 and HDAC2 decreased after DNA damage (Figure 3c). Consistent with a role of PAD4 in mediating histone citrullination and decreasing histone Arg methylation, the dissociation of PAD4 from the p21 promoter after DNA damage was accompanied with a decrease in histone H3 citrullination and an increase in histone H3 Arg17 methylation (Figure 3c). Furthermore, the decrease of HDAC2 association correlates to an increase of histone H3 Lys9/14 acetylation after DNA damage (Figure 3c). These data support a model that PAD4 and HDAC2 associate with p53 at the p21 promoter before DNA damage to inhibit p21 expression, while these two proteins dissociate after DNA damage to allow gene activation.

To test whether the binding of PAD4 and HDAC2 to the p21 promoter is p53 dependent, we performed ChIP experiments in U2OS cells after p53 depletion by shRNA (U2OS/p53-shRNA). The depletion of p53 decreased the amount of p53 and also the induction of p21 by doxorubicin (Figure 3b, lanes 3–4). Compared with the parental U2OS cells, the amount of p53 at the p21 promoter was significantly decreased before and after DNA damage treatment in ChIP assays (Figure 3d). Moreover, after p53 depletion, the amount of PAD4, histone citrullination, and histone Arg methylation was greatly decreased (Figure 3d), suggesting that p53 is a major factor recruiting histone Arg modifying enzymes to the p21 promoter. In contrast, HDAC2 and histone Lys acetylation were decreased to a less extent (Figure 3d), suggesting that additional mechanism(s) is present to regulate HDAC2 recruitment and histone acetylation.

The interaction of PAD4 and HDAC2 implicates that these two proteins may simultaneously associate with the p21 promoter. To test this idea, we performed re-ChIP experiments, a method to determine if two proteins are on the same promoter at the same time. First, we used M2 agarose beads to perform ChIP from MCF-7 cells expressing FLAG-PAD4 or control parental MCF-7 cells. The N-terminal FLAG tag allows efficiently enrichment of PAD4 associated promoters by ChIP. PCR analyses revealed that the M2 beads recovered p21 promoter from MCF-7 cells expressing FLAG-PAD4 but not the control MCF-7 cells (Figure 3e). For the second step ChIP, we used an HDAC2 mAb to perform ChIP using DNA/protein complex eluted from the M2 agarose beads by the FLAG peptide, while the PAD4 antibody was used as a positive control. The HDAC2 antibody was able to re-ChIP the p21 promoter, while no signal was detected when control protein A agarose beads were used for re-ChIP (Figure 3e), indicating that PAD4 and HDAC2 are recruited simultaneously to the p21 promoter.

We have previously found that PAD4 inhibits the expression of other p53 target genes, such as GADD45 and PUMA (Li et al., 2008). To further analyze PAD4 and HDAC2 promoter association at other p53 target genes during DNA damage response, we performed ChIP experiments to measure the recruitment of these factors at GADD45 and PUMA gene promoters in U2OS cells with or without the depletion of p53 (Figure 4a and 4b). We found that: First, before and after DNA damage treatment, the depletion of p53 by shRNA decreased p53 association with these two promoters in U2OS cells. Second, PAD4 and HDAC2 dissociate from these two promoters after DNA damage with a concomitant increase in histone acetylation and histone Arg methylation and a decrease in histone

citrullination. Third, levels of PAD4 promoter association, histone citrullination, and histone Arg17 methylation were decreased after p53 depletion. In contrast, significant amount of HDAC2 associated with these two promoters after p53 depletion. Overall, the dynamics of factor association and histone modifications at GADD45 and PUMA promoters is similar to that of the p21 promoter, suggesting a common theme of p53 target genes regulation by HDAC2 and PAD4.

To further analyze the mechanism of HDAC2 and PAD4 in regulating of p21 expression, we used siRNAs to deplete PAD4, HDAC2, or both in U2OS cells. Consistent with our previously published results (Li *et al.*, 2008) and those by others (Harms and Chen, 2007), singular depletion of PAD4 or HDAC2 increased p21 expression in Western blot analyses (Figure 4c, lane 2 and 3 compared with lane 1). Interestingly, when both PAD4 and HDAC2 were depleted (Figure 4c, lane 4), we did not detect a consistent further increase in the amount of p21 protein compared to the singular depletion of these two proteins. It is likely that the efficacy of complete depletion of both proteins in the same cells was low or that additional factors contribute to the inhibition of p21 expression.

Additionally, we analyzed protein association and histone modifications at the p53 binding site-1 and -2 of the p21 promoter after siRNA treatment of U2OS cells by ChIP (Figure 4d). Strikingly, after the depletion of HDAC2, we found that PAD4 association and histone citrullination were decreased with a concomitant increase in histone Arg methylation and Lys acetylation at both sites (Figure 4d, **green bars**). On the other hand, depletion of PAD4 affected histone Arg methylation and citrullination but not HDAC2 association or histone H3 acetylation (Figure 4d, **red bars**). Above results reveal an interesting crosstalk between histone acetylation and Arg modifications, i.e., a regulatory role of HDAC2 in PAD4 recruitment and function.

To further test the relationship between histone citrullination and deacetylation, we treated U2OS cells with PAD4 and HDAC inhibitors, Cl-amidine and SAHA, respectively. Inhibition of PAD4 by Cl-amidine led to increased binding of p53 at p21, GADD45, and PUMA promoters (Figure 5a and 5b) concomitantly with a decrease of histone H3 citrullination and an increase in histone H3 Arg17 methylation at these gene promoters. SAHA is a general inhibitor of the HDAC family proteins (Gui *et al.*, 2006). Histone H3 acetylation was highly elevated at the p21, GADD45, and PUMA promoters after SAHA treatment (Figure 5a and 5b). Moreover, inhibition of HDAC activity by SAHA not only led to an increase in histone H3 acetylation but also a decrease in histone citrullination and an increase in histone H3 Arg17 methylation at these promoters (Figure 5a and 5b, **bottom four panels**). These results support that histone H3 acetylation regulates histone H3 Arg17 modifications.

To analyze the effects of PAD4 and HDAC inhibition on p21 protein expression, we performed western blot experiments in U2OS cells without or with p53 depletion after Cl-amidine and SAHA treatment. Consistent with previously published results from us (Li *et al.*, 2008) and others (Gui *et al.*, 2006), treatment of with either Cl-amidine or SAHA increased p53 and p21 protein expression (Figure 5c, lanes 2 and 3 compared with lane 1). Interestingly, when both inhibitors were used (Figure 5c, lane 4), the amount of p21 was further elevated compared to the singular inhibitor treatment. Compared with siRNAs, PAD4 and HDAC inhibitors are more effective in elevating histone Arg methylation and Lys acetylation at the p21 promoter as shown by ChIP efficacy of H3 K9/14 acetylation and H3R17 methylation antibodies (Figure 5a compared to that in Figure 4d), which may contribute to a further increased p21 expression. In contrast, after p53 depletion, the basal level of p21 and its expression after PAD4 and HDAC inhibition was dramatically lowered, indicating that the expression of p21 after Cl-amidine and SAHA treatment is largely if not

only dependent on p53. Consistent with the western blot results, we found that SAHA and Cl-amidine increased the expression of p21, GADD45, and PUMA in a p53 dependent manner in quantitative reverse transcription PCR analyses (Supplementary Figure 1). Furthermore, SAHA and Cl-amidine additively increased the expression of these three genes (Supplementary Figure 1).

The further increase of p53 target gene expression after both Cl-amidine and SAHA treatment suggests that a combination of these two inhibitors may more effectively inhibit cancer cell growth. To test this idea, we analyzed the effect of Cl-amidine, SAHA, or a combination of both inhibitors on the growth of U2OS cells. Compared to the control untreated cells, SAHA and Cl-amidine individually inhibited cell growth at 2 and 3 days after treatment (Figure 5d). Consistent with the additive effects of Cl-amidine and SAHA on the expression of p21, GADD45, and PUMA, these two inhibitors cooperatively inhibited U2OS cell growth (Figure 5d). In contrast, the cell growth inhibitory effects of these inhibitors were significantly reduced after p53 depletion in U2OS cells (Growth curves in Figure 5e compared to those in Figure 5d). In particular, after treatment with both inhibitors, U2OS cells grew only ~1.3 fold while U2OS/p53-shRNA cells grew about 2.7 fold at 3 days after treatment. The additive growth inhibitory effect of Cl-amidine, SAHA, or both inhibitors in U2OS cells and U2OS/p53-shRNA cells was further analyzed by phase contrast microscopy (Supplementary Figure 2). Taken together, above results indicate that a combination of PAD4 inhibitor Cl-amidine and HDAC inhibitor SAHA may offer a novel strategy in preventing cancer cell growth by activating the p53 pathway.

DISCUSSION

Post-translational histone modifications are thought to regulate gene expression by facilitating the formation of open chromatin structure or serve as binding platform for additional effector proteins (Jenuwein and Allis, 2001; Kouzarides, 2007). A common theme is emerging that covalent histone modifications often cross talk with each other to govern gene expression (Fischle et al., 2003). We report here that PAD4 and HDAC2 interact with each other, and they are simultaneously recruited to the p21 promoter and serve as corepressors for gene expression. After DNA damage, PAD4 and HDAC2 dissociate with a subsequent increase in histone Arg methylation and histone Lys acetylation. Our data favor a model that a dynamic association of PAD4 and HDAC2 ensures a low level of p21 expression before DNA damage. Histone Arg methylation and Lys acetylation were first shown to cooperatively activate p53-mediated transcription in biochemical analyses (An et al., 2004). Reversely, we found that PAD4 functions as a corepressor to regulate p53 target gene expression by counteracting the function of histone Arg methylation (Li et al., 2008; Yao et al., 2008). Here, our studies at the p21 promoter offered a novel link between histone citrullination and deacetylation. Of particular interest, we found that inhibition of HDAC functions affects PAD4 association, histone citrullination and Arg methylation. On the other hand, HDAC2 association and histone Lys acetylation are not much affected by PAD4 inhibition. The ordered and cooperative function of the histone H3 acetyltransferase p300 and H3 Arg methyltransferase CARM1 was previously reported wherein the action of histone H3 acetylation precedes H3 Arg17 methylation during p53-mediated transcription (An et al., 2004). Our studies of PAD4 and HDAC2 inhibition/depletion in U2OS cells are consistent with this previous report, indicating that increased histone H3 Lys acetylation facilitates histone H3 Arg17 methylation, but not vice versa.

It has been previously reported that the HDAC inhibitor SAHA (suberoylanalide hydroxamic acid) activates p21 expression by increasing histone acetylation at the p21 promoter (Gui et al., 2004). SAHA was recently approved by the US FDA (Food and Drug Administration) for treatment of cutaneous T-cell lymphoma (Marks and Breslow, 2007),

which serves as a harbinger for cancer treatment by targeting epigenetic mechanisms (Minucci and Pelicci, 2006). PAD4 is overexpressed various type of cancers (Chang et al., 2006; Chang et al., 2009), suggesting that PAD4 is a putative drug target for cancer treatment. Our studies showed that PAD4 inhibitor Cl-amidine and HDAC inhibitor SAHA has additive effects in growth inhibition of osteosarcoma U2OS cells, suggesting that simultaneous targeting these two types of enzymes in cancer treatment is worth of future exploration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Identification of HDAC2 as a PAD4 interacting protein

(a) Affinity purification of FLAG-PAD4 together with HDAC2 and p53 using M2 agarose beads. To facilitate the purification of PAD4, we established 293T cells and MCF-7 cells stably expressing FLAG-PAD4. M2 agarose beads were used to affinity purify proteins from 293T/FLAG-PAD4 cells, MCF-7/FLAG-PAD4, or the respective control parental cells following a method previously described (Li et al., 2008). Proteins retained by M2 agarose beads were eluted by FLAG-peptide. Western blot experiments were performed to analyze other proteins affinity-purified with FLAG-PAD4. PAD4 polyclonal antibody (1:2000), p53 (1:2000, Clone BP53-12, Sigma-aldrich), and HDAC2 (1:2000, Clone 3F3, Abcam, Ab51832) were used for Western blot. **(b)** Different percentages of input as indicated in lanes 1-4 were loaded with affinity purified proteins by the M2 beads to evaluate the efficacy of copurification of HDAC2 with FLAG-PAD4. **(c)** GST-HDAC2 but not GST expressed in *E.coli* BL-21 can pull down HA-tagged PAD4 expressed in Cos7 cells. GST pull down experiments were performed as previously described (Li et al., 2008). **(d)** GST-PAD4 but not GST beads pulled down HDAC2. **(e)** Co-immunoprecipitation (Co-IP) of HDAC2 with α -PAD4 rabbit antibody but not normal rabbit IgG. Co-IP was performed as previously described (Li et al., 2008).

Figure 2. Detection of PAD4 and HDAC2 activities and protein-protein interaction domain mapping between PAD4, HDAC2, and p53

(a) GST-PAD4 purified from *E.coli* BL-21 and FLAG-PAD4 purified from 293T cells citrullinated free histone H3 in biochemical analyses *in vitro*. Histone H3 citrullination was detected by a histone H3 citrullination specific antibody (Abcam, Ab5103) in western blot analyses. PAD4 enzymatic assays were performed as previously described (Wang et al., 2004). Coomassie blue staining showed a change in histone H3 behavior during electrophoresis after citrullination similarly as previously described (Wang et al., 2004). (b) GST-PAD4 citrullinated nucleosomes purified from HeLa cells. Concomitant with an increase in histone H3 citrullination, a decrease in histone H3 Arg17 methylation was detected. (c) GST and GST-PAD4 derivative fusion proteins were used to perform pull down using 293T nuclear extracts. Proteins retained by beads were eluted using 10 mM glutathione. HDAC activities purified with various fusion proteins were analyzed using a fluorometric HDAC assay kit (Upstate, #17-356) by following the manufacturer's instructions. Full-length GST-PAD4 and GST-Ig1&2 fusion protein beads retained HDAC activities (lanes 9 and 10). Data presented are the averages of fluorometric values from three experiments with standard deviation within 10% of the average. (d) The amount of GST and GST-PAD4 fusion proteins purified from *E.coli* BL-21 and used in the pull down experiments was shown by Ponceau S staining. (e) GST-PAD4 full length and GST-PAD4 IgL domains 1 and 2 (GST-Ig1&2) were able to retain HDAC2 in GST-pull down assays. (f) The amount of GST and GST-p53 derivative fusion proteins used in pull down experiments was shown by Ponceau S staining. (g) GST-p53 and its N- or C-terminal truncation derivatives interacted with HDAC2, suggesting that HDAC2 interacts with multiple parts of p53. (h) A diagram illustrating the interaction of p53, PAD4, and HDAC2 via different domains (see text for further discussion).

Figure 3. PAD4 and HDAC2 dynamically associate with the p21 promoter during DNA damage response

(a) Illustration of the p21 gene structure and its two p53-binding sites (PBS1 and PBS2). (b) The amount of p53, p21, PAD4, and HDAC2 was analyzed by western blot in U2OS cells (lanes 1 and 2) or U2OS/p53-shRNA cells (lanes 3 and 4) before and after doxorubicin treatment. β -actin was probed as a control to ensure equal loading. (c–d) Chromatin immunoprecipitation (ChIP) experiments were performed to analyze the level of p53, PAD4, HDAC2, histone H3 K9/14 acetylation (H3K9/14Ac), H3 citrullination (H3Cit), or H3 Arg 17 methylation (H3R17Me) in U2OS cells (c) or U2OS/p53-shRNA cells (d) before or after 0.4 μ M doxorubicin treatment for 6 hr at the two p53 binding sites (PBS1 and PBS2) as well as the transcription start site (TSS). ChIP samples were analyzed using real-time PCR as previously described (Li et al., 2008). ChIP signals were calculated as percentages of the input (% IP). ChIP primers are available upon request. Protein A agarose beads were used to measure the background signals. Averages and standard deviations were shown. Throughout this manuscript, ChIP experiments were generally performed from two to three independent experiments of sample preparation and PCR reactions were performed at least 6 times. For those ChIP results with small fold of changes but key to the conclusion, p values were analyzed by student t-test. * denotes $p < 0.02$. (e) Re-ChIP experiments detected simultaneous binding of PAD4 and HDAC2 to the p21 promoter. For re-ChIP analyses, the first step ChIP was performed using the M2 agarose beads to recover FLAG-PAD4 associated chromatin fragments. After elution with 200 μ g/ml FLAG peptide, the second step ChIP was performed using the affinity purified PAD4 antibody or anti-HDAC2 antibody (Upstate, 05–814).

Figure 4. Promoter association of PAD4 and HDAC2 during DNA damage or after depletion of PAD4 or HDAC2

(a–b) ChIP experiments were performed to analyze the level of p53, PAD4, HDAC2, histone H3 K9/K14 acetylation (H3K9/14Ac), H3 citrullination (H3Cit), or H3 Arg 17 methylation (H3R17Me) in U2OS cells or U2OS/p53-shRNA cells before or 6 hr after 0.4 μ M doxorubicin treatment at the p53 binding site of GADD45 **(a)** or PUMA **(b)**. ChIP signals were calculated as percentages of the input (% IP). Protein A agarose beads were used to measure the background signals. Averages and standard deviations shown were measured (n=6). p values were analyzed by student t-test. * denotes p<0.02. **(c)** The effects of PAD4 and/or HDAC2 depletion on the expression of p21 in U2OS cells were analyzed by western blot assays. β -actin was probed as a control to ensure equal loading. **(d)** ChIP experiments were performed to analyze the level of p53, PAD4, HDAC2, histone H3 K9/K14 acetylation (H3K9/14Ac), H3 citrullination (H3Cit), or H3 Arg 17 methylation (H3R17Me) in U2OS cells after the depletion of HDAC2 and/or PAD4 at the p21 promoter. ChIP signals were calculated as percentages of the input (% IP). Averages and standard deviations were shown (n=6). Note the depletion of HDAC2 decreased PAD4 binding and histone H3 citrullination with a simultaneous increase of histone H3 Lys9/14 acetylation and H3 Arg17 methylation.

Figure 5. Inhibition of PAD4 and/or HDACs impacts on histone modifications at p53 target gene promoters and cancer cell growth in a p53-dependent manner
(a–b) ChIP experiments were performed to analyze the level of p53, PAD4, HDAC2, histone H3 K9/K14 acetylation (H3K9/14Ac), H3 citrullination (H3Cit), or H3 Arg 17 methylation (H3R17Me) in U2OS cells before and after treatment with the PAD4 inhibitor Cl-amidine (200 μ M) and/or the HDAC inhibitor SAHA (0.4 μ M) at the p53 binding sites of p21 **(a)**, GADD45 **(b)**, and PUMA **(b)**. ChIP signals were calculated as percentages of the input (% IP). Averages and standard deviations were shown (n=6). Cl-amidine was prepared via a solution-phase strategy following reported procedures (Causey and Thompson, 2008). The final compound was purified by silica gel flash chromatography. The structure and purity (>95%) was characterized by $^1\text{H-NMR}$ and mass spectrometry. **(c)** The effect of PAD4 and/or HDAC2 inhibition on the expression of p21 in U2OS cells and U2OS/p53-shRNA cells was analyzed by western blot assays. β -actin was probed as a control to ensure equal loading. **(d)** The growth inhibitory effect of the PAD4 inhibitor Cl-amidine (200 μ M), the HDAC inhibitor SAHA (0.4 μ M), or both inhibitors was analyzed in U2OS cells. **(e)** The growth inhibitory effect of the PAD4 inhibitor Cl-amidine (200 μ M), the HDAC inhibitor SAHA (0.4 μ M), or both inhibitors was analyzed in U2OS/p53-shRNA cells. To obtain cell growth curve, 60,000 U2OS cells or U2OS/p53-shRNA cells were seeded in each well of the 6-well plate. Cl-amidine and SAHA were used at 200 μ M and 0.4 μ M, respectively, to treat cells singularly or in combination. The cell numbers were counted at the time points indicated after inhibitor treatment using a hemocytometer at time points indicated. The fold of cell number increase and standard deviations at each time point were obtained from three independent experiments.