

# **DBC1 phosphorylation by ATM/ATR inhibits SIRT1 deacetylase in response to DNA damage**

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**Abstract**

Human DBC1 (deleted in breast cancer-1; KIAA1967) is a nuclear protein that, in response to DNA damage, competitively inhibits the NAD<sup>+</sup>-dependent deacetylase SIRT1, a regulator of p53 apoptotic functions in response to genotoxic stress. DBC1 depletion in human cells increases SIRT1 activity, resulting in deacetylation of p53 and protection from apoptosis. However, the mechanisms regulating this process have not yet been determined. Here we report that, in human cell lines, DNA damage triggered the phosphorylation of DBC1 on Thr454 by ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) kinases. Phosphorylated DBC1 bound to and inhibited SIRT1, resulting in the dissociation of the SIRT1-p53 complex and stimulating p53 acetylation and p53-dependent cell death. Indeed, DBC1-mediated genotoxicity, which was shown in knockdown experiments to be dependent on SIRT1 and p53 expression, was defective in cells expressing the phospho-mutant DBC1<sup>T454A</sup>. This study describes the first post-translational modification of DBC1 and provides new mechanistic insight linking ATM/ATR to the DBC1-SIRT1-p53 apoptotic axis triggered by DNA damage.

Keywords: DBC1, phosphorylation, SIRT1 deacetylase, DNA damage

## **Introduction**

SIRT1, the mammalian ortholog of the yeast silent information 2 (Sir2), is a NAD<sup>+</sup>-dependent class III histone deacetylase that participates in the regulation of various cellular processes including apoptosis, stress response, metabolism and tumorigenesis (Brooks et al., 2009; Nakagawa and Guarente, 2011). SIRT1 deacetylates histones H1, H3 and H4 to promote heterochromatin formation and transcriptional suppression. It also deacetylates several non-histone proteins, leading to either tumor suppression or tumor promotion (Deng, 2009). A key target of SIRT1 is the tumor suppressor p53 (Vaziri et al., 2001), a protein that plays a critical role in initiating cell cycle arrest and apoptosis. Activation or overexpression of SIRT1 in human and murine cell lines deacetylated and inactivated p53, resulting in fewer cells going into apoptosis after DNA damage (Chen et al., 2005; Kim et al., 2008; Zhao et al., 2008). Given the important yet complex role that SIRT1 plays in cell survival and cancer development, there is great interest in understanding how its cellular activity is regulated.

A recently identified inhibitor of SIRT1 in human cells is DBC1 (deleted in breast cancer 1; KIAA1967; Kim et al., 2008; Zhao et al., 2008). This protein was initially discovered as a possible tumor suppressor in an analysis of genes that were homozygously deleted in breast cancer specimens (Hamaguchi et al., 2002), but it was identified as a regulator of SIRT1 by the fact that it was co-purified with this enzyme (Kim et al., 2008; Zhao et al., 2008). DBC1 overexpression in human cells repressed SIRT1 activity, leading to increased p53 acetylation (Zhao et al., 2008). In contrast, DBC1 down-regulation enhanced p53 deacetylation by SIRT1, resulting in fewer apoptotic cells after stress (Kim et al., 2008; Zhao et al., 2008). DBC1 is a nuclear protein that inhibits SIRT1 by binding to its deacetylase core and disrupting its

association with substrates (Kim et al., 2008; Zhao et al., 2008). The binding site on DBC1 has been localized to residues 243–264 (Kim et al., 2008). The inhibitory action of DBC1 was recently attributed to its ability to displace, from the deacetylase core, a bound peptide loop of the C-terminus of SIRT1 essential for SIRT1 activity (ESA; Kang et al., 2011). DBC1 may also be able to regulate SIRT1 activity by binding  $\text{NAD}^+$ , an inhibitory mechanism suggested by the finding of a catalytically inactive Nudix hydrolase domain in the sequences of DBC1 (residues 339-462) and homologs (Anantharaman and Aravind, 2008). Thus, DBC1 may modulate SIRT1 activity by a direct association with the enzyme and by potentially interacting with  $\text{NAD}^+$  metabolism. However, how the SIRT1-DBC1 association is modulated has not yet been determined.

The interplay between SIRT1 and DBC1 is often studied in cellular models of DNA damage induced by genotoxic agents such as etoposide, a molecule that interferes with topoisomerase II resulting in DNA double-stranded breaks (DSBs). Such DNA damage activates the DNA damage response (DDR), a complex network of pathways, that senses DNA damage and replication stress and that activates a coordinated response to prevent genome instability (Ciccio and Elledge, 2010). The key step of this response is cell cycle checkpoint initiation leading to either DNA repair or apoptosis. DDR is mainly mediated by members of the family of phosphatidylinositol 3-kinase-like protein kinases (PIKKs), mostly ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR) kinases (Shiloh, 2006). Following the recognition of DNA damage by sensor proteins, ATM/ATR phosphorylate both mediator and effector proteins. Mediator proteins then amplify the signal by recruiting ATM/ATR substrates (Shiloh, 2006), whereas effector proteins determine the fate of a damaged cell by regulating physiological processes, for

example by activating cell cycle checkpoints to promote DNA repair, apoptosis or senescence (Ciccia and Elledge, 2010). Substrates of ATM/ATR contain serine or threonine residues followed by glutamine (SQ/TQ motif) (Abraham, 2004). Two large-scale searches for ATM/ATR substrates, using a contextual computational approach (Linding et al., 2007) or phosphoproteomic screening (Stokes et al., 2007), both identified threonine-454 in DBC1 as a putative ATM/ATR target. Whether DBC1 is phosphorylated *in vivo* in response to DNA damage has not yet been studied.

Here we demonstrate that, in human cells treated with genotoxic agents, DBC1 is phosphorylated by ATM/ATR on threonine-454 and this functional modification enhances SIRT1-DBC1 binding. The resulting inactivation of SIRT1 has a direct, activating impact upon the p53-mediated apoptotic response, linking ATM/ATR kinases to the DBC1-SIRT1-p53 apoptotic axis for the first time.

## Results

### *DBC1 is a novel ATM/ATR substrate in DDR*

To test if DBC1 is an ATM substrate, we immunoprecipitated the FLAG-tagged protein from U2OS cells and examined its phosphorylation before and after genotoxic treatments (Figure 1A, B). The anti-phospho-SQ/TQ antibody detected a faint band of FLAG-DBC1<sup>WT</sup> in immunoprecipitates from 1-h etoposide treatment and a stronger signal at 3 h (Figure 1A). When we immunoprecipitated the point-mutated FLAG-DBC1<sup>T454A</sup>, no phosphorylated band was detected at any time point. Similarly, a commercially available anti-phospho-DBC1 (Thr454) antibody reacted with FLAG-DBC1<sup>WT</sup> but not FLAG-DBC1<sup>T454A</sup> (Figure 1B). These results indicate that etoposide-induced genotoxic stress results in the phosphorylation of DBC1 on Thr454 and not on any of the other six SQ/TQ motifs in the protein (Supplementary Figure S1). When

the ATM inhibitor KU-55933 was added prior to etoposide treatment (Figure 1B), we observed a marked suppression but not abrogation of DBC1-Thr454 phosphorylation. Similarly, DBC1-Thr454 phosphorylation in the ATM-negative cell line AT52RM (Delia et al., 2000), was only partially reduced compared to that in a normal cell line (Figure 1C). These results suggest that ATM phosphorylates DBC1 and another kinase is also involved.

Besides ATM, etoposide treatment can also activate ATR (Ding et al., 2003). Thus, to determine if this kinase is implicated in DBC1 phosphorylation, U2OS cells were transfected with siRNA to knockdown ATR, treated with different combinations of etoposide and KU-55933, and analyzed by western blot (Figure 1D). Etoposide treatment of cells transfected with control siRNA (siLUC) resulted in the phosphorylation of endogenous DBC1 on Thr454; knockdown of ATR reduced the extent of phosphorylation while additional treatment with KU-55933 entirely abolished it. These data indicate that Thr454 of DBC1 is an *in vivo* ATM/ATR substrate in response to DNA damage caused by etoposide. This effect is both time- and dose-dependent (Figure 1E) and can be reproduced by treatment with H<sub>2</sub>O<sub>2</sub> (Supplementary Figure S2A) and ionizing radiation (Supplementary Figure S2B-D). Although radiation is thought to primarily activate ATM (Pandita et al., 2000; Myers and Cortez, 2006), the phosphorylation of DBC1 did not appear to be limited to this kinase, as it was not completely abrogated by KU-55933 treatment and it was detected in the ATM-negative cell line AT52RM (Supplementary Figure S2B and C). However, radiation-induced phosphorylation of DBC1 was rapid and transient, peaking around 15-30 min, and also dose-dependent (Supplementary Figure S2D).

To further verify that DBC1 is a direct ATM/ATR target, we performed *in vitro* kinase assays using immunoprecipitated ATM or ATR proteins and recombinant

full-length GST-DBC1 as substrate. As shown in Supplementary Figure S3, we found that both these kinases can directly phosphorylate DBC1 on Thr454.

Hence, DBC1 is phosphorylated by ATM and ATR in response to various genotoxic stresses.

A basal phosphorylation of endogenous DBC1 was detected on western blot of undamaged U2OS cells (Figure 1F, lane 1). Moreover, immunofluorescence analysis of undamaged cells overexpressing ectopic DBC1, in the absence of DNA damage, indicated that about 17% of the transfected cells were positive for phospho-DBC1 (Figure 1G). Combined suppression of ATM and ATR abrogated the phosphorylation of endogenous and ectopic DBC1, whereas suppression of just one kinase did not (Figure 1F and G). To determine if the cells containing phosphorylated DBC1 carried genomic lesions, we analyzed  $\gamma$ H2AX foci, a marker of DSBs (Figure 1H). Cells positive for DBC1 phosphorylation on Thr454 had more of these structures (mean,  $22.7 \pm 10.1$  foci/nucleus) than did cells lacking phospho-DBC1 ( $6.8 \pm 3.2$  foci/nucleus) and this increase was specifically mediated by DBC1<sup>WT</sup> since overexpression of DBC1<sup>T454A</sup> did not affect the number of foci (Figure 1I). Additional experiments showed that ectopic DBC1 was phosphorylated in S-phase cells (Supplementary Figure S4). Hence, in undamaged cells, DBC1 is phosphorylated on Thr454 by ATM and ATR, likely during S-phase, and these cells are characterized by an increased number of DSBs.

Noteworthy, sequence analysis (using SIM + JalnView) indicated that human and mouse DBC1 proteins share 91.4% identity, but that Thr454 is not conserved in the mouse (Supplementary Figure S5). This observation suggests a different regulation or function for DBC1 in humans and mice.

*Thr454 phosphorylation does not affect DBC1 protein stability or subcellular localization*

Upon phosphorylation by ATM/ATR, many proteins undergo changes in activity, stability or subcellular localization (Shiloh, 2006; Bensimon et al., 2010). To determine whether phosphorylation of DBC1 on Thr454 affected any of these properties, we compared the stability of DBC1<sup>WT</sup> and DBC1<sup>T454A</sup> in U2OS cells treated for up to 6 h with the protein synthesis inhibitor cycloheximide (CHX) in the absence (Figure 2A) or presence (Figure 2B) of etoposide. Under these conditions, no measurable changes in DBC1 protein levels were detected, excluding a role of phospho-Thr454 in protein stability. Efficient CHX treatment was confirmed by western blot analyses of p53 protein levels in the same lysates used for Figure 2A (Supplementary Figure S6). We then examined the subcellular localization of DBC1<sup>WT</sup> and DBC1<sup>T454A</sup> by immunofluorescence (Figure 2C). FLAG-DBC1<sup>WT</sup>, detected with anti-FLAG, had a nuclear localization and this was unaffected by the Thr454 mutation. When probed with anti-phospho-DBC1 (Thr454), we found phosphorylated DBC1<sup>WT</sup> in 17.7% of untreated FLAG-positive cells and this value increased to 100% after etoposide treatment, without any change in subcellular localization. In cells expressing FLAG-DBC1<sup>T454A</sup>, etoposide treatment induced a low level of phosphorylation of the endogenous native protein, again only in the nucleus. This pattern was also observed in untransfected cells, in which endogenous DBC1 phosphorylated on Thr454 was nuclear (Figure 2D). Considering that other ATM/ATR substrates, such as H2AX and 53BP1, are recruited to DNA lesions where they form foci (Bekker-Jensen and Mailand, 2010), we exposed cells to ionizing radiation and pre-extracted them prior to fixation before dual immunofluorescence labeling (Figure 2E). The merged image indicated that phosphorylated DBC1 did not



cluster in these structures, but was nonetheless associated with chromatin. Altogether, these results indicate that Thr454 phosphorylation does not modify the subcellular localization of DBC1.

*ATM/ATR phosphorylation of DBC1 inhibits SIRT1 activity and increases p53 acetylation*

To understand how Thr454 phosphorylation affects DBC1 function, HEK-293T cells were transfected with plasmids encoding DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> or with empty vector (mock), treated with etoposide for 1 h and then assayed for endogenous SIRT1 activity. Compared to mock-transfected cells, SIRT1 activity was reduced by 40% in cells expressing DBC1<sup>WT</sup> but only by 10% in cells expressing DBC1<sup>T454A</sup> (Figure 3A). Since DBC1 depletion affects p53 acetylation (Kim et al., 2008; Zhao et al., 2008; Supplementary Figure S7), we analyzed this modification in similarly transfected U2OS cells, pretreated with MG132 to stabilize p53 and exposed or not to etoposide (Figure 3B). No acetylated p53 was found in undamaged cells while 1-h etoposide treatment resulted in a low level of p53 acetylation on Lys-382 (p53-Ac-K382) in mock-transfected cells, which increased 2.5-fold in cells overexpressing DBC1<sup>WT</sup> but not DBC1<sup>T454A</sup> (Figure 3B, right). Of note, in the same cells, p53 phosphorylation was unaffected by expression of either wild type or mutant DBC1 (Supplementary Figure S8).

To determine if phosphorylated DBC1 regulates p53 acetylation through SIRT1, we analyzed the levels of p53-Ac-K382 in U2OS depleted of SIRT1 and expressing either DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> (Figure 3C). In the absence of SIRT1, p53 acetylation increased 10-fold in mock-transfected cells and a similar pattern was observed in cells overexpressing wild type or mutant DBC1. These findings confirm that DBC1 regulates p53 acetylation by inhibiting SIRT1. However, the inhibition of

SIRT1 by DBC1 was incomplete, as attested by the greater amount of acetylated p53 in cells depleted of SIRT1 than in cells overexpressing DBC1<sup>WT</sup> (Figure 3C, compare lanes 2 and 3).

Since acetylation activates p53, we investigated whether DBC1 phosphorylation resulted in a concomitant increase in p53 activity in U2OS cells co-transfected with a PUMA promoter-driven luciferase reporter gene and with plasmids encoding DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> or with empty vector (Figure 3D). In cells expressing DBC1<sup>WT</sup>, we observed an approximately 2-fold increase in PUMA promoter activity after etoposide treatment, whereas in cells expressing DBC1<sup>T454A</sup> the induction was less strong. Accordingly, a greater induction of endogenous PUMA protein was seen in cells expressing DBC1<sup>WT</sup> than DBC1<sup>T454A</sup> (Figure 3E).

Altogether, these results indicate that ATM/ATR phosphorylation of DBC1 inhibits the deacetylase function of SIRT1, enhancing p53 acetylation and transcriptional activity.

#### *Thr454 phosphorylation is required for DBC1-induced apoptosis*

To further investigate the outcome of DBC1 phosphorylation on SIRT1 inhibition and p53 activation, we analyzed cellular levels of apoptotic markers. In etoposide-treated U2OS cells overexpressing DBC1<sup>WT</sup>, the levels of cleaved caspase-9 were about 2-fold higher than in mock-transfected cells (Figure 4A and B, compare lanes 2 and 4). However, these levels did not change upon depletion of SIRT1 or p53 (Figure 4A and B, compare lanes 6 and 8). Moreover, immunofluorescence analyses for cleaved PARP-1 showed a greater induction of apoptosis in etoposide-treated DBC1<sup>WT</sup>-expressing cells only when both SIRT1 and p53 were expressed (Figure 4C). These results indicate that DBC1-induced apoptosis is completely mediated by SIRT1 and p53.

Furthermore, we investigated the role of DBC1 phosphorylation in apoptosis. U2OS cells were transfected with vectors encoding DBC1<sup>WT</sup>, DBC1<sup>T454A</sup> or mock control, treated or not with etoposide for 30 h, and harvested to assess cell viability and levels of apoptotic markers (Figure 4D and E). Etoposide treatment increased the percentage of dead cells to about 26% of total in mock transfected and DBC1<sup>T454A</sup>-overexpressing samples, but to 39% in cells overexpressing DBC1<sup>WT</sup> (Figure 4D). Similarly, higher levels of cleaved PARP-1 and cleaved caspase-9 were seen by western blot in cells overexpressing DBC1<sup>WT</sup>, compared to mock transfected or DBC1<sup>T454A</sup>-expressing cells (Figure 4E). Immunofluorescence analyses using anti-cleaved-PARP-1 and anti-FLAG antibodies, in U2OS cells expressing FLAG-DBC1<sup>WT</sup> or FLAG-DBC1<sup>T454A</sup>, also revealed, after etoposide treatment, a greater induction of apoptosis among all cells expressing DBC1<sup>WT</sup> than in mock-transfected or DBC1<sup>T454A</sup>-expressing samples (Figure 4F). Finally, in colony formation assays of transfected U2OS cells treated with increasing doses of etoposide and analyzed 10-15 days later, the clonogenic survival of mock transfected and DBC1<sup>T454A</sup>-expressing cells was similar, but that of DBC1<sup>WT</sup>-expressing cells was significantly lower (Figure 4G). Altogether, these results indicate that Thr454 phosphorylation of DBC1 by ATM/ATR increases cellular sensitivity to genotoxic agents by markedly enhancing apoptosis and decreasing long-term clonogenic survival after DNA damage.

#### *DBC1 phosphorylation on Thr454 regulates DBC1-SIRT1 interaction*

As previously reported (Kim et al., 2008) and as also found here (Figure 5A) DBC1-SIRT1 association increases after etoposide treatment. Thus, to investigate the underlying mechanism of SIRT1 inhibition by phosphorylated DBC1, co-immunoprecipitations of SIRT1 and FLAG-DBC1<sup>WT</sup> or FLAG-DBC1<sup>T454A</sup> were performed on extracts from cells treated or not with etoposide (Figure 5B). Although

SIRT1 levels were similar in all cell extracts, etoposide treatment increased the amount of SIRT1 that immunoprecipitated with FLAG-DBC1<sup>WT</sup>, as already reported (Kim et al., 2008), whereas it decreased the amount that immunoprecipitated with DBC1<sup>T454A</sup> (Figure 5B). To study the importance of DBC1 phosphorylation on the interaction between SIRT1 and p53, FLAG-SIRT1 was immunoprecipitated from U2OS cells transfected with vectors encoding p53, FLAG-SIRT1 and DBC1 (wild type or point mutated) and treated with etoposide (Figure 5C). We found that the SIRT1-p53 association was reduced by DBC1<sup>WT</sup> expression, but unaffected by DBC1<sup>T454A</sup>. Altogether, these findings suggest that Thr454 phosphorylation promotes DBC1-SIRT1 binding, thus dissociating the SIRT1-p53 complex. Finally, to determine whether ATM/ATR directly target and somehow regulate SIRT1, we examined FLAG-SIRT1 immunoprecipitated from untreated or etoposide-treated cells with an anti-phospho-S/TQ antibody, but despite having two TQ motifs, SIRT1 was not phosphorylated by these kinases (Figure 5D).

To further assess the role of Thr454 phosphorylation on SIRT1 activities, we tested whether the FLAG-DBC1<sup>T454D</sup> phosphomimic mutant in undamaged U2OS cells behaves like DBC1<sup>WT</sup> following DNA damage. As shown in Supplementary Figure S9, FLAG-DBC1<sup>T454D</sup> significantly increased its association with SIRT1 and induced p53 acetylation and apoptosis, but only in response to DNA damage. These results indicate that DBC1<sup>T454D</sup> only partially mimics the effect of the phosphorylatable DBC1<sup>WT</sup>.

Overall, these results suggest a model in which, in cells without DNA damage, SIRT1 maintains p53 in a hypoacetylated, inactive state, but after genotoxic stress, ATM/ATR signaling activates the p53 pathway in two ways. On one hand, it phosphorylates p53 on Ser15 and primes it for acetylation (Lambert et al., 1998;

Sakaguchi et al., 1998). On the other, it phosphorylates DBC1 on Thr454, enhancing its binding to SIRT1 and leading to increased SIRT1 inhibition, greater p53 acetylation and activation, and induction of apoptosis (Figure 6).

## **Discussion**

Here, we report that the nuclear protein DBC1, in response to genotoxic stress, is directly phosphorylated on Thr454 by ATM and ATR. This finding indicates that this protein is a new member of the DDR pathway. Thr454 phosphorylation is the first known post-translational modification of DBC1 and any activity linked to this phosphorylation site may be specific to the human protein, since Thr454 is not conserved in murine DBC1.

The phosphorylation of DBC1 does not affect protein stability or subcellular localization, but rather its inhibitory activity on SIRT1 deacetylase. Indeed, cells expressing DBC1<sup>WT</sup>, but not DBC1<sup>T454A</sup>, have reduced SIRT1 activity, as demonstrated by SIRT1 deacetylase assays and by the increased acetylation and transcriptional activation of its substrate p53, thus underscoring the importance of ATM/ATR phosphorylation of DBC1 for SIRT1 inhibition. Of note, DBC1 only partially inhibited SIRT1 in response to DNA damage, as demonstrated by the increased p53 acetylation in SIRT1-depleted cells compared to DBC1-overexpressing cells. This result is concordant with the existence of other endogenous SIRT1 inhibitors (Chen et al., 2005; Liu et al., 2011; Yuan et al., 2011), but we cannot also exclude that DBC1 could more efficiently inhibit SIRT1 in response to stimuli different from etoposide treatment. In agreement with previous reports about SIRT1 inhibition or depletion (Wang et al., 2006; Wang et al., 2008; Fan and Luo, 2010), we found that DBC1 phosphorylation increased genotoxic sensitivity, as evident by the greater viability and long-term clonogenic survival of cells expressing DBC1<sup>T454A</sup>

than DBC1<sup>WT</sup>. In addition, we demonstrated that cell death induced by DBC1 is entirely mediated by SIRT1, and ultimately p53. These findings lend support to the reported role of DBC1 in etoposide-induced apoptosis (Kim et al., 2008; Zhao et al., 2008) and contribute to understanding the underlying mechanisms.

Notably, ectopic DBC1 was phosphorylated on Thr454 in a fraction of undamaged cells corresponding to those in S-phase. We further demonstrated that only the simultaneous inhibition and depletion of ATM/ATR can abrogate this phosphorylation, and that cells overexpressing phosphorylated DBC1 have an increased number of  $\gamma$ H2AX foci, indicating a stress condition for DNA. This observation correlates well with a previous report showing that SIRT1 depletion induces an accumulation of DNA damage in S-phase cells (Yuan et al., 2009) and further supports our model in which SIRT1 activity is inhibited by DBC1 phosphorylation on Thr454. Currently, however, it is unknown whether endogenous DBC1 is phosphorylated during normal S-phase, because of the low sensitivity of the anti-phospho-DBC1 antibody and of the limited amount of phosphorylated DBC1 in undamaged cells.

As many ATM/ATR substrates accumulate in  $\gamma$ H2AX nuclear foci after DNA damage, we examined whether phosphorylated DBC1 localized to these structures. However, even after a pre-extraction procedure, we were unable to detect a co-localization between  $\gamma$ H2AX and phosphorylated DBC1, which was clearly distributed throughout the chromatin. These results could indicate a possible involvement of phosphorylated DBC1 in chromatin structure regulation, also through SIRT1, as previously suggested (Li et al., 2009).

We further investigated the mechanism linking DBC1 phosphorylation to SIRT1 inhibition. Starting from the evidence that etoposide potentiates the DBC1-

SIRT1 association (Kim et al., 2008 and Figure 5A), we determined the requirement of Thr454 phosphoresidue for this interaction. We found that while the association between SIRT1 and DBC1<sup>WT</sup> increased, the interaction of SIRT1 with DBC1<sup>T454A</sup> was significantly reduced, suggesting that Thr454 phosphorylation is required for the interaction with SIRT1. Since Thr454 is not located in the DBC1 region involved in SIRT1 interaction, it is possible that phosphorylation of this residue induces a DBC1 conformational change creating a new binding site for SIRT1. The role of Thr454 phosphorylation in DBC1-SIRT1 interaction was further confirmed by the finding that in cells overexpressing DBC1<sup>WT</sup> the association between SIRT1 and p53 was reduced compared to mock transfected or DBC1<sup>T454A</sup>-expressing cells. However, as demonstrated by experiments with DBC1<sup>T454D</sup>, Thr454 phosphorylation alone is not sufficient to induce DBC1-SIRT1 association after DNA damage, since the phosphomimic mutant only slightly increases its association with SIRT1 in undamaged cells.

Collectively, our results suggest that in unperturbed cells SIRT1 deacetylates p53 to prevent apoptosis, whereas in cells with DNA damage ATM and ATR phosphorylate p53 and DBC1, promoting p53 acetylation and transcriptional activity, also through the enhanced DBC1-SIRT1 interaction and inhibition of SIRT1 deacetylase activity towards p53 and promotion of apoptosis (Figure 6). Previous findings indicated that SIRT1 activity is regulated by fluctuations in NAD<sup>+</sup> levels. However, our model suggests a new mechanism of SIRT1 regulation, possibly independent from changes in cellular levels of NAD<sup>+</sup> that could more rapidly modulate SIRT1 activity modulation as previously suggested (Gerhart-Hines et al., 2011).

Beside the regulation of SIRT1 and apoptosis, we cannot exclude other functions for ATM/ATR phosphorylation of DBC1. For instance, Thr454 phosphorylation might regulate SIRT1 activity towards substrates different from p53, affecting other cellular processes like senescence or metabolism. In this context, it has been shown that DBC1 knock-out mice have metabolic defects (Escande et al., 2010) and that ATM is involved in the regulation of the pentose phosphate pathway (Cosentino et al., 2011) and of mTOR complex 1 (mTORC1) that coordinates cell growth and metabolism (Cam et al., 2010). Thus, DBC1, ATM and SIRT1 could be further linked in the control and regulation of cellular metabolism after genotoxic stress. Regarding the impact of DBC1 on SIRT1-mediated histone deacetylation, our preliminary experiments do not demonstrate an effect on H3-K9 acetylation (data not shown), suggesting that DBC1 selectively regulates the activity of SIRT1 towards a subset of targets.

Of note, the ATM/ATR substrate Chk2 was found to phosphorylate the RNA binding protein HuR in response to H<sub>2</sub>O<sub>2</sub>, causing the dissociation of the HuR-SIRT1 mRNA complex and the decay of SIRT1 mRNA (Abdelmohsen et al., 2007). These data, together with our findings, indicate that in response to DNA damage ATM/ATR signaling finely regulates SIRT1 by different mechanisms and in an indirect manner, given that SIRT1 does not appear to be directly targeted by these kinases.

While revising this manuscript, data similar to ours on DBC1-Thr454 phosphorylation by ATM after genotoxic stress were reported (Yuan et al., 2012). Our research confirms and extends these findings by also demonstrating the involvement of ATR in this response and the phosphorylation of DBC1-Thr454 in undamaged S-phase cells.



In summary, our study expands recent observations implicating SIRT1 in DDR (Wang et al., 2008; Fan and Luo, 2010; Uhl et al., 2010) by elucidating the molecular mechanisms by which ATM/ATR phosphorylation of DBC1 inhibits SIRT1 activity.

## **Materials and methods**

### *Cells, transfections, treatments and antibodies*

U2OS human osteosarcoma cell line and HEK-293T human embryonic kidney cell line were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Normal lymphoblastoid cell line (LCL-N) and the ATM-negative cell line (AT52RM, provided by Luciana Chessa, La Sapienza University, Rome) were cultured in RPMI medium supplemented with 15% fetal bovine serum. Cells were transfected using Lipofectamine 2000 (Invitrogen) and lysed in Laemmli buffer for western blot analysis. Etoposide treatments were performed using a final 20  $\mu$ M concentration unless otherwise indicated. The ATM inhibitor KU-55933 (Kudos, R&D Chemicals) and the proteasome inhibitor MG132 were used, respectively, at 10 and 20  $\mu$ M, and added to cells 1 h or 20 min before genotoxic treatments (Buscemi et al., 2009). Cycloheximide (CHX) was used at 100  $\mu$ g/ml for 1, 3 or 6 h. Cell viability was tested by trypan blue (Sigma) exclusion. Antibodies used were against DBC1 (Bethyl Laboratories, A300-434A), p53 (DO7, Zymed), cyclin B1 (BD-Biosciences),  $\gamma$ H2AX (Upstate, Millipore), SIRT1, FLAG-M2,  $\beta$ -actin (Sigma), phospho-DBC1 (Thr454), phospho-SQ/TQ, PUMA, p53, p53-AcK382, cleaved PARP-1, and cleaved caspase-9 (Cell Signaling Technology).

### *Plasmids, site-directed mutagenesis and siRNAs*

pCMV-Sport-6 encoding full-length DBC1 (DBC1<sup>WT</sup>) was obtained from Source BioScience Life Sciences (Germany). DBC1 cDNA was successively cloned in pcDNA3-FLAG vector (FLAG-DBC1<sup>WT</sup>) and mutants DBC1<sup>T454A</sup> (in pCMV-Sport-6) and FLAG-DBC1<sup>T454A</sup> (in pcDNA3-FLAG) were obtained using the QuikChange II XL Site Directed Mutagenesis Kit (StrataGene, Agilent Technologies). Forward and reverse primers were, respectively, 5'-GCAGCTCCCCCAGCCCAGGAGGCAC-3' and 5'-GTGCCTCCTGGGCTGGGGGAGCTGC-3'. Sequences were verified by automated DNA sequencing. FLAG-SIRT1 was previously described (Kim et al., 2008). HA-p53 was obtained by cloning full-length p53 cDNA in pcDNA3-HA vector. siRNAs against ATR, p53 and SIRT1 were ON-TARGETplus SMARTpool reagents (Thermo Scientific Dharmacon). siRNA against luciferase (siLUC) was custom made by Thermo Scientific Dharmacon.

### *Immunoprecipitations*

FLAG-tagged proteins were immunoprecipitated with the anti-FLAG-M2 antibody from extracts of transfected U2OS cells and analyzed by western blot as previously described (Zannini et al., 2009). SIRT1 protein was immunoprecipitated using the specific antibody. FLAG-SIRT1 immunoprecipitates were washed with ELB buffer containing 750 mM LiCl<sub>2</sub> and 1% NP-40 to dissociate SIRT1-DBC1 complex.

### *Luciferase reporter assays*

U2OS cells were transfected with a mixture containing p53-responsive PUMA promoter-driven firefly luciferase reporter vector (PUMA-Luc), pRL-TK (encoding *Renilla* luciferase) and either DBC1<sup>WT</sup> or DBC1<sup>T454A</sup>. After 48 h, cells were treated

with 20  $\mu$ M etoposide for 3 h or left untreated. Cells were then lysed and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

#### *Colony formation assays*

U2OS were transfected with empty (mock), DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> expression plasmids, plated in triplicate (500 cells/well in 6-well plates), treated with increasing doses of etoposide (from 0 to 80 nM) and allowed to grow for about 2 weeks. Plates were fixed in methanol and stained with 0.5% crystal violet solution. Colonies were counted and results were expressed as percentages of the untreated control.

#### *Immunofluorescence*

Cells grown on glass coverslips were fixed with 4% paraformaldehyde, blocked in PBS, 5% normal goat serum, 0.3% Triton X-100 for 60 min and incubated overnight at 4°C with antibodies against FLAG, phospho-DBC1 (Thr454),  $\gamma$ H2AX or cleaved PARP-1, diluted in PBS, 1% BSA, 0.3% Triton X-100. After washing, slides were incubated with secondary antibodies, counterstained with DAPI, mounted and scored by fluorescence microscopy. For the pre-extraction procedure, cells were treated with 0.1% Triton X-100 in PBS for 30 sec before fixation.

#### *SIRT1 activity assay*

SIRT1 activity was measured with the SIRT1 Direct Fluorescent Screening Assay kit (Cayman Chemical) as described (Escande et al., 2010). Briefly, 293T cells were transfected with empty vector (mock) or with DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> expression plasmids. After 24 h, cells were treated with etoposide for 1 h and lysed in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Lysates (30  $\mu$ g/well) were then incubated in SIRT1 assay buffer in the presence of SIRT1

Direct Peptide, 5  $\mu$ M TSA and 200  $\mu$ M NAD<sup>+</sup> for 45 min at room temperature in a half-volume 96-well white plate. Reactions were terminated by adding Stop/Developing solution, and fluorescence was measured with a Tecan Ultra microplate reader (excitation 360 nm, emission 465 nm).

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### Figure legends

**Figure 1** Human DBC1 is phosphorylated at Thr454 after DNA damage. **(A)** FLAG-DBC1<sup>WT</sup> and FLAG-DBC1<sup>T454A</sup> were expressed in U2OS cells and immunoprecipitated before and 1 and 3 h after etoposide treatment. Immunocomplexes were analyzed by western blot with anti-phospho-SQ/TQ and re-probed with anti-FLAG. Input, total lysate from untreated cells; Ip  $\alpha$ -FLAG, immunoprecipitates; PC, pre-cleared negative control. Asterisk indicates a non-specific band. **(B)** As in **A**, ectopic FLAG-DBC1<sup>WT</sup> and FLAG-DBC1<sup>T454A</sup> were immunoprecipitated from cells treated or not with the ATM inhibitor KU-55933 and then with etoposide (Eto). Immunocomplexes were probed with anti-phospho-DBC1 (Thr454) and then with anti-FLAG. **(C)** LCL-N and the ATM negative AT52RM cells were exposed or not to etoposide and analyzed by western blot for DBC1 Thr454 phosphorylation. **(D)** U2OS cells were transfected with siRNA targeting ATR or with siLUC control, pretreated or not with KU-55933 and then exposed to etoposide. Cell extracts were then analyzed by western blot. **(E)** Time-dependent (top) and dose-dependent (bottom) effects of etoposide on the phosphorylation of endogenous DBC1. Unt, untreated. **(F)** Phosphorylation of endogenous DBC1 in cells transfected with siLUC control or with ATR siRNA, in the absence or presence KU-55933. Eto, etoposide-treated cells used as positive control. **(G)** Percentage of cells scoring positive for phospho-DBC1<sup>WT</sup> among all DBC1-overexpressing cells after ATR depletion and KU-55933 (KU) treatment. Values are mean $\pm$ SD of foci/nucleus within the phospho-DBC1<sup>WT</sup> (pT454) positive cells. **(H)** In undamaged samples, the small fraction of cells containing phosphorylated DBC1 has more  $\gamma$ H2AX nuclear foci than cells without phosphorylated DBC1. Scale bar, 10  $\mu$ m. **(I)** Cells were transfected with mock, FLAG-DBC1<sup>WT</sup> or FLAG-DBC1<sup>T454A</sup> vectors and  $\gamma$ H2AX foci were

enumerated. Data report the mean $\pm$ SD of foci detected in the FLAG-positive nuclei. \* $P=0.0003$ , \*\* $P=0.03$ . Western blot (right) shows the levels of the overexpressed proteins.

**Figure 2** Thr454 phosphorylation does not affect DBC1 stability or subcellular localization. Total levels of FLAG-DBC1<sup>WT</sup> and FLAG-DBC1<sup>T454A</sup> were analyzed by western blot after 0-6 h of cycloheximide (CHX) treatment (**A**) or CHX and etoposide (Eto) treatment (**B**). (**C**) Immunofluorescence analysis of U2OS cells expressing FLAG-DBC1<sup>WT</sup> or FLAG-DBC1<sup>T454A</sup> revealed a nuclear localization, as detected with anti-FLAG. Anti-phospho-DBC1 (Thr454) revealed a nuclear fluorescence signal in 17.7% of undamaged transfected cells and in all FLAG-DBC1<sup>WT</sup>-positive cells after etoposide exposure. Phosphorylation signal in etoposide-treated cells expressing FLAG-DBC1<sup>T454A</sup> is due to endogenous DBC1. (**D**) In etoposide-treated cells, endogenous phosphorylated DBC1 has a nuclear localization, as detected with anti-phospho-DBC1 (Thr454). (**E**) Radiation-induced  $\gamma$ H2AX nuclear foci do not appear to co-localize with phosphorylated DBC1, which is nonetheless chromatin bound as demonstrated by pre-extraction procedure prior to fixation. Scale bar, 10  $\mu$ m.

**Figure 3** Thr454 phosphorylation is required for DBC1-mediated inhibition of SIRT1. (**A**) HEK293T cells were transfected with mock, DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> vectors, exposed to etoposide for 1 h and then assayed for SIRT1 activity using a SIRT1 fluorometric kit. Western blot (right) shows the levels of the overexpressed proteins. (**B**) U2OS cells transfected with mock, DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> vectors were incubated with 20  $\mu$ M MG132 for 20 min prior to 1 h etoposide treatment. p53 acetylation at K382 was analyzed by western blot (left). Densitometric analysis (right) shows the ratios of acetylated p53-K382/total p53 normalized to the value of mock-

transfected cells. Values are mean±SD from three independent western blots. (C) Cells were transfected with control (siLUC) or SIRT1 siRNA and then with mock, DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> encoding vectors and assessed by western blot 1 h after etoposide and MG132 treatment (for each lane, the fold induction of acetylated p53 relative to total p53 is indicated). Asterisk indicates a non-specific band. (D) PUMA promoter transactivation was measured by luciferase assay, before and after etoposide treatment, on cells co-transfected with PUMA-Luc reporter gene and with plasmids encoding DBC1<sup>WT</sup> or DBC1<sup>T454A</sup>. The expression of these proteins was verified (bottom). (E) Endogenous PUMA protein assessed by western blot on lysates from untreated and etoposide-treated cells expressing wild type or point-mutated DBC1.

**Figure 4** Thr454 phosphorylation is involved in apoptosis induction and long-term cell survival. (A) U2OS cells transfected with control (siLUC) or SIRT1 siRNA and then with DBC1-encoding plasmid were exposed or not to 20 μM etoposide for 30 h and examined on western blot for levels of cleaved caspase-9. (B) The same experiment was repeated using siRNA against p53. (C) U2OS cells were transfected with siLUC, SIRT1 or p53 siRNA and then with FLAG-DBC1. 30 h after etoposide treatment cells were analyzed by two-color immunofluorescence with anti-cleaved-PARP-1 and anti-FLAG antibodies. Cleaved PARP-1-positive cells were counted and expressed as a fraction of FLAG-positive cells; these data were used to determine the induction of apoptosis of DBC1<sup>WT</sup>-expressing cells compared to mock. (D) U2OS cells transfected with mock, DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> vectors and treated or not with etoposide for 30 h, were scored for viability with trypan blue staining. Values are mean±SD of three independent experiments. \**P*=0.024. (E) Western blot of cleaved-PARP and cleaved caspase-9 in cells overexpressing DBC1<sup>WT</sup> or DBC1<sup>T454A</sup>. The values below cleaved-PARP and cleaved caspase-9 blots represent the fold induction

of these apoptotic markers (normalized to the levels of  $\beta$ -actin) after etoposide exposure. **(F)** Cells transfected with mock, FLAG-DBC1<sup>WT</sup> and FLAG-DBC1<sup>T454A</sup> vectors were harvested 18 and 30 h after etoposide treatment and probed with antibodies against FLAG and cleaved PARP-1. Cleaved PARP-1-positive cells are expressed as a fraction of FLAG-positive cells; these data were used as in **C** to determine the induction of apoptosis relative to mock. Values are mean $\pm$ SD from three independent experiments. **(G)** Colony formation assays were performed on cells transfected as in **C** treated with increasing doses of etoposide. Colonies were counted and expressed as a percentage of the untreated sample. Results are mean $\pm$ SD from three independent experiments. \* $P=0.0001$ , \*\* $P=0.0015$ , \*\*\* $P=0.0041$ .

**Figure 5** Thr454 phosphorylation regulates DBC1 binding to SIRT1. **(A)** Western blot analyses of endogenous SIRT1 immunocomplexes from U2OS cells treated or not with etoposide (top). Protein levels were assessed by western blot (bottom). Ip, immunoprecipitates; IgG, negative control; Input, total lysates. **(B)** FLAG-tagged DBC1<sup>WT</sup> or DBC1<sup>T454A</sup> immunoprecipitates from untreated and etoposide-treated cells were analyzed by western blot (top) with anti-SIRT1 and anti-FLAG antibodies. Protein levels were analyzed by immunoblotting of total lysates (middle). PC, pre-cleared negative control. Densitometric quantification of SIRT1 co-immunoprecipitated with DBC1 (bottom). Data from etoposide-treated cells were normalized to those from untreated samples. Values are mean $\pm$ SD from three independent experiments. \* $P=0.0023$ , \*\* $P=0.038$ . **(C)** FLAG-SIRT1 was immunoprecipitated from U2OS cells transfected with the indicated combinations of plasmids and treated for 3 h with etoposide. Ips were then analyzed by immunoblot with anti-p53 and anti-FLAG antibodies (top). Total lysates were analyzed for p53, FLAG, DBC1 and  $\beta$ -actin antibodies (middle). Densitometric quantification of

relative levels of p53 bound to SIRT1 from three independent experiments (bottom). \* $P=0.0051$ . (D) FLAG-SIRT1 or FLAG-DBC1, used as positive control, were immunoprecipitated from transfected U2OS 3 h after etoposide exposure. Ips were then probed with anti-phospho-SQ/TQ and anti-FLAG.

**Figure 6** Role of DBC1 in the DNA damage response. In response to DNA damage, ATM/ATR kinases contribute to the activation of p53 in two ways, (i) by directly phosphorylating p53 on Ser15, an event which facilitates its subsequent acetylation, and (ii) phosphorylating DBC1 on Thr454, which enhances the inhibition of SIRT1 deacetylase. Altogether, these phosphorylation events promote the acetylation-dependent activation of p53 and enhance the p53-mediated apoptotic process.













