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The coordination of nuclear and mitochondrial communication during aging and calorie restriction

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Abstract

Mitochondria are dynamic organelles that integrate environmental signals to regulate energy production, apoptosis and Ca^{2+} homeostasis. Not surprisingly mitochondrial dysfunction is associated with aging and the pathologies observed in age-related diseases. The vast majority of mitochondrial proteins are encoded in the nuclear genome, and so communication between the nucleus and mitochondria is essential for maintenance of appropriate mitochondrial function. Several proteins have emerged as major regulators of mitochondrial gene expression, capable of increasing transcription of mitochondrial genes in response to the physiological demands of the cell. In this review, we will focus on PGC-1α, SIRT1, AMPK and mTOR and discuss how these proteins regulate mitochondrial function and their potential involvement in aging, calorie restriction and age-related disease. We will also discuss the pathways through which mitochondria signal to the nucleus. Although such retrograde signaling is not well studied in mammals, there is growing evidence to suggest that it may be an important area for future aging research. Greater understanding of the mechanisms by which mitochondria and the nucleus communicate will facilitate efforts to slow or reverse the mitochondrial dysfunction that occurs during aging.

Keywords

mitochondria; aging; SIRT1; PGC-1α; metabolism; AMPK

1. Introduction

Mitochondria are dynamic organelles that play critical roles in energy production, apoptosis, and intracellular signaling (Finkel and Holbrook, 2000; Wallace, 2005; Ryan and Hoogenraad, 2007). Mammalian tissues vary widely in mitochondrial number and bioenergetic capacity, and these parameters adapt in response to various physiological conditions, such as diet, exercise, temperature and hormones (Scarpulla, 2002). Mitochondria serve many metabolic roles, including ATP production, fatty acid oxidation, metabolism of acetyl-CoA by the tricarboxylic acid cycle, maintenance of intracellular calcium levels and biosynthesis of various molecules. Proper mitochondrial function is crucial for maintenance of homeostasis and engagement of appropriate stress responses both

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at the level of the cell and the entire organism. Not surprisingly, changes in mitochondrial number and activity are implicated in aging and age-related diseases, including diabetes, neurodegenerative diseases and cancer (Wallace, 2005).

Although mitochondria contain their own genome encoding 13 subunits involved in electron transport, the vast majority of the estimated 1000-1500 mitochondrial proteins are nuclear encoded (Johnson, et al., 2007; Pagliarini, et al., 2008). Mitochondrial biogenesis requires a sophisticated transcriptional program capable of responding to the energetic demands of the cell by coordinating expression of both nuclear and mitochondrial encoded genes (Scarpulla, 2002). Defects in regulation of mitochondrial biogenesis and gene expression leads to damaging consequences for the ability of the organism to cope with basal energetic demands or physiological stressors.

Mitochondria generate ATP through a process of oxidative phosphorylation (OXPHOS). As high-energy electrons derived from metabolic fuels are passed through a series of protein complexes (I-IV) embedded in the inner-mitochondrial membrane, their energy is used to pump protons from the mitochondrial matrix through the inner membrane into the inter-membrane space, generating a proton gradient known as the mitochondrial membrane potential ($\Delta\psi_m$) (Figure 1). Ultimately, the electrons reduce oxygen to form water, and the protons flow down their gradient through ATP synthase, driving the formation of ATP from ADP. Alternatively, protons can flow through uncoupling proteins, dissipating their potential energy as heat. Reactive oxygen species (ROS), formed when electrons inappropriately combine with O_2 to form the highly-reactive superoxide radical, are an inevitable byproduct of this process (Wallace, 2005). An increase in $\Delta\psi_m$, whether caused by impaired OXPHOS or an overabundance of nutrients relative to ADP, will stall electrons in the early steps of the electron transport chain and result in elevated ROS production (Wallace, 2005). SOD2 converts mitochondrial superoxide to hydrogen peroxide, which can then diffuse out of the mitochondria. ROS react with lipids, protein and DNA to generate oxidative damage, and so cells must guard carefully against an increase in oxidative stress accompanying ROS production.

Mitochondria are the primary site of ROS production within the cell, and increased oxidative stress is proposed to be one of the causes of mammalian aging (Harman, 1956; Finkel and Holbrook, 2000; Wallace, 2005). As a result, there is much interest in mitochondrial bioenergetic changes that occur with aging, and the topic has been extensively scrutinized (Merry, 2004; Hunt, et al., 2006; Navarro and Boveris, 2007). Major mitochondrial age-related changes are observed in multiple tissues and include decreased $\Delta\psi_m$, increased ROS production and an increase in oxidative damage to mtDNA, proteins and lipids (Sastre, et al., 1996; Hagen, et al., 1997; Bevilacqua, et al., 2005; Short, et al., 2005).

Importantly, many of these changes can be mitigated or prevented by the dietary regimen of calorie restriction (CR) (Merry, 2002; Merry, 2004; Hunt, et al., 2006). CR extends the lifespan of a wide variety of organisms, from yeast to rodents, and in higher organisms CR reduces the incidence of age-related diseases such as diabetes, cancer and cardiovascular disease (Loeb and Northrop, 1917; Comfort, 1963; Weindruch and Walford, 1988; McCay,

et al., 1989; Lin, et al., 2002b; Bordone and Guarente, 2005; Haigis and Guarente, 2006). Although the molecular mechanism by which CR exerts its anti-aging effects remains unknown, its beneficial impact on mitochondrial function is well established. Studies using cell culture models of CR and in rodents illustrate that CR causes a decrease in ROS production and attenuates the accrual of age-related oxidative damage, especially in postmitotic tissues such as brain, heart and skeletal muscle (Sohal, et al., 1994; Sohal and Weindruch, 1996; Gredilla, et al., 2001; Sanz, et al., 2005; Lopez-Lluch, et al., 2006). There is also evidence that CR may increase mitochondrial turnover in the liver, facilitating the removal of damaged mitochondria (Miwa, et al., 2008).

The effects of CR on mitochondrial respiratory function and ATP generation are quite controversial (Hunt, et al., 2006). Several studies in rodents suggest that CR can block an age-related decline in OXPHOS enzyme activity in skeletal muscle and can increase mitochondrial respiration in white adipose tissue (Nisoli, et al., 2005; Baker, et al., 2006; Hepple, et al., 2006). However, Lambert et al. studied rats on a CR diet for 4.5 and 15 months and found no change in the respiration rate of mitochondria from liver, heart, brain or kidney, although the authors did see an increase in respiration in brown adipose tissue (Lambert, et al., 2004). Other studies have shown that CR for 2, 6 and 12 months, but not for 18 months, decreases mitochondrial oxygen consumption in skeletal muscle (Bevilacqua, et al., 2004; Bevilacqua, et al., 2005). Such discrepancies could be due to differences in diet or experimental protocol, tissue-specific effects, or subtle changes in the physiological state of the animal at the time of the experiment. The relationship between mitochondrial OXPHOS activity and longevity is equally controversial. Some studies have found that CR in yeast increases mitochondrial respiration, and that respiration is required for CR-induced longevity (Lin, et al., 2002b; Easlon, et al., 2007), although another report did not observe this requirement (Kaeberlein, et al., 2005a). CR also increases mitochondrial respiration in worms, although paradoxically, knockdown of a large number of genes critical for mitochondrial function extends lifespan in worms (Lee, et al., 2003; Bishop and Guarente, 2007). Clearly, more work needs to be done to address how changes in mitochondrial function affect aging and longevity.

Transcriptional profiles of skeletal muscle, white adipose tissue, kidney, brain, and heart consistently show a decrease in expression of mitochondrial genes with age (Lu, et al., 2004; Zahn, et al., 2006; Linford, et al., 2007; Melov, et al., 2007). At the same time, mitochondrial turnover may be reduced, resulting in an age-related accumulation of old, damaged, or nonfunctional mitochondria (Terman, 2006). A study comparing transcriptional profiles of humans, mice, flies and worms found that genes involved in OXPHOS were downregulated during aging in all four organisms, suggesting that these genes represent a conserved marker of aging (Zahn, et al., 2007). Additionally, transcriptional profiling and analysis of mitochondrial content have demonstrated that CR can stimulate mitochondrial biogenesis and increase the expression of mitochondrial enzymes involved in OXPHOS (Lambert, et al., 2004; Nisoli, et al., 2005; Lopez-Lluch, et al., 2006; Civitarese, et al., 2007). Many studies show that fatty acid oxidation in rodents and humans declines in a variety of tissues with age, and that this decline can be linked to an age-dependent increase in fat accumulation (Abu-Erreish, et al., 1977; Melanson, et al., 1997; Tucker and Turcotte,

2002; Park, et al., 2006; Solomon, et al., 2008). CR can prevent an age-related decline in the expression of genes involved in fatty acid oxidation (Lee, et al., 2002; Zhu, et al., 2004).

Given the grave consequences of mitochondrial dysfunction seen during aging and in age-related disease, including falling ATP production, increased ROS generation and accumulation of oxidatively-damaged macromolecules, it is crucial to understand the signaling pathways that regulate mitochondrial number and function. Communication between the nucleus and mitochondria is essential for maintenance of appropriate mitochondrial activity, and disruption of this coordination often accompanies aging and age-related disease. In this review, we will address major signaling pathways between the nucleus and mitochondria that are responsible for regulating mitochondrial number, fuel utilization and energy production, and how this regulation is affected by aging and age-related diseases.

2. Transcriptional pathways controlling mitochondria

2.1 PGC-1 α

Several transcription factors play a role in the regulation of mitochondrial gene expression. For example, mitochondrial transcription factor A (TFAM) is responsible for transcription of genes encoded by mtDNA and is essential for mtDNA maintenance (Scarpulla, 2008). Although there is no single nuclear transcription factor responsible for coordinated expression of all genes encoding mitochondrial proteins, the binding sites of several transcription factors, including Sp1, YY1, CREB, MEF-2/E-box, and the nuclear respiratory factors NRF-1, NRF-2, ERRα, REBOX/OXBOX and MT1-4, are common in nuclear-encoded mitochondrial gene promoters (Goffart and Wiesner, 2003; Scarpulla, 2008). Since none of these transcription factors alone can account for the regulation of most mitochondrial proteins, coordinated changes in nuclear-encoded mitochondrial gene expression likely requires integration of multiple transcriptional pathways, a feat achieved largely by the peroxisome-proliferator-activated receptor γ coactivator-1 (PGC-1) family of transcriptional coactivators. PGC-1 coactivators interact with chromatin modifying enzymes, basal transcription machinery and splicing factors and trigger a robust induction of transcriptional activity (Handschin and Spiegelman, 2006). Members of the PGC-1 family, PGC-1α, PGC-1β and PRC, have emerged as master transcriptional regulators of mitochondrial function. Although PGC-1β has been shown to exert powerful effects on mitochondrial biogenesis and function, including regulating genes involved in fatty acid oxidation and ROS scavenging (Lin, et al., 2003a; St-Pierre, et al., 2003; Arany, et al., 2007), PGC-1α is the best studied of this coactivator family.

PGC-1α was originally identified as a transcriptional regulator of PPARγ capable of stimulating UCP-1 expression in brown adipose tissue (Puigserver, et al., 1998). Subsequently, PGC-1α has been shown to coactivate a wide variety of nuclear receptors, including PPARs, HNF4α, GR and ERRα, in addition to transcription factors such as NRF-1, MEF2C, FOXO1 and YY1 (Handschin and Spiegelman, 2006; Rodgers, et al., 2008). This wide variety of binding partners empowers PGC-1α with the ability to induce context-specific coordinated sets of gene expression that are tailored to the immediate physical demands of the organism. Moreover, the ability of PGC-1α to coactivate major

transcription factors involved in mitochondrial gene expression, including NRF-1, NRF-2, ERRα and PPARα, endows PGC-1α with the ability to direct the complex program of mitochondrial biogenesis (Handschin and Spiegelman, 2006; Ryan and Hoogenraad, 2007). By coactivating NRF-1 directly on the TFAM promoter and the promoters of mitochondrial transcription specificity factors TFB1M and TFB2M, PGC-1α ensures the coordination of nuclear and mitochondrial genomes during biogenesis (Wu, et al., 1999; Gleyzer, et al., 2005). Consistent with its role as a master-regulator of mitochondrial biogenesis, PGC-1α expression closely correlates with mitochondrial content and is induced by cold exposure, short-term exercise and fasting—all conditions that increase tissue demands on mitochondrial function (Kelly and Scarpulla, 2004). Indeed, overexpression of PGC-1α in skeletal muscle, white adipose cells and the heart is sufficient to induce mitochondrial biogenesis, elevate oxygen consumption and increase fatty acid oxidation (Puigserver, et al., 1998; Wu, et al., 1999; Lin, et al., 2002a; Kelly and Scarpulla, 2004; Russell, et al., 2004).

Given the apparent decline in mitochondrial biogenesis and function during aging, it is important to understand how PGC-1α activity changes with age. Several lines of evidence link PGC-1α and aging: expression of PGC-1α is regulated by aging; PGC-1α is capable of improving or rescuing mitochondrial dysfunction, and PGC-1α is implicated in the pathogenesis of several age-related diseases, including diabetes, heart failure and neurodegeneration. First, PGC-1α levels decline with age in rodent skeletal muscle and this decline is blunted by CR (Baker, et al., 2006; Hepple, et al., 2006). Human subjects engaged in CR or CR plus exercise demonstrated increased PGC-1α levels (Civitarese, et al., 2007). A cell culture model of CR, in which cells were cultured with serum from calorie restricted humans or rats, similarly induced increased PGC-1α expression (Lopez-Lluch, et al., 2006; Civitarese, et al., 2007; Allard, et al., 2008). Furthermore, several genetic models of longevity show increased levels of PGC-1α accompanied by improvements in mitochondrial function. Fat-specific insulin receptor knock-out (FIRKO) mice have an increased lifespan in addition to elevated levels of PGC-1α and PGC-1β and increased mitochondrial DNA content in white adipose tissue (Katic, et al., 2007). While expression of nuclear-encoded mitochondrial genes tended to decline with age in the white adipose tissue of the control mice, expression of these genes was maintained, or even increased, in the long-lived FIRKO mice (Katic, et al., 2007). Likewise, long-lived growth hormone receptor knock-out mice have higher levels of PGC-1α than their controls, while bovine growth hormone transgenic mice have reduced levels of PGC-1α and shortened lifespan (Al-Regaiey, et al., 2005). Taken together, these studies suggest that modulation of mitochondrial function by PGC-1α may play an important role in lifespan regulation.

Secondly, PGC-1α improves mitochondrial dysfunction, a major phenotype of aging and age-related diseases. The biological changes effected by exercise provide an important example of the ability of PGC-1α to exert anti-aging benefits through a program of mitochondrial biogenesis. Exercise increases skeletal muscle mitochondrial number and enzyme activity (Holloszy and Booth, 1976). Moreover, exercise mitigates the age-related decline of both of these parameters in skeletal muscle (Short, et al., 2003; Menshikova, et al., 2006; Conley, et al., 2007). For example, compared with young subjects, the gene expression profile of elderly subjects showed striking downregulation of genes involved in mitochondrial function. Six months of strength training reversed many of these

transcriptional changes, particularly for genes encoding mitochondrial proteins (Melov, et al., 2007). Similarly, skeletal muscle mitochondrial ATP production was improved by exercise in elderly subjects (Jubrias, et al., 2001; Williams, et al., 2007). The mitochondrial benefits of exercise may be mediated in large part by PGC-1α; indeed, many studies have documented an increase in PGC-1α levels following both acute exercise and exercise training, although the exercise-induced increase in PGC-1α is enhanced by prior training (Goto, et al., 2000; Baar, et al., 2002; Pilegaard, et al., 2003; Russell, et al., 2003; Terada, et al., 2005; Winder, et al., 2006). Consistent with this hypothesis, mice with muscle-specific PGC-1α overexpression display improved performance in a variety of exercise challenges in addition to increased mitochondrial gene expression, mtDNA content, and enzyme activity (Calvo, et al., 2008). Moreover, transgenic overexpression of PGC-1α in the skeletal muscle of a mouse model of mitochondrial myopathy resulted in increased mitochondrial biogenesis and improved ATP levels and was sufficient to improve clinical symptoms and prolong lifespan (Wenz, et al., 2008).

Many studies suggest that mitochondrial dysfunction is associated with age-related diseases such as diabetes (Lowell and Shulman, 2005). For example, insulin-resistant offspring of patients with type 2 diabetes have reduced muscle mitochondrial density and substrate oxidation (Petersen, et al., 2004; Morino, et al., 2005). Additionally, a study by Petersen et al. showed that elderly subjects had a marked increase in insulin resistance in muscle accompanied by decreases in mitochondrial substrate oxidation and ATP synthesis (Petersen, et al., 2003). Gene expression changes corroborate these observations: expression of genes involved in OXPHOS is reduced in diabetic patients, their first-degree family members and individuals with glucose intolerance (Mootha, et al., 2003; Patti, et al., 2003). The same studies showed that PGC-1α (and PGC-1β) expression levels were also decreased, suggesting that a decline in PGC-1-mediated coactivation of transcription of genes encoding mitochondrial proteins is a major cause of the decline in mitochondrial number and function during diabetes (Mootha, et al., 2003; Patti, et al., 2003). Supporting this hypothesis, Mootha et al. demonstrated that PGC-1α regulates OXPHOS gene expression through induction and coactivation of ERRα and NRF-2, and that disruption of this interaction in cultured muscle cells elicits some aspects of a type 2 diabetes-like phenotype (Mootha, et al., 2004). A pair of recent studies challenge the model that decreased PGC-1α expression plays a causal role in the development of diabetes. PGC-1α skeletal muscle specific knockout mice show normal peripheral insulin sensitivity and conversely, transgenic mice overexpressing PGC-1α in muscle are more prone to insulin resistance (Handschin, et al., 2007; Choi, et al., 2008). It will be important for future studies to examine how specific changes in mitochondrial function affect the pathogenesis of diabetes. Since both PGC-1α and PGC-1β expression in skeletal muscle decreases with age, PGC-1α may serve as an important bridge that links age-related decline in mitochondrial function with a concurrent increase in the incidence of type 2 diabetes (Ling, et al., 2004).

Cardiovascular disease is another hallmark of aging, and PGC-1α has been linked to the pathogenesis of heart failure (Finck and Kelly, 2006; Handschin and Spiegelman, 2006). PGC-1α is decreased in some forms of heart failure that are associated with a decrease in fatty acid oxidation and an increased reliance on glucose oxidation (Huss and Kelly, 2004; Huss and Kelly, 2005). For example, in mouse models of chronic pressure overload (a

model for hypertension), PGC-1α and PPARα target genes are downregulated (Finck and Kelly, 2006). End-stage heart failure is often associated with reduced mitochondrial respiration and ATP production, and PGC-1α null mice display both of these characteristics (Arany, et al., 2005; Huss and Kelly, 2005). Hearts from mice deficient for PGC-1α have an impaired ability to increase work output in response to stimulation and display an age-dependent increase in cardiac dysfunction (Arany, et al., 2005). However, chronic cardiac-specific overexpression of PGC-1α causes mitochondrial proliferation that ultimately results in ultrastructurally abnormal mitochondria and cardiomyopathy (Lehman, et al., 2000; Russell, et al., 2004). Thus, although increased PGC-1α expression may not prove a panacea for all diseases of aging, there is much evidence to suggest that falling PGC-1α levels may play a role in age-related heart failure.

Several studies suggest a link between PGC-1α and neurodegeneration. Impaired mitochondrial function, including decreased expression of genes involved in OXPHOS, is linked to a number of neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's disease (HD) (Schon and Manfredi, 2003). Mice lacking PGC-1α display behavioral changes and neurodegeneration, including lesions in the striatum, the primary brain region affected by HD (Lin, et al., 2004a; Leone, et al., 2005). More direct evidence of a role for PGC-1α in HD was provided by Cui et al., who showed that mutant huntingtin can directly interfere with transcription of PGC-1α, triggering mitochondrial dysfunction (Cui, et al., 2006). Exogenous PGC-1α can partially reverse the deleterious effects of mutant huntingtin in cultured cells, and lentiviral-mediated overexpression of PGC-1α in the striatum is neuroprotective in a transgenic model of HD (Cui, et al., 2006). Furthermore, mitochondria from HD mouse brain show reduced PGC-1α levels, OXPHOS gene expression and oxygen consumption, and stable expression of PGC-1α increases the resistance of mutant neurons to mitochondrial toxins (Weydt, et al., 2006). Increased PGC-1α expression has also been shown to reduce neuronal cell death due to oxidative stress (St-Pierre, et al., 2006). This study found that PGC-1α null mice are hypersensitive to neurodegeneration triggered by oxidative stressors such as MPTP and kainic acid, and that induction of anti-oxidant genes requires PGC-1α activity (St-Pierre, et al., 2006). With the ability to increase mitochondrial biogenesis, function and anti-oxidant defenses, PGC-1α is powerfully equipped to combat many of the suspected causes of aging and age-related decline.

While changes in PGC-1α expression have an important impact on mitochondrial number and bioenergetics, PGC-1α is subject to a wide variety of regulatory mechanisms, including subcellular localization, turnover and post-translational modification, all of which have important consequences for its activity (Handschin and Spiegelman, 2006). A study by Anderson et al. suggested a model in which all of these mechanisms of regulation converge to form a transcriptional response tailored to specific physiological conditions (Anderson, et al., 2008). In their model, PGC-1α is found in both the nucleus and the cytoplasm under basal conditions, and translocates to the nucleus during stress. Nuclear SIRT1 deacetylates and activates PGC-1α at the same time that GSK3β phosphorylates PGC-1α, priming it for ubiquitination and degradation. The net result is transient PGC-1α activity, enabling a rapid transcriptional stress response that is quickly shut off by subsequent degradation of the coactivator. Under chronic stress conditions such as CR, however, GSK3β does not

phosphorylate PGC-1α, and PGC-1α is thus perpetually active in the nucleus, facilitating a sustained increased in transcription of genes involved in mitochondrial function (Anderson, et al., 2008). In this manner, post-translational regulation provides an additional mechanism whereby PGC-1α can regulate mitochondrial function according to the specific needs of the cell.

2.2 SIRT1

Conserved from bacteria to mammals, sirtuins are a family of proteins that regulate metabolism and longevity in a variety of model organisms (Table 1) (Haigis and Guarente, 2006; Schwer and Verdin, 2008). Sirtuins are protein deacetylases and/or ADP-ribosyltransferases whose absolute requirement for NAD^+ suggests that their activity may be tied to the metabolic state of the cell (Guarente, 2006; Schwer and Verdin, 2008). In yeast, increased dosage of silent information regulator 2 (Sir2) extends lifespan by 50% while Sir2 deletion shortens lifespan, and some studies demonstrate that lifespan extension by CR requires Sir2 (Kaeberlein, et al., 1999; Lin, et al., 2000). Similarly, increased levels of Sir2 extend lifespan in worms and flies (Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). Of the seven mammalian sirtuins (SIRT1-7), SIRT1 is most closely related to yeast Sir2 and the best studied to date (Frye, 2000; Bordone and Guarente, 2005). Through deacetylation of a variety of proteins, including PGC-1α, FOXO1, FOXO3, Ku70, NF-κB, AceCS1, MEF2 and p53, SIRT1 can regulate physiological processes, such as stress resistance and energy metabolism, that are known to be affected during aging and moderated by CR (Haigis and Guarente, 2006; Schwer and Verdin, 2008).

Intriguingly, mammalian sirtuins have diverse subcellular localizations, raising the possibility that sirtuins may provide a mechanism for inter-organelle coordination under various stress conditions (Michishita, et al., 2005). SIRT1, SIRT6 and SIRT7 are found predominantly in the nucleus; SIRT3, SIRT4 and SIRT5 localize to mitochondria, and SIRT2 is cytoplasmic (Michishita, et al., 2005). Growing evidence suggests that sirtuins may shift localization in response to specific signals. SIRT3 may translocate from the mitochondria to the nucleus during cellular stress, and both SIRT1 and SIRT2 shuttle between the nucleus and cytoplasm (North and Verdin, 2007; Scher, et al., 2007; Tanno, et al., 2007; Nakamura, et al., 2008). In addition, sirtuins likely orchestrate inter-organelle communication independent of physical shuttling. For example, SIRT3 overexpression results in increased levels of phospho-CREB, which may result in numerous transcriptional changes, including elevated PGC-1α transcription (Shi, et al., 2005). It will be interesting to see if changes in the activity of mitochondrial sirtuins can influence signaling pathways outside of the mitochondria. Furthermore, although SIRT1 physically binds nuclear and cytosolic proteins, its activity has profound effects on mitochondrial number and oxidative capacity (Haigis and Guarente, 2006; Schwer and Verdin, 2008).

There are few studies directly linking SIRT1 to mammalian aging, but evidence suggests that SIRT1 may be a pro-longevity factor. In some studies, SIRT1 expression mirrors the proliferative state of the cell: SIRT1 levels decline as cells senesce but are restored with immortalization (Sasaki, et al., 2006). Similarly, SIRT1 protein expression decreases with age in tissues which mitotic activity declines over time, but tissues with constant mitotic

activity demonstrate no change in SIRT1 levels (Sasaki, et al., 2006). SIRT1 activity, but not protein levels, decline with age in pancreatic β cells, and this may be due to a decline in systemic NAD^+ biosynthesis (Ramsey, et al., 2008). It will be interesting to see if this systemic decline in NAD^+ biosynthesis translates into lower SIRT1 activity in a variety of tissues. A recent study by Oberdoerffer et al. suggests that SIRT1 represses repetitive DNA and thus may promote genomic stability in mammalian cells in a manner analogous to that of yeast Sir2 (Oberdoerffer, et al., 2008). Loss of SIRT1 repression results in transcriptional changes that are similar to those seen during aging in the brain, and overexpression of SIRT1 can suppress these age-dependent transcriptional changes (Oberdoerffer, et al., 2008). A fraction of the genes regulated by SIRT1 may encode mitochondrial proteins, and so it will be interesting to see how changes in regulation of genomic stability by SIRT1 affect mitochondrial function.

Rodent studies of CR support the hypothesis that SIRT1 is a conserved mediator of several benefits of CR. Several groups have found that CR upregulates SIRT1 expression in a variety of tissues, such as brain, kidney, liver, white adipose tissue and skeletal muscle, although the effects of CR on SIRT1 expression remain controversial and may be tissue specific (Cohen, et al., 2004; Nisoli, et al., 2005; Civitarese, et al., 2007; Barger, et al., 2008; Chen, et al., 2008). Consistent with the idea that SIRT1 may be responsible for some of the benefits of CR, transgenic mice overexpressing SIRT1 display several similarities with CR mice. Whole body SIRT1 knock-in mice were leaner, more glucose tolerant, and displayed reduced levels of blood cholesterol, adipokines and insulin compared to WT controls (Bordone, et al., 2007). A separate model of SIRT1 overexpression found that increased levels of SIRT1 did not improve basal glucose tolerance, but were protective against obesity-induced glucose intolerance (Banks, et al., 2008). Additionally, while CR causes an increase in activity in WT mice, restricted SIRT1 null mice do not increase their activity, suggesting that SIRT1 is necessary for this phenotype of CR (Chen, et al., 2005; Boily, et al., 2008). Moreover, CR was not able to increase the lifespan of SIRT1 null mice, although the short lifespan of SIRT1 null mice complicate the interpretation of this observation (Boily, et al., 2008; Li, et al., 2008). Furthermore, adipose tissue size and distribution change with age, and a reduction in white adipose tissue (WAT) mass is presumed to be responsible for at least part of the lifespan increase by CR (Bluher, et al., 2003; Cartwright, et al., 2007). SIRT1 has been shown to bind PPARγ and repress transcription of its target genes involved in fat storage (Picard, et al., 2004). As a result, upregulation of SIRT1 in differentiated fat cells causes lipolysis and fat loss, suggesting that an increase in SIRT1 levels in WAT during CR may be in part responsible for increased mobilization of fatty acids and reduced adiposity (Picard, et al., 2004).

Several studies lead to the conclusion that SIRT1 exerts its control over mitochondrial number and fuel oxidation primarily by regulating PGC-1α activity. Rodgers et al. showed that in the fasted liver, SIRT1 interacts with and deacetylates PGC-1α, enabling a transcriptional switch from glycolytic to gluconeogenic gene expression and enhancing hepatic glucose output (Rodgers, et al., 2005). Subsequent studies have shown that SIRT1 can orchestrate a coordinated shift in mitochondrial substrate usage, allowing the cell to preserve glucose while oxidizing fatty acids, reminiscent of the substrate switch seen during CR. In skeletal muscle, deacetylation of PGC-1α by SIRT1 promotes transcription of

mitochondrial fatty acid oxidation genes, and SIRT1 is required for the switch to fatty acid oxidation under low-glucose conditions (Gerhart-Hines, et al., 2007). Additionally, this study found that deacetylation of PGC-1α by SIRT1 produces an increase in transcription of genes involved in OXPHOS, suggesting that under certain conditions, SIRT1 can have an important impact on mitochondrial substrate oxidation and energy production (Gerhart-Hines, et al., 2007). Mitochondria isolated from livers of SIRT1 null mice displayed lower rates of respiration and ROS production (Boily, et al., 2008). Indeed, compared to WT counterparts, SIRT1 null mice had increased levels of active AMPK after a 24 hour fast, implying that the SIRT1 null mice have difficulty maintaining ATP levels (Boily, et al., 2008). Together, these studies suggest that SIRT1 is necessary for appropriate mitochondrial substrate oxidation during energy depletion.

Studies with pharmacological activators also support a role for SIRT1 in the control of mitochondrial function. Resveratrol, a naturally occurring polyphenol, was identified in a screen for SIRT1 activators and has subsequently been shown to increase lifespan and delay aging in worms and flies in a Sir2-dependent manner (Howitz, et al., 2003; Wood, et al., 2004; Viswanathan, et al., 2005). Resveratrol can increase survival of mice fed a high-calorie diet, and although it does not increase lifespan of mice fed a standard chow diet, resveratrol does reduce the severity of physical hallmarks of aging, including cataract formation, loss of motor coordination, decline in bone mineral density and increased aortic inelasticity (Baur, et al., 2006; Pearson, et al., 2008). Mitochondrial number declines in mice fed a high-calorie diet, and reseveratrol treatment can reverse this decline. Consistent with its role as a SIRT1-activating compound, resveratrol causes a decline in PGC-1α acetylation levels, providing a potential mechanism for the increase in mitochondrial number (Baur, et al., 2006). Treating mice with resveratrol activates PGC-1α and induces genes involved in OXPHOS and mitochondrial biogenesis, resulting in increased mitochondrial size, number, and oxidative activity (Lagouge, et al., 2006). Similar effects in cell culture were prevented with SIRT1 deletion, arguing for a direct role for SIRT1 in regulation of mitochondrial number and oxidative capacity (Lagouge, et al., 2006). Notably, microarray analyses revealed that both every other day feeding (a dietary regimen that produces lifespan extensions similar to CR) and resveratrol treatment produce significant upregulation of mitochondrial gene expression (Pearson, et al., 2008). It is important to note that resveratrol can activate a variety of proteins, including AMPK, and this lack of specificity complicates interpretation of the above studies (Baur, et al., 2006; Zang, et al., 2006). Recently, SRT1720 has been identified as a specific activator of SIRT1 (Milne, et al., 2007; Feige, et al., 2008). While SRT1720 treatment results in PGC-1α deacetylation and induction of genes involved in fatty acid oxidation, unlike resveratrol, SRT1720 did not affect mitochondrial number (Feige, et al., 2008). Thus, resveratrol's effects on mitochondrial density may stem from alternate targets, complex signaling networks, or dose-dependent interactions. In contrast, the ability of SIRT1 to cause a global shift towards fatty acid oxidation is supported by a number of pharmacologic and genetic models (Hallows, et al., 2006; Feige, et al., 2008; Rodgers, et al., 2008).

An increasing body of evidence points to the beneficial impact of SIRT1 on age-related disease, particularly diabetes. Resveratrol treatment has been shown to increase insulin sensitivity and protect mice against diet-induced obesity and insulin resistance (Baur, et al.,

2006; Lagouge, et al., 2006). SRT1720, a specific activator of SIRT1, similarly lowers plasma glucose and improves insulin sensitivity in diet-induced and genetically obese mice (Milne, et al., 2007). Moreover, SRT1720 can improve glucose tolerance in chow fed mice, protect against diet-induced obesity and improve lipid and cholesterol profiles of mice on a high fat diet (Feige, et al., 2008). Supporting the link between SIRT1 and insulin resistance, a study by Sun et al. found that experimentally inducing insulin resistance in cultured cells or in mice results in downregulation of SIRT1 expression (Sun, et al., 2007). Importantly, knock-down of SIRT1 expression was sufficient to induce insulin resistance, and overexpression of SIRT1 under conditions of insulin resistance improved insulin sensitivity (Sun, et al., 2007). The authors found that SIRT1 can repress expression of protein tyrosine phosphatase 1B (PTP1B), a negative regulator of insulin signaling, providing a potential mechanism for the effect of SIRT1 on insulin signaling in peripheral tissues (Sun, et al., 2007). Furthermore, SIRT1 can regulate insulin secretion from pancreatic β cells, at least in part by regulating expression of uncoupling protein 2 (UCP2). Increased UCP2 expression reduces OXPHOS efficiency and ATP production, thus reducing insulin secretion, which is sensitive to the ATP/ADP ratio. Mice with β cell-specific SIRT1 overexpression have reduced UCP2 levels and increased ATP production, enabling enhanced insulin secretion in response to glucose stimulation, although this effect is blunted when mice reach 18-24 months of age (Moynihan, et al., 2005; Ramsey, et al., 2008). Conversely, knock down of SIRT1 in β cell lines induces UCP2, lowers the ATP/ADP ratio and blunts insulin secretion, while SIRT1 null mice have constitutively elevated UCP2 expression and low levels of blood insulin (Bordone, et al., 2006). The effects of SIRT1 on insulin secretion and glucose homeostasis are likely not limited to regulation of PTP1B and UCP2 expression, and it will be important for future studies to address how the effects of SIRT1 on other targets, including PGC-1α, FOXO1 and NF-κB, affect context-specific responses to insulin signaling.

SIRT1 protects against some models of neurodegeneration, although unlike CR, which convincingly delays neurodegeneration (Bruce-Keller, et al., 1999; Duan, et al., 2003; Maswood, et al., 2004), the protective role of SIRT1 remains controversial. Although mitochondrial dysfunction plays an important role in many models of neurodegeneration, studies linking SIRT1 and neuronal survival have not addressed the potential for SIRT1 to ameliorate pathological changes in mitochondrial function. Because SIRT1 activates PGC-1α, which can improve mitochondrial bioenergetics and resistance to oxidative damage, it will be important to investigate whether SIRT1 can mitigate neurodegeneration via PGC-1α. Instead, many studies have implicated SIRT1 in stress response pathways: SIRT1 deacetylates p53 and represses its proapoptotic activity (Luo, et al., 2001; Vaziri, et al., 2001) and SIRT1 can deacetylate FOXO3, potentiating its ability to induce resistance to cell stress but inhibiting its ability to induce cell death (Brunet, et al., 2004; Motta, et al., 2004). In contrast, a recent study showed that SIRT1 inhibition increased neuronal resistance to oxidative damage and the brains of aged SIRT1-deficient mice displayed fewer markers of oxidative damage, suggesting that SIRT1 inhibition provides protection against oxidative stress *in vivo* (Li, et al., 2008). Taken together, these studies suggest that SIRT1 may play diverse, context-specific roles in neuronal stress response pathways, and that careful investigation is needed to elucidate the conditions under which enhanced SIRT1

activity will be beneficial. Furthermore, while current studies emphasize targets such as p53, FOXO and NF-κB as mediators of SIRT1-driven neuroprotection, it will be interesting to see if changes in mitochondrial number or bioenergetics play a role in the neuronal benefits of enhanced SIRT1 activity, given that defective mitochondrial bioenergetics are implicated in many neurodegenerative disorders (Wallace, 2005).

2.3 AMPK

Activity of the conserved AMP-activated protein kinase (AMPK) depends on the intracellular AMP/ATP ratio, enabling the kinase to serve as a sensor of cellular energy status. Stresses such as glucose deprivation, oxidative or hyperosmotic stress, ischemia, hypoxia and exercise deplete cellular ATP and increase AMPK activity (Kahn, et al., 2005). AMPK activation triggers transcriptional and post-translational responses that coordinate appropriate changes in mitochondrial bioenergetics in order to respond to the stress of low energy (Nilsson, et al., 2006; Osler and Zierath, 2008). For example, stimulation of AMPK activity induces transcription of PPARα and PGC-1α target genes, enabling a switch towards reliance on oxidation of fatty acids to replenish energy stores (Lee, et al., 2006). Recently, AMPK has been shown to phosphorylate and activate transcriptional activity of FOXO3 (Greer, et al., 2007a), and AMPK has been implicated in regulation of a wide variety of transcription factors, including HDAC5, p300, HNF4α, TRIP6 and p53 (McGee and Hargreaves, 2008).

As a central regulator of pathways affected by aging and CR, including energy storage and oxidative metabolism, AMPK is increasingly studied as a potential longevity regulator in model organisms (Table 1). In support of this hypothesis, two studies using independent models of CR in worms found that lifespan extension with dietary restriction requires the AMPK homolog aak-2 (Greer, et al., 2007b; Schulz, et al., 2007). Similarly, overexpression of aak-2 extended worm lifespan while worms with mutant aak-2 have shortened lifespan (Apfeld, et al., 2004). Interestingly, many common low-level stressors, such as high temperature pulse, caused an increase in the AMP/ATP ratio and resulted in lifespan extension, but the same treatment did not affect the lifespan of aak-2 mutant worms (Apfeld, et al., 2004). These studies suggest that increases in the AMP/ATP ratio may play an important role in the response to CR by signaling through aak-2 to permit lifespan extension. In contrast, it appears that an increase in activity of Snf1p, the yeast homolog of AMPK, causes accelerated aging by increasing recombination at rDNA loci and encouraging the formation of extrachromsomal rDNA circles (ERC) (Ashrafi, et al., 2000; Lin, et al., 2003b). Notably, yeast Sir2 extends lifespan in yeast by suppressing ERC formation (Sinclair and Guarente, 1997; Kaeberlein, et al., 1999). However, Snf1p is clearly necessary for the response to glucose restriction in yeast: in the absence of fermentable carbon sources, yeast derepress expression of genes involved in mitochondrial oxidative metabolism, and Snf1p is required for this metabolic switch (Hardie, 2004). Further study is necessary to resolve this apparent contradiction between the effect of AMPK homologs on lifespan in yeast and worms.

Given its ability to modulate mitochondrial number and substrate oxidation, mammalian AMPK is also a strong candidate for mediating mitochondrial changes during aging and CR.

Nevertheless, two groups have shown that CR does not change AMPK activity in the heart or in skeletal muscle, and may even decrease AMPK activity in the liver (Gonzalez, et al., 2004; To, et al., 2007). On the other hand, AMPK activity does appear to change with age. One group found that the activity of AMPK decreases with age in rat skeletal muscle, and that this decline is associated with an increase in insulin resistance which can be blocked by treatment with AICAR, an AMP analog known to activate AMPK (Qiang, et al., 2007). In contrast, Reznick et al. did not observe an age-related change in basal AMPK activity in mouse skeletal muscle, but they did find that stimuli such as AICAR, β-guanidinopropionic acid (β-GPA) feeding and exercise were able to increase AMPK activity in young, but not old, mice (Reznick, et al., 2007). Consequently, while β-GPA feeding resulted in a 38% increase in mitochondrial density in young mice, this effect was not seen in old mice, suggesting that impaired AMPK activation is linked to reduced mitochondrial biogenesis during aging (Reznick, et al., 2007). It is unclear if these results can be generalized to tissues besides skeletal muscle, as one study showed that basal AMPK activity is increased in the liver of 24 month old mice compared to 5 month old mice, although this study also suggested that the responsiveness of AMPK activity declines with age (Mulligan, et al., 2005). Suggestively, resveratrol activates AMPK as well as SIRT1, and would be interesting to know whether AMPK contributes to the lifespan extension conferred by resveratrol treatment (Baur, et al., 2006; Zang, et al., 2006). More research is needed to determine how changes in AMPK activity may affect aging and if any of the benefits of CR are mediated by AMPK.

Growing evidence suggests that reductions in AMPK activity may be an important factor associated with the decreased mitochondrial biogenesis and fatty acid oxidation associated with aging. Certainly, modulations of AMPK activity can greatly influence these parameters. For example, several groups have found that pharmacological activation of AMPK through β-GPA or AICAR administration results in mitochondrial biogenesis in skeletal muscle, measured by increased NRF-1 binding activity and upregulation of key mitochondrial enzymes (Winder, et al., 2000; Bergeron, et al., 2001). Importantly, β-GPA feeding did not affect mitochondrial number in mice expressing a dominant negative form of AMPK in skeletal muscle (Zong, et al., 2002). Furthermore, a transgenic mouse overexpressing a gain-of-function mutant of the AMPK γ3 subunit in skeletal muscle ($\gamma3^{225Q}$) demonstrated elevated AMPK activity, resulting in increased expression of NRF-1, NRF-2, TFAM and PGC-1α and increased mitochondrial biogenesis (Garcia-Roves, et al., 2008).

Only recently have studies begun to shed light on the mechanism by which AMPK activity causes increased expression of transcriptional activators of mitochondrial biogenesis. Jager et al. showed that in cultured muscle cells many of the transcriptional effects of AMPK activity, including the increase in expression of genes encoding mitochondrial proteins, require PGC-1α (Jager, et al., 2007). AMPK directly phosphorylates PGC-1α, enhancing its activity at its own promoter and triggering a transcriptional cascade that increases expression of PGC-1α and its target genes involved in mitochondrial biogenesis (Jager, et al., 2007). PGC-1α regulates expression of genes involved in fatty acid oxidation in skeletal muscle, and since AMPK can enhance PGC-1α levels, it is not surprising that AMPK activation results in increased levels of enzymes required for fatty acid oxidation (Long, et

al., 2005). Accordingly, mice with the AMPK γ3 subunit deleted in skeletal muscle have decreased expression of several genes involved in fatty acid oxidation, while ($\gamma3^{225Q}$) mice have increased levels of these same genes (Nilsson, et al., 2006).

Additionally, AMPK can regulate mitochondrial substrate oxidation at the post-translational level. AMPK phosphorylates and modulates the activity of a variety of metabolic proteins, including TSC2, PFK2, ACC1/2 and glycogen synthase, resulting in a general cellular switch away from biosynthesis towards ATP generating processes such as glucose uptake, glycolysis and fatty acid oxidation (Hardie, 2004). AMPK powerfully regulates mitochondrial fatty acid oxidation through phosphorylation of ACC1 and ACC2. As the rate-limiting enzyme in mitochondrial fatty acid uptake, carnitine palmitoyltransferase-1 (CPT1) is an important regulator of mitochondrial β-oxidation. CPT1, in turn, is allosterically inhibited by malonyl CoA. ACC1/2 synthesize malonyl CoA from acetyl CoA, and it is thought that the malonyl CoA generated by the cytoplasmic ACC1 is used for fatty acid synthesis, while the malonyl CoA generated by the mitochondrially-associated ACC2 is responsible for inhibition of mitochondrial fatty acid oxidation (Abu-Elheiga, et al., 2001; Abu-Elheiga, et al., 2005). Activated AMPK phosphorylates and inhibits both ACC1 and ACC2, simultaneously blocking fatty acid synthesis and activating mitochondrial fatty acid oxidation (Merrill, et al., 1997; Vavvas, et al., 1997; Hardie and Pan, 2002). Thus, during conditions of energy depletion, the switch from fatty acid synthesis to oxidation is mediated in a large part by the increased activity of AMPK. Although there is no evidence yet to suggest that mitochondrial changes that occur with aging or CR are mediated by AMPK, the fact that AMPK can positively influence many of the parameters that are negatively affected by aging makes it an important target for future study.

Increasingly, AMPK is viewed as a central player in mediating some of the beneficial effects of exercise and as a potential target for the treatment of diabetes. AMPK is activated by endurance training and pharmacological activation of AMPK by AICAR causes transcriptional changes similar to those seen during endurance exercise (Winder, et al., 2006; Jorgensen and Rose, 2008). The importance of AMPK is controversial, as a dominant negative form of AMPK prevents the transcriptional changes induced by β-GPA, but exercise induced increases in transcription were not blocked in mice lacking AMPK activity in skeletal muscle (Zong, et al., 2002; Jorgensen, et al., 2005). A recent study found that treating sedentary mice with AICAR alone was sufficient to induce genes involved in oxidative metabolism and to enhance running endurance by 44% (Narkar, et al., 2008). Similarly, increasing AMPK activity is believed to be a central mechanism for type 2 diabetes therapies (Hardie, 2008). In addition to its ability to increase mitochondrial number and fatty acid oxidation and to reduce fatty acid synthesis, AMPK can also stimulate GLUT4 translocation in skeletal muscle, thus increasing glucose uptake and metabolism. Since all of these processes are important parts of diabetes treatments, it is not surprising that many of the drugs used for type 2 diabetes, such as metformin and the thiazolidinediones, result in increased AMPK activity (Hardie, 2008). Genetic studies support the therapeutic potential of AMPK. For example, the ability of AICAR treatment to lower whole body glucose levels is abolished in mice lacking the catalytic α2 subunit of AMPK, and $\gamma3^{225Q}$ mice are protected against diet-induced skeletal muscle insulin resistance (Mu, et al., 2001; Barnes, et al., 2004; Jorgensen, et al., 2004; Long and Zierath,

2006). Clearly, AMPK has beneficial effects on pathways associated with aging and age-related disease and will continue to serve as an important therapeutic target in the treatment of metabolic diseases (McCarty, 2004).

2.4 mTOR

The conserved kinase target of rapamycin (TOR) integrates environmental cues to coordinate cell growth and metabolism (Wullschleger, et al., 2006; Cunningham, et al., 2007). Recent studies have begun to explore the link between mTOR and mitochondrial energy production and have found that mTOR can increase both mitochondrial number and oxygen consumption. In mammals, mTOR exists in two distinct complexes, mTORC1 and mTORC2. mTORC1, including mTOR, RAPTOR, PRAS40 and mLST8, exerts effects on cell growth, protein synthesis and degradation, ribosome biogenesis and autophagy through targets including S6K1 and 4E-BP1 (Guertin and Sabatini, 2007). mTORC2 comprises mTOR, RICTOR, mSIN1, PROTOR and mLST8 and can affect growth, proliferation and survival, although to date, the only known target of mTORC2 is Akt (Guertin and Sabatini, 2007).

In model organisms, TOR has emerged as a powerful regulator of lifespan and the response to CR (Table 1). A large-scale screen for genes involved in yeast longevity yielded 10 hits, six of which correspond to components of the TOR and Sch9 (the yeast homolog of S6K1) pathways (Kaeberlein, et al., 2005b). Deletion of TOR and Sch9 extended replicative lifespan, and CR could not further extend lifespan in these strains, suggesting that TOR and Sch9 are important effectors of the response to CR (Kaeberlein, et al., 2005b). Additionally, both mutations in the conserved TOR pathway and pharmacological inhibition of TOR signaling increase yeast chronological life span (Powers, et al., 2006; Wei, et al., 2008). The pathway through which TOR exerts its effects on longevity in yeast are controversial: some reports suggest that it acts through Sir2 to stabilize rDNA, while others indicate that inhibition of protein synthesis is the primary factor driving lifespan extension (Medvedik, et al., 2007; Steffen, et al., 2008). Downregulation of TOR or components of TOR signaling similarly increase lifespan in worms and flies (Vellai, et al., 2003; Jia, et al., 2004; Kapahi, et al., 2004; Honjoh, et al., 2008). Several studies suggest that inhibition of protein synthesis extends lifespan in worms and flies, providing a potential mechanism for the increased longevity conferred by reduction of TOR signaling (Kapahi, et al., 2004; Chen, et al., 2007; Hansen, et al., 2007; Pan, et al., 2007; Steffen, et al., 2008).

Because mTOR activity is suppressed by conditions of nutrient limitation, reduction in mTOR signaling is a logical candidate mechanism for the anti-aging benefits of CR. Nevertheless, it has proved extremely difficult to extend the results from model organisms to mammals, and there is surprisingly little evidence to link mTOR to aging or CR in mammals. One study found that mTOR signaling was reduced in the Ames Dwarf mouse, a model of extended longevity, but few other studies provide direct links between mTOR signaling and mammalian aging (Sharp and Bartke, 2005). Unexpectedly, transcriptional profiling of heart and white adipose tissue from old mice fed either an ad lib (AL) or CR diet revealed that aging reduced expression of genes associated with mTOR activity and that CR could block this reduction (Linford, et al., 2007). Indeed, genetic models suggest that

inhibition of mTOR activity has fatal consequences in mammals. Deletion of raptor, rictor, mLST8 or mTOR in mice results in embryonic lethality (Gangloff, et al., 2004; Guertin, et al., 2006; Shiota, et al., 2006), and even skeletal muscle-specific deletion of raptor results in premature death between 4 and 7 months of age (Bentzinger, et al., 2008). Intriguingly, these mice display reduced oxidative capacity and downregulated expression of PGC-1α (Bentzinger, et al., 2008).

Although mTORC1 is best known as a central controller of cell growth, recent studies have begun to uncover both direct and indirect roles for mTOR in the regulation of mitochondrial metabolism. Several studies associate downregulation of mTORC1 activity with increased fatty acid oxidation. Inhibition of mTORC1 by rapamycin reduces glycolysis and increases fatty acid oxidation in skeletal muscle cells and enables increased fatty acid oxidation coupled with reduced lipid synthesis in primary hepatocytes (Sipula, et al., 2006; Brown, et al., 2007). Compared to wild type counterparts, mice deficient for S6K1 have higher expression of genes involved in OXPHOS and fatty acid oxidation in both white adipose tissue and skeletal muscle (Um, et al., 2004). Consistent with the hypothesis that these mice display elevated rates of fatty acid oxidation, $S6K1^{-/-}$ mice have reduced fat stores and are protected from weight gain on a high fat diet (Um, et al., 2004). Conversely, mice lacking 4E-BP1 and 4E-BP2, targets that are inhibited by mTORC1, show decreased lipolysis, increased fatty acid esterification and enhanced sensitivy to diet-induced obesity (Le Bacquer, et al., 2007). All of these studies are consistent with the model that downregulation of mTORC1 activity and subsequent decrease in translation result in increased fatty acid catabolism. mTORC1 can also enhance mitochondrial function independently of its regulation of protein translation. Shieke et al. first found that mTORC1 activity can stimulate mitochondrial oxygen consumption and oxidative capacity (Schieke, et al., 2006). Treating cells with rapamycin reduced $\Delta\psi_m$, oxygen consumption and maximum rate of OXPHOS independently of previously identified targets of mTORC1 (Schieke, et al., 2006). The authors used 2D gel electrophoresis to examine mitochondrial proteins, and although they did not find changes in expression of proteins involved in OXPHOS or substrate oxidation, they did detect isoelectric shifts consistent with dephosphorylation of proteins involved in intermediate metabolism and OXPHOS (Schieke, et al., 2006). Finally, two groups found that a fraction of mTOR physically localized with mitochondria, suggesting that mTOR can directly regulate mitochondrial metabolism (Desai, et al., 2002; Schieke, et al., 2006). An alternative model for regulation of mitochondrial activity by mTOR was provided by a recent study that showed that mTORC1 can positively influence transcription of genes important for mitochondrial biogenesis and substrate oxidation (Cunningham, et al., 2007). Rapamycin treatment decreased transcription of genes controlling mitochondrial function, such as PGC-1α, PGC-1β, NRF-1 and ERRγ. As a result, transcription of genes involved in OXPHOS declined, oxygen consumption fell and mitochondrial number was reduced with rapamycin treatment. Under normal conditions mTORC1 formed a complex with PGC-1α and the transcription factor YY1 and this complex enabled transcription of nuclear-encoded mitochondrial genes. Upon rapamycin treatment, PGC-1α broke away from the complex, and transcription of mitochondrial genes was downregulated (Cunningham, et al., 2007). Both studies provide exciting evidence that mTOR coordinates cell growth and mitochondrial metabolism. Clearly, future work is needed to elucidate the role of mTOR in

regulating mitochondrial activity and to determine how such changes in mitochondrial respiration and substrate oxidation may be influenced by aging and CR.

3. Retrograde signaling

In order for nuclear proteins to regulate mitochondrial activity appropriately, mitochondria must be able to signal to the nucleus to alert the cell to changes in mitochondrial function. This retrograde (RTG) signaling enables the cell to adapt and compensate for perturbations in mitochondrial metabolism. Unlike anterograde transcriptional control of mitochondria, the retrograde response is not well studied and represents an important area of future research. Unicellular eukaryotes are capable of retrograde signaling and many of the genes involved have been identified in yeast. However, the molecular mechanisms driving retrograde signaling do not appear conserved across species (Liu and Butow, 2006).

3.1 Retrograde signaling in yeast

Disruption of energy production, whether by mutations in the TCA cycle or interruption of OXPHOS, trigger the retrograde response in yeast. In this event, the main function of the RTG pathways is the maintenance of glutamate supplies, which would otherwise be compromised as a result of blockage in the TCA cycle (Butow and Avadhani, 2004). The main RTG pathway in yeast relies on a family of Rtg proteins, of which Rtg2p senses mitochondrial dysfunction through an unknown mechanism. Under basal conditions, phosphorylation of Rtg3p sequesters both Rtg3p and Rtg1p in the cytoplasm. Upon mitochondrial dysfunction, activation of Rtg2p results in partial dephosphorylation of Rtg3p, with the result that Rtg3p and Rtg1p can translocate to the nucleus, where they heterodimerize to activate transcription of specific RTG target genes (Liu and Butow, 2006). Interestingly, TOR seems to play a regulatory role in the RTG pathway: rapamycin treatment results in upregulation of genes in the RTG pathway, and Lst8p, a component of TORC1 and TORC2, can negatively regulate RTG signaling (Liu and Butow, 2006). While the direct involvement of TOR is controversial, at least one study has found that TOR negatively regulates RTG signaling (Dilova, et al., 2002). Furthermore, the retrograde response appears to play a direct role in the regulation of longevity in yeast. Growing yeast on medium containing raffinose will activate retrograde regulation and will also extend yeast replicative lifespan (Kirchman, et al., 1999). The same study showed that yeast strains lacking mtDNA have increased longevity, and that deletion of a gene essential for RTG signaling eliminated the lifespan extension (Kirchman, et al., 1999). The authors concluded that yeast lifespan extension, at least in response to mtDNA deletion, is proportional to the degree of RTG signaling (Kirchman, et al., 1999). While the precise cues responsible for inducing the RTG pathway are unknown, it is clear that mitochondrial signaling may play an important role in yeast aging.

3.2 Retrograde signaling in mammals

Our understanding of mammalian retrograde signaling, also known as mitochondrial stress signaling, is much murkier. Mitochondria respond to accumulation of mutant or unfolded proteins with a mitochondrial unfolded protein response (UPR) analogous to the ER UPR. The mitochondrial UPR is regulated, at least in part, by the transcription factors CHOP and

C/EBPβ that increase expression of chaperones and proteases that are specific to mitochondria, such as Hsp60, Hsp10 and ClpP (Zhao, et al., 2002; Ryan and Hoogenraad, 2007). The specific signal that activates the mitochondrial UPR is still unknown, and it is unclear if this pathway is affected by aging or CR. Other forms of mitochondrial stress, such as perturbations in energy production or structural damage, signal to the nucleus through alternate mitochondrial stress response (MSR) pathways (Ryan and Hoogenraad, 2007). These pathways are largely believed to be triggered by changes in mitochondrial release of metabolites and molecules, such as Ca^{2+}, nitric oxide (NO), ATP, NAD^{+}/NADH and ROS (Figure 1).

Mitochondria are important regulators of Ca^{2+} storage and homeostasis and cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) is perhaps the best studied of the mammalian mitochondrial messengers. Mitochondrial membrane potential serves as a gauge of mitochondrial function: disruption of OXPHOS, interruption in the supply of nutrients or their catabolism and loss of mitochondrial structural integrity generally will result in a drop in $\Delta\psi_m$. A fall in $\Delta\psi_m$ disrupts mitochondrial Ca^{2+} uptake, producing a rise in $[Ca^{2+}]_c$ (Butow and Avadhani, 2004). As a result, depleting mitochondrial DNA with ethidium bromide or direct uncoupling with CCCP results in elevated cytosolic free Ca^{2+} and upregulation of genes involved in Ca^{2+} transport and storage (Luo, et al., 1997; Biswas, et al., 1999; Amuthan, et al., 2002). Cybrid cells with mutant mitochondria or cells lacking mitochondrial DNA have elevated intracellular calcium, accompanied by activation of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) (Arnould, et al., 2002). Importantly, these studies showed that activation of Ca^{2+}-sensitive factors and subsequent changes in nuclear transcription were blocked by the removal of free Ca^{2+} with specific cheators (Butow and Avadhani, 2004). A rise in cytosolic free Ca^{2+} activates calcineurin and several Ca^{2+}-dependent kinases, such as PKC, CamKIV, JNK and MAPK (Butow and Avadhani, 2004). Ultimately, Ca^{2+} signaling affects a wide variety of transcription factors, including NFAT MEF2, TORC, CREB and NFκB, to produce appropriate cell-specific transcriptional responses (Mellstrom, et al., 2008). Ca^{2+} signaling can in turn regulate mitochondrial function by activating PGC-1α, providing an important regulatory loop for maintenance of mitochondrial activity. Mammalian cells treated with a chemical uncoupler suffer a temporary decline in ATP production but undergo an increase in transcription of mitochondrial genes and an increase in OXPHOS that serves to restore cellular ATP levels (Rohas, et al., 2007). This compensatory response requires PGC-1α and is mediated by Ca^{2+}-responsive CREB and TORCs associating at the PGC-1α promoter, resulting in increased PGC-1α transcription (Rohas, et al., 2007). Similarly, overexpression of CamKIV in skeletal muscle results in increased expression of PGC-1α and enhanced mitochondrial biogenesis, OXPHOS and fatty acid oxidation (Wu, et al., 2002).

Although cytosolic free Ca^{2+} is an important signal governing mitochondrial and cellular physiology, very few studies have investigated whether aging is associated with disruptions in Ca^{2+} homeostasis. There is scattered evidence to suggest that this is a promising area for future research. Calcineurin and CaMKIV activity decline with age in rat T cells, and CR diminishes the age-related decline in calcineurin activity (Pahlavani and Vargas, 1999; Pahlavani and Vargas, 2000). Suggestively, during aging in rats, several methionine residues of calmodulin are oxidized, disrupting normal interactions with target proteins

(Anbanandam, et al., 2005). The bulk of the research involving Ca^{2+} and aging has been carried out in neurons because of the critical role that Ca^{2+} plays in regulating neurotransmitter release, excitability and apoptosis, and subtle defects in Ca^{2+} homeostasis are implicated in neuronal aging and degeneration (Buchholz, et al., 2007). While the values of resting intracellular Ca^{2+} do not appear to change with age, aging does slow the rate at which neurons can restore resting Ca^{2+} levels after large stimulation (Toescu, 2005). The role of mitochondria in neuronal Ca^{2+} regulation during aging is controversial, but a number of studies suggest that the Ca^{2+} buffering capacity of mitochondria does not decrease with age and may even increase with age in order to compensate for reduced function of other Ca^{2+}-buffering systems (Buchholz, et al., 2007). Increased Ca^{2+} uptake may place stress upon mitochondria and increase susceptibility to mitochondrially-induced apoptosis (Buchholz, et al., 2007). The intimate relationship between Ca^{2+} signaling, Ca^{2+} storage and mitochondrial function highlights this as an important area for future aging research.

Nitric oxide (NO) is synthesized from arginine and O_2 by NO synthase, of which there are several isoforms: endothelial (eNOS), neuronal (nNOS), inducible (iNOS) and mitochondrial (mtNOS) (Nisoli and Carruba, 2006). NO exerts systemic effects on energy metabolism by inducing vasodilation thereby increasing nutrient supply, and by regulating the binding of O_2 to hemoglobin. NO can more directly influence mitochondrial energy production by competing with cytochrome *c* oxidase for O_2, and this block in OXPHOS lowers O_2 consumption, $\Delta\psi_m$ and ATP production (Cleeter, et al., 1994). Inhibition of cytochrome *c* oxidase by NO is highly sensitive to the rate of OXPHOS, suggesting that regulation by NO is coupled to the metabolic status of the cell (Brookes, et al., 2002). As all isoforms of NOS require Ca^{2+}, an elegant feedback loop results whereby mitochondrial NO decreases $\Delta\psi_m$, which in turn reduces mitochondrial Ca^{2+} uptake and thus blocks mtNOS activity (Ghafourifar and Cadenas, 2005). Outside the mitochondria, NO plays an important role in mitochondrial biogenesis. NO synthesized by eNOS signals through cGMP to activate SIRT1 and PGC-1α, resulting in an increase in mitochondrial number and oxidative capacity (Nisoli and Carruba, 2006). CR increases eNOS expression and cGMP production in various tissues, and deletion of eNOS abrogates increases in PGC-1α expression and mitochondrial biogenesis typically induced by CR (Nisoli, et al., 2005). While it is difficult to measure NO directly, indirect measures suggest that NO synthesis or secretion decline with age, and mtNOS activity is thought to decrease in the aging brain (Toprakci, et al., 2000; Drew and Leeuwenburgh, 2002; Navarro and Boveris, 2008)

Mitochondrial NO can also signal to the nucleus by generating ROS. NO can combine with the superoxide radical to form peroxynitrite, a reactive radical capable of causing nitrosative damage to lipids, proteins and DNA (Drew and Leeuwenburgh, 2002). By blocking cytochrome *c* oxidase, NO can increase the reduction state of the electron transport chain, thus increasing the rate of electron leakage and superoxide production (Brookes, et al., 2002). Given the damaging consequences of ROS activity, it is essential for cells to sense and respond to changes in ROS production. A variety of transcription factors are redox sensitive, including p53, AP-1, NF-kB, Sp-1, glucocorticoid receptor and Egr-1 (Sun and Oberley, 1996). In most cases, oxidation or reduction of highly conserved cysteines in the DNA binding domain of the protein causes changes in transcriptional activity (Sun and

Oberley, 1996). Additionally, oxidants can activate a variety of signaling pathways with diverse outcomes. Activation of ERK, PI3K/Akt and NF-kB by oxidative stress triggers pro-survival pathways, while p53, JNK and p38 activation favor apoptosis, although there are numerous exceptions to this paradigm (Finkel and Holbrook, 2000).

Changes in redox state can directly affect pathways that control mitochondrial number and activity, providing an important feedback mechanism. Activity of NRF-1 and NRF-2 are modulated by changes in redox state. In the case of NRF-1, oxidants signal through the PI3KAkt pathaway to activate NRF-1 and induce transcription of mitochondrial genes (Martin, et al., 1996; Suliman, et al., 2003; Piantadosi and Suliman, 2006). Changes in redox state can also affect the stability of the mTOR complex, with oxidizing agents increasing phosphorylation of S6K1 by mTOR (Sarbassov and Sabatini, 2005). Alterations in mitochondrial ROS production have profound and varied effects on cell signaling in order to tailor changes in cellular and mitochondrial activity appropriately. Because mitochondrial ROS production increases with age, changes in redox signaling pathways can have important consequences during aging. Indeed, enhanced stress resistance is linked to extended life span in worms, flies and mice (Finkel and Holbrook, 2000).

ATP is another mitochondrial product with potent signaling capacities. Mitochondrial ATP is exchanged for cytosolic ADP through the mitochondrial adenine nucleotide transporter (ANT), and a rise in mitochondrial ATP production will increase the cytosolic ATP/ADP ratio. This change will reduce AMPK activity and relieve the repression of anabolic processes. Additionally, AMPK can regulate mTOR at several levels: AMPK phosphorylates and activates the mTOR repressor TSC2 and AMPK can phosphorylate raptor to inhibit mTORC1 directly (Inoki, et al., 2003; Shaw, et al., 2004; Gwinn, et al., 2008). An increase in the ATP/ADP ratio will therefore increase mTOR activity, and there is some evidence that ATP can directly stimulate mTOR kinase activity as well (Dennis, et al., 2001). Conversely, a variety of stresses that disrupt mitochondrial OXPHOS inhibit mTOR kinase activity (Kim, et al., 2002). Thus, ATP can serve as an important signal to coordinate cell growth and proliferation with mitochondrial energy production. Determining whether basal ATP levels or the rate of ATP production change during aging has proved very controversial. Many studies have examined activity of specific complexes of the respiratory chain in an effort to understand how mitochondrial OXPHOS capacity varies with age. Respiratory complexes do not respond to aging uniformly and there is no common pattern between various tissues (Yen, et al., 1989; Kwong and Sohal, 2000). Physical fitness, activity and body fat are potentially confounding variables that render analysis of mitochondrial OXPHOS activity especially contentious in skeletal muscle, and different studies report either no change a decline in mitochondrial respiratory capacity during aging (Cooper, et al., 1992; Chretien, et al., 1998; Conley, et al., 2000; Petersen, et al., 2003; Rasmussen, et al., 2003; Tonkonogi, et al., 2003; Lanza, et al., 2005). Direct measures of ATP production and content in the heart, brain and skeletal muscle showed that ATP content and production declined with age only in skeletal muscle, and that CR did not affect these parameters in any of the tissues studied (Drew and Leeuwenburgh, 2003; Drew, et al., 2003). It will be important for future studies to address not only how ATP levels change with age, but also whether aging is associated with alterations in the ATP/ADP ratios and in the sensitivity of cellular signaling pathways to perturbations in the ratio.

NAD^+ and NADH are crucial metabolites intimately connected with mitochondrial energy production that can also affect mitochondrial function by regulating the mitochondrial permeability transition pore, voltage-dependent anion channels and calcium homeostasis (Ying, 2006). Changes in the cellular NAD^+/NADH ratio can change the redox state of the cell and alter the activity of enzymes such as poly-ADP-ribose polymerases and sirtuins, with subsequent effects on signaling cascades and gene expression (Rodgers, et al., 2005; Ying, 2006). The NAD^+/NADH ratio changes in a tissue-specific pattern during CR and has been linked to aging in yeast (Lin, et al., 2004b; Chen, et al., 2008). However, it is unclear if changes in the mitochondrial NAD^+/NADH ratio can have any effect on the cytosolic NAD^+/NADH ratio. The inner mitochondrial membrane is impermeable to NAD^+ and NADH, enabling the mitochondrial NAD^+/NADH ratio to fall between 7-8:1 while the cytosolic NAD^+/NADH ratio is approximately 700:1 (Ying, 2006). Indeed, this is the basis for the “mitochondrial oasis theory” proposed by Yang et al., which suggests that cells can survive the depletion of cytosolic NAD^+ that occurs following genotoxic stress because mitochondrial NAD^+ levels are preserved (Yang, et al., 2007). However, the mitochondrial malate-asparatate shuttle provides a mechanism whereby cytosolic NADH can transfer reducing equivalents to mitochondrial NAD^+, generating mitochondrial NADH, and there is some evidence that changes in the mitochondrial energy state can modify the extramitochondrial redox state (Bremer and Davis, 1975). Overexpressing components of the malate-aspartate shuttle in yeast extends lifespan and deleting the shuttle components blocks both the increase in lifespan and decrease in cytosolic NADH characteristic of CR (Easlon, et al., 2008). It is possible, therefore, that under certain conditions, changes in mitochondrial energy state can be translated into subtle differences in the cytosolic NAD^+/NADH ratio, with consequences for aging and longevity. A NAD^+-carrier protein was recently discovered in yeast, raising the intriguing possibility that such a shuttle may exist in mammals and provide a more direct mechanism for mitochondria to influence the cytosolic NAD^+/NADH ratio (Todisco, et al., 2006).

4. Perspectives and future directions

Communication between the mitochondria and nucleus is crucial for maintenance of appropriate mitochondrial function. As a result, mitochondrial-nuclear signaling plays an integral role in normal energy homestasis and disruption contributes to aging and age-related disease. Elucidating the molecular mechanisms that regulate these pathways will be essential for understanding the dysregulation of mitochondrial function that is associated with aging. Recent studies have begun to uncover the complex integration of the various signaling pathways that coordinate to regulate mitochondrial gene expression (Figure 1). While the major players regulating transcription of mitochondrial genes, namely PGC-1α, SIRT1, AMPK and mTOR, have begun to be studied in great detail, the signals through which mitochondria communicate to the nucleus are largely unknown. Although Ca^{2+}, NO, ATP/ADP, ROS and NAD^+/NADH are suspected to be important interorganelle messengers, mammalian retrograde signaling remains a major unexplored avenue for aging research. It will be important for future studies to invest how aging affects the signals that mitochondria emit in addition to the responsiveness of downstream signaling pathways. A better understanding of this bidirectional communication will enable a new avenue of therapies

that improve nuclear-mitochondrial communication. Finally, a new generation of therapeutics such as AICAR, resveratrol and SRT1720 demonstrate the promise of targeting pathways that promote the pathways involved in the cross-talk between the nucleus and mitochondria.

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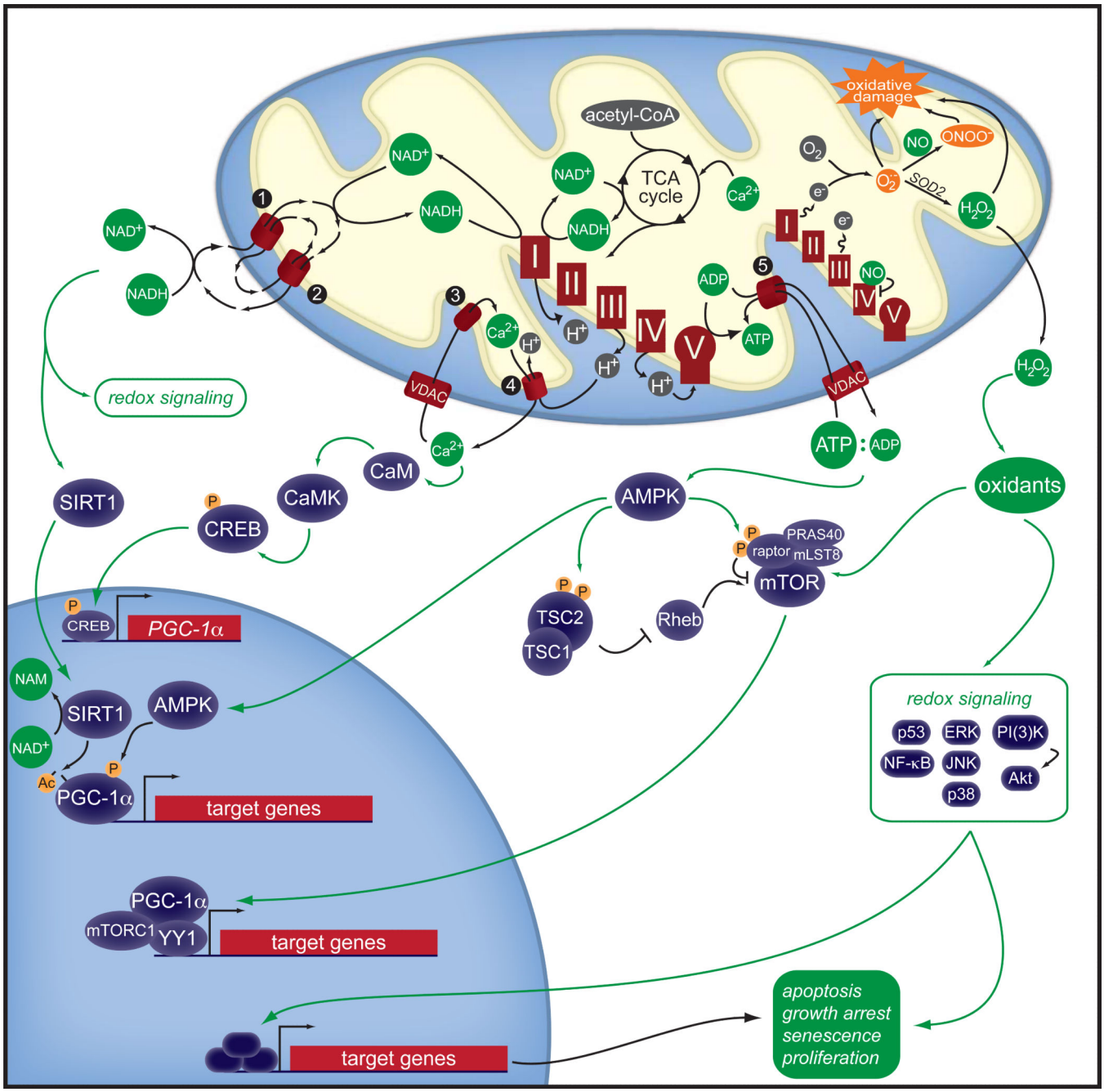

Figure 1.

Mammalian mitochondrial retrograde signaling pathways. Mitochondrial activity can influence cellular NAD^+/NADH ratios, cytosolic free Ca^{2+} concentrations, ATP/ADP ratios and cellular oxidative stress. Electrons from cytosolic NADH can be transferred to mitochondrial NAD^+ to form NADH via the malate-aspartate shuttle (*1*,*2*). This NADH, along with the NADH generated by the TCA cycle, donates electrons to complex I of the mitochondrial electron transport chain, regenerating NAD^+. Electrons are passed between the complexes of OXPHOS (I, II, III, IV) and their energy is used to pump protons into the intermembrane space. Complex V uses this proton gradient to synthesize ATP, which is then

exchanged for cytosolic ADP by ANT (*5*). Small molecules, such as ADP, ATP and Ca^{2+} diffuse cross the outer mitochondrial membrane through VDAC. Mitochondrial membrane potential drives Ca^{2+} entry into the matrix through a Ca^{2+} uniporter (3), and Ca^{2+} is transferred out through an antiporter that exchanges Na^{+}/H^{+} for Ca^{2+}(*4*). Ca^{2+} can stimulate activity of three enzymes in the TCA cycle, resulting in increased electron transport. Electrons can leak out of the electron transport chain at complex I and complex III, combining with O_2 to form superoxide, which can be converted by SOD2 to H_2O_2 or combine with NO to form $ONOO^{-}$. Superoxide, $ONOO^{-}$ and H_2O_2 can all cause oxidative damage, and H_2O_2 can diffuse out of the mitochondria to cause oxidative stress and trigger redox signaling. Ca^{2+} is required for synthesis of NO, and NO can inhibit complex IV, increasing electron leakage. Each of these outputs (shown in green) can act as signaling molecules and influence the activity of various signaling pathways with the potential to regulate transcription of mitochondrial genes. Increases in the NAD/NADH ratio are thought to activate SIRT1, which can deacetylate and activate PGC-1α. Increased cytosolic Ca^{2+} triggers a signaling cascade that increases phosphorylation of CREB, stimulating its ability to activate transcription of the PGC-1α gene. A rise in the ADP/ATP ratio activates AMPK, which can inhibit mTORC1 activity by phosphorylation of TSC2 and raptor. AMPK also phosphorylates and activates PGC-1α. mTORC1 itself can interact with PGC-1α and YY1 to stimulate transcription of mitochondrial genes. Finally, changes in cellular redox state, whether by changes in the NAD^{+}/NADH ratio or increased oxidant production, can activate several redox signaling cascades. *1*, Malate—γ-ketoglutarate transporter; *2*, Glutamate—aspartate transporter; *3*, Calcium uniporter; *4*, Na^{+}/H^{+} -dependent Ca^{2+} antiporter; *5*, Adenine nucleotide translocator (ANT).

Table 1

Summary of role of major mitochondrial regulators on lifespan and aging. Arrows indicate that a given protein is involved in the corresponding process. All four proteins are capable of increasing measures of mitochondrial function, including mitochondrial number, OXPHOS capacity or fatty acid oxidation. Expression of the SIRT1 ortholog, Sir2, increases lifespan in yeast, worms and flies; AMPK increases lifespan in worms but decreases yeast lifespan, and inhibition of mTOR increases lifespan in all three model organisms. For mammalian aging and CR, arrows indicate how a given protein's expression or activity changes.

	mitochondrial function	lifespan			mammalian aging	mammalian CR
		yeast	worms	flies		
PGC-1α	↑	?	?	?	↓	↑
SIRT1	↑	↑	↑	↑	↓	↑
AMPK	↑	↓	↑	?	↓	no change
mTOR	↑	⊣↑	⊣↑	⊣↑	?	?