CyclinB1/Cdk1 phosphorylates mitochondrial antioxidant MnSOD in cell adaptive response to radiation stress

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Manganese superoxide dismutase (MnSOD), a major antioxidant enzyme within the mitochondria, is responsible for the detoxification of free radicals generated by cellular metabolism and environmental/therapeutic irradiation. Cell cycle-dependent kinase Cdk1, along with its regulatory partner CyclinB1, plays important roles in the regulation of cell cycle progression as well as in genotoxic stress response. Herein, we identified the presence of the minimal Cdk1 phosphorylation consensus sequence ([S/T]-P; Ser106) in human MnSOD, suggesting Cdk1 as a potential upstream kinase of MnSOD. A substantial amount of CyclinB1/Cdk1 was found to localize in the mitochondrion upon irradiation. The enhanced Cdk1/MnSOD interaction and MnSOD phosphorylation were detected in both the irradiated human cells and mouse tissues. We report that CyclinB1/Cdk1 can regulate MnSOD through reversible Ser106 phosphorylation, both in vivo and in vitro. The CyclinB1/Cdk1-mediated MnSOD Ser106 resulted in increased MnSOD activity and stability, along with improved mitochondrial function and cellular resistance to radiation-induced apoptosis. These results demonstrate a unique pro-survival mechanism by which cells enhance the survival via CyclinB1/Cdk1-mediated MnSOD activation under genotoxic stress conditions.

Keywords: MnSOD, CyclinB1/Cdk1, radioadaptive response

Introduction

Manganese superoxide dismutase (MnSOD) is a primary mitochondrial antioxidant enzyme for detoxification of reactive oxygen species (ROS), namely superoxide free radicals, generated as by-products of oxidative phosphorylation or under oxidative stress (Oberley and Buettner, 1979). MnSOD catalyzes the conversion of two molecules of superoxide anion (O₂⁻) into hydrogen peroxide (H₂O₂) that is further oxidized to water (McCord et al., 1971). The translation of the SOD2 gene starts with a mitochondrial targeting sequence at the N terminus of the protein, which allows a direct transport of the newly synthesized MnSOD protein into the mitochondrion (Borgstahl et al., 1992). Once entering the mitochondrion, MnSOD can self-associate into an active homotetramer that incorporates Mn³⁺ ion. The misfolded MnSOD or folded without manganese shows no cellular functions (Culotta et al., 2005). The loss of the MnSOD expression or its enzymatic activity leads to a high risk of cell transformation (Oberley et al., 1989; St Clair and Holland, 1991; Church et al., 1993; Li et al., 1995; Zhong et al., 1997; Van Remmen et al., 2001; Zhang et al., 2010) and also high sensitivity of cells to oxidative stress and therapeutic radiation (Epperly et al., 2000a; Van Remmen et al., 2004; Murley et al., 2008). These observations highlight the important role of MnSOD in cancer prevention and in normal tissue protection under cancer radiotherapy.

In addition to providing >90% of cellular ATP, mammalian mitochondria play crucial roles in organizing signaling transduction and apoptosis, in which MnSOD plays a key role in balancing the ROS and protecting mitochondrial normal function (Fujimura et al., 1999; Spitz et al., 2004). For example, the MnSOD activity is inducible in the heart and intestine after irradiation (Summers et al., 1989) and in mouse skin cells exposed to low-dose ionizing radiation (LDIR) (Fan et al., 2007); and overexpression of MnSOD protects mouse epithelial tissues from radiation injury (Epperly et al., 2000b). Moreover, mice with the MnSOD haplo insufficiency have abnormal mitochondrial function correlated with increased oxidative damage (Williams et al., 1998). Thus, MnSOD is an important determinant for intact mitochondrial functions, and for defending cells against ROS injury during oxygen metabolism and radiation stress.
In mammals, the expression and activation of cell-cycle proteins Cyclins and Cyclin-dependent kinases (Cdks) are tightly controlled during cell-cycle progression (Eldège, 1996). CyclinB1 and its catalytic partner, Cdk1, form an active complex to regulate the G2/Mmitosis transition by regulating both the nuclear and cytoplasmic factors for a successful entrance into the mitosis (Solomon et al., 1990; Pines, 2006; Lindqvist et al., 2007). CyclinB1/Cdk1 protects mitotic cells against apoptosis (Allan and Clarke, 2007) and promotes mitochondrial fission, a critical step for an equal distribution of mitochondria to the newly formed daughter cells (Cerveny et al., 2007). MnSOD also contains a minimal Cdk1 phosphorylation consensus sequence ([S/T]T-P) (Runner and Murray, 2000; Ubersax et al., 2003) at Ser106, we propose that CyclinB1/Cdk1 can phosphorylate MnSOD to regulate its enzymatic activity. Our results showed that the mitochondria-translocated Cdk1 could phosphorylate MnSOD at Ser106, stabilize MnSOD protein and enhance its antioxidant activity, leading to reductions in mitochondrial superoxide levels and mitochondrial-mediated apoptosis under LDIR-induced adaptive radioprotection.

**Results**

*CyclinB1/Cdk1 translocates to mitochondria and interacts with MnSOD after irradiation*

Cells receiving an exposure of LDIR (10 cGy) are able to induce SOD2 gene expression and adaptive radioprotection (Fan et al., 2007). To investigate whether CyclinB1/Cdk1 is involved in LDIR-induced adaptive radioprotection, we first measured CyclinB1, Cdk1 and MnSOD protein levels in whole cell lysates and mitochondrial fractions of human breast epithelial MCF10A cells after exposure to LDIR. Mitochondrial translocation of Cdk1 and CyclinB1 has been previously shown following IR exposure (Nantajit et al., 2010). We found that although Cdk1 levels in whole cell extracts remained constant, CyclinB1 levels were increased several folds in response to IR. Substantial amounts of both proteins, especially CyclinB1, were also detected in the mitochondria after a single low dose (10 cGy) of irradiation, starting at 2 h and peaking at 8 h post-irradiation (Figure 1A). MnSOD protein levels were also increased after exposure to LDIR, and had the highest in the mitochondria at 8 h post-irradiation, but the increment was moderate compared with its basal levels (Figure 1A). LDIR-induced mitochondrial translocation of CyclinB1 and Cdk1 with limited increase in the MnSOD protein in the mitochondria indicates that MnSOD enzymatic activity, rather than its protein availability in the mitochondria, may be regulated by the cell-cycle kinase CyclinB1/Cdk1 in response to LDIR. To address whether Cdk1 physically interacts with MnSOD, we performed reciprocal co-immunoprecipitation (co-IP) experiments and showed their interaction in *vitro* in MCF10A cells (Figure 1B) with or without 10 cGy of irradiation. The interaction seems to be more prominent post-irradiation (8 h post-10 cGy of IR). Cdk1 and MnSOD also interact in MEFs (Figure 1C) and human keratinocytes (HK18; unpublished data) suggesting their likely commonality. Although the interaction is universal in different cell lines, the degree of interactions showed deviations suggesting that the nature of Cdk1-MnSOD interaction may be cell and stimulus type dependent.

*CyclinB1/Cdk1 phosphorylates MnSOD at Ser106*

The fact that MnSOD contains a minimum Cdk1 phosphorylation site led to the analysis of whether Cdk1 could phosphorylate MnSOD. *In vitro* kinase assay using immunoprecipitated flag-tagged MnSOD derived from transfected cells as the substrate and commercial Cdk1 enzyme as the kinase revealed that Cdk1 phosphorylates MnSOD. However, it failed to phosphorylate mutant MnSOD S106A, where the serine 106 phosphorylation site of the MnSOD was replaced with alanine (Figure 1D and Supplementary Figure S1), supporting that Cdk1 phosphorylates MnSOD at Ser106. To further confirm the Cdk1-dependent phosphorylation of MnSOD, we overexpressed mitochondrion-targeted wild-type (WT) or dominant-negative Cdk1 (van den Heuvel and Harlow, 1993) in MCF10A cells (Supplementary Figure S2) and measured the phosphorylation levels of MnSOD by pulling down the MnSOD protein via IP and detecting the phosphoprotein levels using phospho-serine antibody via western blotting. The results showed that the phosphorylation levels of MnSOD were increased in cells expressing WT mitochondrial Cdk1 but not in cells transfected with dominant-negative mitochondrion-targeted Cdk1 (Figure 1E and Supplementary Figure S2). These results provide the early evidence that mitochondrial Cdk1 is able to phosphorylate MnSOD protein at Ser106 residue.

We then examined the *in vivo* Cdk1-mediated MnSOD phosphorylation using sham or whole-body irradiated mice to detect the Cdk1-MnSOD interaction and MnSOD phosphorylation. The results demonstrated that compared with the sham-irradiated animals, the interaction between Cdk1 and MnSOD was enhanced in 10 cGy-irradiated mouse tissues, especially in the tissues of heart and muscles (Figure 2A). Moreover, using the same mouse tissues, the enhanced serine phosphorylation of MnSOD was detected *in vivo* by pulling down MnSOD with anti-MnSOD antibody and performing an immunoblot with phospho-serine antibody (Figure 2B). The IPs with IgG were included in all co-IP assays as negative controls to detect non-specific staining in the samples, which were taken into account while evaluating the level of interaction in experimental preparations. Taken together, the *in vivo* and *in vitro* data strongly suggest that Cdk1 physically interacts with MnSOD and the interaction likely results in the phosphorylation of MnSOD.

*Cdk1-mediated phosphorylation enhances MnSOD enzymatic activity*

Protein phosphorylation is an essential covalent modification that can regulate the protein functions post-transcriptionally. To determine the effect of Cdk1-dependent phosphorylation of MnSOD on its enzymatic activity, we measured the MnSOD activity in cells expressing either WT or mutant MnSOD with or without LDIR. MnSOD activity was much higher in cells expressing WT MnSOD where MnSOD was phosphorylated compared with the
cells transfected with mutant MnSOD S106A where the phosphorylation of MnSOD was attenuated (Figure 3A), indicating that the phosphorylation of MnSOD at Ser106 is required for MnSOD activity. Consistent with this, LDIR significantly induced the activity of MnSOD, but failed to do so when Ser106 was mutated (Figure 3B). To further determine whether Cdk1 specifically regulates the MnSOD activity in the mitochondria, we measured the MnSOD activity in the cells transfected with mitochondrion-targeted WT or mutant Cdk1 constructs. MnSOD activity was doubled in cells transfected with WT Cdk1 compared with the vector transfecants, whereas the MnSOD activity was significantly inhibited in cells expressing the mitochondrion-targeted dominant-negative mutant Cdk1 (Figure 3C). The presence of active MnSOD in the cells transfected with WT versus mutant MnSOD or WT versus mutant Cdk1 was shown by activity gels, where the achromatic bands obtained corresponds to the active MnSOD sites. In consistent with the results from the biochemical activity assays, activity gels also revealed the higher activity of WT MnSOD compared with MnSOD S106A mutant as well as higher activity of MnSOD in the WT Cdk1-expressing cells compared with mutant Cdk1-bearing cells (Figure 3D). These results indicate that Cdk1-dependent phosphorylation of MnSOD at Ser106 is an activating phosphorylation event that enhances the dismutase activity of MnSOD antioxidant.

Phosphorylation of MnSOD at Ser106 stabilizes MnSOD

Reversible phosphorylation of enzymes results in the conformational change in their structures, causing them to become activated or deactivated. We addressed whether Cdk1-mediated MnSOD phosphorylation could affect MnSOD conformation and stability. To assess the tetrameric conformation of MnSOD protein, the active form of this enzyme, we transfected the cells with WT or mutant MnSOD and resolved the cell lysates on both denatured and native gels. The results showed that mutation of MnSOD at Ser106 markedly reduced its tetramer protein levels compared with WT MnSOD (Figure 4A, lower panel), which was calculated by normalizing the WT and mutant protein levels on the native gel to their expression levels detected in the denatured gels (Figure 4A, top and middle panels). The results indicate that the Cdk1-mediated phosphorylation of Ser106 of MnSOD is required for the tetramer formation and/or for MnSOD protein stability. In agreement with the decreased tetramer protein levels of mutant MnSOD, the half-life of the mutant MnSOD was decreased compared with that of the WT MnSOD (Figure 4B). In order to test whether the lack of stability of mutant MnSOD

Figure 1 MnSOD and Cdk1/CyclinB1 interaction and MnSOD phosphorylation in the mitochondria. (A) Immunoblotting (IB) of CyclinB1, Cdk1 and MnSOD in total cell lysates (upper panel) or mitochondrial fractions (lower panel) from human breast epithelial MCF10A cells after irradiation with 10 cGy X-ray at indicated times. β-actin and CoxIV were included as the loading control for the whole cell lysates and mitochondria fractions, respectively. (B) The interaction of mitochondrial Cdk1 and MnSOD. MCF10A cells were either left untreated (sham) or exposed to 10 cGy of X-ray before reciprocal IP assays. IP is performed 8 h post-irradiation. IgG was used as a negative control. (C) The interaction of Cdk1 and MnSOD in MEFs. (D) The phosphorylation of MnSOD by commercial Cdk1. Upper panel: the flag-tagged WT MnSOD (MnSOD-flag) or mutant (MnSOD S106A-flag) proteins derived from transfected MCF10A cells were used as a substrate for in vitro kinase assay. Histone was used as a positive control substrate. A specific Cdk1 inhibitor olomoucine was included to indicate specificity of the kinase. Lower panel: the expression of flag-tagged WT and mutant MnSOD in MCF10A cells assessed by western blotting. (E) The phospho-MnSOD levels in MCF10A cells transfected with mitochondrion-targeted WT (MTS-Cdk1) or dominant-negative mutant Cdk1 (MTS-dnCdk1). MnSOD protein was pulled down using the IP anti-MnSOD antibody and the phospho-protein levels were determined by the anti-phospho-serine antibody. The input (4%) for IP reactions was used as a loading control.
is because of its inability to interact with Cdk1, we carried out IP reactions and found the mutant MnSOD was still able to interact with Cdk1 (Figure 4C), indicating that the interaction between MnSOD and Cdk1 is independent from the Ser106 residue and the phosphorylation status of MnSOD.

Cdk1-phosphorylated MnSOD inhibits mitochondrial apoptosis

The enzymatic function of tetrameric MnSOD protein in the mitochondria is to convert superoxide into hydrogen peroxide that can then be further metabolized to water. This way, the mitochondrial antioxidative enzyme that converts the highly reactive superoxide anions (O$_2^-$) into less reactive hydrogen peroxide (H$_2$O$_2$), the superoxide radicals are generated as by-products of mitochondrial oxidative respiration as well as by oxidative stress including IR, both of which can cause the primary damages in mitochondrial DNA (mtDNA) and other mitochondrial macromolecules (Henderson et al., 2009). Superoxide anions in the mitochondrial matrix that arise from the reduced MnSOD activity lead to damaged mitochondria, which in turn release more ROS, increasing oxidative damage to the mitochondrial, cytosolic and nuclear compartments (Van Remmen and Richardson, 2001). Excessive amounts of ROS and imbalanced redox are believed to induce a large scale of age-related pathologies such as carcinogenesis, cardiovascular impairments, and many other diseases (Balaban et al., 2005). Therefore, an enzymatic faultless MnSOD protein must be sustained for mitochondrial functions and efficient scavenging of superoxide radicals. We have previously identified the

The adaptive response was induced in cells by LDIR (10 cGy) followed by exposure to a single high lethal dose of 10 Gy 8 h post-10 cGy of radiation (10 Gy + 10 Gy; Fan et al., 2007). Irradiations with sham, 10 cGy or 10 Gy alone were included as controls. Cells expressing the mutant MnSOD S106A (Figure 5C) or the mitochondria-targeted mutant Cdk1 (Figure 5D) showed enhanced apoptosis rates compared with cells expressing WT MnSOD or WT Cdk1 when treated with 10 cGy + 10 Gy irradiation. Moreover, the mutant MnSOD transfectants were radiosensitive under 10 Gy of high-dose irradiation (Figure 5C), supporting the radioprotective role of WT MnSOD. However, no radioprotection was detected in the WT Cdk1 transfectants following 10 Gy of high-dose radiation (Figure 5D), indicating that MnSOD but not Cdk1 is a limiting factor in mediating mitochondrial apoptosis. Additionally, clonogenic survival analysis of WT MnSOD or mutant MnSOD-transfected cells were performed to further elucidate the role of Cdk1-mediated MnSOD regulation in the radioadaptive response of MCF10A cells. Consistently, the mutant MnSOD-bearing cells showed lower levels of clonogenic ability compared with the WT MnSOD-bearing cells when exposed to 10 Gy of radiation (Figure 5E). Radioadaptive protection was observed in cells with WT MnSOD upon exposure to 10 cGy of LDIR prior to 10 Gy of higher dose of irradiation; however, mutant-MnSOD transfected cells failed to exhibit such radioadaptive response (Figure 5E).

**Discussion**

This study reveals a mechanism by which the mitochondrial antioxidant MnSOD enzyme is activated by phosphorylation at Ser106 residue by mitochondria-located cell-cycle kinase, Cdk1 (Figure 6). The Cdk1-phosphorylated MnSOD showed enhanced enzymatic activity and protein stability that is required for the adaptive radioprotection. Using the adaptive radiation protocol, i.e. LDIR followed by high-dose irradiation, we found a substantial increase in mitochondrial apoptosis in S106A mutant MnSOD transfectants versus WT MnSOD transfectants. Moreover, the cells harboring the mutant MnSOD S106A had elevated superoxide levels under a stress of single high-dose (10 Gy) radiation exposure and decreased ATP production.

MnSOD has long been recognized as the primary mitochondrial antioxidant enzyme that converts the highly reactive superoxide ions (O$_2^-$) into less reactive hydrogen peroxide (H$_2$O$_2$). The superoxide radicals are generated as by-products of mitochondrial oxidative respiration as well as by oxidative stress including IR, both of which can cause the primary damages in mitochondrial DNA (mtDNA) and other mitochondrial macromolecules (Henderson et al., 2009). Superoxide anions in the mitochondrial matrix that arise from the reduced MnSOD activity lead to damaged mitochondria, which in turn release more ROS, increasing oxidative damage to the mitochondrial, cytosolic and nuclear compartments (Van Remmen and Richardson, 2001). Excessive amounts of ROS and imbalanced redox are believed to induce a large scale of age-related pathologies such as carcinogenesis, cardiovascular impairments, and many other diseases (Balaban et al., 2005). Therefore, an enzymatic faultless MnSOD protein must be sustained for mitochondrial functions and efficient scavenging of superoxide radicals. We have previously identified the
cell cycle-dependent kinases in the regulation of mitochondrial proteins/functions (Nantajit et al., 2010). In this regard, the translocation of Cdk1 into the mitochondria appears to not only promote ATP production for cell division, but also equip the cells with enhanced superoxide scavenging capacity by activating MnSOD via phosphorylation, supporting a potential link between cell cycle G2/M transition and mitochondrial metabolism.

Recent evidence indicates the mitochondrial translocation of p53 as a coping mechanism adopted by cells under increased oxidative stress (Zhao et al., 2002; Mihara et al., 2003; Zhao et al., 2005). Mitochondrial-localized p53 induces apoptosis by binding to multiple targets on the outer membrane of the mitochondria such as Bcl-2/Bcl-xL (Mihara et al., 2003), p53API1 (Oda et al., 2000), and Bax (Chipuk et al., 2004). Further studies by Zhao et al., 2005 showed that p53 also localizes into the matrix of the mitochondria and physically interacts with MnSOD to inhibit its superoxide scavenging activity, showing a direct role for mitochondrial p53 in the regulation of MnSOD activity and oxidative stress. Analysis of downstream effects and possible modulators of p53-MnSOD interaction will largely improve our knowledge of the molecular mechanisms of p53-mediated protection of cellular and potentially mitochondrial genome. A recent study demonstrated that p53 translocates into the mitochondria upon UVB radiation-induced mtDNA damage and enhances mtDNA repair by acting on mtDNA polymerase Poly (Bakthavatchalu et al., 2012). Interestingly, this study also suggested MnSOD as a fidelity protein that enhances the repair of mtDNA by physically interacting with the mtDNA repair enzyme Poly and protecting it from the deleterious effects of UVB radiation. In this regard, our study implies that the phosphorylation of MnSOD by CyclinB1/Cdk1 may promote its mtDNA repair-promoting functions in addition to its superoxide scavenging capacity to ensure the passage of error-free mtDNA onto the daughter cells. Apart from directly acting on MnSOD, CyclinB1/Cdk1 complex may indirectly regulate the MnSOD functions by targeting mitochondrial p53. CyclinB1/Cdk1 is among the upstream effectors of p53 in the mitochondria (Nantajit et al., 2010). Nantajit et al. (2010) showed that mitochondrial Cdk1 interacts with and phosphorylates mitochondrial p53 at Ser315 in colon cancer cells upon exposure to IR. Interestingly, the phosphorylation of p53 at Ser315 by mitochondrial Cdk1 resulted in increased survival of the cells, suggesting an anti-apoptotic role for mitochondrial phospho-p53. Further elucidation of the role of mitochondrial matrix p53 and the effect of Ser315 phosphorylation on pro-apoptotic functions of p53 is still

Figure 3 Phosphorylation of MnSOD at Ser106 by Cdk1 enhances its dismutase activity. (A) MnSOD activity in MCF10A cells transfected with flag-tagged WT or mutant MnSOD measured by the superoxide dismutase assay (left panel; n = 3, **P < 0.01). Phosphorylation levels of the WT or mutant MnSOD proteins measured by IP with an anti-flag antibody followed by IB with an anti-phospho-serine antibody (right panel). (B) MnSOD activity and phosphorylation 8 h post-10 cGy of irradiation determined as indicated in A (n = 3, **P < 0.01). (C) MnSOD activity in the MCF10A cells transfected with mitochondrion-targeted WT or dnCdk1 with 10 cGy irradiation. The activities were measured 8 h post-LDIR (n = 3, **P < 0.01). Data are represented as the mean ± SEM. (D) Active MnSOD levels in MCF10A cells transfected with flag-tagged WT or mutant MnSOD (left panel) and with mitochondria-targeted WT or mutant Cdk1 (right panel), measured by in gel activity assays. Commercially available active MnSOD tetrameric enzyme is included as a positive control. Lower panel: equal loading of the samples assessed by western blotting using α-tubulin antibody (n = 3).
elusive. The studies demonstrating mitochondrial localization of p53 and CyclinB1/Cdk1 in oxidative stress conditions, together with our present findings that Cdk1 can directly phosphorylate and enhance MnSOD activity may point to a Cdk1-p53-MnSOD regulatory loop in genotoxic stress response.

The phosphorylation of antioxidant proteins has already been linked with the growth in mammalian cells. The phosphorylation of the human antioxidant peroxiredoxin I (Prx I) by Cdk1 results in the inactivation of this antioxidant and affects the cell-cycle progression (Chang et al., 2002). Peroxiredoxins are the enzymes involved in the elimination of H2O2 and present in organisms from all kingdoms (Rhee et al., 2001). The transient inhibition of Prx1 activity via Cdk1-dependent phosphorylation results in H2O2 accumulation, which is then thought to stimulate the transition from G2 to M phase (Chang et al., 2002). Cdk1 forms a complex with its regulatory partner, CyclinB1, at the late G2 phase to regulate the transition from G2 to M (Nigg, 1995).

Thus, it is logical to assume that Cdk1 phosphorylates MnSOD to enhance its superoxide dismutase activity, which will further contribute to the accumulation of H2O2 and assist the progression through G2 phase. Nevertheless, further studies are needed to fully understand the role of CyclinB1/Cdk1-mediated phosphorylation of MnSOD in cell-cycle progression and other critical cellular outcomes.

The spatiotemporal regulation of cyclins and Cdks is pivotal for the normal cell cycle and is subject to multiple control steps. CyclinD1 is the first cell-cycle regulatory protein to respond to mitogenic stimuli and facilitates entry into S-phase. CyclinB1 regulates the progression from G2 to M. Besides our present data showing Cdk1-mediated regulation of MnSOD activity, MnSOD activity and ROS levels were shown to be linked to the changes in both CyclinD1 and CyclinB1 protein levels (Sarsour et al., 2008), suggesting a bidirectional regulation between Cdk1 and MnSOD activities. Findings from Sarsour et al. (2008) also suggested that MnSOD activity regulates the transitions between quiescent and proliferative growth in MEFs via these changes in CyclinD1.

**Figure 4** Cdk1-mediated phosphorylation of MnSOD at Ser106 postpones the MnSOD protein degradation. (A) The homo-tetrameric conformation detected with the flag-tagged WT or mutant MnSOD. MCF10A cells were transfected with the WT or S106A mutant MnSOD and the cell lysates collected 24 h after LDIR were subjected to denatured (SDS-PAGE) or non-denaturing native gel electrophoresis and the membrane was blotted with anti-tubulin (loading control) and anti-flag to determine the protein levels and the homo-tetrameric conformations of WT and mutant MnSOD. The expression levels of the transfected WT or mutant MnSOD-flag proteins were determined by densitometry using Image J software and normalized with α-tubulin from SDS-PAGE immunoblots. The level of the tetrameric flag tagged WT or mutant MnSOD proteins (detected in native gel) were normalized to the expression levels of flag-tagged transfected proteins. (B) The stability of S106A mutant MnSOD compared with WT MnSOD. MCF10A cells were transfected with flag-tagged WT or mutant MnSOD for 24 h and then treated with cycloheximide (CHX, 10 μg/ml) at indicated time points. The exogenous MnSOD levels were determined by western blot with anti-flag antibody (upper panel) and the degradation rates of WT and mutant MnSOD proteins were determined by densitometry and normalized to β-actin levels (lower panels). (C) Cdk1 interacts with both of the WT and mutant MnSOD proteins. MCF10A cells were transfected with flag-tagged WT or mutant MnSOD and Cdk1/MnSOD interactions were detected by IP with anti-flag followed by IB with anti-Cdk1. The MnSOD input (4%) for IP reactions was used as a loading control.
and CyclinB1 protein levels. The regulation of cell-cycle regulatory protein expressions according to cellular redox environment supports the presence of a redox cycle within the cell cycle (Menon and Goswami, 2007; Burhans and Heintz, 2009).

Lysine acetylation has recently emerged as an important post-translational modification used to regulate mitochondrial proteins that are associated with energy homeostasis (Choudhary et al., 2009; Ozden et al., 2011). Herein, we showed that phosphorylation may provide an alternative means to modulate mitochondrial functions, highly likely to match the energy requirements of cells by inducing ATP production as well as to provide adaptive radioprotection via modulation of mitochondria-mediated apoptosis.

Low MnSOD activity has been associated with the malignant phenotype. Many cancer cells have low MnSOD activity due to lack of SOD2 promoter activity (Xu et al., 2002). The present study suggests that low MnSOD activity may also result from deficient Cdk1-mediated phosphorylation of MnSOD enzyme.

The results of this study demonstrate a novel post-translational modification of MnSOD in the control of the MnSOD enzymatic activity and protein stability in response to LDIR. This new feature of MnSOD regulation by cell-cycle proteins, CyclinB1/Cdk1, represents a tight correlation between cell-cycle progression and mitochondrial antioxidant status, which contributes to anti-apoptotic status and survival advantage over genotoxicity of high-dose radiation.

Materials and methods

Reagents and antibodies
Antibodies against MnSOD, Cdk1 and CyclinB1 were purchased from Santa Cruz Biotechnology, β-actin was purchased from Sigma Chemical Co., anti-phospho serine antibody was

Figure 5 Cdk1-mediated phosphorylation of MnSOD regulates the mitochondrial function and inhibits high-dose radiation-induced mitochondrial apoptosis. (A) Mitochondrial superoxide levels in MCF10A cells transfected with WT or mutant flag-tagged MnSOD measured 8 h after 10 cGy LDIR (n = 3, **P < 0.01). (B) ATP levels in MCF10A cells transfected with WT or mutant flag-tagged MnSOD (n = 3, **P < 0.01). (C) Apoptosis levels in MCF10A cells transfected with flag-tagged WT or mutant MnSOD measured by Annexin V–PI staining. The transfected cells were treated with sham, LDIR (10 cGy) or high-dose irradiation (10 Gy), or pre-exposed to 10 cGy and 8 h later irradiated with 10 Gy (10 cGy + 10 Gy). (D) Apoptosis levels in the MCF10A cells transfected with mitochondrion-targeted WT or dominant-negative Cdk1 measured as in C. Data are represented as the mean ± SEM. (E) Clonogenic survival of MCF10A cells transfected with flag-tagged WT or mutant MnSOD. The transfected cells were treated with high-dose irradiation (10 Gy), or pre-exposed to 10 cGy and irradiated with 10 Gy 8 h post-10 cGy (10 cGy + 10 Gy) (n = 3, **P < 0.01).
CyclinB/Cdk1-mediated phosphorylation of MnSOD

**Figure 6** Schematic representation of the putative mechanism of the regulation of MnSOD via Ser106 phosphorylation by mitochondrial CyclinB/Cdk1 complex in mammalian cells under LDIs. CyclinB1 and Cdk1 translocate to the mitochondria upon radiation and phosphorylates MnSOD at Ser106. The phosphorylation of MnSOD enhances its tetrameric conformation, stability and enzymatic activity, resulting in decreased ROS levels and apoptosis. By this pathway, cells are able to sense a low level genotoxic stress and induce an adaptive radioprotection by Cdk1-mediated MnSOD activation.

Cytosolic and mitochondrial fractions were extracted from exponentially growing MCF10A cells at 50%–80% confluence using a mitochondria isolation kit (Thermo Scientific). Briefly, cells were incubated in ice-cold hypotonic buffer containing 10 mM NaCl, 1.5 mM MgCl₂, and 10 mM Tris–HCl, pH 7.5, for 20 min and the cell membranes were disrupted by glass pestle in buffer containing 2 M sucrose, 35 mM EDTA and 50 mM Tris–HCl, pH 7.5. The mitochondrial fraction was then separated by centrifugation at 12000 rpm 10000 g for 20 min. For in vivo Cdk1 kinase assay, fusion proteins were extracted from transfected cells by IP with mouse monoclonal anti-flag antibody, and incubated in kinase buffer containing purified Cdc2 (Cdk1/CyclinB1 complex) kinase (New England Biolabs), 1 μl [γ-32P] ATP (10 μCi/μl) (Perkin Elmer), and 0.1-M cold ATP was used for control reaction. Histone H1 (10 μM, Gibco BRL) was added for kinase control. Highly specific Cdk1/CyclinB1 complex inhibitor, olomoucine (Calbiochem), was used to assess the specificity of the reaction. The reaction was incubated at 30°C for 30 min and stopped by boiling in 5 × LSB for 5 min.

**MnSOD enzyme activity**

MnSOD activity was measured using the Superoxide Dismutase Assay Kit ( Trevigen). Briefly, cells were collected in cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM α-glycerophosphate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and sonicated at 5 × 10/ sec pulses followed by centrifugation at 12000 g for 10 min at 4°C. MnSOD activity was determined by the colorimetric cytochrome c xanthine/xanthine oxidase assay as previously described (Murley et al., 2008).

**MnSOD native gel-activity assay**

MnSOD activity was assayed by native gel-electrophoresis following previously published protocol (Sarsour et al., 2005; Weydart and Cullen, 2010). Briefly, equal amounts of total protein extracts were resolved on a 8% non-denaturing polyacrylamide gel at 100 V for 3 h. The gels were then incubated with nitroblue tetrazolium (2.43 mM) for 30 min followed by riboflavin–TEMED (riboflavin 2.8 × 10⁻³ M and TEMED 28 mM) for 30 min. The gels were then rinsed with water before placing under a fluorescent light source until the achromobands of MnSOD were visualized by ECL western blotting detection system (Amersham).

**Cdk1 kinase assay**

To determine the mitochondrial ROS production (Mukhopadhyay et al., 2007), briefly, cells were incubated with the MitosOX Red at a final concentration of 5 μM for 10 min at 37°C, and ROS generation was determined by an increase in the

**IP and western blots**

Protein extracts were purified by IP using indicated antibodies and followed by the addition of protein A/G-agarose beads, and resolved on SDS-PAGE, or non-denatured native PAGE gels and then transferred onto nitrocellulose membranes. The membranes were probed with primary antibodies overnight at 4°C, followed by secondary antibody conjugated with horseradish peroxidase, and visualized with ECL western blotting detection system (Amersham).
fluorescence intensity measured with a micro-plate reader at an excitation wavelength of 510 nm and emission wavelength of 580 nm. The data were expressed as the percent of fluorescence generated in control cells.

**Measurement of ATP production**

The cells (1 x 10^6 cells/well) were seeded on 96-well plates and transfected with WT or mutant MnSOD for 24 h. Ten microliters of TCA extract was neutralized with 140 µl of 250 mM Tris-acetate (pH 7.75) for ATP assay using ATP Determination Kit (Molecular Probes) with a standard curve known ATP concentrations ranging from 100 pM to 1 mM.

**Flow cytometry-Annexin V staining**

The sham- or irradiation-treated cells were harvested at 8 h post-irradiation. Cell pellets were suspended in 500 µl of Annexin-binding buffer (BioSource, Invitrogen) at a population of 2 x 10^6 cells/ml. One microliter of Annexin-V FITC (BioSource, Invitrogen) and propidium iodide (Sigma) were added to 100 µl of cell suspensions, and incubated at room temperature for 15 min, and refilled with 400 µl of Annexin-binding buffer. The cell population was analyzed on FACSCan (BD).

**Cisplatin survival assay**

Standard radiation clonogenic survival assays were performed as previously described (Guo et al., 2004). Survival fraction was assessed by colony formation following exposure to 0 Gy (sham), 10 cGy, 10 Gy or 100 cGy + 10 Gy of radiation. An equal number of the irradiated and control cells were seeded on 60-mm dishes and cultured for 14 days. The colonies were stained with Coomassie blue and colonies containing >50 cells were counted as surviving colonies and normalized to the plating efficiency of cells without radiation.

**Statistical analysis**

The results were analyzed by Student’s t-test, with data considered significant at P < 0.05. All data are presented as the mean ± standard error of the mean of three individual experiments.

**Supplementary material**

Supplementary material is available at *Journal of Molecular Cell Biology* online.

**Funding**

This work was supported by National Institutes of Health (CA152313 to J.L.L.) and the Department of Energy Office of Science (DE-SC0001271 to G.W., J.L.L., D.J.G.).

**Conflict of interest:** none declared.

**References**


