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Study on the Role of SIRT2 in Stem Cell Aging and Chronic Inflammation

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Study on the Role of SIRT2 in Stem Cell Aging and Chronic Inflammation

By

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Professor Danica Chen, Chair
Professor Hei Sook Sul
Professor Daniel Nomura
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Abstract

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Aging is among the top known risk factors for most human diseases. Understanding the biology of aging holds the promise to prevent or treat a wide range of seemingly unrelated diseases. Originally being viewed as a passive and irreversible accumulation of changes over time, aging is currently perceived as a progressive biological decline that succumbs to genetic manipulations. Hallmarks of aging have been identified, including but not limited to, stem cell exhaustion, mitochondrial dysfunction, deregulated nutrient sensing, and genomic instability. However, the molecular mechanisms determining the aging process remain elusive. In particular, the cell/tissue specific responses to aging-associated damages still await investigation. This knowledge is pivotal to understand the molecular basis of the heterogeneous effects of aging on diverse tissues. The aim of this dissertation work was to gain molecular insights into this knowledge by studying the two striking aspects of aging: stem cell dysfunction and chronic inflammation.

Adult stem cells maintain tissue homeostasis throughout life. It has been known for decades that adult stem cell function declines with age, however, the exact mechanisms contributing to this degeneration remain tentative. We found that SIRT2, a primarily cytosolic NAD^+-dependent deacetylase, is required for hematopoietic stem cell (HSC) maintenance at old age. Mechanistic studies demonstrated that SIRT2 exerts its role through modulating cell death processes in HSCs. SIRT2 expression is significantly reduced in old HSCs, which is consistent with increased cell death in HSCs during aging. Enforced SIRT2 expression reverses the increased cell death observed in HSCs during physiological aging. Further, we show that restoring SIRT2 expression can rejuvenate the functionality of old HSCs, suggesting the reversibility of the functional decline in HSCs with age.

SIRT2 has been reported to suppress inflammation in multiple inflammation-inducing mouse models. Based on these findings, we investigated SIRT2's role in chronic sterile inflammation associated with physiological aging. Chronic NLRP3 inflammasome activation during aging has a causal role in developing pathological inflammation in sterile inflammatory diseases, such as atherosclerosis, Alzheimer's disease, Parkinson's disease, obesity, diabetes, multiple sclerosis, and cancer. In light of this, we explored whether SIRT2 suppresses aging-associated chronic inflammation through regulating the NLRP3 inflammasome activity. We found that SIRT2 specifically inhibits NLRP3 inflammasome in macrophages. NLRP3 inflammasome is activated in macrophages with age, with a concomitant reduction in SIRT2 levels. Enforced SIRT2 expression in macrophages from old mice reverses aging-associated NLRP3 inflammasome activation, suggesting a potential reversible mechanism for the aging-associated inflammation phenotype.

Our studies demonstrate that down-regulation of SIRT2 levels plays significant roles in different cells and tissues during aging. In HSCs, SIRT2 suppresses the activation of cell death processes and preserves HSC regenerative capacity, while in macrophages, SIRT2 mediated NLRP3 inhibition prevents the development of chronic inflammation. These results not only broaden the physiological relevance of the cytosolic NAD^+ protein deacetylase SIRT2 to include stem cell homeostasis, but also exemplify the heterogeneity in tissue responses to aging-associated down-regulation of the SIRT2 protein expression. Further, from the therapeutic standpoints, these findings also open novel avenues to explore the potential reversibility of both stem cell aging and systemic low-grade inflammation associated with aging.

This dissertation is dedicated to

my parents, for their unconditional love, trust and support

Table of Contents

List of Figures

Acknowledgements

This dissertation encloses my 6-year learning experience: an unforgettable journey with an exciting yet bumpy start, a struggling yet stimulating transition, and (hopefully) a fruitful end. I am indebted to many who have nurtured and guided my intellectual development, as well as my maturation as a scientist.

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Chapter 1: Introduction

Aging was once believed to be an inevitable degenerative process. However, it is now widely recognized that aging process is regulated genetically and lifespan or healthspan can be extended by molecular interventions simply targeting one gene. Multiple molecular and cellular hallmarks of aging have been identified recently, including genomic instability, epigenetic alterations, loss of proteostasis, etc. However, key questions remain in the field to elucidate the biological identity of aging causing damages, their tissue specificity and reversibility. This chapter serves as an introduction to my PhD thesis. In this introduction, I will review on the most effective regime in lifespan and healthspan extension: calorie restriction, and its implications in cellular nutrient sensing, stress response pathways, as well as normal physiology and disease (previously published article, reproduced with permission). Next, I will review on two prominent aspects of physiological aging: stem cell dysfunction and chronic inflammation, with discussions on the mitochondrial basis and inflammasome regulation respectively.

Part I: Nutrient sensing and the oxidative stress response[1]

The simplicity and effectiveness of calorie restriction (CR) in lifespan and healthspan extension have fascinated generations searching for the Fountain of Youth. CR reduces levels of oxidative stress and damage, which have been postulated in the free radical theory of aging as a major[1] cause of aging and diseases of aging. This reduction has long been viewed as a result of passive slowing of metabolism. Recent advances in nutrient sensing have provided molecular insights into the oxidative stress response and suggest that CR triggers an active defense program involving a cascade of molecular regulators to reduce oxidative stress. Physiological studies have provided strong support for oxidative stress in the development of aging-associated conditions and diseases but have also revealed the surprising requirement for oxidative stress to support normal physiological functions and, in some contexts, even slow aging and prevent the progression of cancer. Deciphering the molecular mechanisms and physiological implications of the oxidative stress response during CR will increase our understanding of the basic biology of aging and pave the way for the design of CR mimetics to improve healthspan.

Introduction

In the 1930s, fear of the adverse health consequences of limited availability of food triggered the first CR experiment, which led to the surprising finding that CR increases lifespan in rodents [1]. Since this initial discovery, the link between nutrient uptake and longevity has been expanded across species. A growing number of studies in model organisms have extrapolated the effects of CR and fasting in decreasing the onset of age-associated pathologies [2-6]. The prolongevity, prohealth effect of CR was viewed as a result of the passive effect of food limitation and slow metabolism resulting in reduced production of reactive oxygen species (ROS) and prevention of oxidative stress [7]. However, it has become increasingly appreciated that the organismal effects of CR are actively regulated processes and that CR triggers a robust defense program involving a cascade of molecular regulators to reduce oxidative stress[8-10]. Path- ways regulating ROS signaling and antioxidant activity have thus become promising focal points for uncovering mechanisms through which nutrient sensing networks employ a pro- health phenotype.

Historically ROS were thought to be produced as byproducts of cellular respiration and have deleterious effects in cells. Following the induction of Denham Harman's free radical theory of aging, a large body of evidence has emerged supporting the notion that oxidative stress causes the deterioration of cellular integrity and tissue functions and underlies the etiology of numerous diseases [11, 12]. However, recent studies also

[1] Previously published material 'Luo, H., Chiang, H., Louw, M., Susanto, A., Chen, D. (2017). Nutrient Sensing and the Oxidative Stress Response. Trends Endocrinol Metab. DOI: http://dx.doi.org/10.1016/j.tem.2017.02.008' is included in Chapter 1 part I.

reveal ROS as signaling molecules essential to support normal physiological functions [13]. Some studies even caution that the effects of oxidative stress in aging and disease are context dependent.

In this review we summarize recent advances in our molecular understanding of the CR response to reduce oxidative stress and highlight the actions of nutrient sensors in coordinating metabolic reprogramming and the oxidative stress response. Furthermore, we critically review recent studies that illustrate the two faces of ROS as signaling molecules and damaging agents under physiological conditions and discuss how cancer cells hijack the oxidative stress response mechanisms to promote tumorigenesis.

Nutrient Sensors and Oxidative Stress

Oxidative stress originates from an offsetting of the ratio of pro-oxidants to antioxidants in favor of pro-oxidants. On nutrient deprivation cells experience a metabolic switch from energy- inefficient glycolysis to energy-efficient oxidative phosphorylation [14-16]. At the organismal level, animals also switch their energy source from glycolysis to oxidative phosphorylation during fasting or CR [17]. This metabolic switch requires cells to be reliant on increased mitochondrial activity, which is associated with increased ROS production [18]. It is therefore plausible that nutrient sensors regulate not only intermediary metabolism to facilitate the metabolic switch but also the oxidative stress response to cope with the associated ROS production.

Regulation of Oxidative Stress by Sirtuins

The clearest evidence that nutrient sensors are critically required to reduce oxidative stress and damage during CR has come from studies of SIRT3 [9, 10]. SIRT3 belongs to the sirtuin family of NAD-dependent deacylases. Their dependence on NAD links their activity to cellular metabolic status [19]. There are seven sirtuins in mammals, SIRT1–7, that localize to various cellular compartments. CR reduces the accumulation of oxidative damage and protects against the development of oxidative damage-related pathologies such as hearing loss in wild-type (WT) mice [9, 10]. However, these protective effects are blunted in mice deficient in SIRT3, a mitochondrial sirtuin. It is interesting to note that the levels of oxidative damage and hearing loss are indistinguishable between WT and SIRT3-knockout (KO) mice fed ad libitum and the SIRT3 expression level is induced by CR in WT mice [20, 21], indicating that the SIRT3 protective program is specifically turned on during CR to reduce oxidative stress. More broadly these observations highlight a new paradigm for nutrient sensing and the oxidative stress response: instead of passively slowing the metabolic rate, CR initiates an active regulatory program to reduce oxidative stress, with nutrient sensors centrally positioned in such regulation.

It is striking that, in addition to hearing loss, SIRT3 prevents a wide spectrum of aging-associated conditions and diseases. SIRT3-KO mice develop spontaneous cancer, insulin resistance, diet-induced obesity, inflammation, hyperlipidemia, steatohepatitis,

reduced hematopoietic stem cell (HSC) number and function, and sarcopenia [22-30]. Furthermore, SIRT3 prevents neuronal death in mouse models of Huntington's disease [31]. It has been argued that induction of SIRT3 and reduction of oxidative stress contribute to the profound prohealth effects of CR, underscoring the free radical theory of aging.

Extensive biochemical studies have provided mechanistic insights into the oxidative stress response regulated by SIRT3. The critical antioxidant enzyme superoxide dismutase 2 (SOD2) is modified by acetylation in cells and SIRT3 targets key lysine residues on SOD2 for deacetylation and activation [9, 32]. SIRT3 also deacetylates isocitrate dehydrogenase 2 (IDH2), resulting in an increased level of the reducing equivalent NADPH [10]. NADPH is used by glutathione reductase to convert oxidized glutathione (GSSG) to reduced glutathione (GSH), the cofactor used by glutathione peroxidase (GPX) to detoxify ROS. In addition to the antioxidants, SIRT3 deacetylates a large number of mitochondrial enzymes and proteins controlling the major metabolic pathways, such as fatty acid oxidation, ketogenesis, amino acid metabolism, acetyl CoA metabolism, and oxidative phosphorylation, to coordinate the directionality and the rate of the metabolic flux on changes in nutritional input [33]. The concurrent activation of the mitochondrial metabolic pathways and antioxidants by SIRT3 is a clear indication that cells have evolved mechanisms to reduce oxidative stress when mitochondrial activity is turned on under nutrient deprivation.

Much data have accumulated to suggest that the nuclear SIRT1 mediates aspects of the CR response, including increased physical activity, disease protection, and lifespan extension [34-36]. Although there is no direct evidence that SIRT1 mediates the CR response at least in part by reducing oxidative stress, biochemical studies have convincingly demonstrated that SIRT1 regulates cellular redox status by deacetylating the longevity factor forkhead box O 3a (FOXO3a), a transcription factor that governs the expression of several antioxidant genes [37, 38]. It is likely that SIRT1 reduces oxidative stress to influence cellular and physiological outcomes during CR. The convergence of SIRT1 and SIRT3 on the regulation of antioxidants suggests synergistic activation of antioxidants at the transcriptional and post-translational levels to allow effective quenching of oxidative stress under conditions of CR.

Regulation of Oxidative Stress by AMP-Activated Protein Kinase (AMPK), mTOR, and General Amino Acid Control Non-Derepressible 2 (GCN2)

Besides sirtuins, nutrient sensors that have been shown to be required for lifespan extension under CR regimens or fasting include AMPK [39-42], the TOR signaling network [43, 44], and GCN2 [45, 46]. AMPK is an energy sensor [47] while TOR and GCN2 sense the availability of intracellular amino acids [48, 49]. These nutrient sensors have been intensively studied in metabolic regulation, but evidence for their regulation of oxidative stress has begun to emerge. It has recently been noted that cancer cells are dependent on AMPK for suppression of oxidative stress and promotion of cell survival under energy-stress conditions such as glucose limitation, anchorage-

independent growth, and solid tumor formation [50, 51]. Aberrant regulation of the mTOR signaling network results in increased mitochondrial biogenesis and ROS level in HSCs [52] while genetic deletion of GCN2 in antigen-presenting cells or intestinal epithelial cells also results in increased ROS [53].

These observations further solidify the link between nutrient sensing and the oxidative stress response and, importantly, offer an opportunity to deepen our mechanistic understanding of this regulation. AMPK has been proposed to reduce oxidative stress by inhibiting acetyl-CoA carboxylase 1 (ACC1) and ACC2, decreasing NADPH consumption in fatty acid synthesis, and subsequently maintaining the NADPH level [50]. GCN2 and mTOR inhibition reduce oxidative stress by activating autophagy and recycling damaged mitochondria [53-55]. Thus, nutrient sensors could engage in multiple ways to influence the activity of antioxidants, the integrity of mitochondria, and the metabolic flux to modulate ROS production and scavenging (Figure 1).

Interestingly, evidence is emerging to suggest that, in addition to nutritional input, nutrient sensors can be activated in response to ROS. The expression of SIRT3 is induced by nutrient deprivation and ROS [56]. AMPK can also be activated by ROS [57-59]. Thus, in addition to the well-established function of AMPK in energy sensing and reestablishing energy homeostasis, AMPK senses oxidative stress and induces the oxidative stress response. The intimate connection between nutrient sensing and oxidative stress sensing underscores a previously underappreciated crosstalk: the oxidative stress response is engaged under nutrient-deprived conditions to dispose of the increased production of ROS associated with the metabolic switch to mitochondrial metabolism and, similarly, the metabolic reprogramming typically associated with nutrient deprivation also occurs under oxidative stress conditions to maintain the energy reserve and cell survival.

Oxidative Stress in Aging and Disease

Numerous studies have aimed to test the free radical theory of aging. Surprisingly, lifespan studies of various antioxidant enzymes show that only one of 18 genetic manipulations influenced lifespan [60]. However, new evidence supporting a causal role of oxidative stress in aging continues to emerge from studies in various model systems. In yeast, overexpression of the H-scavenging enzyme Tsa1 extends lifespan [61]. Mice overexpressing glucose-6 phosphate dehydrogenase, a rate-limiting enzyme of the pentose phosphate pathway that generates the reducing agent NADPH, are protected from oxidative damage and exhibit improved healthspan [62]. Repressed NRF2 activity and increased oxidative stress recapitulate Hutchinson–Gilford progeria syndrome (HGPS) defects, while NRF2 activation decreases oxidative stress and reverses cellular HGPS defects [63]. These findings add a surprising twist to the free radical theory of aging and form the foundation for the formulation of new hypotheses to scrutinize this half-century-old theory of aging.

A common approach to test an aging theory is the lifespan analysis. While critically important and informative, these studies do not offer the resolution necessary to tease out the numerous variables that act together to influence the organismal lifespan, which include but are not limited to the rate of aging. It is therefore imperative to dissect the impact of oxidative stress on aging-associated conditions in specific tissues or even cell types. Further, lifespan is influenced by life-threatening diseases such as cancer. Admittedly, the incidence of cancer increases with aging and these two rival demons are likely to share some common origins, such as oxidative stress [64]. However, recent advances in cancer research also reveal that oxidative stress prevents aspects of cancer development and can complicate the effect of oxidative stress on lifespan [65-69]. Our constantly improving capacity to study physiology and pathology with increasing spatial and temporal resolution ushers in a new era for the free radical theory of aging.

Stem Cell Aging and Tissue Degeneration

Adult stem cells or tissue-specific stem cells persist throughout the lifespan to maintain and repair tissues. This lifetime commitment requires stem cells to develop robust cellular protective mechanisms to ensure their integrity. Using the hematopoietic system as an example, the cellular ROS level is considerably lower in HSCs than in their differentiated progeny [23]. The low cellular ROS level in HSCs is in part due to reduced ROS production. Adult HSCs mostly remain in a quiescent state, which is associated with low metabolic rate and mitochondrial number [70]. Quiescent HSCs primarily rely on glycolysis for energy production [71, 72]. Compared with mitochondrial oxidative phosphorylation, glycolysis is much less efficient for energy production but is sufficient to support the low energy requirement of quiescent HSCs. This metabolic feature is essential for the maintenance of HSCs, because less ROS are produced [70]. HSCs are also armed with heightened capacity for ROS disposal. Oxidative stress regulators are highly enriched in HSCs and activate robust oxidative stress responses to scavenge ROS. FOXOs tend to be enriched in the nucleus of HSCs but excluded from the nucleus of differentiated progeny [73]. SIRT3 is highly expressed in HSCs but its expression is much repressed in differentiated hematopoietic cells [23].

The transition of HSCs from the quiescent state to proliferation is regulated by a metabolic checkpoint that monitors the health of mitochondria and repairs mitochondrial damage before the cells progress through the restriction point and enter the cell cycle [74, 75]. Mitochondrial damage beyond repair leads to cell death. A tight correlation has been observed between increased ROS levels and HSC proliferation and death in numerous mouse models. Increased ROS production due to aberrant activation of the TSC–mTOR pathway [52, 76] or defective DNA damage response [77, 78], failure to engage the oxidative stress response resulting from defective FOXOs, SIRT3, Nrf2, or thioredoxin-interacting protein (Txnip) [23, 79-82], and dysregulation of ROS signaling such as the SIRT7-mediated mitochondrial unfolded protein response [75, 83] or p38 MAPK signaling [84] all result in loss of HSC quiescence and maintenance and attrition of HSC regenerative capacity. These studies suggest that ROS act as a signal that dictates the balance between HSC quiescence, proliferation, and survival. The ROS

level is increased in HSCs with aging [84], consistent with the role of ROS as a trigger of the functional deterioration of HSC aging. ROS are also essential regulators of neural stem cells [85-88] and intestinal stem cells [89]. Thus, ROS regulation of stem cell fate decision appears to be conserved across tissues.

ROS management differs between HSCs and their progeny. Hematopoietic progenitor cells (HPCs) display increased levels of ROS under physiological conditions. Scavenging ROS from HPCs prevents their differentiation into mature blood cells while increasing the ROS level triggers precocious differentiation into mature blood cells, indicating that ROS function as signaling molecules that prime the differentiation of HPCs [90]. How ROS signaling leads to cell fate decision-making remains unclear, but different cellular programs are likely to be activated by ROS signaling to render distinct cell fate decisions in HSCs and HPCs (Figure 2).

If ROS are indeed a cause of stem cell aging, it is tempting to ask whether ROS cause aging due to chronic accumulation of oxidative damage over the lifetime or the acute effects of high levels of ROS, and whether ROS-induced physiological aging is reversible. The expression of SIRT3 and SIRT7 is reduced in aged HSCs, which may contribute to the increased ROS level and defective ROS signaling in aged HSCs [23, 75]. Importantly, overexpression of SIRT3 or SIRT7 improves the regenerative capacity of aged HSCs [23, 75]. These studies suggest that ROS- induced physiological aging is likely to be acute and reversible. It appears that HSC aging is due not to the passive accumulation of cellular damage over the lifetime but to the regulated repression of cellular protective programs, giving hope for targeting the deregulated cellular protective programs to reverse HSC aging and rejuvenate tissue homeostasis.

Innate Immunity

Host defense is dependent on the burst of oxidative stress in phagocytes as part of the innate immune response during infection. Various pathways are employed by immune cells to produce ROS and facilitate the immediate killing of pathogens. Neutrophils and inflammatory monocytes produce ROS by metabolic enzymes such as NADPH oxidases and myeloperoxidases [91]. By contrast, killer lymphocytes deliver cytotoxic granules containing granzymes into the invading bacterial strains, where granzymes disrupt the electron transport chain proteins as well as the antioxidant proteins resulting in increased ROS [92]. It was shown recently that activated macrophages undergo metabolic reprogramming and the resulting increase in mitochondrial oxidation of succinate and an elevation of mitochondrial membrane potential combine to drive mitochondrial ROS production [93]. Interestingly, pathogens also develop defense mechanisms against the oxidative stress generated by the host immune system [94, 95]. The diverse pathways employed by innate immune cells to produce ROS and the various strategies developed by pathogens to defend against oxidative stress are a strong indication that oxidative stress is crucial in supporting innate immunity (Figure 3).

Although oxidative stress is necessary to rid host cells of foreign pathogens, the host may suffer tissue damage and dysfunction due to the over-accumulation of ROS that is

characteristic of the inflammatory processes. Mice deficient in NRF2 display enhanced host defense 4 h after *Streptococcus pneumoniae* challenge but exhibit increased lung injury and death in 24 h [96]. Similarly, mice deficient in negative regulator of ROS (NRROS), a negative regulator of ROS production in phagocytes, exhibit enhanced bactericidal activity but develop severe experimental autoimmune encephalomyelitis due to oxidative damage in the central nervous system [97]. Thus, oxidative stress levels in the innate immune cells require tightly controlled regulation to ensure effective host defense while maintaining tissue integrity.

The findings in stem and progenitor cells as well as innate immunity have challenged the view that ROS are produced as the byproducts of cellular respiration and supported the idea that ROS can act as signaling molecules and support physiological functions. These new findings make us rethink the evolution of ROS production and motivate future studies to explore new roles of ROS in supporting cellular physiology. Along these lines, it is likely that the robustness of oxidative stress regulation but not the inhibition of ROS is optimal for slowing aging.

Cancer

ROS cause DNA damage and genomic instability, a major driving force for tumorigenesis. Thus, ROS have long been thought to play a critical role in tumor initiation. SIRT3-KO cells exhibit increased genomic instability and susceptibility to transformation and SIRT3-KO mice develop spontaneous tumors [98]. Complementary to this traditional view, recent studies have revealed several novel roles that ROS play in tumor initiation. SIRT3 deficiency results in ROS stabilization and upregulation of hypoxia-inducible factor-1 alpha (HIF1a) [25], a transcription factor that triggers metabolic reprogramming characterized by increased glycolysis in the presence of oxygen, a hallmark of tumor cells known as the Warburg effect [99]. This metabolic feature provides tumor cells with the substrates required for biomass generation. Furthermore, ROS alter cellular signaling events to promote cancer cell proliferation [100]. Finally, heightened ROS levels can result in necrosis, an unprogrammed cell death pathway that triggers the recruitment of macrophages followed by the secretion of inflammatory mediators. The chronic inflammatory state in the tumor niche provides a warm house for malignant transformation [101].

Although oxidative stress has a pro-cancer role at the tumor-initiation stage by promoting transformation, proliferation, and establishment of the tumor microenvironment, cancer cells seem to hijack the oxidative stress response to survive (Figure 4). As a result oxidative stress also plays an anticancer role at the development stage by limiting tumor progression and metastasis. In lung cancer cells, the regulatory property of the glycolytic enzyme pyruvate kinase M2 (PKM2) in ROS detoxification is essential for PKM2 to promote tumor progression [65]. The transition into anchorage-independent growth is associated with the induction of isocitrate dehydrogenase-1 (IDH1), which mitigates ROS and promotes anchorage-independent growth [66]. Further, melanoma cells that are capable of distant metastasis undergo metabolic changes during metastasis to increase their capacity to withstand oxidative stress [68],

and age-related changes in the tumor microenvironment also affect cancer cell metastasis through modulation of the oxidative stress response pathways [67]. In line with the importance of redox control of tumor development, inducing heightened levels of oxidative stress in cancer cells has been explored as a potential therapy for certain cancers [69].

Concluding Remarks and Future Perspectives

Recent molecular links between nutrient sensors and the oxidative stress response support the notion that CR triggers an active defense program to reduce oxidative stress. It would be important to determine whether nutrient sensors are required to reduce oxidative stress during CR and, if so, whether they reduce oxidative stress in particular cell types or ubiquitously. Much work is needed to demonstrate that the physiological functions of these nutrient sensors do indeed impact the aging process and the development of diseases at least in part by reducing oxidative stress. In this regard the studies on SIRT3 are the most advanced and SIRT3 KO mice have been extensively characterized in various cell types and tissues for oxidative stress and tissue functions. Gain-of-function studies would be of particular interest, as this information is relevant for therapeutic implications. Mouse models for other nutrient-sensing pathways have been extensively characterized, although oxidative stress has not been analyzed extensively in these mouse models and whether redox regulation contributes to the physiological functions of these nutrient-sensing pathways remains largely unknown [54, 102-104]. Compelling evidence supports the new role of ROS as signaling molecules supporting physiological functions in addition to being damaging agents. It is also increasingly appreciated that the effects of ROS on aging and cancer are more complex than previously thought. Future studies will unravel new biological processes and physiological functions that require ROS as signaling molecules and elucidate whether CR interferes with these processes. Much work is needed to scrutinize the contexts in which ROS promote or prevent aging or cancer. So far the evidence that ROS promote cancer initiation but suppress cancer progression and metastasis derives from studies of different genetic manipulations in different cancers. Studies of the same genetic manipulations of ROS using diverse cancer models are necessary to crystallize the differential effects of ROS at the various developmental stages of cancers. It is also tempting to ask whether CR has differential effects at different stages of cancer development. This knowledge builds a solid foundation for the design of CR mimetics and antioxidants with maximal health benefits.

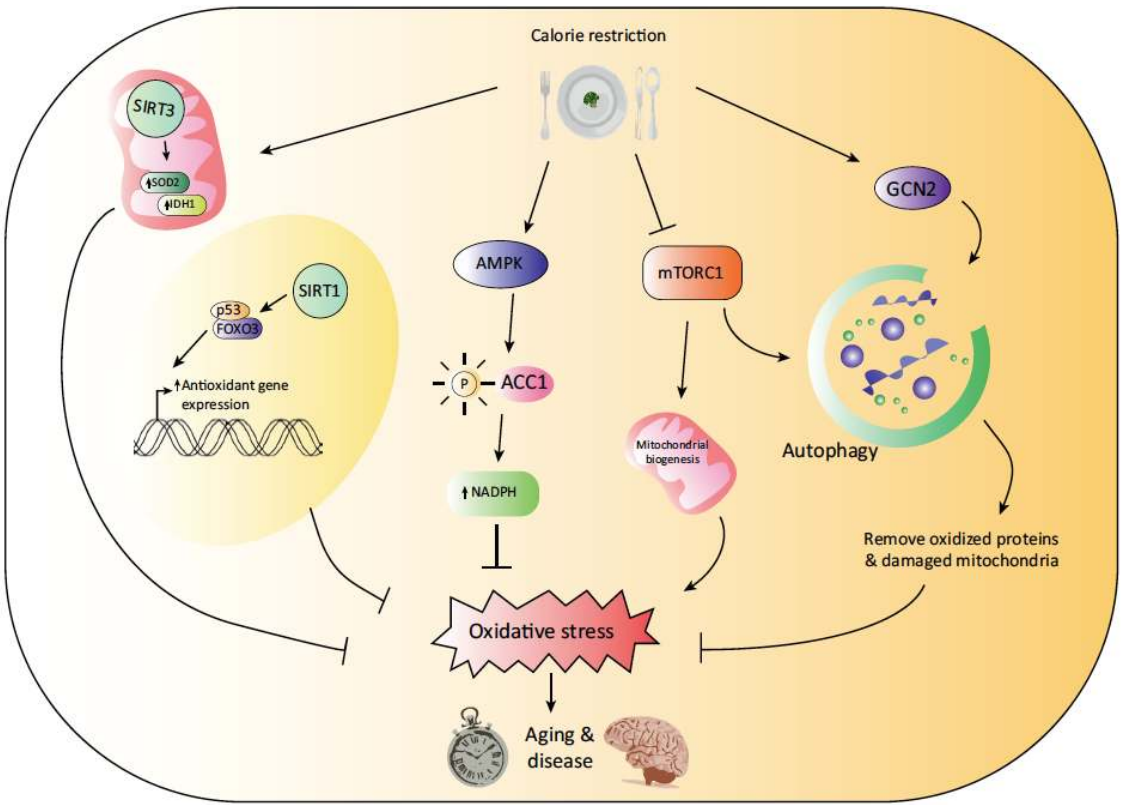

Figure 1. Calorie restriction triggers an active defense program to reduce oxidative stress and prevent aging and aging-associated diseases. SIRT1 and SIRT3 reduce oxidative stress by regulating the transcriptional expression or post-translational modification of antioxidants. AMP-activated protein kinase (AMPK) reduces fatty acid synthesis and increases the NADPH level to combat oxidative stress. Inhibition of the mTOR kinase suppresses mitochondrial biogenesis. Both inhibition of mTOR and activation of general amino acid control non-derepressible 2(GCN2) facilitate the clearance of oxidized proteins and damaged mitochondria through autophagy.

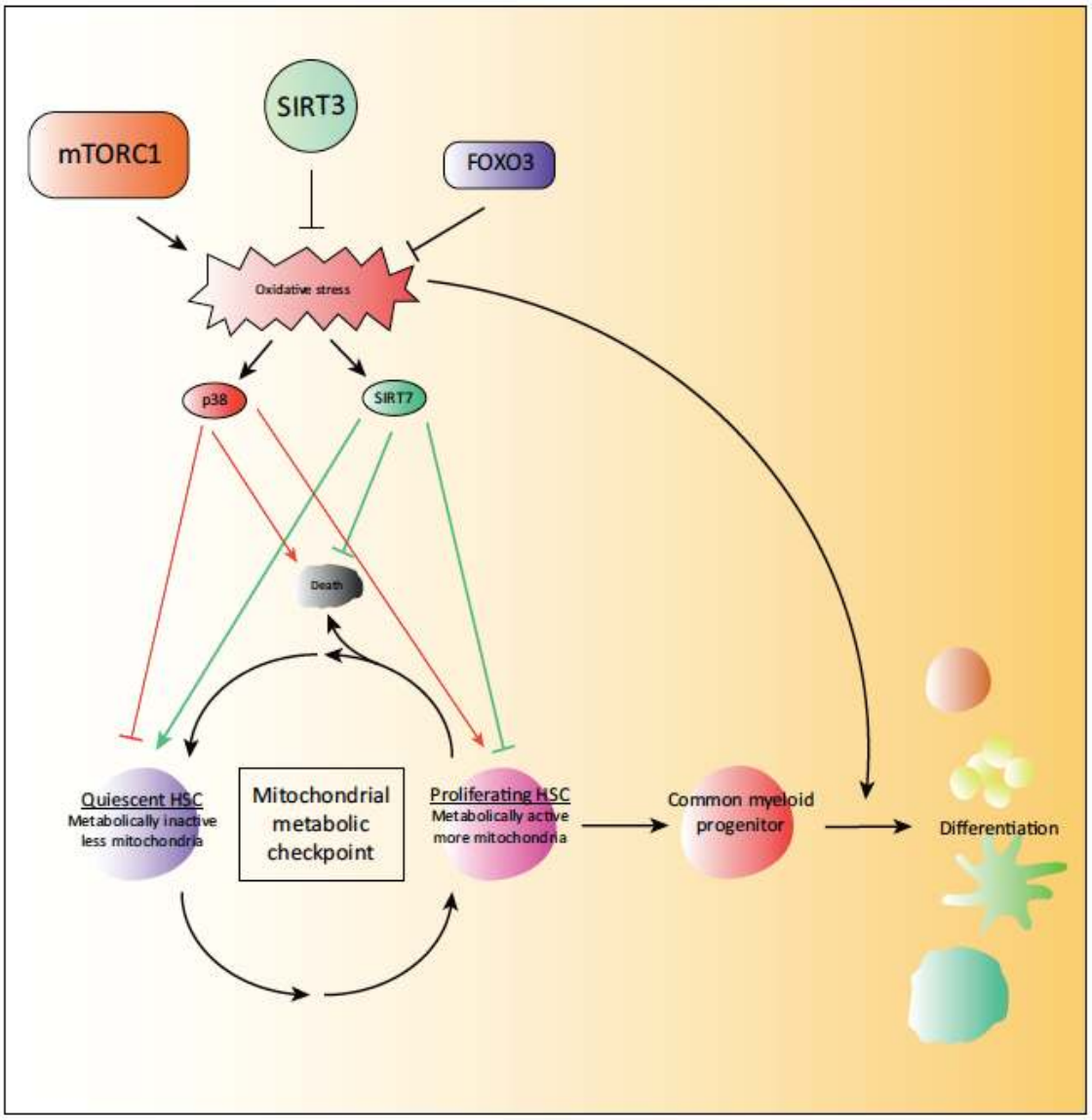

Figure 2. Reactive oxygen species (ROS) govern cell fate decision-making in hematopoietic stem progenitor cells. The transition of hematopoietic stem cells (HSCs) from quiescence to proliferation is associated with increased mitochondrial biogenesis and oxidative stress, which is monitored by the mitochondrial metabolic checkpoint that determines the cell fate decision on quiescence, proliferation, or death through the SIRT7-mediated mitochondrial unfolded protein response or p38 signaling. ROS prime common myeloid progenitor cells for differentiation.

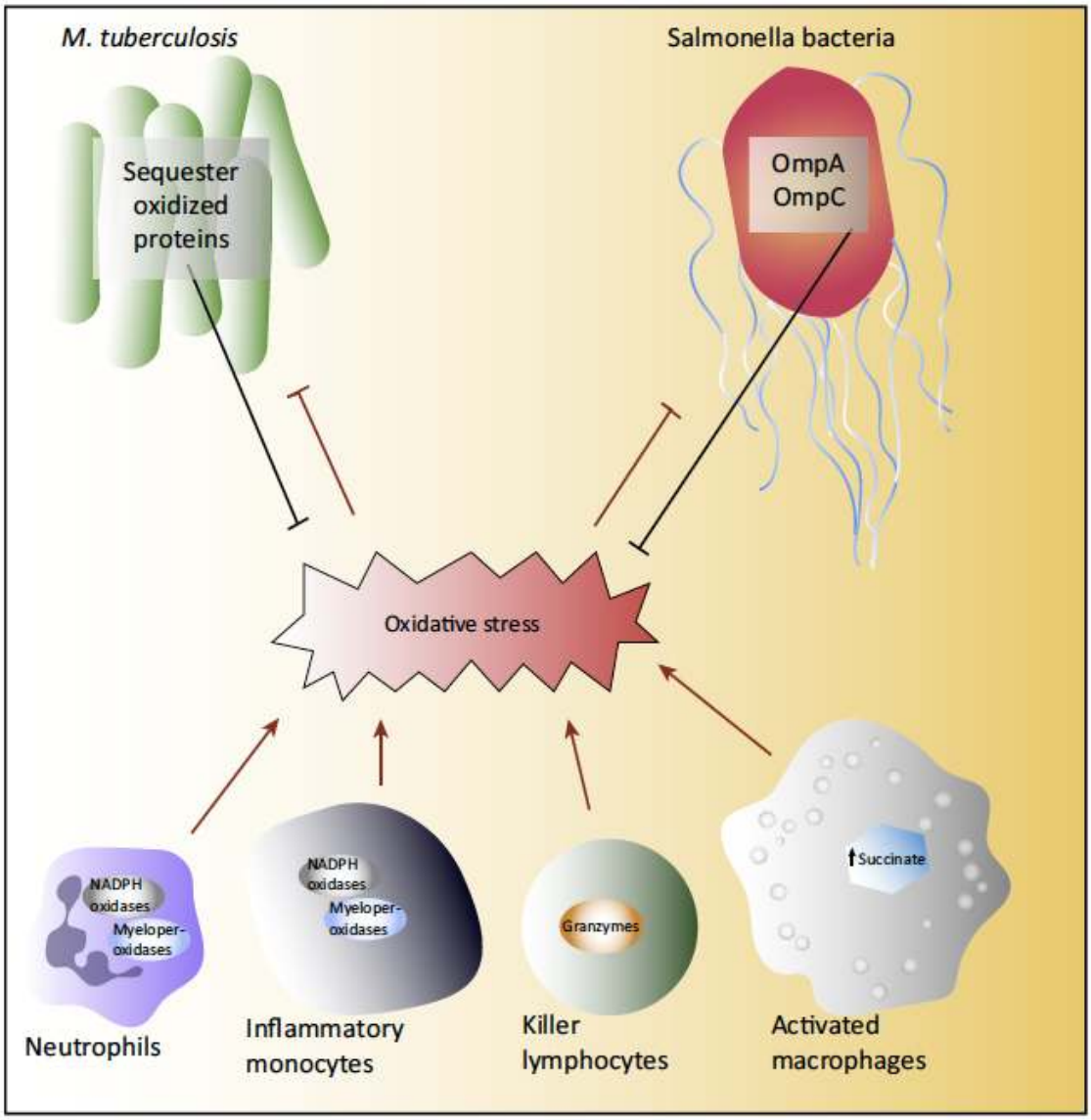

Figure 3. Oxidative stress supports innate immunity. Phagocytes employ various mechanisms of reactive oxygen species (ROS) production to facilitate pathogen clearance, while pathogens develop strategies to alleviate ROS damage.

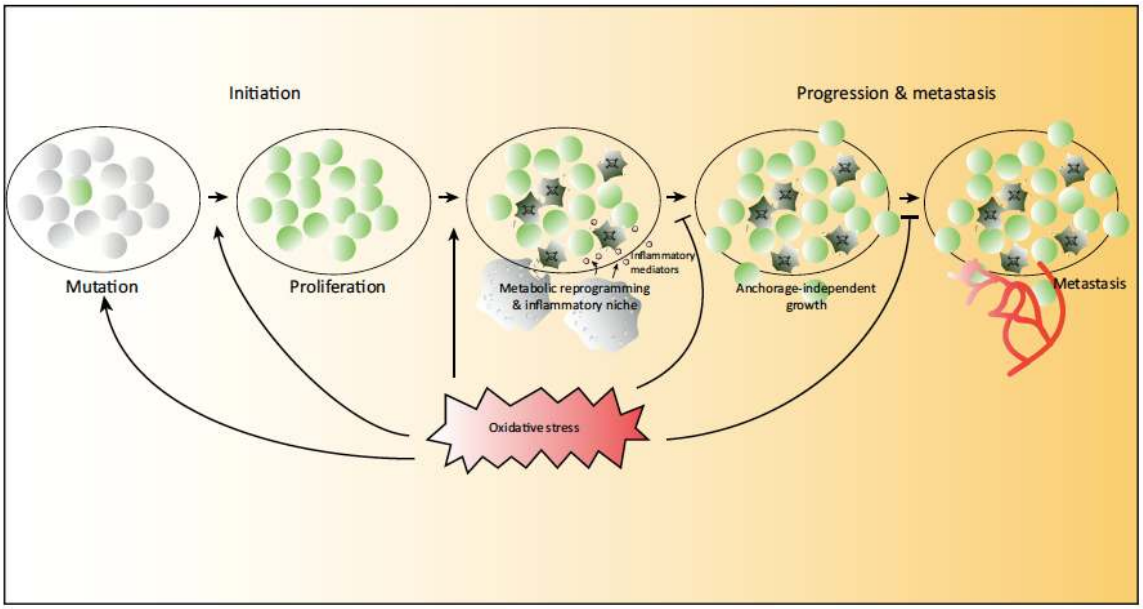

Figure 4. Oxidative stress regulation of cancer. Although oxidative stress has a pro cancer role at the tumor-initiation stage by promoting DNA damage, proliferation, metabolic reprogramming, and the establishment of the tumor microenvironment, cancer cells also hijack the oxidative stress responses to survive, progress, and metastasize.

Part II: Stem cell aging, the mitochondrial perspective

Adult stem cells maintain tissue homeostasis throughout life. They are capable to both self-renewal and differentiation in order to remodel and replenish our organs under physiological or stress conditions. Mounting evidence has pointed to the fact that adult stem cell functions decline with age [105-108]. These deterioration processes are highly tissue-specific, leading to the onset of multifaceted phenotypes of adult stem cell aging [105-108]. Nonetheless, the single commonality shared among diverse adult stem cells aging patterns is the loss of two key potentials of adult stem cells, which are the self-renewal and differentiation abilities. The decline in stem cell functions with age is believed to be causal to loss of tissue maintenance, tissue degeneration, and the ultimate death of organisms. However, current investigations on these devastating deterioration processes reveal a more complex and dynamic model of stem cell aging [105-108], and further studies are needed to unveil the molecular mechanisms.
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In this section, I will focus on the hematopoietic system as the model system and review the current knowledge on the aging processes in hematopoietic stem cells. Next, I will summarize the recent advances on the pivotal role of mitochondrial biology in hematopoietic stem cell aging.

Aging of the hematopoietic stem cells

The maintenance of our hematopoietic system highly depends on the functions of hematopoietic stem cells (HSCs). Phenotypes of HSC aging are well characterized, including a myeloid-skewed differentiation potential, and a significant reduction in regenerative ability [109-113]. These cellular changes further translate into an increased incidence of myeloid cancers, a decrease in immune competency, as well as the development of anemia in the elderly population [114, 115].

Recent advances have highlighted both cellular intrinsic and extrinsic mechanisms contributing to the aging of HSCs [110, 116-123]. Transcriptome analysis comparing genes that are differentially expressed in young and old HSCs have uncovered the loss of sirtuin expressions in HSCs during aging [124]. To follow up on the biological significance of these changes, our lab have identified that mitochondrial stress increases during aging in HSCs, potentially due to the reduced expressions of SIRT3 and SIRT7 [23, 74, 75]. SIRT3, a mitochondrial localized protein deacetylase, deacetylates and activates SOD2 to reduce mitochondrial oxidative stress, while SIRT7, a nuclease localized sirtuin, represses NRF1 activity to inhibit mitochondrial protein translation, alleviating mitochondrial protein folding stress [23, 74, 75]. These molecular regulations of mitochondrial stress levels in HSCs are particularly important, as an increase in mitochondrial stress is associated with a burst of mitochondrial biogenesis that occurs during G0 to G1 transition. A failure to restore and relieve mitochondrial stress levels results in an impairment of mitochondrial integrity in HSCs and defects in G1 to G0 conversion, in other words, loss of quiescence [74]. Indeed, reduced levels of SIRT3 and SIRT7 lead to a deregulation of the mitochondrial stress surveillance

mechanisms, resulting in an accumulation of mitochondrial stress and a loss of quiescence in old HSCs [23, 74, 75]. Remarkably, overexpressing SIRT3 or SIRT7 in old HSCs improves the functional capacity of the stem cells [23, 74, 75]. These studies set nice examples dissecting the roles that nutrient sensors play in HSC aging.

Other known intrinsic mechanisms regulating HSC aging include increased genomic instability and epigenetic alterations, both of which contribute to the lineage bias and loss of regenerative potential in HSCs in aging hematopoiesis [117, 119-123]. Mounting evidence has also pointed to the aging HSC niche that serves as an extrinsic regulator of HSC aging [118]. HSCs reside in the bone marrow niche surrounded by different types of cells, including vascular-forming endothelial cells and macrophages [118, 125]. Together with cellular matrix proteins, these supporting cells form the hematopoietic microenvironment interact with HSCs and maintain HSC function. Deregulation in HSC polarity due to a change in cellular CDC42 levels, alterations in bone matrix formation, and fluctuations in secreted chemokine ligands in the bone marrow niche have all been shown to contribute to the aging-associated decline in HSC-niche interaction, leading to deterioration in HSC function with age [118].

Although numerous studies have featured the intrinsic and extrinsic roles of the aging process in HSCs, there is much to be learned about the relationship between various defined intrinsic and extrinsic regulations, as well as exploring undefined mechanisms. Further mechanistic studies will pave the way for formulating therapeutic approaches to rejuvenate the aging hematopoietic system.

Mitochondrial regulation in hematopoietic stem cell function

Mitochondria are the powerhouse of cells, which is the essential site for oxidative phosphorylation and ATP production. The quantity and quality of mitochondria in cells not only reflect the cellular bioenergetics status, but also shape cellular signaling networks, thus acting as cell fate determinants [70, 126, 127]. The capacities to both self-renew and differentiate as adult stem cells suggest the existence of a delicate system in order to facilitate cell fate decision via regulating mitochondrial homeostasis. Indeed, recent advances have demonstrated diverse roles of mitochondria in HSC functions [81, 128-133].

Quiescent HSCs have few mitochondria, which is recognized as an attempt to maintain low metabolic rates and to produce little toxic byproducts of respiration [134]. Under homeostatic conditions, nearly 90% of adult HSCs remain in the quiescent state, which serves as a regulated protective mechanism preventing unwanted cell death and a depletion of HSC pool. Upon stimulation of intrinsic or extrinsic cues, HSCs exit quiescence and enter cell cycle. One major event that occurs upon G0 to G1 transition is a burst of mitochondrial biogenesis, associated with increases in mitochondrial stresses. As previously mentioned, studies from our lab have identified SIRT3 and SIRT7 reduces mitochondrial oxidative stress and mitochondrial protein folding stress respectively to surveil mitochondrial stress levels in HSCs [9, 23, 74, 75]. Loss of SIRT3

and SIRT7 expression in HSCs during aging results in an accumulation of mitochondrial stress and a loss of quiescence in HSCs [23, 75].

Consistent with our findings, mitochondrial integrity has been increasingly appreciated as a safeguard for adult stem cell function. HSCs with mitochondrial DNA mutations acquire aberrant differentiation potential, driving premature HSC aging phenotypes [135]. Defect in mitophagy process, the mitochondrial quality control pathway aiming to clear damaged mitochondria in cells, leads to failure of self-renewal capacity in HSCs [130, 133]. Alterations in mitochondrial metabolism, prominently a deregulation in the PPARδ mediated fatty acid oxidation pathway, results in a failure of self-renewal in HSCs as well [131]. Moreover, the choice of lineage fate in HSCs turns out to be highly dependent on the proper tethering of mitochondria to ER membranes [132]. Studies in human mammary stem-like cells also identified that during cell division, the asymmetrical apportioning of young and old mitochondria, but not other cellular organelles, governs the fate determination of two daughter cells [129]. However, whether this asymmetrical segregation of age in mitochondria occurs in HSCs remains to be tested.

Summary

There is no doubt about the closely intertwined relationship between mitochondrial health and HSC homeostasis. Despite the rising evidence supporting mitochondria' roles in regulating HSC function during aging, further investigation are needed to unveil the molecular basis of these regulations mediated by mitochondria. Understanding stem cell aging from the mitochondrial perspective will offer novel insights to the study of aging at both molecular and organellar levels.

Part III: Chronic Inflammation, the inflammasome perspective

Aging-associated chronic inflammatory state is key contributor to the chronic diseases of aging, including metabolic disorders, cardiovascular disease, neurodegeneration, and cancer [136]. This systemic low-grade inflammation associated with aging, which has been termed as “inflammaging”, is characterized by elevated levels of inflammatory biomarkers such as C reactive protein and interleukin-6 (IL-6). This mild increase in inflammatory cytokines is predictive of a range of aging-related phenotypes, including perturbations in glucose homeostasis, energy metabolism, cardiovascular health, cognitive function and tumor suppression [137]. Understanding the molecular mechanisms behind the development of chronic inflammation with age and how it contributes to chronic diseases of aging is quintessential to the management of diseases of aging and the promotion of healthy aging.

In this section, I will discuss the current understandings on the potential causes of aging-associated chronic inflammation. Next, I will focus on inflammasomes and the recent advances about inflammasome-mediated regulation in aging-associated chronic inflammation.

What are the causes of aging-associated inflammation?

One cause of inflammaging may be the remodeling of the innate and adaptive immune systems that occurs during aging [137, 138]. One key characteristic of the remodeling process is the lineage bias towards the myeloid cells against the lymphoid cells in the peripheral blood of the elderly population. The increase in myeloid cells, including macrophages and monocytes, is a major contributor to the secretion of pro-inflammatory cytokines without an overt infection.

From a qualitative angle, innate immune cells gradually lose phagocytic and antigen clearance capacity, while B and T lymphocytes in the adaptive immune system undergo reduced expansion and differentiation upon antigen stimulation during aging [136-138]. Both of these qualitative declines result in a compromised immune surveillance environment against pathogen infections. Meanwhile, aging-associated accumulation of environmental or self-derived stressors such as oxidized or damaged macromolecules, self-debris, or even metabolites including ATP, fatty acids, cholesterol, or urate crystal, all serve as danger-associated molecular patterns (DAMPs) that activate innate immunity responses followed by secretion of pro-inflammatory cytokines, contributing to inflammaging [137, 139].

Cellular senescence may also underlie the chronic inflammatory phenotype associated with aging [140-142]. Cells undergo senescence in response to damage and stress as a protective measure in order to suppress proliferation and prevent cancer [64, 140, 142]. On the downside, however, senescent cells are found to secrete numerous inflammatory cytokines, which are termed as senescence-associated secretion

phenotype or SASP [64, 140, 142]. The accumulation of senescent cells with age may also drive the development of systemic low-grade inflammation.

Other possible causes of inflammaging include the changes that occur in gut commensal bacteria composition [143-145], as well as the increased permeability in intestinal epithelial lining [146], which may result in pathogens leaking into circulation, leading to stimulation of the innate immune system.

Inflammasomes and chronic inflammation

Inflammasomes are intracellular multiprotein complexes that play an important role in the innate immunity [147, 148]. Consisting of Nod-like receptors such as NLRP3, NLRC4, Aim2, adapter protein ASC, and pro-Caspase1, inflammasomes are caspase-1 activation platforms that act to respond to a variety of stimuli [147-149]. Nod-like receptors are intracellular sensors of pathogen-associated molecular patterns (PAMPs) or DAMPs [147-149]. These pattern recognition receptors recognize stressors, which thereby stimulates the assembly and oligomerization of Nod-like receptors with ASC and pro-Caspase-1, which ultimately activates caspase-1. Activated Caspase-1 then proteolytically cleaves pro-IL1b and pro-IL18 into mature cytokines for secretion.

Despite their profound roles in pathogen sensing and clearance, inflammasomes have been implicated in mediating the systemic, low-grade inflammation that occurs during aging [149]. Studies have focused on the NLRP3 inflammasome, as the NLRP3 protein is unique among the Nod-like receptors due to the fact that it is the only sensor that responds to non-pathogen-associated activators [148]. The sterile activators of NLRP3 include self-derived molecules, ROS and fatty acids, as well as environment-derived molecules such as asbestos and silica [150-152]. Interestingly, these sterile activators of NLRP3 have found to accumulate in humans during aging [149, 152]. Therefore, it is plausible that the increasing levels of sterile activators of the NLRP3 inflammasome contribute to the chronic activation of NLRP3 inflammasome assembly, leading to chronic inflammatory cytokine secretion with stress conditions such as aging or over-nutrition. Genetic evidence also lends support to this hypothesis. Mice deficient in NLRP3 are protected from high-fat diet induced inflammatory states, while aged NLRP3 knockout (KO) mice are protected from aging-associated inflammation [153, 154]. Chronic inflammation is causal to a range of chronic diseases, with the most prominent examples being metabolic disorders [155]. NLRP3 KO mice are protected from developing insulin resistance under over-nutrition or aging stresses [153, 154]. These studies highlight the potential of targeting the NLRP3 inflammasome as a therapeutic approach to tackling aging-associated chronic inflammation.

Several regulators of NLRP3 inflammasome activation have been reported, including double-stranded RNA-dependent protein kinase (PKR), guanylate-binding protein 5 (GBP5), and serine–threonine kinase Nek7 [156, 157]. Ubiquitination of NLRP3 by multiple E3 ubiquitin ligases negatively regulates its activation, which can be counteracted by deubiquitinase BRCC3 [157]. Moreover, recruitment of NLRP3 to the

mitochondria via mitochondrial antiviral signaling protein (MAVS) activates NLRP3 activity [158]. However, the diverse mechanisms of NLRP3 regulation still remain elusive. Thus, understanding the biology of the NLRP3 inflammasome offers a unique angle in order to probe inflammaging.

Summary

Emerging evidence has suggested that aging-associated chronic inflammation can be derived from complex and interconnected causes. Concomitantly, the NLRP3 inflammasome has been increasingly appreciated as a crucial mediator of the chronic inflammatory state. However, it still remains an open question as to identify the core inflammatory components that cause inflammatory damage over the wide spectrum of chronic diseases that accompany aging. Furthermore, challenges lie ahead to explore interventions that can act to reverse chronic inflammation and prevent the development of chronic diseases. Recent advances in the inflammasome field have revealed strategies that target NLRP3 inflammasome activation by investigating the biology of NLRP3 inflammasome regulation. More mechanistic insights are needed to broaden our understanding of NLRP3 inflammasome biology, and also to facilitate the functional targeting of the NLRP3 inflammasome as a potential therapeutic avenue for chronic diseases of aging.

Chapter 2: Hematopoietic Stem Cell Aging Modulated by SIRT2

Summary

Adult stem cells maintain tissue homeostasis throughout life. It has been known for decades that adult stem cell function declines with age, however, the exact mechanisms contributing to this degenerative process remain tentative. We found that SIRT2, a primarily cytosolic NAD+-dependent deacetylase, is required for hematopoietic stem cell (HSC) maintenance at an old age. Mechanistic studies demonstrated that SIRT2 exerts its role through modulating cell death processes in HSCs. SIRT2 expression is significantly reduced in old HSCs, which is consistent with increased cell death in HSCs during aging. Enforced SIRT2 expression reverses the increased cell death observed in HSCs during physiological aging. Further, we show that restoring SIRT2 expression can rejuvenate the functionality of old HSCs, suggesting the reversibility of the functional decline in HSCs with age.

Introduction

The degeneration and dysfunction of aging tissues are attributable to the deterioration of adult stem cells [108, 159, 160]. Stem cell aging is thought to be due to the cumulative cellular damages [114, 161-163]. Cellular damages are monitored by cell cycle checkpoints that sense defects that occur during essential cellular processes, either inducing a cell cycle arrest in response until the defects are repaired or leading to cell death when damages are beyond repair [164]. Adult stem cells are maintained in a metabolically inactive quiescent state for prolonged periods of time [70, 165]. The transition from the quiescent state to proliferation is associated with increased mitochondrial biogenesis to facilitate a metabolic switch from glycolysis to oxidative phosphorylation to support the increased energy requirement of proliferation [72, 166, 167]. This transition is monitored by the restriction point that surveils the mitochondrial health [23, 74, 75, 132, 133, 168]. Defective mitochondrial metabolic checkpoint results in loss of stem cell quiescence and susceptibility to death [23, 75]. The mitochondrial metabolic checkpoint is dysregulated in stem cells during physiological aging, contributing to their functional deterioration [23, 75]. Apoptosis has been widely proposed to be the mechanism of death for aged hematopoietic stem cells (HSCs) [114, 169], but recent studies reveal that caspase 3 is not activated in aged HSCs [166]. Thus, how the cell death processes are regulated in aged HSCs remains elusive.

Sirtuins are a family of protein deacylases that regulate diverse cellular pathways that control metabolism, stress resistance, and genome maintenance [83, 103, 179, 180].

SIRT2 is a mammalian sirtuin that resides in the cytosol and possesses deacetylase activity [181]. We show here that SIRT2 inhibits the cell death processes in HSCs and is required for HSC maintenance and regenerative capacity at an old age. The expression of SIRT2 reduces with age in HSCs and SIRT2 overexpression improves the maintenance and regenerative capacity of aged HSCs. These results suggest that down-regulation of SIRT2 protein levels is causal to the functional decline of HSC aging and highlight the possibility of reversing HSC aging to improve healthspan.

Results

SIRT2 is not required for HSC maintenance at a young age

Transcriptome analysis comparing HSCs from young and old mice have identified genes that are differentially regulated with age in HSCs [124]. SIRT2 is among the genes that are downregulated most in aged HSCs. To validate the microarray finding, we isolated young and old HSCs (immunophenotypically defined as $Lin^-c\text{-}Kit^+Sca1^+CD150^+CD48^-$) from bone marrow of young (3-month-old) and old (24-month-old) mice (Figure 1A). Briefly, bones (including pelvis, femur, tibia and arms) are isolated via surgical dissection from mice. The extraction of bone marrow cells is achieved by crushing the collected bones followed by serial filtering to rid the bone chips and debris. Purified HSCs are isolated via multicolor influx sorter based on its immunophenotypically-defined markers. SIRT2 mRNA level is compared between young and old HSCs by quantitative real-time PCR. We found that SIRT2 mRNA level was reduced by 4-fold in HSCs isolated from old mice compared to those from young mice (Figure 2A). The protein levels of SIRT2 were also reduced in old HSCs by Immunocytochemistry analyses of HSCs from young and old mice (Figure 2B).

To further understand whether the reduced expression of SIRT2 in old HSCs contribute to HSC aging, we analyzed the hematopoietic phenotypes of WT and SIRT2 knockout (KO) mice. SIRT2 KO mice models have been reported [182, 183]. Mice deficient in SIRT2 are viable and they are born at the Mendelian ratio, and interestingly, SIRT2 KO mice are susceptible to inflammation-associated conditions, such as experimental colitis and brain inflammation [182, 183]. We started off analyzing WT and SIRT2 KO mice at young age. SIRT2 KO mice showed approximately 75% lymphoid cells (immunophenotypically-defined as $B220^+$ and $CD3^+$ cells) and 25% myeloid cells (immunophenotypically-defined as $Mac1^+Gr1^+$ cells) in the peripheral blood, which was comparable with young WT controls (Figure 3). No difference in bone marrow cellularity was observed in young SIRT2 KO mice and WT controls (Figure 4A). Similarly, no difference in HSC number was found in the bone marrows of young SIRT2 KO mice and WT controls (Figure 4B). These results suggest that SIRT2 is not required to maintain HSC number at a young age.

To determine whether loss of SIRT2 affects HSC function, we performed a gold-standard competitive bone marrow transplantation assay (Figure 5A). Briefly, CD45.2

donor HSCs were isolated from SIRT2 KO and WT mice. These donor HSCs were subsequently mixed with CD45.1 competitor bone marrow cells respectively. Mixtures of cells were then transplanted into lethally irradiated recipient mice through retro-orbital injections. Donor contributions in the peripheral blood of the recipient mice were monitored over 16 weeks. Donor engraftments in the bone marrow of the recipient mice were evaluated at 16 weeks post transplantation. HSCs isolated from young WT and SIRT2 KO mice were equally adept in reconstituting the hematopoietic system of the recipient mice (Figure 5B). Altogether, these results suggest that SIRT2 is not required for HSC maintenance at a young age.

SIRT2 is required for HSC maintenance at an old age

Because Sirtuins are widely known as stress response proteins, their roles in mediating enhanced healthspan are highly age-dependent [83, 103, 179, 180]. One example is SIRT3, a mitochondrial Sirtuin, which deacetylates numerous mitochondrial metabolic enzymes and proteins to rewire mitochondrial metabolism [33]. SIRT3's protective roles in reducing mitochondrial oxidative stress and maintaining HSC function are only observed at an old age but not at a young age [23]. It has been postulated that the accumulation of a wide range of stressors during physiological aging results in a heightened cellular dependence on stress response proteins, including Sirtuins, in order to resolve stress and maintain homeostasis. In light of this hypothesis, we aged SIRT2 KO and WT control mice for 24 months, and evaluated their hematopoietic phenotypes.

At 24 months old, WT mice developed lineage bias towards the myeloid cells (around 40% lymphoid cells and 60% myeloid cells) in the peripheral blood, which a key feature of the aging hematopoietic system (Figure 6). However, aged SIRT2 KO mice showed severer myeloid bias (Figure 6). There was almost two-fold reduction in the lymphoid cell population and significant increase in the myeloid cell population in the aged SIRT2 KO peripheral system, indicating an accelerated aging phenotype of the hematopoietic system. The bone marrow cellularity was reduced by 40% in aged SIRT2 KO mice compared to WT controls (Figure 7A). The HSC quantities in the bone marrow of SIRT2 KO mice were reduced by 2-fold compared to the WT littermates under homeostatic condition (Figure 7B). These results suggest that SIRT2 is required to maintain HSC number at an old age.

To further test SIRT2's role in HSC maintenance at an old age, we isolated HSCs from aged SIRT2 KO mice and WT littermate controls. The ability for aged SIRT2 KO HSCs to reconstitute the blood system of lethally irradiated recipient mice also decreased by 2-fold compared to their WT counterparts (Figure 8). Together, these data suggest that SIRT2 has an age-dependent effect on HSC maintenance and hematopoiesis.

SIRT2 prevents cell death in aged HSCs

To understand how SIRT2 exerts its age-dependent effect on HSC maintenance, we characterized the phenotypes of SIRT2 KO HSCs. We first sought to determine if the

absence of SIRT2 affects cell survival to account for the age-associated changes in HSC number and repopulating capacity. In flow cytometry analyses of bone marrow cells derived from aged WT and SIRT2 KO mice using 7-Aminoactinomycin D (7AAD) to assess cell viability, we observed increased percentage of cell death in the HSC population of SIRT2 KO mice compared to their WT counterparts (Figure 9). Increased cell death resulting from SIRT2 deficiency was specific to the HSC compartment but not the differentiated populations. While SIRT2 deficient HSCs had increased levels of 7-AAD staining compared to WT controls, the differentiated progeny from both genotypes displayed similar staining (Figure 9). SIRT2 is ubiquitously expressed in various subpopulations in the hematopoietic system (Figure 1B). This expression pattern cannot explain the preferential effect of SIRT2 deletion on HSCs. However, HSCs have greatly reduced levels of NADH [134], a competitive inhibitor of Sirtuins [189]. We speculate that this may lead to increased SIRT2 activity in the HSC compartment. Consistently, deficiency in either SIRT3 or SIRT7 preferentially impacts the cellular activities of HSCs but not their differentiated progeny [23, 75]. These results suggest loss of SIRT2 leads to increased cell death in HSCs, which could potentially explain the reduced HSC number and function in aged SIRT2 KO HSCs.

Under homeostatic conditions, almost 90% of HSCs remain in the quiescent state [70-72,74]. Quiescence is quintessential for HSC maintenance during aging [70-72,74]. Loss of quiescence has been found to be causal to the deterioration of HSC function with aging [70-72,74]. To determine whether the SIRT2 deficiency affects cell cycle status of HSCs, we stained bone marrow cells with HSC and cell cycle markers. The Ki67 protein is highly associated with cellular proliferation and it is expressed at G1 state [23,75]. The 7AAD dye only stains DNA, which is not only used to measure cell death, but also used to distinguish S-G2-M phase of cells [23,75]. Therefore, we incorporate Ki67/7AAD positivity to distinguish different cell cycle stages of HSCs. Aged SIRT2 KO HSCs had reduced G0 population and increased G1 and S-G2-M phases when compared to WT controls (Figure 10). These results suggest that SIRT2 deficiency at an old age reduced quiescence and increased proliferation of HSCs, which probably is a compensatory effect due to increased cell death with loss of SIRT2 protein.

SIRT2 overexpression reverses the increased cell death in HSCs during physiological aging

To investigate whether the SIRT2 mediated cell death regulation is relevant to physiological aging, we evaluated cell death phenotypes of different hematopoietic compartments between young and old WT mice. HSCs from old WT mice showed a two-fold increase in cell death compared to young WT HSCs (Figure 11A). To examine the reduced HSC viability observed in HSCs from freshly isolated bone marrow cells are due to an intrinsic defect in HSC survival, we isolated young and old donor HSCs from WT mice and competitively transplanted the donor HSCs into lethally irradiated young recipient mice. A two-fold increase in cell death was observed in the donor old WT

HSCs compared to donor young HSCs, demonstrating that the defect in survival is intrinsically regulated in old HSCs (Figure 11B).

We next examined whether SIRT2 overexpression reduces the increased cell death in HSCs associated with physiological aging. We purified old WT HSCs using multicolor influx sorter, followed by culturing in cytokine-rich stem cell media for 4 hours to recover the physical stresses that HSCs might experience under sorting procedure. After 4 hours resting, old WT HSCs were subject to lentiviral infection procedure in order to overexpress SIRT2 protein or control empty vector in old HSCs. Briefly, lentiviral-containing media were prepared by transfecting viral constructs and packaging vectors in 293T cells. Then, lentivirus were concentrated by ultra-speed centrifugation, resuspended in the cytokine-rich stem cell media and applied to the sorted old HSCs with polybrene. Two days after initial infection, cells were harvested, stained for HSC markers and analyzed for cell death. SIRT2 overexpression in old WT HSCs significantly reduced cell death percentage in HSCs compared to control infection (Figure 12). In all, these results suggest that SIRT2 overexpression reverses the increased cell death associated with physiological aging in HSCs.

SIRT2 regulates HSC maintenance cell-autonomously

To investigate whether the increased cell death in HSCs from SIRT2 KO mice is due to SIRT2 deficiency in HSCs or the systemic effect of SIRT2 deletion, we reintroduced SIRT2 in aged SIRT2 KO HSCs via lentiviral transduction. Reintroduction of SIRT2 in aged SIRT2 KO HSCs significantly inhibited cell death process (data not shown), indicating that SIRT2 promotes HSC survival cell-autonomously.

We further asked whether SIRT2 regulates HSC maintenance in a cell-autonomous manner. We overexpressed SIRT2 or control protein in isolated aged SIRT2 KO HSCs via lentiviral transduction. These aged SIRT2 KO HSCs that reconstituted with control or SIRT2 protein were then assayed for their function using the competitive bone marrow transplantation method. SIRT2 overexpression in aged SIRT2 KO HSCs lead to a significant increase in donor engraftment in the bone marrow of the recipient mice 16 weeks post transplantation (Figure 13). Together, these results suggest that SIRT2 regulates cell death and HSC maintenance cell-autonomously.

SIRT2 overexpression rejuvenate the functional decline of aged HSCs

SIRT2 levels reduce in HSCs during physiological aging (Figure 2). To determine whether repression of SIRT2 is causal to the functional deterioration of aged HSCs, we overexpressed SIRT2 in aged WT HSCs via lentiviral transduction. We performed competitive bone marrow transplantation assay to compare aged donor WT HSCs transduced with SIRT2 or control protein. Reintroduction of SIRT2 in aged WT HSCs resulted in increased HSC engraftment in the bone marrow of the recipient mice and increased reconstitution capacity in the peripheral blood of the recipients (Figure 14A,

B). Remarkably, restoring SIRT2 levels in aged WT HSCs reversed myeloid biased differentiation of donor cells when examined under competitive transplantation conditions (Figure 14C). In contrast, SIRT2 overexpression in young WT HSCs did not affect the reconstitution capacity of the HSCs (Figure 15A). The functional output of SIRT2 overexpressing young WT HSCs showed similar lineage percentage compared to control (Figure 15B). In all, these results suggest that restoration of SIRT2 levels in aged WT HSCs rejuvenates the functional decline in HSC aging.

Discussion

Collectively, our results establish down-regulation of SIRT2 protein levels is causal to the functional decline of HSC aging. Characteristics of the functional deterioration of aged HSCs are reduced repopulating activity per cell, myeloid biased differentiation, and increased cell death with stress [114, 169]. SIRT2 modulates specific age-associated HSC functions. SIRT2 deficiency had no effect on HSCs at a young age (Figure S2). However, in the absence of SIRT2, reduced repopulating activity, myeloid biased differentiation, and increased cell death are further exacerbated in aged mice (Figure 2, 3). The age-dependent effect of SIRT2 on HSC maintenance is consistent with the observations that mitochondrial stress increases with age in HSCs [75, 84]. At a young age when the mitochondrial stress is low, the protection of SIRT2 from mitochondrial stress associated damages is dispensable. Only at an old age when the mitochondrial stress is heightened, the protection of SIRT2 becomes necessary. Mitochondrial stress preferentially depletes HSCs with lymphoid potential [75, 132], contributing to aging-associated myeloid biased differentiation. SIRT2 overexpression in aged HSCs improved regenerative capacity, and reversed myeloid-biased differentiation (Figure 5). Notably, functional deterioration of aged HSCs observed in mice is conserved in humans [112]. Our findings are particularly exciting from the therapeutics standpoint of view, since activation of SIRT2 may offer opportunities to reverse the physiological impact of aging on HSCs and thereby improve the repair and maintenance of the aging tissue.

Cell death process is prominently activated in HSCs but not as much in their differentiated progeny during physiological aging in the bone marrow, suggesting that physiological aging particularly burdens the HSC compartment and aged HSCs endure substantially higher levels of stresses due to their long lifespan relative to their differentiated shorter-lived progeny. Alternatively, HSCs may have lower threshold for cell death activation, as an evolved adaptation to remove damaged cells and remain pristine.

The detailed molecular mechanisms on how SIRT2 regulates cell death processes in HSCs remain an open question. One possible approach to tackle this question is to examine the degree of diverse caspases activation pathways. Recent advancement in cell death processes reveals the existence of multiple cell death pathways, including the apoptosis, necrosis, necroptosis, and pyroptosis [188,193-194]. The canonical apoptosis process involves activation of initiator caspases, and effector caspases

(caspase 3, 6 and 7). Apoptosis is a programmed and non-inflammatory cell death process, while other types of cell death processes represent a messier and pro-inflammatory form of death [188,193-194]. Apoptosis has been widely proposed to be the mechanism of death for aged hematopoietic stem cells (HSCs) [114, 169], but recent studies reveal that caspase 3 is not activated in aged HSCs [166]. Thus, the mode of death for aged HSCs remains elusive. It will be of great interest to identify the cell death pathway regulated by SIRT2 in HSCs during physiological aging.

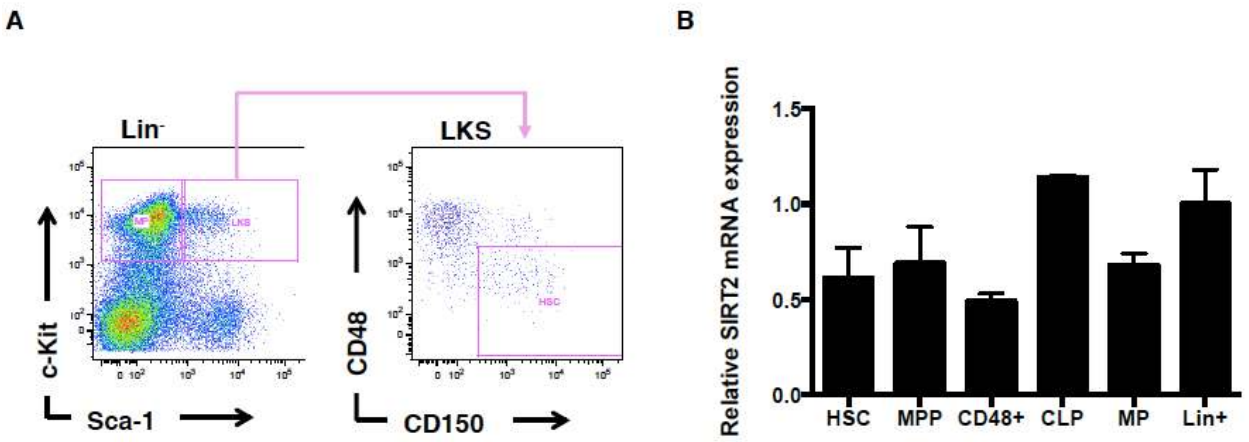

Figure 1. SIRT2 is ubiquitously expressed in various hematopoietic cellular compartments in the bone marrow. A, Gating strategy. Lin-, lineage negative cells. LKS, Lin-c-Kit+Sca1+ cells. MP, myeloid progenitor cells. B, Various cell populations in the bone marrow were isolated via cell sorting based on cell surface markers. HSC, Lin-c-Kit+Sca1+CD150+CD48-; multipotent progenitors (MPPs), Lin-c-Kit+Sca1+CD150-CD48-; CD48+, Lin-c-Kit+Sca1+CD48+; CLP, Lin−IL7Rα+c-kitmed/Sca1med; myeloid progenitors (MPs), Lin-c-Kit+Sca1-; and differentiated blood cells, Lin+. The expression of SIRT2 was determined by qPCR. n=3.

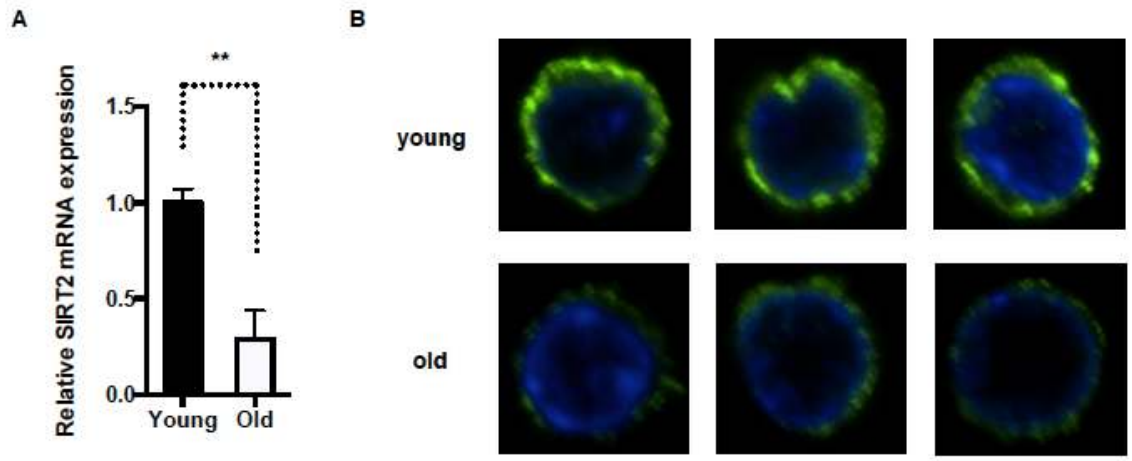

Figure 2. SIRT2 expression reduces with age in HSCs. A, B, The expression of SIRT2 in HSCs isolated from the bone marrow of young (3 months old) and old mice (24-month- old) was quantified by qPCR (A, n=4) or detected by immunocytochemistry (B, n=3). Blue: DAPI. Green: SIRT2. Error bars represent SE. **: $p<0.01$. Student's *t* test.

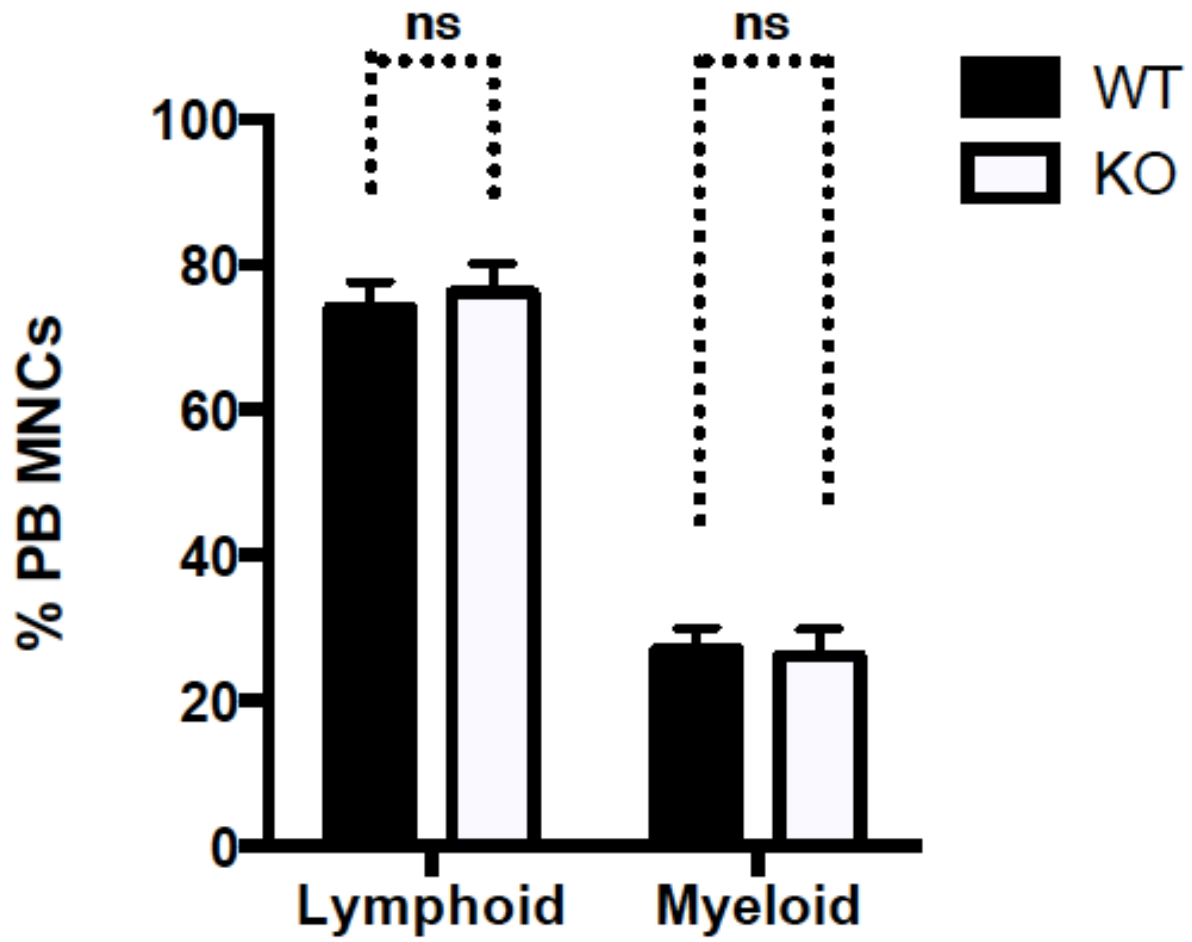

Figure 3. Lineage differentiation in the peripheral blood of 3-month-old WT and SIRT2 KO mice. Lymphoid: $B220^+$ and $CD3^+$ cells, Myeloid: $Mac1^+GR1^+$, MNCs: mononuclear cells. n=9. Error bars represent SE. ns: $p>0.05$. Student's t test.

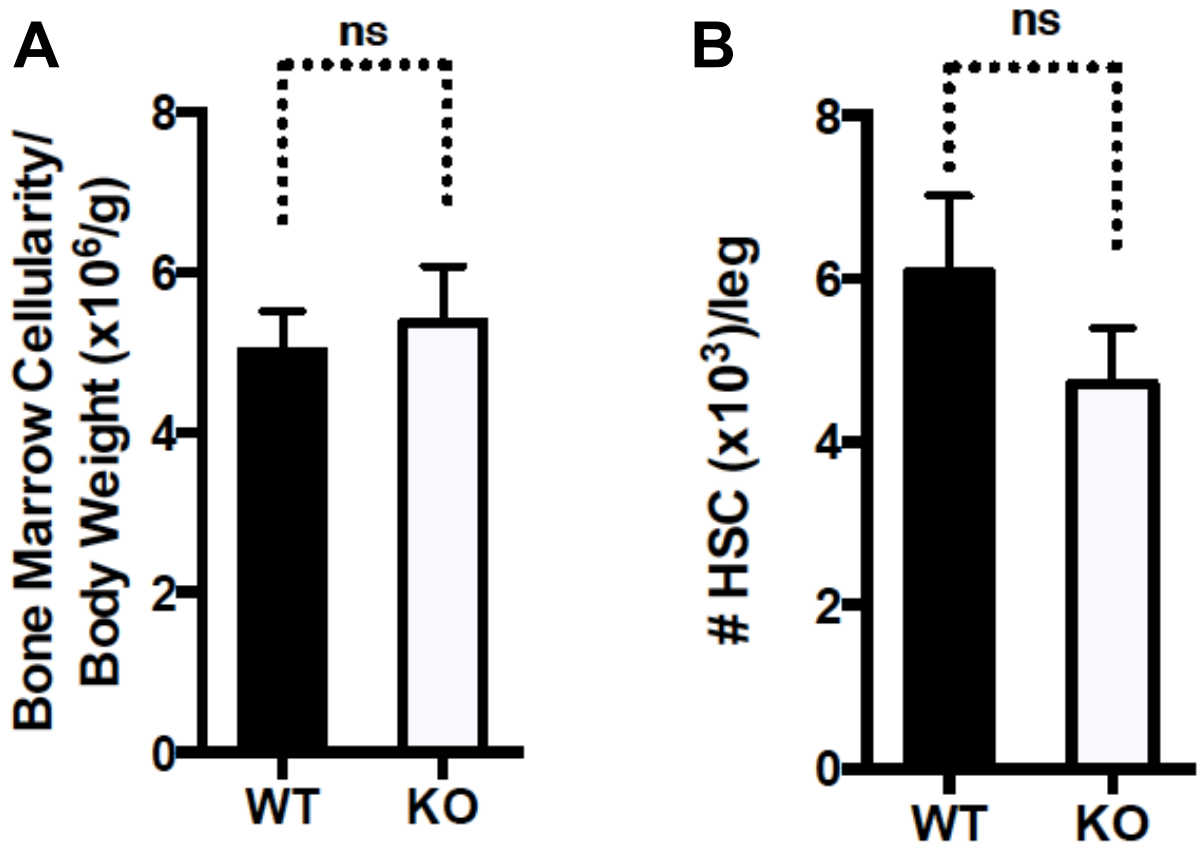

Figure 4. SIRT2 is not required for HSC number at a young age. A, Bone marrow cellularity in 3-month-old WT and SIRT2 KO mice. n=9. B, The frequency of HSCs in the bone marrow of 3-month-old WT and SIRT2 KO mice was determined via flow cytometry. Data presented are the number of HSCs per leg. n=9. Error bars represent SE. ns: $p>0.05$. Student's t test.

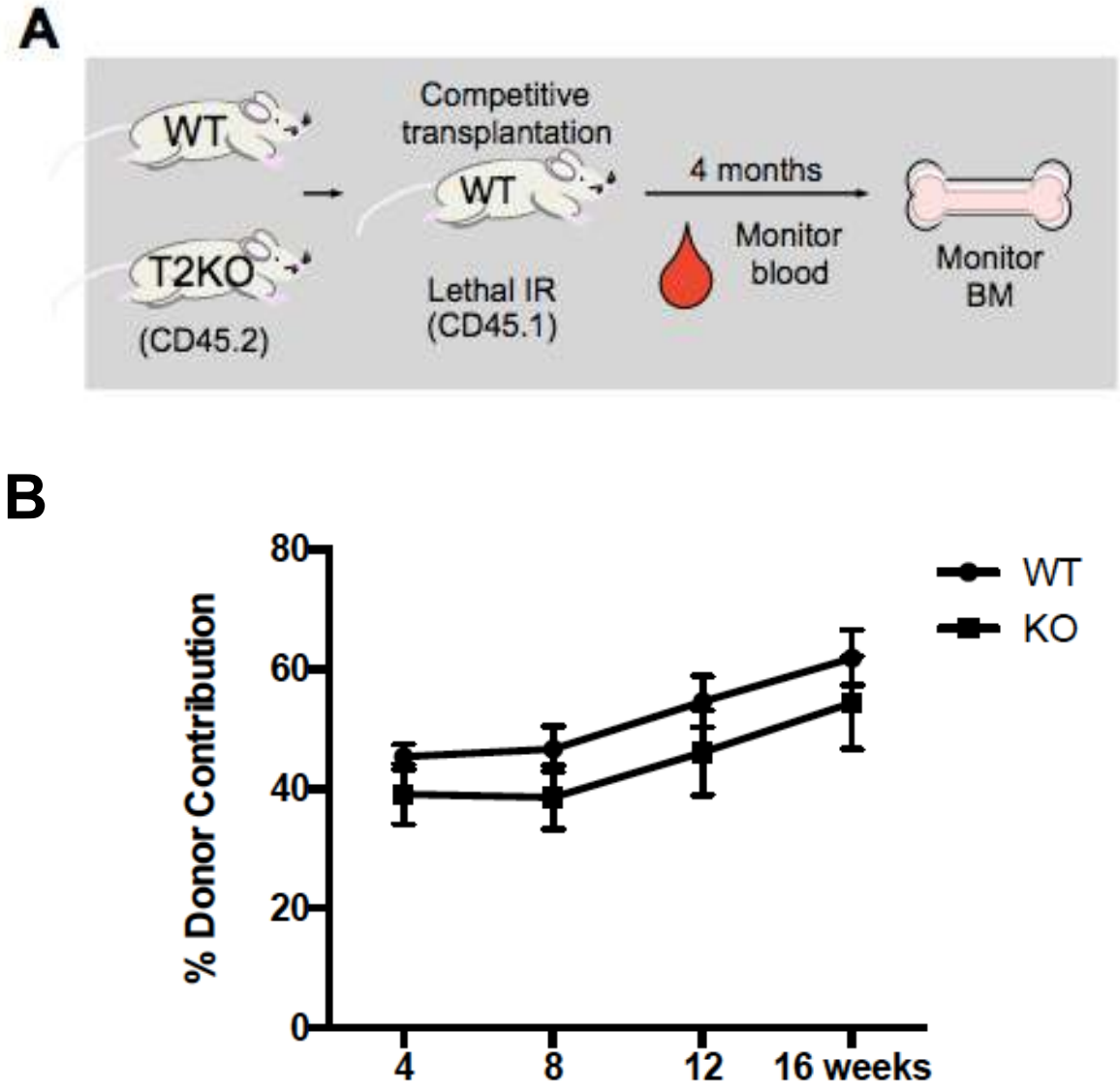

Figure 5. SIRT2 is not required for HSC function at a young age. A, Schematic design for Fig 5B; B, Competitive transplantation using HSCs isolated from 3-month-old WT and SIRT2 KO mice as donors. Data shown are the percentage of total donor-derived cells in the peripheral blood (PB) of the recipients. n=15, Error bars represent SE. ns: $p>0.05$. Student's t test.

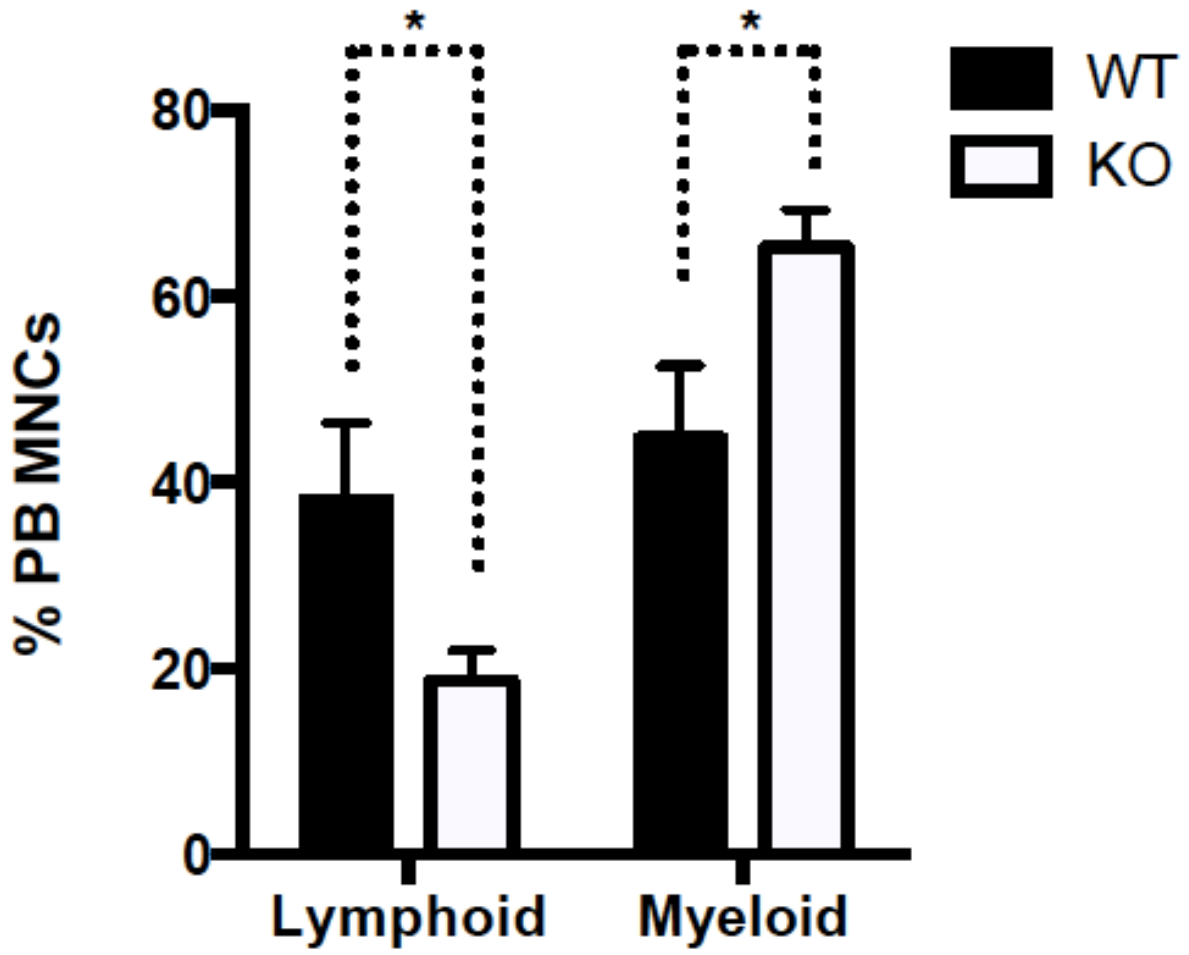

Figure 6. Lineage differentiation in the peripheral blood of 24-month-old WT and SIRT2 KO mice. Lymphoid: $B220^+$ and $CD3^+$ cells, Myeloid: $Mac1^+GR1^+$, MNCs: mononuclear cells. n=9. Error bars represent SE. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. Student's *t* test.

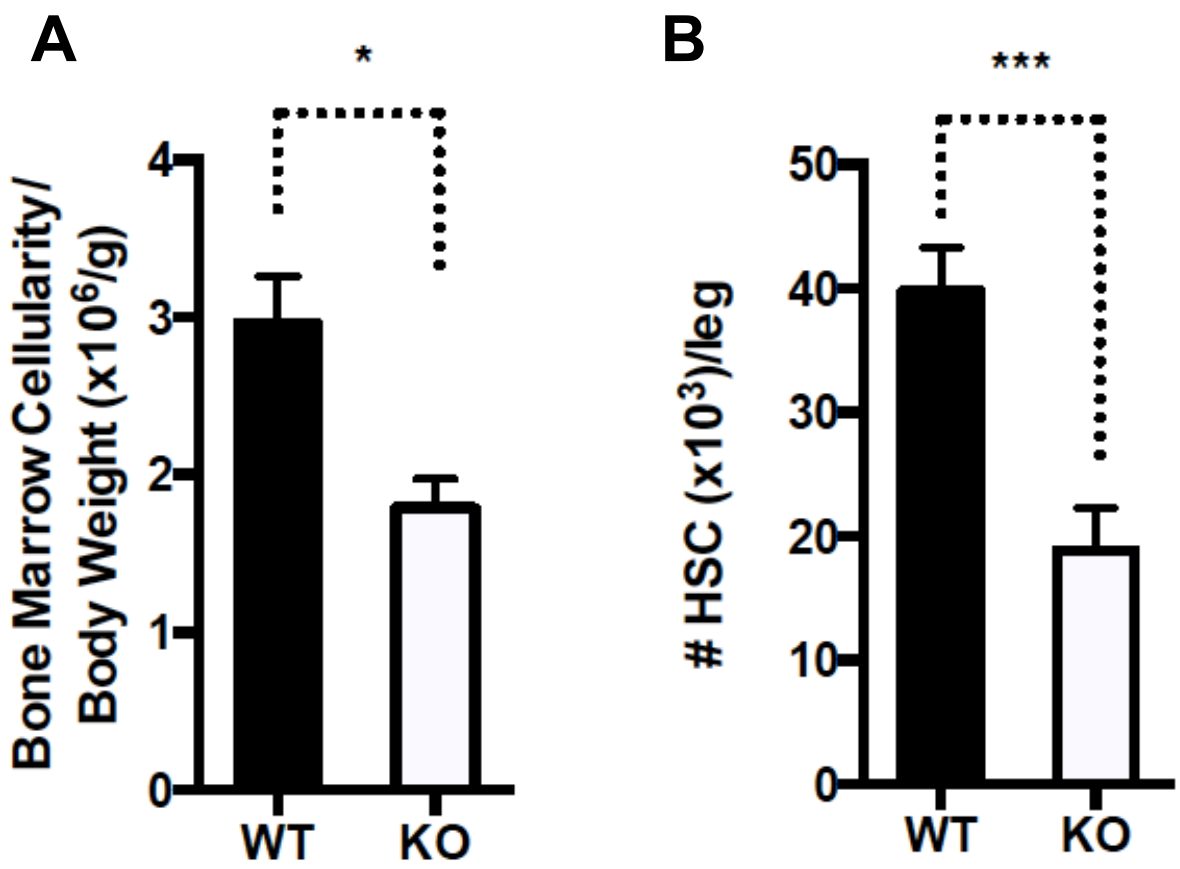

Figure 7. SIRT2 is required for HSC number at an old age. A, Bone marrow cellularity in 24-month-old WT and SIRT2 KO mice. n=6. B, The frequency of HSCs in the bone marrow of 24-month-old WT and SIRT2 KO mice was determined via flow cytometry. Data presented are the number of HSCs per leg. n=6. Error bars represent SE. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. Student's *t* test.

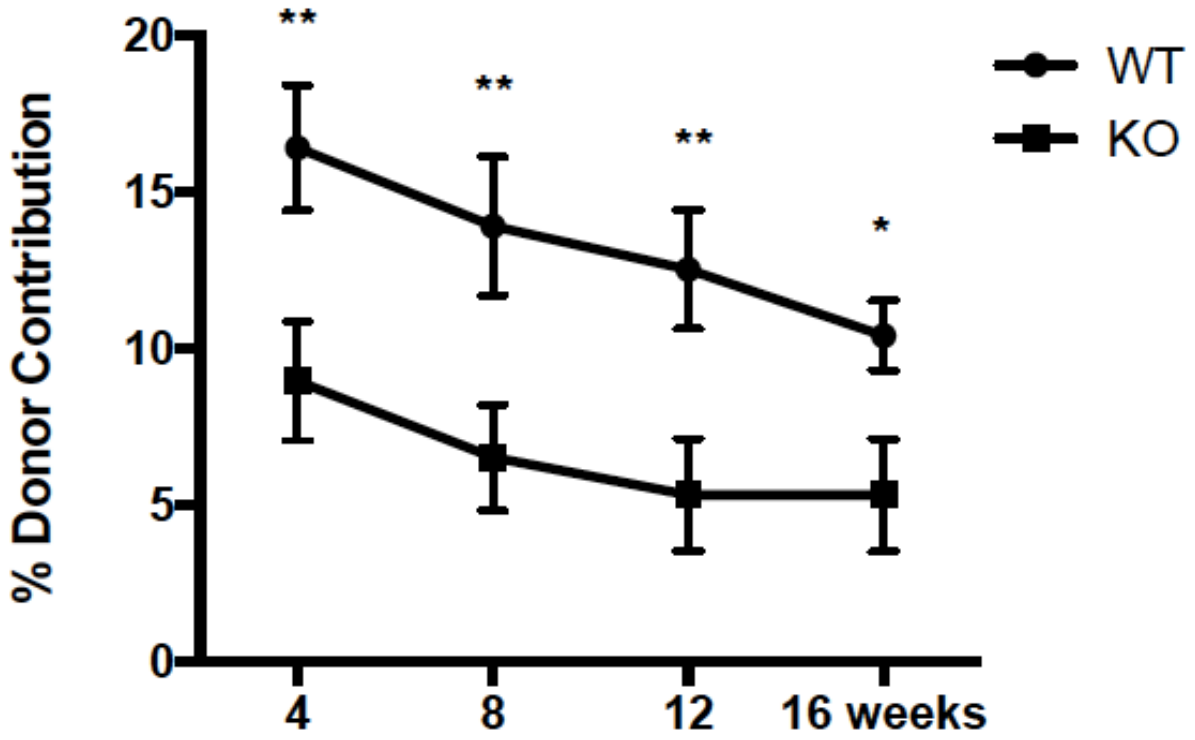

Figure 8. SIRT2 is required for HSC maintenance at an old age. Competitive transplantation using HSCs isolated from 24-month-old WT and SIRT2 KO mice as donors. Data shown are the percentage of total donor-derived cells in the peripheral blood (PB) of the recipients. n=15. Error bars represent SE. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. Student's *t* test.

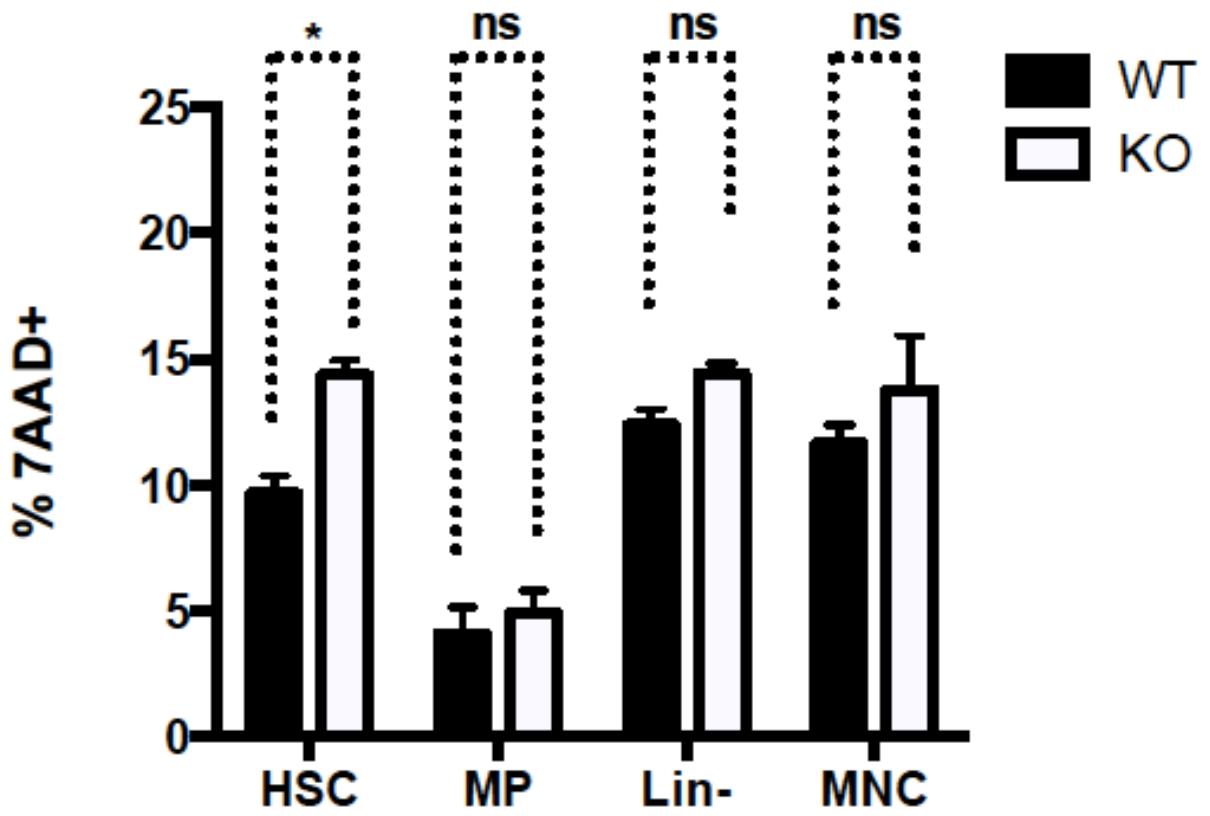

Figure 9. SIRT2 prevents cell death in aged HSCs. Flow cytometry analyses of staining for 7AAD of bone marrow cells derived from aged WT and SIRT2 KO. n=6, Error bars represent SE. *: p<0.05. **: p<0.01. ***: p<0.001. Student's *t* test.

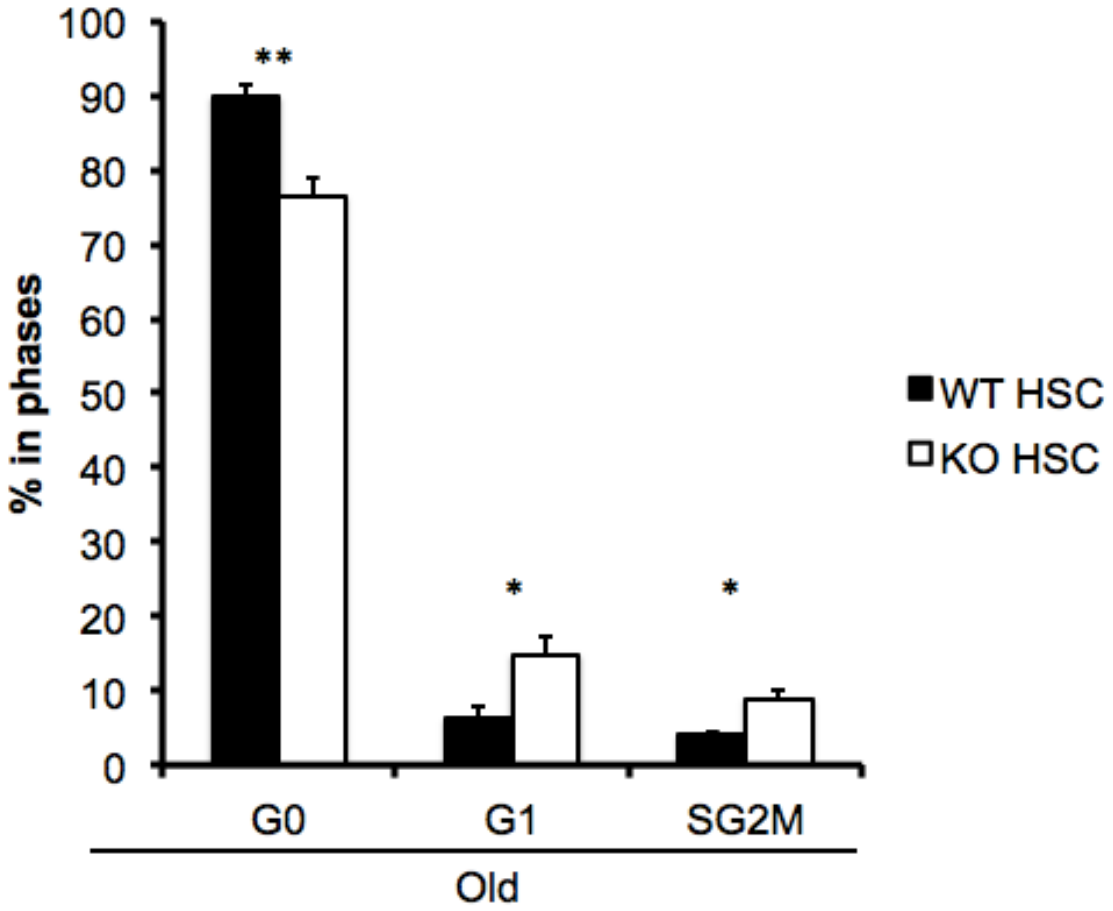

Figure 10. SIRT2 regulates cell cycle status in aged HSCs. Flow cytometry analyses of staining for Ki67/7AAD of bone marrow cells derived from aged WT and SIRT2 KO. n=6, Error bars represent SE. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. Student's *t* test.

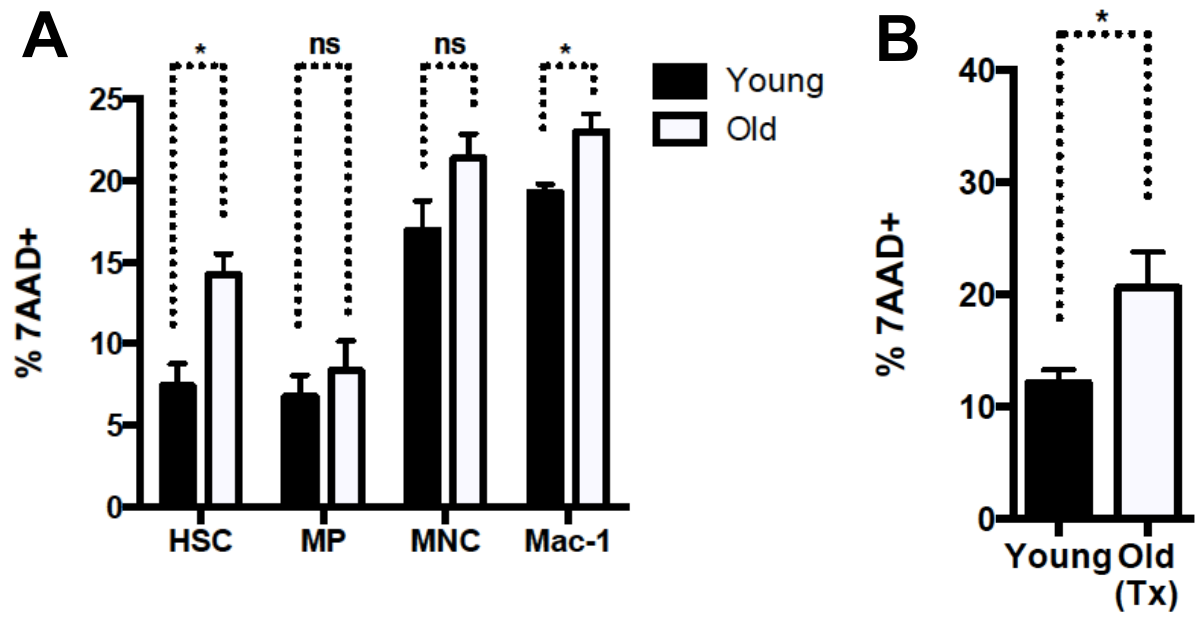

Figure 11. Induction of cell death in aged HSCs. A, Flow cytometry analyses of staining for 7AAD of bone marrow cells derived from young and aged WT mice. n=6, B, Flow cytometry analyses of staining for 7AAD of young and aged WT donor HSCs in bone marrow cells derived from recipient mice. n=13, Error bars represent SE. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. Student's *t* test.

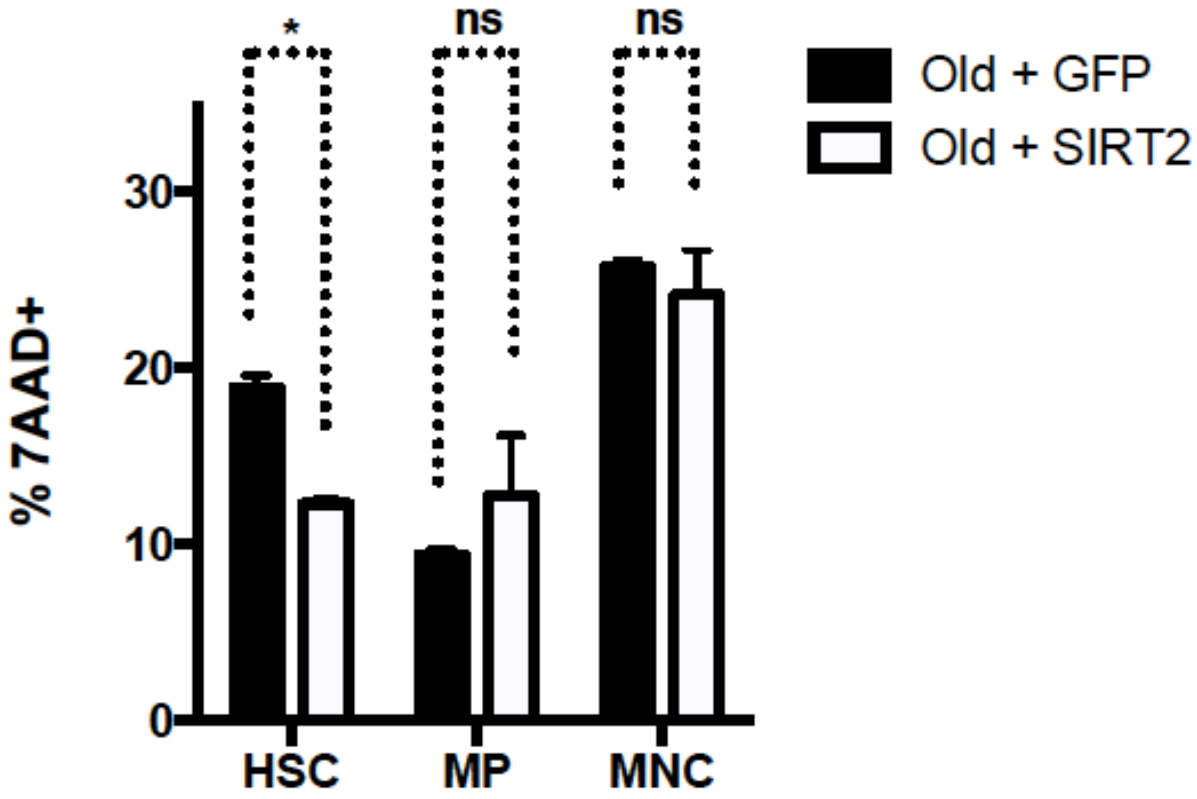

Figure 12. SIRT2 overexpression reverses the increased cell death in HSCs associated with physiological aging. Aged HSCs transduced with SIRT2 or control lentivirus were stained for 7AAD, and analyzed by flow cytometry. n=6. Error bars represent SE. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. Student's *t* test.

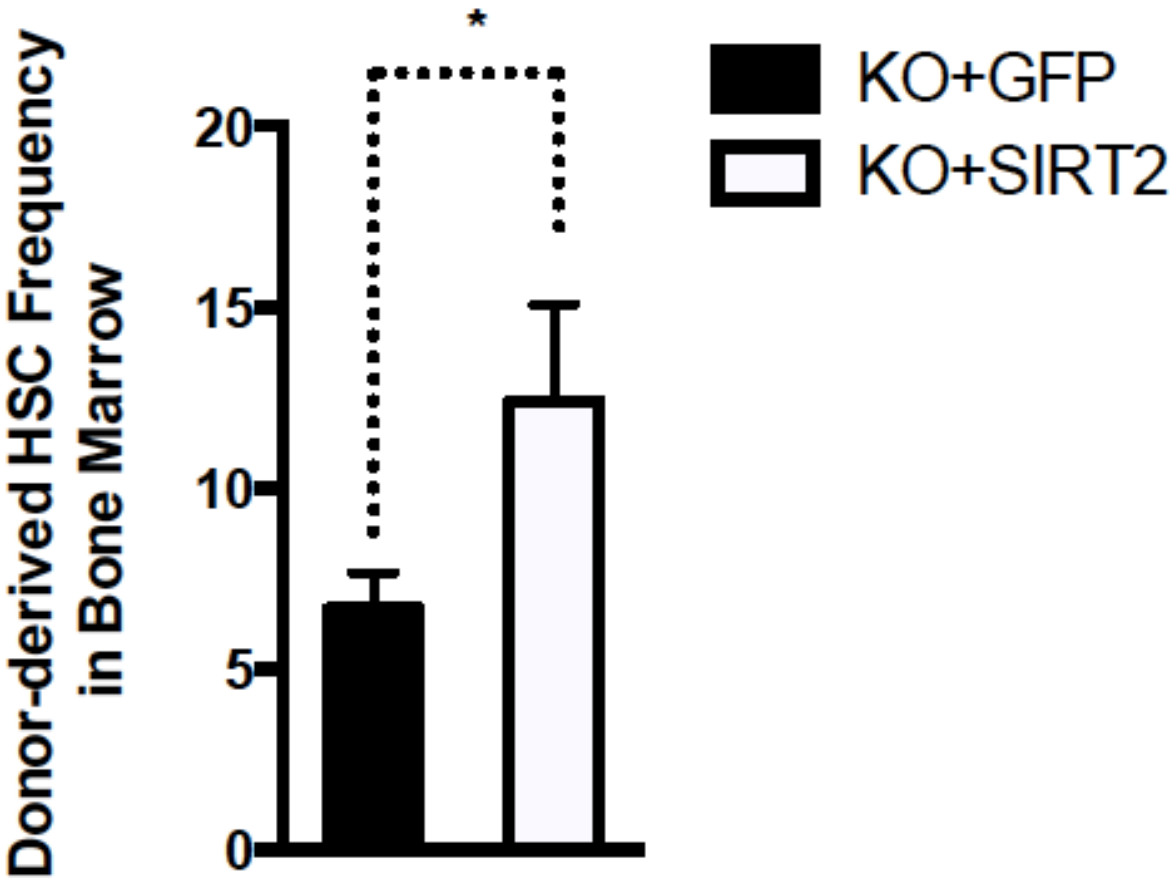

Figure 13. SIRT2 regulates HSC maintenance cell-autonomously. Aged SIRT2 KO HSCs transduced with SIRT2 or control lentivirus were used as donors in a competitive transplantation assay. Data shown are the percentage of donor-derived HSC contribution in the bone marrow of the recipients, n=6. Error bars represent SE. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. Student's *t* test.

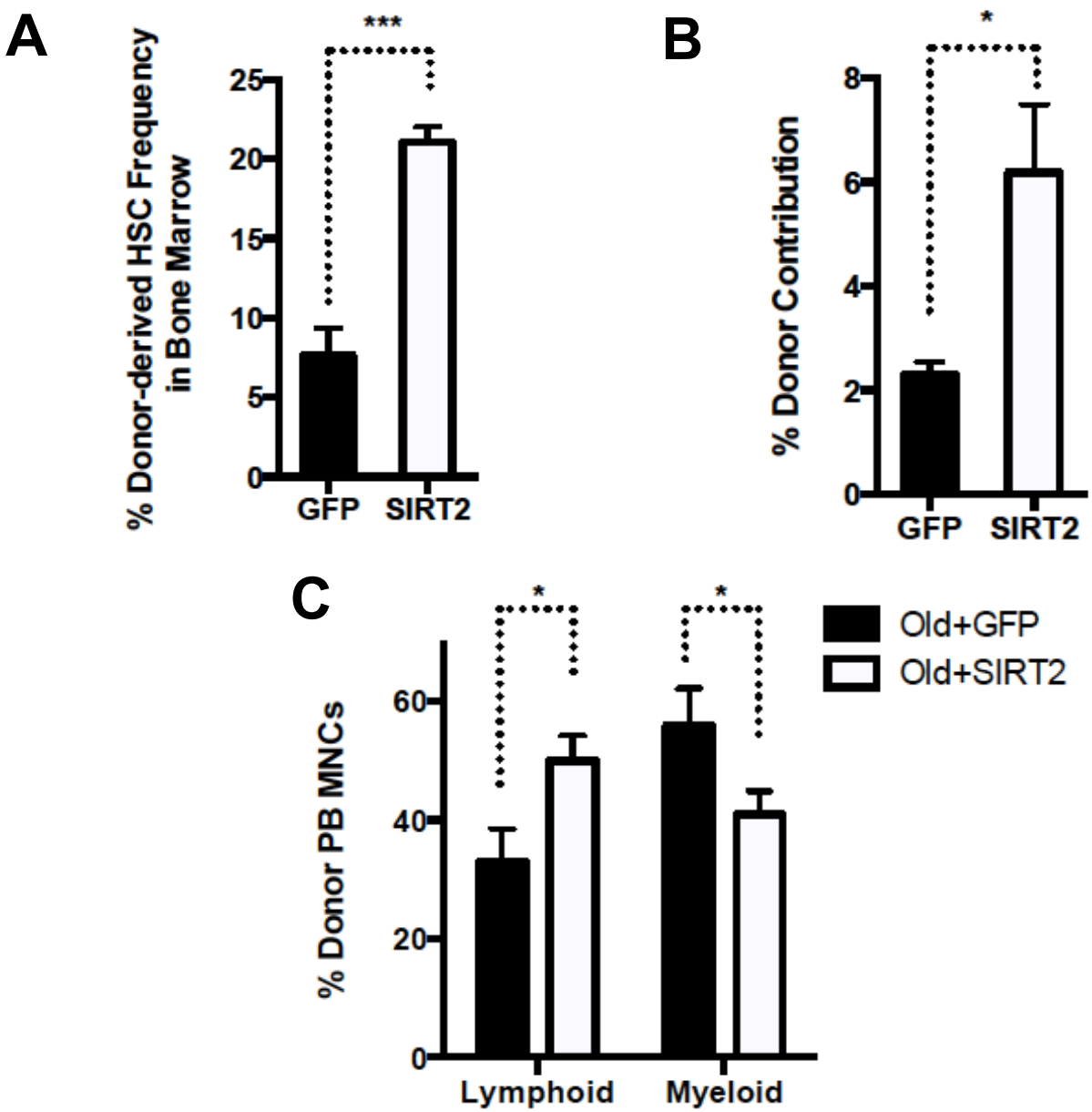

Figure 14. SIRT2 overexpression rejuvenates the functional decline in HSC aging. Competitive transplantation using aged HSCs transduced with SIRT2 or control lentivirus as donors. Data shown are the donor-derived HSC engraftment in the bone marrow (A), the percentage of donor-derived cells in the peripheral blood (B), and donor-derived lineage differentiation in the peripheral blood (C). n=6. Error bars represent SE. *: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. Student's *t* test.

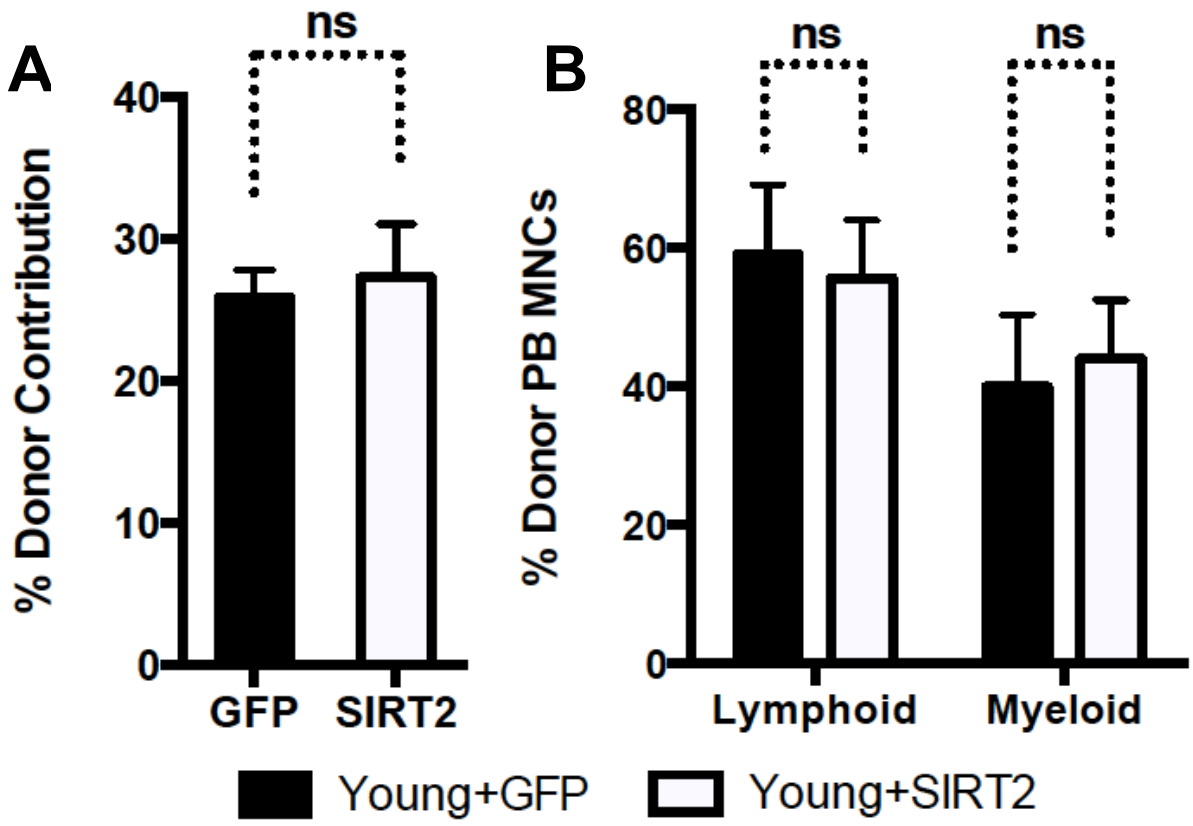

Figure 15. SIRT2 overexpression has no effect on young WT HSC function. Competitive transplantation using young HSCs transduced with SIRT2 or control lentivirus as donors. Data shown are the percentage of donor-derived cells in the peripheral blood of the recipients (A) and donor-derived lineage differentiation in the peripheral blood (B). n=6. Error bars represent SE. *: $p<0.05$. ns: $p>0.05$. Student's t test.

Chapter 3: SIRT2 reverses aging associated chronic inflammation in macrophages

Summary

In Chapter 2, we found that the down-regulation of SIRT2 is causal to the functional decline in HSC aging, and importantly, restoring SIRT2 levels in old HSCs can reverse this process. Since SIRT2 KO mice are reported to be susceptible to inflammation-associated conditions, including experimental colitis and brain inflammation [182, 183], we are interested to test SIRT2's role in chronic inflammation in this chapter. Chronic NLRP3 inflammasome activation during aging induced stress has a causal role in developing pathological inflammation in sterile inflammatory diseases, such as atherosclerosis, Alzheimer's disease, Parkinson's disease, obesity, diabetes, multiple sclerosis, and cancer [149, 171-175]. In light of this, we explored whether SIRT2 suppresses aging-associated chronic inflammation through regulating the NLRP3 inflammasome activity. We found that SIRT2 specifically inhibits NLRP3 inflammasome in macrophages. NLRP3 inflammasome is activated in macrophages with age, with a concomitant reduction in SIRT2 levels. Enforced SIRT2 expression in macrophages from old mice reverses aging-associated NLRP3 inflammasome activation, suggesting a potential reversible mechanism for the aging-associated inflammation phenotype.

Introduction

The low-grade systemic, sterile inflammation underlies multiple aging-associated diseases, including metabolic disorders, neurodegenerative diseases, and cancer [136]. Remodeling of the innate and acquired immune systems that occur during aging results in chronic inflammatory cytokine production [136-138]. This process turns the beneficial role of the inflammatory response towards pathogenic agents early in life into a detrimental incubator for the aforementioned aging-associated chronic disorders later in life. It still remains elusive how remodeling of the immune system during aging is regulated. Myeloid bias, a hallmark of hematopoietic stem cell aging that is characterized by a lineage differentiation bias towards myeloid cells, could partially explain this immune system remodeling in a quantitative perspective [109-113]. From a qualitative angle, the accumulation of environmental and self-derived stressors that serve as chronic activators of the innate immune system may promote the remodeling process with age [109-113]. Nonetheless, the appealing questions currently are to define the molecular basis of immune system remodeling during aging and to explore the potential reversibility of low-grade systemic inflammation that comes with aging.

Inflammasomes are cytosolic multiprotein complexes that act as innate immune system receptors and sensors, in which they play critical roles in the host defense mechanisms against infectious pathogens [147, 148]. The assembly of inflammasomes in response

to diverse stimuli activates caspase-1 and induces secretion of inflammatory cytokines [147-149]. Thus, aberrant inflammasome activations are believed to be involved in a variety of inflammatory disorders [147-149]. The NLRP3 inflammasome is unique in that it can be activated in response to the widest array of stimuli, including sterile metabolic activators in the absence of overt infection [148-152]. These sterile metabolic activators of the NLRP3 inflammasome seem to accumulate in humans during aging or over-nutrition [149,152]. Mice deficient in NLRP3 are protected from either aging-induced or obesity-induced inflammation and insulin resistance [153,154]. These findings raise the possibility that targeting the regulation of NLRP3 activity during aging may hold promise to potentially reversing the low-grade systemic inflammation that is seen in the elder population.

Studies from the Akira group demonstrated that the microtubule arrangement of mitochondria is the key to activating NLRP3 inflammasome complex [192]. Loss of intracellular NAD+ or inhibition of SIRT2 activity promotes activation of NLRP3 inflammasome via indirect microtubule-mediated mechanisms [192]. These results highlight the possibility that SIRT2 may modulate NLRP3 inflammasome activity in macrophages to regulate sterile inflammation. Indeed, SIRT2 KO mice are reported to be susceptible to inflammation-associated conditions, including experimental colitis and brain inflammation [182, 183]. Therefore, we set out to explore whether SIRT2 suppresses aging-associated inflammation through modulating NLRP3 inflammasome activity.

Previous studies in our lab have confirmed that SIRT2, a cytosolic NAD+-dependent deacetylase, prevents caspase 1 activation and IL1b secretion in a NLRP3 inflammasome-dependent manner. SIRT2 levels are reduced with aging in macrophages, while the NLRP3 inflammasome is specifically activated during aging. Here, we report a cell-based system that can faithfully recapitulate aging-associated inflammation. Using this system, we show that enforced expression of SIRT2 reduces NLRP3 activity in macrophages derived from old mice. Thus, our results highlight a novel and reversible mechanism for the NLRP3 inflammasome-mediated activation of low-grade systemic inflammation that occurs during physiological aging stress.

Results

SIRT2 specifically inhibits the NLRP3 inflammasome activation in macrophages

SIRT2 has been reported to suppress NLRP3 inflammasome activation in macropahges through indirectly modulating intracellular microtubule dynamics [192]. To confirm this finding, we primed bone marrow derived macrophages (BMDM) with LPS and then activated inflammasomes by their specific inducers (nigericin or ATP for NLRP3 inflammasome; flagellin for NLRC4 inflammasome; dsDNA for AIM2 inflammasome) [150]. SIRT2 KO macrophages presented increased processing of caspase 1 and secreted more IL-1β compared to WT macrophages only upon NLRP3 inflammasome

stimulation, but not upon NLRC4 or AIM2 inflammasome stimulation (data not shown). Overexpressing of SIRT2 in THP1 cells, a human monocyte cell line, by lentiviral transduction, lead to a reduction in caspase 1 processing under NLRP3 inflammasome stimulation (data not shown). Together, these data indicate that SIRT2 specifically inhibits the NLRP3 inflammasome.

SIRT2 reverses aging-associated inflammation in macrophages

To test whether SIRT2 is involved in regulating aging-associated inflammation in macrophages, we isolated primary BMDMs from young (2-month-old) and old (2-years-old) mice. SIRT2 level is significantly reduced in primary BMDMs isolated from old mice (data now shown). Reduced level of SIRT2 in old primary BMDMs lead us to examine whether the NLRP3 inflammasome is specifically activated in macrophages during aging. BMDMs from old mice showed increased cleavage of IL-1b in response to NLRP3 stimuli but not NLRC4 or Aim2 stimuli compared to young BMDMs (data not shown). These results suggest that NLRP3 inflammasome is specifically activated during aging.

To investigate whether restoring SIRT2 level in old macrophages reverses aging-associated inflammatory phenotypes, we immortalized myeloid progenitors from young and old mice using Hoxb8 oncoprotein via retroviral transduction (Figure 1A) [200]. Macrophages differentiated from old immortalized myeloid progenitors exhibited increased caspase 1 processing and mature IL1b secretion compared to young controls under NLRP3 stimulation (Figure 1B). These results demonstrate that the cell-based system can faithfully recapitulate the aging-associated inflammatory phenotypes in macrophages. Using this system, we overexpressed SIRT2 in old immortalized myeloid progenitors via lentiviral transduction, further differentiated them into mature macrophages, and compared the response to diverse inflammasome stimulation (Figure 2A). SIRT2 overexpression significantly reduced caspase 1 cleavage and p17 IL-1β upon ATP but not flagellin stimulation (Figure 2B). Together, these results suggest that SIRT2 overexpression reverses aging-associated NLRP3 activation in macrophages.

Discussion

Collectively, our results establish the deregulation of SIRT2-NLRP3 inflammasome axis as a driver of chronic inflammation during aging. SIRT2, a primarily cytosolic NAD+-dependent deacylase, suppresses chronic inflammation through suppressing the NLRP3 inflammasome in cells. Recent advances in the field have highlighted the role of mitochondria in NLRP3 inflammasome activation [158, 192, 201-203]. Mitochondria serve as a platform for assembling the NLRP3 inflammasome complex and house the effector molecules that directly activate the NLRP3 inflammasome in macrophages [158, 192, 201-203]. Studies from the Akira group demonstrated that the microtubule arrangement of mitochondria is the key to activating NLRP3 inflammasome complex

[192]. Loss of intracellular NAD+ or inhibition of SIRT2 activity promotes activation of NLRP3 inflammasome via indirect microtubule-mediated mechanisms [192]. Consistent with their findings, we provide a novel and reversible regulation on NLRP3 inflammasome activation during stress conditions, such as physiological aging.

Post-translational modifications play pivotal roles in regulating inflammasome activities [156]. NLRC4 inflammasome activity is stimulated by PKCδ mediated phosphorylation and ubiquitination [178]. Similarly, multiple post-translational modifications of NLRP3 inflammasomes have been identified, including phosphorylation, ubiquitination, deubiquitination, ADP-ribosylation, S-nitrosylation, and proteolytic processing [178]. However, modification sites of most these modifications on NLRP3 inflammasome remain unclear, leaving many open questions for further efforts to map and elucidate the functional domain of NLRP3 inflammasome activation. In an independent study under Chapter 2, we found that the NLRP3 inflammasome is acetylated in macrophages. Further, we determined the lysine residues on NLRP3 that are modified by acetylation using mass spectrometry analysis. Mutagenesis study of the identified lysine residues elucidates NLRP3 inflammasome acetylation functionally facilitates pyroptosome formation and cytokine secretion. Our systematic molecular and biochemical analysis of NLRP3 acetylation provides significant insights into inflammasome regulation under various pathophysiological conditions. However, whether SIRT2-mediated deacetylation of the identified lysine residues is responsible for NLRP3 inflammasome activation during aging remains to be tested.

The SIRT2-mediated NLRP3 inflammasome regulation of chronic inflammation has broad implications in physiology and disease. It will be of great interest to characterize the inflammation and metabolic phenotypes of SIRT2 KO mice. If our model stays correct, mice deficient in SIRT2 are expected to be susceptible to aging-associated or over-nutrition-associated glucose intolerance. Interestingly, SIRT2 levels are reduced in macrophages with age, which could potentially explain the aging-associated inflammation phenotypes in macrophages. Our work is particularly intriguing from the therapeutic standpoint, as enforced SIRT2 expression in macrophages derived from old mice rescues the aging-associated NLRP3 inflammasome activation, which leads to a revert of the aging-associated inflammatory phenotype. Further studies are needed to elucidate whether SIRT2 overexpression in macrophages with old age reverses the development of chronic inflammation in vivo. Our results raise the possibility of developing therapeutic molecules that target the SIRT2-NLRP3 inflammasome regulatory network to treat pathological inflammation conditions associated with aging.

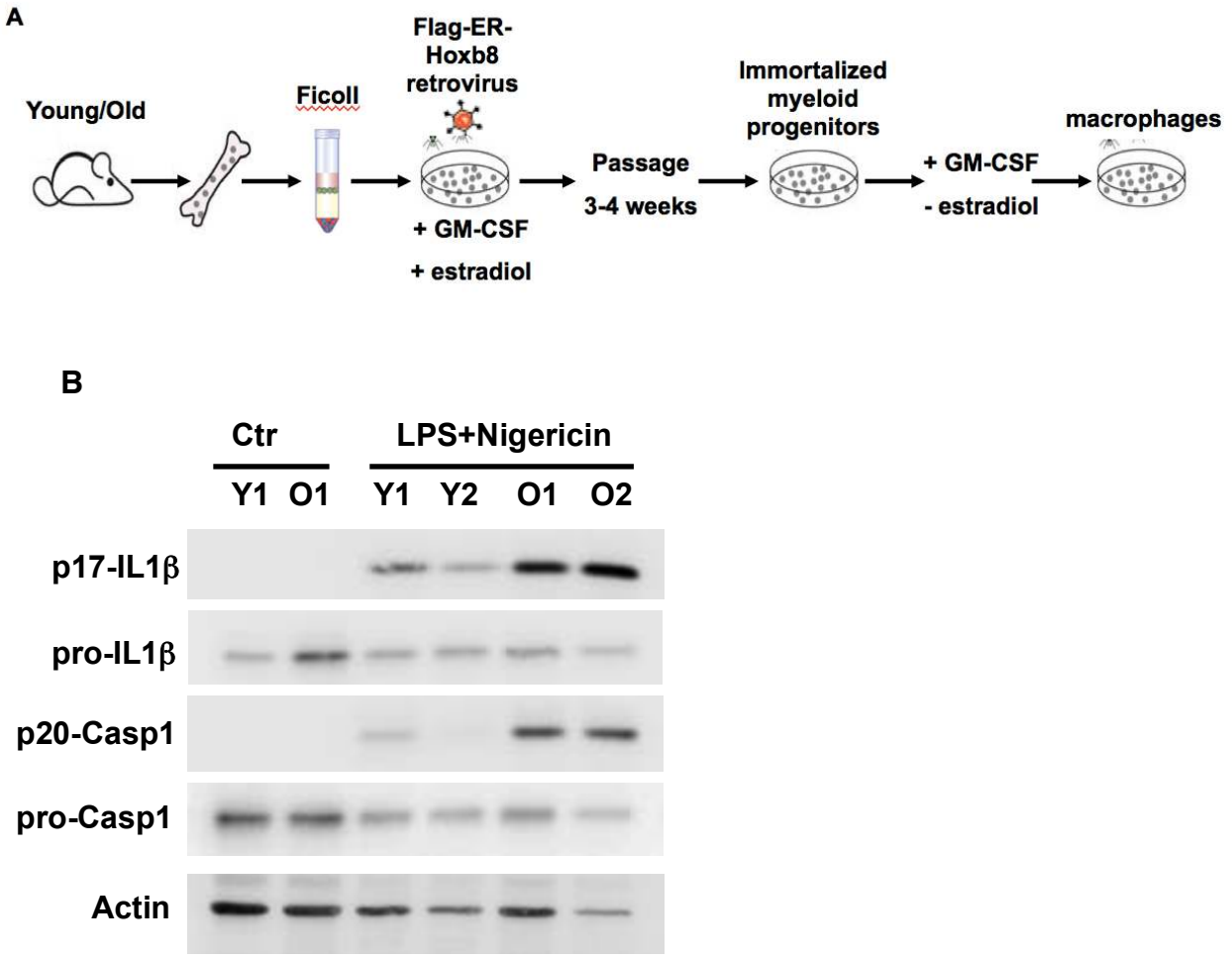

Figure 1. A cell-based system that faithfully recapitulates aging-associated inflammation in macrophages. (A) Schematic experimental design for Figure 1B. (B) Progenitors from young (2 months old) or old (2 years old) wild type mice were immortalized with ER-Hoxb8, differentiated into macrophages, and treated with or without LPS and nigericin.

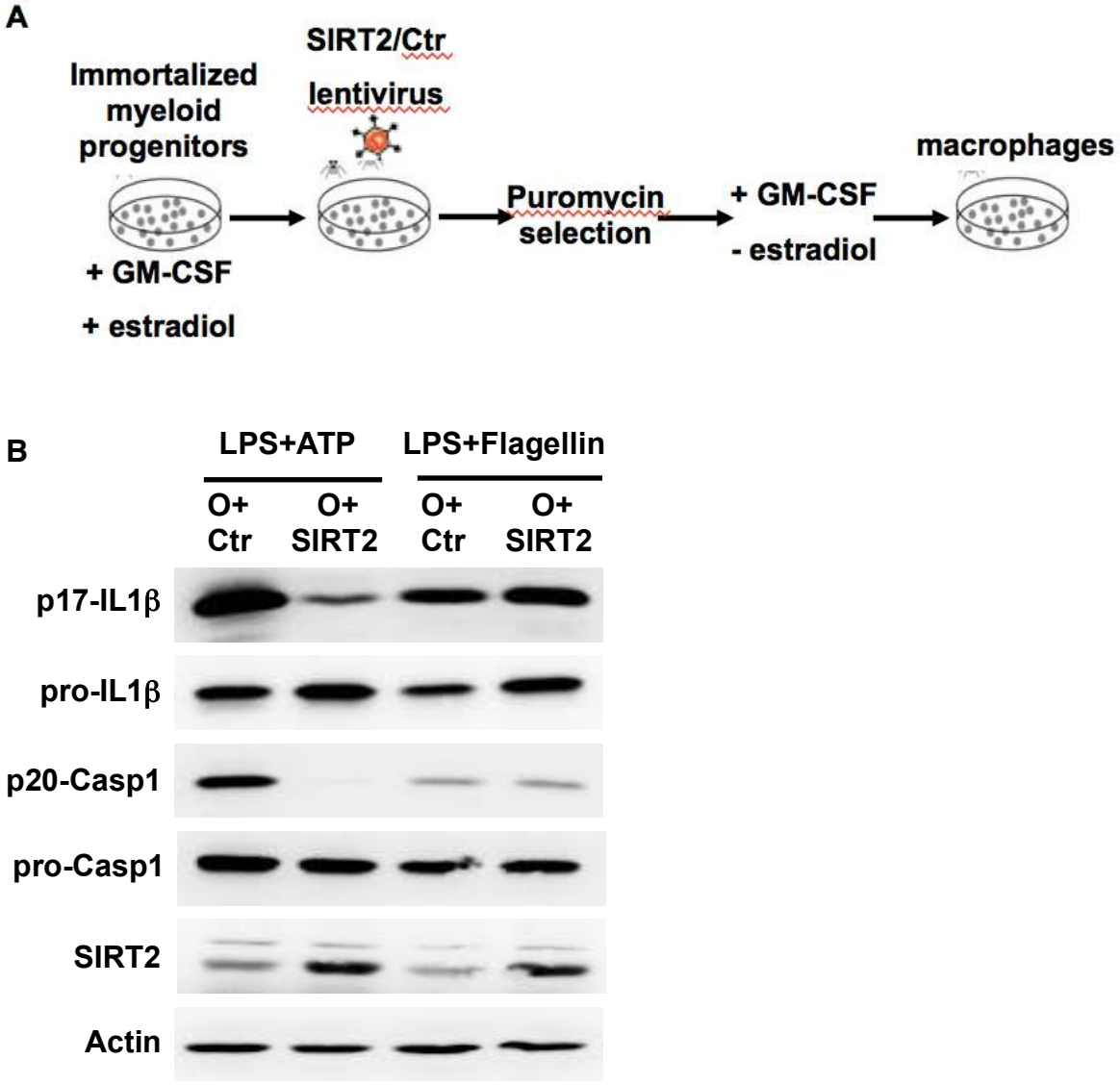

Figure 2. SIRT2 overexpression reduces NLRP3 activity in macrophages derived from aged mice. (A) Schematic experimental design for Figure 2B. (B) Immortalized progenitors from old wild type mice were transduced with control or SIRT2 Lentivirus, differentiated into macrophages, treated with LPS/ATP or LPS/flagellin. Cell lysates were used for Western analyses for pro-IL1b and pro-caspase 1 and culture supernatants used for p17-IL1b and p20-caspase 1 Western analyses.

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Chapter 4: Concluding remarks and future directions

The goal of this dissertation work was to advance current knowledge on the three fundamental questions in the aging field: Why do we age? How do we age? Can we age healthily? Using mouse genetics, molecular biology, cell biology and biochemical approaches, this investigation has elucidated the role of one member of nutrient sensors, SIRT2, in maintaining hematopoietic stem cell function and suppressing chronic inflammation during physiological aging.

In Chapter 2, we focused on hematopoietic stem cells and aimed to understand the molecular basis underlying changes in HSCs during aging. We found that SIRT2, a cytosolic member of the Sirtuin protein deacylases family, suppresses cell death processes in HSCs. SIRT2 levels are downregulated in old HSCs, resulting in the aberrant activation cell death processes in HSCs during physiological aging. The activation of cell death pathways in HSCs requires physiological intrinsic cues, as the increased cell death in old HSCs persisted in young microenvironment under transplantation setting. Our lab has previously demonstrated that mitochondrial stress accumulates with age in HSCs. It remains to be tested whether the accumulation of mitochondrial stress serves as a trigger for increased cell death observed in HSCs during physiological aging. Importantly, restoring SIRT2 levels in old HSCs reduces cell death activation, and reverses the functional decline in HSCs. Taken together, this study highlighted down-regulation of SIRT2 protein levels as a reversible driver of HSC aging.

In Chapter 3, we focused on another prominent aspect of aging, which is chronic inflammation. Consistent with its previously reported roles in suppressing inflammation, SIRT2 constrains NLRP3 inflammasome activation in macrophages. Interestingly, SIRT2 levels are reduced in macrophages. This reduction in SIRT2 expression contributes to the activation of the NLRP3 inflammasome in macrophages during aging. Forced expression of SIRT2 in old macrophages reverses the aging-associated inflammation phenotypes.

Studies in both Chapter 2 and Chapter 3 demonstrated that the down-regulation of SIRT2 protein is causal to two major aging-associated physiological conditions: functional decline in HSCs and chronic inflammation associated metabolic disorders (Fig 1). The decline in SIRT2 protein levels in HSCs and primary macrophages with age result in the exacerbated decline in tissue homeostasis. Forced expression of SIRT2 in both old HSCs and macrophages reverses the functional decline associated with aging. Without a doubt, these studies open up exciting therapeutic avenues to treat diseases of aging.

Multiple intriguing questions remain to be answered, as knowledge has no limit when it comes to healthy aging. One outstanding question involves the regulation of SIRT2 expression during aging. Why do SIRT2 levels decrease with age? Potential alterations in epigenetic programming or changes in RNA splicing and processing machinery during aging awaits exploration. What are the molecular mechanisms for SIRT2's role in

cell death regulation in HSCs? Can we rejuvenate old HSCs by simply blocking cell death processes? Does SIRT2 have conserved functions in different stem cell types? Can we develop specific SIRT2 activators to boost its activity at old age? Can a SIRT2 activator reverse aging-associated functional declines? Further research endeavors will hopefully provide insights into these questions.

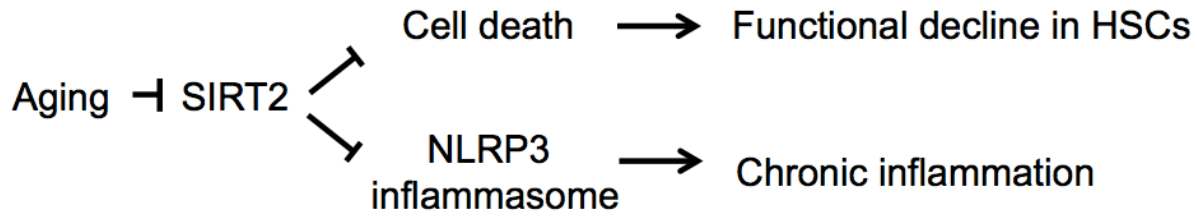

Figure 1. Overall model. The cytosolic NAD^+-dependent deacetylase SIRT2 protein is involved in the development of two physiological aging conditions: functional decline in HSCs and chronic inflammation. SIRT2 levels reduce with age. Forced expression of SIRT2 in both old HSCs and macrophages reverses the functional decline associated with aging.

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Appendix: Materials and Methods

Materials and Methods for Chapter 2

Mice

SIRT2 knockout mice have been described previously [182, 204]. All mice were housed on a 12:12 hr light:dark cycle at 25°C. Animal procedures were performed in accordance with the animal care committee.

Flow Cytometry and Cell Sorting

Bone marrow cells were obtained by crushing the long bones with sterile PBS without calcium and magnesium supplemented with 2% FBS. Lineage staining contained a cocktail of biotinylated anti-mouse antibodies to Mac-1 (CD11b), Gr-1 (Ly-6G/C), Ter119 (Ly-76), CD3, CD4, CD8a (Ly-2), and B220 (CD45R) (BioLegend). For detection or sorting, we used streptavidin conjugated to APC-Cy7, c-Kit-APC, Sca-1-Pacific blue, CD48-FITC, and CD150-PE (BioLegend). For congenic strain discrimination, anti-CD45.1 PerCP and anti-CD45.2 PE-Cy7 antibodies (BioLegend) were used. To determine intracellular activation of specific caspases, fluorescent labelled inhibitors of caspases (FLICA) probe assays (ImmunoChemistry Technologies) and active caspase 3 detection kits (BD Pharmingen) were used based on the manufacturer's instructions. All data were collected on a Fortessa (Becton Dickinson) and data analysis was performed with FlowJo (TreeStar). For cell sorting, lineage depletion or c-kit enrichment was performed according to the manufacturer's instructions (Miltenyi Biotec). Cells were sorted using a Cytopeia INFLUX Sorter (Becton Dickinson).

Lentiviral Transduction of HSCs

As previously described [205], sorted HSCs were prestimulated for 5-10 hr in a 96 well U bottom dish in StemSpan SFEM (Stem Cell Technologies) supplemented with 10% FBS (Stem Cell Technologies), 1% Penicillin/Streptomycin (Invitrogen), IL3 (20ng/ml), IL6 (20ng/ml), TPO (50ng/ml), Flt3L (50ng/ml), and SCF (100ng/ml) (Peprotech). SIRT2 was cloned into the pFUGw lentiviral construct. shRNAs were cloned into pFUGw-H1 lentiviral construct. Lentivirus was produced as described [9], concentrated by centrifugation, and resuspended with supplemented StemSpan SFEM media. The lentiviral media were added to HSCs in a 96 well plate, spinoculated for 90 min at 270G in the presence of 8ug/ml polybrene. This process was repeated 24 hr later with a fresh batch of lentiviral media.

Transplantation Assays

250 sorted HSCs from CD45.2 donor mice were mixed with $5x10^5$ CD45.1 B6.SJL competitor bone marrow cells and injected into lethally irradiated B6.SJL recipient mice. To assess multilineage reconstitution of transplanted mice, peripheral blood was

collected every month for 4 months by retroorbital bleeding. Red blood cells were lysed and the remaining blood cells were stained with CD45.2 FITC, CD45.1 PE, Mac1 PerCP, Gr1 Cy7PE, B220 APC, and CD3 PB (Biolegend). Bone marrow cells were analyzed 4 months posttranplantation.

mRNA analysis
RNA was isolated from cells using Trizol reagent (Invitrogen). cDNA was generated using the qScript™ cDNA SuperMix (Quanta Biosciences). Gene expression was determined by real time PCR using Eva qPCR SuperMix kit (BioChain Institute) on an ABI StepOnePlus system. All data were normalized to b-Actin expression.

Immunocytochemistry
Immunocytochemistry of HSCs was performed as previously described [80]. Briefly, cells were directly sorted onto a glass slide, fixed with 4% paraformaldehyde (PFA), and stained with SIRT2 antibody. Nuclei were identified by staining with DAPI. Subcellular localizations were determined using confocal microscopy.

Statistical Analysis
No statistical methods were used to predetermine sample size. The number of mice chosen for each experiment is comparable to published literature for the same assays performed. Mice were randomized to groups and analysis of mice and tissue samples was performed by investigators blinded to the treatment or the genetic background of the animals. No data were excluded. Statistical analysis was performed with Excel (Microsoft). Means between two groups were compared with two-tailed, unpaired Student's t-test. Error Bars represent standard errors. In all corresponding figures, * represents $p<0.05$. ** represents $p<0.01$. *** represents $p<0.001$. ns represents $p>0.05$.

Materials and Methods for Chapter 3

Mice
SIRT2 knockout mice has been described previously [182,204]. All mice were housed on a 12:12 hr light:dark cycle at 25°C. The mice were fed with ad libitum normal chow diet consisting of 4.5% fat or high fat diet consisting of 60% calories from fat (Harland Teklad) starting from weaning at 3 weeks of age. Animal procedures were performed using gender-matched littermates. All experiments and animal use were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at University of California, Berkeley.

Cell culture
To induce caspase 1 activation, primary macrophages were primed with 100ng/ml LPS for 12 hours and then stimulated with 3mM ATP for 0.5 hour, or 20uM nigericin for 1hr, or 1ug/ml Flagellin for 1hr or 1ug/ml dsDNA for 1hr. Proteins from culture media were trichloroacetic acid (TCA) precipitated for Western analyses.

Isolation and immortalization of myeloid progenitors

We isolated bone marrow from the femurs of mice after ammonium-chloride-potassium lysis of red blood cells and centrifugation onto a cushion of Ficoll-Paque. Ficoll-purified progenitors were pre-stimulated for 48 hours in 50ng/ml SCF, 25ng/ml IL-3 and 25ng/ml IL-6. We infected progenitors with ER-Hoxb8 retrovirus and the culture them in medium containing 1uM estrogen [200]. We selected immortalized myeloid progenitors by moving nonadherent progenitor cells every 3 days to a new culture well for 3 weeks. Differentiation to macrophages was performed by removal of estrogen from the culture medium.

Lentiviral and retroviral transduction

To overexpress SIRT2 in myeloid progenitors, SIRT2 was cloned into the pFUGw lentiviral construct. Lentivirus was produced as described [23, 75], concentrated by centrifugation, and resuspended with culture medium. Retrovirus was generated by transfecting 293T cells (ATCC) with pMSCVgfp retroviral constructs as well as VSV-G and gag/pol expression vectors using Lipofectamine 2000 transfection kit (Invitrogen). 48 hours posttransfection, culture supernatant was filtered through 0.45-mm-pore cellulose acetate filters, supplemented with 10 μg/ml of polybrene, and was applied to target cells. The cells were subjected to another cycle of infection on the next day.

Statistical Analysis

The differences between genotypes or treatments were compared with two-tailed, unpaired Student's t-test. Error Bars represent standard errors. In all corresponding figures, * represents $p<0.05$. ** represents $p<0.01$. *** represents $p<0.001$. ns represents $p>0.05$. The results were presented as means ± s.e.m. No statistical methods were used to predetermine sample size. The number of mice chosen for each experiment is comparable to published literature for the same assays performed. No animals were excluded from the analyses.