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The SirT3 Divining Rod Points to Oxidative Stress

Eric L. Bell¹ and Leonard Guarente^{1,*}

¹Paul F. Glenn Laboratory and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

*Correspondence: leng@mit.edu

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Sirtuins are NAD⁺ dependent deacetylases that counter aging and diseases of aging. Sirtuin research has focused on SirT1, which deacetylates transcription factors and cofactors in the nucleus. More recent findings highlight SirT3 as a mitochondrial sirtuin that regulates metabolism and oxidative stress. This review focuses on new data linking SirT3 to management of reactive oxygen species from mitochondria, which may have profound implications for aging and late-onset diseases.

Introduction

The molecular era of aging research can be said to have begun with the oxidative damage theory of aging in the 1950s (Harman, 1956). According to this theory, reactive oxygen species (ROS) generated by leakage of electrons during electron transport damage macromolecules within and outside the mitochondria. This effect would be cumulative with aging and lead to functional decline. This theory has followed a rocky path as recent genetic studies provide evidence both in favor of it (Schirner et al., 2005) and against it (Pérez et al., 2009; Van Remmen et al., 2003). Clearly, other kinds of damage occur during aging, such as telomere shortening, protein aggregation, etc. (Garcia et al., 2007; Morimoto and Cuervo, 2009), but it remains possible that these are consequences of the primary trigger of cumulative oxidative stress.

A burgeoning area of aging research has involved genes that control the pace of aging, initially identified in lower organisms. Data indicate that these gene products, including FOXO and sirtuin proteins, can also slow aging phenotypes in mammals. It was suggested that a full understanding of the function of these genes in mammals might be a divining rod pointing to the most important causes of aging (Guarente, 2003).

Sirtuins are NAD⁺ dependent protein deacetylases that slow aging in lower organisms (Imai et al., 2000; Kaeberlein et al., 1999; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001; Viswanathan et al., 2005). Much data now indicate that mammalian sirtuins adapt animal physiology to dietary extremes, such as fasting and calorie restriction (CR) (Finkel et al., 2009). The mammalian Sir2 ortholog SirT1 has been shown to deacetylate scores of critical transcription factors in the nucleus to mediate a broad range of physiological effects (Haigis and Sinclair, 2010; Imai and Guarente, 2010). In general, pathways controlled by SirT1 govern two domains, metabolic adaptations and stress response. Thus, increases in SirT1 activity via transgenic or pharmacological activation may slow aging by a variety of criteria and combat diseases, such as diabetes (Banks et al., 2008), Alzheimer's (Donmez et al., 2010), and cancer (Herranz et al., 2010). However, whole-body SirT1 gain-of-function mice have not been reported to live longer on a normal chow diet, although they do show protection against aging-induced pathologies (Herranz et al., 2010).

Of the seven mammalian sirtuins, three are targeted to the mitochondrial matrix (Verdin et al., 2010), where they deacetylate

and/or ADP-ribosylate enzymes to trigger metabolic adaptation to fuel sources such as amino acids and fatty acids during energy limitation (Table 1 and below). It has been argued that these metabolic adaptations, such as the oxidation of fatty acids to produce energy, may per se have salutary effects on cell maintenance (discussed in SirT3 and Metabolism, below).

Beyond these metabolic functions, SirT3 has been recently shown to control the levels of ROS themselves by multiple mechanisms. The implications of these findings, if confirmed in a broad range of cell types, may redirect the focus of attention back to mitochondrial oxidative damage as a primary cause of aging, unite some of the central ideas on aging, and suggest novel ways to intervene pharmacologically.

Unity at Last—SirT3, CR, ROS, and Hearing Loss

There has been some debate over the role of sirtuins in CR, mainly centered on systems contrived in yeast, *Caenorhabditis elegans*, and *Drosophila* to simulate rodent CR. In the case of mammalian sirtuins, considerable evidence has accumulated, which suggests that by deacetylating many nuclear factors, SirT1 mediates many outputs of CR, including increased physical activity (Chen et al., 2005), increased protection against disease (Herranz and Serrano, 2010; Kume et al., 2010), regulation of central neuroendocrine control (Cohen et al., 2009), and longevity (Boily et al., 2008; Li et al., 2008). As mentioned above, mitochondrial sirtuins deacetylate (or ADP-ribosylate) mitochondrial metabolic enzymes that may be important for adaptation to CR.

The clearest example relating CR, ROS, sirtuins, and aging has come from a recent study on hearing loss in mice (Figure 1) (Someya et al., 2010). C57BL/6 mice sustain cumulative oxidative damage to hair cells and spiral ganglia neurons of the inner ear cochlea, resulting in hearing loss in 12-month-old animals. CR completely protected against both oxidative damage to these cells and hearing loss in wild-type mice. However, SirT3^{-/-} mice were completely refractory to protection by CR; i.e., SirT3^{-/-} CR mice sustained the same level of oxidative damage and hearing loss as ad libitum-fed animals. Note that young SirT3^{-/-} mice are generally indistinguishable from wild-type mice by many physiological criteria (Lombard et al., 2007). Because CR is known to induce SirT3 protein levels in wild-type mice (Hallows et al., 2011; Pérez et al., 2009; Shi et al., 2005), it is likely that this sirtuin can mediate ROS management, mitochondrial integrity, and sensory function, at least in those neurons that govern hearing.

Table 1. Mitochondrial Sirtuin Targets

Enzyme	Sirtuin	Pathways	References
Glutamate dehydrogenase (GDH)	SirT3, SirT4	Amino acid catabolism, NADPH production	Haigis et al., 2006; Lombard et al., 2007; Schlicker et al., 2008
Carbamoyl phosphate synthase 1 (CPS1)	SirT5	Amino acid catabolism, urea cycle	Nakagawa et al., 2009; Ogura et al., 2010
Acetyl CoA synthase 2 (ACS2)	SirT3	Acetate metabolism	Hallows et al., 2006; Schwer et al., 2006
Ornithine transcarbamoylase (OTC)	SirT3	Amino acid catabolism, urea cycle	Hallows et al., 2011
3-Hydroxy-3-methylglutaryl CoA synthase 2 (HMGCS2)	SirT3	Ketogenesis	Shimazu et al., 2010
Long-chain acyl-CoA dehydrogenase (LCAD)	SirT3	β -oxidation	Hirschey et al., 2010
NADH quinone oxidoreductase (Complex I)	SirT3	Oxidative phosphorylation	Ahn et al., 2008
Succinate dehydrogenase (Complex II)	SirT3	Oxidative phosphorylation	Cimen et al., 2010; Lombard et al., 2007
Isocitrate dehydrogenase 2 (IDH2)	SirT3	TCA cycle, NADPH production	Schlicker et al., 2008; Someya et al., 2010
Manganese superoxide dismutase (MnSOD or SOD2)	SirT3	Antioxidant	Qiu et al., 2010; Tao et al., 2010
Cytochrome C	SirT5	Oxidative phosphorylation, apoptosis	Schlicker et al., 2008
Mitochondrial ribosomal protein L19	SirT3	Mitochondrial protein translation	Yang et al., 2010
Cyclophilin D (CypD)	SirT3	Apoptosis, glycolysis	Hafner et al., 2010; Shulga et al., 2010

It is striking that this example brings together major threads running through the history of modern research on aging. It would appear to resuscitate the importance of the oxidative damage theory and mitochondria in aging. Further, it suggests that CR may function, at least in part, by controlling oxidative damage, thereby preventing aging-induced decline. Finally, it shows that genes identified because they slow aging in lower organisms such as sirtuins can fit centrally into the control of

oxidative damage by CR. The SirT3 divining rod has thus brought us back to familiar themes as we contemplate future interventions. Mechanisms by which SirT3 may govern production or levels of ROS are discussed below.

Will these findings on hearing loss extend to other cell types, and, if so, what are the implications for the science of aging? The roles of ROS and oxidative damage in aging are probably important in the aging of postmitotic cells, e.g., neurons, skeletal muscle, and cardiac cells. Further, the upregulation of SirT3 by CR has been observed in a variety of tissues (Hallows et al., 2011; Pérez et al., 2009; Shi et al., 2005), suggesting that this sirtuin may mediate a broad spectrum of tissue protection against ROS-induced aging. It will be important to test whether SirT3 gain-of-function transgenic mice that express CR-levels of the protein are protected against oxidative damage as they age. If so, it is possible that SirT3 activation will slow aging itself and many of the effects of aging on health decline and disease. An important issue in considering SirT3 gain of function is whether ROS can be reduced or eliminated without compromising normal physiological functions of free radicals. For example, the induction of hypoxia-inducible factor HIF-1 α in embryogenesis during periods of hypoxia is essential for development (Maltepe et al., 1997; Ryan et al., 1998) and numerous reports indicate that ROS plays a major role in hypoxic induction of HIF-1 α (Bell et al., 2007; Brunelle et al., 2005; Guzy et al., 2005; Mansfield et al., 2005). ROS can also play a mitogenic role, are necessary for proper immune response, and can guide differentiation of tissue-specific stem cells (Esposito et al., 2004; Pervaiz et al., 2009).

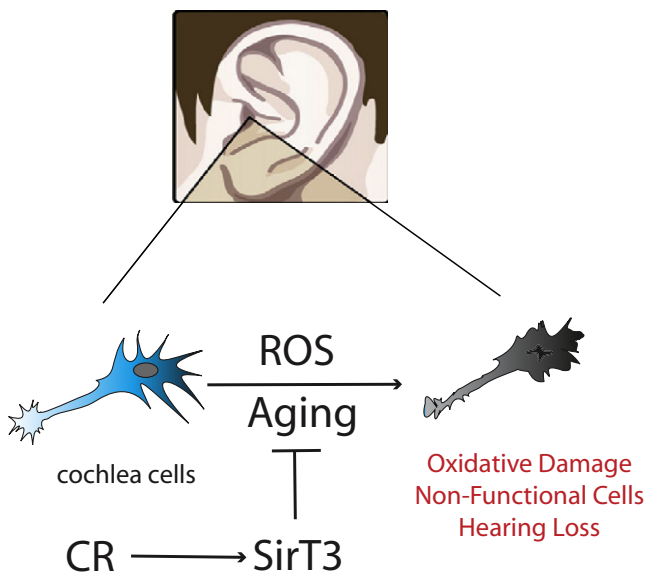


Figure 1. SirT3 Is Necessary for Protection from Hearing Loss by CR Aging induces ROS damage to inner ear cochlea neurons. In conditions of calorie restriction (CR), SirT3 inhibits ROS-mediated damage of cochlea neurons to delay hearing loss.

Mechanisms of Protection against Oxidative Damage by SirT3

Recent studies have revealed a surprisingly large number of antioxidant functions for SirT3 in mitochondria. Accordingly, a large

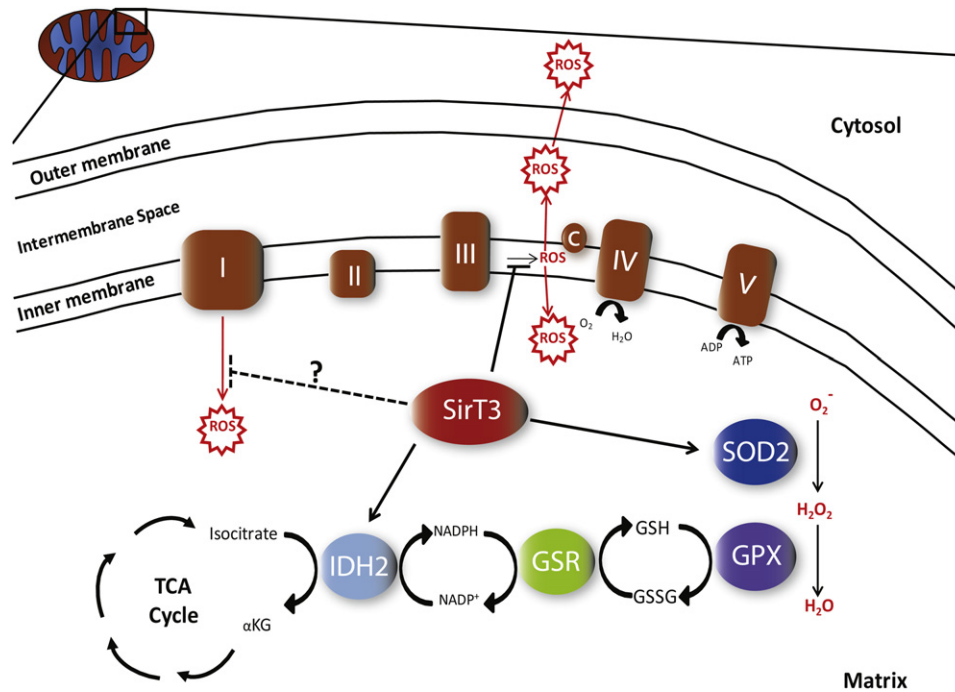


Figure 2. SirT3 Protects against Damage from Mitochondrially Derived ROS

ROS are primarily generated by complex I (matrix) and complex III (matrix, intermembrane space, and cytosol). Superoxides ($O_2^{\cdot-}$) localized to the matrix are detoxified by manganese superoxide dismutase (SOD2) into hydrogen peroxide (H_2O_2), which is subsequently converted into water by glutathione peroxidase (GPX). GPX requires reduced glutathione (GSH) for enzymatic activity. Oxidized glutathione (GSSG) is reduced by glutathione reductase (GSR), which requires NADPH. NADPH is generated from $NADP^+$ by isocitrate dehydrogenase 2 (IDH2). By activating SOD2 and IDH2 and inhibiting ROS generation by complex III and potentially complex I, SirT3 may be a major determinant of oxidative damage in cells.

number of mitochondrial proteins implicated in the aging process are acetylated (Kim et al., 2006), and SirT3, but not the other mitochondrial sirtuins SirT4 and SirT5, deacetylates a substantial subset of mitochondrial proteins (Lombard et al., 2007). The study of Someya et al. (2010) proposes a specific mechanism by which SirT3 protects cochlea cells against oxidative damage (Figure 2). SirT3 deacetylates and activates the TCA cycle enzyme isocitrate dehydrogenase 2 (IDH2), which produces NADPH in the mitochondria. The elevated NADPH, in turn, is necessary for glutathione reductase, which converts oxidized glutathione (GSSG) into reduced glutathione (GSH), the cofactor used by mitochondrial glutathione peroxidase (GPX) to detoxify ROS. Interestingly, another target of SirT3, glutamate dehydrogenase (GDH) (Lombard et al., 2007; Schlicker et al., 2008), also produces NADPH and may contribute to the increased pool of GSH that is available for GPX to detoxify ROS within mitochondria.

Two other studies demonstrate that SirT3 deacetylates the critical antioxidant enzyme manganese superoxide dismutase (SOD2) in the mitochondrial matrix (Figure 2) (Qiu et al., 2010; Tao et al., 2010). Deacetylation of this enzyme results in increased specific activity and enhanced scavenging of ROS. Strikingly, the two studies revealed different lysines in SOD2 that are deacetylated by SirT3 (K53 and K68 [Qiu et al., 2010] and K122 [Tao et al., 2010]). In both studies, mutating the lysine(s) to arginine gave rise to a hyperactive SOD2 that was not further enhanced by SirT3, suggesting that all of these

lysines may be important. Qiu et al. (2010) also showed that CR itself results in the deacetylation and activation of SOD2, presumably by upregulating SirT3. This finding may help explain why CR has consistently been associated with a reduction in ROS levels in mitochondria (Weindruch and Roy, 1988). It is important to note that the role of SirT3 on ROS detoxification through the generation of NADPH and activation of SOD2 is confined to the mitochondrial matrix and does not represent a boost in antioxidant capacity in the nucleus or cytosol. However, SirT3 does suppress ROS responsive pathways that are induced by cardiac hypertrophy in the cytosol such as RAS, AKT, and MAPK signaling (Sundaresan et al., 2009). Another example of SirT3 suppressing cytosolic ROS involves HIF-1 α , as discussed below.

How might SirT3 influence ROS in the cytoplasm? It is intriguing that SirT3 has been shown to deacetylate numerous components of the electron transport chain, suggesting that SirT3 may directly affect the production of ROS (Figure 2). For example, SirT3 was shown to deacetylate subunits of complex I (NADH dehydrogenase) (Ahn et al., 2008) and complex II (succinate dehydrogenase) (Cimen et al., 2010), while mitochondria from SirT3^{-/-} mouse embryo fibroblasts (MEFs) had reduced complex I activity, complex II activity, and complex III activity (Ahn et al., 2008; Cimen et al., 2010; Kim et al., 2010). Crucially, complex III directs ROS to both the matrix and the cytoplasm (Muller et al., 2004). Thus, by regulating complex III SirT3 could affect cytoplasmic levels of ROS.

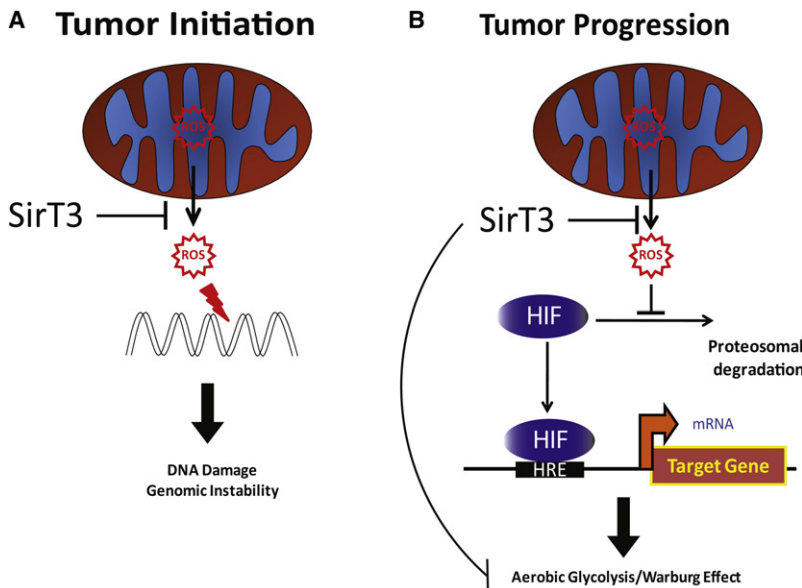


Figure 3. SirT3 Acts as a Tumor Suppressor through the Regulation of ROS

(A) SirT3 suppresses mitochondrially generated ROS to reduce DNA damage and genomic instability. This may be important in the initiation of tumorigenesis.

(B) By suppressing mitochondrially derived ROS, SirT3 also inhibits a signaling cascade resulting in the stabilization and activation of HIF-1 α protein and activation of transcriptional targets that promote aerobic glycolysis, angiogenesis, and the progression of tumorigenesis.

SirT3^{-/-} cells are known to reduce steady-state ATP levels, which may result from a defect in deacetylating electron transport chain components (Ahn et al., 2008). While SirT3 has not been demonstrated to deacetylate subunits of the ATP-generating complex V, it does physically interact with complex V subunits (Law et al., 2009). The net effect of SirT3 deficiency on electron transport may result in reduced efficiency of electron transfer within the chain, thereby increasing the probability of electrons being transferred to molecular oxygen to generate ROS at the expense of ATP production. The fact that SirT3 has apparently evolved multiple mechanisms to reduce ROS hints of a close relationship between this sirtuin, CR, and aging.

Finally, SirT3 and Sirt4 are necessary for nutrient-sensitive protection from genotoxic stress facilitated by increased mitochondrial NAD⁺ levels (Yang et al., 2007). In addition, SirT3 deacetylates cyclophilin D to inhibit apoptosis induced by opening of the mitochondrial permeability transition pore (Hafner et al., 2010). Reduction of the ROS burden coupled with an increase in stress resistance may help maintain cells, such as the cochlea cells of the inner ear, during CR.

SirT3, HIF-1 α , and Cancer

The first indication that SirT3 may function as a tumor suppressor was the observation that SirT3^{-/-} mice develop mammary tumors at 24 months old (Kim et al., 2010). Consistent with the idea that SirT3 is a tumor suppressor, SirT3^{-/-} MEFs were easier to transform (requiring a single oncogene) compared to wild-type MEFs (which also require inactivation of a tumor suppressor gene). SirT3^{-/-} cells also show increased superoxide levels and greater chromosome instability when stressed. All these findings suggest that SirT3 suppresses tumor initiation *in vivo* by preventing chromosome instability (Figure 3A).

Additional insight into the role of SirT3 as a tumor suppressor came from two studies examining the relationship between SirT3 and the transcription factor hypoxia-inducible factor, HIF-1 α . As

mentioned above, ROS-mediated stabilization of HIF-1 α is one major adaptive response to hypoxia. In one study, the inactivation of SirT3 by shRNA in several tumor cell lines gave rise to higher ROS and HIF-1 α activation in normoxia and hyperactivation of HIF-1 α in hypoxic conditions. This is consistent with the idea that SirT3 suppresses ROS, which are necessary for the hypoxic induction of HIF-1 α . Moreover, transgenic overexpression of SirT3 prevents the induction of HIF-1 α by hypoxia (Bell et al., 2011). This finding is interesting because

HIF-1 α is known to enhance the growth of tumors by adapting them to hypoxic conditions that occur during tumor development (Majmundar et al., 2010). These adaptations include induction of aerobic glycolysis for biosynthetic intermediates (Warburg effect) (Vander Heiden et al., 2009) and induction of angiogenesis to bring additional glucose and oxygen to the tumor. In fact, in xenografts of tumor cells with normal, reduced, or elevated SirT3 levels, the tumors with reduced SirT3 were the largest, while tumors with SirT3 overexpression were the smallest (Bell et al., 2011). Interestingly, antioxidant treatment was able to normalize the size of the tumors lacking SirT3. Moreover, inducing SirT3 expression after initiation of tumor formation sufficed to arrest tumor growth, suggesting that SirT3 is important not only in preventing the initiation of tumors, as suggested above, but also in preventing the maintenance and progression of tumors (Figure 3B).

In the other study, SirT3^{-/-} MEFs consumed more glucose, generated more lactate, had higher levels of glycolytic intermediates, and decreased levels of some TCA cycle intermediates (Finley et al., 2011). These data are consistent with the altered cellular metabolism that is similar to the Warburg effect in cancer cells. Consistent with this metabolic reprogramming, transcriptional profiling of SirT3^{-/-} MEFs demonstrated hyperactivation of HIF-1 α targets, which was reversed in the presence of shRNA's targeting HIF-1 α . In a complementary finding, overexpression of SirT3 reversed the Warburg effect in different breast cancer cell lines. The above data demonstrate that SirT3 suppresses the Warburg effect through the ROS-HIF-1 α axis. In addition, the deacetylation of cyclophilin D by SirT3 may contribute to this suppression because it also promotes the dissociation of hexokinase II from the mitochondria (Shulga et al., 2010), thereby decreasing the entry of glucose into glycolysis. It will be important to determine how generally SirT3-mediated control of ROS governs the Warburg effect in other kinds of tumors.

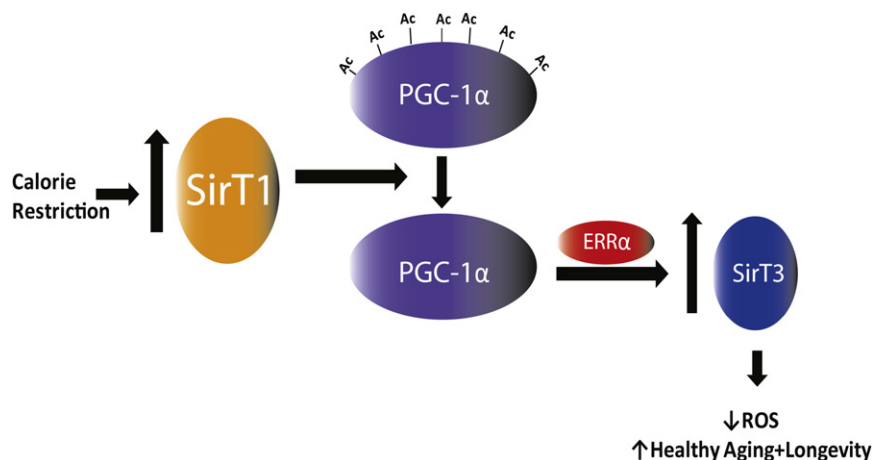


Figure 4. Model by which SirT1 and SirT3 Cooperate during CR

In conditions of decreased caloric intake, SirT1 protein levels and activity are increased, leading to the deacetylation and activation of PGC-1 α . Transactivation of ERR α by PGC-1 α increases transcription of SirT3, resulting in a decrease in ROS and the promotion of healthy aging.

Finally, a broad survey indicated SirT3 gene deletion and reduction in protein levels in human breast and ovarian carcinomas (40%) and other tumors (20%–30%) compared to non-cancer control (Finley et al., 2011; Kim et al., 2010). These data suggest that SirT3 is an important tumor suppressor in human cancers. While it has been suggested that aging may be the price for zealous tumor suppression early in life (e.g., by p53; Tyner et al., 2002), the above findings suggest that the tumor suppressor SirT3 involves no such trade-off and actually links tumor suppression to a slowing of aging.

SirT3 and Metabolism

As alluded to above, SirT3 promotes mitochondrial oxidative metabolism of amino acids and fatty acids during energy limitation. To wit, SirT3-mediated deacetylation and activation of acetyl CoA synthase 2 (ACS2) allow incorporation of acetate into central metabolism while deacetylation and activation of long-chain acyl-CoA dehydrogenase (LCAD) promote β -oxidation of fatty acids (Hirschey et al., 2010), which may be reinforced by SirT3-mediated regulation of the LKB1-AMPK axis (Pillai et al., 2010; Shi et al., 2010). Moreover, this could explain why SirT3 modulates susceptibility to lipotoxicity (Bao et al., 2010). Likewise, deacetylation of GDH allows amino acids to be converted into α KG for metabolism. A recent proteomics screen identified many potential SirT3 mitochondrial protein substrates, including ornithine transcarbamoylase (Hallows et al., 2011). Deacetylation of this urea cycle enzyme by SirT3 activates the urea cycle for disposal of ammonia when amino acids are catabolically stripped of their carbon. The fact that SirT5 was already known to deacetylate and activate another urea cycle enzyme, CPS1 (Nakagawa et al., 2009; Ogura et al., 2010), reinforces the importance of the urea cycle in sirtuin-mediated adaptations to energy limitation. Starvation also induces the liver to synthesize ketones to help bridge energy deficits for the brain and SirT3 was also shown to deacetylate and activate 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCS2), the rate-limiting enzyme for the synthesis of the ketone β -hydroxybutyrate (Shimazu et al., 2010).

These metabolic adaptations all allow the organism to use fuels that might otherwise be stored (fat and amino acids) or

ignored (acetate) and globally shift energy production away from carbohydrate catabolism. How might this be beneficial for health and longevity? It was suggested that garnering energy from fat might per se reduce ROS production (Guarente, 2008). This follows, because some of the electrons derived from oxidation of fatty acids feed into

the electron transport chain via FADH₂ (and not NADH), thus bypassing one of the sources of ROS production, complex I. Of course, this strategy does not bypass the other source of ROS production, complex III. However, the induction of SirT3 might do so, because this sirtuin appears to suppress ROS from complex III (Bell et al., 2011).

Do SirT1 and SirT3 Cooperate in CR?

Given the strong data that both SirT1 and SirT3 are required for the responses to CR, is there any single unifying pathway that links these two sirtuins? Interestingly, SirT3 expression has been linked to the activity of the coactivator PGC-1 α via the estrogen-related receptor alpha (ERR α), which binds to the SirT3 promoter (Giralt et al., 2011; Kong et al., 2010). SIRT1 has been convincingly shown to deacetylate PGC-1 α to increase its potential to activate transcription (Nemoto et al., 2005; Rodgers et al., 2005). For example, in CR, SirT1 protein levels and NAD⁺ levels increase in muscle and white adipose tissue, which results in an increase in mitochondrial biogenesis by the activated PGC-1 α (Nisoli et al., 2005). It is thus tempting to draw a linear pathway in which CR triggers an increase in SirT1 activity, thereby activating PGC-1 α and SirT3 expression (Figure 4). This strategy would coordinate the CR response between the nucleus and the mitochondria and might be a core mechanism driving the anti-aging effects of this dietary regimen.

However, it is also possible that the activity of SirT3 would be induced by an increase in the mitochondrial NAD⁺/NADH ratio during CR (Guarente, 2000; Nakagawa et al., 2009) even without an increase in the levels of this sirtuin. The observed increase in SirT3 protein levels may only magnify such an increase in activity. One test of the model in Figure 4 would be to determine whether CR can induce expression of SirT3 in tissues knocked out for SirT1. If not, this would confirm the model linking SirT1 to SirT3 expression.

Conclusions

Sirtuins have been proposed to be regulators of aging and diseases of aging. For the nuclear SirT1, there are strong data supporting disease mitigation and slowing of aging by several

criteria, although life-span extension has not yet been observed. This latter finding may be because SirT1 has so many important functions in mammalian physiology that global upregulation can exert opposing effects. Alternatively, global upregulation may indeed slow aging globally, but may not affect the proximal cause limiting life span in the mouse strains tested.

In contrast, SirT3 is a mitochondrial protein (Onyango et al., 2002; Schwer et al., 2002) and knockout mice present with no obvious phenotypes, at least as young animals. However, more subtle analyses have shown that CR induces SirT3 levels resulting in lower ROS and oxidative damage to mitochondria. So far, most of the evidence linking SirT3 to these processes involves loss of function, which triggers greater ROS, mitochondrial oxidative damage, and loss of protection by CR. Cell-based studies have recently provided the first evidence that gain of function has the potential to exert opposite effects (Bell et al., 2011). It will be important to bolster these studies with transgenic mice that exhibit increased SirT3 expression.

Interestingly, SirT3 is the only sirtuin for which a human polymorphism has been associated with extreme longevity. This allele is reported to create an enhancer in intron 5 of the SirT3 gene and is highly enriched in a long-lived population in southern Italy (Bellizzi et al., 2005, 2009; Rose et al., 2003). It will be important to carry out comprehensive analysis of SirT3 polymorphisms in other populations to secure the link between SirT3 and human longevity. Finally, a recent study indicates that SirT3 is critical in protecting mouse preimplantation embryos against oxidative damage (Kawamura et al., 2010). Testing whether sirtuins play roles in early embryogenesis or even gametogenesis is fertile ground for study based on the hypothesis that antiaging mechanisms protecting the soma of adults may also maintain youthfulness of the species in transitioning from one generation to the next.

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