Oncogene (2010) 29, 3153–3162
© 2010 Macmillan Publishers Limited All rights reserved 0950-9232/10 $32.00
www.nature.com/onc

SHORT COMMUNICATION

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

P Li[1], D Wang[1], H Yao[1,6], P Doret[1], G Hao[2], Q Shen[1], H Qiu[3], X Zhang[4], Y Wang[3], G Chen[5] and Y Wang[1]

[1]*Department of Biochemistry and Molecular Biology, Center for Eukaryotic Gene Regulation, Pennsylvania State University, University Park, PA, USA;* [2]*Department of Pharmacology, Weill Medical College of Cornell University, New York, NY, USA;* [3]*Department of Chemistry-027, University of California, Riverside, CA, USA;* [4]*Department of Pathology and Cell Biology, University of South Florida College of Medicine, Tampa, FL, USA and* [5]*Department of Chemistry, Pennsylvania State University, University Park, PA, USA*

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 histone deacetylase (HDAC2) and PAD4 interact with p53 through 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 (for example, 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 suberoylanilide hydroxamic acid show 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.
Oncogene (2010) **29,** 3153–3162; doi:10.1038/onc.2010.51; published online 1 March 2010

Keywords: histone citrullination; histone deacetylation; PAD4; HDAC2; p53

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 (Luger *et al.*, 1997; Kornberg and Lorch, 1999). Various posttranslational modifications, such as methylation, acetylation, phosphorylation, ubiquitination, and citrullination, are believed to have a significant role in chromatin activities, such as transcription (Shilatifard, 2006; Barski *et al.*, 2007; Berger, 2007; Klose and Zhang, 2007; Li *et al.*, 2007). In light of the biological significance of histone modifications in cellular activities, a ‘histone code’ hypothesis was proposed (Strahl and Allis, 2000; Turner, 2000; Jenuwein and Allis, 2001), 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 through crosstalks (Fischle *et al.*, 2003). For example, histone acetyltransferases catalyze histone acetylation, whereas histone deacetylases (HDACs) remove the pre-existing acetyl groups. Histone Arg methylation is catalyzed by protein arginine methyltransferases (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 demethylimination (Wang *et al.*, 2004).

The tumor suppressor protein p53 functions at the center of an extremely complicated signaling network in human cells (Vogelstein *et al.*, 2000; Laptenko and Prives, 2006). 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

Correspondence: Dr Y Wang, Department of Biochemistry and Molecular Biology, Center for Eukaryotic Gene Regulation, Pennsylvania State University, 454 North Frear, University Park, PA 16802, USA.
E-mail: yuw12@psu.edu
[6]Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA.
Received 28 April 2009; revised 27 December 2009; accepted 25 January 2010; published online 1 March 2010

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 (Vogelstein *et al*., 2000; Harris and Levine, 2005). 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 has 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 histone acetyltransferases have been found as p53 coactivators (Barlev *et al*., 2001; Espinosa and Emerson, 2001), whereas HDACs function as p53 corepressors by counteracting the function of histone acetyltransferases (Luo *et al*., 2000; Dannenberg *et al*., 2005; Harms and Chen, 2007). 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

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 an earlier 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 whether 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).

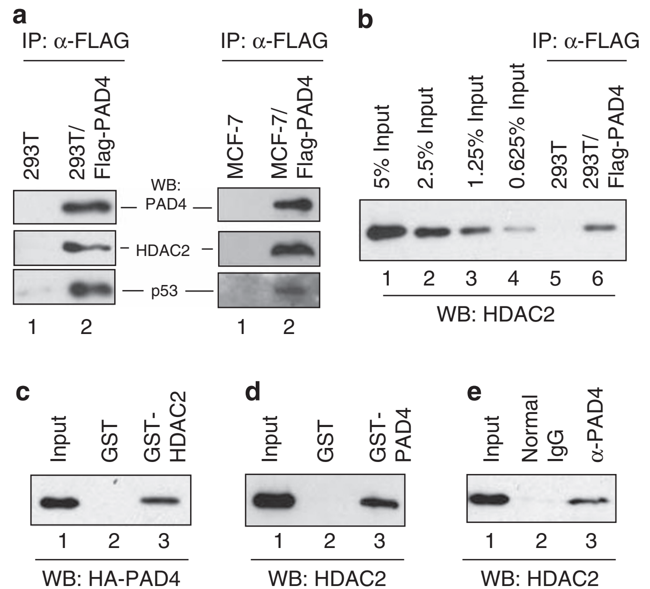


Figure 1 Identification of HDAC2 as a PAD4 interacting protein. (**a**) Affinity purification of FLAG-PAD4 together with HDAC2 and p53 using M2 agarose beads. To facilitate the purification of PAD4, we established 293T cells and MCF-7 cells stably expressing FLAG-PAD4. M2 agarose beads were used to affinity purify proteins from 293T/FLAG-PAD4 cells, MCF-7/FLAG-PAD4 cells, or the respective control parental cells following a method described earlier (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, St Louis, MO, USA), and HDAC2 (1:2000, Clone 3F3, Abcam, Cambridge, MA, USA, 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 described earlier (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 described earlier (Li *et al*., 2008).

The interaction of PAD4 and HDAC2 prompted us to test whether histone deacetylase and histone citrullination activities associate with each other. First, GST-PAD4 purified from *Escherichia 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 showed earlier that PAD4 demethyliminates free histone H3 in *in vitro* biochemical assays (Wang *et al*., 2004). However, the demethylimination activity has not been tested using nucleosomal substrate *in vitro*. Here, we further tested GST-PAD4 activity using oligo nucleosomes purified form Hela cells as substrates. Concomitant with an increase in histone citrullination, a decrease of histone H3 Arg17 methylation was detected in a GST-PAD4

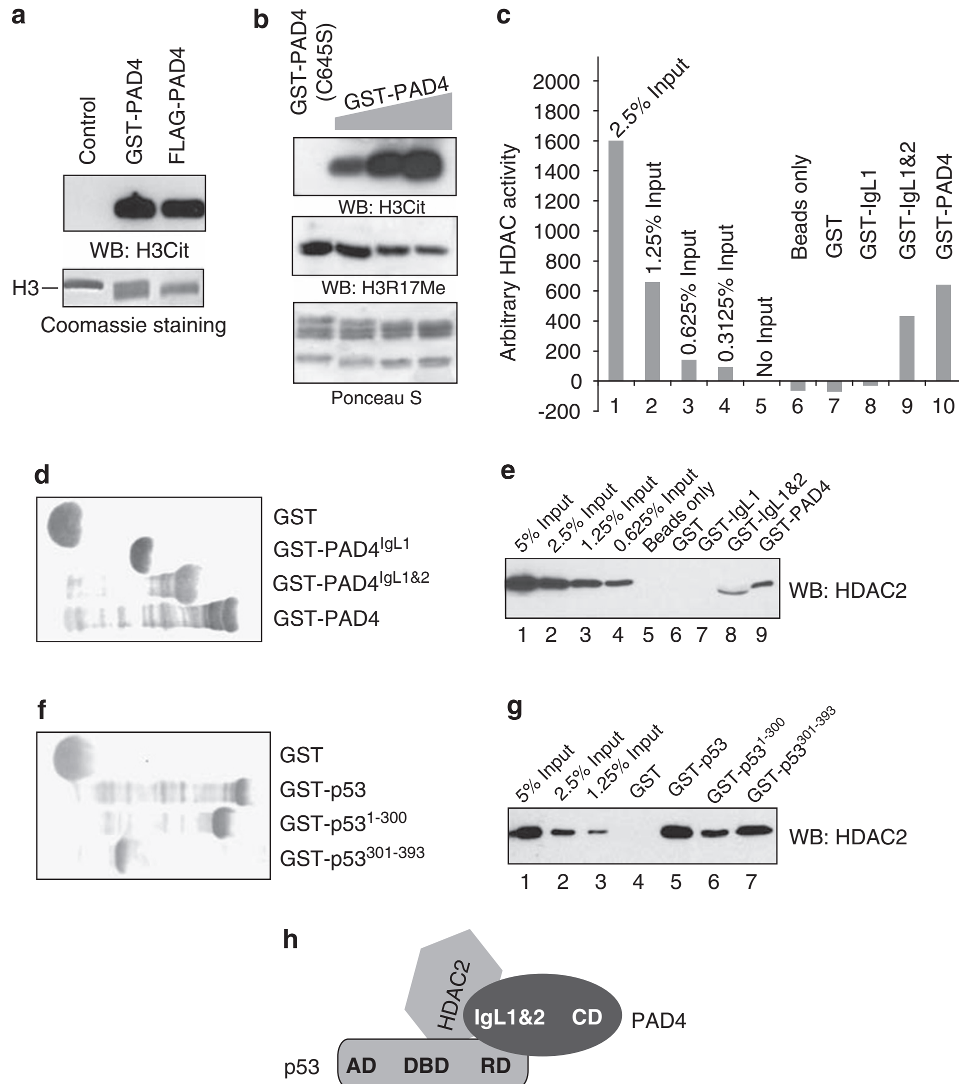


Figure 2 Detection of PAD4 and HDAC2 activities and protein–protein interaction domain mapping between PAD4, HDAC2, and p53. (**a**) GST-PAD4 purified from *E. coli* BL-21 and FLAG-PAD4 purified from 293T cells citrullinated free histone H3 in biochemical analyses *in vitro*. Histone H3 citrullination was detected by a histone H3 citrullination specific antibody (Abcam, Ab5103) in western blot analyses. PAD4 enzymatic assays were performed as described earlier (Wang *et al*., 2004). Coomassie blue staining showed a change in histone H3 behavior during electrophoresis after citrullination similarly as described earlier (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, Billerica, MA, USA, #17–356) by following the manufacturer's instructions. Full-length GST-PAD4 and GST-IgL1&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-IgL1&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 through different domains (see text for further discussion).

dose-dependent manner (Figure 2b), indicating the demethylimination activity of PAD4. As GST-PAD4 pulled down HDAC2, we further tested whether 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 through 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 (IgL)-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 IgL-like domains of PAD4 interact with the C-terminal regulatory domain of p53 (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 IgL-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 IgL-like domains of PAD4 interact with the C-terminal regulatory domain of p53 (Li *et al*., 2008) and HDAC2 (Figure 2e), whereas p53 and HADC2 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 through 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 h 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 h. 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 in HDAC2 association correlates to an increase in 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, whereas 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 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 whether 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 efficient 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, whereas the PAD4 antibody was used as a positive control. The HDAC2 antibody was able to re-ChIP the p21 promoter, whereas 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 found earlier 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 (Figures 4a and b). 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.

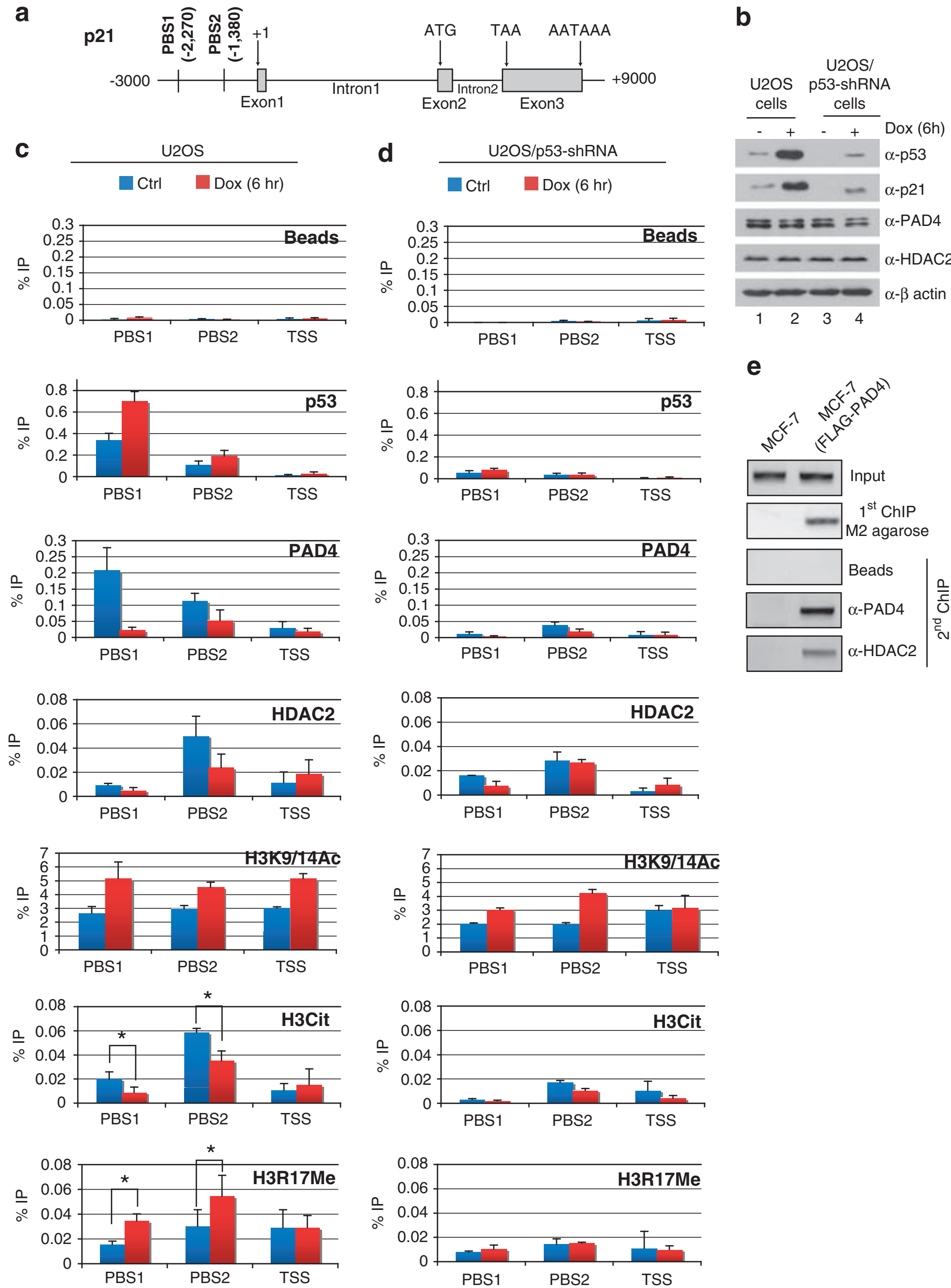
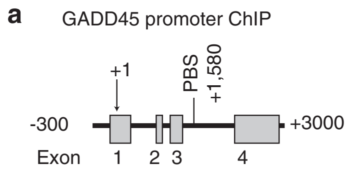
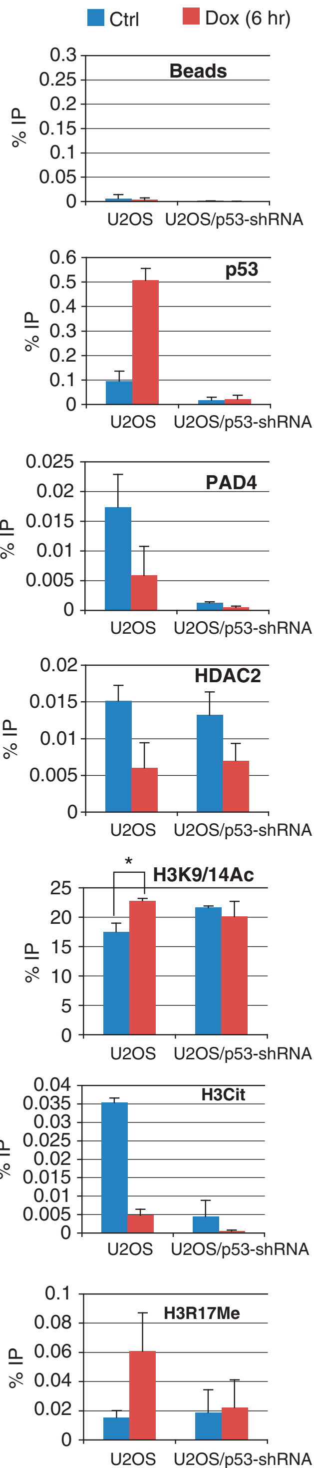


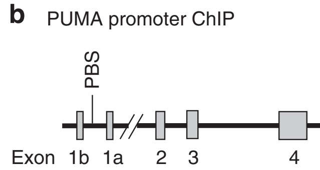
Figure 3 PAD4 and HDAC2 dynamically associate with the p21 promoter during DNA damage response. (**a**) Illustration of the p21 gene structure and its two p53-binding sites (PBS1 and PBS2). (**b**) The amount of p53, p21, PAD4, and HDAC2 was analyzed by western blot in U2OS cells (lanes 1 and 2) or U2OS/p53-shRNA cells (lanes 3 and 4) before and after doxorubicin treatment. β-actin was probed as a control to ensure equal loading. (**c**, **d**) 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 μM doxorubicin treatment for 6 h 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 described earlier (Li *et al*., 2008). ChIP signals were calculated as percentages of the input (% IP). ChIP primers are available on 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 six times. For those ChIP results with small fold of changes but key to the conclusion, *P* values were analyzed by Student's *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 μg/ml FLAG peptide, the second step ChIP was performed using the affinity purified PAD4 antibody or anti-HDAC2 antibody (Upstate, 05-814).

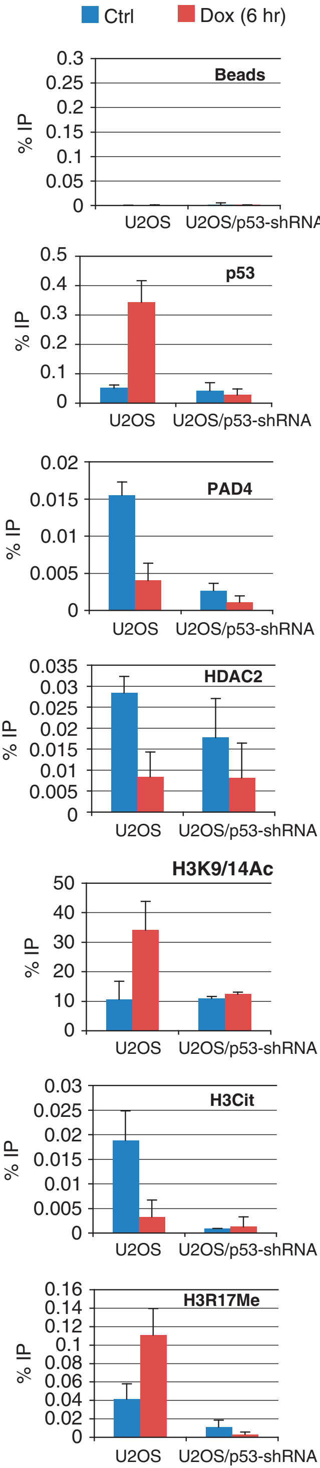
a GADD45 promoter ChIP



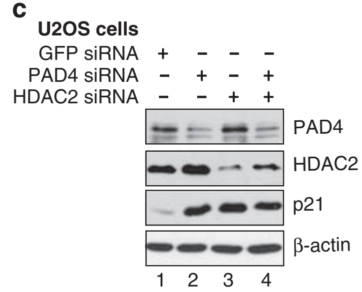


b PUMA promoter ChIP

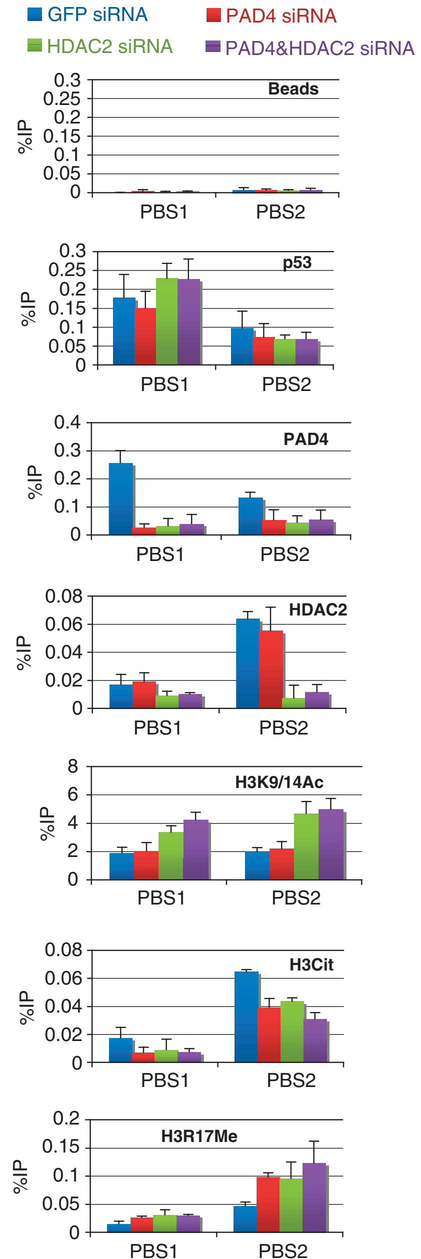




c



d p21 promoter ChIP in U2OS cells



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, lanes 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 with 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, that is, 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 suberoylanalide hydroxamic acid (SAHA), respectively. Inhibition of PAD4 by Cl-amidine led to increased binding of p53 at p21, GADD45, and PUMA promoters (Figures 5a and b) concomitantly with a decrease in 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*., 2004). Histone H3 acetylation was highly elevated at the p21, GADD45, and PUMA promoters after SAHA treatment (Figures 5a and b). 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 (Figures 5a and b, 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*., 2004), treatment 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 with 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 with 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 in 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 with 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 with those in Figure 5d). In particular, after treatment with both inhibitors, U2OS cells grew only $\sim$1.3-fold whereas 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

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 Arg17 methylation (H3R17Me) in U2OS cells or U2OS/p53-shRNA cells before or 6 h after 0.4 μM 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 were shown ($n=6$). *P*-values were analyzed by Student's *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 in histone H3 Lys9/14 acetylation and H3 Arg17 methylation.

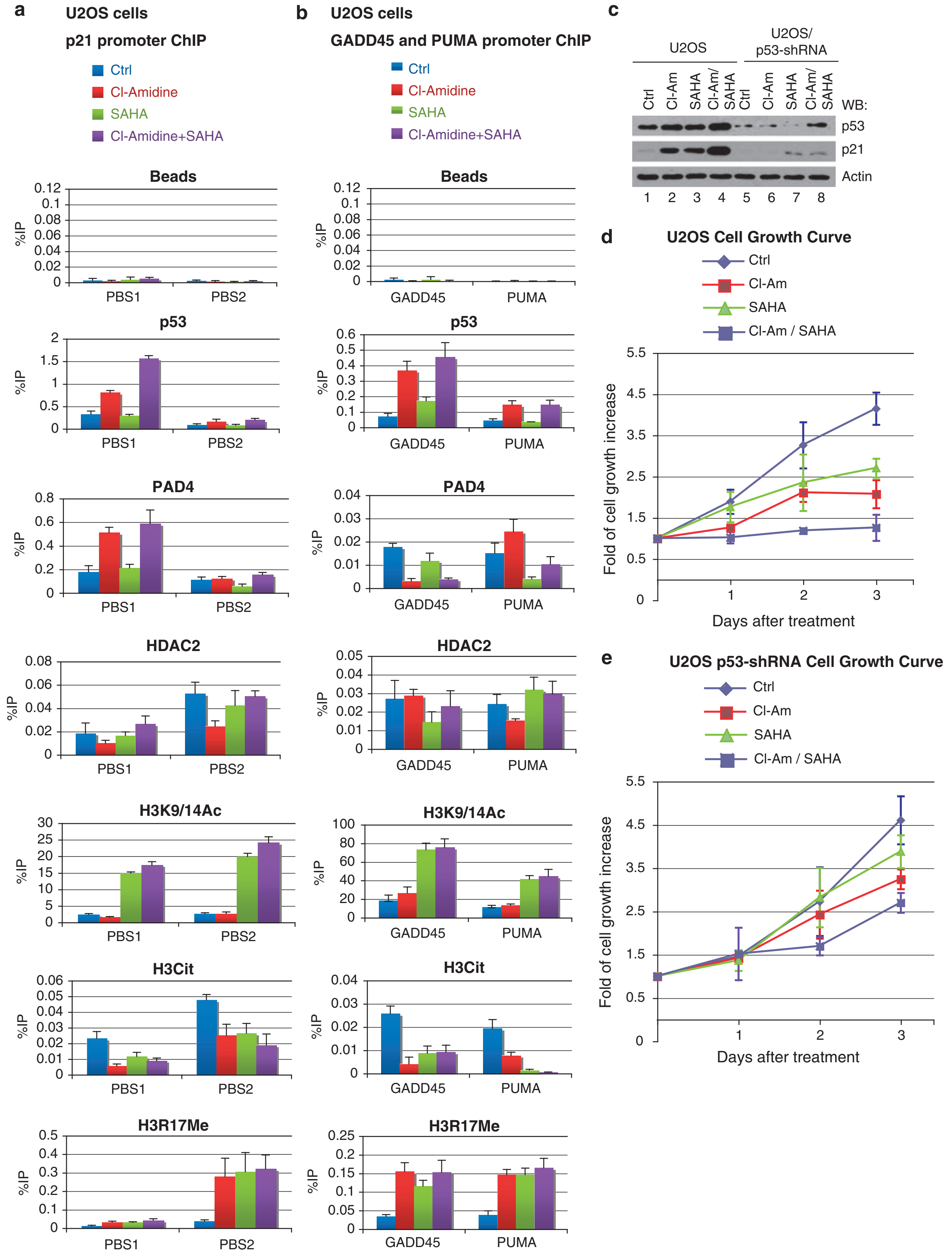

a
U2OS cells
p21 promoter ChIP
Ctrl
Cl-Amidine
SAHA
Cl-Amidine+SAHA
Beads
p53
PAD4
HDAC2
H3K9/14Ac
H3Cit
H3R17Me
%IP
PBS1
PBS2
b
U2OS cells
GADD45 and PUMA promoter ChIP
GADD45
PUMA
c
U2OS
U2OS/
p53-shRNA
Ctrl
Cl-Am
SAHA
Cl-Am/
SAHA
WB:
p53
p21
Actin
1 2 3 4 5 6 7 8
d
U2OS Cell Growth Curve
Ctrl
Cl-Am
SAHA
Cl-Am / SAHA
Fold of cell growth increase
Days after treatment
e
U2OS p53-shRNA Cell Growth Curve

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 μM) and/or the HDAC inhibitor SAHA (0.4 μM) 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 through 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}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 μM), the HDAC inhibitor SAHA (0.4 μM), or both inhibitors was analyzed in U2OS cells. (**e**) The growth inhibitory effect of the PAD4 inhibitor Cl-amidine (200 μM), the HDAC inhibitor SAHA (0.4 μM), 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 six-well plate. Cl-amidine and SAHA were used at 200 and 0.4 μM, respectively, to treat cells singularly or in combination. The cell numbers were counted at the time points indicated after inhibitor treatment using a hemocytometer. The fold of cell number increase and standard deviations at each time point were obtained from three independent experiments.

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 crosstalk 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 reported earlier 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 reported earlier that the HDAC inhibitor SAHA activates p21 expression by increasing histone acetylation at the p21 promoter (Gui *et al*., 2004). SAHA was recently approved by the US 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 in various type of cancers (Chang and Han, 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 have 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.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We are grateful to Drs JC Reese, DS Gilmour, BF Pugh, and S Tan for discussions and helpful comments. Research is supported in part by a PSU start-up fund and NIH Grants R01 CA136856 to YW (PSU) and R01 CA116522 to YW (UC Riverside).

References

An W, Kim J, Roeder RG. (2004). Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53. *Cell* **117**: 735–748.

Arita K, Hashimoto H, Shimizu T, Nakashima K, Yamada M, Sato M. (2004). Structural basis for Ca(2+)-induced activation of human PAD4. *Nat Struct Mol Biol* **11**: 777–783.

Baek HJ, Malik S, Qin J, Roeder RG. (2002). Requirement of TRAP/mediator for both activator-independent and activator-dependent transcription in conjunction with TFIID-associated TAF(II)s. *Mol Cell Biol* **22**: 2842–2852.

Barlev NA, Liu L, Chehab NH, Mansfield K, Harris KG, Halazonetis TD *et al*. (2001). Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. *Mol Cell* **8**: 1243–1254.

Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z *et al*. (2007). High-resolution profiling of histone methylations in the human genome. *Cell* **129**: 823–837.

Bedford MT, Clarke SG. (2009). Protein arginine methylation in mammals: who, what, and why. *Mol Cell* **33**: 1–13.

Berger SL. (2007). The complex language of chromatin regulation during transcription. *Nature* **447**: 407–412.

Causey CP, Thompson PR. (2008). An improved synthesis of haloaceteamidine-based inactivators of protein arginine deiminase 4 (PAD4). *Tetrahedron Lett* **7**: 4383–4385.

Chang X, Han J. (2006). Expression of peptidylarginine deiminase type 4 (PAD4) in various tumors. *Mol Carcinog* **45**: 183–196.

Chang X, Han J, Pang L, Zhao Y, Yang Y, Shen Z. (2009). Increased PADI4 expression in blood and tissues of patients with malignant tumors. *BMC Cancer* **9**: 40.

Cuthbert GL, Daujat S, Snowden AW, Erdjument-Bromage H, Hagiwara T, Yamada M *et al*. (2004). Histone deimination antagonizes arginine methylation. *Cell* **118**: 545–553.

Dannenberg JH, David G, Zhong S, van der Torre J, Wong WH, Depinho RA. (2005). mSin3A corepressor regulates diverse transcriptional networks governing normal and neoplastic growth and survival. *Genes Dev* **19**: 1581–1595.

Espinosa JM, Emerson BM. (2001). Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. *Mol Cell* **8**: 57–69.

Fischle W, Wang Y, Allis CD. (2003). Histone and chromatin cross-talk. *Curr Opin Cell Biol* **15**: 172–183.

Gui CY, Ngo L, Xu WS, Richon VM, Marks PA. (2004). Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. *Proc Natl Acad Sci USA* **101**: 1241–1246.

Harms KL, Chen X. (2007). Histone deacetylase 2 modulates p53 transcriptional activities through regulation of p53-DNA binding activity. *Cancer Res* **67**: 3145–3152.

Harris SL, Levine AJ. (2005). The p53 pathway: positive and negative feedback loops. *Oncogene* **24**: 2899–2908.

Ho J, Benchimol S. (2003). Transcriptional repression mediated by the p53 tumour suppressor. *Cell Death Differ* **10**: 404–408.

Jenuwein T, Allis CD. (2001). Translating the histone code. *Science* **293**: 1074–1080.

Kitayner M, Rozenberg H, Kessler N, Rabinovich D, Shaulov L, Haran TE *et al*. (2006). Structural basis of DNA recognition by p53 tetramers. *Mol Cell* **22**: 741–753.

Klose RJ, Zhang Y. (2007). Regulation of histone methylation by demethylimination and demethylation. *Nat Rev Mol Cell Biol* **8**: 307–318.

Kornberg RD, Lorch Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98**: 285–294.

Kouzarides T. (2007). Chromatin modifications and their function. *Cell* **128**: 693–705.

Laptenko O, Prives C. (2006). Transcriptional regulation by p53: one protein, many possibilities. *Cell Death Differ* **13**: 951–961.

Levesque AA, Eastman A. (2007). p53-based cancer therapies: Is defective p53 the Achilles heel of the tumor? *Carcinogenesis* **28**: 13–20.

Li B, Carey M, Workman JL. (2007). The role of chromatin during transcription. *Cell* **128**: 707–719.

Li P, Yao H, Zhang Z, Li M, Luo Y, Thompson PR *et al*. (2008). Regulation of p53 target gene expression by peptidylarginine deiminase 4. *Mol Cell Biol* **28**: 4745–4758.

Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**: 251–260.

Luo J, Su F, Chen D, Shiloh A, Gu W. (2000). Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature* **408**: 377–381.

Marks PA, Breslow R. (2007). Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. *Nat Biotechnol* **25**: 84–90.

Minucci S, Pelicci PG. (2006). Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nat Rev Cancer* **6**: 38–51.

Shi Y, Downes M, Xie W, Kao HY, Ordentlich P, Tsai CC *et al*. (2001). Sharp, an inducible cofactor that integrates nuclear receptor repression and activation. *Genes Dev* **15**: 1140–1151.

Shilatifard A. (2006). Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. *Annu Rev Biochem* **75**: 243–269.

Strahl BD, Allis CD. (2000). The language of covalent histone modifications. *Nature* **403**: 41–45.

Turner BM. (2000). Histone acetylation and an epigenetic code. *Bioessays* **22**: 836–845.

Vogelstein B, Lane D, Levine AJ. (2000). Surfing the p53 network. *Nature* **408**: 307–310.

Wang Y, Wysocka J, Sayegh J, Lee YH, Perlin JR, Leonelli L *et al*. (2004). Human PAD4 regulates histone arginine methylation levels via demethylimination. *Science* **306**: 279–283.

Yao H, Li P, Venters BJ, Zheng S, Thompson PR, Pugh BF *et al*. (2008). Histone Arg modifications and p53 regulate the expression of OKL38, a mediator of apoptosis. *J Biol Chem* **283**: 20060–20068.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)