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hSIR2^{SIRT1} Functions as an NAD-Dependent p53 Deacetylase

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Summary

DNA damage-induced acetylation of p53 protein leads to its activation and either growth arrest or apoptosis. We show here that the protein product of the gene *hSIR2*^{SIRT1}, the human homolog of the *S. cerevisiae* Sir2 protein known to be involved in cell aging and in the response to DNA damage, binds and deacetylates the p53 protein with a specificity for its C-terminal Lys382 residue, modification of which has been implicated in the activation of p53 as a transcription factor. Expression of wild-type hSir2 in human cells reduces the transcriptional activity of p53. In contrast, expression of a catalytically inactive hSir2 protein potentiates p53-dependent apoptosis and radiosensitivity. We propose that hSir2 is involved in the regulation of p53 function via deacetylation.

Introduction

The Silent Information Regulator (SIR2) family of genes is a highly conserved group of genes present in the genomes of organisms ranging from archaebacteria to eukaryotes (Frye, 2000). The encoded SIR proteins are involved in diverse processes ranging from regulation of gene silencing to DNA repair. The best characterized of these is the product of the *S. cerevisiae SIR2* gene, which is involved in silencing HM loci that contain information specifying yeast mating type, telomere position effects, and cell aging (Guarente, 1999).

In yeast, SIR2 was thought to function as an NAD (nicotinamide adenine dinucleotide)-dependent ADP-ribosyl transferase (Tanny et al., 1999). However, recent biochemical studies have provided evidence that Sir2 functions as a deacetylase which uses NAD as a cofactor (Imai et al., 2000; Smith et al., 2000; Landry et al., 2000).

Among its other functions in *S. cerevisiae*, Sir2 is involved in DNA damage responses (Martin et al., 1999; McAinsh et al., 1999; Mills et al., 1999). These findings caused us to ask whether the related Sir2 protein of mammalian cells is also involved in the DNA damage response. In fact, much is known about this response in mammalian cells, in part through study of the p53 protein, the primary mediator of their DNA damage response (Levine, 1997; Oren, 1999; Vogelstein et al., 2000). Following DNA damage, the p53 protein is protected from rapid degradation and acquires transcription-activating functions, largely as a result of posttranslational modifications (Canman et al., 1998; Shieh et al., 2000; Siliciano et al., 1997). Activation of the p53 protein as a transcription factor allows it, in turn, to upregulate the expression of genes whose products promote cell cycle exit (such as the p21^{WAF1} gene; el-Deiry et al., 1993) or of genes that favor apoptosis (Lin et al., 2000).

The p53 protein is phosphorylated in response to DNA damage (Siliciano et al., 1997). For example, the ATM protein phosphorylates p53 at residue Ser15 (Siliciano et al., 1997) and Chk1/2 kinases modify residue Ser20 (Chehab et al., 1999; Shieh et al., 2000). Recent evidence suggests that the Ser15 phosphorylation does not lead directly to the functional activation of the p53 protein. Instead, it increases the affinity of the p300 acetylase for p53 (Lambert et al., 1998). This association leads, in turn, to the acetylation of p53. Indeed, p53 is acetylated in vitro by p300 at Lys 370–373, 381, and 382 (Gu and Roeder, 1997). Moreover, at least two of these sites, namely residues 320 and 382, are found to be acetylated in vivo in response to DNA damage (Abraham et al., 2000; Sakaguchi et al., 1998). Among other factors that can affect acetylation of p53 is the MDM2 protein, which is involved in the negative regulation of p53 (Oren, 1999) and is able to block acetylation of p53 protein by p300 (Kobet et al., 2000). While the acetylation by p300 and deacetylation by the TSA-sensitive HDAC1 complex (Luo et al., 2000) have been shown to be important in the positive and negative regulation of p53 protein activity, respectively, the remaining factors responsible for its regulation as a transcription factor remain elusive.

We have previously shown that analogs of NAD that inhibit endogenous ADP-ribosylases are also able to overcome p53-dependent senescence (Vaziri et al., 1997). In addition, we demonstrated that p53 protein can bind to the NAD-dependent poly-ADP-ribose polymerase. These observations led us to speculate that other NAD-dependent enzymes, notably Sir2 which was known to be involved in life span regulation in lower eukaryotes, might also be able to interact with p53. For these reasons, we isolated the full-length human homolog of the Sir2 gene and investigated the function of its product in regulating the p53-dependent DNA damage response pathways in mammalian cells. These experiments revealed unanticipated physical and functional interactions between the Sir2 protein and p53. Related results are to be found in the report by Luo et al. (2001) in this issue of *Cell*.

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Results

Physical Interaction of hSir2 with p53

Our initial interest in Sir2 was motivated by its reported involvement in the DNA damage response and its ability to function as an NAD-dependent ADP ribosylase (Tanny et al., 1999); this latter trait allied it with other ADP ribosylases that are activated in response to DNA damage. However, soon after our work began, the function of SIR2 as an NAD-dependent deacetylase was reported (Imai et al., 2000; Smith et al., 2000). Independent of this, research of others had previously indicated that the p53 protein is acetylated in response to DNA damage (Abraham et al., 2000; Sakaguchi et al., 1998). These various findings reoriented our thinking to entertain the possibility that Sir2 acts as a deacetylase of p53, thereby downmodulating its function.

In order to study the possible functional interaction between p53 and hSir2, we isolated and introduced a full-length human *hSIR2*^{SIRT1} cDNA clone (Experimental Procedures) into a pBabe-based retroviral expression vector that also carries a puromycin resistance gene as a selectable marker. The resulting construct was termed pYESir. A retroviral construct bearing a derived, mutant allele of the *hSIR2*^{SIRT1} gene and termed pYESirHY was constructed and used in parallel. This mutant allele specifies an amino acid substitution at residue 363 of the hSir2 protein, replacing the normally present histidine by tyrosine. We reasoned, by analogy with the Sir2 protein of yeast (Tanny et al., 1999), that this substitution would result in alteration of the highly conserved catalytic site of the hSir2 protein, the loss of its deacetylase activity, and the acquisition of a dominant-negative function. These vector constructs were used to express hSir2 by transfection and by retroviral infection.

We also developed a polyclonal rabbit antibody that specifically recognizes the C-terminal portion of hSir2; its specificity was validated both by the results of immunoprecipitation and Western blotting presented in Figure 1A. Both the endogenous and the ectopically expressed hSir2 proteins detected in these assays migrated as a protein species of 120 kilodalton (kDa) (Figure 1A). Immunofluorescent staining of the hSir2 protein using our hSir2 antibody revealed nuclear localization (Figure 1B).

To test for possible physical interactions between hSir2 and p53, we cotransfected the pYESir2wt plasmid and a vector expressing wt p53 under the control of the cytomegalovirus promoter (pCMV-wtp53) transiently into H1299 human lung carcinoma cells. These cells have a homozygous deletion of the p53 gene (Mitsudomi et al., 1992). Cell lysates were subsequently mixed with the rabbit anti-hSir2 antibody and the resulting immune complexes were collected and analyzed by immunoblotting with the Ab-6 anti-p53 antibody (Figure 1D). As indicated in Figure 1D, immunoprecipitation of hSir2 from lysates of these cotransfected cells resulted in coprecipitation of p53. We also detected this interaction reciprocally by using the Ab-6 anti-p53 antibody for immunoprecipitation and our anti-hSir2 antibody for probing of the blotted precipitate (Figure 1C). These observations provided the first indications that hSir2 and p53 can form physical complexes with one another in vivo.

We also determined whether hSir2 could form complexes with p53 protein even without ectopic overexpression of these two proteins. To do this, we performed immunoprecipitation of lysates of primary BJ human fibroblasts or MCF-7L human breast cancer cells with our anti-hSir2 and p53 antibodies as described above (Figures 1C and 1D). These observations indicated that hSir2 and p53 formed physical complexes with each other in vivo under physiological conditions. However, no p53 or Sir2 proteins were detected when either a control antibody or IgG was used. As an additional control, lysates from Saos2 cells (lacking p53 expression) were subjected to immunoprecipitation in the same set of experiments described above. Complexes between hSir2 and p53 protein were not detectable in these experiments (Figures 1C and 1D).

Comparison of the immunoprecipitable hSIR2 protein present in MCF-7L cells with the amount present in precipitates generated with anti-p53 antibody allowed us to estimate that approximately 0.0005% of the total hSir2 protein was present in physical complexes with p53 (Figure 1C). Conversely, we estimated that in MCF-7L cells, approximately 1% of the total p53 could be found in physical complexes with the hSIR2 protein (Figure 1D).

Deacetylation of p53 by hSir2 In Vitro

Since hSir2 could form physical complexes with p53, we sought to determine whether this protein, a known deacetylase, could deacetylate human p53 in vitro. To address this question we used bacterially expressed murine Sir2, substituted for human Sir2 because of low yields of the human protein. As substrate in these reactions, we used a 20 residue-long oligopeptide that contains the sequence corresponding to residues 368-386+Cys of the human p53 protein. The lysine residues in this oligopeptide corresponding to residues 373 and 382 of the p53 protein were synthesized in an acetylated form. These two residues of p53 are known to be acetylated in vivo by p300 (Gu and Roeder, 1997) following either γ- or UV-irradiation (Liu et al., 1999; Sakaguchi et al., 1998; Abraham et al., 2000). After incubation with mSir2α, we detected alterations of this oligopeptide substrate by high-pressure liquid chromatography (HPLC).

As mentioned, the deacetylase activity of Sir2 utilizes NAD as a cofactor (Smith et al., 2000). In the absence of added NAD, incubation of mSir2α with the p53 oligopeptide gave rise to a single prominent peak (peak 1) and a second, minor peak (peak 2) when the incubated material was analyzed by HPLC; these two peaks corresponded to the monomeric and dimeric forms of the acetylated peptide, respectively (Figure 2A). In contrast, incubation of this oligopeptide and mSir2α in the presence of 1 mM NAD produced a singly deacetylated species as the major product (peak 3, Figure 2B). Edman sequencing of this singly deacetylated species revealed that mSir2α preferentially deacetylated the residue corresponding to Lys382 of p53 (Figures 2E–2H) while having relatively little effect on the acetylated Lys373 residue. Furthermore, a catalytically inactive mutant form of mSir2α, termed H355A, was unable to deacetylate the acetylated p53 peptide (Figure 2D). Like the H363Y mutant version of hSir2, this H353A mutant carries a substitution in residue 353, in this case an alanine in place of the normally present histidine. These results

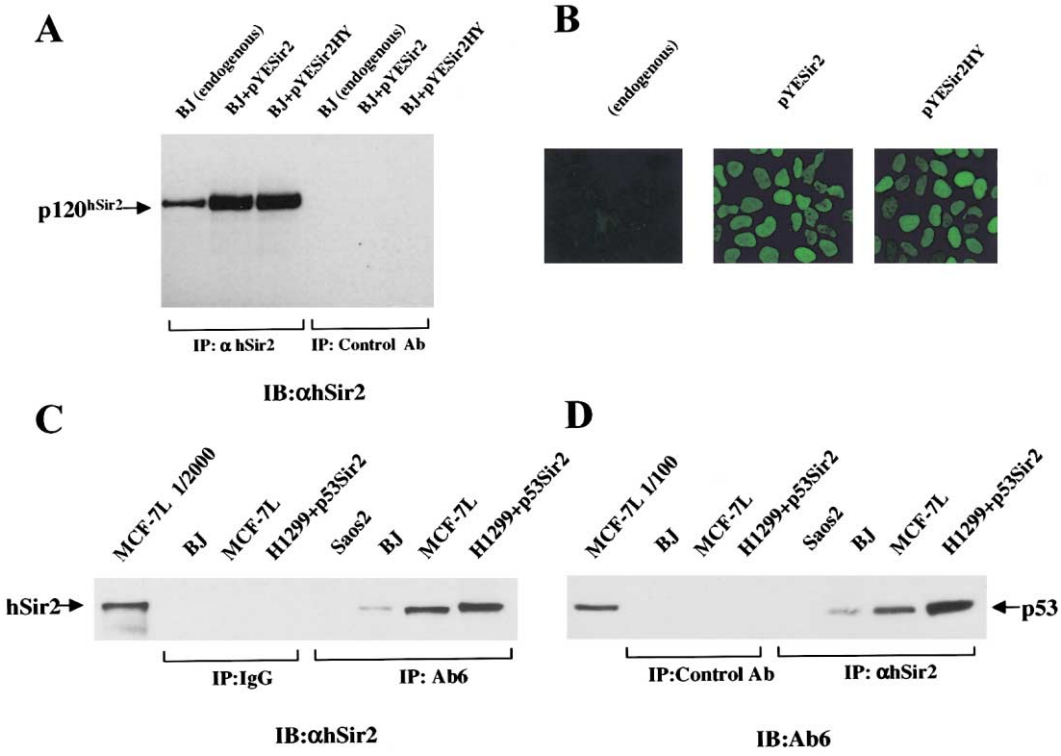


Figure 1. Coprecipitation of hSir2 and p53 Protein

(A) Immunoprecipitation of hSir2 followed by immunoblotting with the same antibody revealed the existence of a 120 kDa protein in normal BJ fibroblasts, and increased levels in these cells expressing the wild-type and HY mutant of hSir2.

(B) Immunofluorescence analysis of hSir2 indicated the existence of a nuclear protein with a nuclear staining pattern.

(C) p53 protein was immunoprecipitated with the Ab-6 (DO-1) anti-p53 antibody from lysates as shown, the blot was probed with anti-hSir2 antibody.

(D) Nuclear lysates from H1299 cells, BJ, Saos2, and MCF-7L were precipitated with the anti-hSir2 antibody. The blot was probed with the anti-p53 antibody Ab-6. H1299 cells were ectopically expressing hSir2 and wt p53.

provided evidence that the acetylated p53 peptide could serve as a substrate for mSir2α and indicated that the de-acetylation of p53 at Lys382 by mammalian Sir2 is specific and not the result of an indiscriminate deacetylase function.

Deacetylation of p53 by hSir2 In Vivo

We proceeded to test the ability of hSir2 to deacetylate full-length p53 protein in vivo. Previous studies had shown that acetylated p53 can be produced in vivo by cotransfection of a p53 expression plasmid together with one expressing p300 in the absence of exposure to DNA-damaging agents (Luo et al., 2000). We have found, however, that under our conditions of transient transfection, ectopically expressed p53 is acetylated, even in the absence of ectopically expressed p300 acetylase (Figure 3). We suspect that the transfection procedure may induce a DNA damage response, likely due to fragmented DNA introduced into the cells during this procedure, and that this damage response causes endogenous cellular acetylases, notably p300, to modify the ectopically expressed p53. We took advantage of the resulting acetylated p53 in subsequent experiments, since it allowed us to assess the influence of hSir2 on acetylated p53 in the presence of endogenous, physiologically relevant concentrations of p53 acetylases.

We used this system to determine whether hSir2 could deacetylate the ectopically expressed p53 protein at its K382 residue in H1299 cells. To gauge the levels of acetylation of p53 at Lys382, we used a rabbit polyclonal antibody, termed Ab-1 which had been raised against the acetylated K382 of p53 protein. Its specificity had been validated previously (Sakaguchi et al., 1998).

Introduction of the CMVp53 plasmid expressing wild-type p53 into the H1299 cells confirmed that p53 protein in these cells is acetylated at K382, as demonstrated by probing the immunoblot with the Ab-1 antibody (Figure 3A, lanes 3, 6). Recognition of this acetylated form of p53 by the Ab-1 antibody was specific, since a mutant K382A p53 protein was not recognized by the Ab-1 antibody (Figure 3A, lane 2).

Cotransfection of the hSir2- and p53-expression plasmids substantially decreased the acetylated p53 that could be detected by the Ab-1 antibody (Figure 3A, lane 4). The residual amount of acetylated p53 could be further reduced by increasing the dose of cotransfected hSir2 expression plasmid (lane 5).

Since the overall level of p53 was largely unaffected by ectopic expression of hSir2 (Figure 3A), we concluded that hSir2 acts in vivo to specifically reduce the level of the K382 acetylated form of p53. In light of the previously demonstrated deacetylase activity of hSir2, we con-

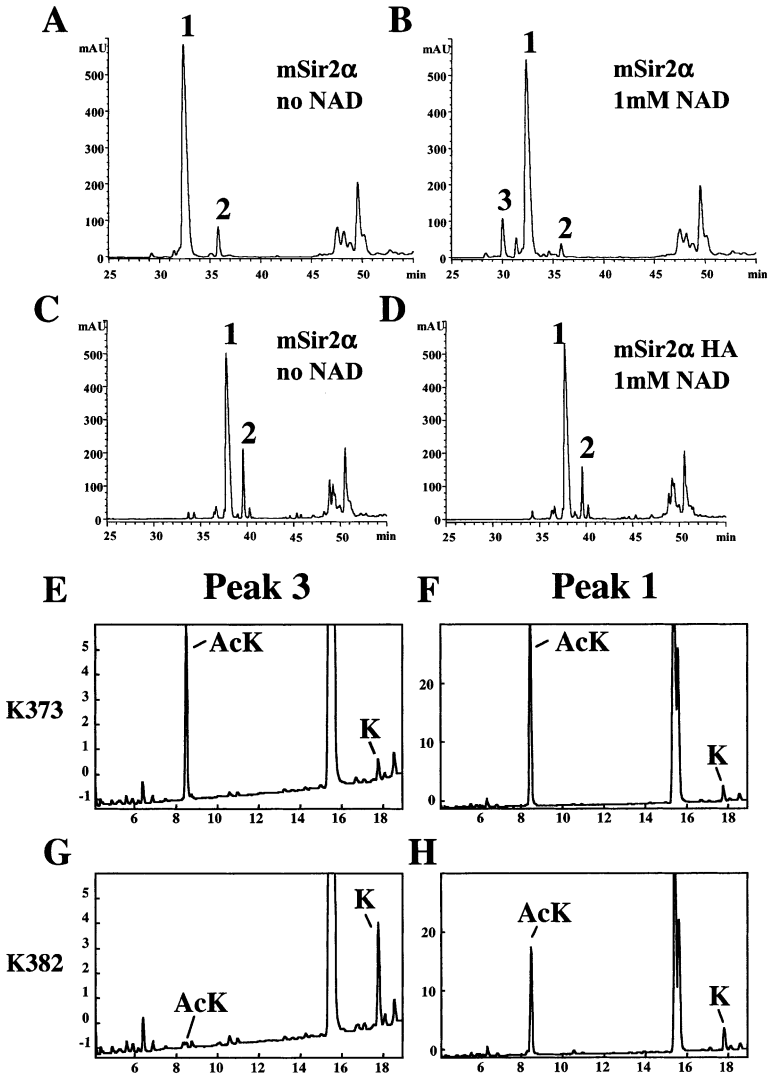


Figure 2. Effect of hSir2 Expression on p53 Acetylation In Vitro

The deacetylation activity of mSir2α on the human p53 C-terminal peptide (residues 368-386) diacetylated at positions 373 and 382. (A and B) HPLC chromatograms of products of deacetylation assays with mSir2. (C) HPLC chromatogram of wild-type mSir2 in absence of NAD as a control for (D). (D) HPLC chromatograms of products from a mutant mSir2α protein in presence of NAD. (C) and (D) are from a different set of HPLC analysis performed independently from that for (A) and (B). Peaks 1 and 2 correspond to the monomeric and dimeric forms of the p53 peptide, respectively. Peak 3 corresponds to the singly deacetylated monomer identified by mass spectroscopy. (E–H) Amino-terminal Edman sequencing of peaks 1 and 3. Chromatograms of positions 373 and 382 are shown. Peaks of acetyl-lysine (AcK) and simple lysine (K) are indicated in each panel. Small peaks of lysine are due to residual fractions of previous lysines at positions 372 and 381.

cluded further that this reduction of acetylated p53 levels was achieved by the direct action of the hSir2 enzyme on acetylated p53 substrate.

We also introduced into these H1299 cells the hSir2HY vector which expresses the mutant, catalytically inactive hSir2. In our assay, this mutant failed to deacetylate p53 efficiently, indicating that the catalytic activity of the introduced wild-type hSIR2 gene product was required for specific deacetylation of p53 Lys382 (Figure 3A, lane 7). However, we did observe a small amount of deacetylase activity at 2-fold higher hSir2HY plasmid concentrations (lane 8).

Finally, as a measure of the substrate specificity of hSir2, we gauged its effects on acetylated histone, specifically the acetylated lysine 9 residue of histone H3. We monitored H3 Lys9 acetylation through use of the 9671S monoclonal antibody which specifically recognizes histone H3 that is acetylated at this position (Experimental Procedures). hSir2 overexpression did not alter the acetylation of histone H3Lys9 (Figure 3B). Taken together, these results provide evidence that the deacetylation of p53 Lys382 in vivo reflects a defined substrate specificity of hSir2 and not a nonspecific consequence of its overexpression.

Effects of TSA on hSir2

Detection of endogenous acetylated p53 in most cell types is a difficult task due to the metabolic instability of p53 protein and the resulting need to process and analyze large amounts of extracted protein. It appears that the deacetylase inhibitor trichostatin A (TSA) can increase the levels of acetylated p53 protein in cells (Sakaguchi et al., 1998). This is likely due to inhibition by TSA of non-hSir2 deacetylases that target p53 protein. At the same time, in vitro studies have indicated that Sir2 belongs to a class of deacetylases that are resistant to TSA inhibition (Imai et al., 2000). Together these observations suggested the usefulness of including TSA in our in vivo assays. To confirm the resistance of hSir2 to this drug and examine the utility of using TSA, we subjected MCF-7L (Experimental Procedures) cells and their derivatives infected with either control vector, hSir2wt or hSir2HY retroviral vectors to 6 Gy

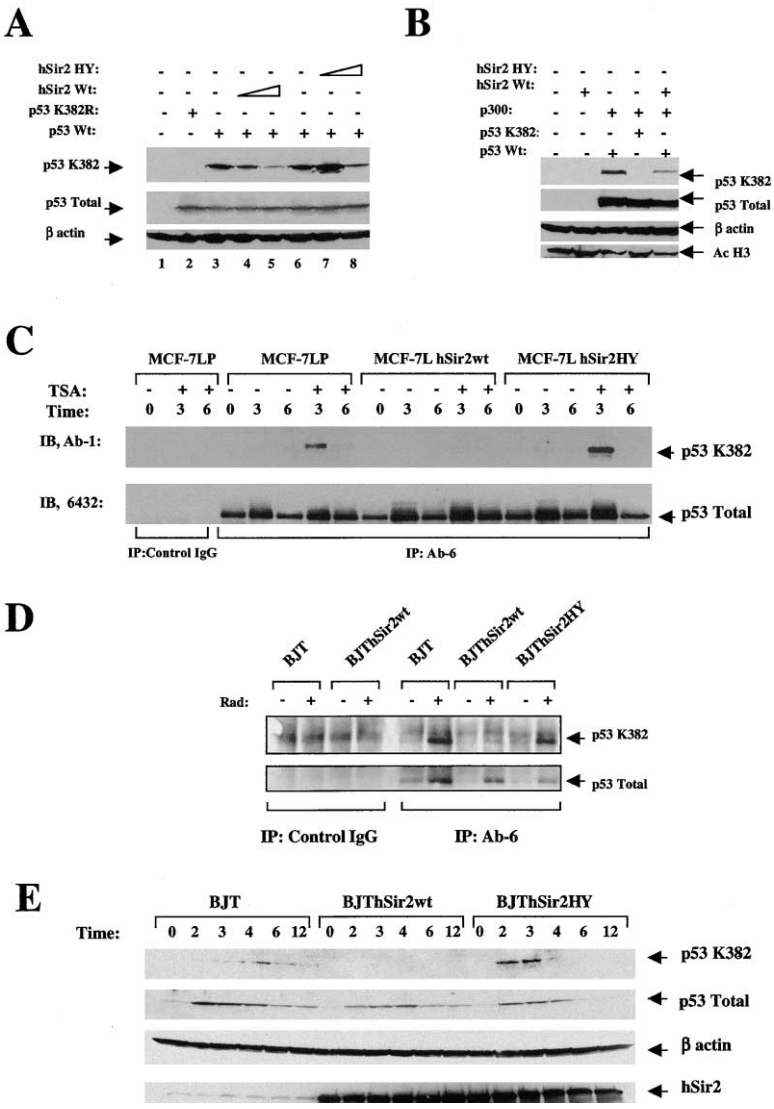


Figure 3. hSir2 Effects on p53 Acetylation In Vivo

(A) Reconstitution of the acetylation and deacetylation in H1299 cells by transient cotransfection. After cotransfection of the constructs, the cellular lysates were analyzed by western blot analysis, using Ab-1 to detect K382 p53, 6243 for total p53 or β actin for loading control. Lanes 3, 6, CMVwtp53 by itself generates acetylated p53 at K382. Lane 2, tranfection of the acetylation mutant K382R of p53. Lanes 4, 5, with cotransfected wild-type hSir2. Lanes 7, 8, cotransfected mutant hSir2HY.

(B) Similar to (A), except for Histone H3 acetylation was assessed before and after addition of wild-type hSir2 and the mentioned constructs.

(C) MCF-7LP, MCF-7LhSir2wt and MCF-7LhSir2HY cells were treated with or without 0.1 μM concentrations of TSA and subjected to 6 Gy of ionizing radiation. Acetylated levels of p53 were detected using Ab-1. Total p53 levels were measured using 6243 antibody (bottom).

(D) BJT control cells and their derivatives BJThSir2wt and BJhSir2HY were treated with 6Gy of ionizing radiaton. 1 mg of lysates were immunoprecipitated with either a control whole IgG fraction or with Ab-6. The resulting immunoblot was probed with Ab-1 (top) for acetylated p53 and for total p53 protein.

(E) BJT cells were stably infected with either a wild-type expressing hSir2 or a mutant hSir2HY virus. Cells were subjected to 6 Gy of ionizing radiation in presence of low concentrations of TSA (0.1 μM), and the p53 acetylation was measured at indicated time points by immunoblotting with Ab-1 that recognizes specifically the deacetylated K382 p53 protein. The blots were subsequently probed with anti-p53, anti-β actin and anti-hSir2 antibodies. Time (hr) post 6 Gy of irradiation is shown inside the brackets.

of ionizing radiation in the presence or absence of 0.1 μM TSA.

As indicated in Figure 3C, wild-type hSir2 was able to efficiently deacetylate p53 either in the presence or absence of TSA. In contrast, the mutant hSir2HY was unable to deacetylate p53. These results indicated that TSA treatment did not affect the ability of hSir2 to deacetylate its in vivo target, p53 protein. These results also indicate that in vivo hSir2 acts as a TSA-resistant deacetylase. Having validated the use of TSA in our experiments, we proceeded to use this drug to facilitate the investigation of the effect of hSir2 on p53 acetylation following DNA damage.

hSir2 as an Antagonist of the Radiation-Induced p53 Acetylation

The observation that p53 is acetylated at K382 raised the question of whether hSir2 could antagonize and reverse this acetylation of p53, doing so through its deacetylase function. We chose to investigate this possible interaction in BJT cells, polyclonal derivatives of human BJ fibroblasts that have been immortalized by ectopic expression of the telomerase enzyme. We note that our previous studies indicated that expression of telomerase in these cells, undertaken to extend their life span, had no effect on either the activation of p53 protein or on their responses to DNA damage (Vaziri et al., 1999).

We expressed either wild-type hSir2 or the mutant form specified by the hSir2HY vector in the BJT cells and examined subsequent responses to ionizing radiation. To monitor the anticipated acetylation of p53, we used, as before, the Ab-1 antibody which recognizes the acetylated p53 protein. Indeed, upon exposure of the cells to 6 Gy of ionizing radiation, we observed a marked increase in the level of acetylated p53 protein that could be immunoprecipitated by the Ab-6 antibody and detected by immunoblotting with the Ab-1 antibody (Figure 3D). This increase in acetylated p53 protein was blocked effectively in BJT cells that stably overexpressed wild-type hSir2. In contrast, cells expressing the hSir2HY mutant protein showed acetylated p53 protein levels comparable to those of control BJT cells while

the level of total immunoprecipitable p53 in these cells was slightly decreased.

Evidence for Dominant-Negative Effects of hSir2HY on Acetylated p53 following DNA Damage

Experiments in the previous section suggested that p53 protein can be deacetylated by hSir2 in BJT cells. However, these experiments did not address the kinetics with which maximal acetylation/deacetylation at K382 residue occurs in the presence of wild-type or mutant hSir2. We also wished to measure the levels of wild-type and mutant hSir2 that were required to alter the acetylated state of p53 in the BJT cells ectopically expressing one or the other of these hSir2 proteins.

To address these issues, we exposed the BJT cells and their derivatives to 6 Gy of ionizing radiation in the presence of 0.1 μM TSA. We subsequently collected cells before irradiation and at five time points post-irradiation. Cells were lysed and equal amounts of protein were resolved by gel electrophoresis and then transferred to immunoblots. As in earlier experiments, we used as a probe in these immunoblots the polyclonal rabbit antiserum (Ab-1) which specifically recognizes the acetylated K382 form of p53. These blots were sequentially reprobed with a polyclonal p53 antibody, with a β actin antibody as a loading control, and with the anti-hSir2 antibody.

As anticipated, following 6 Gy of ionizing radiation we observed a 1.5- to 2-fold increase in the level of acetylated p53 protein, indicated by the level of p53 protein recognized by the Ab-1 antiserum (Figure 3E). A 4-fold increase in hSir2 level, achieved through ectopic expression of hSir2, sufficed to block the radiation-induced increase in acetylated K382 p53 protein (Figure 3E). In contrast, we observed that the ectopic expression at comparable levels of the catalytically inactive hSir2HY increased the radiation-induced levels of p53 acetylated at residue K382 (Figure 3B). This observation provided support for the notion that the hSir2HY mutant protein was able to act in a dominant-negative fashion in BJT cells.

We considered it possible that the observed inhibition of acetylated p53 described above might be attributable to a reduction in the levels of total p53 in these cells. However, a reprobing of this immunoblot with a polyclonal anti-p53 antibody showed normal stabilization of p53 in control cells in response to DNA damage and at most very slightly reduced steady-state levels of p53 in the presence of ectopically expressed wild-type hSir2 (Figure 3E). This lower level of p53 could not account for the much larger decrease in acetylated p53 observed in the presence of wild-type hSir2. Moreover, the mutant hSir2HY also reduced the stability of p53 protein slightly despite its ability to cause increased acetylation of this protein (Figure 3E). Hence, while hSir2 was able to reverse the radiation-induced acetylation of p53 in these cells, it had only minimal effects on the steady state level of p53 induced by exposure to radiation.

We also made several observations regarding the kinetics of p53 acetylation (Figure 3E). Ectopic expression of hSir2 was able to efficiently block p53 acetylation. In contrast, expression of the mutant hSir2HY in BJT cells had the opposite effect: it caused premature induction of p53, seen already at 2 hr post-irradiation, compared to induction at 3–4 hr post-irradiation in control, parental BJT cells. On the basis of these experiments, we concluded that wild-type hSir2 protein is able to deacetylate p53 protein efficiently at the K382 residue. Moreover, ectopic expression of a mutant hSir2HY protein at only 4-fold above endogenous background levels of hSir2 is able to exert a significant dominant-negative effect.

Effects of hSir2 on the Transcriptional Activity of p53 Protein

We also investigated the possible effects of hSir2 on the activity of p53 as a transcription factor. To do so, we cotransfected H1299 cells transiently with a p53 expression plasmid and a reporter construct in which the promoter of the *p21^{WAF1}* gene (el-Deiry et al., 1993), a known target of transcriptional activation by p53, drives expression of a luciferase reporter gene (Vaziri et al., 1997). As indicated in Figure 4A, luciferase activity increased in response to increasing amounts of cotransfected wild-type p53 expression vector. Conversely, the activity of the p21 promoter was suppressed in a dose-dependent fashion by coexpression of wild-type hSir2. We note that there remains a possibility that hSir2 can also suppress the p21 promoter in a p53-independent fashion, since luciferase activity was also reduced below that of the control (lane 1) cells in which no ectopic p53 was expressed. The catalytically inactive hSir2HY mutant had no effect on the activity of this promoter (Figure 4A). To gauge the specificity of hSir2 in affecting promoter activity, we used as control a constitutively active SV40 promoter linked to the luciferase gene. Expression of this control construct was not affected by increasing amounts of the hSir2 vector expressed at any level (Figure 4B).

The above experiments relied on ectopic gene expression and may not necessarily reflect how p21^{WAF1} expression is affected by hSir2 under more physiologic conditions including exposure to ionizing radiation. To address this issue, we used MCF-7L cells cultured in the absence of TSA. Like the parental MCF-7 cells, those from the MCF-7L subline can induce p53 protein normally in response to radiation. These cells were infected stably with the retroviral vectors expressing wild-type hSir2 and dominant-negative hSir2HY. We then exposed these cells to 6 Gy of ionizing radiation and subsequently measured total p53 and p21^{WAF1} protein levels (Figure 4C). p53 protein levels increased normally in these polyclonal cell populations in response to irradiation. A 4-fold overexpression of wild-type hSir2 resulted in a slight but clearly detectable decrease of p21^{WAF1} protein in these cells (Figure 4C). This decrease was not sufficient to abrogate the p53-dependent G1 checkpoint as measured by analysis of DNA content (data not shown). In contrast, the levels of p21^{WAF1} protein in irradiated MCF-7L cells expressing the hSir2HY protein were significantly (~3-fold) higher when compared with parental MCF-7LP cells infected with an empty control vector.

Consistent with this effect on p21 protein levels, we found that measurements of the K382-acetylated fraction of p53 in lysates of irradiated hSir2HY-expressing MCF-7LP cells showed a large increase when compared with the irradiated controls that did not express hSir2HY

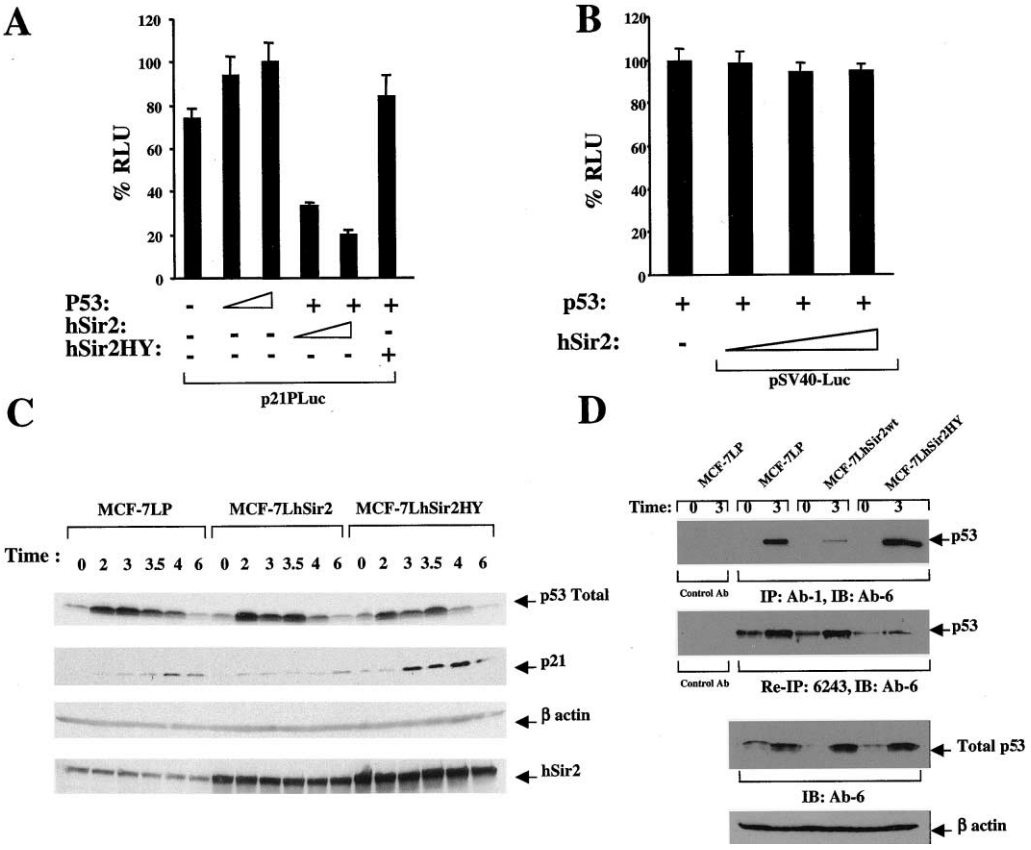


Figure 4. hSir2 Expression and Its Influence on p53 Activity and p21^{WAF1}

(A) Transcriptional activity of p53 protein was measured in H1299 cells by cotransfection p53 with a p21^{WAF1} promoter-luciferase construct (p21Pluc) and hSir2wt or hSir2HY.

(B) A control SV40-Luciferase was cotransfected with CMVp53 and increasing amounts of wild-type hSir2 into H1299 cells and luciferase activity was measured.

(C) Levels of p21^{WAF1} in MCF-7L cells expressing wt hSir2 or hSir2HY protein in response to 6 Gy of ionizing radiation in absence of TSA. The blot was probed with Ab-6 for detection of total p53 and β actin for loading control.

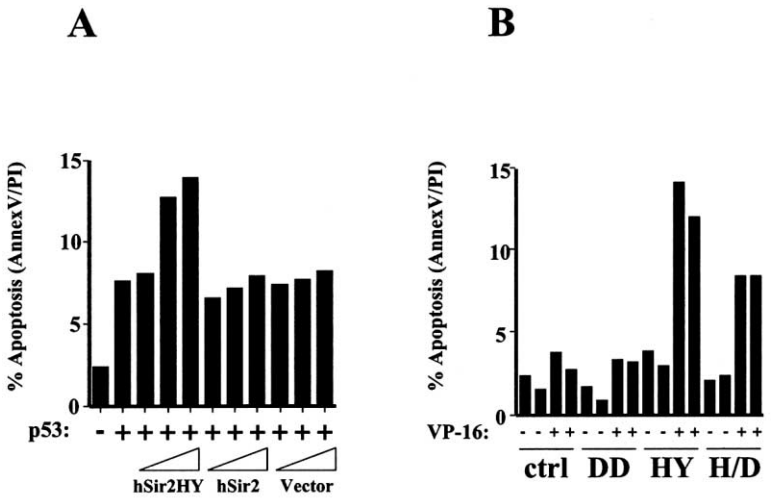
(D) Deacetylation of p53 in vivo in MCF-7LP cells. Ectopic expression of wild-type hSir2 or hSir2HY mutant in MCF-7L cells radiated with 6 Gy of ionizing radiation and its effect on p53 acetylation at K382. 1 mg of total protein lysates was subjected to immunoprecipitation with Ab-1 (top panel), and the blot was probed for p53 with Ab-6. The remaining lysates were immunoprecipitated with SC6423 polyclonal rabbit antibody for total p53 protein, and the resulting p53 in the precipitate was detected by Ab-6 (second panel). Equal amounts of protein from the aforementioned experiment were also quantified to ensure equal total protein was loaded and that total p53 levels were comparable to each other using Ab-6 and β actin antibodies.

(Figure 4D, upper panel). Furthermore, immunoprecipitation with a polyclonal anti-p53 rabbit antibody of these lysates, following depletion by incubation with the Ab-1 antibody indicated that the majority of p53 in the irradiated MCF-7LhSir2HY cells was in an acetylated state (Figure 4D). Taken together, these experiments converged on the conclusion that the hSir2HY mutant functions as a dominant-negative protein by quantitatively inhibiting the p53 deacetylation at K382.

Potentiation of p53-Dependent Apoptosis by hSir2HY

Given that dominant-negative inhibition of hSir2 can potentiate the ability of p53 to induce p21^{WAF1}, we sought to ascertain whether hSir2 inhibition could potentiate p53-dependent apoptosis. To address this possibility, we relied on the fact that restoration of wild-type p53 function in H1299 cells, achieved via introduction of a wt p53-expressing vector, induces apoptosis as gauged by the display of the cell surface annexin V antigen (Figure 5A). The apoptotic response in H1299 cells transfected with p53 was potentiated in a dose-dependent manner in cells cotransfected the dominant-negative hSir2HY expression plasmid. Expression of hSir2 on its own did not significantly change the level of apoptosis.

Although this assay is accepted widely for measuring p53-dependent apoptosis, it relies on ectopic expression of p53 under nonphysiologic conditions. In order to address this problem, we made use of BJT cells which contain a functional endogenous p53 protein. These cells undergo p53-dependent apoptosis upon exposure to high concentration of the chemotherapeutic drug VP-16 (etoposide), that induces double-strand DNA breaks (Figure 5B). Exposure of BJT and BJThSir2HY cells to 40 μM VP-16 and TSA induced apoptosis, as gauged by the display of the cell surface annexin V antigen



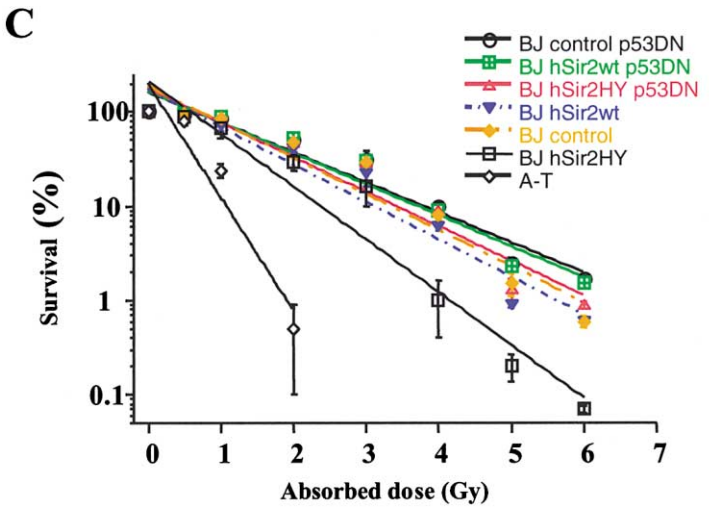


Figure 5. Effects of hSir2 on p53-Dependent Apoptosis and Radiosensitivity

(A) Ectopic expression of hSir2HY and its dose-dependent influence on p53-dependent apoptosis in H1299 cells. H1299 cells were transfected with a wild-type p53 expression construct to induce p53-dependent apoptosis. Annexin V positive and propidium iodide negative cells were measured after 48 hr.

(B) BJT and BJThSir2HY cells were exposed to 40 μM VP16 in presence of TSA and p53-dependent apoptosis was measured as in (A) after 72 hr. Ctrl (BJT cells), DD (BJT cells expressing dominant-negative p53DD), HY (BJThSir2HY cells), H/D (BJThsir2HY cells expressing dominant-negative p53DD).

(C) Comparison of γ-ray survival. Dose-response curves are shown for different types of BJ cells treated with ionizing radiation while growing exponentially and asynchronously. Twelve days after radiation the colonies were counted and survival calculated.

(Figure 5B). We found that expression of hSir2HY in BJT cells potentiated apoptosis by VP-16 significantly. Ectopic expression of a dominant-negative p53 construct reduced hSir2HY-induced apoptosis, indicating its dependence on p53 function. These results indicated that hSir2HY specifically potentiated apoptosis induced by DNA damage. Moreover, these observations are consistent with our previous results indicating that hSir2HY potentiates p53 function via inhibition of deacetylation.

Effects of Mutant hSir2HY on Radiosensitivity of Human Fibroblasts

Human fibroblasts become relatively radioresistant upon loss or inactivation of their p53 function (Tsang et al., 1995). This behavior suggested an additional test of the ability of hSir2 to antagonize p53 function, which depended on measuring the long-term survival of human BJT fibroblasts cells following exposure to various doses of low-level ionizing radiation. Ectopic expression of wild-type hSir2 to a level 4-fold above that of the endogenous protein in these irradiated cells had no significant effect on long-term survival (Figure 5C), while expression of the mutant hSir2HY sensitized BJT cells to radiation. As anticipated, control ataxia-telangiecstasia cells were found to be highly radiosensitive (Figure 5C).

The specific role played by p53 in these various survival responses was demonstrated by the behavior of three sublines of BJT fibroblasts that expressed a dominant-negative form of p53 (Shaulian et al., 1992) in addition to either hSir2wt, hSir2HY, or a control vector. Expression of the DNp53 construct abrogated the radiosensitive behavior of BJhSir2HY cells and rendered them once again radioresistant. This provided strong evidence that the dominant-negative behavior of hSir2HY in BJT cells is dependent on p53 function. Moreover, these responses support the previous conclusion that the hSir2HY mutant specifically potentiates the activity of p53 protein.

Discussion

The SIR2 complex in *S. cerevisiae* was originally identified through its involvement in the maintenance of chromatin silencing at mating type loci and telomeres. It is

composed of three component subunits, Sir2-4p, which are normally found localized to yeast telomeres. In response to DNA damage, the SIR complexes relocate to the site of double-strand breaks where they participate in the repair of these lesions. This DNA damage response is dependent on the function of the MEC1/RAD9 DNA checkpoint pathway. MEC1 is related to the ATM protein which coordinates the DNA damage response in mammalian cells, in part by triggering the cascade of events that lead to stabilization of the p53 protein.

Double-strand DNA breaks in the genomes of mammalian cells trigger a cascade of signaling events that ultimately result in phosphorylation and subsequent stabilization of the p53 protein. In addition, these breaks lead to activation of p53 protein as a transcription factor. This functional activation depends in substantial part on its acetylation. The resulting stabilized, activated p53 protein contributes to the upregulation of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ and thus to the cytostatic effects of p53. Alternatively, depending on the cellular background and the degree of DNA damage, the proapoptotic effects of p53 may predominate.

These various phenomena indicate that specific components of the machinery that monitor the integrity of the genome are able to alert p53 to the presence of genetic damage and cause its functional activation. Conversely, in the event that damage has been successfully repaired, signals must be conveyed to p53 in order to deactivate it. Thus, a blockage of cell cycle progression that has been imposed by p53 in order to allow DNA repair to proceed must be relieved following completion of repair, enabling the cell to return to its active growth state. In such a situation, the inactivation of p53 becomes as important physiologically as its activation.

In the present work, we have observed that hSir2 directly binds the human p53 protein in vivo and specifically deacetylates the K382 residue of p53. One of the observed functional consequences of this deacetylation is an attenuation of the p53 protein's activity as a transcription factor at the $p21^{WAF1}$ promoter. In other cellular contexts, in which the DNA damage response leads to apoptosis, hSir2 activity inhibits the p53-dependent apoptotic response (Luo et al., 2001).

With these observations in mind, we propose that in mammalian cells, signals indicating the successful completion of DNA repair are relayed via hSir2 to acetylated proteins like p53 that have been charged with the task of imposing a growth arrest following DNA damage. In some way, these signals enable hSir2 to reverse in part the damage-induced activation of p53 by deacetylating the K382 residue of p53. In doing so, hSir2 in collaboration with other deacetylases reduces the likelihood of subsequent apoptosis and, at the same time, makes it possible for cells to return to the physiological state they enjoyed prior to sustaining damage to their genomes.

Alterations of the p53 signaling pathway are presumed to be found in most human tumors (Hollstein et al., 1994). In about half of these tumors, mutation of the *p53* gene suffices to derail function. In some of the remaining tumors, loss of $p14^{ARF}$, which acts to downregulate p53 protein levels, has been implicated. The present observations raise the possibility that a third mechanism by which an incipient cancer cell can rid itself of p53 functions may involve overexpression of hSir2 which, like the other two genetic strategies, should contribute to the inactivation of p53. Furthermore, chemical inhibition of deacetylases like hSir2 may potentiate apoptosis of tumor cells that have been challenged with DNA damage-inducing drugs.

Experimental Procedures

Cell Culture and Derivation of Cell Lines

All cells were grown in presence of 20% O_2 and 5% CO_2 at 37°C in humidified chambers. BJ cells, MCF-7, and H1299 were grown in DME +10% FCS. PBS(−/−) (phosphate buffered saline) without magnesium or calcium was used in this work.

Amphotrophic viruses were produced by transient cotransfection of pCL-Ampho with the LTR-containing pBabe vectors (Morgenstern and Land, 1990), pYESir2 or pYESir2HY in to 293T cell line using Fugene6 (Roche). Primary BJ cells or MCF-7L cells were infected with retrovirus containing media in presence of 8 μg/ml of polybrene overnight. 48 hr later cells were selected in puromycin at 1 μg/ml and 5 μg/ml, respectively. These selected BJ cells were subsequently infected and selected with a pBabe-hTERT virus carrying the hygromycin resistance gene (200 μg/ml). The resulting cells were: BJT (carrying pBabe control vectors), BJThSir2wt (carrying pYESir2 and pBhTERT) and BJThSir2HY (pYESir2HYand pBhTERT). MCF-7 cells were transfected with the vector p21P-luc (Vaziri et al., 1997) and pCMVneo, clones were selected in 500 μg/ml of G418 and the clone designated MCF-7L was selected that was able to upregulate the $p21^{WAF1}$ promoter-luciferase in response to treatment with 6 Gy of ionizing radiation. MCF-7L cells were infected with the same viruses as described before to yield the following cell lines: MCF-7LP (carrying pBabe Y-puro backbone), MCF-7L-hSir2wt, and MCF-7L-hSir2HY. Cells were kept under appropriate selection throughout experiments.

Isolation of *hSIR2*^{SIRT1} Gene

hSIR2^{SIRT1} gene was obtained by PCR amplification using 5′ primer-1 GGATCCACCATGGCGGACGAGGCGGCCCTCGCC and 3′ primer-2 GTCTAGAGTGGAACAATTCCTGTACCTGCAC from a human spleen Marathon cDNA library (Clontech) to obtain a majority of the *hSIR2*^{SIRT1} cDNA including the C terminus. Due to GC-rich nature of the 5′ end of $hSIR2^{SIRT1}$, we used a human genomic clone (Accession number: AL133551, clone RP11-57G10) as a template to obtain the 5′ end. We used 10 PCR cycles using pfu-turbo PCR (denaturation at 98°C, 1M betaine and 10% DMSO were added to the stratagene pfu buffer) using Primer-1 and Primer-3, GAGGAGGAGATGCGCAGTTCCGGC CGCCC. The PCR product was cloned into pcr4blunt-TOPO (Invitrogen) and sequenced. This exon-1 on $hSIR2^{SIRT1}$ was used to complete the sequence of $hSIR2^{SIRT1}$ amplified previously by PCR. To generate the hSir2H363Y mutant, the PCR overlap primer method was used to create the point mutation (CAT to TAT) at codon 363.

Construction of Expression Plasmids

A BamHI/SnaBI fragment of *hSIR2*^{SIRT1} cDNA isolated from a cDNA library (Clontech) was inserted into pBabe-Y-Puro, the resulting plasmid was called pYESir2-puro. Similarly a BamHI/SnaBI fragment of hSir2 that was mutated at residue 363 from Histidine (H) to Tyrosine (Y) by site-directed mutagenesis (Stratagene) was used to create the retroviral vector pYESir2HY. pBhTERT contained an EcoRI/SalI fragment of hTERT cloned into EcoRI/SalI site of pBabe-Hygro.

Immunoblot Analysis

For detection of acetylated forms of p53 in BJT cells and MCF-7L cells, we plated an equal number of cells 24 hr before the experiment. 1.5×10^6 BJT cells or 10^7 MCF-7L were exposed to 6 Gy of ionizing radiation (^{137}Cesium). Cell pellets were frozen on dry ice at the appropriate time point. These pellets were lysed on ice at once by adding 0.5% NP40, 150 mM NaCl, 50 mM Tris in presence of protease inhibitor mix (Roche), for 30 min, and vortexing. Cell lysates were prepared by centrifugation for 20 min at 4°C. Protein content of lysates were measured by BioRad D_C protein assay. 300 μg of pro-

tein was resolved on gradient 4%–20% criterion Tris-HC gels (Biorad), transferred to nitrocellulose and blocked in 10% skim milk.

The resulting membrane was incubated overnight in 1:400 dilution of (PAbLys(Ac)382) (termed here Ab-1) (Oncogene Science, peptide based rabbit polyclonal anti K382 p53). This membrane was then washed twice in PBS(−/−) containing 0.05% Tween 20 for 15 min. Secondary Goat anti-rabbit antibody conjugated to HRP (Pierce) was used at a concentration of 1:30,000 for 1 hr in 1% Milk. After washing, the membrane was then incubated with Supersignal west femto maximum substrate (Pierce) for 2 min and exposed to X-OMAT sensitive film (Kodak) for up to 30 min. The membrane was subsequently blotted with a monoclonal $p21^{WAF1}$ antibody (F5, Santa Cruz Biotech), p53 antibody (SC6243, polyclonal rabbit, Santa Cruz) (Ab-6, Oncogene Science), anti-hSir2 (polyclonal rabbit). β actin was used (Abcam) for loading control. 9671S is an anti-acetyl H3; Lys9 was a monoclonal antibody (Cell Signaling).

Immunoprecipitation and Immunofluorescence

Lysates of H1299 cells transiently expressing p53 and hSir2 were incubated with 1 μl of anti-hSir2 antibody overnight. For endogenous p53 immunoprecipitation up to 2 mg of protein were precipitated by either use of NP40 lysis buffer (including 4× higher protease mix) or dounce homogenization. 50 μl of protein G-sepharose beads were added to the lysates and rotated at 4°C for 3 hr. The immune complexes were collected, washed 3–6 times, and resolved using the Nupage gradient 4%–12% with anti-oxidant (Novex). Nitrocellulose membranes were probed with anti-p53 antibody (Ab-6 (DO-1) Oncogene Science). For immunoprecipitation in BJ cells, up to 2 mg of protein per reaction were incubated with 1 μl of Ab-6 and immunoprecipitaion was performed as described above except that the time of incubation in primary antibody was 2 hr due to the high instability of p53 protein in BJT cells. Immune complexes were resolved and membranes were exposed to polyclonal anti-hSir2 antibody.

Immunofluorescence of U20S cells was undertaken by fixing the cells in microchamber slides (LabTek) in 8% paraformaldehyde for 15 min followed by 4% paraformaldehyde for 45 min in PBS and subsequent staining with anti-hSir2 antibody at 1:500 dilution. A secondary goat anti-rabbit FITC antibody at 0.5 μg/ml was used for detection of signal.

Luciferase and Apoptosis Assays

H1299 cells were transfected using Fugene6 (Roche) with pCMVwtp53, pYESir, pYESirHYand p21P-Luc (containing a 2.4 kb fragment of p21 linked to luciferase gene) and luciferase assays performed (Promega). Apoptosis was measured at approximately 48 hr post-transfection using FITC-conjugated anti-annexinV antibody and propidium iodide exclusion (Clontech laboratories). For BJT cells, apoptosis was assayed after incubation of cells in presence of 40 μM VP16 and 0.1 μM TSA for 72 hr. Radiation survival curves of BJ cells were performed as described previously (Dhar et al., 2000).

Deacetylation Assay of the p53 C-Terminal Peptide

The human p53 C-terminal peptide (residues 368-386+Cys; HLKSK(AcK)GQSTSRHK(AcK)LMFKC) diacetylated at positions 373 and 382 was synthesized and purified with HPLC. Deacetylation assays of this peptide by Sir2 and analyses of the reaction products were performed as described previously (Imai et al., 2000).

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