Manganese superoxide dismutase is a mitochondrial fidelity protein that protects Polγ against UV-induced inactivation

Vasudevan Bakthavatchalu, M.V.Sc[1], **Swatee Dey, Ph.D.**[2], **Yong Xu, Ph.D.**[1], **Teresa Noel, B.S.**[1], **Paiboon Jungsuwadee, Ph.D.**[1,3], **Aaron K. Holley, Ph.D.**[1], **Sanjit K. Dhar, Ph.D.**[1], **Ines Batinic-Haberle, Ph.D.**[4], and **Daret K. St Clair, Ph.D.**[1,*]

[1]Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40536

[2]Proctor & Gamble, Cincinnati, OH 45202

[3]Department of Pharmacology and Toxicology, Husson University, Bangor, ME 04401

[4]Duke University Medical Center, Durham, NC 27705

Abstract

Manganese superoxide dismutase is a nuclear encoded primary antioxidant enzyme localized exclusively in the mitochondrial matrix. Genotoxic agents, such as UV radiation, generates oxidative stress and cause mitochondrial DNA (mtDNA) damage. The mitochondrial DNA polymerase (Polγ), a major constituent of nucleoids, is responsible for the replication and repair of the mitochondrial genome. Recent studies suggest that mitochondria contain fidelity proteins and MnSOD constitutes an integral part of the nucleoid complex. However, it is not known whether or how MnSOD participates in the mitochondrial repair processes. Using skin tissue from C57/BL6 mice exposed to UVB radiation, we demonstrate that MnSOD plays a critical role in preventing mtDNA damage by protecting the function of Polγ. Q-PCR analysis shows an increase in mtDNA damage after UVB exposure. Immunofluorescence and immunoblotting studies demonstrate p53 translocation to mitochondria and interaction with Polγ after UVB exposure. The mtDNA immunoprecipitation assay with Polγ and p53 antibodies in $p53^{+/+}$ and $p53^{-/-}$ mice demonstrates an interaction between MnSOD, p53, and Polγ. The results suggest that these proteins form a complex for the repair of UVB-associated mtDNA damage. The data also demonstrate that UVB exposure injures the mtDNA D-loop in a p53-dependent manner. Using MnSOD-deficient mice we demonstrate that UVB-induced mtDNA damage is MnSOD-dependent. Exposure to UVB results in nitration and inactivation of Polγ, which is prevented by addition of the MnSOD mimetic $Mn^{III}TE-2-PyP^{5+}$. These results demonstrate for the first time that MnSOD is a fidelity protein that maintains the activity of Polγ by preventing UVB-induced nitration and inactivation of Polγ. The data also demonstrate that MnSOD plays a role along with p53 to prevent mtDNA damage.

*Address correspondence to: Daret K. St. Clair, Ph.D., Graduate Center for Toxicology, University of Kentucky, 1095 VA Drive, HSRB 454, Lexington, KY 40536-0298, Phone: (859) 257-3720, FAX: (859) 323-1059, dstcl00@uky.edu.

Conflict of Interest

The authors declare no conflict of interest.

Introduction

Ultraviolet (UV) radiation is a pro-oxidant and carcinogen that induces oxidative stress and DNA damage (Aitken *et al.,* 2007, Bickers and Athar 2006). UV irradiation leads to increased stabilization and accumulation of tumor suppressor protein p53 in the skin. The main contributing factor to non-melanoma skin cancer is UVB-induced signature mutations in the p53 gene (Brash *et al.,* 1991, Hall *et al.,* 1993, Liu *et al.,* 1994). N-acetyl cysteine (NAC), superoxide dismutase, and catalase mimetic attenuate UVB-induced p53 stabilization without altering the transcriptional activation and cell cycle arrest functions of p53, suggesting a role for oxidative stress in UVB-induced p53 stabilization and accumulation (Decraene *et al.,* 2004, Renzing *et al.,* 1996). Increased cellular stress by ROS triggers p53 translocation to mitochondria, leading to apoptosis and mtDNA repair (Mihara *et al.,* 2003, Mihara and Moll 2003, Waster and Ollinger 2009, Zhao *et al.,* 2002, Zhao *et al.,* 2005).

mtDNA is organized in the inner mitochondrial membrane as nucleoids. The nucleoids consist of mtDNA-protein macromolecular complexes containing 2–8 mtDNA molecules associated with various proteins such as mitochondrial transcription factor A (mTFA), a mitochondrial single-strand DNA-binding protein (mtSSB) and Polγ (Chen and Butow 2005, Garrido *et al.,* 2003, Legros *et al.,* 2004). mtDNA is more susceptible to UV-induced damage than nuclear DNA is because it lacks histone and an elaborate repair system (Brown *et al.,* 1979, Shokolenko *et al.,* 2009, Yakes and Van Houten 1997).

Polγ is the only known polymerase enzyme responsible for replication and repair of mtDNA (Bogenhagen *et al.,* 2001, Bolden *et al.,* 1977, Hubscher *et al.,* 1979, Longley *et al.,* 1998, Stuart *et al.,* 2004). The Polγ holoenzyme is a heterotrimer consisting of 1 catalytic subunit and 2 accessory subunits (Carrodeguas *et al.,* 1999, Gray and Wong 1992, Yakubovskaya *et al.,* 2006). The Polγ catalytic subunit has polymerase and proof-reading activity for mtDNA replication, and dRP (5′-deoxyribose-5-phosphate) lyase activity for base excision repair. The accessory subunits bind nucleotide to mtDNA for faster replication, increased processivity and protection of the catalytic subunit from ROS-mediated oxidative damage (Johnson *et al.,* 2000). Polγ is susceptible to oxidative modifications due to the presence of 31 tyrosine residues in the catalytic subunit, including the two highly conserved tyrosine residues in the active site responsible for catalytic efficiency (Graziewicz *et al.,* 2002, Graziewicz *et al.,* 2004, Lewis *et al.,* 2006, Lim *et al.,* 2003, Van Goethem *et al.,* 2001).

UV irradiation triggers nitric oxide production in keratinocytes, which combines with superoxide to form the powerful oxidant peroxynitrite (Maglio *et al.,* 2005, Wu *et al.,* 2010). Inactivation of proteins by tyrosine nitration is regarded as a marker of nitrosative stress. The importance of nitration to protein structure or function depends on the location of tyrosine residues in the proteins; for example, its location in a loop or hydrophobic milieu such as the active site of an enzyme (Alvarez and Radi 2003).

ROS produced in mitochondria are detoxified by enzymatic and non-enzymatic antioxidant defense systems. The major constituents of the enzymatic system are MnSOD (Weisiger and Fridovich 1973), glutathione peroxidase (Esworthy *et al.,* 1997) and members of the thioredoxin family (Holmgren 1985). MnSOD forms the first line of defense against the superoxide produced in the mitochondria. Lack of MnSOD causes accumulation of oxidative mtDNA damage as well as inactivation of respiratory and Krebs cycle enzymes (Li *et al.,* 1995b, Melov *et al.,* 1999). Recent studies have identified MnSOD as a nucleoid complex component that may protect mtDNA and proteins associated with mtDNA from oxidative damage (Kienhofer *et al.,* 2009). Further MnSOD enzymatic activity is regulated by mitochondrial SIRT3 to maintain ROS levels and mitochondrial homeostasis (Ozden et al., 2011, Tao et al., 2010). We propose that epidermal cells use the following novel dual-step strategy to counteract a UVB insult involving tumor suppressor protein p53 and MnSOD 1) Damaged mtDNA recruit p53 to mitochondria where p53 might provide support to mitochondrial DNA polymerase Polγ in repairing the damaged mtDNA; and 2) MnSOD may act as a fidelity protein by interacting with Polγ and protecting it from oxidative stress-induced inactivation.

Results

UVB induces mtDNA damage in mouse skin

To assess mtDNA damage by UVB, we performed a time course study in which wild-type C57BL/6 mice were exposed to 5kJ/m^2 of UVB radiation. mtDNA isolated from skin at 1 and 24 hr after exposure to UVB was subjected to quantitative PCR analysis. The mtDNA damage was analyzed based on the difference in amplification due to blockage of the recombinant Thermus thermophilus (rTth) DNA polymerase by UVB-induced lesions. PCR negative control was used as a blank reference to eliminate background fluorescence. The relative amplification plot in Fig.1 shows a significant decrease in amplification of 10 kb mtDNA at 1 hr and 24 hr after UVB treatment compared to undamaged control template, indicating an increase in mtDNA damage. A quantitative analysis of lesions induced by UVB treatment in the 10 kb mtDNA is shown in Table 1. We normalized mtDNA copy number variation using fluorescence values from amplified short mtDNA fragments (117 bp), as there is a very low probability that UVB treatment will introduce a lesion in small mtDNA segments. This analysis indicates a significant increase in lesions at 1 hr and 24 hr after UVB treatment, suggesting mtDNA is highly prone to damage by UVB treatment.

UVB induces p53 translocation to mitochondria in mice epidermal cells

We have previously observed p53 translocation to mitochondria in a multistage skin cancer model using a known mutagenic chemical initiator, 7,12-dimethyl-benz(a)-anthracene (DMBA), followed by repetitive treatment with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is a known tumor promoter (Zhao *et al.,* 2002). It has also been shown that p53 translocated into mitochondria after exposure to a stress inducing agent including ionizing radiation (Vaseva and Moll 2009). To determine whether UVB-induced mtDNA damage triggers p53 translocation to mitochondria *in vivo*, skin mitochondrial fractions devoid of cytosolic and nuclear contamination were prepared from mouse skin by a sucrose density gradient ultracentrifugation process after UVB

treatment and subjected to immunoblot analysis. As shown in Fig. 2A & 2B, the p53 level increased significantly in both whole skin lysates and mitochondrial fractions at 1 hr and at 24 hr after UVB treatment. The absence of cytosolic and nuclear contamination was confirmed by immunoblotting of the mitochondrial fractions with antibodies against cytosolic and nuclear marker proteins IκBα and PCNA, respectively.

UVB enhances physical interaction of p53 with Polγ and mtDNA, and the role of p53 in Polγ and mtDNA interaction

Previous studies have shown that p53 can directly interact with Polγ and mtDNA and enhance Polγ repair efficiency (Achanta *et al.,* 2005, Bakhanashvili *et al.,* 2008, Heyne *et al.,* 2004). Here, we tested whether UVB could enhance p53 interaction with Polγ and mtDNA. Mitochondria were isolated from the skin of wild-type C57BL/6 mice exposed to $5kJ/m^2$ of UVB radiation and the interaction between p53 and Polγ was assessed by co-immunoprecipitation with an antibody specific for p53 with subsequent detection of p53 or Polγ by Western analysis. As shown in Fig. 3A, Polγ and p53 proteins pulled down from the mitochondrial lysates by both p53 and Polγ antibodies increased at 1 hr and 24 hr after UVB treatment. We also determined UVB-induced p53 and mtDNA interaction by isolating mtDNA from the skin of C57BL/6 wild-type mice exposed to $5kJ/m^2$ of UVB radiation. The mtDNA immunoprecipitated with p53 antibody were purified and amplified by real-time PCR with primers specific for mtDNA D-loop region to probe the relationship between mtDNA and Polγ function. As shown in Fig. 3B, the amplification of the D-loop region of mtDNA immunoprecipitated by p53 antibody increased significantly at 1 hr and 24 hr after UVB treatment. To verify the role of p53 in mtDNA repair by Polγ after UVB treatment, mtDNA immunoprecipitation was performed on $p53^{+/+}$ and $p53^{-/-}$ mice at 1 hr and 24 hr after UVB treatment. As shown in Fig. 3C, the amplification of the D-loop region of mtDNA immunoprecipitated by Polγ antibody in $p53^{+/+}$ mice increased significantly at 1 hr and 24 hr after UVB treatment. The amplification level of the D-loop in $p53^{-/-}$ mice was lower than that in the $p53^{+/+}$ mice and did not change significantly in response to UVB treatment. Thus, the significant increase in amplification of the D-loop region after UVB treatment was limited to the $p53^{+/+}$ genotypes, suggesting a role for p53 in the interaction of Polγ with mtDNA.

UVB induces physical interaction of MnSOD with p53 and Polγ, and the effect of MnSOD on Polγ and mtDNA

We have previously observed an interaction between p53 and MnSOD in the mitochondria of epidermal cells exposed to DMBA and TPA (Zhao *et al.,* 2002, Zhao *et al.,* 2005). To determine whether UVB causes p53 and MnSOD to interact in mitochondria, skin mitochondrial lysates from UVB-treated wild-type C57BL/6 mice were immunoprecipitated with p53 and MnSOD antibodies, respectively. As shown in Fig. 4A, the amount of MnSOD and p53 proteins pulled down from mitochondrial lysates by p53 and MnSOD antibodies increased at 1 hr and 24 hr after UVB treatment. Previous studies have demonstrated that MnSOD is an integral part of the nucleoid complex (Kienhofer *et al.,* 2009). Hence, we tested whether UVB induces MnSOD and Polγ interaction using co-immunoprecipitation. As shown in Fig. 4B, the amount of MnSOD and Polγ proteins pulled down from mitochondrial lysates by Polγ and MnSOD antibodies increased at 1 hr and 24 hr after UVB

treatment. We further used $p53^{+/+}$ and $p53^{-/-}$ mice to perform a co-immunoprecipitation study to determine whether UVB induces interaction between MnSOD and Polγ and whether it is dependent on p53. As shown in Fig. 4C, the amount of MnSOD and Polγ proteins pulled down from mitochondrial lysates by Polγ and MnSOD antibodies increased at 1 hr and 24 hr after UVB treatment in both genotypes. To assess the role of MnSOD in mtDNA and Polγ interaction after UVB treatment, mtDNA immunoprecipitation was performed on $MnSOD^{+/+}$ and $MnSOD^{+/-}$ mice. As shown in Fig. 4D, amplification of the D-loop region of mtDNA immunoprecipitated by the Polγ antibody resulted in more than 1.5 fold increase at 1 hr after UVB treatment in both genotypes, and when compared between the genotypes. Although the increase was less than 1.5 fold at 24 hr after UVB treatment in both genotypes, the increase was significant in the $MnSOD^{+/+}$ genotype at 24 hr after UVB treatment. The results suggest that MnSOD interacts with both p53 and Polγ, but the interaction between MnSOD and Polγ appears to be independent of p53. The results also show that MnSOD enhances the Polγ/mtDNA interaction 24 hr after UVB-irradiation, implying a role for MnSOD in maintaining Polγ activity.

MnSOD deficiency enhances UVB-mediated Polγ inactivation

To determine whether UVB treatment induces an alteration in Polγ protein level in the mitochondria of $MnSOD^{+/+}$ and $MnSOD^{+/-}$ mice, Western blotting for Polγ protein was performed using skin mitochondrial lysates from UVB- treated wild-type C57BL/6 mice. As shown in Fig. 5A, there was no apparent change in the Polγ protein level at 1 hr and 24 hr after UVB treatment in either genotype. UVB induces $OONO^-$ production in skin by the reaction of $NO^\bullet$ with $O_2^{\bullet-}$ which nitrates proteins and that Polγ protein undergoes oxidative damage-induced inactivation (Graziewicz *et al.,* 2002, Wu *et al.,* 2010). Polγ reverse transcriptase activity assay was performed using the skin mitochondrial lysates from UVB- treated $MnSOD^{+/+}$ and $MnSOD^{+/-}$ mice to determine whether UVB treatment results in a change in Polγ activity in the mitochondria. As shown in Fig. 5B, Polγ reverse transcriptase activity decreased significantly in $MnSOD^{+/-}$ genotype at 1 hr and 24 hr after UVB treatment compared to $MnSOD^{+/+}$ mice, where a significant decrease in activity was observed only at 24 hr after UVB treatment. To determine whether UVB-induced Polγ inactivation is associated with nitration, we performed a co-immunoprecipitation study using 3-nitrotyrosine antibody. As shown in Fig. 5C & 5D, the amount of Polγ protein pulled down from the mitochondrial lysates by 3-nitrotyrosine antibody significantly increased at 1 hr and 24 hr after UVB treatment in $MnSOD^{+/-}$ mice. There was a significant difference between the genotypes at 24 hr after UVB treatment, as shown in Fig. 5D. These results suggest MnSOD may be important in preventing nitration and subsequent inactivation of Polγ after UV exposure.

Mn^{III}TE-2-PyP^{5+} rescues Polγ from inactivation by UVB-mediated nitration

Previous studies have clearly established $Mn^{III}TE\text{-}2\text{-}PyP^{5+}$ as a potent $O_2^{\bullet-}$ and $OONO^-$ scavenger (Batinic-Haberle *et al.,* 2010, Batinic-Haberle 2010). To test if Polγ could be rescued from UV-induced inactivation, $MnSOD^{+/-}$ mice were pre-treated twice daily with $Mn^{III}TE\text{-}2\text{-}PyP^{5+}$ at 5mg/kg body weight in 250 μl volume before UVB exposure. Polγ reverse transcriptase activity assay was performed in skin mitochondrial lysates from saline and $Mn^{III}TE\text{-}2\text{-}PyP^{5+}$ treated $MnSOD^{+/-}$ mice after UVB exposure. As shown in Fig. 6A,

Polγ reverse transcriptase activity in Mn^{III}TE-2-PyP^{5+} pre-treated MnSOD$^{+/-}$ mice increased significantly at 24 hr after UVB treatment and compared with saline pre-treated MnSOD$^{+/-}$ mice. There was a significant decrease in activity in saline pre-treated MnSOD$^{+/-}$ mice at 24 hr after UVB treatment. To determine whether Mn^{III}TE-2-PyP^{5+} pre-treatment reduces UVB-mediated Polγ inactivation by nitration, co-immunoprecipitation was performed using 3-nitrotyrosine antibody. As shown in Fig. 6B, the amount of Polγ protein pulled down from the mitochondrial lysates by 3-nitrotyrosine antibody in saline pre-treated MnSOD$^{+/-}$ mice increased significantly at 1 hr and 24 hr after UVB treatment. There was no significant change in nitration levels in Mn^{III}TE-2-PyP^{5+} pre-treated MnSOD$^{+/-}$ mice. When compared with saline pre-treated MnSOD$^{+/-}$ mice, the decrease in nitration in Mn^{III}TE-2-PyP^{5+} pre-treatment was significant at 24 hr after UVB treatment, as shown in Fig. 6C.

Discussion

The present study demonstrates that UVB-induced mtDNA damage triggers p53 translocation to mitochondria, that mitochondrial p53 interacts with both mtDNA and Polγ after UVB exposure, and that the mtDNA/Polγ association is dependent on the availability of p53. These results support a previous report on the role of p53 in the mtDNA repair process (Achanta *et al.,* 2005, Bakhanashvili *et al.,* 2008). We have previously shown that oxidative stress induces p53 translocation to mitochondria and its subsequent interaction with MnSOD in a multistage chemical carcinogenesis model (Zhao *et al.,* 2002, Zhao *et al.,* 2005). The present study confirms these findings using UVB treatment to cause DNA damage, and extends to demonstrate that UVB treatment leads to increased interaction between MnSOD and Polγ and that the interaction between MnSOD and Polγ is p53 independent. These results suggest that MnSOD may participate in mtDNA repair processes by acting as a fidelity protein protecting the function of the major DNA repair enzyme in the mitochondria. This conclusion is supported by the findings that 1) MnSOD deficiency leads to a decrease in mtDNA-Polγ interaction and 2) inactivation of Polγ by nitration is prevented by treatment with Mn^{III}TE-2-PyP^{5+}, a potent peroxynitrite inhibitor. The findings of this study verify that MnSOD is an integral part of the nucleoid complex and extend to demonstrate that the antioxidant property of MnSOD serves to enhance the fidelity of mtDNA repair by salvaging the DNA polymerase (Polγ) in mitochondria from UV-induced peroxynitrite mediated inactivation.

Skin is the primary target of UVB radiation. UVB exposure has wide-ranging effects in skin, such as erythema and epidermal hyperplasia, which can eventually lead to photocarcinogenesis or photoaging. The underlying cellular processes for these conditions include DNA damage, apoptosis and oxidative stress. Both nuclear and mitochondrial DNA can be damaged by UVB radiation. Owing to its uniqueness, mtDNA has been used as a reliable biomarker for UV-induced DNA damage (Birch-machin *et al.,* 1998, Harbottle and Birch-Machin 2006, Krishnan *et al.,* 2004). Our Q-PCR analysis of mitochondrial genome indicates that UVB causes damage to mtDNA in mouse skin. The relative amplification ratio of the 10 kb mtDNA fragment at 1 hr and 24 hr after UVB treatment was significantly less than that of the reference control, which indicates the presence of DNA damage that blocks the amplification process. There were 0.15 lesions per 10 kb per strand at both the 1 hr and

24 hr treatments compared to the reference control. An earlier report that used Q-PCR to analyze UVB-exposed epidermal mtDNA from human skin shows deletions ranging from 4 to 10.5 kb in a 11.1 kb PCR fragment (Ray *et al.,* 2000). Thus, in our model UVB-induced mtDNA damage is being repaired.

UVB-induced oxidative stress and DNA damage stabilize p53 protein in the skin, leading to DNA repair in proliferating basal keratinocytes but cell cycle arrest and apoptosis in differentiated keratinocytes (Berg *et al.,* 1996, Li *et al.,* 1997, Ouhtit *et al.,* 2000, Renzing *et al.,* 1996, Tron *et al.,* 1998). UVB signature mutations in the p53 gene may lead to dysregulation of apoptosis and DNA repair, resulting in skin carcinogenesis (Li *et al.,* 1995a). Our results demonstrating p53-dependent UVB-induced mtDNA repair are consistent with these findings. The increased physical interaction of p53 with mtDNA and Polγ might be a p53 response to mtDNA damage after UVB treatment. A basal level of interaction between mtDNA and Polγ has been observed in both $p53^{+/+}$ and $p53^{-/-}$ genotypes. This interaction can be attributed to a p53-independent mtDNA replication process, since the very same basal level interaction between mtDNA and Polγ was observed 1 hr and 24 hr after UVB treatment in the $p53^{-/-}$ phenotype. In contrast, increased interaction between mtDNA and Polγ was observed 1 hr and 24 hr after UVB treatment in $p53^{+/+}$ mice. This increase from basal level can be attributed to mtDNA repair and indicates that the interaction requires p53. The presence of p53 enhances the accuracy of mtDNA repair by proof reading and by reducing the misincorporation of dNTP into mtDNA by Polγ (Bakhanashvili *et al.,* 2008). Recent studies have identified p53-regulated p53R2 as a subunit of ribonucleotide reductase (RNR), a rate-limiting enzyme in the denovo synthesis of deoxyribonucleotides (dNTPs) (Bourdon *et al.,* 2007, Kulawiec *et al.,* 2009, Lebedeva *et al.,* 2009). The UVB induced p53 stabilization triggers transcriptional up-regulation of p53R2, and increases output of dNTPs required for the UVB induced mtDNA repair process. This may be attributed to the increase in amplification of the D-loop region after UVB treatment observed in Fig. 3B.

The diverse functions of mitochondrial p53, which range from maintaining mtDNA integrity to apoptosis, led us to further explore our previous finding concerning MnSOD and p53 interaction in mitochondria. The physical interaction between p53 and MnSOD is observed with UVB treatment as was seen in chemical carcinogenesis studies. In light of new evidence that establishes MnSOD as an integral component of nucleoids (Kienhofer *et al.,* 2009), and our findings that a physical interaction occurs between MnSOD and Polγ after UVB treatment and that this interaction is p53-independent, these results suggest that MnSOD may enhance mtDNA stability by serving as a fidelity protein protecting the function of the key mitochondrial DNA repair enzyme. The interaction between MnSOD and Polγ and subsequent protection of Polγ from UVB-induced inactivation support a new role for mitochondrial antioxidant enzyme MnSOD in maintaining mtDNA fidelity. Our previous studies with MnSOD overexpressing and knockdown mice treated with DMBA/TPA have clearly established that MnSOD prevents protein oxidation in mitochondria (Zhao *et al.,* 2001, Zhao *et al.,* 2002). The decrease in the interaction between mtDNA and Polγ after UVB treatment in $MnSOD^{+/-}$ mice might be attributed to increased oxidative modification of Polγ. Oxidative stress in yeast due to a lack of MnSOD leads to

oxidation of various mitochondrial proteins such as aconitase, pyruvate, keto-acid dehydrogenase reductoisomerase, acetolactate synthase, α-ketoglutarate dehydrogenase, HSP60, glyceraldheyde-3-phosphate, and cytosolic fatty acid synthase (Cabiscol *et al.,* 2000, O'Brien *et al.,* 2004). The Polγ protein is known to be sensitive to oxidative modification when exposed to hydrogen peroxide (Graziewicz *et al.,* 2002). The Polγ exonuclease proof reading mutator mice have increased point mutations, mtDNA deletions, and increased apoptosis that leads to accelerated aging (Kujoth *et al.,* 2005, Trifunovic *et al.,* 2004, Vermulst *et al.,* 2008). $NO^{\bullet}$ and $O_2^{\bullet-}$ generated by UVB can result in $OONO^-$ production in skin, leading to nitration of proteins (Wu *et al.,* 2010). In this study we provide evidence for the first time that Polγ nitration and the consequent inactivation after UVB exposure are enhanced when MnSOD is deficient, and that pre-treatment of MnSOD with $Mn^{III}TE-2-PyP^{5+}$ results in decreased nitration and inactivation of Polγ.

In summary, our study shows that MnSOD plays an important role in protecting the fidelity of mtDNA against UVB-induced mtDNA damage in keratinocytes. We propose a model in Fig. 7 to illustrate a novel dual-step strategy adapted by keratinocytes in response to UVB insult that enhances the repair of mtDNA and protects the mtDNA repair enzyme from being inactivated. On one hand, UVB-induced mtDNA damage triggers p53 translocation to mitochondria. The mitochondrial p53 interacts with mtDNA and enhances the polymerase and exonuclease activities of Polγ, which plays a major role in the mtDNA repair process with its proof reading ability. On the other hand, mitochondrial antioxidant MnSOD plays a novel role by interacting with Polγ and protecting it from peroxynitrite-mediated inactivation. Circumstances that lead to increased oxidative stress by depleting MnSOD level and/or decrease MnSOD activity by acetylation can result in Polγ inactivation. Increasing MnSOD levels and/or activity by Sirt3 deacetylation enhances mitochondrial $O_2^{\bullet-}$ scavenging and reduces ROS-mediated insult on vital mitochondrial proteins such as Polγ. Additional studies aimed at identification of the specific tyrosine residues of Polγ that are nitrated after UVB exposure, tyrosine residues that are responsible for reduced Polγ activity and/or protected by MnSOD will provide further biochemical insight into the mechanisms by which the nuclear encoded, mitochondrial localized protein, MnSOD, serves to maintain the fidelity of the mtDNA and will establish a nuclear mitochondrial feed forward loop in cellular adaptive responses against oxidative stress mediated mitochondrial DNA instability.

Material and Methods

Materials

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) with exception of the following: protease inhibitor set III from Calbiochem (La Jolla, CA), dithiothreitol from BioRad (Carlsbad, CA), rabbit poly-clonal anti-MnSOD from Upstate Technology (Lake Placid, NY), Protein A/G agarose from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit poly-clonal anti-nitrotyrosine antibody from Cayman Chemical (Ann Arbor, MI), rabbit poly-clonal anti-Polγ antibody from Pierce Biotechnology (Rockford, IL), Monoclonal p53 antibody (DO-1) from Santa Cruz Biotechnology (Santa Cruz, CA),

poly(rA).oligo(dT)$_{12-18}$ from Midland Certified Reagent Company (Midland, TX), and DNase I from New England Biolabs Inc. (Ipswich, MA).

Animal studies

Heterozygous MnSOD knockdown mice (MnSOD$^{+/-}$) (Li *et al.,* 1995b, Van Remmen *et al.,* 1999) and p53 knock-out mice (p53$^{-/-}$) (Jacks *et al.,* 1994) were generated and genotyped as described. The animal experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

UV exposure

Depilated mice in resting phase of hair cycle and JB6 cells were exposed to ultraviolet irradiation in a Plexiglass cabinet (Plastic Design Corporation, MA). A single dose of 5kJ/m^2 (mice) or 400mJ/cm^2 (JB6 cells) was delivered by UVB lamps (Black light blue lamp, Sankyo Denkco Ltd., Japan). The UV emittance was measured using a UVB photometer radiometer (International Light Technologies, Peabody, MA) equipped with UVB measuring head.

Isolation of mitochondrial fraction from mouse skin tissue

The skin mitochondria were isolated as previously described (Zhao *et al.,* 2002).

mtDNA isolation and mtDNA damage analysis using quantitative PCR (Q-PCR)

The Q-PCR to analyze mtDNA damage using Pico-Green dye was performed as described previously (Kovalenko and Santos 2009). mtDNA was isolated from mouse skin tissue using a genomic DNA extraction kit (QIAGEN, Chatsworth, VA). The mtDNA and PCR products were quantified using the PicoGreen dsDNA Quantitation kit (Invitrogen Corp., Carlsbad, CA). The fluorescence values of PCR products from UVB-treated samples and the control samples were used to calculate relative amplification and lesion frequency.

Mitochondrial fractionation

The subcellular fractionation of enriched mitochondria was performed as described previously (Mihara and Moll 2003). Briefly, mouse skin was homogenized with a polytron homogenizer in calcium reticulocyte buffer (10mM Tris pH 7.6, 1.5 mM CaCl$_2$, 10 mM NaCl) and mixed with 2ml of ice-cold mannitol-sucrose buffer (210 mM mannitol, 70 mM sucrose, 5 mM EDTA, 5 mM Tris, pH 7.6). Two hundred microlitres of whole skin homogenate was stored at −20°C for further use. The remainder of the whole skin homogenate was centrifuged thrice at 1000 × g for 5 min at 4°C to clear nuclear, cytosolic and intact skin cell fraction. The mitochondria were isolated by discontinuous sucrose gradient centrifugation.

Co-Immunoprecipitation

Immunoprecipitation was performed as previously described (Bonifacino *et al.,* 2001). Briefly, the protein A/G-agarose beads (40 μL) were conjugated overnight to Polγ, MnSOD, p53 (DO-1), and 3-nitrotyrosine antibodies (3 μg) at 4°C in 0.5 mL ice-cold PBS. Appropriate control antibody was used as negative control. The mitochondrial pellets were

lysed in denaturing buffer 1% w/v sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 7.4, 10 mM dithiothreitol, 5 mM EDTA, 15 U/ml DNase I, and 1 μL/mL Protease inhibition by heat at 95°C for 5 min and the DNA was sheared. After denaturation and shearing, 250–500 μg of precleared mitochondrial protein were immunoprecipitated with antibody conjugated protein A/G-agarose beads with 10 μL of 10% BSA incubated overnight at 4°C in tube rotator. The immunoprecipitates were analyzed with 4–10% gradient gel.

mtDNA immunoprecipitation

The ChIP-IT system (Active Motif, Carlsbad, CA) was used to investigate the interaction of Polγ, MnSOD, p53 (DO-1) and mtDNA. Briefly, skin mtDNA exposed to UVB was immunoprecipitated with p53 and Polγ antibodies, and the mtDNA D-loop region was quantified by real-time PCR (LightCycler 480 Real-Time PCR System, Roche). The following primer sets were designed and used to amplify the mtDNA D-loop region: 5′-ACTATCCCCTTCCCCATTTG-3′ a n d 5′-TGTTGGTCATGGGCTGATTA-3′. Equal amounts of mtDNA from each treatment were used as input loading control, and mtDNA pulled down by IgG served as the negative antibody control.

Polγ reverse transcriptase activity assay

The RNA-dependent DNA polymerase activity of Polγ was measured as described previously (Longley and Copeland 2002, Taanman *et al.,* 2010). Briefly, the mitochondrial lysates prepared in extraction buffer (100 mM NaCl, 25 mM HEPES-KOH pH 8.0, 1% v/v Triton-X 100) were assayed at 37°C for 10 min in 50 μL reaction mixture with 10 μg of the mitochondrial protein, 25 mM HEPES-KOH pH 8.0, 0.5 mM $MnCl_2$, 100 mM NaCl, 2.5 mM β-mercaptoethanol, 50 μg/mL poly(rA).oligo(dT)$_{12–18,}$ 100 μg/mL acetylated bovine serum albumin (Ac-BSA), 0.1 mM aphidicolin, 500 μg/mL RNasin® RNase inhibitor (Promega, Madison, WI) and 5 μM [α-^{32}P]thymidine 5′-triphosphate (dTTP; specific activity: 5 Ci/mmol) (Amersham Corp., Piscataway, NJ). The reaction was stopped with 1.0 ml of stop solution (500 mM NaOH, 100 mM sodium pyro-phosphate, 0.1 mg/mL sonicated calf thymus DNA, 0.5 mg/ml BSA). The DNA precipitated with 20% TCA was filtered through GF/C filters and washed thrice with 1N HCl, rinsed with 95% ethanol and dried. The TCA-insoluble radioactivity was measured by liquid scintillation counting.

Treatment with Mn-based porphyrin

The Mn(III) meso-tetrakis(*N*-ethylpyridinium-2-yl) porphyrin (Mn^{III}TE-2-PyP^{5+}) was synthesized as previously described (Batinic-Haberle *et al.,* 1999, Ferrer-Sueta *et al.,* 1999). The mice were injected IP with 5 mg/kg of Mn^{III}TE-2-PyP^{5+} in saline twice daily for 2 days before UVB treatment.

Data analysis

The data are represented as mean ± SEM from replicate samples (n = 3), and were analyzed by one-way and two-way analyses of variance using Prism software (GraphPad, San Diego, CA). An α level of $P<0.05$ was considered significant.

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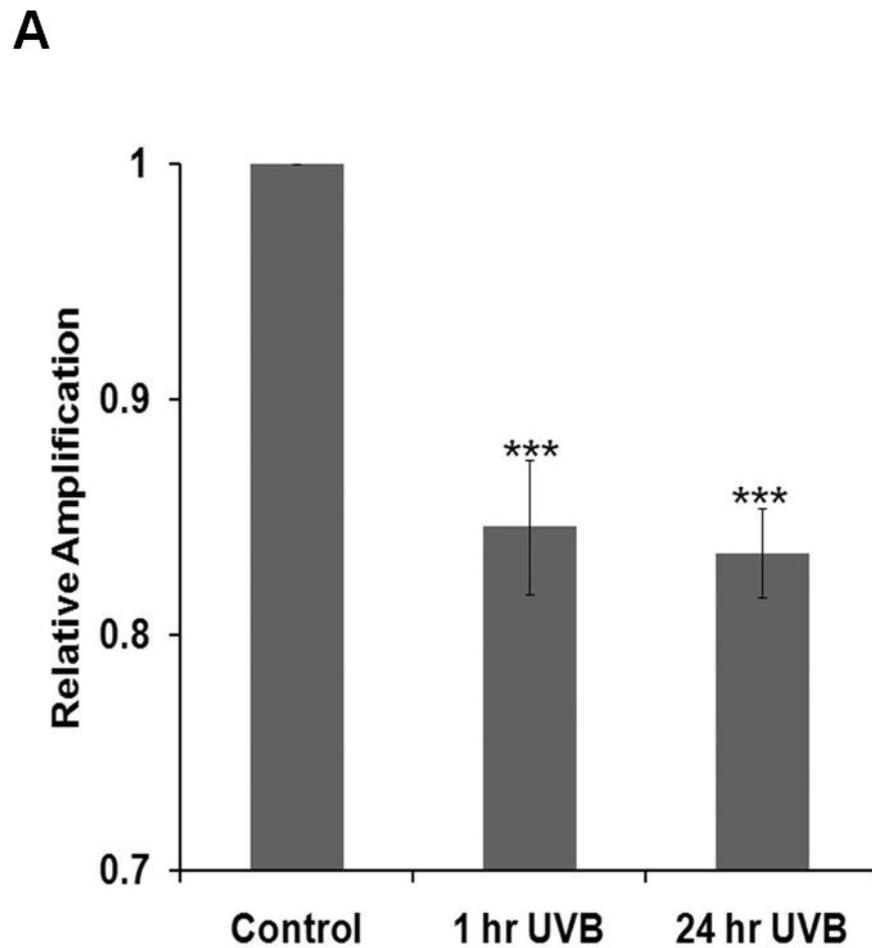
Figure 1. Quantification of mtDNA damage in mouse skin induced by UVB radiation
Wild-type C57BL/6 mice were exposed to 5kJ/m^2 of UVB radiation. mtDNA was isolated and analyzed with Q-PCR using primers specific for mouse mtDNA. The 10 kb mtDNA fragment was amplified and the blockage of rTth DNA polymerase amplification by damage in mtDNA was quantified in PCR products with Pico-Green dye. The relative amplification levels at 1 hr and 24 hr after UVB treatment were normalized with untreated control. ***P < 0.001 compared with control.
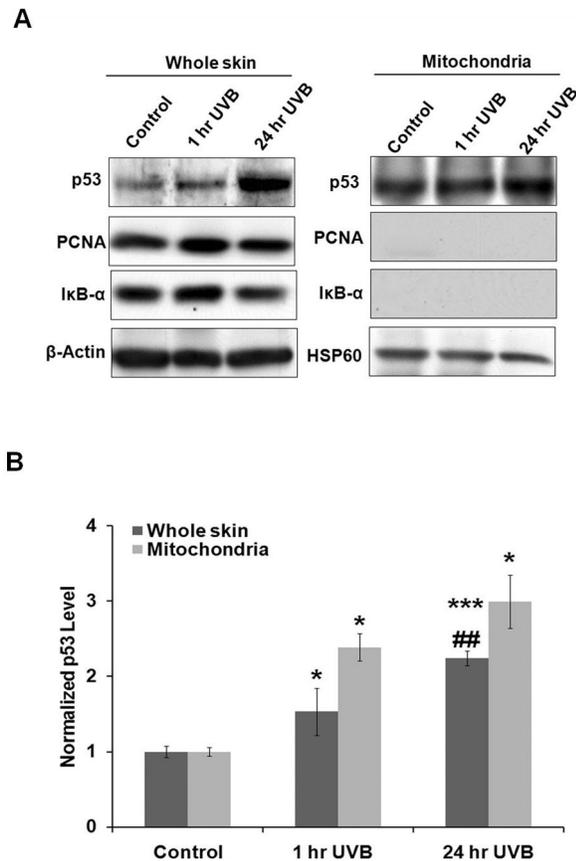
Figure 2. UVB enhances p53 mitochondrial translocation

(A) C57BL/6 mice were exposed to 5kJ/m^2 of UVB radiation. Whole skin tissue lysates and fractionated mitochondrial lysates were immunoblotted for p53 antibody (DO-1). Ponceau staining was used to confirm equal loading and uniform transfer of protein. Monoclonal anti-β-actin and anti-HSP60 antibodies were used as internal loading control. **(B)** The p53 accumulations in whole skin lysates and mitochondrial lysates at 1 hr and 24 hr after UVB exposure were quantified. Results were averaged from 3 sets of independent experiments. *P < 0.05, ***P < 0.001, compared with control, ##P < 0.01, comparison of 1 hr and 24 hr UVB treatment.
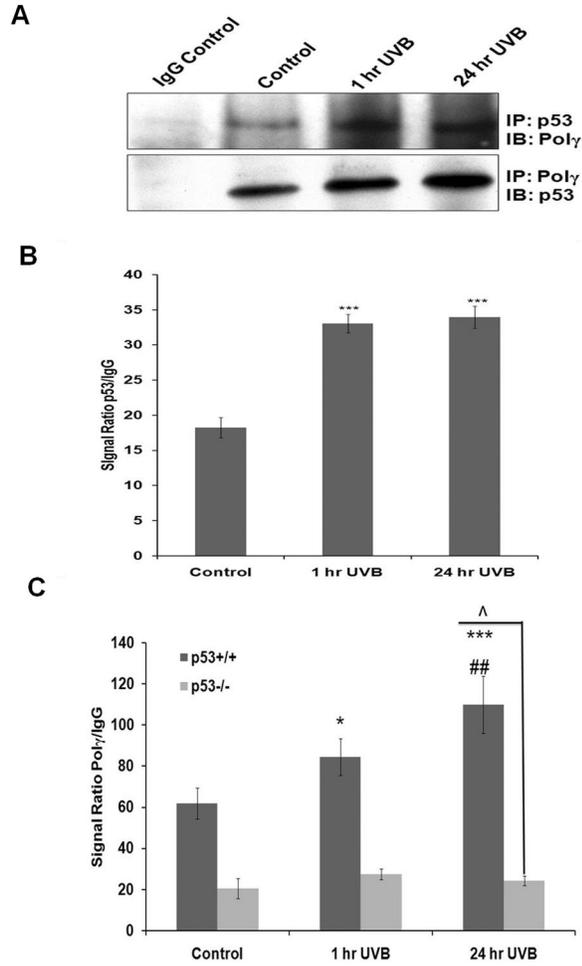
Figure 3. UVB-induced physical interaction of p53-Polγ-mtDNA

(**A**) Skin mitochondrial lysates from wild-type C57BL/6 mice exposed to 5kJ/m^2 of UVB radiation were immunoprecipitated with p53 (DO-1), Polγ antibodies, and control IgG. (**B**) mtDNA isolated from wild-type C57BL/6 mice skin exposed or sham exposed to 5 kJ/m^2 of UVB radiation was immunoprecipitated with p53 antibody (DO-1) and control IgG. The input mtDNA from control at 1 hr and 24 hr after UVB treatment was used as internal control. Real-Time PCR was used to amplify mtDNA D-loop region from input and immunoprecipitated mtDNA. ***$P < 0.001$. (**C**) mtDNA isolated from p53$^{+/+}$ and p53$^{-/-}$ mice skin exposed or not exposed to 5kJ/m^2 of UVB radiation was immunoprecipitated with Polγ antibody. Real-time PCR was used to amplify the immunoprecipitated mtDNA D-loop. *$P < 0.05$, ***$P < 0.001$, compared with control; ##$P < 0.01$, compared at 1 hr and 24 hr after UVB treatment; ^$P<0.05$, compared between p53$^{+/+}$ and p53$^{-/-}$ genotypes.
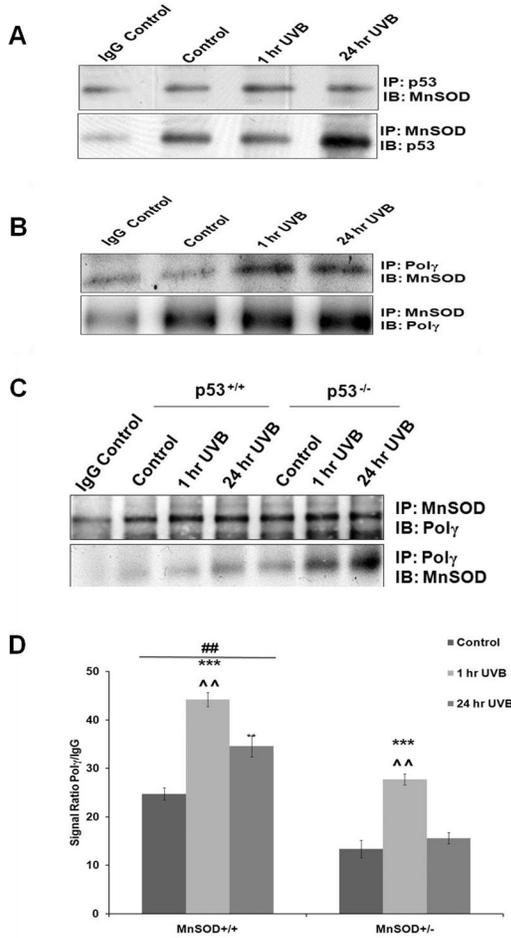
Figure 4. UVB-induced physical interaction of MnSOD-p53-Polγ

(A) Skin mitochondrial lysates from wild-type C57BL/6 mice exposed to 5kJ/m^2 of UVB radiation were immunoprecipitated with p53 (DO-1), MnSOD antibodies, and control IgG. Co-immunoprecipitated MnSOD and p53 were quantified by immunoblotting with specific antibodies. **(B)** Co-immunoprecipitation of Polγ and MnSOD. Mitochondrial lysates from mice skin exposed to UVB at 1 hr and 24 hr were immunoprecipitated with Polγ, MnSOD antibodies, and control IgG. Co-immunoprecipitated Polγ and MnSOD were quantified by immunoblotting with specific antibodies. **(C)** Mitochondrial lysates from p53$^{+/+}$ and p53$^{-/-}$ mice skin exposed to UVB were immunoprecipitated with Polγ, MnSOD antibodies and control IgG. Co-immunoprecipitated Polγ and MnSOD were quantified by immunoblotting with specific antibodies. **(D)** mtDNA isolated from MnSOD$^{+/+}$ and MnSOD$^{+/-}$ mice skin exposed to 5kJ/m^2 of UVB radiation was immunoprecipitated with Polγ antibody. Real-time PCR was used to amplify the precipitated mtDNA D-loop. ***$P < 0.001$, **$P < 0.01$, compared with control; ^^$P < 0.01$ compared at 1 hr and 24 hr after UVB treatment; ##$P < 0.01$ compared between MnSOD$^{+/+}$ and MnSOD$^{+/-}$ mice.
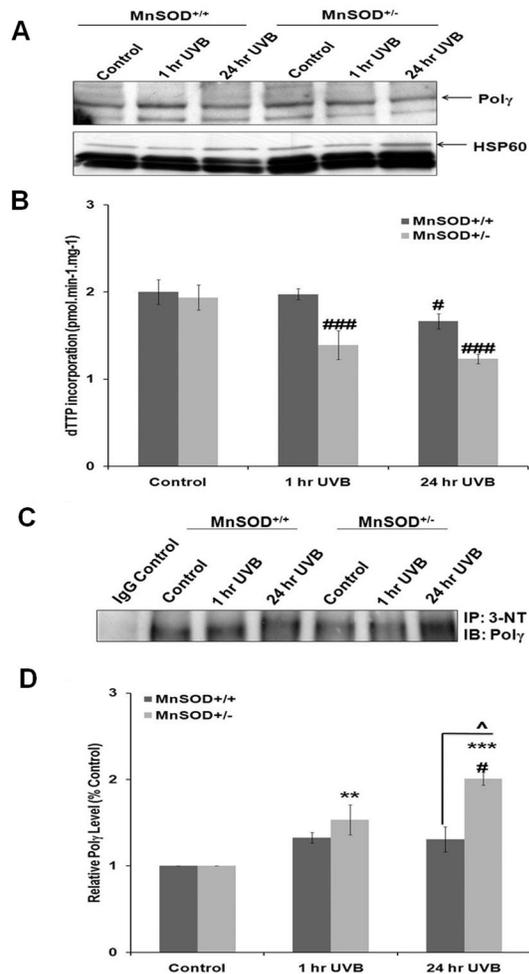
Figure 5. UVB induced Polγ inactivation by nitration

(**A**) Immunoblot analysis of Polγ protein levels in MnSOD^{+/+} and MnSOD^{+/−} mice skin mitochondria exposed to 5kJ/m^{2} of UVB radiation. (**B**) Polγ reverse transcriptase activity assay in MnSOD^{+/+} and MnSOD^{+/−} mice skin mitochondria exposed or not exposed to 5 kJ/m^{2} of UVB radiation #$P < 0.05$, ###$P < 0.001$ compared with control. (**C**) MnSOD^{+/+} and MnSOD^{+/−} mouse skin exposed to 5 kJ/m^{2} of UVB radiation was co-immunoprecipitated with 3-nitrotyrosine antibody. Co-immunoprecipitates were immunoblotted with Polγ antibody. (**D**) Quantification of Polγ co-immunoprecipitation by 3-nitrotyrosine antibody. **$P < 0.01$, ***$P<0.001$ compared with control; #$P < 0.05$ compared between 1 hr and 24 hr after UVB treatment; ^$P < 0.05$ compared between MnSOD^{+/+} and MnSOD^{+/−} mice.
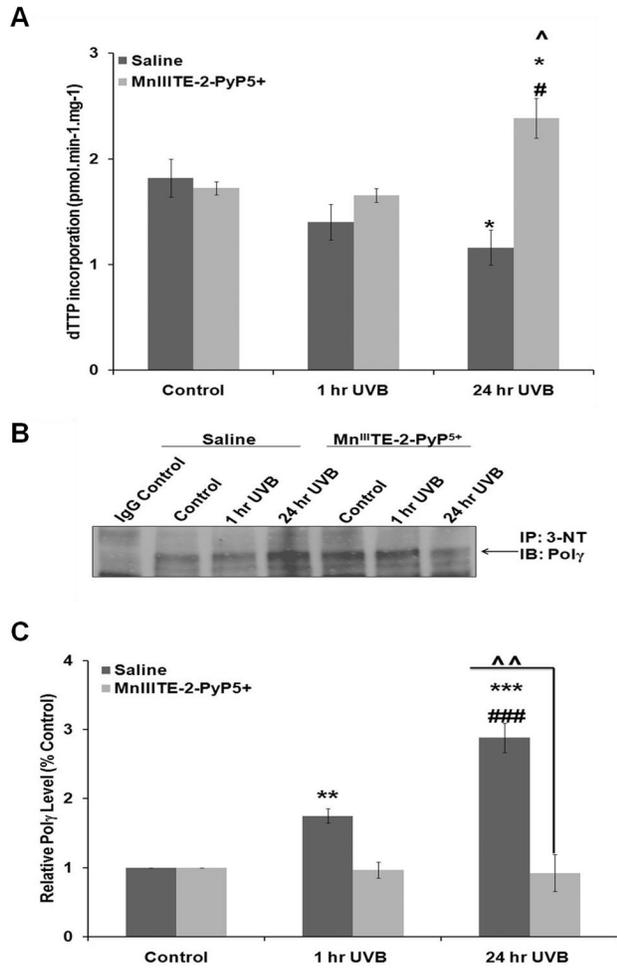
Figure 6. Mn^{III}TE-2-PyP^{5+} protects UVB induced Polγ inactivation by nitration in MnSOD$^{+/-}$ mice

(**A**) Polγ reverse transcriptase activity assay in Mn^{III}TE-2-PyP^{5+} and saline pre-treated MnSOD$^{+/-}$ mice skin mitochondria exposed to 5 kJ/m^2 of UVB radiation *P < 0.05 compared with control; #P < 0.05 compared between 1 hr and 24 hr after UVB treatment; ^P < 0.05 compared between Mn^{III}TE-2-PyP^{5+} and saline pre-treatment. (**B**) Mn^{III}TE-2-PyP^{5+} and saline pre-treated MnSOD$^{+/-}$ mice skin exposed to 5 kJ/m^2 of UVB radiation were co-immunoprecipitated with 3-nitrotyrosine antibody and immunoblotted with Polγ antibody. (**C**) Quantification of Polγ co-immunoprecipitation by anti-3-nitrotyrosine antibody in Mn^{III}TE-2-PyP^{5+} and saline pre-treated MnSOD$^{+/-}$ mice skin exposed to 5 kJ/m^2 of UVB radiation. **P < 0.01, ***P < 0.001 compared with control; ###P < 0.001 compared between 1 hr and 24 hr after UVB treatment; ^^P < 0.01 compared between Mn^{III}TE-2-PyP^{5+} and saline pre-treatment.
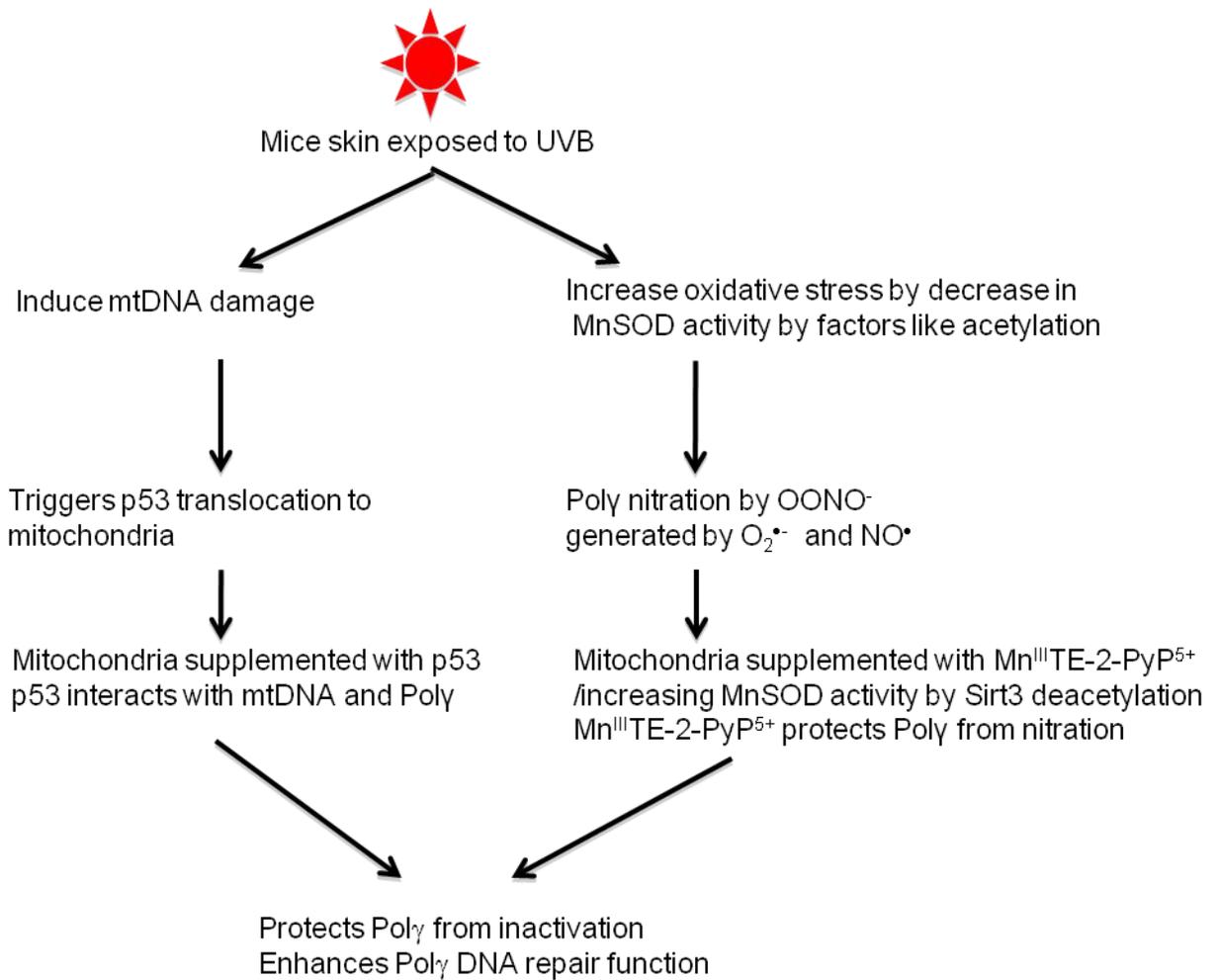
Figure 7.
Schematic illustration of novel dual-step strategy adapted by keratinocytes to survive UVB insult

Table 1

The number of lesions per 10 kb mtDNA at 1 hr and 24 hr after UVB treatment was quantified after normalizing with mitochondrial copy number

Quantitiative analysis of mtDNA lesions induced by UVB

	Control	1 hr UVB	24 hr UVB
Lesions/10kb	0	0.17 ± 0.03**	0.18 ± 0.002***

The data are presented as mean ± S.E. and n = 4,

**
P < 0.01,

***
P < 0.001 compared with control.