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RETRACTION

Retraction: Sirtuin-3 modulates Bak- and Bax-dependent apoptosis

Manish Verma, Nataly Shulga and John G. Pastorino

Retraction of: *J. Cell Sci.* (2013) **126**, 274-288

This article has been retracted at the request of the corresponding author, John G. Pastorino.

This notice updates and replaces a recent Expression of Concern, published on 15 February 2016.

Journal of Cell Science was alerted to potential blot duplication and reuse in the following five papers published in Journal of Cell Science by John G. Pastorino:

Sirtuin-3 deacetylation of cyclophilin D induces dissociation of hexokinase II from the mitochondria
Nataly Shulga, Robin Wilson-Smith and John G. Pastorino
J. Cell. Sci. (2010) **123**, 894-902

Ethanol sensitizes mitochondria to the permeability transition by inhibiting deacetylation of cyclophilin-D mediated by sirtuin-3
Nataly Shulga and John G. Pastorino
J. Cell. Sci. (2010) **123**, 4117-4127

GRIM-19-mediated translocation of STAT3 to mitochondria is necessary for TNF-induced necroptosis
Nataly Shulga and John G. Pastorino
J. Cell. Sci. (2012) **125**, 2995-3003

Sirtuin-3 modulates Bak- and Bax-dependent apoptosis
Manish Verma, Nataly Shulga and John G. Pastorino
J. Cell. Sci. (2013) **126**, 274-288

Mitoneet mediates TNFα-induced necroptosis promoted by exposure to fructose and ethanol
Nataly Shulga and John G. Pastorino
J. Cell. Sci. (2014) **127**, 896-907

These concerns were relayed to Dr Pastorino, the corresponding author, who responded with an explanation and original data. Following review of these data, we felt unable to resolve this matter at a distance, so contacted the authors' institution (Rowan University) and requested that they investigate further.

Following their assessment, Rowan University required that Dr Pastorino retract all of the above named papers published in Journal of Cell Science. Dr Pastorino also entered a Voluntary Exclusion Agreement with The Office of Research Integrity (ORI); the agreement can be found here: http://ori.hhs.gov/content/case-summary-pastorino-john-g.

ORI found that Dr Pastorino intentionally falsified and/or fabricated data and, specifically, that he "duplicated images, or trimmed and/or manipulated blot images from unrelated sources to obscure their origin, and relabelled them to represent different experimental results in:"

- Figures 2A,C; 3B; 5A; 7B; 8A in *J. Cell. Sci.* (2010a), **123**, 894-902.
- Figures 2B; 5A; 6A,B in *J. Cell. Sci.* (2010b), **123**, 4117-4127.
- Figures 1A; 2A,B; 4C; 5A,B; 6A; 7A–C in *J. Cell. Sci.* (2012) **125**, 2995-3003.
- Figures 4F; 5H; 6A in *J. Cell. Sci.* (2013) **126**, 274-288.
- Figures 1B; 2B,C; 3A,B; 4D in *J. Cell. Sci.* (2014) **127**, 896-907.

Sirtuin-3 modulates Bak- and Bax-dependent apoptosis

Manish Verma, Nataly Shulga and John G. Pastorino*

Department of Molecular Biology, School of Osteopathic Medicine, University of Medicine and Dentistry of New Jersey, Stratford, NJ 08084, USA

*Author for correspondence (pastorjg@umdnj.edu)

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Summary

Sirtuin-3 exhibits properties of a tumor suppressor partly emanating from its ability to control the rate of mitochondrial metabolism, with depletion of sirt-3 increasing tumor cell survival. In the present study we demonstrate that depletion of sirtuin-3 brings about an anti-apoptotic phenotype via stimulating cyclophilin-D activity, which promotes the binding of hexokinase II to the mitochondria, thereby preventing Bak/Bax dependent mitochondrial injury and cell death. By contrast, increased expression of sirtuin-3 decreases cyclophilin-D activity, resulting in detachment of hexokinase II from the mitochondria and potentiation of Bak- and Bax-induced mitochondrial injury and loss of cell viability.

Key words: Sirtuin-3, Mitochondria, Hexokinase-2, Bak, Bax, Apoptosis, Cisplatin

Introduction

The mitochondrial deacetylase, sirtuin-3, controls multiple aspects of mitochondrial function. Sirtuin-3 deacetylates a number of mitochondrial matrix proteins including constituents of the respiratory chain complexes and anti-oxidant effectors such as isocitrate dehydrogenase-2 (IDH2) and mitochondrial manganese superoxide dismutase (MnSOD) (Ahn et al., 2008; Tao et al., 2010; Yu et al., 2010). The increased acetylation of respiratory chain components by depletion of sirt-3 brings about a decrease in oxidative phosphorylation. Similarly, the increased acetylation of MnSOD and IDH2 in sirt-3 depleted cells inhibits their activity as anti-oxidants. These two effects of sirt-3 depletion conspire to elevate ROS levels. The increased ROS is thought to promote tumorigenesis in part by generating genomic instability (Kim et al., 2010). Additionally, once transformed, the stimulation of ROS due to sirt-3 depletion helps to promote glycolysis, a characteristic of transformed cells, by increasing the stability of hypoxia inducible factor-1α (HIF-1α) (Bell et al., 2011; Schumacker, 2011). Elevated levels of HIF-1α bring about increased expression of glycolytic enzymes including hexokinase II, which is a rate limiting step of glycolysis (Marín-Hernández et al., 2009; Mathupala et al., 2001; Riddle et al., 2000). Moreover, suppression of sirtuin-3 expression also promotes the binding of hexokinase II to the mitochondria by stimulating the activity of cyclophilin-D (Shulga et al. 2010).

Cyclophilin-D is localized to the mitochondrial matrix and is best known as a positive modulator of the mitochondrial permeability transition pore (Baines et al., 2005; Basso et al., 2005). Onset of the permeability transition is a critical event in necrotic cell death, with knock-down of cyclophilin-D preventing necrosis. As in necrosis, mitochondrial injury is also a central mediator of apoptotic cell death, but with the mechanisms differing vastly. In contrast to necrosis some studies indicate that cyclophilin-D prevents apoptotic cell death (Li et al., 2004; Lin and Lechleiter, 2002; Schubert and Grimm, 2004). The duality of cyclophilin-D effects in necrosis versus apoptosis may lie in the ability of cyclophilin-D to modulate the binding of hexokinase II to the mitochondria (Machida et al., 2006). We and others have demonstrated that mitochondrial bound hexokinase II prevents mitochondrial injury during apoptosis by inhibiting the ability of pro-apoptotic proteins Bak and Bax from disrupting the integrity of the outer mitochondrial membrane (Gall et al., 2011; Majewski et al., 2004a; Majewski et al., 2004b; Pastorino et al., 2002). In the present report we demonstrate that depletion of sirt-3 promotes tumor cell survival via a cyclophilin-D dependent pathway; through stabilizing the binding of hexokinase II to the mitochondria, thereby preventing Bak/Bax induced mitochondrial injury and cell death.

Results

Sirt-3 modulates loss of cell viability induced by Mcl-1 and Bcl-X_L depletion

Mcl-1 and Bcl-X_L are critical anti-apoptotic proteins localized to the outer mitochondrial membrane. As shown in Fig. 1A, siRNAs targeting Mcl-1 or Bcl-X_L are able to selectively suppress their respective targets, with tandem transfection suppressing expression of both Mcl-1 and Bcl-X_L. Fig. 1B demonstrates that downregulation of Mcl-1 and Bcl-X_L separately gave little loss of cell viability as assessed by Yo-Pro-1 staining, which selectively stains apoptotic cells (Boffa et al., 2005; Idziorek et al., 1995). However, the tandem suppression of Mcl-1 and Bcl-X_L expression resulted in 78% of the cell undergoing apoptosis following 48 hours (Fig. 1B). These results agree with previous studies indicating that suppression of both Mcl-1 and Bcl-X_L are required to induce mitochondrial injury and subsequent apoptosis. Bak and Bax are pro-apoptotic proteins localized to the outer mitochondrial membrane and kept in a tonic state of inhibition by interaction with Mcl-1 and Bcl-X_L. We wanted to determine if cell death induced by depletion of Mcl-1 and Bcl-X_L was dependent on Bak/Bax. As shown in Fig. 1C, left panel, siRNAs targeting Bak and Bax are able to selectively suppress the expression of their respective targets, with tandem transfection lowering the levels of both Bak and Bax. Significantly, concomitant suppression of Bak and Bax expression prevented induction of cell death induced by Mcl-1 and Bcl-X_L depletion, with only an 18% loss of cell viability after

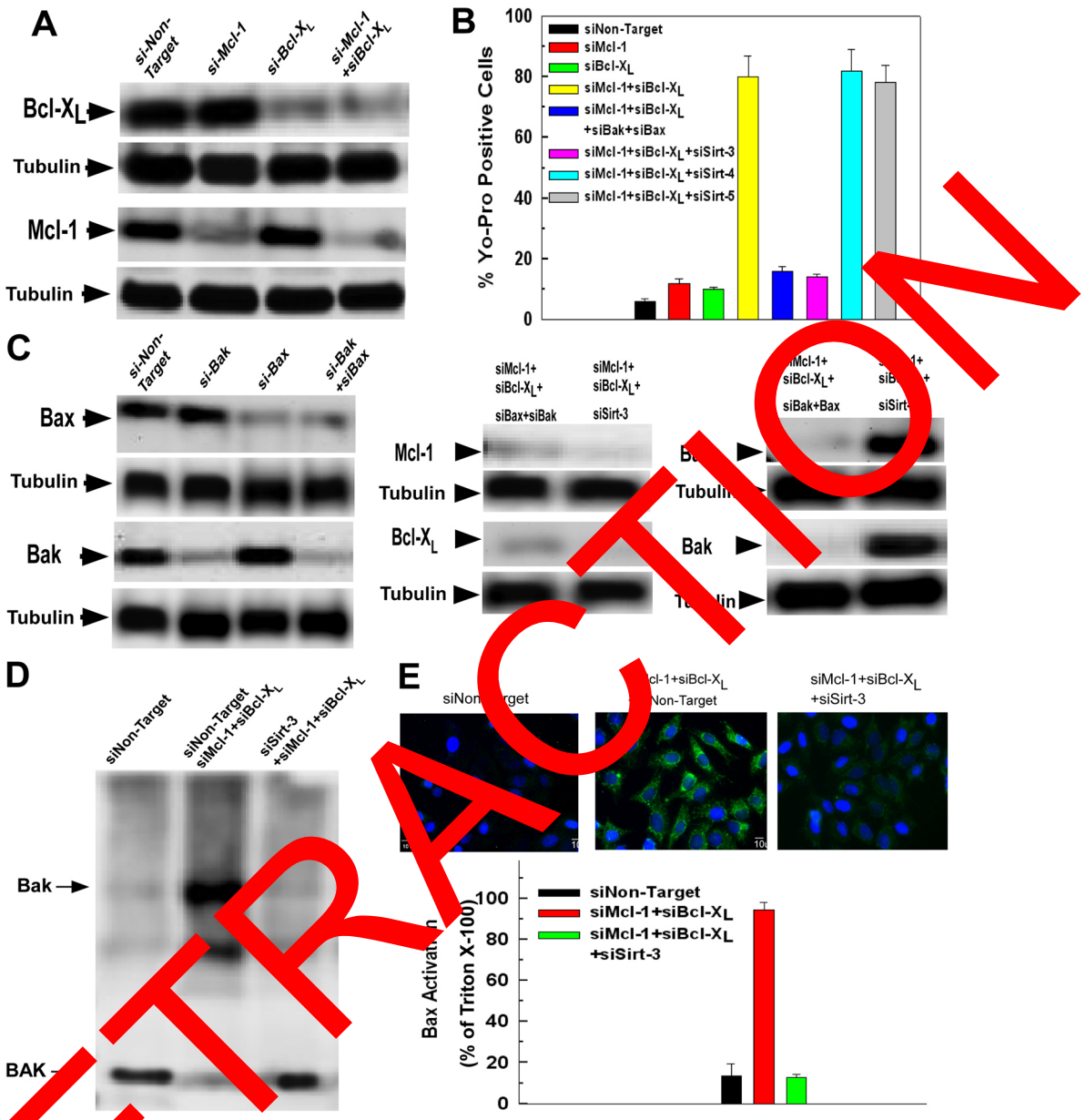

Fig. 1. Sirt-3 modu[lates] Bak[/Ba]x dependent apoptosis induced by Mcl-1 and Bcl-X_L depletion. (**A**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA[/]NAs targeti[ng] Mcl-1 or Bcl-X_L, either alone or in tandem. Following 24 hours incubation, the cells were harvested and the levels of Mcl-1, Bcl[-X_L] tubulin[/] were determ[ined] by western blotting. The results are typical of three independent experiments. (**B**) HeLa cells were transfected with 50 nM [of] the indic[ated] siRNA[s] individually or in tandem. Following 48 hours, the cells were harvested and the degree of apoptosis determined by staining with [Yo]-Pro-1 as [de]scribed in M[at]erials and Methods. Values are the means of three independent experiments with the error bars indicating standard deviations. (**C**) [He]a cells [were] transfected with 50 nM of a non-targeting control siRNA or siRNAs targeting Bak, Bax, sirtuin-3, Mcl-1 or Bcl-X_L, either alone or in tandem. Follow[ing] 24 hours incubation, the cells were harvested and the levels of Bak, Bax, Mcl-1, Bcl-X_L and tubulin were determined by western blotting. The results are typical [of] three independent experiments. (**D**) HeLa cells were transfected with 50 nM of the indicated siRNAs. Following 24 hours incubation, the cells from four wells we[re] harvested and the mitochondrial fraction isolated. The isolated mitochondria were incubated with 0.1 mM of the cross-linking reagent BMH for 30 minutes. The[n] samples were then run out on 12% SDS-PAGE gels and Bak oligomerization was assessed by western blotting. (**E**) HeLa cells were plated on 12 mm coverslips (Fisher Scientific) at 5.0×10^4 cells and allowed to attach overnight. The next day, cells were transfected with the indicated siRNAs. Following 24 hours incubation, the cells were washed twice with PBS, fixed and immunostained with anti-Bax clone 6A7 as described in Materials and Methods. Quantification was done by drawing a region of interest around each cell in the acquired images (200–250 cells). The intensity was determined by SlideBook and is expressed as the percentage of Bax immunostaining induced by Triton X-100.

48 hours (Fig. 1B). Importantly, as shown in Fig. 1C, middle panel, lane 1, simultaneous transfection with siRNAs targeting Bak/Bax did not interfere with the ability of siRNAs to target and deplete Mcl-1 and Bcl-X_L.

Sirtuin-3 (sirt-3) has been proposed to be a tumor suppressor, whose depletion enhances cancer cell survival. We wanted to determine if sirt-3 expression had any effect on the Bak/Bax dependent cell death brought about by depletion of Mcl-1 and

Bcl-X_L. As shown in Fig. 1B, concurrent transfection with siRNA targeting sirt-3 prevented the loss of cell viability brought about by depletion of Mcl-1 and Bcl-X_L to the same degree as did suppression of Bak/Bax expression. Importantly, depletion of sirt-3 had no effect on the ability to suppress expression of Mcl-1 and Bcl-X_L when utilizing their respective siRNAs (Fig. 1C, middle panel, lane 2). Intriguingly, the protective effect exerted by suppression of sirt-3 is not mediated by depletion of Bak or Bax. As demonstrated in Fig. 1C, right panel, lane no. 2, transfection with siRNAs targeting sirt-3 in combination with siRNAs against Mcl-1 and Bcl-X_L had no effect on the level of Bak or Bax expression. In contrast to sirt-3, suppression of sirtuin-4 or sirtuin-5 expression, both localized to the mitochondria, did not prevent cell death induced by Mcl-1 and Bcl-X_L depletion (Fig. 1B).

Depletion of sirt-3 also prevented Bak oligomerization. When unrestrained by Mcl-1 or Bcl-X_L, Bak undergoes activation and oligomerization. As show in Fig. 1D, lane 2, depletion of Mcl-1 and Bcl-X_L induced Bak oligomerization that was not prevented by concomitant transfection with a non-targeting siRNA, but was prevented by depletion of sirt-3 (lane 3). Similarly, when activated, Bax undergoes a conformational alteration that exposes it N-terminus, making it detectable utilizing the Bax6A7 antibody. As shown by immunostaining and quantification, cells transfected with non-targeting control siRNA displayed minimal Bax activation (Fig. 1E, left micrograph). However, there was robust immunostaining 24 hours following transfection with siRNAs targeting Mcl-1 and Bcl-X_L (Fig. 1E, middle micrograph). Importantly, like Bak activation, depletion of sirtuin-3 also inhibited the activation of Bax induced by suppression of Mcl-1 and Bcl-X_L expression (Fig. 1E, right micrograph).

Bak/Bax induced mitochondrial damage brings about an increase in the generation of reactive oxygen species (ROS) and eventual loss of mitochondrial membrane potential. As shown in Fig. 2A,B, following 24 hours of incubation, simultaneous suppression of Mcl-1 and Bcl-X_L expression provoked a reduction of mitochondrial membrane potential and stimulation of ROS production that was prevented by suppression of Bak/Bax expression. Importantly, depletion of sirt-3 also prevented the loss of mitochondrial membrane potential and stimulation of ROS production induced by suppression of Mcl-1 and Bcl-X_L to the same extent as did Bak/Bax depletion. Mitotracker-Green labels mitochondria independent of membrane potential, making it useful to determine if there are any effects of Mcl-1, Bcl-X_L or sirt-3 depletion on mitochondrial mass. As shown in Fig. 2A, there were no changes to Mito-tracker Green labeling in any of the conditions studied, suggesting that the time frames under scrutiny, there are no alterations in mitochondrial mass. Also, in supplementary material Fig. S1, depletion of sirt-3 is shown to have no effect on expression of UQCRC1, a core component of complex III of the mitochondrial respiratory complex. Also, suppression of Mcl-1 and Bcl-X_L did not alter levels of UQCRC1 in the presence or absence of sirt-3, indicating that there is no discernible effect on mitochondrial biogenesis in the time frame under study.

Downstream effects of mitochondrial injury during the progression of apoptosis include caspase-3 activation and externalization of phosphatidylserine (PS) on the exterior leaflet of the plasma membrane. As shown in Fig. 2C, as assessed by cleavage of a fluorometric caspase substrate (DEVD-Nunc View 488), 36 hours following transfection suppression of Mcl-1 and Bcl-X_L brings about caspase activation that is inhibited by depletion of Bak/Bax. Importantly, depletion of sirt-3 also prevented cleavage of DEVD-NuncView 488 brought about by suppression of Mcl-1 and Bcl-X_L expression (Fig. 2C). Similarly, the endogenous caspase-3 substrate, PARP, was cleaved and degraded by suppression of Mcl-1 and Bcl-X_L expression, which in turn was prevented by a caspase-3 inhibitor or depletion of sirt-3 (Fig. 2C, inset). Externalization of PS on the exterior leaflet of the plasma membrane promotes phagocytosis and is utilized as a marker for apoptosis. As assessed by annexin V binding, suppression of Mcl-1 and Bcl-X_L induced externalization of PS on the plasma membrane, which was prevented by depletion of Bak/Bax or sirt-3 (Fig. 2D). These above results indicate that by preventing the proximal activation of Bak/Bax, depletion of sirt-3 prevents the mitochondrial injury mediated by suppression of Mcl-1 and Bcl-X_L expression and the resultant onset of downstream apoptotic events such as depolarization of the mitochondria, mitochondrial ROS generation, caspase activation, PS externalization and eventual loss of viability.

We next wanted to determine if the results that we obtained with HeLa cells were applicable to other cancer cell line with a high glycolytic rate. Cells of the breast cancer cell line, MDA-MB-231, also express a high level of hexokinase II (Furtado et al., 2012; Zaoli et al., 2010). As shown in Fig. 3A, simultaneous suppression of Mcl-1 and Bcl-X_L in the presence of non-targeting siRNA induced an 80% and 60% increase in the number of cells staining positive for Yo-Pro or annexin V, respectively. Suppression of Bak and Bax largely suppressed the loss of cell viability induced by depletion of Mcl-1 and Bcl-X_L. Importantly, as in HeLa cells, suppression of sirt-3 expression prevented the loss of cell viability brought about by depletion of Mcl-1 and Bcl-X_L to the same degree as suppression of Bak/Bax expression. As shown in Fig. 3B,C, the loss of cell viability induced by depletion of Mcl-1 and Bcl-X_L in MDA-MB-231 cells was accompanied by depolarization of the mitochondrial membrane potential and increase of ROS formation as measured by TMRM and MitoSOX or DCF fluorescence, respectively. Both mitochondrial depolarization and ROS formation was prevented by depletion of Bak/Bax or sirt-3. Importantly, as shown in Fig. 3B, there were no changes in Mito-Tracker Green fluorescence, indicating that there are no alterations in the mitochondrial mass of the MDA-MB-231 cells under the conditions and time course under study. Accompanying the ability to preserve mitochondrial integrity, depletion of sirt-3 also prevented the activation of Bax induced by suppression of Mcl-1 and Bcl-X_L expression (Fig. 3D).

Hexokinase II mediates Sirt-3 control of Bak/Bax dependent mitochondrial injury

To more directly assess the mechanism(s) by which sirt-3 modulates mitochondrial injury, we selectively permeabilized the plasma membrane utilizing digitonin as described in Materials and Methods. As shown in Fig. 4A, over an 18-minute time course, as assessed by the retention of the potentiomeric fluorophore, tetramethyl-rhodamine methyl ester (TMRM), digitonin permeabilized cells maintain a relatively steady mitochondrial membrane potential. Upon addition of truncated Bid (t-Bid), a BH3 domain only pro-apoptotic protein, mitochondria underwent depolarization. However, when cells were depleted of Bak/Bax, mitochondria became insensitive to t-Bid induced depolarization; consistent with the notion that t-Bid

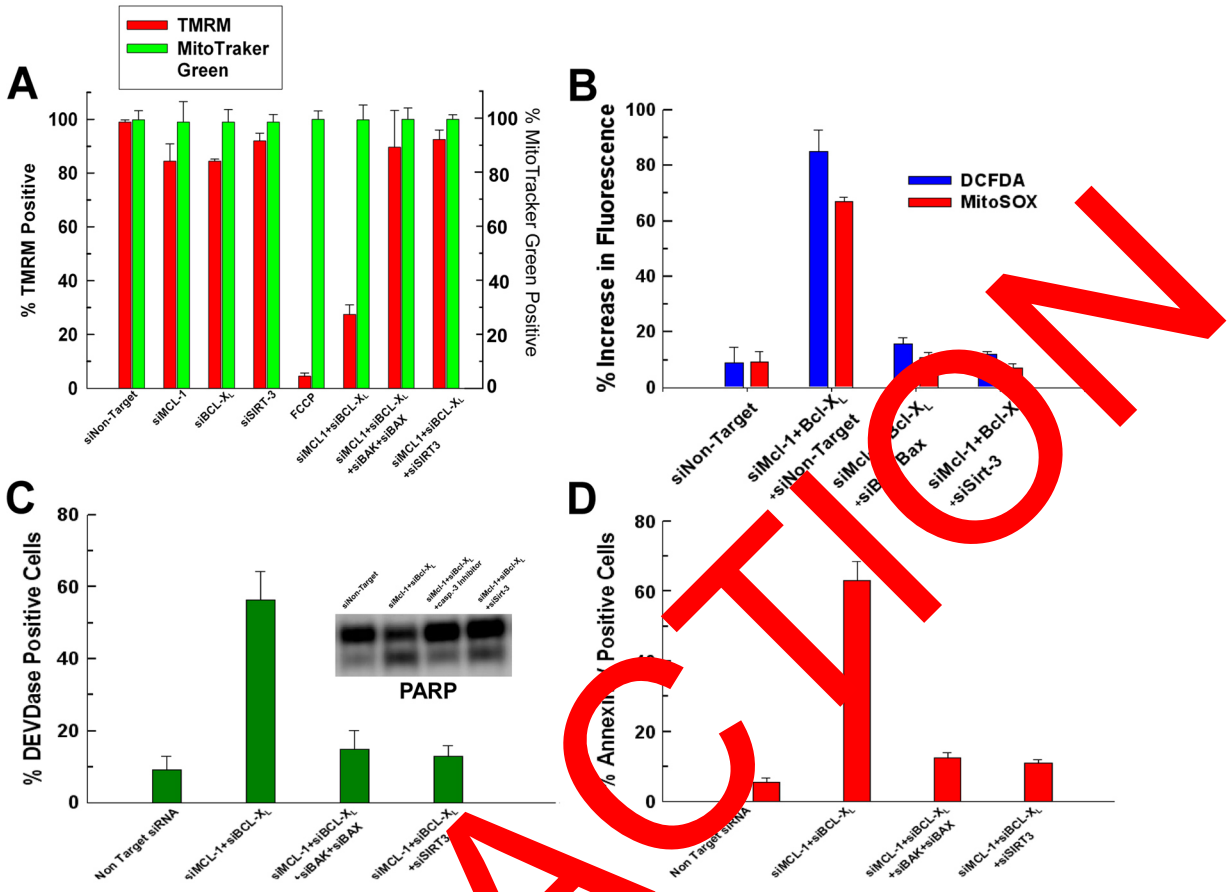

Fig. 2. Depletion of sirt-3 prevents Bak/Bax dependent mitochondrial injury, caspase activation and phosphatidylserine externalization. (**A**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA or siRNAs targeting Mcl-1, Bcl-X_L, Bak, Bax, sirtuin-3; either alone or in tandem. Forty-eight hours after siRNA transfection, 200 nM TMRM and 200 nM of Mito-Tracker Green was added to each well and the cells were incubated at 37℃ for 30 minutes. After incubation, floating and attached cells were collected and washed twice with ice cold PBS. The cells were suspended in ice cold PBS and analyzed immediately using flow cytometry as described in Materials and Methods. Values are the means of three independent experiments with the error bars indicating standard deviations. (**B**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA or siRNAs targeting Mcl-1, Bcl-X_L, Bak, Bax, or sirtuin-3, either alone or in tandem. Forty-eight hours after siRNA transfection, 200 nM of MitoSOX or 10 μM DCFDA was added to each well and the cells were incubated for 30 minutes at 37℃. After incubation, floating and attached cells were collected and washed twice with ice cold PBS. After the final wash, cells were suspended in ice cold PBS and analyzed immediately by flow cytometry as described in Materials and Methods. Values are the means of three independent experiments with the error bars indicating standard deviations. (**C**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA or siRNAs targeting Mcl-1, Bcl-X_L, Bak, Bax, sirtuin-3; either alone or in tandem. Following 24 hours incubation, the cells were harvested. The samples were separated by SDS-PAGE and immunoblotted onto PVDF membranes to detect PARP cleavage. Alternatively, caspase activity was determined using NucView 488 Caspase-3 activity kit as described in Materials and Methods. Values are the means of three independent experiments with the error bars indicating standard deviations. (**D**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA or siRNAs targeting Mcl-1, Bcl-X_L, Bak, Bax, sirtuin-3; either alone or in tandem. Following 48 hours incubation, the cells were harvested. Floating and attached cells were collected and resuspended in 100 μl of binding buffer at 1.0×10^6 cells/ml. FITC-Annexin V (5 μl) was added to determine phosphatidylserine (PS) externalization, and the cells were incubated for 15 minutes at room temperature. PS positive cells were determined by flow cytometry. Values are the means of three independent experiments with the error bars indicating standard deviations.

induce mitochondrial injury by activating Bak/Bax, either directly or by antagonizing the inhibition on Bak/Bax exerted by Mcl-1 and Bcl-X_L. Significantly, depletion of sirt-3 rendered mitochondria refractory to t-Bid induced mitochondrial depolarization to the same extent as did depletion of Bak/Bax.

The addition of t-Bid also brought about secondary manifestations of mitochondrial injury, such as generation of ROS and release of cytochrome c. As shown in Fig. 4B, t-Bid stimulated a 95% increase in the fluorescence of MitoSOX, a mitochondria localized fluorophore sensitive to ROS. Importantly, as with mitochondrial membrane potential, depletion of sirt-3 prevented the t-Bid induced stimulation of ROS to the same extent as did suppression of Bak/Bax expression. The stimulation of ROS and depolarization of mitochondria are thought to be due in part to release of cytochrome c from the mitochondrial inter-membrane space. As shown in Fig. 4C, t-Bid induced a complete loss of mitochondrial cytochrome c, which was prevented by suppression of Bak/Bax expression (lanes 2 and 3, respectively). Markedly, depletion of sirt-3 also prevented the loss of mitochondrial cytochrome c induced by t-Bid (lane 4).

The data indicate that depletion of sirt-3 prevents t-Bid dependent mitochondrial injury that is mediated by Bak/Bax. We next wanted to determine the mechanism(s) by which sirt-3 modulates mitochondrial injury. We and others have demonstrated that hexokinase II, when bound to the mitochondria, inhibits Bak/Bax induced mitochondrial dysfunction (Majewski et al., 2004a;

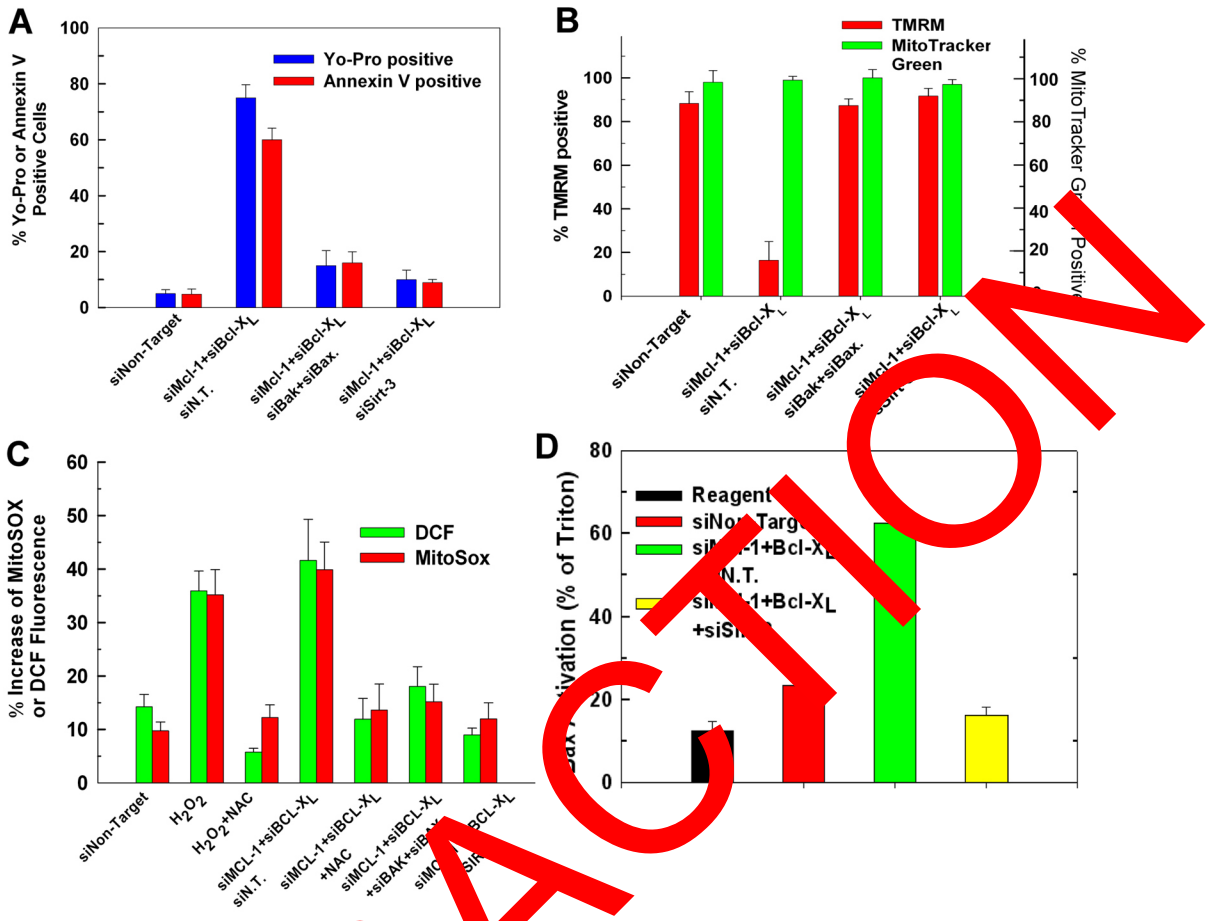

Fig. 3. Sirt-3 is required for Bak/Bax dependent a[p]optosis in MDA-M[B]-231 breast cancer cells. (**A**) MDA-MB-231 cells were transfected with 50 nM of the indicated siRNAs, either individually or in tandem. Following 4[8] hours, the cells were harvested and the degree of apoptosis determined by staining with Yo-Pro-1 as described in Materials and Methods. Values are the means of three independent experiments with the error bars indicating standard deviations. For annexin V staining, floating and attached cells were collected and re-suspended in 100 µl binding buffer at 1.0×10^6 cells/ml. FITC-Annexin-V (5 µl) was added to determine phosphatidylserine (PS) externalization, and the cells were incubated for 15 minutes at room temperature. PS positive cells were determined by flow cytometry. Values are the means of three independent experiments with the error bars indicating standard deviations. (**B**) MDA-MB-231 cells were transfected with 50 nM of a non-targeting control siRNA or siRNAs targeting Mcl-1, Bcl-X_L, Bak, Bax, sirtuin-3; either alone or in tandem. Forty-eight hours after siRNA transfection, 200 nM TMRM and 200 nM of Mito-Tracker Green was added to each well and the cells were incubated at 37℃ for 30 minutes. After incubation, floating and attached cells were collected and washed twice with ice cold PBS. The cells were suspended in ice cold PBS and analyzed immediately using flow cytometry as described in Materials and Methods. Values are the means of three independent experiments with the error bars indicating standard deviations. (**C**) MDA-MB-231 cells were transfected with 50 nM of a non-targeting control siRNA or siRNAs targeting Mcl-1, Bcl-X_L, Bak, Bax, sirtuin-3; either alone or in tandem. Forty-eight hours after siRNA transfection, 200 nM MitoSOX or 10 µM DCFDA was added to each well and the cells were incubated for 30 minutes at 37℃. After incubation, floating and attached cells were collected and washed twice with ice cold PBS. After the final wash, cells were suspended in ice cold PBS and analyzed immediately by flow cytometry as described in Materials and Methods. Values are the means of three independent experiments with the error bars indicating standard deviations. (**D**) MDA-MB-231 cells were isolated on 12 mm coverslips (Fisher Scientific) at 5.0×10^4 cells and allowed to attach overnight. The next day, cells were transfected with the indicated siRNAs. Forty-eight hours incubation, the cells were washed twice with PBS, fixed and immunostained with anti-Bax clone 6A7 as described in Materials and Methods. Quantification was done by drawing a region of interest around each cell in the acquired images. The intensity was determined by SlideBook and expressed as percentage of Bax immunostaining induced by Triton X-100. The results are mean of three independent experiments ± s.d.

Pastorino et al., 2002; Vyssokikh et al., 2002). Moreover, we have demonstrated that depletion of sirt-3 increases the binding of hexokinase II to mitochondria, suggesting a possible mechanism for the protective effects of sirt-3 depletion (Shulga et al., 2010). Indeed as shown in Fig. 4D, suppression of hexokinase II expression reversed the ability of sirt-3 depletion to prevent t-Bid induced mitochondrial depolarization. Similarly, pre-treatment with clotrimazole (CTZ), an agent that detaches hexokinase II from the mitochondria, also neutralized the ability of sirt-3 depletion to prevent t-Bid induced mitochondrial depolarization. Moreover, downregulation of hexokinase II or pre-treatment with CTZ, reversed the ability of sirt-3 depletion to prevent t-Bid induced mitochondrial ROS generation and loss of mitochondrial cytochrome c (Fig. 4E,F).

We have shown that suppression of sirt-3 expression stimulates the binding of hexokinase II to the mitochondria by increasing the acetylation and activity of cyclophilin-D (CyP-D) (Shulga et al., 2010). Therefore, suppression of CyP-D expression should reverse the protective effect of sirt-3 depletion. Indeed, Fig. 5A demonstrates that suppression of CyP-D expression reversed the protective effect exerted by sirt-3 depletion against t-Bid induced mitochondrial depolarization. Additionally, inhibition of

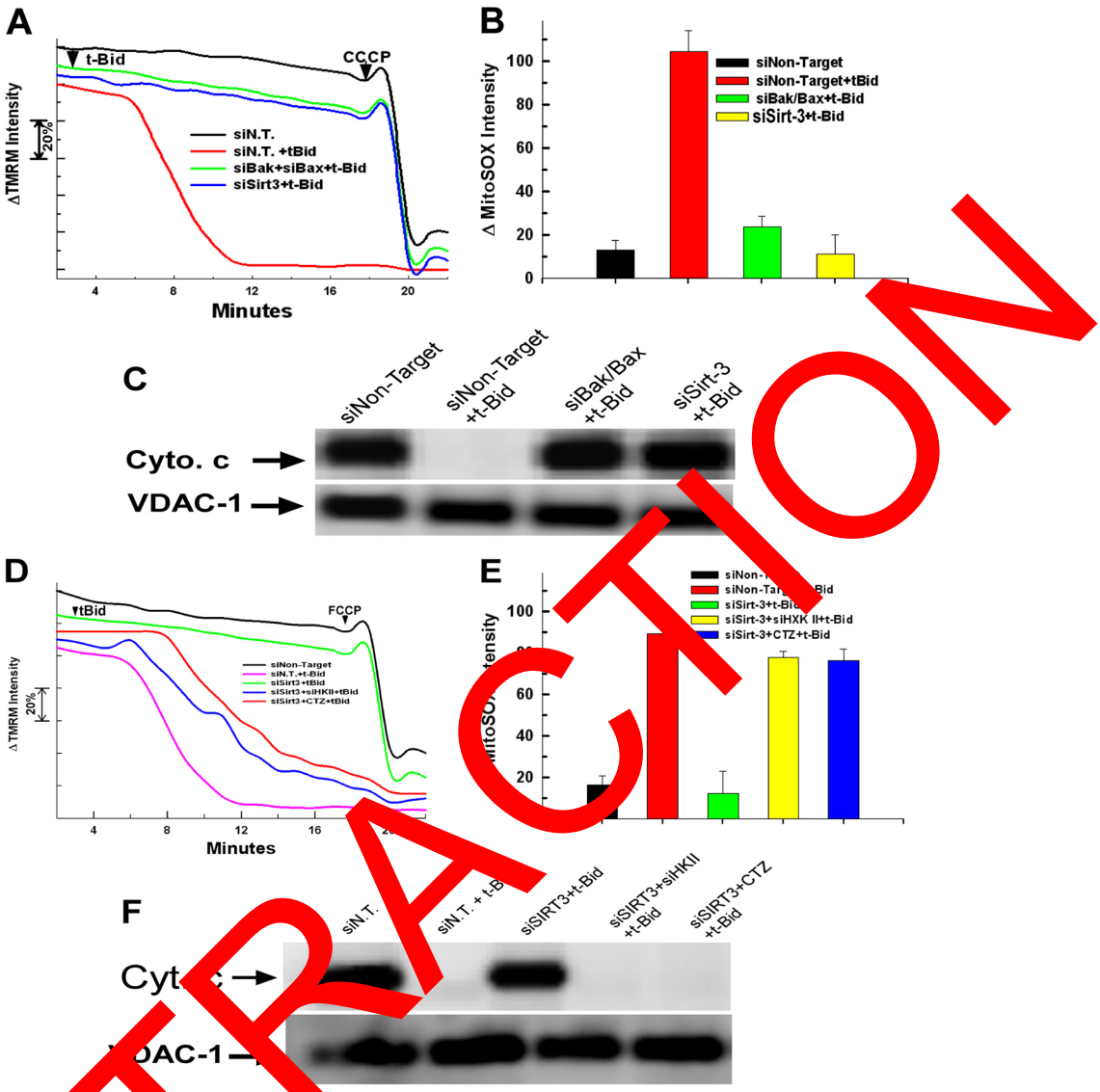

Fig. 4. The protective effect of sirt-3 depletion is dependent on hexokinase II binding to mitochondria. (**A**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA or siRNA targeting Bak/Bax or sirtuin-3. Following 48 hours incubation, HeLa cells were loaded with 200 nM of TMRM for 30 minutes. The cells were then washed twice and placed in respiratory buffer. The cells were mounted on a heated stage kept at 37℃. Digitonin (2.5 μg/ml final concentration) was then added to permeabilize the plasma membrane and TMRM fluorescence was monitored over a 20 minute time course. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at the 2-minute time point and 5 μM of CCCP was added at the 18 minute time point. The result is the average of three independent experiments. (**B**) HeLa cells were plated and then transfected with siRNAs targeting Bak/Bax or sirt-3. Following 48 hours, the cells were loaded with MitoSOX and mounted on a heated microscopy stage. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM. Time-lapse microscopy was conducted over a 20 minute time course with MitoSOX fluorescence intensity assessed as described in Materials and Methods. The results are mean of three independent experiments±s.d. (**C**) Following time lapse microscopy, cells from four wells for each condition were pooled and mitochondria isolated. Cytochrome c content was determined by western blotting using mouse anti-cytochrome c antibody (BD Pharmingen) at 1:1000 dilution. The results are representative of three independent experiments. (**D**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA, siRNA targeting sirtuin-3 or siRNA targeting sirt-3 and hexokinase II in tandem. Following 48 hours incubation, the cells were loaded with 200 nM of TMRM for 30 minutes. The cells were mounted on a heated microscopy stage kept at 37℃. The cells were then washed twice and placed in respiratory buffer. Where indicated, the cells were pre-treated with 10 μM of clotrimazole (CTZ) for 10 minutes. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane and TMRM fluorescence was monitored over a 20 minute time course. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at 2 minutes and 5 μM of CCCP was added at the 18 minute time point. The result is the average of three independent experiments. (**E**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA, siRNA targeting sirtuin-3 or siRNAs targeting sirt-3 and hexokinase II in tandem. Following 48 hours incubation, the cells were loaded with MitoSOX for 30 minutes and then mounted on a heated microscopy stage kept at 37℃. Cells were then washed twice and placed in respiratory buffer. Where indicated, the cells were pre-treated with 10 μM of clotrimazole (CTZ) for 10 minutes. Digitonin at 2.5 μg/ml was then added to permeabilize the plasma membrane. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at 2 minutes. Time-lapse microscopy was conducted over a 20 minute time course with MitoSOX fluorescence intensity assessed as described in Materials and Methods. The results are mean of three independent experiments±s.d. (**F**) Following time lapse microscopy, cells from four wells for each condition were harvested and mitochondria isolated. Cytochrome c content was detected by western blotting using mouse anti-cytochrome c antibody (BD Pharmingen) at 1:1000 dilution. The results are representative of three independent experiments.

cyclophilin-D peptidyl-prolyl cis-trans isomerase activity with cyclosporin A (CsA) also neutralized the ability of sirt-3 depletion to protect against t-Bid induced mitochondrial depolarization. Moreover, suppression of CyP-D and inhibition of CyP-D activity reversed the protective effects exerted by depletion of sirt-3 on t-Bid induced mitochondrial ROS generation (Fig. 5B) and loss of mitochondrial cytochrome c (Fig. 5C).

Importantly, downregulation of CyP-D or inhibition of CyP-D activity with CsA failed to reverse the protective effect exerted by Bak/Bax depletion against t-Bid induced mitochondrial depolarization (Fig. 5D). Similarly, whereas the protection afforded by sirt-3 depletion against t-Bid induced mitochondrial injury is dependent on mitochondrial hexokinase II, suppression of hexokinase II expression or treatment with clotrimazole (CTZ) failed to reverse the protective effect exerted by Bak/Bax depletion against t-Bid induced mitochondrial depolarization (Fig. 5E). Likewise, the inhibition of t-Bid induced mitochondrial ROS production or loss of cytochrome c, mediated by Bak/Bax depletion, was not reversed by suppressing or inhibiting CyP-D or hexokinase II (Fig. 5F,G, respectively). The dichotomy between the ability to reverse the protective effect of sirt-3 depletion versus the insensitivity of Bak/Bax depletion to reversal, indicate that the protective effect exerted by sirt-3 depletion lies upstream of Bak/Bax. To support this notion, suppression CyP-D or hexokinase II expression reversed the ability of sirt-3 depletion to prevent t-Bid induced Bak oligomerization (Fig. 5H, lanes 4 and 5, respectively).

Sirt-3 modulates sensitivity to cisplatin induced cytotoxicity

We have demonstrated that cisplatin induces Bid dependent cytotoxicity, which is potentiated by mitochondrial translocation of hexokinase II from the mitochondria (Shul[illegible] et al., 200[illegible]. Therefore we wanted to determine if sirtuin-3 can modulate sensitivity to cisplatin induced cytotoxicity. HeLa cells were treated with 30 μM of cisplatin over a 48 hour time course. As shown in Fig. 6A, lane 2, at 24 hours, treatment with cisplatin induced Bak oligomerization that was not prevented by transfection with non-targeting siRNA.

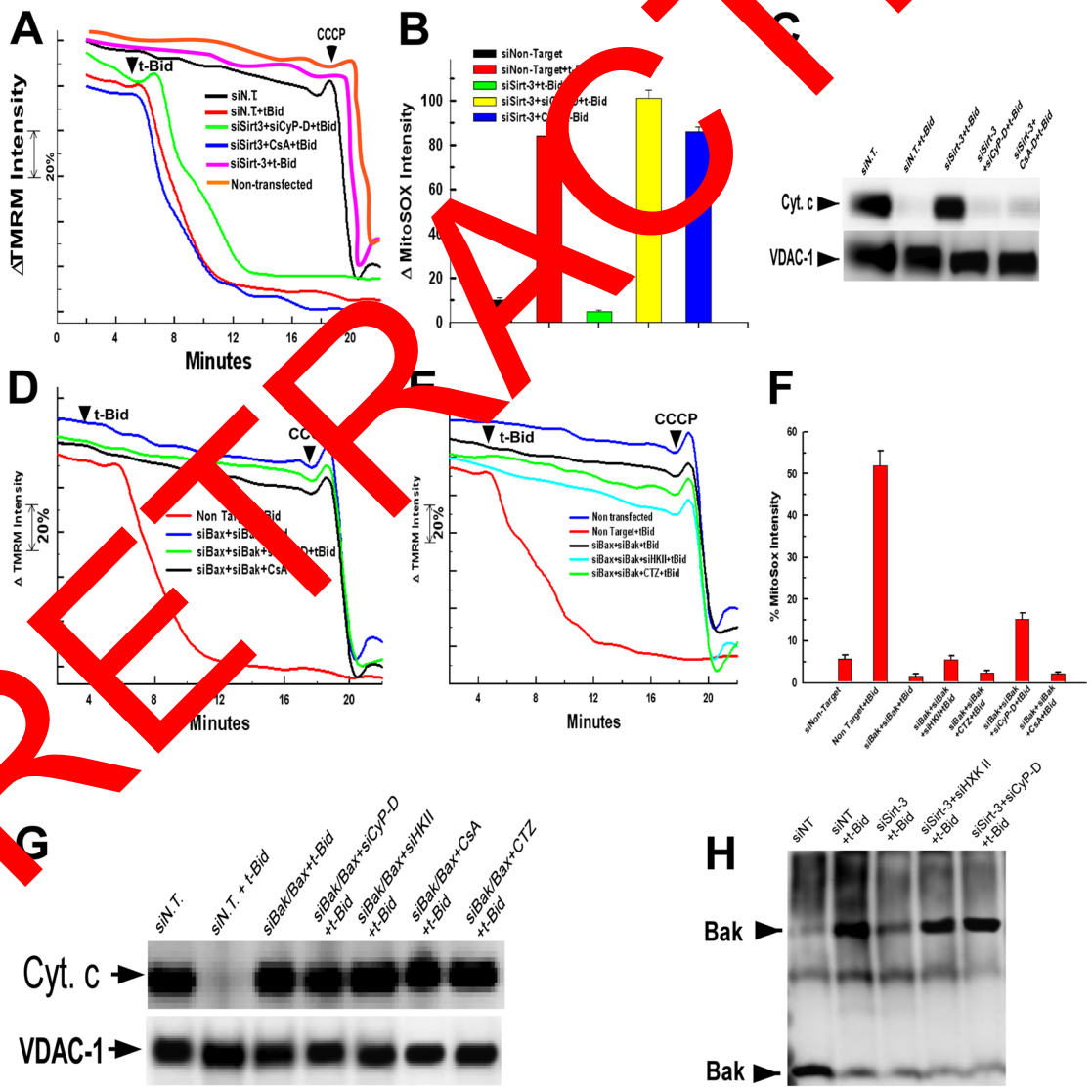

Fig. 5. See next page for legend.

However, cisplatin induced Bak oligomerization was prevented by siRNAs targeting sirt-3 or Bid (lanes 3 and 4, respectively). Fig. 6B demonstrates that treatment with 30 μM of cisplatin brought about a 36% loss of cell viability at 24 hours and a 74% loss of cell viability at 48 hours. As expected, suppressing Bid expression reduced the degree of cytotoxicity induced by cisplatin, as did suppression of Bak/Bax expression. Significantly, depletion of sirt-3 also reduced cytotoxicity induced by cisplatin, with only a 20% loss of cell viability in cells treated with cisplatin over 48 hours (Fig. 6B).

If sirt-3 depletion protects against cisplatin induced cytotoxicity by enhancing the binding of hexokinase II to the mitochondria, then suppression of hexokinase II or cyclophilin-D expression should reverse the protective effect of sirt-3 depletion. As shown in Fig. 6C, 24 hour treatment with 30 μM of cisplatin brought about Bak oligomerization that was prevented by sirt-3 depletion (lane 2 versus lane 3). However, suppression of hexokinase II or cyclophilin-D expression reversed the ability of sirt-3 depletion to prevent cisplatin induced Bak oligomerization (Fig. 6C, lanes 4 and 5, respectively). Moreover, as demonstrated in Fig. 6D, right and left graphs respectively, suppression of hexokinase II or cyclophilin-D expression reversed the ability of sirt-3 depletion to prevent cisplatin induced cytotoxicity. Similarly, detachment of hexokinase II from the mitochondria with clotrimazole or inhibition of cyclophilin-D activity with cyclosporin A (CsA) also reversed the protective effect of sirt-3 depletion against cisplatin induced cytotoxicity. Importantly, suppression of CyP-D or hexokinase II or their activity failed to reverse the protective effect exerted by suppressing Bid expression.

The osteosarcoma cell line U2OS, also displayed a dependence on sirt-3 expression during t-Bid induced mitochondrial injury. As shown in Fig. 7A, the addition of t-Bid to permeabilized U2OS cells transfected with non-targeting siRNA brought about complete depolarization of the mitochondria over an 18 minute time course. The t-Bid induced depolarization was dependent on Bak/Bax expression, and was prevented by depletion of sirt-3. The depolarization induced by the addition of t-Bid was preceded by stimulation in the production of ROS. Fig. 7B shows a sharp increase of MitoSOX fluorescence induced by t-Bid that was largely prevented by depletion of sirt-3 or Bak/Bax. Fig. 7C demonstrates that the ability of sirt-3 depletion to prevent t-Bid induced mitochondrial depolarization is dependent on hexokinase II. Suppression of hexokinase II expression reverses the protective effect of sirt-3 depletion against t-Bid induced depolarization as does detachment of hexokinase II from the mitochondria with clotrimazole. The protective effect of sirt-3 depletion was also dependent on the activity of cyclophilin-D, as suppression of cyclophilin-D expression or treatment with CsA restored the ability of t-Bid to bring about mitochondrial depolarization (Fig. 7D). Importantly, the ability of Bak/Bax depletion to prevent t-Bid induced mitochondrial depolarization was independent of hexokinase II or cyclophilin-D expression (Fig. 7E). Depletion of sirt-3 also rendered the U2OS cells resistant to cisplatin induced cytotoxicity. As shown in Fig. 7F, cisplatin at a dose of 30 μM, induced a progressive increase in the number of dead cells over a 48 hour time course, reaching an 80% loss of cell viability after 48 hours of exposure. Importantly, depletion of sirt-3 greatly ameliorated the cytotoxicity brought about by cisplatin (Fig. 7F). Moreover, the ability of sirt-3 depletion to protect against cisplatin induced cytotoxicity is dependent on hexokinase II and cyclophilin-D expression or activity. Suppression of hexokinase II or cyclophilin-D expression or treatment with clotrimazole or CsA, reversed the protective effect of sirt-3 depletion against cisplatin induced

Fig. 5. Cyclophilin-D is required for sirt-3 depletion to protect against mitochondrial injury. (**A**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA, siRNA targeting sirtuin-3 or siRNAs targeting sirt-3 and CyP-D in tandem. Following 48 hours incubation, the cells were loaded with 200 nM of TMRM for 30 minutes. The cells were then mounted on a heated microscopy stage kept at 37℃. The cells were then washed twice and placed in respiratory buffer. Where indicated, the cells were pre-treated with 10 μM of cyclosporin A (CsA) for 10 minutes. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane. TMRM fluorescence was monitored over a 20 minute time course. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at 2 minutes and 5 μM of CCCP was added at the 18 minute time point. The result is the average of three independent experiments. (**B**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA, siRNA targeting sirtuin-3 or siRNAs targeting sirt-3 and cyclophilin-D in tandem. Following 48 hours incubation, the cells were loaded with MitoSOX for 30 minutes. The cells were then washed twice, placed in respiratory buffer and mounted on a heated microscopy stage kept at 37℃. Where indicated, the cells were pre-treated with 10 μM of CsA (CsA) for 10 minutes. Digitonin at 2.5 μg/ml was then added to permeabilize the plasma membrane. Recombinant truncated Bid (t-Bid) was added at a final concentration of 5 μM at 2 minutes. Time-lapse microscopy was conducted over a 20 minute time course with MitoSOX fluorescence intensity assessed as described in Materials and Methods. The results are mean±s.d. of three independent experiments. (**C**) Following time lapse microscopy, cells from four wells per condition were harvested and mitochondria isolated. Cytochrome c content was detected by western blotting using mouse anti-cytochrome c antibody (BD Pharmingen) at 1:1000 dilution. The result is representative of three independent experiments. (**D**,**E**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA, siRNAs targeting Bak/Bax or siRNAs targeting Bak/Bax and CyP-D or HXK II in tandem. Following 48 hours incubation, the cells were loaded with 200 nM of TMRM for 30 minutes. The cells were washed twice, placed in respiratory buffer and mounted on a heated microscopy stage kept at 37℃. Where indicated, the cells were pre-treated with 10 μM of cyclosporin A (CsA) or clotrimazole (Clt) for 10 minutes. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane. TMRM fluorescence was monitored over a 20 minute time course. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at 2 minutes and 1.5 μM of CCCP was added at the 18 minute time point. The results are the average of three independent experiments. (**F**) HeLa cells were transfected with 50 nM of a non-targeting control siRNA, siRNAs targeting Bak/Bax or siRNAs targeting Bak/Bax and CyP-D or HXK II in tandem. Following 48 hours incubation, the cells were loaded with MitoSOX for 30 minutes. The cells were then washed twice, placed in respiratory buffer and mounted on a heated microscopy stage kept at 37℃. Where indicated, the cells were pre-treated with 10 μM of cyclosporin A (CsA) or clotrimazole (Clt) for 10 minutes. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at 2 minutes. Time-lapse microscopy was conducted over a 20 minute time course with MitoSOX fluorescence intensity assessed as described in Materials and Methods. The results are the mean±s.d. of three independent experiments. (**G**) Following time lapse microscopy, cells from four wells for each condition were harvested and mitochondria isolated. Cytochrome c content was detected by western blotting using mouse anti-cytochrome c antibody (BD Pharmingen) at 1:1000 dilution. The results are representative of three independent experiments. (**H**) HeLa cells were transfected with 50 nM of the indicated siRNAs, either individually or in tandem. Following 48 hours incubation, the cells were permeabilized with digitonin (2.5 μg/ml). Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at 18 minutes. The cells were then harvested from four wells per condition and the mitochondria fraction isolated. The isolated mitochondria were incubated with 0.1 mM of the cross-linking reagent BMH for 30 minutes. The samples were then run out on 12% SDS-PAGE gels and Bak oligomerization was assessed by western blotting. The results are representative of three independent experiments.

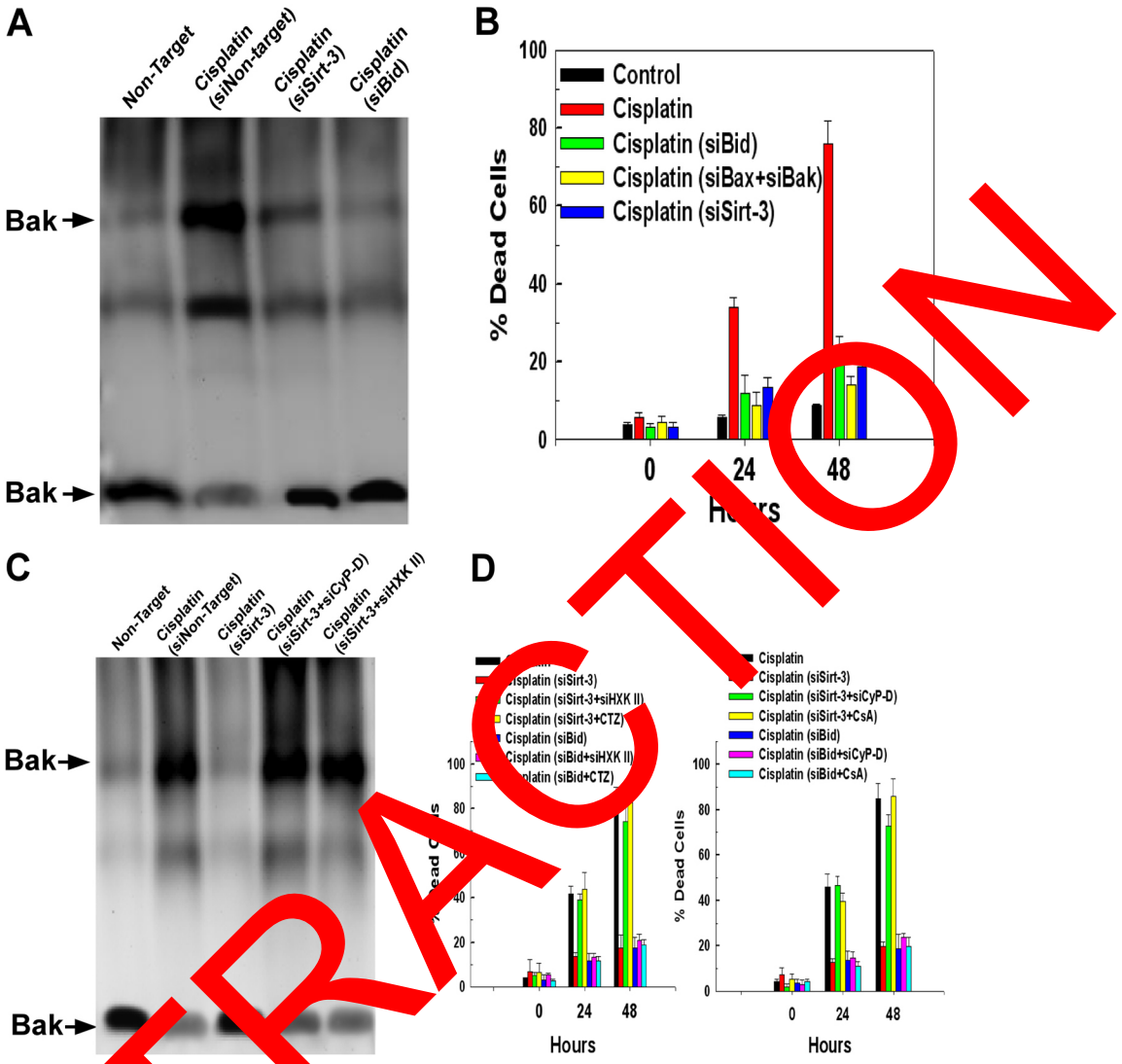

Fig. 6. Sirt-3 depletion protect gainst latin induced toxicity by regulating the binding of hexokinase II to the mitochondria. (**A**) HeLa cells were transfected with 50 nM of the indicated siR Following 24 hours incubation, the cells were treated with 30 μM of cisplatin. Following 24 hours exposure to cisplatin, the cells from fo wells per condition e harvested and the mitochondrial fraction isolated. The isolated mitochondria were incubated with 0.1 mM of the cross-linking reage MH for 30 minutes. The mples were then run out on 12% SDS-PAGE gels and Bak oligomerization was assessed by western blotting. The result is repres tative of th independent experiments. (**B**) HeLa cells were transfected with 50 nM of the indicated siRNAs. Following 24 hours incubation, the cell re trea with 30 μM of cisplatin. At the time points indicated, the cells were harvested and viability determined utilizing Yo-Pro-1 as described in Materials hods. Valu re the mean of three independent experiments with the error bars indicating standard deviations. (**C**) HeLa cells were transfected 0 nM o indicat iRNAs. Following 24 hours incubation, the cells were treated with 30 μM of cisplatin. After 24 hours exposure to cisplati he cell om four ells each condition were harvested and the mitochondrial fraction isolated. The isolated mitochondria were incubated with 0.1 of the cr s-linking reag t BMH for 30 minutes. The samples were then run out on 12% SDS-PAGE gels and Bak oligomerization was assessed by we blottin lts are representative of three independent experiments. (**D**) HeLa cells were transfected with 50 nM of siRNAs targeting sirt-3 and Bid, either y or in tandem with siRNAs targeting HXK II or CyP-D. Following 24 hours incubation, the cells were treated with 30 μM of cisplatin in the absence esence of 10 μM of CsA or 10 μM of CTZ. At the time points indicated, the cells were harvested and viability determined utilizing Yo-Pro-1 as described in terials and Methods. Values are the mean of three independent experiments with the error bars indicating standard deviations.

cytotoxicity, but did not reverse the protective effect of depleting Bid expression.

Increased expression of Sirt-3 enhances sensitivity to cell death

We next wanted to determine if increasing the expression of sirt-3 enhances sensitivity to mitochondrial injury. Galactose cannot be readily utilized for glycolysis. We have shown that the substitution of galactose for glucose in the culture media of transformed cells increases the expression of sirt-3, bringing about detachment of hexokinase II from the mitochondria (Shulga et al., 2010). As shown in Fig. 8A, a fivefold lower dose of t-Bid (1 μM) did not induce mitochondrial depolarization in cells kept in glucose based media. However, the sub-threshold dose of t-Bid induced rapid and complete mitochondrial depolarization in cells incubated in galactose based media. Further, suppression of sirt-3 expression prevented t-Bid induced mitochondrial depolarization in galactose based media, even at

the fivefold higher concentration of t-Bid (5 μM) which brings about mitochondrial depolarization in cells incubated in glucose based media. Incubation in galactose based media also sensitized to cisplatin induced cytotoxicity. As show in Fig. 8B, treatment with half the dose of cisplatin (15 μM) utilized in glucose based media induced an over 80% loss of cell viability following only 16 hours of exposure. Importantly, the galactose induced potentiation of cisplatin induced cell killing was prevented by depletion of sirt-3 (Fig. 8B). Moreover, the protection afforded by sirt-3 depletion was dependent on the expression of hexokinase II and cyclophilin-D activity, with suppression of hexokinase II expression or treatment with CsA reversing the protective effect of sirt-3 depletion (Fig. 8B). Importantly, neither CsA nor suppression of hexokinase II reversed the protective effect of Bid depletion against cisplatin induced cytotoxicity in galactose based media. We next attempted to determine if increased expression of sirt-3 in cells incubated in glucose had any effect on cell viability. Significantly, overexpression of sirt-3 in cells incubated in medium containing glucose brought about Bak oligomerization and loss of cell viability (Fig. 8C,D, respectively). However, overexpression of an inactive sirt-3 mutant, sirt-3 (H248Y) did not [illegible]g about Bak oligomerization and had little effect on cell viability.

Discussion

The results of the present study indicate [illegible] sirtuin-3 exerts a cyclophilin-D dependent tumor [illegible]sor effect by regulating the binding of hexokinase II [illegible] the mito[illegible]ondria, which in turn

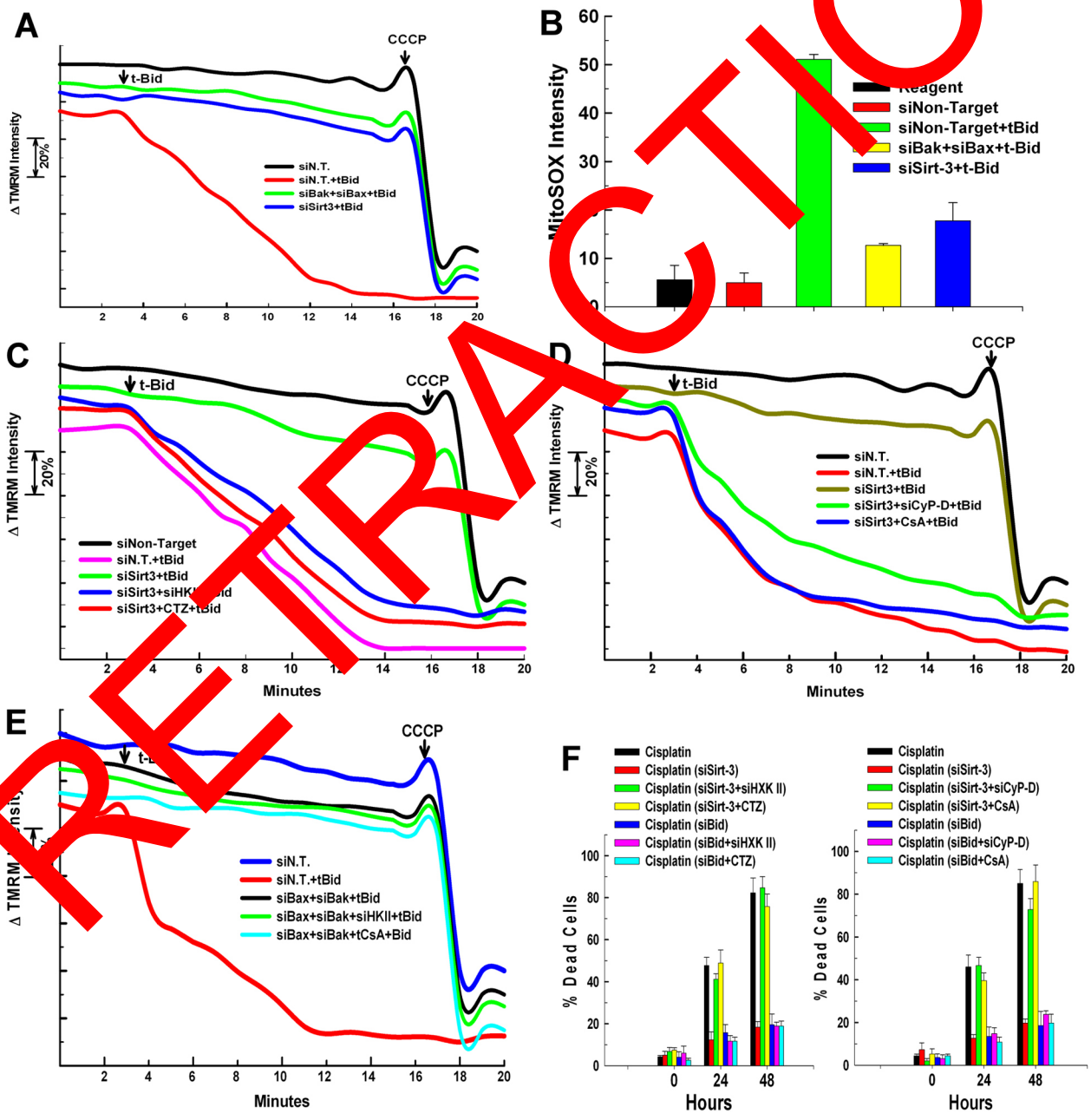

Fig. 7. See next page for legend.

controls the ability of Bak/Bax to bring about mitochondrial injury and loss of cell viability. Suppression of sirt-3 prevented all of the manifestations of Bak/Bax dependent mitochondrial injury and apoptosis induction; including mitochondrial depolarization, release of cytochrome c, stimulation of ROS production, caspase-3 activation, PS externalization and activation of Bak and Bax. Moreover depletion of sirtuin-3 protected against Bak/Bax dependent mitochondrial injury and cell death in three models and three different cell types; downregulation of Mcl-1 and Bcl-X_L expression in HeLa and MDA-MB-231 cells and exposure of mitochondria to t-Bid and treatment with cisplatin in HeLa and U2OS cells. Importantly, the ability of sirt-3 depletion to protect against mitochondrial injury and subsequent cell death was dependent on hexokinase II and cyclophilin-D. Suppression of hexokinase II expression or forced detachment of hexokinase II from mitochondria with clotrimazole reversed the protective effect of sirt-3 depletion. Similarly, suppression of cyclophilin-D expression or inhibition of cyclophilin-D cis-trans isomerase activity with CsA negated the protective effect of sirt-3 depletion. Importantly, suppression of Bak/Bax expression protected under all conditions and was not reversible by suppression or inhibition of hexokinase II or cyclophilin-D, indicating that sirt-3 depletion exerts a protective effect up-stream of Bak/Bax.

Sirtuin-3 has emerged as a central regulator of mitochondrial metabolism. The acetylation-deacetylation cycle impacts on the stability and activity of a multitude of mitochondrial components that directly or indirectly effect cell survival. Paradoxically, sirt-3 has been demonstrated to protect against loss of cell viability while also being identified as a tumor suppressor, whose absence enhances cellular transformation and the ability of transformed cells to survive. The protective effects of sirt-3 against cellular stress include a decrease in ROS levels, mediated by sirt-3 stimulating anti-oxidant capacity and diminishing ROS generation from the mitochondrial respiratory chain. Indeed, it has been shown that sirt-3 mediates a reduction of oxidative damage during age-related hearing loss in part by deacetylating and activating IDH-2, which increases the levels of reduced glutathione (Someya et al., 2010; Yu et al., 2012). Sirt-3 also activates mitochondrial manganese superoxide dismutase, another key element of the mitochondrial ROS detoxification pathway (Tao et al., 2010). Importantly, sirt-3 has been demonstrated to exert a protective effect against hypoxia and staurosporine induced cell death by inhibiting the mitochondrial permeability transition and dampening intracellular acidification (Pellegrini et al., 2012; Zamzami et al., 1997). Carbonic anhydrase helps to regulate intracellular pH by catalyzing the conversion of carbon dioxide to bicarbonate. In particular, carbonic anydrase VB, localized to the mitochondria, requires sirt-3 expression to maintain activity.

The mitochondrial permeability transition is a central event in the onset of necrotic cell death (Baines et al., 2005; Halestrap et al., 2004; Zamzami et al., 1997). The constituents of the permeability transition pore (PTP) are ill defined, but cyclophilin-D is a key regulator of PTP sensitivity. We have shown that sirt-3 deacetylates and inactivates the peptidyl-prolyl cis-trans isomerase activity of cyclophilin-D, thereby dampening PTP induction and preventing TNF mediated necrotic cell death in hepatocytes (Shulga and Pastorino, 2010). All of these processes regulated by sirt-3 converge to make the mitochondria and cell more resilient against necrotic induced damage. However sirt-3 also acts as a tumor suppressor, whose loss increases the chances of cellular transformation and the ability of cancer cells to survive.

Loss of sirt-3 is thought to promote cellular transformation by stimulating ROS generation. The increased ROS contributes to

Fig. 7. Sirt-3 is required for Bak/Bax dependent apoptosis in U2OS osteosarcoma cells. (**A**) U2OS cells were transfected with 50 nM of a non-targeting control siRNA or siRNA targeting Bak/Bax or sirtuin-3. Following 48 hours incubation, U2OS cells were loaded with 200 nM of TMRM for 30 minutes. The cells were then washed twice and placed in respiratory buffer. The cells were mounted on a heated stage kept at 37℃. Digitonin (2.5 μg/ml final concentration) was then added to permeabilize the plasma membrane and TMRM fluorescence was monitored over a 20 minute time course. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at the 2 minute time point and 5 μM of CCCP was added at the 18 minute time point. The result is the average of three independent experiments. (**B**) U2OS cells were plated and then transfected with siRNAs targeting Bak/Bax or sirt-3. Following 48 hours, the cells were loaded with MitoSOX and mounted on a heated microscopy stage. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM. Time-lapse microscopy was conducted over a 20 minute time course with MitoSOX fluorescence intensity assessed as described in Materials and Methods. The results are mean of three independent experiments±s.d. (**C**) U2OS cells were transfected with 50 nM of a non-targeting control siRNA, siRNA targeting sirtuin-3 or siRNA targeting sirt-3 and hexokinase II in tandem. Following 48 hours incubation, the cells were loaded with 200 nM of TMRM for 30 minutes. The cells were mounted on a heated microscopy stage kept at 37℃. The cells were then washed twice and placed in respiratory buffer. Where indicated, the cells were pre-treated with 10 μM of clotrimazole (CTZ) for 10 minutes. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane and TMRM fluorescence was monitored over a 20 minute time course. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at 2 minutes and 5 μM of CCCP was added at the 18 minute time point. The result is the average of three independent experiments. (**D**) U2OS cells were transfected with 50 nM of non-targeting control siRNA, siRNA targeting sirtuin-3 or siRNA targeting sirt-3 and CyP-D in tandem. Following 48 hours incubation, the cells were loaded with 200 nM of TMRM for 30 minutes. The cells were then mounted on a heated microscopy stage kept at 37℃. The cells were then washed twice and placed in respiratory buffer. Where indicated, the cells were pre-treated with 10 μM of cyclosporin A (CsA) for 10 minutes. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane. TMRM fluorescence was monitored over a 20 minute time course. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at 2 minutes and 5 μM of CCCP was added at the 18 minute time point. The result is the average of three independent experiments. (**E**) U2OS cells were transfected with 50 nM of a non-targeting control siRNA, siRNAs targeting Bak/Bax or siRNAs targeting Bak/Bax and HXK II in tandem. Following 48 hours incubation, the cells were loaded with 200 nM TMRM for 30 minutes. The cells were then washed twice, placed in respiratory buffer and mounted on a heated microscopy stage kept at 37℃. Where indicated, the cells were pre-treated with 10 μM of cyclosporin A (CsA) for 10 minutes. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane. Recombinant truncated Bid (t-Bid) was added at a concentration of 5 μM at 2 minutes. Time-lapse microscopy was conducted over a 20 minute time course with TMRM fluorescence intensity assessed as described in Materials and Methods. The result is the average of three independent experiments. (**F**) U2OS cells were transfected with 50 nM of siRNAs targeting sirt-3 and Bid, either separately or in tandem with siRNAs targeting HXK II or CyP-D. Following 24 hours incubation, the cells were treated with 30 μM of cisplatin in the absence or presence of 10 μM of CsA or 10 μM of CTZ. At the time points indicated, the cells were harvested and viability determined utilizing Yo-Pro-1 as described in the Materials and Methods. Values are the mean of three independent experiments with the error bars indicating standard deviations.

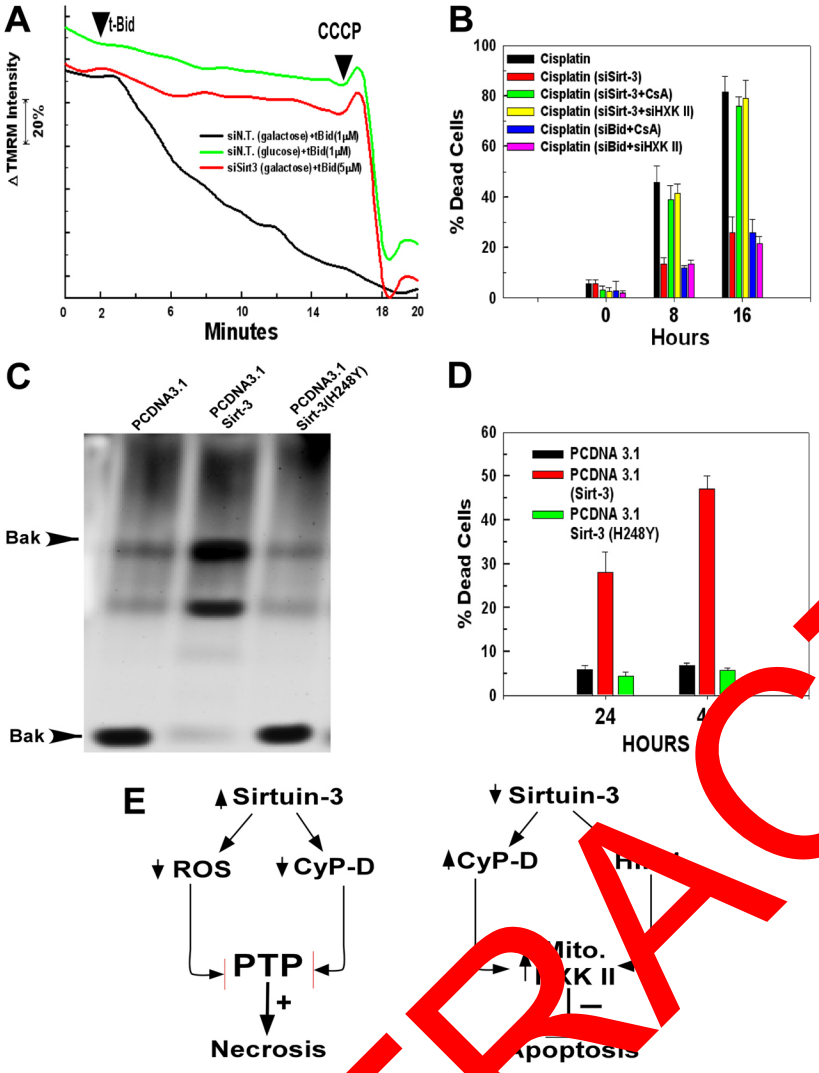

Fig. 8. Increased sirt-3 expression sensitizes cells to cisplatin induced cytotoxicity. (**A**) HeLa cells were incubated in DMEM containing glucose (4.5 g/l) or transferred to DMEM containing galactose at 4.5 g/l. Following 24 hours incubation, HeLa cells were transfected with 50 nM of non-targeting siRNA or siRNA targeting sirt-3. After 48 hours incubation, the cells were loaded with 200 nM of TMRM for 30 minutes. The cells were washed twice, placed in respiratory buffer and mounted on a heated microscopy stage kept at 37°C. Digitonin (2.5 μg/ml) was then added to permeabilize the plasma membrane and TMRM fluorescence was monitored over a 20 minute time course. Recombinant t-Bid was added at a concentration of 5 μM after 2 minutes and 5 μM of CCCP was added at the 18 minute time point. The result is the average of three independent experiments. (**B**) HeLa cells were transferred to DMEM containing galactose at 4.5 g/l. Following 24 hours incubation, HeLa cells were transfected with 50 nM of siRNA targeting sirt-3 and Bid, either separately or in tandem with siRNA against HXK II. The cells were incubated for another 24 hours. Where indicated, the cells were pre-treated for 30 minutes with 10 μM of CsA. The cells were then treated with 5 μM of cisplatin and cell viability determined at the time points indicated. Values are the means of three independent experiments with the error bars indicating standard deviations. (**C**) U2OS cells were transfected with 250 nM of the indicated plasmids. Following 24 hours incubation, the cells were harvested and the mitochondrial fraction isolated from four wells for each condition. The isolated mitochondria were incubated with 0.1 mM of the cross-linking reagent BMH for 30 minutes. The samples were then run out on 12% SDS-PAGE gels and Bak oligomerization was assessed by western blotting. (**D**) U2OS cells were co-transfected with 250 nM of the indicated plasmids and 250 nM of EGFP. Following 48 hours, the cells were harvested and the number of EGFP positive cells stained with Yo-Pro-1 determined as described in Materials and Methods. Values are the means of three independent experiments with the error bars indicating standard deviations. (**E**) Sirtuin-3 exerts opposing effects on apoptosis and necrosis by mediating the localization and activity of cyclophilin-D and hexokinase II.

cellular transformation by promoting genomic instability, with sirt-3−/− mouse embryonic fibroblasts displaying a transformation-permissive phenotype (Hallows et al., 2012). Moreover, once transformed a cancer cell must survive in a hostile environment. By increasing the basal level of ROS, sirt-3 depletion enhances the stability of hypoxia factor 1α (HIF-1α) (Bell et al., 2007; Schumacker, 2011). The increased stability of HIF-1α contributes to the increased rate of glycolysis exhibited by many cancers. Even in the presence of adequate oxygen, a phenomena known as the Warburg effect (Pedersen, 2007). Aside from producing ATP under hypoxic conditions, the stimulation of glycolysis maybe more critical for generating glucose-6-phosphate (G-6-P) that is a ready conduit to a variety of biosynthetic pathways required for cellular proliferation, such as lipid synthesis and shunting to the pentose phosphate pathway for the generation of NADPH.

The first and rate controlling enzymes of glycolysis are hexokinase I or II, which catalyzes the conversion of glucose to glucose-6-phosphate utilizing ATP. Hexokinase II is highly expressed in cancer cells, in part due to transcriptional activation of the hexokinase II gene by HIF-1α (Gwak et al., 2005; Marín-Hernández et al., 2009). Moreover, in cancer cells, hexokinase I or II activity is accelerated by their ncreased binding to the mitochondria (Azoulay-Zohar et al., 2004; Pastorino and Hoek, 2008). Hexokinase I or II binds to the mitochondria via an interaction with the voltage dependent anion carrier (VDAC), embedded in the mitochondrial outer membrane (Shoshan-Barmatz et al., 2009). In this position, hexokinase I or II gains preferential access to ATP generated by oxidative phosphorylation, thereby accelerating G-6-P production and utilization. Indeed, the binding of hexokinase II to the mitochondria and its ability to funnel ATP produced in the mitochondria to form G-6-P is a primary mechanism driving the phenomena of aerobic glycolysis.

In addition to promoting aerobic production of G-6-P, the binding of hexokinase I or II to mitochondria interferes with Bak/Bax dependent mitochondrial injury (Gall et al., 2011; Majewski et al., 2004a; Majewski et al., 2004b; Pastorino et al., 2002; Shulga et al., 2009; Tajeddine et al., 2008; Vyssokikh et al., 2002). The mechanism by which mitochondrial bound hexokinase I or II interferes with Bak/Bax induced mitochondrial damage is not fully clear. However, a number of reports indicate that Bak/Bax interacts with or are modulated by VDAC, thereby supporting the notion that hexokinase I or II prevents Bak/Bax activation by interfering with their ability to interact with VDAC (Abu-Hamad

et al., 2008; Adachi et al., 2004; Cheng et al., 2003; Lazarou et al., 2010; Roy et al., 2009; Tajeddine et al., 2008).

The binding of hexokinase II to mitochondria is mediated in part by cyclophilin-D (Machida et al., 2006; Shulga et al., 2010). Cyclophilin-D has been demonstrated to exert an anti-apoptotic effect in a variety of contexts (Eliseev et al., 2009; Li et al., 2004; Schubert and Grimm, 2004). Cyclophilin-D expression and activity were found to prevent Bax dependent apoptosis, which in turn was reversed by detachment of hexokinase II from the mitochondria (Machida et al., 2006). This is in contrast with cyclophilin-D promoting opening of the PTP and onset of necrotic cell death. Indeed, it has been shown that cyclophilin-D can exert opposite effects on apoptosis versus necrosis. These observations are in keeping with the ability of cyclophilin-D ablation to protect against necrotic cell death but not apoptosis. Indeed, it has been demonstrated that a developmental shift occurs with regards to the role of cyclophilin-D in neuronal survival (Wang et al., 2009). In the immature brain, cyclophilin-D exerts a protective effect, whereas in the adult brain, cyclophilin-D promotes loss of neuronal viability. Intriguingly, hexokinase II is expressed at much higher concentrations in the developing brain than in the adult brain, perhaps accounting in this instance for the dichotomy of cyclophilin-D effects on cell viability.

The regulation exerted on cyclophilin-D by sirtuin-3 may help to explain some of the paradoxical effects of both on necrosis versus apoptosis. We have shown that sirtuin-3 deacetylates and inactivates cyclophilin-D (Shulga et al., 2010). By contrast when sirt-3 is depleted, cyclophilin-D becomes acetylated and exhibits increased activity, which in turn promotes increased binding of hexokinase II to the mitochondria (Shulga and Pastorino, 2010). Therefore, when sirtuin-3 is expressed and active, it prevents onset of permeability transition pore opening and necrosis by dampening cyclophilin-D activity. Sirt-3 activity also inhibits PTP opening by lowering ROS levels through stimulating the anti-oxidant activity of MnSOD and IDH2. By contrast, when sirt-3 is down-regulated, cyclophilin-D becomes more active, increasing the probability of PTP opening and vulnerability to necrosis. However, in a transformed cell, depletion of sirt-3 leads to HIF-1α activation that stimulates expression of hexokinase II. Moreover, the increased acetylation of cyclophilin-D by sirt-3 depletion stimulates cyclophilin-D peptidyl-prolyl cis-trans isomerase activity, which promotes hexokinase II binding to the mitochondria where it prevents Bak/Bax dependent mitochondrial injury and apoptosis. Therefore, sirt-3 depletion has the twofold effect of increasing hexokinase II expression and the binding of hexokinase to the mitochondria. These effects of sirt-3 depletion promote the proliferative capacity of transformed cells and their ability to survive austere conditions, thus placing sirt-3 in a key position to regulate sensitivity or resistance to chemotherapeutic agents (cisplatin, 5FC).

Materials and Methods

Cell culture

HeLa, U2OS and MDA-MB-231 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in 25 cm^2 flasks with 5 ml Dulbecco's modified Eagle's medium (Gibco) containing 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% heat-inactivated fetal bovine serum and incubated at 37℃ under an atmosphere of 95% air and 5% CO_2. For most experiments, cells were plated in 24 well plates (Corning Costar, Corning, NY, USA) at 5.0×10^4 cells/well. Where indicated, cells were transferred to DMEM containing galactose at 4.5 g/l.

Mitochondrial isolation

Following treatment, cells from four individual wells (~200,000 cells total) were harvested by trypsinization and centrifuged at 600 ***g*** for 10 minutes at 4℃. The cell pellets were washed once in PBS and then resuspended in three volumes of isolation buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM dithiothreitol, and 10 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin) in 250 mM sucrose. After chilling on ice for 3 minutes, the cells were disrupted by 40 strokes of a glass homogenizer. The homogenate was centrifuged twice at 1500 ***g*** at 4℃ to remove unbroken cells and nuclei. The mitochondria-enriched fraction (heavy membrane fraction) was then pelleted by centrifugation at 12,000 ***g*** for 30 minutes. Mitochondrial integrity was determined by the respiratory control ratio as oxygen consumption in state 3 and state 4 of respiration using a Clark oxygen electrode with 5 mM glutamate and 1 mM malate as respiratory substrates. The supernatant was recovered and filtered through 0.2 μm and then 0.1 μm Ultrafree MC filters (Millipore) to give cytosolic protein.

RNA interference and plasmid transfection

siRNAs targeting sirtuin-3 (sirt3), Mcl-1, Bcl-X$_L$, Bax, Bak, Hexokinase-II (HKII), Cyclophilin-D and the non-target control were delivered into cells using TransIT-TKO at a final concentration of 5 nM. Twenty-four hours after plating cells, siRNA-liposome complexes were added and incubated for 24 hours after which the cells were washed twice with phosphate-buffered saline (PBS) and fresh complete medium was added. Where indicated, the cells were incubated for additional 24 hours.

For transfection of plasmids, U2OS cells were plated at 50,000 cells per well in 24 well plates. Following 24 hours, the cells were co-transfected with a plasmid encoding enhanced green fluorescent protein (EGFP) and the mammalian expression vector PCDNA 3.1 containing sirt-3 or the sirt-3 mutant [sirt-3(H248Y)]. Following 24 hours, the cells were either un-treated or treated with 30 μM of cisplatin. After 16 hours exposure to cisplatin, the cells were harvested and the number EGFP expressing cells staining positive for Yo-Pro-1 was determined on a Cellometer Vision.

Cell viability assay

Cell viability was determined utilizing Yo-Pro-1 that is selectively taken up by apoptotic cells (Ruffa et al., 2005; Idziorek et al., 1995). Briefly, 48 hours post siRNA transfection or following treatment with cisplatin, attached and floating cells were collected and washed once with PBS. Yo-Pro-1 (5 μg/ml) was added, incubated 20 minutes and analyzed using the Cellometer Vision.

Determination of caspase activity and phosphatidylserine exposure

Caspase activity was determined using NucView 488 Caspase-3 activity kit (Biotium Hayward, CA, USA). Forty-eight hours after siRNA transfection, floating and attached cells were collected and resuspended in DMEM containing 5 μM of the NucView 488 substrate and then incubated at room temperature for 30 minutes protected from light. After incubation, the cells were washed once with ice cold PBS, and then resuspended in PBS. Caspase activity was detected by an increase in the intensity of the DNA binding dye using Cellometer Vision. For determination of phosphatidylserine (PS) externalization, 48 hours after siRNA transfection, floating and attached cells were collected and resuspended in 100 μl of binding buffer at 1.0×10^6 cells/ml. FITC- Annexin-V (5 μl) was added, and the cells were incubated for 15 minutes at room temperature. PS positive cells were determined by flow cytometry.

Mitochondrial membrane potential and ROS production

Mitochondrial membrane potential after transfection with siRNA was determined using the potentiometric dye TMRM and MitoTracker green for mitochondrial mass. Forty-eight hours after siRNA transfection, 200 nM TMRM and 200 nM of MitoTracker-green was added to each well and the cells were incubated at 37℃ for 30 minutes. After incubation, floating and attached cells were collected and washed twice with ice cold PBS. The cells were then suspended in ice cold PBS and analyzed immediately using flow cytometry as described in Materials and Methods. For ROS determination, MitoSOX and DCFDA [5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate] were utilized (Life Technologies). Forty-eight hours after siRNA transfection, 5 μM MitoSOX or 10 μM of DCFDA was added to each well and the cells were incubated for 30 minutes at 37℃. After incubation, floating and attached cells were collected and washed twice with ice cold PBS. After the final wash, the cells were suspended in ice cold PBS and analyzed immediately utilizing flow cytometry.

BAX and Bak activation

For Bax activation, cells were plated on 12 mm coverslips at 5.0×10^4 and allowed to attach overnight. The cells were transfected with the indicated siRNAs for 24 hours. Following siRNA transfection, the cells were washed twice with PBS, fixed with 3.7% formaldehyde in PBS for 5 minutes, and then permeabilized with 0.2% CHAPS in PBS for 10 minutes. Non-specific antibody binding was blocked with 5% goat serum and 1% BSA in PBS for 30 minutes. Following another wash, cells were incubated for 1 hour in primary antibody solution (1:100, mouse anti-BAX, clone 6A7; Sigma). The cells were then incubated in the secondary antibody

solution (1:750 Alexa 488-conjugated goat anti-mouse IgG) for 1 hour. For nuclear staining, the coverslips were incubated with 2 μg/ml Hoechst 33342 in PBS for 10 minutes. The coverslips were mounted on glass slides using 80% glycerol in PBS. The stained cells were viewed on an Olympus IX51 (Olympus, Center Valley, PA, USA). Images were captured with a Hamamatsu C-10600-10B camera (Hamamatsu, Bridgewater, NJ, USA) and SlideBook software (Intelligent Imaging Innovations, Denver, CO, USA). Quantification was done by drawing a region of interest around each cell in the acquired images. The intensity was determined by the SlideBook software, and was expressed as the percentage of Bax activation and staining induced by the Triton X-100.

For Bak oligomerization, mitochondria were isolated from ~400,000 cells. Briefly, cell pellets were washed once with phosphate-buffered saline and then resuspended in three volumes of isolation buffer (20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM aprotinin) in 250 mM sucrose. The cells were disrupted in a glass homogenizer and centrifuged twice at 1500×***g*** at 4℃ to remove unbroken cells and nuclei. The mitochondrial fraction was then pelleted by centrifugation at 12,000×***g*** for 30 minutes. Cellular fractionation was assessed for cross contamination by utilizing lamin A/C (1:1000 Cell Signaling), prohibitin (1:1000, EMD) and GADPH (1:1000, Cell Signaling) as nuclear, mitochondrial and cytosolic markers, respectively (supplementary material Fig. S2). After various treatments in intact cells and isolated mitochondria, Bak oligomerization was determined by incubating isolated mitochondria with 0.1 mM bismaleimidohexane (BMH) for 30 minutes at room temperature. The samples were then separated on Novex 10% Tris/Glycine precast gels in the XCell II module with MagicMark XP (all from Invitrogen) as the molecular weight marker. Proteins were immune-blotted onto PVDF membrane and probed with anti-Bak antibody (Cell Signaling).

Time-lapse fluorescence microscopy

HeLa cells or U2OS cells were plated in 8-well chamber slides (Nunc, Rochester, NY, USA) at 25,000 cells/well. Cells were transfected with siRNA as described above. Where indicated, cells were pre-treated with cyclosporin A or clotrimazole for 10 minutes before the start of image acquisition. For determination of mitochondrial energization, cells were loaded with 200 nM TMRM for 30 minutes. For determination of ROS formation, cells were loaded with 200 nM of MitoSOX for 30 minutes. The cells were then washed twice [illegible] Tris, 1 mM EGTA, pH 7.5). The cells were placed in respiratory medium containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/l [illegible], 2 μM oligomycin, 1 mM of succinate and either 20 nM [illegible] or 20 nM of MitoSOX. The cells were then incubated in the respiratory medium for 5 minutes on a heated stage maintained at 37℃. Digitonin (2.5 μg/ml) was added to permeabilize the plasma membrane. Images containing 200-250 cells were taken at 1 minute intervals for 20 minutes on the Olympus IX81 microscope using a 20× objective. Recombinant truncated Bid was added after 2 minutes, and CCCP was added after 18 minutes to induce complete depolarization. For superoxide anion production, cells were loaded with 5 μM MitoSOX for 30 minutes. Treatment and image acquisition was similar to TMRM except that an increase in ROS dependent MitoSOX fluorescence was measured over 20 minutes. Images were analyzed using Slidebook software. For TMRM, regions of interest were drawn around cells in the acquired images, pixel intensities were measured and averaged for each image. A line graph for each condition was drawn in SigmaPlot using the formula: % intensity=(observed intensity-final image intensity)/(first image intensity-final image intensity)*100. For MitoSOX, intensity measurements were done similar to TMRM experiments. The increase in fluorescence intensity at one minute intervals was compared to intensity of the first image acquired (at zero minute time point) and represented as percent using the formula: % increase in fluorescence intensity=(observed image intensity-first image intensity)/(first image intensity)*100. A line graph was made for the 2 and 20 minutes time points. At the end of each experiment, cells from four wells per condition were collected and used to determine cytochrome c retention.

Western blotting

Protein samples (20 μg/lane) were separated on Novex 10% Tris/Glycine precast gels in the XCell II module with MagicMark XP (all from Invitrogen) as the molecular weight marker. Proteins were immuno-blotted onto PVDF membranes using the XCell II Blot module (Invitrogen). Mcl-1, Bcl-X_L, SIRT3, hexokinase II, VDAC-1, tubulin, UQCRC-1, prohibitin, lamin A/C, βactin, GAPDH and PARP were detected using mouse monoclonal antibodies (from Sigma, Cell Signaling and MitoSciences) at 1:1000 dilution. BAK monoclonal antibody also was used at 1:1000 dilution (Cell Signaling). Cytochrome c in the pellets collected after time lapse fluorescence microscopy was detected using mouse anti-cytochrome c antibody (BD Pharmingen) at 1:1000 dilution. Appropriate horseradish peroxidase-labeled secondary antibodies (1:10,000) were used to detect the relevant proteins by enhanced chemiluminescence.

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