

Regulation of SIRT1 activity by genotoxic stress

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SIRT1 regulates a variety of cellular functions, including cellular stress responses and energy metabolism. SIRT1 activity is negatively regulated by DBC1 (Deleted in Breast Cancer 1) through direct binding. However, how the DBC1–SIRT1 interaction is regulated remains unclear. We found that the DBC1–SIRT1 interaction increases following DNA damage and oxidative stress. The stress-induced DBC1–SIRT1 interaction requires the ATM-dependent phosphorylation of DBC1 at Thr 454, which creates a second binding site for SIRT1. Finally, we showed that the stress-induced DBC1–SIRT1 interaction is important for cell fate determination following genotoxic stress. These results revealed a novel mechanism of SIRT1 regulation during genotoxic stress.

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SIRT1, a mammalian homolog for yeast silent information regulator 2 (SIR2), is a NAD⁺-dependent deacetylase that belongs to the class III histone deacetylases (Imai et al. 2000). SIRT1 and its orthologs were initially implicated in the regulation of life span in lower organisms, including yeast, *Caenorhabditis elegans*, and *Drosophila melanogaster* (Lin et al. 2000; Tissenbaum and Guarente 2001; Wood et al. 2004), although recent studies suggested that some of the reported effects may be due to confounding effects of genetic assays (Burnett et al. 2011). In mammals, SIRT1 participates in various cellular functions ranging from differentiation and development to metabolism and cell survival by deacetylating various proteins, including histones, transcription factors, and cell cycle and apoptosis regulatory proteins (Bordone and Guarente 2005; Schwer and Verdin 2008; Finkel et al. 2009; Haigis and Sinclair 2010; Yu and Auwerx 2010). Given its role in human health, SIRT1 activities in vivo are tightly regulated (Nemoto et al. 2004; Chen et al. 2005; Wang et al. 2006; Abdelmohsen et al. 2007; Kim et al. 2007; Yang et al. 2007; Sasaki et al. 2008). Recently, we and others have demonstrated that SIRT1's activity is modu-

lated by protein–protein interaction through the DBC1 (Deleted in Breast Cancer 1) protein (Kim et al. 2008; Zhao et al. 2008; Kang et al. 2011). Using DBC1 knockout mice, we have also shown that DBC1 is a major regulator of SIRT1 in vivo (Escande et al. 2010). However, how the DBC1–SIRT1 interaction is regulated remains unclear. In this study, we found that, following DNA damage and oxidative stress, DBC1 binds more tightly to SIRT1. We further characterized the mechanism underlying this stress-induced DBC1–SIRT1 interaction and its functional significance.

Results and Discussion

DBC1–SIRT1 interaction increased following cellular stress

Previous studies have shown that p53 acetylation, which is deacetylated by SIRT1, increases following DNA damage (Luo et al. 2001; Vaziri et al. 2001). In addition to p53 acetylation, the acetylation of other SIRT1 target proteins also increases, suggesting that SIRT1 activity is inhibited by DNA damage (Fig. 1A). When we examined the protein levels of DBC1 and SIRT1 following various genotoxic stresses, we found that the protein levels of DBC1 and SIRT1 did not change (Fig. 1B), suggesting that other mechanisms besides protein expression regulate SIRT1 activity following genotoxic stress. Previous studies have suggested that decreased NAD⁺ levels caused by PARP activation could contribute to decreased SIRT1 activity (Bai et al. 2011). To test whether there were other mechanisms that might be responsible for SIRT1 inhibition following DNA damage, we immunoprecipitated SIRT1 protein from cells and performed an in vitro deacetylation assay. As shown in Figure 1C, DNA damage resulted in decreased SIRT1 activity in vitro. Since we used equal amounts of NAD⁺ in the in vitro assay, we reasoned that factors other than NAD⁺ level also contribute to SIRT1 inhibition following DNA damage. Furthermore, when we treated cells with a PARP inhibitor (ABT-888) (Penning et al. 2009), which prevents NAD⁺ depletion caused by PARP activation (Bai et al. 2011), we still detected increased p53 acetylation (Fig. 1D), although the acetylation levels were moderately less than the mock-treated cells. These results suggest that, at the condition we used, NAD⁺ depletion accounts for only a fraction of SIRT1 inhibition, and SIRT1 activity could be regulated by genotoxic stress through mechanisms other than NAD⁺ depletion. Interestingly, the DBC1–SIRT1 interaction increased following genotoxic stresses in a dose-dependent manner (Fig. 1E; Supplemental Fig. 1A,C). Since DBC1 functions as a cellular inhibitor for SIRT1 (Kim et al. 2008; Zhao et al. 2008), we hypothesized that the genotoxic stress-induced DBC1–SIRT1 interaction is one of the mechanisms to regulate SIRT1 activity.

It is well-known that phosphorylation is a major post-translational modification of the DNA damage response pathway and has been shown to regulate protein activity and protein–protein interactions. We tested whether the phosphorylation of these proteins might be responsible for the inducible increase of the DBC1–SIRT1 interaction following DNA damage. As shown in Supplemental Figure 1B, λ-phosphatase treatment reversed the increase

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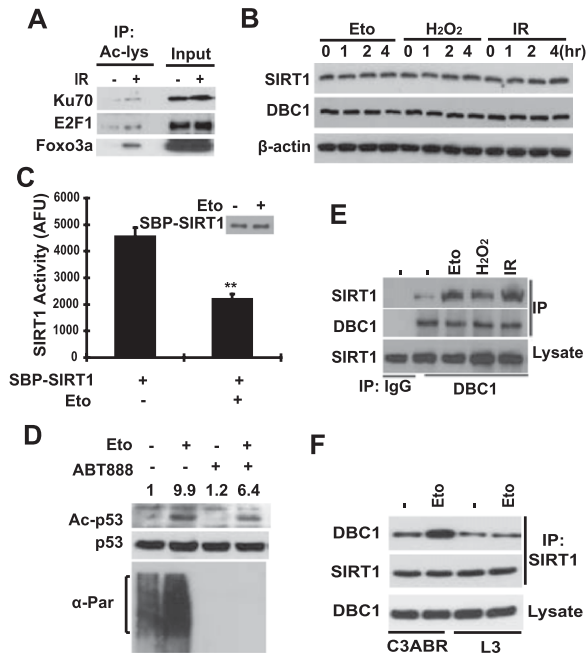


Figure 1. DBC1-SIRT1 interaction increased following cellular stress. (A) A549 cells were irradiated (10 Gy); 2 h later, cell lysates were subjected to immunoprecipitation with Ac-Lys antibodies. The immunoprecipitates were blotted with the indicated antibodies. (B) A549 cells were left untreated or were treated with etoposide (Eto) (20 μ M), H₂O₂ (500 μ M), or irradiation (10 Gy). Cells were harvested at the indicated times, and cell lysates were blotted with the indicated antibodies. (C) Cells transfected with SBP-tagged SIRT1 were left untreated or were treated with etoposide (20 μ M). Two hours later, SBP-tagged SIRT1 was immunoprecipitated from cells and used in the *in vitro* deacetylation assay. (AFU) Arbitrary fluorescence units. Error bar represents the SEM of triplicate experiments. (**) $P < 0.01$ two-tailed Student's test. (D) A549 cells were pretreated with the PARP inhibitor ABT-888 for 1 h, then left untreated or treated with etoposide (20 μ M). An additional 2 h later, cells were lysed, and cell lysates were blotted with the indicated antibodies. Numbers represent relative intensity of Ac-p53 signals compared with the control sample. (E) A549 cells treated as in B were subjected to immunoprecipitation with control IgG or anti-DBC1 antibodies. The immunoprecipitates were blotted with the indicated antibodies. (F) ATM-proficient cells (C3ABR) and ATM-deficient cells (L3) were left untreated or were treated with etoposide. After 2 h, cells were lysed, and cell lysates were subjected to immunoprecipitation and immunoblotted with the indicated antibodies.

in the DBC1-SIRT1 interaction following DNA damage. On the other hand, λ -phosphatase inhibitors reinstated the inducible increase in the DBC1-SIRT1 interaction. These results suggest that stress-induced phosphorylation events regulate the DBC1-SIRT1 interaction. Since ATM is an important protein kinase that initiates a cascade of signal transduction events following DNA damage and oxidative stress (Kitagawa and Kastan 2005), we next examined whether ATM regulates the genotoxic stress-induced DBC1-SIRT1 interaction. We found that treating with KU55933, a specific ATM inhibitor (Hickson et al. 2004), reduced DNA damage-induced DBC1-SIRT1 interaction (Supplemental Fig. 1D). Furthermore, the DBC1-SIRT1 interaction increased in ATM-proficient cells but not in ATM-deficient cells (Fig. 1F). These results suggest that an ATM-dependent phosphorylation event is responsible for the increased DBC1-SIRT1 interaction following cellular stress.

DBC1 is phosphorylated by ATM at Thr 454 following cellular stress

We next examined whether DBC1 or SIRT1 could be phosphorylated by ATM following DNA damage. Using an antibody against consensus ATM phosphorylation sites (anti-phospho-SQ/TQ), we did not find phosphorylation of SIRT1 at SQ/TQ motifs following DNA damage (data not shown). However, DBC1 became phosphorylated at SQ/TQ motifs following etoposide treatment, while λ -phosphatase treatment abolished the phosphorylation at these motifs (Fig. 2A). In addition, KU55933 inhibited DBC1 phosphorylation following etoposide or H₂O₂ treatments (Fig. 2B). Furthermore, we found that DBC1 interacted with ATM, and the interaction was enhanced after genotoxic stresses (Fig. 2C). These results suggest that DBC1 is phosphorylated by ATM following genotoxic stresses. Recent large-scale mass spectrometry analysis of potential ATM substrates also identified DBC1 as an ATM/ATR substrate, confirming our results (Matsuoka et al. 2007; Stokes et al. 2007). These studies suggested Thr 454 of DBC1 as an ATM/ATR phosphorylation site. To confirm this, we mutated Thr 454 to Ala (T454A). The T454A mutation totally abolished DBC1 phosphorylation induced by DNA damage (Fig. 2D), confirming that T454 is a major DNA damage-induced phosphorylation site. To

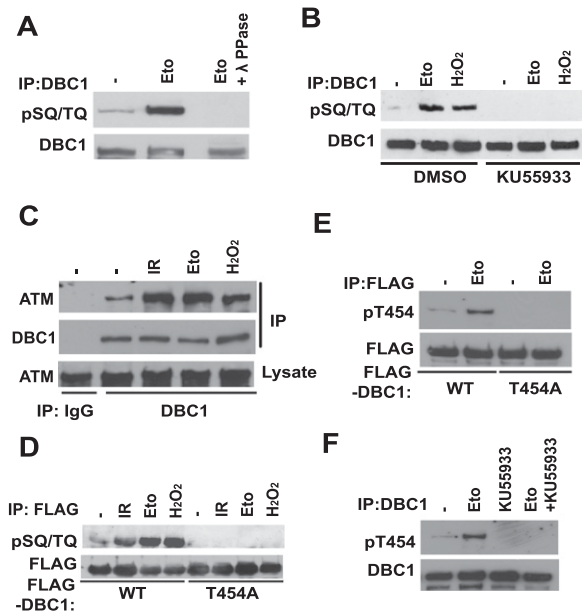


Figure 2. DBC1 is phosphorylated by ATM at Thr 454 following cellular stress. (A) U2OS cells were treated with etoposide (20 μ M) for 1 h, and cells were lysed. Cell lysates were then left untreated or were treated with λ -phosphatase for 30 min, and then subjected to immunoprecipitation with anti-DBC1 antibody and immunoblotted with phospho-SQ/TQ (pSQ/TQ) antibody. (B) U2OS cells were pretreated with DMSO or 25 μ M KU55933 for 2 h, then treated with the indicated agents. After an additional 1 h, cells were harvested. DBC1 phosphorylation was evaluated as in A. (C) U2OS cells were treated as indicated. Cells were lysed, and the DBC1-ATM interaction was evaluated by coimmunoprecipitation. (D,E) U2OS cells transfected with Flag-DBC1 (wild type [WT] or T454A) were treated as indicated. DBC1 phosphorylation was then evaluated with the pSQ/TQ antibody (D) or phosphor-T454 (pT454) antibody (E). (F) U2OS cells were treated as in B, and DBC1 phosphorylation was examined with the pT454 antibody.

further confirm that T454 is phosphorylated in cells, we examined DBC1 phosphorylation using a phospho-specific antibody against T454. As shown in Figure 2, E and F, T454 was phosphorylated following DNA damage, while Ku55933 treatment or T454A mutation abolished T454 phosphorylation. Furthermore, knockdown of ATM in cells significantly decreased T454 phosphorylation following DNA damage (Supplemental Fig. 2A). The increase of stress-induced DBC1 phosphorylation was dose-dependent and correlated with increased ATM phosphorylation, DBC1-SIRT1 interaction, and p53 acetylation, consistent with an inhibition of SIRT1 activity (Supplemental Figs. 1C, 2B). Furthermore, DNA damage induced DBC1 phosphorylation at very early time points (Supplemental Fig. 2C). These results established that DBC1 is phosphorylated at T454 following various cellular stresses, such as DNA damage and oxidative stress, and might act as a switch in response to cellular stresses to regulate SIRT1 activity and cell fate.

DBC1 phosphorylation by ATM creates a second binding site for SIRT1

Our previous work showed that DBC1 binds to SIRT1's catalytic domain through its leucine zipper (LZ) motif (amino acids 243–264), which mediates a basal interaction between DBC1 and SIRT1 (Kim et al. 2008). We hypothesized that the phosphorylation of DBC1 is important for stress-induced interaction between DBC1 and SIRT1. In support of our hypothesis, we found that mutation at T454 abolished DNA damage-induced SIRT1-DBC1 interaction, while it had no effect on the constitutive SIRT1-DBC1 interaction (Fig. 3A). How does T454 phosphorylation enhance the SIRT1-DBC1 interaction? One possibility is that phosphorylation of DBC1 T454 creates a second binding site for SIRT1. As shown in Figure 3B, when p-T454 or a T454 peptide was used to incubate with cell lysates, the p-T454 peptide alone was able to pull down SIRT1, suggesting that in addition to the LZ motif, phosphorylated T454 is able to interact with SIRT1. Interestingly, we found that deletion of the catalytic domain of SIRT1, the same domain that mediates the constitutive SIRT1-DBC1 interaction, is also required for the DNA damage-induced interaction (data not shown). In addition, the SIRT1 catalytic domain is sufficient for the DNA damage-induced interaction with DBC1 (Fig. 3C), while T454A mutation abolished this DNA damage-induced interaction without affecting the constitutive interaction with the SIRT1 catalytic domain. These results suggest that the catalytic domain of SIRT1 mediates both constitutive and stress-induced interaction with DBC1. To demonstrate the direct interaction between the SIRT1 catalytic domain and phosphorylated Thr 454 of DBC1, T454 or p-T454 peptide was incubated with purified GST-SIRT1 catalytic domain in vitro. As shown in Figure 3D, only the p-T454 peptide bound to the SIRT1 catalytic domain. It is possible that the p-T454 motif and the LZ motif of DBC1 bind different regions of the SIRT1 catalytic domain. To further map the interaction regions with the SIRT1 catalytic domain, we generated three deletion mutants within the SIRT1 catalytic domain and performed pull-down assays using the LZ domain and the pT454 peptide. As shown in Supplemental Figure 3A, all three deletions abolished the interaction with the DBC1-LZ motif and pT454 peptide. It is likely that the internal deletions within the SIRT1 catalytic domain change the overall conformation of the

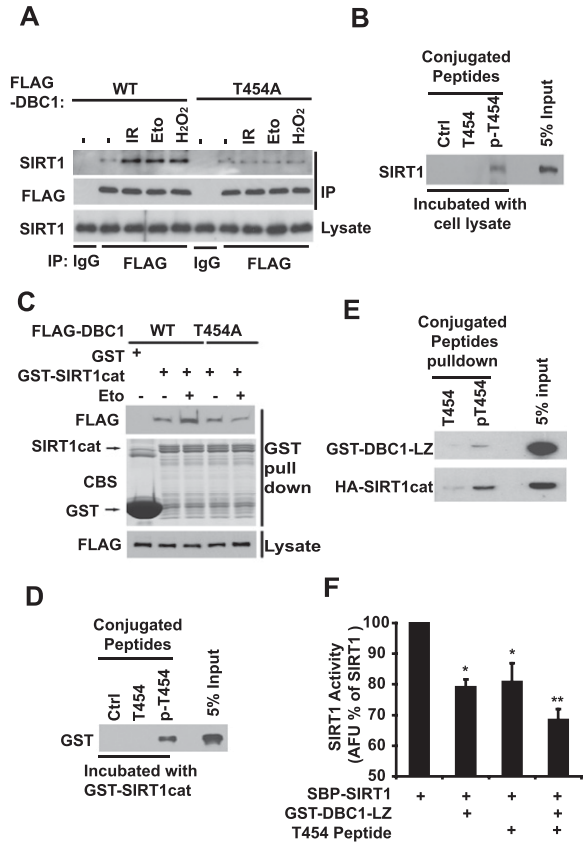


Figure 3. DBC1 phosphorylation by ATM creates a second binding site for SIRT1. (A) A549 cells stably expressing siRNA-resistant Flag-DBC1 (wild type [WT] or T454A) were transfected with DBC1 siRNA. Seventy-two hours later, cells were treated as indicated, and the DBC1-SIRT1 interaction was examined by coimmunoprecipitation. (B,D,E) Nonphosphorylated or phosphorylated Thr 454 peptide (T454 or p-T454, respectively) was conjugated to Sepharose beads and incubated with cell lysates (B) or purified GST-SIRT1-catalytic domain (cat) in NETN buffer (D) or purified GST-DBC1-LZ and HA-SIRT1-catalytic domain in NETN buffer (E). After washing, proteins bound on beads were blotted with the indicated antibodies. (C) Cells as in A were treated with etoposide, and cell lysates were incubated with Sepharose coupled with GST or GST-SIRT1-catalytic domain. After washing, proteins bound on Sepharose were blotted with the indicated antibodies. (F) GST-DBC1-LZ or p-T454 peptides were used to test SIRT1 inhibition by *in vitro* deacetylation activity. The AFU value of the control (SBP-SIRT1 alone) was set as 100%. The error bar represents the SEM of triplicate experiments. (*) $P < 0.05$; (**) $P < 0.01$; two-tailed Student's test.

catalytic domain, making it difficult to further define the pT454-binding site. To test whether both the p-T454 epitope and the LZ motif could bind the SIRT1 catalytic domain, we incubated p-T454 peptide, purified SIRT1 catalytic domain, and DBC1-LZ motif *in vitro*. We found that the p-T454 peptide was able to pull down the SIRT1 catalytic domain and DBC1-LZ motif (Fig. 3E), while the p-T454 peptide could not directly bind the DBC1-LZ motif in the absence of the SIRT1 catalytic domain (data not shown). These results suggest that the p-T454 epitope and the LZ motif could both bind the catalytic domain of SIRT1 and form a complex (Fig. 3E). On the other hand, the ability of DBC1-LZ motif to pull down SIRT1 was not affected by a high concentration of p-T454 peptide (Supplemental Fig. 3B), supporting the notion that the p-T454

epitope is not competing with the LZ motif at the same region of the SIRT1 catalytic domain. Furthermore, we performed the *in vitro* SIRT1 deacetylation assay with the DBC1-LZ motif and p-T454 peptide. As shown in Figure 3F and Supplemental Figure 3C, both the DBC1-LZ and the pT454 peptide alone inhibited SIRT1 activity, while the T454 peptide had no effect on SIRT1. DBC1-LZ and the pT454 peptide together could further inhibit SIRT1 activity. Overall, our results suggest that phosphorylated Thr 454 of DBC1 acts as a second binding site for the SIRT1 catalytic domain, which might be one of the mechanisms that enhance the DBC1–SIRT1 interaction and the inhibitory effect of DBC1 on SIRT1. However, we could not completely exclude the possibility that phosphorylated Thr 454 of DBC1 changes the overall DBC1 structure, which in turn increases the DBC1–SIRT1 interaction.

DBC1 phosphorylation is important for cellular stress response

Since SIRT1 is an important regulator of cellular stress and cell fate (Luo et al. 2001; Vaziri et al. 2001; Brunet et al. 2004; Daitoku et al. 2004; Motta et al. 2004; van der Horst et al. 2004), the regulation of the SIRT1–DBC1 interaction by DBC1 phosphorylation could regulate SIRT1's activity and cell fate under stress. Consistent with this, T454 phosphorylation correlated with enhanced DBC1–SIRT1 interaction, p53 acetylation, and PUMA expression following cellular stress in a dose-dependent manner (Supplemental Figs. 1C, 2B). To further confirm the functional significance of DBC1 phosphorylation, we depleted endogenous DBC1 by siRNA, then reconstituted cells with siRNA-resistant wild-type DBC1 or DBC1 T454A. As shown in Figure 4A, p53 acetylation was induced by DNA damage, while knocking down DBC1 by siRNA abolished DNA damage-induced p53 acetylation. Reconstitution of wild-type DBC1 but not DBC1 T454A rescued p53 acetylation. These results suggest that DBC1 phosphorylation by ATM is important for DNA damage-induced SIRT1 inhibition and p53 acetylation. p53 acetylation plays an important role in its activation (Tang et al. 2008). We also examined the expression of p53 target genes following DNA damage. As shown in Figure 4B, DBC1 phosphorylation was also important for the DNA damage-induced expression of p53 target genes, consistent with the p53 acetylation status. In addition to p53, we examined other SIRT1 target proteins, such as Foxo1. As shown in Supplemental Figure 4A, reconstitution of wild-type DBC1 but not DBC1 T454A rescued Foxo1 acetylation following genotoxic stress. Furthermore, cells reconstituted with wild-type DBC1 but not DBC1 T454A displayed decreased SIRT1 activity following DNA damage by *in vitro* SIRT1 deacetylation assay (Fig. 4C; Supplemental Fig. 4B), supporting the notion that T454 phosphorylation is important for SIRT1 inhibition by DNA damage. To assess the role of DBC1 phosphorylation in stress response, we tested whether DBC1 was involved in cell death induced by DNA damage. As shown in Figure 4D, knockdown of DBC1 inhibited DNA damage-induced apoptosis, while reconstitution cells with wild-type DBC1 dramatically rescued the DNA damage-induced apoptosis. Reconstitution cells with the DBC1 T454A mutation partially rescued the DNA damage-induced apoptosis, which might be due to the constitutive inhibition effect on SIRT1. Similar results were obtained when we used DBC1 knockout cells

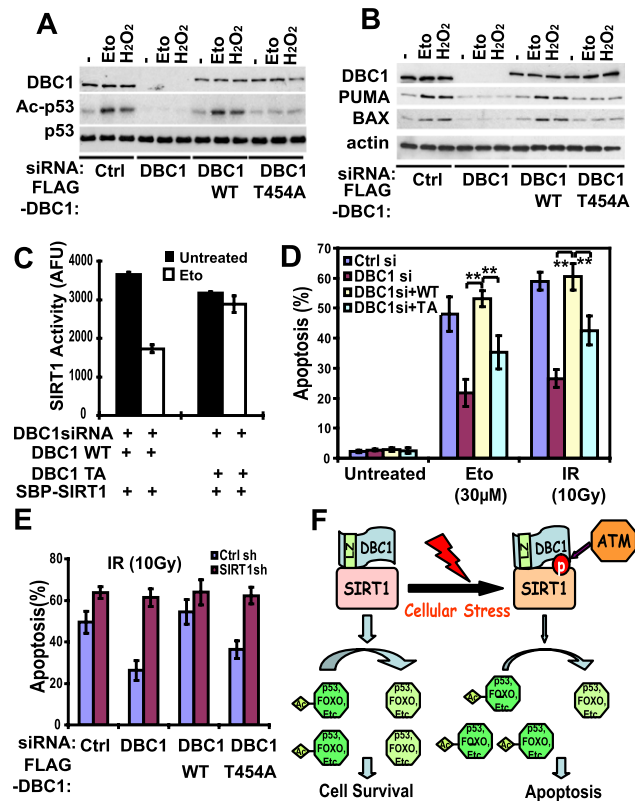


Figure 4. DBC1 phosphorylation is important for cellular stress response. (A,B) A549 cells stably expressing vector, siRNA-resistant pBABE-Flag-DBC1 (wild type [WT] or T454A) were transfected with DBC1 siRNA. Seventy-two hours later, cells were treated with etoposide or H₂O₂ for 2 h. Cells were pretreated with MG132 to equalize p53 levels in A and B. Cell lysates were then blotted with indicated antibodies. (C) A549 cells stably expressing siRNA-resistant Flag-DBC1 (wild type [WT] or T454A) were transfected with DBC1 siRNA and SBP-tagged SIRT1. After 72 h, cells were left untreated or were treated with etoposide (20 μM). An additional 2 h later, SBP-tagged SIRT1 was immunoprecipitated from cells and used in the *in vitro* deacetylation assay. (D) Cells as in A were treated as indicated. Forty-eight hours later, the apoptotic population was determined. (E) A549 cells stably infected with the indicated shRNA and transfected with the indicated constructs were transfected with DBC1 siRNA. Cells were then treated with ionizing radiation, and the apoptotic population was determined 48 h later. (F) The working model of regulation SIRT1 by DBC1 following DNA damage. (C–E) The error bar represents the SEM of triplicate experiments. (***) $P < 0.01$; two-tailed Student's test.

reconstituted with DBC1 wild type and T454 mutants (Supplemental Fig. 4C) or examined cell survival by colony formation assay (Supplemental Fig. 4D,E). These results suggested that the phosphorylation of DBC1 T454 is important for SIRT1 regulation and cell fate determination in response to genotoxic stresses. The different effects of DBC1 wild type and TA mutants in DNA damage-induced apoptosis could not be observed in cells depleted of SIRT1 (Fig. 4E), suggesting that DBC1 regulates cell survival through SIRT1.

Overall, our studies suggest that DBC1, as the negative regulator of SIRT1, binds to SIRT1 more tightly following cellular stress. This increase of binding relies on ATM-dependent phosphorylation of DBC1 at T454, which creates a second binding site for SIRT1. Finally, T454 phosphorylation of DBC1 contributes to SIRT1 regulation

and cell fate determination in response to cellular stress (Fig. 4F). These findings have a significant impact on our understanding of the molecular mechanism that regulates SIRT1 during cellular stress.

Materials and methods

Cell culture, plasmids, antibodies, and reagents

A549, U2OS, and HEK293T cells were cultured in RPMI 1640 with 10% FBS.

The cloning of DBC1 and SIRT1 was previously described (Kim et al. 2008). Deletion mutants were generated by site-directed mutagenesis (Stratagene).

The generation of rabbit anti-SIRT1 and anti-DBC1 antibodies was previously described (Kim et al. 2008). The following antibodies were purchased: anti-Flag (m2) (Sigma); anti-HA (Covance); anti-p53 (DO-1) and anti-GST (Santa Cruz Biotechnology); anti-PUMA, anti-BAX, anti-SMCL1, anti-p-SMCL1, anti-pS15-p53, anti-pT68-Chk2, anti-Ku70, anti-E2F1, anti-Chk2, anti-ATM/ATR substrates, and anti-phospho-DBC1 (Cell Signaling); anti-Ac-K373-p53 (Abcam); anti-ATM and anti-phospho-ATM (Epitomics); anti-Foxo3a (Bethyl Laboratories); and anti-Ac-Lys (Upstate Biotechnologies).

ABT-888 (veliparib) was a gift from Dr. Scott H. Kaufmann (Mayo Clinic). Etoposide, H₂O₂, and caffeine were purchased from Sigma. KU55933 was purchased from Tocris Bioscience.

siRNA

siRNAs against DBC1 were synthesized by Dharmacon, Inc. The siRNA duplexes were 21 base pairs (bp) as follows: DBC1 siRNA #1 sense strand, 5'-CAGCUUGCAUGACUACUUUUU-3'; DBC1 siRNA #2 sense strand, 5'-AAACGGAGCCUACUGAACAUU-3'. SIRT1 and ATM siRNAs were from Dharmacon SmartPool. Transfections were performed twice, 24 h apart, with 200 nM siRNA using oligofectamine reagent according to the manufacturer's instructions (Invitrogen).

Coimmunoprecipitation assay

Cells were lysed with NETN buffer (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 50 mM β -glycerophosphate, 10 mM NaF, and 1 mg/mL each pepstatin A and aprotinin. Whole-cell lysates obtained by centrifugation were incubated with 2 μ g of antibody and protein A or protein G Sepharose beads (Amersham Biosciences) for 2 h at 4°C. The immunocomplexes were then washed with NETN buffer three times and separated by SDS-PAGE. Immunoblotting was performed following standard procedures.

GST pull-down

GST fusion proteins were prepared following a standard protocol. For in vitro binding assays, GST fusion proteins bound to the GSH Sepharose were incubated with cell lysates. After washing, the bound proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Peptide-binding assay

DBC1 T454 non-phospho-peptides (AEAAPPPTQEAQGE) or phospho-peptides (AEAAPP(p)TQEAQGE) were conjugated to agarose beads according to the manufacturer's instructions (Thermo Scientific, no. 44999). Beads conjugated with peptides were incubated with cell lysates or purified proteins in NETN buffer for 2 h. After incubation, beads were washed four times with NETN buffer and boiled with SDS loading buffer.

In vitro SIRT1 deacetylation assay

SIRT1 activity in vitro was determined with a SIRT1 Fluorometric kit (Biomol International) according to the manufacturer's instructions. This assay uses a small lysine-acetylated peptide, corresponding to K382 of

human p53, as a substrate. The lysine residue is deacetylated by SIRT1, and this process is dependent on the addition of exogenous NAD⁺. Addition of exogenous NAD⁺ was necessary, most likely because endogenous NAD⁺ was lost during sample preparation. Cultured cells were transfected with the indicated constructs. After 2 d, cells were untreated or treated with etoposide (20 μ M). Two hours later, cells were lysed in NETN buffer as described above. SBP-tagged SIRT1 was immunoprecipitated from cells and eluted with Biotin (Sigma). An equal amount of SIRT1 was incubated with 50 μ M Fluor de Lys-SIRT1 substrate and 500 μ M NAD⁺ in 50 μ L of reaction buffer (Biomol International, BML-K1286). The mixture was incubated for 60 min at 37°C, and the reaction was terminated by adding a solution containing Fluor de Lys Developer (Enzo Life Sciences) and 2 mM nicotinamide. Plates were incubated for 1 h at 37°C. Values were determined by reading fluorescence on a fluorometric plate reader (Spectramax Gemini XPS, Molecular Devices) with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Calculation of net fluorescence included the subtraction of a blank consisting of buffer containing no NAD⁺.

Apoptosis assay

Cells stably expressing siRNA-resistant DBC1 wild type and DBC1 TA were transfected with DBC1 siRNA. After 2 d, cells were treated with etoposide (30 μ M) or ionizing radiation (10 Gy). After an additional 24 h, cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature and then stained with DAPI. The number of apoptotic cells with nuclear morphology typical of apoptosis were analyzed by fluorescence microscopy and scored in at least 400 cells per sample by an analyst blinded to the sample groups.

Cell viability assay

A colony formation assay was used to measure cell viability following cellular stress. Cells were plated in triplicate into 35-mm dishes at various cell densities, with a target number of surviving colonies at 50–100 per dish. Treatment with etoposide or H₂O₂ was carried out 14–18 h after cell plating. After 2 h of exposure to the drug, cells were rinsed three times with PBS, and then regular medium was added. After 2 wk, colonies were fixed with methanol and stained with Giemsa. The surviving fractions were calculated as the plating efficiency of treated cells relative to the plating efficiency of untreated control cells.

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