Modulation of p53 C-Terminal Acetylation by mdm2, p14ARF, and Cytoplasmic SirT2

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Abstract

Acetylation of C-terminal lysine residues in the p53 tumor suppressor is associated with increased stability and transcription factor activity. The function, protein level, and acetylation of p53 are downregulated by mdm2, which in its turn is inhibited by the p14ARF tumor suppressor. Here, we show that p14ARF increases the level of p53 acetylated at lysine 382 in a nuclear chromatin-rich fraction. Unexpectedly, this accumulation of p53AcK382 is dramatically enhanced in the presence of ectopic mdm2. In light of these observations, we propose that p14ARF increases the binding of p53–mdm2 complexes to chromatin, thereby limiting the access of protein deacetylases to p53. Supporting this notion, we show that p53AcK382 can be deacetylated in the cytoplasm and that sirtuin SirT2 catalyzes this reaction. These results help understand why inhibition of both SirT1 and SirT2 is needed to achieve effective activation of p53 by small-molecule sirtuin inhibitors. Mol Cancer Ther; 12(4): 1–10. ©2013 AACR.

Introduction

p53 is subjected to a variety of posttranslational modifications that modulate its function as a tumor suppressor (1). Acetylation of p53 at C-terminal lysine residues reduces binding to mdm2 and prevents ubiquitination and subsequent degradation of p53 (2). In addition, C-terminal acetylation increases the affinity of p53 for DNA (3). Thus, p53 acetylation at the C-terminus not only leads to stabilization but also promotes the function of p53 as a transcription factor. Acetylation of p53 is carried out by several protein acetyltransferases including p300 and CREB-binding protein (CBP), whereas deacetylation is catalyzed by protein deacetylases such as class I and II histone deacetylases (HDAC) and SirT1 of the sirtuin family of class III HDACs (4).

Sirtuins possess a high degree of homology in their central regions (5) and catalyze deacetylation of lysine residues or ADP ribosylation reactions. Their activity is dependent on NAD+, a property that links sirtuin function to metabolic status. SirT1, which is primarily located in the nucleus, deacetylates p53 at lysine 382, thereby enabling its ubiquitination by ubiquitin ligases such as mdm2 and subsequent proteasomal degradation (4). SirT2 is primarily cytoplasmic, and it has been shown to reduce p53 acetylation at K382 in adenovirus-positive HEK293 cells (6), where the adenoviral E1B55KD protein is known to lead to the accumulation of large amounts of p53 in a cytoplasmic juxtanuclear body (7). SirT2 has also been implicated in regulating p53 in a study using HeLa cells (8), which are infected with human papillomavirus (HPV). In HeLa cells, p53 degradation is governed by the action of the HPV protein E6 instead of mdm2. To date, there is no data about a possible effect of SirT2 on p53 acetylation in a non–virus-infected background. A recent study has reported that p53 is a substrate for mammalian SirT3 in the mitochondria (9). Finally, SirT7, a nuclear sirtuin preferentially localized in the nucleolus, has been shown to deacetylate p53 in vitro (10). The remaining members of the sirtuin family, SirT4, SirT5, and SirT6, have not been implicated in regulating p53 acetylation (reviewed in ref. 11).

mdm2 is the major negative regulator of the function of p53 and therefore is regarded as an attractive target for cancer therapy (12). mdm2 binds directly to p53, obstructing the transactivation domain of p53 and interferes with the function of p53 as a transcription factor (13). In addition, mdm2 contains a RING finger domain that catalyzes the ubiquitination of p53 at lysine residues (14). This ubiquitination leads to export of p53 from the nuclear compartment and subsequent degradation. In addition, mdm2 has been reported to inhibit acetylation of p53 by protein acetyltransferases (15–17) as well as to promote deacetylation by HDACs (18).

The p14ARF tumor suppressor is a small protein that binds directly to the acidic domain of mdm2. It constitutes the major inhibitor of mdm2 and is also a very effective
activator of the transcription factor function of p53 (reviewed in ref. 19). p14\(^{ARF}\) is very rich in arginine residues and has an isoelectric point of 12.91, which is higher than the 11.54 of histone H3. There are several possible models attempting to explain the mechanism by which p14\(^{ARF}\) has such a strong influence on both p53 stability and activity. p14\(^{ARF}\) has been proposed to sequester mdm2 in the nucleolar compartment, inhibit export of p53 from the nucleus, and/or downregulate p53 ubiquitination by mdm2 (20–22). These models, although not incompatible, have fuelled considerable debate since their proposal more than a decade ago (23, 24).

Here, we present new studies on the effects of mdm2 and p14\(^{ARF}\) on the stability, localization, ubiquitination, and acetylation of p53. We found that expression of ectopic mdm2 caused an increase in p53 acetylation at lysine 382 when ectopic p14\(^{ARF}\) was also present. We propose that mdm2–p14\(^{ARF}\) complexes sequester p53 in a nuclear chromatin-rich fraction. As a consequence, p53 is protected from the action of protein deacetylases that are not linked to chromatin, such as cytoplasmic deacetylases. Supporting this model, we show that p53KAc382 is a substrate for the cytoplasmic deacetylase SirT2. This is the first time that p53 deacetylation in the cytoplasm has been shown. These findings provide an insight into why inhibition of both SirT1 and SirT2 seems to be required to achieve effective p53 activation in a nuclear chromatin-rich fraction. As a consequence, p53 is protected from the action of protein deacetylases that are not linked to chromatin, such as cytoplasmic deacetylases. Supporting this model, we show that p53KAc382 is a substrate for the cytoplasmic deacetylase SirT2. This is the first time that p53 deacetylation in the cytoplasm has been shown. These findings provide an insight into why inhibition of both SirT1 and SirT2 seems to be required to achieve effective p53 activation with small-molecule sirtuin inhibitors (25). Finally, our observations are in line with previously suggested tumor suppressor roles for mdm2 (26), and the recently described positive effect of mdm2 on p53-dependent transcription when the E3 ubiquitin ligase function of mdm2 is impaired (27).

Materials and Methods

Cells, reagents, and antibodies

MCF7 cells [purchased from American Type Culture Collection (ATCC)] and ARN8 cells (28) were grown in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with FBS and penicillin/streptomycin. H1299 cells (purchased from ATCC) were cultivated in RPMI supplemented in the same manner. H1299-pcDNA3 control cells and H1299-SirT2–overexpressing cells are described previously (29). Cells were grown at 37°C and 5% CO\(_2\) in a humidified atmosphere. The authors did not authenticate the cell lines for the present study. However, H1299s are p53-null and express high levels of endogenous p14\(^{ARF}\), whereas MCF7 cells bear wild-type p53 that can be activated by nutlin-3 treatment. These features were checked on a regular basis, and cell lines were also routinely tested for mycoplasma (Mycoplasma Alert Detection Kit, Lonza). Leptomycin B (LMB) was obtained from Cancer Research UK and stored at −80°C as a 10 mmol/L solution in absolute ethanol. Etoposide, nutlin-3 (racemic), EX527, and MG132 were purchased from Sigma. Tenovin (tnv1, tnv6, and tnvD3) were synthesized as described previously (30, 31) and stored at −20°C as 40 mmol/L solutions in dimethyl sulfoxide (DMSO). The following antibodies were used: p53 [DO1, mouse monoclonal (ref. 32), and CM-1, rabbit polyclonal (ref. 33)], p53 acetylated at K382 (BioLegend #614202, rabbit polyclonal, and AbCam #ab75754, rabbit monoclonal), mdm2 [4B2, mouse monoclonal (34)], p14\(^{ARF}\) (Santa Cruz #53392, mouse monoclonal), β-galactosidase (AbCam #ab616, rabbit polyclonal), α-tubulin (Sigma #T6199, mouse monoclonal), 20S proteasome subunit α-6 (Affiniti #PW8100, mouse monoclonal), histone H3 (AbCam #ab1791, rabbit polyclonal), histone H3 acetylated at K9 (AbCam #ab12179, mouse monoclonal), histone H4 (Upstate, #07-108, rabbit polyclonal), SirT2 (AbCam #ab51023, rabbit monoclonal), and paxillin (AbCam #ab32084, rabbit monoclonal).

Transient transfections and plasmids

Cells were seeded on 10-cm tissue culture dishes and transfected using the calcium phosphate precipitation method, as described previously (21). At least 1 hour before transfection, the medium was replaced by fresh high-glucose DMEM supplemented with 10% FBS only. For H1299 cells, the medium was changed back to RPMI-based medium 18 hours after transfection. Cells were harvested for Western blot analysis (see below) 36 hours posttransfection. The following plasmids were used: pcDNA3 constructs expressing p14\(^{ARF}\) (kind gift from K.H. Vousden, The Beatson Institute for Cancer Research, Glasgow, United Kingdom), human wild-type p53, p53-R273H, p53-K320T, p53-6KR, SirT1-H363Y, and SirT2-H187Y; pCMV constructs expressing human mdm2 (hdm2, kind gift from A.J. Levine, Institute for Advanced Study, Princeton, NJ) and hdm2-C464A (21); and pCOC-X2 constructs expressing mdm2 (kind gift from M. Oren, The Weizmann Institute of Science, Rehovot, Israel) and mdm2 N-terminal fragments (residues 1–244, 1–258, and 1–342, ref. 35). The vector expressing hdm22EVEAA mutant was generated by standard site-directed mutagenesis of the pCMV-hdm2 plasmid. Vectors expressing HAUSP/ys and His\(_e\)-tagged ubiquitin were kind gifts from D.P. Xiropoulos (The University of Dundee, Dundee, UK) and S. Mittnacht (University College of London, London, UK), respectively. BK5 and pcDNA3 empty vectors were used to equalize the total amount of DNA. β-Galactosidase was used as a control for transfection, viability, and loading efficiency.

Nuclear extraction

Cell cultures with washed twice with PBS and then the cells were scraped into 1 mL of PBS and harvested by centrifugation. The cells were resuspended in homogenization buffer (10 mmol/L Tris-HCl, pH 7.5, 10 mmol/L NaCl, and 1.5 mmol/L MgCl\(_2\) supplemented with protease inhibitor cocktail) and lysed in a Dounce homogenizer fitted with a loose pestle. Samples were centrifuged at 16,100 × g, and the pellets (nuclear chromatin-rich, N\(_a\)) and the supernatants (cytoplasmic soluble, C\(_a\)) transferred to separate tubes. The N\(_a\) fractions were washed in homogenization buffer. Samples for Western blot analysis
(see below) were prepared with 1× SDS sample buffer (Invitrogen).

**Analysis of cell extracts by Western blotting**

Western blot analysis was carried out as described previously (36). Dithiothreitol (Sigma #D06032) was added to the samples to a final concentration of 100 mmol/L. Proteins were separated on NuPAGE 4%–12% Bis–Tris gels (Invitrogen) in 1× NuPAGE MOPS buffer (Invitrogen #NP000102) supplemented with NuPAGE antioxidant (Invitrogen #NP0005). The mobility of the proteins of interest was determined using molecular weight markers (SeeBlue Plus2, Invitrogen #LC5925). Protein transfer to polyvinylidene difluoride (PVDF) membranes (Millipore #IPVH00010) was done with 1× NuPage transfer buffer (Invitrogen #NP0006). Following incubation with primary and horseradish peroxidase-conjugated secondary antibodies, chemiluminescent signals were detected with ECL solution (Amersham Biosciences). Adobe Photoshop was used to adjust the brightness and contrast of scanned blot images. All lanes were treated in the same manner.

**Immunofluorescence staining and cell imaging**

Cells were seeded on 2-well glass slide chambers (Nunc), incubated for 24 hours, and then treated as indicated. After rinsing twice with PBS, cells were fixed with ice-cold methanol–acetone (1:1), rehydrated in serum-free DMEM, and then incubated with anti-p53 (DO1 diluted in serum-free DMEM) antibody for 1 hour at room temperature. Thereafter, cells were washed 4 times and incubated with secondary fluorescently labeled antibody (FITC donkey anti-mouse IgG, Jackson ImmunoResearch Laboratories) in the dark for 45 minutes at room temperature. Cells were counterstained with Hoechst (Sigma #H1155) for 1 minute, and then the slides were mounted using Hydromount (National Diagnostics #HS106). Cells were visualized using an Axiovert 200M microscope from Zeiss powered with the Volocity software from Improvison and a Hamamatsu Orca ER camera.

**SirT2 expression and purification**

SirT2 cDNA (residues 34–356) was cloned into the PGE6-6P-1 vector (GE Life Sciences) and overexpressed in BL21(DE3)T1R bacterial cells (Sigma) as a glutathione S-transferase (GST) fusion protein. Purification of the GST-SirT2 was achieved using glutathione sepharose (GE LifeSciences) followed by cleavage of the fusion protein using PreScission protease (GE LifeSciences). SirT2 was separated from GST via glutathione sepharose chromatography (GSTrap 4B, GE LifeSciences) and the SirT2 containing fractions pooled and further purified via gel filtration chromatography (HiLoad 16/60 Superdex 75, GE Healthcare).

**Sirtuin activity assays**

**Fluorescence-based assay.** Deacetylation assays were conducted as described previously (29) using recombinant SirT2 prepared as described and components from the Fluor de Lys Fluorescent Assay System (Enzo Life Sciences kits BML-AK555 and BML-AK556). SirT2 was used at a final concentration of 0.01 mg/mL and the FdL-SirT1/T2 substrates at 15 μmol/L. The FdL-SirT1 substrate is a peptide consisting of residues 379–382 of human p53 [Arg-His-Lys(Lys(Ac))] whereas the FdL-SirT2 peptide is composed of residues 317–320 of human p53 [Gln-Pro-Lys(Lys(Ac))].

**Assay using membrane-bound acetylated p53.** Whole-cell extracts were separated by electrophoresis using 4%–12% Bis–Tris gels (Invitrogen) and transferred to PVDF membranes (Millipore). Membrane pieces containing the protein substrates of interest were cut and incubated in 10× Complete EDTA-free protease inhibitor (Roche), 527 mmol/L Tris pH 7.5, 5 mmol/L dithiothreitol, 750 mmol/L NaCl in the absence or presence of SirT2 (0.023 mg/mL), and 5 mmol/L NAD⁺ for 90 minutes at 37°C. Relevant antigens were detected by chemiluminescence following incubation with primary and horseradish peroxidase-conjugated secondary antibodies.

**Results**

**mdm2 increases p53 C-terminal acetylation in the presence of p14ARF**

According to the most accepted model, p53 is mono-ubiquitinated by mdm2 in the nuclear compartment and then exported to the cytoplasm where it is polyubiquitinated and subsequently degraded by the proteasome (37). However, some degree of p53 polyubiquitination and degradation can also occur in the nuclear compartment (38). We conducted cytoplasmic and nuclear fractionations based on the classic Dignam method (39), which separates cytoplasmic proteins such as α-tubulin and paxillin from chromatin-bound proteins such histones H3 and H4. Using this method, we observed that p53, as well as its ubiquitinated forms, accumulates primarily in the cytoplasmic compartment upon a short treatment with the proteasome inhibitor MG132 (Fig. 1A and B). This suggests that the degradation of ubiquitinated forms of p53 occurs mainly in the cytoplasm. However, we detected a substantial leakage of nuclear p53, and in particular ubiquitinated p53, during sample preparation, as evidenced in Fig. 1C. Therefore, we conclude that the Dignam extraction method separates a fraction containing mainly cytoplasmic p53, but also soluble nuclear p53, and a nuclear chromatin-enriched fraction containing insoluble p53 that is likely to be tightly bound to nucleic acids. We named these fractions Cᵣ and Nᵢₑᵣ, respectively. In agreement with this interpretation, the nuclear export inhibitor and p53 activator LMB (40) has no effect on the Cᵣ and Nᵢₑᵣ distribution of p53 obtained by the Dignam method (Fig. 1D). However, among the extraction methods tested, the Dignam protocol provided the best separation.
The p14ARF tumor suppressor is a highly positively charged protein with a high affinity for chromatin. As can be seen in Fig. 1D, in the presence of ectopic p14ARF, most p53 forms accumulate in the Nch compartment, but this does not occur when a dominant-negative mutant of the HAUSP p53 deubiquitinase (41) is added to the system. Furthermore, leakage of ubiquitin-conjugated p53 from the Nch fraction is substantially diminished in the presence of p14ARF (Fig. 1C).

When an antibody against p53 acetylated at lysine 382 was used, we observed that the expression of ectopic p14ARF leads to a slight increase in the levels of p53AcK382 in the Nch fraction (Fig. 2A). In addition, we noted that the level of p53AcK382 in Nch fractions from cells expressing human mdm2 together with p14ARF was much higher than the levels observed in counterparts expressing p14ARF alone (Fig. 2A). The large increase in p53AcK382 induced by combining ectopic mdm2 and p14ARF occurred independently of the transcriptional activity of p53 (Fig. 2B).

A strong joint effect of p14ARF and mdm2 on the level of p53 acetylation also occurs with the hdm2–C464A mutant (Fig. 2A), indicating that ubiquitin and nedd8 ligase activities of mdm2 are dispensable in this context. The hdm2–C464A construct has a critical mutation in the RING finger domain of mdm2 that impairs ubiquitin/nedd8 conjugation. However, the regions of mdm2 that interact with p14ARF are essential, as shown by the weak effect of the

Figure 1. Localization of ubiquitin-conjugated and unconjugated forms of p53. Cells were transiently transfected with plasmids expressing p53, p53-R273H, human mdm2 (hdm2), His6-tagged ubiquitin, and bacterial β-galactosidase. Cytoplasmic (Cs) and nuclear (Nch) fractions were separated as described in Materials and Methods, and protein levels analyzed by Western blotting. MCF7 cells (A) and H1299 cells (B) were treated with 20 μmol/L MG132 for 1 hour before harvesting or left untreated. Blots were probed with antibodies against p53, β-galactosidase, histone H3, and alpha-6 proteasomal subunit. C, the ubiquitinated forms of p53 become strongly linked to the Nch fraction in the presence of hdm2 and p14ARF. H1299 cells were transfected with plasmids expressing p53-R273H and hdm2 or with empty control vectors. Cs and Nch fractions from transfected cells were mixed as indicated and, thereafter, the resulting samples were subjected to a new centrifugation to separate the Cs and Nch fractions again. D, H1299 cells expressing ectopic p53-R273H were also transfected with plasmids expressing hdm2 wild-type or hdm2-EVEAAA (residues 250–252 in hdm2 acidic domain substituted by alanine, which prevents polyubiquitination and degradation of p53). Where indicated, cells were also transfected with plasmids expressing p14ARF and a dominant-negative HAUSP construct (HAUSPcys) or treated with 20 nmol/L LMB for 3 hours.
deletion mutant mdm2-D4 on the p14arf-mediated increase in the level of acetylated p53 in the nucleus (Fig. 2C). Unlike mdm2 and hdm2-C464A, mdm2-D4 does not bind to p14arf (35). In addition, as can be seen in Fig. 2D, the action of p14arf on the level of acetylation of mutant p53. The p53-R273H mutant is transcriptionally inactive. The p53-6KR construct, where the 6 C-terminal lysines have been mutated to arginine, serves to assess the specificity of the antibody against p53AcK382. C, cells were transfected with plasmids expressing 3 different mdm2 deletion mutants: Δ11 (residues 1–342), Δ18 (residues 1–258), or Δ4 (residues 1–244). The Δ11 and the Δ18 mdm2 deletion mutants, but not the Δ4 mutant, bind to p14arf (35). D, cells were transfected as indicated and incubated with 20 μmol/L MG132 for 1 hour before harvesting or left untreated. α-Tubulin and paxillin were used as cytoplasmic markers and histone H3 as a nuclear marker.

Figure 2. Effect of p14arf on the levels of p53 acetylated at lysine 382. H1299 cells were transfected with plasmids expressing p53, human mdm2 (hdm2), p14arf, and β-galactosidase. Cytoplasmic (C) and nuclear (N) fractions were separated as described in Materials and Methods, and protein levels analyzed by Western blotting. A, comparison of the effect of p14arf on acetylated p53 in the presence or absence of hdm2. The hdm2-C464A construct is defective for both ubiquitination and neddylation. B, effect of p14arf on the level of acetylation of mutant p53. The p53-R273H mutant is transcriptionally inactive. The p53-6KR construct, where the 6 C-terminal lysines have been mutated to arginine, serves to assess the specificity of the antibody against p53AcK382. C, cells were transfected with plasmids expressing 3 different mdm2 deletion mutants: Δ11 (residues 1–342), Δ18 (residues 1–258), or Δ4 (residues 1–244). The Δ11 and the Δ18 mdm2 deletion mutants, but not the Δ4 mutant, bind to p14arf (35). D, cells were transfected as indicated and incubated with 20 μmol/L MG132 for 1 hour before harvesting or left untreated. α-Tubulin and paxillin were used as cytoplasmic markers and histone H3 as a nuclear marker.

Deacetylation of p53 by SirT2

According to Fig. 2, p14arf causes accumulation of acetylated p53 in the N fraction and this is enhanced by mdm2. However, on the basis of existing experimental data, mdm2 is unlikely to have a direct positive effect on the acetylation of p53 at the biochemical level even in the presence of p14arf. For instance, it has been shown that mdm2 inhibits p300-mediated p53 acetylation by forming a ternary complex with these 2 proteins (15) and that mdm2–HDAC1 complexes enhance p53 deacetylation (18).

As mentioned in the introduction, SirT1 is an important p53 deacetylase. However, as shown in Supplementary Figs. S1B and S2, results obtained by overexpressing SirT1 or a dominant-negative mutant SirT1 suggest that SirT1 is not the only factor modulating p53 deacetylation in
our model system. Therefore, to explain our data about the levels of acetylated p53 in the N_c fraction, we hypothesized that p14 ARF, through its ability to retain p53–mdm2 complexes in the nucleus, could be protecting p53 from deacetylation by cytoplasmic deacetylases. Thus, we set out to test whether SirT2, a deacetylase known to be located primarily in the cytoplasmic compartment (42), had any effect on p53 acetylation.

There is previous evidence that SirT2 can affect p53 acetylation (6, 8). However, these studies were conducted in cell lines in which viral proteins govern the degradation of p53. Furthermore, these studies did not establish whether acetylated p53 is a substrate for SirT2. Here, we show that SirT2 can deacetylate full-length p53 directly in a biochemical assay. This can be seen in Fig. 3A, where membrane-bound acetylated p53 was incubated in the presence and absence of purified SirT2 and NAD^+. In addition, SirT2 deacetylates a p53 peptide containing acetylated K382 (Fig. 3B).

Tenovin-6 is a small-molecule inhibitor of both SirT1 and SirT2 (29) that can increase the levels of p53 acetylated at K382 as shown in Fig. 3C. Supporting that inhibition of SirT2 by tenovin-6 contributes to this effect, overexpression of SirT2 reduces p53 acetylation level (Fig. 3C). In contrast, tenovin-1 and -6 lead to an increase in both nuclear and cytoplasmic p53AcK382 (Fig. 4D).

In agreement with previous reports, in our system, SirT2 is mainly cytoplasmic (Fig. 4A and C), whereas SirT1 is primarily nuclear (Supplementary Fig. S3). SirT2 is actively exported from the nucleus in a Crm1-dependent manner and, following LMB treatment, SirT2 accumulates rapidly in the nucleus (44). However, in our experiments, we were not able to detect an effect of LMB on the distribution of SirT2 or p53 (Fig. 4C). This is possibly due to leakage of non–chromatin-bound proteins from the N_c fraction during the extraction procedure.

The data presented in Figs. 3 and 4 indicate that SirT2 is involved in the deacetylation of p53 in the cytoplasmic compartment. Further supporting this postulation, we observed that in cells stably transfected with a plasmid expressing SirT2 the levels of p53AcK382 in the C_c fraction are reduced while the levels of p53AcK382 in the N_c compartment, where SirT2 levels are lower, remain unaffected by SirT2 expression (Fig. 5A). Accordingly, expression of the deacetylase-

**Figure 3.** Identification of p53AcK382 as a SirT2 substrate. A, ARN8 cells were treated with 1 μmol/L EX527 and 20 μmol/L etoposide for 6 hours to raise the levels of acetylated p53. Whole-cell extracts were separated by electrophoresis and transferred to PVDF membranes. Membrane pieces containing the protein substrates of interest were cut and incubated in the absence or presence of SirT2 (0.023 mg/mL) and 5 mmol/L NAD^+ for 90 minutes at 37 °C. B, SirT2 deacetylates both SirT1 and SirT2 Fluor de Lys (FdL) fluorogenic substrates derived from acetylated lysine residues within p53. The FdL p53-382 and p53-320 peptides contain acetylated residues K382 and K320, respectively. Recombinant SirT2 was incubated with 15 μmol/L of each substrate and 1 mmol/L NAD^+ and deacetylation detected by measuring the fluorescence generated. C, H1299 were transiently transfected with plasmids expressing p53 (wild-type), SirT2, and β-galactosidase and incubated with the indicated doses of tenovin-6 for 6 hours. Whole-cell extracts were analyzed by Western blotting.
defective SirT2-H187Y dominant-negative mutant led to an increase in p53AcK382 (Fig. 5B). Possibly because of the presence of some ectopic SirT2-H187Y in the nucleus as a consequence of overexpression (Fig. 5B), we also observed a slight increase in p53-dependent transcription in the presence of this SirT2 mutant (Fig. 5C). An equivalent mutant of SirT1 had a much stronger effect (Fig. 5C).

Discussion

Recent reports show that the acetylation status of p53 plays a crucial role in the modulation of p53 levels and transcription factor activity (4). For example, acetylation of C-terminal lysines in p53 is associated with stability and increased transcription factor activity of this tumor suppressor. mdm2 impairs p53 transcription factor activity, catalyzes p53 ubiquitination, promotes its export from the nucleus, and targets it for degradation. In addition, mdm2 downregulates p53 acetylation by protein acetyltransferases (15–17) and favors deacetylation of p53 by HDACs such as HDAC1 (18). Therefore, it is not unexpected that there is an enhanced accumulation of p53 and p53AcK382 in whole-cell extracts in the presence of mdm2 inhibitor p14ARF (16, 45).

According to published data, p14ARF expression reverses the negative effect of mdm2 on p300-mediated acetylation of p53 (16). In addition, p14ARF activates p53 acetyltransferases such as p300, thus resulting in further stabilization and acetylation of p53 (45). Here, we confirm that p14ARF does indeed increase p53 acetylation. Correspondingly, we show that this accumulation of p53AcK382 occurs in chromatin-rich fractions, which explains further why p14ARF is a very efficient activator of p53-dependent transcription. The p14ARF-mediated accumulation of p53 and acetylated p53 in chromatin fractions confirms and completes previous studies,
suggesting that p14 ARF inhibits the export of p53 from the nucleus (20). However, it was striking that the levels of p33AcK382 in the Nch fraction reached in the presence of both p14ARF and mdm2 were higher than those observed in the absence of mdm2. This observation was confirmed by 2 different chromatin extraction methods (Fig. 2A and Supplementary Fig. S1A). Hence, contrary to expectations, our results show that the effect of p14 ARF on p53 acetylation at lysine 382 is enhanced, rather than diminished, by the presence of mdm2. Given this paradoxical observation, we hypothesize that p14 ARF increases the association of p53-mdm2 complexes to chromatin and that this limits the accessibility to p53 of protein deacetylases, including cytoplasmic deacetylases.

Here, we show for the first time that p53 can be deacetylated in the cytoplasm and that SirT2 is involved in p53 deacetylation in this compartment. SirT2 expression has previously been implicated in regulating p53 (6, 8); however, these studies were conducted with whole-cell extracts from virally infected HEK293 and HeLa cells where p53 modulation is strongly influenced by viral factors. Our data are the first to characterize the effect of SirT2 on p53 in a virus-free background. Furthermore, unlike previous reports, we provide evidence that SirT2 deacetylates p53 directly.

In summary, we propose a model in which all forms of p53 (including acetylated p53) accumulate in the chromatin-enriched compartment in the presence of p14 ARF, an event that requires the presence of mdm2 to bridge the interaction between p53 and p14 ARF. As a consequence, p14 ARF–mdm2 complexes protect p53 from deacetylases, including cytoplasmic deacetylases such as SirT2. Our vision of the modulation of p53 C-terminal acetylation by mdm2, p14 ARF, and SirT2 is illustrated in Fig. 6. Whether binding of mdm2–p14ARF to p53 can also prevent deacetylation of p53 by other cytoplasmic deacetylases remains to be determined. Similarly, deacetylation of p53 in the nucleus could also be mediated by deacetylases other than SirT1. In this regard, p53 has been shown to be regulated by several sirtuin and non-sirtuin HDACs (see Introduction and ref. 4).

Previous studies have reported possible tumor suppressor roles for mdm2 (26, 46). Interestingly, an intact p53/mdm2/p14 ARF axis may be needed to detect an antitumor effect of mdm2 (47). Altogether our work on acetylated p53 also suggests that under conditions where p14 ARF expression is induced, mdm2 could play a positive role in the activation of p53. We analyzed p53-dependent transcription in the presence of p14 ARF and increased amounts of ectopic mdm2. However, this experiment did not lead to an increase in p53 transcriptional activity by mdm2 (Supplementary Fig. S4). This is not unexpected in this type of assays using overexpressed proteins. One reason could be that binding of mdm2 to p53 suffices to
reduce p53-dependent transcription even when the level of p53 acetylation is high. We speculate that additional mechanisms are likely to be required to enable a positive role for mdm2 in p53-dependent transcription in our system. In a recent groundbreaking publication, Clegg and colleagues (27) obtained enhanced expression of p53 target genes in mouse embryonic fibroblasts from an mdm2–C462A knock-in mouse model. This extremely interesting observation shows that in this model system, there are mechanisms that make it possible for mdm2 to enhance p53 transcriptional activity when mdm2 E3 ubiquitin ligase function is impaired. We believe that our results showing that mdm2 promotes p53 acetylation in chromatin-rich fractions when p14ARF is present (p14ARF inhibits ubiquitination of p53 by mdm2) contribute to elucidating such mechanisms further.

In addition to contributing to a further understanding of the regulation of p53 acetylation, the results presented here also provide an insight into why inhibition of both SirT1 and SirT2 is necessary to achieve effective activation of p53 by small-molecule sirtuin inhibitors such as the tenovins. This is in full agreement with results published previously using sirtuin inhibitors unrelated to the tenovins (25). It is worth mentioning here that tenovin-6, which has antitumor activity in vivo as a single agent (29, 48), has been recently shown to induce p53 in imatinib-resistant leukemic stem cells and achieve cure in a murine model for chronic myelogenous leukemia (CML) in combination with imatinib (49, 50).

Finally, these results show that p53 acetylation in the cytoplasm is modulated by a metabolism-sensitive protein deacetylase. Now that roles for cytoplasmic p53 in cell death are becoming consolidated (51–53), our observation that it is possible to protect p53 with small molecules in this cellular compartment may have therapeutic implications and be important in elucidating the transcription-independent effects of p53.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: S. Lain
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