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Methylation-Acetylation Interplay Activates p53 in Response to DNA Damage▽

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p53, an important tumor suppressor protein, exerts its function mostly as a sequence-specific transcription factor and is subjected to multiple posttranslational modifications in response to genotoxic stress. Recently, we discovered that lysine methylation of p53 at K372 by Set7/9 (also known as SET7 and Set9) is important for transcriptional activation and stabilization of p53. In this report we provide a molecular mechanism for the effect of p53 methylation on transcription. We demonstrate that Set7/9 activity toward p53, but not the nucleosomal histones, is modulated by DNA damage. Significantly, we show that lysine methylation of p53 is important for its subsequent acetylation, resulting in stabilization of the p53 protein. These p53 modification events can be observed on the promoter of p21 gene, a known transcriptional target of p53. Finally, we show that methylation-acetylation interplay in p53 augments acetylation of histone H4 in the promoter of p21 gene, resulting in its subsequent transcriptional activation and, hence, cell cycle arrest. Collectively, these results suggest that the cross talk between lysine methylation and acetylation is critical for p53 activation in response to DNA damage and that Set7/9 may play an important role in tumor suppression.

The product of the TP53 gene (p53) is an important tumor suppressor mutated in more than half of all human cancers (19, 20). In addition, p53 regulates the response of cells to DNA-damaging agents, including those commonly used for cancer therapy (28, 32). In response to genotoxic stress, p53 exerts its function mostly as a sequence-specific transcription factor by activating expression of its downstream transcription targets, the products of which are involved in induction of cell cycle arrest, apoptosis, and DNA repair (24, 35). Accordingly, p53 activates transcription of the p21/WAF1/CIP gene that encodes an inhibitor of cyclin-dependent kinases (15) and to a significant extent accounts for the ability of p53 to induce cell cycle arrest in response to DNA damage (33). In addition to cell cycle arrest, p53 can also facilitate apoptosis by transactivating a number of proapoptotic genes, including Bax, a proapoptotic Bcl-2 family member (37); PUMA, a "BH3-only" member of the Bcl-2 family that induces Bax-dependent cytochrome *c* release (57); Killer/DR5 (49); and a group of genes (PIGs) acting during apoptosis (43) (for the full list of p53-regulated genes, see reference 62).

p53 is regulated mostly at the posttranslational level. Both the amino and carboxy termini of p53 are subjected to multiple posttranslational modifications (1, 59). These modifications contribute to the stabilization and activation of p53. Phosphorylation of serines 15 and 20 at the N terminus of p53 attenuates its interaction with MDM2, which targets p53 for ubiquitin-mediated degradation. The carboxyl terminus of p53 is subjected to acetylation, ubiquitination, sumoylation, neddylation, and phosphorylation. Acetylation of the C terminus was shown to protect p53 from ubiquitination. Moreover, acetylation of p53 at lysines 373 and 382 stimulates its DNA-binding activity in vivo (18, 34) and promotes its association with p300/CBP (2, 39). Recently, acetylation of p53 was shown to be important for its ability to block cell cycle progression in G_2 phase. This block is apparently achieved through NF-Y-p53-dependent repression of the G_2-responsive genes (3, 23).

p53 was also shown to be sumoylated at lysine 386, although the exact role of this modification in the regulation of p53 is not yet clear (17, 27). The role of phosphorylation in the C terminus of p53 is less well defined. Dephosphorylation of constitutively modified serine 376 and phosphorylation of serine 378 apparently promotes binding of the 14-3-3 protein, which serves as a chaperone to many phosphorylated proteins (56). Phosphorylation at serine 392 was shown to positively regulate DNA binding and tetramerization of p53 in vitro (22, 46). Taken together, these data suggest very complex regulatory mechanisms. However, there is growing evidence that different posttranslational modifications may affect each other. For example, phosphorylation of serines 33 and 37 in the amino terminus of p53 promotes acetylation at lysine 320 in the carboxyl terminus (45). Similarly, phosphorylation of serines 6, 9, 15, and threonine 18 enhances the acetylation of

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p53 mediated by p300/CBP (44). Collectively, these results suggest that the assortment of modifications appearing on the p53 molecule is nonrandom and may occur in an ordered fashion.

Set7/9 was originally identified as a lysine-specific histone methyltransferase that targets lysine 4 in histone H3 (H3-K4) (41, 55). Although Set7/9 was unable to modify nucleosomes, it was highly active on free histone H3. H3-K4 methylation was shown to enhance K9 and K14 acetylation by precluding K9 methylation and hence potentiating serine 10 (S10) phosphorylation (41, 61). Recently, Set7/9 was also reported to methylate nonhistone substrates, such as p53, TAF10, RPL29a, and others (12, 26, 38). Set7/9-dependent methylation of p53 on K372 resulted in stabilization and transcriptional activation of the latter (12). Importantly, the carboxyl terminus of p53 undergoes yet another lysine methylation on the K370 residue (21). This methylation is mediated by SMYD2 (SET/MYND Domain-2), which in vitro was also shown to methylate K36 of histone H3 (8). Methylation of p53 on K370 prevents its binding to DNA. In response to DNA damage, K370 methylation is inhibited by Set7/9-dependent K372 methylation (21). These findings suggest that lysine methylation is a dynamic posttranslational modification involved in complex regulation of p53 activity.

To investigate the molecular mechanism of p53 regulation by Set7/9, we analyzed the role of Set7/9 in methylation of nucleosomal histone H3 and p53 both in vitro and in vivo. We found that the lysine methylation activity of Set7/9 was enhanced by DNA damage. Using Set7/9-specific small hairpin RNA (shRNA)-expressing cells we demonstrated that the lack of functional Set7/9 did not affect H3-K4 methylation but specifically attenuated p53 K372 methylation. Moreover, in the absence of p53-lysine methylation, DNA damage-induced acetylation of p53 was also impaired. Thus, the role of p53 methylation at K372 is, at least in part, to stimulate its subsequent acetylation, thus enhancing the stability and activity of p53, which ultimately results in transcriptional upregulation of the p21/WAF/CIP gene and cell cycle arrest.

MATERIALS AND METHODS

Cell cultures and DNA manipulations. U2-OS, a human osteosarcoma cell line (ATCC HTB-96); HEK293, a human embryonic renal epithelial cell line transformed with Ad5 (ATCC CRL-1573); and H1299 cells, a human lung carcinoma cell line lacking endogenous p53 (ATCC CRL-5803) were cultured in Dulbecco modified minimal essential medium supplemented with 10% fetal bovine serum, and 100 nM glutamine and penicillin-streptomycin (all from Gibco) in a humidified atmosphere with 5% CO_2. U2-OS cells stably expressing Flag-His_6-Set7/9 wild type, H297A mutant, and shRNA-Set7/9 were generated as described previously (12). Control U2-OS cell line was generated by stable infection of the cells with nonrelevant shRNA as described earlier (54). The shRNA-resistant Flag-His_6-Set7/9 construct was generated by site-directed mutagenesis using the oligonucleotide 5-GCG GTG CAA GGG CAT TTA GAC GAT GGC CTG CCG CAC GGG TTC TGC-3.

Transient transfections into H1299 cells were performed using DOSPER liposomal transfection reagent (Roche Diagnostics) and 10 μg of DNA containing either pCDNA3-p53 wild type or pCDNA3-p53/K372R mutant. After transfection, the cells were treated with 0.5 μM adriamycin (Adr) and harvested 12 h later.

Cell cycle analysis. For cell cycle analysis, cells were treated with 10 Gy of gamma irradiation, 2 μM Adr, or 300 nM 5-fluorouracil for the indicated periods of time, harvested, washed, and fixed with 70% ethanol. The cells were then treated with RNase and stained with propidium iodine, followed by flow cytometry analysis of DNA content using CellQuest and ModFit software essentially as described earlier (2).

Ubiquitination in vivo. H1299 cells lacking endogenous p53 were transiently cotransfected with PCDNA3—FLAG-His_6-Ubiquitin and either PCDNA3-p53wt or PCDNA3—p53/K372R in the presence or absence of PCDNA3–FLAG-MDM2 (obtained from A. Kinev, University of North Carolina), using DOSPER liposomal transfection reagent (Roche Applied Science). At 24 h after a change of media, a proteosomal inhibitor MG132 (2 μM) was added to cells for additional 12 h to reduce degradation of the ubiquitin-conjugated p53 protein. Cells were then harvested, lysed with 8 M urea (10 mM Tris-HCl, 100 mM Na H_2PO_4, 20 mM imidazole; pH 8.0), and sonicated, and whole-cell extracts were incubated with Ni-NTA agarose beads (QIAGEN) for 2 h at 4°C. Bead-bound materials were washed three times with 8 M urea (pH 8.0), followed by a wash with 8 M urea (pH 6.3), and then eluted from beads by 5 min of boiling in the Laemmli sodium dodecyl sulfate (SDS) sample buffer. The eluates were collected and analyzed by Western blotting with α-p53 (AB-6; Oncogene) mouse monoclonal antibodies.

Analysis of Set7/9 activity. U2-OS cells stably expressing Flag-His_6-Set7/9 were treated with 0.5 μM Adr for the indicated time periods before harvesting. Nuclear extracts were then prepared as described earlier (13) in the presence of phosphatase inhibitor cocktail. Then, 2-mg portions of nuclear extracts were subjected to immunoprecipitation with M-2 monoclonal antibodies conjugated to agarose beads (Sigma). After washes, 15 μl of Flag-Set7/9 beads were subsequently used in methylation assay. Portions (2 μg) of purified recombinant GST-$p53_{300\text{-}393}$, GST-histone $H3_{1\text{-}27}$K4R, and purified nucleosomes (a gift from G. Schnitzler, Tufts University) were used as substrates for Set7/9-dependent methylation. To assess the effect of phosphorylation on Set7/9 activity, Flag-Set7/9-containing beads were treated or mock treated (processed without the enzyme) with 40 U of alkaline phosphatase for 1 h at 37°C. After washes with methylation reaction buffer (50 mM NaCl, 50 mM Tris [pH 8.3], 10% glycerol, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), Set7/9 beads were incubated with substrates in the presence of 1.5 μl of *S*[^{3}H]adenosylmethionine ([^{3}H]SAM) (Amersham).

Analysis of chromatin for monomethylation of K4-H3. Control and shRNA-Set7/9 U2-OS cells were treated with 0.5 μM Adr and collected at the indicated time points. The nuclear pellet fraction was purified as described previously (12). The salt concentration was adjusted to 500 mM NaCl by dilution with buffer B, and 7 μg of the each fraction was analyzed by Western blotting using anti-monomethyl K4 H3 antibodies (Abcam) at a dilution of 1:1,000.

Immunoprecipitation of methylated p53. Various derivatives of U2-OS cells were harvested at the indicated times after treatment with Adr, and whole-cell extracts were then prepared. Typically, 1.5 mg of whole-cell extract was incubated with 5 μg of antigen-purified rabbit polyclonal anti-methyl-K372 p53 antibodies (12) at 4°C for 16 h. Protein A-agarose beads (Repligen) were added to the sample, followed by incubation for an additional 4 h at 4°C. Agarose beads were collected by centrifugation at 12,000 × *g* for 1 min, washed three times with wash buffer (0.5% Tween 20, 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA), resuspended in 30 μl of lysis buffer (100 mM $Na_2H_2PO_4$, 150 mM NaCl, 2 mM EDTA, 5 mM dithiothreitol, 1% Triton X-100, 3% SDS, 1 mM sodium orthovanadate and 150 mM β-mercaptoethanol), and then incubated for 30 min at room temperature to release methyl-K372-p53 protein from the antibody and protein A-beads. Alternatively, methylated p53 was released from the antibody by incubation with an excess amount of the competitor methyl-p53 peptide (Abcam). Samples were then separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with p53-specific antibody (Ab-6; Oncogene). The acetylation status of p53 in vivo was tested by Western blotting with either polyclonal acetyl-382 p53 antibodies (Cell Signaling) or polyclonal acetyl-373/382 p53 antibodies (Santa Cruz).

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (12). Briefly, the cross-linked extracts made out of 5×10^6 to 10×10^6 cells were used in ChIP. The following antibodies were used in the assay: 2 μg of polyclonal antigen-purified anti-methylated p53, 1 μg of polyclonal anti-p53 and anti-acetylated K373/382 p53 (Santa Cruz), 2 μg of polyclonal anti-acetylated H4 (Upstate), and 2 μg of polyclonal anti-K4H3-Me1 (Abcam). After purification, immunoprecipitated DNAs were analyzed by quantitative PCR (MJR, DNA Engine Opticon 2) using p21- and GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-specific primers in the presence of CyberGreen dye (ABI). The plotted values represent the ratio of p21 to GAPDH signals.

Reverse transcription-PCR. Total RNA was extracted by using the TRI reagent (Sigma, Poole, United Kingdom). First-strand cDNA was synthesized by using Ready-To-Go You-Prime First-Strand beads (Amersham Biosciences). The reactions were processed in a DNA thermal cycler under the following conditions: denaturation at 94°C for 1 min, annealing at 67°C for 30 s, and extension at 72°C for 45 s. The number of cycles was 36. PCR primers for human

p21 (Cip1/WAF1) and GAPDH (GenBank accession numbers NM_000389 and NP_002037, respectively) were designed by using the Primer3 software.

Interplay between p53 modifications. Methylation and acetylation interplay using different p53 peptides was performed as described previously (42). For the methylation assay 2.5 nmol of each peptide was methylated with 0, 0.5, 1, or 2.5 pmol of the recombinant purified His_6-Set7/9 protein in a 20-μl reaction mixture at 30°C for 10 min. Reaction mixtures were then incubated with 20 μl of streptavidin-conjugated Sepharose (Pharmacia Biotech) in the presence of excess of unlabeled SAM at 4°C for 1 h. After extensive washing with BC500 and 1% Triton X-100, the bound materials were subjected to scintillation counting.

In the acetylation assay, 2.5 nmol of each peptide was acetylated with 0.2 μl of purified, baculovirus-expressed p300 for the indicated times in a 20-μl reaction at 30°C. The acetylation reaction was then stopped by dilution with BC300 containing an excess of unlabeled acetyl-coenzyme A (CoA). Reaction mixtures were then incubated with 20 μl of streptavidin-conjugated Sepharose (Pharmacia Biotech) in the presence of excess of unlabeled acetyl-CoA at 4°C for 1 h. After extensive washing with BC500 and 1% Triton X-100, the bound materials were subjected to scintillation counting. The following peptides were used in the reactions: p53 unmodified, NH_2-SHLKSKKGQSTSRHKKLMFK (biotin); p53 acetyl-K373-K382, NH_2-CSHLKSKK-(Ac)GQSTSRHKK-(Ac)LMFK (biotin); and p53 methyl-K372, NH_2-CSHLKSK-$(Me)_2$-KGQSTSRHKKLMFK (biotin).

Analysis of 1Me-K372-p53 antibody. The efficiency of 1Me-K372 p53-specific antibody to recognize double-modified p53 (K372 methylated/K373 acetylated) was tested in vitro by immunoblotting. To this end, 1 μg of the recombinant purified glutathione *S*-transferase (GST)-p53 protein (amino acids 300 to 393) was incubated for 1.5 h at 30°C in the presence of either 0.5 μg of recombinant purified His_6-Set7/9 and 2 pmol of [^{3}H]SAM (methylation) or 100 ng of recombinant p300 (PROTEINONE, USA) and 2 nmol of [^{3}H]acetyl-CoA. To prepare the double-modified protein, GST-p53 was first methylated and then acetylated as described above. Mock-treated p53 supplemented with both SAM and acetyl-CoA was used as a negative control. The resulting proteins were then subjected to SDS-polyacrylamide gel electrophoresis, transferred, and immunoprobed with the following antibodies: anti-GST (Santa Cruz) to evaluate the levels of total GST-p53 in reactions, anti-1Me-K372-p53 (Abcam) to detect methylated p53, and anti-acK373-p53 (Upstate Biotech) to control the efficiency of acetylation.

RESULTS

In order to elucidate the functional consequences of p53 methylation by Set7/9 in vivo, we decided to examine the effect of Set7/9 on the dynamics of protein accumulation for p53 and its major transcriptional target p21/WAF/CIP in response to DNA damage. To this end, a time course experiment was performed using two U2-OS-derived cell lines stably expressing either nonspecific or Set7/9-specific small hairpin RNA (U2-OS-control or U2-OS-shRNA-Set7/9, respectively) treated with a radiomimetic drug, Adr.

As expected, we found that the level of Set7/9 expression in U2-OS-shRNA-Set7/9 cells was diminished fourfold compared to that in U2-OS-control cells (Fig. 1A1 and B, left). Treatment with Adr did not affect the levels of Set7/9 expression in either of these cell lines, as judged by Western blotting with Set7/9-specific antisera (Fig. 1A1 and B, left). On the contrary, Adr treatment of the control and shRNA-Set7/9 cells induced differential stabilization and accumulation of p53 in response to DNA damage. The levels of p53 protein peaked at 6 and 12 h post-DNA damage treatment in control cells (Fig. 1A2), whereas in shRNA-Set7/9 cells stabilization of p53 was both delayed and attenuated (Fig. 1A2, and Fig. 1B, middle). These data suggest that Set7/9 indeed contributes to the stabilization of p53 upon DNA damage in agreement with our previously published results (12). Notably, compared to control cells, the low levels of p53 protein in shRNA-Set7/9 cells were paralleled by attenuated expression of the p21/WAF/CIP protein (Fig. 1A3 and B, right).

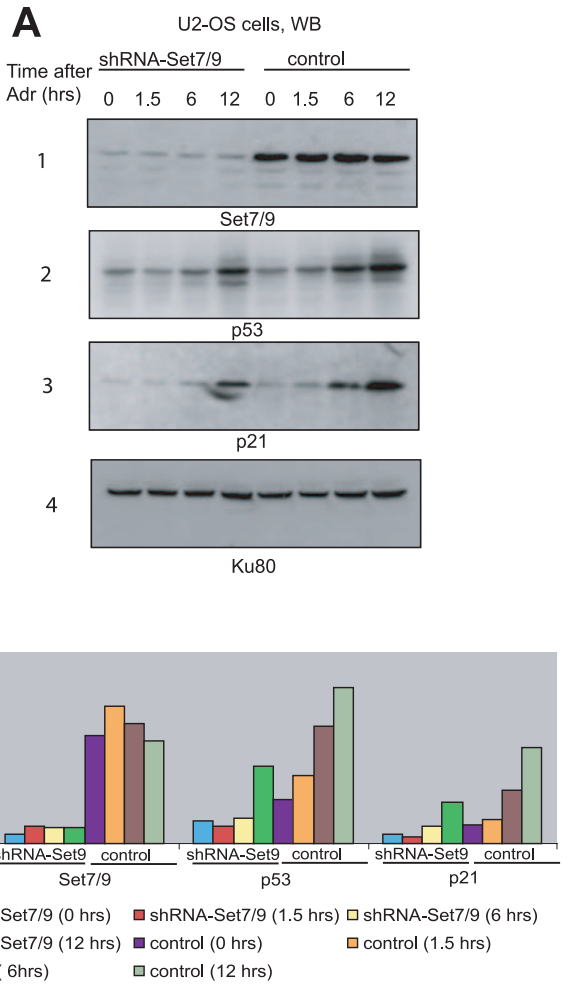

FIG. 1. Knockdown expression of Set7/9 causes destabilization of p53 and attenuates p21 expression in response to DNA damage. (A) Immunoblot analysis of Set7/9 (panel 1), p53 (panel 2), p21 (panel 3), and Ku80 (panel 4) in U2-OS cells stably infected either with scrambled shRNA (54) (control) or with Set7/9-specific shRNA (shRNA-Set7/9). Both cell lines were analyzed for the expression of the above proteins at different time points after DNA damage treatment with 0.5 μM Adr. (B) Quantification of the Western blot signals shown in panel A. All signals were normalized to the Ku80 signal.

Since p21/WAF/CIP is a potent inhibitor of cyclin-dependent kinases and is modulated by the p53-Set7/9 axis, we next analyzed the effect of Set7/9 on cell cycle progression. U2-OS cells engineered to express normal or diminished levels of Set7/9 were treated separately with Adr and 10 Gy of gamma irradiation, which causes double-strand breaks in the DNA (Fig. 2). U2-OS cells transfected with a Set7/9 expression vector and treated with Adr were used as a positive control. As expected, Adr treatment of control U2-OS cells transfected with an empty pCDNA vector resulted in p53-dependent G_1 and G_2/M arrest (Fig. 2A1 and 2). Ectopic expression of Set7/9 (Fig. 2D left, upper panel) generated even more pronounced arrest in G_2/M compared to control U2-OS cells (Fig. 2A, compare panels 2 and 4). Intriguingly, U2-OS shRNA-Set7/9 cells, expressing diminished levels of Set7/9 (Fig. 2D middle, upper panel), exhibited less pronounced arrest in G_2/M com-

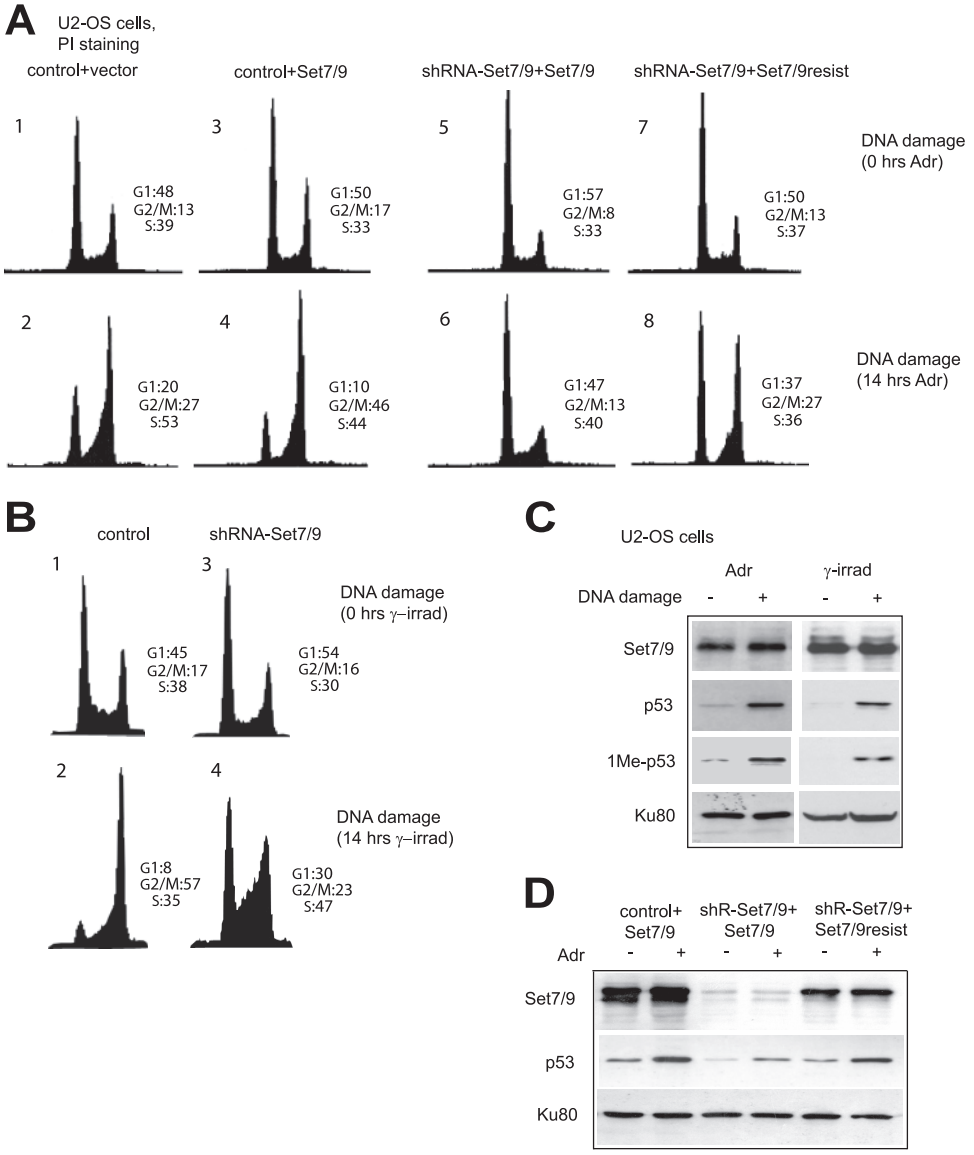

FIG. 2. Set7/9 controls p53-dependent cell cycle arrest in the G_2 phase in response to DNA damage. (A) Fluorescence-activated cell sorting (FACS) analysis of control- and shRNA-Set7/9-infected U2-OS cells transfected with either empty or Set7/9-expressing vectors after DNA damage treatment with 2 μM Adr. Panels 1, 3, 5, and 7 and panels 2, 4, 6, and 8 represent untreated and Adr-treated cells, respectively. Mock-treated or Adr-treated cells were harvested, fixed, and stained with propidium iodide (PI) before sorting. The percentage of cells in G_1, G_2/M, and S phases are indicated in the right corner of each panel. (B) FACS analysis of control- and shRNA-Set7/9-infected cells treated or mock-treated with 10 Gy of gamma irradiation. Panels 1 and 3 and panels 2 and 4 represent untreated and treated cells, respectively. (C) Western blot analysis of U2-OS cells treated with Adr and gamma irradiation (γ-irrad). The status of Set7/9 (upper), p53 (lower), 1Me-K372-p53 (middle), and Ku80 (bottom) was analyzed by Western blotting with the corresponding antibodies. (D) Western blots of the corresponding cell lines analyzed in panel A. The amounts of Set7/9, p53, and Ku80, used as a loading control, are shown in cell lines treated with Adr as in panel A. Note that ectopic Set7/9 migrates more slowly than the endogenous protein because of the Flag- and His_6 tag epitopes.

pared to the control cells (Fig. 2A, compare panels 2 and 6). To ensure that the observed phenotype was not due to a nonspecific, off-target effect of Set7/9-shRNA, we transfected Set7/9 knockdown cells with vector expressing shRNA-resistant Set7/9 (Fig. 2D right, upper panel). Reintroduction of Set7/9 into these cells rescued the phenotype of G_2/M arrest in response to Adr treatment (Fig. 2A, compare panels 6 and 8).

To test the notion that Set7/9 affects cell cycle specifically in response to DNA damage, we analyzed the cell cycle distribution of wild-type and Set7/9 knockdown cells treated with gamma irradiation. Similar to Adr, gamma irradiation treatment caused a robust G_2/M arrest in control cells (Fig. 2B, compare panels 1 and 2). The G_2/M-phase arrest was impaired in Set7/9 knockdown cells (Fig. 2B, compare panels 2 and 4). In agreement with our previously published results, the expression levels of Set7/9 directly correlated with the cellular levels of p53 (Fig. 2D, middle panel), indicating that these effects are mediated through p53. Moreover, both Adr and gamma irradiation induced p53-K372 methylation in U2-OS cells (Fig. 2C, third row). Collectively, these results suggest that Set7/9-me-

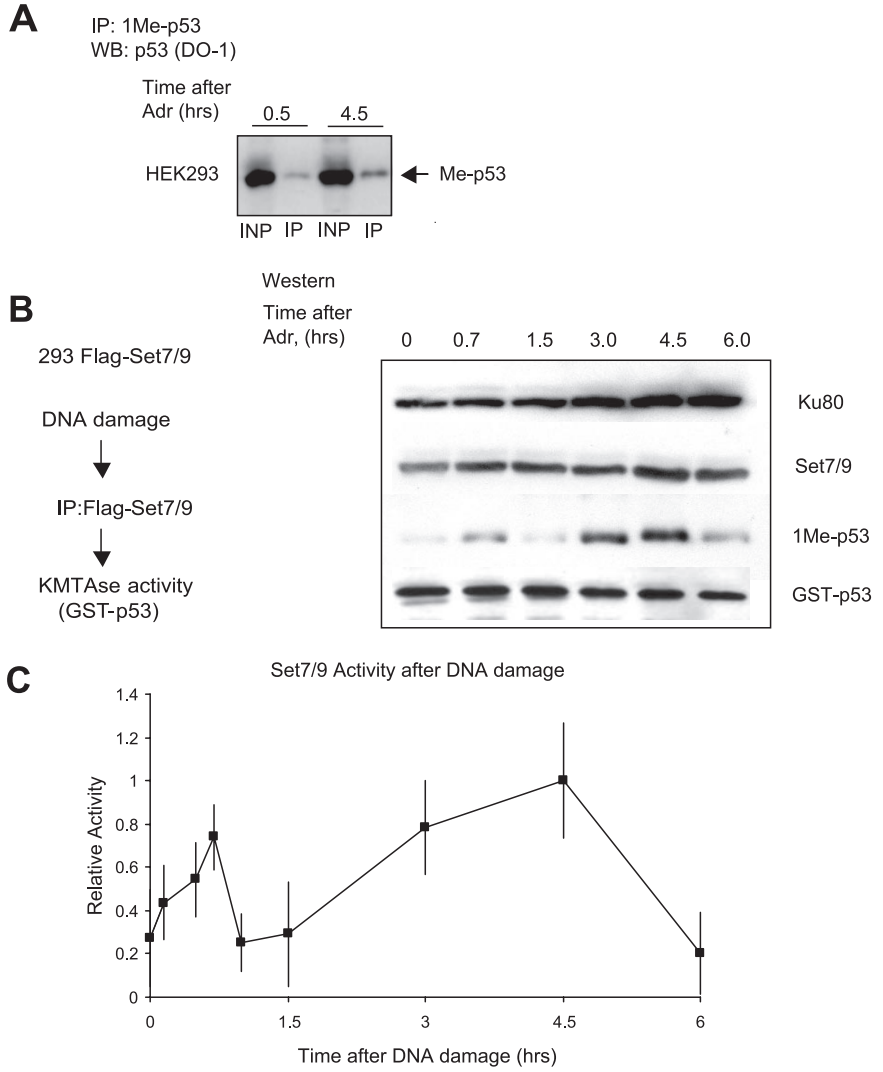

FIG. 3. KMTase activity of Set7/9 is regulated by DNA damage. (A) HEK293 cells were treated with Adr for the indicated periods of time and methylated p53 was immunoprecipitated by using anti-K372Me1-p53 serum. The precipitated material was analyzed by immunoblotting with anti-p53 (Ab-6) monoclonal antibodies. (B) On the left is shown an experimental scheme to study the effect of DNA damage on regulation of Set7/9 activity. The right side shows that p53 methylation by Set7/9 is regulated by DNA damage. Ectopic Flag-Set7/9 immunopurified from HEK293 cells treated with Adr for the indicated time periods was examined for the ability to methylate the recombinant GST-$p53_{300-393}$ protein. Shown are the input amounts of the loading control Ku80 (top row), the amounts of Set7/9 used in the reaction (second row), and the levels of methylated p53 (third row), as well as the levels of total p53 used in reactions as judged by immunoblotting with the corresponding antibodies (fourth row). (C) Summary plot of the densitometry measurements of the p53 methylation signal generated by Set7/9 after DNA damage. The values were obtained from at least four independent experiments and were normalized to the protein amount of Set7/9. The maximal Set7/9 activity was arbitrarily set as 1. Standard deviations are shown as vertical bars.

diated methylation of p53 is activated by DNA damage and is important for the ability of p53 to induce cell cycle arrest in G_2/M phase.

We have previously demonstrated that Set7/9-dependent methylation of p53 was induced by DNA damage (12). A time course experiment in HEK293 cells showed differential K372 p53 methylation at 0.5 and 4.5 h after Adr treatment (Fig. 3A). This fact prompted us to examine the regulation of the lysine methyltransferase (KMTase) activity of Set7/9 in response to DNA damage. To this end, HEK293 cells stably expressing Flag-Set7/9 were treated with Adr for the indicated periods of time (Fig. 3B and C). The Flag-Set7/9 protein was immunopurified from these cells and assayed for KMTase activity against the recombinant purified GST-p53 protein used as substrate. A 1.5-fold increase in Set7/9 activity was readily detected at 15 min and was further elevated 2.5-fold at 45 min after DNA damage compared to the basal activity at the 0-h time point. Another peak of activity was observed at 4.5 h (4-fold increase over the basal activity) (Fig. 3B and C). Importantly, neither the protein level nor subcellular localization of Set7/9 changed upon DNA damage (Fig. 3B and data not shown). Thus, we concluded that DNA damage regulated the activity of Set7/9 in a biphasic manner.

We have previously demonstrated that Set7/9 failed to methylate nucleosomal histones (41, 61). Given that the KMTase activity of endogenous Set7/9 against p53 is increased after

DNA damage, we revisited Set7/9 activity toward nucleosomal histones under these conditions. We analyzed the ability of ectopic Set7/9 immunopurified from cells harvested at different time points after DNA damage (see the scheme in Fig. 4A) to methylate purified nucleosomes in vitro (Fig. 4A). GST-$H3_{1-27}$ bearing the K4R substitution mutation and GST-$p53_{300-393}$ fusion proteins were used as negative and positive controls, respectively. The methylation signal of nucleosomal H3 treated by Set7/9 was at least fivefold weaker than the p53-specific methylation signal using the same preparations of Set7/9 (Fig. 4A and B). A methylation signal was undetectable when the histone H3 tail with the K4R mutation was used as a substrate, serving as a specificity control for the methylation reaction (Fig. 4A and B). Although the nucleosomal H3 methylation signal was not affected by DNA damage, the activity of Set7/9 toward p53 peaked at 1.5 and 4.5 h after DNA damage (Fig. 4A and B). This result is in a good agreement with the results shown in Fig. 3 derived from a different cell type, suggesting that regulation of Set7/9 activity by DNA damage is rather a general phenomenon. Collectively, these data suggest that, at least in vitro, the nucleosomal histones, which are considered to be the "physiological" substrate for histone-specific methyltransferases, cannot be efficiently methylated by Set7/9.

It was plausible that Set7/9 might act cooperatively with other histone modification enzymes to efficiently monomethylate H3-K4 in vivo. If this were the case, then the immunopurified Set7/9 protein alone would not be expected to methylate nucleosomes in vitro. Therefore, we next assessed the effect of DNA damage on global Set7/9-dependent H3-K4 monomethylation in vivo (Fig. 4C). To this end, bulk chromatin extracted from control and shRNA-Set7/9 U2-OS cells was analyzed for monomethylation of H3-K4 as a function of DNA damage by immunoblotting. Both cell lines showed similar levels of H3-K4 methylation irrespective of the presence of Set7/9 and DNA damage. These results suggest that Set7/9, even after DNA damage, is not able to methylate bulk nucleosomal H3-K4 in vivo.

It was still possible, however, that Set7/9 did methylate histones in only a small subset of promoters. Such a subtle promoter-specific effect may be masked by the global H3-K4 methylation of genomic chromatin. To test this possibility, we examined the effect of Set7/9 on the level of H3-K4 methylation in the promoter of the p21/WAF/Cip gene, which we previously identified as a transcriptional target for Set7/9 (12). ChIP assays were performed on the previously characterized U2-OS versus U2-OS-shRNA-Set7/9 cells (Fig. 4D) before and after DNA damage using antibody specific to 1Me-K4-H3. Quantitative reverse transcription-PCR with p21-specific primers showed comparable levels of H3-K4 methylation in the p21 promoter in U2-OS and U2-OS-shRNA-Set7/9 cells, although the peak of maximal K4-H3 monomethylation was slightly shifted in U2-OS-shRNA-Set7/9 cells compared to the control cells (Fig. 4D). We concluded that ablation of Set7/9 did not have any direct effect on H3-K4 monomethylation. Importantly, the shRNA-Set7/9 cells exhibited low levels of Set7/9 expression compared to normal U2-OS cells, which was also accompanied by the diminished amounts of the p21 transcript after DNA damage (Fig. 4E, compare the 12- and 24-h time points in control versus shRNA-Set7/9 cells). Furthermore, the downregulation of p21 transcription observed in shRNA-Set7/9 cells versus normal U2-OS cells was specific as the levels of GAPDH transcription were unaffected (Fig. 4E, middle row). Significantly, the attenuated transcription of p21 in Set7/9 knockdown cells resulted in a significant decrease of its protein levels (Fig. 4E, bottom). Collectively, these data strongly suggest that Set7/9 regulates the expression of p21/WAF/CIP and other genes not through histone methylation but rather via methylation of their relevant transcription regulators, such as p53 in the case of p21.

Set7/9-mediated methylation of p53 at K372 results in stabilization and "activation" of p53 as a transcription factor (12) without affecting its DNA-binding activity, at least in vitro (data not shown). One possibility is that K372 methylation of p53 directly ablates the ubiquitination of five other lysine residues present in the carboxyl terminus of p53. We reasoned that if methylation directly counteracts ubiquitination, then the nonmethylatable p53 might be a better substrate for in vivo ubiquitination than the wild-type methylatable protein. In direct contradiction to this hypothesis, the results of the experiment shown in Fig. 5 indicate that the wild-type and K372R mutant ectopic p53 are ubiquitinated with similar efficiency in the absence of DNA damage (Fig. 5A, compare lanes 4 and 5). In addition, another p53 mutant, bearing a mutation in one of the acetylation sites, K373R, showed a similar ubiquitination pattern, strongly suggesting that ubiquitination targets several lysine residues in the carboxyl terminus of p53 and that mutation of just one lysine is not sufficient to alter stability of the p53 protein. Thus, we concluded that K372 methylation affects the stability of p53 indirectly, possibly by modulating other modifications in the C terminus of p53.

Acetylation of p53 has been reported to stabilize the protein by blocking ubiquitination of the target lysine residues in its C terminus. The site of p53 methylation by Set7/9 (K372) is surrounded by several lysines subject to acetylation by p300/CBP in vitro (K370, K373, K381, and K382) and in vivo (K373 and K382). In the case of histone H3, methylation of K4 results in increased acetylation of K9 and K14 (61). We hypothesized that, similar to histone H3, methylation of K372 in p53 may affect the efficiency of its acetylation. Therefore, we tested whether the C-terminal lysines of p53 also exhibit similar interplay between the extent of their methylation and acetylation.

To investigate the causal link between p53 methylation and acetylation, we compared the levels of p53 acetylation in HEK293 cells stably expressing either wild-type Set7/9 or its K297A mutant, which is dominant negative toward the endogenous protein (12). Overexpression of Set7/9 wild type, but not of the K297A mutant, increased the level of p53 acetylation in HEK293 cells (Fig. 5B). These results indicate that K372 methylation of p53 by Set7/9 is likely required for efficient acetylation of p53 in vivo.

We reasoned that if methylation was required for acetylation of p53 in vivo, then the methylation-impaired p53 should be compromised for acetylation. To test this hypothesis, we transfected p53-negative H1299 cells stably expressing Set7/9 with plasmids expressing either p53 wild-type or K372R nonmethylatable mutant protein. After 12 h of treatment with Adr, the total protein levels of p53 wild type and its K372R mutant were normalized, and their acetylation statuses were examined by immunoblotting with the corresponding antibodies (Fig. 5C).

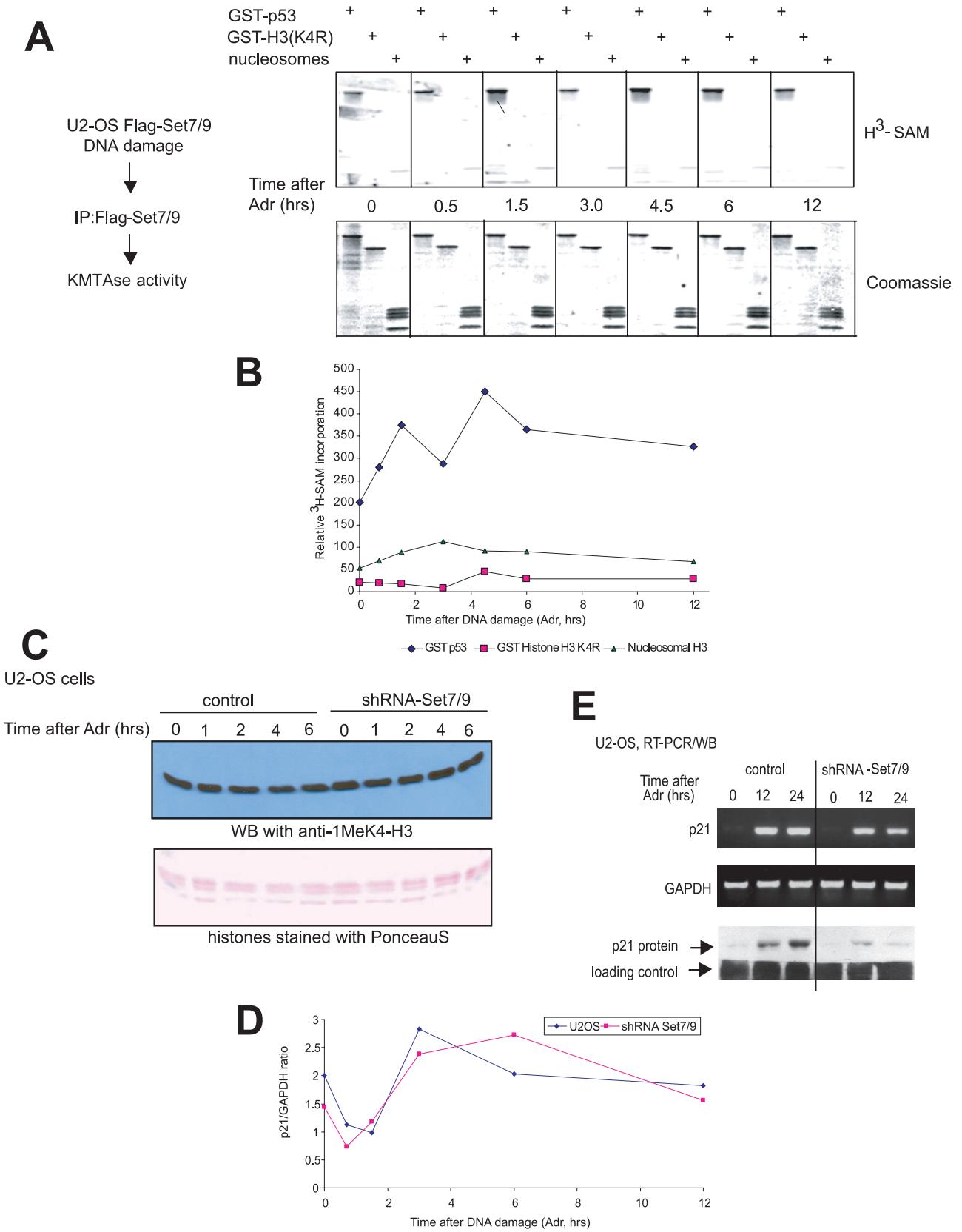

FIG. 4. DNA damage does not activate nucleosomal HMTase activity of Set7/9. (A) Flag-Set7/9 immunopurified from U2-OS-Flag-Set7/9 cells, which were treated with DNA damage for the indicated periods of time, was tested in the methylation reaction in the presence of [^{3}H]SAM with the following substrates: GST-p53$_{300-393}$, GST-H3$_{1-27}$ (K4R), and nucleosomes purified from MCF-7 cells (top). The efficiency of protein

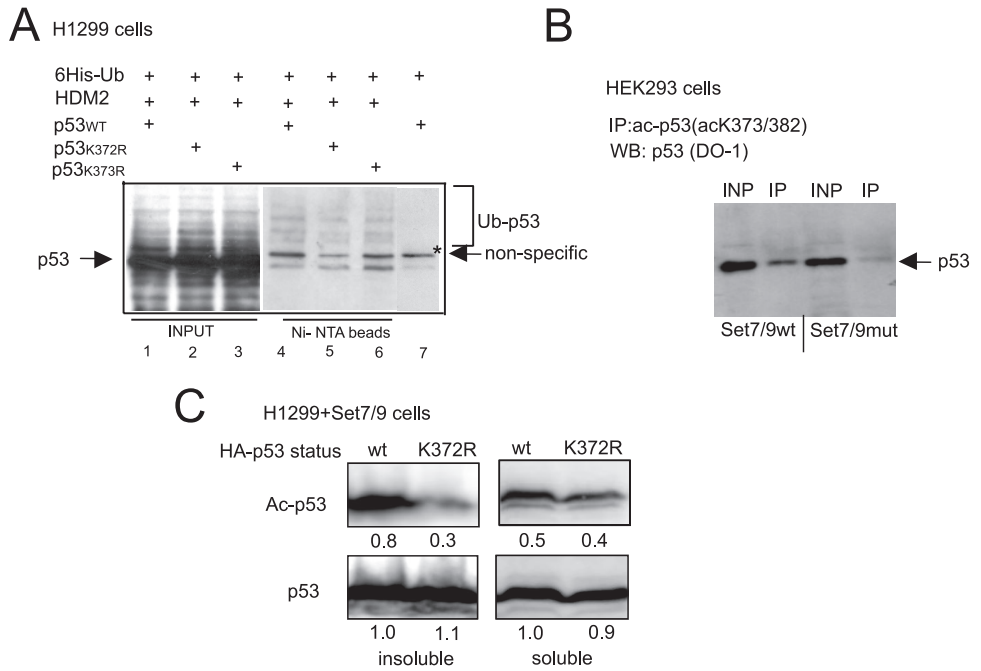

FIG. 5. p53 acetylation is impaired in the absence of K372 methylation. (A) The methylation-deficient mutant of p53 does not exhibit increased ubiquitination in the absence of DNA damage. H1299 cells were transiently transfected with vectors expressing His_6-ubiquitin and either p53 wild type or K372R methylation- or K373R acetylation-defective mutants. These cells were also transfected with HDM2-expressing plasmid where indicated. After incubation with proteosomal inhibitor MG132 for 12 h, cells were lysed with 8 M urea, and His_6-ubiquitin-containing proteins were purified on Ni-NTA beads. The bead-bound proteins were eluted and analyzed by immunoblotting with p53-specific antibody (Ab-6). The positions of p53 and its ubiquitinated ladder are indicated. Lanes 1 to 3 (INPUT) represent 10% of the starting material, and lanes 4 to 6 (Ni-NTA beads) represent the bead-bound material eluted from Ni-agarose beads. (B) p53 acetylation depends on the KMTase activity of Set7/9. HEK293 cells stably expressing Set7/9 wild type or its catalytically impaired mutant (H297A) were subjected to immunoprecipitation with acetyl-p53-specific antibody. The resulting proteins were analyzed by immunoblotting with (Ab-6) p53-specific antibody. Input (INP) lanes represent 10% of the starting material. (C) Mutation in p53 methylation site diminishes acetylation. H1299 (p53-) cells were transfected with vectors expressing either wild type or K372R nonmethylatable mutant p53. After DNA damage, cells were fractionated into soluble and insoluble chromatin fractions. The amounts of both p53 variants were normalized after Western blotting. The levels of acetylation in the wild-type and mutant p53 protein present in soluble and insoluble fractions were analyzed by immunoblotting with specific antibodies and quantified. The relative strength of p53 acetylation signal was quantified by arbitrarily setting the p53 wild-type signal as 1.

Since the most robust methylation of p53 was observed in the chromatin-enriched insoluble fraction of whole-cell extract (12), the transfected cells were fractionated into soluble and chromatin-containing insoluble fractions, and the resulting materials were analyzed separately. Consistent with our previous results, methylated p53 was observed mostly in the insoluble fraction. Moreover, in the same fraction we observed a significant difference in the levels of acetylation between the wild-type p53 and its methylation-deficient mutant (Fig. 5C, upper left panel). No appreciable difference in acetylation was detected in the soluble fractions. This result indicates that methylation specifically affects acetylation in only a subfraction of chromatin-bound p53 (Fig. 5C, bottom row). Notably, the cellular amounts of wild-type and K372R mutant p53 were normalized prior to their comparison (Fig. 5C, bottom left panel) and thus do not account for the difference observed.

To directly address the question of methylation-acetylation interplay, we used chemically modified p53 peptides, either methylated or acetylated, with the intent to measure the ability of p300/CBP and Set7/9 to utilize these premodified peptides

methylation was determined by exposure of the ^3H-labeled autoradiograph to film followed by densitometry. The protein levels were verified by Coomassie staining (bottom). (B) Quantification analysis of the experiment shown in panel A. (C) The levels of K4-H3 monomethylation in U2-OS versus U2-OS shRNA-Set7/9 cells. Cells were treated with 0.1 μM Adr for the indicated time periods, followed by chromatin extraction. Chromatin samples were probed for K4-H3 monomethylation by immunoblotting with specific antibody (Abcam). The input protein levels were analyzed by Ponceau-S staining. (D) The level of K4-H3 monomethylation in the p21 promoter is independent of the presence of Set7/9. A ChIP assay using K4H3-Me1-specifc antibody was performed on U2-OS and U2-OS shRNA-Set7/9 cells treated with 0.1 μM Adr. The levels of K4H3-Me1 in the promoter regions of p21 were determined by quantitative PCR using specific primers. The values represent the ratio of p21 to GAPDH signals. (E) p21 mRNA expression depends on Set7/9 activity. U2-OS cells stably infected with either scrambled shRNA (control) or vectors expressing shRNA-Set7/9 (shRNA-Set9) were treated with 0.5 μM Adr for 0, 12, and 24 h to induce DNA damage. Total RNA from these cells was then isolated, converted into cDNA by reverse transcriptase and analyzed for the presence of the p21 coding sequence by PCR using specific primers. GAPDH signal served as a loading control. In parallel, the same samples were subjected to Western blot analysis to measure the levels of p21 protein.

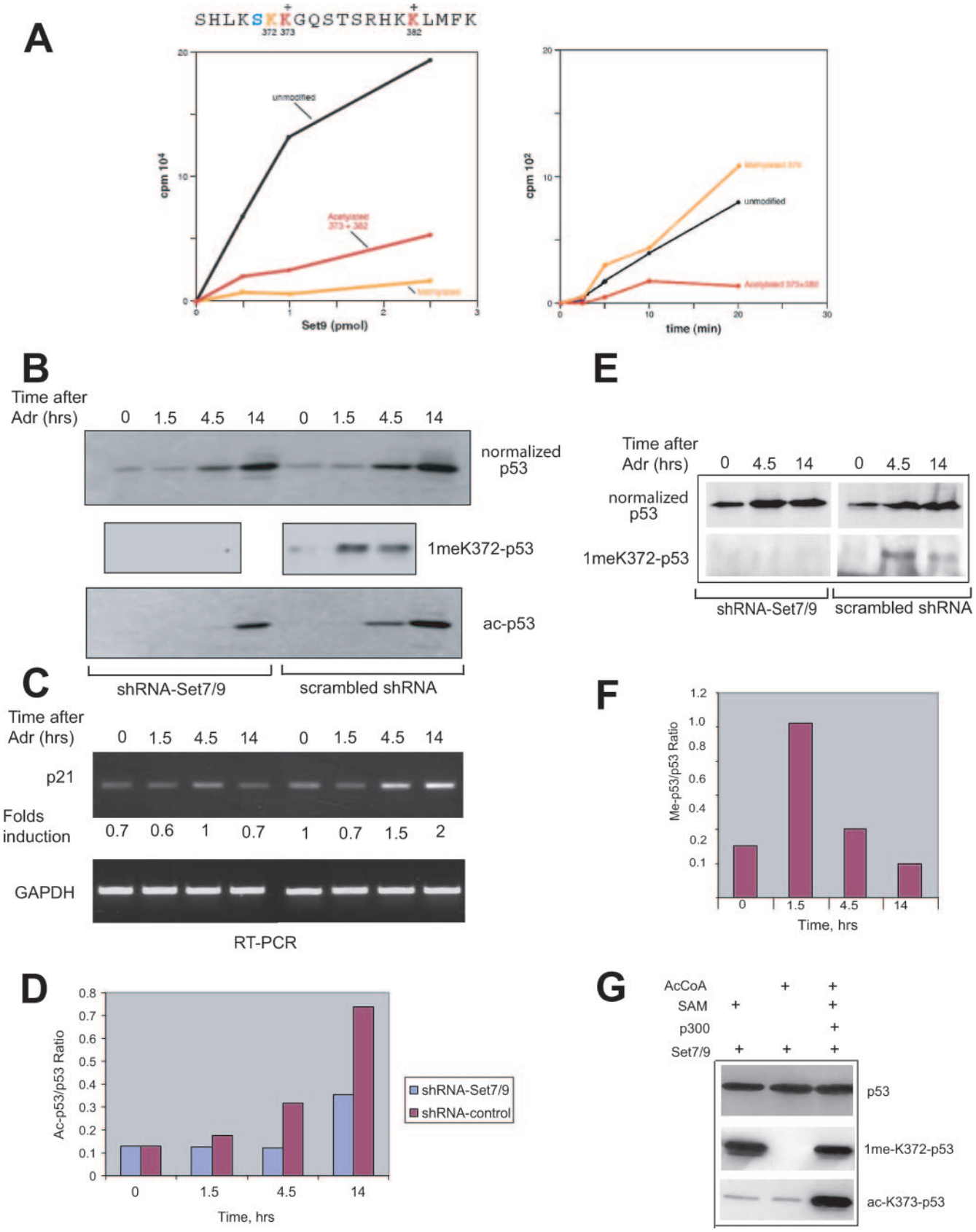

FIG. 6. Methylation of p53 on K372 promotes its subsequent acetylation. (A) In vitro interplay between acetylation and methylation modifications of p53. Chemically nonmodified, premethylated, and preacetylated p53 peptides were incubated with Set7/9 (left panel) or p300 (right

as substrates in the corresponding enzymatic reactions (Fig. 6A). We first compared the ability of Set7/9 to methylate either nonmodified, K372/382 acetylated, or K372 methylated p53 peptides in vitro (Fig. 6A, left). As expected, the recombinant Set7/9 protein effectively methylated the nonmodified p53 peptide. The K372-me p53 peptide served as a poor substrate for Set7/9-mediated methylation, confirming that K372 is the only methylation site in the C terminus of p53 (12). Importantly, preacetylation of the p53 peptide at K373/382 also inhibited subsequent methylation of K372 by Set7/9.

In parallel, the premodified p53 peptides were subjected to acetylation by purified, baculovirus-expressed p300 (Fig. 6A, right). Unmodified peptide was efficiently acetylated by p300. Preacetylation of the p53 peptide at K373 and K382 dramatically reduced subsequent acetylation by p300, indicating that these lysines are the major sites of acetylation. Importantly, premethylation at K372 did not inhibit, but rather slightly enhanced subsequent acetylation of p53 by p300.

These results led us to conclude that methylation and acetylation can exist on the same molecule of p53, but that methylation must precede acetylation. To test the validity of these predictions in vivo, we compared the kinetics of DNA damage-induced methylation and acetylation of the endogenous p53. First, we compared the kinetics of p53 methylation in vivo after DNA damage in U2-OS versus U2-OS shRNA-Set7/9 cells (Fig. 6B). Methylated p53 in control U2-OS cells was readily detected at 1.5 h after Adr treatment (Fig. 6B, middle right). In contrast to methylation, the kinetics of p53 acetylation in the same samples was much slower, showing an appreciable level of p53 acetylation only after 4.5 h of exposure to Adr (Fig. 6B, bottom panel). These results suggest that in vivo methylation of p53 precedes its acetylation. Another important observation made in this experiment was that the lack of p53 methylation in shRNA-Set7/9 cells (Fig. 6B, compare the middle left and middle right panels) was paralleled by attenuation of the DNA damage-induced p53 acetylation (compare the 1.2-fold increase in p53 acetylation in shRNA-Set7/9 cells versus the 3-fold increase in control cells at 4.5 h after DNA damage) (Fig. 6D). Importantly, the lack of efficient methylation of p53 in the absence of Set7/9 resulted in attenuation of p21/WAF/CIP transcription, a known p53 transcriptional target (Fig. 6C, upper panel).

To corroborate our results and to determine the levels of p53 K372 methylation at later time points after DNA damage, we expanded the time course experiment shown in Fig. 6B by including an additional, 14-h time point after Adr treatment (Fig. 6E). The p53 expression levels at 0, 4.5, and 14 h in both shRNA-Set7/9- and scrambled shRNA- expressing cell lines were normalized to each other before assessing the levels of K372 methylation (Fig. 6E, upper and lower panels, respectively). In agreement with the results of our previous experiment, the ratio of methylated to total p53 in control cells, after peaking at 1.5 h (Fig. 6B), started to decline at later time points after DNA damage (Fig. 6E, lower right panel, and Fig. 6F). These results correlate with our in vitro data, which demonstrate that acetylation inhibits subsequent methylation of p53 (Fig. 6A, left). The attenuated expression of Set7/9 in shRNA-Set7/9 cells consistently resulted in the loss of K372 methylation signal in p53 (Fig. 6B, middle left panel, and Fig. 6E, lower left panel), confirming the identity of Set7/9 as the 1Me-K372-specific KMTase.

The decreased K372-p53 methylation signal in vivo at 4.5 and 14 h after DNA damage could be a result of interference between 1Me-K372-specific antibody and acetylation mark on the adjacent K373 residue. To test this possibility, we analyzed the ability of 1Me-K372-specific antibody to recognize an in vitro-methylated/acetylated p53 peptide. As evident from Fig. 6G (middle panel), acetylation of p53 on K373 had no significant effect on the ability of 1Me-K372-specific antibody to recognize methylated K372 residue. This result strongly supports the notion that attenuation of p53-K372 methylation does occur in vivo as DNA damage-induced acetylation of p53 progresses.

Since p53 exerts its function mostly as a DNA-binding transcription factor, we next compared the levels of methylated and of acetylated p53 bound to the promoter of its target gene, p21 as a function of DNA damage and the presence of Set7/9. To accomplish this task, we performed a time course ChIP experiment using Adr-treated U2-OS control- and shRNA-Set7/9-expressing cells (Fig. 7). As expected, DNA damage increased the levels of p53 bound to the p21 promoter in both U2-OS

panel) in the presence of [^{3}H]SAM or [^{3}H]AcCoA, respectively. The efficiency of de novo methylation or acetylation was determined by peptide incorporation of radioactive tritium using scintillation counting. The results were plotted as a function of Set7/9 protein concentration in the case of methylation and incubation time in the case of acetylation. (B) The dynamics of p53 methylation in response to DNA damage. U2-OS and U2-OS shRNA-Set7/9 cells were treated with Adr for the indicated time periods, followed by immunoblotting with p53-specific antibody to normalize the levels of p53 between the two cell lines. Adjusted amounts of cells were then used to prepare nuclear extracts. Insoluble, chromatin-enriched pellets were solubilized by sonication and used for immunoprecipitation with methylated or acetylated p53-specific antibody. Western blot analysis of both input and immunoprecipitated material was performed with monoclonal antibody against p53 (Ab-6; Oncogene). To avoid the interference between the p53 signal and the signal from the heavy chain of immunoglobulin G (in the case of immunoprecipitations), κ-light chain-specific antibody conjugated to peroxidase was used as a source of the secondary antibody. (C) p21 mRNA expression depends on Set7/9 activity. U2-OS cells stably infected with either control shRNA (scrambled shRNA) or shRNA-Set7/9 (shRNA-Set9) vectors were treated with 0.5 μM Adr as in panel B. The total RNA from these cells was then isolated, converted into cDNA by using reverse transcriptase, and analyzed for the presence of the p21 coding sequence by PCR using specific primers. GAPDH signal served as a loading control. (D) Quantification of the results shown in Fig. 6B. The values shown in the plot represent the ratio between acetylated and total p53. (E) The experiment shown in panel B was repeated using an additional 14-h time point after DNA damage. (F) Quantification of the levels of methylated p53 in control U2-OS cells treated with Adr for the indicated periods of time. The values shown in the plot represent the ratio between methylated and total p53 from the experiments shown in panels B and C. (G) Effect of K373 acetylation on the recognition efficacy of methylated p53 by 1Me-K372-p53-specific antibody. Recombinant purified GST-p53$_{300\text{-}393}$ proteins were either mock treated, methylated, or sequentially methylated by Set7/9 and acetylated by p300 HAT. The resulting proteins were analyzed by immunoblotting with GST-, 1Me-K372-p53-, or ac-K373-p53-specific sera to assess the levels of total, methylated, and methylated/acetylated p53, respectively.

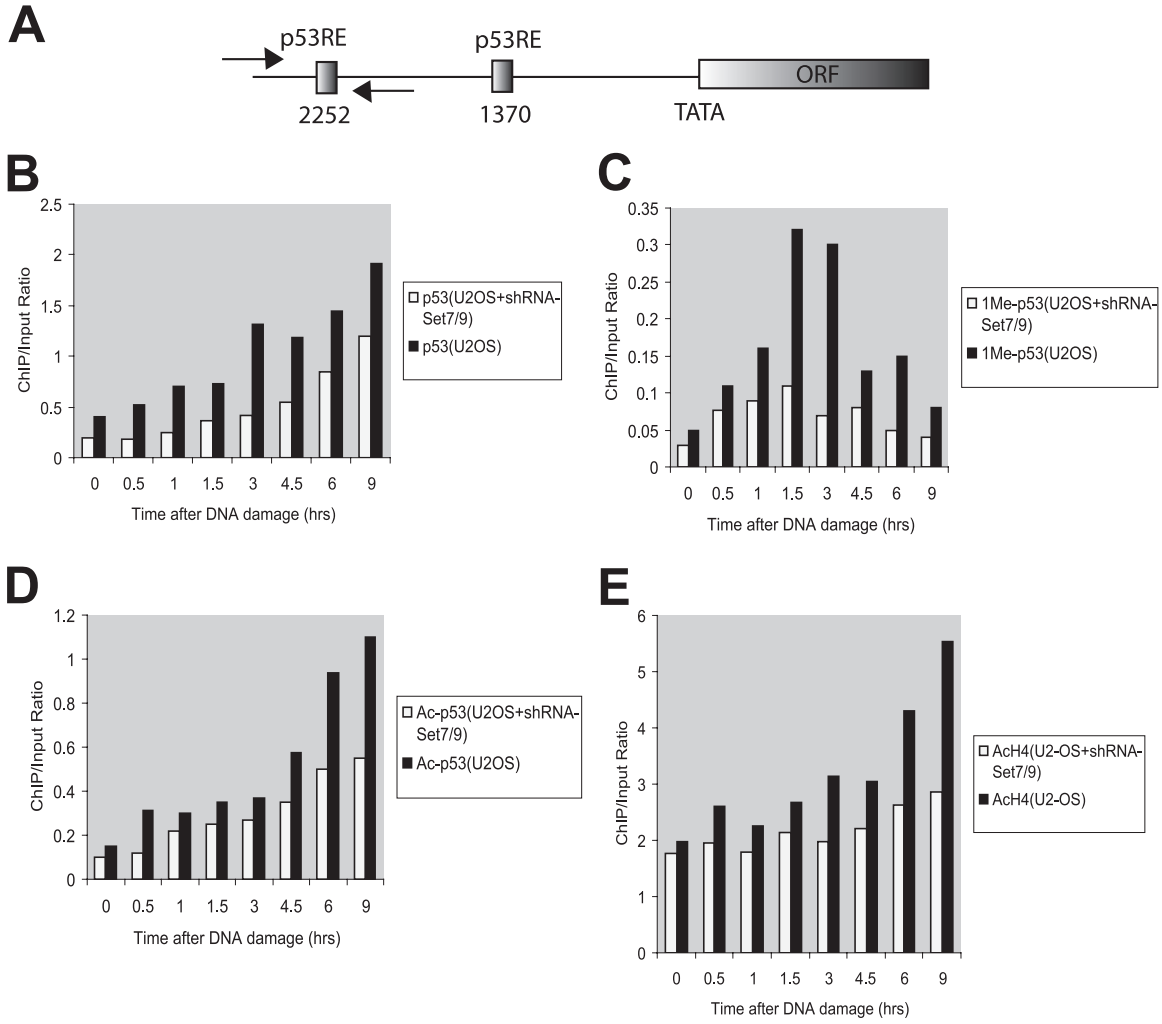

FIG. 7. The loss of p53 methylation attenuates promoter acetylation of the p21 gene. (A) Schematic representation of the p21 gene. The binding sites of p53 and the transcription start site are indicated. Positions of the PCR primers used in the Q-PCRs are indicated. (B) The p21 promoter occupancy by p53 is decreased in the absence of Set7/9. A ChIP assay using p53-specific antibody was performed on U2-OS and U2-OS shRNA-Set7/9 cells treated with Adr for the indicated time periods. Immunoprecipitated DNA was analyzed by quantitative PCR for the presence of p21 and GAPDH using specific primers. Values were normalized to the GAPDH signal. (B, C, D, and E) ChIP analysis of the p21 promoter as performed in panel B for the presence of methylated p53 using methylated p53-specific antibody (C), acetylated p53 using acetylated p53-specific antibody (D), and acetylated histone H4 using acetylated histone H4-specific antibody (E).

and shRNA-Set7/9 cells, although the latter exhibited less p21 DNA-p53 association (Fig. 7B). Next, we analyzed the dynamics of p53 methylation in these two cell lines. In agreement with the results shown in Fig. 4A, the maximal accumulation of methylated p53 on the promoter was detected at approximately 1.5 h after DNA damage. The occupancy of the p21 promoter by methylated p53 was greatly diminished in shRNA-Set7/9 cells compared to that in the control cells (Fig. 7C). We also analyzed the levels of acetylated p53 at the p21 promoter in U2-OS and shRNA-Set7/9 cells (Fig. 7D). Supporting the notion that methylation of p53 should precede acetylation, Adr-induced accumulation of acetylated p53 on the p21 promoter in U2-OS control cells was delayed relative to the appearance of methylated p53 (compare Fig. 7C and D). Importantly, the levels of acetylated p53 bound to the p21 promoter were reduced in shRNA-Set7/9 cells compared to the control cells.

One of the consequences of p53 acetylation is enhanced recruitment of p300/CBP through the recognition of acetyllysines by the bromodomain of p300/CBP (2, 39). The latter results in increased histone H4 acetylation and gene activation (29, 39, 52). In accordance with this, the ChIP data showed higher levels of histone H4 acetylation at the promoter of p21/WAF/Cip in the control cells compared to the shRNA-Set7/9 cells (Fig. 7E).

Collectively, the results of the present study indicate that in response to DNA damage Set7/9 directly methylates the p53 protein, promoting its acetylation and thus rendering it transcriptionally active.

DISCUSSION

The role of posttranslational modifications such as phosphorylation, acetylation, or ubiquitination in regulation of p53 tran-

scriptional activity is well documented (5). Recently, we discovered that in response to DNA damage p53 undergoes yet another modification, lysine methylation at K372, mediated by KMTase Set7/9 (12). In the present study, we provide evidence that DNA damage-induced Set7/9-mediated K372 methylation renders p53 stable and transcriptionally active via facilitating the appearance of a subsequent posttranslational modification, acetylation. Acetylation of the C-terminally located lysines is known to enhance the activity and stability of p53 through direct competition with ubiquitination mediated by Mdm2 (6).

A chief task for p53 as a tumor suppressor is to protect cells from abnormal proliferation during genotoxic stress. To achieve this goal, p53 induces cell cycle arrest of damaged cells in both G_1 and G_2 phases by activating the expression of the cyclin-dependent kinase inhibitor, p21/WAF/CIP (9, 53), and/or by directly repressing the transcription of genes involved in G_2/M progression (3, 10, 23, 60). The p21 protein inhibits the enzymatic activity of cyclin-dependent kinases cdk2 and cdk1 (cdc2), and their respective cyclin partners E and B, which are required for enzymatic activation of these kinases. Cdk2 and Cdk1 (cdc2) promote cell cycle progression through the G_1/S and G_2/M stages, respectively (36). Therefore, the inability of Set7/9 knockdown cells to undergo G_2/M arrest in response to DNA damage (Fig. 2A, compare panels 1 and 2 and panels 5 and 6) may be explained by the fact that the expression of p21 in these cells is significantly attenuated compared to wild-type cells (Fig. 1A and 4E). Importantly, reintroduction of Set7/9 into the Set7/9 knockdown cells rescued the G_2/M phase arrest, confirming that the observed phenotype was specific to Set7/9, as well as pointing to Set7/9 as a critical regulator of p53-mediated checkpoint arrest in G_2/M (Fig. 2A, compare panels 1, 5, and 7 to panels 2, 6, and 8).

At present, we do not know whether Set7/9 operates only through p21 to activate the DNA damage checkpoint in G_2/M or whether it also enhances the ability of p53 to directly repress cdc2, cyclin B, cdc25, and 14-3-3 genes, which are required for G_2/M transition.

Irrespective of the mechanism, the present study demonstrates that the efficiency of DNA damage-induced G_2/M arrest depends on Set7/9 and correlates with the K372 methylation status of p53. In line with this notion, Set7/9 had no effect on the cell cycle arrest elicited by 5-fluorouracil treatment, which induces ribosomal stress rather than DNA damage per se (data not shown). Collectively, these results strongly suggest that methylation of p53 on K372 by Set7/9 is a DNA damage-specific modification. Future experiments will determine whether p53 methylation is specific to single-strand or double-strand DNA breaks and whether other forms of genotoxic stress known to induce p53 operate through K372 methylation.

The fact that methylation of p53 is dynamic prompted us to investigate the role of DNA damage in regulation of Set7/9. Since neither the level of Set7/9 protein expression nor its subcellular localization changed in response to DNA damage (Fig. 1A, 3C, and data not shown), we concluded that it was the activity of Set7/9 that was altered. Interestingly, the regulation of Set7/9 KMTase activity by DNA damage seems to be biphasic. The physiological significance of this phenomenon in the case of Set7/9 is yet to be determined; however, several reports describe biphasic transcriptional regulation in response to DNA damage (4, 48). One plausible explanation of this effect is that the activity of Set7/9 itself is subject to regulation by posttranslational modifications. Our preliminary data indicate that in response to Adr treatment, the DNA-bound fraction of Set7/9 becomes phosphorylated. Interestingly, phosphorylation of Set7/9 also occurs in a biphasic manner. It is tempting to speculate that phosphorylation regulates the enzymatic activity of Set7/9 and/or the protein-protein interactions with its partner(s), e.g., p53. Of note is the fact that the in vivo methylation profile of p53 coincides with the peaks of Set7/9 activity (data not shown). In addition to activation, in vivo phosphorylation of Set7/9 may target the latter for degradation, thereby protecting cells from potentially hazardous accumulation of "super-active" Set7/9. A similar mechanism was described for ubiquitination, which stimulates the activity of several transcription factors, at the same time targeting these proteins for proteosome-dependent degradation (25, 47). If true, then phosphorylated hyperactive Set7/9 molecules tagged for degradation should at some point be replenished by the "naive" nonphosphorylated and hypoactive form of Set7/9, allowing the cell to decide whether to continue the program of p53 activation. Irrespective of the physiological consequences, the fact that KMTase activity of Set7/9 is biphasic indicates that it is subject to regulation by the DNA damage-induced signal transduction pathways.

Importantly, our extensive analysis of Set7/9-dependent methylation upon DNA damage revealed that Set7/9, even in its "active" state, was unable to efficiently methylate nucleosomal histone H3, which is a bona fide substrate for chromatin-related enzymes. Collectively, our data strongly suggest that Set7/9 exerts its function as a factor methyltransferase but not as a histone methyltransferase.

How does methylation of p53 on K372 lead to p53 activation and stabilization as a transcription factor? One possibility is that methylation of p53 at K372 directly interferes with its ubiquitination at the same lysine residue and therefore stabilizes the protein. However, at least in vitro, p53 was shown to undergo ubiquitination at multiple lysines, including K370, K372, K373, K381, and K382, whereas Set7/9 methylates p53 only at one residue, K372. Therefore, it is unlikely that the monomethylation of one lysine residue is able to efficiently abolish ubiquitination at five other sites.

A plausible alternative to this scenario is that K372 methylation may stabilize p53 through triggering another modification rival to ubiquitination, e.g., acetylation. Acetylation is well documented to inhibit ubiquitination of the same target lysines in the C terminus of p53 (7, 38). Our data, which suggest that methylation precedes and enhances acetylation of p53 in vivo, support the latter scenario. The exact molecular mechanism of this methylation-acetylation interplay is still unclear but is likely to be complex. The fact that methylation of the p53 peptide at K372 failed to enhance the efficiency of its subsequent acetylation by the recombinant p300 protein in vitro strongly indicates that this connection is indirect and may involve other accessory protein(s). In addition, our in vivo data indicate that the dynamics of p53 methylation overlap with, but do not exactly parallel, acetylation (Fig. 6B and 7). This observation is in agreement with our in vitro acetylation and methylation data, whereby robust acetylation inhibits methylation of p53 (Fig. 6A). Collectively, these results suggest that K372 methylation plays a positive role in acetylation of p53

only at the early stage of DNA damage and later becomes dispensable for acetylation.

Why is K372 methylation important for acetylation of p53 immediately after DNA damage? One possibility is that K372 methylation is required to counteract the negative effect of K370 methylation, which, in turn, inhibits p53 binding to DNA (21). In line with this assumption, it was shown that DNA damage-induced methylation of K372 by Set7/9 repressed p53-K370 methylation by Smyd2 (21). Thus, methylation of p53 on K372 may facilitate its DNA binding in vivo. It is well documented that DNA-bound, tetrameric p53 represents a better substrate for p300/CBP-mediated acetylation compared to its unbound, monomeric form (11, 14). In agreement with this hypothesis, we observed a stimulatory effect of K372 methylation on acetylation only in the subfraction of insoluble, DNA-bound p53, whereas in the soluble fraction, methylation had no obvious effect on p53 acetylation (Fig. 5C). This finding strongly argues that Set7/9-dependent methylation plays a role in regulation of p53 only in the context of chromatin. Moreover, our cell cycle results, which revealed the critical role of Set7/9 in regulation of the p53 ability to induce G_2/M arrest (Fig. 2A), along with the fact that p53 acetylation is also crucial for this process (3, 23), strongly imply the existence of a functional cross talk between K372 methylation and acetylation in the p53 molecule.

It is also important to note that similar positive interplay between lysine methylation and acetylation has already been described for histone H3 (41, 61). Specifically, K4-H3 methylation blocks the deacetylation or methylation of K9-H3, hence, allowing S10-H3 phosphorylation to occur (16, 41, 61). The latter modification, in turn, enhances K14-H3 acetylation (30, 31).

Lastly, it should be noted that the exact mechanism of methylation-dependent acetylation of p53 remains to be described. After the parallel between p53 and histone modifications, it is tempting to speculate that one of the members of the ING (for inhibitor of growth) family, which were recently discovered to bind methylated K4 of histone H3 (50, 58) via their plant homeodomains, can also specifically recognize methylated K372 of p53 to recruit histone acetyltransferase(s) to enforce subsequent p53 acetylation. On a related note, ectopic expression of ING2 and ING4/5 were shown to increase the levels of p53 acetylation in vivo (40, 51). Future studies will test this intriguing possibility as well as elucidate the complex cascade of posttranslational modifications that precisely regulate the critical function of p53 in response to genotoxic stress.

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REFERENCES

1. **Appella, E., and C. W. Anderson.** 2001. Post-translational modifications and activation of p53 by genotoxic stresses. Eur. J. Biochem. **268:**2764–2772.
2. **Barlev, N. A., L. Liu, N. H. Chehab, K. Mansfield, K. G. Harris, T. D. Halazonetis, and S. L. Berger.** 2001. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol. Cell **8:**1243–1254.
3. **Basile, V., R. Mantovani, and C. Imbriano.** 2006. DNA damage promotes histone deacetylase 4 nuclear localization and repression of G_2/M promoters, via p53 C-terminal lysines. J. Biol. Chem. **281:**2347–2357.
4. **Benton, M. G., S. Somasundaram, J. D. Glasner, and S. P. Palecek.** 2006. Analyzing the dose-dependence of the *Saccharomyces cerevisiae* global transcriptional response to methyl methanesulfonate and ionizing radiation. BMC Genomics **7:**305.
5. **Bode, A. M., and Z. Dong.** 2004. Post-translational modification of p53 in tumorigenesis. Nat. Rev. Cancer **4:**793–805.
6. **Brooks, C. L., and W. Gu.** 2006. p53 ubiquitination: Mdm2 and beyond. Mol. Cell **21:**307–315.
7. **Brooks, C. L., and W. Gu.** 2003. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr. Opin. Cell Biol. **15:**164–171.
8. **Brown, M. A., R. J. Sims III, P. D. Gottlieb, and P. W. Tucker.** 2006. Identification and characterization of Smyd2: a split SET/MYND domain-containing histone H3 lysine 36-specific methyltransferase that interacts with the Sin3 histone deacetylase complex. Mol. Cancer **5:**26.
9. **Bunz, F., A. Dutriaux, C. Lengauer, T. Waldman, S. Zhou, J. P. Brown, J. M. Sedivy, K. W. Kinzler, and B. Vogelstein.** 1998. Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. Science **282:**1497–1501.
10. **Ceribelli, M., M. Alcalay, M. A. Vigano, and R. Mantovani.** 2006. Repression of new p53 targets revealed by ChIP on chip experiments. Cell Cycle **5:**1102–1110.
11. **Ceskova, P., H. Chichger, M. Wallace, B. Vojtesek, and T. R. Hupp.** 2006. On the mechanism of sequence-specific DNA-dependent acetylation of p53: the acetylation motif is exposed upon DNA binding. J. Mol. Biol. **357:**442–456.
12. **Chuikov, S., Y. Kurash, J. R. Wilson, B. Xiao, N. Justin, G. Ivanov, K. McKinney, P. Tempst, C. Prives, S. Gamblin, N. Barlev, and D. Reinberg.** 2004. Regulation of p53 activity through lysine methylation. Nature **432:**353–360.
13. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
14. **Dornan, D., H. Shimizu, N. D. Perkins, and T. R. Hupp.** 2003. DNA-dependent acetylation of p53 by the transcription coactivator p300. J. Biol. Chem. **278:**13431–13441.
15. **el Deiry, W., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
16. **Fischle, W., Y. Wang, and C. D. Allis.** 2003. Binary switches and modification cassettes in histone biology and beyond. Nature **425:**475–479.
17. **Gostissa, M., A. Hengstermann, V. Fogal, P. Sandy, S. E. Schwarz, M. Scheffner, and G. Del Sal.** 1999. Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. EMBO J. **18:**6462–6471.
18. **Gu, W., and R. Roeder.** 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell **90:**595–606.
19. **Harris, C. C.** 1993. p53: at the crossroads of molecular carcinogenesis and risk assessment. Science **262:**1980–1981.
20. **Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris.** 1991. p53 mutations in human cancers. Science **253:**49–53.
21. **Huang, J., L. Perez-Burgos, B. J. Placek, R. Sengupta, M. Richter, J. A. Dorsey, S. Kubicek, S. Opravil, T. Jenuwein, and S. L. Berger.** 2006. Repression of p53 activity by Smyd2-mediated methylation. Nature **444:**629–632.
22. **Hupp, T. R., D. W. Meek, C. A. Midgley, and D. P. Lane.** 1992. Regulation of the specific DNA binding function of p53. Cell **71:**875–886.
23. **Imbriano, C., A. Gurtner, F. Cocchiarella, S. Di Agostino, V. Basile, M. Gostissa, M. Dobbelstein, G. Del Sal, G. Piaggio, and R. Mantovani.** 2005. Direct p53 transcriptional repression: in vivo analysis of CCAAT-containing G_2/M promoters. Mol. Cell. Biol. **25:**3737–3751.
24. **Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R. W. Craig.** 1991. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. **51:**6304–6311.
25. **Kim, S. Y., A. Herbst, K. A. Tworkowski, S. E. Salghetti, and W. P. Tansey.** 2003. Skp2 regulates Myc protein stability and activity. Mol. Cell **11:**1177–1188.
26. **Kouskouti, A., E. Scheer, A. Staub, L. Tora, and I. Talianidis.** 2004. Gene-specific modulation of TAF10 function by SET9-mediated methylation. Mol. Cell **14:**175–182.
27. **Kwek, S. S., J. Derry, A. L. Tyner, Z. Shen, and A. V. Gudkov.** 2001. Functional analysis and intracellular localization of p53 modified by SUMO-1. Oncogene **20:**2587–2599.
28. **Leonardo, A. D., S. P. Linke, K. Clarkin, and G. M. Wahl.** 1994. DNA damage triggers a prolonged p53-dependent G_1 arrest and a long-term induction of Cip1 in normal human fibroblasts. Genes Dev. **8:**2540–2551.
29. **Liu, G., T. Xia, and X. Chen.** 2003. The activation domains, the proline-rich domain, and the C-terminal basic domain in p53 are necessary for acetylation of histones on the proximal p21 promoter and interaction with p300/CREB-binding protein. J. Biol. Chem. **278:**17557–17565.
30. **Lo, W. S., E. R. Gamache, K. W. Henry, D. Yang, L. Pillus, and S. L. Berger.**

2005. Histone H3 phosphorylation can promote TBP recruitment through distinct promoter-specific mechanisms. EMBO J. **24:**997–1008.
31. **Lo, W.-S., R. C. Trievel, J. R. Rojas, L. Duggan, J.-Y. Hsu, C. D. Allis, R. Marmorstein, and L. Berger.** 2000. Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14. Mol. Cell **5:**917–926.
32. **Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Housman.** 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74:**957–967.
33. **Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne, and T. Jacks.** 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature **362:**847–849.
34. **Luo, J., M. Li, Y. Tang, M. Laszkowska, R. G. Roeder, and W. Gu.** 2004. Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. Proc. Natl. Acad. Sci. USA **101:**2259–2264.
35. **Maltzman, W., and L. Czyzyk.** 1984. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell. Biol. **4:**1689–1694.
36. **Massague, J.** 2004. G_1 cell-cycle control and cancer. Nature **432:**298–306.
37. **Miyashita, T., S. Krajewski, M. Krajewska, H. G. Wang, H. K. Lin, D. A. Liebermann, B. Hoffman, and J. C. Reed.** 1994. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene **9:**1799–1805.
38. **Morgunkova, A., and N. A. Barlev.** 2006. Lysine methylation goes global. Cell Cycle **5:**1308–1312.
39. **Mujtaba, S., Y. He, L. Zeng, S. Yan, O. Plotnikova, Sachchidanand, R. Sanchez, N. J. Zeleznik-Le, Z. Ronai, and M. M. Zhou.** 2004. Structural mechanism of the bromodomain of the coactivator CBP in p53 transcriptional activation. Mol. Cell **13:**251–263.
40. **Nagashima, M., M. Shiseki, K. Miura, K. Hagiwara, S. P. Linke, R. Pedeux, X. W. Wang, J. Yokota, K. Riabowol, and C. C. Harris.** 2001. DNA damage-inducible gene p33ING2 negatively regulates cell proliferation through acetylation of p53. Proc. Natl. Acad. Sci. USA **98:**9671–9676.
41. **Nishioka, K., S. Chuikov, K. Sarma, H. Erdjument-Bromage, C. D. Allis, P. Tempst, and D. Reinberg.** 2002. Set9, a novel histone H3 methyltransferase that facilitates transcription by precluding histone tail modifications required for heterochromatin formation. Genes Dev. **16:**479–489.
42. **Nishioka, K., J. C. Rice, K. Sarma, H. Erdjument-Bromage, J. Werner, Y. Wang, S. Chuikov, P. Valenzuela, P. Tempst, R. Steward, J. T. Lis, C. D. Allis, and D. Reinberg.** 2002. PR-Set7 is a nucleosome-specific methyltransferase that modifies lysine 20 of histone H4 and is associated with silent chromatin. Mol. Cell **9:**1201–1213.
43. **Polyak, K., Y. Xia, J. L. Zweier, K. W. Kinzler, and B. Vogelstein.** 1997. A model for p53-induced apoptosis. Nature **389:**300–305.
44. **Saito, S., H. Yamaguchi, Y. Higashimoto, C. Chao, Y. Xu, A. J. Fornace, Jr., E. Appella, and C. W. Anderson.** 2003. Phosphorylation site interdependence of human p53 posttranslational modifications in response to stress. J. Biol. Chem. **278:**37536–37544.
45. **Sakaguchi, K., J. E. Herrera, S. Saito, T. Miki, M. Bustin, A. Vassilev, C. W. Anderson, and E. Appella.** 1998. DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev. **12:**2831–2841.
46. **Sakaguchi, K., H. Sakamoto, D. Xie, J. W. Erickson, M. S. Lewis, C. W. Anderson, and E. Appella.** 1997. Effect of phosphorylation on tetramerization of the tumor suppressor protein p53. J. Protein Chem. **16:**553–556.
47. **Salghetti, S. E., A. A. Caudy, J. G. Chenoweth, and W. P. Tansey.** 2001. Regulation of transcriptional activation domain function by ubiquitin. Science **293:**1651–1653.
48. **Shah, G., R. Ghosh, P. A. Amstad, and P. A. Cerutti.** 1993. Mechanism of induction of c-fos by ultraviolet B (290–320 nm) in mouse JB6 epidermal cells. Cancer Res. **53:**38–45.
49. **Sheikh, M. S., T. F. Burns, Y. Huang, G. S. Wu, S. Amundson, K. S. Brooks, A. J. Fornace, Jr., and W. S. el-Deiry.** 1998. p53-dependent and -independent regulation of the death receptor KILLER/DR5 gene expression in response to genotoxic stress and tumor necrosis factor alpha. Cancer Res. **58:**1593–1598.
50. **Shi, X., T. Hong, K. L. Walter, M. Ewalt, E. Michishita, T. Hung, D. Carney, P. Pena, F. Lan, M. R. Kaadige, N. Lacoste, C. Cayrou, F. Davrazou, A. Saha, B. R. Cairns, D. E. Ayer, T. G. Kutateladze, Y. Shi, J. Cote, K. F. Chua, and O. Gozani.** 2006. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature **442:**96–99.
51. **Shiseki, M., M. Nagashima, R. M. Pedeux, M. Kitahama-Shiseki, K. Miura, S. Okamura, H. Onogi, Y. Higashimoto, E. Appella, J. Yokota, and C. C. Harris.** 2003. p29ING4 and p28ING5 bind to p53 and p300, and enhance p53 activity. Cancer Res. **63:**2373–2378.
52. **Su, L., Y. Sai, R. Fan, S. W. Thurston, D. P. Miller, W. Zhou, J. C. Wain, T. J. Lynch, G. Liu, and D. C. Christiani.** 2003. P53 (codon 72) and P21 (codon 31) polymorphisms alter in vivo mRNA expression of p21. Lung Cancer **40:**259–266.
53. **Taylor, W. R., and G. R. Stark.** 2001. Regulation of the G_2/M transition by p53. Oncogene **20:**1803–1815.
54. **Wang, C., A. Ivanov, L. Chen, W. J. Fredericks, E. Seto, F. J. Rauscher III, and J. Chen.** 2005. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. EMBO J. **24:**3279–3290.
55. **Wang, H., R. Cao, L. Xia, H. Erdjument-Bromage, C. Borchers, P. Tempst, and Y. Zhang.** 2001. Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase. Mol. Cell **8:**1207–1217.
56. **Waterman, M. J., E. S. Stavridi, J. L. Waterman, and T. D. Halazonetis.** 1998. ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. Nat. Genet. **19:**175–178.
57. **Willis, S. N., and J. M. Adams.** 2005. Life in the balance: how BH3-only proteins induce apoptosis. Curr. Opin. Cell Biol. **17:**617–625.
58. **Wysocka, J., T. Swigut, H. Xiao, T. A. Milne, S. Y. Kwon, J. Landry, M. Kauer, A. J. Tackett, B. T. Chait, P. Badenhorst, C. Wu, and C. D. Allis.** 2006. A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodeling. Nature **442:**86–90.
59. **Xu, Y.** 2003. Regulation of p53 responses by posttranslational modifications. Cell Death Differ. **10:**400–403.
60. **Yun, J., H. D. Chae, H. E. Choy, J. Chung, H. S. Yoo, M. H. Han, and D. Y. Shin.** 1999. p53 negatively regulates cdc2 transcription via the CCAAT-binding NF-Y transcription factor. J. Biol. Chem. **274:**29677–29682.
61. **Zegerman, P., B. Canas, D. Pappin, and T. Kouzarides.** 2002. Histone H3 lysine 4 methylation disrupts binding of nucleosome remodeling and deacetylase (NuRD) repressor complex. J. Biol. Chem. **277:**11621–11624.
62. **Zhao, R., K. Gish, M. Murphy, Y. Yin, D. Notterman, W. H. Hoffman, E. Tom, D. H. Mack, and A. J. Levine.** 2000. Analysis of p53-regulated gene expression patterns using oligonucleotide arrays. Genes Dev. **14:**981–993.