Title

The Role of Mitochondrial Deacetylase SIRT3: Delivering Benefits of Calorie Restriction and Promoting Adult Stem Cell Function

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The Role of Mitochondrial Deacetylase SIRT3: Delivering Benefits of Calorie Restriction and Promoting Adult Stem Cell Function

by

Katharine van Dyke Brown

A dissertation submitted in partial satisfaction of the
requirements for the degree of
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of the
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Committee in Charge:

Prof. Danica Chen, Chair
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Abstract

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With the increase in the aging population, there has been a growing interest in understanding the process of age-related physical decline and increased disease risk. Research in model organisms has shown that aging, far from being a spontaneous development, is actually a controlled process, with molecular mechanisms that can alter the pace of cellular and tissue decline. The aim of this dissertation work was to gain insight into the molecular mechanisms that can control this aging rate.

We found that SIRT3, a mitochondrial NAD+-dependent deacetylase, performs a vital role during calorie restriction in mice to decrease oxidative damage in tissues. These results led us to identify superoxide dismutase 2(SOD2) as a target protein of SIRT3, and confirmed that SIRT3 can deacetylate SOD2 at two critical lysine residues (K53 and K89). Deacetylation of these residues on SOD2 leads to an increase in SOD2 detoxification activity. Furthermore, SOD2 is more deacetylated in the tissues of mice on calorie restriction, but this effect is abrogated in mice that are deficient for SIRT3. These results led us to develop a model whereby calorie restriction upregulates SIRT3 expression and activity, leading to an increased deacetylation of SOD2. The increased activity level of the deacetylated SOD2 has the effect of decreasing oxidative damage in tissues.

Concomitantly, we found that SIRT3 is required for the switch to fatty acid utilization during calorie restriction. SIRT3 KO mice on calorie restriction have reduced β- oxidation, lower long chain-acyl CoA dehydrogenase activity (LCAD), and a preference for glucose uptake and carbohydrate metabolism. Our findings indicate that other molecular adaptations that occur during calorie restriction are insufficient to compensate for a SIRT3 deficiency in this metabolic context.

We also present our findings that SIRT3 is highly expressed in hematopoietic stem cells (HSCs) as compared to differentiated hematopoietic cells, which led us to explore the role SIRT3 was playing in this stem cell population. Our results indicate that SIRT3 is required

as a stress-responsive protein that can protect stem cell function during conditions of oxidative stress. These conditions can include serial transplant, chemical treatment, or the increased oxidative stress associated with aging. Furthermore, we show that SIRT3 expression is decreased in HSCs from aged mice, and enforced expression of SIRT3 can improve the function of aged HSCs, suggesting the potential for rejuvenation of aged HSCs.

Our studies confirm the role that SIRT3 plays in protecting cells and tissues from oxidative stress, as well as offer. Although to date, no lifespan data has been published on SIRT3 KO mice, the results presented here indicate that a relatively shortened lifespan or healthspan would not be unexpected. These findings also open avenues for understanding the role of SIRT3 in stem cell biology, both other stem cell types, and potentially in human stem cell systems.

To the various teachers, mentors, and role models who have encouraged my love of science throughout my life. Most especially to my parents, Walter and Patricia Brown, for their unwavering support, and to my younger brother, Ian, who inspires me as much as I hope inspire I him.

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CHAPTER I

INTRODUCTION

Aging and the associated physical decline are considered a normal part of the lifespan progression in most organisms. This physical decline leads to a decrease in tissue maintenance and repair, and also increased rates of occurrence for a plethora of age-related diseases. In humans, considering the unprecedented increase in average lifespan and the expansion of the aging population, there has been considerable interest in understanding the aging process. Although it was once believed that aging was a spontaneous development, it is now widely understood that aging in a controlled process, with molecular mechanisms which can alter the pace of aging. In this introduction, there are presented two previously published articles, reproduced with permissions, which discuss some aspects of causal factors in determining the balance between youthful maintenance and aging.

[1]PART I: AGING: THE MITOCHONDRIAL CONNECTION

Abstract

Mitochondria are the powerhouses of the cell, providing the vast majority of cellular ATP under aerobic conditions. However, this essential function comes at the cost of reactive oxygen species (ROS) production, which can cause cellular damage. With age, mitochondrial function declines, resulting in decreased ATP production and increased ROS production, which may contribute to the aging process. In this review, we explore the evidence linking impaired mitochondrial function to age-related degeneration, highlight methods that have been shown to improve mitochondrial function, and speculate on whether they can lead to rejuvenation.

Introduction

Aging is broadly defined as a decline in the functional capacity of both individual cells and entire tissues over time. This decline in function leads not only to an increase in disease risk, but also to the eventual demise of the organism. Although the aging process is still not fully understood, events at the subcellular level are increasingly implicated in promoting or allowing the progressive deterioration during aging. The mitochondrion, an organelle typically presented as the “powerhouse of the cell,” has received a great deal of attention as a driving cause of aging in cells and tissues. With increased understanding of how mitochondria decline with age, there are increased

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opportunities for therapeutic interventions that could not only improve mitochondrial function, but benefit the entire cell, tissue, and organism. Here, we review different aspects of mitochondrial changes with aging (Figure 1), as well as attempts to limit or reverse mitochondrial dysfunction to prevent aging (Figure 2).

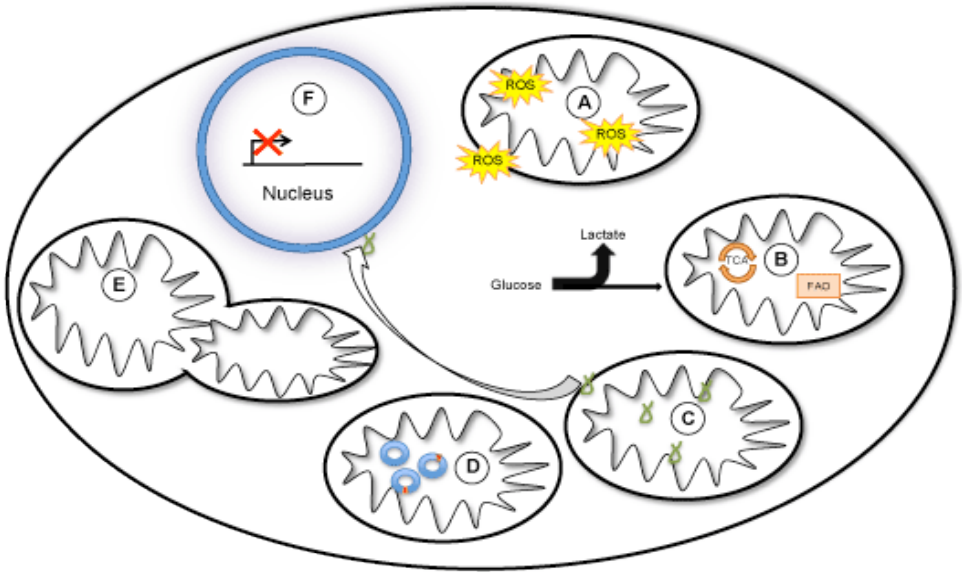

Figure 1: Mitochondria experience several functional and morphological changes with age. These include an increase in oxidative stress and damage (A), metabolic shift towards glycolysis and extra-mitochondrial energy metabolism (B), reduced retrograde signaling from mitochondria to nucleus (C), increased mtDNA mutation/deletions (D), altered mitochondrial dynamics (E), decreased expression of mitochondrial biogenic genes in the nucleus (F).

Changes in Mitochondria with Aging ROS

A prominent theory proposes that sometime in the early evolution of eukaryotes, an endosymbiotic event led to the formation of mitochondria [1,2]. This event allowed eukaryotic cells to perform oxidative respiration, conferring an evolutionary advantage as evidenced by the explosion of eukaryotic cell life forms. However, mitochondria also became the major site of reactive oxygen species (ROS) production that could, if uncontrolled, level considerable damage on the mitochondria and potentially the rest of the cell [3]. Extrapolated to a multi-cellular organism, ROS could be detrimental to tissue integrity and function, especially if oxidative damage were allowed to accumulate over time. In order to combat this problem, the mitochondrion has a well-developed cadre of antioxidant enzymes and molecules to defend against ROS production. For example, it has its own isoforms of superoxide dismutase (SOD2 or MnSOD), and glutathione peroxidase [4,5].

The ability of mitochondria to defend against oxidative stress is not necessarily maintained throughout aging. Endogenous antioxidant systems, especially in the mitochondria, are often found to be downregulated with age [6,7]. Aged tissues often have high levels of oxidative damage, and oxidative stress has been implicated in the

progression of age-related diseases such as diabetes, heart disease, hearing loss, cancer, and various neurological disorders [7-11]. These observations support the “Mitochondrial Free Radical Theory of Aging”, which proposes that oxidative stress and increasing mitochondrial dysfunction create a vicious cycle that promotes the deterioration of the cell and eventually, the deterioration of tissues and the whole organism [12,13].

Despite the elegant simplicity behind the “Mitochondrial Free Radical Theory of Aging”, it is not without controversy. Numerous studies have shown that the relationship between oxidative stress, aging, and longevity is tenuous at best. A comparison of rats and pigeons, similarly sized endotherms that have a seven-fold difference in longevity, showed no overt difference in ROS production and antioxidant levels in various tissues [14]. However, rats, which are shorter-lived, show increased oxidative damage to fatty acids. The naked mole rat (Heterocephalus glaber), a curious rodent with a lifespan of up to 30 years or more, shows intriguing patterns in oxidative stress and damage. Compared to closely related species with much shorter lifespans, such as mice and rats, the naked mole rats produce comparable amounts of oxygen radicals and, surprisingly, exhibits higher biomarkers of oxidative damage [15,16]. However, the naked mole rats differ in their susceptibility to oxidative stress: cells from the naked mole rats have increased resistance to oxidative stress-induced apoptosis [17]. Thus, a confounding factor to oxidative stress is the capacity of cells to resist oxidative stress.

If oxidative stress and damage are indeed causal to aging, reducing oxidative stress and damage should slow or reverse aging. However, several studies indicate just the opposite. Exogenous antioxidant treatments are not helpful in promoting longevity or preventing age-related diseases [18-22]. Strikingly, overexpression of several major antioxidants, including the various isoforms of SODs, as well as catalase, an antioxidant enzyme in the peroxisome, did not lead to increased lifespan [23].

Some explanations as to why antioxidant treatments remain ineffectual include technical considerations, such as permeability through membranes and bioavailability of the antioxidant to the sites of oxidative stress [24]. In support of this idea, the targeted expression of catalase to the mitochondria clearly offers protection against mitochondrial oxidative stress and age-related decline in mitochondrial function in mice [25]. Similarly, the antioxidant compound SkQ1, whose positive charge allows targeting to the negatively charged intermembrane space of the mitochondria, is successful in reducing mitochondrial oxidative stress and decelerating senescence [26,27]. Additionally, overexpression of the cytosolic antioxidant thioredoxin 1 in mice conferred clear protection against oxidative damage, as well as increased survivability at earlier life stages [28].

Another possibility is that merely increasing the levels of the antioxidative enzymes is not sufficient to effectively increase antioxidative capacity. The antioxidative enzymes may need to be modified to function at full potential. For example, overexpression of SOD2 only modestly reduces cellular ROS levels. However, deacetylated SOD2 has dramatically increased capacity to dampen cellular ROS [29]. Thus, despite some

evidence inconsistent with the Free Radical Theory of Aging, oxidative stress is still likely to be a major cause of aging.

mtDNA Damage

Mitochondria are unique among the organelles in that they sustain their own genomes. The mitochondrial genome is limited to the expression of rRNAs, tRNAs, and genes coding for mitochondrial proteins, most of which are subunits in the complexes of the electron transport chain (ETC) (as reviewed in [30]). Damage or mutations to the mtDNA can accumulate clonally, leading to respiratory chain deficiencies in tissues [31-33]. mtDNA damage is widely believed to be created by ROS: the proximity of the mtDNA to the ROS-producing ETC induces damage and mutations in the mtDNA. These mtDNA mutations, if accumulated to a sufficient level, can lead to inefficient ETC and increased ROS production. This process becomes a vicious cycle [34].

Much has been learned about mtDNA mutation and aging from the mtDNA mutator mice, which express an error-prone version of the catalytic subunit of mtDNA polymerase. These mice show accumulation of mtDNA mutations and accelerated aging phenotypes [35]. Strikingly, in non-dividing tissues, the amount of ROS produced is normal and there is no increased accumulation of oxidative damage [36]. However, the mtDNA mutator mice contain dysfunctional somatic stem cells, which can be rescued by antioxidant treatment [37]. Thus, mtDNA mutations cause aging by increasing cellular ROS levels in somatic stem cells, which are particularly sensitive to oxidative stress and crucial for the aging process. In addition to stem cell defects, mtDNA mutator mice also have deficiencies in several ETC complexes [38], which indicates that dysregulated energy production and metabolism may also contribute to their premature aging phenotypes.

Energy Production and Metabolism

Mitochondria are often affectionately nicknamed “ the powerhouse of the cell.” The epithet is well earned, as one of the most important contributions of the mitochondria to the cell is the production of ATP through oxidative phosphorylation. It has been observed that aged mitochondria have diminished ATP production [39]. The expression of mitochondrial genes is usually downregulated in aged rats and mice, which may account for diminished mitochondrial ATP production [40,41]. Interestingly, the long-lived naked mole rats do not have a reduction in mitochondrial gene expression with age.

The diminished ATP production by aged mitochondria poses a problem for the cell: aged cells must either be able to survive on less ATP or they must shift their metabolism toward extra-mitochondrial energy production. Recent work by Houtkooper et al. indicates that the latter may be occurring [42]. Among their findings was evidence that aged mouse liver and muscle have reduced glycolytic intermediates, but more lactate, indicating a higher reliance on anaerobic metabolism through glycolysis. This reliance on anaerobic glycolysis is in keeping with their observations that several genes

that code for mitochondrial proteins are transcriptionally downregulated with age, including genes involved in fatty acid import and oxidation, as well as genes in the ETC. These findings are consistent with work done on dog liver tissue, where glycolytic genes were found to be upregulated, but ATPase was downregulated [43]. This increased dependence on glycolysis with age may be a conserved phenomenon in humans, as suggested by work on aged skin keratinocytes [44].

Consistent with changes in metabolism, the cellular redox status changes with age. In several tissues of aged wistar rats, both the total cellular NAD+ pool and the NAD+:NADH ratio were diminished compared to young rats [45]. These changes in redox status can have dramatic effects on aging. For example, this environment is prohibitive for the activity of NAD+-dependent enzymes, such as the sirtuins, which extend lifespan in model organisms. It would be interesting to know if the mitochondrial NAD+ and NADH pools are altered with age.

Structure and Dynamics

Mitochondria have very distinct structures, consisting of a double membrane construction and a highly folded internal membrane forming the “cristae”. Mitochondria form adaptable networks, which fuse and break apart in turn. These mitochondrial dynamics have been increasingly appreciated for their role in healthy mitochondrial maintenance. Deficient fusion or fission causes severe mitochondrial dysfunction, which has profound physiological relevance, such as neurodegeneration and muscle atrophy [46-50]. Aged tissues were found to harbor giant mitochondria [51-53], suggesting that mitochondrial dynamics may be altered in aged tissues, contributing to mitochondrial decline with age.

Maintaining structure within the mitochondria also has functional consequences. The major site for oxidative phosphorylation (OXPHOS) is the cristal membrane. The organization of the cristae into welldefined cristal junctions creates a thermodynamically favorable environment for efficient ETC and ATP production [54]. Whereas the muscle mitochondria of young rats have well delineated cristal structures, the muscle mitochondria of aged rats display undefined cristae [55]. These structural defects may result in an age-dependent decline in mitochondrial function.

Mitochondria not only interact with each other, but can physically interface with other organelles, such as the endoplasmic reticulum (ER) [56]. This interface can affect calcium pools and calcium-related signaling within these organelles. The ER-mitochondria foci also allow for direct exchange of phospholipids necessary to maintain the mitochondrial inner and outer membranes. These interactions may represent a potentially important mitochondrial maintenance mechanism during aging. Indeed, an alternate explanation for the agerelated swollen “giant mitochondria” is increased calcium signaling and aberrant opening of the mitochondrial permeability transition pores [57].

Mitochondrial-nuclear Crosstalk

Although mitochondria have their own DNA, the coding regions are only responsible for a small fraction of the total mitochondrial proteins; nuclear genomic DNA code for the remainder. Therefore, it is necessary for the nucleus and mitochondria to communicate their adaptive needs as the cell experiences different stressors. “Mitohormesis” is a concept that arose to describe the retrograde cellular response to mild stresses in the mitochondria. A mild insult occurring in the mitochondria, such as low levels of oxidative stress, results in the cell mounting a protective response to manage the insult. The net outcome is actually beneficial to the cell. This process is very much dependent upon mitochondrial-nuclear communication and is thought to underlie the life-extension benefits of low glucose dietary interventions [58,59].

The rhomboid protease PARL, found in the inner mitochondrial matrix, has the ability to cleave itself, creating a PARLb peptide, which is targeted to the nucleus to promote the transcription of mitochondrialrelated nuclear factors (PGC1-b, NRF1, and MFN1) and increase mitochondrial mass [60]. PARL appears to be relevant to mitochondrial aging as PARL expression was found to be downregulated in aged human skeletal muscle biopsies [61]. The decreased expression of PARL with age may lead to a decreased ability to communicate mitochondrial bioenergetic signals back to the nucleus through the PARLb peptide.

Another mitochondrial-nuclear crosstalk pathway was uncovered in Retinal Pigment Epithelial (RPE) cells. Under oxidative stress conditions, the mitochondrial protein prohibitin is shuttled from the mitochondria to the nucleus, creating anti-apoptotic conditions to protect the cells from premature cell death [62]. Interestingly, prohibitin expression is decreased in RPE cells with age, indicating that this defense mechanism is suppressed with age.

The Promise of Rejuvenation

The possible causal role of declining mitochondrial function to aging raises the question of whether rejuvenation can be achieved by improving mitochondrial function. This section of the review will focus on methods to improve mitochondrial function and speculate on whether any of these methods could potentially be used to rejuvenate cells and extend lifespan (Figure 2).

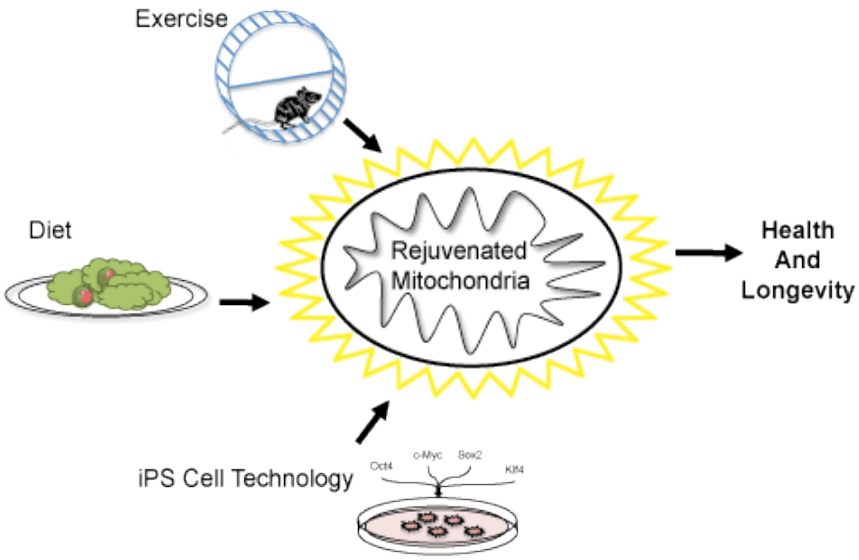

Figure 2: Possible interventions for mitochondrial rejuvenation. Exercise serves to improve mitochondrial function and reduce pathologies even in mice with inborn mitochondrial dysfunction. Calorie restriction (CR), a dietary intervention that increases longevity in a variety of organisms, improves mitochondrial function. Induced pluripotent stem (iPS) cell technology "reset" the clock in the mitochondrial compartment. Improving mitochondria function may have benefits at the cellular and organismal levels, leading to improved health and potentially increased longevity.

CR, SIRT3, and Mitochondria

It has been known for decades that Calorie Restriction (CR), a diet that consists of reduced caloric intake without malnourishment, extends lifespan in rodents, and recent work has shown that CR extends lifespan in yeast, worms, and flies among other organisms [63]. CR has even been shown to dramatically improve health and cognition and reduce age-related mortality in primates [64]. While the mechanisms behind how

CR improves health and extends lifespan are not completely known, studies have consistently shown that CR leads to a reduction in mitochondrial oxidative stress [65]. Originally, the reduced oxidative stress in mitochondria was thought to be due to a decreased metabolic rate, resulting in a lower production of ROS, and some evidence supports this theory [66,67]. However, other studies indicate that mitochondrial activity actually increases in organisms undergoing CR [68,69]. Indeed, CR promotes mitochondrial biogenesis via eNOS [68]. Thus, CR increases the amount of mitochondria and can lead to an increase in their metabolic rate. How then do mitochondria reduce oxidative stress under CR?

Clues to the resolution of this puzzle came from reports that during CR, the ability of mitochondria to scavenge ROS improves, and this ability depends on the activity of the sirtuin SIRT3 [10,29]. The sirtuins are an evolutionarily conserved family of NAD+-depended deacetylases that have been shown to promote lifespan in model organisms when overexpressed [70]. SIRT3 is one of seven mammalian sirtuins and one of three

that are localized to the mitochondria [71]. CR increases the expression and activity of SIRT3 [29,72]. SIRT3 deacetylates superoxide dismutase 2 (SOD2) and isocitrate dehydrogenase 2 (IDH2), increasing their activities, which are essential for reduction of oxidative stress in the mitochondria. In the absence of SIRT3, the reduction in oxidative stress during CR is abrogated [10,29].

From a physiological standpoint, SIRT3 has been shown to be crucial for the ability of CR to stave off age-related hearing loss [10]. Hearing loss is a degenerative condition that occurs with aging and is at least partially driven by oxidative damage arising from mitochondria. Mice fed a CR diet show a delay in the deterioration of hearing that is abrogated in the absence of SIRT3 [10]. The study directly implicated oxidative stress from mitochondria in a pathology of aging and showed that this condition can be ameliorated by a dietary regimen.

The importance of SIRT3 for mitochondrial health is also demonstrated by studies in the heart and the kidneys. Mice deficient in SIRT3 show premature aging in the heart, due to increased oxidative stress and dysregulation of the mitochondrial permeability transition pore. This results in hypertrophy and fibrosis in the heart well before the age at which they normally occur in wild type hearts [73,74]. Oxidative stress from the mitochondria also plays a detrimental role in kidney function as evidenced by mice fed a high fat diet, which exhibit strong kidney inflammation. This inflammation was abrogated by enforced expression of SIRT3, which improved mitochondrial function and the health of the kidney cells [75].

The benefits of CR are at least partially mediated by improved function of mitochondria. SIRT3 is crucial for maintaining mitochondrial health and for reducing oxidative stress in mitochondria under CR. Thus, SIRT3 serves as an intriguing target for mitochondrial rejuvenation. While CR poses practical limitations in human implementation due to its effect on the quality of life, pharmaceutical activators of proteins activated by CR, such as SIRT3, may provide most of the beneficial effects of CR with none of the hunger pains. A study of mitochondrial health and lifespan in SIRT3 transgenic mice will be informative with regards to assessing the feasibility of this approach.

Rejuvenation through Exercise

The idea of rejuvenation through exercise has been tested with the mitochondrial mutator mice [35]. When the mice were placed on an endurance exercise regimen for 5 months, they showed substantial improvements in mitochondrial function along with much improved physiology [76]. Although the molecular mechanisms underlying these drastic systemic changes are not well understood, it is thought that endurance exercise may increase the activity of PGC-1α, which has been speculated to increase mitochondrial biogenesis and improve clearance of damaged mitochondria [76].

Next-generation Interventions

More advanced molecular interventions hold even more promise for rejuvenation. A cocktail of 4 transcription factors has been shown to restore the differentiation capacity of cells, including cells from very aged donors, into cells that have attributes of embryonic stem cells (ESCs), termed induced pluripotent stem (iPS) cells [77]. Recent work indicates that the process of inducing pluripotency also rejuvenates the energetic capacity of the aged cells, and dramatically improves their mitochondrial function [78,79]. Mitochondria from the iPSC cells of centenarian donors are functionally and morphologically indistinguishable from the mitochondria of Embryonic Stem Cells (ESCs). Thus, the decline in mitochondrial function with age is fully reversible.

Spatial and Temporal Mitochondrial Regulation of Aging

While it is clear that increasing mitochondria dysfunction contributes to the decline of tissue integrity and the progression of aging, it is unclear whether mitochondrial health is equally important across all tissue and cell types. Tissue-specific stem cells are responsible for the maintenance and repair of the tissues of an organism throughout its lifespan. Given this monumental task, it is probable that these cell populations are more sensitive to age-related mitochondrial dysfunction. Aged stem cells have fewer mitochondria, reduced oxidative metabolism, and increased oxidative stress [80]. Numerous mouse models with defective management of ROS have compromised stem cell functions, which can be rescued by antioxidant treatments [81-83], indicating the critical importance of mitochondrial oxidative stress in stem cell and tissue maintenance during the aging process. As discussed above, stem cells are particularly sensitive to mitochondrial damage and oxidative stress compared to post-mitotic tissues.

Mitochondria in different tissues may differentially contribute to organismal longevity. In D. melanogaster, overexpression of PGC-1 in the digestive tract has been shown to improve mitochondrial function and increase longevity [84]. Yet, there is no increased longevity for overexpression in neurons, muscle, or upon ubiquitous overexpression of PGC-1.

Elegant studies in C. elegans provide high resolution spatial and temporal views of mitochondrial regulation during the aging process [85]. Although compromised mitochondrial function is thought to contribute to aging, lifespan can also be extended by reducing mitochondrial function, and in particular, the function of ETC components. However, reduced ETC modulates the aging process in tissue-specific and temporal-specific manners. The L3/L4 larval developmental period, when mitochondria undergo dramatic proliferation, is a critical period in which reduced ETC modulates the aging process. Suppressing the ETC components before but not after this developmental stage induces the mitochondrial stress response and extends lifespan. Not all tissues are equally responsive to the ETC-induced lifespan extension. For example, reducing the ETC in neuronal and intestinal tissues but not muscle extends lifespan. Strikingly, mitochondrial stress in one tissue can produce a signal that is transmitted to a distal tissue to induce mitochondrial stress. Thus, mitochondrial perturbation can modulate the aging process in a non cell-autonomous fashion.

Conclusion and Future Perspectives

The mysteries shrouding the aging process are slowly being unraveled by the powerful tools of molecular and cell biology. As we have learned more about the mechanisms that contribute to aging at the molecular level, we have begun to appreciate the complexity of aging and have come to familiarize ourselves with the key players in the process. Mitochondria are surely among the most important of these players. However, the role of mitochondria in aging is complex, as highlighted by observations that reducing oxidative stress may not be sufficient to extend lifespan. Indeed, extension of lifespan may require improved mitochondrial function, which paradoxically, can be induced by mildly stressing the mitochondria, a concept known as mitohormesis.

The question then is whether there is an optimal level of oxidative stress in mitochondria to induce peak function. And, if that optimal level of stress exists, which tissues would benefit most from improved mitochondrial function and at what timeframe? At a smaller scale, what cell types in the tissues would make the best targets? Somatic stem cells, which are responsible for maintaining tissues throughout the life of an organism, would appear to be prime targets for interventions to improve mitochondrial function. Much work remains to be done to answer these questions, which will form the basis for developing mitochondrial interventions to improve health and extend lifespan.

References

1. Sagan L (1967) On the origin of mitosing cells. J Theor Biol 14: 255-274.

2. Bhattacharya D, Archibald JM, Weber AP, Reyes-Prieto A (2007) How do endosymbionts become organelles? Understanding early events in plastid evolution. Bioessays 29: 1239-1246.
3. Whiteman M, Dogra Y, Winyard PG, Armstrong JS (2009) Detection and measurement of reactive oxygen intermediates in mitochondria and cells. Methods Mol Biol 476: 28-49.
4. Melov S (2000) Mitochondrial oxidative stress. Physiologic consequences and potential for a role in aging. Ann N Y Acad Sci 908: 219-225.
5. Panfili E, Sandri G, Ernster L (1991) Distribution of glutathione peroxidases and glutathione reductase in rat brain mitochondria. FEBS Lett 290: 35-37.
6. Bai XY, Ma Y, Ding R, Fu B, Shi S, et al. (2011) miR-335 and miR-34a Promote renal senescence by suppressing mitochondrial antioxidative enzymes. J Am Soc Nephrol 22: 1252-1261.
7. Castro Mdel R, Suarez E, Kraiselburd E, Isidro A, Paz J, et al. (2012) Aging increases mitochondrial DNA damage and oxidative stress in liver of rhesus monkeys. Exp Gerontol 47: 29-37.
8. Zhang L, Ebenezer PJ, Dasuri K, Fernandez-Kim SO, Francis J, et al. (2011) Aging is associated with hypoxia and oxidative stress in adipose tissue: implications for adipose function. Am J Physiol Endocrinol Metab 301: E599-607.
9. Cocheme HM, Quin C, McQuaker SJ, Cabreiro F, Logan A, et al. (2011) Measurement of H2O2 within living drosophila during aging using a ratiometric

mass spectrometry probe targeted to the mitochondrial matrix. Cell Metab 13: 340-350.
10. Someya S, Yu W, Hallows WC, Xu J, Vann JM, et al. (2010) Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell 143: 802-812.
11. Finley LW, Carracedo A, Lee J, Souza A, Egia A, et al. (2011) SIRT3 opposes reprogramming of cancer cell metabolism through HIF1a destabilization. Cancer Cell 19: 416-428.
12. HARMAN D (1956) Aging: a theory based on free radical and radiation chemistry. J Gerontol 11: 298-300.
13. Harman D (1972) The biologic clock: the mitochondria? J Am Geriatr Soc 20: 145-147.
14. Montgomery MK, Hulbert AJ, Buttemer WA (2011) The long life of birds: the rat-pigeon comparison revisited. PLoS One 6: e24138.
15. Pérez VI, Buffenstein R, Masamsetti V, Leonard S, Salmon AB, et al. (2009) Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat. Proc Natl Acad Sci U S A 106: 3059-3064.
16. Andziak B, O'Connor TP, Qi W, DeWaal EM, Pierce A, et al. (2006) High oxidative damage levels in the longest-living rodent, the naked mole-rat. Aging Cell 5: 463-471.
17. Labinskyy N, Csiszar A, Orosz Z, Smith K, Rivera A, et al. (2006) Comparison of endothelial function, O2-* and H2O2 production, and vascular oxidative stress resistance between the longest-living rodent, the naked mole rat, and mice. Am J Physiol Heart Circ Physiol 291: H2698-2704.
18. Bernhard D, Wang XL (2007) Smoking, oxidative stress and cardiovascular diseases--do anti-oxidative therapies fail? Curr Med Chem 14: 1703-1712.
19. Magwere T, West M, Riyahi K, Murphy MP, Smith RA, et al. (2006) The effects of exogenous antioxidants on lifespan and oxidative stress resistance in Drosophila melanogaster. Mech Ageing Dev 127: 356-370.
20. (1994) The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. N Engl J Med 330: 1029-1035.
21. Bjelakovic G, Nikolova D, Simonetti RG, Gluud C (2004) Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. Lancet 364: 1219-1228.
22. Heart Protection Study Collaborative Group (2002) MRC/BHF heart protection study of antioxidant vitamin supplementation in 20,536 high-risk individuals: A randomised placebo-controlled trial. Lancet 360: 23-33.
23. Pérez VI, Van Remmen H, Bokov A, Epstein CJ, Vijg J, et al. (2009) The overexpression of major antioxidant enzymes does not extend the lifespan of mice. Aging Cell 8: 73-75.
24. GaÅ¡perlin M, Gosenca M (2011) Main approaches for delivering antioxidant vitamins through the skin to prevent skin ageing. Expert Opin Drug Deliv 8: 905-919.

25. Lee HY, Choi CS, Birkenfeld AL, Alves TC, Jornayvaz FR, et al. (2010) Targeted expression of catalase to mitochondria prevents age-associated reductions in mitochondrial function and insulin resistance. Cell Metab 12: 668-674.
26. Skulachev MV, Antonenko YN, Anisimov VN, Chernyak BV, Cherepanov DA, et al. (2011) Mitochondrial-targeted plastoquinone derivatives. Effect on senescence and acute age-related pathologies. Curr Drug Targets 12: 800-826.
27. Shipounova IN, Svinareva DA, Petrova TV, Lyamzaev KG, Chernyak BV, et al. (2010) Reactive oxygen species produced in mitochondria are involved in age-dependent changes of hematopoietic and mesenchymal progenitor cells in mice. A study with the novel mitochondria-targeted antioxidant SkQ1. Mech Ageing Dev 131: 415-421.
28. Pérez VI, Cortez LA, Lew CM, Rodriguez M, Webb CR, et al. (2011) Thioredoxin 1 overexpression extends mainly the earlier part of life span in mice. J Gerontol A Biol Sci Med Sci 66: 1286-1299.
29. Qiu X, Brown K, Hirschey MD, Verdin E, Chen D (2010) Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell Metab 12: 662-667.
30. Falkenberg M, Larsson NG, Gustafsson CM (2007) DNA replication and transcription in mammalian mitochondria. Annu Rev Biochem 76: 679-699.
31. Bua E, Johnson J, Herbst A, Delong B, McKenzie D, et al. (2006) Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers. Am J Hum Genet 79: 469-480.
32. Hartmann N, Reichwald K, Wittig I, Dröse S, Schmeisser S, et al. (2011) Mitochondrial DNA copy number and function decrease with age in the short-lived fish Nothobranchius furzeri. Aging Cell 10: 824-831.
33. Mao P, Gallagher P, Nedungadi S, Manczak M, Shirendeb UP, et al. (2012) Mitochondrial DNA deletions and differential mitochondrial DNA content in Rhesus monkeys: implications for aging. Biochim Biophys Acta 1822: 111-119.
34. Greaves LC, Reeve AK, Taylor RW, Turnbull DM (2012) Mitochondrial DNA and disease. J Pathol 226: 274-286.
35. Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, et al. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature 429: 417-423.
36. Trifunovic A, Hansson A, Wredenberg A, Rovio AT, Dufour E, et al. (2005) Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production. Proc Natl Acad Sci U S A 102: 17993-17998.
37. Ahlqvist KJ, Hämäläinen RH, Yatsuga S, Uutela M, Terzioglu M, et al. (2012) Somatic progenitor cell vulnerability to mitochondrial DNA mutagenesis underlies progeroid phenotypes in Polg mutator mice. Cell Metab 15: 100-109.
38. Edgar D, Shabalina I, Camara Y, Wredenberg A, Calvaruso MA, et al. (2009) Random point mutations with major effects on protein-coding genes are the driving force behind premature aging in mtDNA mutator mice. Cell Metab 10: 131-138.
39. Ghosh S, Lertwattanarak R, Lefort N, Molina-Carrion M, Joya-Galeana J, et al. (2011) Reduction in reactive oxygen species production by mitochondria from

elderly subjects with normal and impaired glucose tolerance. Diabetes 60: 2051-2060.
40. Kim EB, Fang X, Fushan AA, Huang Z, Lobanov AV, et al. (2011) Genome sequencing reveals insights into physiology and longevity of the naked mole rat. Nature 479: 223-227.
41. Yu C, Li Y, Holmes A, Szafranski K, Faulkes CG, et al. (2011) RNA sequencing reveals differential expression of mitochondrial and oxidation reduction genes in the long-lived naked mole-rat when compared to mice. PLoS One 6: e26729.
42. Houtkooper RH, Argmann C, Houten SM, Cantó C, Jeninga EH, et al. (2011) The metabolic footprint of aging in mice. Sci Rep 1: 134.
43. Kil DY, Vester Boler BM, Apanavicius CJ, Schook LB, Swanson KS (2010) Age and diet affect gene expression profiles in canine liver tissue. PLoS One 5: e13319.
44. Prahl S, Kueper T, Biernoth T, Wöhrmann Y, Münster A, et al. (2008) Aging skin is functionally anaerobic: importance of coenzyme Q10 for anti aging skin care. Biofactors 32: 245-255.
45. Braidy N, Guillemin GJ, Mansour H, Chan-Ling T, Poljak A, et al. (2011) Age related changes in NAD+ metabolism oxidative stress and Sirt1 activity in wistar rats. PLoS One 6: e19194.
46. Lee Y, Lee HY, Hanna RA, Gustafsson ÅB (2011) Mitochondrial autophagy by Bnip3 involves Drp1-mediated mitochondrial fission and recruitment of Parkin in cardiac myocytes. Am J Physiol Heart Circ Physiol 301: H1924-1931.
47. Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, et al. (2008) Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J 27: 433-446.
48. Chen H, Vermulst M, Wang YE, Chomyn A, Prolla TA, et al. (2010) Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. Cell 141: 280-289.
49. Malena A, Loro E, Di Re M, Holt IJ, Vergani L (2009) Inhibition of mitochondrial fission favours mutant over wild-type mitochondrial DNA. Hum Mol Genet 18: 3407-3416.
50. Chen H, McCaffery JM, Chan DC (2007) Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell 130: 548-562.
51. Beregi E, Regius O (1987) Comparative morphological study of age related mitochondrial changes of the lymphocytes and skeletal muscle cells. Acta Morphol Hung 35: 219-224.
52. Shaposhnikov VM (1985) The ultrastructural features of secretory cells of some endocrine glands in aging. Mech Ageing Dev 30: 123-142.
53. Sato T, Tauchi H (1975) The formation of enlarged and giant mitochondria in the aging process of human hepatic cells. Acta Pathol Jpn 25: 403-412.
54. Gilkerson RW, Selker JM, Capaldi RA (2003) The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. FEBS Lett 546: 355-358.
55. Lopes GS, Mora OA, Cerri P, Faria FP, Jurkiewicz NH, et al. (2004) Mitochondrial alterations and apoptosis in smooth muscle from aged rats. Biochim Biophys Acta 1658: 187-194.

56. Kornmann B, Currie E, Collins SR, Schuldiner M, Nunnari J, et al. (2009) An ER-mitochondria tethering complex revealed by a synthetic biology screen. Science 325: 477-481.
57. Crompton M (2004) Mitochondria and aging: a role for the permeability transition? Aging Cell 3: 3-6.
58. Tapia PC. Sublethal mitochondrial stress with an attendant stoichiometric augmentation of reactive oxygen species may precipitate many of the beneficial alterations in cellular physiology produced by caloric restriction, intermittent fasting, exercise and dietary phytonutrients: "mitohormesis" for health and vitality. Med Hypotheses 2006 66: 832-43.
59. Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, et al. (2007) Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress. Cell Metab 6: 280-293.
60. Sík A, Passer BJ, Koonin EV, Pellegrini L (2004) Self-regulated cleavage of the mitochondrial intramembrane-cleaving protease PARL yields Pbeta, a nuclear-targeted peptide. J Biol Chem 279: 15323-15329.
61. Civitarese AE, MacLean PS, Carling S, Kerr-Bayles L, McMillan RP, et al. (2010) Regulation of skeletal muscle oxidative capacity and insulin signaling by the mitochondrial rhomboid protease PARL. Cell Metab 11: 412-426.
62. Sripathi SR, He W, Atkinson CL, Smith JJ, Liu Z, et al. (2011) Mitochondrial-nuclear communication by prohibitin shuttling under oxidative stress. Biochemistry 50: 8342-8351.
63. Guarente L (2008) Mitochondria--a nexus for aging, calorie restriction, and sirtuins? Cell 132: 171-176.
64. Colman RJ, Anderson RM, Johnson SC, Kastman EK, Kosmatka KJ, et al. (2009) Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 325: 201-204.
65. Sohal RS, Weindruch R (1996) Oxidative stress, caloric restriction, and aging. Science 273: 59-63.
66. Heilbronn LK, de Jonge L, Frisard MI, DeLany JP, Larson-Meyer DE, et al. (2006) Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. JAMA 295: 1539-1548.
67. Blanc S, Schoeller D, Kemnitz J, Weindruch R, Colman R, et al. (2003) Energy expenditure of rhesus monkeys subjected to 11 years of dietary restriction. J Clin Endocrinol Metab 88: 16-23.
68. Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, et al. (2005) Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310: 314-317.
69. Selman C, Phillips T, Staib JL, Duncan JS, Leeuwenburgh C, et al. (2005) Energy expenditure of calorically restricted rats is higher than predicted from their altered body composition. Mech Ageing Dev 126: 783-793.
70. Longo VD, Kennedy BK (2006) Sirtuins in aging and age-related disease. Cell 126: 257-268.

71. Onyango P, Celic I, McCaffery JM, Boeke JD, Feinberg AP (2002) SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria. Proc Natl Acad Sci U S A 99: 13653-13658.
72. Shi T, Wang F, Stieren E, Tong Q (2005) SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. J Biol Chem 280: 13560-13567.
73. Hafner AV, Dai J, Gomes AP, Xiao CY, Palmeira CM, et al. (2010) Regulation of the mPTP by SIRT3-mediated deacetylation of CypD at lysine 166 suppresses age-related cardiac hypertrophy. Aging (Albany NY) 2: 914-923.
74. Sundaresan NR, Gupta M, Kim G, Rajamohan SB, Isbatan A, et al. (2009) Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. J Clin Invest 119: 2758-2771.
75. Koyama T, Kume S, Koya D, Araki S, Isshiki K, et al. (2011) SIRT3 attenuates palmitate-induced ROS production and inflammation in proximal tubular cells. Free Radic Biol Med 51: 1258-1267.
76. Safdar A, Bourgeois JM, Ogborn DI, Little JP, Hettinga BP, et al. (2011) Endurance exercise rescues progeroid aging and induces systemic mitochondrial rejuvenation in mtDNA mutator mice. Proc Natl Acad Sci U S A 108: 4135-4140.
77. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663-676.
78. Suhr ST, Chang EA, Tjong J, Alcasid N, Perkins GA, Goissis MD, Ellisman MH, Perez GI, Cibelli JB. Mitochondrial rejuvenation after induced pluripotency. PLoS One 2010 Nov 23 5: e14095.
79. Lapasset L, Milhavet O, Prieur A, Besnard E, Babled A, et al. (2011) Rejuvenating senescent and centenarian human cells by reprogramming through the pluripotent state. Genes Dev 25: 2248-2253.
80. Stoll EA, Cheung W, Mikheev AM, Sweet IR, Bielas JH, et al. (2011) Aging neural progenitor cells have decreased mitochondrial content and lower oxidative metabolism. J Biol Chem 286: 38592-38601.
81. Miyamoto K, Araki KY, Naka K, Arai F, Takubo K, et al. (2007) Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell 1: 101-112.
82. Ito K, Hirao A, Arai F, Takubo K, Matsuoka S, et al. (2006) Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat Med 12: 446-451.
83. Mortensen M, Soilleux EJ, Djordjevic G, Tripp R, Lutteropp M, et al. (2011) The autophagy protein Atg7 is essential for hematopoietic stem cell maintenance. J Exp Med 208: 455-467.
84. Rera M, Bahadorani S, Cho J, Koehler CL, Ulgherait M, et al. (2011) Modulation of longevity and tissue homeostasis by the Drosophila PGC-1 homolog. Cell Metab 14: 623-634.
85. Durieux J, Wolff S, Dillin A (2011) The cell-non-autonomous nature of electron transport chain-mediated longevity. Cell 144: 79-91.

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[2]PART II: SIRTUIN REGULATION IN CALORIE RESTRICTION

Abstract
The beneficial effects of calorie restriction diet in extending lifespan and preventing diseases have long been recognized. Recent genetic and molecular studies in model organisms began to uncover the molecular regulation of calorie restriction response, with the gene SIR2 playing an essential role. This article summarizes the latest development on how mammalian SIR2 homologs coordinately regulate the calorie restriction response.

Keywords
Sirtuin; Calorie restriction; Aging

1. Introduction

Metabolic syndrome, cancer, neurodegenerative diseases, and immune dysfunction are the major threats to human health. These seemingly unrelated diseases share one common theme: their incidence increases with age. It is intriguing that interventions slowing the aging process may have the potential to prevent a wide range of diseases associated with aging. This notion is supported by the beneficial effects of calorie restriction (CR) diet. 30–40% reduction of food intake increases lifespan up to 50% in laboratory rodents. Indeed, this dietary intervention of aging ameliorates many late-onset diseases [1].

An important question is whether the multifaceted effects of CR are mediated by regulated biological pathways that are amenable to studies and can be harnessed for disease prevention. Recent genetic and molecular studies in model organisms began to uncover the molecular regulation of CR response, with the gene SIR2 playing an essential role. SIR2 is highly conserved from yeast to mammals [2]. SIR2-like genes are collectively known as “sirtuins” (reviewed in [3] and several articles in this issue of BBA). In this review, we will discuss the regulation of sirtuins during CR, the effects of such regulation on aging and diseases of aging, and the implications on drug development.

2. Sirtuins as mediators of calorie restriction response

The lifespan of the budding yeast Saccharomyces cerevisiae can be measured by the number of daughter cells that one mother cell can produce (replicative lifespan) [4]. SIR2 promotes yeast replicative lifespan by inhibiting the generation of extrachromosomal ribosomal DNA circles (ERCs), maintaining intact telomeric chromatin, and ensuring appropriate distribution of oxidatively damaged proteins [5], [6], [7] and [8]. It was shown that the replicative lifespan of S. cerevisiae can be extended by reducing the available sugar in the medium [9]. Lifespan extension by this model of

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CR was found to be mediated by SIR2, as CR increases the silencing activity of SIR2, and does not extend the lifespan when SIR2 is deleted [10].

However, SIR2-independent lifespan extension by CR has been observed in a yeast strain in which the SIR2 paralog HST2 is also capable of inhibiting ERC generation [11]. Lifespan extension by CR is abolished when both SIR2 and HST2 are deleted. These findings have interesting implications in lifespan regulation in higher organisms, which have multiple members of the SIR2 family. For instance, there are 4 SIR2 homologs in Caenorhabditis elegans and 7 in mammals [2]. Multiple members of SIR2-like proteins may regulate lifespan cooperatively in response to diet. Evidence is emerging for elaborate cooperation between 7 mammalian sirtuins in regulating CR response (see below).

Another model of yeast aging has been developed, in which lifespan is measured by the length of time that cells survive in a nondividing state when exposed to a hypocaloric environment (chronological lifespan) [12]. In contrast to its effect on replicative aging, SIR2 is not required for chronological life extension by CR [13] and [14]. These studies generate another layer of complexity in lifespan regulation by SIR2, which may have interesting implications in higher organisms. While chronological aging may be a good model for postmitotic cells, replicative aging may more closely mirror mitotic cells in mammals [15]. It has been proposed that nutrient-responsive signaling pathways regulate chronological aging and replicative aging through different downstream effectors: increased stress resistance for chronological aging and decreased ribosome biogenesis for replicative aging (Fig. 1). It is logical that in mammals, aging in mitotic cells and postmitotic cells are regulated through different mechanisms. In mitotic cells, such as stem cells, interventions that increase their proliferation cause aging and the exhaustion of the stem cell pool [16] and [17].

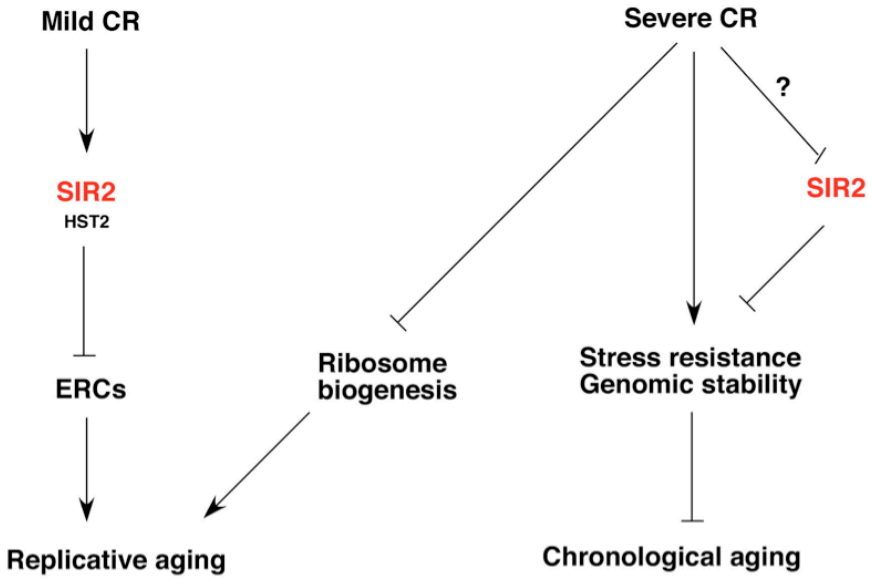

Fig. 1. CR regulation in yeast. Mild CR suppresses the ERCs and prevents replicative aging dependent on SIR2. Severe CR suppresses ribosome biogenesis and prevents replicative aging independent of SIR2. Chronological aging can be prevented by increased stress resistance and genomic stability induced by severe CR and SIR2 deficiency.

Yet, this does not apply to postmitotic cells, such as neurons, where increased ability to combat cell death and to increase stress resistance plays a larger role [18].

The hypothesis that yeast chronologic aging (which is not delayed by SIR2) represents a model for aging in post-mitotic cells, whereas yeast replicative aging (which is delayed by SIR2) models aging in mitotic cells may explain some puzzling observations in SIR2 regulation of aging. Overexpression of the SIR2 homolog Sir-2.1 in C. elegans extends lifespan by up to 50% [19]. However, resveratrol, a compound produced by plants under stress and an activator of SIR2, increases lifespan by inhibiting Sir-2.1-mediated repression of ER stress genes [20]. One possibility is that under normal conditions, mitotic cells may play a larger role in lifespan determination than post-mitotic cells in worms. Thus, overexpression of Sir-2.1 extends lifespan. However, under stress conditions, post-mitotic cells may be more sensitive to stress and become the determining factor in lifespan. Therefore, Sir-2.1 inactivation leads to lifespan extension.

The CR condition used in these studies (switching from minimum medium plus 2% glucose to water) is drastically different from the one in which SIR2-dependent lifespan extension by CR is observed (switching from 2% glucose medium to 0.5% glucose medium) [10], [21] and [22]. Therefore, it is likely that different pathways are activated in mild versus severe CR conditions (Fig. 1). Mild CR increases oxygen consumption and respiration, and leads to a reduction in NADH, an electron donor for respiration and a competitive inhibitor of SIR2 [21]. In addition, the NAD salvage pathway is upregulated to synthesize NAD from nicotinamide (NAM) and ADP-ribose, resulting in decreased levels of NAM, another inhibitor of SIR2 [23]. As a result, the silencing activity of SIR2 is activated by mild CR. However, under severe CR condition, it is likely that downregulation of SIR2 contributes to increased chronological lifespan [24]. sir2Δ mutants show increased expression of many genes involved in DNA repair and stress resistance, which are also activated by severe CR. Furthermore, SIR2 deficiency, like severe CR, results in increased resistance to heat and oxidative damage and elevated genomic stability. Thus, the role of SIR2 in regulating CR response in yeast is context-dependent. For an extensive discussion on CR response in yeast, please refer to Refs. [25] and [26].

Which CR condition, mild or severe, is more relevant to the one applied to mammals (30–40% reduction in calorie intake), which exhibits the maximum lifespan extension? One possibility is that when mammals are calorie restricted, tissues with different energy demands experience varying degrees of CR. When food is scarce, mammals may have to redistribute the limited resource to maintain survival and shut down unnecessary energy expenditures, such as growth, synthesis and reproduction. Thus, tissues necessary for basic survival, such as the muscle, the heart, and certain brain regions are protected from starvation and experience mild CR. However, tissues for synthesis, such as the liver and the pancreas, and the reproductive system are likely to experience severe CR. Although in the circulation, levels of glucose are roughly the same throughout the body, tissues may experience varied degrees of CR due to

different capacities for glucose uptake. As an example, hepatocytes and pancreatic beta cells use glucose transporter GLUT2, which has a high Km and therefore a low affinity for glucose. Thus, hepatocytes and beta cells may experience more severe CR compared to tissues like muscle and adipose tissue, which use glucose transporter GLUT4.

Another possibility is that mild and severe CR conditions may also occur at different time points during the day. CR animals are normally fed once a day of their daily quota or once every two days of double of their daily quota. After long-term CR, animals are trained to gorge their entire food supply within half an hour of feeding. During the course of each feeding cycle, CR animals may experience a fed state, a mild CR state and then a severe CR state. Thus, CR in mammals may resemble both mild and severe CR in yeast depending on tissue type and time of day.

Despite the lack of evidence for ERCs in organisms other than yeast, the activity of Sir2 homologs seem to affect lifespan in flies, worms, and mammals. In Drosophila melanogaster, increased expression of the Sir2 homolog dSir2 extends lifespan [27], and dSir2 deficiency blocks the lifespan-extending effect of CR. A non-sirtuin deacetylase, Rpd3, which is considered specific for histones, has also been suggested to mediate the effect of CR in Drosophila[28] and may interact with dSir2. Decreasing rpd3 expression results in elevated transcription levels of dSir2, and mutations in dSir2 blocks the lifespan-extending effect of rpd3 mutations. These observations suggest that dSir2 may act downstream of rpd3 in mediating the effects of CR in Drosophila[27].

It has been suggested that Sir-2.1 extends lifespan in C. elegans via activation of the transcription factor DAF-16 and probably also through additional targets [29]. Nevertheless, there are contradictions regarding the role of Sir-2.1 in mediating CR effects in C. elegans: whereas some studies support this role [30], others dismiss it [31], [32] and [33]. These differences may originate from the different approaches to limit nematode feeding, as Wang and Tissenbaum (2006) relied on a mutant (eat-2) with defects in the nematode's pharynx that resulted in lower feeding rate, while in the other studies it was the amount of food that was limited. Since there are 4 SIR2 homologs in nematode, it would be interesting to see whether the other SIR2 homologs have functions redundant to Sir-2.1 and whether deletion of all 4 genes would completely abolish CR-induced lifespan extension.

The ability of sirtuins to extend lifespan in yeast, flies, and nematodes via different pathways is intriguing from the evolutionary perspective. This begs a question: do mammalian sirtuins extend lifespan and mediate CR response? In mammals, there are 7 sirtuins, SIRT1-7, localized in various cellular compartments [34]. The lifespans of sirtuin knockout or transgenic mice are yet to be determined. However, it is intriguing that polymorphisms of certain sirtuins have been linked to human longevity [35], although this study requires further confirmation [36]. In addition, emerging genetic evidence suggests that mammalian sirtuins are required for the CR response. SIRT1 may be required for increased physical activity of CR mice, as SIRT1 knockout mice do not have increased activity [37]. It is likely that SIRT1 in the hypothalamus is necessary

to sense hunger and trigger the foraging response. In addition, genetic, molecular and pharmacological studies discussed below are all consistent with the role of mammalian sirtuins in regulating the CR response.

3. SIRT1 and metabolic regulation

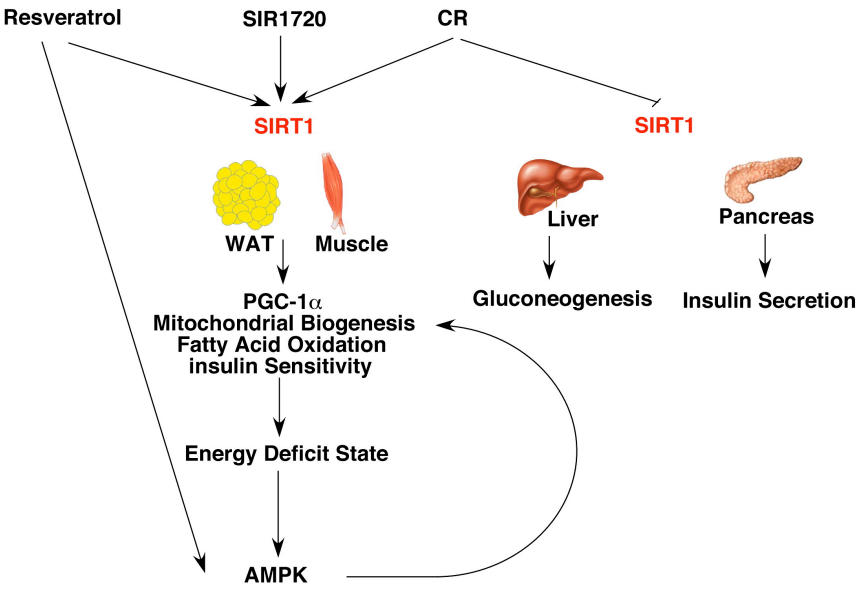

Fig. 2. Tissue-specific regulation of SIRT1 by CR. SIRT1 is downregulated in the liver and pancreas of CR mice, resulting in decreased gluconeogenesis and insulin secretion. It is upregulated in the muscle and the WAT, leading to increased mitochondrial biogenesis, fatty acid oxidation and insulin sensitivity. These metabolic changes cause a cellular energy-deficit state and activation of AMPK. Activated AMPK further amplifies these metabolic changes. SIRT1 activators mimic the CR response by activating PGC-1a in the muscle.

It has been thought that CR extends lifespan by decreasing metabolism and the associated production of damaging reactive oxygen species. However, this traditional view has been challenged by recent findings indicating that CR in fact increases metabolism. CR yeast switches metabolism from fermentation toward the mitochondrial tricarboxylic acid cycle and increased respiration [22]. Similarly, increased oxygen consumption has been observed in CR worms [38]. Evidence for changes in oxygen consumption in CR mice is controversial, as the mice experience drastic alterations in body weight and composition [39]. However, CR mice do experience a metabolic switch that mirrors the one observed in CR yeast. When energy is abundant, instead of storing excess energy in the form of ethanol, animals store it as fat. During CR, animals turn on fatty acid oxidation and switch fuel usage from glucose to fatty acids. Since fatty acids are more reduced than glucose, fatty acid oxidation will consume more oxygen per carbon than glycolysis. At the cellular level, increased mitochondrial biogenesis in CR tissues suggests a tissue-specific increase in metabolic rate of CR animals [40].

How do sirtuins regulate these metabolic changes? SIRT1, a nuclear sirtuin, coordinates the metabolic switch in multiple tissues by regulating different molecular targets (Fig. 2). In the white adipose tissue (WAT), SIRT1 negatively regulates the nuclear receptor PPARγ, a key regulator of adipocyte differentiation and fat storage [41]. Thus, activation of SIRT1 suppresses fat storage and promotes the mobilization of fat from the WAT to be utilized by other tissues. In the muscle, SIRT1 activates PGC-1α, a master regulator of mitochondrial biogenesis and fatty acid oxidation [42]. Consistent with the role of SIRT1 in promoting fat mobilization and fatty acid oxidation,

SIRT1 activity seems to be upregulated in the muscle and the WAT of CR mice. Both SIRT1 expression and NAD level are increased in these two tissues [43].

CR animals have decreased levels of blood insulin in response to limited food intake. A low insulin level suppresses glycolysis and maintains blood glucose levels, allowing a switch to fatty acids as an energy source. In the pancreatic β-cells, SIRT1 suppresses UCP2, a mitochondrial uncoupler, and permits efficient ATP production [44] and [45]. As ATP is required to trigger insulin secretion from the β-cells, SIRT1 is a positive regulator of this biological process. Low insulin levels in CR mice suggest that the activity of SIRT1 is likely reduced in the pancreatic beta cells. Although the NAD levels in the pancreatic beta cells during CR have not been reported, they are decreased during fasting, when insulin secretion is also suppressed [44].

The liver synthesizes fat for storage when there is excessive food intake, and produces glucose from non-carbohydrate carbon substrates in response to fasting. PGC-1α is also a key regulator of gluconeogenesis [46]. By deacetylating and activating PGC-1α, SIRT1 increases the expression of enzymes catalyzing the rate-limiting steps of gluconeogenesis. It would be logical, then, if SIRT1 activity is also increased in the livers of CR mice to activate PGC-1α and promote glucose production. However, decreased SIRT1 expression and increased NADH levels have been observed in the livers of CR mice, suggesting that the activity of this deacetylase is actually decreased [43]. Adding support to this observation, liver-specific SIRT1 knockout mice respond to CR normally and induce gluconeogenesis to the same degree as their wild type counterparts [43].

How do we make sense of the decreased SIRT1 activity in the liver of CR mice? Active gluconeogenesis is an acute response to the fasting signal glucagon immediately following food withdrawal. Hepatic glucose production decreases after fatty acid oxidation is activated and the ketone bodies produced by the liver supply compensatory fuel for glucose-dependent tissues, such as the brain [47]. The expression of PGC-1α and another transcription factor regulating gluconeogenesis, TORC2, are both increased upon acute fasting to promote glucose production [47] and [48]. During prolonged fasting, TORC2 is quickly deacetylated and inactivated by SIRT1, which is not active during acute fasting but is activated during prolonged fasting. Moreover, the transcription of PGC-1α is also decreased to suppress gluconeogenesis when glucose is no longer in high demand. At this stage, only FOXO1, whose gluconeogenic activity is activated by SIRT1, is active to maintain the basal level of glucose.

The expression of PGC-1α is greatly induced by CR, which is essential to induce mitochondrial biogenesis [40]. Increased mitochondrial biogenesis is a hallmark of CR and is not observed under other energy limiting conditions, such as fasting. This high level of PGC-1α is likely to be the dominant factor regulating gluconeogenesis in CR liver and triggers more glucose production than what is needed by the CR animals. After long-term CR, animals become adapted to efficient fatty acid usage and effectively rely on ketone bodies. In addition, after the initial adaptation stage of the CR diet, the

animals do not lose weight any more. This suggests that there is no further break down of muscle to use their amino acids as supplies for glucose production. Thus, CR animals do not have as high of a demand for glucose as animals on acute fasting, and the downregulation of SIRT1 in the liver may be necessary to suppress glucose production induced by high level of PGC-1α. In this case, TORC2 may stay active to support basal levels of glucose production.

Inactivation of SIRT1 during CR in the liver is further supported by increased apoptosis [49] and [50], as SIRT1 suppresses p53 [51] and [52], FOXO [53] and [54], Ku70 [55], E2F [56] and promotes cell survival. This can also explain the puzzling observation that while the SIRT1 activator resveratrol mimics many aspects of CR response, resveratrol and CR regulate cell cycle checkpoints and apoptosis in opposite directions in the liver [57]. Thus, as discussed earlier, increased SIRT1 in the muscle and the WAT promotes cell survival and increases stress resistance, which are crucial for the lifespan of postmitotic cells. However, hepatocytes are mitotic cells that retain tremendous proliferation potential. Slowing the proliferation by cell cycle arrest may result in increased lifespan.

How is SIRT1 regulated in a tissue-dependent manner during CR? CR increases metabolism, and CR animals have increased energy demand. This pushes NADH to enter the electron transport chain to produce ATP and be converted into NAD, resulting in increased NAD level observed in the CR muscle and WAT. However, the liver differs from other metabolic tissues in that under fed conditions, it is engaged in fatty acid synthesis, which increases the cellular redox state. During CR, fatty acid synthesis is shut down and the cellular redox state is decreased, consistent with the increased NADH level observed in the CR liver.

Interestingly, the changes in the redox state of the CR tissues correlate with the changes in the expression level of SIRT1 [43]. In muscle and WAT, the NAD/NADH ratio is increased and the expression of SIRT1 is also upregulated. In the liver, both the NAD/NADH ratio and the SIRT1 expression are reduced. Since the same changes in the NAD/NADH ratio occur in the SIRT1 KO mice on CR diet, this correlation suggests that the expression of SIRT1 is regulated by the cellular redox state. Indeed, the transcription factor HIC1 represses SIRT1 expression when coupled with the redox sensing co-repressor CtBP [58]. When the cellular redox state is increased, CtBP is dissociated from HIC1, resulting in a reduction in transcriptional repression of SIRT1 mediated by HIC1. Thus, NAD and NADH not only regulate the enzymatic activity of SIRT1 but also its expression at the transcriptional level, allowing a tight control of its deacetylase activity by the cellular redox state.

These studies are consistent with a model whereby SIRT1 is a master regulator of CR in many tissues. It is worth noting that SIRT1 whole body knockout mice have pleiotropic metabolic phenotype. In this case, tissue-specific SIRT1 knockout mice will be valuable tools to dissect the role of SIRT1 in mediating CR response in vivo.

4. Calorie restriction: insights from SIRT1 activators

The observations that SIRT1 increases mitochondrial biogenesis and fatty acid oxidation suggest that activation of SIRT1 may mimic many aspects of CR response and improve metabolic homeostasis. Consistent with this idea, SIRT1 transgenic mice display some phenotypes similar to CR mice. One transgenic line has decreased body weight, increased metabolic rate, and improved glucose tolerance. The other two lines have less overt metabolic phenotype on a chow diet but are protected against high fat diet feeding despite the same weight gain [59], [60] and [61]. It is worth noting that three existing SIRT1 transgenic lines are driven by different promoters and have different overexpression patterns/levels of SIRT1. Despite differences in certain phenotypes, they all have improved metabolic homeostasis. Thus, small molecule SIRT1 activators may be the best candidates for the coveted CR mimetics to delay age-related diseases. Interestingly, although resveratrol does not increase lifespan of mice fed a chow diet, it significantly improves the physiology and survival of mice fed a high fat diet [57] and [62]. Resveratrol-treated mice have increased numbers of mitochondria, decreased levels of blood glucose and insulin, increased glucose tolerance and insulin sensitivity, and improved motor activity, reminiscent of CR effects.

In addition to its interaction with SIRT1, resveratrol is known to interact with many other proteins and pathways involved in energy balance, such as mitochondrial ATP synthase, complex III, fatty acid synthase, and AMP kinase [63]. An important question is whether the CR-mimicking effects of resveratrol are mediated by SIRT1 in vivo. Newly identified SIRT1 activators structurally unrelated to resveratrol, such as SIR1720, have similar effects in obese rodents, such as improved insulin sensitivity and mitochondrial capacity [64] and [65]. These specific SIRT1 activators are 1000-fold more potent than resveratrol, suggesting that SIRT1 activation alone is sufficient to induce the beneficial metabolic effects.

Among the growing list of SIRT1 substrates, PGC-1α seems to be one of the most critical downstream effectors of resveratrol [62] and [64]. Resveratrol enhances muscle oxidative capacity and endurance due to its ability to engender a switch from fast-twitch/glycolytic fibers to slow-twitch/oxidative muscle fibers. This switch is coupled with an increased expression of mitochondrial genes and genes involved in oxidative phosphorylation, consistent with the known function of PGC-1α in regulating muscle fiber types and mitochondrial biogenesis [66] and [67]. Resveratrol increases PGC-1α activity by enhancing its SIRT1-mediated deacetylation. The effects of resveratrol on the expression of mitochondrial genes are abolished in cells with SIRT1 knocked down or with PGC-1α containing mutated acetylation sites [62].

Diet-induced obesity is a major risk factor in the development of insulin resistance, characterized by reduced glucose clearance in the peripheral tissues. High doses of SIRT1 activators protect mice from weight gain when fed a high fat diet, along with improving their insulin resistance [62] and [64]. However, it is worth noting that low doses of resveratrol improve insulin resistance even when there is no change in body weight and body fat [57], [64] and [65]. Thus, increased mitochondrial capacity may be the key to combat insulin resistance.

AMPK, a stress and energy sensor that is activated during low energy states to maintain glucose, fatty acids, and energy homeostasis, is also upregulated in mice treated with SIRT1 activators [57] and [64]. The activation of AMPK by SIR1720 is an indirect effect, since SIR1720 does not activate AMPK in tissue culture cells or in mice via acute treatment. Rather, SIRT1 activation leads to increased energy expenditure, and the resulting energy-deficit state, reflected by decreased ATP and ADP levels, leads to AMPK activation. Activated AMPK can further amplify the increase in fatty acid usage and mitochondrial capacity. In addition to this SIRT1-dependent mechanism, AMPK may also be directly activated by resveratrol. These mechanistic insights on the actions of SIRT1 activators shed light on how the interplay of CR and SIRT1 regulates energy balance at the molecular level (Fig. 2).

How can SIRT1 activators mimic the CR response if SIRT1 is inactivated in certain CR tissues such as the liver? It is likely that muscle is the dominant effector mediating the metabolic changes when small molecular SIRT1 activators are administered. For example, increased mitochondrial biogenesis is observed in muscles and brown adipose tissues, but not in the livers of resveratrol treated mice [62]. Moreover, the insulin-stimulated glucose uptake is improved in muscles but not the livers of SIR1720-treated mice [64]. Following oral administration, SIRT1 activators are likely to be removed in the liver.

5. Mitochondria: the centers of CR response

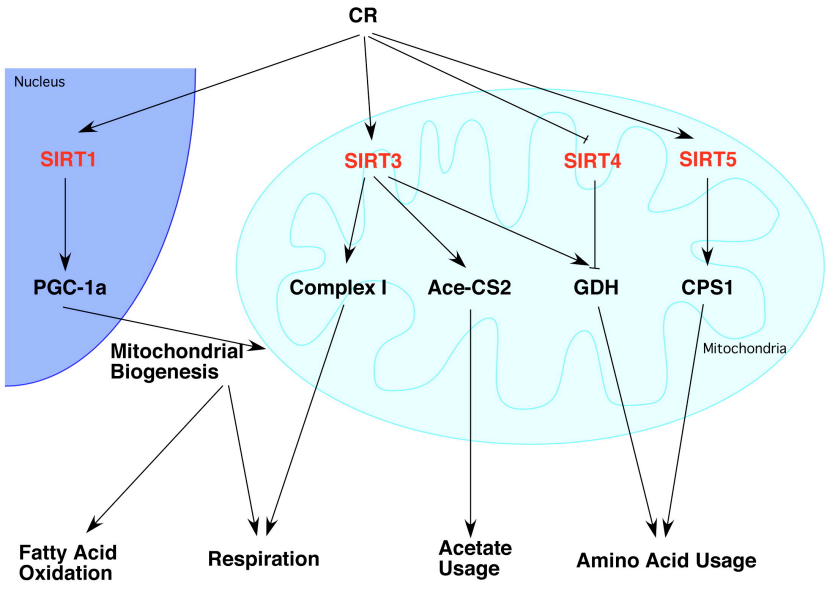

Fig. 3. The central role of mitochondria in CR response. CR increases mitochondrial biogenesis, fatty acid usage, and respiration via the nuclear sirtuin SIRT1. Mitochondrial sirtuins, SIRT3, 4, and 5, also mediate the CR-induced metabolic shift toward acetate and amino acid usage.

Mitochondria are the hubs of metabolism. At least 20% of mitochondrial proteins are acetylated [68]. This observation underscores the importance of mitochondrial deacetylases in metabolic regulation. Many mitochondrial proteins are hyperacetylated in mice deficient in SIRT3, a mitochondrial sirtuin, suggesting that SIRT3 may have broad impacts in metabolic control [69]. One substrate of SIRT3 is acetyl-CoA synthetase 2 (AceCS2), which converts acetate into acetyl-CoA [70] and [71]. Under ketogenic conditions such as CR, the liver releases large amounts of acetate into the blood. Tissues such as the muscle and the heart express AceCS2 and effectively utilize acetate as an energy supply. Since

deacetylation of AceCS2 activates its enzymatic activity, SIRT3 is likely to be activated during CR [72]. SIRT3 also deacetylates many subunits of complex I and increases its respiration rate [73]. This allows efficient entry of NADH into the electron transport chain to generate ATP. Thus, activation of SIRT3 during CR may also contribute to increased respiration. Other SIRT3 substrates and their roles in CR response remain to be elucidated.

Under CR conditions, amino acid catabolism is activated and the urea cycle has to be upregulated to remove excess amounts of ammonia. SIRT5, another mitochondrial sirtuin, deacetylates and activates carbamoyl phosphate synthetase 1 (CPS1), an enzyme catalyzing the first step of the urea cycle [74]. The expression level of SIRT5 is unchanged but the level of NAD is increased by 50% in the mitochondria of the CR liver. This increased SIRT5 activity may upregulate the urea cycle during CR by deacetylating and activating CPS1.

SIRT4 is also localized to the mitochondria. It has no detectable deacetylase activity but possesses NAD-dependent mono-ADP-ribosyltransferase activity. One known substrate of SIRT4 is glutamate dehydrogenase (GDH), which converts glutamate to α-ketoglutarate and allows it to enter the TCA cycle [75]. By ADP-ribosylating GDH, SIRT4 suppresses its activity and prevents the utilization of amino acids as an energy source. During CR, although the NAD level is increased in the mitochondria, the expression of SIRT4 is decreased. The decreased SIRT4 activity results in the activation of GDH and the utilization of glutamate and glutamine to generate ATP. This is particularly important for the pancreatic β-cells of CR mice. Due to the low blood glucose level, glucose-stimulated insulin secretion is suppressed. However, the activation of GDH allows amino acid-stimulated insulin secretion to maintain the basal level of insulin. GDH can also be deacetylated by SIRT3, although the functional link between acetylation and ADP-ribosylation of this enzyme is not established [69].

The center of CR response is mitochondrial activation, which allows metabolic adaptation to chronic energy deficit (Fig. 3). One beneficial effect of mitochondrial activation is increased respiration and more efficient ATP production. In addition, it also permits a fuel usage switch from glucose to non-carbohydrates since glycolysis and fatty acid synthesis occur in the cytosol while fatty acid oxidation, amino acid metabolism and ketogenesis are confined to the mitochondria. SIR2 has evolved to have diverse paralogs in mammals; at least four of them are directly or indirectly involved in mitochondrial activation, emphasizing the importance of the mitochondria and sirtuins in mediating CR response. These four sirtuins, SIRT1, 3, 4, and 5, are found in various cellular compartments, have different enzymatic activities, and coordinate with each other to regulate mitochondrial activity. It is interesting to speculate why mammals evolved to have three mitochondrial sirtuins and how their substrate specificity is achieved.

6. Regulation of sirtuin activities by CR

It is striking that the activities of all sirtuins are subject to the regulation of NAD. Yet, sirtuin activities are differentially regulated during CR. Activation of SIRT3 and SIRT5 is

in contrast to the inactivation of SIRT1 in the CR liver. The mitochondria and the cytoplasm/nucleus have separate NAD pools, since NAD(H) cannot pass through the mitochondrial membranes. For instance, following genotoxic stress, nuclear and cytoplasmic NAD is depleted, but mitochondrial NAD still remains at the physiological level [76]. This allows for differential activation of sirtuins based on their subcellular localization. As discussed above, the redox state of the CR liver is reduced by fatty acid synthesis, which is confined to the cytoplasm. The increased NAD level in the mitochondria results from the upregulation of the NAD biosynthetic enzyme Nampt [74] and [76]. It still remains to be clarified whether it is the mitochondrial or the cytoplasmic Nampt that is activated. The second model proposes that increased cytoplasmic Nampt elevates the level of nicotinamide mononucleotide (NMN), the precursor of NAD. The mitochondrial membrane is permeable to NMN, and the translocation of NMN into the mitochondria increases the NAD level.

Furthermore, different sirtuins have different Km values for NAD [77], [78] and [79]. This is relevant because the same redox state may have different effects on different sirtuins. This is particularly important for sirtuins reacting to the same NAD pool, such as SIRT3, 4, and 5. Other than HIC1, the transcription of SIRT1 is also regulated by p53 and Foxo3a in response to nutrient conditions [80]. In addition to NAD, NADH, and regulation at the transcriptional level, other regulatory mechanisms are also worth exploring. SIRT1 was found to have 13 phosphorylation sites and one sumoylation site, and in vitro deacetylation activity assays showed that dephosphorylated or desumoylated SIRT1 has decreased activity [81] and [82]. In addition, an endogenous activator (AROS) [83]and inhibitor (DBC1) [73] and [84]of SIRT1 have been identified. It would be interesting to see whether posttranscriptional modifications or endogenous regulators of sirtuins also contribute to sirtuin regulation during CR.

7. Cancer

CR is known to prevent the incidence of cancer. The observation that SIRT1 suppresses the apoptosis pathway mediated by p53, FOXO, and Ku70 and increases stress resistance raises a tantalizing question: does SIRT1 activation promote cancer? If so, how can we reconcile CR-induced SIRT1 activation and cancer prevention by CR? Many lines of evidence do support the cancer-promoting potential of SIRT1. First, Deleted in Breast Cancer 1 (DBC1), likely a tumor suppressor, promotes p53-mediated apoptosis through specific inhibition of SIRT1 [73] and [84]. Second, newly identified small molecule activators of p53 with the potential to decrease tumor growth act through inhibiting SIRT1 and SIRT2 [3]. Third, the loss of tumor suppressor HIC1 promotes tumorigenesis via SIRT1 activation and the subsequent attenuation of p53 [85]. Finally, SIRT1 knockout mice show increased p53 hyperacetylation and radiation-induced apoptosis [86].

However, in vivo evidence suggests that SIRT1 is a tumor suppressor. In a p53+/− background, mice with deficient SIRT1 develop cancers in multiple tissues [87]. BRCA-1 mutant mice develop mammary tumors due to the suppression of SIRT1 [88]. In addition, SIRT1 overexpression reduces colon cancer formation in the APCmin/+ mouse model [89]. How can SIRT1 prevent cancer when it suppresses p53-induced apoptosis?

First, SIRT1 modifies histones, ensures proper chromosome condensation and segregation during mitosis, and prevents genetic instability [87]. SIRT1 reverses acetylation of histone H3 on lysine 56 (H3K56), which is critical in packaging DNA into chromatin following DNA replication and repair [90]. In addition, SIRT1 maintains genomic stability by silencing repetitive DNA [91]. SIRT1 also promotes DNA damage repair through NBS1 [92]. Increased genomic stability, together with enhanced DNA damage repair, puts less demand on the cellular surveillance system, such as p53-dependent apoptosis. Second, despite its general anti-apoptotic activity, SIRT1 may promote cancer-specific cell death by suppressing Survivin, an inhibitor of apoptosis that is highly expressed in most human tumors but is completely absent in terminally differentiated cells [88]. Finally, SIRT1 also prevents cancer by suppressing oncogenes, such as beta-catenin [89]. Thus, SIRT1 activation during CR may increase stress resistance by suppressing apoptosis and at the same time, prevent cancer by increasing genomic stability, promoting cancer-specific cell death and suppressing oncogenes (Fig. 4).

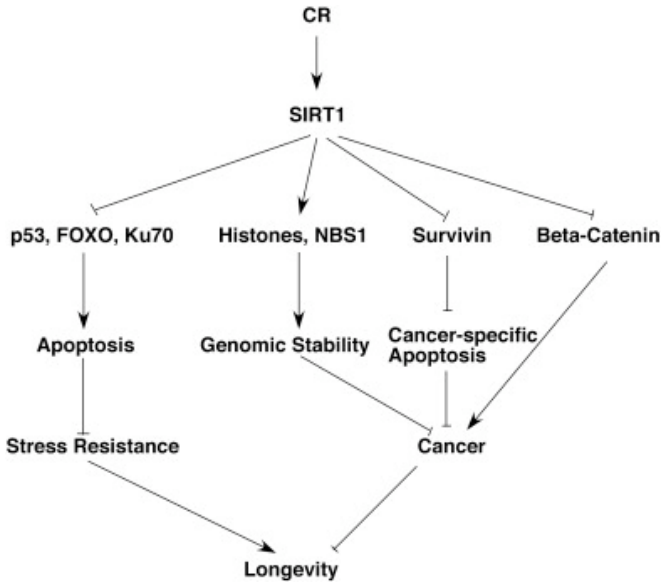

Fig. 4. SIRT1 increases stress resistance yet prevents cancer. SIRT1 increases stress resistance by suppressing apoptosis. However, SIRT1 prevents cancer by increasing genomic stability, promoting cancer-specific apoptosis, and suppressing certain oncogenes.

8. Inflammation

Inflamm-aging, a term first coined by Claudio Franceschi [93] and [94], refers to the phenomenon that aging is accompanied by a low-grade chronic, systemic up-regulation of the pro-inflammatory response. Inflammaging is caused by overactive innate immune response and repressed adaptive immunity. Genes involved in inflammation are upregulated with age [95], [96], [97] and [98], and these age-related changes can be reverted by CR regimen [97], [98] and [99]. Evidence is emerging for a role of sirtuins in dampening inflammation, as both SIRT1 and SIRT6 inhibit NF-KB, a key factor for the

inflammatory response [100] and [101]. This is further supported by the observation that resveratrol attenuates inflammation and SIRT1 KO mice develop inflammatory lesions [102], [103], [104] and [105]. However, more in vivo evidence is needed to support this idea.

9. Future directions

The groundbreaking studies on SIRT1 activators in metabolic disease mouse models are the first proof of principle that it is possible to design small molecule CR mimetics to combat aging. Despite the unequivocal effects of SIRT1 activators on metabolic regulation and aging-related deterioration, it does not extend the lifespan of mice fed a regular diet [106]. Tissue-specific regulation of SIRT1 and coordination between multiple sirtuins to regulate CR also suggest that a more complete understanding of sirtuin regulation by CR is necessary before we can fully take advantage of the beneficial effects of this dietary regimen. In addition, the central role of mitochondria in CR response raises the possibility of targeting mitochondria for preventing metabolic diseases. Finally, despite the intense studies on sirtuins and metabolic regulation, the physiological role of sirtuins in regulating biological pathways related to other diseases of aging are still awaiting further validation. These studies will serve as the basis for using sirtuin activators or inhibitors to prevent or treat other diseases.

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References

[1]
J. Koubova, L. Guarente
How does calorie restriction work?
Genes Dev., 17 (2003), pp. 313–321
[2]
R.A. Frye
Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins
Biochem. Biophys. Res. Commun., 273 (2000), pp. 793–798
[3]
L. Guarente
Sirtuins in aging and disease
Cold Spring Harbor Symp. Quant. Biol., 72 (2007), pp. 483–488
[4]
R.K. Mortimer, J.R. Johnston
Life span of individual yeast cells
Nature, 183 (1959), pp. 1751–1752
[5]
H. Aguilaniu, L. Gustafsson, M. Rigoulet, T. Nystrom
Asymmetric inheritance of oxidatively damaged proteins during cytokinesis
Science, 299 (2003), pp. 1751–1753
[6]

W. Dang, K.K. Steffen, R. Perry, J.A. Dorsey, F.B. Johnson, A. Shilatifard, M. Kaeberlein, B.K. Kennedy, S.L. Berger
Histone H4 lysine 16 acetylation regulates cellular lifespan
Nature, 459 (2009), pp. 802–807
[7]
M. Kaeberlein, M. McVey, L. Guarente
The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms
Genes Dev., 13 (1999), pp. 2570–2580
[8]
D.A. Sinclair, L. Guarente
Extrachromosomal rDNA circles—a cause of aging in yeast
Cell, 91 (1997), pp. 1033–1042
[9]
I. Muller, M. Zimmermann, D. Becker, M. Flomer
Calendar life span versus budding life span of Saccharomyces cerevisiae
Mech. Ageing Dev., 12 (1980), pp. 47–52
[10]
S.J. Lin, P.A. Defossez, L. Guarente
Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae
Science, 289 (2000), pp. 2126–2128
[11]
D.W. Lamming, M. Latorre-Esteves, O. Medvedik, S.N. Wong, F.A. Tsang, C. Wang, S.J. Lin, D.A. Sinclair
HST2 mediates SIR2-independent life-span extension by calorie restriction
Science, 309 (2005), pp. 1861–1864
[12]
P. Fabrizio, V.D. Longo
The chronological life span of Saccharomyces cerevisiae
Aging Cell., 2 (2003), pp. 73–81
[13]
M. Jimenez-Hidalgo, C. Santos-Ocana, S. Padilla, J.M. Villalba, G. Lopez-Lluch, A. Martin-Montalvo, R.K. Minor, D.A. Sinclair, R. de Cabo, P. Navas
NQR1 controls lifespan by regulating the promotion of respiratory metabolism in yeast
Aging Cell, 8 (2009), pp. 140–151
[14]
D.L. Smith Jr., J.M. McClure, M. Matecic, J.S. Smith
Calorie restriction extends the chronological lifespan of Saccharomyces cerevisiae independently of the Sirtuins
Aging Cell, 6 (2007), pp. 649–662
[15]
B.K. Kennedy, E.D. Smith, M. Kaeberlein
The enigmatic role of Sir2 in aging
Cell, 123 (2005), pp. 548–550
[16]

K. Miyamoto, K.Y. Araki, K. Naka, F. Arai, K. Takubo, S. Yamazaki, S. Matsuoka, T. Miyamoto, K. Ito, M. Ohmura, C. Chen, K. Hosokawa, H. Nakauchi, K. Nakayama, K.I. Nakayama, M. Harada, N. Motoyama, T. Suda, A. Hirao
Foxo3a is essential for maintenance of the hematopoietic stem cell pool
Cell Stem Cell, 1 (2007), pp. 101–112
[17]
O.H. Yilmaz, R. Valdez, B.K. Theisen, W. Guo, D.O. Ferguson, H. Wu, S.J. Morrison
Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells
Nature, 441 (2006), pp. 475–482
[18]
Y.A. Kim, B.T. Choi, Y.T. Lee, D.I. Park, S.H. Rhee, K.Y. Park, Y.H. Choi
Resveratrol inhibits cell proliferation and induces apoptosis of human breast carcinoma MCF-7 cells
Oncol. Rep., 11 (2004), pp. 441–446
[19]
H.A. Tissenbaum, L. Guarente
Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans
Nature, 410 (2001), pp. 227–230
[20]
M. Viswanathan, S.K. Kim, A. Berdichevsky, L. Guarente
A role for SIR-2.1 regulation of ER stress response genes in determining C. elegans life span
Dev. Cell, 9 (2005), pp. 605–615
[21]
S.J. Lin, E. Ford, M. Haigis, G. Liszt, L. Guarente
Calorie restriction extends yeast life span by lowering the level of NADH
Genes Dev., 18 (2004), pp. 12–16
[22]
S.J. Lin, M. Kaeberlein, A.A. Andalis, L.A. Sturtz, P.A. Defossez, V.C. Culotta, G.R. Fink, L. Guarente
Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration
Nature, 418 (2002), pp. 344–348
[23]
R.M. Anderson, K.J. Bitterman, J.G. Wood, O. Medvedik, D.A. Sinclair
Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae
Nature, 423 (2003), pp. 181–185
[24]
P. Fabrizio, C. Gattazzo, L. Battistella, M. Wei, C. Cheng, K. McGrew, V.D. Longo
Sir2 blocks extreme life-span extension
Cell, 123 (2005), pp. 655–667
[25]
M. Kaeberlein, D. Hu, E.O. Kerr, M. Tsuchiya, E.A. Westman, N. Dang, S. Fields, B.K. Kennedy
Increased life span due to calorie restriction in respiratory-deficient yeast

PLoS Genet., 1 (2005), p. e69
[26]
M. Kaeberlein, R.W. Powers III
Sir2 and calorie restriction in yeast: a skeptical perspective
Ageing Res. Rev., 6 (2007), pp. 128–140
[27]
B. Rogina, S.L. Helfand
Sir2 mediates longevity in the fly through a pathway related to calorie restriction
Proc. Natl. Acad. Sci. U. S. A., 101 (2004), pp. 15998–16003
[28]
B. Rogina, S.L. Helfand, S. Frankel
Longevity regulation by Drosophila Rpd3 deacetylase and caloric restriction
Science, 298 (2002), p. 1745
[29]
B. Hamilton, Y. Dong, M. Shindo, W. Liu, I. Odell, G. Ruvkun, S.S. Lee
A systematic RNAi screen for longevity genes in C. elegans
Genes Dev., 19 (2005), pp. 1544–1555
[30]
Y. Wang, H.A. Tissenbaum
Overlapping and distinct functions for a Caenorhabditis elegans SIR2 and DAF-16/FOXO
Mech. Ageing Dev., 127 (2006), pp. 48–56
[31]
M. Hansen, S. Taubert, D. Crawford, N. Libina, S.J. Lee, C. Kenyon
Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans
Aging Cell, 6 (2007), pp. 95–110
[32]
T.L. Kaeberlein, E.D. Smith, M. Tsuchiya, K.L. Welton, J.H. Thomas, S. Fields, B.K. Kennedy, M. Kaeberlein
Lifespan extension in Caenorhabditis elegans by complete removal of food
Aging Cell, 5 (2006), pp. 487–494
[33]
T.J. Schulz, K. Zarse, A. Voigt, N. Urban, M. Birringer, M. Ristow
Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress
Cell. Metab., 6 (2007), pp. 280–293
[34]
M.C. Haigis, L.P. Guarente
Mammalian sirtuins—emerging roles in physiology, aging, and calorie restriction
Genes Dev., 20 (2006), pp. 2913–2921
[35]
D. Bellizzi, G. Rose, P. Cavalcante, G. Covello, S. Dato, F. De Rango, V. Greco, M. Maggiolini, E. Feraco, V. Mari, C. Franceschi, G. Passarino, G. De Benedictis
A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages
Genomics, 85 (2005), pp. 258–263

[36]
F. Lescai, H. Blanche, A. Nebel, M. Beekman, M. Sahbatou, F. Flachsbart, E. Slagboom, S. Schreiber, S. Sorbi, G. Passarino, C. Franceschi
Human longevity and 11p15.5: a study in 1321 centenarians
Eur. J. Hum. Genet. (2009) [April 15, Electronic publication ahead of print]
[37]
D. Chen, A.D. Steele, S. Lindquist, L. Guarente
Increase in activity during calorie restriction requires Sirt1
Science, 310 (2005), p. 1641
[38]
N.A. Bishop, L. Guarente
Two neurons mediate diet-restriction-induced longevity in C. elegans
Nature, 447 (2007), pp. 545–549
[39]
L. Guarente, F. Picard
Calorie restriction—the SIR2 connection
Cell, 120 (2005), pp. 473–482
[40]
E. Nisoli, C. Tonello, A. Cardile, V. Cozzi, R. Bracale, L. Tedesco, S. Falcone, A. Valerio, O. Cantoni, E. Clementi, S. Moncada, M.O. Carruba
Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS
Science, 310 (2005), pp. 314–317
[41]
F. Picard, M. Kurtev, N. Chung, A. Topark-Ngarm, T. Senawong, R. Machado De Oliveira, M. Leid, M.W. McBurney, L. Guarente
Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma
Nature, 429 (2004), pp. 771–776
[42]
Z. Gerhart-Hines, J.T. Rodgers, O. Bare, C. Lerin, S.H. Kim, R. Mostoslavsky, F.W. Alt, Z. Wu, P. Puigserver
Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha
EMBO J., 26 (2007), pp. 1913–1923
[43]
D. Chen, J. Bruno, E. Easlon, S.J. Lin, H.L. Cheng, F.W. Alt, L. Guarente
Tissue-specific regulation of SIRT1 by calorie restriction
Genes Dev., 22 (2008), pp. 1753–1757
[44]
L. Bordone, M.C. Motta, F. Picard, A. Robinson, U.S. Jhala, J. Apfeld, T. McDonagh, M. Lemieux, M. McBurney, A. Szilvasi, E.J. Easlon, S.J. Lin, L. Guarente
Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells
PLoS Biol., 4 (2006), p. e31
[45]
K.A. Moynihan, A.A. Grimm, M.M. Plueger, E. Bernal-Mizrachi, E. Ford, C. Cras-Meneur, M.A. Permutt, S. Imai

Increased dosage of mammalian Sir2 in pancreatic beta cells enhances glucose-stimulated insulin secretion in mice
Cell. Metab., 2 (2005), pp. 105–117
[46]
J.T. Rodgers, C. Lerin, W. Haas, S.P. Gygi, B.M. Spiegelman, P. Puigserver
Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1
Nature, 434 (2005), pp. 113–118
[47]
Y. Liu, R. Dentin, D. Chen, S. Hedrick, K. Ravnskjaer, S. Schenk, J. Milne, D.J. Meyers, P. Cole, J. Yates 3rd, J. Olefsky, L. Guarente, M. Montminy
A fasting inducible switch modulates gluconeogenesis via activator/coactivator exchange
Nature, 456 (2008), pp. 269–273
[48]
J.C. Yoon, P. Puigserver, G. Chen, J. Donovan, Z. Wu, J. Rhee, G. Adelmant, J. Stafford, C.R. Kahn, D.K. Granner, C.B. Newgard, B.M. Spiegelman
Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1
Nature, 413 (2001), pp. 131–138
[49]
B. Grasl-Kraupp, W. Bursch, B. Ruttkay-Nedecky, A. Wagner, B. Lauer, R. Schulte-Hermann
Food restriction eliminates preneoplastic cells through apoptosis and antagonizes carcinogenesis in rat liver
Proc. Natl. Acad. Sci. U. S. A., 91 (1994), pp. 9995–9999
[50]
S.J. James, L. Muskhelishvili, D.W. Gaylor, A. Turturro, R. Hart
Upregulation of apoptosis with dietary restriction: implications for carcinogenesis and aging
Environ. Health Perspect., 106 (Suppl. 1) (1998), pp. 307–312
[51]
J. Luo, A.Y. Nikolaev, S. Imai, D. Chen, F. Su, A. Shiloh, L. Guarente, W. Gu
Negative control of p53 by Sir2alpha promotes cell survival under stress
Cell, 107 (2001), pp. 137–148
[52]
H. Vaziri, S.K. Dessain, E. Ng Eaton, S.I. Imai, R.A. Frye, T.K. Pandita, L. Guarente, R.A. Weinberg
hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase
Cell, 107 (2001), pp. 149–159
[53]
A. Brunet, L.B. Sweeney, J.F. Sturgill, K.F. Chua, P.L. Greer, Y. Lin, H. Tran, S.E. Ross, R. Mostoslavsky, H.Y. Cohen, L.S. Hu, H.L. Cheng, M.P. Jedrychowski, S.P. Gygi, D.A. Sinclair, F.W. Alt, M.E. Greenberg
Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase
Science, 303 (2004), pp. 2011–2015
[54]

M.C. Motta, N. Divecha, M. Lemieux, C. Kamel, D. Chen, W. Gu, Y. Bultsma, M. McBurney, L. Guarente
Mammalian SIRT1 represses forkhead transcription factors
Cell, 116 (2004), pp. 551–563
[55]
H.Y. Cohen, C. Miller, K.J. Bitterman, N.R. Wall, B. Hekking, B. Kessler, K.T. Howitz, M. Gorospe, R. de Cabo, D.A. Sinclair
Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase
Science, 305 (2004), pp. 390–392
[56]
C. Wang, L. Chen, X. Hou, Z. Li, N. Kabra, Y. Ma, S. Nemoto, T. Finkel, W. Gu, W.D. Cress, J. Chen
Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage
Nat. Cell Biol., 8 (9) (2006), pp. 1025–1031
[57]
J.A. Baur, K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu, J.S. Allard, G. Lopez-Lluch, K. Lewis, P.J. Pistell, S. Poosala, K.G. Becker, O. Boss, D. Gwinn, M. Wang, S. Ramaswamy, K.W. Fishbein, R.G. Spencer, E.G. Lakatta, D. Le Couteur, R.J. Shaw, P. Navas, P. Puigserver, D.K. Ingram, R. de Cabo, D.A. Sinclair
Resveratrol improves health and survival of mice on a high-calorie diet
Nature, 444 (2006), pp. 337–342
[58]
Q. Zhang, S.Y. Wang, C. Fleuriel, D. Leprince, J.V. Rocheleau, D.W. Piston, R.H. Goodman
Metabolic regulation of SIRT1 transcription via a HIC1:CtBP corepressor complex
Proc. Natl. Acad. Sci. U. S. A., 104 (2007), pp. 829–833
[59]
A.S. Banks, N. Kon, C. Knight, M. Matsumoto, R. Gutierrez-Juarez, L. Rossetti, W. Gu, D. Accili
SirT1 gain of function increases energy efficiency and prevents diabetes in mice
Cell. Metab., 8 (2008), pp. 333–341
[60]
L. Bordone, D. Cohen, A. Robinson, M.C. Motta, E. van Veen, A. Czopik, A.D. Steele, H. Crowe, S. Marmor, J. Luo, W. Gu, L. Guarente
SIRT1 transgenic mice show phenotypes resembling calorie restriction
Aging Cell, 6 (2007), pp. 759–767
[61]
P.T. Pfluger, D. Herranz, S. Velasco-Miguel, M. Serrano, M.H. Tschop
Sirt1 protects against high-fat diet-induced metabolic damage
Proc. Natl. Acad. Sci. U. S. A., 105 (2008), pp. 9793–9798
[62]
M. Lagouge, C. Argmann, Z. Gerhart-Hines, H. Meziane, C. Lerin, F. Daussin, N. Messadeq, J. Milne, P. Lambert, P. Elliott, B. Geny, M. Laakso, P. Puigserver, J. Auwerx
Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha

Cell, 127 (2006), pp. 1109–1122
[63]
J.A. Baur, D.A. Sinclair
Therapeutic potential of resveratrol: the in vivo evidence
Nat. Rev., Drug Discov., 5 (2006), pp. 493–506
[64]
J.N. Feige, M. Lagouge, C. Canto, A. Strehle, S.M. Houten, J.C. Milne, P.D. Lambert, C. Mataki, P.J. Elliott, J. Auwerx
Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation
Cell. Metab., 8 (2008), pp. 347–358
[65]
J. Milne, P. Lambert, C. Westphal, J. Milne, P. Lambert, S. Schenk, D. Carney, J. Smith, D. Gagne, L. Jin, O. Boss, R. Perni, C. Vu, J. Bemis, R. Xie, J. Disch, P. Ng, J. Nunes, A. Lynch, H. Yang, H. Galonek, K. Israelian, W. Choy, A. Iffland, S. Lavu, O. Medvedik, D. Sinclair, J. Olefsky, M. Jirousek, P. Elliott, C. Westphal
Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes
Nature, 450 (2007), pp. 712–716
[66]
J. Lin, H. Wu, P.T. Tarr, C.Y. Zhang, Z. Wu, O. Boss, L.F. Michael, P. Puigserver, E. Isotani, E.N. Olson, B.B. Lowell, R. Bassel-Duby, B.M. Spiegelman
Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres
Nature, 418 (2002), pp. 797–801

[67]
Z. Wu, P. Puigserver, U. Andersson, C. Zhang, G. Adelmant, V. Mootha, A. Troy, S. Cinti, B. Lowell, R.C. Scarpulla, B.M. Spiegelman
Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1
Cell, 98 (1999), pp. 115–124
[68]
S.C. Kim, R. Sprung, Y. Chen, Y. Xu, H. Ball, J. Pei, T. Cheng, Y. Kho, H. Xiao, L. Xiao, N.V. Grishin, M. White, X.J. Yang, Y. Zhao
Substrate and functional diversity of lysine acetylation revealed by a proteomics survey
Mol. Cell, 23 (2006), pp. 607–618
[69]
D.B. Lombard, F.W. Alt, H.L. Cheng, J. Bunkenborg, R.S. Streeper, R. Mostoslavsky, J. Kim, G. Yancopoulos, D. Valenzuela, A. Murphy, Y. Yang, Y. Chen, M.D. Hirschey, R.T. Bronson, M. Haigis, L.P. Guarente, R.V. Farese Jr., S. Weissman, E. Verdin, B. Schwer
Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation
Mol. Cell. Biol., 27 (2007), pp. 8807–8814
[70]
W.C. Hallows, S. Lee, J.M. Denu
Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases
Proc. Natl. Acad. Sci. U. S. A., 103 (2006), pp. 10230–10235

[71]
B. Schwer, J. Bunkenborg, R.O. Verdin, J.S. Andersen, E. Verdin
Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2
Proc. Natl. Acad. Sci. U. S. A., 103 (2006), pp. 10224–10229
[72]
T. Shi, F. Wang, E. Stieren, Q. Tong
SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes
J. Biol. Chem., 280 (2005), pp. 13560–13567
[73]
B.H. Ahn, H.S. Kim, S. Song, I.H. Lee, J. Liu, A. Vassilopoulos, C.X. Deng, T. Finkel
A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis
Proc. Natl. Acad. Sci. U. S. A., 105 (2008), pp. 14447–14452
[74]
T. Nakagawa, D.J. Lomb, M.C. Haigis, L. Guarente
SIRT5 deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle
Cell, 137 (2009), pp. 560–570
[75]
M.C. Haigis, R. Mostoslavsky, K.M. Haigis, K. Fahie, D.C. Christodoulou, A.J. Murphy, D.M. Valenzuela, G.D. Yancopoulos, M. Karow, G. Blander, C. Wolberger, T.A. Prolla, R. Weindruch, F.W. Alt, L. Guarente
SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells
Cell, 126 (2006), pp. 941–954
[76]
H. Yang, T. Yang, J.A. Baur, E. Perez, T. Matsui, J.J. Carmona, D.W. Lamming, N.C. Souza-Pinto, V.A. Bohr, A. Rosenzweig, R. de Cabo, A.A. Sauve, D.A. Sinclair
Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival
Cell, 130 (2007), pp. 1095–1107
[77]
M.T. Borra, M.R. Langer, J.T. Slama, J.M. Denu
Substrate specificity and kinetic mechanism of the Sir2 family of NAD+-dependent histone/protein deacetylases
Biochemistry, 43 (2004), pp. 9877–9887
[78]
K.T. Howitz, K.J. Bitterman, H.Y. Cohen, D.W. Lamming, S. Lavu, J.G. Wood, R.E. Zipkin, P. Chung, A. Kisielewski, L.L. Zhang, B. Scherer, D.A. Sinclair
Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan
Nature, 425 (2003), pp. 191–196
[79]
M.T. Schmidt, B.C. Smith, M.D. Jackson, J.M. Denu
Coenzyme specificity of Sir2 protein deacetylases: implications for physiological regulation
J. Biol. Chem., 279 (2004), pp. 40122–40129
[80]

S. Nemoto, M.M. Fergusson, T. Finkel
Nutrient availability regulates SIRT1 through a forkhead-dependent pathway
Science, 306 (2004), pp. 2105–2108
[81]
T. Sasaki, B. Maier, K.D. Koclega, M. Chruszcz, W. Gluba, P.T. Stukenberg, W. Minor, H. Scrable
Phosphorylation regulates SIRT1 function
PLoS One, 3 (2008), p. e4020
[82]
Y. Yang, W. Fu, J. Chen, N. Olashaw, X. Zhang, S.V. Nicosia, K. Bhalla, W. Bai
SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress
Nat. Cell Biol., 9 (2007), pp. 1253–1262
[83]
E.J. Kim, J.H. Kho, M.R. Kang, S.J. Um
Active regulator of SIRT1 cooperates with SIRT1 and facilitates suppression of p53 activity
Mol. Cell, 28 (2007), pp. 277–290
[84]
W. Zhao, J.P. Kruse, Y. Tang, S.Y. Jung, J. Qin, W. Gu
Negative regulation of the deacetylase SIRT1 by DBC1
Nature, 451 (2008), pp. 587–590
[85]
W.Y. Chen, D.H. Wang, R.C. Yen, J. Luo, W. Gu, S.B. Baylin
Tumor suppressor HIC1 directly regulates SIRT1 to modulate p53-dependent DNA-damage responses
Cell, 123 (2005), pp. 437–448
[86]
H.L. Cheng, R. Mostoslavsky, S. Saito, J.P. Manis, Y. Gu, P. Patel, R. Bronson, E. Appella, F.W. Alt, K.F. Chua
Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice
Proc. Natl. Acad. Sci. U. S. A., 100 (2003), pp. 10794–10799
[87]
R.H. Wang, K. Sengupta, C. Li, H.S. Kim, L. Cao, C. Xiao, S. Kim, X. Xu, Y. Zheng, B. Chilton, R. Jia, Z.M. Zheng, E. Appella, X.W. Wang, T. Ried, C.X. Deng
Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice
Cancer Cell, 14 (2008), pp. 312–323
[88]
R.H. Wang, Y. Zheng, H.S. Kim, X. Xu, L. Cao, T. Luhasen, M.H. Lee, C. Xiao, A. Vassilopoulos, W. Chen, K. Gardner, Y.G. Man, M.C. Hung, T. Finkel, C.X. Deng
Interplay among BRCA1, SIRT1, and Survivin during BRCA1-associated tumorigenesis
Mol. Cell, 32 (2008), pp. 11–20
[89]

R. Firestein, G. Blander, S. Michan, P. Oberdoerffer, S. Ogino, J. Campbell, A. Bhimavarapu, S. Luikenhuis, R. de Cabo, C. Fuchs, W.C. Hahn, L.P. Guarente, D.A. Sinclair
The SIRT1 deacetylase suppresses intestinal tumorigenesis and colon cancer growth
PLoS One, 3 (2008), p. e2020
[90]
C. Das, M.S. Lucia, K.C. Hansen, J.K. Tyler
CBP/p300-mediated acetylation of histone H3 on lysine 56
Nature, 459 (2009), pp. 113–117
[91]
P. Oberdoerffer, S. Michan, M. McVay, R. Mostoslavsky, J. Vann, S.K. Park, A. Hartlerode, J. Stegmuller, A. Hafner, P. Loerch, S.M. Wright, K.D. Mills, A. Bonni, B.A. Yankner, R. Scully, T.A. Prolla, F.W. Alt, D.A. Sinclair
SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging
Cell, 135 (2008), pp. 907–918
[92]
Z. Yuan, X. Zhang, N. Sengupta, W.S. Lane, E. Seto
SIRT1 regulates the function of the Nijmegen breakage syndrome protein
Mol. Cell, 27 (2007), pp. 149–162
[93]
C. Franceschi, M. Bonafe, S. Valensin, F. Olivieri, M. De Luca, E. Ottaviani, G. De Benedictis
Inflamm-aging. An evolutionary perspective on immunosenescence
Ann. N.Y. Acad. Sci., 908 (2000), pp. 244–254
[94]
C. Franceschi, S. Valensin, M. Bonafe, G. Paolisso, A.I. Yashin, D. Monti, G. De Benedictis
The network and the remodeling theories of aging: historical background and new perspectives
Exp. Gerontol., 35 (2000), pp. 879–896
[95]
A. Csiszar, Z. Ungvari, A. Koller, J.G. Edwards, G. Kaley
Aging-induced proinflammatory shift in cytokine expression profile in coronary arteries
FASEB J., 17 (2003), pp. 1183–1185
[96]
K.J. Kyng, A. May, T. Stevnsner, K.G. Becker, S. Kolvra, V.A. Bohr
Gene expression responses to DNA damage are altered in human aging and in Werner syndrome
Oncogene, 24 (2005), pp. 5026–5042
[97]
C.K. Lee, R. Weindruch, T.A. Prolla
Gene-expression profile of the ageing brain in mice
Nat. Genet., 25 (2000), pp. 294–297
[98]
R. Weindruch, T. Kayo, C.K. Lee, T.A. Prolla

Microarray profiling of gene expression in aging and its alteration by caloric restriction in mice
J. Nutr., 131 (2001), pp. 918S–923S
[99]
W.R. Swindell
Comparative analysis of microarray data identifies common responses to caloric restriction among mouse tissues
Mech. Ageing Dev., 129 (2008), pp. 138–153
[100]
T.L. Kawahara, E. Michishita, A.S. Adler, M. Damian, E. Berber, M. Lin, R.A. McCord, K.C. Ongaigui, L.D. Boxer, H.Y. Chang, K.F. Chua
SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span
Cell, 136 (2009), pp. 62–74
[101]
F. Yeung, J.E. Hoberg, C.S. Ramsey, M.D. Keller, D.R. Jones, R.A. Frye, M.W. Mayo
Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase
EMBO J., 23 (2004), pp. 2369–2380
[102]
A.A. Bertelli, R. Baccalini, E. Battaglia, M. Falchi, M.E. Ferrero
Resveratrol inhibits TNF alpha-induced endothelial cell activation
Therapie, 56 (2001), pp. 613–616
[103]
C.A. de la Lastra, I. Villegas
Resveratrol as an anti-inflammatory and anti-aging agent: mechanisms and clinical implications
Mol. Nutr. Food Res., 49 (2005), pp. 405–430
[104]
A.R. Martin, I. Villegas, M. Sanchez-Hidalgo, C.A. de la Lastra
The effects of resveratrol, a phytoalexin derived from red wines, on chronic inflammation induced in an experimentally induced colitis model
Br. J. Pharmacol., 147 (2006), pp. 873–885
[105]
J. Sequeira, G. Boily, S. Bazinet, S. Saliba, X. He, K. Jardine, C. Kennedy, W. Staines, C. Rousseaux, R. Mueller, M.W. McBurney
Exp. Cell Res., 314 (2008), pp. 3069–3074
[106]
K.J. Pearson, J.A. Baur, K.N. Lewis, L. Peshkin, N.L. Price, N. Labinskyy, W.R. Swindell, D. Kamara, R.K. Minor, E. Perez, H.A. Jamieson, Y. Zhang, S.R. Dunn, K. Sharma, N. Pleshko, L.A. Woollett, A. Csiszar, Y. Ikeno, D. Le Couteur, P.J. Elliott, K.G. Becker, P. Navas, D.K. Ingram, N.S. Wolf, Z. Ungvari, D.A. Sinclair, R. de Cabo
Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span
Cell. Metab., 8 (2008), pp. 157–168

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http://www.sciencedirect.com/science/article/pii/S1570963909002635

Part III: Project Goals

It is with these concepts in mind that research was undertaken to understand the contribution of mitochondrial functioning, and the role of SIRT3 in particular, to the process of aging. The following chapters describe experimental research that was developed based on the hypothesis that SIRT3 functions in a protective manner at the mitochondrial level.

Chapter II represents work that was undertaken in order to understand the role and function that SIRT3 serves during calorie restriction, and reports findings that SIRT3 has a major impact on oxidative stress and damage reduction during calorie restriction. The results from this study helped to inform the projects described in Chapter III and IV.

Chapter III presents data collected in concert with the project described in Chapter II, and indicates that substrate utilization and metabolism is also affected by SIRT3 deficiency during calorie restriction.

The project described in Chapter IV focuses on an adult stem cell, in this case the well-characterized hematopoietic stem cell as a model system for stem cell aging and function. This project reveals the role of SIRT3 in maintaining adult stem cell function, especially under conditions of increased oxidative stress.

CHAPTER II

CALORIE RESTRICTION REDUCES OXIDATIVE STRESS BY SIRT3-MEDIATED SOD2 ACTIVATION[3]

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SUMMARY

A major cause of aging and numerous diseases is thought to be cumulative oxidative stress, resulting from the production of reactive oxygen species (ROS) during respiration. Calorie restriction (CR), the most robust intervention to extend life span and ameliorate various diseases in mammals, reduces oxidative stress and damage. However, the underlying mechanism is unknown. Here, we show that the protective effects of CR on oxidative stress and damage are diminished in mice lacking SIRT3, a mitochondrial deacetylase. SIRT3 reduces cellular ROS levels dependent on superoxide dismutase 2 (SOD2), a major mitochondrial antioxidant enzyme. SIRT3 deacetylates two critical lysine residues on SOD2 and promotes its antioxidative activity. Importantly, the ability of SOD2 to reduce cellular ROS and promote oxidative stress resistance is greatly enhanced by SIRT3. Our studies identify a defense program that CR provokes to reduce oxidative stress and suggest approaches to combat aging and oxidative stress-related diseases.

HIGHLIGHTS

► CR reduces oxidative stress by inducing SIRT3
► SIRT3 activates SOD2 via deacetylation
► SIRT3 reduces cellular ROS and promotes stress resistance by deacetylating SOD2
► SOD2 is activated by SIRT3 via deacetylation during CR

INTRODUCTION

The free radical theory of aging postulates that the production of reactive oxygen species (ROS) is the major determinant of life span ([Balaban et al., 2005], [Merry, 2004] and [Sohal and Weindruch, 1996]). ROS are produced as a natural byproduct of cellular respiration. Antioxidant enzymes, such as superoxide dismutases (SODs), scavenge ROS and maintain a reducing environment in the cell. An imbalance between the production of ROS and the cell's ability to readily detoxify ROS disturbs the cellular reducing environment and results in oxidative stress. ROS can damage various components of the cell, including DNA, RNA, proteins, and lipids. Accumulated oxidative stress is thought to be a major cause of aging ([Balaban et al., 2005] and [Merry, 2004]). Consistent with this, animals with reduced levels of oxidative stress, such as animals fed a calorie restriction (CR) diet or overexpressing mitochondrially targeted catalase, have extended life spans, while animals with high levels of oxidative stress, such as mice lacking SODs, have shortened life spans ([Merry, 2004], [Schriner et al., 2005] and [Wallace and Fan, 2009]).

CR is the most robust intervention to extend life span in mammals, and delays the onset of numerous age-associated diseases including cancer, diabetes, and neurodegenerative diseases ([Colman et al., 2009], [Van Remmen et al., 2001] and [Weindruch and Walford, 1988]). CR was hypothesized to extend life span by slowing metabolism and reducing mitochondrial ROS (Sohal and Weindruch, 1996). However, metabolic rate normalized to body weight does not decrease in CR mice (Masoro et al., 1982). In fact, mitochondrial activity is increased during CR (Nisoli et al., 2005). Thus, the molecular mechanism underlying CR-induced reduction of oxidative stress remains elusive.

In the budding yeast Saccharomyces cerevisiae and the fruit fly Drosophila melanogaster, CR extends life span by increasing the activity of the Sir2 protein ([Lin et al., 2000] and [Rogina and Helfand, 2004]). Sir2p has nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase activity ([Frye, 2000] and [Imai et al., 2000]). An extra copy of SIR2 extends life span in yeast, flies, and worms ([Kaeberlein et al., 1999], [Rogina and Helfand, 2004] and [Tissenbaum and Guarente, 2001]). Increasing evidence suggests that the function of SIR2 in mediating the CR response is conserved in mammals. SIRT1, the mammalian ortholog of SIR2, is upregulated in many tissues of CR mice and is an essential mediator of many aspects of the CR response, such as increased cell survival and physical activity, and decreased somatotropic signaling ([Chen et al., 2005], [Cohen et al., 2004], [Cohen et al., 2009] and [Li et al., 2008]).

Given the role of SIR2 in mediating the CR response and the observation that animals fed a CR diet have reduced oxidative stress, we speculate that mammalian SIR2 homologs might play a role in regulating oxidative stress. Since 90% of cellular ROS are produced in the mitochondria (Balaban et al., 2005), we set out to investigate whether SIRT3, a mammalian SIR2 homolog localized in the mitochondria ([Onyango et al., 2002] and [Schwer et al., 2002]), mediates the CR response and reduces oxidative stress.

RESULTS

Reduction of Oxidative Stress and Damage by CR Requires SIRT3

CR induces an increase in the expression of SIRT3 ([Palacios et al., 2009] and [Shi et al., 2005]) and mitochondrial NAD+ levels (Nakagawa et al., 2009), suggesting that SIRT3 activity is likely to be upregulated during CR. To investigate whether SIRT3 is required for CR to reduce oxidative stress, we fed SIRT3 knockout (KO) mice (Lombard et al., 2007) and wild-type (WT) littermates a CR diet for 6 months. We compared oxidative stress and damage between WT and SIRT3 KO mice fed ad libitum (AL) or CR diets. Consistent with earlier reports, CR significantly reduced oxidative stress and damage in WT mice, as shown by levels of 4-hydroxy-2-nonenal (HNE), a marker for lipid peroxidation (Figure 1A), protein carbonyl content, a protein oxidative modification (Figure 1B), and the GSH:GSSG ratio, a common measure of oxidative stress (Figure 1C) ([Merry, 2004] and [Rebrin et al., 2003]). However, the reduction in oxidative stress and damage under CR was not observed in SIRT3 KO mice (Figures 1A–1C), suggesting that SIRT3 is required for reducing oxidative stress during CR.

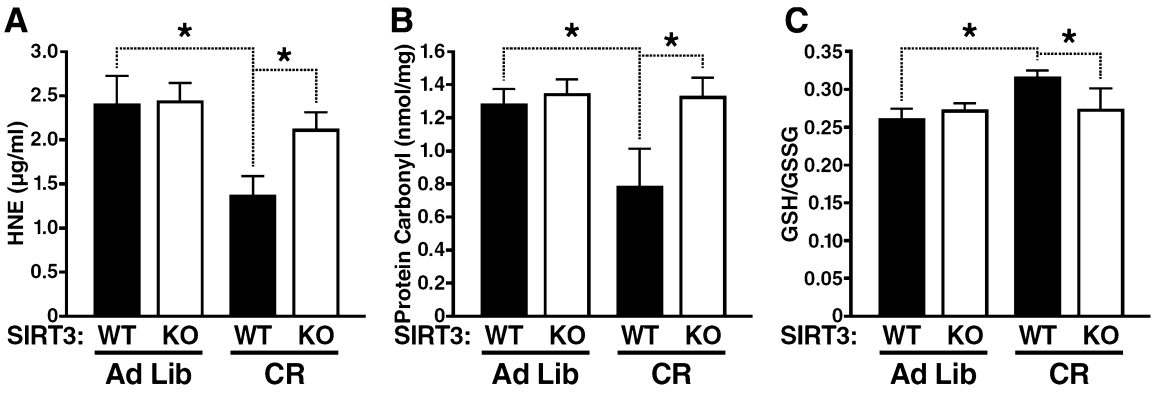

Figure 1. Reduction of Oxidative Stress and Damage by Calorie Restriction Requires SIRT3(A–C) Liver lysates from WT and SIRT3 KO mice fed AL or CR diet were assayed for lipid peroxidation (A), protein carbonyl formation (B), and the GSH:GSSG ratio (C). ∗p < 0.05.

SIRT3 Reduces Cellular ROS via SOD2

In mammals, there are three forms of SODs localized in various cellular compartments (Balaban et al., 2005). SOD2 is located in the mitochondria. To determine whether SOD2 plays a role in the reduction of cellular ROS mediated by SIRT3, we overexpressed SIRT3 in WT and SOD2 KO mouse embryonic fibroblasts (MEFs) via lentiviral transduction and assessed cellular ROS levels. Overexpression of SIRT3 in WT MEFs reduced cellular ROS by 40%. However, reduction of cellular ROS mediated by SIRT3 was blunted in SOD2 KO MEFs (Figure 2A), indicating that SOD2 is the major downstream mediator of SIRT3 in reducing cellular ROS.

SIRT3 Activates SOD2 via Deacetylation

SOD2 was identified in mass spectrometry-based screens for acetylated peptides ([Choudhary et al., 2009], [Kim et al., 2006], [Kim et al., 2010] and [Schwer et al., 2009]). We tested the possibility that SIRT3 regulates the acetylation state of SOD2. To test whether SIRT3 interacts with SOD2, we overexpressed Flag-tagged SIRT3 in 293T cells and SIRT3-associated proteins were immunopurified (anti-Flag). The association of SOD2 with SIRT3 was detected by western blotting with SOD2 antibody (Figure 2B). Additionally, we also transfected Flag-tagged SOD2 into 293T cells and the presence of SIRT3 in the immunopurified SOD2 complex was confirmed by western blotting with SIRT3 antibody (Figure 2C). Finally, we examined whether the interaction between SIRT3 and SOD2 is physiologically relevant by carrying out immunoprecipitation with liver extracts. SIRT3 was coimmunoprecipitated with SOD2 antibody (Figure 2D).

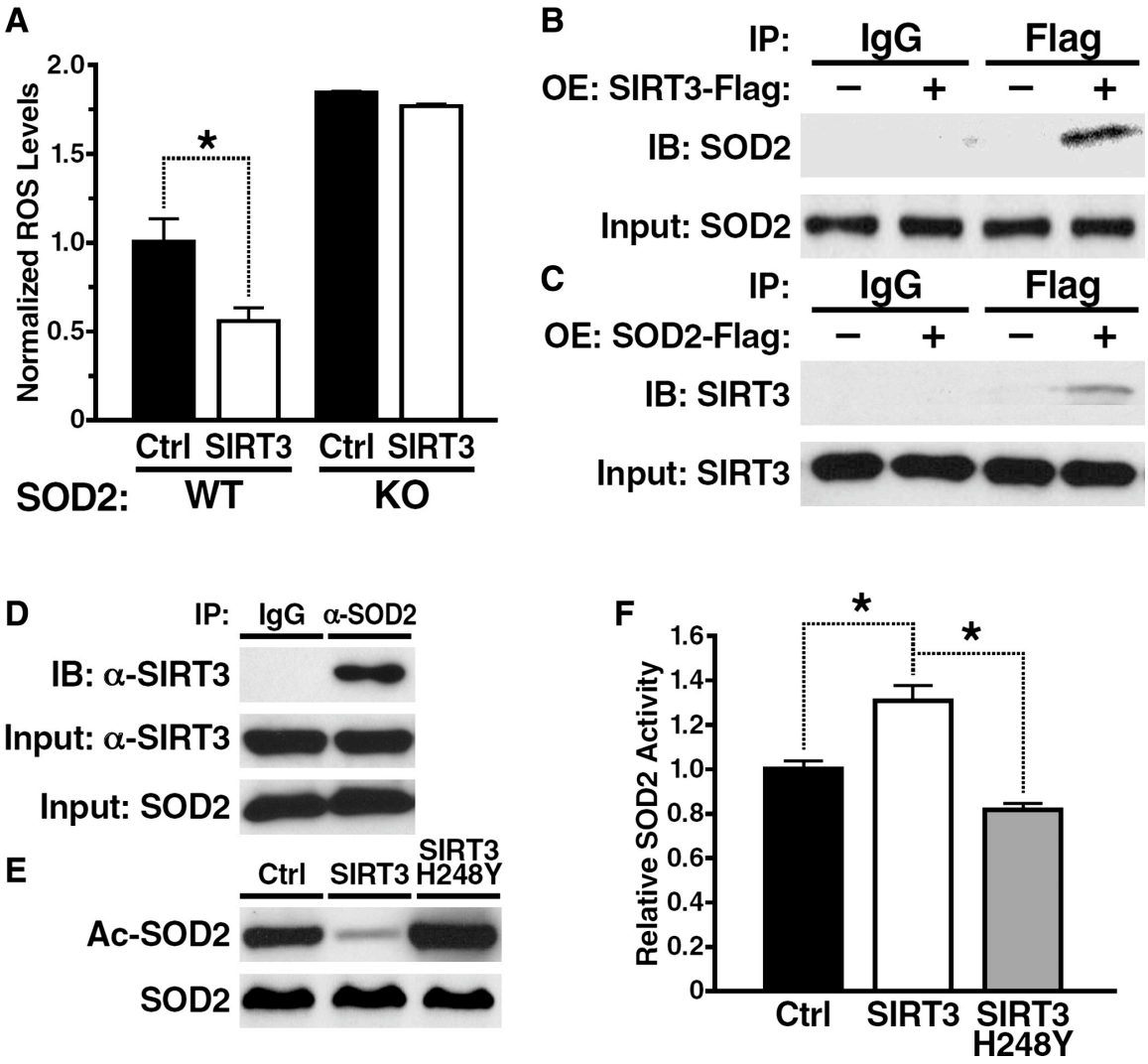

Figure 2. SIRT3 Deacetylates and Activates SOD2(A) SIRT3 reduces cellular ROS via SOD2. SIRT3 was overexpressed in WT and SOD2 KO MEFs via lentiviral transduction and cellular ROS levels were quantified by MitoSox staining. $*p < 0.05$.(B–D) SIRT3 physically interacts with SOD2 in vivo. Flag-tagged SIRT3 was transfected into 293T cells, immunopurified, followed by Western blotting with anti-SOD2 (B). Flag-tagged SOD2 was transfected into 293T cells. The association of SIRT3 with immunopurified Flag-SOD2 was detected by Western blotting with anti-SIRT3 antibody (C). Endogenous SOD2 was immunopurified from liver lysates with anti-SOD2 antibody, followed by western blotting with anti-SIRT3 antibody (D).(E and F) SIRT3 deacetylates and activates SOD2. Flag-tagged SOD2 was transfected into 293T cells with a control vector, SIRT3, or enzymatically inactive SIRT3-H248Y. SOD2 was immunopurified. Its acetylation status was detected by western blotting with acetyl-lysine antibody (E), and its enzymatic activity was quantified by conversion of superoxide colormetrically as described (Schisler and Singh, 1985) (F). $*p < 0.05$.

To test whether SIRT3 deacetylates SOD2, we cotransfected Flag-tagged SOD2 with SIRT3 or enzymatically inactive SIRT3-H248Y into 293T cells. Acetylation levels for SOD2 were measured after immunoprecipitation by western blotting with anti-acetyl-lysine antibody. SIRT3, but not SIRT3-H248Y, reduced acetylation levels of SOD2 (Figure 2E). To determine whether the acetylation state of SOD2 modifies its enzymatic activity, we overexpressed Flag-tagged SOD2 in 293T cells with SIRT3 or SIRT3-H248Y, immunopurified SOD2, and determined its enzymatic activity by measuring superoxide conversion colorimetrically (Schisler and Singh, 1985). Overexpression of WT SIRT3 decreased the acetylation of SOD2 (Figure 2E) and significantly increased its enzymatic activity (Figure 2F). In contrast, overexpression of SIRT3-H248Y had the opposite effect, suggesting that SIRT3 activates SOD2 via deacetylation.

To identify which lysine residue(s) on SOD2 are targeted by SIRT3 for deacetylation, we mutated lysines (K68 and K130) that have been shown to be acetylated in mass spectrometry-based acetylation proteomic surveys ([Choudhary et al., 2009], [Kim et al., 2006] and [Schwer et al., 2009]). Surprisingly, mutating these lysine residues did not significantly reduce the overall acetylation levels of SOD2 (Figure 3A). Additionally, SIRT3 reduced acetylation levels of these SOD2 mutants, indicating that these lysines are unlikely to be the major acetylation sites on SOD2.

Protein sequence alignment studies showed that two lysines (K53 and K89) adjacent to the active site of SOD2 are highly conserved across species (Figure 3B). Acetylation levels of SOD2 were decreased when these two lysines were mutated individually (K53R and K89R) or simultaneously (K53/89R) (Figures 3A and 3C). SIRT3 further decreased acetylation levels of K53R and K89R, but not K53/89R. Collectively, these studies identified K53 and K89 as the acetylation sites on SOD2 targeted by SIRT3.

To determine how deacetylation of these two lysines on SOD2 affects its antioxidative activity, we determined the antioxidative activity of K53/89R, which mimics the constitutively deacetylated state. The enzymatic activity of SOD2 K53/89R was 100% higher than the WT control and SIRT3 did not further increase its enzymatic activity (Figure 3D). Thus, SIRT3 promotes the enzymatic activity of SOD2 by deacetylating two critical lysine residues adjacent to the active site. Conceivably, these two lysine residues, when exposed, increase the positive charge around the active site and improve the efficiency of trapping the negatively charged superoxide.

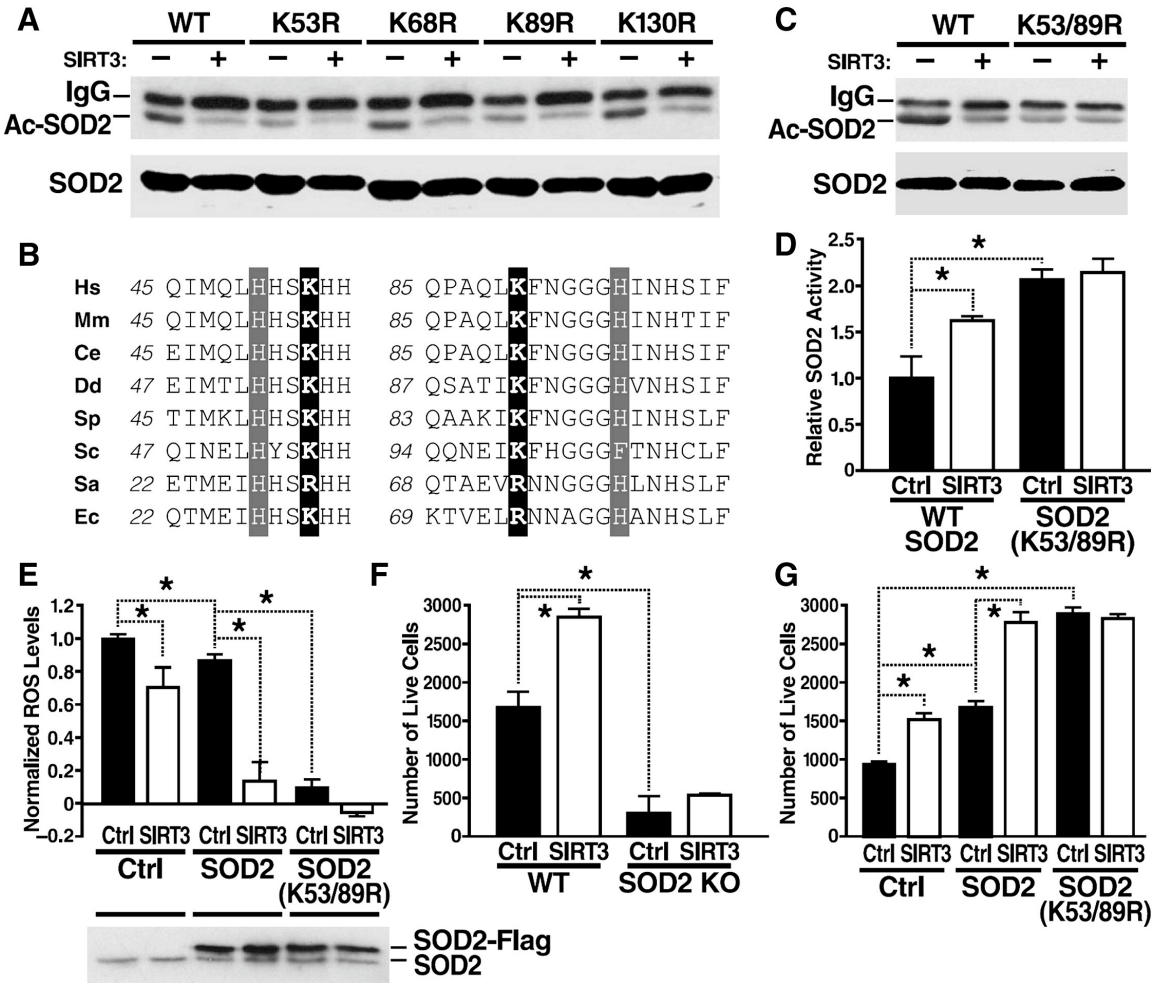

Figure 3. SIRT3 Reduces Cellular ROS Levels and Promotes Oxidative Stress Resistance by Deacetylating Two critical Lysine Residues on SOD2(A and C) SIRT3 deacetylates two critical lysine residues on SOD2 in vivo. Flag-tagged SOD2 or SOD2 mutants were cotransfected with a control vector or SIRT3 into 293T cells. Immunopurified Flag-tagged SOD2 was examined for its acetylation levels by western blotting with anti-acetyl-lysine antibody. Sequence alignment of SOD2 from various species is shown in (B). Residues (H50 and H95) coordinating the metal center are shaded. Conserved lysines (K53 and K89) are in bold.(D) SIRT3 activates SOD2 by deacetylating two critical lysine residues in vivo. Flag-tagged SOD2 or SOD2 K53/89R was cotransfected with a control vector or SIRT3 into 293T cells. The antioxidative activity of immunopurified SOD2 was determined. $*p < 0.05$.(E) SIRT3 reduces cellular ROS by deacetylating SOD2. SOD2 or SOD2 K53/89R mutant was overexpressed with or without SIRT3 in SIRT3 KO MEFs, and cellular ROS levels were determined by MitoSox staining. $*p < 0.05$.(F and G) SIRT3 promotes oxidative stress resistance by deacetylating SOD2. SIRT3 was overexpressed in WT or SOD2 KO MEFs (F). SOD2 or SOD2 K53/89R mutant was overexpressed with or without SIRT3 in SIRT3 KO MEFs (G). Ten thousand cells were treated with 200 μM paraquat and live cells were counted. See also Figure S1. $*p < 0.05$.

SIRT3 Reduces Cellular ROS by Deacetylating SOD2

To determine whether SIRT3 reduces cellular ROS levels by deacetylating SOD2, we overexpressed SOD2 or SOD2 K53/89R with or without SIRT3 into SIRT3 KO MEFs and assessed cellular ROS levels. We used SIRT3 KO MEFs in this assay to avoid the influence of endogenous SIRT3. Surprisingly, overexpression of SOD2 6-fold above the

endogenous levels only marginally decreased cellular ROS (10%) (Figure 3E). However, coexpression of SIRT3 and SOD2 depleted 90% of cellular ROS. Additionally, constitutively deacetylated SOD2 K53/89R alone also diminished cellular ROS by 90%. These results indicate that increasing SOD2 expression alone can only modestly reduce cellular ROS. SIRT3 deacetylation significantly enhances the ability of SOD2 to reduce cellular ROS.

SIRT3 Increases Oxidative Stress Resistance by Deacetylating SOD2

To determine whether SIRT3-mediated SOD2 activation results in increased oxidative stress resistance, we overexpressed SIRT3 in WT or SOD2 KO MEFs, treated the cells with paraquat, a superoxide-generating compound, and assayed cell survival. SIRT3 overexpression in WT MEFs doubled the number of surviving cells (Figures 3F and S1). However, SIRT3 overexpression in SOD2 KO MEFs had no effect on cell survival, indicating that SIRT3 promotes oxidative stress resistance and SOD2 is the mediator of this process. To determine whether SIRT3 increases oxidative stress resistance by deacetylating SOD2, we overexpressed WT SOD2 or SOD2 K53/89R with or without SIRT3 into SIRT3 KO MEFs, and assessed oxidative stress resistance of these cells. Although overexpression of SOD2 alone only modestly reduced cellular ROS (10%) (Figure 3E), it increased the cell survival rate by 50% upon paraquat treatment (Figure 3G). It is worth noting that the condition for measuring cellular ROS level (Figure 3E) is different from the condition for assessing oxidative stress resistance (Figures 3F and 3G). The former is for untreated cells, while the latter is for cells treated with paraquat when ROS levels are significantly higher than physiological levels. Importantly, coexpression of SOD2 and SIRT3 resulted in a nearly 3-fold increase in cell survival (Figure 3G). Moreover, constitutively deacetylated SOD2 K53/89R increased cell survival to the same level as coexpression of SOD2 and SIRT3. Coexpression of SIRT3 did not further increase cell survival mediated by SOD2 K53/89R. These results indicate that SIRT3 promotes oxidative stress resistance by deacetylating SOD2.

SOD2 Is Activated by SIRT3 via Deacetylation during Calorie Restriction

Given that SIRT3 is induced by CR and that SIRT3 activates SOD2 via deacetylation, we speculated that SIRT3 might deacetylate SOD2 and increase its antioxidative activity in CR animals. We compared acetylation levels of SOD2 and its antioxidative activity in WT and SIRT3 KO mice fed AL or CR diets. To assess acetylation levels of SOD2 in mouse tissues, endogenous proteins were immunoprecipitated with anti-SOD2 antibody and analyzed by western blotting using acetyl-lysine antibody. Endogenous SOD2 was acetylated and became deacetylated during CR in WT mice (Figure 4A). However, CR-induced SOD2 deacetylation was not observed in SIRT3 KO mice, demonstrating that SIRT3 is necessary for SOD2 deacetylation during CR. We next determined endogenous SOD2 activity using tissue lysates as described (Schisler and Singh, 1985). CR induced a 50% increase in SOD2 activity in the white adipose tissues of WT mice (Figure 4B). Importantly, this increase in SOD2 activity under CR was lost in

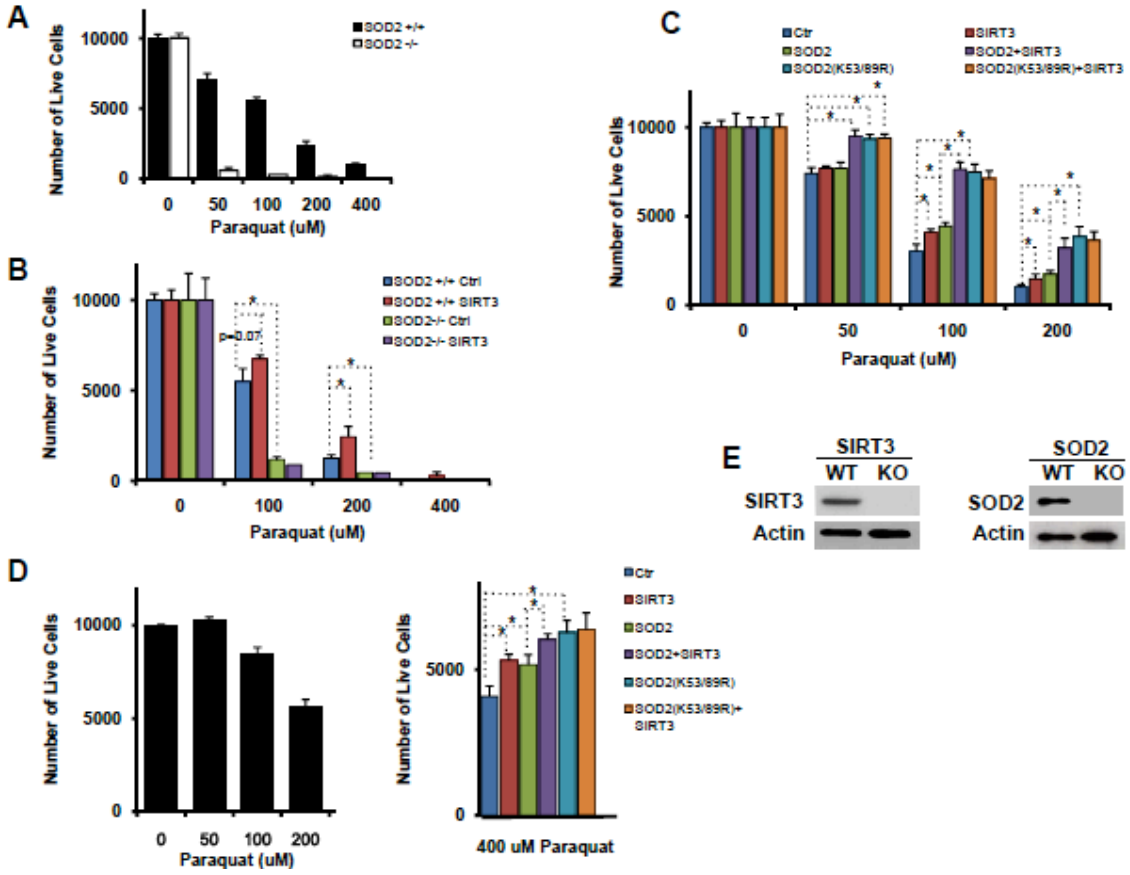

Figure S1. SIRT3 Promotes Oxidative Stress Resistance via SOD2, Related to Figure 3
(A) A dose curve response to paraquat treatment. WT and SIRT3 KO MEFs were treated with increasing doses of paraquat and live cells were counted. (B, C) SIRT3 was overexpressed in WT or SOD2 KO MEFs (B). SOD2 or SOD2 K53R mutant was overexpressed with or without SIRT3 in SIRT3 KO MEFs (C). Cells were treated with paraquat and live cells were counted. (D) A dose curve response of 293T cells to paraquat treatment (left). SOD2 or SOD2 K53R mutant was overexpressed with or without SIRT3 in 293T cells. Cells were treated with paraquat and live cells were counted (right). (E) Western blots confirming the identity of SIRT3 KO MEFs and SOD2 KO MEFs.

SIRT3 KO mice. These results suggest that during CR, SIRT3 reduces oxidative stress by activating SOD2 and promoting the detoxification of ROS. It is worth noting that SIRT3-mediated SOD2 deacetylation and activation was not observed in mice fed AL (Figures 4A and 4B). Consistent with this, oxidative stress and damage were comparable in WT and SIRT3 KO mice fed AL (Figure 1), suggesting that SIRT3 is not active under AL conditions.

DISCUSSION

Our studies identify an active defense program CR provokes to reduce oxidative stress. CR induces an increase in SIRT3 expression ([Palacios et al., 2009] and [Shi et al., 2005]). Activation of SIRT3 during CR reduces oxidative stress by activating the mitochondrial antioxidant enzyme SOD2. SOD2 activation and increased oxidative stress resistance have been linked to numerous long-lived mouse models ([Baba et al., 2005], [Hauck et al., 2001], [Taguchi et al., 2007] and [Yamamoto et al., 2005]). We

speculate that the loss of protection from oxidative stress in the SIRT3 KO mice could abrogate CR-induced life-span extension.

Despite the prevalence of the free radical theory of aging, it is intriguing that SOD2 transgenic mice do not have increased life spans (Pérez et al., 2009). Our studies suggest that increasing SOD2 expression alone can only modestly reduce cellular ROS. The ability of SOD2 to reduce cellular ROS is greatly enhanced by SIRT3 deacetylation. Transgenic mice overexpressing both SIRT3 and SOD2 might have extended life spans.

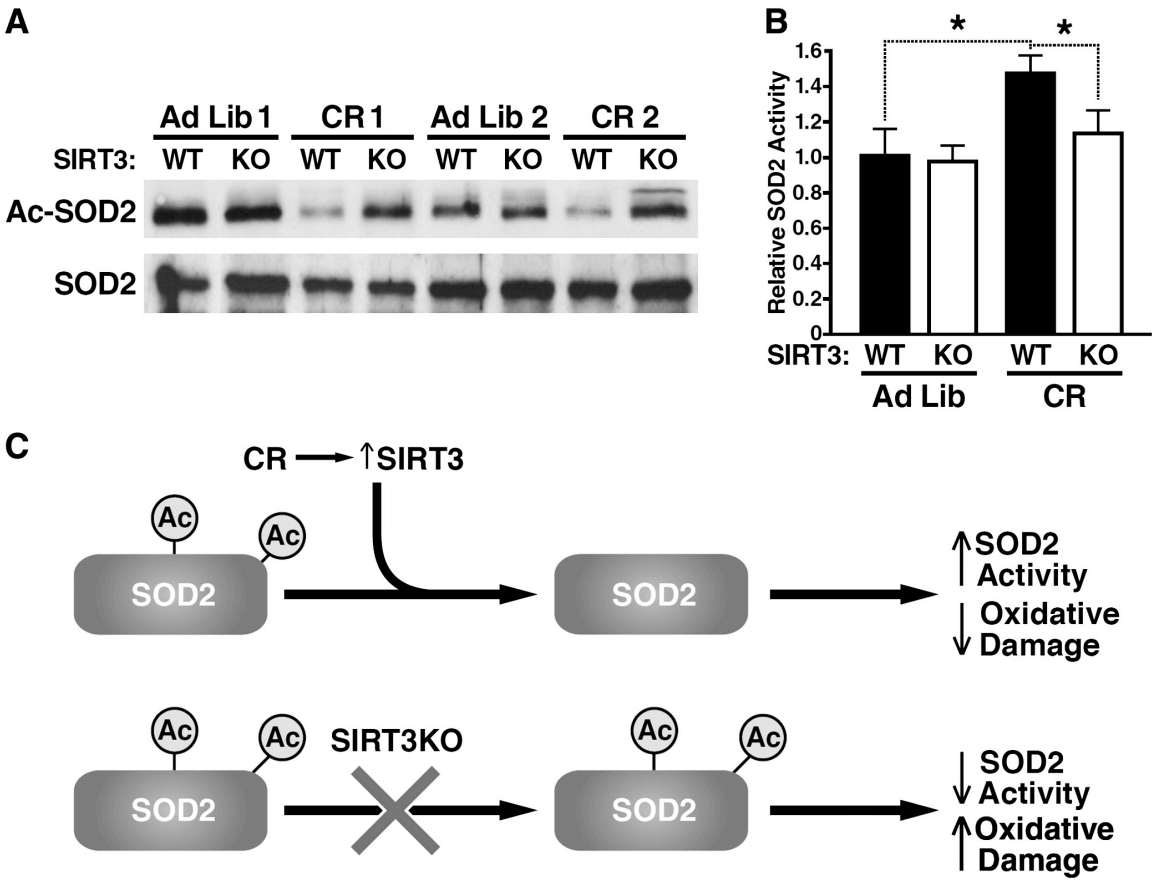

Figure 4. SIRT3 Activates SOD2 via Deacetylation during Calorie Restriction(A) SIRT3 deacetylates SOD2 in CR mice. Acetylation levels of SOD2 in WT and SIRT3 KO mice fed an AL or CR diet were determined. Endogenous SOD2 was isolated by immunoprecipitation with anti-SOD2 antibody followed by western blotting with anti-acetyl-lysine antibody.(B) SIRT3 increases the antioxidative activity of SOD2 in CR mice. The antioxidative activity of SOD2 in white adipose tissues of WT and SIRT3 KO mice fed an AL or CR diet were determined (Schisler and Singh, 1985). $*p < 0.05$.(C) A model on SIRT3-mediated SOD2 activation.

Although mitochondrial activity is increased by CR (Nisoli et al., 2005), oxidative stress and damage are reduced in CR animals (Merry, 2004). Evidence is emerging to support the mitochondrial hormesis or “mitohormesis” concept, which hypothesizes that induction of mitochondrial metabolism and the increased formation of ROS trigger an active defense program, resulting in increased stress resistance and possibly extended life span ([Schulz et al., 2007] and [Sinclair, 2005]). Perhaps activation of SIRT3 in CR animals is a key step of this defense program against oxidative stress.

In light of the importance of mitochondrial ROS production in the onset and progression of diverse diseases associated with aging (Wallace and Fan, 2009), our findings have important implications for developing CR mimetics for treating the diseases of aging. Notably, SIRT3 has been shown to protect mice from developing cancer and cardiac hypertrophy ([Kim et al., 2010] and [Sundaresan et al., 2009]). Future studies will determine the impact of SIRT3 in other pathological conditions and explore therapeutic strategies to treat human diseases with SIRT3 activators or SOD2 variants.

EXPERIMENTAL PROCEDURES

Mice

SIRT3−/− mice have been described (Lombard et al., 2007). All mice were housed on a 12:12 hr light:dark cycle at 25°C. Six-month-old animals (n = 8) were either fed AL or a 30% CR diet, which was provided daily for six months. All animal procedures were in accordance with the animal care committee at the University of California, Berkeley.

Protein Preparation and Analysis

Proteins from mouse tissues were extracted in lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM MgCl2, 1 mM DTT and 1% NP40) supplemented with a complete protease inhibitor cocktail (Roche), trichostatin A, and nicotinamide. Protein extracts were subjected to centrifugation at 14,000 rpm for 10 min. Protein lysates were precleared with protein A beads for 30 min before immunoprecipitation with specified antibodies for 2 hr or overnight. Immunoprecipitates were extensively washed with lysis buffer and eluted with 100 mM glycine, pH 3.0, or Flag peptides. Acetyl-lysine antibody was provided by Cell Signaling, BioLegend; SOD2 antibody was provided by Santa Cruz Biotechnology; Flag antibody was provided by Sigma.

Lentiviral Production and Transduction

SIRT3 and SOD2 were cloned into pFUGW lentiviral vector. Lentiviruses were produced by transient transfection of pFUGW and packaging vectors into 293T cells with lipofectamine. Lentiviruses were harvested 48 hr posttransfection and filtered through

0.45-μm-pore cellulose acetate filters. Virus containing media was mixed with fresh media (1:1) and added to MEF cells in the presence of 8 μg/ml polybrene.

Measurement of Mitochondrial Superoxide Levels

Cells were incubated with 3 μM of Mito-SOX at 37°C for 15 min prior to flow cytometry analysis.

Enzyme Assays

Superoxide dismutase activity was measured as the inhibition of nitroblue tetrazolium (NBT) reduction in a xanthine-xanthine oxidase system. The assay was performed as described (Schisler and Singh, 1985), and SOD2 specific activity was determined in the presence of 5 mM sodium cyanide.

Carbonyl Content Measurement

Protein carbonyls were spectrophotometrically quantified with a carbonyl specific reagent, 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1994). Briefly, 1 ml of 0.5 mg protein was treated with 200 μl of 10 mM of DNPH (dissolved in 2M HCl) for 1 hr, and then precipitated by 10% trichloroacetic acid. The pellets were washed with 1:1 (v/v) ethanol:ethyl acetate for three times, and solublized in 0.5 ml 0.2% SDS, 20 mM Tris-Cl, pH 6.8. Protein concentration in the final solution was then determined with a BCA kit (Piercenet), and the absorbance at 360 nm was measured to calculate the carbonyl content. Protein samples treated with HCl but not with DNPH were used as blanks.

Glutathione Redox Measurement

Glutathione was measured in mitochondrial fractions isolated from liver ([Merry, 2004] and [Rebrin et al., 2003]). The GSH: GSSG ratio was determined by Glutathione Assay Kit (BioVision), following the manufacturer's instructions.

HNE Measurement

HNE levels were measured in indicated liver samples with an OxiSelect HNE-His Adduct ELISA Kit (Cell Biolabs, Inc. San Diego, CA) following the instructions.

Cell Survival Assay

Ten thousand cells were incubated with increasing doses of paraquat (Sigma) (0, 50, 100, 200, 400 μM) for 48 hr. Remaining adherent cells were trypsinized and counted using a hemocytometer. A dose-curve response was determined (Figure S1A).

Statistical Analysis

Student's t test was used for statistic analysis and null hypotheses were rejected at 0.05.

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REFERENCES

Baba et al., 2005
T. Baba, T. Shimizu, Y. Suzuki, M. Ogawara, K. Isono, H. Koseki, H. Kurosawa, T. Shirasawa
Estrogen, insulin, and dietary signals cooperatively regulate longevity signals to enhance resistance to oxidative stress in mice
J. Biol. Chem., 280 (2005), pp. 16417–16426

Balaban et al., 2005
R.S. Balaban, S. Nemoto, T. Finkel
Mitochondria, oxidants, and aging
Cell, 120 (2005), pp. 483–495

Chen et al., 2005
D. Chen, A.D. Steele, S. Lindquist, L. Guarente
Increase in activity during calorie restriction requires Sirt1
Science, 310 (2005), p. 1641

Choudhary et al., 2009
C. Choudhary, C. Kumar, F. Gnad, M.L. Nielsen, M. Rehman, T.C. Walther, J.V. Olsen, M. Mann
Lysine acetylation targets protein complexes and co-regulates major cellular functions
Science, 325 (2009), pp. 834–840

Cohen et al., 2004
H.Y. Cohen, C. Miller, K.J. Bitterman, N.R. Wall, B. Hekking, B. Kessler, K.T. Howitz, M. Gorospe, R. de Cabo, D.A. Sinclair
Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase
Science, 305 (2004), pp. 390–392

Cohen et al., 2009
D.E. Cohen, A.M. Supinski, M.S. Bonkowski, G. Donmez, L.P. Guarente
Neuronal SIRT1 regulates endocrine and behavioral responses to calorie restriction
Genes Dev., 23 (2009), pp. 2812–2817

Colman et al., 2009
R.J. Colman, R.M. Anderson, S.C. Johnson, E.K. Kastman, K.J. Kosmatka, T.M. Beasley, D.B. Allison, C. Cruzen, H.A. Simmons, J.W. Kemnitz, R. Weindruch
Caloric restriction delays disease onset and mortality in rhesus monkeys
Science, 325 (2009), pp. 201–204

Frye, 2000
R.A. Frye
Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins
Biochem. Biophys. Res. Commun., 273 (2000), pp. 793–798

Hauck et al., 2001
S.J. Hauck, W.S. Hunter, N. Danilovich, J.J. Kopchick, A. Bartke
Reduced levels of thyroid hormones, insulin, and glucose, and lower body core temperature in the growth hormone receptor/binding protein knockout mouse
Exp. Biol. Med. (Maywood), 226 (2001), pp. 552–558

Imai et al., 2000
S. Imai, C.M. Armstrong, M. Kaeberlein, L. Guarente
Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase
Nature, 403 (2000), pp. 795–800

Kaeberlein et al., 1999
M. Kaeberlein, M. McVey, L. Guarente
The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms
Genes Dev., 13 (1999), pp. 2570–2580

Kim et al., 2006
S.C. Kim, R. Sprung, Y. Chen, Y. Xu, H. Ball, J. Pei, T. Cheng, Y. Kho, H. Xiao, L. Xiao et al.
Substrate and functional diversity of lysine acetylation revealed by a proteomics survey
Mol. Cell, 23 (2006), pp. 607–618

Kim et al., 2010
H.S. Kim, K. Patel, K. Muldoon-Jacobs, K.S. Bisht, N. Aykin-Burns, J.D. Pennington, R. van der Meer, P. Nguyen, J. Savage, K.M. Owens et al.
SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress

Cancer Cell, 17 (2010), pp. 41–52

Levine et al., 1994
R.L. Levine, J.A. Williams, E.R. Stadtman, E. Shacter
Carbonyl assays for determination of oxidatively modified proteins
Methods Enzymol., 233 (1994), pp. 346–357

Li et al., 2008
Y. Li, W. Xu, M.W. McBurney, V.D. Longo
SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons
Cell Metab., 8 (2008), pp. 38–48

Lin et al., 2000
S.J. Lin, P.A. Defossez, L. Guarente
Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae
Science, 289 (2000), pp. 2126–2128

Lombard et al., 2007
D.B. Lombard, F.W. Alt, H.L. Cheng, J. Bunkenborg, R.S. Streeper, R. Mostoslavsky, J. Kim, G. Yancopoulos, D. Valenzuela, A. Murphy et al.
Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation
Mol. Cell. Biol., 27 (2007), pp. 8807–8814

Masoro et al., 1982
E.J. Masoro, B.P. Yu, H.A. Bertrand
Action of food restriction in delaying the aging process
Proc. Natl. Acad. Sci. USA, 79 (1982), pp. 4239–4241

Merry, 2004
B.J. Merry
Oxidative stress and mitochondrial function with aging—the effects of calorie restriction
Aging Cell, 3 (2004), pp. 7–12

Nakagawa et al., 2009
T. Nakagawa, D.J. Lomb, M.C. Haigis, L. Guarente
SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle
Cell, 137 (2009), pp. 560–570
Nisoli et al., 2005
E. Nisoli, C. Tonello, A. Cardile, V. Cozzi, R. Bracale, L. Tedesco, S. Falcone, A. Valerio, O. Cantoni, E. Clementi et al.
Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS
Science, 310 (2005), pp. 314–317

Onyango et al., 2002

P. Onyango, I. Celic, J.M. McCaffery, J.D. Boeke, A.P. Feinberg
SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria
Proc. Natl. Acad. Sci. USA, 99 (2002), pp. 13653–13658

Palacios et al., 2009
O.M. Palacios, J.J. Carmona, S. Michan, K.Y. Chen, Y. Manabe, J.L. Ward 3rd, L.J. Goodyear, Q. Tong
Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle
Aging (Albany NY), 1 (2009), pp. 771–783

Pérez et al., 2009
V.I. Pérez, A. Bokov, H. Van Remmen, J. Mele, Q. Ran, Y. Ikeno, A. Richardson
Is the oxidative stress theory of aging dead?
Biochim. Biophys. Acta, 1790 (2009), pp. 1005–1014

Rebrin et al., 2003
I. Rebrin, S. Kamzalov, R.S. Sohal
Effects of age and caloric restriction on glutathione redox state in mice
Free Radic. Biol. Med., 35 (2003), pp. 626–635

Rogina and Helfand, 2004
B. Rogina, S.L. Helfand
Sir2 mediates longevity in the fly through a pathway related to calorie restriction
Proc. Natl. Acad. Sci. USA, 101 (2004), pp. 15998–16003

Schisler and Singh, 1985
N.J. Schisler, S.M. Singh
Tissue-specific developmental regulation of superoxide dismutase (SOD-1 and SOD-2) activities in genetic strains of mice
Biochem. Genet., 23 (1985), pp. 291–308

S.E. Schriner, N.J. Linford, G.M. Martin, P. Treuting, C.E. Ogburn, M. Emond, P.E. Coskun, W. Ladiges, N. Wolf, H. Van Remmen et al.
Extension of murine life span by overexpression of catalase targeted to mitochondria
Science, 308 (2005), pp. 1909–1911

Schulz et al., 2007
T.J. Schulz, K. Zarse, A. Voigt, N. Urban, M. Birringer, M. Ristow
Glucose restriction extends Caenorhabditis elegans life span by inducing mitochondrial respiration and increasing oxidative stress
Cell Metab., 6 (2007), pp. 280–293

Schwer et al., 2002
B. Schwer, B.J. North, R.A. Frye, M. Ott, E. Verdin

The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase
J. Cell Biol., 158 (2002), pp. 647–657

Schwer et al., 2009
B. Schwer, M. Eckersdorff, Y. Li, J.C. Silva, D. Fermin, M.V. Kurtev, C. Giallourakis, M.J. Comb, F.W. Alt, D.B. Lombard
Calorie restriction alters mitochondrial protein acetylation
Aging Cell, 8 (2009), pp. 604–606

Shi et al., 2005
T. Shi, F. Wang, E. Stieren, Q. Tong
SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes
J. Biol. Chem., 280 (2005), pp. 13560–13567

Sinclair, 2005
D.A. Sinclair
Toward a unified theory of caloric restriction and longevity regulation
Mech. Ageing Dev., 126 (2005), pp. 987–1002

Sohal and Weindruch, 1996
R.S. Sohal, R. Weindruch
Oxidative stress, caloric restriction, and aging
Science, 273 (1996), pp. 59–63

Sundaresan et al., 2009
N.R. Sundaresan, M. Gupta, G. Kim, S.B. Rajamohan, A. Isbatan, M.P. Gupta
Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice
J. Clin. Invest., 119 (2009), pp. 2758–2771

Taguchi et al., 2007
A. Taguchi, L.M. Wartschow, M.F. White
Brain IRS2 signaling coordinates life span and nutrient homeostasis
Science, 317 (2007), pp. 369–372

Tissenbaum and Guarente, 2001
H.A. Tissenbaum, L. Guarente
Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans
Nature, 410 (2001), pp. 227–230

Van Remmen et al., 2001
H. Van Remmen, Z. Guo, A. Richardson
The anti-ageing action of dietary restriction
Novartis Found Symp., 235 (2001), pp. 221–230 discussion 230–223

Wallace and Fan, 2009
D.C. Wallace, W. Fan
The pathophysiology of mitochondrial disease as modeled in the mouse
Genes Dev., 23 (2009), pp. 1714–1736

Weindruch and Walford, 1988
R. Weindruch, R.L. Walford
The Retardation of Aging and Disease by Dietary Restriction
Charles C. Thomas, LTD, Springfield, IL (1988)

Yamamoto et al., 2005
M. Yamamoto, J.D. Clark, J.V. Pastor, P. Gurnani, A. Nandi, H. Kurosu, M. Miyoshi, Y. Ogawa, D.H. Castrillon, K.P. Rosenblatt, M. Kuro-o
Regulation of oxidative stress by the anti-aging hormone klotho
J. Biol. Chem., 280 (2005), pp. 38029–38034

Available online at:

http://www.sciencedirect.com/science/article/pii/S1550413110004079

CHAPTER III

SIRT3 IS REQUIRED FOR THE CALORIE RESTRICTION ADAPTIVE SWITCH IN SUBSTRATE UTILIZATION

Introduction

As the average waistlines of Americans have expanded, so has the need for understanding the impact of fat, both in the diet and in metabolism, on health and disease. Although the so-called "western diet" is high in dietary fats, how the body actually metabolizes these fats may be more relevant to health outcomes. Indeed, many metabolic pathological states have reduced β- oxidation capacity[1, 2]. Recent evidence indicates that β- oxidation may actually be a positive factor in healthspan, and possibly lifespan and some model organisms.[3, 4].

SIRT3, a mitochondrial NAD-dependent deacetylase, is downregulated during high fat feeding, and a deficiency of SIRT3 leads to an accelerated development of the metabolic syndrome[5]. On the other hand, SIRT3 is upregulated during calorie restriction (CR), a dietary intervention with reduced energy intake without malnourishment. Upregulation of SIRT3 during CR reduces oxidative stress and oxidative damage, linking SIRT3 to positive indicators of physiological health and the adaptive benefits during CR [6, 7]. However, SIRT3 also targets enzymes involved in mitochondrial energy substrate metabolism[8-10]. Therefore, we investigated the impact SIRT3 might have on whole body metabolism and substrate utlization during CR.

Methods

Mice

SIRT3 KO mice have been described [6]. All mice were housed on a 12:12-hr light:dark cycle at 25°C. Six-month-old animals were subjected to a 30% calorie restriction (CR) diet, which was provided daily for 6 months. Respiration exchange ratio (RER), movement and oxygen consumption were measured in metabolic cages (Columbus Instruments) for 24 hours, according to the manufacturer's instruction. Mice were weighed before and after being placed in the cages, and were fed their allotted meals at the time they were placed in the cages. Measurements were taken every 10 minutes. All animal procedures were in accordance with the animal care committee at the University of California, Berkeley.

Plasma chemical analysis:

Blood was collected from the tail veins of 8 pairs of littermates and kept on ice until centrifugation (5000rpm, 10min at 4°C). Glucose concentrations were determined using a glucometer (Acensia Contour, Bayer). Insulin (Alpco Diagnostics), free fatty acids and triglyceride (Wako) were measured using ELISA and colorimetric kits.

For the glucose tolerance test, mice were fasted overnight and injected intraperitoneally with a saline glucose solution at 1g/kg body weight. Plasma glucose levels were measured before and 20, 40, 60 and 120 minutes after glucose injection. For the triglyceride tolerance test, 400 uL of vegetable oil were administered by oral gavage, and blood was collected at baseline(0 hours), 1, 2.5 and 5 hours after injection.

Enzyme Assays:
Pentose phosphate pathway activity was determined as previously described [11]The reaction was measured on a Spectramax 190 (Molecular Devices) for up to 30 minutes, with readings taken every 15 seconds.

ACAD activity was measured as previously described [12, 13]. The reaction mixture contained 50 mM potassium phosphate, pH 7.4, 35 µM DCPIP, 1 mM N-ethylmaleimide (NEM), 1.6 mM PMS and 500µg liver lysate with or without 50 µM palmitoyl-CoA. OD600 was measured with Spectramax 190 (Molecular Devices) for up to 30 minutes, with readings taken every 1-2 minutes. ΔOD600 values derived from reactions in the absence of 50µM of palmitoyl-coA (Sigma) was considered as background and subtracted.

Fatty Acid Oxidation Assay:
Liver sections (0.4g in total) (Huang W. Endocrinology *147*, 1480 (2006)) or isolated primary white adipocytes (100 µl) [14] were incubated with 1ml of Krebs-Ringer buffer supplemented with 3 mM glucose, 1% BSA and [^{14}C]palmitic acid (0.2 µCi/ml) for 30min or 1 h respectively at 37°C with gentle shaking. The buffer was then acidified with 200 µl of H_2SO_4 (0.5N) and maintained sealed at 37°C for an additional 30 min. $^{14}CO2$ was trapped by 200µl of 2-phenylethylamine/methanol (1:1 ratio) and radioactivity was quantified by liquid scintillation.

Statistic analysis:
Student's t-test was used for statistic analysis and the null hypothesis was rejected when $P<0.05$.

Results

CR mice that are meal fed undergo a very specific metabolic pattern that can be determined by metabolic cages. It begins with a high respiratory exchange ratio(RER ~ 1) directly after feeding, reflecting carbohydrate use, that eventually declines as β-oxidation increases[15]. We found that the WT mice on CR display this distinct RER pattern; however, the KO mice exhibited a right-shifted RER curve until 10 hours post feeding (Fig 1A).Furthermore, KO mice had a small but significant bias towards using carbohydrate sourced kCals (Fig 1 B.) Additional investigation of the metabolic parameters of the CR mice indicated that the KO mice had higher triglyceride levels in both plasma and liver, as well as higher free fatty acids in the serum (Fig 1 C-E). A triglyceride tolerance test showed a trend of decreased triglyceride clearance from the blood over a 5 hour period (Fig 1 F). Furthermore, ex vivo labeled palmitate oxidation assays showed a decrease in oxidation rates (38% decrease in the liver; 39 % decrease WAT (Fig 1 G and H). Collectively, these data support the RER data and demonstrate that the KO mice display a substrate utilization preference away from fatty acids.

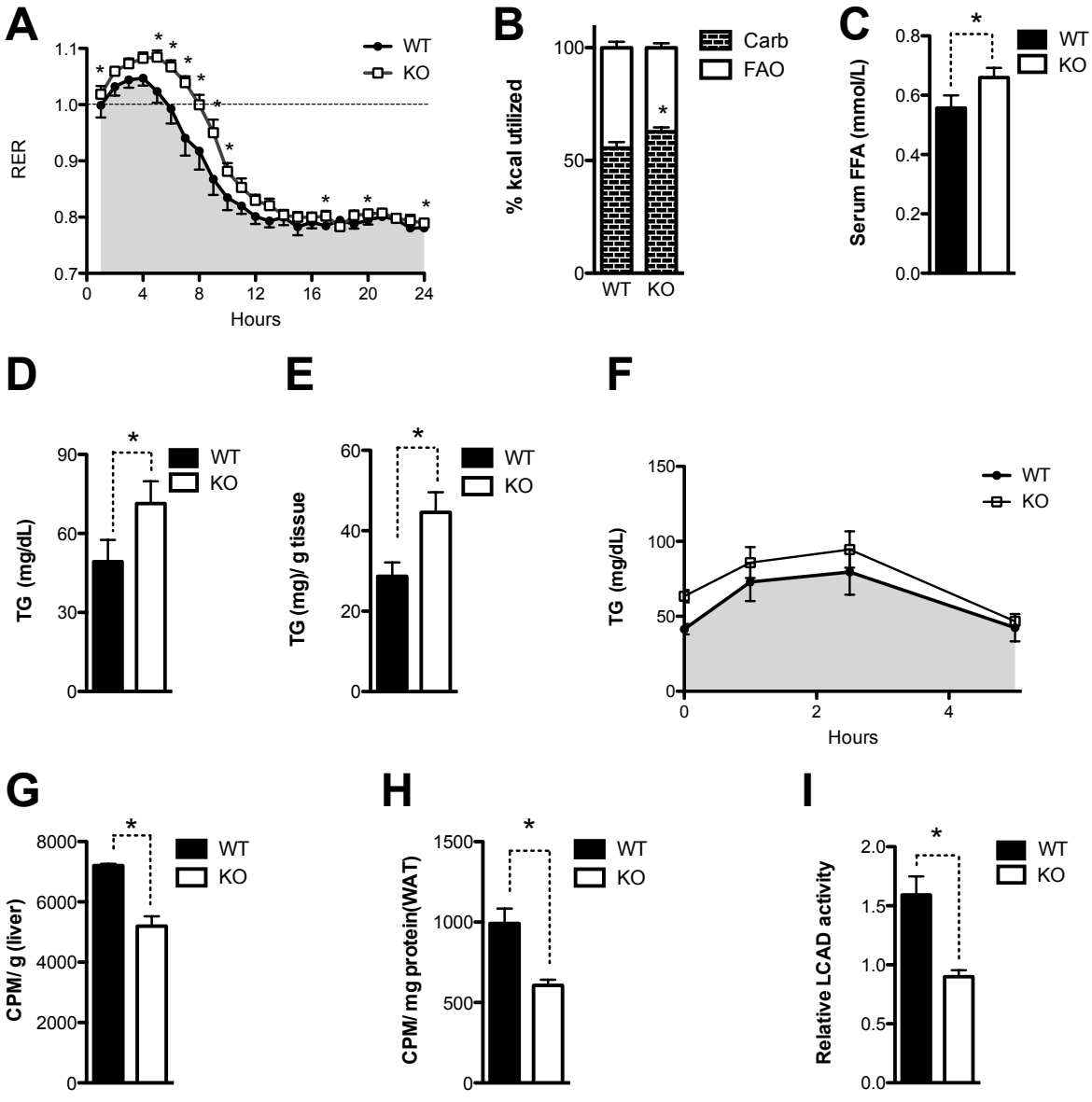

Figure 1. SIRT3 deficiency results in altered substrate utilization during long term CR. A) 24-hour RER data from mice that had been maintained on CR for 6 months. N=8 mice B) Breakdown of substrate utilization as a percentage of kCal oxidized C) serum free fatty acids in fasted CR mice. D) Plasma triglyceride levels E) Liver triglyceride content in livers of CR mice. N=4 mice F) Triglyceride tolerance test in fasted CR mice. G) and H) β- oxidation rates as determined by labeled palmitate oxidation and CO_2 capture in freshly excised liver and white adipose tissue, respectively. N= 3 mice I) Relative LCAD activity in the liver of calorie restricted (CR) mice, as determined by spectrophotometric assay * indicates $p \leq 0.05$

LCAD catalyzes the rate-limiting step of β- oxidation, and is also a characterized target of SIRT3[16]. SIRT3 increases the activity of LCAD during fasting when β- oxidation is upregulated. In keeping with these published studies, we found LCAD activity was upregulated in CR WT mouse livers compared to normal ad libitum mice (data not shown), but CR KO mouse livers had significantly lower LCAD activity compared to CR WT livers (Fig. 1 I).

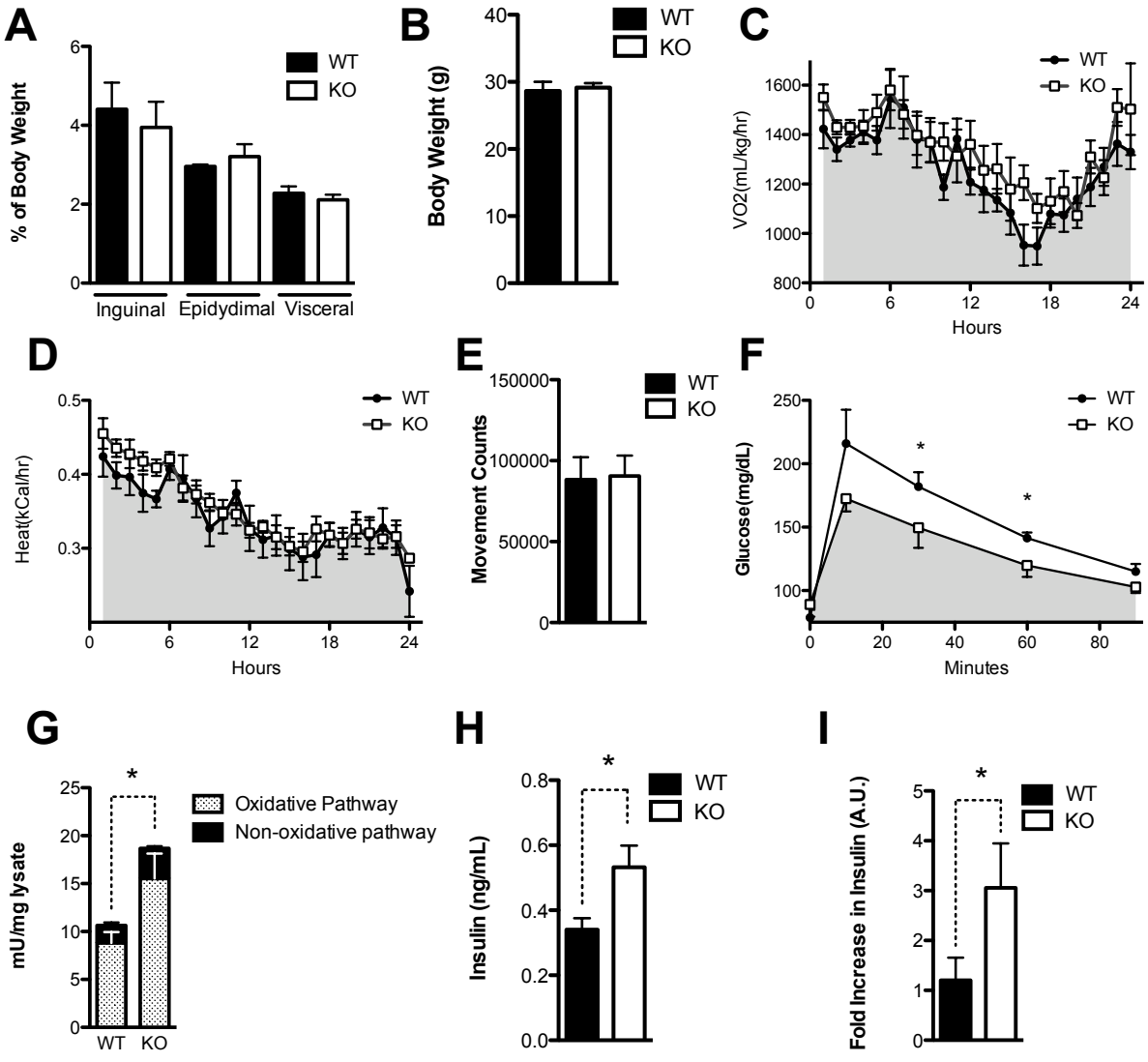

Figure 2. SIRT3 KO mice on CR have same metabolic rate, but a preference for carbohydrate utilization. A) Relative weights of various WAT depots. B) Total mouse body weights after 6 months of CR. N=8 mice C) Hourly average VO_2 and D) heat measurements collected by metabolic cage. E) Total movement counts over a 24-hour period. F) Glucose tolerance test. G) Measurement of Pentose Phosphate Pathway activity from mouse livers taken at 4 hours post feeding. N=4 H) Plasma insulin levels in fasted CR mice. N=8 I) Relative increase in insulin after GTT * indicates $p \leq 0.05$

Despite a reflection of differential substrate utilization by the RER curves (Fig 1A), we found no difference in fat pad weights, nor overall body weight in the CR mice (Fig 2 A-B). Consistent with these observations, the mice showed no difference in measurements that reflect total energy use and metabolism (Oxygen consumption (VO_2), heat produced, and movement (Figure 2 C,D, and E)).

The lack of difference in metabolic rate and body weights indicated that the KO mice were utilizing the same total energy. Glucose tolerance testing (GTT) indicated that the CR KO mice had faster clearance of glucose from the blood stream (Fig 2 H). This faster clearance and uptake may in part be due to upregulated activity in the pentose phosphate pathway (PPP). Indeed, we found that the CR KO livers had 40% increased activity in both the oxidative and non-oxidative arms of the PPP (Fig. 2 J). These results indicate an upregulation of carbohydrate metabolism to compensate for decreased fatty acid utilization for energy demands. Interestingly, despite the ability to clear glucose from the bloodstream, the KO mice had higher circulating insulin levels, and a larger relative increase in insulin after GTT (Fig 2 D and I), both data suggesting insulin resistance in the CR KO mice.

Discussion

Although the CR WT and KO mice appeared physically indistinguishable from each other, our metabolic investigations indicate that a SIRT3 deficiency does alter substrate utilization in favor of carbohydrate oxidation. CR is thought to streamline metabolism, enhancing mitochondrial metabolism, therefore it was unclear whether the impact of SIRT3 deficiency could be overcome by other adaptive molecular changes that occur during this dietary regimen. The relative reliance on carbohydrate metabolism is consistent with expectations, as SIRT3 has been shown to repress pathways such as PPP [17]. There has been considerable debate on whether the increase in β- oxidation during fasting, and especially during CR is responsible for many of the health benefits of this regimen. For example, it is clear that a build up of lipids in tissues leads to "lipotoxicity," and conditions with increased β- oxidation prevent this lipid build up[4]. Increasingly, it is understood that intermediate metabolites can act as signaling molecules, and it is probable that β- oxidation creates a particular signaling environment which may enhance subcellular health, with a cascading positive impact to the tissue and whole-organism level[18]. Our results hint at possible contributions by substrate utilization to the health benefits of CR. Future studies could elucidate the role that fatty acid utilization plays in healthspan or lifespan extension in calorie restriction.

References

1. Mensink M, Blaak EE, van Baak MA, Wagenmakers AJ, Saris WH. Plasma free fatty acid uptake and oxidation are already diminished in subjects at high risk for developing type 2 diabetes. Diabetes 2001 Nov;50(11):2548-54.

2. He J, Watkins S, Kelley DE. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. Diabetes 2001 Apr;50(4):817-23.

3. Orellana-Gavalda JM, Herrero L, Malandrino MI, Paneda A, Sol Rodriguez-Pena M, Petry H, Asins G, Van Deventer S, Hegardt FG, Serra D. Molecular therapy for obesity and diabetes based on a long-term increase in hepatic fatty-acid oxidation. Hepatology 2011 Mar;53(3):821-32.

4. Berdichevsky A, Nedelcu S, Boulias K, Bishop NA, Guarente L, Horvitz HR. 3-ketoacyl thiolase delays aging of caenorhabditis elegans and is required for lifespan extension mediated by sir-2.1. Proc Natl Acad Sci U S A 2010 Nov 2;107(44):18927-32.

5. Hirschey MD, Shimazu T, Jing E, Grueter CA, Collins AM, Aouizerat B, Stancakova A, Goetzman E, Lam MM, Schwer B, et al. SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. Mol Cell 2011 Oct 21;44(2):177-90.

6. Qiu X, Brown K, Hirschey MD, Verdin E, Chen D. Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. Cell Metab 2010 Dec 1;12(6):662-7.

7. Someya S, Yu W, Hallows WC, Xu J, Vann JM, Leeuwenburgh C, Tanokura M, Denu JM, Prolla TA. Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell 2010 Nov 24;143(5):802-12.

8. Schwer B, Bunkenborg J, Verdin RO, Andersen JS, Verdin E. Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2. Proc Natl Acad Sci U S A 2006 Jul 5;103(27):10224-9.

9. Finley LW, Haas W, Desquiret-Dumas V, Wallace DC, Procaccio V, Gygi SP, Haigis MC. Succinate dehydrogenase is a direct target of sirtuin 3 deacetylase activity. PLoS One 2011;6(8):e23295.

10. Ahn BH, Kim HS, Song S, Lee IH, Liu J, Vassilopoulos A, Deng CX, Finkel T. A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. Proc Natl Acad Sci U S A 2008 Sep 23;105(38):14447-52.

11. Cabezas H, Raposo RR, Melendez-Hevia E. Activity and metabolic roles of the pentose phosphate cycle in several rat tissues. Mol Cell Biochem 1999 Nov;201(1-2):57-63.

12. Izai K, Uchida Y, Orii T, Yamamoto S, Hashimoto T. Novel fatty acid beta-oxidation enzymes in rat liver mitochondria. I. purification and properties of very-long-chain acyl-coenzyme A dehydrogenase. J Biol Chem 1992 Jan 15;267(2):1027-33.

13. Dommes V, Kunau WH. A convenient assay for acyl-CoA-dehydrogenases. Anal Biochem 1976 Apr;71(2):571-8.

14. Ahmadian M, Duncan RE, Varady KA, Frasson D, Hellerstein MK, Birkenfeld AL, Samuel VT, Shulman GI, Wang Y, Kang C, et al. Adipose overexpression of desnutrin promotes fatty acid use and attenuates diet-induced obesity. Diabetes 2009 Apr;58(4):855-66.

15. Bruss MD, Khambatta CF, Ruby MA, Aggarwal I, Hellerstein MK. Calorie restriction increases fatty acid synthesis and whole body fat oxidation rates. Am J Physiol Endocrinol Metab 2010 Jan;298(1):E108-16.

16. Hirschey MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, Grueter CA, Harris C, Biddinger S, Ilkayeva OR, et al. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature 2010 Mar 4;464(7285):121-5.

17. Finley LW, Carracedo A, Lee J, Souza A, Egia A, Zhang J, Teruya-Feldstein J, Moreira PI, Cardoso SM, Clish CB, et al. SIRT3 opposes reprogramming of cancer cell metabolism through HIF1alpha destabilization. Cancer Cell 2011 Mar 8;19(3):416-28.

18. Samuel VT, Shulman GI. Mechanisms for insulin resistance: Common threads and missing links. Cell 2012 Mar 2;148(5):852-71.

CHAPTER IV

SIRT3 REGULATES STRESS-RESPONSIVE MITOCHONDRIAL HOMEOSTASIS IN HEMATOPOIETIC STEM CELLS AND REVERSES AGING-ASSOCIATED DEGENERATION

SUMMARY

Sirtuins are evolutionarily conserved deacetylases that have been shown to extend lifespan in yeast, nematodes, fruitflies, and very recently, mice, although the role of sirtuins in regulating lifespan in nematodes and fruitflies is controversial. Whether sirtuins can reverse, as opposed to simply slow, aging-associated degeneration is unknown. Tissue-specific stem cells persist throughout the entire lifespan of an organism to repair and maintain tissues, but during the aging process, their self-renewal capacity and differentiation potential become dysregulated. We show that SIRT3, a mammalian mitochondrial sirtuin, is highly enriched in hematopoietic stem cells (HSCs) where it regulates a stress response. Deletion of the SIRT3 gene in mice has no effect on the HSC pool at a young age under homeostatic conditions, but causes compromised self-renewal of HSCs under stress or at an old age. SIRT3 expression and activity in HSCs decrease with age. Importantly, forced expression of SIRT3 in aged HSCs improves their regenerative capacity. Our study illuminates the plasticity of mitochondrial homeostasis controlling stem cell maintenance and tissue homeostasis during the aging process, and shows that aging-associated degeneration can be reversed by a sirtuin family member.

HIGHLIGHTS

- SIRT3 is highly enriched in HSCs and is suppressed in differentiated hematopoietic cells
- SIRT3 regulates HSC self-renewal under stress or at an old age
- SIRT3 regulates mitochondrial metabolic homeostasis and reduces ROS in HSCs.
- SIRT3 is suppressed with age and its upregulation rejuvenates aged HSCs

INTRODUCTION

Aging is a multifaceted degenerative process. Remarkably, lifespan can be extended by single gene mutations (Guarente and Kenyon, 2000). A key regulator of organismal longevity is SIR2 (silencing information regulator 2). An extra copy of SIR2 extends lifespan in yeast, worms, and flies (Guarente, 2007). However, its role in worms and flies has recently become controversial (Burnett et al., 2011). In mammals, there are seven SIR2 homologs (sirtuins), SIRT1-7, localized in various cellular compartments (Haigis and Sinclair, 2010). Recently, mice overexpressing SIRT6 have been shown to have increased lifespan (Kanfi et al., 2012), providing additional evidence that the role of SIR2 in lifespan extension is conserved throughout evolution. However, it is unclear whether sirtuins can reverse, as opposed to simply slow, aging-associated degeneration.

A hallmark of aging is compromised tissue maintenance (Rando, 2006). Tissue-specific stem cells self-renew and persist throughout an organism's lifespan to repair and maintain tissues. The self-renewal potential and differentiation capacity of stem cells

become dysregulated with age (Morrison et al., 1996; Rossi et al., 2008; Sahin and Depinho, 2010). Stem cell aging is thought to be due to cumulative cellular and genomic damages, resulting in permanent cell cycle arrest, apoptosis, or senescence (Janzen et al., 2006; Rossi et al., 2008; Sahin and Depinho, 2010). A major source of cellular damage is reactive oxygen species (ROS), a natural byproduct of cellular respiration (Balaban et al., 2005). ROS levels in stem cells increase dramatically with age (Ito et al., 2006). Deficient intracellular management of ROS results in increased stem cell cycling and apoptosis, as well as compromised self-renewal and differentiation, resembling essential aspects of aged stem cells (Ito et al., 2004; Ito et al., 2006; Miyamoto et al., 2007; Paik et al., 2009; Renault et al., 2009; Tothova et al., 2007). Despite compelling evidence supporting the essential role of ROS in regulating stem cell aging, outstanding questions still remain unanswered. How do ROS levels increase with age in stem cells? Is stem cell aging a chronic result of cumulative oxidative damage or an acute effect of increased ROS levels? Is ROS-induced physiological stem cell aging and tissue degeneration reversible?

Approximately 90% of cellular ROS are produced in the mitochondria (Balaban et al., 2005). ROS levels are thought to increase with age due to the accumulation of damaged mitochondria in a self-perpetuating cycle. ROS-induced impairment of mitochondria results in increased ROS production, which in turn leads to further mitochondrial damage. However, nutrient intake and numerous genetic mutations alter the rate of aging with concomitant alteration of mitochondrial metabolism and ROS accumulation, suggesting that mitochondrial homeostasis is amenable to regulation during the aging process (Balaban et al., 2005).

Metabolic pathways are coordinated through reversible acetylation of metabolic enzymes in response to nutrient availability (Wang et al., 2010; Zhao et al., 2010). The sirtuin family has emerged as key regulators of the nutrient-sensitive metabolic regulatory circuit (Shin et al., 2011). Prominently, SIRT3 regulates the global acetylation landscape of mitochondrial proteins, and SIRT3-initiated metabolic adaptations enhance mitochondrial management of ROS (Bell and Guarente, 2011; Lombard et al., 2007). SIRT3 increases the activity of antioxidants, such as superoxide dismutase 2 (SOD2) and reduced glutathione, and promotes ROS scavenging (Chen et al., 2011; Qiu et al., 2010; Someya et al., 2010; Tao et al., 2010). Additionally, SIRT3 initiates metabolic reprogramming toward more efficient electron transport and fuel usage away from carbohydrate catabolism, which are thought to result in reduced ROS production (Ahn et al., 2008; Bell et al., 2011; Bell and Guarente, 2011; Finley et al., 2011; Hirschey et al., 2010; Shimazu et al., 2010). Thus, SIRT3 provides a unique tool to understand mitochondrial metabolism and management of ROS.

In this study, we use hematopoietic stem cells (HSCs) to identify SIRT3 as an essential regulator of physiological stem cell aging. SIRT3 is downregulated with age, contributing to increased ROS levels in aged HSCs. Evidence is provided to show that increased ROS levels in aged HSCs have acute effects on HSC function and that ROS-mediated physiological HSC aging is reversible. These data shed new light on the plasticity of mitochondrial homeostasis in stem cell maintenance and tissue homeostasis during the

aging process and have profound implications for understanding aging and rejuvenation.

RESULTS

SIRT3 deficiency has no effect on the hematopoietic stem cell pool at a young age under homeostatic conditions

We compared the expression of SIRT3 in various immunophenotypically-defined subpopulations of mouse bone marrow (BM) cells (In this paper, cell populations are defined as HSC: $Lin^-c\text{-}Kit^+Sca1^+CD150^+CD48^-$, multipotent progenitors (MPP): $Lin^-c\text{-}Kit^+Sca1^+CD150^-CD48^-$, hematopoietic stem/progenitor cells (HSPC or LKS): $Lin^-c\text{-}Kit^+Sca1^+$, myeloid progenitors (MP): $Lin^-c\text{-}Kit^+Sca1^-$, common lymphoid progenitors (CLP): $Lin^-IL7R\alpha^+c\text{-}kit^{med}/Sca1^{med}$, differentiated blood cells: Lin^+) (Kiel et al., 2005). Strikingly, SIRT3 mRNA levels were about 3000-fold higher in HSCs and MPPs than in differentiated blood cells (Figure 1A-C). In contrast to the high expression levels of SIRT3, the expressions of the other mitochondrial sirtuins, SIRT4 and SIRT5, were too low to be detected in HSCs (data not shown). Thus, SIRT3 is highly enriched in HSPCs and its expression decreases dramatically in differentiated hematopoietic cells.

To assess the functional role of SIRT3 in HSCs, we compared the quantity and quality of HSCs in wild type (WT) and SIRT3 knockout (KO) mice. SIRT3 KO mice fed *ad libitum* do not have overt phenotypes at a young age (Lombard et al., 2007). In young animals (3-month old), no difference in the number of enriched HSPCs or highly enriched HSCs was observed between WT and SIRT3 KO mice (Figure 1D, E, F). BM cellularity was also comparable (Figure 1G). To assess whether SIRT3 affects HSC regeneration capacity *in vivo*, we performed a competitive transplantation assay. Donor BM cells were transplanted with an equal number of $CD45.1^+$ competitor BM cells to reconstitute the hematopoietic compartment of lethally irradiated recipient mice (Figure 1H). BM cells from young WT and SIRT3 KO mice were equally adept at hematopoietic reconstitution (Figure 1I). When differentiated, HSCs give rise to all blood cell types including myeloid and lymphoid lineages. To determine whether SIRT3 regulates lineage differentiation, we assayed donor-derived mature hematopoietic subpopulations in the transplanted recipients. No significant difference was observed in the percentages of B cells ($B220^+$), granulocytes ($Gr1^+$), and macrophages ($Mac\text{-}1^+$) in the blood (Figure 1J). Additionally, we performed *in vitro* colony-forming assays in which isolated BM mononuclear cells (MNCs) were cultured in methylcellulose medium supplemented with growth factors. The numbers of colonies derived from young WT and SIRT3 KO BM cells were comparable (Figure 1K). Thus, SIRT3 is not required to maintain the HSC pool size and regenerative capacity at a young age.

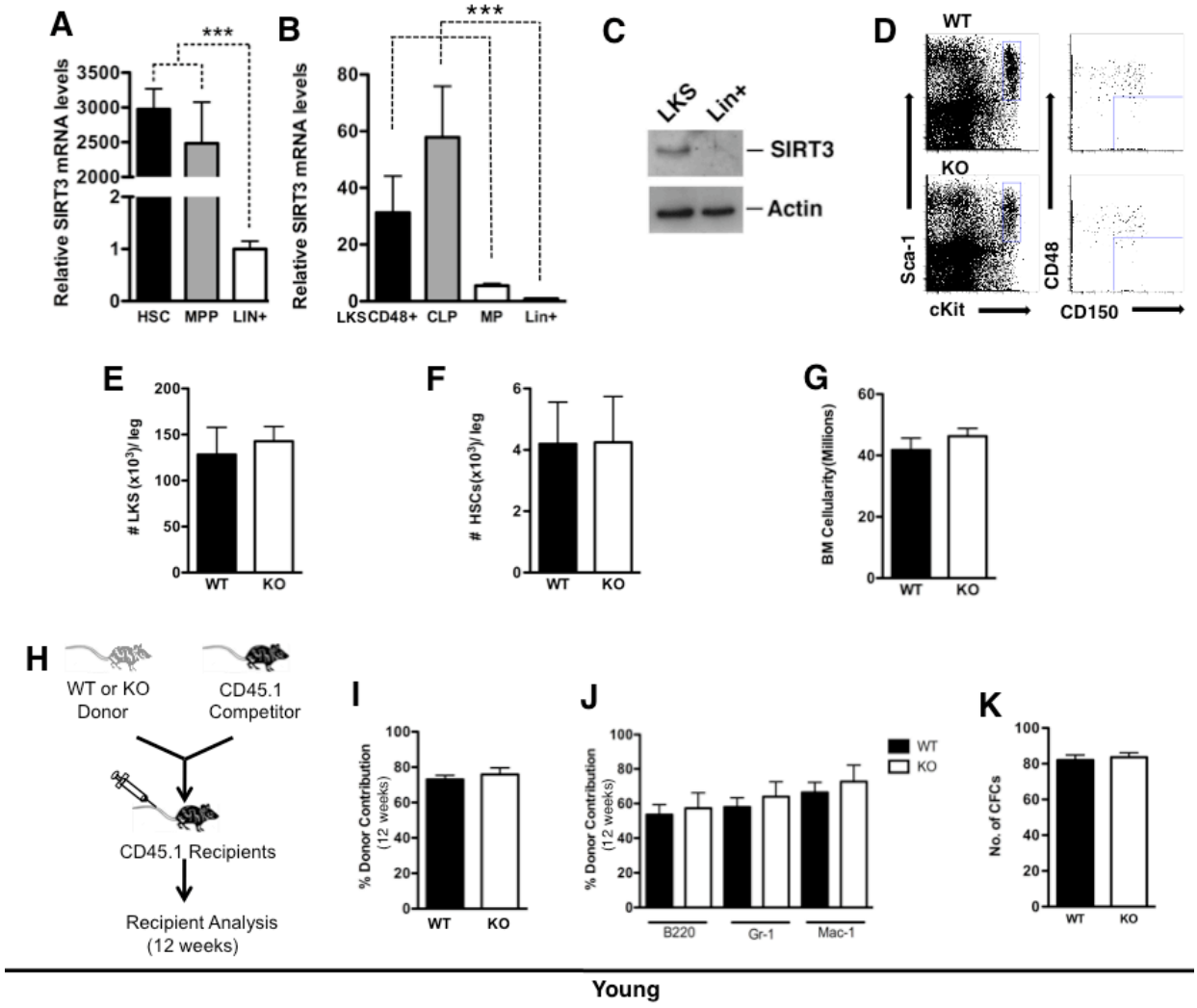

Figure 1. SIRT3 is highly enriched in HSCs and SIRT3 deficiency does not affect the HSC pool at a young age. A, B, C. BM subpopulations were isolated based on cell surface markers. SIRT3 expression levels were quantified by real time PCR (A, B) or western blot (C). n=5. **D, E, F.** The frequency of HSPCs and HSCs in the BMs of young mice was determined via flow cytometry (n=3). Flow cytometry plots are gated on Lin^- BM cells. Data presented are the numbers of specified cell populations per leg. **G.** The numbers of total BM cellularity per leg of young WT and SIRT3 KO mice (n=3). **H, I, J**. Bone marrow transplantation. Schematic representation of competitive transplantation assays using BM cells from WT and SIRT3 KO mice as donors (H). Data shown are the percentage of total donor-derived cells (I) and donor-derived individual lineages (B cells ($B220^+$), granulocytes ($Gr\text{-}1^+$), macrophages ($Mac\text{-}1^+$)) (J) in the peripheral blood of the recipients 12 weeks posttransplant, using young mice as donors. Donors: n=3/group. Recipients: n=15/group. **K.** Colony forming assay. $2x10^4$ BM MNCs from young WT and SIRT3 KO mice (non-transplanted) were cultured with cytokines in methylcellulose medium. Data shown are the number of colonies formed (n=6). Error bars represent standard errors. ***: $p<0.001$.

SIRT3 deficiency results in reduced HSC pool at an old age

Given that SIRT3 functions to trigger mitochondrial reprogramming toward reduced oxidative stress (Bell and Guarente, 2011), we investigated whether SIRT3 regulates HSCs under conditions of elevated oxidative stress, such as aging (Ito et al., 2006). The size of both HSPC and HSC compartments were 50% smaller in aged (18-24-month

old) SIRT3 KO mice compared to their WT littermates (Figure 2A, B, C). BM cellularity of aged SIRT3 KO mice was 15% lower (Figure 2D). In a competitive BM transplantation assay, the reconstitution ability of donor cells from aged SIRT3 KO mice decreased 30% in comparison to age-matched WT controls, with B cells, T cells, granulocytes, and macrophages all significantly reduced (Figure 2E, F). The reduced reconstitution capacity of aged SIRT3-deficient cells is not due to compromised homing, as WT and SIRT3 KO HSPCs have comparable homing efficiency (Figure S1). In a colony-forming assay, aged SIRT3 KO BM cells gave rise to 20% fewer colonies than WT controls (Figure 2G). Thus, SIRT3 is required to maintain HSC pool size and regenerative capacity at an old age.

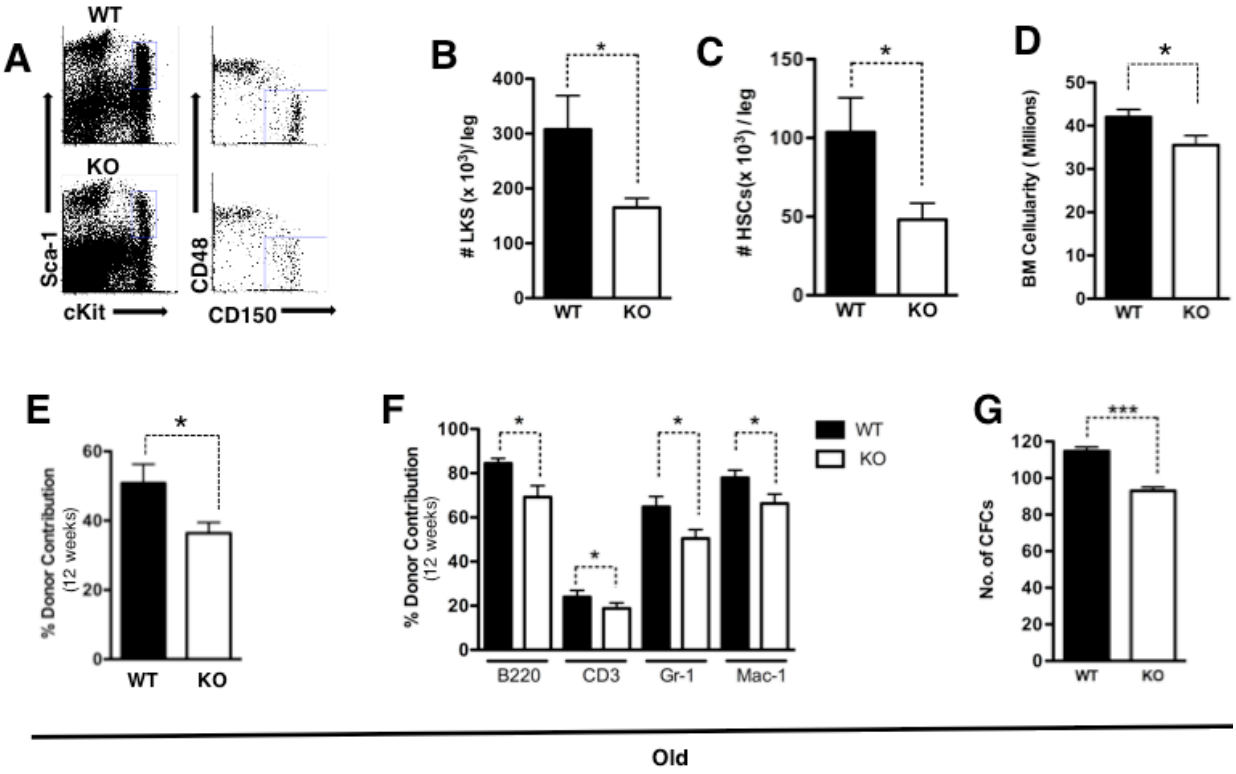

Figure 2. SIRT3 deficiency results in compromised HSC self-renewal with age. A, B, C. The frequency of HSPCs and HSCs in the BMs of aged mice was determined via flow cytometry (n=4). Data presented are the numbers of specified cell populations per leg. **D.** The numbers of total BM cellularity per leg of aged WT and SIRT3 KO mice (n=4). **E, F.** 12 weeks after competitive transplantation using aged mice as donors, the percentage of total donor-derived cells (E) and donor-derived individual lineages (F) in the peripheral blood of the recipients are shown. Donors: n=3/group. Recipients: n=15/group. **G.** The number of colonies formed in a colony-forming assay using $2x10^4$ BM MNCs from aged WT and SIRT3 KO mice (non-transplanted) (n=6). Error bars represent standard errors. *: p<0.05. ***:p<0.001.

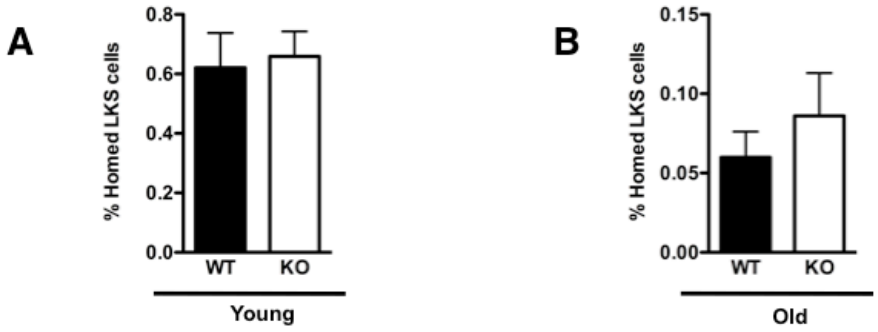

Figure S1. WT and SIRT3 KO HSPCs cells have comparable homing efficiency. Young (A) and old (B) WT and SIRT3 KO BM cells (CD45.2) were transplanted into lethally irradiated CD45.1 recipient mice. Data shown are percentage of donor-derived LKS cells homed to the BM of the recipients. Donor: n=3. Recipient: n=6. Error bars represent standard errors.

SIRT3 deficiency causes compromised HSC self-renewal upon serial transplantation stress

A hallmark of stem cells is their ability to self-renew, allowing them to maintain and repair tissues throughout life. HSCs are able to reconstitute lethally irradiated hosts in secondary and tertiary transplants. ROS levels in HSCs increase modestly after the primary and the secondary transplants but increase dramatically after the tertiary transplant (Ito et al., 2006). We investigated whether SIRT3 is required to sustain HSC function upon serial transplantation (Figure 3A). No difference was observed in HSC self-renewal and hematopoietic reconstitution derived from BM cells of young WT or SIRT3 KO mice in the secondary transplant recipients (Figure 3B, C). However, in the tertiary transplant, SIRT3 KO BM cells resulted in a 50% reduction in HSC self-renewal and reconstitution (Figure 3B, C, D). Together with the results from aged mice, these data suggest that SIRT3 is required to preserve HSCs under oxidative stress conditions, such as aging and serial transplant.

SIRT3 reduces oxidative stress in HSCs

To assess whether SIRT3 regulates HSPC function by reducing oxidative stress, we evaluated ROS levels in HSPCs of WT and SIRT3 KO mice. While HSPCs from young WT and SIRT3 KO mice had comparable ROS levels (Figure S2A), increased ROS levels were detected in HSPCs of aged SIRT3 KO mice compared to WT controls under homeostatic conditions (Figure S2B) and under transplant stress (Figure 4A). There was a 60% increase in ROS levels in HSPCs derived from SIRT3 KO mice compared to the WT controls in the recipients of BM transplants. These data indicate that SIRT3 reduces oxidative stress in HSPCs under stress.

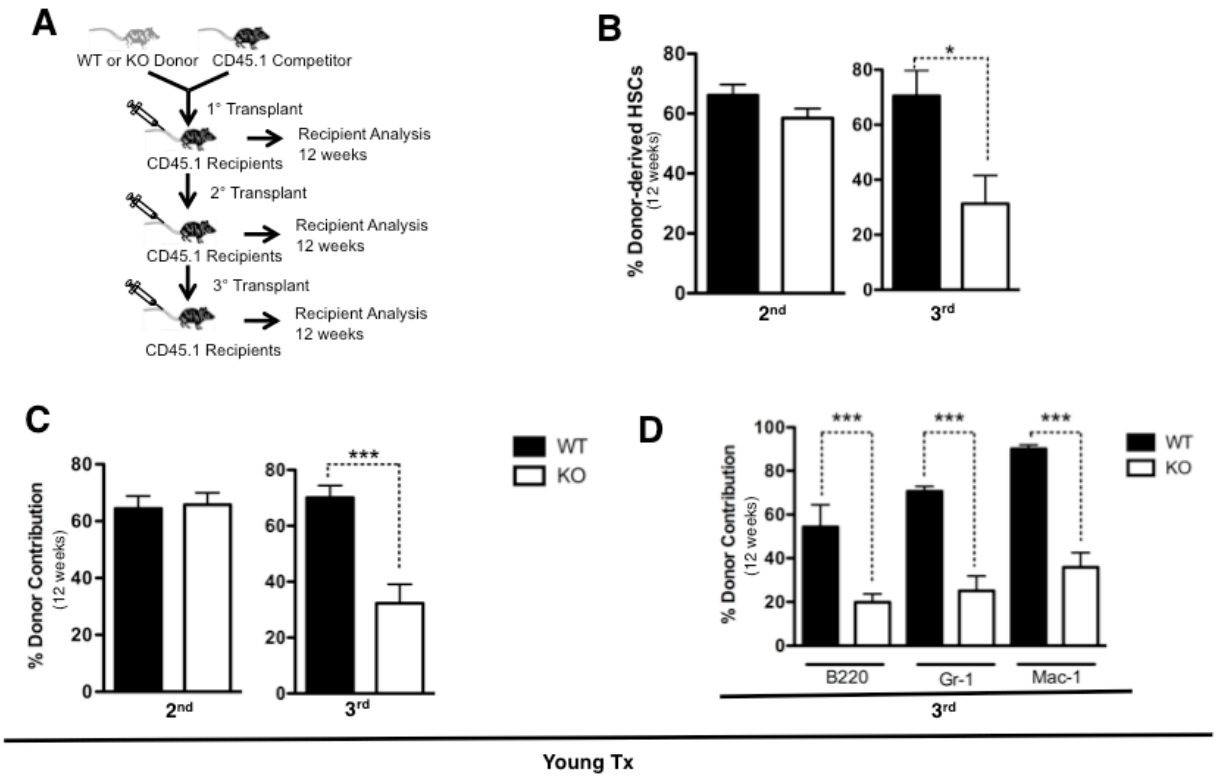

Figure 3. SIRT3 regulates HSC self-renewal under transplantation stress. Schematic representation of competitive serial transplantation assays. BM cells from the competitive transplant recipients were used as donors for the next round of transplantation (A). Data shown are the percentage of donor-derived HSCs (LKSCD150+) in the BM (B), total donor-derived cells (C) and donor-derived individual lineages (D) in the peripheral blood using BM cells from young WT and SIRT3 KO mice as donors. Donors: n=3/group. Recipients: n=15/group. Error bars represent standard errors. *: $p<0.05$. ***:$p<0.001$.

We next investigated whether SIRT3 promotes oxidative stress resistance in HSCs. BM cells from WT and SIRT3 KO mice were cultured with or without paraquat, a superoxide-generating compound. The cell survival rates for the SIRT3 KO HSC and LKS populations were 37% lower than WT controls (Figure 4B), suggesting that SIRT3 promotes HSC survival in response to oxidative stress. Next, we determined whether the defects in SIRT3-deficient HSCs are due to increases in cell death. No significant difference was detected between various BM cell populations of young WT and SIRT3 KO mice (Figure S2C). However, in aged SIRT3 KO mice, the percentage of dead cells in the HSC and HSPC populations doubled relative to the WT controls, but no difference in the Lin- and Lin+ fractions was observed (Figure 4C, S2D), consistent with the SIRT3 expression pattern in these populations (Figure 1A).

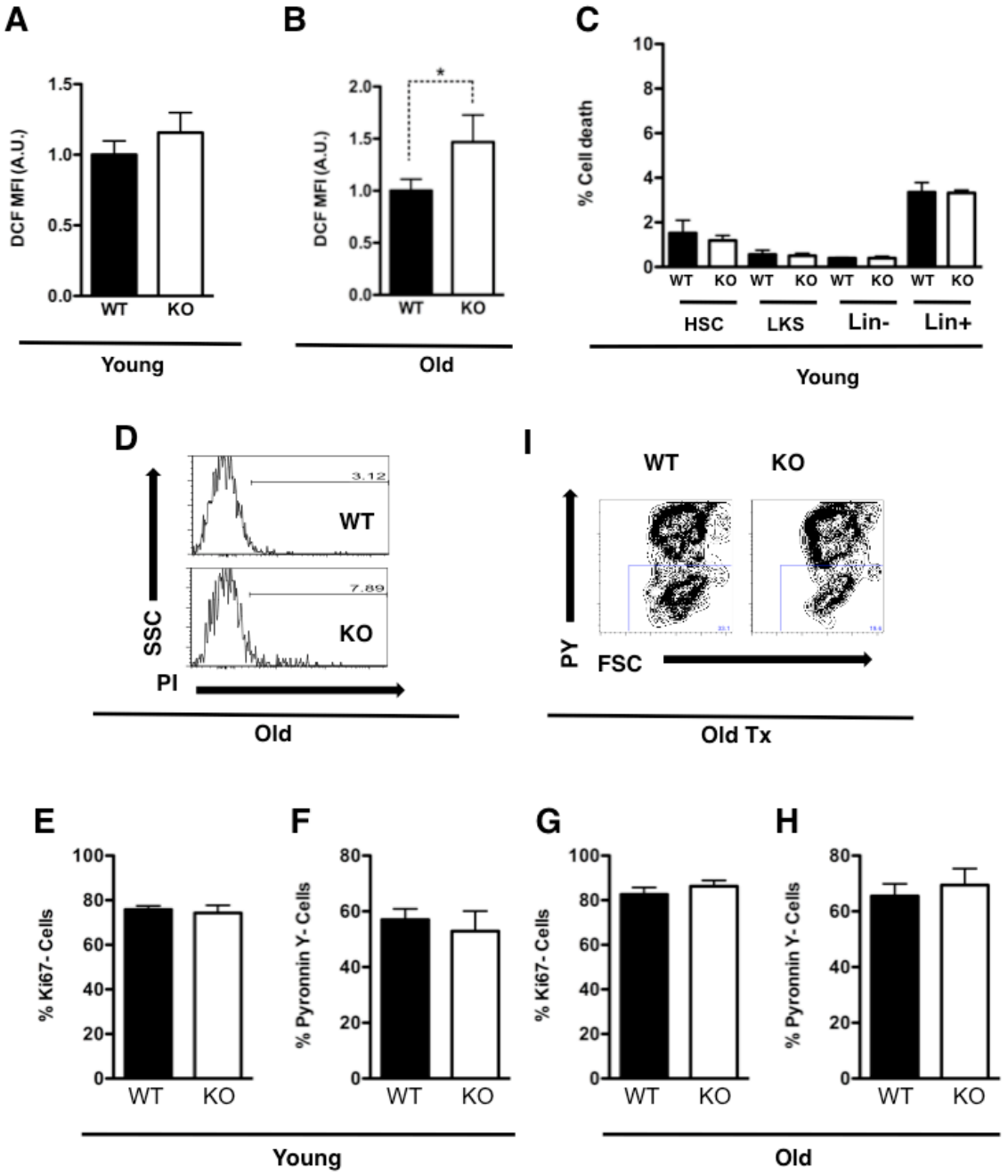

Figure S2. SIRT3 regulates oxidative stress in HSCs. A, B. SIRT3 deficiency results in increased cellular ROS in HSPCs under stress. Intracellular ROS levels were determined by H_2DCFDA staining in LKS cells of young (A) or old (B) WT and SIRT3 KO mice. **C, D.** SIRT3 deficient HSCs are prone to cell death. Dead cells were quantified in HSCs of young (C) and old (D) WT and SIRT3 KO mice by propidium iodide staining. Data presented are the representative flow cytometry plots (D). n=4. **E-H.** The effect of SIRT3 on HSC cycling. Proliferation of HSCs (LKSCD150+) from young (E, F) and old (G, H) mice was determined by Ki-67 staining or Pyronin Y staining. n=4. **I.** SIRT3 deficiency results in increased cell cycling under stress. Cycling of LKS cells derived from old mice was assessed in transplant recipients using PY staining. Data presented are representative flow cytometry plots. n=4. Error bars represent standard errors. *:$p<0.05$.

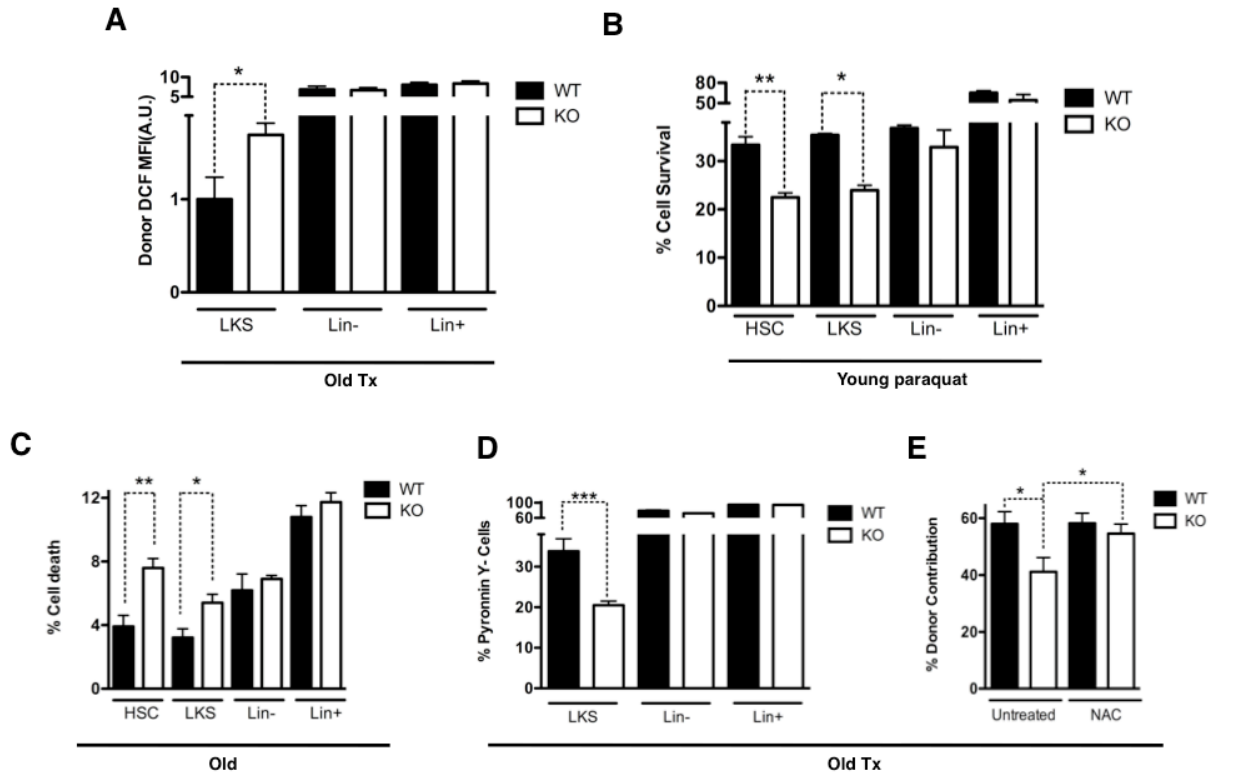

Figure 4. SIRT3-mediated mitochondrial management of ROS regulates HSC function. A. Intracellular ROS levels were determined by H_2DCFDA staining in various subpopulations of BMs of old WT and SIRT3 KO mice in a transplant setting. n=4. **B.** BM cells isolated from WT and SIRT3 KO mice were treated with 200 uM paraquat and cell survival in various cell populations were scored by flow cytometry (HSC: LKSCD150+). n=3. **C.** Dead cells were quantified in various subpopulations of BM cells of old WT and SIRT3 KO mice by propidium iodide staining (HSC: LKSCD150+). n=4. **D.** Cycling of BM cells derived from old mice was assessed in transplant recipients using PY staining. n=4. **E.** Competitive transplantation assays using BM cells from old WT or SIRT3 KO mice as donors. Recipient mice were either untreated or supplemented with NAC throughout the entire experiment. Data shown are the percentage of donor-derived cells in the peripheral blood 12 weeks post transplant. Donors: n=3/group. Recipients: n=15/group. Error bars represent standard errors. *: p<0.05. **: p<0.005. ***: p<0.001.

HSCs are normally maintained in a quiescent state, which protects HSCs from losing their self-renewal capacity (Morrison et al., 1996; Rossi et al., 2008). Oxidative stress drives HSCs out of quiescence (Ito et al., 2006; Miyamoto et al., 2007; Tothova et al., 2007). We evaluated cell cycling by staining with Ki67, a cell proliferation marker, and with Pyronin Y (PY), a chemical that stains double-stranded RNA. PY negative populations quantitatively represent quiescent cells (Miyamoto et al., 2007). No difference in cell cycling was noted in HSCs of WT and SIRT3 KO mice (Figure S2E-H). However, differences in cycling were observed under stress. There was a 40% reduction in PY negative HSPCs derived from SIRT3 KO BM compared to WT controls in a transplant setting (Figure 4D, S2I). Thus, SIRT3 deficiency results in increased cycling and reduced survival, which may account for the compromised HSC self-renewal.

To determine whether increased oxidative stress is the underlying cause of compromised function in HSCs lacking SIRT3, we examined whether antioxidant treatment could restore the repopulating ability of aged SIRT3 KO cells. We performed a competitive transplant with bone marrow cells from aged WT and SIRT3 KO mice, and the transplant recipients were supplemented daily with the antioxidant N-acetyl-L-cysteine (NAC), which has been shown to effectively reduce ROS levels in HSCs (Ito et al., 2006; Jang and Sharkis, 2007; Miyamoto et al., 2007; Tothova et al., 2007). NAC treatment rescued reconstitution defects of aged SIRT3 KO HSCs (Figure 4E), demonstrating that oxidative stress is indeed compromising HSC function in the absence of SIRT3.

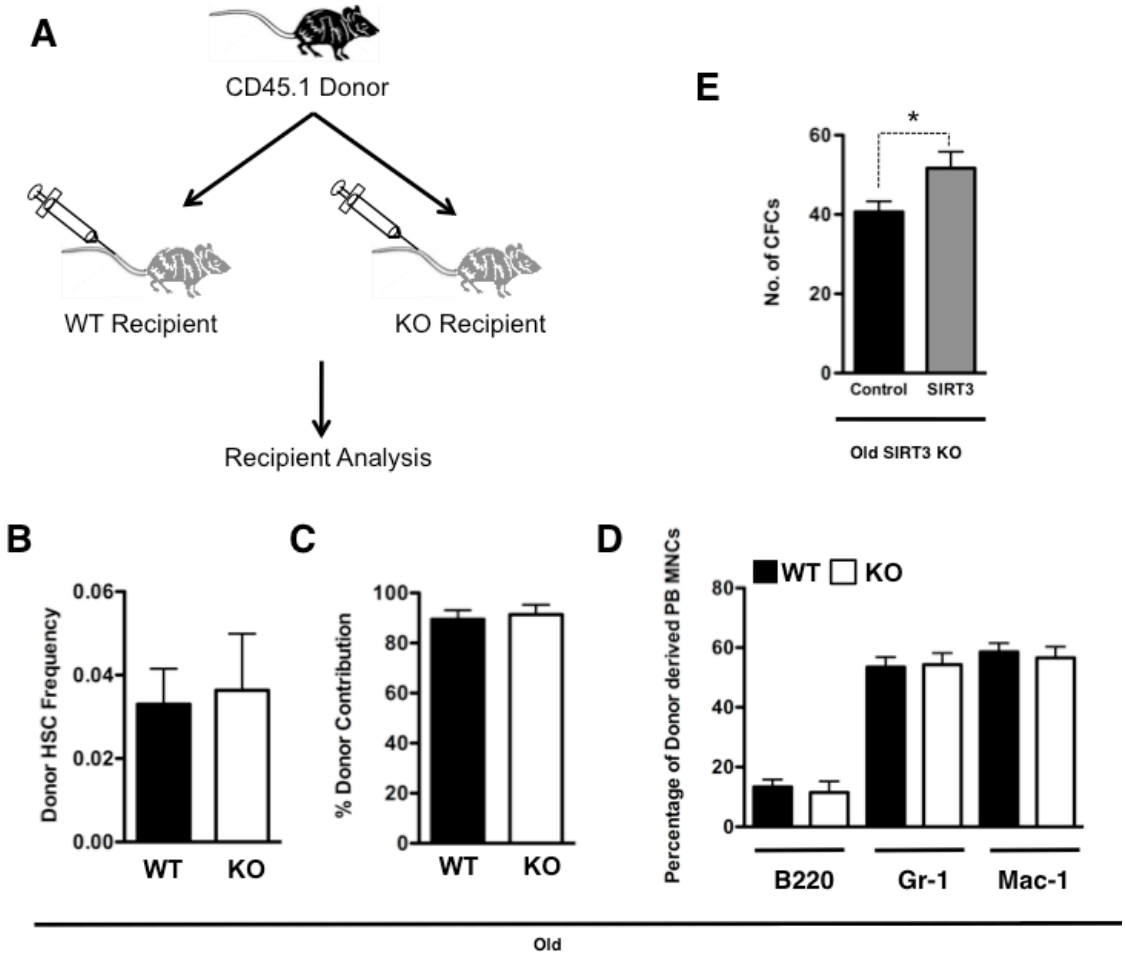

Figure S3. SIRT3 acts cell-autonomously to regulate HSC function. WT BM cells (CD45.1) were transplanted into lethally irradiated WT or SIRT3 KO mice (CD45.2) (A). Data shown are the frequency of donor derived HSCs in the BM (B), relative donor contribution in the peripheral blood (C), and donor derived lineage profiles in the peripheral blood (D). n=5. (E) SIRT3 rescues the functional defect of HSCs derived from SIRT3 KO mice. Lin^- cells derived from old SIRT3 KO mice were transduced with control virus or SIRT3 virus. Colony-forming activity was determined in a colony-forming assay. n=6. Error bars represent standard errors. *: $p<0.05$.

SIRT3 regulates HSCs autonomously

Since the SIRT3 KO mice employed in this study have whole body SIRT3 deletion, we investigated whether the HSC defects observed in this mouse model are due to HSC-autonomous effects of SIRT3 or to a non-autonomous role of SIRT3, e.g. the role of SIRT3 in regulating the HSC microenvironment or the niche. The transplantation studies comparing WT and SIRT3 KO BM-derived donors suggest that SIRT3 acts cell-autonomously to maintain HSC self-renewal (Figure 3). Additionally, when aged WT donors were transplanted into lethally irradiated WT or SIRT3 KO mice, comparable HSC self-renewal, reconstitution, and differentiation were observed (Figure S3A-D). Thus, SIRT3 is not required in the niche to support HSC function. Furthermore, SIRT3 overexpression increased the colony-forming activity of aged SIRT3 KO cells by 25% (Figure S3E). These data suggest that the functional defects of HSCs derived from SIRT3 KO mice can be rescued by SIRT3, providing additional support that SIRT3 regulates HSCs autonomously.

SIRT3 regulates mitochondrial metabolism in HSCs

We next determined how SIRT3 regulates mitochondrial metabolism in HSPCs. SOD2, a key mitochondrial antioxidant, is a substrate of SIRT3 (Qiu et al., 2010; Tao et al., 2010). We tested whether SIRT3 reduces oxidative stress in HSCs by activating SOD2. SIRT3 enhances the enzymatic activity of SOD2 via a posttranscriptional mechanism (Qiu et al., 2010; Tao et al., 2010). Consistently, SOD2 mRNA levels were comparable in WT and SIRT3 KO HSPCs (Figure 5A). However, the enzymatic activity was 50% lower in SIRT3 KO HSPCs compared to WT controls (Figure 5B). To determine the effects of oxidative stress on mitochondrial function, we used two mitochondria-specific labels to distinguish respiring (MitoTracker Red) versus total (MitoTracker Green) mitochondria. Dysfunctional non-respiring mitochondria (low MitoTracker Red relative to MitoTracker Green) increase with age ((Balaban et al., 2005) and Figure S4). There was a 40% increase in dysfunctional non-respiring mitochondria in aged SIRT3 KO HSCs compared to the age-matched control (Figure 5C, S4). Thus, SIRT3 regulates mitochondrial metabolic homeostasis and integrity in HSPCs.

SIRT3 expression reduces with age in HSCs

Next, we examined how SIRT3 is regulated with age. We compared expression of SIRT3 in HSPCs isolated from the bone marrows of young and old mice. SIRT3 mRNA levels were 70% lower in HSPCs of old mice compared to those in young mice (Figure 5D, E). Thus, SIRT3 expression is suppressed with age. To determine whether SIRT3 activity is also decreased with age, we compared expression and the enzymatic activity of SOD2 in HSPCs of young and old mice. SOD2 mRNA levels were unchanged with age (Figure 5F). However, the enzymatic activity was reduced by 30% in aged HSPCs compared to young HSPCs (Figure 5G), suggesting that SOD2 activity is suppressed with age via a posttranscriptional mechanism. Together, these data indicate that SIRT3 expression and activity decrease with age and that suppression of SIRT3-mediated mitochondrial homeostasis contributes to increased ROS in aged HSCs.

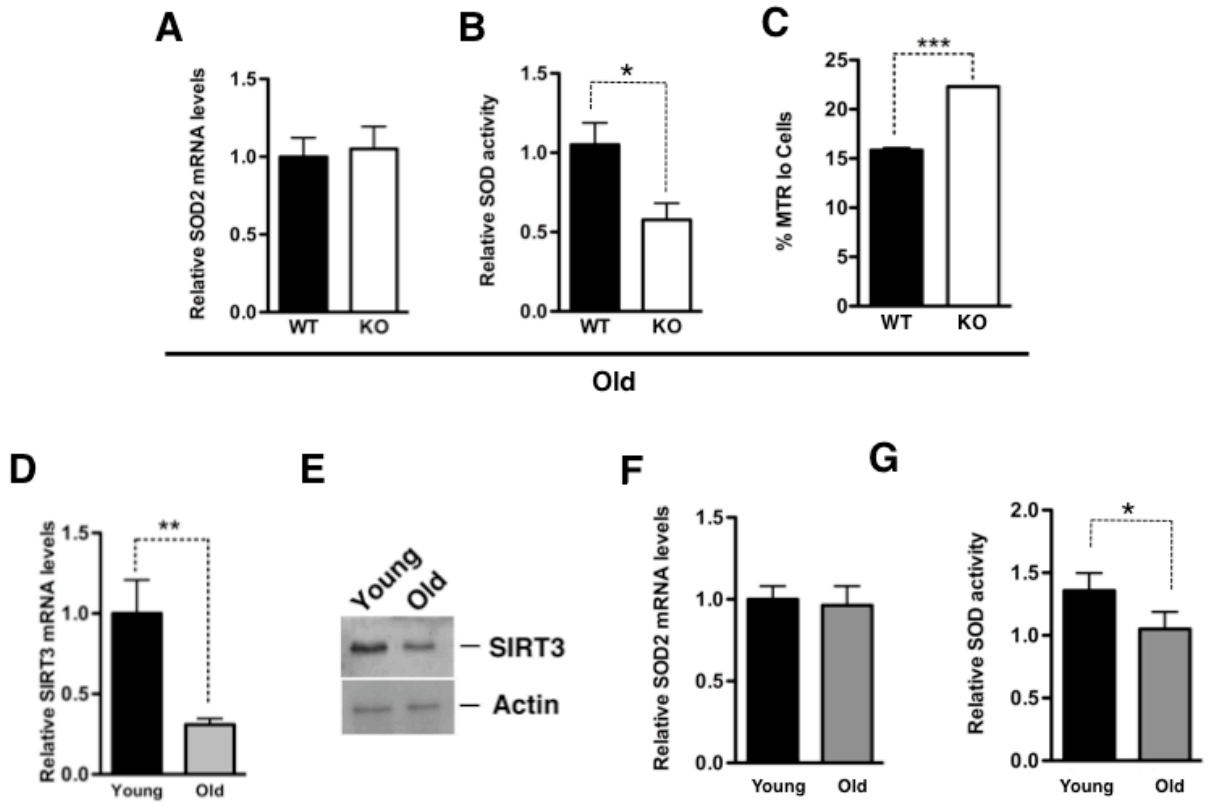

Figure 5. SIRT3 regulates mitochondrial metabolic homeostasis in HSCs but SIRT3 reduces with age. A, B. SOD2 mRNA levels (A) and the enzymatic activity (B) in HSPCs of old WT and SIRT3 KO mice were determined. n=4. **C.** Dysfunctional non-respiring mitochondria in HSCs of old WT and SIRT3 KO mice were determined by MitoTracker Green and MitoTracker Red staining. **D, E.** HSPCs were isolated from the BMs of young or old mice. SIRT3 expression levels were quantified by real time PCR (D) and western blotting (E) (n=3). **F, G.** SOD2 mRNA levels (F) and the enzymatic activity (G) in the HSPCs of young and old mice were determined. n=4.

SIRT3 upregulation rescues functional defects of aged HSCs

We next determined whether SIRT3 upregulation is sufficient to rescue the functional defects of aged HSCs and reverse aging-associated degeneration. Consistent with the role of SIRT3 in reducing oxidative stress (Bell and Guarente, 2011), SIRT3 overexpression reduced the ROS levels in HSCs (Figure 6A, S5A, B). While the expression of p19 and BAX increased in HSPCs of aged SIRT3 KO mice compared to WT controls, SIRT3 overexpression in aged HSPCs suppressed their expression (Figure 6B). Two assays confirmed functional rescue of aged HSCs by SIRT3 upregulation. In a colony-formation assay, SIRT3 overexpression increased the colony-forming activity of aged HSCs by 40% (Figure 6C). In a competitive transplantation assay, SIRT3 overexpression resulted in a 5-fold increase in functional reconstitution, with B cells, granulocytes, and macrophages all increased (Figure 6D, E, F). These data indicate that forced SIRT3 expression can reduce oxidative stress and rejuvenate aged HSCs.

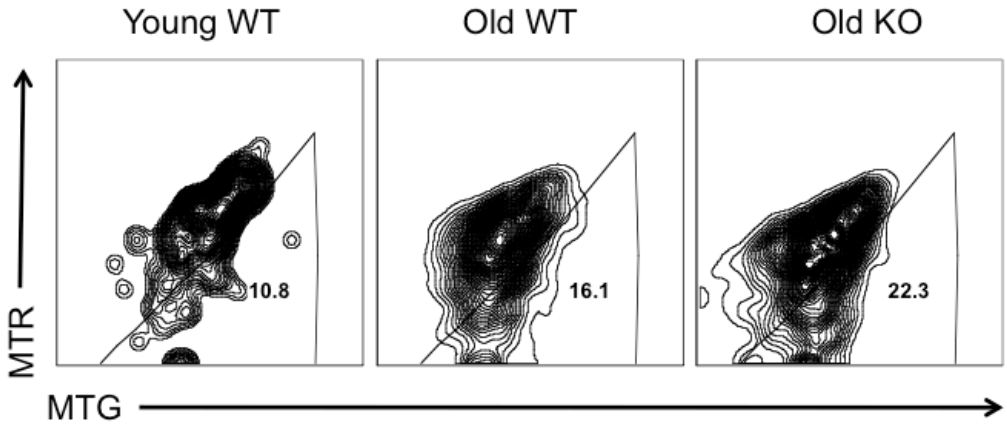

Figure S4. SIRT3 regulates mitochondrial metabolism in HSCs. HSCs from young and old WT and SIRT3 KO HSCs were stained with MitoTracker Green and MitoTracker Red. Dysfunctional non-respiring mitochondria (low MitoTracker Red relative to MitoTracker Green) were determined by Flow Cytometry.

SIRT3 deacetylates critical lysine residues on SOD2 and improves the antioxidative activity of SOD2 (Qiu et al., 2010; Tao et al., 2010). We next examined whether constitutively deacetylated SOD2 can improve the functional capacity of aged HSCs without SIRT3. WT or mutant SOD2 with lysines 53 and 89 mutated to arginines (K53/89R) to mimic the constitutively deacetylated form were ectopically expressed via lentiviral infection in Lin^- cells isolated from aged SIRT3 KO mice. Infected cells were treated with paraquat to assay oxidative stress resistance. Compared to control virus, SOD2 K53/89R improved the survival of HSCs by 67% while WT SOD2 had no effect (Figure 6G). Furthermore, in a colony-formation assay, SOD2 K53/89R increased colony forming activity by 75% while WT SOD2 infected cells showed comparable activity as cells infected with control virus (Figure 6H). These data suggest that constitutively deacetylated SOD2 bypasses SIRT3 to improve the function of aged HSCs and provide additional support that reducing oxidative stress improves the functional capacity of aged HSCs.

Discussion

The study presented here provides important insights into mitochondrial metabolism in stem cell maintenance, and illuminates the previously underappreciated plasticity of mitochondrial homeostasis in stem cell maintenance and tissue homeostasis during the aging process. Using oxidative stress as a readout for various mitochondrial processes regulated by SIRT3 (Bell and Guarente, 2011), we show that SIRT3-mediated

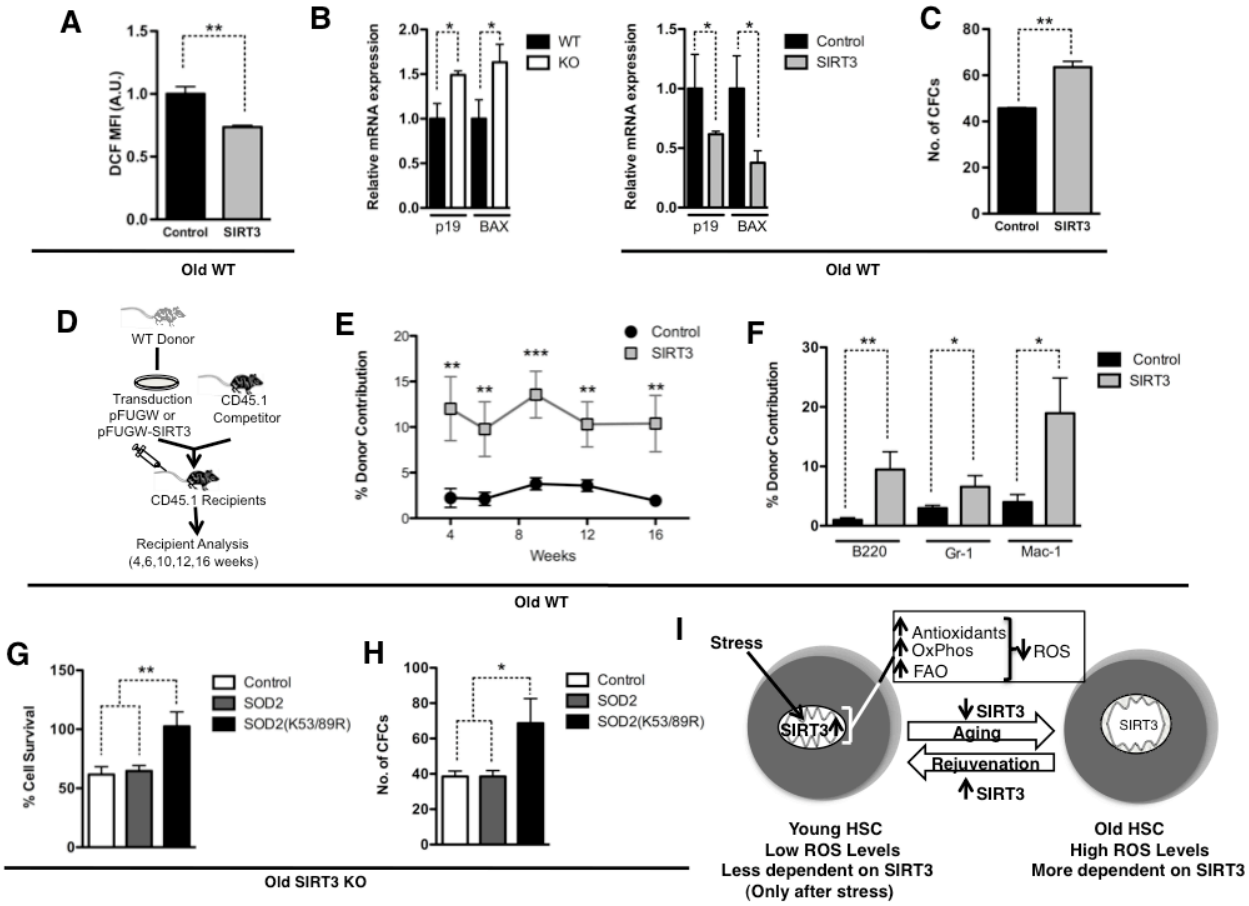

Figure 6. SIRT3 overexpression rescues functional defects of aged HSCs. A-F. SIRT3 was overexpressed in Lin^- cells isolated from old mice via lentiviral transduction (control: pFUGW. SIRT3: pFUGW-SIRT3). The cellular ROS levels in the HSC population (LKSCD150+) were determined by H_2DCFDA staining (n=3) (A). The expression of p19 and BAX was compared in LKS cells of aged WT and SIRT3 KO mice, and aged LKS cells transduced with control or SIRT3 lentivirus by RT-PCR (B). Colony-forming activity was determined in a colony-forming assay (n=6) (C). Schematic representation of a competitive transplantation assay to compare in vivo reconstitution activity of HSCs transduced with control lentivirus or SIRT3 lentivirus (D). Data shown are the percentage of total donor-derived cells (E) and donor-derived individual lineages (F) in the peripheral blood at 4, 6,10, 12, 16 weeks posttransplant. Donor: n=3. Recipients: n=15. **G, H.** Lin^- cells isolated from old SIRT3 KO mice were infected with a control virus (pFUGW), WT SOD2 virus (pFUGW-SOD2), or SOD2 K53/89R virus (pFUGW-SOD2 K53/89R). Cells were treated with paraquat and HSC survival was scored by flow cytometry (G). n=3. Colony-forming activity was determined in a colony-forming assay (H). n=6. **I.** A proposed model on stem cell aging and rejuvenation regulated by SIRT3-mediated mitochondrial homeostasis. SIRT3-mediated mitochondrial homeostasis plays an essential role in HSC maintenance and tissue homeostasis in response to stress. With advancing age, oxidative stress increases in HSCs, and aged HSCs rely more on the SIRT3-mediated mitochondrial stress response. However, as SIRT3 is downregulated in aged HSCs, this stress response becomes less effective, further contributing to increased oxidative stress. Overexpression of SIRT3 reverses stem cell aging and tissue degeneration. OxPhos: oxidative phosphorylation. FAO: fatty acid oxidation. Error bars represent standard errors. *: $p<0.05$. **: $p<0.005$. ***: $p<0.001$.

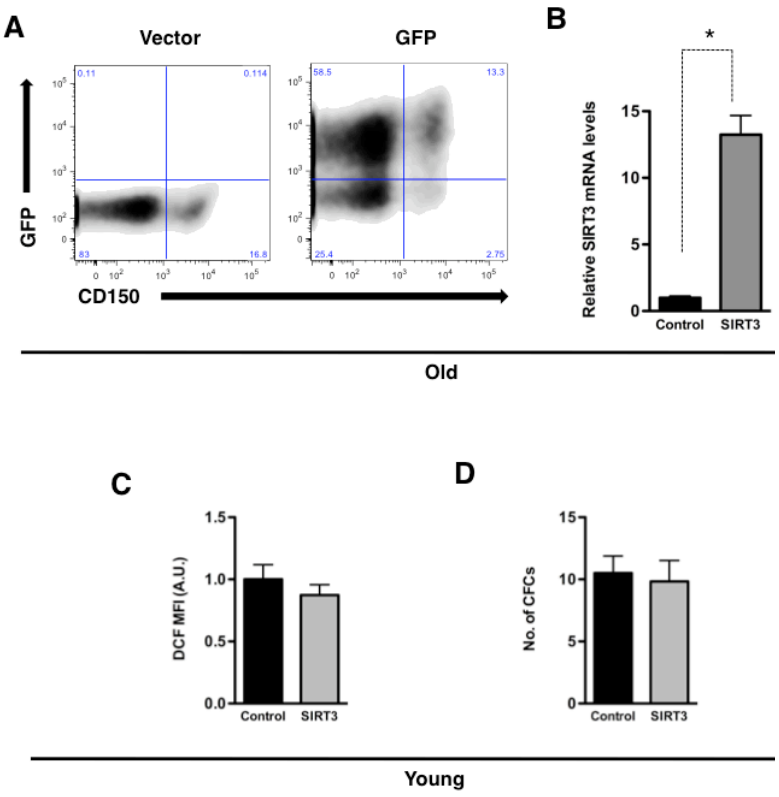

Figure S5. SIRT3 overexpression improves functional capacity of aged HSCs. A, B. SIRT3 was ectopically expressed in Lin$^-$ cells derived from old WT mice via lentiviral transduction. The transduction efficiency was quantified based on GFP-expressing lentiviral transduction. Flow cytometry plots are gated on Lin$^-$c-Kit$^+$Sca1$^+$ cells (A). SIRT3 mRNA levels in Lin$^-$ cells transduced with control virus or SIRT3-expressing lentivirus were quantified by RT-PCR (B). n=3. **C, D.** Overexpression of SIRT3 in young HSCs does not affect young HSCs. SIRT3 was overexpressed in Lin$^-$ cells derived from young WT mice via lentiviral transduction. The cellular ROS levels in the HSC population were determined by H_2DCFDA staining (n=3) (C). Colony-forming activity was determined in a colony-forming assay (n=6) (D). Error bars represent standard errors. *: $p<0.05$.

mitochondrial homeostasis is essential for HSC maintenance under stress (Figure 1, 2, 3), and that this regulatory program is downregulated with age (Figure 5D-G). Together, these data suggest that suppression of SIRT3-mediated mitochondrial homeostasis contributes to increased oxidative stress in aged HSCs. This regulatory process complements the view that passive accumulation of damaged mitochondria with age results in increased ROS and underlies the plasticity of mitochondrial homeostasis in stem cell maintenance and tissue homeostasis (Figure 6I).

The more surprising finding of our study is that upregulation of SIRT3 rescues functional defects of aged HSCs (Figure 6), providing direct evidence that physiological stem cell aging can be an acute casualty of high levels of oxidative stress and that oxidative stress-induced physiological stem cell aging and tissue degeneration are reversible. Although we do not rule out the possibility that chronic oxidative damage to cellular components contributes to the functional decline of aged stem cells, our data suggest that ROS-initiated signaling events are the likely regulators of physiological stem cell aging, providing the basis for reducing oxidative stress to rejuvenate aged stem cells and improve tissue regeneration.

It is intriguing that HSC defects are only apparent in aged but not young SIRT3 KO mice (Figure 1, 2, 3). This is consistent with our observation that SIRT3 preserves HSC function by reducing oxidative stress (Figure 4). Young mice have low levels of cellular ROS, which can be managed by antioxidants in the absence of SIRT3 (Figure S2A). With advancing age or stress, such as serial transplants, the levels of ROS increase (Ito et al., 2006). High levels of cellular ROS require a more robust antioxidative defense system. SIRT3 mediates metabolic reprogramming to reduce ROS production and enhances the antioxidant system to counteract oxidative stress (Figure 4A, B). These data suggest that SIRT3-mediated mitochondrial homeostasis is particularly important for stem cell maintenance under stress conditions. With advancing age, oxidative stress increases in HSCs, and aged HSCs rely more on the SIRT3-mediated mitochondrial stress response. However, as SIRT3 is downregulated in aged HSCs, this stress response becomes less effective, further contributing to increased oxidative stress.

In summary, we have shown that SIRT3 regulates a stress-responsive mitochondrial homeostasis, and more importantly, SIRT3 upregulation rejuvenates aged HSCs. We speculate that SIRT3 may regulate stem cells in other tissues. Experiments are in progress to determine the role of SIRT3 in other tissue-specific stem cells. Given that adult stem cells are thought to be central to tissue maintenance and organismal survival, SIRT3 may promote organismal longevity by maintaining the integrity of tissue-specific stem cells. Future studies will determine the effect of SIRT3 on lifespan. While evidence is emerging to indicate that mammalian sirtuins slow aging (Kanfi et al., 2012; Kawahara et al., 2009; Mostoslavsky et al., 2006), our study demonstrates that a sirtuin can also reverse aging-associated degeneration. The understanding of the plasticity of mitochondrial homeostasis in stem cell maintenance and tissue homeostasis should provide new insights into mammalian aging and rejuvenation, and the development of novel approaches for regenerative medicine.

EXPERIMENTAL PROCEDURES

Mice

SIRT3$^{-/-}$ mice have been described (129) (Lombard et al., 2007). All mice were housed on a 12:12-hr light:dark cycle at 25°C. Experiments were performed using age-matched littermates. All animal procedures were in accordance with the animal care committee at the University of California, Berkeley.

Flow Cytometry and Cell Sorting

BM cells were obtained by crushing the long bones (tibias and femurs) with sterile PBS without calcium and magnesium supplemented with 2% FBS. Lineage staining contained a cocktail of biotinylated anti-mouse antibodies to Mac-1α (CD11b), Gr-1 (Ly-6G/C), Ter119 (Ly-76), CD3, CD4, CD8a (Ly-2), and B220 (CD45R) (BD Biosciences). For detection or sorting, we used streptavidin conjugated to Percp, c-Kit-APC, CD48-FITC, CD150-PE-Cy7, and Sca-1-Pacific blue (Biolegend). For congenic strain discrimination, anti-CD45.1-PE or PERCP and anti-CD45.2 FITC or PE-Cy7 antibodies (BD Biosciences) were used. For cell cycle analysis, Pyronnin Y staining was performed as described (Miyamoto et al., 2007) and Ki-67 staining was performed according to the manufacturer's recommendation (BD Biosciences). For cell death, we used propidium

iodide staining (Biolegend) with the same antibodies as described above, except Streptavidin-APC-Cy7, c-Kit-FITC, and CD150-APC (Biolegend). ROS levels were detected using H_2DCFDA (Invitrogen) at 10 μM for 30 minutes at 37° in the dark (Miyamoto et al., 2007). All data were collected on an LSRII (Beckon, Dickinson) and data analysis performed with FlowJo (Treestar). For cell sorting, lineage depletion was performed according to the manufacture's instructions (Miltenyi Biotech). Cells were sorted using a Cytopeia INFLUX Sorter.

Transplantation Assays

$5x10^5$ whole BM cells from WT or SIRT3 KO CD45.2 littermates were mixed with $5x10^5$ CD45.1 B6.SJL (Jackson Laboratory) wild-type competitor cells and injected into lethally irradiated (950 Gy) CD45.1 B6.SJL recipient mice (15 recipients each). For the niche experiment, $1x10^6$ BM cells from WT CD45.1 B6.SJL were injected into lethally irradiated WT or SIRT3 KO CD45.2 recipients. Multilineage reconstitution was assayed by flow cytometry analysis of peripheral blood 12 to 16 weeks post transplant. For the serial transplantation assay, cells were pooled from all the recipients derived from the same donor. $1x10^6$ whole BM cells were transplanted into each secondary recipient. The tertiary transplant was performed in the same way as the secondary transplant, using the secondary recipients as donors (Miyamoto et al., 2007). Homing efficiency was analyzed as previously described (Christopherson et al., 2004). $5x10^6$ BM cells from WT or SIRT3 KO CD45.2 littermates were transplanted into lethally irradiated CD45.1 B6.SJL recipients. 16 hours later, homing efficiency was determined by the number of donor-derived LKS cells in the BM of the recipients divided by the number of LKS cells in the donors.

Cell culture

$2x10^4$ BM cells were used for colony formation assay in methylcellulose-based medium following the manufacture's instruction (Stem Cell Technologies). Colonies were counted 12 days after plating.

For paraquat treatment, $1x10^6$ BM cells were cultured in StemSpan SFEM (Stem Cell Technologies) supplemented with 20 ng/mL thrombopoietin, 100 ng/mL SCF, and 100 ng/mL FLT3 ligand (Invitrogen), and 1% Penicillin Streptomycin (Invitrogen), with or without 200 uM paraquat for 2 days. BM cells were stained with cell surface markers for various cell populations and counted via flow cytometry. The percentage of cell survival was determined by the number of cells after paraquat treatment divided by the number of cells without treatment.

Gene expression

mRNA was extracted from fluorescence-activated cell sorting (FACS) BM subpopulations using Trizol (Invitrogen). Reverse transcription was performed using qScript™ cDNA SuperMix (Quanta Biosciences). Gene expression was determined by real time PCR using Eva qPCR SuperMix kit (BioChain Institute) on an ABI StepOnePlus. GAPDH or b-actin were used as an internal controls. SIRT3 primers: Forward 5'-ACAGCTACATGCACGGTCTG-3'; Reverse 5'-ACACAATGTCGGGTTTCACA-3'. SOD2 primers: Forward 5'-

CCCAGACCTGCCTTACGACTAT-3'; Reverse 5'-TCTCCCAGTTGATTACATTCCA-3'. BAX primers: Forward 5'-CAA TAT GGA GCT GCA GAG GAT G-3'; Reverse 5'-CTG ATC AGC TCG GGC ACT TTA-3'. p19 primers: Forward 5'-TGC TTC TGG AAG AAG TCT GCG-3'; Reverse 5'-CTT CAG GAG CTC CAA AGC AAC-3'.

Immunoblot

$3x10^4$ freshly sorted LKS or lineage positive cells were resuspended in 1x Laemmli sample buffer and boiled for 5 minutes. The lysates were then loaded onto a Tris-SDS–PAGE gel. Protein transfer was performed onto nitrocellulose membrane, and the immunoblots were then probed with antibodies against SIRT3 (kind gift from Verdin lab) and B-actin (Sigma).

SOD Activity

Superoxide dismutase activity was measured as the inhibition of nitroblue tetrazolium (NBT) reduction in a xanthine-xanthine oxidase system as described (Schisler and Singh, 1985). $2.5x10^4$ HSPCs were used for each reaction.

Lentiviral Transduction

Stem and progenitor cells from an aged mouse were enriched using a lineage depletion kit (Miltenyi Biotec) according to manufacturer's instructions. The transduction procedure was adapted from methods as previously described (Ye et al., 2008). Lineage depleted BM cells were pre-stimulated for 24 hours in StemSpan SFEM (Stem Cell Technologies) supplemented with 20 ng/mL thrombopoietin, 100 ng/mL SCF, and 100 ng/mL FLT3 ligand (Invitrogen), and 1% Penicillin Streptomycin (Invitrogen). SIRT3, SOD2, SOD2 K53/89R were cloned into pFUGW lentiviral construct. Lentivirus was produced as previously described (Qiu et al., 2010), concentrated by centrifugation, and resuspended with fresh supplemented StemSpan SFEM media. The lentiviral media was then added to the enriched stem and progenitor cells, which were then spinoculated for 90 minutes at 270 x *g* in the presence of 8μg/ml polybrene. This process was repeated 24 hours later with a fresh batch of lentiviral media. After an additional 24 hours, $5x\ 10^4$ transduced cells were mixed with $5x\ 10^5$ competitor cells (CD45.1) for competitive transplantation, as described above. 750 transduced cells were used for colony formation assay, as described above. $2.5x10^5$ transduced cells were used for paraquat treatment and oxidative stress resistance, as described above.

Statistical analysis

Student's t-test was used for statistical analyses and null hypotheses were rejected at 0.05. The error bars represent standard errors.

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REFERENCES

Ahn, B.H., Kim, H.S., Song, S., Lee, I.H., Liu, J., Vassilopoulos, A., Deng, C.X., and Finkel, T. (2008). A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. Proc Natl Acad Sci U S A 105, 14447-14452.

Balaban, R.S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging. Cell 120, 483-495.

Bell, E.L., Emerling, B.M., Ricoult, S.J., and Guarente, L. (2011). SirT3 suppresses hypoxia inducible factor 1alpha and tumor growth by inhibiting mitochondrial ROS production. Oncogene.

Bell, E.L., and Guarente, L. (2011). The SirT3 divining rod points to oxidative stress. Mol Cell 42, 561-568.

Burnett, C., Valentini, S., Cabreiro, F., Goss, M., Somogyvari, M., Piper, M.D., Hoddinott, M., Sutphin, G.L., Leko, V., McElwee, J.J., Vazquez-Manrique, R.P., Orfila, A.M., Ackerman, D., Au, C., Vinti, G., Riesen, M., Howard, K., Neri, C., Bedalov, A., Kaeberlein, M., Soti, C., Partridge, L., and Gems, D. (2011). Absence of effects of Sir2 overexpression on lifespan in C. elegans and Drosophila. Nature 477, 482-485.

Chen, Y., Zhang, J., Lin, Y., Lei, Q., Guan, K.L., Zhao, S., and Xiong, Y. (2011). Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. EMBO Rep 12, 534-541.

Christopherson, K.W., 2nd, Hangoc, G., Mantel, C.R., and Broxmeyer, H.E. (2004). Modulation of hematopoietic stem cell homing and engraftment by CD26. Science 305, 1000-1003.

Finley, L.W., Carracedo, A., Lee, J., Souza, A., Egia, A., Zhang, J., Teruya-Feldstein, J., Moreira, P.I., Cardoso, S.M., Clish, C.B., Pandolfi, P.P., and Haigis, M.C. (2011). SIRT3 Opposes Reprogramming of Cancer Cell Metabolism through HIF1alpha Destabilization. Cancer Cell 19, 416-428.

Guarente, L. (2007). Sirtuins in aging and disease. Cold Spring Harb Symp Quant Biol 72, 483-488.

Guarente, L., and Kenyon, C. (2000). Genetic pathways that regulate ageing in model organisms. Nature 408, 255-262.

Haigis, M.C., and Sinclair, D.A. (2010). Mammalian sirtuins: biological insights and disease relevance. Annu Rev Pathol 5, 253-295.

Hirschey, M.D., Shimazu, T., Goetzman, E., Jing, E., Schwer, B., Lombard, D.B., Grueter, C.A., Harris, C., Biddinger, S., Ilkayeva, O.R., Stevens, R.D., Li, Y., Saha, A.K., Ruderman, N.B., Bain, J.R., Newgard, C.B., Farese, R.V., Jr., Alt, F.W., Kahn, C.R., and Verdin, E. (2010). SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature 464, 121-125.

Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N., Ikeda, Y., Mak, T.W., and Suda, T. (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature 431, 997-1002.

Ito, K., Hirao, A., Arai, F., Takubo, K., Matsuoka, S., Miyamoto, K., Ohmura, M., Naka, K., Hosokawa, K., Ikeda, Y., and Suda, T. (2006). Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat Med 12, 446-451.

Jang, Y.Y., and Sharkis, S.J. (2007). A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche. Blood 110, 3056-3063.

Janzen, V., Forkert, R., Fleming, H.E., Saito, Y., Waring, M.T., Dombkowski, D.M., Cheng, T., DePinho, R.A., Sharpless, N.E., and Scadden, D.T. (2006). Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature 443, 421-426.

Kanfi, Y., Naiman, S., Amir, G., Peshti, V., Zinman, G., Nahum, L., Bar-Joseph, Z., and Cohen, H.Y. (2012). The sirtuin SIRT6 regulates lifespan in male mice. Nature doi:10.1038/nature10815.

Kawahara, T.L., Michishita, E., Adler, A.S., Damian, M., Berber, E., Lin, M., McCord, R.A., Ongaigui, K.C., Boxer, L.D., Chang, H.Y., and Chua, K.F. (2009). SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. Cell 136, 62-74.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121, 1109-1121.

Lombard, D.B., Alt, F.W., Cheng, H.L., Bunkenborg, J., Streeper, R.S., Mostoslavsky, R., Kim, J., Yancopoulos, G., Valenzuela, D., Murphy, A., Yang, Y., Chen, Y., Hirschey, M.D., Bronson, R.T., Haigis, M., Guarente, L.P., Farese, R.V., Jr., Weissman, S., Verdin, E., and Schwer, B. (2007). Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. Mol Cell Biol 27, 8807-8814.

Miyamoto, K., Araki, K.Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M., Chen, C., Hosokawa, K., Nakauchi, H., Nakayama, K., Nakayama, K.I., Harada, M., Motoyama, N., Suda, T., and Hirao, A. (2007). Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell Stem Cell 1, 101-112.

Morrison, S.J., Wandycz, A.M., Akashi, K., Globerson, A., and Weissman, I.L. (1996). The aging of hematopoietic stem cells. Nat Med 2, 1011-1016.

Mostoslavsky, R., Chua, K.F., Lombard, D.B., Pang, W.W., Fischer, M.R., Gellon, L., Liu, P., Mostoslavsky, G., Franco, S., Murphy, M.M., Mills, K.D., Patel, P., Hsu, J.T., Hong, A.L., Ford, E., Cheng, H.L., Kennedy, C., Nunez, N., Bronson, R., Frendewey, D., Auerbach, W., Valenzuela, D., Karow, M., Hottiger, M.O., Hursting, S., Barrett, J.C., Guarente, L., Mulligan, R., Demple, B., Yancopoulos, G.D., and Alt, F.W. (2006). Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. Cell 124, 315-329.

Paik, J.H., Ding, Z., Narurkar, R., Ramkissoon, S., Muller, F., Kamoun, W.S., Chae, S.S., Zheng, H., Ying, H., Mahoney, J., Hiller, D., Jiang, S., Protopopov, A., Wong, W.H., Chin, L., Ligon, K.L., and DePinho, R.A. (2009). FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. Cell Stem Cell 5, 540-553.

Qiu, X., Brown, K., Hirschey, M.D., Verdin, E., and Chen, D. (2010). Calorie Restriction Reduces Oxidative Stress by SIRT3-Mediated SOD2 Activation. Cell Metab 12, 662-667.

Rando, T.A. (2006). Stem cells, ageing and the quest for immortality. Nature 441, 1080-1086.

Renault, V.M., Rafalski, V.A., Morgan, A.A., Salih, D.A., Brett, J.O., Webb, A.E., Villeda, S.A., Thekkat, P.U., Guillerey, C., Denko, N.C., Palmer, T.D., Butte, A.J., and Brunet, A. (2009). FoxO3 regulates neural stem cell homeostasis. Cell Stem Cell 5, 527-539.

Rossi, D.J., Jamieson, C.H., and Weissman, I.L. (2008). Stems cells and the pathways to aging and cancer. Cell 132, 681-696.

Sahin, E., and Depinho, R.A. (2010). Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature 464, 520-528.

Schisler, N.J., and Singh, S.M. (1985). Tissue-specific developmental regulation of superoxide dismutase (SOD-1 and SOD-2) activities in genetic strains of mice. Biochem Genet 23, 291-308.

Shimazu, T., Hirschey, M.D., Hua, L., Dittenhafer-Reed, K.E., Schwer, B., Lombard, D.B., Li, Y., Bunkenborg, J., Alt, F.W., Denu, J.M., Jacobson, M.P., and Verdin, E. (2010). SIRT3 deacetylates mitochondrial 3-hydroxy-3-methylglutaryl CoA synthase 2 and regulates ketone body production. Cell Metab 12, 654-661.

Shin, J., Zhang, D., and Chen, D. (2011). Reversible Acetylation of Metabolic Enzymes Celebration: SIRT2 and p300 Join the Party. Mol Cell 43, 3-5.

Someya, S., Yu, W., Hallows, W.C., Xu, J., Vann, J.M., Leeuwenburgh, C., Tanokura, M., Denu, J.M., and Prolla, T.A. (2010). Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell 143, 802-812.

Tao, R., Coleman, M.C., Pennington, J.D., Ozden, O., Park, S.H., Jiang, H., Kim, H.S., Flynn, C.R., Hill, S., Hayes McDonald, W., Olivier, A.K., Spitz, D.R., and Gius, D. (2010). Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. Mol Cell 40, 893-904.

Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., Armstrong, S.A., Passegue, E., DePinho, R.A., and Gilliland, D.G. (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell 128, 325-339.

Wang, Q., Zhang, Y., Yang, C., Xiong, H., Lin, Y., Yao, J., Li, H., Xie, L., Zhao, W., Yao, Y., Ning, Z.B., Zeng, R., Xiong, Y., Guan, K.L., Zhao, S., and Zhao, G.P. (2010). Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. Science 327, 1004-1007.

Ye, Z., Yu, X., and Cheng, L. (2008). Lentiviral gene transduction of mouse and human stem cells. Methods Mol Biol 430, 243-253.

Zhao, S., Xu, W., Jiang, W., Yu, W., Lin, Y., Zhang, T., Yao, J., Zhou, L., Zeng, Y., Li, H., Li, Y., Shi, J., An, W., Hancock, S.M., He, F., Qin, L., Chin, J., Yang, P., Chen, X., Lei, Q., Xiong, Y., and Guan, K.L. (2010). Regulation of cellular metabolism by protein lysine acetylation. Science 327, 1000-1004.

CHAPTER V

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The goal of this dissertation work was to elucidate molecular mechanisms that could be responsible for controlling the pace of age-related physical decline. Using model systems including calorie restriction and adult stem cells, this investigation has shown that SIRT3 is a crucial stress-response gene, which appears to protect against physical decline. During calorie restriction, SIRT3 is up-regulated as part of an endogenous system to suppress oxidative stress. The study in Chapter IV also indicates that SIRT3 plays a protective role in adult HSCs, again through protection from oxidative stress.

Although disparate systems, calorie restriction and HSC biology may hold at least one thing in common: preservation of cellular structures. During calorie restriction, "famine-like" conditions appear to streamline the economy of cellular maintenance. In other words, proteins and other molecular structures may generally not be replaced at the same rate as during higher energy states. Therefore, the cell responds by up-regulating processes that can protect proteins and structures already in place. On the other hand, adult stem cells, which have the ability to self-renew and are generally quiescent for long periods of time, also may experience this cellular maintenance economy. Protection of DNA structures would be necessary to prevent detrimental mutations from being clonally propagated to progenitor cells. Furthermore, quiescence would increase the time during which damage could accumulate in stem cells to various cellular structures. Conceivably, this may affect the ability of the cell to divide and differentiate properly when it is finally signaled to do so.

The focus of both studies presented in Chapters II and IV was on the protection from oxidative stress. However, as established in the introduction and in Chapter III, it is clear that SIRT3 has other targets that it can modify in the mitochondria. A substantial number of these targets are major components of metabolic pathways. The ability to adapt metabolic response to changes in cellular energy status or proliferation signals (calorie restriction and stem cell biology respectively), is likely to be an important component of SIRT3's function. Future studies may be able to elucidate the effects of SIRT3 on energy metabolism (i.e. in fatty acid oxidation) and its impact on cellular health, maintenance, and function.

Understanding metabolism is increasingly gaining interest, especially in stem cell function. Chapter IV shows findings in mouse HSCs, but it would also be valuable to determine whether the SIRT3 mechanism is also conserved in other tissue-specific cell types. HSCs are a high turnover stem cell, constantly replenishing the blood system, even under homeostatic conditions. But what about stem cells that are in low-turnover tissues, such as muscle stem cells? Does SIRT3 also help to protect their function? And would SIRT3 also have conserved function in human stem cells? Future endeavors will hopefully provide answers to these questions.