

## RETRACTION

# Retraction: Sirtuin-3 deacetylation of cyclophilin D induces dissociation of hexokinase II from the mitochondria

Nataly Shulga, Robin Wilson-Smith and John G. Pastorino

Retraction of: *J. Cell Sci.* **123**, 894-902.

This article has been retracted at the request of the corresponding author, John G. Pastorino.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 TNF $\alpha$ -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 deacetylation of cyclophilin D induces dissociation of hexokinase II from the mitochondria

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## Summary

We demonstrate that the transition from a reliance on glycolysis to oxidative phosphorylation in a transformed cell line is dependent on an increase in the levels and activity of sirtuin-3. Sirtuin-3 deacetylates cyclophilin D, diminishing its peptidyl-prolyl cis-trans isomerase activity and inducing its dissociation from the adenine nucleotide translocator. Moreover, the sirtuin-3-induced inactivation of cyclophilin D causes a detachment of hexokinase II from the mitochondria that is necessary for stimulation of oxidative phosphorylation. These results might have important implications for the role of sirtuin-3 in the metabolism of some cancer cells and their susceptibility to mitochondrial injury and cytotoxicity.

**Key words:** Sirtuin-3, Hexokinase II, Mitochondria, Cyclophilin D

## Introduction

Sirtuin (SIRT) proteins are class III NAD<sup>+</sup>-dependent histone deacetylases that have been implicated in a variety of functions including aging and regulation of metabolism (Saunders and Verdin, 2007; Schwer and Verdin, 2008). There are currently seven known sirtuin family members. Sirtuin-3, sirtuin-4 and sirtuin-6 are localized to the mitochondria. Sirtuin-3 is the best characterized of the mitochondrial sirtuin proteins and is localized to the mitochondrial matrix. Sirtuin-3 has been shown to deacetylate and activate acetyl-CoA synthetase 2 and glutamate dehydrogenase (GDH), leading to an enhancement of the tricarboxylic acid cycle (Hallows et al., 2006; Schlicker et al., 2008). The activation of acetyl-CoA synthetase 2 and GDH is in line with the ability of sirtuin-3 to regulate mitochondrial oxidative phosphorylation. Sirtuin-3-knockout mice exhibit massive acetylation of mitochondrial proteins, along with a suppression of oxidative phosphorylation and a decrease in ATP levels under stress conditions (Ahn et al., 2008).

Many cancers exhibit a decrease in mitochondrial oxidative phosphorylation (Ma et al., 2007; Modica-Napolitano et al., 2007; Pan and Mak, 2007). The basis for the reduction in oxidative phosphorylation is not clear, but is compensated for by a greatly accelerated rate of glycolysis. The increased rate of glycolysis not only helps to maintain ATP levels, but also provides the growing and dividing cancer cell with required biosynthetic precursors. This aspect of cancer cell metabolism is partly reliant on the binding of hexokinase II to the mitochondria mediated by the outer mitochondrial membrane protein VDAC (voltage-dependent anion channel) (Pedersen, 2007). The increased binding of hexokinase II also inhibits damage to the mitochondria and is associated with increasing the resistance of cancer cells to cytotoxicity (Abu-Hamad et al., 2008; Azoulay-Zohar et al., 2004; Shoshan-Barmatz et al., 2008).

The amplified binding of hexokinase II to mitochondria is mediated in part by the Akt signaling pathway, which is frequently deregulated in transformed cells (Majewski et al., 2004). However,

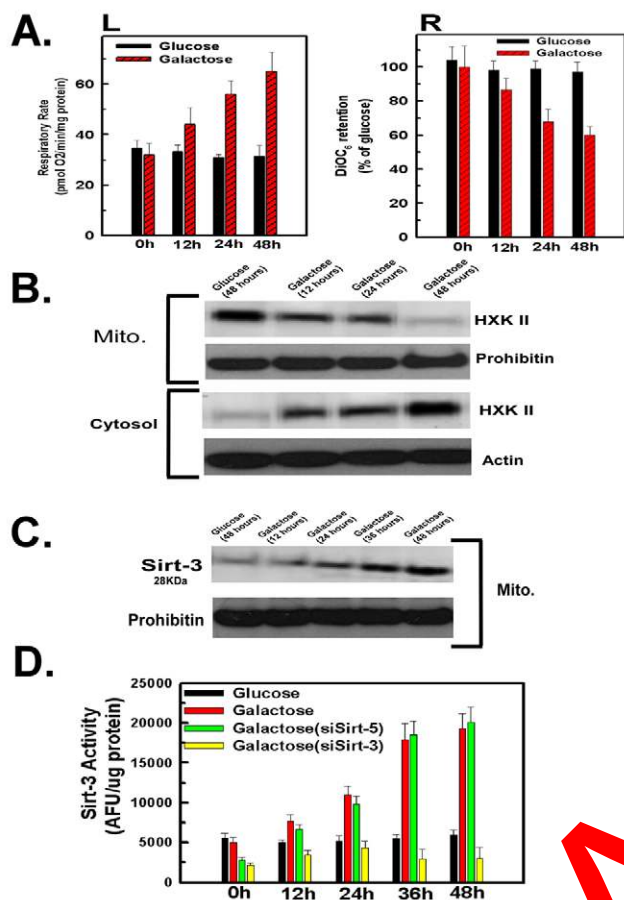
it has been demonstrated that cyclophilin D (also known as peptidyl-prolyl cis-trans isomerase D or PPID), which localizes to the mitochondrial matrix, is also necessary to promote the binding of hexokinase II to VDAC. Inhibition of the peptidyl-prolyl cis-trans isomerase activity of cyclophilin D causes detachment of hexokinase II from the mitochondria (Machida et al., 2006).

The present study demonstrates that when HeLa, HCT-116 or MDA-MB-231 cells use galactose rather than glucose as an energy substrate, thereby increasing the need for oxidative phosphorylation, some of the features that are characteristic of transformed cells are reversed, such as an increase in mitochondrial respiratory rate and decrease in mitochondrial membrane potential. Importantly, the transition from a reliance on glycolysis to oxidative phosphorylation requires an increase in the expression and activity of sirtuin-3. Moreover, the increased sirtuin-3 activity causes a marked redistribution of hexokinase II from the mitochondria to the cytosol. This is mediated in part by a sirtuin-3-dependent deacetylation of cyclophilin D, which causes an inhibition of its peptidyl-prolyl cis-trans isomerase activity and its dissociation from the adenine nucleotide translocator.

## Results

### Galactose stimulates mitochondrial respiration and the release of hexokinase II from the mitochondria

As shown in Fig. 1A, left panel, HeLa cells displayed a steady increase in respiratory rate when transferred from a glucose-based medium to a galactose-based medium, reaching a nearly twofold increase of respiratory rate after 48 hours. The increase in respiratory rate was mirrored by a decrease in the mitochondrial membrane potential as assessed by DiOC<sub>6</sub> fluorescence, decreased by 50% over 48 hours when the cells were incubated in galactose as opposed to glucose (Fig. 1A, right panel). Such results suggest that incubation in a galactose-based medium compels the cell to enhance the activity of mitochondrial oxidative phosphorylation.



**Fig. 1. Transfer to galactose-based medium stimulates mitochondrial oxidative phosphorylation, sirtuin-3 expression and release of hexokinase II from the mitochondria.** (A) HeLa cells incubated in DMEM containing glucose (4.5 g/l) or transferred to DMEM containing galactose (4.5 g/l). Oxygen consumption was measured in a thermostatically controlled chamber equipped with a Clarke-type oxygen electrode. For mitochondrial membrane potential measurements, 40 nM of DiOC<sub>2</sub> was added during the last 30 minutes of incubation. Fluorescence intensity was measured on a fluorescent plate reader. (B,C) The mitochondrial or cytosolic fractions were used for western blotting and probed with antibodies against hexokinase II (HXK2) or sirtuin-3 (Sirt-3). Actin and prohibitin were used as loading controls for the cytosolic and mitochondrial fractions, respectively. (D) Mitochondrial extracts were prepared and sirtuin-3 activity was measured using a fluorometric assay. All values are the means of triplicate samples; error bars indicate s.d.

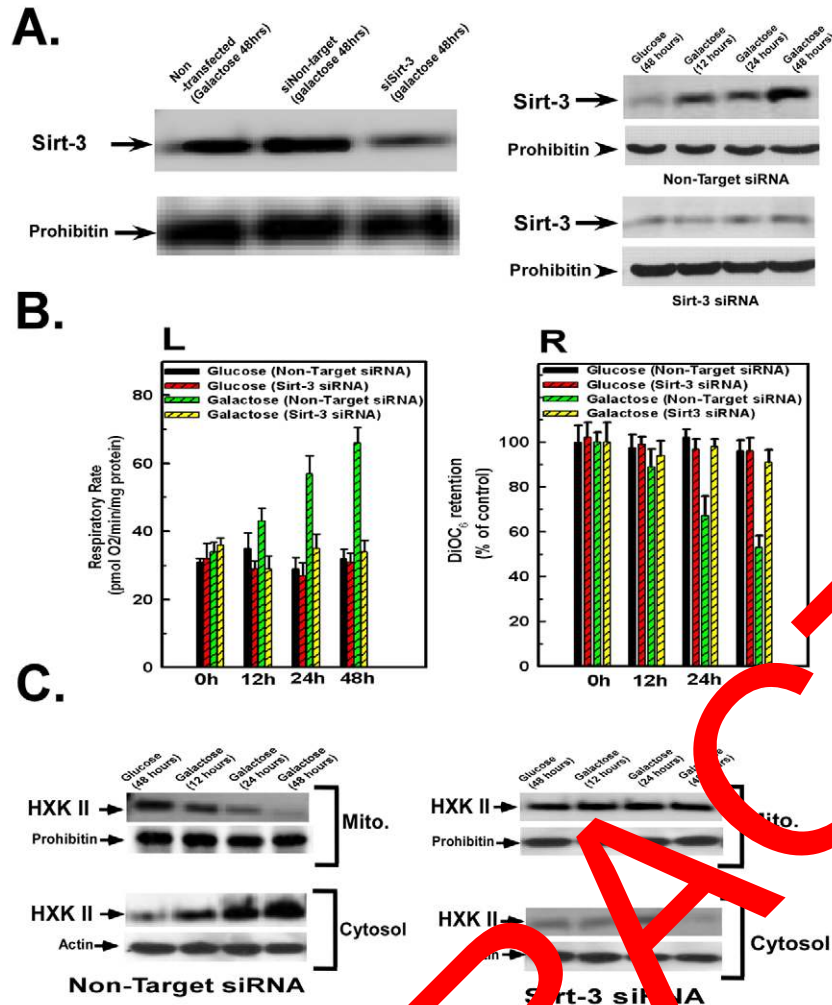
Many cancer cells display a greatly increased binding of hexokinase II to the mitochondria that aids in the accelerated rate of glycolysis (Pedersen, 2007; Pedersen et al., 2002). Therefore, we wanted to determine whether the shift to a greater dependence on oxidative phosphorylation had any influence on the expression or localization of hexokinase II. When the cells were incubated in glucose-based medium for 48 hours, the majority of the hexokinase II was associated with the mitochondrial fraction (Fig. 1B, lane 1). By contrast, transfer to galactose-based medium provoked a marked redistribution of hexokinase II from the mitochondria to the cytosol over a 48 hour time course (Fig. 1B, lanes 2-4). Importantly, despite the redistribution of hexokinase II from the mitochondria to the cytosol, transfer to galactose-based medium did not alter the level of hexokinase II expression.

Transfer to galactose-based medium caused a dramatic upregulation in the expression of the 28 kDa form of sirtuin-3 in the mitochondria at 48 hours, compared with levels in cells maintained in a glucose-based medium over the same time period (Fig. 1C, lane 1 versus lane 5). Notably, the increased expression of sirtuin-3 in galactose was matched with an elevation of sirtuin-3 activity in the mitochondrial fraction (Fig. 1D). HeLa cells incubated in galactose demonstrate a fourfold elevation of sirtuin-3 activity after 48 hours incubation compared with cells incubated in glucose-based medium. Sirtuin-5 was also localized to the mitochondria. To determine whether the effect was specific for sirtuin-3, siRNA was used to suppress the expression of sirtuin-5 or sirtuin-3. Importantly, suppression of sirtuin-3 expression largely prevented the increase in sirtuin-3 activity measured in mitochondrial extracts when the cells were transferred to a galactose-based medium (Fig. 1D). By contrast, suppression of sirtuin-5 only had an effect on the basal sirtuin-3 activity seen in cells incubated in glucose-based medium.

### The galactose-induced increase of mitochondrial oxidative phosphorylation and hexokinase II detachment from mitochondria is Sirtuin-3 dependent

The concomitant increase in respiration rate and sirtuin-3 expression upon transfer to galactose infers that sirtuin-3 might mediate the adaptation from a reliance on glycolysis to oxidative phosphorylation. Therefore, we used siRNA-mediated suppression of sirtuin-3 levels to determine its role. Transfection of HeLa cells with siRNA targeting sirtuin-3 suppressed the induction of sirtuin-3 expression brought about by transfer to galactose-based medium (Fig. 2A, left panel), whereas the non-targeting control siRNA had no effect. The induction of sirtuin-3 expression started at 12 hours after transfer to galactose-based medium and was maximal at 48 hours (Fig. 2A, right panel), and was suppressed by transfection with siRNA targeting sirtuin-3, but not the non-targeting control, all time points. Importantly, the suppression of sirtuin-3 expression prevented the stimulation in respiratory rate that occurred over 48 hours in HeLa cells transferred to galactose, whereas the non-targeting control did not (Fig. 2B, left panel). Similarly, the suppression of sirtuin-3 expression prevented the decline that occurs in mitochondrial membrane potential when the cells were incubated in galactose for 48 hours (Fig. 2B, right panel), indicating that sirtuin-3 expression is necessary for the mitochondrial membrane potential to be utilized. By contrast, suppression of sirtuin-3 expression had no effect on mitochondrial membrane potential in cells that were maintained in glucose-based medium (Fig. 2B, right panel).

Since sirtuin-3 expression was necessary for the increase in oxidative phosphorylation, we wanted to determine whether it was also required for the dissociation of hexokinase II from the mitochondria. Transfection of HeLa cells with non-target control siRNA did not prevent the redistribution of hexokinase II from the mitochondria to the cytosol over 48 hours upon transfer to galactose (Fig. 2C, left panels, lanes 2-4). However, when sirtuin-3 expression was repressed by siRNA, the dissociation of hexokinase II from the mitochondria induced by incubation in galactose-based medium was prevented (Fig. 2C, right panels, lanes 2-4). Suppression of sirtuin-3 had no effect on hexokinase II distribution when the cells were incubated in glucose-based medium for 48 hours (Fig. 2C, left and right panels, lane 1).



**Fig. 4. Sirtuin-3 is required for the induction of oxidative phosphorylation and detachment of hexokinase II from the mitochondria.** (A) HeLa cells were transfected with siRNA targeting sirtuin-3 or with a non-targeting control siRNA. Cells were then incubated in DMEM containing glucose or galactose. At the times indicated, the cells were harvested and mitochondria isolated. The mitochondrial fractions were then western blotted and probed with antibodies against sirtuin-3 or prohibitin as a loading control. (B) Oxygen consumption was measured in a thermostatically controlled chamber equipped with an oxygen electrode. To measure mitochondrial membrane potential, the cells were washed and 40 nM DiOC<sub>6</sub> was added during the last 30 minutes of incubation. Values are the means from triplicate samples; error bars indicate s.d. (C) The mitochondrial or cytosolic fractions were western blotted and probed with antibodies against hexokinase II. Actin and prohibitin were used as loading controls for the cytosolic and mitochondrial fractions, respectively.

Importantly, as shown in Fig. 4A, two transformed cell lines (HCT-116 and MDA-MB-231) that displayed an increase in glycolysis and hexokinase II expression also displayed an increase in sirtuin-3 expression when transferred from a glucose- to galactose-based medium. Additionally, transfer to galactose triggered a redistribution of hexokinase II from the mitochondria to the cytosol (Fig. 4B). These effects of galactose on MDA-MB-231 and HCT-116 cells were paralleled by an increase and decrease, respectively, in the respiratory rate and mitochondrial membrane potential (Fig. 4B). However, mouse embryonic fibroblasts, an immortalized non-transformed cell line, did not display a significant change in respiration or mitochondrial membrane potential when transferred from glucose to galactose. Significantly, MEFs did not overexpress hexokinase II, unlike many transformed cells.

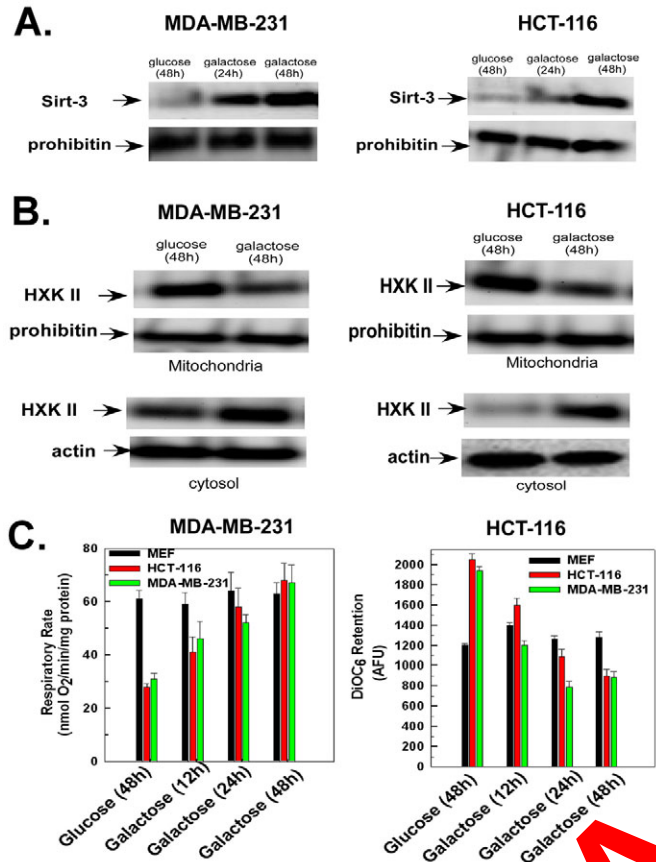
#### Sirtuin-4 and sirtuin-5 are not required for increased mitochondrial respiration or hexokinase detachment from the mitochondria

Sirtuin-4 and sirtuin-5 were also localized to the mitochondria. However, as shown in Fig. 4A, left panel, incubation of HeLa cells in galactose for 24 and 48 hours did not induce an increase in the expression of sirtuin-4 or sirtuin-5, as it did the expression of sirtuin-3. The expression of sirtuin-4 or sirtuin-5 was suppressed selectively

with siRNA (Fig. 4A, right panel). Importantly, as opposed to sirtuin-3, suppression of sirtuin-4 or sirtuin-5 expression did not prevent an increase and decrease, respectively, of respiratory rate or mitochondrial membrane potential when cells were transferred to galactose-based medium (Fig. 4B). Additionally, suppression of expression of sirtuin-4 or sirtuin-5 did not prevent the galactose-induced redistribution of hexokinase II from mitochondria to the cytosol (Fig. 4C).

#### Sirtuin-3 deacetylates and inactivates cyclophilin D

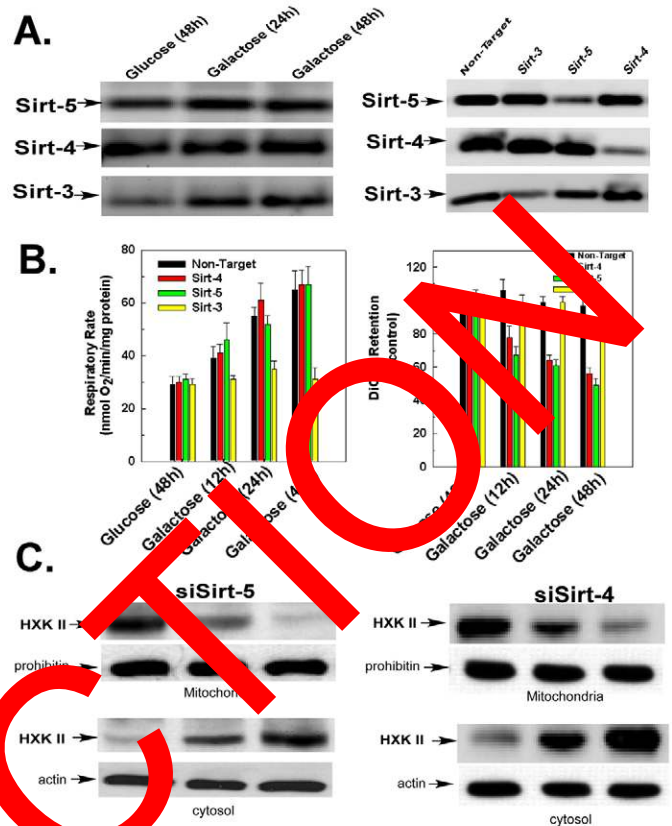
Hexokinase II binds to the VDAC on the outer mitochondrial membrane (Nakashima, 1989; Wilson, 2003). Sirtuin-3 is localized to the mitochondrial matrix, indicating that its modulation of hexokinase II binding must be indirect. Cyclophilin D has been shown to affect the binding of hexokinase II to the mitochondria and is also localized to the mitochondrial matrix (Machida et al., 2006). Therefore, cyclophilin D was immunoprecipitated and its acetylation status determined with anti-acetylated-lysine antibodies. Cyclophilin D was acetylated in HeLa cells when incubated in glucose-based medium for 48 hours (Fig. 5A, top panels, lane 1). However, cyclophilin D underwent progressive deacetylation when the cells were transferred to galactose-based medium, with near complete deacetylation by 48 hours, which was not prevented by transfection with non-targeting siRNA (Fig. 5A, left panel, lanes



**Fig. 3.** HCT-116 and MDA-MB-231 cells display an increase in sirtuin-3 expression when transferred to galactose-based medium. (A) HCT-116 or MDA-MB-231 cells incubated in DMEM containing glucose or galactose for the times indicated. The mitochondrial or cytosolic fractions were used for western blotting and probed with antibodies against hexokinase II and sirtuin-3. Actin and prohibitin were used as loading controls for the cytosolic and mitochondrial fractions, respectively. (B) HCT-116 and MDA-MB-231 cells or mouse embryonic fibroblasts (MEF) incubated in DMEM containing glucose for 48 hours or galactose for the times indicated. Oxygen consumption was measured in a thermostatically controlled chamber equipped with an oxygen electrode. Mitochondrial membrane potential was measured after addition of DiOC<sub>6</sub> for the last 30 minutes of incubation. Values are the means from triplicate samples and the error bars indicate s.d.

2-4). By contrast, transfection with siRNA targeting sirtuin-3 inhibited cyclophilin D deacetylation induced by transfer to galactose, indicating that the deacetylation of cyclophilin D is dependent on sirtuin-3 activity (Fig. 5A, right panel). Significantly, transfer of HCT-116 and MDA-MB-231 cells from glucose to galactose also caused an increase in cyclophilin D acetylation that was prevented by suppression of sirtuin-3 expression (Fig. 5B).

Cyclophilin D possesses peptidyl-prolyl cis-trans isomerase activity. Fig. 5C shows that the activity of cyclophilin D was unchanged when incubated in glucose for 48 hours in the presence or absence of sirtuin-3 expression. However, in parallel with its deacetylation in galactose, the peptidyl-prolyl cis-trans isomerase activity of cyclophilin D diminished over 48 hours, when the cells were transferred to galactose, and this was not prevented by transfection with non-targeting siRNA. However, suppression of sirtuin-3 expression by transfection with siRNA targeting sirtuin-3

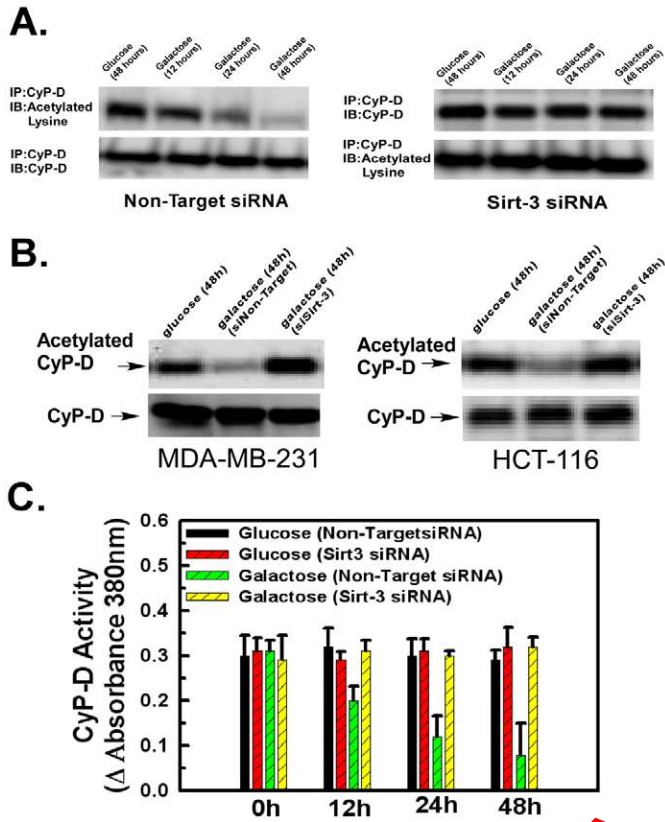


**Fig. 4.** Sirtuin-5 and sirtuin-4 are not upregulated by transfer to galactose-based medium and do not mediate an increase in mitochondrial function. (A) HeLa cells transfected with siRNA targeting sirtuin-3, sirtuin-4 or sirtuin-5 incubated in DMEM containing glucose or galactose (left panel). Mitochondrial fractions were western blotted and probed with antibodies against sirtuin-3, sirtuin-4 or sirtuin-5. (B) Oxygen consumption was measured in a thermostatically controlled chamber equipped with an oxygen electrode. Mitochondrial membrane potential was determined after addition of DiOC<sub>6</sub> for the last 30 minutes of incubation. The values are the means from triplicate samples and the error bars indicate s.d. (C) HeLa cells transfected with siRNA targeting sirtuin-4 or sirtuin-5 incubated in DMEM containing glucose or galactose. The mitochondria or cytosolic fractions were western blotted and probed with antibodies against hexokinase II. Actin and prohibitin were used as loading controls for the cytosolic fractions and mitochondrial fractions, respectively.

largely prevented the decrease in cyclophilin D peptidyl-prolyl cis-trans isomerase activity upon transfer to galactose (Fig. 5C), thereby indicating that deacetylation of cyclophilin D by sirtuin-3 decreases its enzymatic activity.

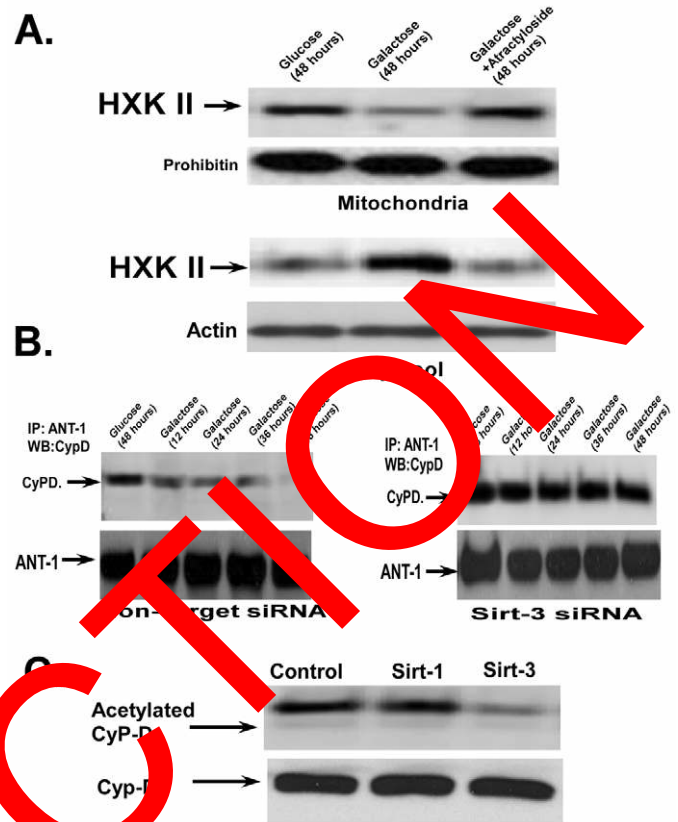
#### Sirtuin-3 induces dissociation of cyclophilin D from the adenine nucleotide translocator

Hexokinase II binds to VDAC localized to contact sites between the outer and inner mitochondrial membranes, which are formed by the adenine nucleotide carrier (ANT) (Beutner et al., 1998; Brdiczka et al., 2006). Additionally, cyclophilin D interacts with and promotes conformational alterations in the ANT (Bauer et al., 1999; Woodfield et al., 1998). Therefore, we wanted to determine whether modulation of the ANT could influence the ability of sirtuin-3 to detach hexokinase II from the mitochondria. Atractyloside binds to the ANT and locks the ANT in the cytosolic



**Fig. 5. Sirtuin-3 deacetylates and inhibits the peptidyl-prolyl cis-trans isomerase activity of cyclophilin D.** HeLa cells (A) or HCT-116 and MDA-MB-231 cells (B) were transfected with siRNA targeting sirtuin-3 or with non-targeting siRNA incubated in DMEM containing glucose or galactose. Cyclophilin D was immunoprecipitated. The western blot was probed with antibodies against acetylated lysine or cyclophilin D. (C) HeLa cells transfected with siRNA targeting sirtuin-3 or with non-targeting siRNA. Cyclophilin D was immunoprecipitated from mitochondrial lysates and its peptidyl-prolyl cis-trans isomerase activity determined. Values are the means from triplicate samples, and the error bars indicate s.d.

conformation, a configuration that promotes the binding of hexokinase II to VDAC (Brdiczka et al., 2000; Vieira et al., 2000). As shown in Fig. 6A, incubation of the cells with atractyloside prevented the dissociation of hexokinase II from the mitochondria when the cells were incubated in galactose-based medium for 48 hours, indicating that sirtuin-3 mediates its effects on hexokinase II binding through the ANT1. Importantly, cyclophilin D has been shown to interact with the ANT1 (Sauter et al., 1996). Cyclophilin D co-immunoprecipitates with ANT1 protein when the cells are incubated in glucose-based medium for 48 hours, indicating that cyclophilin D binds to ANT1 when acetylated (Fig. 6B, left panels, lane 1). However, when the cells were transferred to galactose, there was a progressive decrease, over 48 hours, in the level of cyclophilin D co-immunoprecipitated with ANT1 (Fig. 6B, left panels, lanes 2-5). Importantly, this was not prevented by transfection with non-targeting siRNA. By contrast, suppression of sirtuin-3 expression prevented the dissociation of cyclophilin D from ANT1 when the cells were incubated in galactose (Fig. 6B, right panels). These results suggest that the sirtuin-3-mediated deacetylation of cyclophilin D is necessary for the interaction between cyclophilin D and ANT1 to be disrupted.



**Fig. 6. Sirtuin-3 prevents binding of cyclophilin D to the adenine nucleotide translocator.** (A) HeLa cells incubated in DMEM containing glucose or transferred to medium containing galactose in the absence or presence of 10  $\mu$ M atractyloside. The western blot was probed with antibodies against hexokinase II. Actin and prohibitin were used as loading controls for the cytosolic fractions and mitochondrial fractions, respectively. (B) HeLa cells transfected with siRNA targeting sirtuin-3 or with a non-targeting siRNA incubated in DMEM containing glucose or galactose. ANT1 was immunoprecipitated from mitochondrial extracts. The western blot was then probed with antibodies against cyclophilin D or ANT1. (C) HeLa cells incubated in DMEM containing glucose were harvested and the mitochondrial fraction isolated. Immunoprecipitated cyclophilin D was incubated with recombinant sirtuin-1 or sirtuin-3. Following incubation, the reaction was western blotted and probed with antibodies against acetylated lysine or cyclophilin D.

We then determined whether sirtuin-3 could deacetylate cyclophilin D *in vitro*. Cyclophilin D was immunoprecipitated from HeLa cells grown in glucose-based medium and then incubated with recombinant sirtuin-1 or sirtuin-3 followed by western blotting with anti-acetylated-lysine antibody. As shown in Fig. 6C, sirtuin-3 was able to deacetylate cyclophilin D *in vitro*, whereas sirtuin-1 was ineffective.

#### Deacetylation of Lys145 controls cyclophilin D activity and its interaction with ANT1

A database search indicated that cyclophilin A is possibly acetylated at Lys125. Cyclophilin D displays a homologous lysine at position 145. Therefore Lys145 of cyclophilin D was mutated to a glutamine or arginine residue to preserve the neutral and positive charge of acetylation or deacetylation, respectively and stably transfected into HeLa cells. FLAG-tagged wild-type cyclophilin D, cyclophilin D

(K145Q) and cyclophilin D (K145R) were expressed in HeLa cells that were incubated in glucose- or galactose-based medium for 48 hours. The cyclophilin D proteins were then immunoprecipitated with anti-FLAG antibody and their peptidyl-prolyl cis-trans isomerase activity was determined.

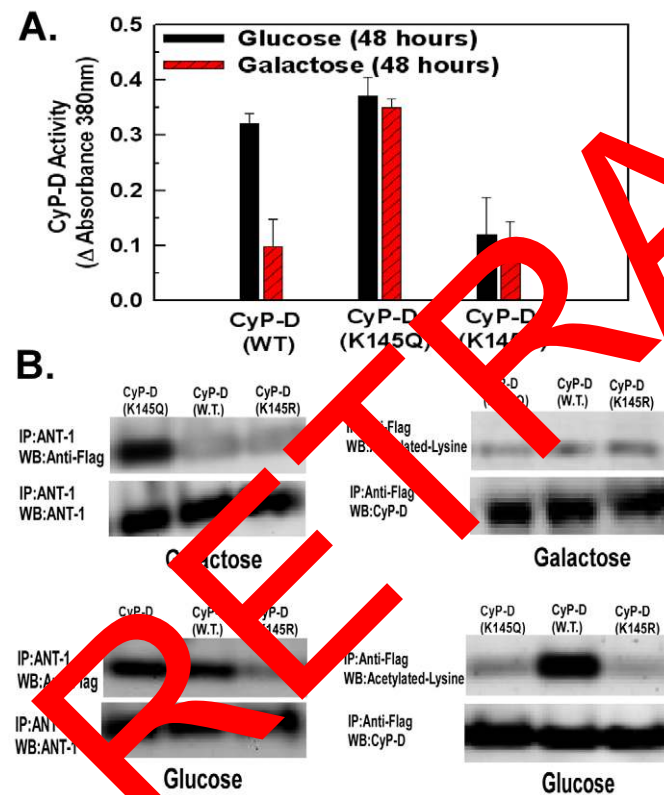
As shown in Fig. 7A, wild-type cyclophilin D displayed a decrease in activity when the cells were incubated in galactose-based medium, similarly to the decrease of native cyclophilin D activity detected in mitochondrial extracts of cells incubated in galactose medium (Fig. 5B). Conversely, cyclophilin D (K145Q) did not exhibit a decrease in enzymatic activity when incubated in galactose-based medium, in agreement with the ability of a glutamine residue to negate the negative charge of a lysine residue that is acetylated. By contrast, cyclophilin D (K145R) displayed reduced activity in both glucose- and galactose-based medium, indicating that the constitutive positive charge of arginine suppresses cyclophilin D activity.

The ability of wild-type cyclophilin D and the mutants to bind ANT1 and their acetylation status in glucose- or galactose-based medium was then determined. Wild-type cyclophilin D bound to ANT1 in glucose but not galactose, (Fig. 7B, right panels). By contrast, cyclophilin D (K145Q) remained bound to ANT1 in

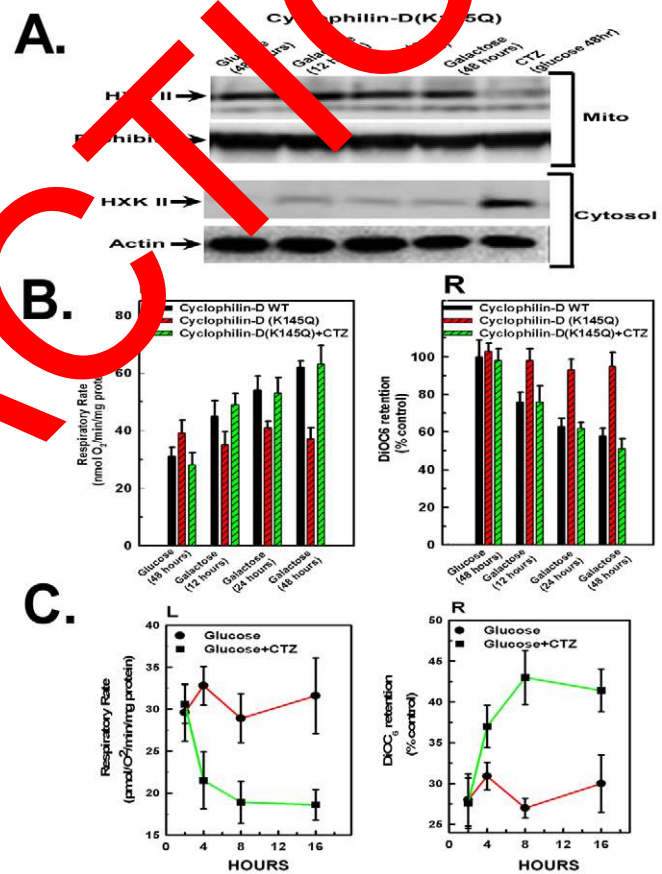
galactose-based medium, indicating the importance of cyclophilin D deacetylation for dissociation from ANT1. However, as anticipated, cyclophilin D (K145R) did not bind to ANT1 in either glucose- or galactose-based medium. Neither of the mutants was acetylated in glucose-based medium, whereas wild-type cyclophilin D was acetylated in glucose-based medium, but was deacetylated in galactose-based medium (Fig. 7B, right panels).

### Deacetylation of cyclophilin D is necessary for dissociation of hexokinase II from the mitochondria and enhanced oxidative phosphorylation

As demonstrated in Fig. 8A, expression of cyclophilin D (K145Q) prevented the redistribution, over a 48-hour time period, of hexokinase II from the mitochondria to the cytosol when the cells



**Fig. 7. Acetylation of Lys145 of cyclophilin D modulates enzyme activity and interaction with ANT1.** (A) HeLa cells overexpressing wild-type cyclophilin D, cyclophilin D (K145Q) or cyclophilin D (K145R) were transferred to DMEM medium containing glucose or galactose. Recombinant cyclophilins were isolated using anti-FLAG antibody for immunoprecipitation. The peptidyl-prolyl cis-trans isomerase activity of the cyclophilins was determined. The values are the means from triplicate samples, and the error bars indicate s.d. (B) Mitochondrial extracts were immunoprecipitated with anti-ANT1 or anti-FLAG antibody and immunoblotted with anti-FLAG or anti-acetylated-lysine antibody, respectively.



**Fig. 8. Sirtuin-3-induced detachment of hexokinase II from the mitochondria is necessary but not sufficient for stimulation of oxidative phosphorylation.** (A) HeLa cells overexpressing cyclophilin D (K145Q) incubated in DMEM containing glucose in the absence or presence of 15  $\mu$ M clotrimazole or transferred to galactose. The western blots were probed with antibodies against hexokinase II. Actin and prohibitin were used as loading controls for the cytosolic and mitochondrial fractions, respectively. (B) HeLa cells overexpressing cyclophilin D (WT) or cyclophilin D (K145Q). Oxygen consumption was measured in a thermostatically controlled chamber equipped with a Clarke-type oxygen electrode. For measurement of mitochondrial membrane potential, the cells were incubated in 40 nM DiOC<sub>6</sub> for the final 30 minutes. (C) HeLa cells were incubated in DMEM containing glucose, in the absence or presence of 15  $\mu$ M clotrimazole. Oxygen consumption and mitochondrial membrane potential are shown. Values are the means of triplicate samples; error bars indicate s.d.

were transferred to galactose-based medium. Moreover, cyclophilin D (K145Q) expression suppressed the increase in respiratory rate (Fig. 8B, left panel) and prevented the decline of the mitochondrial membrane potential (Fig. 8B, right panel) that was initiated when the cells were transferred to galactose medium, suggesting that the dissociation of cyclophilin D from ANT1 and subsequent hexokinase II detachment from the mitochondria is necessary for an increase of oxidative phosphorylation. By contrast, expression of wild-type cyclophilin D did not prevent the galactose-induced stimulation or decline, respectively, of mitochondrial respiration or membrane potential (Fig. 8B, panels L and R).

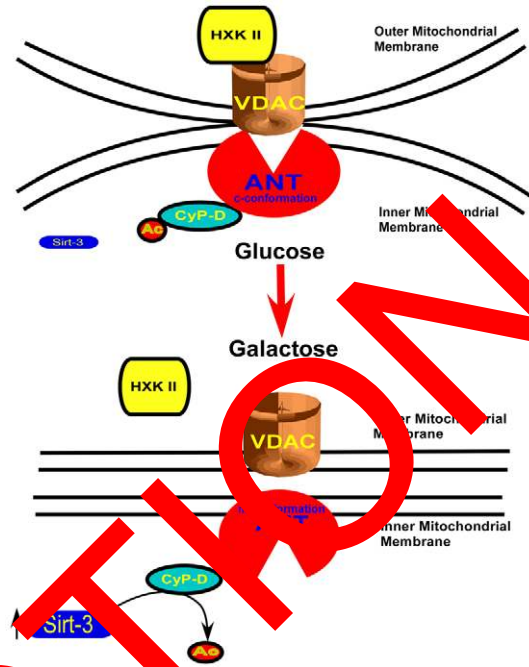
Clotrimazole has been shown to promote the dissociation of hexokinase II from the mitochondria (Penso and Beitner, 1998). Clotrimazole forced the dissociation of hexokinase II from the mitochondria to the cytosol in HeLa cells expressing cyclophilin D (K145Q) when the cells were incubated in glucose-based medium (Fig. 8A, lane 5). Intriguingly, enforced detachment of hexokinase II from the mitochondria with clotrimazole restored the increase in respiratory rate and decrease in mitochondrial membrane potential in HeLa cells expressing cyclophilin D (K145Q) when the cells were transferred to galactose-based medium (Fig. 8B, panels L and R, respectively). However, detachment of hexokinase II from the mitochondria does not in itself increase oxidative phosphorylation. As shown in Fig. 8C, when incubated in glucose-based medium, clotrimazole did not provoke an increase in mitochondrial respiratory rate or utilization of membrane potential. On the contrary, when the cells were incubated in glucose medium, the clotrimazole induced detachment of hexokinase II from the mitochondria rapidly provoked a further decrease and decrease, respectively, of the mitochondrial respiratory rate and membrane potential, above that of nontreated controls (Fig. 8C). These results demonstrate that sirtuin-3-induced dissociation of hexokinase II from the mitochondria is necessary, but not sufficient, for an increase of mitochondrial activity, and that sirtuin-3 must act on additional targets for stimulation of oxidative phosphorylation to occur.

## Discussion

The present study demonstrates that sirtuin-3 is required for adaptation from a primarily glycolytically based metabolism to one with an enhanced component of mitochondrial respiration. Additionally, the sirtuin-3-mediated deacetylation of cyclophilin D provokes a dissociation of hexokinase II from the mitochondria, which when prevented, inhibits the increase in mitochondrial respiration induced by transfer to a galactose-based medium.

Transformed cells are capable of high rates of glycolysis, even in the presence of adequate oxygenation. The corollary to the dramatic increase in glycolysis and lactate production is an attenuation of oxidative phosphorylation. The reduction of oxidative phosphorylation exhibited by many cancer cells is manifested by a decrease in mitochondrial respiratory rate and increase of mitochondrial membrane potential (Fantin et al., 2006). However, HeLa cells incubated in medium containing galactose as an alternate sugar source display prominent oxidative phosphorylation (Reitzer et al., 1979; Rossignol et al., 2004). The present study indicates that the mechanistic basis for the control exerted by metabolic substrates depends in part on regulation of sirtuin-3 expression and the effect that sirtuin-3 activity has on cyclophilin D, and in turn, hexokinase II binding to the mitochondria.

Sirtuin-3 was identified as a nuclear-encoded, mitochondria-localized homologue of the human silent information regulator SIR2 (Onyango et al., 2002; Schwer et al., 2002). Sirtuin-3 is processed



**Fig. 9. Sirtuin-3-mediated cyclophilin D deacetylation triggers hexokinase II detachment.** Transfer to galactose-based medium causes an increase in the expression and activity of sirtuin-3. In turn, sirtuin-3 (Sirt-3) promotes deacetylation of cyclophilin D (Cyp-D), causing it to dissociate from ANT1, which then promotes the detachment of hexokinase II (HXK II) from the mitochondria. VDAC, voltage-dependent anion channel.

into a 28 kDa protein, the predominant form that we identified in cells. However there is controversy regarding the localization of sirtuin-3. One report finds that sirtuin-3 is localized to the nucleus as a 44 kDa precursor that is processed to the 28 kDa form when translocated to the mitochondria under stress conditions (Scher et al., 2007). However, another study reports that the localization of sirtuin-3 is exclusively mitochondrial (Cooper and Spelbrink, 2008). We did find low expression of a full-length 44 kDa form in the nucleus. However, its expression was not influenced by the change in metabolic profile induced by incubation in galactose-based medium.

Sirtuin-3 was shown to activate thermogenesis in brown adipocytes, suggesting that sirtuin-3 has a role in mitochondrial energy metabolism (Shi et al., 2005). The first identified substrate of sirtuin-3 was acetyl-CoA synthetase 2 (AceCS2), which it deacetylates and activates (Hallows et al., 2006; Schwer et al., 2006). AceCS2 produces an increase in tricarboxylic acid cycle activity. Recently, glutamate dehydrogenase (GDH) and isocitrate dehydrogenase have also been found to be deacetylated and activated by sirtuin-3 (Lombard et al., 2007; Schlicker et al., 2008). However, sirtuin-3-knockout mice displayed no metabolic differences from control mice under feeding or fasting conditions (Lombard et al., 2007). By contrast, another study using mice lacking sirtuin-3 identified marked depletion of ATP levels in the heart, kidney and liver, suggesting that sirtuin-3 is crucial in mediating oxidative phosphorylation (Ahn et al., 2008). Additionally, sirtuin-3 was found to deacetylate and associate with complex I of the mitochondrial respiratory chain, suggesting that complex I activity is also regulated by acetylation status.



Hexokinase II binds to VDAC that is localized to contact sites, where VDAC interacts with the adenine nucleotide translocator (ANT) (Ardail et al., 1990; Beutner et al., 1996; Brdiczka et al., 1998). In turn, cyclophilin D interacts with and binds to the ANT, where it alters the ANT conformation (Crompton et al., 1998). The ANT can assume a cytosolic or matrix conformation. Binding of hexokinase II to VDAC is promoted when the ANT is in the cytosolic conformation. Indeed, in the present study, arcteoside, which promotes the cytosolic conformation of the ANT, prevented hexokinase II detachment when the cells were incubated in galactose, indicating that cyclophilin D exerts its effects on hexokinase II binding by controlling the conformation of ANT. A transition in the ANT conformation upon a switch from glucose to galactose is consistent with observations in electron micrographs demonstrating a change in mitochondrial structure from an orthodox configuration to a condensed one (Rossignol et al., 2004). A restructuring of the mitochondria to a condensed configuration in galactose-based medium with the ANT assuming the matrix conformation would not be conducive to hexokinase II binding to the mitochondria, whereas incubation in glucose maintains the orthodox configuration when the ANT is in the cytosolic conformation, which is more conducive to hexokinase II interaction with the mitochondria.

Expression of cyclophilin D (K145Q), where Lys145 is mutated to glutamine, thereby preserving the neutral charge and binding of cyclophilin D to ANT1, prevented the resulting increase in mitochondrial respiration, decrease in mitochondrial membrane potential and the detachment of hexokinase II from the mitochondria, brought about by transfer to galactose-based medium (Fig. 8B). However, enforced detachment of hexokinase II with clotrimazole restored the increase in mitochondrial oxidative phosphorylation in cells expressing cyclophilin D (K145Q), indicating that by remaining bound to VDAC, hexokinase II was precluding the sirtuin-3-mediated increase in oxidative phosphorylation (Fig. 8B). Nevertheless, whereas hexokinase II dissociation was necessary for an increase in oxidative phosphorylation, it was not sufficient. Detachment of hexokinase II with clotrimazole in cells incubated in glucose-based medium did not bring about an increase in mitochondrial oxidative phosphorylation. These results indicate that sirtuin-3 activation is essential for the observed stimulation of mitochondrial activity and although necessary, hexokinase II dissociation from the mitochondria is not sufficient.

Paradoxically, although cyclophilin D overexpression potentiates necrotic cell injury, stimulation of onset of the mitochondrial permeability transition, in some instances it has been reported to prevent apoptotic cell killing (Chen et al., 2004; Schubert and Grimm, 2006). The inhibition of apoptosis by cyclophilin D has been linked to its promotion of hexokinase II binding to the mitochondria and its interaction with Bcl-2 (Eliseev et al., 2009; Machida et al., 2006). Therefore, activation of sirtuin-3 in a cancer cell would have the effect of detaching hexokinase II from the mitochondria and inhibiting Bcl-2, thereby increasing the susceptibility of the cell to apoptotic cell killing. By contrast, activation of sirtuin-3 in a non-transformed cell relying on oxidative phosphorylation would inhibit cyclophilin D, as a result restraining onset of the mitochondrial permeability transition and preventing necrotic cell death. Consequently, selective activation of sirtuin-3 could have the beneficial effects of enhancing the susceptibility of cancer cells to chemotherapeutic-induced apoptosis, while preventing non-transformed cells from undergoing necrosis resulting from insults such as ischemia.

In summary, sirtuin-3 is required for an increase in mitochondrial activity when certain transformed cells are transferred to a galactose-based medium. Sirtuin-3 deacetylates and inactivates cyclophilin D, promoting the detachment of hexokinase II from the mitochondria, a process that is necessary, but not sufficient, for the resulting stimulation of mitochondrial activity (Fig. 9).

## Materials and Methods

### Cell culture conditions in glucose and galactose medium

HeLa HCT-116 and MDA-MB-231 cells (American Type Culture Collection) were maintained in 25-cm<sup>2</sup> flasks (Corning Costar, Oneonta, NY) with 5 ml of Dulbecco's modified Eagle's medium containing 100 U/ml penicillin, 100 U/ml streptomycin and 10% heat-inactivated fetal bovine serum and incubated under an atmosphere of 95% air and 5% CO<sub>2</sub>.

Cells were plated in six- or 24-well plates at 500,000 cells/well and 50,000 cells/well, respectively, in DMEM containing glucose at 4.5 g/l. After 24 hours, the cells were washed twice with phosphate-buffered saline and incubated in DMEM containing glucose (4.5 g/l) or DMEM containing galactose at 4.5 g/l for the times indicated.

### Isolation of mitochondrial and cytosolic fractions

Following treatment, cells were harvested by trypsinization and centrifuged at 600 *g* for 10 minutes at 4°C. The cell pellets were washed once in PBS and then resuspended in the volumes of isolation buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM sodium DTTA, 1 mM dithiothreitol, and 10 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin) in 250 mM sucrose. After chilling on ice for 30 minutes, the cells were disrupted by 40 strokes of a glass homogenizer. The homogenate was centrifuged twice at 1500 *g* at 4°C to remove broken 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 respiration using a Clark oxygen electrode with 1 mM glutamate and 1 mM malate respiratory substrates. The supernatant was removed and filtered through 0.2 μm and then 0.1 μm Ultrafree MC filters (Millipore) to give cytosolic preparations.

### Detection of hexokinase II, sirtuin-3 and acetylated lysine by western blotting

Samples were separated on 12% SDS-polyacrylamide gels and electroblotted onto PVDF membranes. Membranes were incubated with anti-hexokinase II at 1:1000, sirtuin-3 at 1:1000 (Cell Signaling) and cyclophilin D (MitoSciences) at 1:500. In each case, the relevant protein was visualized by staining with the appropriate horseradish-peroxidase-labeled secondary antibody (1:10,000) and detected by enhanced chemiluminescence.

### Immunoprecipitation of ANT1 and detection of cyclophilin D

ANT1 was immunocaptured from mitochondrial extracts using monoclonal antibodies to ANT1 crosslinked to agarose beads (MitoSciences). The immunocomplexes were eluted with SDS buffer and were separated on 12% SDS-polyacrylamide gels and electroblotted onto PVDF membranes. The western blots were then probed with antibody against cyclophilin D and visualized by staining with horseradish-peroxidase-labeled secondary antibody (1:10,000), with detection by enhanced chemiluminescence.

### Measurement of sirtuin-3 and cyclophilin D activity

Sirtuin-3 activity was measured in mitochondrial extracts using the Cyclex sirtuin-3 assay kit (MBL). A sirtuin-3 peptide substrate that is acetylated and fluorescently labeled is mixed with the mitochondrial extract. Deacetylation of the peptide by sirtuin-3 activity sensitizes it to lysyl endopeptidase that cleaves the peptide releasing a quencher of the fluorophore. Fluorescence intensity was measured on a fluorescence plate reader with excitation at 340 nm and emission at 440 nm.

Cyclophilin D was immunoprecipitated from mitochondrial extracts. Cyclophilin D PPLase activity was determined colorimetrically using a peptide in which the rate of conversion of *cis* to *trans* of a proline residue in the peptide makes it susceptible to cleavage by chymotrypsin, resulting in the release of the chromogenic dye, p-nitroanilide. The absorbance change at 380 nm was monitored over a 2 minute period with data collected every 0.2 seconds. Additionally, cyclophilin D was immunoprecipitated from mitochondrial extracts isolated from cells incubated in glucose based media. The immunoprecipitated cyclophilin D was incubated with recombinant sirtuin-1 or sirtuin-3 (Biomol) in sirtuin reaction buffer (50 mM Tris-HCl, pH 8.8, 4 mM MgCl<sub>2</sub>, 0.5 mM DTT). The reaction was then run on SDS-PAGE gels and electroblotted onto PVDF membranes. The western blots were developed with anti-acetylated-lysine antibody (Cell Signaling).

### Measurement of mitochondrial respiration and membrane potential

Mitochondrial energization was determined as the retention of the dye DiOC<sub>6</sub>. Cells were loaded with 40 nM of DiOC<sub>6</sub> during the last 30 minutes of incubation. The

cells were then washed twice in PBS. The level of retained DiOC<sub>6</sub> was measured on a plate reader at 488 nm excitation and 500 nm emission.

Cellular oxygen consumption was monitored using intact cells. The cells were incubated at  $5.0 \times 10^6$  cells/ml at 37°C in a thermostatically controlled chamber. The oxygen consumption was measured with a Clarke oxygen electrode. The respiratory medium was DMEM containing glucose or galactose.

#### siRNA-mediated knockdown of sirtuin-3

The Dharmacon SMART selection and SMART pooling technologies were used for RNA interference studies. The siRNAs targeting sirtuin-3 were delivered by a lipid-based method supplied from a commercial vendor (Gene Therapy Systems) at a final siRNA concentration of 50 nM. After formation of the siRNA-liposome complexes, the mixture was added to the HeLa cells for 4 hours. The medium was then aspirated, and complete medium containing glucose or galactose at 4.5 g/l was added. The sequences for the siRNA were: non-target, 5'-AAUUCUCCGACGUGUGUC-ACGU-3'; Sirt-3, 5'-GCAAUAGAUUUUAGACAG-3'; Sirt-5, 5'-GGAGAU-CCAUGGUUA-3'; Sirt-4, 5'-GAGAGUUGUUUCCAGUAU-3'.

#### Generation of cyclophilin D mutant and transfection

The cDNA of human cyclophilin D was isolated by RT-PCR and subcloned into the mammalian expression vector, pcDNA3. The QuikChange site-directed mutagenesis kit (Stratagene) was utilized to generate cyclophilin D with Lys145 mutated to a glutamine or arginine residue. Plasmid DNA was transfected in 24-well plates into HeLa cells using Lipofectamine reagent. Following 72 hours of incubation, the cells were transferred to six-well plates into medium containing Zeocin (200 µg/ml). The surviving cells were grown for 2 weeks. Surviving colonies were transferred to 25 cm<sup>2</sup> flasks and grown under restrictive conditions.

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