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SIRT3 is a Mitochondrial Tumor Suppressor: A Scientific Tale that Connects Aberrant Cellular ROS, the Warburg Effect, and Carcinogenesis

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Abstract

Tumors exhibit metabolic reprogramming characterized by increased cellular reactive oxygen species (ROS) and the preferential use of glucose, as first published by Otto Warburg in 1956, referred to as the “Warburg effect.” However, the mechanism(s) linking these processes remain largely elusive. Murine tumors lacking *Sirt3* exhibit abnormally high levels of ROS that directly induce genomic instability and increase HIF-1α protein levels. The subsequent transcription of HIFα-dependent target genes results in cellular metabolic reprogramming and increased cellular glucose consumption. In addition, agents that scavenge ROS or reverse the Warburg effect prevent the transformation and malignant phenotype observed in cells lacking *Sirt3*. Thus, mice lacking *Sirt3* provide a model mechanistically connecting aberrant ROS, the Warburg effect, and carcinogenesis.

Keywords

Sirt3; Warburg; Metabolism; Mitochondria; ROS; MnSOD; HIF-1α; Acetylation; Acetylome; Cancer; Metabolic Reprogramming

The Maintenance of Mitochondrial Metabolic Homeostasis by Fidelity Proteins

While the first identified tumor suppressors (TSs) were confined to either nucleus and/or cytoplasm, it seemed logical to hypothesize that the mitochondria would also contain fidelity sensing proteins that serve as TSs. A central role of the mitochondria is to generate ATP from ADP and organophosphate via oxidative phosphorylation (OXPHOS), which generates ROS as a toxic byproduct. Failure to match the rate of OXPHOS with nutrient supply or energy demand would cause decreased energy availability or excess ROS production, resulting in cellular stress. Therefore, fidelity proteins within the mitochondria should be critical for sensing and responding to changes in ATP demand as well as elevations in ROS production, and the processes of ATP production, ROS production, and clearance and/or removal of damaged molecules should be regulated by specific sensing or watchdog proteins so that energy production closely matches cellular energy requirements.

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Mitochondrial aerobic respiration is an efficient method of generating energy in biological systems. However, as a side product of electron transfer reactions, aerobic cells continuously produce ROS from the incomplete reduction of dioxygen molecules (1). The steady state levels of ROS are a function of ATP production and incomplete removal by detoxification enzymes. Oxidative stress occurs when the physiological balance between the production and scavenging of ROS is disturbed (2). While low levels of cellular ROS are well tolerated by the cell and are a key part of homeostatic signaling pathways, abnormally high levels can induce oxidative stress, which has been implicated as a causative agent in several degenerative diseases such as amyotrophic lateral sclerosis and rheumatoid arthritis, as well as in genomic instability, aging, and most importantly, carcinogenesis (1).

Cellular signaling networks often contain critical or central “sensor” proteins that activate downstream targets in response to environmental conditions (1, 3, 4). In this regard, lysine acetylation has emerged as an important post-translational modification employed to activate mitochondrial signaling proteins (5, 6). The idea that the acetylome may be critical in regulating mitochondrial metabolism is based on several proteomic surveys identifying a high number of acetylated proteins that direct mitochondrial metabolism (6, 7). Given the tight link between metabolism, energy production and ROS, it is likely that the mitochondrial acetyltransferases and deacetylases may coordinate, at least in part, the balance between energy production and ROS detoxification.

Sirt3 is a Mitochondrial Tumor Suppressor and Metabolic Regulator

Sirtuin genes are the homologs of the *S. cerevisiae Sir2* gene that directs downstream processes involved in longevity, and while it has not been shown that these genes determine longevity in mammals, they do appear to regulate critical pathways and physiologies that are implicated in age-related diseases (8, 9). Sirtuins share a 275-amino acid catalytic deacetylase domain and are localized to the nucleus (SIRT1, 6, and 7), cytoplasm (SIRT2), and mitochondria (SIRT3, 4, and 5), respectively (9). Sirt3 is a mitochondrial deacetylase that acts on numerous substrates to activate fat oxidation, amino acid metabolism and electron transport (10). Several manuscripts published in the last year provided convincing evidence that Sirt3, the primary mitochondrial deacetylase, is a *bona fide* TS (11-13).

In support of Sirt3’s role as a fidelity or TS gene, mouse embryonic fibroblasts (MEFs) lacking *Sirt3* exhibited stress-induced genomic instability and were immortalized by infection with a single oncogene (11). By contrast, wild-type cells required both *Myc* and *Ras* to achieve a similar phenotype. Moreover, $Sirt3^{-/-}$ MEFs expressing only *Myc* or *Ras* grew in soft agar and developed into subcutaneous tumors in nude mice. Thus, Sirt3 functions as an in vitro TS and loss of Sirt3 amplifies the phenotypic effects of oncogene expression. In vivo overexpression of *Sirt3* decreased tumorigenesis in xenografts, even when induction of the Sirt3 occurred after tumor initiation (13). In addition, mice lacking *Sirt3* developed estrogen- and progesterone-(ER/PR-) positive mammary tumors (11). Finally, human breast cancer data sets consisting of genomic, RNA, and tissue data from 992 human breast cancer samples also showed that SIRT3 is decreased in human breast cancers (11). Together, the knockout mice, tissue culture, and human tumor data provide genetic evidence that a mitochondrial protein can function as a TS.

With respect to its role in mitochondrial metabolism, MEFs lacking *Sirt3* exhibited increased ROS (11) and in vivo overexpression of *Sirt3* suppressed cellular ROS levels (13). These findings raised the question: what is the mechanistic link between loss of *Sirt3* and aberrant mitochondrial ROS production? Several studies have shown that cells lacking *Sirt3* exhibit aberrant or decreased activity of oxidative phosphorylation proteins, including complex I (14) and complex III of the electron transport chain (11, 12). Altered flux through

the electron transport chain directly influences ROS production: electrons can leak out of complexes I and III, resulting in one-electron reductions of oxygen to produce the superoxide radical (15). Thus, when electrons are flowing quickly and efficiently through electron transport chains, opportunities for ROS production are diminished. In contrast, when electrons are flowing slowly or inefficiently, as proposed in cells lacking *Sirt3*, there is a greater possibility for one-electron reductions of oxygen, presenting the opportunity for forming ROS.

While these results account for increased ROS production, cells also contain detoxification enzymes that should scavenge the increased ROS in cells lacking *Sirt3*. Thus, in accord with recent studies, cells lacking *Sirt3* may have dysfunctional coordination of both electron transport and detoxification enzymes that, when combined, results in aberrant and potentially damaging levels of ROS.

Two recent manuscripts showed that MnSOD, the primary mitochondrial superoxide detoxification enzyme, contains a lysine that is deacetylated by caloric restriction, fasting, and SIRT3 overexpression (16, 17). Further analysis by Tao et al. (2010) showed that lysine 122, which is conserved in multiple species, is directly deacetylated by SIRT3. When lysine 122 was changed to arginine (to mimic the deacetylated state; $MnSOD^{K122\text{-}R}$), enzymatic activity was increased, intracellular ROS were decreased, and stress-induced genomic instability was prevented. In contrast, when lysine 122 was changed to a glutamine (to mimic the acetylated state; $MnSOD^{K122\text{-}Q}$), MnSOD activity was decreased, suggesting that the acetylation status directs MnSOD enzymatic activity and cellular ROS levels.

The role of acetylation in cells lacking *Sirt3* was confirmed by experiments showing that $Sirt3^{-/-}$ MEFs expressing $MnSOD^{K122\text{-}R}$, but not $MnSOD^{K122\text{-}Q}$, are resistant to in vitro transformation by infection with a single oncogene or exposure to irradiation. Thus, decreased Sirt3 deacetylation activity appears to increase ROS levels by two mechanisms: decreased electron transport and decreased MnSOD enzymatic detoxification activity. These results establish a connection between mitochondrial metabolism, i.e., increased production and decreased detoxification of ROS by MnSOD in cells lacking *Sirt3*, and tumor suppression, i.e., the genomic instability observed in *Sirt3* knockouts (Fig. 1A).

Sirt3 Directs Cellular Metabolic Reprogramming that Mirrors the Warburg Effect

A fundamental observation in oncology is that tumor cells exhibit a reprogramming of cellular metabolism that involves an increase in glucose consumption. This is referred to as the “Warburg effect” as described by Otto Warburg 60 years ago (18). In this regard, Finley et al. (2011) have recently linked aberrant ROS regulation by SIRT3 to the Warburg effect. In this manuscript it was shown that the increased glucose consumption in cells lacking *Sirt3* promoted a tumor permissive phenotype both in vitro and in vivo (12). They also demonstrated that elevated glucose metabolism was the direct result of increased hypoxia-inducible factor 1α (HIF-1α) stability in response to aberrant increased ROS levels.

HIF-1α is stabilized in response to low oxygen levels and subsequently increases the expression of more than 200 genes (19). The activation of HIF-1α protein is linked to multiple cellular pathways including metabolic reprogramming, cell survival, proliferation, progression, and metastasis, all of which are essential to the process of carcinogenesis. Finally, increased HIF-1α levels are associated with a poor prognosis in breast cancer (20).

Two independent studies by Finley et al. (12) and Bell et al. (13) demonstrated a new link between Sirt3 and HIF-1α. They found that loss of *Sirt3* increased cellular HIF-1α levels by

inhibiting prolyl hydroxylase (PHD) via a mechanism dependent upon an increase in cellular ROS levels (Fig. 1B). This group also showed that this regulatory node regulated glucose metabolism; *SIRT3* deletion increased HIF-1α target gene expression, glycolytic metabolism and glucose-dependent cellular proliferation. In vivo, brown adipose tissue of the $Sirt3^{-/-}$ mice had elevated glucose uptake and the gene expression profile was very similar to that observed in cells exposed to low oxygen, suggesting a direct regulation of gene expression. In this regard, one could hypothesize that the induction of HIF-1α in response to increased ROS may be an adaptive response to increase glucose metabolism to detoxify hydroperoxides. This response could activate a series of necessary adaptive pro-survival signaling pathways to protect against cell death while also increasing metabolic oxidative stress due to increased flux of ROS. Thus, this compensative increase in cellular ROS, over time, could lead to genomic instability, a mutator phenotype, and progression to the malignantly transformed phenotype. Finally, at least one copy of the *SIRT3* gene is deleted in 20% of all human cancers and 40% of breast and ovarian cancers, and the majority of genomic *SIRT3* deletions are heterozygous. Thus, this work suggests a model in which increased ROS stabilize HIF-1α, reprogramming the cell toward Warburg conditions as well as driving cells already containing genomic instability toward cell proliferation that amplifies or propagates DNA damage (Fig. 1A)

Model Connecting ROS, HIF-1α, and the Warburg Effect in Carcinogenesis

The results presented above suggest that increased levels of cellular ROS, stemming from both increased production and decreased detoxification, may be an early initiating event allowing genomic instability and establishing a DNA damage permissive phenotype. This is based on the Hanahan and Weinberg multi-hit model (21) for carcinogenesis proposing that transformation requires an initiating event (Fig. 1C). Based on the above studies, we propose that Sirt3 is well poised to mediate the balance between energy generation and ROS scavenging. Loss of or a decrease in Sirt3 activity results in increased mitochondrial ROS, due to decreased ROS detoxification, via hyper-acetylation of proteins such as MnSOD (16, 17), and via the inefficient electron transport chain function (11, 13), creates a cellular environment favoring the development of genomic instability. This model is well supported by experiments from three studies showing that agents scavenging ROS prevent in vitro transformation as measured by contact inhibition, proliferation, and tumor cell growth in soft agar and nude mice (11-13). These observations are consistent with studies (1, 2) showing that abnormal levels of cellular ROS are a strong genotoxic stress that, over time, results in genomic instability. Thus, increased ROS in cells lacking *Sirt3* plays a role in the establishment of a DNA damage permissive cellular phenotype.

While it is proposed that increased ROS and genomic instability are potential early events in tumorigenesis, it is clear that additional promoting genetic events are also required that induce proliferation. It has been suggested that the hallmarks of carcinogenesis (Fig. 1C) comprise a series of biological capabilities that occur via a multistep de-differentiation pathway (21). In this regard, loss of *Sirt3* stabilizes HIF-1α via a ROS dependent mechanism, resulting in the reprogramming of cellular metabolism that may be one such promoter event. The subsequent increase in HIF-1α increases the expression of a series of HIF-1α dependent pro-proliferative and pro-survival genes/proteins (Fig. 1B-C). Cell proliferation, over time, expands the pool of cells and allows for the eventual selection of one or more cells with additional genetic mutations, leading to activation of oncogenes and/or inactivation of tumor suppressors. Thus, the combination of genomic instability and proliferation results in a pro-mutation and pro-division phenotype that may allow one individual cell, with the necessary “carcinogenic” combination of mutations, to progress through several additional events that ultimately lead to a malignant cell phenotype (Fig. 1C).

While the results reviewed here suggest a mechanism by which absent or decreased Sirt3 activity results in a HIF-1α dependent pro-proliferative phenotype, they do not, *a priori*, explain the genomic instability observed in murine cells lacking *Sirt3*. One possible mechanism may be due to ROS leakage into the cytoplasm and/or nucleus. These oxidative ROS could physically interact with any of a number of cellular targets and damage cells by peroxidizing lipids, disrupting proteins, and/or inducing DNA damage as well as activating transformation driver gene mutations (1). Alternatively, increased cellular ROS may induce pro-metabolism and/or pro-proliferative signaling networks that activate downstream oncogenes that are transformation driver proteins. While these or other mechanisms are possible, it is clear that conditions that scavenge ROS, such as exposure to NAC or enforced MnSOD expression, reverse both the increased cellular ROS levels and the tumor permissive phenotype.

Conclusions

Investigations of the mechanism of carcinogenesis in mice lacking *Sirt3* have resulted in several important observations regarding the mechanistic connection between aberrant mitochondrial metabolism and carcinogenesis: (1) Sirt3 regulates HIF-1α activity, resulting in altered cellular metabolism which supports cell proliferation; (2) Sirt3 directly regulates MnSOD enzymatic activity via acetylation and the aberrant acetylation of MnSOD in cells lacking Sirt3 increases cellular ROS; and (3) *Sirt3* knockout mice develop ER-positive mammary tumors. Furthermore, *SIRT3* expression is decreased in many different types of human cancers and heterozygous loss of *SIRT3* occurs in 40% of human breast malignancies, suggesting that the knockout mice are the first murine model for the most common subtype of breast cancer observed in older, post-menopausal women.

However, if this is the case, why is spontaneous tumorigenesis only found in the mammary tissue? While this could be due, at least in part, to functional redundancy of different members of the sirtuin family, especially those localized to the mitochondria, recent studies demonstrated that the deacetylase activity of sirtuins can be changed under the influence of different forms of cellular stress. Thus it is plausible that tumor suppressor functions of Sirt3 in other cell types might only become obvious under various stress conditions, and/or after accumulation of different oncogenic driver mutations. In conclusion, these new findings reveal several therapeutic opportunities, including the identification of potential molecular targets and biomarkers to determine tumor risk and the development of agents that can selectively inhibit ROS-dependent aberrant cellular signaling networks in cancer.

Acknowledgments

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Perspective: This work shows for the first time that the genetic deletion of a mitochondrially localized protein results in a tumor permissive phenotype in mice and connects ROS and HIF-1α in a murine model of ER+ breast cancer.

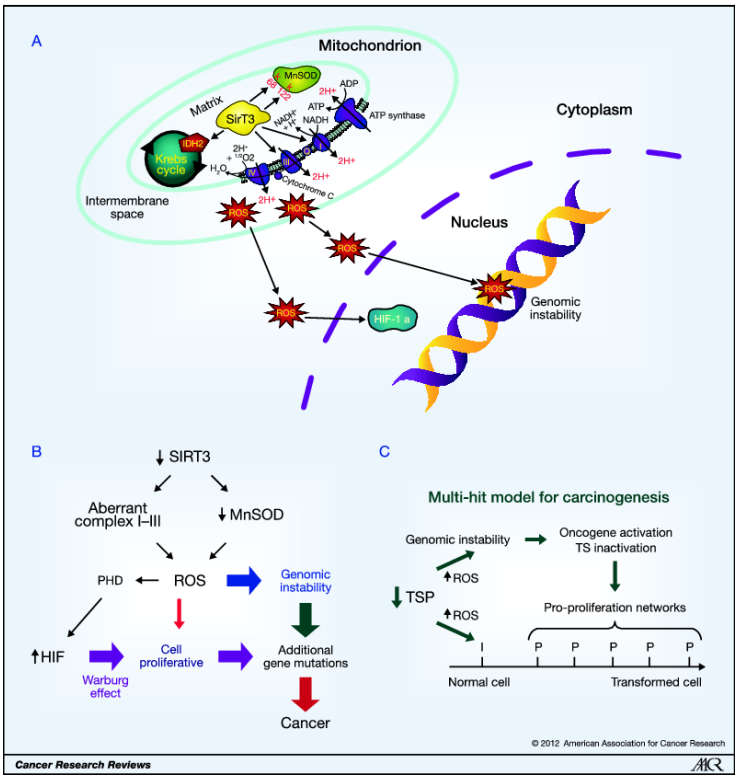

Figure 1

. **(A)** The Biochemical connection between ROS and a Sirt3$^{-/-}$ tumor permissive phenotype. In cells lacking Sirt3, increased ROS induces HIF-1α expression as well as genomic instability. (B) Proposed model describing the aberrant signaling networks in cells lacking ***Sirt3*** that result in a pro-proliferative and tumor permissive phenotype. In this model, loss of Sirt3 deacetylation activity results in increased ROS production and decreased activity of mitochondrial detoxifying enzymes. The increase in cellular ROS results in a tumorigenic phenotype by (1) directly interacting with DNA to induce mutations and genomic instability and indirectly inducing pro-proliferative pathways (orange arrow) or (2) inducing HIF-1α DNA binding and transcriptional activity and increasing the cellular levels of a series of pro-proliferative proteins that favor aberrant proliferation/division leading to tumorigenesis. (C) Outline for the multi-hit model for solid tumor carcinogenesis. In this model, proposed by Hanahan and Weinberg (21), an early “initiating” genetic event involves the inactivation or deletion of a fidelity or TS gene that results in a new cellular phenotype permissive for the accumulation of genomic instability due to an increase in cellular ROS. In addition, a series of secondary pro-proliferative and pro-survival events are required favoring cell division that, over time, allow the development of addition mutations to oncogenes and the subsequent transformation from a normal to a malignant cell.