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Toward the Understanding of the Metabolic Regulation of Sirtuins

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Toward the Understanding of the Metabolic Regulation of Sirtuins

By

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Abstract

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The ability to sense and respond to fluctuations in environmental nutrient levels is a requisite for life. Therefore, multiple nutrient sensing mechanisms are developed though evolution. Silent information regulator 2 (Sir2) proteins, or sirtuins, are highly conserved nicotinamide adenine dinucleotide (NAD+) dependent protein deacetylases. Their dependence on NAD+ links their activity to cellular metabolic status to serve as critical intracellular nutrient sensors. There are seven sirtuins in mammals and these proteins may mediate some of the benefits of caloric restriction by modulating energy metabolism, genomic stability and stress resistance. However, the molecular mechanisms by which sirtuins exert their physiological or pathological influences are not fully understood. The aim of this dissertation work was to gain molecular insights into this knowledge by studying the two aspects of biology: chronic inflammation-induced insulin resistance, and mitochondrial unfolded protein response (mtUPR).

Chronic low-grade inflammation has been shown to contribute to the pathophysiology of many diseases, such as atherosclerosis, diabetes, Alzheimer's disease, Parkinson's disease, and cancer. In addition, SIRT2, a cytosol localized sirtuin, has been reported to suppress inflammation in multiple inflammation-inducing mouse models. Based on these findings, we investigated whether SIRT2 suppresses chronic inflammation through regulating inflammasome activity. We found that SIRT2 specifically inhibits the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in macrophages by directly deacetylating NLRP3. We confirmed that NLRP3 acetylation enhances its inflammasome function by mutating the deacetylated lysine residues to mimic the constitutively deacetylated state. Under conditions of aging and overnutrition, two prominent risk factors associated with insulin resistance, SIRT2 ablation in mice leads to increased systematic chronic inflammation and insulin resistance. These

results establish a novel regulation of the NLRP3 inflammasome by SIRT2, and identify a nodal control point at the interface of nutrient sensing and innate immunity.

Perturbation of mitochondrial protein homeostasis (proteostasis), also known as mitochondrial protein folding stress (mtPFS), activates the mtUPR, a retrograde signaling pathway leading to transcriptional upregulation of mitochondrial chaperones, repression of translation, and stress relief. The mtUPR is a nascent cellular pathway: the molecular components and physiological relevance of which are not well understood. Our lab has previously shown that SIRT7, a nucleus localized sirtuin, alleviates the mtPFS. Here we set up the experimental system of mtUPR by treating the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) in HEK293T and Hela cells. We found that CDDO treatment leads to mitochondrial aggregation to a focus nearby the nucleus, and widespread increased electron density in the mitochondrial matrix under electron microscopy. We are going to analyze whether genetic manipulation of SIRT7 will also change these two newly identified mtPFS markers. In addition, we demonstrated global transcriptional downregulation of genes encoded by mitochondrial DNA in response to mtPFS, which can potentially relieve the stress and serve as a novel branch of mtUPR.

These results not only broaden the understanding of inflammasome regulations and inflammasome-related diseases, but also provides important insights into detailed molecular mechanisms of mtUPR. Further, from the therapeutic standpoints, these findings also open novel avenues to treat diseases characteristic of chronic inflammation or mitochondrial dysfunction.

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Chapter 1: Introduction

It is essential for life to have the ability to sense and respond to fluctuations in environmental nutrient levels. Therefore, multiple nutrient sensing mechanisms and pathways are developed though evolution. However, nutrient-sensing pathways are commonly dysregulated in human diseases that are characteristic of chronic inflammation or mitochondrial dysfunction, such as metabolic disorders, neurodegenerative diseases, and cancer. This indicates the negative impacts of chronic overnutrition in our modern lifestyle. On the other hand, calorie restriction has been shown to increase lifespan and healthspan across multiple species. Therefore, understanding how dysregulated nutrient sensing pathways lead to chronic inflammation and mitochondrial dysfunction is critical to deal with these chronic degenerative diseases. 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 nutrient sensing pathways: chronic inflammation and mitochondrial dysfunction, with discussions on the inflammasomes and mitochondrial stresses respectively.

Part I: Nutrient sensing and the oxidative stress response

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 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 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 oxidative burst 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 burst 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 procancer 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-indepen- dent 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.

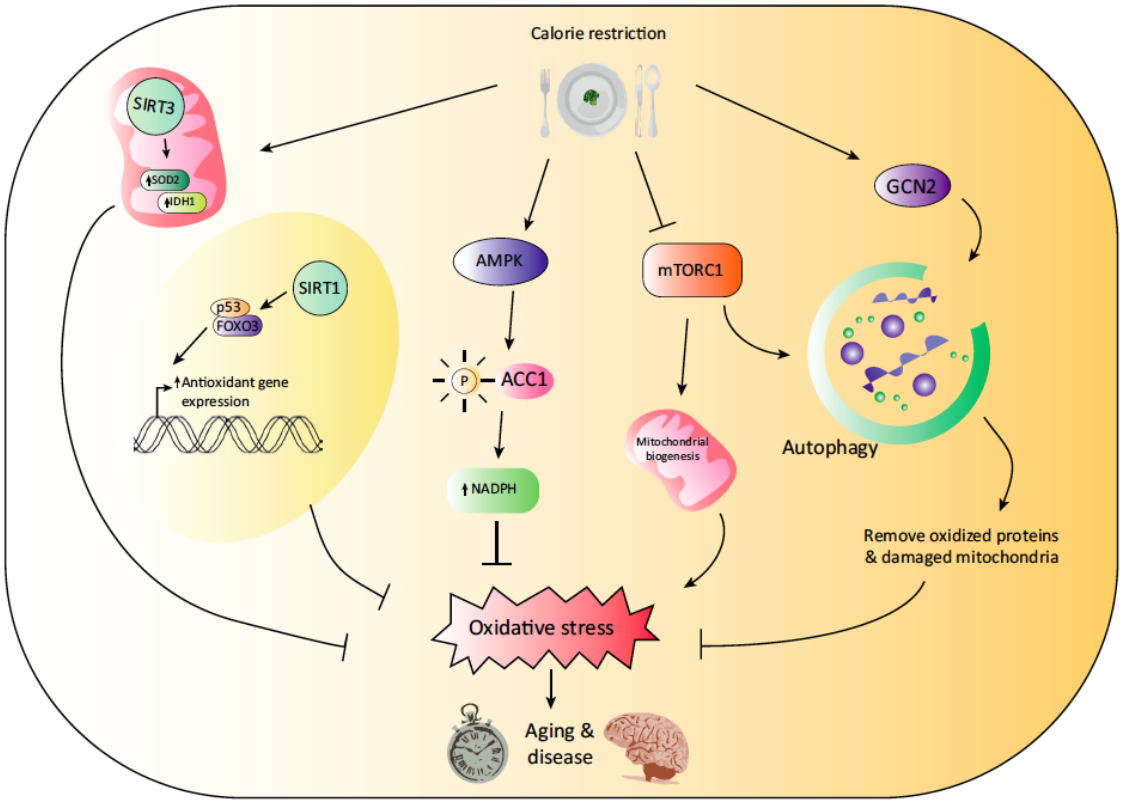

Figure 1. Calorie restriction triggers an active defense program to reduce oxidative stress and prevent aging and aging-associated diseases. SIRT1 and SIRT3 reduce oxidative stress by regulating the transcriptional expression or post-translational modification of antioxidants. AMP-activated protein kinase (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.

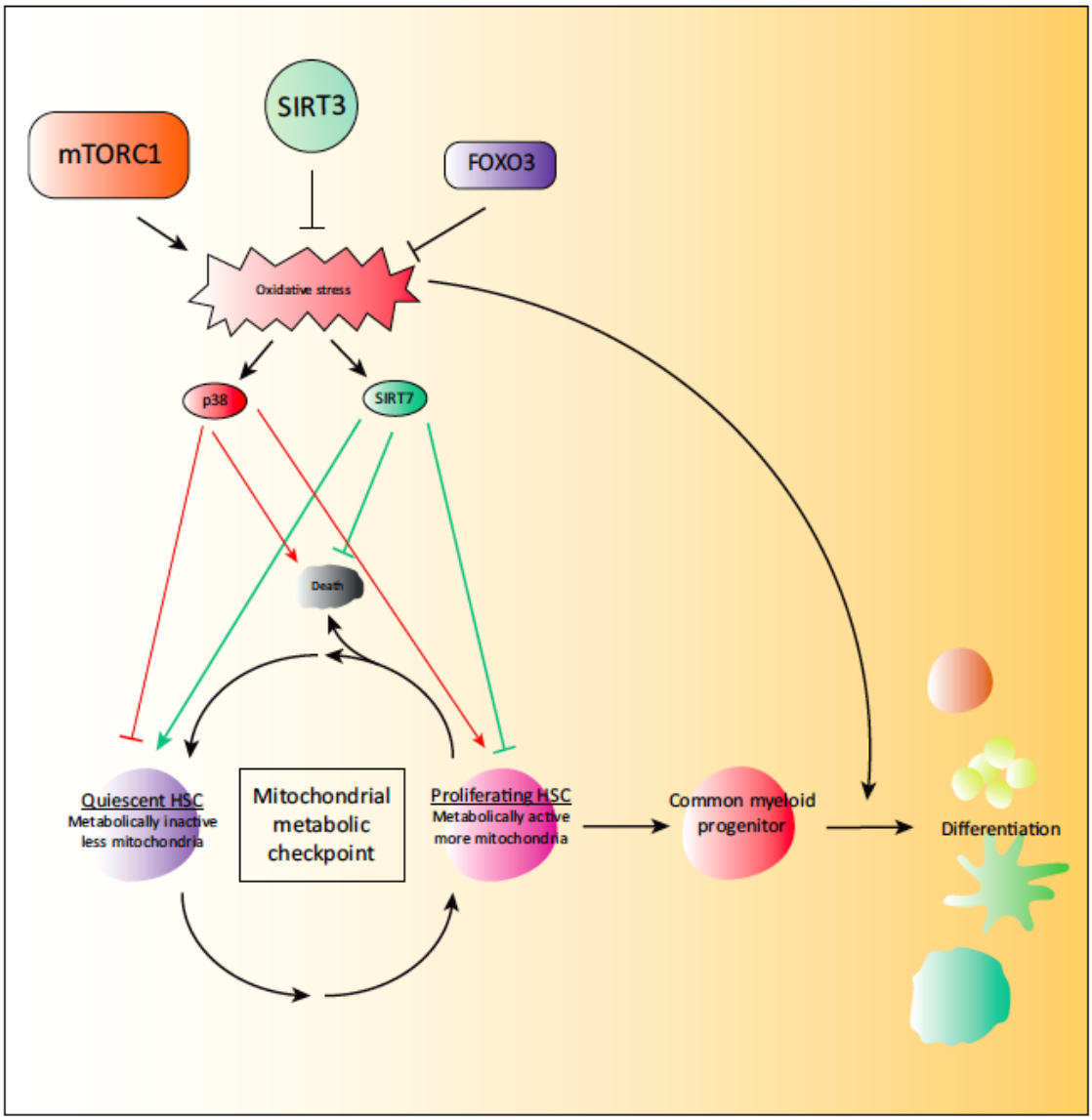

Figure 2. Reactive oxygen species (ROS) govern cell fate decision-making in hematopoietic stem progenitor cells. The transition of hematopoietic stem cells (HSCs) from quiescence to proliferation is associated with increased mitochondrial biogenesis and oxidative stress, which is monitored by the mitochondrial 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.

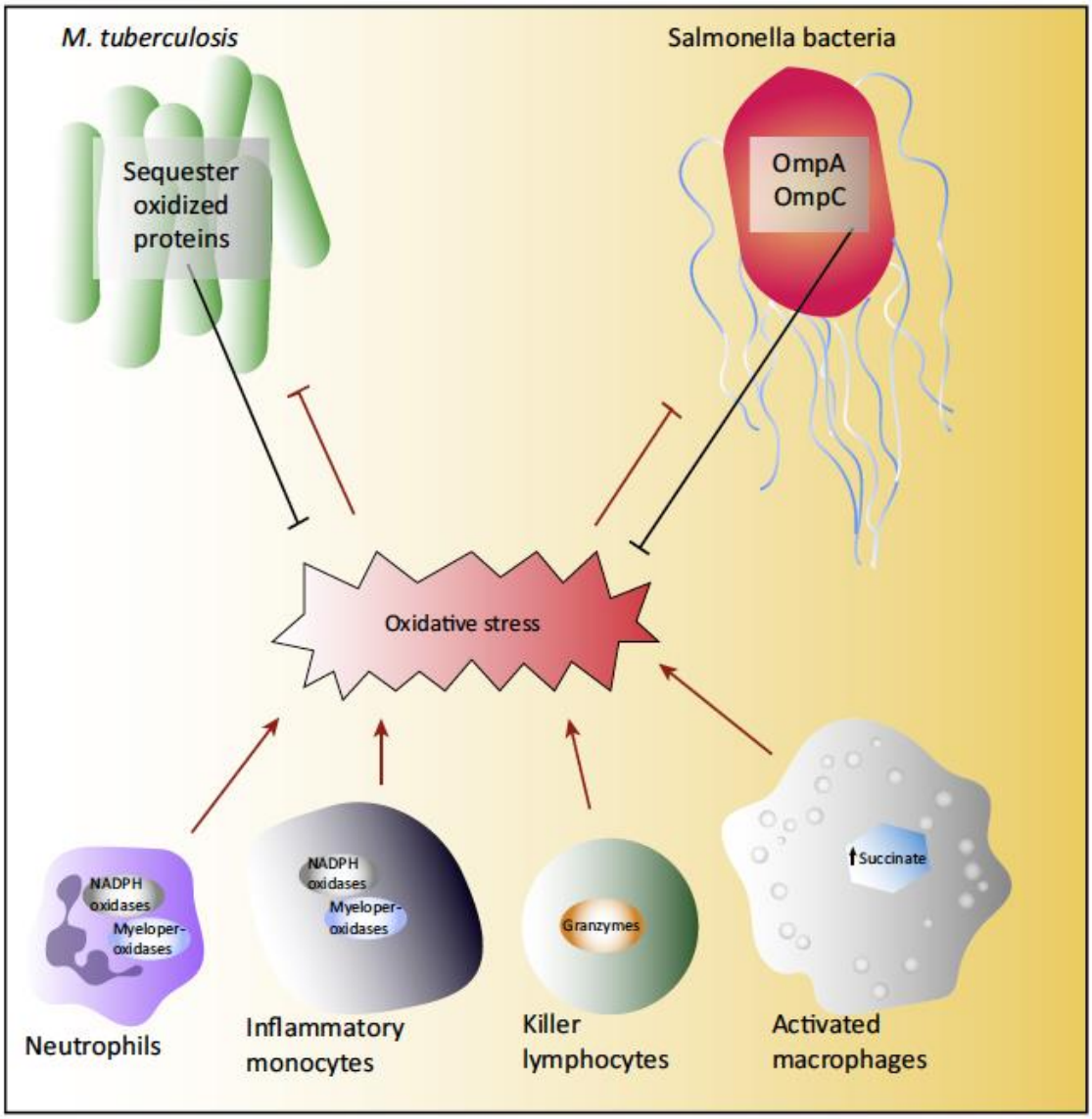

Figure 3. Oxidative stress supports innate immunity. Phagocytes employ various mechanisms of reactive oxygen species (ROS) production to facilitate pathogen clearance, while pathogens develop strategies to alleviate ROS damage.

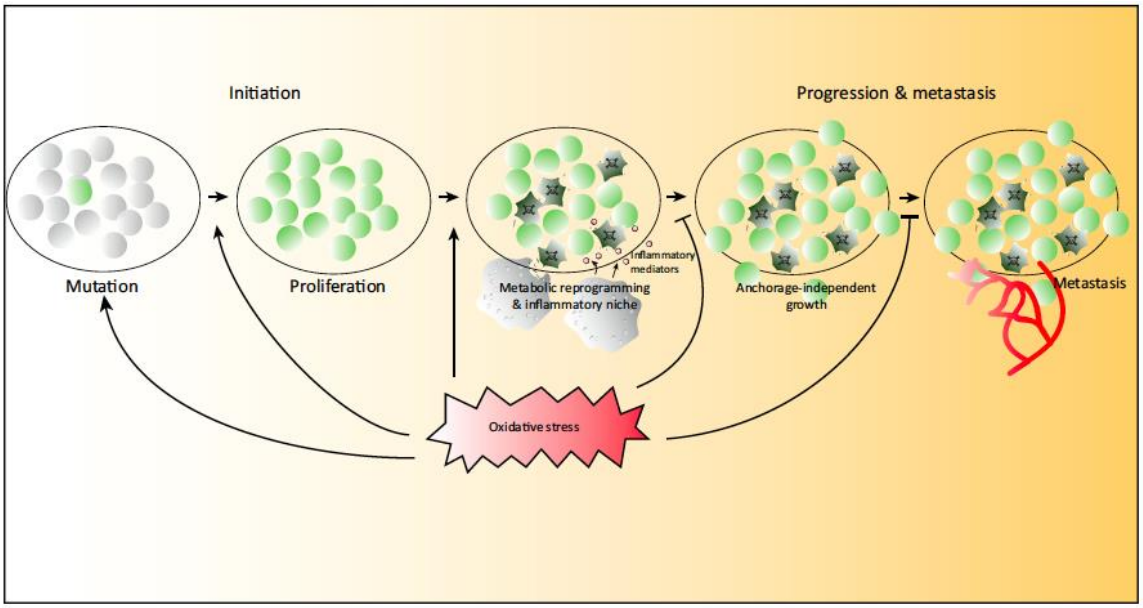

Figure 4. Oxidative stress regulation of cancer. Although oxidative stress has a pro cancer role at the tumor-initiation stage by promoting DNA damage, proliferation, metabolic reprogramming, and the establishment of the tumor microenvironment, cancer cells also hijack the oxidative stress responses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

Part II: Nutrition sensing and chronic inflammation

The ability to sense and respond to fluctuations in environmental nutrient levels is a requisite for life [48]. Therefore, multiple nutrient sensing mechanisms and pathways are developed though evolution, such as sirtuins, AMPK, mTOR and GCN2. However, nutrient-sensing pathways are commonly dysregulated in human diseases that are characteristic of chronic inflammation. In this section, I will discuss the current understandings on inflammasomes which triggers inflammation. Next, I will focus on recent evidence of nutrient excess and chronic inflammation.

Inflammasomes and inflammation

Inflammation is a normal defense mechanism that protects the host from infection and other insults; it initiates pathogen killing as well as tissue repair processes and helps to restore homeostasis at infected or damaged sites. Inflammasomes are components of the innate immune system which play critical roles in the host defense mechanisms by triggering inflammation [105].

Inflammasomes are intracellular multiprotein complexes consisting of Nod-like receptors such as NLRP3, NLRC4, Aim2, adapter protein ASC, and pro-Caspase1 [106,107]. inflammasomes act as caspase-1 activation platforms to respond to a variety of stimuli [106-108]. Nod-like receptors are intracellular sensors of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) [106-108]. 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-interleukin-1β (pro-IL-1β) 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 [108]. Studies have focused on the NOD-like receptor family, pyrin domain containing 3 (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 [107]. The sterile activators of NLRP3 include self-derived molecules, ROS and fatty acids, as well as environment-derived molecules such as asbestos and silica [109-111]. It is believed 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. NLRP3 knockout mice are protected from high-fat diet induced inflammatory states, while aged NLRP3 knockout mice are protected from aging-associated inflammation [112,113]. Chronic inflammation is causal to a range of chronic diseases, with the most prominent examples being metabolic disorders [114]. NLRP3 knockout mice are protected from developing insulin resistance under over-nutrition or aging stresses [112-113]. These studies highlight the

potential of targeting the NLRP3 inflammasome as a therapeutic approach to tackling aging-associated chronic inflammation.

Nutrient excess, nutrient sensing and chronic inflammation

There is a substantial amount of evidence to suggest that many foods and nutrients modulate inflammation both acutely and chronically [115]. The postprandial inflammatory response lasts for only few (4–8) hours but it recurs several times a day following eating. Several cells in the body associated with the innate immune system, including abdominal adipocytes and macrophages, respond to acute postprandial elevation of several components of a meal by mounting a transient inflammatory response. The most efficient triggers of the postprandial inflammatory response appear to be triacylglyceride and glucose [116-119]. Meals or food components may contain lipopolysaccharide (LPS) which directly triggers systemic inflammation. Related to this effect, the absorptive process may allow translocation of LPS from gut bacteria into the systemic circulation [120]. Postprandial hyperglycemia can suppress antioxidant capacity and induce the production of free radicals which themselves initiate an inflammatory reaction [121]. Although the postprandial inflammatory response has been known for several years [122], it is only recently that its probable importance in the generation of insulin resistance and atherosclerosis has been appreciated [123-125].

In the setting of chronic overnutrition, obese people have higher circulating concentrations of many inflammatory markers than lean people do, including Tumor necrosis factor α (TNFα), IL-6 and C-reactive protein (CRP) [126]. These are believed to play a role in insulin resistance and other metabolic disturbances [127]. Obesity is characterized by an expansion of the mass of adipose tissue and dramatic changes in its distribution in the body. The increase in abdominal fat mass is associated with a chronic elevation of the circulating concentrations of inflammatory mediators including several acute-phase inflammatory proteins such as CRP [128.129], pro-inflammatory cytokines, adhesion molecules and pro-thrombotic molecules [130-132]. Thus, adipose tissue becomes a major producer resulting in a chronic and constant local and systemic inflammatory milieu.

Summary

Considering ample evidence that overnutrition induces chronic inflammation-associated diseases, it is not difficult to imagine that our modern lifestyle switches the nutrient sensing pathways from nutrient scarcity homeostatic mechanisms to chronic detrimental inflammation. That leads to common belief that health benefits of caloric restriction come from compensating our modern lifestyle. In the real world, however, reduced caloric intake 30-40 percent for a lifelong time is never easy to achieve for most people.

In the molecular level, the NLRP3 inflammasome has been increasingly appreciated as a crucial mediator of the chronic inflammatory state. Recent advances in the inflammasome field have revealed strategies that target NLRP3 inflammasome activation

by investigating the biology of NLRP3 inflammasome regulation. Based on the above knowledge, we are interested in targeting nutrient sensing mechanisms or the NLRP3 inflammasome as a potential therapeutic avenue for chronic inflammatory diseases.

Part III: Nutrition sensing and mitochondria stress

Mitochondria are the main cellular powerhouses. They generate the majority of cellular energy by coupling nutrient oxidation to ATP synthesis via the respiratory chain and ATP synthase. Mitochondria also have other essential metabolic functions. Thus, mitochondria integrity is critical to the whole cell and several mechanisms have been evolved to manage mitochondrial stresses.

In this section, I will introduce the common mitochondrial stresses. Next, I will focus on a newly recognized mitochondrial stress—mitochondrial protein folding stress (mtPFS) and recent evidence that links mtPFS to cellular nutrient sensing mechanisms.

Mitochondrial stresses

Mitochondria are double-membrane organelles found in nearly all eukaryotic cells. The mitochondrial proteome comprises approximately 1100 proteins, which are encoded by genes located in both the nuclear and the mitochondrial DNA (mtDNA) [133]. Mitochondria are well-known the essential sites for oxidative phosphorylation and ATP production. In addition to energy production, mitochondria have other essential metabolic roles, including in amino acid and nucleotide synthesis, iron–sulfur cluster biogenesis, intermediate metabolite biogenesis and calcium homeostasis. Thus, the quantity and quality of mitochondria are critical to the whole cell and several mechanisms have been evolved to deal with mitochondrial stresses, including oxidative stress, which originates from byproducts of oxidative phosphorylation respiration, and mtPFS, which is accumulation of unfolded proteins within the mitochondrial matrix [134]. Furthermore, because approximately 99% of mitochondrial proteins are encoded by the nucleus, these stress defense mechanisms very often involve signaling pathways communicating between the two organelles [135].

Mitochondrial unfolded protein response

The mitochondrial unfolded protein response (mtUPR) is a transcriptional response that is activated by accumulation of unfolded proteins within the mitochondrial matrix and regulated by mitochondrial-to-nuclear communication [136-139]. The discovery of this stress response that functions to alleviate the accumulation of unfolded proteins within mitochondria was reported more than 15 years ago in cultured mammalian cells: Overexpression of an irreversibly misfolded mitochondrial protein resulted in increased accumulation of nuclear transcripts encoding several mitochondrial chaperones and proteases [140]. It is well accepted now that mtUPR leads to transcriptional upregulation of mitochondrial chaperones and repression of translation, in order to maintain protein homeostasis (proteostasis) within mitochondria [140,141]. This mitochondrial-specific response is conceptually similar to the transcriptional response activated by protein misfolding in the endoplasmic reticulum known as the UPR, it was named the mtUPR [142,143].

The physiological importance of mtUPR has been demonstrated: Uncontrolled perturbation of mitochondrial proteostasis will lead to mitochondrial dysfunction and ultimately apoptotic cell death. In addition, Caenorhabditis elegans lacking mtUPR signaling components exhibit impaired development and survival during conditions that perturb mitochondrial function [139]. In terms of its molecular mechanisms, three bZIP transcription factors, C/EBP homologous protein (CHOP), Activating transcription factor 4 (ATF4) and ATF5 have been proposed to mediate the unfolded protein response in mammalian cells [144-146]. However, the mtUPR is a still nascent cellular pathway: the molecular components and physiological relevance of which are not well understood.

Nutrient sensing and mitochondrial protein stresses

Since mitochondria are the main cellular powerhouses and have other essential metabolic roles, their functions and quality are closed linked to the cellular nutrient sensing mechanisms. Our lab has previously shown that SIRT3, a mitochondrial localized protein deacetylase, deacetylates and activates superoxide dismutase 2 (SOD2) to reduce mitochondrial oxidative stress [9,23]. We also demonstrated that SIRT7, a nucleus localized sirtuin, represses NRF1 activity to inhibit mitochondrial protein translation, alleviating mitochondrial protein folding stress [147]. These molecular regulations of mitochondrial stress levels in hematopoietic stem cells (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].

Summary

Our lab recently identified SIRT7-NRF1 axis as a regulatory branch of mtUPR. In that particular paper, we demonstrated that SIRT7 knockdown results in upregulation of mitochondrial chaperons and proteases, as well as inefficient clearance of mutant ornithine transcarbamylase, an overexpressed aggregation-prone mitochondrial protein [140, 147]. We are interested in looking for additional biomarkers, in order to better define mtUPR.

Meanwhile, further investigations are needed to unveil more detailed molecular mechanisms of mtUPR. Considering the close linkage of mitochondria and metabolism, nutrient sensing mechanism is likely to play more roles in mtUPR. Understanding mitochondria from the mtUPR perspective will offer novel insights to the study of metabolic diseases at both molecular and organellar levels.

Chapter 2: SIRT2 Regulates Aging- and Overnutrition-associated Chronic Inflammation and Insulin Resistance by Deacetylating the NLRP3 Inflammasome

Summary

Chronic inflammation has been shown to contribute to many age-related diseases, such as metabolic disorders, neurodegenerative diseases, and cancer [148-153]. SIRT2, a more obscure sirtuin members, knockout mice have been reported to be susceptible to inflammation-associated conditions, including experimental colitis and brain inflammation [154,155]. Therefore, we are interested to test the role of SIRT2 in chronic inflammation in this chapter. We explored whether SIRT2 suppresses chronic inflammation through regulating inflammasome activities. We found that SIRT2 specifically inhibits the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in macrophages by directly deacetylating NLRP3. Under conditions of aging and overnutrition, two prominent risk factors associated with insulin resistance, SIRT2 ablation in mice leads to increased systematic chronic inflammation and insulin resistance. These results suggest a novel treatment strategy of inflammation-associated diseases by modulating SIRT2 activity.

Introduction

The immune system has evolved to launch robust yet acute responses necessary to effectively eliminate pathogens. However, chronic, low-grade, sterile inflammation has been found in age- or overnutrition-related conditions [156-165] and diseases [166-176]. In these conditions, the inflammation has no beneficial role for survival, but becomes detrimental incubator for aging-associated chronic disorders later in life, including atherosclerosis, diabetes, Alzheimer's disease, Parkinson's disease, and cancer [148-153]. It remains elusive how this remodeling of the immune system is regulated. Therefore, the appealing question currently is to define the molecular basis of immune system remodeling during these aging-associated pathologies.

Inflammasomes, components of the innate immune system, are cytosolic multiprotein complexes that detect pathogenic microorganisms [105]. They play critical roles in the host defense mechanisms against infectious pathogens [106,107]. Once these protein complexes have formed, the inflammasomes activate caspase 1, which proteolytically activates the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. In addition, inflammasome activation causes a rapid, pro-inflammatory form of cell death called pyroptosis [106-108].

The formation of inflammasome complexes is triggered by a range of substances that emerge not only during infections, but also upon tissue damage or metabolic imbalances. Thus, aberrant inflammasome activations are believed to be involved in a variety of inflammatory disorders [106-108]. Among these inflammasomes, the NLRP3 inflammasome can be activated by a wide array of stimuli, including sterile metabolic activators in the absence of overt infection, including excess ATP, reactive oxygen species, cholesterol crystals, and amyloid fibrils. These sterile metabolic activators of the NLRP3 inflammasome seem to accumulate in humans during aging or overnutrition [108,109,111]. NLRP3 knockout mice are protected from either aging-induced or obesity-induced inflammation and insulin resistance [112,113]. These findings raise the possibility that targeting the regulation of NLRP3 activity may hold promise to potentially reverse the chronic low-grade inflammation and its associated diseases.

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 [177,178]. Ubiquitination of NLRP3 by multiple E3 ubiquitin ligases negatively regulates its activation, which can be counteracted by deubiquitinase BRCC3 [178]. Moreover, recruitment of NLRP3 to the mitochondria via mitochondrial antiviral signaling protein (MAVS) activates NLRP3 activity [179]. However, the diverse mechanisms of NLRP3 regulation remain elusive. Thus, understanding the biology of the NLRP3 inflammasome offers a unique angle to probe chronic inflammation.

Silent information regulator 2 (Sir2) proteins, or sirtuins, are highly conserved nicotinamide adenine dinucleotide (NAD+) dependent protein deacetylases found in organisms ranging from bacteria to humans. Their dependence on NAD+ links their activity to cellular metabolic status to serve as critical intracellular nutrient sensors [180-187]. There are seven sirtuins in mammals, SIRT1-7, that localize to various cellular compartments. A more obscure family member, SIRT2 is known to localize to the cytosol. Studies from the Akira group demonstrated that loss of intracellular NAD+ or pharmacological inhibition of SIRT2 activity promotes activation of NLRP3 inflammasome [188]. These results highlight the possibility that SIRT2 may modulate NLRP3 inflammasome activity in macrophages to regulate sterile inflammation. Indeed, SIRT2 knockout mice are reported to be susceptible to inflammation-associated conditions, including experimental colitis and brain inflammation [154,155]. Therefore, we set out to explore the molecular mechanism by which SIRT2 suppresses NLRP3 inflammasome activity, and whether this suppression modulates aging-associated pathologies.

Results

SIRT2 specifically inhibits the NLRP3 inflammasome in macrophages.

Previous studies have shown that pharmacological inhibition of SIRT2 activity suppresses

NLRP3 inflammasome in macrophages through indirectly modulating intracellular microtubule dynamics [188]. To extend this finding, we isolated bone marrow derived macrophages from wild type and SIRT2 knockout mice. We stimulated them with lipopolysaccharide (LPS) and then activated multiple inflammasomes by their specific inducers (nigericin or ATP for NLRP3 inflammasome; flagellin for NLRC4 inflammasome; dsDNA for AIM2 inflammasome) [109]. The expression of pro-caspase 1 and pro-IL-1β in stimulated wild type and SIRT2 knockout macrophages was comparable (data not shown). SIRT2 knockout macrophages had increased production of cleaved caspase 1 and IL-1β compared to wild type controls in response to NLRP3 inducers, such as nigericin and ATP (data not shown). However, there was no difference in caspase 1 and IL-1β cleavage between wild type and SIRT2 knockout macrophages in response to flagellin, a NLRC4 inflammasome inducer, or dsDNA, an AIM2 inflammasome inducer (data not shown).

To test whether SIRT2 regulates the NLRP3 inflammasome in human cells, we constructed THP1 cells, a human monocytic cell line, that stably overexpressed SIRT2 (Figure 1A). In THP1-derived macrophages, SIRT2 overexpression resulted in reduced NLRP3 inflammasome activation upon induction, as evidenced by reduced caspase 1 cleavage (Figure 1B). Together, these data indicate that SIRT2 specifically inhibits the NLRP3 inflammasome in macrophages.

NLRP3 is modified by acetylation in macrophages and is deacetylated by SIRT2.

Sirtuins are NAD+-dependent deacylases that regulate diverse metabolic processes [37-46]. Thus, we hypothesized that SIRT2 directly deacetylates NLRP3. To this end, we firstly investigated the posttranslational modifications of NLRP3. We immunopurified NLRP3 from NG5 cells, an immortalized NLRP3 knockout macrophages stably expressing Flag-NLRP3 [178]. Western analyses with acetylated lysine antibody confirmed that NLRP3 was modified by acetylation (Figure 2A).

Next, we knocked down sirtuins in NG5 cells via small interfering RNA (siRNA). Knocking down SIRT1 in NG5 cells did not affect the acetylation level of NLRP3 (Figure 3). In contrast, knocking down SIRT2 in NG5 cells increased the acetylation level of NLRP3 determined by Western analyses (Figure 2B). Mass spectrometry analyses revealed several lysine residues modified by acetylation, located in pyrine domain (PYD) nuclear binding domain (NBD) and leucine-rich repeat (LRR) (Figure 4A, B). Furthermore, there are markedly increased number of acetylated lysine residues in SIRT2 knockdown cells compared to control cells (Figure 4A). Most of the lysine residues targeted for deacetylation are highly conserved in mammals, suggesting that these lysine residues are important throughout evolution (Figure 5). Together, these results suggest that SIRT2 deacetylates multiple lysine residues of NLRP3 in macrophages.

NLRP3 acetylation enhances its inflammasome activity.

To investigate whether acetylation regulates the activity of the NLRP3 inflammasome, we mutated the lysine residues at the acetylation sites of NLRP3 to arginine to mimic the

constitutively deacetylated state and reconstituted NLRP3 knockout macrophages with wild type or mutant forms of NLRP3 via retroviral transduction. Wild type NLRP3 and NLRP3 mutants were expressed to comparable levels (Figure 6A). Upon priming with LPS followed by ATP stimulation, cells reconstituted with wild type NLRP3 produced more cleaved caspase 1 and IL-1β. In contrast, cells reconstituted with NLRP3 mutants, except for NLRP3 K970R, had significantly reduced level of caspase 1 and IL-1β cleavage (Figure 6A), consistent with the notion that acetylation of some but not all lysine residues have functional consequences [9]. In summary, these results suggest that acetylation of NLRP3 enhances the NLRP3 inflammasome activity and SIRT2 represses the NLRP3 inflammasome activity at least in part by deacetylating NLRP3.

NLRP3 acetylation facilitates the assembly of inflammasome complexes.

NLRP3 complexes with Apoptosis-Associated Speck-Like Protein Containing CARD (ASC) to form inflammasome [189-190]. Therefore, we hypothesized that acetylation of the lysine residues targeted by SIRT2 facilitates the formation of inflammasome. To visualize and quantify the formation of inflammasome complexes, we reconstitute ASC-EGFP and wild type or mutant NLRP3 in 293T cells [191]. In contrast to wild type NLRP3, which triggered the formation of speck-like foci containing ASC in close to 30% of GFP positive cells, K21/22/24R and K234/243/248R NLRP3 mutants were compromised in forming speck-like foci with ASC (Figure 7A, B), indicating that acetylation of NLRP3 facilitates the assembly and activation of the NLRP3 inflammasome.

The pyrin domain (PYD) domain is believed to mediate the interaction between NLRP3 and ASC, and the formation of the inflammasome [189]. We therefore focused on K21/22/24R NLRP3 mutant, which lysine residues are in the PYD domain, and made single mutations to arginine. K21R and K22R mutants, but not K24R mutant, resulted in reduced NLRP3 inflammasome activity and compromised formation of speck-like foci with ASC (Figure 6B, 7C), consistent with our hypothesis that K21 and K22 mediate the PYD-PYD interaction.

SIRT2 deficiency cause inflammation and insulin resistance during aging or overnutrition.

Previous studies in our lab have confirmed that young SIRT2 knockout mice were phenotypically unremarkable. Compared to the wild type littermates, SIRT2 knockout mice fed a chow diet for 6 months had normal body fat, similar levels of plasma IL-18 and glucose, and responded comparably in a glucose tolerance test (data not shown). Because the NLRP3 inflammasome can be activated by endogenous metabolic signals associated with obesity and aging [108,109,111], we characterized SIRT2 knockout mice fed a high fat diet for 6 months or fed a chow diet for 2 years.

SIRT2 knockout mice fed a high fat diet had more cleaved caspase 1 in the liver and adipose tissue compared to the wild type controls (Figure 9), consistent with increased activation of the NLRP3 inflammasome under the condition of diet-induced obesity in the

absence of SIRT2.

Aged SIRT2 knockout mice fed a chow diet also had more cleaved caspase 1 in the liver compared to wild type controls (Figure 10). In addition, aged SIRT2 knockout mice showed impaired insulin signaling in insulin responsive tissues, as evidenced by reduced phosphorylation of Akt after the mice were infused with insulin, indicating that SIRT2 is also required to maintain insulin sensitivity during aging (Figure 11).

The SIRT2-NLRP3 axis is dysregulated upon aging.

Chronic inflammation characterizes many aging associated pathologies [156-165]. It has been found that sterile metabolic activators of the NLRP3 inflammasome seem to accumulate in humans during aging [108,109,111]. Here, we test another possible mechanism of aging-associated inflammation, the SIRT2-NLRP3 acetylation axis. We isolated primary bone marrow-derived macrophage (BMDM)s from young (2-month-old) and old (2-years-old) mice. SIRT2 expression is significantly reduced in primary BMDMs isolated from old mice (data now shown). In addition, we immunopurified NLRP3 from differentiated immortalized myeloid progenitors from young (2-month-old) and old (2-years-old) mice. Western analyses demonstrated the acetylation level of NLRP3 increases upon aging [Figure 8]. Together, these data indicate dysregulation of the SIRT2-NLRP3 axis during aging.

Discussion

Type 2 diabetes mellitus is nowadays acknowledged as a low-grade chronic inflammatory condition characterized by the over-secretion of pro-inflammatory cytokines. A growing body of evidence currently points at IL-1β, which is a major player in a wide array of auto-inflammatory diseases, to also act as key promoter of systemic and tissue inflammation in type 2 diabetes [192-196]. Pharmacological blockage of IL-1β can achieve not only a sustained reduction in inflammatory markers, but also a persistent improvement in glucose homeostasis and β-cell function [197,198]. Although the results from IL-1β blockage were encouraging, there are still characteristics that make the drug unsuitable for long-term treatment of type 2 diabetes, such as immunosuppression [199,200]. Therefore, immunological modulation of more specific nodes holds the promise to offer long-term control of these chronic inflammatory diseases.

In the current study, SIRT2 knockout mice suffered from increased inflammation and insulin resistance in metabolic organs during aging and overnutrition, indicating that SIRT2 suppresses meta-inflammation. Interestingly, SIRT2 levels are reduced in macrophages with age, which could potentially explain the aging-associated inflammation and insulin resistance. Collectively, dysregulation of SIRT2-NLRP3 inflammasome axis could serve as a driver of chronic inflammation during aging and overnutrition. From the therapeutic standpoint, it would be intriguing to elucidate whether SIRT2 overexpression or NAD+ supplement in old macrophages can rescues the aging-associated NLRP3

inflammasome activation, and whether SIRT2 overexpression or NAD+ supplement in macrophages can reverse the development of chronic inflammation and associated phenotypes in mice models. 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 and overnutrition.

Post-translational modifications play pivotal roles in regulating inflammasome activities [177]. Multiple post-translational modifications of NLRP3 inflammasomes have been identified, including phosphorylation, ubiquitination, ADP-ribosylation, S-nitrosylation, and proteolytic processing [177,178,201]. 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 domains of NLRP3 inflammasome activation. In addition, the crystal structure of NLRP3 has not been solved yet, except the PYD domain [202], making it difficult to study the regulation of the inflammasome activity. In this chapter, we found that the NLRP3 inflammasome is acetylated in macrophages. Furthermore, we confirmed that NLRP3 acetylation is required for inflammasome function by mutating the deacetylated lysine residues to mimic the constitutively deacetylated state, reconstituting NLRP3 knockout macrophages with wild type or mutant forms of NLRP3, and evaluating their inflammasome functions. Finally, we found that acetylation of NLRP3 facilitates the assembly of the NLRP3 inflammasome, providing a structural mechanism of the acetylation. Our systematic molecular and biochemical analysis of NLRP3 acetylation provides significant insights into inflammasome regulation under various pathophysiological conditions.

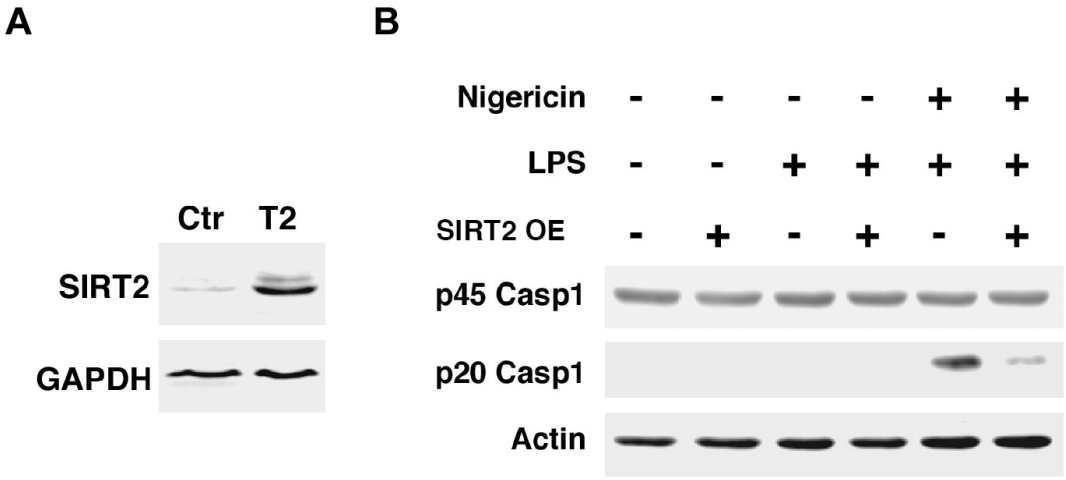

Figure 5. SIRT2 inhibits the NLRP3 inflammasome in macrophages.
(A) THP1 cells were transduced with control or SIRT2 lentivirus and selected by puromycin. The expression of SIRT2 was confirmed by Western analyses. (B) Control and SIRT2 overexpressing THP1 cells were differentiated into mature macrophages and stimulated with LPS and nigericin. Cell lysates were used for Western analyses for pro-caspase 1 (p45) and culture supernatants were used for cleaved caspase 1 (p20) Western analyses.

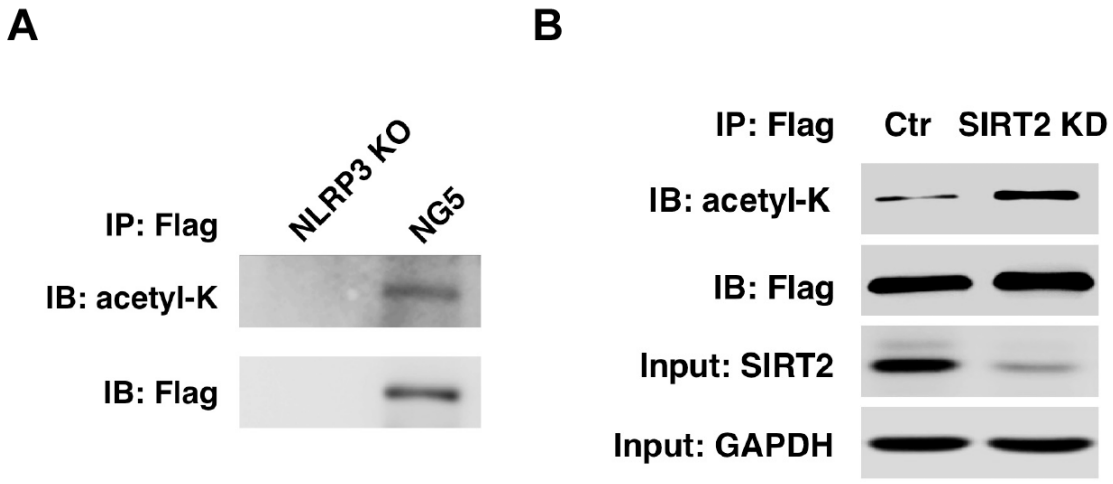

Figure 6. NLRP3 is modified by acetylation in macrophages and is deacetylated by SIRT2. (A) NLRP3-Flag was immunopurified from NG5 cells followed by Western analyses. NLRP3 knockout cells were used as a negative control. (B) NLRP3-Flag was immunopurified from NG5 cells treated with control or SIRT2 siRNA followed by Western analyses.

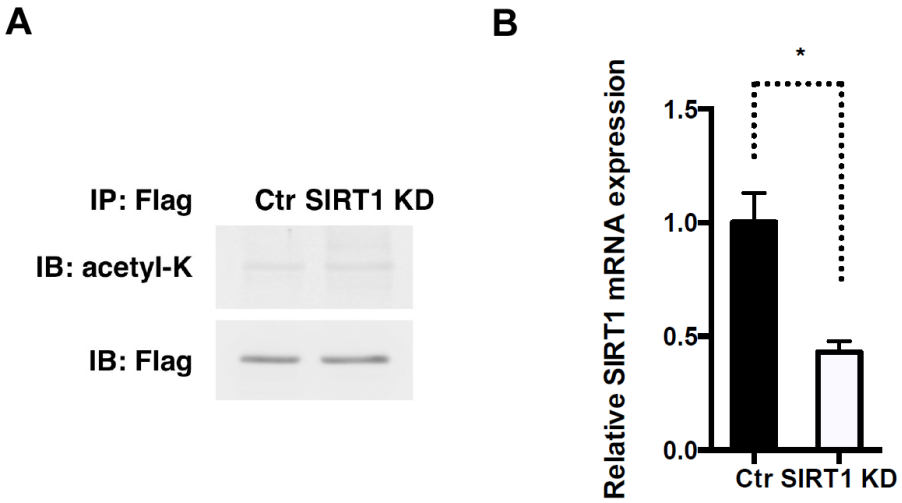

Figure 7. SIRT1 does not affect NLRP3 acetylation.
(A) Western analyses of immunopurified NLRP3-Flag from NG5 cells treated with control or SIRT1 siRNA. (B) Validation of SIRT1 siRNA knockdown efficiency. Data shown are the SIRT1 mRNA levels in NG5 cells treated with control or SIRT1 siRNA. n=3. Error bars represent SE. *: $p<0.05$. Student's *t* test.

A

Acetylated lysine peptides	WT	SIRT2 KD	
K*(21)		*	LK*(21)K*(22)FKMHLEDYPPEKG
K*(22)		*	LK*(21)K*(22)FKMHLEDYPPEKG
K* (24)	*	*	KLAQYLEDLEDVDLKKFK*MHLE
K*(234)		*	FQGAAGIGKTILARK*(234)IMLDWALGK*(243)LF
K*(243)		*	FQGAAGIGKTILARK*(234)IMLDWALGK*(243)LF
K*(248)		*	KDK*FDYLFFIHCRE
K*(543)	*	*	RK*GPGGCSDLLNRD
K*(593)	*		YLEKK*LSCKISQQVRLELLKW
K*(597)		*	YLEKKLSCK*ISQQVRLE
K*(875)		*	RLYIGENALGDSGVQVLCEK*MKDPQCNLQ
K*(877)		*	LYIGENALGDSGVQVLCEKMK*DPQCNLQ RLYIGENALGDSGVQVLCEKMK*DPQCNL
K*(970)		*	RK*LNLGNNDLGDLCVVTLCEVLKQ

B

Figure 8. Mapping of NLRP3 lysine residues targeted by SIRT2 for deacetylation. (a) NLRP3-Flag was immunopurified from NG5 cells treated with control or SIRT2 siRNA and analyzed by mass spectrometry. (b) Domain structure of NLRP3. Acetylation sites are marked with *.

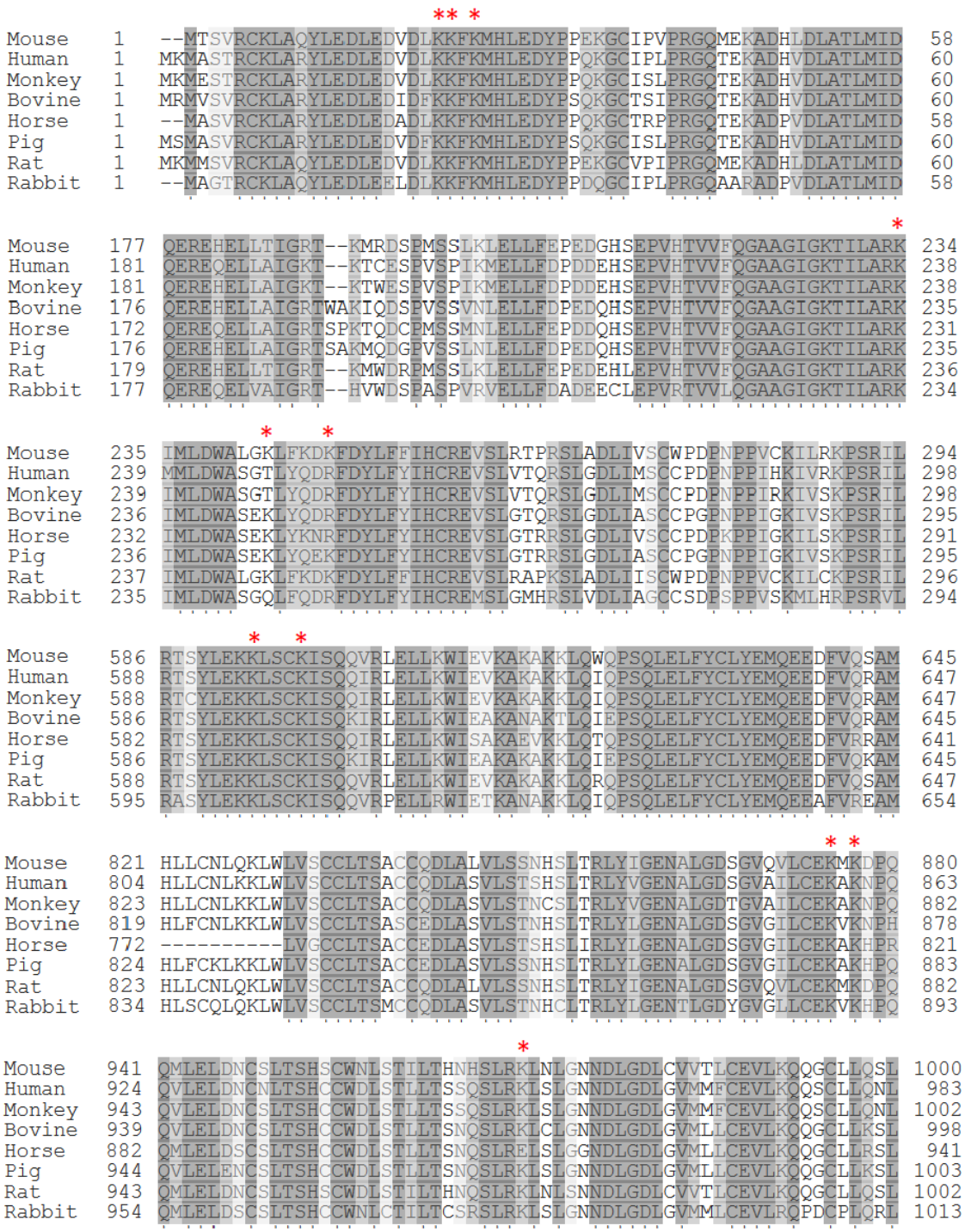

Figure 9. The acetylation sites on NLRP3 are highly conserved in mammals. Sequence alignment of NLRP3 from various mammalian species is shown. Acetylated lysine residues are labeled with *.

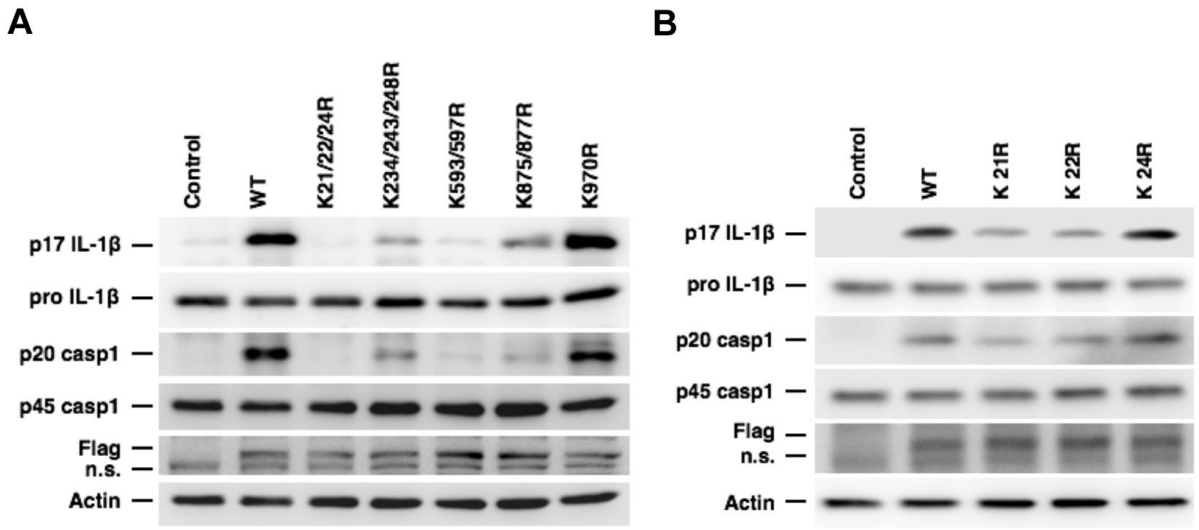

Figure 10. Acetylation of NLRP3 enhances the NLRP3 inflammasome activity. NLRP3 knockout macrophages were reconstituted with wild type or mutant NLRP3 by retroviral transduction, and then stimulated with LPS and ATP. Cell lysates were used for Western analyses for pro-caspase 1 (p45) and pro-IL-1β. Culture supernatants were used for cleaved caspase 1 (p20) and IL-1β (p17) Western analyses. n.s., non-specific band.

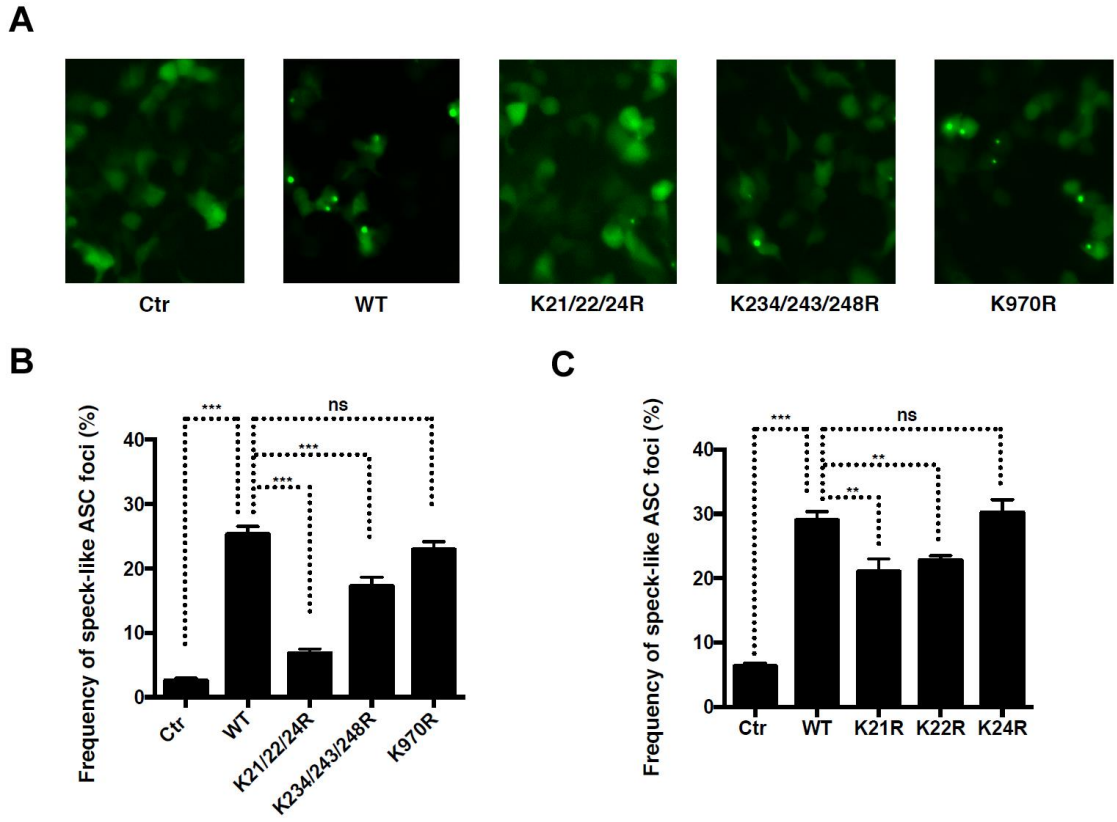

Figure 11. NLRP3 acetylation facilitates the assembly of inflammasome complexes. 293T cells were co-transfected with ASC-EGFP and control vector or wild type or constitutively deacetylated NLRP3 mutants. Data shown are the fluorescence images of speck-like ASC foci (A) and quantification of the frequency of speck-like ASC foci in GFP positive cells (B, C).

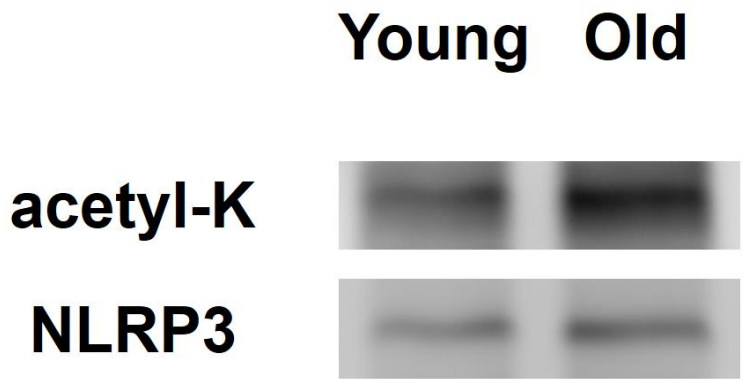

Figure 12. NLRP3 acetylation in macrophages increase upon aging.
we immunopurified NLRP3 from differentiated immortalized myeloid progenitors from young (2-month-old) and old (2-years-old) mice.

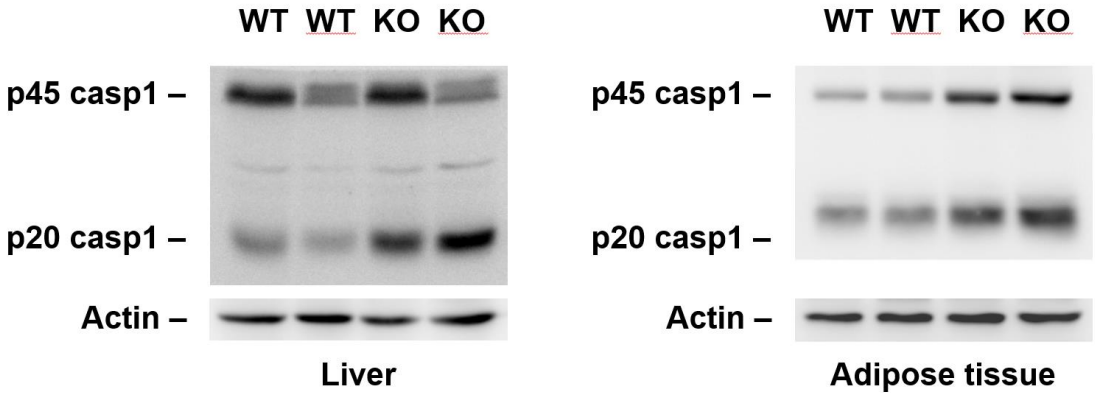

Figure 13. SIRT2 knockout mice fed a high fat diet have increased inflammation in liver and adipose tissue.
SIRT2 knockout mice were fed a high fat diet for 6 months. Liver and adipose tissue were used for Western analyses for pro-caspase 1 (p45) and cleaved caspase 1 (p20).

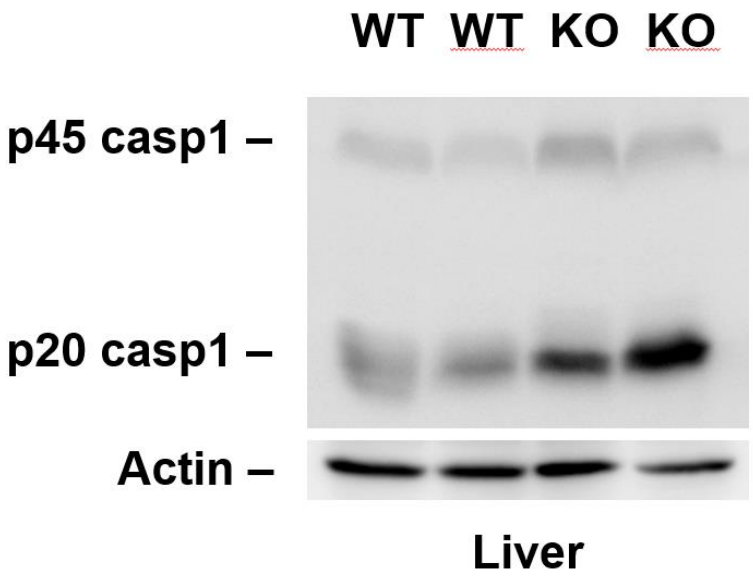

Figure 14. Aged SIRT2 knockout mice have increased inflammation in liver. SIRT2 knockout mice were fed a chow diet for 2 years. Liver was used for Western analyses for pro-caspase 1 (p45) and cleaved caspase 1 (p20).

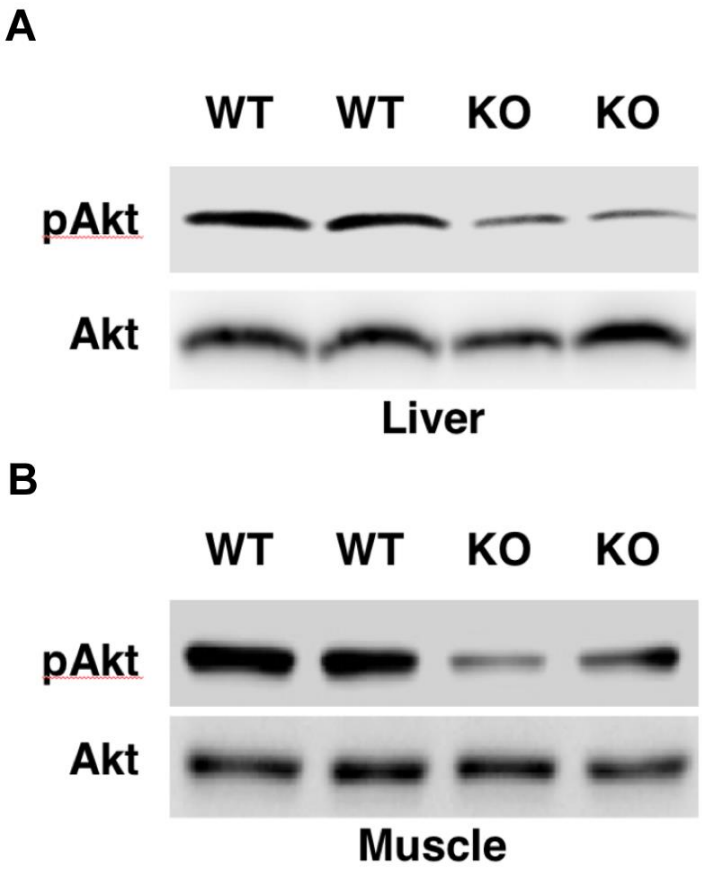

Figure 15. Aged SIRT2 knockout mice have increased inflammation in liver. SIRT2 knockout mice were fed a chow diet for 2 years. After intraperitoneal injection of insulin, insulin responsive tissues such as liver and muscle were harvest for Western analyses for Akt and phospho-Akt (Ser473).

Chapter 3: Transcriptional Downregulation of Genes Encoded by Mitochondrial DNA is a Novel Branch of the Mitochondrial Unfolded Protein Response

Summary

Diabetes and its complications, such as blindness, kidney failure, nerve damage, coronary heart disease, stroke, and even cancer, cause tremendous health hazards and economic burdens [203]. The international epidemic of obesity and type 2 diabetes makes it an even worse public health crisis [204]. Although the primary cause of this disease is not well understood, insulin resistance plays an early role in its pathogenesis. Mitochondrial dysfunctions caused by varies insults plays a critical role in insulin resistance through suppression of insulin signaling [205]. Perturbation of mitochondrial proteostasis, also known as mitochondrial protein folding stress (mtPFS), activates the mitochondrial unfolded protein response (mtUPR), a retrograde signaling pathway leading to transcriptional upregulation of mitochondrial chaperones, repression of translation, and stress relief [134]. The mtUPR is a nascent cellular pathway: the molecular components and physiological relevance of which are not well understood. Here we found two novel mtPFS biomarkers: mitochondrial aggregation and increased electron density in the mitochondrial matrix. We also identified a novel branch of mtUPR that involves transcriptional downregulation of genes encoded by mitochondrial DNA (mtDNA).

Introduction

Over the past three decades, the number of people with diabetes mellitus has more than doubled globally, making it one of the most important public health challenges to all nations [206]. The rapidly rising prevalence of obesity and diabetes worldwide is believed to a result of population aging, urbanization and increasing sedentary lifestyle [207]. Diabetes and its complications, such as blindness, kidney failure, nerve damage, coronary heart disease, stroke, and even cancer, cause tremendous health hazards and economic burdens [203].

90% of diabetes mellitus are type 2, which is linked to overnutrition and obesity. Although the primary cause of type 2 diabetes is not well understood, insulin resistance plays an early role in its pathogenesis and defects in insulin secretion by pancreatic β cells make the progression to overt hyperglycemia. Evidence suggests that mitochondrial content and functions are altered in insulin responsive tissues of patients with insulin resistance [208,209]. As a result, humans with insulin resistance exhibit impaired fasting fatty acid oxidation and accumulation of intracellular fatty acid

metabolites. There are ample mechanistic studies indicating that mitochondrial dysfunctions lead to insulin resistance through direct suppression of insulin signaling, for instance, insulin receptor substrate-1 (IRS-1), by accumulated fatty acid metabolites [205].

Mitochondria are double-membrane organelles found in nearly all eukaryotic cells. The mitochondrial proteome comprises approximately 1100 proteins, which are encoded by genes located in both the nuclear and mitochondrial DNA [133]. Mitochondria are the main cellular powerhouses and have other essential metabolic functions. Because approximately 99% of mitochondrial proteins are encoded by the nucleus, and mitochondrial activities impact many essential cellular processes, signaling pathways to communicate between the two organelles have been evolved [135].

MtUPR is a retrograde signaling pathway from the mitochondria to the nucleus that occurs during mitochondrial stress caused by the accumulation of unfolded or misfolded proteins within the mitochondrial matrix. MtUPR leads to transcriptional upregulation of mitochondrial chaperones, repression of translation, and stress relief [140,141]. The physiological importance of mtUPR has been demonstrated: Uncontrolled perturbation of mitochondrial proteostasis will lead to mitochondrial dysfunction and ultimately apoptotic cell death [211]. In addition, Caenorhabditis elegans lacking mtUPR signaling components exhibit impaired development and survival during conditions that perturb mitochondrial function [139]. The mtUPR is a nascent cellular pathway: the molecular components and physiological relevance of which are not well understood. Therefore, we set up the experimental system of mtUPR and explored its potential molecular mechanisms.

Our lab has previously shown that SIRT7, a nucleus localized sirtuin, represses NRF1 activity to inhibit mitochondrial protein translation, alleviating mitochondrial protein folding stress [147]. In that particular paper, we demonstrated that SIRT7 knockdown results in upregulation of mitochondrial chaperons and proteases, as well as inefficient clearance of mutant ornithine transcarbamylase, an overexpressed aggregation-prone mitochondrial protein [140, 147]. An appealing question currently is to look for additional biomarkers, in order to better define mtUPR. Meanwhile, further investigations are needed to unveil more detailed molecular mechanisms of mtUPR.

Results

CDDO treatment decreases expression of genes encoded by mitochondrial DNA

There are several experimental conditions that have been reported to induce the mtPFS, including small molecule chemicals and genetic manipulations [134]. However, specificity is always a concern, that is, whether the condition specifically induces mtPFS or mitochondrial stress in general. Studies from the Harper group demonstrated the specificity of the synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), a synthetic oleanane triterpenoid that inhibits mitochondrial matrix protease

LON, to mtPFS [210,211]. Thus, we tested CDDO in HEK293T and Hela cells. Treatment of CDDO 5uM for 6 hours successfully increased the expression of HSPD1/HSP60 and HSPE1/HSP10, two mitochondrial matrix chaperones traditionally used as mtUPR readouts (Figure 12,13).

Mitochondria aggregate and electron density increases upon mitochondrial protein folding stress

Then we asked how mtPFS would affect mitochondrial distribution and morphology. Studies from the Germain group showed that MG132, a proteasomal inhibitor, and overexpression of mutant intermembrane space endonuclease G leads to mitochondrial aggregation compared to an even mitochondrial distribution in wild type cells [212]. When visualized by electronic microscopy, cells with the above treatments showed dark condensed mitochondria [212,213]. We used MitoTracker Red to stain mitochondria in HEK293T cells. CDDO treatment leads to mitochondrial aggregation to a focus nearby the nucleus, which is consistent with the literature (Figure 14). Under electron microscopy, HEK293T cells with CDDO and MG132 treatment both showed widespread increased electron density in the mitochondrial matrix (Figure 15).

Expression of genes encoded by mitochondrial DNA decreases globally upon mitochondrial protein folded stress

Endoplasmic reticulum (ER) UPR inhibits global cytosolic translation through phosphorylation of eIF2α to reduce the protein folding load [88]. In contrast, mechanisms underlying protein translation in response to mtPFS, was poorly understood. Studies from the Harper group demonstrated that acute mtPFS reduced matrix-localized protein synthesis through inhibition of pre-RNA processing and translation [211]. However, whether transcriptional expression of mtDNA-encoded genes changes in the mRNA level was not mentioned. Thus, we randomly analyzed mRNAs of four mtDNA-encoded genes (ND1, COI, mito-tRNA Leu and ATPase6) in response to CDDO treatment in HEK293T cells. The results showed decreased expression in all the genes analyzed (Figure 16). To consider possible change of mtDNA copy number after CDDO treatment, we measured mitochondrial-to-nuclear DNA ratios by qPCR. There was no significant change in mitochondrial-to-nuclear DNA ratios after CDDO treatment for 6 hours, confirming the transcriptional downregulation of mtDNA-encoded genes (Figure 17).

Discussion

Mammalian mtDNA is a gene-dense, double-stranded DNA molecule of 16.6 kb, which encodes 11 mRNAs (translated to 13 proteins), 2 ribosomal RNAs, and 22 tRNAs [214]. The 13 mtDNA-encoded subunits constitute only a minority of the oxidative phosphorylation subunits, but they are nevertheless essential because oxidative

phosphorylation collapses in the absence of mtDNA expression [215]. The reduction in mitochondrial oxidative-phosphorylation activity in insulin-resistant individuals could be due not to mitochondrial loss but rather to a defect in mitochondrial function. This hypothesis is supported by muscle biopsy studies where the activity of mitochondrial oxidative enzymes was found to be lower in type 2 diabetic subjects [216]. In this chapter, we found global decreased expression of mtDNA-encoded genes upon mtPFS. It would be a great question to ask whether the reduction of mitochondrial oxidative enzymes in type 2 diabetes subjects are due to higher mtPFS.

Under electron microscopy, HEK293T cells with CDDO and MG132 treatment both showed widespread increased electron density in the mitochondrial matrix. Our findings are not exactly the same as the aggregated unfolded proteins reported previously [212,213]. It would require labeling of specific proteins and/or DNA to dissect the nature of the increased electron density in the mitochondrial matrix. Since our lab previous showed that SIRT7 knockdown leads upregulation of mitochondrial chaperons and proteases, as well as inefficient clearance of an overexpressed aggregation-prone mitochondrial protein. It is interesting to analyze whether genetic manipulation of SIRT7 will also change mitochondrial aggregation and electron density. These two morphological readouts could be used as novel biomarkers of mtPFS in the future.

The mtUPR is a nascent cellular pathway. Recent studies suggest the involvement of three bZIP transcription factors, C/EBP homologous protein (CHOP), Activating transcription factor 4 (ATF4) and ATF5, associated with the integrated stress response (ISR) [144-146]. However, the entire unfolded protein responses are not well understood yet. In this chapter, we set up the experimental system of mtUPR by treating CDDO in HEK293T and Hela cells. In addition, we found global transcriptional downregulation of mtDNA-encoded genes upon mtFPS, which can potentially relieve the protein folding stress and serve as a novel branch of mtUPR. Since the transcriptional regulation of mtDNA-encoded genes is largely unknown, further studies are needed to elucidate the mechanism of decreased expression of mtDNA-encoded genes in response to mtPFS.

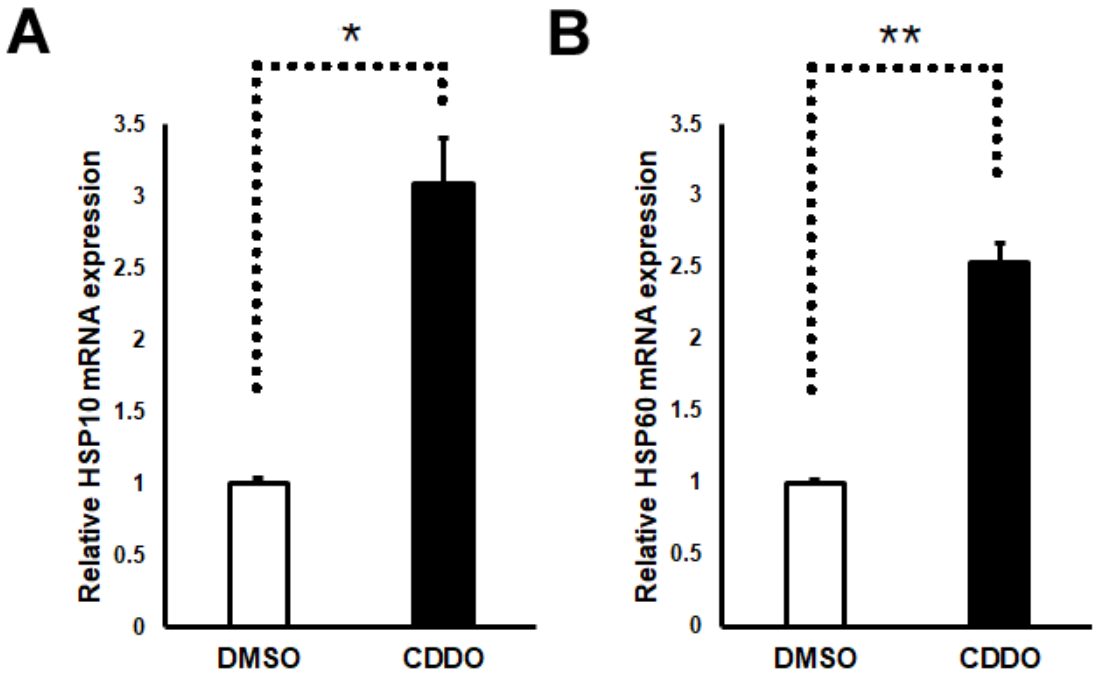

Figure 16. CDDO treatment induces mtUPR in HEK293T cells.
Data shown are the mRNA levels in HEK293T cells treated with DMSO or CDDO. n=3. Error bars represent SE. *: $p<0.05$. **: $p<0.01$. Student's *t* test.

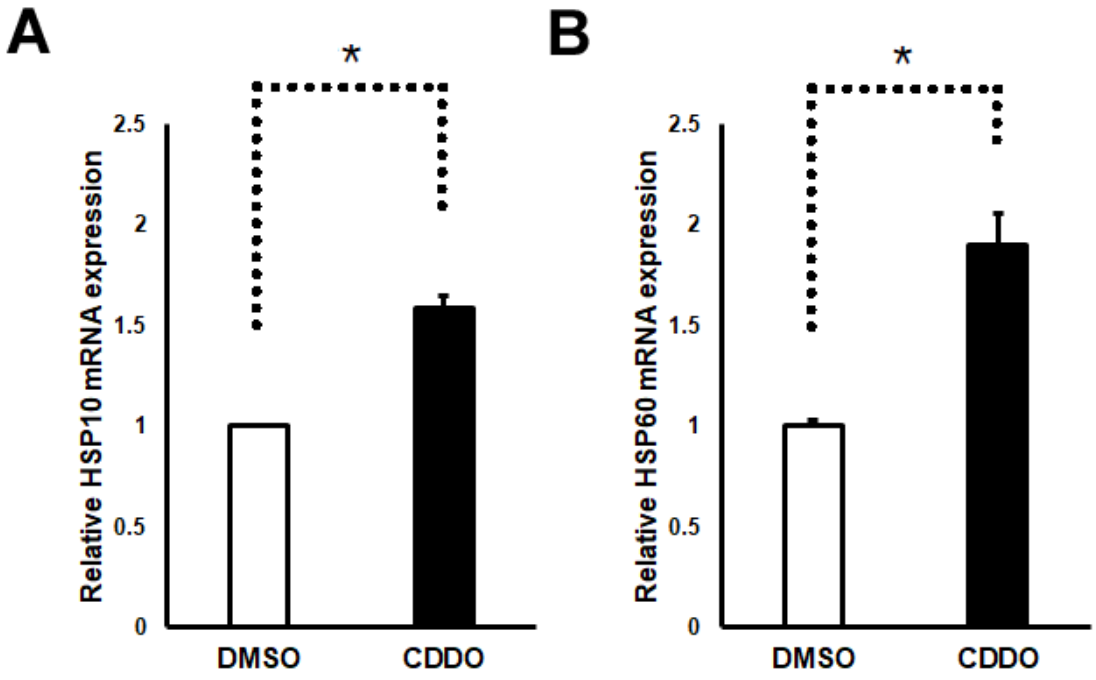

Figure 17. CDDO treatment induces mtUPR in Hela cells.
Data shown are the mRNA levels in Hela cells treated with DMSO or CDDO. n=3. Error bars represent SE. *: $p<0.05$. Student's *t* test.

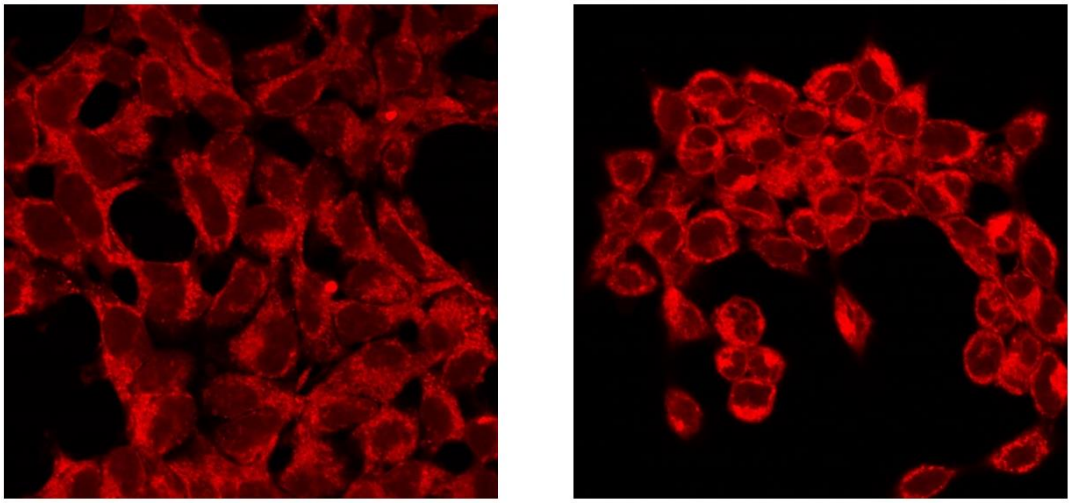

Figure 18. Mitochondria aggregate upon mtPFS.
HEK293T cells treated with DMSO or CDDO were stained by MitoTracker Red. Images were obtained by confocal microscopy.

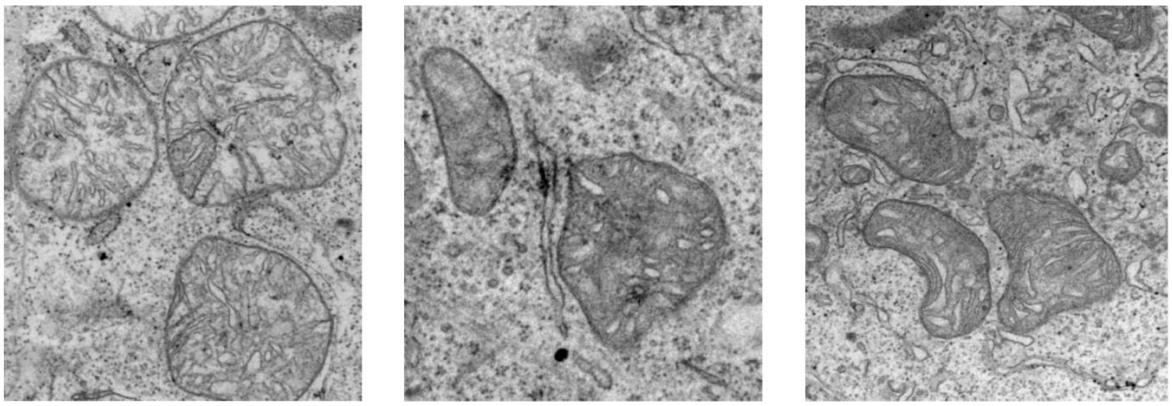

Figure 19. Electron density increases in the mitochondrial matrix upon mtPFS. HEK293T cells were treated with DMSO or CDDO or MG132. Cells were processed and observed by electron microscopy.

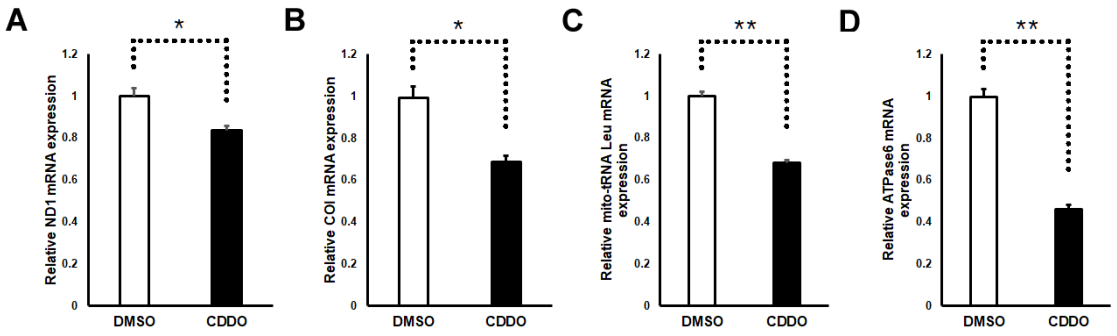

Figure 20. Expression of mtDNA-encoded genes decreases upon mtPFS. Data shown are the mRNA levels of randomly selected four mtDNA-encoded genes (ND1, COI, mito-tRNA Leu and ATPase6) in HEK293T cells treated with DMSO or CDDO. n=3. Error bars represent SE. *: $p<0.05$. **: $p<0.01$. Student's *t* test.

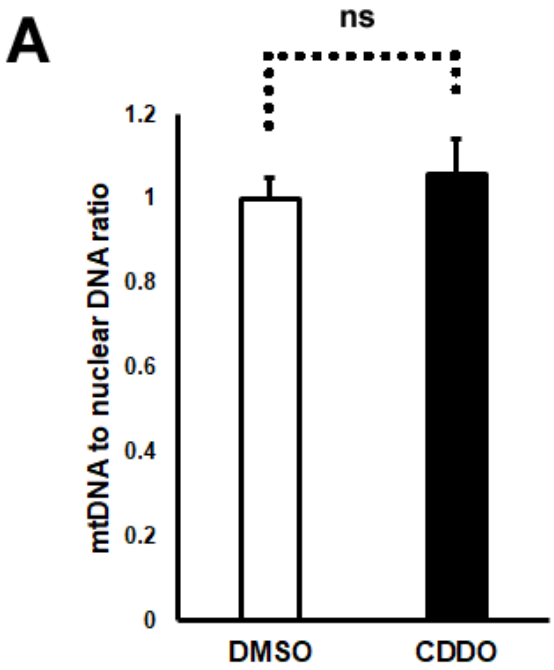

Figure 21. Short-term CDDO treatment does not alter mtDNA copies.
Mitochondrial-to-nuclear DNA ratios were analyzed by qPCR in HEK293T cells after DMSO or CDDO treatment for 6 hours. n=3. Error bars represent SE. n.s.: $p>0.05$. Student's *t* test

Chapter 4: Concluding remarks and future direction

The goal of this dissertation work was to advance current knowledge on the two aspects of biology: inflammasome regulations, and mitochondrial unfolded protein response (mtUPR). These two emerging fields are conceptually associated with nutrient sensing mechanisms. Using mouse genetics, molecular biology, cell biology and biochemical approaches, this investigation has elucidated the role of one member of nutrient sensors, SIRT2, in modulating the NLRP3 inflammasome activity. By setting up the experimental system of mtUPR, we also identified novel biomarkers of mitochondrial protein folding stress (mtPFS) as well as a novel branch of mtUPR.

In Chapter 2, we focused on SIRT2 and inflammasome regulations. We found that SIRT2 specifically constrains the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome in macrophages by directly deacetylating NLRP3. We confirmed that NLRP3 acetylation enhances its inflammasome function by mutating the deacetylated lysine residues to mimic the constitutively deacetylated state. Under conditions of aging and overnutrition, two prominent risk factors associated with insulin resistance, SIRT2 ablation in mice leads to increased systematic chronic inflammation and insulin resistance.

In Chapter 3, we focused on mtUPR and aimed to understand its underlying molecular basis and biomarkers. We set up the cellular experimental condition of mtUPR in our lab. We found two novel mtPFS biomarkers: mitochondrial aggregation and increased electron density in the mitochondrial matrix. We also identified a novel branch of mtUPR that involves transcriptional downregulation of genes encoded by mitochondrial DNA (mtDNA).

Multiple intriguing questions remain to be answered. One outstanding question involves the rescue of the SIRT2-NLRP3 axis. Can SIRT2 overexpression or NAD+ supplement in old macrophages reverse the aging-associated NLRP3 inflammasome activation and associated insulin resistance in mice models? Does SIRT2 expression decrease in the model of diet-induced obesity, and can SIRT2 overexpression rescue the phenotypes? Does the SIRT2-NLRP3 axis play a role in other chronic inflammatory diseases? Regarding the mtUPR, how sensitive and specific are the mitochondrial aggregation and increased electron density to mtPFS? Does genetic manipulation of SIRT7 change the above two biomarkers? And what is the molecular mechanism of decreased expression of mtDNA-encoded genes in response to mtPFS? Further research endeavors will hopefully provide insights into these questions.

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Appendix: Materials and Methods

Mice

SIRT2 knockout mice has been described previously [217]. 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 and RNAi

Double-stranded siRNAs were purchased from Qiagen and were transfected into cells via RNAiMax (Invitrogen) according to manufacturer's instructions. Mouse SIRT2 siRNA targeting sequence is 5'-CCAGAATAAGGCATTTCTCTA-3'. Mouse SIRT1 siRNA targeting sequence is AAGCGGCTTGAGGGTAATCAA. The control siRNA is non-targeting control (Qiagen). To induce caspase 1 activation, macrophages were primed with 100ng/ml LPS for 12 hours and then stimulated with 3mM ATP for 30mins. Proteins from culture media were trichloroacetic acid (TCA) precipitated for Western analyses of p17 IL-1β and p20 caspase 1. Proteins from cell lysates were analyzed for pro-IL-1β and pro-caspase 1. For NLRP3 acetylation, cells were glucose starved for 6 hours before immunoprecipitation.

Induction of mtPFS

HEK293T cells (ATCC) were treated with CDDO (Cayman Chemical) 5uM for 6 hours to induce mtPFS, or MG132 (Sigma) 20uM for 18 hours to inhibit proteasome.

Determination of mtDNA copy number

Simultaneous measurement of mitochondrial and nuclear DNA ratio was done as described [218]. DNA was isolated from cells using DNeasy Blood & Tissue Kits (Qiagen). DNA was quantified by real time PCR using Eva qPCR SuperMix kit (BioChain Institute) on an ABI StepOnePlus system.

Visualization of inflammasome complexes.

Inflammasome assembly was performed as previously described [219]. Briefly, HEK293T cells were co-transfected with ASC-EGFP and WT or mutant NLRP3 or control vector. 48 hours post transfection, cells were observed under fluorescence microscope for foci formation.

Immunoprecipitations

Immunoprecipitations were performed as previously described with Flag-resin (Sigma) [83]. Elution was performed with either Flag peptide (Sigma) for western analyses or 100mM Glycine solution (pH 3) for mass spectrometry analyses.

Insulin signaling in vivo
Mice were fasted for 5 hours, injected with either phosphate-buffered saline or insulin (Sigma) at 2 mU/g body weight for 15 min [220]. Mice were then euthanized by carbon dioxide. Muscle and liver were harvested for Western analyses with Akt and phospho-Akt Ser473 antibodies.

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 [221]. 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 THP1 cells, SIRT2 was cloned into the pFUGw lentiviral construct. Lentivirus was produced as described [23,75] concentrated by centrifugation, and resuspended with culture medium. To reconstitute NLRP3 knockout macrophages with wild type or NLRP3 mutants, wild type or NLRP3 mutants were cloned into pMSCVgfp retroviral construct. Retrovirus was generated by transfecting HEK293T cells (ATCC) with pMSCVgfp retroviral constructs as well as VSV-G and gag/pol expression vectors using Lipofectamine 2000 transfection kit (Invitrogen). 48 hours post transfection, culture supernatant was filtered through 0.45-mm-pore cellulose acetate filters, supplemented with 10 μg/ml of polybrene, and was applied to target macrophages (a gift from E. Alnemri). The cells were subjected to another cycle of infection on the next day.

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 β-Actin expression.

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. Statistical analysis was performed with Excel (Microsoft). 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 $p<0.01$. ns represents $p>0.05$. The results were presented as means ± s.e.m.

Supplementary Table 1: Antibodies used in this study

Antibodies	**Source**	**Catalog #**
SIRT2	Proteintech	15345-1-AP
Beta Actin	Sigma	A2066
Flag	Sigma	F1804
GAPDH	Santa Cruz	SC 25778
Acetyl-K	Biolegend	623402
Caspase 1	eBioscience	14-9832
IL-1β	R&D Systems	AF-401-NA
Akt	Cell Signaling	9272
p-Akt Ser473	Cell Signaling	4060