

Negative Control of p53 by Sir2 α Promotes Cell Survival under Stress

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Summary

The NAD-dependent histone deacetylation of Sir2 connects cellular metabolism with gene silencing as well as aging in yeast. Here, we show that mammalian Sir2 α physically interacts with p53 and attenuates p53-mediated functions. Nicotinamide (Vitamin B3) inhibits an NAD-dependent p53 deacetylation induced by Sir2 α , and also enhances the p53 acetylation levels in vivo. Furthermore, Sir2 α represses p53-dependent apoptosis in response to DNA damage and oxidative stress, whereas expression of a Sir2 α point mutant increases the sensitivity of cells in the stress response. Thus, our findings implicate a p53 regulatory pathway mediated by mammalian Sir2 α . These results have significant implications regarding an important role for Sir2 α in modulating the sensitivity of cells in p53-dependent apoptotic response and the possible effect in cancer therapy.

Introduction

The p53 tumor suppressor exerts anti-proliferative effects, including growth arrest, apoptosis, and cell senescence, in response to various types of stress (Levine, 1997; Prives and Hall, 1999; Vogelstein et al., 2000). Mutations within the p53 gene have been well documented in more than half of all human tumors. Accumulating evidence further indicates that, in the cells that retain wild-type p53, other defects in the p53 pathway also play an important role in tumorigenesis (Prives and Hall, 1999). The molecular function of p53 that is required for tumor suppression involves its ability to act as a transcriptional factor in regulating downstream target gene expression (reviewed in Nakano and Vousden, 2001; Yu et al., 2001).

p53 is a short-lived protein whose activity is maintained at low levels in normal cells. Tight regulation of p53 is essential for its effect on tumorigenesis as well as maintaining normal cell growth. The precise mechanism by which p53 is activated by cellular stress is not completely understood; it is generally thought to involve mainly posttranslational modifications of p53, including phosphorylation and acetylation (reviewed in Appella

and Anderson, 2000). Early studies demonstrated that CBP/p300, a histone acetyl-transferase (HAT), acts as a coactivator of p53 and potentiates its transcriptional activity as well as biological function in vivo (Gu et al., 1997; Lill et al., 1997; Avantaggiati et al., 1997). Significantly, the observation of functional synergism between p53 and CBP/p300 together with its intrinsic HAT activity led to the discovery of an FAT (transcriptional factor acetyl-transferase) activity of CBP/p300 on p53; this finding also predicted that acetylation may represent a general functional modification for nonhistone proteins in vivo (Gu and Roeder, 1997).

By developing site-specific acetylated p53 antibodies, CBP/p300 mediated acetylation of p53 was further confirmed in vivo by a number of studies (reviewed in Appella and Anderson, 2000). Significantly, the steady-state levels of acetylated p53 are stimulated in response to various types of stress, indicating the important role of p53 acetylation in stress response (reviewed in Ito et al., 2001). By introducing a transcriptional defective p53 mutant (p53^{Q255E26}) into mice, it was found that the mutant mouse thymocytes and ES cells failed in undergoing DNA damage-induced apoptosis (Chao et al., 2000; Jimenez et al., 2000). Interestingly, this mutant protein was phosphorylated normally at the N terminus in response to DNA damage but could not be acetylated at the C terminus (Chao et al., 2000), supporting a critical role of p53 acetylation in p53-dependent apoptotic response (Chao et al., 2000; Luo et al., 2000). Furthermore, it has been found that oncogenic Ras as well as PML can upregulate the levels of acetylated p53 in normal primary fibroblasts, and also induce premature senescence in a p53-dependent manner (Pearson et al., 2000; Ferbeyre et al., 2000). p53 acetylation may also play a critical role in protein stabilization (Rodriguez et al., 2000; Nakamura et al., 2000; Ito et al., 2001). In addition, another independent study showed that acetylation, but not phosphorylation of the p53 C terminus, may be required to induce metaphase chromosome fragility in the cell (Yu et al., 2000).

The yeast silent information regulator 2 (Sir2) protein belongs to a family of histone deacetylases (reviewed in Guarente, 2000; Shore, 2000). Sir2 activity is nicotinamide adenine dinucleotide (NAD)-dependent, but can not be inhibited by TSA (Imai et al., 2000; Landry et al., 2000a; Smith et al., 2000). The NAD-dependent deacetylase activity of Sir2 is essential for its functions, and this activity also connects its biological role with cellular metabolism in yeast (Guarente, 2000). Recently, mammalian Sir2 homologs have been found to also contain the NAD-dependent histone deacetylase activity (Imai et al., 2000; Smith et al., 2000), further supporting that the enzymatic activity is key to elucidate the molecular mechanism for its mediated functions. Among Sir2 and its homolog proteins (HSTs) in yeast, Sir2 is the only protein exclusively localized in nuclei, whose activity is critical for both gene silencing and extension of yeast life span (reviewed in Guarente, 2000). Based on protein sequence homology analysis, mouse Sir2 α and its human ortholog SIRT1 (or human Sir2 α) are the closest

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homologs to yeast Sir2 (Imai et al., 2000; Frye, 1999, 2000). Both of them exhibit nuclear localization (S.I. and L.G. unpublished data; also in Figure 6C), but their biological functions remain unclear.

Since homologs of Sir2 have been identified in almost all organisms examined including bacteria, which has no histone proteins (reviewed in Guarente, 2000; Frye, 1999, 2000), it is likely that Sir2 also targets nonhistone proteins for functional regulation. Our preliminary results uncovered an activity in protein fractions from mammalian nuclear extract that could effectively deacetylate p53 in the presence of TSA. In support of the possibility that mammalian Sir2 α targets p53 for functional regulation, we present evidence that mouse Sir2 α as well as human SIRT1 can directly bind p53 both in vitro and in vivo, and promotes cell survival under stress by specifically repressing p53-dependent apoptotic response. This discovery has important implications for elucidating a biological role for Sir2 α in mammalian cells.

Results

Mammalian Sir2 α Interacts with p53 Both In Vitro and In Vivo

Since mouse Sir2 α shares a highly conserved region at the C terminus with human SIRT1 (Figure 1A), but not with any other mammalian Sir2 homologs (Frye, 1999, 2000), we developed a polyclonal antibody against the C terminus (amino acid 480–737) of mouse Sir2 α . By Western blot analysis, this antibody can detect both mouse Sir2 α and human SIRT1 proteins, but not other human Sir2 homologs (J.L. and W.G., unpublished data, also see Figures 1B and 1C). Next, we used this antibody to investigate whether p53 interacts with Sir2 α or hSIRT1 in normal cells. Cell extracts from both human (H460) and mouse cells (F9), which express wild-type p53 proteins, were immunoprecipitated with α -Sir2 α , or with the preimmune serum. Western blot analysis revealed that this antibody immunoprecipitated both Sir2 α and hSIRT1 (lower panels, Figures 1B and 1C). More importantly, both human and mouse p53 were clearly detected in the respective α -Sir2 α immunoprecipitations from cell extracts, but not in the control immunoprecipitations with the preimmune serum, indicating that p53 interacts with mammalian Sir2 α in normal cells. Interestingly, this interaction was strongly detected in the cells after DNA damage treatment (Figure 1D), suggesting that the possible regulation of p53 by mammalian Sir2 α may be still effective after DNA damage.

Furthermore, we tested whether Sir2 α directly interacts with p53 in vitro. As shown in Figure 1E, ³⁵S-labeled in vitro-translated Sir2 α strongly bound to immobilized GST-p53 but not to immobilized GST alone (lane 1 versus 6). Moreover, Sir2 α tightly bound to the C-terminal domain of p53 (GST-p53CT) (lane 4, Figure 1E), also bound to the central DNA binding domain (GST-p53M), but showed no binding to the N-terminal domain of p53 (GST-p53NT) (lane 3 versus 2, Figure 1E). Thus, the above findings demonstrate that p53 interacts with mammalian Sir2 α both in vitro and in vivo.

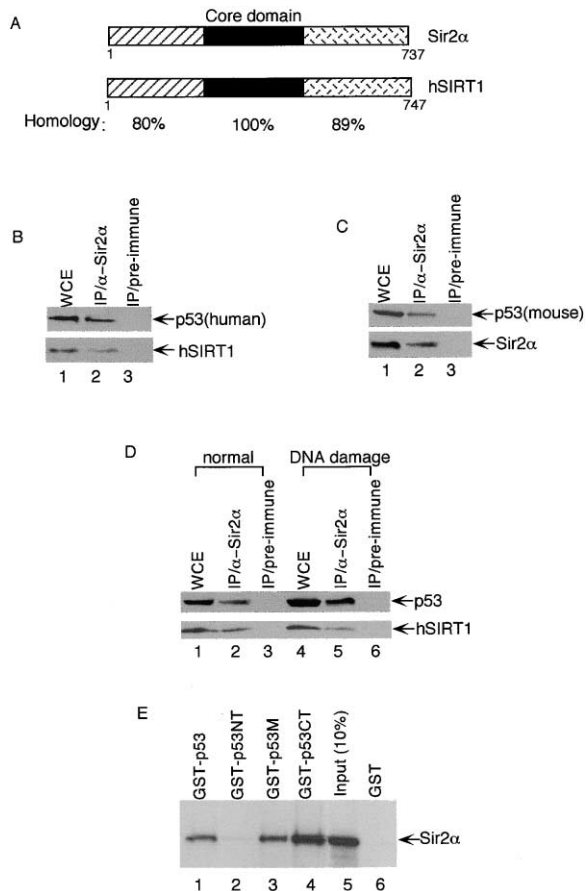


Figure 1. Interactions between p53 and Mammalian Sir2 α Both In Vitro and In Vivo

(A) Schematic representation of the high homology regions between mouse Sir2 α and human SIRT1 (hSIRT1). The core domain represents the very conserved enzymatic domain among all Sir2 family proteins (Frye, 1999, 2000).

(B) The interaction between p53 and hSIRT1 in H460 cells.

(C) The interaction between p53 and Sir2 α in F9 cells.

(D) The interaction between p53 and hSIRT1 in HCT116 cells either at the normal condition (lanes 1–3) or after DNA damage treatment by etoposide (lanes 4–6). Western blot analyses of the indicated whole cell extract (WCE) (lanes 1, 4), or immunoprecipitates with anti-Sir2 α antibody (IP/anti-Sir2 α) (lanes 2, 5) prepared from different cell extracts, or control immunoprecipitates with preimmunoserum from the same extracts (lanes 3, 6), with anti-p53 monoclonal antibodies (DO-1 for human p53, 421 for mouse p53), or anti-sir2 α antibody.

(E) Direct interactions of Sir2 α with GST-p53. The GST-p53 full-length protein (GST-p53) (lane 1), the N terminus of p53 protein (1–73) (lane 2), the middle part of p53 (100–290) (lane 3), the C terminus of p53 (290–393) (lane 4), and GST alone (lane 6) were used in GST pull-down assay with in vitro translated ³⁵S-labeled full-length mouse Sir2 α .

Deacetylation of p53 by Mammalian Sir2 α

In order to test whether p53 could be specifically deacetylated by mammalian Sir2 α in vitro, the mouse Sir2 α protein was expressed with the N-terminal Flag epitope in cells and purified to near homogeneity on the M2-agarose affinity column (lane 3, Figure 2A). As shown in Figure 2B, ¹⁴C-labeled acetylated p53 was efficiently

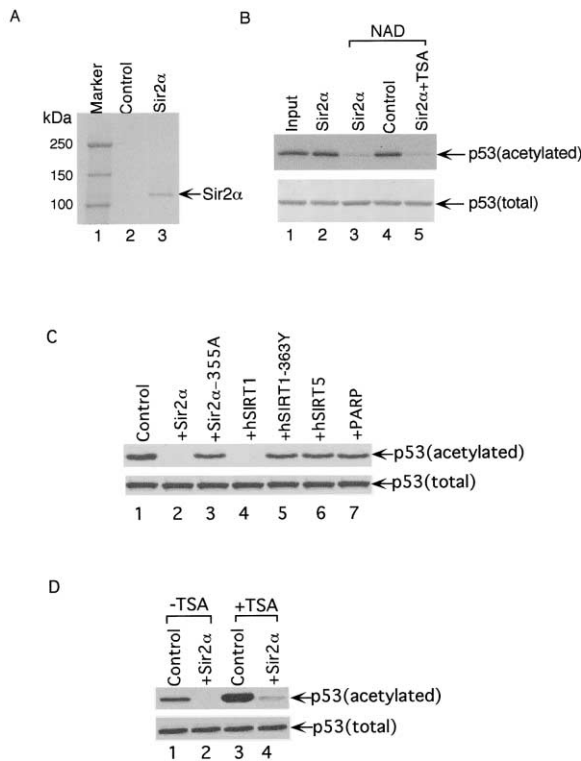


Figure 2. TSA-Insensitive Deacetylation of p53 by Mammalian Sir2 α
(A) Colloidal blue staining of a SDS-PAGE gel containing protein Marker (lane 1), a control eluate from M2 loaded with untransfected cell extract (lane 2), and 100 ng of the highly purified Flag-tagged Sir2 α recombinant protein (lane 3).
(B) Deacetylation of p53 by Sir2 α . 2.5 μ g of 14 C-labeled acetylated p53 (lane 1) was incubated with either the control eluate (lane 4), the purified 10 ng of Sir2 α (lanes 2 and 3), or the same amount of Sir2 α in the presence of 500 nM TSA (lane 5) for 60 min at 30°C. NAD (50 μ M) was also added in each reaction except lane 2. The proteins were analyzed by resolution on SDS-PAGE and autoradiography (upper) or Coomassie blue staining (lower).
(C) Reduction of the steady-state levels of acetylated p53 by both mouse Sir2 α and human SIRT1 expression. Western blot analysis of H1299 cell extracts from the cells cotransfected with p53 and p300 (lane 1), or in combination with Sir2 α (lane 2), or in combination with hSIRT1 (lane 4), or Sir2 α -355A (lane 3), or hSIRT1-363Y (lane 5), or hSIRT5 (lane 6), or PARP (lane 7) by acetylated p53-specific antibody (upper) or DO-1 for total p53 (lower). The highly conserved histidine residue at the core domain was replaced by alanine for mouse Sir2 α (aa 355) (Sir2 α -355A), or replaced by tyrosine for human SIRT1 (aa 363) (hSIRT1-363Y).
(D) Deacetylation of p53 by Sir2 α in the presence of TSA. The acetylated p53 levels in the cells cotransfected with p53 and p300 (lanes 1, 3), or cotransfected with p53, p300 and Sir2 α (lanes 2, 4). Cells were either not treated (lanes 1,2) or treated with 500 nM TSA (lanes 3, 4).

deacetylated by purified Sir2 α (lane 3), but not by a control eluate (lane 4). Importantly, NAD is required for Sir2 α -mediated deacetylation of p53 (lane 2 versus lane 3, Figure 2B). In addition, the deacetylase inhibitor TSA, which significantly abrogates HDAC1-mediated deacetylase activity on p53 (Luo et al., 2000), had no apparent effect on Sir2 α -mediated p53 deacetylation (lane 5, Figure 2B). These results indicate that the Sir2 α can strongly deacetylate p53 in vitro, and that this activity depends on NAD.

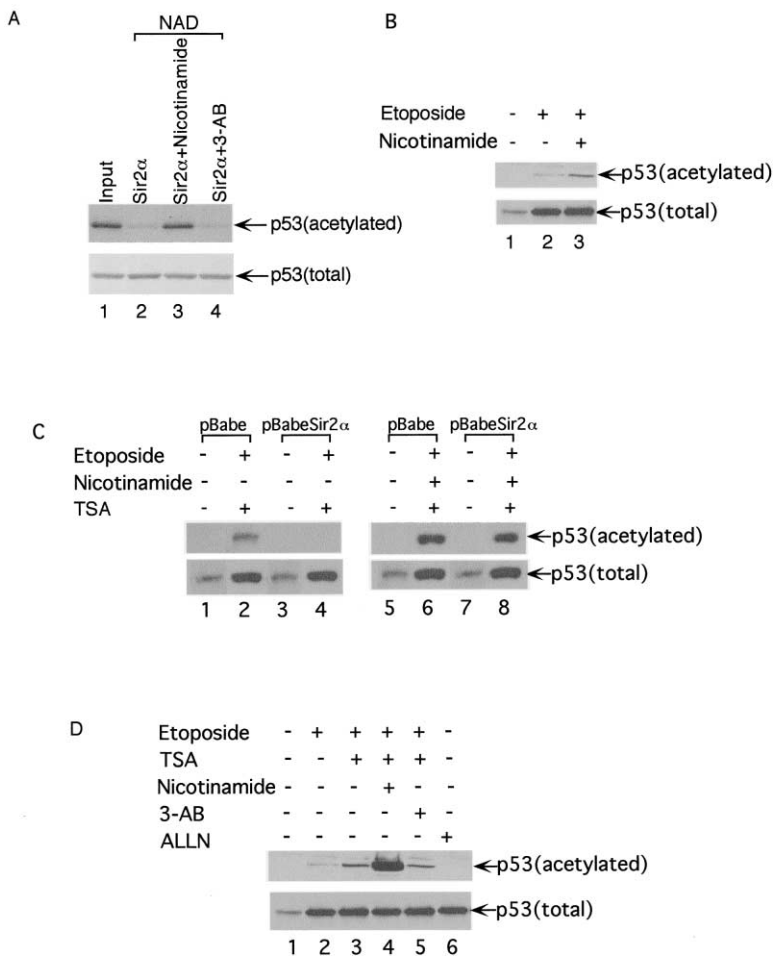
To establish the role for mammalian Sir2 α in deacetylating p53 in cells, we used an acetylated p53-specific antibody to monitor the steady-state levels of acetylated p53 in vivo (Luo et al., 2000). As indicated in Figure 2C, a high level of acetylated p53 was found in the cells cotransfected with p300 and p53 (lane 1); however, p53 acetylation levels were significantly abolished by expression of either Sir2 α or hSIRT1 (lanes 2, 4). In contrast, a point mutation at the highly conserved histidine residue at the core domain (Sir2 α -355A and hSIRT1-363Y) effectively abolished the deacetylase activity (lane 3 versus 2, lane 5 versus 4, Figure 2C). Furthermore, neither SIRT5, another human Sir2 homolog, nor poly (ADP-ribose) polymerase (PARP), whose activity is also NAD-dependent (reviewed in Vaziri et al., 1997), had any significant effect on p53 acetylation (lanes 6,7, Figure 2C). In addition, in contrast to HDAC-mediated deacetylation of p53 (Luo et al., 2000), Sir2 α still strongly deacetylated p53 in the presence of TSA (lane 4 versus 3, Figure 2D) even though the steady state level of acetylated p53 was elevated when the cells were treated with TSA (lane 3 versus 1, Figure 2D). Taken together, these data implicate a strong TSA-independent p53 deacetylation activity of mammalian Sir2 α .

Inhibition of Sir2 α -Mediated p53 Deacetylation by Nicotinamide

To further elucidate the in vivo effect by endogenous Sir2 α , we tried to identify an inhibitor for Sir2 α -mediated deacetylase activity on p53. Deacetylation of acetyllysine by Sir2 α is tightly coupled to NAD hydrolysis, producing nicotinamide and an acetyl-ADP-ribose compound (1-O-acetyl-ADP-ribose) (Landry et al., 2000b; Tanner et al., 2000; Tanny and Moazed, 2001). Although the molecular mechanism of Sir2-mediated NAD-dependent deacetylation needs to be detailed, it was proposed that formation of an enzyme-ADP-ribose intermediate through NAD hydrolysis is critical for this chemical reaction (Landry et al., 2000b). Since nicotinamide is the first product from hydrolysis of the pyridinium-N-glycosidic bond of NAD, it may function as an inhibitor for its deacetylase activity (Landry et al., 2000b). We thus tested whether nicotinamide is able to inhibit the deacetylase activity of Sir2 α on acetylated p53 in vitro.

Similar reactions as described above (Figure 2B), were set up by incubating labeled p53 substrate, recombinant Sir2 α and NAD (50 μ M) alone, or in combination with nicotinamide (5 mM). As shown in Figure 3A, 14 C-labeled acetylated p53 was efficiently deacetylated by Sir2 α (lane 2); however, the deacetylation activity was completely inhibited in the presence of nicotinamide (lane 3 versus 2). As a negative control, 3-AB (3-aminobenzamide), a strong inhibitor of PARP which is involved in another type of NAD-dependent protein modifications (Vaziri et al., 1997), showed no significant effect on Sir2 α -mediated deacetylation (lane 4 versus 3, Figure 3A).

Furthermore, we examined the effect of Sir2 α expression on endogenous levels of acetylated p53. Mouse embryonic fibroblast (MEF) cells, which express the wild-type of p53, were infected with either a pBabe retrovirus empty vector or a pBabe retrovirus containing Sir2 α . We first examined the protein levels of p53 activation



in response to DNA damage in these cells by Western blot analysis. Similar protein levels of p53 activation were induced in both types of cells after etoposide treatment for 6 hr (lanes 3, 4 versus lanes 1, 2, lower panel, Figure 3C). In the mock-infected cells, as expected, the acetylation level of p53 was significantly enhanced by DNA damage (lane 2 versus 1, upper panel, Figure 3C). However, the same DNA damage treatment failed to stimulate the p53 acetylation in the pBabe-Sir2α infected cells even in the presence of TSA (lane 4 versus 2, upper panel, Figure 3C), indicating that Sir2α expression results in deacetylation of endogenous p53. Notably, this Sir2α-mediated effect was completely abrogated by nicotinamide treatment (lane 8 versus 6, Figure 3C). Thus, these data indicate that Sir2α-mediated deacetylation of p53 can be inhibited by nicotinamide both in vitro and in vivo.

Maximum Induction of p53 Acetylation Levels in Normal Cells Requires Inhibition of Endogenous Sir2α Activity

After we found that nicotinamide has a strong inhibitory effect on Sir2α-mediated deacetylation in vivo (Figure 3C), we further tested whether the endogenous Sir2α is critical in regulating the p53 acetylation levels in normal cells during the DNA damage response. As indicated in

Figure 3. Abrogation of Mammalian Sir2α-Mediated Deacetylation of p53 by Nicotinamide

(A) Sir2α-mediated deacetylation of p53 is inhibited by nicotinamide. 2.5 μg of ¹⁴C-labeled acetylated p53 (lane 1) was incubated with 10 ng of purified Sir2α and 50 μM NAD alone (lane 2), or in the presence of either 5 mM of nicotinamide (lane 3) or 3 mM of 3-AB (3-aminobenzamide) (lane 4) for 60 min at 30°C. The proteins were analyzed by resolution on SDS-PAGE and autoradiography (upper) or Coomassie blue staining (lower).

(B) Enhancement of endogenous p53 acetylation levels by nicotinamide. Western blot analysis of cell extracts from untreated H460 cells (lane 1), or the cells treated with etoposide alone (lane 2), or in combination with nicotinamide (lane 3).

(C) The Sir2α-mediated deacetylation of endogenous p53 was abrogated in the presence of nicotinamide. Cell extracts from the mock-infected MEF p53^{+/+} cells (lanes 1–2, 5–6), or the pBabe-Sir2α infected cells (lanes 3–4, 7–8), either untreated (lanes 1, 3, 5, 7), or treated with etoposide and TSA (lanes 2, 4), or in combination with nicotinamide (lanes 6, 8) for 6 hr were analyzed by Western blot with acetylated p53-specific antibody (upper) or DO-1 for total p53 (lower).

(D) Synergistic induction of p53 acetylation levels by TSA and nicotinamide during DNA damage response. Western blot analysis of cell extracts from the H460 cells treated with etoposide alone (lane 2), or in combination with TSA (lane 3), or TSA and nicotinamide (lane 4), or TSA and 3-AB (lane 5) for 6 hr by acetylated p53-specific antibody (upper) or DO-1 for total p53 (lower). The cell extracts from untreated cells (lane 1), or treated with ALLN (50 μM) were also included (lane 6).

Figure 3B, after the wild-type p53-containing human lung carcinoma cells (H460) were treated by etoposide, acetylation of p53 was indeed induced (lane 2, versus 1). Significantly, the cellular levels of acetylated p53 induced by DNA damage were enhanced when the cells were treated with nicotinamide (lane 3 versus 2, Figure 3B), indicating an inhibitory effect of nicotinamide on endogenous Sir2α-mediated p53 deacetylation.

Furthermore, we have previously shown that p53 can be deacetylated by a PID/MTA2/HDAC1 complex, whose activity is completely abrogated in the presence of TSA (Luo et al., 2000). Therefore, the enhancement of the acetylation level of p53 by TSA during DNA damage response may be due mainly to its inhibitory effect on endogenous HDAC1-mediated deacetylase activity (lane 3 versus 2, Figure 3D). Since no significant p53 acetylation was detected in the cells treated with a proteasome inhibitor ALLN (lane 6, Figure 3D), indicating that the observed stimulation of p53 acetylation is induced by DNA damage, not through p53 stabilization. Strikingly, a super induction of p53 acetylation was evident when the cells were treated with both TSA and nicotinamide (lane 4 versus 3, Figure 3D). In contrast, 3-AB treatment had no effect on the level of p53 acetylation (lane 5 versus 3, Figure 3D), indicating that PARP-mediated poly-ADP ribosylation has no effect on p53 acetylation.

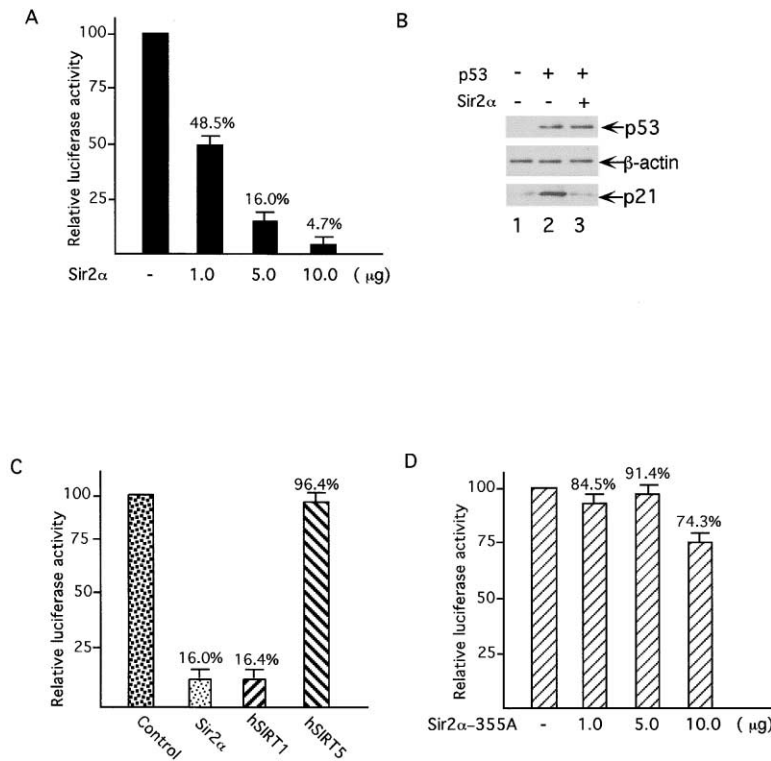


Figure 4. Repression of p53-Mediated Transcriptional Activation by Mammalian Sir2 α

(A) MEF ($p53^{-/-}$) cells were transiently transfected with 10 ng of CMV-p53 alone, or in combination with indicated amount of Sir2 α together with the PG13-Luc reporter construct by calcium phosphate precipitation essentially as previously described (Luo et al., 2000).

(B) Western blot analysis of indicated transfected H1299 cell extracts with anti-p53 (DO-1), anti-p21 (C-19) and anti- β -actin.

(C and D) MEF ($p53^{-/-}$) cells were transiently transfected with 10 ng of CMV-p53 alone, or in combination with 5 μ g of either CMV-Sir2 α , or CMV-hSIRT1, or CMV-hSIRT5 (C), or CMV-Sir2 α -355A as indicated (D) together with the PG13-Luc reporter construct. All transfections were done in duplicate and representative experiments depict the average of three experiments with standard deviations indicated.

Thus, these data clearly indicate that maximum induction of p53 acetylation requires inhibitors for both types of deacetylases (HDAC1 and Sir2 α), and that endogenous Sir2 α plays an important role in the regulation of the p53 acetylation levels induced by DNA damage.

Repression of p53-Mediated Functions by Mammalian Sir2 α Requires Its Deacetylase Activity

To determine the functional consequence of mammalian Sir2 α -mediated deacetylation of p53, we tested its effect on p53-mediated transcriptional activation. A mammalian p53 expression vector (CMV-p53), alone or in combination with different amounts of mouse Sir2 α expression vector (CMV-Sir2 α), was cotransfected into MEF ($p53^{-/-}$) cells along with a reporter construct containing synthetic p53 binding sites placed upstream of the luciferase gene (PG13-Luc). As shown in Figure 4A, Sir2 α strongly repressed p53-mediated transactivation in a dose-dependent manner (up to 21-fold), and expression of human SIRT1 showed a similar effect on the p53 target promoter (Figure 4C). Furthermore, an acetylation-defective p53 mutant (Luo et al., 2000) was partially impaired in the Sir2 α -mediated repression (see Supplemental Figures S1 and S2; <http://www.cell.com/cgi/content/full/107/2/137/DC1>). Significantly, Sir2 α expression attenuated p53 sequence-specific DNA binding activity (see Supplemental Figure S3), and also abrogated p53-dependent induction of endogenous p21 expression (lane 3 versus 2, Figure 4B). In contrast, neither the Sir2 α -355A mutant nor SIRT5, both of which are defective in p53 deacetylation (Figure 2C), had any effect on the p53-mediated transactivation (Figures 4C and 4D). These data suggest that mammalian Sir2 α spe-

cifically represses p53-dependent transactivation, and that this repression requires its deacetylase activity.

To further test the biological role of mammalian Sir2 α , we examined its modulation on p53-dependent apoptosis. p53 null cells (H1299) were transfected with p53 alone or cotransfected with p53 and Sir2 α . The transfected cells were fixed, stained for p53, and analyzed for apoptotic cells (SubG1) (Luo et al., 2000). As indicated in Figure 5A, overexpression of p53 alone induced significant apoptosis (32.3% SubG1). However, cotransfection of p53 with Sir2 α significantly reduced the level of apoptosis (16.4% SubG1), while the mutant Sir2 α -355A was severely impaired in this effect (29.5% SubG1) (Figures 5A and 5B). Similar results were also obtained by using the Annexin V assay (see Supplemental Figure S4). Taken together, these data demonstrate that mammalian Sir2 α is involved in the regulation of both p53-mediated transcriptional activation and p53-dependent apoptosis, and that the deacetylase activity is required for these Sir2 α -mediated effects on p53.

The Role of Mammalian Sir2 α in Stress-Induced Apoptotic Response

Our data have indicated that mammalian Sir2 α can deacetylate p53 both in vitro and in vivo (Figure 2); more importantly, Sir2 α can attenuate p53-mediated transcriptional activation (Figure 4). To elucidate the physiological significance for this Sir2 α -mediated regulation, we examined its effect on DNA damage-induced apoptotic response. For this study, we chose the same MEF ($p53^{+/+}$) cells as described above (Figure 3C), which were infected with either a pBabe retrovirus empty vector or a pBabe retrovirus containing Sir2 α . After the DNA damage treatment by etoposide, the cells were stained

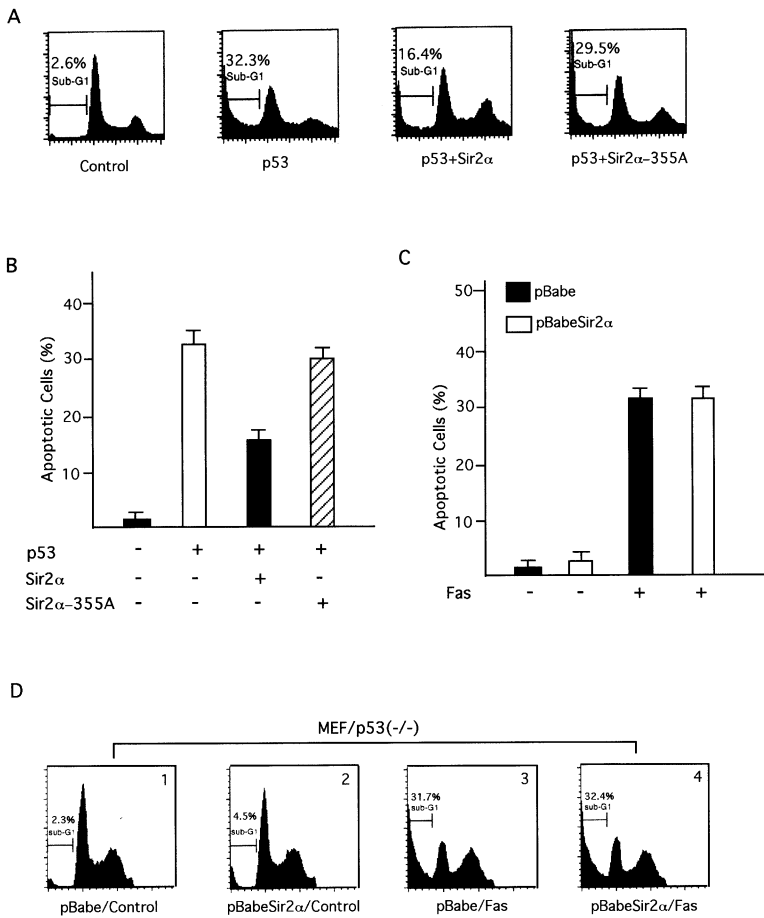


Figure 5. Inhibition of p53-Dependent Apoptosis by Sir2α

(A and B) H1299 cells were transfected with p53 alone, or cotransfected with p53 and Sir2α, or cotransfected with p53 and Sir2α-355A. After transfection, the cells were fixed, stained for p53 by FITC-conjugated α-p53 antibody, analyzed for apoptotic cells (subG1) according to DNA content (PI staining).

(C and D) Mammalian Sir2α has no effect on the Fas mediated apoptosis. Both mock infected cells and pBabe-Sir2α infected MEF p53^{-/-} cells were either not treated (1 and 2) or treated with 100 ng/ml Fas antibody in presence of actinomycin D (0.25 μg/ml) (3 and 4).

The experiments were repeated more than three times and the results depict the average of three experiments with standard deviations indicated (B and C).

with PI and analyzed by flow cytometric analysis for apoptotic cells (SubG1) according to DNA content. As shown in Figure 6A, the cells mock infected with the pBabe-vector, were susceptible to etoposide-induced cell death, with about 48% of the cells apoptotic after exposure to 20 μM of etoposide (3 versus 1, Figure 6A). In contrast, the pBabe-Sir2α infected MEF (p53^{+/+}) cells were more resistant to apoptosis induced by the same dose of etoposide, with only 16.4% apoptotic cells (4 versus 3, Figures 6A and 6B). Since no significant apoptosis was detected in MEF (p53^{-/-}) cells by the same treatment (D.C. and W.G., unpublished data), the induced apoptosis observed in MEF (p53^{+/+}) cells is totally p53-dependent. Thus, these results indicate that Sir2α significantly inhibits p53-dependent apoptosis in response to DNA damage.

Since stimulation of p53 acetylation as well as p53-dependent apoptosis have also been implicated in many other types of stress response (reviewed in Ito et al., 2001), we examined the role of mammalian Sir2α in the oxidative stress response. Recent studies have indicated that oxidative stress-induced cell death is p53-dependent (Yin et al., 1998; Migliaccio et al., 1999). We chose early-passage normal human fibroblast (NHf) IMR-90 cells for this study since it has been demonstrated that p53-dependent apoptosis can be strongly induced by hydrogen peroxide treatment in these cells (Chen et al., 2000). IMR-90 cells were infected with either

a pBabe retrovirus empty vector or a pBabe retrovirus containing Sir2α. By immunofluorescence staining, we found that p53 in these infected cells was induced significantly after hydrogen peroxide treatment, along with Sir2α localized in the nuclei detected by immunostaining with specific antibodies (Figure 6C). Importantly, Sir2α expression significantly promotes cell survival under oxidative stress. As indicated in Figure 6D, the cells mock infected with the pBabe-vector, were susceptible to H₂O₂-induced cell death, with more than 80% of the cells being killed after 24 hr exposure to 200 μM H₂O₂ (II versus I). In contrast, the pBabe-Sir2α infected cells were much more resistant to death by the same dose of H₂O₂, with about 70% of the cells surviving after 24 hr of H₂O₂ treatment (IV versus III, Figure 6D).

Taken together, these results suggest that mammalian Sir2α promotes cell survival under stress by inhibiting p53-dependent apoptosis.

Mammalian Sir2α Has No Effect on p53-Independent Cell Death Induced by Anti-Fas

In order to determine the specificity of mammalian Sir2α-mediated protection of cells from apoptosis, we examined whether Sir2α has any effect on p53-independent, Fas-mediated apoptosis. The MEF (p53^{-/-}) cells were infected with either a pBabe retrovirus empty vector or a pBabe retrovirus containing Sir2α. After the treatment by anti-Fas (100 ng/ml) for 24 hr, the cells were harvested

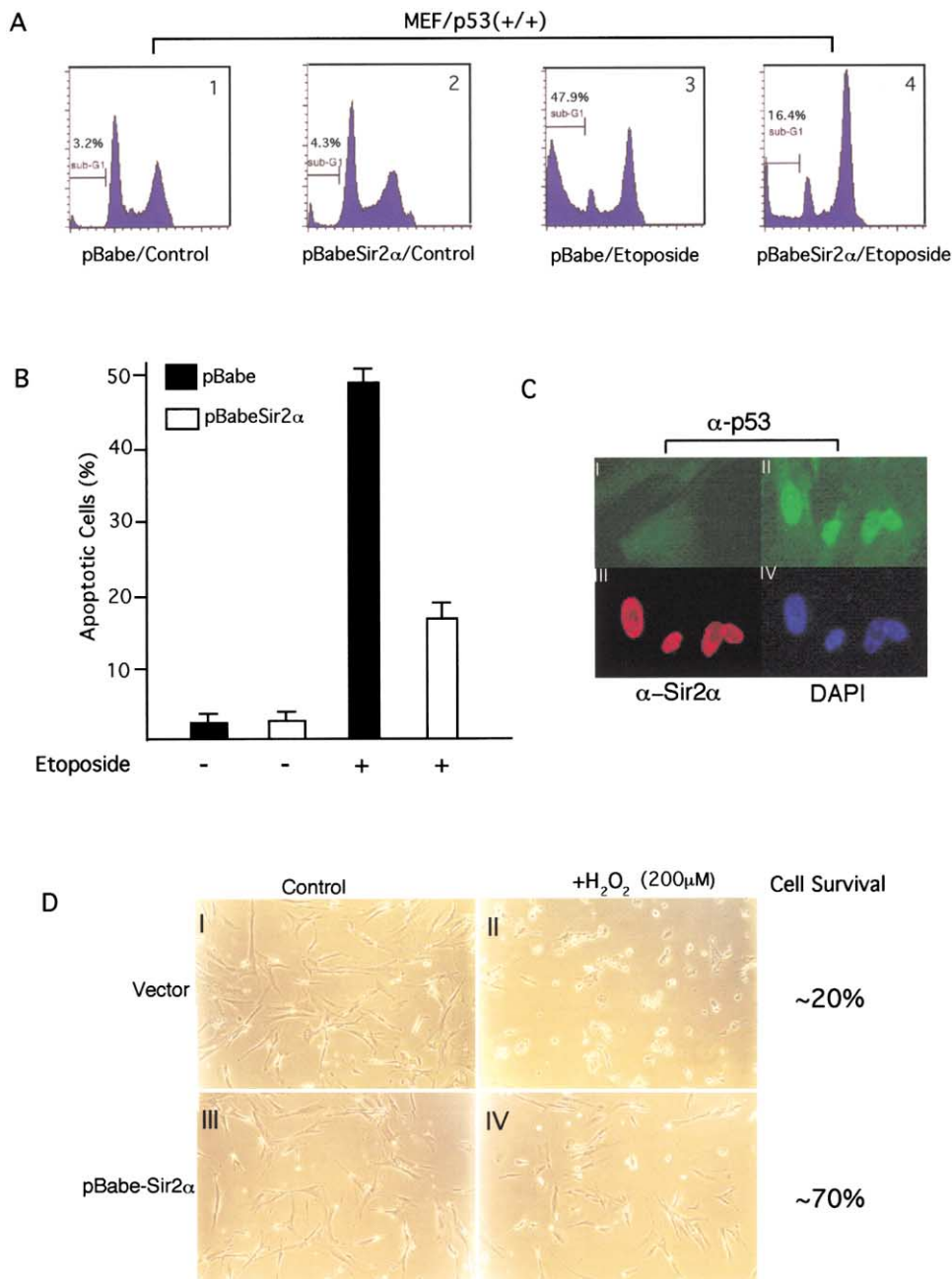


Figure 6. Inhibition of the p53-Dependent Apoptosis in Response to Stress by Mammalian Sir2 α

(A) Repression of the apoptotic response to DNA damage by Sir2 α . Both mock infected cells and pBabe-Sir2 α infected MEF p53^{+/+} cells were either not treated (1 and 2) or treated with 20 μ M etoposide. The cells were analyzed for apoptotic cells (subG1) according to DNA content (PI staining). Similar results were obtained for three times, and the representative data depict the average of three experiments with standard deviations indicated (B). (C) Subcellular localization of p53 and Sir2 α in the pBabe-Sir2 α infected IMR-90 cells. p53 and Sir2 α were detected with either α -p53 (DO-1) (visualized by green fluorescence from secondary antibody staining with anti-mouse IgG-FITC), or affinity purified α -Sir2 α antibody (visualized by red fluorescence from secondary antibody staining with anti-rabbit IgG conjugated to Alexa 568). The cells were counterstained with DAPI to visualize the nuclei as essentially described before (Guo et al., 2000). Cells were either not treated (I) or treated with 100 μ M H₂O₂ (II, III, IV) for 24 hr. (D) Inhibition of the apoptotic response to oxidative stress by mammalian Sir2 α . Both mock infected cells and pBabe-Sir2 α infected cells were either not treated (I and III) or treated with 200 μ M H₂O₂ (II and IV). 24 hr later, the cells were photographed under a microscope.

and further analyzed for apoptotic cells (SubG1). As shown in Figure 5D, the cells mock infected with the pBabe vector, were susceptible to anti-Fas induced cell death, with about 31.7% of the cells becoming apo-

ptotic. However, in contrast to the strong protection of p53-dependent apoptosis by Sir2 α during DNA damage response in the MEF (p53^{+/+}) cells (Figures 6A and 6B), Sir2 α expression had no significant effect on Fas-mediated

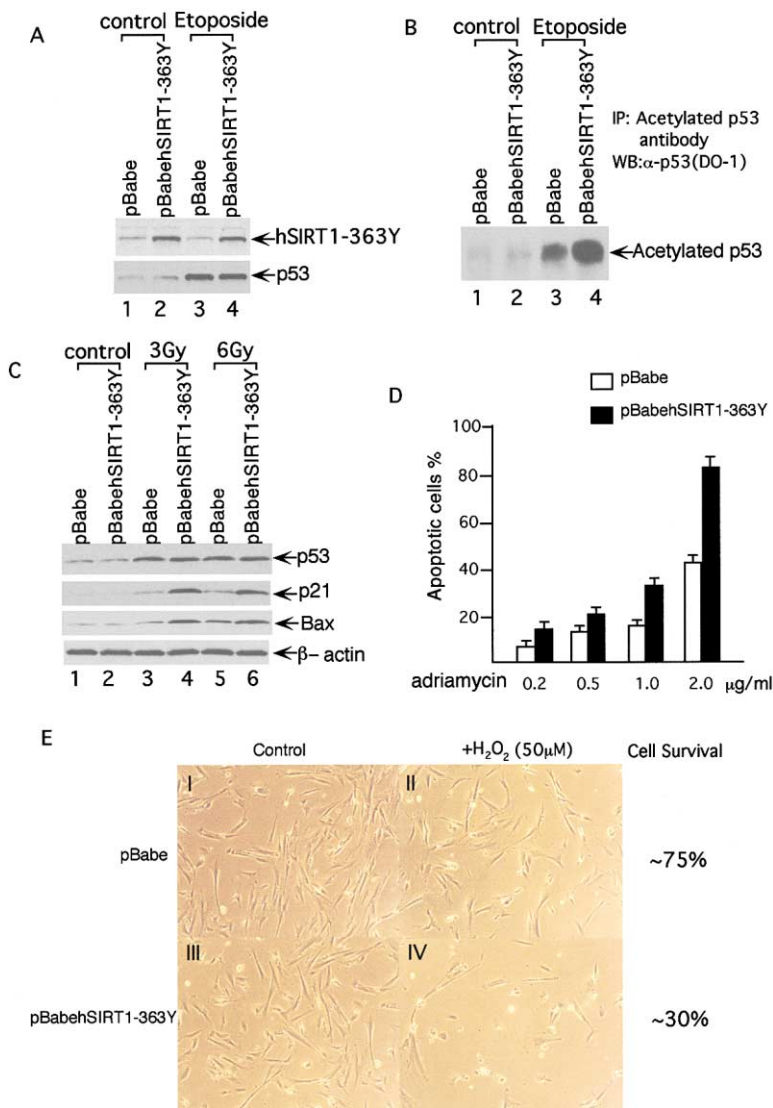


Figure 7. Expression of a Sir2 α Point Mutant (hSIRT1-363Y) Increases the Sensitivity of the Cells in Response to Stress

(A) The levels of p53 are induced by DNA damage in both mock infected and pBabe-hSIRT1-363Y infected cells. Western blot analysis of the cell extracts from both types of cells by anti-Sir2 polyclonal antibody (upper) or anti-p53 monoclonal antibody (DO-1) (lower). Cells were either not treated (lanes 1, 2) or treated with 20 μ M of etoposide (lanes 3, 4) for 6 hr.

(B) Expression of the Sir2 α mutant enhances the acetylated p53 levels induced by DNA damage. The cell extracts obtained from treated or untreated cells were first immunoprecipitated with anti-acetylated p53 antibody and the immunoprecipitates were analyzed by Western blot with α -p53 (DO-1).

(C) DNA damage induced expression of p21 and Bax in both mock infected and pBabe-hSIRT1-363Y infected cells. Both types of cells were γ -radiated (3 or 6 Gy); 3 hr later, the cells were collected for Western blot analysis for p53, p21, Bax, and β -actin.

(D and E) Expression of the Sir2 α mutant increases the sensitivity of the cells in stress-induced apoptotic response. Both mock infected cells and pBabe-hSIRT1-363Y infected cells were either not treated (I and III) or treated with 50 μ M H₂O₂ (II and IV) (E), or treated with different concentration of adriamycin as indicated; 48 hr later, the cells were collected for analysis (D).

ated apoptosis in the MEF (p53^{-/-}) cells (Figures 5C and 5D). Thus, these results further support a specific role for mammalian Sir2 α in regulating p53-mediated apoptosis.

Expression of a Sir2 α Point Mutant Increases the Sensitivity of Cells in the Stress Response

To further demonstrate that endogenous Sir2 α regulates endogenous p53 under normal conditions, we introduced a Sir2 α point mutant (hSIRT1-363Y), which is functional-defective in p53 deacetylation (Figure 2C), into normal human cells. IMR-90 cells were infected with either a pBabe retrovirus empty vector or a pBabe retrovirus containing hSIRT1-363Y. As indicated in Figure 7A, the pBabe-hSIRT1-363Y infected cells expressed a significant amount of the mutant protein whereas similar levels of p53 were induced in both types of cells after DNA damage treatment (lanes 3, 4 versus lanes 1, 2, Figure 7A). Significantly, the levels of acetylated p53 were strongly enhanced in the pBabe-hSIRT1-363Y infected cells, indicating that hSIRT1-363Y functions as a dominant negative mutant and inhibits

endogenous Sir2 α -mediated deacetylation of p53 (lane 4 versus 3, Figure 7B). We further tested whether hSIRT1-363Y expression has any effect on p53 target genes induced by DNA damage. As indicated in Figure 7C, both p21 and Bax were induced in the cells after DNA damage; interestingly however, the expression levels of both p21 and Bax in hSIRT1-363Y infected cells were significantly higher than those in mock-infected cells, indicated that hSIRT1-363Y expression abrogates the endogenous Sir2 α -mediated repression on p53-dependent transactivation. Moreover, although the IMR-90 cells were susceptible to H₂O₂-induced cell death after exposure to 200 μ M H₂O₂ (Figure 6D), the cells were relatively resistant to the treatment with a lower concentration of H₂O₂ (50 μ M) (II versus I, Figure 7E). In contrast, hSIRT1-363Y expression led to the cells very sensitive to such a mild treatment (50 μ M H₂O₂); less than 30% of the cells were able to survive after the treatment (IV versus III, Figure 7E). In order to corroborate these results, we also test whether hSIRT1-363Y expression increases the sensitivity of the cells in DNA damage-

induced cell death. As shown in Figure 7D, the pBabe-hSIRT1-363Y infected cells are much more sensitive to DNA damage-induced cell death when the cells were treated with different concentrations of a DNA damage-inducing reagent adriamycin.

Taken together, these results suggest that endogenous Sir2 α is critically involved in deacetylating p53 as well as regulating p53-mediated biological functions under physiological conditions.

Discussion

The present data reveal the existence of a p53 regulatory pathway that is controlled by mammalian Sir2 α . Sir2 α is involved in gene silencing and extension of life span in yeast and *C. elegans* (reviewed in Guarente, 2000; Tissenbaun and Guarente, 2001). Although the mammalian homolog has also been shown to contain a unique histone deacetylase activity (Imai et al., 2000; Smith et al., 2000), its biological function remains to be elucidated. We show here (1) that p53 strongly binds to mouse Sir2 α as well as its human ortholog hSIRT1 both in vitro and in vivo, (2) that p53 is a substrate for the NAD-dependent deacetylase of mammalian Sir2 α , (3) that the Sir2 α -mediated deacetylation antagonizes p53-dependent transcriptional activation and apoptosis, (4) that the Sir2 α -mediated deacetylation of p53 is inhibited by nicotinamide both in vitro and in vivo, (5) that Sir2 α specifically inhibits p53-dependent apoptosis in response to DNA damage as well as oxidative stress, but not the p53-independent, Fas-mediated cell death, and (6) expression of a Sir2 α point mutant increases the sensitivity of the cells in response to stress. These results are especially relevant to the multiple regulatory pathways of p53 in vivo and, since the acetylation levels of p53 are stimulated in response to various types of stress, to the role of mammalian Sir2 α in stress response.

The Sir2 α -Mediated Pathway Is Critical for Cells Under Stress

Our study implicates an Mdm2-independent, negative regulatory pathway for p53, which further supports the views that there are multiple pathways in cells for tight regulation of p53 function (Prives and Hall, 1999; Appella and Anderson, 2000). In normal cells, Mdm2 is the major negative regulator for p53, and Mdm2-mediated repression appears sufficient to downregulate p53 activity. Interestingly, while no obvious effect by Sir2 α expression was observed in cells at normal conditions, however, Sir2 α became critical in protecting cells from apoptosis when cells were either treated by DNA damage or under oxidative stress (Figure 6). Therefore, we propose that this Sir2 α -mediated pathway is critical for cell survival when the p53 negative control mediated by Mdm2 is severely attenuated in response to DNA damage or other types of stress.

In this regard, p53 is often found as latent forms and the levels of p53 protein are very low in unstressed cells, mainly due to the tight regulation by Mdm2 through functional inhibition and protein degradation mechanisms. However, in response to DNA damage, p53 is phosphorylated at multiple sites at the N terminus, and

these phosphorylation events contribute to p53 stabilization and activation by preventing the binding with Mdm2 (reviewed in Appella and Anderson, 2000; Shieh et al., 1997). Mdm2 itself is also phosphorylated by ATM during DNA damage response, and this modification attenuates its inhibitory potential on p53 (Maya et al., 2001). Furthermore, while p53 is strongly stabilized and highly acetylated in stressed cells, acetylation of the C-terminal multiple lysine sites may occupy the same sites responsible for Mdm2-mediated ubiquitination (Rodriguez et al., 2000; Nakamura et al., 2000), and the highly acetylated p53 can not be effectively degraded by Mdm2 without deacetylation (Ito et al., 2001). Thus, in contrast to unstressed cells, the main p53 negative regulatory pathway mediated by Mdm2 is severely blocked at several levels in response to DNA damage (Maya et al., 2001). Under these circumstances, the Sir2 α -mediated regulation may become a major factor in controlling p53 activity, making it possible for cells to adjust the p53 activity for DNA repair before committing to apoptosis.

Interestingly, using a different approach, Vaziri et al. (2001 [this issue of *Cell*]) found that γ -radiation-induced p53-dependent apoptosis is also inhibited by Sir2 α expression. Thus, our findings together with their results further imply that Sir2 α may modulate the sensitivity of cells in various types of stress response through inhibiting p53-mediated function.

Attenuation of p53-Mediated Transactivation by Sir2 α

Earlier studies indicated that p53-mediated transcriptional activation is sufficient and also absolutely required for its effect on cell growth arrest, while both transactivation-dependent and -independent pathways are involved in p53-mediated apoptosis (reviewed in Prives and Hall, 1999). However, there is now growing evidence showing that p53 can effectively induce apoptosis by activating proapoptotic genes in vivo (reviewed in Nakano and Vousden, 2001; Yu et al., 2001). Thus, tight regulation of p53-mediated transactivation is critical for its effect on both cell growth and apoptosis (Chao et al., 2000; Jimenez et al., 2000).

Recent studies indicate that the intrinsic histone deacetylase activity of Sir2 α is essential for its mediated functions (reviewed in Gurante, 2000). Reversible acetylation was originally identified in histones; however, accumulating evidence indicates that transcriptional factors are also functional targets of acetylation (reviewed in Sterner and Berger, 2000; Kouzarides, 2000). Thus, the transcriptional attenuation mediated by histone deacetylases may act through the effects on both histone and nonhistone transcriptional factors (Sterner and Berger, 2000). Interestingly, microarray surveys for transcriptional effects of Sir2 in yeast revealed that Sir2 appears to repress amino acid biosynthesis genes, which are not located at traditional "silenced" loci (Bernstein et al., 2000). Thus, in addition to silencing (repression) at telomeres, mating type loci and ribosomal DNA (reviewed in Guarente, 2000; Shore, 2000), Sir2 may also be targeted to specific endogenous genes for transcriptional regulation in yeast.

The precise mechanism by which mammalian Sir2 α

inhibits p53-mediated functions needs to be further elucidated; however, our results suggest that mammalian Sir2 α inhibits p53-mediated apoptosis through attenuation of the transcriptional activation potential of p53. A speculative model would be that the interaction between p53 and Sir2 α leads to deacetylation of p53 and—since p53 sequence-specific DNA binding can also be activated by other mechanisms such as phosphorylation and single-stranded DNA—recruitment of the p53-Sir2 α complex to the target promoter; the subsequent transcription repression may act both through decreasing p53 transactivation capability and through Sir2 α -mediated histone deacetylation at the target promoter region. Our study also predicts that other cellular factors may use a similar mechanism to recruit Sir2 family proteins for TSA-insensitive transcriptional regulation in mammalian cells.

Implications for Cancer Therapy?

Inactivation of p53 functions has been well documented as a common mechanism for tumorigenesis (Vogelstein et al., 2000). Many cancer therapy drugs have been designed based on either reactivating p53 functions or inactivating p53 negative regulators. Since p53 is strongly activated in response to DNA damage mainly through attenuation of the Mdm2-mediated negative regulatory pathway (Maya et al., 2001), many DNA damage-inducing drugs such as etoposide are very effective antitumor drugs in cancer therapy (reviewed in Chresta and Hickman, 1996; Lutzker and Levine, 1996). Based on our results that the maximum induction of p53 acetylation in normal cells requires both types of deacetylase inhibitors in addition to DNA damage, there are at least three different p53 negative regulatory pathways in mammalian cells. Interestingly, inhibitors for HDAC-mediated deacetylases, including sodium butyrate, TSA, SAHA and others, have been also proposed as antitumor drugs (reviewed in Marks et al., 2001; Butler et al., 2000; Yoshida et al., 1995). Thus, we propose that the combining DNA damage drugs, HDAC-mediated deacetylase inhibitors, and Sir2 α -mediated deacetylase inhibitors, may have synergistic effects in cancer therapy for maximally activating p53.

The differential roles between HDAC1-dependent and Sir2 α -mediated regulation on p53 need to be further elucidated; however, in contrast to PID/HDAC1-mediated p53 regulation (Luo et al., 2000), our results have shown that mammalian Sir2 α -mediated effect on p53 is NAD-dependent, indicating that this type of regulation is closely linked to cellular metabolism (reviewed in Guarente 2000; Campisi, 2000). In fact, null mutants of NPT1, a gene that functions in NAD synthesis, show phenotypes similar to that of Sir2 mutants in silencing (Smith et al., 2000) and in life extension in response to caloric restriction in yeast (Lin et al., 2000). Thus, metabolic rate may play a role in Sir2 α -mediated regulation of p53 function and, perhaps, modulate the sensitivity of cells in p53-dependent apoptotic response.

Experimental Procedures

Plasmids and Antibodies

To construct Sir2 α expression constructs, the full-length cDNA was subcloned from pET28a-Sir2 α (Imai et al., 2000) into pcDNA3 or

pBabepuro vector. Site-directed mutation was generated in the plasmid pRS305-Sir2 α using the Gene Edit system (Promega). To construct the human SIRT1 expression construct, DNA sequences corresponding to the full-length hSIRT1 (Frye, 1999) were amplified by PCR from Marathon-Ready Hela cDNA (Clontech), and initially subcloned into pcDNA3.1/V5-His-Topo vector (Invitrogen), and then subcloned with a Flag-tag into a pCIN4 vector for expression (Gu et al., 1999). To prepare the Sir2 α antibody that can recognize both human and mouse Sir2 α , we made a polyclonal antibody against the highly conserved C terminus of Sir2 α . DNA sequences corresponding to this region (480-737) were amplified by PCR and subcloned into pGEX-2T (Pharmacia). α -Sir2 α antisera were raised in rabbits against the purified GST-Sir2 α (480-737) fusion protein (Covance), and further affinity-purified on both protein-A and antigen columns.

In Vitro p53 Deacetylation Assay

The Flag-tagged Sir2 α cells were established and expanded in DMEM medium, and cell extracts were prepared essentially as previously described (Luo et al., 2000; Gu et al., 1999). The 14 C-labeled acetylated p53 (2.5 μ g) was incubated with purified Sir2 α (10 ng) at 30°C for 1 hr either in the presence of 50 μ M NAD or as indicated. The reactions were performed in a buffer containing 50 mM Tris-HCl (pH 9.0), 50mM NaCl, 4 mM MgCl $_2$, 0.5 mM DTT, 0.2 mM PMSF, 0.02% NP-40, and 5% glycerol. The reactions were resolved on SDS-PAGE and analyzed by Coomassie blue staining and autoradiography.

Virus Infection and Stress Response

All MEF cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, and the IMR-90 cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and nonessential amino acids. The virus infection and selection were essentially as described previously (Ferbeyre et al., 2000). After one-week selection, the cells were either frozen for stock or immediately used for further analysis. About 500,000 MEF cells were plated on a 10 cm dish 24 hr before treatment. The cells were then exposed to etoposide (20 μ M) for 12 hr. After treatment, the cells were washed with PBS and fed with normal medium. Another 36 hr later, the cells were stained with PI and analyzed by flow cytometric analysis for apoptotic cells (SubG1) according to DNA content. In case of the Fas-mediated apoptosis assay, the cells were treated with actinomycin D (0.25 μ g/ml) and Fas antibody (100 ng/ml) as previously described (Di Cristofano et al., 1999). In the case of oxidative stress response, the IMR-90 cells were treated with H $_2$ O $_2$ (50 to 200 μ M) for 24 hr.

Detecting Acetylation Levels of p53 in Cells

For DNA damage response, about 1 million cells were plated on a 10 cm dish 24 hr before treatment. The cells were then exposed to etoposide (20 μ M) and/or other drugs (0.5 μ M of TSA, 5 mM of nicotinamide, and 50 μ M of ALLN) as indicated for 6 hr. After treatment, the cells were harvested for Western blot analysis. In the case of cotransfection assays testing for p53 acetylation levels, H1299 cells were transfected with 5 μ g of CMV-p53 plasmid DNA, 5 μ g of CMV-p300 plasmid DNA, and 10 μ g of pcDNA3-Sir2 α plasmid DNA as indicated. 24 hr after the transfection, the cells were lysed in a Flag-lysis buffer (50 mM Tris, 137 mM NaCl, 10 mM NaF, 1 mM EDTA, 1% Triton X-100 and 0.2% Sarkosyl, 1 mM DTT, 10% glycerol, pH 7.8) with fresh proteinase inhibitors, 10 μ M TSA and 5 mM nicotinamide (Sigma). The cell extracts were resolved by either 8% or 4%–20% SDS-PAGE gels (Novex) and analyzed by Western blot with α -p53(Ac)-C and α -p53(DO-1).

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