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GCN5L1 Functions as a Mitochondrial Acetyltransferase that Regulates Mitophagy

Bradley Roy Webster

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GCN5L1 Functions as a Mitochondrial Acetyltransferase that Regulates Mitophagy

by

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DISSERTATION

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ABSTRACT

Caloric restriction (CR) increases lifespan and promotes both longevity and ‘healthy-aging.’ CR’s benefits are dependent in part on NAD-dependent deacetylases known as sirtuins. NAD levels increase with CR promoting sirtuin-mediated deacetylation of lysine residues. Humans express seven sirtuin isoforms that localize to specific sub-cellular compartments. Sirtuins and acetylation affect numerous cellular processes including mitochondrial function. Sirt3 is the major mitochondrial deacetylase and regulates mitochondrial function namely ATP levels, oxidative stress and metabolism. Given the importance of mitochondrial acetylation and Sirt3, we hypothesized the existence of a mitochondrial-localized acetyltransferase that 1) localizes to the mitochondria; 2) has an acetyltransferase domain; and 3) counters known effects of Sirt3. We found that the protein GCN5L1 localizes to mitochondria, and its depletion or overexpression attenuates and augments mitochondrial protein acetylation, respectively. Sirt3 increases mitochondrial respiration and ATP production. Acute GCN5L1 knockdown also increases mitochondrial respiration and ATP production suggesting unopposed Sirt3 activity. GCN5L1 promotes the acetylation of the electron transport chain proteins NDUFA9 and ATP5 α (known Sirt3 targets). In addition, Sirt3 knockdown

leads to mitochondrial hyperacetylation, which is countered by concurrent GCN5L1 depletion. We propose that GCN5L1 is an essential component of a mitochondrial acetyltransferase complex.

As acetylation levels affect the housekeeping program known as autophagy, we interrogated GCN5L1's role in this program. GCN5L1 knockdown causes autophagic proteins to accumulate on mitochondria, which can be reversed with concurrent Sirt3 depletion. This knockdown selectively promotes the autophagic clearance of mitochondria (mitophagy) without altering global autophagy. Chronic GCN5L1 depletion leads to the diminution of mitochondrial proteins/mass that is dependent on the autophagy proteins ATG5 and p62 but independent of the E3-ligase Parkin. This program is elevated in GCN5L1 knockout MEFs, which is reversed with reconstitution of GCN5L1. GCN5L1 depleted mitochondria generate lower ROS levels and are resistant to MPTP opening. Both mitophagy and mitochondrial biogenesis (i.e. mitochondrial turnover) are simultaneously elevated with GCN5L1 knockdown. Therefore, GCN5L1 functions as a critical component of the mitochondrial acetyltransferase machinery and regulates mitochondrial acetylation-dependent mitophagy/mitochondrial biogenesis. GCN5L1 may play a role in numerous pathologies with known mitochondrial defects such as neuro-degeneration, obesity and aging.

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Chapter 1 Introduction

Acetylation as a post-translational modification

The cell uses numerous mechanisms to control cellular functions. Aside from regulating bulk protein expression levels by alterations at the gene, mRNA and ribosomal stages of production, cells can fine tune protein function through the use of post-translational modifications (PTM). PTMs can alter amongst other things enzymatic function and activity, protein localization, interactions with other proteins, three-dimensional shape and stability. The best characterized PTM is the phosphorylation of protein serine, threonine and tyrosine residues by protein kinases. However, recent advances in proteomics, mass spectrometry and the generation of modification-specific antibodies have highlighted the importance and broad scope of countless other PTMs including, but not limited to, lipidation, ubiquitinylation, methylation, sumoylation and glycosylation. Adding another layer of complexity to the control of proteins by PTMs, a single protein can have numerous sites and types of modifications occurring simultaneously. The tumor suppressor p53 is controlled through acetylation, methylation, phosphorylation, neddylation, sumoylation and ubiquitinylation at > 50 different sites showing how tightly p53 function is controlled by the cell through utilizing numerous signaling pathways [1-3]. It should be noted that not all PTMs have a functional significance on the target protein. These detected PTMs can occur in a non-specific fashion, especially on long lasting proteins that are turned over slowly, effectively making some PTMs merely ‘decorations’ that do not appear to alter the protein’s function [4].

The regulation of PTMs is controlled both enzymatically and through available substrate. For example, phosphorylation is regulated by kinases phosphorylating and phosphatases de-phosphorylating target proteins. Kinases utilize ATP as a substrate for phosphorylation such that the ATP/ADP ratio, essentially substrate availability, also dictates the level of protein phosphorylation. This effect is especially seen in the mitochondrial TCA cycle. High ratios of ATP/ADP slow mitochondrial respiration due to an abundance of ATP levels in a feedback dependent fashion as originally shown by Chance and Williams [5]. This feedback inhibition effectively maintains the ratio of ATP/ADP across a dynamic range, such as cardiac tissue under varying workloads [6]. High ratios of ATP/ADP inhibit mitochondrial proteins both allosterically and through reversible phosphorylation. Within the TCA cycle this inhibition occurs at the levels of pyruvate dehydrogenase (PDH), citrate synthase (CS) and isocitrate dehydrogenase (IDH). High ATP levels inhibit these enzymes allosterically, but a second level of inhibition occurs by the direct phosphorylation of PDH by pyruvate dehydrogenase kinase (PDK) [7]. There is also evidence of direct inhibition of IDH by phosphorylation [8]. Therefore, PTMs are tightly regulated by both enzyme levels but also by substrate availability.

As mentioned previously, phosphorylation is the best characterized PTM to date. However, advances in proteomics have revealed an extensive network of other PTMs. The scope of the PTM acetylation has only begun to be appreciated, an extensiveness that rivals that of phosphorylation. Acetylation of proteins was initially discovered in the 1960's with the discovery of acetyl groups attached to histones [9, 10]. Acetylation of histones is enzymatically mediated by histone acetyltransferases (HATs) and histone

deacetylases (HDACs). Generally, HATs promote gene activation while HDACs mediate gene silencing. Lysine is positively charged when deacetylated which would result in tighter binding of negatively charged DNA to histones and gene silencing. Acetylation by HATs neutralizes this positive charge and weakens the DNA-histone interaction [11]. Work on acetylation therefore focused on the role of acetylation in the regulation of gene expression for the ensuing decades [12]. The expansion of acetylation as a PTM beyond histones was recognized in 1997 with the discovery that p53 is acetylated as well [13]. Acetylation can occur on lysine residues or the amino terminus of proteins. Amino-terminus acetylation is thought to play a role in regulating protein stability and occurs co-translationally [14]. Acetylation of the ϵ -amino group of lysine has been shown to be much more dynamic. Interestingly, aside from being acetylated, the same lysine residue of a protein can be ubiquitinated, mono- bi- tri-methylated, sumoylated and neddylated suggesting extensive cross-talk between numerous signaling cascades [15]. As an example of such cross-talk, p53 acetylation has been shown to block the ubiquitination of lysine residues therefore preventing p53 nuclear export and degradation by the proteasome [16, 17]. However, acetylation can also promote protein degradation as acetylation of the gluconeogenic enzyme, PEPCK, promotes its ubiquitination and degradation whereas its deacetylation promotes PEPCK stability [18].

Similar to phosphorylation, protein acetylation is regulated by substrate availability. Acetyltransferases utilize acetyl-CoA, derived from glucose/pyruvate or fatty acids, as a substrate to acetylate protein targets. With regards to nuclear HATs, acetyl-CoA is generated in the mitochondria and converted to citrate by the TCA cycle. Citrate is then shuttled into the cytosol where ATP-citrate lyase (ACL) cleaves citrate into

oxaloacetate and acetyl-CoA. Cytosolic acetyl-CoA can be used for fatty acid synthesis or move into the nucleus for HAT mediated histone acetylation. Increased histone acetylation in response to growth factors and glucose availability is dependent on ACL generated acetyl-CoA linking gene expression to substrate availability [19].

Concurrently, deacetylation by class III deacetylases (sirtuins-*see below*) is mediated by the substrate nicotinamide adenine dinucleotide (NAD or NADH when reduced). As such the NAD/NADH ratio regulates sirtuin activity and therefore deacetylation of protein targets. Increasing the generation of NAD by overexpression of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme NAD biosynthesis, drives sirtuin activity and protein deacetylation [20]. Overall acetylation status is highly regulated by acetyl-CoA and NAD levels. Feeding and fasting could alter acetyl-CoA levels (higher with feeding) and the NAD/NADH ratio (higher with fasting) therefore linking acetylation to the nutrient status of the cell and implicating acetylation status as a key regulator of cellular metabolism.

By 2006 the number of proteins shown to undergo lysine acetylation had grown to ~100. Using mass spectrometry, three seminal studies greatly expanded the number of proteins known to undergo lysine acetylation. Kim et al. identified 195 protein targets with 388 acetylated lysine sites. Interestingly, acetylation of numerous sites changed when cells were fed or fasted. Also of note ~20% of all mitochondrial proteins (133 proteins) were acetylated. The high number of acetylated mitochondrial proteins further expanded this PTM beyond histones and the nucleus [21]. Another proteomics study identified ~1750 acetylated protein targets with >3600 acetylation sites [22]. Zhou et al. showed >900 non-nuclear proteins are acetylated [23]. Taken together >2000 proteins are

acetylated including structural proteins, kinases, transcription factors, ubiquitin ligases and metabolic enzymes. Of note, many metabolic enzymes involved in most major metabolic pathways such as gluconeogenesis, glycolysis, fatty acid and amino acid metabolism, the urea cycle and glycogen metabolism are found to be acetylated. The mitochondria is involved in most of these pathways suggesting that the 20% of acetylated mitochondrial proteins greatly regulate metabolism [24].

To date, approximately 500 kinases and 700 ubiquitin E3 ligases have been described. However, only 22 acetyltransferases and 18 deacetylases are known [24]. Acetyltransferases fall into three classes: GNAT (GCN5-related N-acetyltransferases), p300/CBP and MYST, which are classified based on conserved motifs. Each acetyltransferase has a highly conserved acetyl-CoA binding site [25]. Protein deacetylases are classified into four classes based on sequence homology with class I, II and IV being zinc dependent and act by deacetylating target proteins with the use of water to generate acetate; while class III consists of the NAD dependent sirtuin deacetylases that target acetylated proteins and generate nicotinamide and 1-O-acetyl-ADP-ribose as products [12] (Figure 1). Class I shows homology to the yeast deacetylase Rpd3 and includes histone deacetylase (HDAC) 1, 2, 3 and 8; Class II shows homology to the yeast enzyme Hda1 and includes HDAC 4, 5, 6,7, 9 and 10; and Class IV consists of HDAC 11. Class III, the sirtuins, consists of Sirt1-7 [15]. It should be noted that numerous HDACs (namely HDAC 1, 2 and 3) and all acetyltransferases function as multi-subunit proteins [26, 27]. The presence of acetylated proteins was discovered in 1964, but the first identification of a protein with acetyltransferase activity did not come

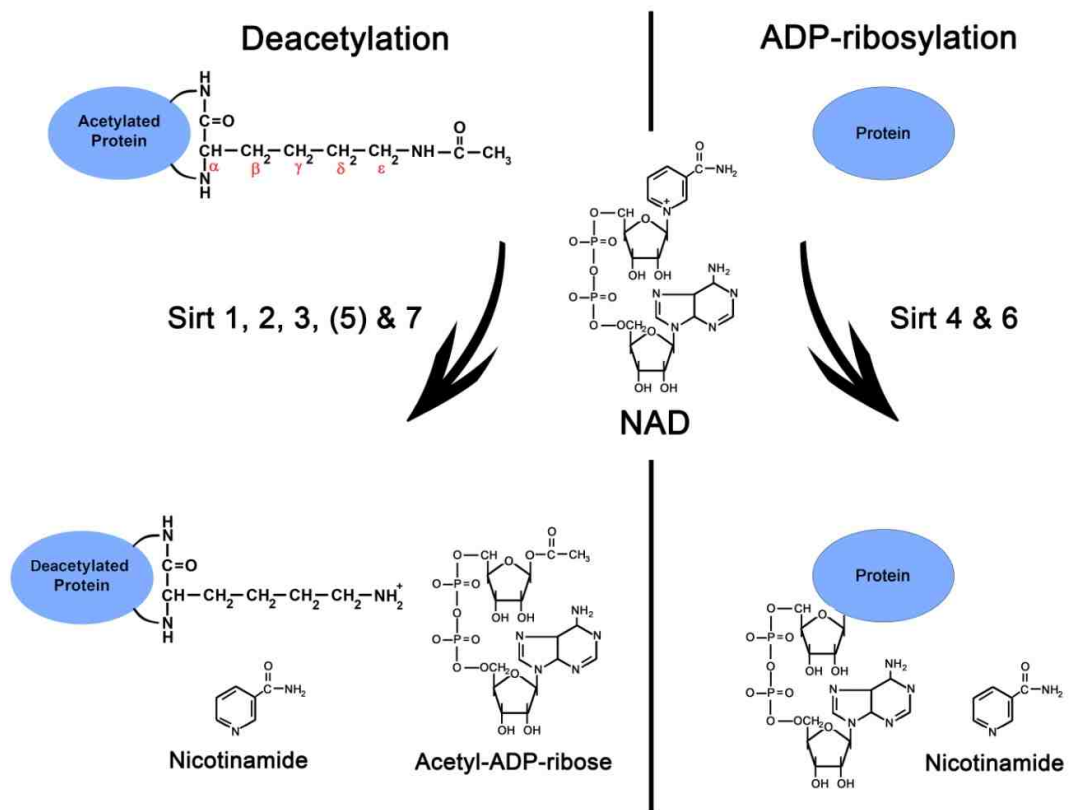


Figure 1: Sirtuin (sirt) mediated deacetylation or ADP-ribosylation of target proteins. Sirt1, 2, 3, 5 and 7 perform deacetylation of cellular proteins utilizing the substrate NAD (nicotinamide adenine dinucleotide) and yields deacetylated protein targets, acetyl-ADP-ribose and nicotinamide. Sirt4 and 6 also use NAD as a substrate but add ADP-ribose to target proteins with the generation of nicotinamide. Sirt5 is also proposed to perform desuccinylation and demalonylation reactions in the mitochondria.

until 1979 with the first acetyltransferase complex characterized in 1997 [27-29].

Therefore, given that greater than 2000 proteins are known to undergo acetylation and the slow progress in identifying the known 22 acetyltransferases and 18 deacetylases, it is likely that more enzymes are yet to be discovered that regulate this PTM.

Caloric restriction and longevity

In the 1930's Clive McCay showed that caloric restriction (CR) increased lifespan in rats by up to 40% [30, 31]. CR is defined as a reduction in daily caloric intake by 30-60%. The benefits of CR have been extended to other organisms including yeast, worms, flies, mice and non-human primates and improve quality of life, delay age-related diseases and reduce mortality [32]. Different CR protocols have been used including a reduction in glucose or amino acid availability, but in general, life span extension in yeast, worms, flies and mice results in a mean and maximal lifespan extension of 20-100% [33]. However, the ability to increase life span in non-human primates has yielded conflicting results in two longitudinal studies. In a 20-year study, Colman et al. showed that CR treated rhesus monkeys (CR by 30%) have a delay in age-related pathologies such as diabetes, cancer, brain atrophy and cardiovascular disease. The incidence of cardiovascular disease and cancer was reduced by 50% in the CR treated group while total age-related disease was reduced approximately three times compared to control animals. When only age-related deaths are considered, survival analysis showed a statistically significant improvement in CR treated groups ($p = 0.03$). However, when all deaths were included mortality rates were statistically insignificant ($p = 0.16$) [34]. In contrast, another longitudinal study failed to show an increase in survival rates in CR treated rhesus monkeys even when only age-related deaths were analyzed. Similar to the

results found by Colman et al., cancer related pathology was reduced with CR, but diabetes and cardiovascular disease only trended downwards. Possible reasons for the discrepancy between the two studies include differences in dietary content and genetic differences between the groups of rhesus monkeys used [35], but the overall differences highlight the complexities of studying how CR may benefit longevity and age-related pathologies.

Despite conflicting results on whether CR may extend lifespan, CR is believed to alter the aging process by regulating cellular metabolism, gene expression and signaling pathways to allow the cell to minimize inflammation, oxidative stress and mitochondrial dysfunction, all of which increase with age. Oxidative stress (ROS) was originally proposed to augment aging by Harman in 1956 by promoting cellular senescence through DNA damage [36]. CR is thought to reduce ROS levels and the accumulation of ROS damaged macromolecules [37]. Levels of peroxidized lipids, carbonylated proteins and damaged DNA are lower with CR treatment possibly due to increased activation of housekeeping programs such as the proteasome and autophagy [38]. The mitochondria are the major source of ROS, and CR improves mitochondrial function and attenuates mitochondrial generated ROS [37, 39]. Antioxidant systems, such as SOD and catalase, are also thought to be augmented with CR [37].

The molecular mechanisms of the benefits of CR are only beginning to be understood. To date, the signaling pathways/proteins coordinating CR's ability to extend lifespan tend to be those that are activated or inhibited in response to nutrient availability including the insulin/IGF-1 pathway, mTOR, AMP-kinase (AMPK) and sirtuins (see below) with extensive cross-talk occurring between numerous signaling pathways leading

to activation of beneficial stress responses; autophagy to removed defective organelles and toxic protein aggregates; improved oxidative stress (ROS) handling; changes in ribosome/protein synthesis; and effects on metabolic rate [33, 37] (Figure 2). In worms, Daf-16 (a member of the Foxo transcription factor family) is phosphorylated in response to activation of Daf-2, an insulin-like receptor, by insulin or IGF-1. This phosphorylation event prevents Daf-16 from entering the nucleus and thus prevents activation of protective anti-oxidant and stress pathways. Therefore mutations that inhibit Daf-2 or activate Daf-16 have been shown to promote longevity [37, 40-42]. Linking nutrient availability to IGF-1 signaling, glucose inhibits Daf-16 and shortens lifespan [43].

Two other nutrient sensing proteins linked to the benefits of CR are AMPK and mTOR. AMPK is activated upon an increase in the AMP/ATP ratio, which would be expected to be higher with CR, and acts as a major regulator of the cell's starvation response. AMPK increases glycolysis and glucose uptake by its action on GLUT4 and phospho-fructo kinase 2. It also promotes fatty acid oxidation by activating CD36, lipoprotein lipase and acetyl-CoA carboxylase (and preventing the formation of the CPT1 inhibitor malonyl-CoA) [44]. AMPK also inhibits the anabolic processes of triglyceride, ketone, glycogen and cholesterol synthesis. To promote appetite, ghrelin signaling increases AMPK activity in the arcuate nucleus of the hypothalamus activating NPY/AgRP neurons [45]. Insulin/IGF-1 signaling inhibits AMPK, and AMPK overexpression increases lifespan [46]. mTOR is a serine/threonine kinase and exists in one of two complexes containing Rictor or Raptor. mTOR is activated by high levels of amino acids (i.e. a fed state) and promotes protein translation by activating or inhibiting p70s6k and 4ebp1, respectively. CR is proposed to inhibit mTOR activity. mTOR

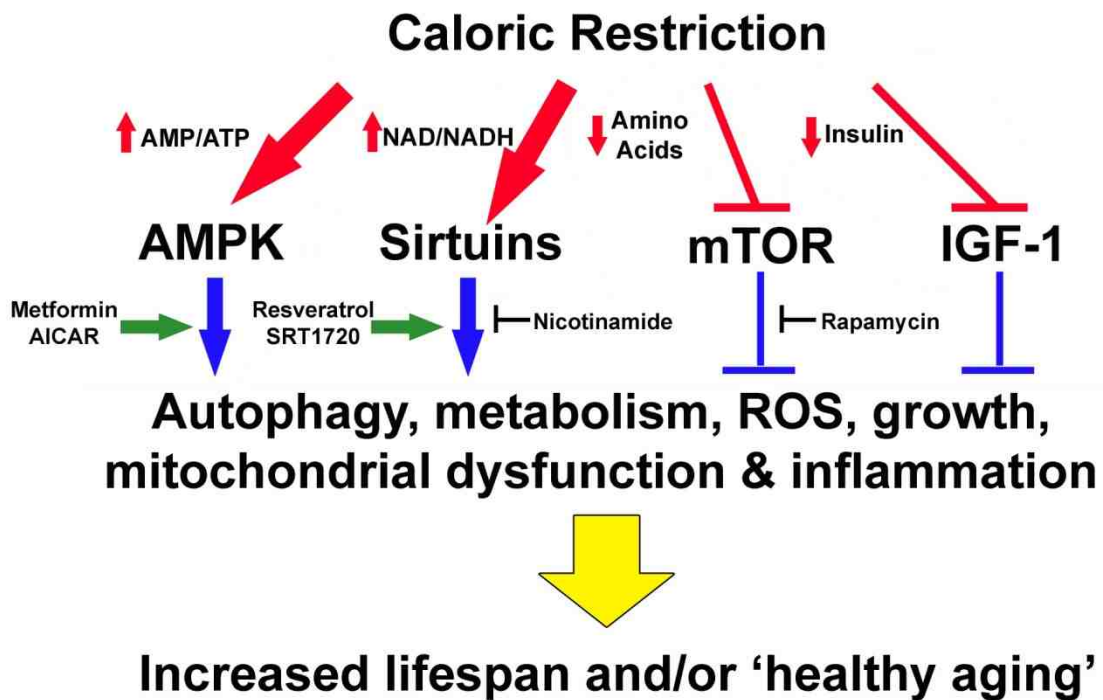


Figure 2: Caloric restriction (CR) augments lifespan in various organisms and utilizes several signaling pathways in a coordinated fashion. CR is believed to change metabolite ratios such as AMP/ATP (adenosine mono/tri-phosphate) and NAD/NADH (nicotinamide adenine dinucleotide), and increases in AMP and NAD levels activate the nutrient sensing enzymes AMPK (AMP-activated kinase) and sirtuins. CR is also thought to lower levels of amino acids and insulin signaling, which attenuates mTOR (mammalian target of rapamycin) and IGF (insulin-like growth factor) signaling. Through these signaling cascades CR can activate housekeeping programs, such as autophagy, lower oxidative stress, attenuate growth and promote mitochondrial function, which all play a role in ameliorating age-associated degeneration. Numerous drugs are being investigated to activate/inhibit these pathways to pharmacologically mimic CR. (5-aminoimidazole-4-carboxamide ribonucleotide, AICAR)

depletion can extend lifespan in yeast [47] or flies [37, 48]. Further promoting mTOR as a mediator of CR mediated longevity, rapamycin, an mTOR inhibitor, treatment extends lifespan in aged mice by 14% for females and 9% for males [49]. AMPK and mTOR also coordinate their activities together with AMPK inhibiting mTOR through activation of TSC-1. In addition, CR is thought to activate autophagy, which is a key cellular housekeeping process to recycle nutrients and to eliminate defective organelles such as ROS generating mitochondria and toxic protein aggregates seen in numerous neurodegenerative disorders (see below). Autophagy has been shown to be reduced with aging [50]. AMPK and mTOR activate and inhibit autophagy induction, respectively, in two ways. Low glucose stimulates AMPK to activate the autophagy protein, ATG1/ULK1, through phosphorylation at Ser 317 and Ser 777. mTOR meanwhile inhibits ATG1/ULK1 by phosphorylating Ser 317 when nutrients are plentiful. Secondly, AMPK can inhibit mTOR directly to promote autophagy [51].

It is unlikely that many people would willingly maintain a CR diet. Therefore attempts have been made to create drugs to mimic the benefits of CR, and many of these drugs target AMPK and mTOR (Figure 2). The drug rapamycin was originally discovered in 1975 [52]. It has been used as an immunosuppressant and cancer treatment, especially for renal carcinomas. Its ability to extend lifespan is believed to be due to its inhibition of mTOR, which is inhibited when amino acids are limited, therefore lowering protein synthesis and activating cellular-component recycling through autophagy [53]. Rapamycin has been shown to reverse the aging phenotype in cells from Hutchinson-Gilford progeria cells, a disease that causes premature aging [54]. The diabetic drug metformin was originally discovered in the 1920s and inhibits hepatic gluconeogenesis

[53]. Metformin is thought to activate AMPK, possibly inhibit mTOR and can act as a CR mimetic by increasing lifespan in mice [53, 55]. Another possible AMPK activator is 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). Therefore the ability to mimic CR pharmacologically may improve the aging process without actually having to maintain a CR diet.

Nuclear and cytosolic sirtuins (Sirt1, 2, 6 and 7)

Along with AMPK, mTOR and IGF-1 signaling another target that CR modulates is the family of deacetylases known as sirtuins. As mentioned, sirtuins are dependent on the metabolite NAD and deacetylate target proteins generating nicotinamide and 1-O-acetyl-ADP-ribose as by-products (Figure 1). It has been proposed that when nutrients are available the NAD/NADH ratio would drop inhibiting sirtuin mediated deacetylation while NAD levels are elevated during fasting or CR thus activating sirtuins. However, the simplicity of this model has been questioned and is more likely tissue specific [56].

Overall, the sirtuins are centrally positioned to regulate the cell's response to CR and therefore possibly longevity. Sirtuins were initially described in yeast as a silencing factor (silencing information regulator), and the protein Sir2 was found to regulate life span in yeast linking the PTM of acetylation to life span [57]. Furthermore, the benefits of CR on lifespan extension are reported to be dependent on Sir2 [58, 59].

Overexpression of Sir2 in worms was also shown to increase lifespan [60]. However, this link has been questioned due to lack of proper controls for genetic background between strains, which when taken into account Sir2 overexpression did not alter lifespan, adding controversy to an already controversial field [61]. Mammals express seven sirtuins (Sirt1-7) that are found in distinct cellular compartments including the nucleus (Sirt1, 6 and 7),

cytosol (Sirt1, 2) and mitochondria (Sirt3, 4 and 5). Sirt1, 2 and 3 show the closest homology to Sir2 and exhibit the most robust deacetylase activity. Sirt4 and 6 are thought to mediate ADP-ribosylation, although Sirt6 also shows weak deacetylase activity [62]. Sirt3 is thought to be the major deacetylase in the mitochondria (see below) while Sirt5 is thought to have desuccinylase and demalonylase activity [63, 64]. The link between sirtuins and aging in mammals has also been inconclusive as Sirt1 overexpression in mice does not extend lifespan but showed diminished DNA damage and aging markers along with lower incidence of carcinomas [65] while Sirt6 overexpression increased lifespan in male mice through attenuated IGF-1 signaling [66]. Although a direct link between sirtuins and increased lifespan has been inconclusive, sirtuin activity does appear to promote 'healthy aging' rather than a direct effect on lifespan extension.

Despite the inability of Sirt1 overexpression to extend lifespan, Sirt1 activity on target proteins is thought to promote healthy aging through its effects on age-related pathologies such as neurodegeneration, cancer, metabolic syndrome, diabetes and cardiovascular disease [59], and the regulation of metabolism is one of the main mechanisms whereby Sirt1 is protective against these aging co-morbidities (Figure 3). One of the key elements of the fasting response is the induction of gluconeogenesis, beta-oxidation and mitochondrial biogenesis. Sirt1 can deacetylate the transcription factor Foxo1 promoting production of the gluconeogenic enzymes PEPCK and glucose-6-phosphatase, and the disruption of Sirt1 mediated deacetylation decreased fasting blood glucose levels in mice [67, 68]. Beta-oxidation and mitochondrial biogenesis induction are mediated by Sirt1 activation of PPAR α (along with Foxo1 which increases triglyceride lipase expression) and PGC1- α , respectively [69-71]. Sirt1 controls

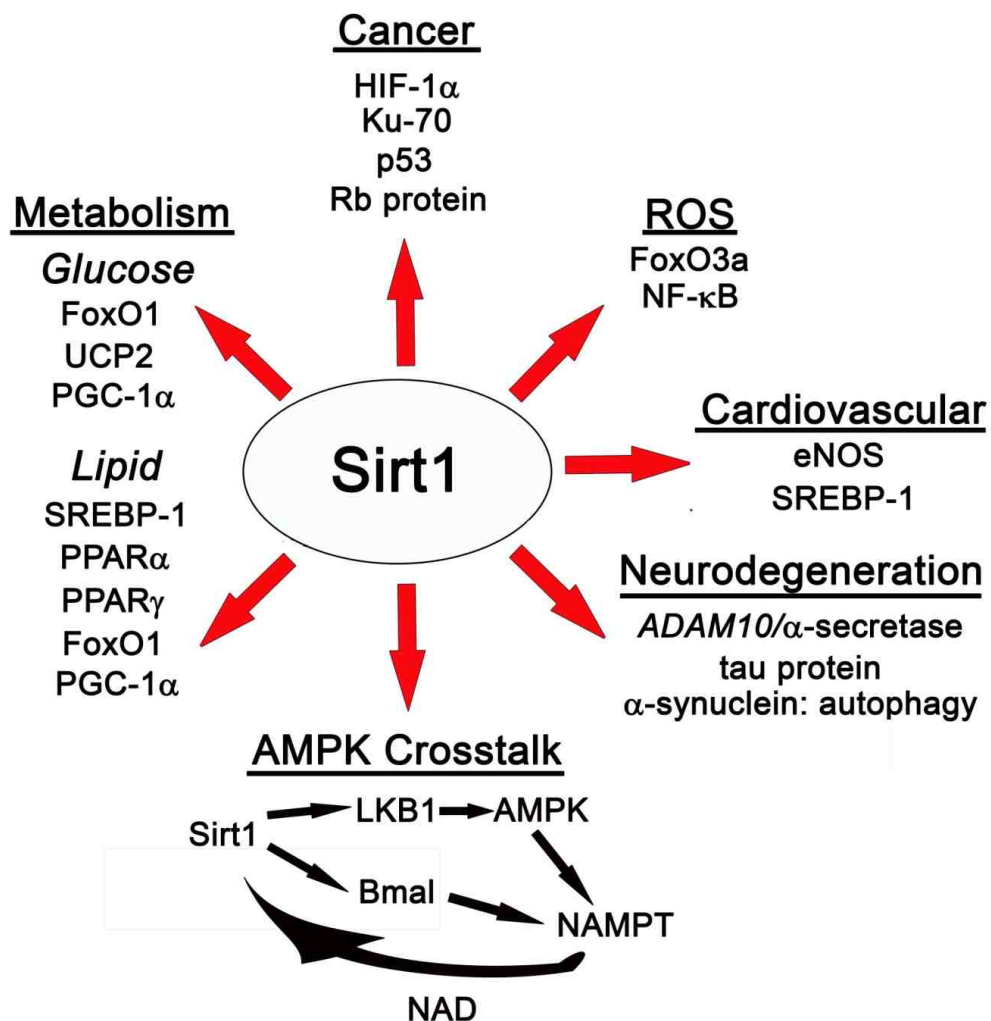


Figure 3: Sirt1 is a cytosolic and nuclear localized sirtuin that regulates numerous cellular processes. Sirt1 deacetylates numerous transcription factors and cellular proteins to coordinate cellular metabolism in response to caloric restriction (CR). Deacetylation of other Sirt1 targets is thought to play a role in the pathogenesis of several age-related diseases. Crosstalk between Sirt1 and AMPK (AMP-activated kinase) further coordinates the response to CR. (uncoupling protein-2,UCP2; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC-1 α ; sterol regulatory element-binding proteins-1, SREBP-1; peroxisome proliferator-activated receptors, PPAR α / γ ; brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like, Bmal; nicotinamide adenine dinucleotide, NAD; liver kinase B1, LKB1, nicotinamide phosphoribosyltransferase, NAMPT; nuclear factor kappa-light-chain-enhancer of activated B cells, NF- κ B; endothelial nitric oxide synthase, eNOS; hypoxia-inducible factor 1, alpha subunit, HIF-1 α)

cholesterol and lipid synthesis by negatively regulating the sterol regulatory element-binding protein-1 (SREBP-1). Fasting attenuated while feeding augmented acetylation levels of SREBP-1 in a Sirt1/p300 dependent manner. Sirt1 deacetylation of SREBP-1 leads to its ubiquitinylation and degradation improving the lipid profile of high fat diet mice [72]. Sirt1 also improves insulin secretion by inhibiting expression of uncoupling protein 2 and inhibits adipocyte maturation by blocking PPAR γ (which is also inhibited by the Sirt1 target FoxO1) activity [71, 73, 74]. In fact, Sirt1 overexpression protects against high fat diet induced insulin resistance [75]. In addition, Sirt1 has been shown to deacetylate and activate eNOS. eNOS generates cardioprotective NO which vasodilates blood vessels, inhibits atherosclerosis and attenuates inflammation [76]. Thus Sirt1 mediated control of glucose, lipid and mitochondrial homeostasis may have implications in diabetes, metabolic syndrome and cardiovascular health.

Aging also increases the risk for cancer, inflammatory disease and neurodegeneration. With regards to cancer, Sirt1 acts on HIF-1 α repressing its pro-angiogenic activity. Tumors tend to be hypoxic, which activates HIF-1 α . Hypoxia lowers Sirt1 levels due to lower NAD levels, which can be reversed by increasing NAD levels. Furthermore, increasing Sirt1 expression is able to suppress HIF-1 α activation with subsequent reductions in tumor growth [77]. Sirt1 also promotes DNA stability by deacetylating the DNA repair protein KU70 [78]. However, the evidence is not linear as numerous lines of evidence suggest that Sirt1 may also promote carcinogenesis as various tumors show elevated Sirt1 levels [79], and Sirt1 inhibits the tumor suppressors p53 and Rb thereby blocking apoptosis and stimulating the cell cycle, respectively [80-82]. Inflammation is also a marker for aging with 2-4 fold elevations in IL-6, C-reactive

protein (CRP) and tumor necrosis factor (TNF)- α , occurring systemically [83]. Sirt1 blocks inflammation by inhibiting NF- κ B [84] and activating Foxo3, which induces expression of the antioxidant enzymes superoxide dismutase and catalase [85]. Sirt1 knockout in mouse macrophages similarly increases LPS induced TNF- α secretion with increased JNK activity while Sirt1 activation diminishes tissue inflammation in obese rats [86]. With regards to neurodegeneration, Sirt1 is vital to cognitive function as Sirt1 depletion in mice diminished spatial learning and memory (Sirt1 expression is high in the hippocampus) with defective synaptic transmission and dendritic branching [87]. Sirt1 increases the expression of α -secretase by inducing the *ADAM10* gene, which acts on β -amyloid aggregates seen in Alzheimer's disease. Interestingly, brain specific Sirt1 knockouts showed increased β -amyloid aggregation while Sirt1 overexpression lowered aggregation [88]. Sirt1 can also deacetylate the tau protein seen in Alzheimer's disease promoting its clearance [89]. Sirt1 is also involved in Parkinson's disease by blocking α -synuclein aggregation, which seems to be mediated by activation of autophagy through deacetylation of numerous autophagy proteins (see below) and the chaperone protein HSP70 through HSF-1 [90, 91]. Sirt1's activation of PGC-1 α may also play a role as PGC-1 α is thought to be a therapeutic target for Parkinson's disease [92].

Interestingly, Sirt1 activity is linked to the other CR regulated enzymes mTOR and AMPK. Sirt1 decreases mTOR activity, which as stated previously is also inhibited by CR, in neurons promoting their survival in fed and nutrient deprived conditions [93]. Sirt1 activity is tightly coupled to AMPK and vice-versa. Sirt1 can deacetylate LKB1, which is an upstream activator of AMPK [94]. Meanwhile AMPK can activate Sirt1 by increasing the de novo production of NAD by increased expression of NAMPT [95, 96].

Of note, NAMPT overexpression, with subsequent increases in NAD levels, can inhibit infarct size following ischemia in cardiomyocytes by inhibiting apoptosis and increasing autophagy presumably through sirtuin activation [97]. This crosstalk between Sirt1 and AMPK would occur with CR, fasting or exercise raising AMP levels to activate AMPK, which would increase already elevated levels of NAD to activate Sirt1 thus creating a positive feedback loop with AMPK through increased activation of LKB1 by Sirt1. Sirt1 and AMPK can then work in tandem to activate PGC1- α to promote gluconeogenesis, β -oxidation and mitochondrial biogenesis [98-100]. Sirt1 and AMPK also work together to regulate circadian rhythms. The circadian rhythm allows a cell to proactively anticipate metabolic changes over the course of a 24-hour daily cycle. Numerous genes are expressed cyclically throughout the day (up to 15% of expressed genes) with a large fraction being metabolic enzymes [101]. In fact, the importance of circadian rhythm to obesity was highlighted when mice were fed a high fat diet either ad libitum or restricted to only 8 hours a day. Both groups consumed the same amount of calories each day. Yet the time restricted group showed significantly less weight gain and maintained insulin sensitivity in part through increased diurnal rhythms of the circadian clock and AMPK activity suggesting that “you are when (as opposed to what) you eat” [102]. Central to the circadian rhythm are core transcription factors whose expression oscillates throughout the day and includes Per1/2, Cry1/2, Bmal and Clock (which interestingly shows HAT activity). Interestingly, NAMPT levels oscillate along with NAD in the liver thereby modulating Sirt1 function [103]. NAMPT expression is inhibited by Per2/Clock/Bmal complex in part by acetylation of Bmal by Clock, which is countered by Sirt1 thus allowing Sirt1 to promote its own activity by increasing expression of NAMPT.

Therefore, Sirt1 directly regulates the circadian rhythm by its action on Bmal and Per2 [101, 103]. AMPK localization to the nucleus also oscillates, and AMPK can act on Cry1 (indirectly inhibiting Per2 and promoting NAMPT expression) promoting its degradation [104]. The circadian rhythm coordinates daily activities, and its disruption can greatly influence disease progression [105]. Therefore, it is not surprising that the crosstalk between longevity proteins influences the circadian rhythm.

The abundant work on Sirt1 has shown that through deacetylation of numerous targets in the cytosol and nucleus Sirt1 can regulate metabolism and cellular homeostasis to greatly affect aging-related pathologies and is centrally located to play a part in the cell's response to CR. Also of note, the other cytosolic and nuclear localized sirtuins (Sirt2, 6 and 7) are emerging as key regulators of cell function. Sirt2 is the only exclusively localized cytosolic sirtuin and mirrors Sirt1 in various ways by regulating metabolism, inflammation and carcinogenesis. For instance, it can deacetylate the Sirt1 targets FoxO1 and FoxO3 promoting their nuclear translocation and promoting lipolysis and gluconeogenesis [106, 107]. CR increases Sirt2 in adipose tissue leading to FoxO1 deacetylation, which then inhibits PPAR γ mediated adipocyte differentiation [106]. FoxO1 expression increases PEPCK expression, and Sirt2 can directly deacetylate PEPCK promoting its stability and countering glucose/p300 mediated acetylation and degradation by ubiquitinylation [18]. It also acts on FoxO3 to promote MnSOD expression to attenuate oxidative stress [108]. Also like Sirt1, it can deacetylate NF- κ B on the p65 subunit therefore regulating inflammatory pathways [109]. With regards to cancer, Sirt2 knockout mice show increased tumorigenesis. Males show increased hepatocellular carcinoma while females had increased mammary tumors [110]. Sirt2's

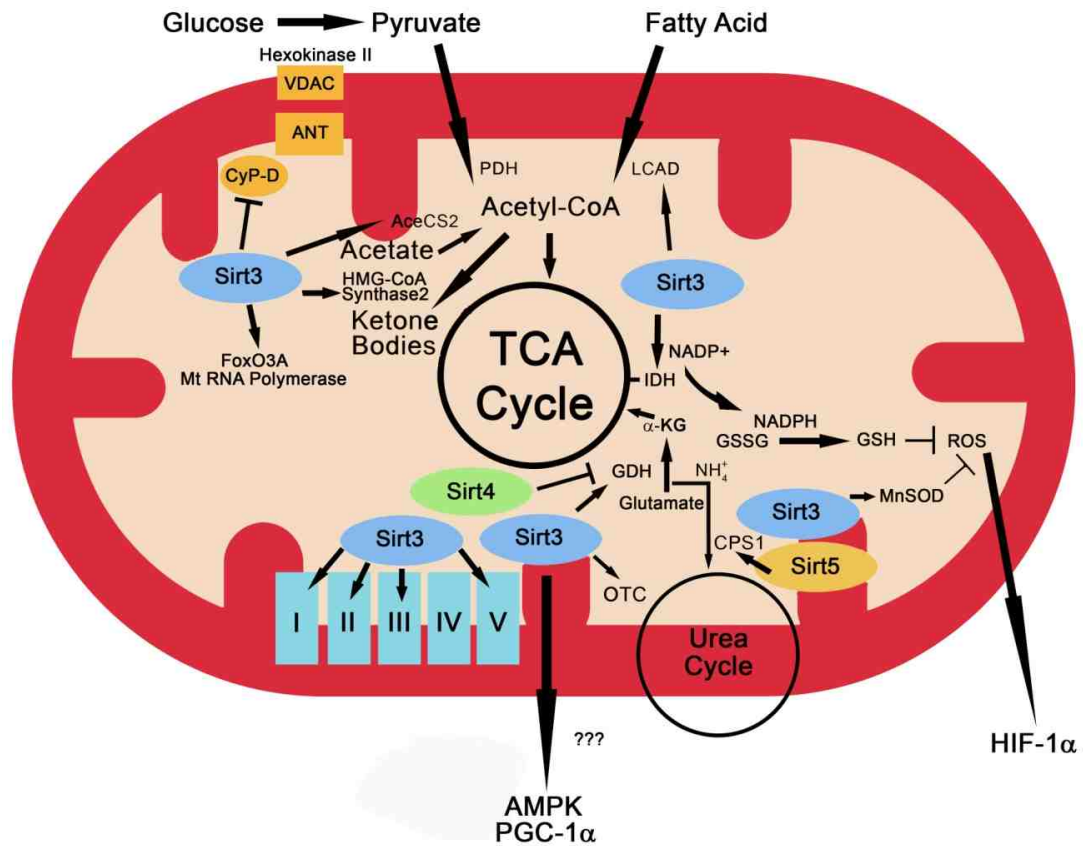
tumor suppressor activity is thought to be mediated through regulation of genomic stability during mitosis and delaying cell cycle progression by deacetylating tubulin [110-112]. It is also thought to augment apoptosis through induction of the pro-apoptotic proteins Bim (by FoxO3 activation) and BAD, via suppression of 14-3-3 ζ expression and cytosolic sequestration of BAD [108] [113]. As a final tumor suppression mechanism, Sirt2 expression is diminished by HIF-1 α , which is inhibited by Sirt1 [114]. Interestingly, Sirt2 was recently shown to be vital to programmed necrosis through its actions on the RIP1-RIP3 complex formation, which are key players in TNF- α -induced programmed necrosis [115].

Sirt6 and Sirt7 along with Sirt1 localize to the nucleus. Rather than deacetylation, Sirt6 performs NAD-dependent ADP-ribosylation. The best known targets of Sirt6 are H3K9 and HK56 [100]. As mentioned, Sirt6 overexpression increased longevity in male mice by 15% [66]. Overexpression of Sirt6 attenuated fat accumulation in mice fed a high fat diet and improved insulin sensitivity by down regulating genes linked to lipid storage [116]. Along with effects on fat accumulation, Sirt6 regulates glucose homeostasis as Sirt6 knockouts die shortly after birth of hypoglycemia due to increased Glut1 expression and glucose uptake into skeletal muscles. This effect is also in part due to increased HIF-1 α activity, glycolysis and diminished mitochondrial respiration reminiscent of the cancer metabolic profile, the Warburg effect [117]. In fact, Sirt6 knockout cells are more glycolytic and show increased tumorigenesis with several cancers showing lower levels of Sirt6 [118]. Sirt6 also promotes the repair of age and oxidative stress-damaged DNA and attenuates senescence by promoting homologous recombination and activating the ADP-polymerase PARP1 [119, 120]. As mentioned,

IGF signaling is linked to diminished lifespan, and Sirt6 attenuates expression of IGF signaling-related genes limiting cardiac hypertrophy [121]. Sirt6 also suppresses hypertrophy through inhibition of NF- κ B signaling [122]. Therefore, Sirt6 increases lifespan by regulating glucose homeostasis, fat accumulation, lowering IGF signaling and functioning as a tumor suppressor. Sirt7 is the least studied sirtuin and localizes to the nucleolus deacetylating histone 3 K18 residues and attenuating tumorigenesis of cancer xenographs in mice [123]. Sirt7 knockouts have diminished lifespan with increased hypertrophy, inflammatory cardiomyopathy, fibrosis and increased p53 acetylation leading to increased apoptosis [124]. In addition, Sirt7 increases RNA polymerase I mediated transcription [125]. Therefore, like Sirt1, the other cytosolic/nuclear sirtuins regulate metabolism, carcinogenesis and inflammation and may mediate many of the benefits of CR.

Mitochondrial sirtuins (Sirt3, 4 and 5)

Three sirtuins (Sirt3, Sirt4 and Sirt5) localize to the mitochondria and greatly regulate mitochondrial function (Figure 4) [126]. Along with Sirt1 and Sirt2, Sirt3 shows strong deacetylase activity and like Sirt1 has been extensively studied. As such, Sirt3 is the major mitochondrial deacetylase while Sirt4 and Sirt5 appear to perform other PTMs (see below) [63]. Mice express both a long and short isoforms of Sirt3 with the long isoform containing a mitochondrial localization sequence [127, 128]. The long isoform is translated in the cytosol as a 44-kDa protein that is transported into the mitochondrial matrix and cleaved to form the final 28-kDa protein [129]. There is also evidence that Sirt3 can localize to the nucleus [130], but this may be an artifact from Sirt3 overexpression. Interestingly, CR and fasting increase expression of Sirt3 in liver,



Sirt3 Regulation of Mitochondrial Function

- Oxidative stress (IDH/MnSOD)
- β-oxidation (LCAD)
- Oxidative phosphorylation (ETC I, II, III & V)
- Urea Cycle (OTC)
- Ketogenesis (HMG-CoAS2)
- Glycolysis/apoptosis (CyP-D)

Figure 4: Mitochondrial sirtuins regulate mitochondrial function. Sirt3, 4 and 5 localize to the mitochondria with Sirt3 being the major deacetylase. Sirt4 and 5 perform ADP-ribosylation and desuccinylation/malonylation, respectively. Sirt3 deacetylates numerous targets to regulate lipid, glucose and amino acid metabolism, oxidative stress and ATP (adenosine triphosphate) generation. (voltage dependent anion channel, VDAC; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC-1α; nicotinamide adenine dinucleotide phosphate, NADP; hypoxia-inducible factor 1, alpha subunit, HIF-1α; AMP-activated kinase, AMPK; manganese superoxide dismutase, MnSOD; reactive oxygen species, ROS; glutamate dehydrogenase, GDH; isocitrate dehydrogenase, IDH; alpha ketoglutarate, α-KG; Ornithine transcarbamylase, OTC; carbamoyl phosphate synthetase 1, CPS1; cyclophilin D, CyP-D; Adenine nucleotide translocator, ANT; 3-hydroxy-3-methylglutaryl-CoA synthase-2, HMG-CoA synthase2; Glutathione disulfide, GSSG; glutathione, GSH)

skeletal muscle, brown adipose tissue, heart and kidney while chronic high fat diet and diabetes lowers expression [131-134]. Sirt3 is also induced at the same time as mitochondrial biogenesis with PGC1- α and ERR α activating the Sirt3 promoter [135, 136]. Sirt3 knockout animals are phenotypically normal but show hyperacetylation of mitochondrial proteins [137]. Despite being phenotypically normal, Sirt3 knockout animals have a >50% reduction in basal ATP levels in heart, liver and kidney tissues [138]. Reductions in ATP levels are caused in part by the hyperacetylation of complex I (NDUFA9) in Sirt3 knockout mitochondria, but this defect can be blocked in Sirt3 knockout MEFs by restoring Sirt3 expression [138]. Sirt3 has also been found to deacetylate other components of the electron transport chain namely Complex I (NDUFA11, NDUFS8) [138, 139]; Complex II (SDHA, SDHB) [139]; Complex III (56-kDa core I subunit) [131]; and Complex V (ATP5A1, ATP5B1, ATP5F1) [100, 139]. Therefore, it appears that Sirt3 greatly affects mitochondrial function and oxidative phosphorylation through regulation of acetylation.

Mitochondria serve as central hubs for metabolism of lipids, glucose and proteins. Non-alcohol fatty liver disease (NAFLD), diabetes and metabolic syndrome can lead to lipotoxicity [136], and there is abundant evidence of a role for Sirt3 in regulating lipid metabolism. Following fasting, livers from Sirt3 knockout animals have elevated triglycerides and β -oxidation intermediates suggesting a defect in lipid metabolism [133]. High fat diet (HFD) also increases mitochondrial protein acetylation (including complexes III and IV) and lowers Sirt3 expression while Sirt3 knockout animals on HFD show accelerated development of metabolic syndrome (obesity, insulin resistance, hyperlipidemia and hepatic steatosis) and reduced mitochondrial function [140-142].

Susceptibility to lipotoxicity is enhanced in primary hepatocytes with Sirt3 depletion, which can be reversed with N-acetylcysteine or Sirt3 reconstitution [141]. These effects on lipid metabolism are mediated in part on Sirt3 activity including the deacetylation and activation of long-chain acyl-CoA dehydrogenase (LCAD) while Sirt3 knockouts have hyperacetylated LCAD [132, 133]. As mentioned, fatty acid metabolism is promoted by AMPK and PGC1- α . There appears to be crosstalk between Sirt3, AMPK and PGC1- α as overexpression of Sirt3 increases PGC1- α levels [143, 144] and increases AMPK activation with diminished lipid accumulation [145]. These effects are reversed by Sirt3 depletion suggesting crosstalk between these two lipid metabolizing enzymes. Sirt3 has also been suggested to deacetylate the upstream AMPK activator LKB1 [146]. Given the crosstalk between Sirt1, AMPK and PGC1- α , it is possible that Sirt1 and Sirt3 also coordinate effects on metabolism. Fatty acids can also be metabolized into ketone bodies during fasting/CR to feed the heart and brain. Sirt3 promotes ketogenesis by deacetylating and activating the rate limiting enzyme 3-hydroxy-3-methylglutaryl-coA synthase 2 with Sirt3 knockouts showing diminished ketone body levels following fasting [147]. Sirt3 can increase fatty acid production using acetate as a substrate by increasing acetyl-CoA levels. It does this by deacetylating acetyl-CoA synthetase-2 (AceCS2), which converts acetate into acetyl-CoA and can then be used for fatty acid synthesis. AceCS2 is mitochondrial, and of note, Sirt1 activates the cytosolic AceCS1 [148]. Interestingly, this same system of regulation is found in the bacteria *Salmonella enteric* with the bacterial sirtuin homolog CobB activating and an identified acetyltransferase Pat inhibiting acetyl-CoA synthetase [149, 150]. It should be noted that no mitochondrial eukaryotic acetyltransferase to counter Sirt3 activity on AceCS2 has been identified.

Overall, Sirt3 greatly regulates lipid metabolism through its actions on numerous protein targets.

In addition to lipid metabolism, Sirt3 affects protein metabolism. Sirt3 positively regulates the enzyme glutamate dehydrogenase (GDH) leading to improved insulin secretion, which counters the activity of Sirt4 on GDH (see below) [151]. GDH converts glutamate into α -ketoglutarate with subsequent generation of ammonia. This ammonia is converted into urea for subsequent elimination by the urea cycle. Sirt3 deacetylates the urea cycle enzyme ornithine transcarbamylase (OTC), and Sirt3 knockout during CR leads to elevated orotic acid levels, which is a signature of the metabolic disorder OTC deficiency [132]. Therefore by activating GDH and OTC Sirt3 is able to regulate amino acid catabolism.

Sirt3 can also influence glucose metabolism through its effects on hexokinase II activity and cyclophilin D. Cyclophilin D is thought to be a component of the mitochondrial permeability transition pore (MPTP). Sirt3 has been shown to deacetylate cyclophilin D inhibiting its peptidyl-prolyl cis-trans isomerase activity causing it to dissociate from adenine nucleotide translocator (ANT) which then promotes the release of hexokinase II from VDAC and the outer mitochondrial membrane (OMM). Mitochondrial localized hexokinase II promotes glycolysis, but its release from the OMM shifts cell metabolism to oxidative phosphorylation, which is inhibited in cancer (i.e. the Warburg effect) [152]. Therefore in combination with deacetylation of electron transport chain proteins, crosstalk with AMPK and PGC1- α and cyclophilin D inhibition, Sirt3 promotes oxidative phosphorylation with a shift away from glycolysis and glucose metabolism.

Sirt3 has also been described as a tumor suppressor. This tumor suppressor role is in part due to its ability to regulate mitochondrial ROS generation. Oxidative stress is thought to be a central mediator of carcinogenesis and aging with 90% of ROS being mitochondrial derived. Mitochondria have inherent anti-oxidant scavenging systems to handle ROS such the Mn superoxide dismutase (MnSOD) [136]. Sirt3 is able to deacetylate and activate MnSOD during CR to attenuate ROS levels [153, 154]. In fact, Sirt3 activation of MnSOD prevented oncogene-mediated immortalization of MEF cells, further suggesting a tumor suppressor function for Sirt3 [155]. In addition to direct deacetylation of MnSOD, there are suggestions that Sirt3 increases expression of MnSOD by increasing FoxO3a activity [156, 157]. A recent report suggests that low glucose levels causes FoxO3a to localize to the mitochondria where it interacts with Sirt3 and mitochondrial RNA polymerase leading to mitochondrial genome activation and increased respiration [158]. Mitochondria also regulate ROS levels through glutathione by generating NADPH by isocitrate dehydrogenase 2 (IDH2). IDH2 converts isocitrate into α -ketoglutarate and generates NADPH, which can then be used to generate reduced glutathione. IDH2 is acetylated in a nutrient dependent manner [21], and Sirt3 can deacetylate IDH2 itself [151]. In fact, Sirt3 activation of IDH2 during CR, with subsequent elevated NADPH and reduced glutathione, was shown to attenuate aging related hearing loss and neuron loss in the cochlea [159]. Further supporting Sirt3 as a tumor suppressor, Sirt3 knockout cells have increased ROS with genomic instability, attenuated contact inhibition and increased carcinogenesis induction while Sirt3 knockout mice have increased breast cancer and human breast cancers have lower Sirt3 levels [160]. As mentioned, Sirt3 inhibits the Warburg effect by promoting oxidative

phosphorylation through cyclophilin D/hexokinase II. Sirt3 knockout cells also show elevated glycolytic and pentose phosphate metabolites, increased glucose uptake with augmented lactate production, which are all markers of a shift to the Warburg effect and carcinogenesis [161]. Another major mediator of the Warburg effect is HIF1 α , which as mentioned is inhibited by Sirt1. Sirt3 inhibits HIF1 α activity indirectly by lowering ROS levels through its actions on IDH2 and MnSOD as ROS can stabilize HIF1 α [161]. Sirt3 levels correlate with HIF1 α stability with subsequent tumor growth in xenograft models [162]. Also of note, Sirt3 deacetylation of cyclophilin D inhibits ethanol mediated MPTP opening but also promotes Bax/Bak mediated apoptosis suggesting another method Sirt3 acts as a tumor suppressor [163, 164]. Therefore, in addition to regulating mitochondrial metabolism, Sirt3 also regulates ROS production and acts as a tumor suppressor.

Sirt3 also influences cardiovascular age-related pathophysiology. The renin-angiotensin system (RAS) plays a major role in hypertension, cardiac hypertrophy and renal health. RAS increases mitochondrial ROS production, attenuates mitochondrial number and function, and RAS blockade mimics the effects of CR [165]. Interestingly, similar to CR, knockout of the Ang II receptor (AT1R) increases lifespan in mice by 26% and show attenuated cardiac fibrosis, hypertrophy, aortic damage, ROS levels with increased mitochondrial density [166]. Linking RAS to sirtuins, AT1R depletion increases Sirt3 and NAMPT (and NAD) levels while Ang II treatment lowers these levels in control animals, which was blocked by candesartan [166]. Also of note, Sirt1 overexpression attenuates AT1R levels in vascular smooth muscle cells [167]. Sirt3 overexpression has been shown to attenuate physiological and chemical induction of cardiac hypertrophy in part through AMPK activation [156, 168]. In summary, Sirt3 is

able to regulate mitochondrial metabolism, ROS production and function leading to an impact on age related pathologies, In fact, population genetics have identified SNPs in the Sirt3 gene that correlate with lifespan [169, 170]. Therefore Sirt3 and mitochondrial acetylation appear to play major roles in aging.

Despite the abundant work on the role of Sirt3 in regulating metabolism and mitochondrial function, relatively little is known about the other mitochondrial localized sirtuins: Sirt4 and Sirt5. As mentioned, Sirt4 mediates NAD-dependent ADP-ribosylation. The best characterized target of Sirt4 is glutamate dehydrogenase (GDH). Again, GDH mediates glutamate metabolism into α -ketoglutarate, NADH and ammonia (which is subsequently eliminated by the urea cycle). This metabolism of glutamate increases ATP production as well as gluconeogenesis. Sirt4 mediated ADP-ribosylation of GDH inhibits its enzymatic activity and lowers ATP production and gluconeogenesis [171]. As mentioned, Sirt3 deacetylation of GDH increases its activity [151]. Therefore in pancreatic β -cells, Sirt4 counters Sirt3 activity and inhibits insulin secretion by diminishing ATP levels leading to inactivation of potassium ATP channels, which can be reversed through CR [171, 172]. Also of note, high fat diet increases the expression of Sirt4 [173]. Interestingly, shRNA mediated knockdown of Sirt4 in primary hepatocytes and myotubes increased expression of Sirt1, AMPK and mitochondrial metabolic and β -oxidation enzymes leading to increased fatty acid oxidation and cellular respiration [174], which again appears to counter Sirt3's augmentation of respiration and fatty acid metabolism. However, it is not known how Sirt3 and Sirt4 are co-regulated given that both use NAD as a substrate yielding opposite effects on cellular metabolism. It does appear that nutritional status has inverse effects on expression levels of both enzymes.

Overall, Sirt4 may negatively regulate mitochondrial metabolism and may be a target for treatment of metabolic syndrome.

Sirt5 was originally described as another mitochondrially-localized deacetylase that acts on the mitochondrial enzyme carbamoyl phosphate synthetase 1 (CPS1). CPS1 catalyzes the rate-limiting step of the urea cycle, which detoxifies ammonia generated during amino acid metabolism that occurs during such conditions as CR and fasting. As mentioned, Sirt3 also promotes the urea cycle through its deacetylation of OTC suggesting these two sirtuins co-regulate the urea cycle and amino acid metabolism [132]. This deacetylation of CPS1 increases its activity with Sirt5 KO mice showing elevated blood ammonia levels during fasting [175]. Overexpression of Sirt5 led to decreased CPS1 acetylation levels and increased urea production [176]. However, the ability of Sirt5 to act as a deacetylase has been questioned as Sirt5 has been shown to act as a lysine demalonylase and desuccinylase with Sirt5 desuccinylating CPS1 and Sirt5 knockout mice having elevated levels of succinylated CPS1 [177-179]. Presumably, this PTM occurs using the high energy metabolic intermediates succinyl-CoA (generated from the TCA cycle or metabolism of branched chain amino acids and long chain fatty acids with propionyl-CoA as an intermediate) and malonyl-CoA (whose formation greatly depends on AMPK regulation of acetyl-CoA carboxylase) similar to acetylation using acetyl-CoA. Interestingly, levels of branched chain amino acids in the blood greatly predict risk for diabetes suggesting that protein succinylation may be altered with diabetes and nutrient status [180]. In addition to CPS1, malate dehydrogenase-2, IDH2, citrate synthase, GDH and HMG-CoA synthase 2 are malonylated and succinylated [177-179], which of note correlates to numerous Sirt3 targets. Therefore the same

mitochondrial enzymes may be both acetylated, succinylated and/or malonylated by Sirt3 and Sirt5. Interestingly, Sirt5 levels increase with CR and this increased expression was linked to improved cognitive ability in mice [181]. Sirt5 appears to regulate some elements of the CR response, and its desuccinylase/demalonylase activity has opened the door to new areas of mitochondrial protein regulation through PTMs. Overall, it is abundantly clear that sirtuin activity within the mitochondria, through changes in acetylation and other PTMs, greatly alters mitochondrial function and plays a major role in aging and the CR response.

Resveratrol and other sirtuin activators

Despite the clear benefits of CR on numerous pathologies and potentially increasing lifespan (in the very least promoting “healthy aging”), population wide CR is not practical. Therefore, attempts to chemically activate the sirtuins are being investigated, similar to attempts to medically alter the other aging pathways involving mTOR (rapamycin) and AMPK (metformin). The first compound found that appeared to activate the sirtuins was the naturally occurring polyphenol resveratrol found in red wine [182]. Initial studies with resveratrol showed that it had anti-inflammatory and anti-oxidizing properties that attenuated mammary gland carcinogenesis in culture and skin cancer in mice [183]. Resveratrol activated Sirt1 *in-vitro* and Sir2 in yeast with a subsequent extension of lifespan by 70%, that was dependent on Sir2 expression, mimicking the benefits for CR [184]. In mice on a high fat diet, resveratrol improved insulin sensitivity, increased AMPK and PGC-1 α activation and reduced IGF-1 signaling [185, 186]. Metabolomics analysis of HepG2 cells treated with resveratrol confirmed a shift from glucose utilization to β -oxidation suggesting Sirt1 and PGC-1 α activation by

resveratrol [187]. Also of note, overexpression of PGC-1 α in skeletal muscle increases lifespan in mice through diminished age-related insulin resistance, inflammation and bone/muscle loss, which further highlights the importance of the resveratrol/Sirt1/ PGC-1 α axis to attenuate aging [188]. Another study found that mice treated with resveratrol have attenuated signs of aging such as inflammation, cataracts formation, bone density and aortic elasticity. However, resveratrol did not increase lifespan in these animals unlike that of lower organisms [189]. Of note, mice fed low dose resveratrol in middle or old age showed a transcriptional profile that greatly overlaps with mice on CR in heart, skeletal muscle and brain and diverges from aged mice [190]. Interestingly, SRT1720, a resveratrol mimetic that is 1000-fold more potent, activates Sirt1, improves insulin sensitivity and enhances mitochondrial function in mice fed a high fat diet with subsequent lifespan extension [191, 192].

The mechanism of action of resveratrol and SRT1720 has come into question as to whether or not they are direct sirtuin activators or act on other targets to induce sirtuin activity [193]. Resveratrol has been shown to activate AMPK, which as previously mentioned acts upstream of Sirt1 by increasing NAD levels, independent of Sirt1 [194]. This activation of AMPK by resveratrol has also been shown to occur via inhibition of phosphodiesterases (PDE4). PDE4 inhibition increases cAMP levels to augment calcium levels activating AMPK by CamKK- β . Interestingly, direct inhibition of PDE4 by rolipram mimicked the benefits of resveratrol in mice by preventing obesity, improving glucose sensitivity and augmenting mitochondrial function [195]. Another study showed that the benefits of resveratrol are lost in AMPK knockout animals fed high fat diet with knockouts failing to show improved insulin sensitivity and mitochondrial function [196].

Therefore resveratrol may in fact be an AMPK activator and an indirect sirtuin agonist.

Regardless of the mechanism, resveratrol has shown beneficial effects in numerous human studies. The benefits of resveratrol in human diets was initially postulated in the 1990's when populations consuming a Mediterranean diets showed lower rates of cardiovascular disease despite consuming high levels of saturated fats, termed the 'French Paradox' [32, 197]. Four weeks of resveratrol treatment (2 x 5 mg/daily) improved insulin sensitivity/signaling and reduced oxidative stress in diabetic patients [198]. Similar to the studies in obese mice, resveratrol treatment (150 mg/day) in human obese patients for 30 days improved mitochondrial function, increased Sirt1 and PGC-1 α levels, activated AMPK, lowered intra-muscular/hepatic lipid content and lowered circulating triglycerides and inflammation markers suggesting that resveratrol can mimic CR in humans [199]. In patients with cardiovascular disease, treatment with resveratrol (10 mg/day for 3 months) improved left ventricular function and vasodilation (possibly due to Sirt1/eNOS activity), lowered LDL levels and attenuated platelet aggregation [200]. Further studies will certainly look at the effects of resveratrol and SRT1720 on other age-related diseases. Despite the uncertainty of its mechanism, resveratrol and subsequent activation of Sirt1 appear to have beneficial effects on numerous pathologies and may offer the benefits of CR without having to actually undergo a CR diet.

Autophagy

Prior to the 1940s, protein turnover was thought to be essentially non-existent with body proteins being static and dietary proteins used primarily for fuel. However, Rudolf Schoenheimer, Harold Urey and David Rittenberg utilized the isotope tagged ¹⁵N-

tyrosine fed to rats and found that only 50% of the ^{15}N was excreted in the urine with the rest deposited in body tissues. Also of note was that the ^{15}N -tyrosine was found in proteins, and the ^{15}N itself was found on other amino acids besides tyrosine suggesting tyrosine metabolism with subsequent generation of new amino acids, i.e. protein turnover. Therefore, it was shown that proteins exist in a dynamic equilibrium rather than a static and stable state [201, 202]. The discovery of the lysosome containing digestive enzymes sequestered from the rest of the cell by Christian de Duve in the 1950s offered a potential mechanism for controlled and regulated protein degradation [201, 203]. To date, two major classes of protein degradation have been described: the lysosomal-independent ubiquitin-proteasome system and the lysosomal-autophagy pathway.

The notion of a non-lysosomal protein degradation system came from the observation that rabbit reticulocytes, which lack lysosomes, perform hemoglobin degradation. A cell-free prep from these cells could degrade hemoglobin at neutral pH and required ATP [204]. Further work showed that this system is composed of multiple proteins rather than a single protease [205]. One of these proteins was found to be ubiquitin/APF-1, which had been previously shown to covalently attach to lysine residues on histones [206], and it was then proposed that ubiquitin covalently tags target proteins for degradation by the other proteins found in the reticulocyte cell-free model [201]. The mechanism of tagging target proteins involves the E1 (ubiquitin-activating) enzyme that uses ATP to adenylate ubiquitin, which then gets transferred to the E2 (ubiquitin-conjugating) enzyme. Finally an E3-ligase transfers the ubiquitin to target proteins from E2. Numerous E3 ligases are expressed in humans and offer specificity for which proteins are ubiquitinated and degraded. Target proteins become poly-ubiquitinated

with chains of ubiquitin forming on target proteins linked together at either K48 or K63 of ubiquitin [201]. The ubiquitin tagged protein target is then degraded by the 26S proteasome (19S and 20S sub-complexes) composed of numerous proteins with the 19S sub-complex recognizing the target protein and the 20S sub-complex having proteolytic activity [207, 208]. To date, numerous pathologies are linked to defects in the ubiquitin-proteasome system highlighting the importance of protein turnover in cellular homeostasis [201].

The ubiquitin-proteasome system is able to degrade individual proteins providing individual proteins with their own half-lives. However, bulk degradation of cellular constituents, protein aggregates and entire organelles, is mediated by the lysosomal-autophagy system thus promoting homeostasis through quality control of cellular components. *Autophagy* or 'self-eating' was proposed by Christian de Duve in the 1960's and is mediated by the engulfment of cellular components into a double-membraned autophagosome with subsequent fusion with lysosomes for degradation of the autophagosome's contents and recycling of basic building blocks [209]. Three forms of autophagy exist which include macroautophagy (termed autophagy), chaperone mediated autophagy and microautophagy. Briefly, chaperone mediated autophagy occurs when individual proteins with a specific peptide sequence are shuttled directly into the lysosome via chaperone proteins and LAMP2a in the lysosome. Microautophagy is the direct engulfment of cytosolic components by the lysosome [210]. Autophagy (macroautophagy) can be broken into three general steps with each step mediated by unique proteins (many of which are termed *ATG* proteins in yeast with several known mammalian homologues) [50, 210, 211] (Figure 5a): 1) initiation/nucleation of the

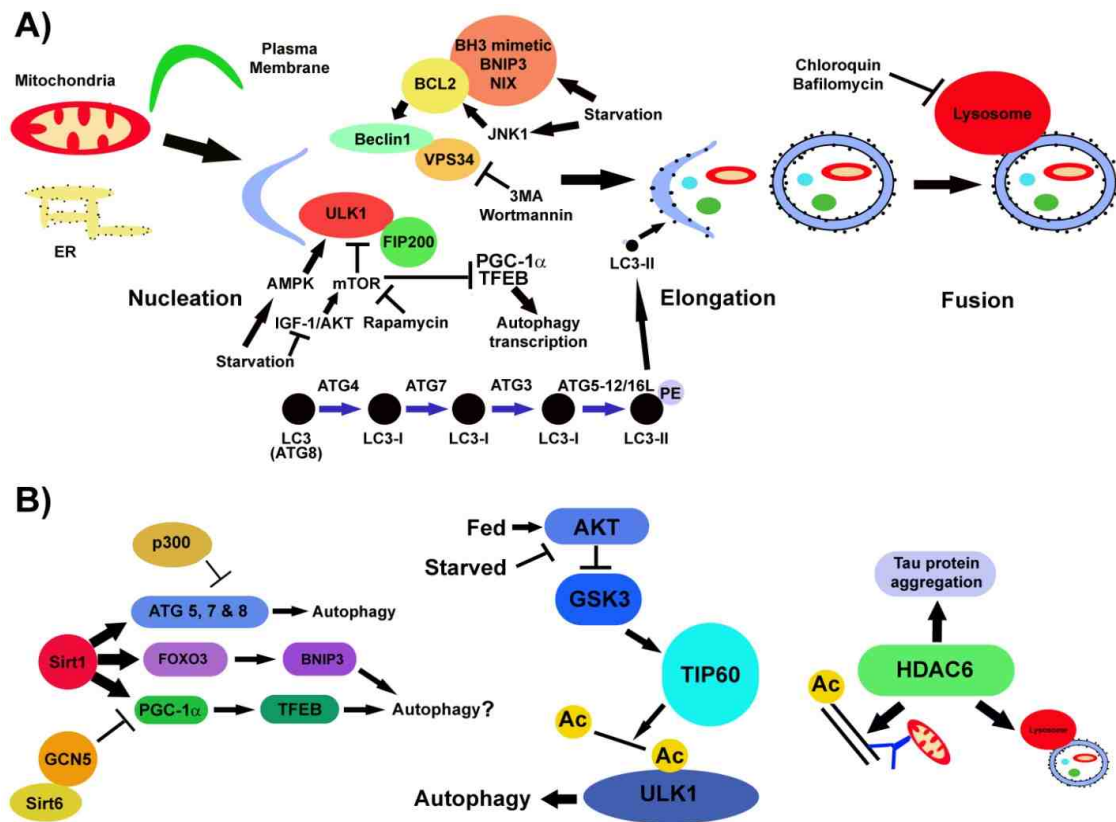


Figure 5: Autophagy is a tightly regulated housekeeping program. (A) Autophagy is triggered in numerous ways including starvation, infection and exercise where the autophagosomal membrane is donated through several possible sources such as the ER, mitochondria or plasma membrane. Phagophore nucleation uses the ULK1 and Beclin1/VPS34 protein complexes, and elongation of the phagophore uses the ubiquitin-like conjugation systems to generate LC3-II. The completed autophagosome with cellular contents fuses with the lysosome forming the autolysosome with subsequent degradation of the autophagosome's cargo. Numerous drugs such as chloroquine, rapamycin and 3MA affect autophagy. (B) Acetylation regulates autophagy in that Sirt1 directly deacetylates and activates ATG proteins, TIP60 acetylates ULK1 and HDAC6 acts on microtubules to affect autophagosome movement and autolysosome formation. (endoplasmic reticulum, ER; B-cell lymphoma 2, BCL2; mammalian target of rapamycin, mTOR; AMP-activated kinase, AMPK; insulin-like growth factor, IGF; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC-1 α ; Tat-interactive protein 60, TIP60; histone deacetylase 6, HDAC6; acetylated lysine, Ac; Glycogen synthase kinase 3, GSK3; General control of amino acid synthesis protein, GCN5; phosphatidylethanolamine, PE; Bcl-2 homology domain 3, BH3)

phagophore; 2) elongation of the phagophore and closure to form the autophagosome; 3) fusion of the autophagosome to the lysosome.

The membrane source of the phagophore used for initiation of autophagy is debated with the ER [212], mitochondria (during starvation) [213] and/or the plasma membrane being potential sources [214]. The class-III PI3 kinase VPS34 is also important for phagophore nucleation and can be inhibited using 3-methyladenine (3MA) or wortmannin. VPS34 interacts with numerous proteins for nucleation including Beclin1 (ATG6), which promotes VPS34 activity [215]. Interestingly, Beclin1 is normally bound to the anti-apoptotic protein BCL-2 preventing its interaction with VPS34 [216]. Also of note, BH3 containing proteins such as BNIP3/NIX (important for some forms of mitochondrial autophagy or 'mitophagy' (see below)) can bind to BCL-2 freeing Beclin1 promoting autophagy [217]. Another important complex for phagophore formation is the FIP200/ULK1 (ATG1) complex, and as mentioned, ULK1 is activated and inhibited by the longevity proteins AMPK and mTOR respectively [51].

Elongation of the phagophore requires two ubiquitin-like conjugation systems. The first one involves ATG5-ATG12 conjugation where ATG12 is first activated by ATG7 (E1-like) and then transferred to ATG10 (E2-like) and finally ATG12 is covalently linked to ATG5. The ATG5-ATG12 conjugate is then linked with ATG16L and a tetramer of the ATG5-ATG12:ATG16L conjugate forms an 800 kDa complex. The second ubiquitin-like system results in phosphatidylethanolamine (PE) lipidation of LC3 (ATG8). LC3 is cleaved by ATG4 yielding LC3-I, which is then processed by ATG7 (E1-like), ATG3 (E2-like) and covalently linked to PE by ATG5-ATG12:ATG16L (E3-like) forming LC3-II. LC3-II is a component of the phagophore membrane and thus is

used as a marker for autophagosome assembly [210].

Finally, fusion of the autophagosome with the lysosome occurs after the autophagosome is moved along microtubules by dynein motor proteins to areas enriched with lysosomes [218]. Lysosomal fusion with the autophagosome utilizes numerous proteins including ESCRT, SNAREs, UVRAG and Rab7 [210]. Lysosomal acidification by H⁺-ATPases is also essential for the completion of the autophagic process. Inhibition of lysosomal acidification by either chloroquine or bafilomycin leads to an accumulation of autophagosomes containing extensive cellular debris. Therefore, these drugs can be used to assess the rate of cellular autophagic flux with increased autophagosome buildup following chloroquine or bafilomycin treatment indicating augmented autophagic flux throughout the cell.

There are numerous mechanisms to induce autophagy, which is also tightly regulated. The classic mechanism for autophagy induction is starvation allowing for degradation of cellular components for use as metabolites to promote survival until nutrients become available again. During starvation, IGF-1 signaling and ATP/amino acid levels would be attenuated leading to downregulation of the AKT/mTOR pathway and augmentation of AMPK thus promoting ULK1 activation and phagophore nucleation [50]. Therefore, the mTOR inhibiting drug rapamycin is thought to promote longevity through autophagy activation as autophagy induction diminishes with age [49, 219]. Recently, the transcription factor TFEB has been shown to be the master regulator for the expression of most proteins involved in the autophagy-lysosome pathway [220, 221]. Interestingly, mTOR can localize to the lysosomal membrane during nutrient abundant conditions where it phosphorylates TFEB promoting its binding to 14-3-3 proteins and

cytoplasmic localization. However, upon starvation mTOR dissociates from the lysosome and TFEB localizes to the nucleus promoting autophagy [222, 223]. Also of note, it was recently shown that PGC-1 α , which as mentioned is activated by Sirt1/AMPK, acts upstream of TFEB to promote autophagy and rescues Huntington's disease proteotoxicity [224]. Therefore, mTOR regulates autophagy through nutrient-sensing and multiple targets such as ULK1 and TFEB. Sirt1 also activates FoxO3, which has been shown to activate autophagy in part by increasing BNIP3 expression [225]. Starvation also activates JNK1 signaling to phosphorylate BCL-2 freeing Beclin1 for autophagy induction [50]. Similarly, starvation increases expression of BNIP3, which promotes the release of Beclin1 from BCL-2, and loss of BNIP3 leads to accumulation of abnormal mitochondria suggesting defects in autophagy [226]. Starvation also induces ROS formation, which is thought to induce autophagy through increased activity of ATG4, AMPK and Beclin1 [210, 227]. Overall, starvation induced autophagy is the most extensively studied mechanism of autophagy induction and is mediated through numerous protein targets/mechanisms.

Besides starvation, other mechanisms for autophagy induction include exercise and infection. Exercise induces autophagy to allow cells to adapt to changing nutritional and energy demands through protein catabolism. Mice deficient in autophagy induction have defective exercise endurance, altered exercise induced glucose metabolism and are not protected from high fat diet insulin resistance by exercise. Therefore, autophagy is essential for the benefits of exercise [228]. The ability to fight off certain pathogens such as salmonella (see below) and tuberculosis also requires autophagy, which has been found to play essential roles in innate and adaptive immunity. Autophagy can directly

eliminate intracellular pathogens, plays a role in antigen presentation by positively regulating MHC-II function and plays a role in immune cell development [210, 229, 230].

Given the numerous mechanisms of autophagy induction, it is not surprising that alterations in autophagic induction play a role in numerous pathologies. Aging is thought to occur in part due to an attenuation of housekeeping autophagic functions leading to accumulation of cellular debris [231]. In fact, promoting autophagy by overexpressing ATG8 in neuronal cells increases lifespan and reduces oxidative stress in *Drosophila* while ATG8 mutations attenuates lifespan [232] suggesting autophagy directly regulates aging. Rapamycin also induces autophagy, increases lifespan in mice and can promote stem cell viability [49, 233]. Autophagy is also thought to be one of the mechanisms by which CR mediates its longevity benefits [50]. Aging greatly affects immunity, the accumulation of protein aggregates in the nervous and cardiovascular systems, cancer progression and the incidence of type-II diabetes. Crohn's disease is an autoimmune disease that is thought to occur in part due to an immune response to commensal bacteria in the gut with defective autophagic clearance of intracellular bacteria and mutations in the autophagy protein ATG16L [234, 235]. Autophagy can also inhibit inflammasome activation, which plays a major role in autoimmune disease, by promoting mitochondrial homeostasis [236]. In addition, autophagy defects play a major role in neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's disease with decreased clearance of the protein aggregates that contribute to these age-related disorders. In Alzheimer's disease reduced Beclin1 expression in the brain leads to accumulation of beta-amyloid plaques [237]. Early onset Parkinson's disease is linked to mutations in the

E3-ligase Parkin, which plays a role in mitochondrial autophagy (see below). Ischemic heart disease is thought to activate autophagy as a protective measure and rapamycin treatment before ischemia decreases infarct size [238, 239]. Autophagy is also believed to inhibit hypertrophic cardiomyopathy [240]. The role of autophagy in cancer is much more complex than its role in cardiovascular and neurodegenerative disease. It is believed that autophagy can act as a tumor suppressor by removing damaged carcinogenic cellular components prior to the onset of cancer, but it can also provide nutrients and promote tumor cell growth in nutrient deprived tumors with poor vascularity [210]. The autophagic protein best characterized as a tumor suppressor is Beclin1. Numerous human cancers show monoallelic deletion of Beclin1, and mice with heterozygous deletion of Beclin1 show increased tumorigenesis and decreased autophagy [241]. Also overexpression of Beclin1 attenuates cancer cell proliferation in cell culture [242]. Knockout of another autophagy protein, ATG4, leads to increased incidence of fibrosarcomas following exposure to carcinogens suggesting autophagy helps prevent cancer [243]. It is believed that a major mechanism for autophagy tumor suppression is through maintaining mitochondrial homeostasis and limiting DNA damaging ROS production [244]. An oncogenic role for autophagy is seen in colorectal cells that have increased rates of autophagy when placed under starved conditions. However, inhibition of autophagy with 3MA or ATG7 knockdown leads to apoptosis [245]. At the clinical level, the autophagy inhibiting drug chloroquine has shown promise as an effective therapy for cancer [246]. Therefore, it appears that autophagy can prevent cancer onset through basic cellular housekeeping, but it also promotes tumor growth once the tumor has become established. Finally, with regards to diabetes, beta-cells from diabetics have

increased numbers of autophagosomes and autophagic inhibition leads to death of beta-cells with impaired insulin secretion [247, 248]. Overall, autophagy appears to play a central role in aging and age-related pathologies [210].

Given the clear roles of autophagy and sirtuins (acetylation) in age-related disorders, there is emerging evidence that the two are intricately linked (Figure 5b). A direct role for Sirt1 in autophagy regulation can be seen in Sirt1 knockout mouse embryonic fibroblasts (MEFs) that fail to fully activate autophagy upon starvation and accumulate damaged organelles while Sirt1 overexpression can increase autophagic flux by directly deacetylating ATG5, 7 and 8 [249]. Interestingly, the acetyltransferase p300 acetylates ATG5, 7, 8 and 12 to inhibit autophagy with overexpression of p300 inhibiting starvation-induced autophagy [250]. Therefore, acetylation can directly induce or inhibit autophagy. As noted, Sirt1 deacetylates PGC-1 α and LKB1, leading to TFEB and AMPK activation, respectively, which could also indirectly promote autophagy. Counter-intuitively, Sirt6 can lead to PGC-1 α hyperacetylation by activating the acetyltransferase GCN5 and may therefore inhibit autophagy, suggesting crosstalk between Sirt1 and Sirt6 as well [70, 99, 251-253]. Another mechanism for indirect autophagy activation by Sirt1 could be FoxO3 mediated increases in BNIP3 expression [225]. Sirt1's regulation of autophagy might contribute to Sirt1 knockout lethality directly after birth since autophagy induction is essential in the period after birth and pre-weaning [50].

Further linking the PTM of acetylation to autophagy, upon starvation or withdrawal of growth factors, the kinase GSK3 phosphorylates the acetyltransferase TIP60. Phosphorylated TIP60 is more active and can then acetylate ULK1 promoting autophagy [254]. Interestingly, TIP60 knockouts are embryonic lethal and die at a time

point of development that is heavily reliant on autophagy [255, 256]. Also the ubiquitin binding histone deacetylase, HDAC6, is essential for lysosome-autophagosome fusion and shuttles autophagic targets on microtubules for delivery to autophagosomes in part due to its ability to regulate tubulin acetylation [257, 258]. During mitochondrial autophagy, also known as mitophagy (see below), damaged mitochondria become ubiquitinated and HDAC6 is essential for clearance by inducing their aggregation near lysosomes [259]. Linking acetylation status, HDAC6 and neurodegenerative disorders is the observation that the acetylation status of mutant Huntington protein affects its autophagic clearance and that HDAC6 activity regulates tau protein aggregation in Alzheimer's disease [260, 261]. Therefore acetylation status of various target proteins by TIP60 and HDAC6, along with Sirt1, appears to regulate autophagy.

Global acetylation patterns in the cytosol and nucleus also appear to affect autophagy induction. Spermidine, an acetyltransferase inhibitor, and resveratrol, a sirtuin activator, both promote autophagy induction through changes in acetylation status [262]. Spermidine treatment extends lifespan in yeast, flies and worms and attenuates oxidative stress in aged mice in part due to decreased acetylation of histones and increased autophagy induction [263]. Autophagy induction by resveratrol, but not spermidine, depends on Sirt1 expression suggesting each drug alters the acetylation status of unique targets. However, both drugs converge to inhibit mTOR activation and hence autophagy induction [262, 264]. Spermidine and resveratrol alter the acetylation status of 375 proteins with 170 (and 27 being mitochondrial) of these proteins being a part of the autophagy protein network [262, 264, 265]. Therefore the acetylation status of cytosolic, nuclear and mitochondrial proteins can trigger autophagy.

Mitophagy

Autophagy was initially described as being the non-selective bulk degradation of cellular components. However, recent evidence suggests that selective autophagy occurs for specific organelles: mitophagy (mitochondria); pexophagy (peroxisomes); ribophagy (ribosomes); and xenophagy (intracellular bacteria). Given the central role of mitochondria in aging, metabolism and cellular homeostasis, it has been proposed that mitophagy degrades functionally impaired mitochondria to prevent numerous pathologies. Mitochondria are the major source of ROS, and the accumulation of dysfunctional mitochondria appears to increase oxidative stress. Increased ROS damages nuclear/mitochondrial DNA, lipids, proteins and mitochondria themselves further promoting oxidative stress in a vicious cycle. Dysfunctional mitochondria also attenuate ATP production promoting necrosis and cytochrome c release leading to apoptosis [266].

Mitochondria are thought to “dilute” the effects of dysfunctional mitochondria through the dynamic processes of fusion/fission. In this way, healthy mitochondria fuse with dysfunctional mitochondria blunting their deleterious effects on the cell [267]. Fission/fusion utilizes specific proteins, and this machinery consists of unique GTPases that fission or fuse the mitochondrial double membrane. Fission is mediated by various proteins, such as Drp1, while fusion occurs through activation of MFN1/2 for the outer membrane and Opa1 for the inner membrane [268]. Interestingly, mutations in MFN1/2 and Opa1 lead to the neurological diseases Charcot Marie Tooth 2a and Optic Atrophy Type 1, respectively, highlighting the importance of functional mitochondrial dynamics. The process of fission/fusion is functionally regulated by nutritional status with starvation or rapamycin treatment promoting mitochondrial fusion in part through inhibition of

fission by Drp1 phosphorylation. This inhibition of fission during short-term starvation promotes global autophagy and lipid/protein metabolism but prevents mitophagy since mitochondria are essential for lipid and protein catabolism [269, 270]. Interestingly, during starvation-mediated autophagy cellular-component degradation occurs in an orderly fashion with cytosolic proteins degraded first within minutes, ribosomes after several hours and mitochondria after nearly a day [271]. It is hypothesized that if starvation is prolonged or if mitochondria are too dysfunctional then these mitochondria are degraded through mitophagy [267]. It has also been shown that mitophagy is dependent on the fission apparatus as Drp1 knockout cells fail to undergo mitophagy [272]. Therefore, it is proposed that fusion during acute starvation protects mitochondria from degradation in part because large elongated mitochondria cannot be engulfed by the autophagosome and mitochondria themselves can donate membrane for the autophagosome during acute starvation [213]. However, after prolonged starvation or extensive mitochondrial damage, fission is induced to generate mitochondrial fragments that are degraded through mitophagy [267, 273]. The overall importance of mitophagy is highlighted by the fact that autophagy mutants in ATG7, ULK1, ATG11 or ATG32 accumulate deformed mitochondria, have increased ROS levels, have damaged mitochondrial DNA, show impaired respiration and have increased senescence, a key marker of aging [272, 274-276]. It is also proposed that mitochondrial turnover through mitophagy and PGC-1 α -mediated biogenesis decreases with age, further emphasizing the importance of mitophagy in cellular homeostasis [277].

In recent years, extensive work has gone into discovering the mechanisms by which mitochondria are selectively degraded through mitophagy (Figure 6a). The best

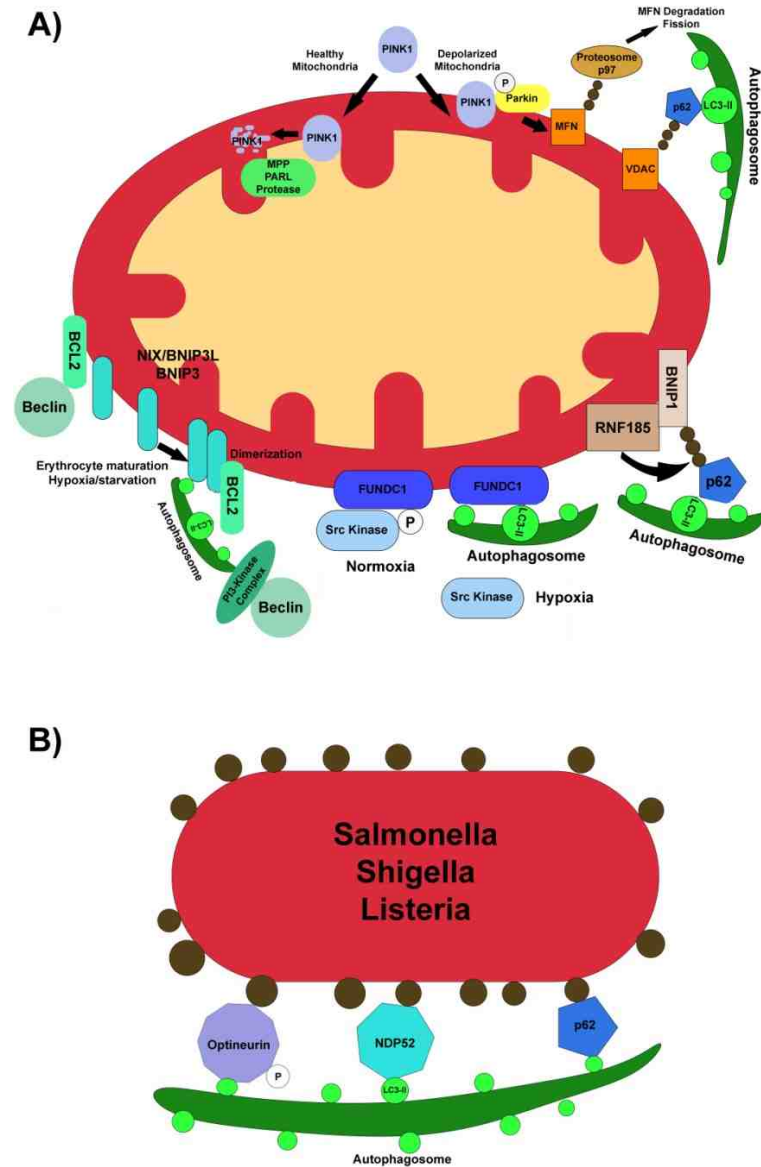


Figure 6: Mitophagy is induced through numerous mechanisms and closely resembles xenophagy. (A) Depolarized mitochondria stabilize Pink1 levels, which activates the E3-ligase Parkin. Parkin ubiquitinylates mitochondrial membrane proteins blocking fusion and targeting mitochondria to autophagosomes by p62 and LC3-II. Hypoxia and cellular differentiation induces mitophagy using NIX and BNIP3, which directly bind to LC3-II. Dimerization is thought to free Beclin1 from BCL-2. Hypoxia also blocks FUNDC1 phosphorylation leading to FUNDC1 binding to LC3-II. The endogenous mitochondrial E3-ligase RNF185 ubiquitinylates BNIP3 targeting mitochondria to autophagosomes. (B) Xenophagy degrades intracellular bacteria through ubiquitylation, adaptor proteins and LC3-II binding. (B-cell lymphoma 2, BCL2; BCL2/adenovirus E1B 19 kDa protein-interacting protein 1/3, BNIP1/3; ring finger protein 185, RNF185; nucleoporin 62, p62; PTEN-induced putative kinase 1, Pink1; voltage dependent anion channel, VDAC; mitofusin, MFN; Presenilins-associated rhomboid-like protein, PARL)

characterized mechanism is that of Pink1/Parkin-induced mitophagy. Pink1 is a serine/threonine kinase and Parkin is an E3 ubiquitin ligase. Both of these enzymes are linked to autosomal-recessive early-onset Parkinson's disease and mitophagy, suggesting mitochondrial quality control plays a role in dopaminergic neurons [278]. Pink1 and Parkin knockouts in *Drosophila* show mitochondrial defects [279, 280]. Mitophagy was initially described to occur following mitochondrial depolarization with subsequent delivery to acidic vesicles [281]. Parkin translocates to depolarized mitochondria treated with the mitochondrial uncoupling drug carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and requires functional Pink1 thus linking Parkin, Pink1 and depolarized mitochondria to mitophagy [282, 283]. Normally, Pink1 is translocated into the inner membrane of the mitochondria where mitochondrial proteases, such as MPP and Parl, mediate its degradation, but upon mitochondrial depolarization, Pink1 is stabilized on the outer mitochondrial membrane and recruits Parkin [284, 285]. Pink1 then phosphorylates and activates Parkin leading to the ubiquitinylation of outer mitochondrial membrane proteins such as VDAC and MFN1/2 [286-288]. Ubiquitinylated outer membrane proteins are then thought to recruit the adaptor protein p62, which targets mitochondria to the autophagosome through interaction with LC3-II in the autophagosome membrane [288]. However, other studies conclude that p62 merely promotes mitochondrial perinuclear clustering and is dispensable for mitophagy [289-291]. Interestingly, the ubiquitinylation of MFN1/2 appears to promote its degradation through p97 and the proteasome, which increases mitochondrial fission, segregation of depolarized mitochondria and mitophagy, while Drp1 knockdown stops Parkin mediated mitophagy [292-294]. HDAC6, which regulates microtubule acetylation, autolysosome formation

and movement of autophagic substrates along microtubules, is essential for Parkin mediated mitophagy through peri-nuclear localization of defective mitochondria [259]. The link between mitochondrial dysfunction and Parkinson's disease is vast as mitochondrial toxins, such as MPTP and rotenone exposure, and mitochondrial protein mutations in complex I are linked the disease [278]. With the addition of Parkin and Pink1 mediated mitophagy, Parkinson's disease is further tied to defects in mitochondrial homeostasis. It also appears that Parkin mediated mitophagy extends beyond Parkinson's disease as ischemic preconditioning in the heart induces Parkin and p62 co-localization to the mitochondria, and Parkin knockout hearts lose the cardioprotective effects of preconditioning [295].

On the opposite arm of mitophagy is mitochondrial biogenesis. Cells overexpressing Pink1 or Parkin and exposed to CCCP completely eliminate mitochondria through mitophagy within 24-96 hours [282, 283, 296]. Therefore, it is believed that after mitophagy induction subsequent mitochondrial biogenesis replaces degraded mitochondria. This mitochondrial turnover is hypothesized to preferentially remove damaged mitochondria first with subsequent biogenesis yielding an overall healthier mitochondrial population with improved respiration, ATP production, attenuated ROS production and resistance to MPTP opening [297]. Fasting and CR induce mitophagy and expression of the master regulator of mitochondrial biogenesis, PGC-1 α , with improved mitochondrial efficiency [296, 298, 299]. Furthermore, the zinc-finger protein Paris, which accumulates in neurons from Parkinson's disease patient neurons, inhibits the expression of PGC-1 α , and Parkin ubiquitinylates Paris for its degradation by the proteasome thus increasing PGC-1 α levels [300]. Therefore, it is hypothesized that

Parkin can mediate both mitophagy and mitochondrial biogenesis through degradation of PGC-1 α with subsequent PGC-1 α activation yielding mitochondrial turnover. This turnover is hypothesized to improve cellular/mitochondrial homeostasis and self-renewal thus attenuating aging [277]. However, the occurrence of Parkin mediating both mitophagy and mitochondrial biogenesis simultaneously in the same cell has yet to be shown.

Interestingly, the process of xenophagy, which is the autophagic degradation of intracellular bacteria such as salmonella or listeria, appears to utilize a similar mechanism to mitophagy by using ubiquitinylation and p62 (Figure 6b). The similarity between mitophagy and xenophagy is interesting from an evolutionary standpoint as mitochondria are believed to have bacterial origins, as stated by the endosymbiotic theory. Putting these together, it is plausible that mitochondria and intracellular bacteria may be degraded by the host-cell in similar ways [301]. In the case of salmonella, upon entering the host-cell, salmonella membrane proteins are ubiquitinated and the autophagy adaptor proteins NDP52 and Optineurin target the bacteria to the autophagosome through LC3-II binding [302, 303]. p62 is also found to localize to ubiquitin labeled bacteria identical to Parkin mediated mitophagy [301, 304]. The E3 ligase(s) mediating the process of xenophagy has not been described.

Other mechanisms for mitophagy have been proposed that do not utilize the Pink1/Parkin pathway but also utilize LC3-II, p62 and ubiquitinylation (Figure 6a). Mammalian cells can induce mitophagy independent of the adaptor protein p62 and ubiquitinylation. Instead 'mitophagy receptors' such as NIX/BNIP3L and BNIP3 incorporate into the mitochondrial membrane, homo-dimerize and directly bind to LC3-II in the autophagosomal membrane [305-307]. Homo-dimerization is also thought to block

BCL-2/Beclin1 interaction to allow Beclin1 to trigger autophagosome nucleation [301]. NIX/BNIP3L-mediated mitophagy is essential for elimination of mitochondria in red blood cells while BNIP3 expression increases and may induce mitophagy during prolonged nutrient withdrawal or hypoxia [217, 226, 305, 307, 308]. As mentioned, Sirt1 deacetylates FoxO3 to promote BNIP3 expression, which may further tie CR to mitophagy [225]. Hypoxia mediates mitophagy through another mechanism involving the protein FUNDC1. Hypoxia is thought to inhibit Src kinase phosphorylation of FUNDC1, which promotes direct binding of FUNDC1 to LC3-II in the autophagosome highlighting another ubiquitinylation/p62 independent mitophagy mechanism [309]. Interestingly, yeast contain a mitophagy receptor, ATG32, that directly targets mitochondria to the autophagosome, but no homologue to ATG32 has been found in mammalian cells [310, 311]. Further complicating things, yet another mitophagy mechanism has been described where a mitochondrial localized ubiquitin E3 ligase, RNF185, ubiquitinylates another outer membrane protein BNIP1, which leads to p62 localization to mitochondria and autophagosome targeting [312]. Therefore mitophagy can be induced through numerous mechanisms utilizing unique proteins. In general, mitophagy uses adaptor proteins, which includes p62, and ubiquitin labeling or mitophagy receptors. like NIX or BNIP3. which directly target mitochondria to the autophagosome through LC3-II binding. It appears that mitophagy is evolutionarily conserved, promotes the regulated elimination of damaged/aged mitochondria, is induced through numerous mechanisms, such as hypoxia and CR, and may play a central role in several aging related pathologies.

Summary and Hypothesis

For over 75 years, caloric restriction (CR) has been known to increase lifespan in

a wide range of organisms. Only recently have the molecular mechanisms, which lead to improved cellular homeostasis, for this augmented lifespan been unraveled. One such mechanism involves CR-mediated activation of lysine deacetylase enzymes, known as sirtuins, with many of the benefits of CR being dependent on sirtuin levels. As such, acetylation has emerged as a major post-translational modification (PTM) rivaling phosphorylation. However, the number of known deacetylase and acetyltransferase enzymes is surprisingly small suggesting there are yet undiscovered enzymes regulating this PTM. Over 20% of mitochondrial proteins are acetylated which is thought to greatly regulate mitochondria function. Sirt3 is the major mitochondrial deacetylase and is activated by CR. It is unknown if mitochondria contain an intrinsic acetyltransferase enzyme complex to counter Sirt3 activity. It is also unknown if changes in expression of such an acetyltransferase would alter mitochondrial turnover and function, countering the effects of Sirt3. The following studies sought to (i) identify the initial components of the mitochondrial acetyltransferase program, (ii) to determine how changes in acetylation alter mitochondrial function, (iii) determine if mitochondrial protein acetylation can induce mitochondrial autophagy or 'mitophagy' considering that cytosolic acetylation status regulates global autophagy and (iv) begin to determine the needed components for acetylation-mediated mitophagy and its effects on mitochondrial homeostasis.

General hypothesis: Global mitochondrial protein acetylation status is regulated enzymatically by an intrinsic mitochondrial acetyltransferase (MAT) complex, which alters mitochondrial function and turnover through changes in acetylation levels and mitophagy.

Specific Aim 1: To determine if mitochondrial protein acetylation is regulated enzymatically.

Hypothesis: Mitochondria contain intrinsic MAT enzymes that utilize acetyl-CoA to regulate global mitochondrial acetylation status.

Specific Aim 2: To evaluate if a MAT affects mitochondrial function.

Hypothesis: A MAT enzyme counters the known acetylation, oxidative phosphorylation and respiratory effects mediated by the major mitochondrial deacetylase, Sirt3.

Specific Aim 3: To determine if changes in mitochondrial acetylation can induce mitochondrial autophagy or 'mitophagy.'

Hypothesis: Global mitochondrial protein deacetylation induces mitophagy and is triggered by the loss of MAT proteins.

Specific Aim 4: Acetylation-mediated mitophagy is regulated by the core autophagy machinery and alters mitochondrial homeostasis.

Hypothesis: Mitophagy induction, through the loss of MAT function, is dependent on known autophagy proteins and improves mitochondrial health due to increased mitochondrial turnover.

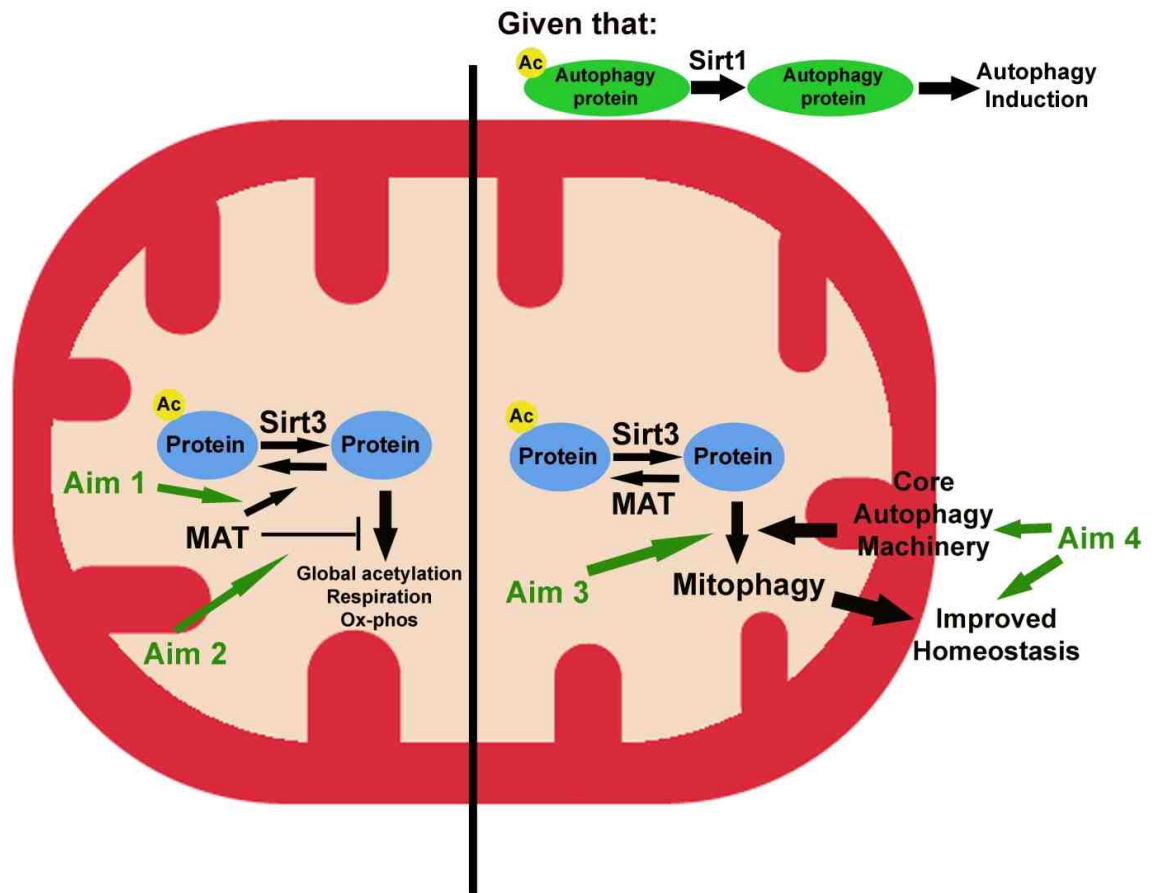


Figure 7: General hypothesis that mitochondrial protein acetylation is controlled enzymatically countering effects mediated by the deacetylase Sirt3. Also, given that Sirt1 deacetylates numerous autophagy proteins to trigger global autophagy, we hypothesize mitochondrial acetylation status regulates 'mitophagy,' increases mitochondrial turnover and improves overall cellular homeostasis.

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Chapter 2

Identification of a molecular component of the mitochondrial acetyltransferase program; a novel role for GCN5L1

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Abbreviations used: Ac-K, acetyl-lysine; ATP5a, ATP synthase subunit 5a; ETC, electron transport chain; GCN5L1, general control of amino acid synthesis 5 (GCN5) like 1; HAT, histone acetyltransferase; NAT, N-terminal acetyltransferase; NDUFA9, NADH dehydrogenase subunit A9; SIRT3, sirtuin family member 3; BtXAT, bacterial xenobiotic acetyltransferase.

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SIRT3 modulates respiration via the deacetylation of lysine residues in electron transport chain proteins. Whether mitochondrial protein acetylation is controlled by a counter-regulatory program has remained elusive. Here we identify an essential component of this previously undefined mitochondrial acetyltransferase system. We show that GCN5L1/Bloc1s1 counters the acetylation and respiratory effects of SIRT3. GCN5L1 is mitochondrial-enriched and displays significant homology to a prokaryotic acetyltransferase. Genetic knockdown of GCN5L1 blunts mitochondrial protein acetylation, and its reconstitution in intact mitochondria restores protein acetylation. GCN5L1 interacts with and promotes acetylation of SIRT3 respiratory chain targets and reverses global SIRT3 effects on mitochondrial protein acetylation, respiration and bioenergetics. These data identify GCN5L1 as a critical, prokaryote-derived component of the mitochondrial acetyltransferase program.

INTRODUCTION

The mitochondrial sirtuin deacetylase SIRT3 plays an important role in regulating oxidative metabolism [1-3] and redox stress [4-6]. Consequently, the regulation of SIRT3 activity is emerging as an important factor in the mitochondrial contribution towards disease susceptibility (reviewed [7]). To understand this system more fully, it is necessary to identify proteins that counteract SIRT3 activity. As such, the discovery of a mitochondrial lysine acetyltransferase has been actively pursued [3].

Given the evolutionary history of mitochondria, one avenue of research has focused on bacteria. In *Salmonella enterica*, the acetyltransferase Pat has been shown to counteract CobB, a sirtuin homologue, in the regulation of acetyl-CoA synthetase [8]. However, eukaryotic orthologs to Pat have not been identified in either the mitochondrial or nuclear genome [9]. An alternate scenario in eukaryotes could be that mitochondrial proteins are acetylated in the cytosol prior to mitochondrial import. However, as fasting and feeding result in a dynamic flux in these post-translational modifications [2, 10], it would be most likely that these modifications occur within mitochondria. Additionally, ATP synthase F_o subunit 8, a mitochondrial-encoded protein, is acetylated under nutrient flux conditions, further supporting the existence of an *in situ* mechanism [10].

In this report, we identify GCN5L1, a protein with significant homology to the nuclear acetyltransferase GCN5 (general control of amino-acid synthesis 5) [11], as a mammalian regulatory protein in the control of mitochondrial protein acetylation and respiration. We show that GCN5L1: includes prokaryote-conserved acetyltransferase substrate and acetyl-CoA binding regions; is localized within mitochondria; modulates mitochondrial electron transport chain (ETC) protein acetylation; alters mitochondrial

oxygen consumption; and counters SIRT3 effects on mitochondrial protein acetylation, respiration and ATP levels.

MATERIALS AND METHODS

Phylogenetic and Structural Analysis

BLAST searches for acetyltransferases related to GCN5L1 identified several prokaryotic proteins, the closest being the xenobiotic streptogramin acetyltransferase from *Burkholderia thailandensis* (ZP_02389502), which we designated BtXAT. The sequences for acetyltransferases (all human, except BtXAT) described were obtained from Genbank and analyzed using the phylogenetic tool PhyML (www.phlogeny.fr.) The XAT-repeat region of GCN5L1 was identified by interrogation of the GCN5L1 protein sequence, looking for a hexapeptide repeat motif matching X-[STAV]-X-[LIV]-[GAED]-X (NCBI cd03349). Data from this region was also used to map the substrate- and acetyl-CoA-binding regions of BtXAT. Predictions of GCN5L1 protein biochemical properties were performed using a Kyte-Doolittle hydrophobicity plot.

Cell Culture and Transfection

HepG2 cells were grown in DMEM supplemented with 10% FBS. Plasmids were transfected using Fugene HD (Roche). For *in vivo* acetylation studies cells were starved for 4 h in HBSS and the re-fed with DMEM for 1.5 h prior to harvesting. Electroporation was used to transfect non-targeting control, GCN5L1 and SIRT3 siRNA (Dharmacon).

Construction of plasmids

The cDNA for GCN5L1 (Open Biosystems) was cloned into p3XFLAG-CMV-14 (Sigma) to create GCN5L1-FLAG. The cDNAs for NDUFA9 and ATP5a (Open Biosystems) were cloned into pCMV3TAG-4A (Stratagene).

Polyclonal Antibody Production

A synthetic peptide corresponding to 16 amino acids of human and mouse GCN5L1, along with a conjugating cysteine at the C-terminus (MLSRLLEKHQAKQNER-C), was produced and injected into NZW rabbits (Covance). Serum was affinity purified against the immunizing peptide, and the antibody validated for recognition of human and mouse GCN5L1.

GCN5L1 Localization, Immunoblot Analysis and Co-Immunoprecipitation

Confocal microscopy was used to localize GCN5L1-FLAG and dsRed-mito (Clontech) in fixed hepatocytes, by indirect immunolabeling of FLAG using Alexa 488 (Invitrogen). Immunogold labeling and electron microscopy was used to localize endogenous GCN5L1 and the ETC protein ATP5a. Sub-mitochondrial localization was performed by osmotic pressure subfractionation and proteinase K protection assays. Antibodies used for westerns include: monoclonal acetyl-lysine (Ac-K), OPA1, GAPDH, VDAC (Cell Signaling); polyclonal Ac-K, ATP5a, NDUFA9, GDH (Abcam); FLAG (Sigma). In co-immunoprecipitation experiments between GCN5L1-FLAG and NDUFA9-Myc or ATP5a-Myc, cells were co-transfected with plasmids for 24 h. Lysates were harvested and incubated with FLAG- or Myc-conjugated beads (Sigma and Cell Signaling, respectively). Beads were washed and analyzed by western blot. Endogenous co-

immunoprecipitation experiments followed a similar protocol, however lysates were incubated with the relevant antibodies overnight, and interacting proteins were captured using protein A/G beads (Santa Cruz). Western blots were quantified using image analysis software, and those shown are representative of at least three independent experiments.

Animal husbandry

The use of mice in this study was approved by the NHLBI Animal Care and Use committee, and animals were maintained according to their guidelines.

Metabolic Measurements

Oxygen consumption measurements were performed on the XF24 analyzer (Seahorse Bioscience). Control- and GCN5L1-transfected siRNA cells were transferred to 24-well plates overnight and incubated in sucrose respiration media for 1 h prior to analysis. To measure the response to the mitochondrial respiration substrates glutamate and malate, cells were permeabilized with digitonin (10 μg per 10^6 cells, 5 min) and incubated in sucrose respiration media. 10 mM glutamate/5 mM malate were added after baseline measurements had been taken. ATP levels in intact cells were measured ($n = 5$) using the EnzyLight assay kit (Bioassay Systems).

In Vitro Acetylation Analysis and In Vitro Immunoprecipitation

An *in vitro* acetylation assay was adapted from a published protocol [12]. HepG2 cells were transfected with control or GCN5L1-FLAG plasmids for 24 h; or control and GCN5L1 siRNA for 72 h; at which time mitochondria were isolated. Samples were

resuspended in reaction buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 4 mM MgCl₂, 5 mM nicotinamide, pH 7.4), sonicated, then incubated for 1.5 h at 37 °C, in the absence or presence of 2.5 mM acetyl-CoA. Mitochondrial proteins were used for global acetylation analysis (reaction stopped by boiling with SDS sample buffer, followed by SDS-PAGE and immunoblot analysis with a monoclonal acetyl-lysine antibody), or immunoprecipitation from non-denatured samples. For the reconstitution of GCN5L1 in mitochondria, HepG2 cells were depleted of GCN5L1 using siRNA as described, after which intact mitochondria were isolated by sucrose gradient centrifugation. 50 µg of pure mitochondria per sample were resuspended in acetyl-CoA assay buffer (100 mM Tris-HCl pH 8.0, 50 mM NaCl, 4 mM MgCl₂, 5 mM nicotinamide, pH 7.4) on ice and sonicated. Samples were incubated at either 25 °C or 95 °C for 5 mins, followed by the addition of 0.5 µg GST-GCN5L1 (Abnova) in PBS or PBS alone (control). After incubation for 1.5 h at 30 °C, the reaction was stopped by boiling in SDS sample buffer. The histone assay followed this protocol, save for the addition of 2.5 µg of recombinant human histone H3 (New England Biolabs) to the mitochondrial samples where indicated. Following the 1.5 h incubation, acetylated H3 was recovered by immunoprecipitation from non-denatured samples with a polyclonal Ac-K antibody. For *in vitro* immunoprecipitation, lysates from the *in vitro* acetylation analysis were incubated overnight with a polyclonal Ac-K antibody. Immunoprecipitated proteins were purified using protein G beads, washed and analyzed by western blot.

Statistical analysis

Where required, data were tested for normality using the Kolmogorov-Smirnov test, followed by a one-tailed Student's t-test or a Mann-Whitney U-test using SigmaPlot 11 (Systat Software). A *P* value of < 0.05 was regarded as significant.

RESULTS

GCN5L1 is identified as a putative mitochondrial counter-regulator of SIRT3

To identify mitochondrial lysine acetyltransferases, we screened the human mitochondrial proteome database MitoCarta [13] for proteins harboring acetyltransferase regions, and used yeast GCN5 as 'bait' in BLAST searches. Fluorescent-tagged candidate proteins were expressed to evaluate mitochondrial localization, and oxygen consumption was measured following siRNA knockdown to identify candidates that counter the known respiratory effects of SIRT3 depletion [1, 14]. The strategy employed is illustrated in Figure S1A. Four candidate proteins identified included GCN5L1 (synonym BLOC1S1), NAT8b, NAT9 and NAT11. Expression of YFP-tagged constructs and dsRed-mito showed that two proteins, namely GCN5L1 and NAT9, co-localized with the mitochondrial marker (Figure S1B). As the knockdown of SIRT3 blunts mitochondrial respiration [1, 15], we reasoned that the genetic depletion of a counter-regulatory component should show the inverse phenotype. siRNA was employed to knockdown GCN5L1 and NAT9; however, only the loss of GCN5L1 resulted in increased oxygen consumption in HepG2 cells (Figure S1C). Subsequent studies focused on the role of GCN5L1 in mitochondrial protein acetylation, and as a putative counter-regulatory protein to SIRT3.

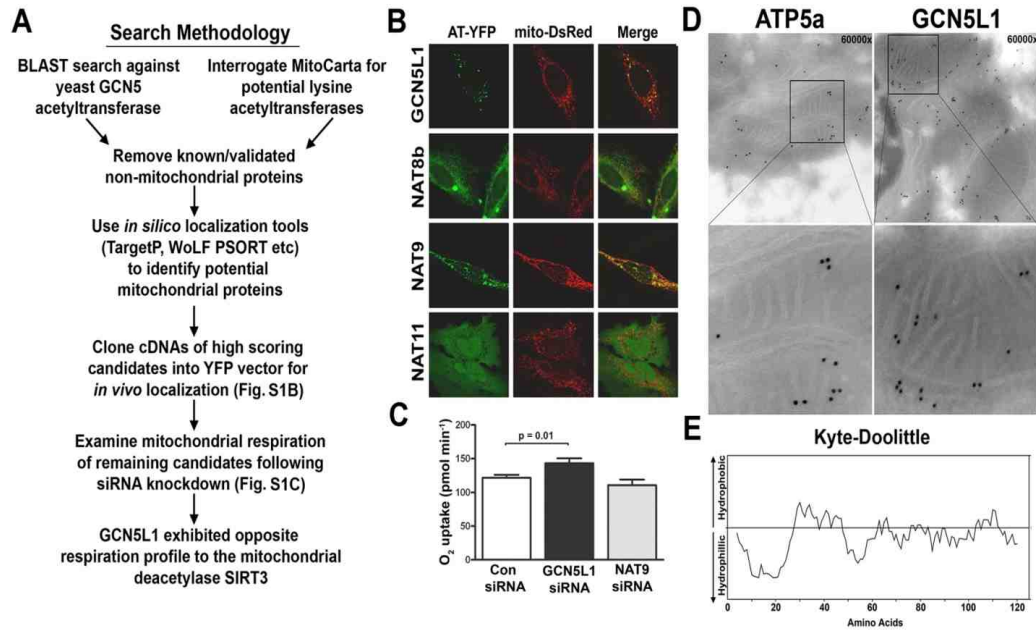


Figure S1. Mitochondrial lysine acetyltransferase search methodology and characterization. (A) *In silico* and *in vivo* methodology used to identify putative mitochondrial acetyltransferases. (B) cDNA of four putative mitochondrial acetyltransferases were cloned into YFP-N1 (Clontech) and expressed in HepG2 cells with dsRed-mito to identify possible mitochondrial localization. (C) Oxygen consumption of two potential mitochondrial acetyltransferases. (D) Immuno-gold labeling of ATP5a (mitochondrial control) and GCN5L1 in fixed mouse liver tissue. (E) Kyte-Doolittle hydropathy plot (window size = 9) shows non-hydrophobicity of GCN5L1, with a predicted hydrophilic surface region at the N-terminus.

GCN5L1 is mitochondrial enriched and more highly expressed in oxidative tissues

GCN5L1 is a previously uncharacterized protein, although its sequence homology to GCN5, the nuclear acetyltransferase, has been described [11, 16]. We first confirmed its mitochondrial localization, using confocal microscopy on HepG2 cells transfected with FLAG-tagged GCN5L1 and dsRed-mito (Figure 1A); and by immuno-gold labeled electron microscopy, which showed that the endogenous protein has similar localization to the alpha subunit of ATP synthase (ATP5a) in mouse liver mitochondria (Figure 1B and S1D). Kyte-Doolittle hydrophathy modeling of the GCN5L1 protein suggests that it is a non-transmembrane globular protein (Figure S1E) [17], suggesting its location within mitochondria to be in either the intermembrane space or matrix soluble fractions. Osmotic pressure subfractionation and proteinase K assays of isolated mouse mitochondria both support that GCN5L1 resides in the soluble matrix and intermembrane space fractions (Figures 1C and 1D). Its mitochondrial enrichment was further supported by the higher levels of GCN5L1 in slow- versus fast-twitch skeletal muscle (Figure 1E).

GCN5L1 possesses prokaryotic features and its knockdown disrupts mitochondrial protein acetylation

Phylogenetic mapping places GCN5L1 in a clade containing a histone acetyltransferase (HAT1), a bacterial xenobiotic acetyltransferase (BtXAT), and the nuclear lysine acetyltransferase GCN5 (Figure 2A). As such, we compared the GCN5L1 amino acid sequence with BtXAT and yeast GCN5. This comparison revealed a 53% similarity to the acetyl-CoA- and substrate-binding motifs of BtXAT. In contrast, homology to yeast GCN5 was limited to the non-catalytic N-terminal region of the

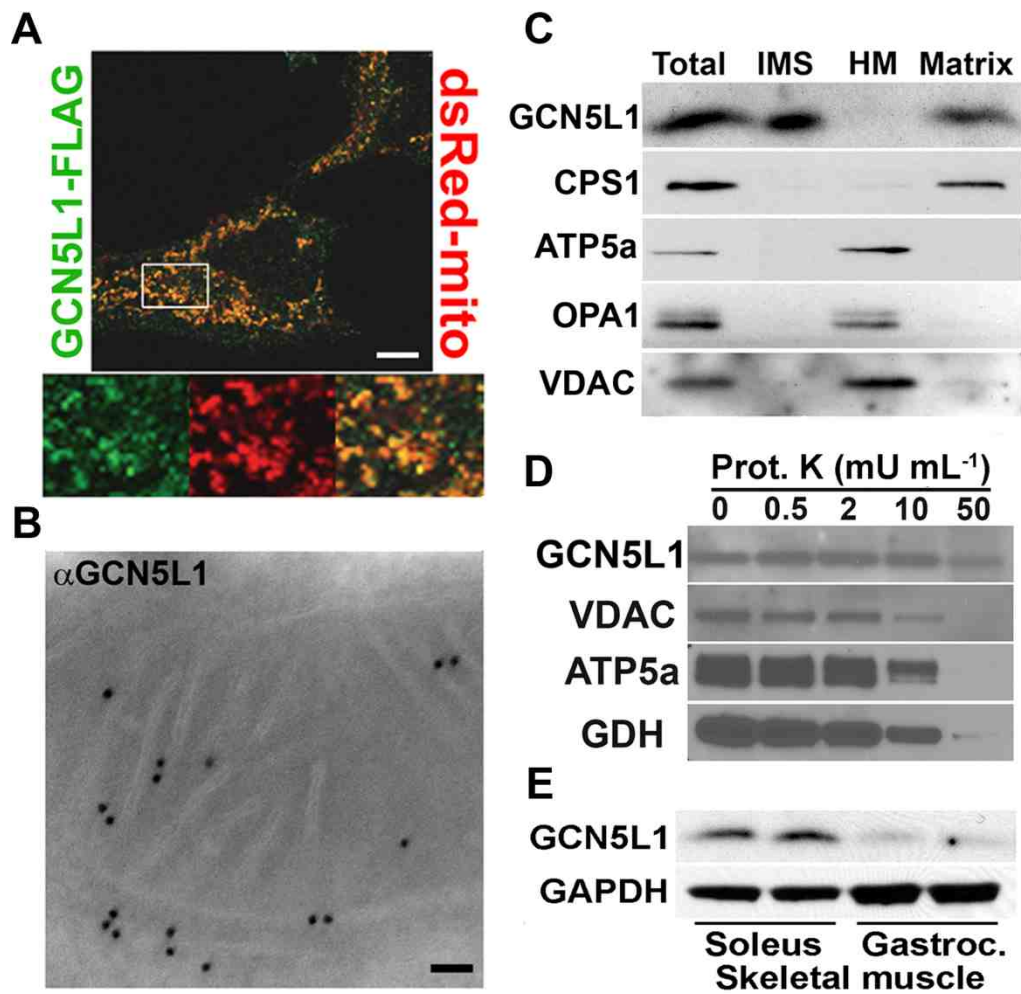


Figure 1. GCN5L1 localizes to mitochondria. (A) Indirect immunofluorescence confocal microscopy of HepG2 cells co-expressing GCN5L1-FLAG and dsRed-mito. Scale bar = 5 μ m. (B) Immuno-gold labeling of endogenous GCN5L1 in fixed mouse liver tissue. Scale bar = 5 nm. (C) Sub-mitochondrial localization of GCN5L1 by osmotic pressure analysis of isolated mouse mitochondria. Abbreviations: IMS – inter-membrane space, HM – heavy mitochondrial membranes. (D) Proteinase K protection assay to establish GCN5L1 sub-mitochondrial localization. (E) Expression of GCN5L1 in soleus (slow-twitch) and gastrocnemius (fast-twitch) skeletal muscle.

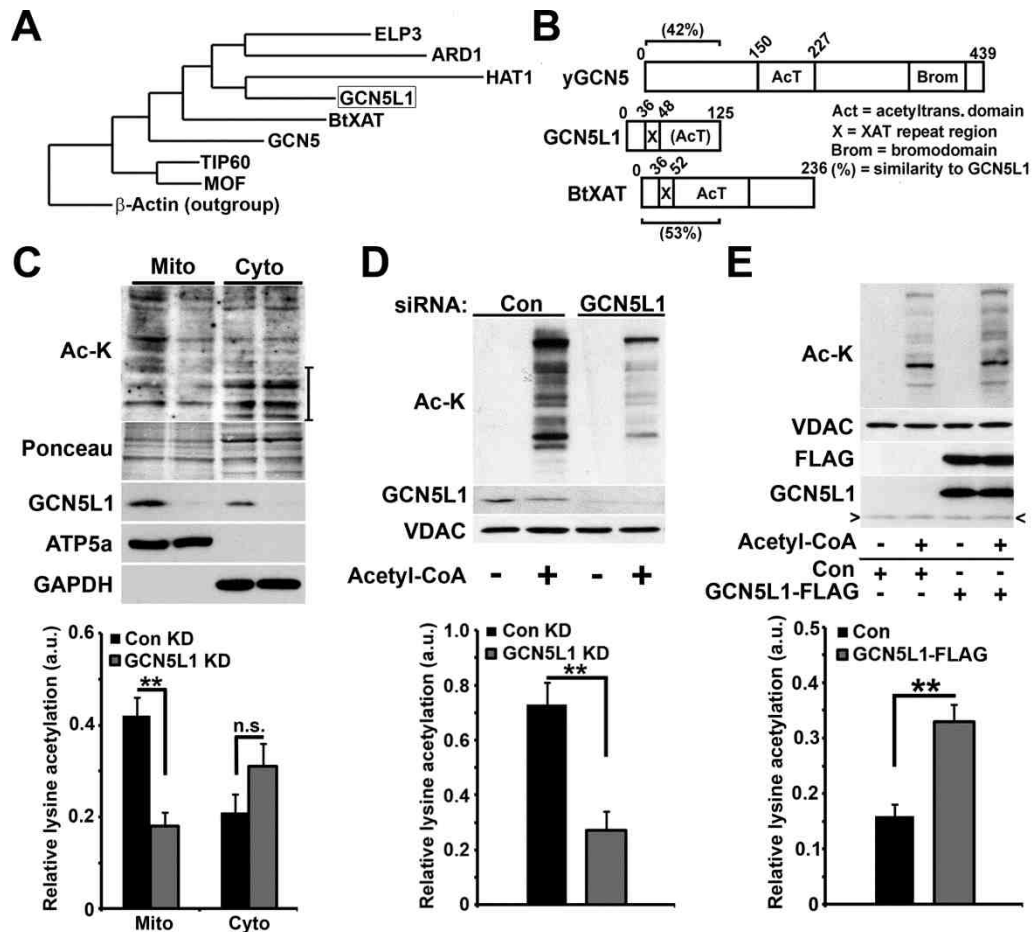


Figure 2. GCN5L1 levels modulate the degree of mitochondrial protein acetylation. (A) Phylogenetic mapping of GCN5L1 against human and bacterial acetyltransferases identifies homology to HAT1 and BtXAT. (B) Alignment of GCN5L1 against yeast GCN5 and BtXAT acetyltransferases. (C) Lysine acetylation (Ac-K) status of endogenous mitochondrial and cytoplasmic proteins following GCN5L1 depletion; bar shows region of Ponceau stain. The accompanying histogram shows relative protein acetylation in the mitochondrial and cytosolic fractions comparing scrambled to GCN5L1 knockdown (KD) (D) Acetylation status of total mitochondrial proteins in control or GCN5L1-depleted HepG2 cells following *in vitro* acetylation assay with the accompanying histogram showing relative protein acetylation. (E) Acetylation status of mitochondrial proteins expressing control or GCN5L1 constructs, following an *in vitro* acetylation assay with the accompanying histogram showing relative protein acetylation. Arrows indicate endogenous GCN5L1. Data are expressed a mean \pm s.e.m. ($n \geq 3$). n.s. – not significant, ** $p < 0.01$, compared to scrambled siRNA treated controls.

protein (Figure 2B). The deduced GCN5L1 protein sequence, and especially the prokaryotic-like XAT repeat region, is highly conserved in metazoans (not shown), suggesting a functional role of this region.

To begin exploring the functional role of GCN5L1, we determined whether siRNA knockdown of this protein would modulate protein acetylation. Although GCN5L1 is predominantly mitochondrial, cytosolic expression was also observed. GCN5L1 levels were depleted in both subcellular compartments by siRNA; however, only the mitochondrial fraction showed a significant reduction in protein acetylation in HepG2 cells, supporting its functional role in mitochondria (Figure 2C). The acetylation function of GCN5L1 was further supported by a $\approx 60\%$ attenuation of mitochondrial protein acetylation, in an *in vitro* acetylation assay, using GCN5L1-depleted cells (Figure 2D). Furthermore, the overexpression of GCN5L1 in HepG2 cells increased mitochondrial protein acetylation to a similar degree in the presence of acetyl-CoA (Figure 2E).

Histone H3 is acetylated by GCN5L1-enriched mitochondrial extracts

To further characterize this effect, *in vitro* assays were performed to assess whether recombinant GCN5L1 can acetylate purified histones, a classic acetylation substrate. In contrast to the *in vivo* studies, GCN5L1 promoted only weak histone acetylation when co-incubated *in vitro* (data not shown). To investigate whether this discrepancy may be due to the requirement of additional mitochondrial factors in the functioning of GCN5L1, we assessed mitochondrial protein acetylation in response to the addition of recombinant GCN5L1 to mitochondrial extracts purified from GCN5L1-depleted cells. Here, the reconstitution of GCN5L1 restored protein acetylation; although

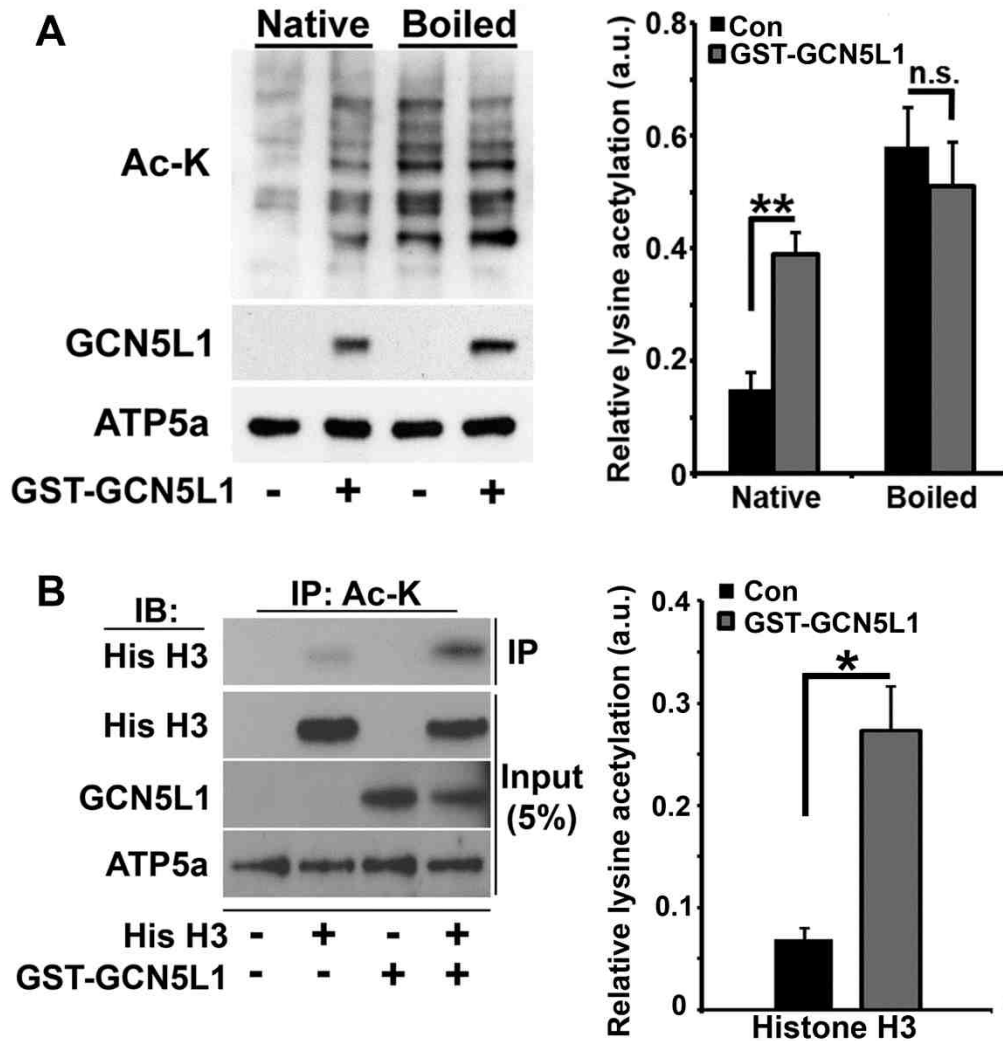


Figure 3. Intact mitochondrial contents are required for GCN5L1 mediated protein acetylation. (A) Effect of the reconstitution of GCN5L1 to native and boiled mitochondrial fractions on protein acetylation (Ac-K). The accompanying histogram shows relative protein acetylation in the native or boiled (denatured) mitochondrial fractions with or without the restoration of GCN5L1. (B) The extent of recombinant histone H3 acetylation in mitochondrial extracts in the presence or absence of GCN5L1. The relative acetylation of histone H3 in the presence or absence of GCN5L1 is shown in the accompanying histogram. Data are expressed as a mean \pm s.e.m. ($n \geq 3$). n.s. – not significant, * $p < 0.05$ and ** $p < 0.01$, compared to scrambled siRNA treated controls.

this effect was limited to native, and not denatured mitochondrial extract samples (Figure 3A). It is interesting to note in Figure 3A that the denaturing of mitochondria appears to facilitate ‘auto-acetylation,’ as the boiled samples show enhanced protein acetylation in the presence and absence of GCN5L1. To further explore the requirement of additional mitochondrial cofactors in the acetylation function of GCN5L1, recombinant histone H3 was added to native GCN5L1-depleted mitochondrial extracts in parallel with the reintroduction, or not, of GCN5L1. The subsequent immunoprecipitation of acetylated lysine proteins, followed by immunoblot analysis with an antibody recognizing histone H3, shows increased H3 acetylation in the presence of GCN5L1 (Figure 3B). These data show that GCN5L1 positively regulates mitochondrial protein acetylation. However, its activity appears to require additional factors that reside in mitochondria.

GCN5L1 Promotes Electron Transport Chain Protein Acetylation

As the predominant mitochondrial protein lysine deacetylase, SIRT3, activates ETC proteins [1, 18], we investigated whether known SIRT3 ETC targets interact with, and are modulated by, GCN5L1. Co-expression of GCN5L1 with Myc-tagged complex I protein NDUFA9, or complex V protein ATP5a, followed by reciprocal immunoprecipitation, shows that there is a physical interaction between both NDUFA9 and ATP5a with GCN5L1 (Figures S2A and S2B). To confirm these interactions occur between the endogenous proteins *in vivo*, untransfected HepG2 cell extracts were used for immunoprecipitation studies. Here the antibody directed against GCN5L1 was employed for immunoprecipitation, and antibodies directed against NDUFA9 and ATP5a were used in immunoblot analysis (Figure 4A). These data confirm the protein-protein

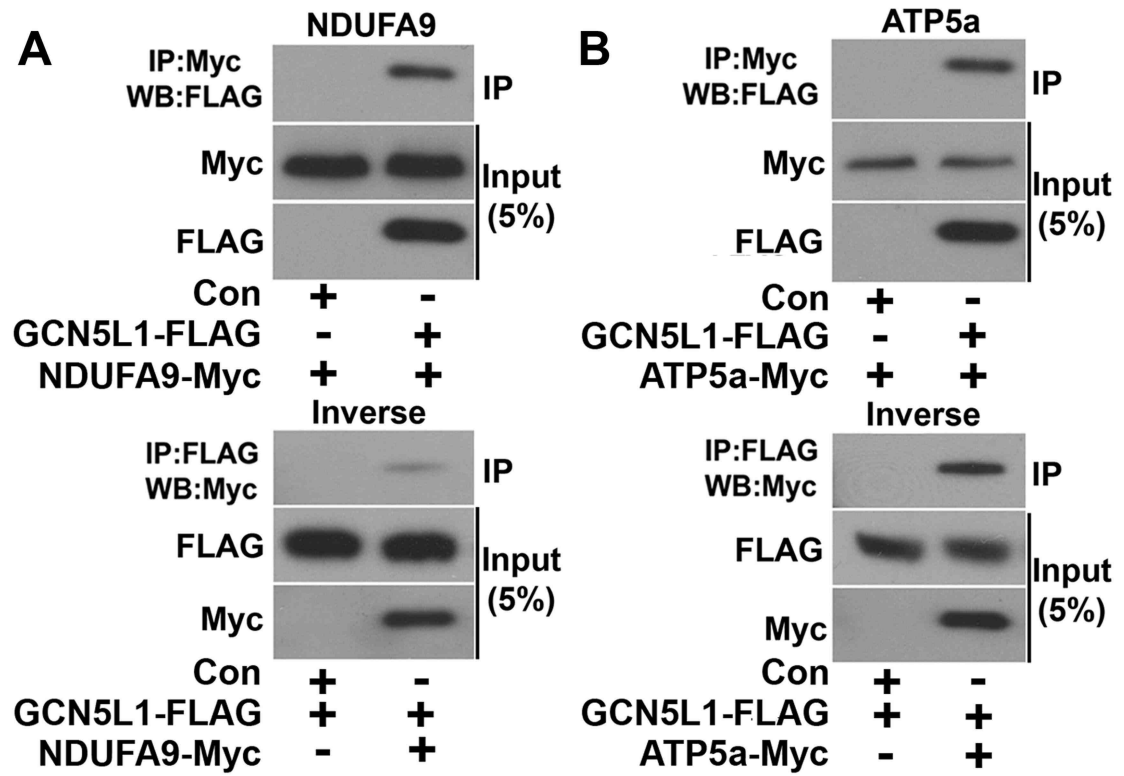


Figure S2. GCN5L1 interacts with mitochondrial electron transport chain proteins. Reciprocal co-immunoprecipitation experiments between GCN5L1-FLAG and Myc-tagged ETC proteins, (A) NDUFA9 and (B) ATP5a. $n \geq 3$ for all experiments.

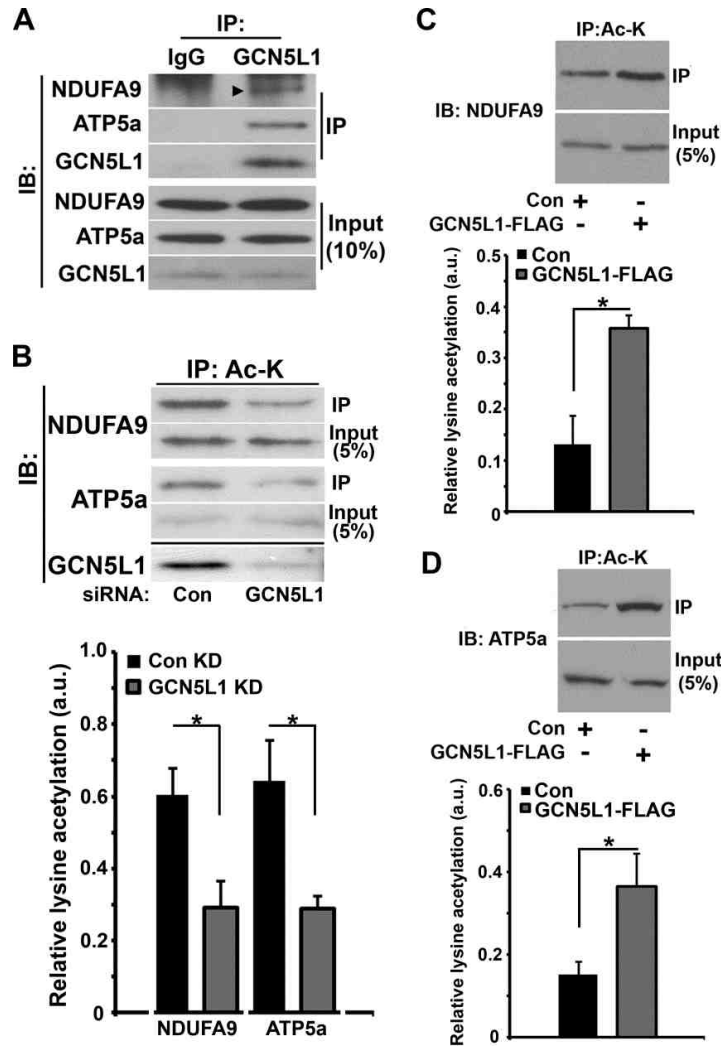


Figure 4. GCN5L1 modulates the acetylation of mitochondrial electron transport chain proteins. (A) *In vivo* interaction of GCN5L1 with the ETC proteins NDUFA9 and ATP5a. The arrow shows the specific band for NDUFA9 relative to the non-specific band seen in the IgG immunoprecipitation control. (B) *In vitro* immunoprecipitation acetylation assay of endogenous NDUFA9 and ATP5a with accompanying histogram showing the relative levels of the respective protein acetylation (Ac-K) levels in control or GCN5L1-depleted HepG2 cells. (C) *In vitro* immunoprecipitation acetylation assay using an acetylated-lysine antibody to assay endogenous NDUFA9 acetylation with the accompanying histogram showing relative acetylation in HepG2 cells expressing control or GCN5L1 plasmids. (D) *In vitro* immunoprecipitation acetylation assay using an acetylated-lysine antibody to assay endogenous ATP5a acetylation with the accompanying histogram showing relative acetylation in HepG2 cells expressing control or GCN5L1 plasmids. $n \geq 3$ for all experiments. * $p < 0.05$, compared to respective controls. Results are mean \pm s.e.m.

interaction between GCN5L1 and the ETC proteins. To functionally characterize whether these ETC proteins are targets of GCN5L1 mediated acetylation, we then undertook *in vitro* acetylation procedures using total mitochondrial protein in cells displaying either knockdown or overexpression of GCN5L1. Using mitochondria from control and GCN5L1-depleted cells, followed by immunoprecipitation with an acetylated lysine antibody and immunoblot analysis, showed that acetylation of endogenous NDUFA9 and ATP5a is diminished following GCN5L1 knockdown (Figure 4B). In parallel, immunoprecipitation with an antibody against acetylated lysine demonstrates that endogenous NDUFA9 and ATP5a exhibit increased acetylation in cells overexpressing GCN5L1 relative to control samples (Figure 4C and 4D).

Mitochondrial Respiration is Attenuated Following GCN5L1 Knockdown

As GCN5L1 targets ETC proteins, we then evaluated the effect of GCN5L1 knockdown on mitochondrial respiration. Following knockdown of GCN5L1 compared to scrambled control siRNA in HepG2 cells (Figure 5A), oxygen consumption was assessed using the Seahorse apparatus. Basal cellular oxygen consumption was increased by GCN5L1 knockdown, as was the maximal oxygen consumption following mitochondrial uncoupling (Figure 5B). The depletion of GCN5L1 in HepG2 cells resulted in a 36% increase in oxygen consumption (Figure 5C). To confirm that this oxygen was consumed in the mitochondria, the HepG2 cells were permeabilized with digitonin and oxygen consumption was determined in the presence of glutamate and malate as specific mitochondrial respiratory substrates. Here, oxygen consumption was increased by 32% in GCN5L1 knockdown cells (Figure 5D). In parallel with these

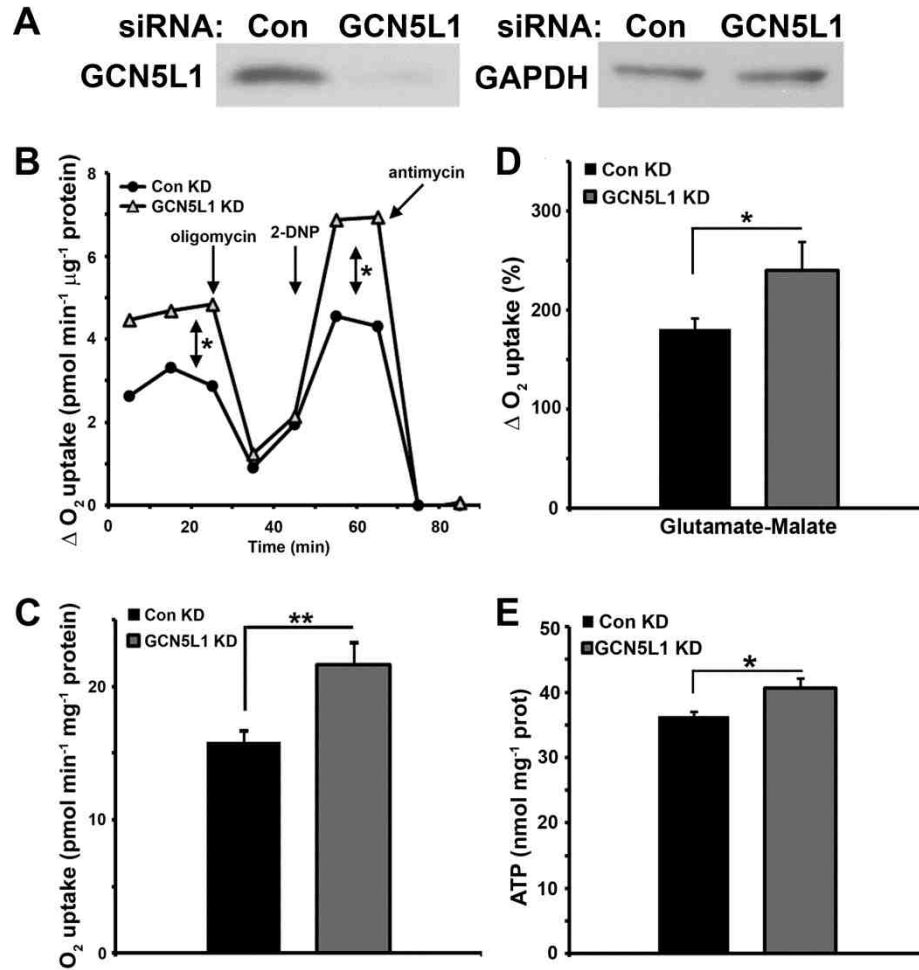


Figure 5. GCN5L1 knockdown increases mitochondrial respiration. (A) Steady-state GCN5L1 levels in HepG2 cells following control or GCN5L1 knockdown (KD). (B) Representative tracing of basal oxygen consumption and maximal oxygen consumption induced by the uncoupler dinitrophenol (2-DNP) comparing control and GCN5L1 KD HepG2 cells. (C) The absolute differences in basal oxygen consumption comparing control and GCN5L1 KD HepG2 cells, taken as a mean of the first three data points. (D) Relative differences in oxygen consumption in control and GCN5L1 KD cells following digitonin administration and the use of glutamate and malate as mitochondrial respiration substrates. (E) Differences in cellular ATP levels in control and GCN5L1 KD HepG2 cells. $n \geq 3$ for all experiments. * $p < 0.05$ and ** $p < 0.01$, compared to respective controls. Results are mean \pm s.e.m.

changes in oxygen consumption, the depletion of GCN5L1 results in significantly higher cellular ATP levels (Figure 5E).

GCN5L1 Functionally Opposes SIRT3 Effects

The above results show that the acetylation and bioenergetic phenotype of GCN5L1 depletion is in direct contrast to that observed following the loss of SIRT3. As these findings suggest that GCN5L1 may counteract SIRT3 function, we explored the response to the combined genetic manipulation of these proteins. We employed an *in vivo* model where SIRT3 was knocked down in HepG2 cells, with or without the concurrent knockdown of GCN5L1. Knockdown of SIRT3 alone increased mitochondrial protein acetylation, which was significantly reversed by the concurrent knockdown of GCN5L1 (Figure 6A). Accordingly, the knockdown of SIRT3 diminished mitochondrial oxygen consumption and cellular ATP levels [1, 14], and the concurrent knockdown of GCN5L1 reversed these metabolic phenotypes (Fig. 6B-C). The ability of GCN5L1 knockdown to reverse SIRT3 acetylation and respiratory phenotypes was confirmed in SIRT3^{-/-} MEF cells (data not shown).

DISCUSSION

Identification of the mitochondrial acetyltransferase machinery has proven elusive. This is in contrast to the nuclear compartment, where the acetyltransferases GCN5, p300 and TIP60 have been found to function as counter-regulatory enzymes to SIRT1 [19]. The identification GCN5L1 in this report reveals a novel mediator of mitochondrial protein acetylation to counter sirtuin deacetylase function.

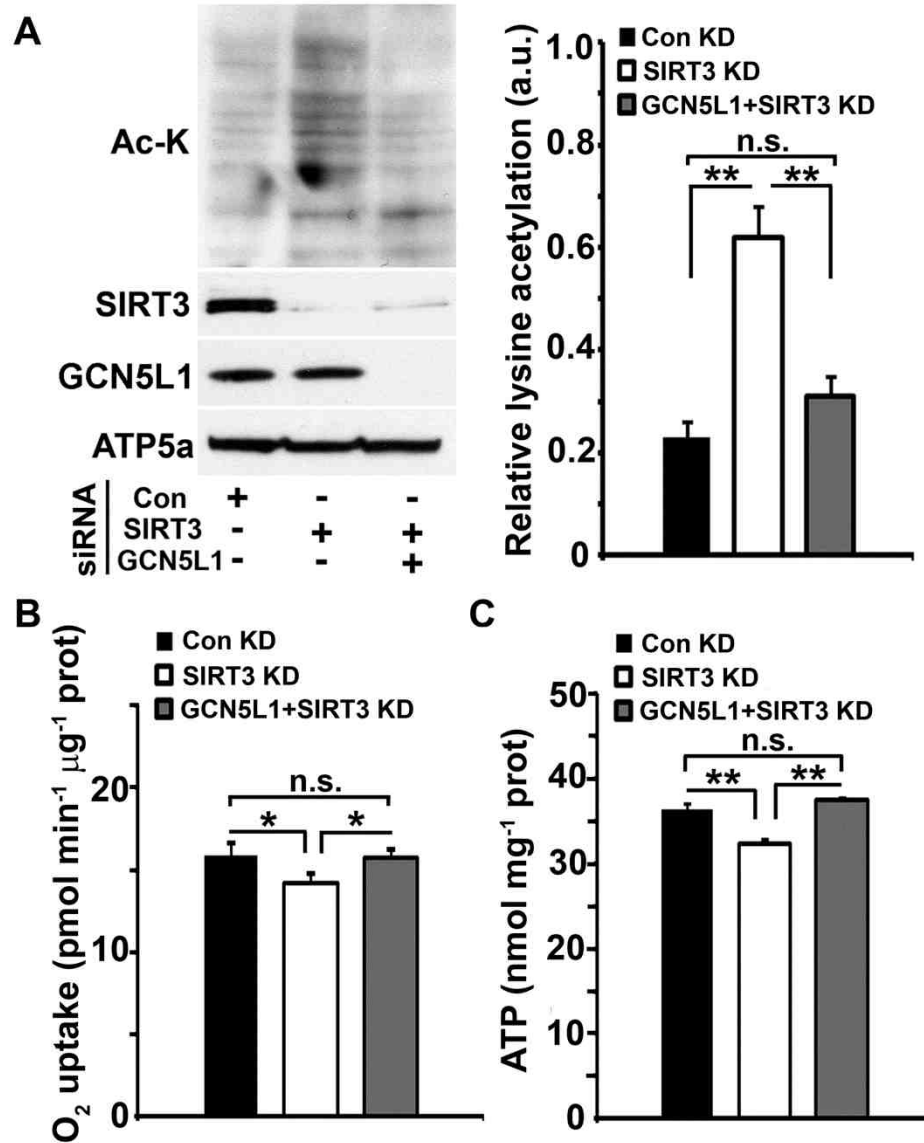


Figure 6. GCN5L1 counteracts the effects of SIRT3. (A) Total mitochondrial protein acetylation (Ac-K) in HepG2 cells transfected with control, SIRT3 or SIRT3+GCN5L1 siRNA with an accompanying histogram showing the relative differences in mitochondrial protein acetylation under the three different conditions. Metabolic measurements of (B) mean total oxygen consumption ($n = 5$) and (C) mean total cellular ATP ($n = 5$) of HepG2 cells transfected with control, SIRT3 or SIRT3+GCN5L1 siRNA. Western analyses of mitochondrial acetylation were performed at least five times, and representative blots are shown. n.s. – not significant, * $p < 0.05$ and ** $p < 0.01$, compared to the defined controls. Results are mean \pm s.e.m.

The maintenance of sequence homology, from prokaryotes to nuclear-encoded eukaryotic mitochondrial proteins, is consistent with the symbiotic hypothesis of mitochondrial evolution [20]. This phylogenetic fidelity is also evident, for example, in eukaryotic mitochondrial kinases [21], and in the iron/sulphur assembly regulatory protein frataxin [22]. As with these examples, the conservation of the BtXAT protein acetyl-CoA- and substrate-binding regions in GCN5L1 supports the strong evolutionary pressure for retention of prokaryotic features in mitochondrial regulatory proteins.

The characterization of GCN5L1 in this study shows that its expression, in isolation, is insufficient to significantly augment protein acetylation. However, the loss of acetylation following GCN5L1 depletion, and the re-establishment of this activity after its reconstitution, both demonstrate the requirement for GCN5L1 in mitochondrial protein acetylation. The findings presented here suggest that GCN5L1 is one critical component of the mitochondrial acetyltransferase machinery. This concept is compatible with the function of yeast GCN5, where integration of this protein into a multi-subunit complex is required for maximal acetyltransferase activity [23]. Together, these data have compelled us to begin to investigate whether additional mitochondrial proteins form multimeric complexes with GCN5L1 in order to orchestrate acetyltransferase activity.

The early characterization of the bioenergetic role of the mitochondrial deacetylase SIRT3 was performed in genetic knockout or knockdown conditions in cell and murine models. The initial studies showed that the absence of SIRT3 resulted in a marked reduction in mitochondrial oxygen consumption and a decrease in cellular ATP levels [1, 14]. Subsequent studies in an *in vivo* context showed that the bioenergetic effects of SIRT3 were most pronounced under fasted conditions, and that the activity of numerous

enzymes in multiple mitochondrial metabolic pathways are modified by SIRT3-mediated protein deacetylation. In this manuscript we employed a similar approach, and identified that the absence of GCN5L1 inversely perturbs mitochondrial oxygen consumption and cellular ATP levels. However, more comprehensive biochemical analysis of the effects of mitochondrial protein acetylation will need to be performed as additional substrates of GCN5L1 are delineated, particularly following the identification of the putative functional partners of GCN5L1.

In summary, this study shows that GCN5L1 functions as an essential component of the mitochondrial lysine acetyltransferase machinery, has phylogenetic linkage to prokaryotic acetyltransferases, and modulates mitochondrial respiration via acetylation of ETC proteins. GCN5L1 also counters the mitochondrial deacetylase function of SIRT3. Further investigation of GCN5L1, its putative interacting proteins and substrates, and the role of acetyl-CoA, should enhance our understanding of how acetylation modulates mitochondrial function.

AUTHOR CONTRIBUTIONS

Iain Scott, Bradley Webster and Michael Sack designed the studies. Iain Scott, Bradley Webster and Jian Li performed the studies and Iain Scott, Bradley Webster and Michael Sack prepared the paper.

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Chapter 3

Restricted mitochondrial protein acetylation initiates mitophagy

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SUMMARY

Mitophagy recycles redundant/dysfunctional mitochondria either selectively or as a component of macroautophagy (autophagy). As nutrient-sensing nuclear/cytosolic acetylation mediates autophagy, we investigated whether Sirt3/GCN5L1 regulated mitochondrial acetylation modulates mitophagy. GCN5L1 siRNA diminished mitochondrial protein acetylation and augmented mitochondrial enrichment of autophagy mediators LC3-II, p62 and ubiquitinylation, independent of macroautophagy. This program was disrupted by Sirt3 knockdown. Chronic GCN5L1 depletion increased the mitophagy rate and reduced mitochondrial protein content/mass. In parallel, GCN5L1 knockdown attenuated respiration with a concomitant increase in glycolysis and activation of mitochondrial biogenesis. Mitochondria also exhibited enhanced 'stress-resistance'. The genetic disruption of p62 and LC3-lipidation abolished, whereas the knockout of the mitochondrial E3-ubiquitin ligase Parkin did not impede, GCN5L1-depletion mitophagy. Together these data support that deacetylation of mitochondrial proteins initiate Sirt3-, Atg5- and p62-dependent but Parkin-independent mitophagy. Furthermore, the regulation of this nutrient-sensing mitochondrial protein acetylation program is sufficient to modulate mitophagy with ameliorative effects on mitochondrial homeostasis.

INTRODUCTION

Cellular macroautophagy controls the regulated turnover of cellular content and damaged organelles to both optimize cellular homeostasis and recycle intracellular energy reserves when external supplies are limited. Interestingly, organelle autophagy can function selectively or in concert with macroautophagy to remove and/or facilitate the turnover of damaged organelles. This regulated program is operational in mitochondria where mitophagy selectively removes mitochondria during erythrocyte maturation and in response to mitochondrial depolarization (Youle and Narendra, 2011). However, the role and contribution of mitophagy to ‘intracellular recycling’ under fasting and/or starvation conditions in mammalia has not been clearly delineated. In yeast the genetic disruption of the mitophagy program results in impaired clearance of mitochondria during prolonged starvation in parallel with the disruption in mitochondrial genomic fidelity (Kurihara et al., 2012).

In the context of nutrient limitation, cellular protein acetylation status plays an important role in adaptation to starvation and/or in response to caloric restriction (Hirschey et al., 2010; Someya et al., 2010). Moreover, the regulation of the acetylome regulates autophagy, and the genetic manipulation of nuclear and cytosolic acetyltransferase/deacetylase enzymes show that changes in acetylation status of autophagy mediators alters macroautophagy induction (Hamai and Codogno, 2012; Lee et al., 2008; Lee and Finkel, 2009; Yi et al., 2012). The nutrient-sensitive deacetylase sirtuin enzymes link the cellular response to starvation, the regulation of the acetylproteome and autophagy. Humans express seven sirtuin isoforms that localize to

various cellular compartments. Of note, the nuclear and cytosolic localized Sirt1 mediated deacetylation of various autophagy proteins is necessary for starvation induced macroautophagy (Lee et al., 2008).

Sirt3 is the major mediator of mitochondrial protein deacetylation (Lombard et al., 2007) and regulates mitochondrial metabolism, redox status and mitochondrial mediated cell death (Webster et al., 2012). Whether the intrinsic acetylation profile of mitochondrial proteins regulates the mitophagy program is uncertain. The tools to interrogate this program have been limited as overexpression of the mitochondrial deacetylase Sirt3 results in its ectopic expression (Bao et al., 2010) with concomitant deacetylation of mitochondrial, cytosolic and nuclear proteins (Lu et al., 2011; Shi et al., 2005; Sundaresan et al., 2008). Recently, GCN5L1 was identified as an essential component of the mitochondrial acetyltransferase program (Scott et al., 2012), and its genetic depletion resulted in the restricted acetylation of mitochondrial proteins. In this context, we interrogated mitochondrial protein acetylation biology to investigate whether mitochondrial-restricted acetylation may function as a ‘molecular signature’ of starvation or caloric restriction, to initiate mitophagy.

In this article we show that i) the absence of the mitochondrial deacetylase Sirt3 attenuates fasting induced mitophagy in-vivo; (ii) the genetic knockdown of GCN5L1 in HepG2 and mouse embryonic fibroblasts (MEFs) restricts acetylation of mitochondrial proteins and initiates the mitochondrial accumulation of autophagy mediators; iii) GCN5L1 knockdown initiated selective mitophagy is dependent on the autophagy mediators p62 and Atg5 but independent of Parkin, an E3-ligase shown to function in mitochondrial depolarization induced mitophagy (Narendra et al., 2008); iv) the

persistent activation of this mitophagy program results in mitochondrial dropout with diminution of oxidative metabolism and the upregulation of the mitochondrial biogenesis regulatory program; and v) the mitochondrial protein deacetylation program augments mitochondrial stress-resistance.

RESULTS

Mitochondrial protein deacetylation promotes mitochondrial accumulation of autophagy factors

To begin to explore whether fasting-induced mitophagy is dependent on mitochondrial protein deacetylation, we measured the mitochondrial enrichment of lipidated ATG8/LC3 (LC3-II), a canonical autophagy mediator recruited to mitochondria (Liu et al., 2012), in response to fasting in wildtype and Sirt3 knockout mice. In wildtype mice, fasting resulted in the accumulation of LC3-II to liver mitochondria in parallel with increased Sirt3 and reduced GCN5L1 levels (Figure 1A). In contrast, the level of mitochondrial-enriched LC3-II was not increased in mitochondrial deacetylation deficient Sirt3 knockout mice liver in response to fasting (Figure 1B and S1A). To evaluate whether the direct change in levels of mitochondrial acetylation regulatory enzymes could modulate the recruitment of autophagy mediators to the mitochondria, we assessed mitochondrial enrichment of LC3-II, p62 and concomitant mitochondrial protein ubiquitinylation in response to siRNA mediated knockdown of either GCN5L1 or Sirt3. Isolated mitochondria from GCN5L1-depleted HepG2 cells showed accumulation of LC3-II, p62 and protein ubiquitinylation (Figure 1C). In contrast, and in parallel to the lack of mitophagy induction in response to fasting in Sirt3 KO mice, Sirt3-depleted cell

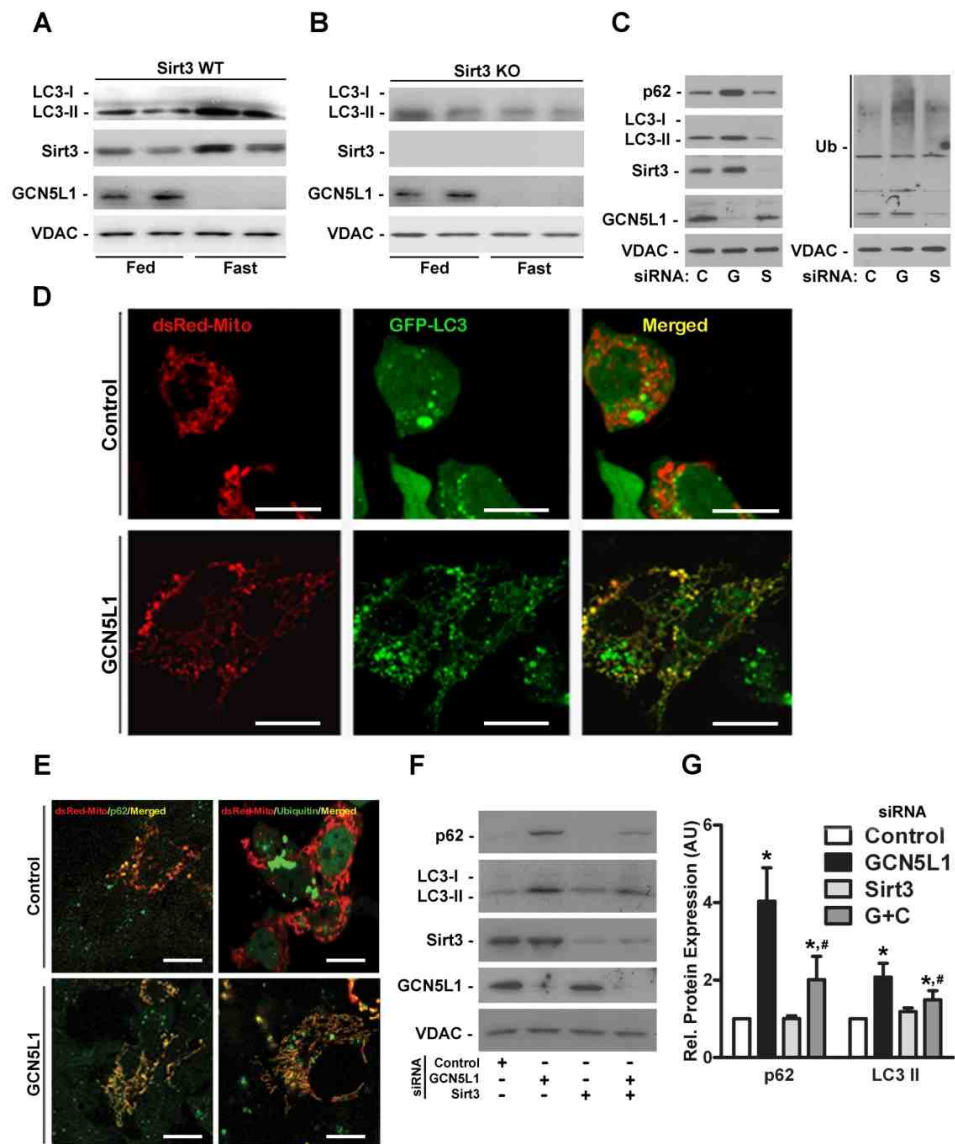
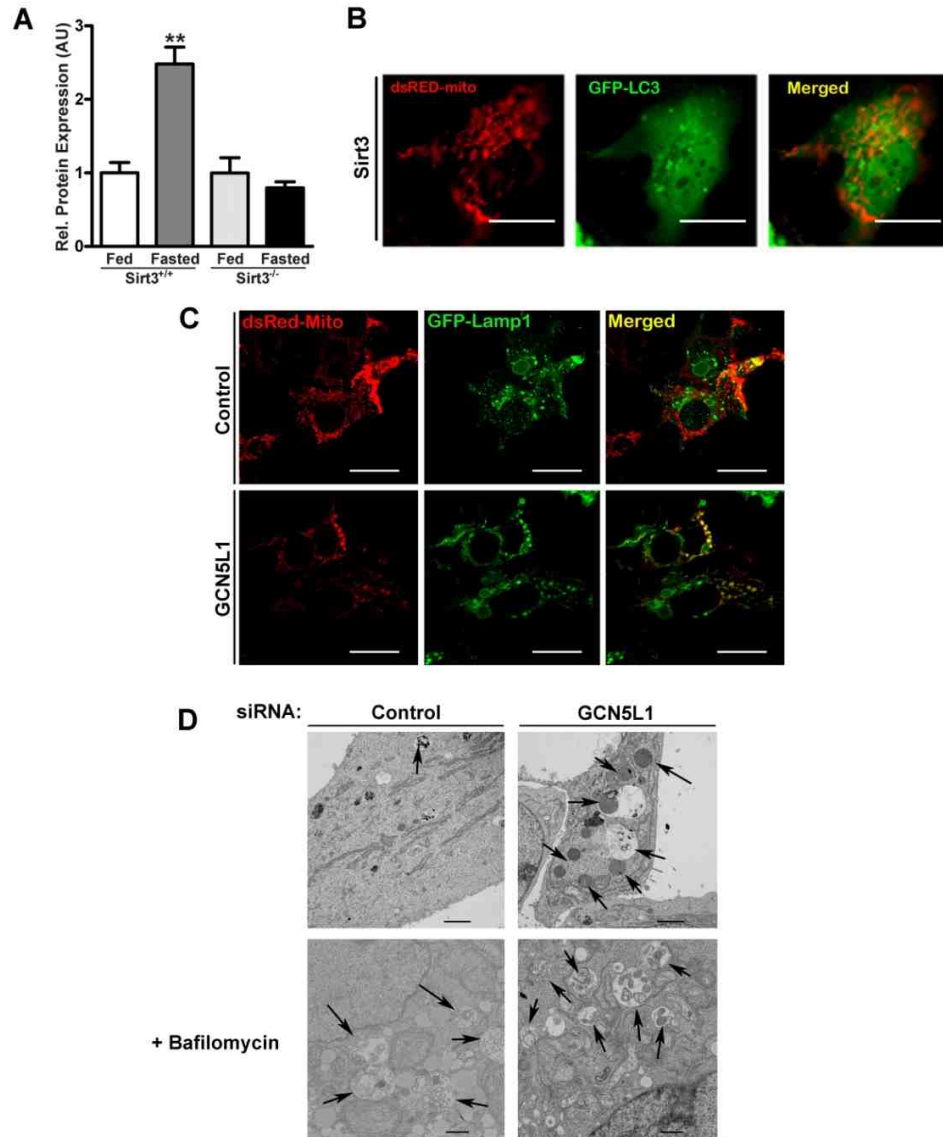


Figure 1. Depletion of GCN5L1 leads to accumulation of autophagy factors on mitochondria. (A-B) Hepatic mitochondria from Sirt3^{+/+} and ^{-/-} mice were analyzed from fed and fasted conditions. Western blotting was performed using antibodies against LC3, Sirt3 and GCN5L1 with VDAC as a loading control (n=4 animals per group). (C) Control and GCN5L1 siRNA HepG2 cell mitochondria were used in western blot analysis with antibodies directed against p62, LC3, Sirt3, GCN5L1 and ubiquitinylation with VDAC as a loading control. (D-E) Confocal microscopy was performed on control or GCN5L1 siRNA treated HepG2 cells. Representative images for co-localization of ds-Red labeled mitochondria with GFP-LC3 (D), GFP-ubiquitin or p62 (E). Scale bar, 10 μ m. (F-G) Representative western blot analysis of the effect of Sirt3 siRNA on GCN5L1 knockdown mediated mitochondrial accumulation of p62 and LC3-II in HepG2 cells. Protein levels were determined relative to VDAC. Control samples were normalized to 1 with Sirt3 and GCN5L1 siRNA treated samples determined relative to control values. Data are expressed as the mean \pm s.e.m. * $p < 0.05$ vs. scrambled siRNA control, # $p < 0.05$ vs. GCN5L1 siRNA levels. All experiments were repeated 3-8 times. AU, arbitrary units.



Supplemental Figure 1. Sirt3 depletion fails to induce mitophagy while GCN5L1 knockdown augments mitochondrial delivery to autophagolysosomes. (A) Quantification of LC3-II levels normalized to VDAC in isolated hepatic mitochondria from fed and 48 hour fasted Sirt3 WT/KO animals. Fed samples were normalized to 1 with protein levels from fasted animals presented relative to controls (n=4 animals per condition). (B) Representative images for co-localization of dsRed labeled mitochondria and GFP-LC3 in HepG2 cells treated with siRNA to Sirt3. Scale bar, 10 μ m. (C) Confocal microscopy of mitochondrial incorporation into the autolysosome via colocalization of dsRed-Mito and GFP-Lamp1 following siRNA to control or GCN5L1 in HepG2 cells. Scale bar, 10 μ m. (D) Electron microscopy of HepG2 cells treated with siRNA to control or GCN5L1. Arrows represent autophagic vacuoles and autolysosomes. To induce accumulation and slow degradation of cellular material in autophagosomes, bafilomycin (10 nM) was added (bottom images). Scale bar, 2 μ m (top) and 1 μ m (bottom). Data are expressed as the mean \pm s.e.m. with ** p<0.01. All experiments were repeated 3-5 times. AU, arbitrary units.

mitochondria showed similar levels of LC3-II, p62 and protein ubiquitinylation as the scrambled control siRNA cells (Figure 1C). To confirm the mitochondrial accumulation of these autophagy mediators, confocal microscopy was performed comparing scrambled control to GCN5L1 siRNA in HepG2 cells. Co-localization was increased between GFP-tagged LC3 and ds-red labeled mitochondria following the knockdown of GCN5L1 compared to scrambled control (Figure 1D), and this effect was not seen in Sirt3-depleted HepG2 cells (Figure S1B). In parallel, p62 and ubiquitin showed similar enhanced localization to mitochondria in response to the knockdown of GCN5L1 (Figure 1E). To determine the extent of mitochondria accumulation in lysosomes, we compared the localization of the GFP-Lamp1 and ds-red labeled mitochondria in scrambled control compared to GCN5L1 knockdown HepG2 cells. Confocal microscopy showed greater overlap of mitochondria with lysosomes following the genetic depletion of GCN5L1 compared to the scrambled control siRNA cells (Figure S1C). Electron micrographs mirrored this biology as siRNA directed against GCN5L1 resulted in the induction of autophagic vacuoles and autolysosomes compared to scrambled siRNA in HepG2 cells. Mitochondrial accumulation within autophagosomes was enhanced following treatment with bafilomycin, which prevents autophagic degradation, in GCN5L1-depleted cells compared to controls (Figure S1D). We previously demonstrated Sirt3 knockdown leads to augmented acetylation, which is blocked by the concurrent knockdown of Sirt3 and GCN5LI (Scott et al., 2012). We exploited this to validate whether GCN5L1-knockdown mediated mitophagy can be attenuated with simultaneous Sirt3 knockdown. We found that the mitochondria enrichment of p62 and LC3-II was blunted following the concurrent knockdown of GCN5L1 and Sirt3 (Figure 1F-G).

Acute GCN5L1 depletion enhances mitochondrial function without perturbing global autophagy or autophagosome-lysosomal flux

The acute induction of mitophagy has been proposed to function to maintain mitochondrial quality control. Previously, we have shown that acute siRNA knockdown of GCN5L1 enhances mitochondrial respiration and cellular ATP levels (Scott et al., 2012). To further interrogate this phenotype, we evaluated the effect of mitophagy on mitochondrial mass, protein content and on the mitochondrial capacity to generate reactive oxygen species. We found that transient GCN5L1 knockdown resulted in a modest reduction in the mitochondrial membrane potential without a change in the mitochondrial mass (Figures 2A and 2B). Also there was no change in levels of mitochondrial proteins including NDUFA9 and VDAC following the acute knockdown of GCN5L1 (Figure S2A). As this mitochondrial bioenergetic profile could be consistent with stress-resilience, we assessed the response to the generation of reactive oxygen species in response to rotenone administration (Zhou et al., 2011). siRNA knockdown of GCN5L1 blunted mitochondrial superoxide production (Figure 2C).

As GCN5L1 has also been shown to associate with lysosomes (Dell'Angelica, 2004), we assessed whether its knockdown altered global autophagy or if it induces 'selective' mitophagy, and whether this program modulates flux through the autophagosome-lysosomal catabolic pathway. To explore this, we evaluated the global autophagy signature in whole cell preparations following control or GCN5L1 siRNA. Phosphorylation of p70s6k, a signaling intermediate in autophagy, p62 and the ratio of LC3-I to LC3-II were not altered by the depletion of GCN5L1 in whole cell preparations

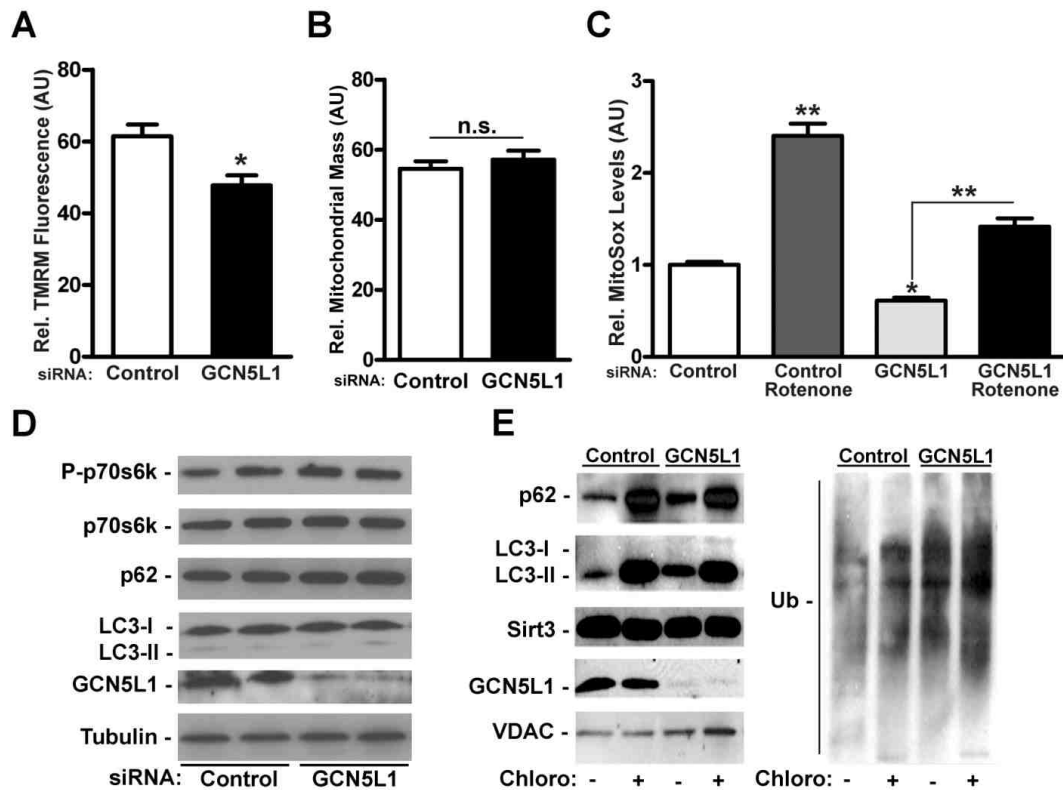
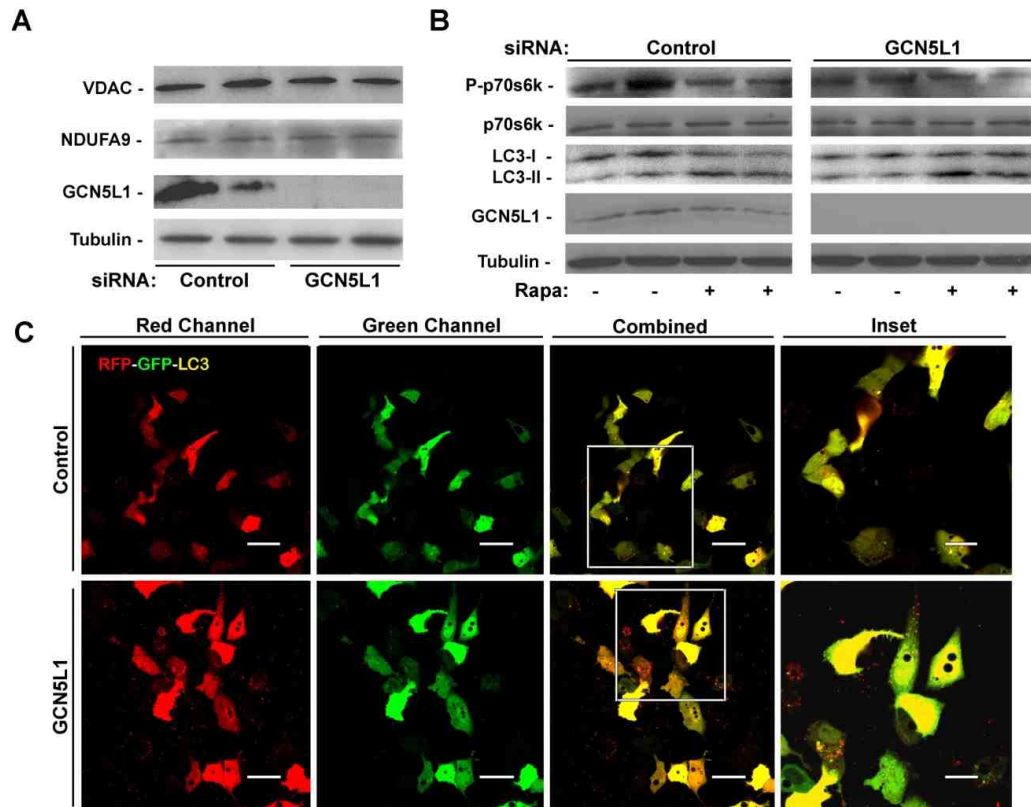


Figure 2. Acute GCN5L1 depletion enhances mitochondrial function without perturbing autophagy. (A) Relative mitochondrial membrane potential measured by cytometric analysis with TMRM (100 nM) fluorescence in scrambled control versus GCN5L1 siRNA in HepG2 cells. (B) Relative mitochondrial mass was measured by cytometric analysis using Mitotracker Green (100 nM) in scrambled control versus GCN5L1 siRNA treated HepG2 cells. (C) Measurement of relative mitochondrial superoxide generation under basal conditions and in response to the inhibition of complex I of the electron transfer chain by the administration of rotenone (15 μ M for 4 hrs) in control and GCN5L1 siRNA HepG2 cells as measured by MitoSox (5 μ M) fluorescence. Control levels were normalized to 1, and levels from rotenone and GCN5L1 knockout samples are relative to normalized controls. (D) Representative immunoblots to determine changes in whole cell autophagy in response to GCN5L1 siRNA by assessing dephosphorylation of p70s6k, the depletion of whole cell p62 or the appearance of LC3-II in HepG2 cells. (E) Assessment of the capacity for mitochondrial accumulation of autophagy mediators (autophagic flux) p62, LC3 and ubiquitinylation in control and GCN5L1 siRNA treated HepG2 cells in response to the inhibition of lysosomal function by chloroquine (60 μ M). Data are expressed as the mean \pm s.e.m. with $n \geq 3$ replicates for each flow cytometry study. * $p < 0.05$ vs. scrambled control studies and ** $p < 0.01$ vs. the respective control group; n.s., not significant. All experiments were repeated 3-7 times. AU, arbitrary units.



Supplemental Figure 2. Acute knockdown of GCN5L1 does not alter mitochondrial protein content or autophagy induction. (A) Mitochondrial proteins VDAC and NDUFA9 levels in whole cell lysates from HepG2 cells treated with control or GCN5L1 siRNA. (B) Representative immunoblot assaying autophagy induction following rapamycin (1 μ M for 24 hours) treatment in HepG2 cells. Autophagy induction was determined by LC3-II and phospho-p70s6k levels in whole cell lysates following GCN5L1 siRNA mediated knockdown. (C) Confocal microscopy of HepG2 cells treated with siRNA to control or GCN5L1 overexpressing dually-tagged RFP-GFP-LC3. Red punctae depict formation of autolysosomes. Scale bar, 10 μ m. All experiments were repeated 3 times.

(Figure 2D). The levels of p62 and LC3-II directly contrast the levels found in isolated mitochondria following GCN5L1 knockdown suggesting ‘selective’ mitophagy induction as opposed to macroautophagy. Furthermore, we confirmed that autophagy induction is intact following GCN5L1 siRNA, as evident by the similar augmentation of this program in response to rapamycin in control and knockdown cells (Figure S2B). Increased levels of autophagic markers could be due to increased induction or a block in autophagic flux leading to protein accumulation. To confirm that autophagosome-lysosomal flux is not impaired following GCN5L1 knockdown, mitochondria were isolated following chloroquine administration. As previously shown, baseline markers of mitophagy were induced in the GCN5L1 knockdown cells compared to controls, and the disruption of lysosomal acidification with chloroquine increased mitophagy markers in both control and GCN5L1 knockdown mitochondria suggesting intact flux (Figure 2E). To further assess whether autophagic flux is disrupted by GCN5L1 knockdown, dual RFP-GFP labeled LC3 was transfected into control and GCN5L1 siRNA treated HepG2 cells. The GFP fluorophore is more susceptible to lysosomal degradation such that RFP labeled punctae would signify successful LC3 delivery to the autolysosome and intact autophagic flux. Confocal microscopy confirmed red punctae formation in both control and GCN5L1 knockdown cells suggesting intact autophagic flux (Figure S2C).

Chronic GCN5L1 depletion diminishes mitochondrial mass and protein levels

As prolonged mitophagy reduces mitochondrial content (Kurihara et al., 2012), we investigated whether the stable knockdown of GCN5L1 using lentiviral shRNA infection replicates this phenotype. Stable depletion of GCN5L1 resulted in the robust

attenuation of mitochondrial protein acetylation while the autophagy markers LC3-II, p62 and protein ubiquitinylation were enriched in the mitochondrial fraction of shRNA infected HepG2 cells (Figure 3A). In contrast to transient GCN5L1-knockdown, the stable knockdown of GCN5L1 resulted in a reduction in mitochondrial mass (Figure 3B). Using whole cell extracts we additionally found that levels of mitochondrial proteins residing in the matrix and in the inner and outer mitochondrial membranes are reduced by $\approx 20 - 50 \%$ in the GCN5L1 shRNA knockdown cells compared to the scrambled shRNA control cells (Figures 3C and 3D). To evaluate whether these reductions in proteins levels are mediated in part via proteosomal or lysosomal degradation, we compared changes in protein levels of GDH and VDAC in response to the proteasome and lysosomal inhibitors MG132 and chloroquine, respectively. Interestingly, in both the control and GCN5L1 knockdown cells, chloroquine markedly increased steady state mitochondrial protein levels above vehicle treated controls with no significant augmentation in protein levels in the presence of the proteosomal inhibitor MG132, suggesting mitochondrial protein degradation is mediated by autophagy rather than the proteasome following GCN5L1 depletion (Figures 3E and 3F).

GCN5L1 shRNA mitophagy is dependent on Atg5 and p62 but independent of Parkin

As the mechanisms underpinning selective mitophagy are beginning to be delineated, we investigated whether canonical autophagy mediators known to be enriched on mitochondria undergoing mitophagy (Geisler et al., 2010; Liu et al., 2012; Mai et al., 2012) are required in response to GCN5L1 knockdown. To interrogate this, we

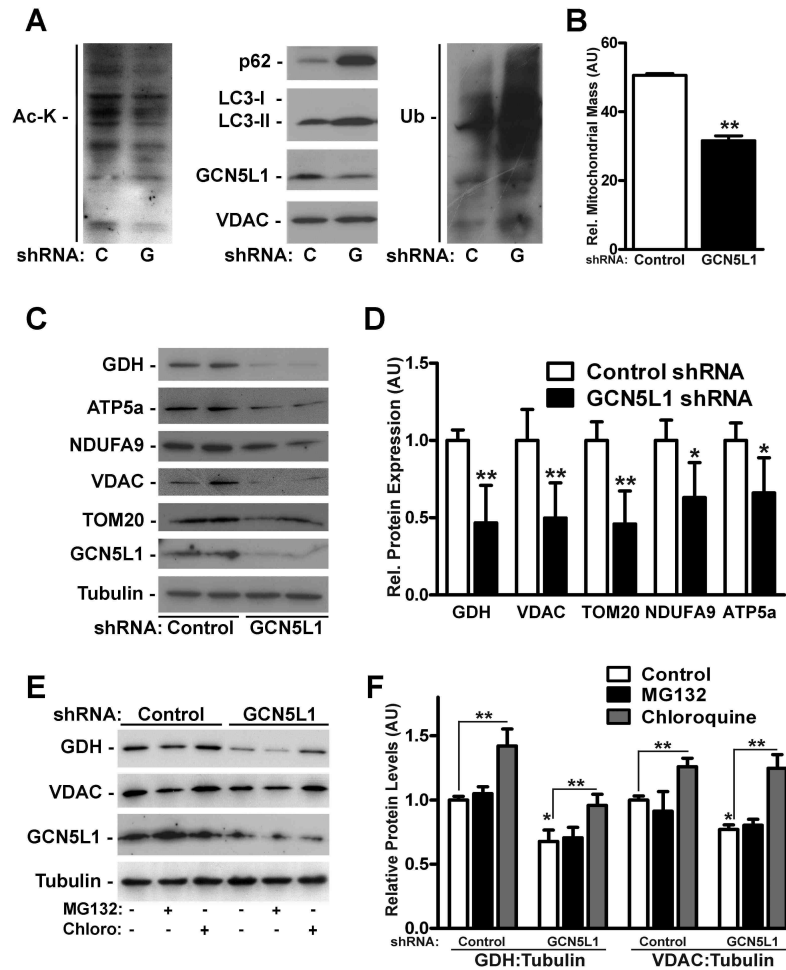
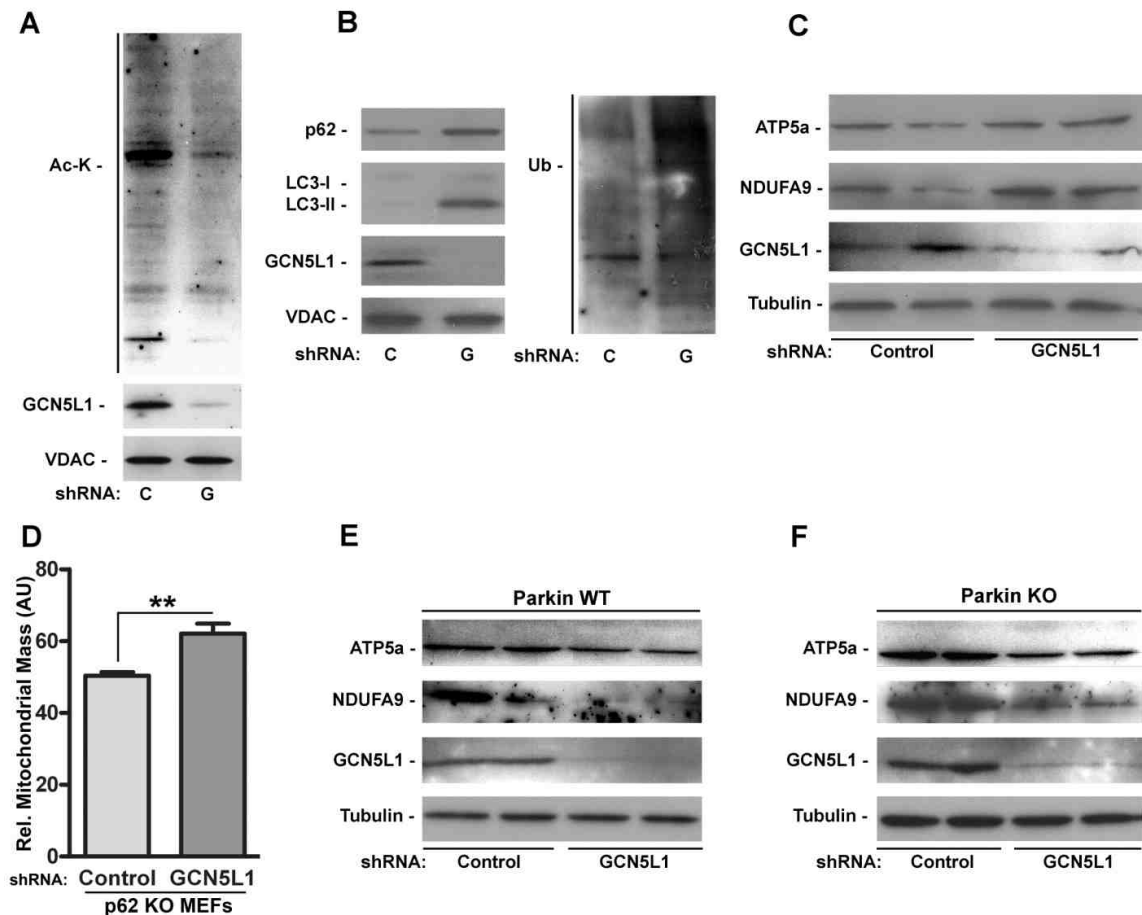


Figure 3. Chronic GCN5L1 depletion attenuates mitochondrial mass and protein levels via autophagic degradation. (A) Representative immunoblots showing mitochondrial protein acetylation and the accumulation of autophagy mediators p62, LC3 and ubiquitinylation in isolated mitochondria following chronic lentiviral shRNA knockdown of GCN5L1 compared to control vector infection in HepG2 cells. (B) Relative mitochondrial mass as measured by cytometric analysis of Mitotracker Green (100 nM) fluorescence in scrambled control versus GCN5L1 lentiviral shRNA infection in HepG2 cells. (C) Western blot analysis of whole cell levels of mitochondrial proteins glutamate dehydrogenase (GDH), ATP5a, NDUFA9 (complex I), VDAC and TOM20 following chronic lentiviral shRNA knockdown of GCN5L1 compared to control vector infection in HepG2 cells. (D) Quantification of mitochondrial protein levels relative to tubulin. Control shRNA samples were normalized to 1, and the GCN5L1 shRNA cell mitochondrial protein levels are represented relative to the control values. (E) Representative immunoblot showing the whole cell levels of mitochondrial proteins following chronic lentiviral shRNA knockdown of GCN5L1 compared to control vector infection in HepG2 cells in response to the proteosomal inhibitor MG132 (7.5 μ M) and lysosome inhibitor chloroquine (60 μ M). (F) Quantification of mitochondrial protein levels relative to tubulin. The control shRNA untreated samples were normalized to 1, and all other mitochondrial protein levels are represented relative to these control values. Data are expressed as the mean \pm s.e.m with $n \geq 3$ replicates for each flow cytometry study. * $p < 0.05$ versus the shRNA control group and ** $p < 0.01$ versus the scrambled or GCN5L1 control shRNA samples. All experiments were repeated 4-12 times. AU, arbitrary units.

employed lentiviral shRNA particles directed against GCN5L1 to generate stable knockdown of this mitochondrial acetyltransferase protein in wildtype MEF cells and in MEF lines deficient in the canonical autophagy/mitophagy mediators: p62, Atg5 and Parkin. As in the HepG2 cells, the stable depletion of GCN5L1 in wildtype MEF cells resulted in a reduction in mitochondrial protein acetylation and the mitochondrial accumulation of autophagy mediators (Figure S3A and S3B). Compared to wildtype MEF cells, the basal mitochondrial levels of p62 were elevated in Atg5 KO MEFs, although further accumulation of p62 was not evident following GCN5L1 knockdown in these cells (Figure 4A). As Atg5 facilitates the lipidation of LC3-I to generate LC3-II, it is not surprising the GCN5L1-depletion in Atg5 KO MEFs does not result in mitochondrial accumulation of LC3-II (Figure 4A). In p62 KO MEFs, neither LC3 species associated with mitochondria. This pattern was not altered by GCN5L1 knockdown (Figure 4A). The chaperone p62 has been shown to link ubiquitinated proteins to LC3-II. It is therefore not surprising that the Atg5 KO MEFs, which have an abundance of mitochondrial p62, show evidence of increased mitochondrial protein ubiquitinylation (Figure 4B). Under these conditions, the additional knockdown of GCN5L1 had no additional effect on mitochondrial protein ubiquitinylation. In stark contrast, GCN5L1 knockdown increased mitochondrial protein ubiquitinylation in both the control and p62 knockout MEF cells supporting that mitochondrial protein ubiquitinylation is upstream of p62 in mitophagy (Figure 4B).

As impaired mitophagy results in the accumulation of mitochondrial content (Kurihara et al., 2012), we compared the mitochondrial protein levels in whole cell preparations in the wildtype, Atg5 and p62 knockout MEFs. We show that both the



Supplemental Figure 3. GCN5L1 knockdown mediated mitophagy in MEF cells is dependent on p62 but not Parkin. (A and B) Representative western blots of isolated mitochondria from wildtype MEF cells following treatment with control or GCN5L1 lenti-viral shRNA. Blots probe for acetyl-lysine, p62, LC3 and ubiquitylation. (C and D) Whole cell lysates (C) and mitochondrial mass levels, as determined by Mitotracker Green (100 nM) cytometric analysis, from p62 knockout MEFs following control or GCN5L1 shRNA treatment. (E and F) Whole cell lysates from Parkin^{+/+} (E) and Parkin^{-/-} (F) MEFs to determine mitochondrial protein levels following GCN5L1 shRNA knockdown. Representative blots are depicted. Data are expressed as the mean \pm s.e.m. with $n \geq 3$ replicates for each flow cytometry. ** $p < 0.01$. All experiments were repeated 3-5 times. AU, arbitrary units.

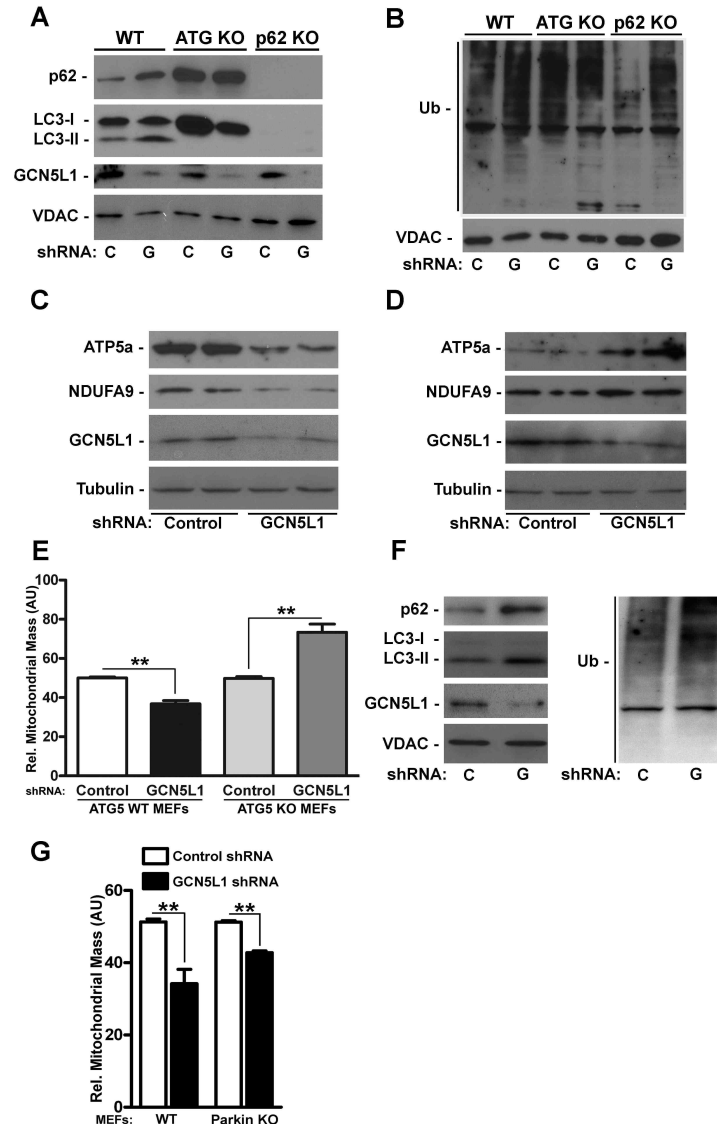


Figure 4. GCN5L1 depletion initiated mitophagy is dependent on Atg5 and p62 but not Parkin. (A-B) Representative mitochondrial protein immunoblots showing the extent of the accumulation of autophagy mediators p62, LC3 and ubiquitylation in isolated mitochondria from wildtype, Atg5 and p62 knockout MEF cells following lentiviral shRNA knockdown of GCN5L1 compared to the control vector infection. (C-D) Representative whole cell immunoblots showing levels of mitochondrial proteins ATP5a and NDUF9 in wildtype (C) and Atg5 knockout (D) MEF cells following lentiviral shRNA knockdown of GCN5L1 compared to control vector infection. (E) Relative mitochondrial mass as measured by cytometric analysis of Mitotracker Green (100 nM) fluorescence in control versus GCN5L1 shRNA lentiviral infection in the wildtype and Atg5 KO MEFs. (F) Representative immunoblots of isolated mitochondria showing autophagic protein levels in Parkin knockout MEF cells following lentiviral shRNA knockdown of GCN5L1 or control vector. (G) Relative mitochondrial mass as measured by cytometric analysis of Mitotracker Green (100 nM) fluorescence in control versus GCN5L1 shRNA lenti-viral infection in wildtype and Parkin KO MEFs. Data are expressed as the mean \pm s.e.m with $n \geq 3$ replicates for each flow cytometry study with $**p < 0.01$. All experiments were repeated 3-7 times. AU, arbitrary units.

Atg5 and p62 KO MEFs accumulate ATP5a and NDUFA9 protein levels in response to GCN5L1 knockdown compared to wildtype control MEFs (Figures 4C, 4D and S3C). In parallel the Atg5 and p62 KO MEF's also show increased mitochondrial protein mass following GCN5L1 shRNA infection compared to the wildtype control MEFs (Figures 4E and S3D).

Numerous E3-ubiquitin ligases have been identified that localize to the mitochondria (Narendra et al., 2008; Tang et al., 2011), and of these, Parkin has been the one most definitively linked to selective mitophagy in response to mitochondrial depolarization. To determine if Parkin is important in deacetylation-mediated mitophagy, we explored the mitochondrial enrichment of p62 and LC3-II in Parkin KO MEFs following GCN5L1 knockdown. In contrast to the results found in Atg5 and p62 KO MEFs, the mitochondrial accumulation of LC3-II and p62 was still evident in the absence of Parkin (Figure 4F). Additionally, knockdown of GCN5L1 in Parkin KO MEFs led to increased mitochondrial protein ubiquitinylation, suggesting an alternative E3 ligase involved in this form of mitophagy (Figure 4F). Parkin KO MEFs also showed a reduction in mitochondrial mass following GCN5L1 shRNA knockdown (Figure 4G). In parallel, the absence of Parkin did not impair the GCN5L1 shRNA-mediated reduction in mitochondrial protein levels (Figures S3E and S3F).

Chronic GCN5L1 knockdown increases mitophagic flux, reduces mitochondrial bioenergetic function and activates mitochondrial biogenesis

Finally, we began to explore the mitochondrial consequences of chronic GCN5L1 knockdown-induced mitophagy. We first explored whether persistent deacetylation of

mitochondrial proteins resulted in the continuous turnover of mitochondria via lysosomal hydrolysis. To quantify the flux of mitochondria through the autophagic degradation pathway, we used flow cytometry to measure the lysosomal acidic pH-dependent loss of the mitochondrial enriched pH-sensitive fluorescent protein mKeima (Katayama et al., 2011). Chronic depletion of GCN5L1 increased the percentage of cells undergoing mitophagy compared to scrambled shRNA HepG2 cells as seen with a change in fluorescence of the mKeima construct upon delivery to the acidic lysosome (Figures 5A and 5B). Interestingly, the addition of bafilomycin, to impair lysosomal acidification, did not alter the basal rate of mitophagy in the control shRNA HepG2 cells, although it did blunt this process in the GCN5L1 knockdown cells (Figures 5B and S4A). To further quantify autophagic flux, we employed flow-cytometry as a confirmatory assay to assess whether the dissipation GFP signal in the RFP-GFP-LC3 construct is similarly accelerated in GCN5L1 shRNA treated cells. We found that chronic GCN5L1 knockdown resulted in a significant reduction in GFP-LC3 signal compared to control shRNA cells (Figures 5C, S4B and S4C). Similar to the acute knockdown of GCN5L1 using siRNA, the shRNA mediated depletion of GCN5L1 led to a reduction in mitochondrial membrane potential (Figure S4D). As the dropout of mitochondria is likely to result in less reliance of oxidative phosphorylation for energy production, we measured basal and maximal rate of oxygen consumption and the rate of glycolysis in control and GCN5L1 depleted cells. Consistent with the 'loss' of mitochondrial content the GCN5L1-depleted cells showed a significantly lower rate of oxygen consumption and reciprocal increase in glycolysis (Figures 5D, 5E, S4E and S4F). We expected that the persistent knockdown of GCN5L1 may result in the progressive reduction in

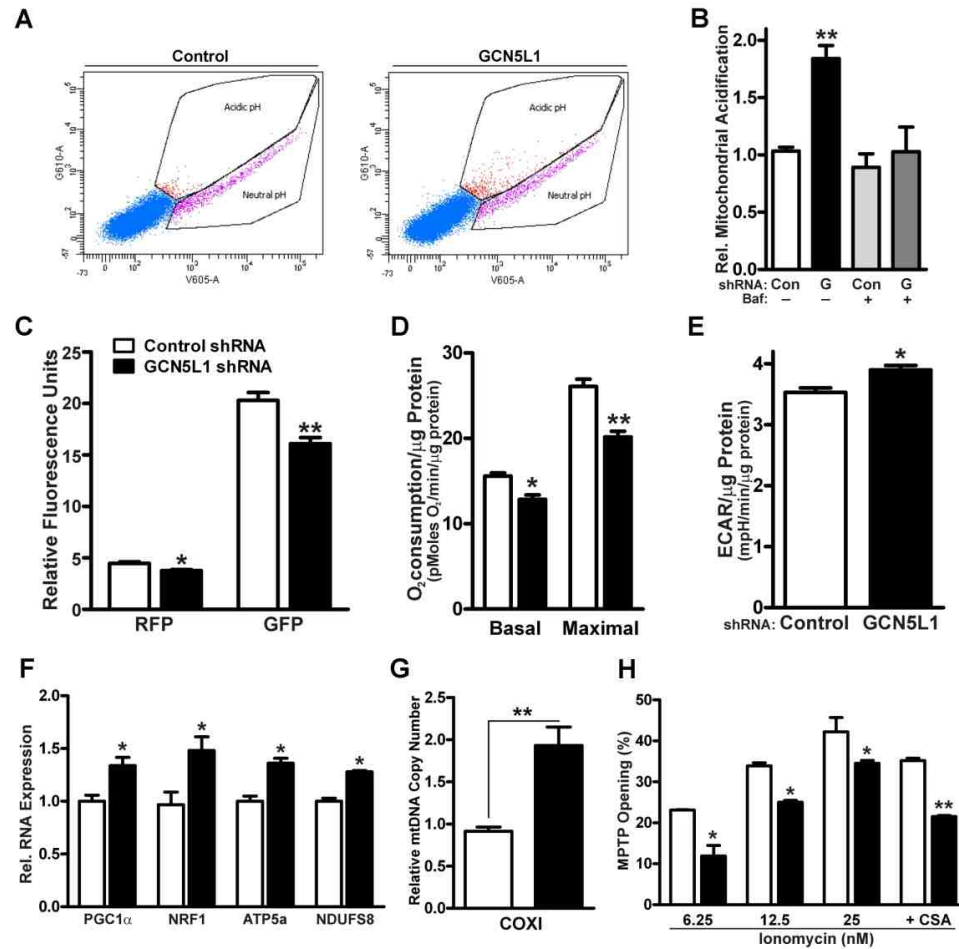
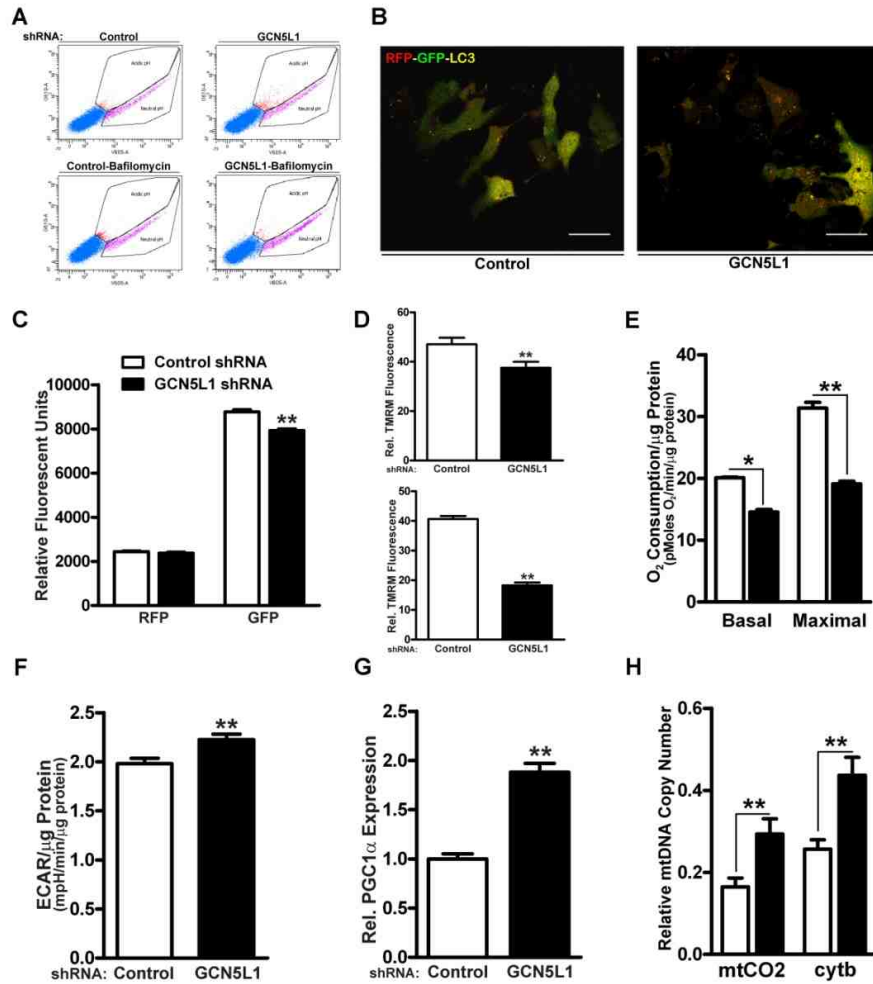


Figure 5. Chronic depletion of GCN5L1 augments mitophagic flux and alters mitochondrial function. (A) Representative cytometric scatter plots showing the relative acidification of the mitochondrial targeted Kiema (mKeima) protein in control and GCN5L1 shRNA infected HepG2 cells. (B) Quantification of mKeima acidification as a measure of mitophagy in control versus GCN5L1 shRNA infected HepG2 cells. Lysosomal acidification was inhibition by bafilomycin (10 nM). Untreated control values were normalized to 1 with treated samples expressed relative to controls. (C) Quantification of autophagy induction by cytometry using RFP-GFP-LC3 as a confirmatory measure of enhanced LC3 incorporation into lysosomes in response to the chronic depletion of GCN5L1 in HepG2 cells. (D-E) Representative Seahorse analysis of basal and maximal oxygen consumption using dinitrophenol (100 μ M) and the rate of glycolysis in response to chronic GCN5L1 depletion in HepG2 cells. (F) Transcript levels of genes encoding mitochondrial biogenesis regulatory proteins and nuclear encoded electron transfer proteins in response to chronic GCN5L1 depletion in HepG2 cells as measured by qPCR. Control values were normalized to 1 with GCN5L1 shRNA values expressed relative to controls. (G) Mitochondrial genomic copy number as measured by the relative content of the mitochondrial genomic encoded cytochrome oxidase I to nuclear-encoded 18S. (H) Assessment of the relative ionomycin (6.25, 12.5 and 25 nM) mediated mitochondrial permeability transition comparing control to GCN5L1 depleted cells. The addition of cyclosporin A (2 μ M for 30 min) is shown to attenuate pore transition in the presence of 25 nM ionomycin. Data are expressed as the mean \pm s.e.m with $n \geq 3$ replicates for each flow cytometry, Seahorse or qPCR study. * $p < 0.05$ and ** $p < 0.01$ vs. respective controls. All experiments were repeated 3-4 times.



Supplemental Figure 4. Chronic depletion of GCN5L1 augments mitophagy, alters mitochondrial respiration and induces mitochondrial biogenesis. (A) Representative FACs scatter plots of shRNA control and GCN5L1 treated HepG2 cells overexpressing mKeima also treated with and without bafilomycin (10 nM) to block lysosomal acidification. (B) Confocal microscopy image of HepG2 cells depleted of GCN5L1 with lentiviral shRNA and overexpressing RFP-GFP-LC3 to monitor autophagic flux. Scale bar – 10μm (C) Quantification of wildtype MEFs treated with control or GCN5L1 shRNA and overexpressing RFP-GFP-LC3. Fluorescence levels of RFP and GFP were determined by FACs. (D) Cytometric analysis with TMRM (100 nM) to assess relative mitochondrial membrane potential in scrambled control versus GCN5L1 shRNA treated MEF (top) and HepG2 (bottom) cells. (E and F) Seahorse analysis of wildtype MEF cells depleted of GCN5L1 with lentiviral shRNA. Basal and maximal respiration (E) and extra-cellular acidification rate (ECAR) (F) were determined. Figures are representative samples. (G) RT-PCR determination of PGC-1α transcript levels in wildtype MEFs treated with control or GCN5L1 shRNA. All reactions were normalized using an 18S endogenous control. Control samples were normalized to 1 with GCN5L1 samples expressed relative to controls. (H) Mitochondrial copy number levels of wildtype MEFs depleted of GCN5L1 following lenti-viral shRNA treatment. DNA copy number was normalized to nuclear encoded 18S. Data are expressed as the mean ± s.e.m. with n≥3 replicates for each flow cytometry, Seahorse or qPCR study. * p<0.05 and ** p<0.01 vs. controls. All experiments were repeated 3-4 times.

mitochondrial mass and oxidative metabolism with each subsequent passage of shRNA knockdown cells. However, it appeared that despite the increased mitophagic flux a lower steady-state mitochondrial content became established in the GCN5L1 knockdown cells (data not shown). To explore whether this new steady-state level resulted from a compensatory induction of the mitochondrial biogenesis program (McLeod et al., 2005), we explored transcript levels of transcriptional mediators of biogenesis, i.e. PGC-1 α and NRF-1, and mitochondrial protein transcripts that would be induced by biogenesis. We found that this regulatory program is indeed induced with a significant augmentation of transcripts for the biogenesis regulatory program (Figures 5F and S4G) and for electron transfer chain proteins ATP5a and NDUFS8 (Figure 5F). To confirm the induction of this program, we measured the mitochondrial genomic content and show that following chronic GCN5L1 knockdown, the relative mitochondrial genomic copy number was increased (Figure 5G and S4H). A higher rate of mitophagic flux may be expected to improve mitochondrial integrity (Kurihara et al., 2012). To explore this, we tested the susceptibility of control versus GCN5L1 shRNA cells to ionomycin-induced mitochondrial permeability transition. GCN5L1 depleted cells due showed greater resilience to permeability transition and required a larger dose of ionomycin to result in the same level of mitochondrial membrane permeability (Figure 5H).

DISCUSSION

Macroautophagy is an intricate program orchestrating cellular recycling and renovation, and autophagy is increasingly recognized as a regulated program to maintain

bioenergetic integrity during nutrient restricted conditions and as a cellular repair program in response to a wide array of biological stressors. Protein acetylation is also highly regulated under nutrient restricted conditions, and therefore it is not surprising that the enzymatic modification of the nuclear and cytosolic ‘acetylomes’ are now linked to the activation of canonical macroautophagy mediators (Lee et al., 2008; Lee and Finkel, 2009; Yi et al., 2012). At the same time, autophagy-specific degradation of cellular structural components and organelle recycling to sustain bioenergetic viability occur in an ordered manner, with the mitochondria being relatively spared during early autophagy given its importance in energy maintenance (Kristensen et al., 2008). As the mitochondrial acetylome is controlled by intrinsic molecular programs, we explored whether the direct modulation of the mitochondrial acetylome is sufficient to initiate and sustain the selective mitochondrial autophagy program. We show that the restricted acetylation of mitochondrial proteins via the genetic depletion of GCN5L1 results in the accumulation of canonical autophagy mediators on mitochondria, without the activation of the macroautophagy program. Moreover this program is dependent on autophagy mediators Atg5 and p62 and is independent of the known stress-induced mitophagy mediator – Parkin. The activation of this selective mitophagy is additionally dependent on the mitochondrial deacetylase Sirt3. Furthermore, we show that chronic mitochondrial protein deacetylation induced mitophagy reduces cellular mitochondrial content and cellular respiratory capacity with the concurrent induction of the reciprocal mitochondrial biogenesis regulatory program. In parallel, these mitochondria exhibit enhanced resilience to mitochondrial stressors.

The concept that the modulation of whole cell acetylation can modulate autophagy has been established using genetic and pharmacologic interventions (Eisenberg et al., 2009; Lee et al., 2008; Lee and Finkel, 2009; Morselli et al., 2011; Yi et al., 2012). Moreover, pharmacologic studies show a synergistic activation of autophagy by the dual administration of a deacetylase activator and an acetylase inhibitor (Morselli et al., 2011). In that study it was noted that numerous proteins residing in the inner and outer mitochondrial membrane and mitochondrial matrix undergo deacetylation as a component of the autophagy-associated modulation of the cellular acetylproteome (Morselli et al., 2011). However, the mechanism(s) and or signaling emanating from mitochondrial protein deacetylation that orchestrate the mitochondrial component of this cellular homeostatic program remain elusive. It is interesting to note though, in an unbiased siRNA library screen, that the depletion of the mitochondrial acetyltransferase protein GCN5L1 (also known as Bloc1s1) was found to modulate Parkin-overexpression mediated mitophagy (Orvedahl et al., 2011).

Mitochondrial localization to the autophagosome is thought to occur through two general mechanisms. The first mechanism results from the direct binding of mitochondrial localized ‘receptors,’ such as FUNDC1, NIX or BNIP3, to LC3-II on the autophagosome (Hanna et al., 2012; Liu et al., 2012; Schweers et al., 2007). The second proposed mechanism is that ubiquitinylation of various mitochondrial proteins by E3-ubiquitin ligases attract the binding of the chaperone p62, which then functions as an adaptor protein for LC3-II to target mitochondria to the autophagosome (15, 21). One possible component of mitophagy initiation by mitochondrial protein deacetylation is that this modification exposes lysine residues to facilitate ubiquitinylation (Glozak and Seto,

2009), a post-translation modification evident following GCN5L1 knockdown. As mentioned, numerous E3-ubiquitin ligases, in a stressor-specific manner, have been identified to modify mitochondrial proteins as a component of mitophagy (Liu et al., 2012; Narendra et al., 2008; Tang et al., 2011). The best characterized is Parkin, which, in response to robust mitochondrial depolarization, is recruited to ubiquitinylate outer mitochondrial membrane proteins (Narendra et al., 2008; Narendra et al., 2010; Suen et al., 2010). Although, we do see evidence of modest mitochondrial depolarization following GCN5L1 knockdown, it is probably insufficient to evoke Parkin-mediated mitophagy, and we find that the mitophagy effects of GCN5L1 depletion remain evident in Parkin null MEF cells. Which E3-ubiquitin ligase(s) is operational in response to the modulation of the mitochondrial acetylome has not been identified to date and will require further study.

Various pathological conditions are associated with concomitant perturbations in mitochondrial function and alterations in the mitochondrial acetylome. Examples include diseases associated with genetic defects in mitochondrial metabolic proteins (Wagner et al., 2012) and in response to nutrient-overload conditions such as high fat feeding (Kendrick et al., 2011). Whether, perturbations in the mitophagy program underpins or contributes to the pathophysiology of these diseases is an interesting hypothesis emanating from our findings, in that the modulation of the mitochondrial acetylome itself is sufficient to initiate selective mitophagy.

The emerging concept of epistatic control of the acetylome has recently been functionally delineated in skeletal muscle following the conditional knockout of Sirt1 (Philp et al., 2011). In that study, the expected attenuation of mitochondrial biogenesis in

the absence of Sirt1 was not evident. Rather, the counter-regulatory acetyltransferase GCN5 was concomitantly downregulated following the Sirt1 knockdown (Philp et al., 2011) suggesting regulatory integration between acetyltransferases and deacetylases in modulating the acetylome and cell function. With respect to autophagy, the same concept was shown with the counter-regulatory effects of Sirt1 and p300 and by the pharmacologic modulation of acetylation and deacetylation (Lee et al., 2008; Lee and Finkel, 2009; Morselli et al., 2011). Our initial fasting study in Sirt3^{+/+} and ^{-/-} mice, suggest that GCN5L1 and Sirt3 work in tandem to control mitophagy. Although GCN5L1 is robustly downregulated in the Sirt3^{-/-} mice in response to fasting, the accumulation of LC3-II to the mitochondria was not evident compared to Sirt3^{+/+} mice.

The role of mitophagy in the broader context of starvation or fasting-induced macroautophagy has also not been extensively explored. Data is emerging showing an important role of the mitochondrial outer membrane in autophagosome membrane biogenesis during amino acid deprivation (Hailey et al., 2010) and the recognition that mitochondrial morphology itself may play a role in modulating mitochondrial turnover during starvation (Gomes et al., 2011). The role of modulating the mitochondrial acetylome in this program requires direct study. Additionally, how mitochondrial and nuclear/cytosolic acetylomes integrates into broader macroautophagy pathways also needs to be explored.

The mitochondrial turnover studies show that chronic knockdown of GCN5L1 almost doubles the rate of mitophagy with a significant reduction in mitochondrial content and bioenergetic function and capacity. In parallel, this increase in the rate of mitophagy is sufficient to activate the mitochondrial biogenesis program and appears to improve

mitochondrial 'health'. Whether this increased mitochondrial turnover would impact pathologies known to be linked with mitochondrial dysfunction requires further study. Nevertheless, our findings expand the concept underpinning fine-tuning of the regulation of mitochondrial homeostatic programs to sustain the optimal mitochondrial content and functioning to most effectively maintain cellular homeostasis.

In conclusion, this study shows that the genetic modulation of the mitochondrial acetylome via the knockdown of the mitochondrial acetyltransferase protein GCN5L1 is sufficient to initiate selective mitophagy. We show that the acute induction of this program has modest ameliorative effects on mitochondrial biology, and chronic GCN5L1 knockdown results in the reduction of mitochondrial content with a concomitant activation of the mitochondrial biogenesis program. We find that this program is dependent on Sirt3 activity and on the canonical autophagy mediators Atg5 and p62 but is independent of Parkin (Figure 6). The manipulation of this program to enhance mitophagy may be a novel approach to ameliorate diseases associated with mitochondrial dysfunction and increased mitochondrial protein acetylation.

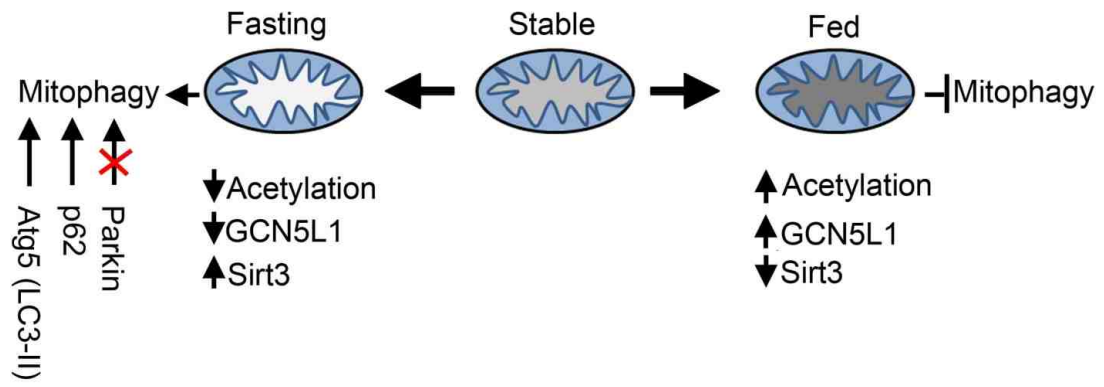


Figure 6. Proposed model for mitochondrial acetylome initiated mitophagy.

EXPERIMENTAL PROCEDURES

***In-vivo* mouse experiments**

3-4 month old littermate wildtype and knockout offspring of C57BL/6 SIRT3^{+/-} mice were used for fasting experiments where mice were either fed *ad-libitum* or fasted for 48 hrs. Mice were euthanized and liver mitochondria isolated via differential centrifugation (Lu et al., 2011). The Animal protocol was approved by the NHLBI Animal Care and Use Committee.

Cell studies

HepG2 cells were obtained from ATCC. MEFs were generated from day 13.5 embryos (Parkin2 and Sirt3^{+/+} and ^{-/-} mice) or obtained from other investigators (Atg5 and p62 knockout MEFs - see acknowledgements). HepG2 and MEF cells were maintained in DMEM (Gibco) supplemented with 10% or 15% FBS, respectively. For autophagy, lysosomal and proteosomal functional studies, chloroquine (60 μ M), rapamycin (1 μ M), bafilomycin (10 nM) or MG132 (7.5 μ M) was added to the culture medium for 24 hrs.

To perform immunoblot analyses, whole cell total protein samples and isolated mitochondria were obtained from cells using RIPA buffer (Pierce) or a Qiagen mitochondrial isolation kit. Samples were sonicated and quantified prior to running 4%–20% Tris-glycine gels (Invitrogen) and transferred onto nitrocellulose membranes (Invitrogen). Antibodies used for immunoblotting were purchased from Sigma (tubulin and LC3), Cell Signaling (ubiquitin, VDAC, acetyl-lysine (monoclonal), p70s6k,

phospho-p70s6k, human Sirt3), Abcam (complex V α , NDUFA9, GDH), Santa Cruz (Tom20), Progen Biotechnik (p62), or custom made by Covance (mouse Sirt3 and GCN5L1).

siRNA transfection was performed in HepG2 cells using scrambled or Smartpool siRNA targeting Sirt3 and GCN5L1 (Dharmacon) via electroporation (Lonza).

Lentiviral shRNA were generated in HEK293T cells. For each plate, 2.6 μ g of scrambled or GCN5L1 shRNA plasmid plus 26 μ L of Lentiviral Packaging Mix (Sigma) were transfected into HEK293T cells with Fugene 6 transfection reagent (Roche). The media was replaced 16 hrs after transfection. Media containing viral particles was harvested twice and combined. To remove the cell debris, media containing viral particles was centrifuged at 2,000 rpm for 10 min at 4°C. Supernatants were transferred to a fresh tube and subjected to centrifuge at 18,000 rpm for 3 hrs at 4 C. Supernatants were then carefully removed. Pellets were resuspended in 500 μ l of ice-cold PBS. Titer of viral particles was determined with a Lenti-X p24 Rapid titer kit (Clontech) and used to infect HepG2 and MEF cells in the presence of puromycin (2 μ g/mL).

Immunofluorescence studies were performed in HepG2 cells transfected with scrambled or GCN5L1 siRNA (Dharmacon). On day three fluorescent-tagged plasmids were transfected into HepG2 cells using FuGene HD (Roche) according to the manufacturer's instructions. Plasmids used were: dsRed-mito (Clontech), RFP-GFP-LC3 and GFP-Ubiquitin (Addgene), GFP-LC3 and RFP or GFP-Lamp1. Samples were viewed on a Zeiss LSM510 confocal microscope. For p62 colocalization studies, siRNA treatment proceeded for 48 hrs at which point dsRed-mito was transfected for 24 hrs.

Indirect immunofluorescence was performed after cell fixation using the p62 antibody (Progen Biotechnik) and Alexa Fluor 488 (Invitrogen).

Flow cytometry studies were performed to assess relative mitochondrial membrane potential, mass, superoxide production, the extent of mitophagy and mitochondrial permeability transition susceptibility. Mitochondrial membrane potential was measured using 100 nM TMRM (Invitrogen). Mitochondrial mass was determined using Mitotracker Green FM (Invitrogen). For mKeima or RFP-GFP-LC3 studies, stable HepG2 GCN5L1 or scrambled controls shRNA cells were transfected with the RFP-GFP-LC3 (or RFP-Lamp1/GFP-LC3 for compensation controls) or mKeima constructs using Fugene HD for 24 hours. For data analysis, geometric means for GFP or RFP signals were determined. For the mKeima studies, samples underwent cytometric analyses while excited simultaneously with a 407nm/605 nm (ex/em) (for neutral staining cells, V605) and at 532 nm/610 nm (ex/em) (for acidic staining cells, V610). The number of cells in each population (acidic or neutral) was determined, and the ratio of acidic labeled vs. total stained cells was compared between control and GCN5L1 shRNA infected cells. Mitochondrial permeability transition was performed using the MitoProbe Transition Pore Assay Kit (Molecular Probes). As a positive control cyclosporine A was used at 2 μ M for 30 min. For oxidative stress studies, HepG2 cells were treated with siRNA for 3 days at which point cells were treated with DMEM + rotenone (15 μ M) for 4 hours. To detect mitochondrial superoxide generation, MitoSox (Invitrogen) was used at 5 μ M for 30 min. Flow cytometry was performed on the FACSCaliber, LSR II Flow or FACSCanto flow instruments (BD Bioscience).

Electron microscopy was performed as previously described (Wu et al., 2009). Briefly, following control or GCN5L1 siRNA in HepG2 cells, electron micrographs were created with ultrathin sections of cells that were fixed in 2% glutaraldehyde/1% paraformaldehyde and post-fixed with 1% OsO₄. Samples were stained en bloc with 1% uranyl acetate and embedded in Em-bed 812 (Electron Microscopy Sciences). Sections were then imaged with a JEOL JEM1200EX transmission electron microscope with AMT 6 megapixel digital camera.

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were performed on the XF24 analyzer (Seahorse Bioscience) as previously described (Scott et al., 2012). HepG2 cells were seeded in 24-well plates overnight and incubated in unbuffered DMEM in a non-CO₂ incubator for 1 h before analysis. Oxygen consumption rate and extracellular acidification rate were automatically calculated by the Seahorse XF-24 software. The maximal oxidative capacity was calculated as the difference in oxygen consumption between dinitrophenol (100 μM) administration and the basal oxygen consumption rate. The metabolic flux analysis was repeated in triplicate and data normalized to protein content using a BCA assay (Thermo Fisher Scientific).

RT-PCR was performed to assess mitochondrial biogenesis. RNA was isolated from HepG2 and MEF cells using the RNeasy Mini Kit (Qiagen) following treatment with scrambled or GCN5L1 shRNA. RNA was transcribed to cDNA using SuperScript III First-Strand Synthesis Supermix (Invitrogen). RT-PCR was performed using primers purchased from predesigned primers (QuantiTect primer assays - Qiagen). All reactions were normalized using an 18S endogenous control. Mitochondrial copy number was

performed as previously described (Pagel-Langenickel et al., 2008; Pagel-Langenickel et al., 2007).

Statistics

Immunoblots were analyzed using ImageJ (NIH). Data are expressed as the mean \pm s.e.m. for the indicated number of experiments. 2-tailed Student t-tests were performed between groups and multiple comparison analysis was performed using ANOVA. $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental information includes four figures.

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Chapter 4 Discussion

Over the past few years lysine acetylation as a post-translational modification (PTM) has moved well beyond its initial site of discovery and function, namely nuclear histones as a mechanism for regulation of gene expression. Progress identifying non-histone acetylated proteins was slow, and prior to 2006, only 100 proteins were known to be acetylated. However, improvements in mass spectrometry have since identified > 2000 acetylated proteins [1-3]. This high number of protein targets rivals phosphorylation, the best characterized PTM, which suggests that acetylation may greatly affect cellular function. What has been perplexing in the field is the lack of enzymes to carry out this PTM with only 22 acetyltransferases and 18 deacetylases having been identified compared to hundreds of protein kinases [4].

Interest in protein acetylation intensified with the discovery of the NAD-dependent deacetylases known as sirtuins. Sirtuins were initially described in yeast and mediate the lifespan extending effects of caloric restriction (CR) [5]. CR activates sirtuins by increasing the NAD/NADH ratio. Humans express seven sirtuin isoforms, which are localized to various cellular compartments and mediate protein deacetylation (Sirt1, 2, 3 and 7), ADP-ribosylation (Sirt4 and 6) and desuccinylation/demalonylation (Sirt5). Sirt1 is the best characterized sirtuin, localizes to the nucleus and cytosol and regulates numerous cellular functions. Sirt1 regulates lipid and glucose metabolism, mitochondrial biogenesis, oxidative stress, autophagy and plays a role in numerous age-related pathologies. Other sirtuin enzymes have also been shown to greatly regulate cellular functions and some have been directly linked to longevity, namely Sirt3 and 6 [6-8]. Sirtuins may therefore link CR to longevity and offer to be a therapeutic target, using

such drugs as resveratrol, for 'healthy aging' as the population ages.

Interestingly, protein acetylation appears to greatly regulate mitochondrial function as ~20% of mitochondrial proteins are acetylated [1, 4]. Also of note, three of the seven sirtuins localize to the mitochondria (Sirt3, 4 and 5) with Sirt3 being the major mitochondrial deacetylase [9]. Sirt3 regulates nearly every aspect of mitochondrial function including oxidative stress, metabolism of lipids, glucose and proteins, apoptosis, oxidative phosphorylation, ketogenesis and may serve as a tumor suppressor [10]. Mitochondrial protein acetylation is also dependent on nutrient status as a high fat diet yields hyperacetylated mitochondria due to increased acetyl-CoA levels [11, 12]. Other diseases have been shown to have increased mitochondrial acetylation levels as well [13]. Conversely, increasing the NAD/NADH ratio, through starvation or increases in NAD synthesis, promotes mitochondrial protein deacetylation [14]. Therefore, given the central importance of mitochondria to cellular metabolism, apoptosis, oxidative stress, and aging interest in acetylation within the mitochondria has exploded in recent years.

The importance of protein acetylation within mitochondria can be attested to by the large number of proteins undergoing this PTM and the presence of a mitochondrial localized deacetylase, Sirt3. It should also be noted that in the nucleus and cytosol Sirt1 activity is opposed by the acetyltransferases p300, TIP60 and GCN5 [15]. It had been hypothesized that mitochondria contain an acetyltransferase enzyme that utilizes acetyl-CoA as a substrate to counter Sirt3, but no such enzyme had been described [16]. Within this context, we began investigating regulation of mitochondrial protein acetylation. The studies presented in this manuscript are the first characterization of such an acetyltransferase complex operating within mitochondria.

Criteria for identifying a potential mitochondrial acetyltransferase include proteins that 1) localize to the mitochondria; 2) have a potential acetyltransferase domain; and 3) counter known effects mediated by Sirt3 as Sirt3 knockdown has been shown to attenuate respiration [17]. As discussed in chapter 2, initial studies show that GCN5L1 and NAT9 fit the first two criteria, but only GCN5L1 knockdown augments mitochondrial respiration as would be expected by unopposed Sirt3 activity. Mitochondrial enrichment of GCN5L1 is further verified by confocal microscopy and immuno-EM. Interestingly, osmotic pressure analysis shows that GCN5L1 localizes to both the intermembrane space and the matrix, while Sirt3 is thought to localize only to the matrix [18]. Acetyl-CoA is generated in the matrix in part by pyruvate dehydrogenase so whether GCN5L1 mediates acetylation in the intermembrane space is not yet known. Also given the bacterial origins of mitochondria, it is interesting to note that GCN5L1's acetyl-CoA and substrate-binding sites have 53% homology with a prokaryotic acetyltransferase suggesting an evolutionary link.

To functionally show that GCN5L1 regulates mitochondrial acetylation status, we utilized multiple approaches namely knockdown or overexpression of GCN5L1 and show attenuated (*in-vivo* and *in-vitro*) and augmented (*in-vitro*) protein acetylation, respectively, and that GCN5L1 uses acetyl-CoA as a substrate. Known acetyltransferases function within protein complexes consisting of multiple subunits [19-22]. It appears that GCN5L1 also functions in combination with other subunits as GCN5L1 fails to increase histone acetylation *in-vitro* when histone H3 and GCN5L1 are incubated together. However, reconstitution of GCN5L1 with mitochondrial extracts depleted of endogenous GCN5L1 promotes histone H3 acetylation suggesting the presence of mitochondrial

factors that work in conjunction with GCN5L1. GCN5L1 working as an acetyltransferase complex makes logical sense as it is only ~15 kDa in molecular weight. Future work will need to identify the other mitochondrial proteins that work with GCN5L1. Returning to the prokaryotic nature of GCN5L1, it is interesting that a unique XAT hexapeptide-repeat region appears to be evolutionarily conserved amongst prokaryotic and eukaryotic acetyltransferases and may play a role in acetyl-CoA or substrate binding. Also of note and as mentioned in appendix 1, deletion of this XAT region blocks *in-vitro* mitochondrial protein acetylation and stops GCN5L1 mediated effects on oxygen consumption. The presence of such a domain may be a unique marker for acetyltransferases and links GCN5L1 to known acetyltransferases such as p300 and GCN5.

Further characterization of GCN5L1 activity was determined by exploring the hypothesis that it opposes Sirt3 activity. As such, Sirt3 has been shown to positively regulate ATP levels and mitochondrial respiration [17]. Therefore using the Sirt3 phenotype as a model, transient GCN5L1 depletion increases ATP levels and oxygen consumption on par with unopposed Sirt3 activity. As chapter 2 shows, GCN5L1 mediates acetylation of complex I protein NDUFA9 and complex V α , which are two known Sirt3 targets [17]. Also as predicted, Sirt3 knockdown attenuates ATP levels, oxygen consumption and increases protein acetylation, but the concurrent knockdown of Sirt3 and GCN5L1 blocks these effects. Therefore GCN5L1 and Sirt3 appear to counter each other's effects similar to that of Sirt1 and other known acetyltransferases [15].

Given the clear 'yin-yang' effects of Sirt3 and GCN5L1 on each other, it will be interesting to see if other known Sirt3 targets are counter regulated by GCN5L1. As

mentioned in chapter 1, Sirt3 regulates ketone body production and β -oxidation through deacetylation of HMG-CoA-2 and LCAD [23-25]. Therefore, it would be interesting to see if GCN5L1 regulates lipid metabolism and ketogenesis in a manner opposite to Sirt3. ROS generation is also attenuated by Sirt3 through deacetylation of both IDH and MnSOD, and this attenuated ROS slows age-related hearing loss [26-28]. Interestingly, as shown in chapter 3, siRNA knockdown of GCN5L1 attenuates mitochondrial ROS levels at baseline and following stress with rotenone. This data was shown in the context that increased mitophagy (see below) may improve mitochondrial health, but this may be in combination with increased activation of MnSOD and IDH, which future work will have to determine if these are targets of GCN5L1 as well. Following this same trend, chapter 3 also shows diminished MPTP opening with GCN5L1 depletion upon exposure to ionomycin. Again this finding may be due to improved mitochondrial health due to mitophagy or it may be due to decreased acetylation of cyclophilin D, which is a target of Sirt3. Therefore, along with finding the functional acetyltransferase components that bind to and work with GCN5L1, future work will have to determine the acetylation targets of GCN5L1 to see if they correlate with known Sirt3 targets such as MnSOD, cyclophilin D, HMG-CoA-2 and LCAD. It will be interesting to see if altering GCN5L1 activity can counter mitochondrial hyperacetylation seen in numerous pathologies such as Freidrich's ataxia [13].

CR restriction elicits numerous cellular responses including changes in metabolism, oxidative stress handling, mitochondrial function and housekeeping programs. Key amongst these housekeeping programs induced by CR is the process known as autophagy [29]. Autophagy allows for cellular recycling of damaged proteins

and organelles and allows the cell to endure periods of nutrient limitations. Therefore, autophagy is thought to be essential for CR mediated increases in lifespan [30]. The nutrient sensing enzymes AMPK and Sirt1 have been shown to increase autophagy through phosphorylation of ULK1/ATG1 and deacetylation of the canonical autophagy mediators ATG5, 7 and 8, respectively. This is in concert with diminished activity of the amino acid sensing enzyme mTOR. Direct induction of autophagy with the mTOR inhibitor rapamycin increases lifespan in aged mice [31]. In addition, acetylation is a major PTM of the autophagy proteome as seen by treatment with the acetyltransferase inhibitor spermidine and sirtuin activator resveratrol with both drugs inducing autophagy and extending lifespan in yeast, flies and worms [32, 33]. Both drugs altered the acetylation status of 375 proteins and 170 (with 27 being mitochondrial) of those being implicated in the autophagy proteome [32-34]. Therefore, it appears that CR, sirtuins, acetylation and autophagy are linked. Given that cytosolic acetylation status can trigger global autophagy and 27 mitochondrial proteins are both differentially acetylated and a part of the autophagy proteome, we hypothesized that mitochondrial protein acetylation status may alter mitochondrial autophagy or 'mitophagy.'

Isolated liver mitochondria from fasted mice show attenuated GCN5L1 levels with increased LC3-II levels suggesting increased mitophagy. Interestingly, in Sirt3 KO animals LC3-II levels are not elevated with fasting. To mimic this effect in cell culture, knockdown of GCN5L1 greatly triggers mitophagy with augmented p62, LC3-II and ubiquitinylation being seen in isolated mitochondria and co-localized to mitochondria. The dual knockdown of GCN5L1 and Sirt3 blocked this mitophagy induction. Electron microscopy shows augmented autophagosome formation with GCN5L1 depletion while

confocal microscopy suggests increased delivery of mitochondria to lysosomes. Overall, this suggests that mitochondrial deacetylation triggers mitophagy and may be dependent on nutritional status. In fact, two other studies have shown in unbiased screens that GCN5L1 plays a role in autophagy and Parkin mediated mitophagy [35, 36].

Furthermore, it will be interesting to see if other tissues besides the liver show similar mitophagy induction and decreased expression of GCN5L1 following fasting.

Preliminary evidence suggests that the brain does not have attenuated GCN5L1 expression *in-vivo* following 48 hours of fasting compared to the liver (Figure 1). It will be interesting to see if tissues such as the heart or skeletal muscle show GCN5L1 depletion following fasting.

It is important to note that this mitochondrial phenotype differs between acute versus chronic GCN5L1 depletion. siRNA mediated knockdown of GCN5L1 triggers mitophagy, increases mitochondrial respiration (as described in chapter 2) and fails to attenuate mitochondrial mass while chronic depletion of GC5L1 with lentiviral shRNA triggers mitophagy leading to diminished mitochondrial respiration and mitochondrial mass. We hypothesize that acute knockdown of GCN5L1 preferentially eliminates damaged mitochondria and mimics a short term fasting condition possibly through Sirt3 deacetylation of electron transport chain proteins. As mentioned, ROS generation is also attenuated with siRNA knockdown of GCN5L1, which could be a combination of Sirt3 deacetylation of MnSOD and IDH and increased mitophagy of damaged mitochondria. It should also be noted that mitochondria are one of the last organelles to be eliminated during fasting induced autophagy as mitochondria become hyper-fused during fasting and fission is necessary for mitophagy [37-39]. Chronic GCN5L1 mimics long term fasting

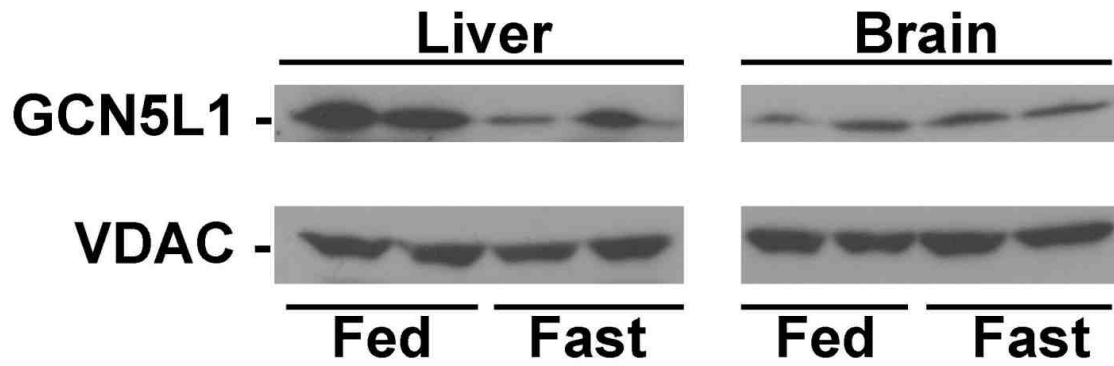


Figure 1: Fasting attenuates GCN5L1 protein levels in liver but not the brain. Isolated liver and brain mitochondria from 48 hour fasted mice show selective attenuation of GCN5L1 in liver samples while brain mitochondria retain GCN5L1 expression.

with mitochondrial mass, protein content and respiration being attenuated. It will be intriguing to evaluate whether mitochondrial fusion and fission correlate with the length of GCN5L1 knockdown with longer periods of GCN5L1 depletion triggering mitochondrial fission as a component of the mitophagy program.

Extensive work described in chapter 3 shows that GCN5L1 depletion leads to selective mitophagy without global autophagy induction or disruption in autophagic flux. Our data also show that mitochondrial protein degradation is mediated by the autophagy pathway but not the proteasome. We also show that p62 and ATG5 are essential components of this mitophagy program. However, the ubiquitin E3-ligase Parkin is non-essential. As mentioned, Parkin mediated mitophagy is the most extensively investigated form of mitophagy. However, other E3 ligases such as Mulan, March5 and RNF185 regulate mitochondrial morphology and mitophagy [40-42]. Therefore, further work will need to determine if a cytosolic or mitochondrial localized E3 ligase is involved in GCN5L1-mediated mitophagy. Further work is also needed to determine how global mitochondrial protein deacetylation signals for mitochondrial protein ubiquitinylation with subsequent mitophagy. However, given the overall robustness of this form of mitophagy with cells perpetually undergoing mitochondrial degradation (as opposed to the artificial system of CCCP/Parkin mediated mitophagy), this system should prove to be a useful tool in determining other proteins needed for mitophagy such as other autophagy proteins like Beclin, HDAC6 and ULK1.

It is believed that aging leads to diminished autophagy and mitophagy leading to the accumulation of dysfunctional mitochondria in part due to accumulation of lipofuscin in lysosomes [43]. CR is able to induce mitochondrial biogenesis while also lowering

mitochondrial membrane potential, respiration and ROS production, which is identical to chronic GCN5L1 knockdown [44]. Therefore, CR induces both autophagy and mitochondrial biogenesis and overall mitochondrial turnover in part through Sirt1 activation and deacetylation of both autophagy proteins (ATG5, 7 and 8) and the master regulator of mitochondrial biogenesis, PGC-1 α [45, 46]. However, it has not been shown that mitophagy induction augments mitochondrial biogenesis leading to mitochondrial turnover. Parkin-mediated mitophagy, following CCCP treatment, is hypothesized to cause turnover since Parkin ubiquitinylates and increases degradation of the PGC-1 α inhibitor protein, Paris [47]. Yet, no study to date has shown that both arms of mitochondrial turnover are simultaneously triggered by Parkin activity. The work presented in this manuscript suggests that GCN5L1 knockdown induces both mitochondrial mitophagy and biogenesis and thus turnover. GCN5L1 knockdown increases mitophagy and also concurrently elevates PGC-1 α and NRF-1 with increased mtDNA copy numbers in HepG2 and MEFs. Recent work in the lab confirms mitochondrial turnover in HepG2 cells depleted of GCN5L1 (Figure 2). The functional consequences of such increased turnover are only hypothetical, but overall mitochondrial health may be improved as seen with diminished MPTP opening upon ionomycin treatment in these cells. It will be interesting to see if the increased mitochondrial turnover shown in this model has applications to pathologies with known mitochondrial defects such as diabetes, obesity and neurodegenerative disorders (Alzheimer's, ALS and Parkinson's).

Highlighting GCN5L1's importance, attempts by our lab and others have confirmed that GCN5L1 knockout is embryonic lethal. However, we have been

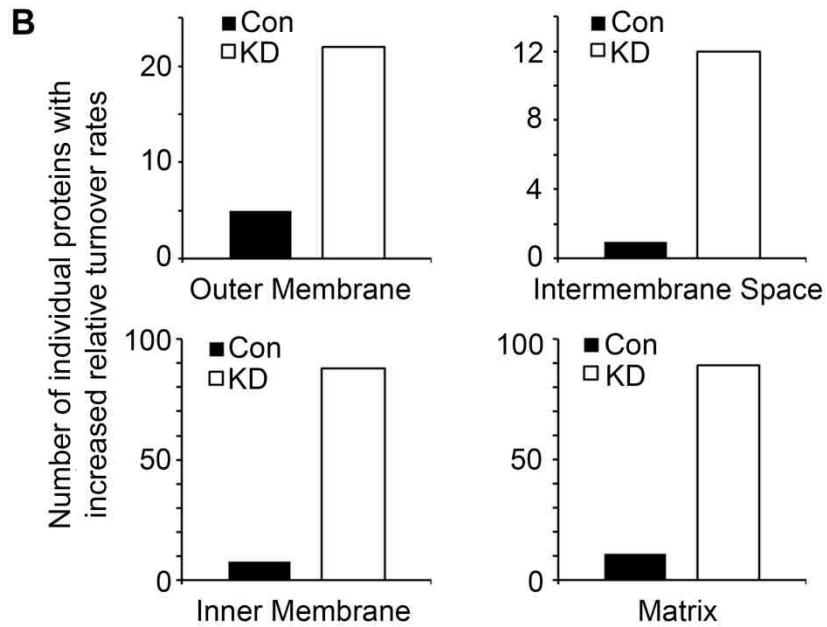
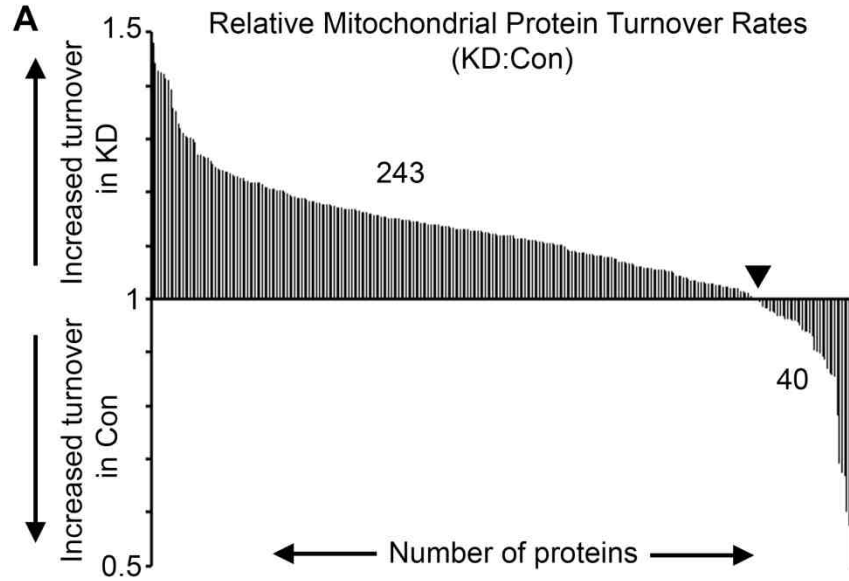


Figure 2: Stable isotope labeling by amino acids (SILAC) in HepG2 cells show that GCN5L1 shRNA treatment leads to increased turnover of mitochondrial proteins compared to control shRNA treated cells. Turnover is greater in GCN5L1 knockdown cells in all mitochondrial compartments suggesting whole mitochondrial mitophagy with subsequent mitochondrial biogenesis.

able to generate GCN5L1 KO MEFs. Work with these cells has confirmed and strengthened our previous findings ruling out any off targets that GCN5L1 siRNA or shRNA might be silencing. As expected, compared to wildtype cells, isolated mitochondria from GCN5L1 KO MEFs show increased levels of mitophagy markers (p62, ubiquitin and LC3-II) (Figure 3A) and selectively attenuates mitochondrial but not cytosolic acetylation levels (Figure 3B). Importantly, reconstitution of GCN5L1 into these KO MEFs using a plasmid encoding Flag-tagged GCN5L1 attenuates the accumulation of mitophagy markers on mitochondria while increasing mitochondrial protein acetylation (Figure 3C-D). Furthermore, electron microscopy of these cells shows increased levels of mitochondria in and being engulfed by autophagosomes (Figure 4).

As mentioned, nutritional status affects mitochondrial fission and fusion with short term fasting causing mitochondrial fusion, which is thought to prevent mitophagy. Longer fasting induces mitophagy, and it is proposed that mitochondrial fission is essential for this process [36-38]. Therefore, we would hypothesize with either chronic GCN5L1 depletion or in the GCN5L1 KO MEFs that mitochondrial fission would be elevated promoting mitophagy. We further hypothesize that the mitochondrial fission program is essential for GCN5L1 regulated mitophagy. Preliminary evidence in GCN5L1 knockout cells show increased fragmented mitochondria using electron (Figure 4) and confocal microscopy (Figure 5). Future studies will have to determine the status of the fission/fusion program in these cells as well as if GCN5L1 expression correlates with the induction of either program.

Recently, the transcription factor Transcription Factor EB (TFEB) has been shown to be the master regulator of autophagy with overexpression of TFEB resulting in

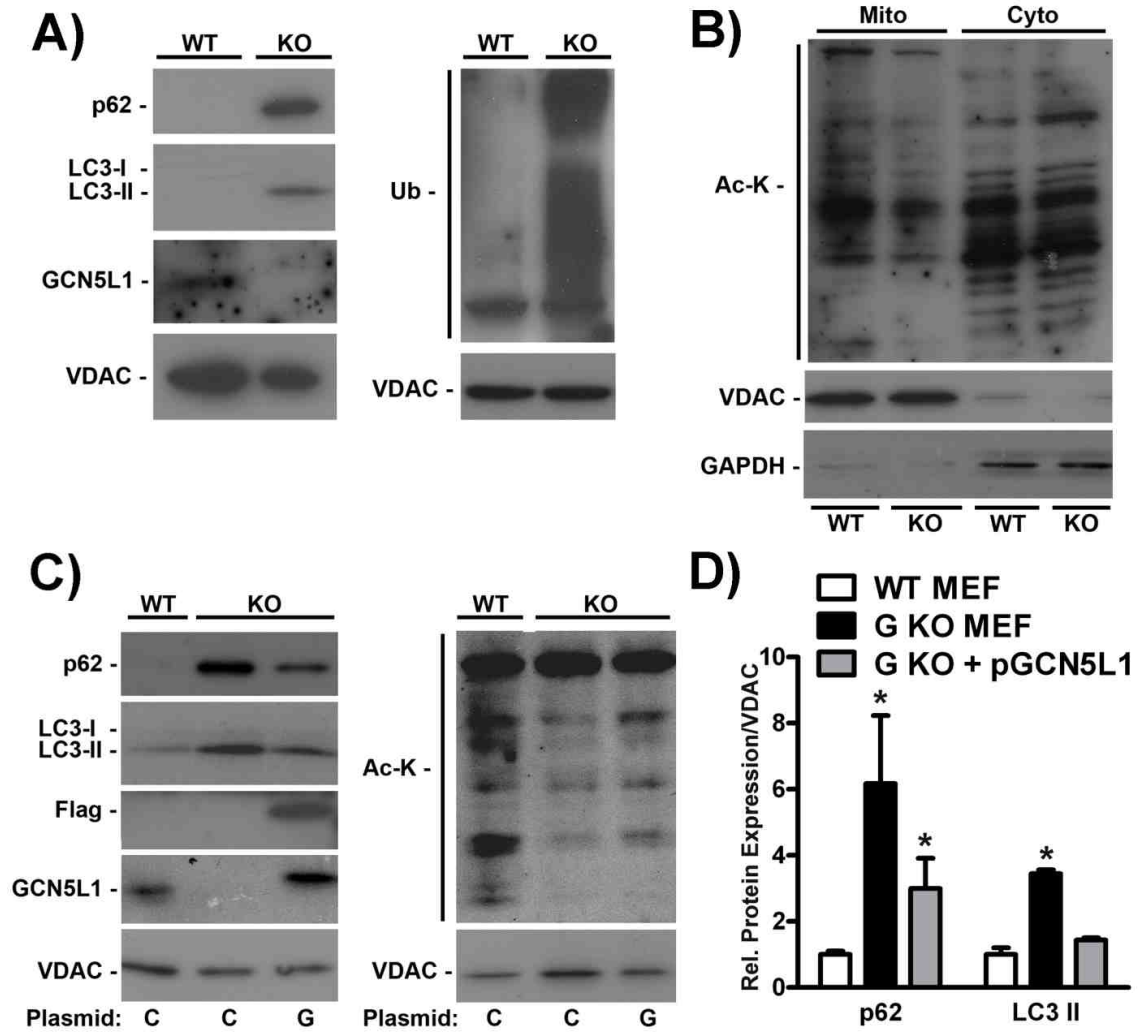


Figure 3: GCN5L1 knockout MEFs show increased mitophagy, which is rescued by reconstitution. (A) Isolated mitochondria from GCN5L1 KO MEFs show elevated p62, LC3-II and protein ubiquitylation. (B) Protein acetylation is selectively attenuated in mitochondria from GC5NL1 KO MEFs with no change found in the cytosolic fraction. (C and D) Reconstitution of Flag-GCN5L1 attenuates mitophagy induction and hypoacetylation in GCN5L1 knockout cells.

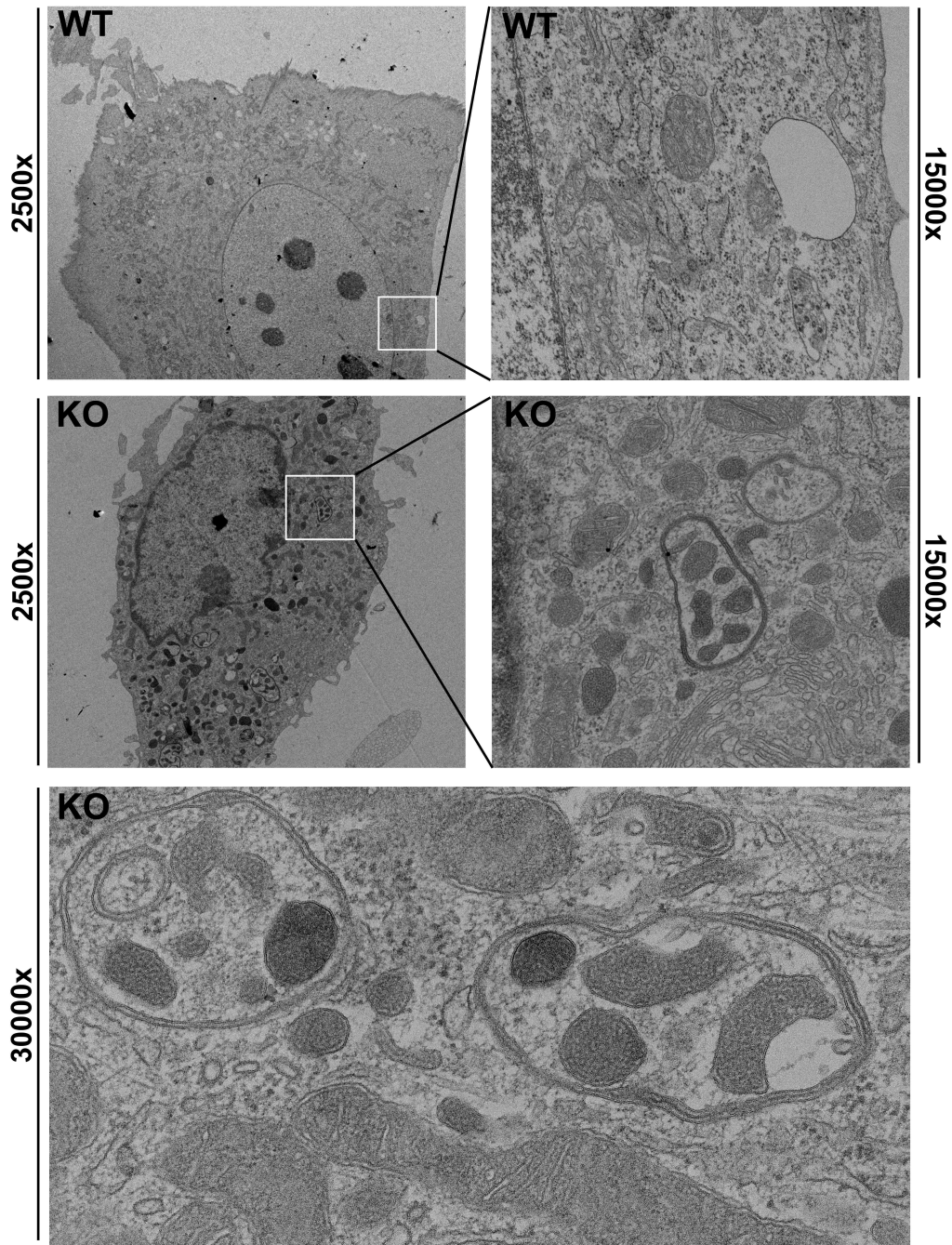


Figure 4: GCN5L1 knockout MEF electron microscopy (EM). EM from GCN5L1 knockout MEFs show augmented mitochondrial accumulation in double-membrane autophagosome and autophagic features compared to wildtype cells. Also mitochondria appear more fragmented with GCN5L1 knockdown.

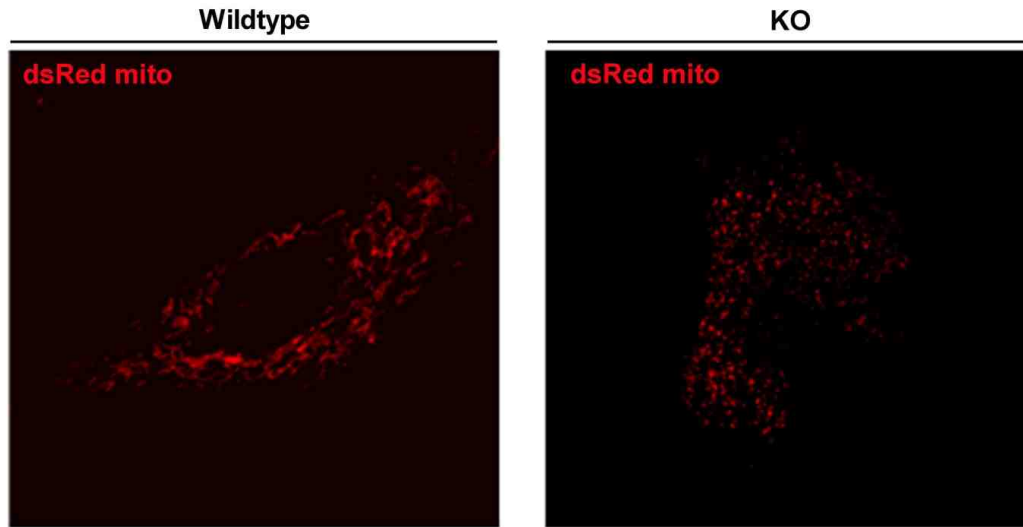


Figure 5: GCN5L1 knockout MEFs may have increased mitochondrial fission. dsRED-mito labeled mitochondria from GCN5L1 knockout MEFs appear more fragmented than wildtype cell mitochondria.

the induction of numerous autophagy genes and lysosomal biogenesis. Furthermore, starvation results in the translocation of TFEB from the cytosol to the nucleus with subsequent autophagy induction, which is dependent on the inhibition of TFEB phosphorylation by mTOR and ERK2, thus linking TFEB activity with nutrient status [48, 49]. Interestingly, PGC-1 α overexpression in a mouse model of Huntington's disease alleviates mutant htt protein aggregation, a hallmark of the disease. It was also shown that PGC-1 α acts upstream of TFEB, and TFEB overexpression alone diminished htt protein aggregation [50]. Also of note is that TFEB has been shown to directly target GCN5L1 expression [51]. As mentioned, GCN5L1 shRNA increases PGC-1 α transcript levels by ~2-fold. In GCN5L1 KO MEFs, PGC-1 α transcript levels are elevated ~12-fold compared to wildtype cells, and reconstitution of GCN5L1 into the KO MEFs attenuates the PGC-1 α transcript levels (Figure 6a). Also in GCN5L1 KO MEFs the expression of TFEB is elevated compared to wildtype cells (Figure 6b). Furthermore, in GCN5L1 KO MEFs GFP-TFEB shows greater nuclear localization compared to WT cells (Figure 6c and 6d). Therefore, we hypothesize that GCN5L1 and nutritional status play a role in this "PGC-1 α /TFEB axis" (Figure 6e). With nutrient withdrawal, GCN5L1 expression is attenuated (in the liver) along with mTOR and ERK2 activity. This leads to the activation of PGC-1 α and promotes TFEB nuclear translocation. PGC-1 α then induces both mitochondrial biogenesis along with TFEB expression therefore increasing GCN5L1 and autophagy gene induction along with mitophagy, effectively creating a positive feedback loop. Our preliminary results suggest that PGC-1 α and TFEB activity are elevated with GCN5L1 depletion. Future work will have to establish the importance of these transcription factors in GCN5L1 mediated acetylation and mitophagy. It will also be

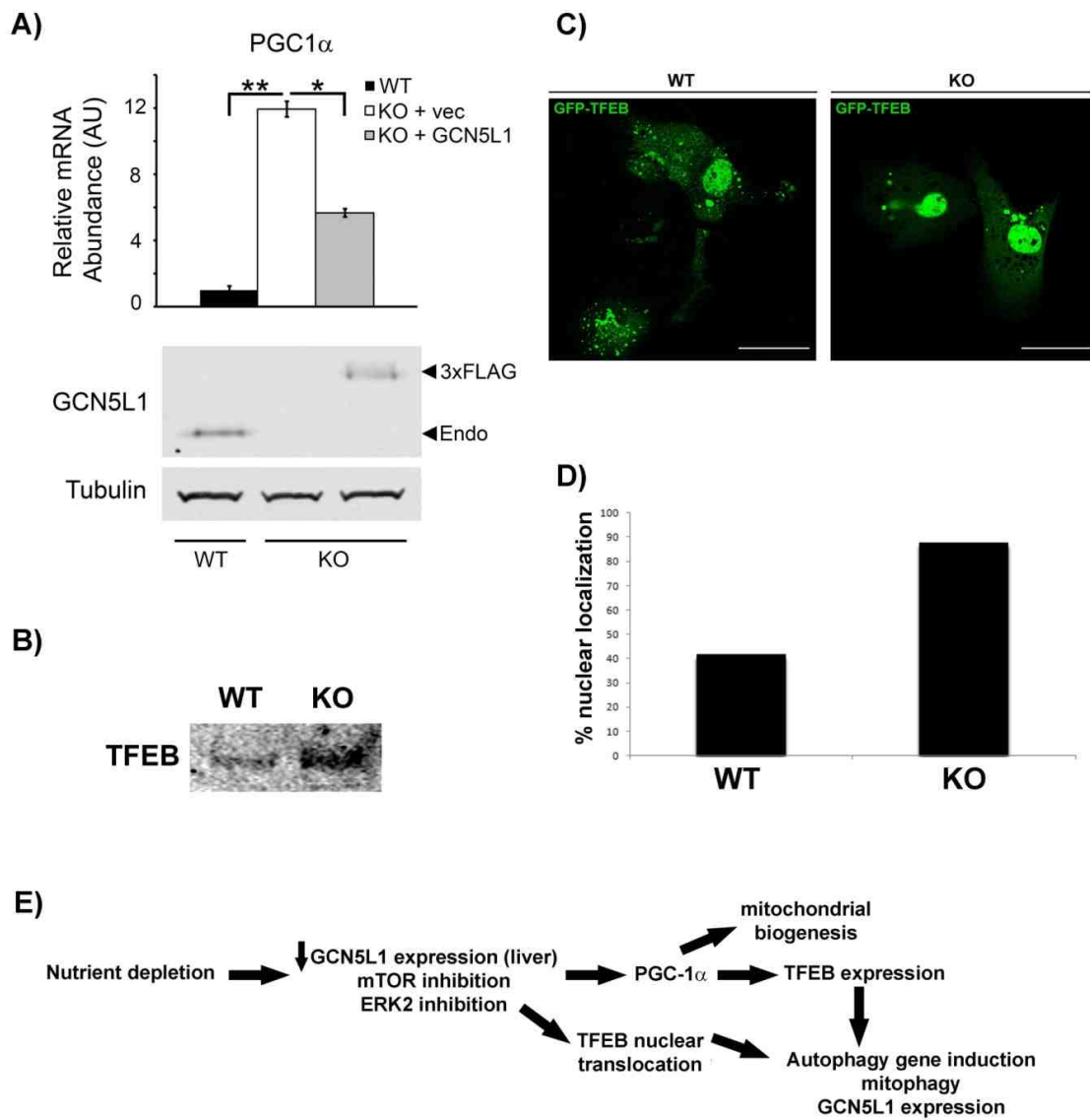


Figure 6: GCN5L1 knockdown triggers the PGC-1 α /TFEB axis. (A) GCN5L1 KO MEFs show greatly elevated PGC-1 α transcript levels, which can be reversed with reconstitution of GCN5L1. (B) Concurrently, whole cell lysates show that TFEB expression appears elevated with GCN5L1 knockdown. (C) GFP-TFEB overexpression in GCN5L1 KO MEFs shows greater nuclear translocation compared to WT cells (scale bar = 10 μ m). (D) Quantification of GFP-TFEB nuclear translocation. (E) Hypothesized PGC-1 α /TFEB axis to promote mitochondrial biogenesis and mitophagy.

interesting to see if this pathway plays a role in pathologies such as Huntington's disease.

Another potential area where GCN5L1 mediated mitochondrial turnover may play a protective role is activation of the inflammasome. The inflammasome is a component of the innate immune response that consists of a multi-protein complex which when formed activates caspase-1 and subsequently inflammatory cytokines such as IL-1 β and IL-18 [52]. Various pathogens such as bacteria and viruses can induce the inflammasome and thus induce the innate immune system for pathogen clearance. However, excessive inflammasome activation is thought to be a key component of numerous autoimmune diseases [53]. Also of note, defective autophagy is thought to promote aberrant inflammasome activation as ATG16L mutants have increased IL-1 β and IL-18 production leading to symptoms that mimic Crohn's disease, an autoimmune disorder of the small intestine [54]. Defective autophagy is thought to induce augmented inflammasome activation in part through an inability to clear out defective mitochondria leading to increased ROS generation and release of mitochondrial DNA into the cytosol. Mitochondrial DNA, which has prokaryotic features, and ROS are key activators of the inflammasome [55, 56]. Therefore mitophagy may play a key role in inflammasome activity. We have therefore hypothesized that mitochondrial deacetylation mediated mitophagy would generate 'healthier' mitochondria leading to attenuated inflammasome activation. Preliminary results in the lab show that in HepG2 cells with GCN5L1 knockdown, mitochondrial DNA release is diminished following treatment with the stressors CCCP or palmitic acid. Work in J774A.1 cells, a mouse derived macrophage cell line, shows that GCN5L1 depletion leads to lower IL-1 β release. Furthermore, Sirt3 KO bone marrow or peritoneal macrophages have increased mitochondrial ROS, IL-1 β

and IL-6 levels. Therefore, mitochondrial acetylation status and mitophagy induction may play a key role in various autoimmune disorders.

Finally, as mentioned in chapter 1, both mitophagy and xenophagy, the autophagy mediated clearance of intracellular pathogens, utilize very similar mechanisms with outer membrane protein ubiquitinylation and p62 binding occurring in both processes [57]. Highlighting this overlap, p62 overexpression decreases growth of the intracellular pathogen *Burkholderia cenocepacia* in macrophages while p62 depletion increases its proliferation with lower co-localization of *Burkholderia cenocepacia* and LC3-II [58]. Therefore, given that GCN5L1 depletion induces mitophagy and p62 activity, it will be interesting to investigate if mitophagy induction also induces clearance of intracellular pathogens concurrently.

Overall, this work has identified the first component of an acetyltransferase program localized to the mitochondria that counters the well-established deacetylase program mediated by Sirt3. GCN5L1 reverses Sirt3 mediated effects on mitochondrial respiration and ATP production in part through antagonizing Sirt3 deacetylation of components of the electron transport chain. Future work will identify the other components of the GCN5L1 acetyltransferase complex and the ‘acetylome’ it targets. It will be interesting to compare these targets with the known targets of Sirt3 to see if GCN5L1 counters Sirt3 activity on mitochondrial function including ROS generation, β -oxidation, ketone body production and the urea cycle. Furthermore, work presented in this manuscript shows that mitochondrial acetylation status regulates mitophagy and suggests that GCN5L1 functions as a ‘mitophagy suppressor.’ Work to date in the field of mitophagy has only identified proteins essential for various forms of mitophagy when

overexpressed such as Parkin, Nix, FUNDC1 and RNF185. GCN5L1 regulated mitophagy is unique in that its expression prevents mitophagy induction. Therefore, by acting as a 'mitophagy suppressor,' its depletion yields a condition where mitophagy is perpetually elevated offering a system that can readily be used to further the understanding of the mechanisms and consequences of mitophagy. Further work is needed to identify how this form of mitophagy is occurring, which includes identifying the E3-ligase that is ubiquitinating mitochondrial proteins, how mitochondrial protein deacetylation signals for mitophagy induction and what autophagy proteins are essential for this process such as HDAC, Beclin and mitochondrial fission proteins. GCN5L1 mediated mitophagy also appears to induce mitochondrial biogenesis thus promoting overall mitochondrial turnover. This is seen with both PGC-1 α and TFEB induction following GCN5L1 depletion, which induce mitochondrial biogenesis and autophagy, respectively. The idea of mitochondrial turnover occurring has for the most part been theoretical thus making our results even more significant. This is the first work, to our knowledge, to show both arms of mitochondrial turnover (mitophagy and biogenesis) functioning simultaneously. Therefore, it will be interesting to alter PGC-1 α and/or TFEB expression to see the effects on mitochondrial mass and function. Future work will further investigate the importance of this PGC-1 α /TFEB axis. This augmented mitochondrial turnover appears to improve mitochondrial 'health' as seen by attenuated ROS generation and MPTP opening. Therefore the functional consequences of this improved mitochondrial health need to be explored in cell culture and in various pathologies. Preliminary work suggests an attenuation of inflammasome activation, which may be beneficial for autoimmune disorders, and GCN5L1 mitophagy may also

affect xenophagy and therefore infections such as tuberculosis. Attempts to generate a GCN5L1 knockout animal have proven elusive thus far but attempts are ongoing. If such an animal is viable, then it would be hypothesized that overall mitochondrial health in these animals would be improved and may be beneficial in the study of mouse models with numerous pathologies where mitochondrial defects are known such as obesity, diabetes, neurodegenerative disorders (Alzheimer's, Parkinson's, Freidrich's Ataxia etc.), heart disease and aging (Figure 7).

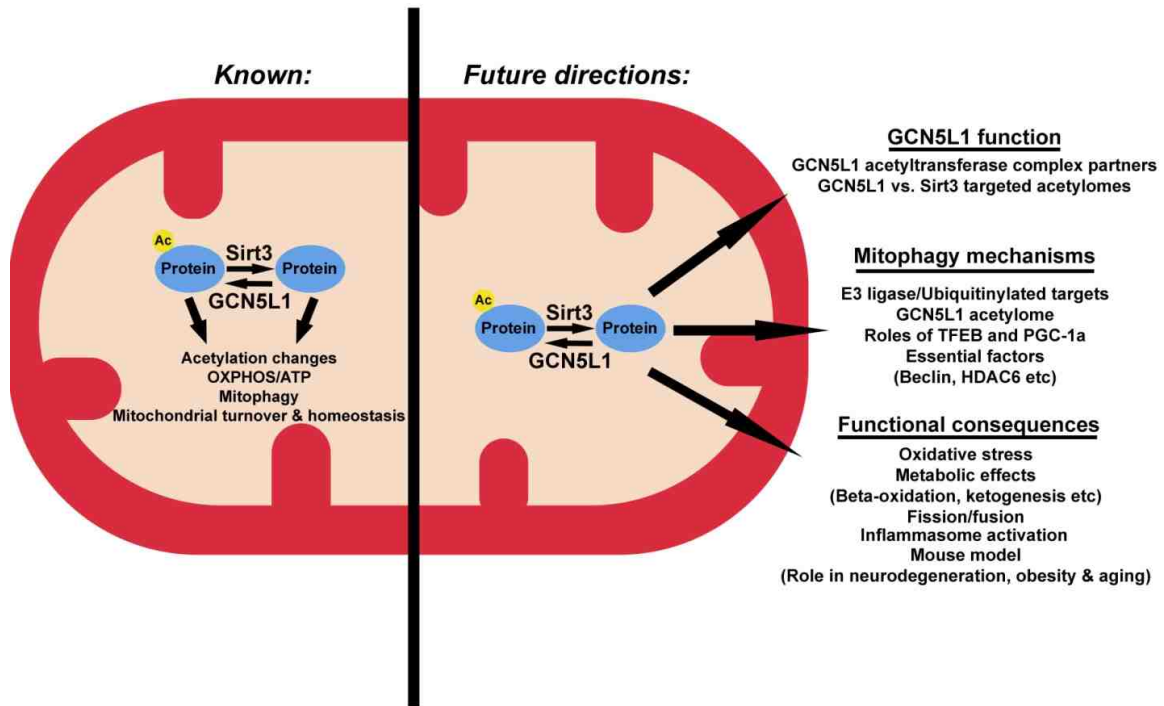


Figure 7: Established mitochondrial functions of GCN5L1 and future research directions. GCN5L1 counters Sirt3 mediated effects on oxidative phosphorylation, respiration and ATP production by acetylating Sirt3 targets. GCN5L1 also functions as a ‘mitophagy suppressor’ promoting mitochondrial turnover and homeostasis. Future work will identify GCN5L1 binding partners that together function as a mitochondrial localized acetyltransferase complex with its own ‘acetyloyme,’ which can then be compared to known Sirt3 targets. In addition, the mechanisms for mitophagy need to be investigated such as finding the E3-ligase mediating mitophagy, roles for TFEB and PGC-1 α and defining which autophagy factors are essential for this process. Finally, potential implications of GCN5L1 acetylation, mitophagy and turnover will explore changes in oxidative stress, metabolism, fission/fusion and overall mitochondrial homeostasis. A mouse model for GCN5L1 knockdown will allow for GCN5L1 function to be explored in the context of various pathologies.

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Appendix 1

THE CONSERVED PROKARYOTE XAT REGION OF GCN5L1 IS REQUIRED FOR MITOCHONDRIAL ACETYLTRANSFERASE FUNCTION

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Running head: XAT region of GCN5L1 and mitochondrial acetylation

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Key Words: GCN5L1, mitochondrial acetylation, acetyltransferase, XAT-hexapeptide
repeat region

This manuscript is currently under review at *J. of Biochemistry*.

Background: GCN5L1 has been identified as a critical component of the mitochondrial acetyltransferase complex.

Results: The hexapeptide XAT-repeat region of GCN5L1 is highly conserved between acetyltransferase enzymes and is necessary for the acetyltransferase function of GCN5L1.

Conclusion: The XAT-repeat region may be a common feature of mammalian acetyltransferase enzymes.

Significance: Our understanding of biological role of this hexapeptide-repeat region may identify a target sequence for the pharmacologic modulation of non-histone acetyltransferases.

SUMMARY

Acetyltransferase enzymes are commonly found in multi-protein complexes, composed of catalytic and non-catalytic subunits, which function in regulatory, adaptor and substrate binding roles. Recently, the first protein component of the mitochondrial acetyltransferase machinery, GCN5L1, was identified, and its initial characterization demonstrated that it countered the regulatory effects of the mitochondrial deacetylase SIRT3. Investigation of GCN5L1 functional regions, and its ‘exploitation’ to identify binding partners, should enable further characterization of the mitochondrial acetyltransferase machinery. In this study we investigate GCN5L1 and demonstrate that while two isoforms of this protein are expressed, only the shorter isoform is enriched within mitochondria. We identify several prokaryote conserved (xenobiotic acetyltransferase - XAT) hexapeptide repeat motifs in GCN5L1, and demonstrate that motif is conserved across many mammalian acetyltransferase proteins. Finally, we show that the genetic disruption of this XAT region disrupts both the acetylation and mitochondrial respiratory effects of GCN5L1, suggesting a novel functional role for this region in mammals.

Lysine acetyltransferases catalyze acetylation of ϵ -amino groups on specific protein lysine residues. Protein acetylation is emerging as a widespread and highly regulated posttranslational modification (PTM) evident from bacteria to mammalia (1-3). The functional effects of protein acetylation include the regulation of gene expression, enzyme activity and protein stability. Within this field, histone acetyltransferase and deacetylase enzymes, which regulate gene expression through changes in histone acetylation status, have been widely characterized (4).

The study of sirtuins, a family of predominantly non-histone lysine deacetylases, has expanded our understanding of the role played by protein acetylation status. In particular, it is now recognized that lysine acetylation regulates functioning in numerous, non-nuclear cellular compartments (5). As the activation of sirtuins is nutrient- and redox-state dependent, the role of this regulatory program in controlling mitochondrial function has come to the fore (6). In mitochondria, the predominant sirtuin deacetylase is SIRT3, and its role in controlling mitochondrial metabolism (7, 8), respiration (9, 10) and responses to mitochondrial stressors (11-13) is well established. However, in contrast to lysine acetyltransferases in the nucleus, characterization of the mitochondrial acetyltransferase program has been elusive.

Lysine acetyltransferase enzymes commonly exist within multi-protein complexes, where subunit integration is required for appropriate structure, function, and regulation (14, 15). Therefore, to uncover the mitochondrial acetyltransferase machinery, a prerequisite was to identify and characterize an 'initial' component of this putative multiprotein complex. The mystery as to whether a mitochondrial acetyltransferase program is operational has begun to be uncovered following the functional

characterization of GCN5L1 (16). This mammalian gene was initially cloned 15 years ago, and was found to have ~ 24% homology with the nuclear acetyltransferase GCN5 (17), in addition to being highly conserved between humans, flies and nematodes (18). However, although it was present in a comprehensive mitochondrial proteome database (19), the function of this protein in mitochondria has only recently begun to be investigated. The initial genetic knockdown studies showed that GCN5L1 is an essential component of the mitochondrial acetyltransferase machinery, and that its depletion counters the acetylation and respiratory phenotype cause by the loss of SIRT3 (16). Consistent with the multi-subunit characteristics of other acetyltransferase enzymes, the *in vitro* acetyltransferase capacity of recombinant GCN5L1 was found to require additional, but as-yet unidentified, mitochondrial components.

In this study we further explore the characteristics of GCN5L1 and find that: (i) it is expressed in multiple tissues; (ii) while two isoforms of the proteins are expressed, only one is found in mitochondria; and (iii) that the prokaryote-conserved xenobiotic acetyltransferase (XAT) region is conserved across numerous mammalian acetyltransferase enzymes, and that this region is necessary for the acetylation and respiratory effects of GCN5L1.

EXPERIMENTAL PROCEDURES

Cells, Plasmids and Transfection Reagents

Human liver HepG2 cells were maintained in DMEM/10%FBS/1xPen-Strep at 37°C/5% CO₂. Cells were transfected using Fugene (Roche) for 48 h, then used according to

experimental need. The deletion constructs used in this study were created from GCN5L1-FLAG (16) using standard PCR cloning techniques, and inserted into the same p3XFLAG-CMV-14 vector (Sigma). Plasmids were sequenced prior to use to ensure fidelity.

Cellular Fractionation and Western Blotting

Whole mouse liver tissue or HepG2 cells were lysed on ice in detergent-free sucrose buffer using a Dounce homogenizer or 25-gauge needle, respectively. Following disruption, cells were centrifuged at 4 °C according to the noted scheme (Fig. 2C) to recover the mitochondrial and cytosolic fractions. For western blotting, proteins were denatured by boiling in SDS sample buffer, and then separated by SDS PAGE. Proteins were transferred to nitrocellulose membranes, followed by incubation with the following antibodies: GCN5L1 (16); FLAG (Sigma); GAPDH, VDAC (Cell Signaling Technology); polyclonal acetyl-lysine (Ac-K), ATP5a (Abcam). Each western blot shown is representative of at least three independent experiments.

In Vitro Acetylation Assay

The *in vitro* acetylation assay was carried out according to Scott et al, (16). Briefly, HepG2 cells were transfected with noted plasmids for 48 h, at which time mitochondria were isolated. Samples were resuspended in reaction buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 4 mM MgCl₂, 5 mM nicotinamide, pH 7.4), sonicated then incubated for 1.5 h at 37 °C, in the absence or presence of 2.5 mM acetyl-CoA. Mitochondrial proteins were

then used for global acetylation analysis (reaction stopped by boiling with SDS sample buffer), followed by SDS-PAGE and immunoblot analysis with an Ac-K antibody.

Metabolic Measurements

Oxygen consumption measurements were performed on the XF24 analyzer (Seahorse Bioscience). Cells transfected with the noted plasmids for 48 h were transferred to 24-well plates overnight and incubated in sucrose respiration media for 1 h prior to analysis. Data are representative of three independent experiments of $n = 5$ replicates per condition.

Bioinformatics and Sequence Analysis

The XAT-repeat regions of GCN5L1 were identified by interrogation of the deduced GCN5L1 protein sequence, looking for a hexapeptide repeat motif matching x-[STAV]-x-[LIV]-[GAED]-x (NCBI cd00208). Human lysine acetyltransferases from the GCN5, p300 and MYST families containing three or more tandem XAT repeats were found by performing a MOTIF search (<http://www.genome.jp/tools/motif/MOTIF2.html>) against the KEGG database for proteins bearing the following pattern: x-[STAV]-x-[LIV]-[GAED]-x(0,100)-x-[STAV]-x-[LIV]-[GAED]-x(0,100)-x-[STAV]-x-[LIV]-[GAED]-x. The XAT regions from these proteins were analyzed using PhyML (www.phylogeny.fr) and separated into phylogenetic clades using the automated function. In any protein displaying multiple, discrete XAT regions, the one containing a conserved acetyltransferase domain was used. Alignment of GCN5L1 with BtXAT from *Burkholderia thailandensis* (ZP_02389502), or with GCN5L1 homologues (current

NCBI accessions) from *Mus musculus*, *Bos taurus*, *Xenopus laevis*, *Danio rerio*, *Drosophila melanogaster* and *Arabidopsis thaliana* were carried out using T-Coffee. The secondary structure of the human HAT1 acetyltransferase domain was modeled using PSIPRED from data contained in Dutnall et al (20).

Animal Husbandry

The use of mice in this study was approved by the NHLBI Animal Care and Use Committee, and animals were treated according to their guidelines.

Statistical analysis

Where required, data were tested for normality using the Kolmogorov-Smirnov test, followed by a one-tailed Student's t-test using the Microsoft Excel statistics package. A *P* value of < 0.05 was regarded as significant

RESULTS

The short form of GCN5L1 is enriched in mitochondria.

GCN5L1, also variously known as RT14, BLOS1 and BLOC1S1, was first cloned as a cDNA from human fetal brain (18, 21). At the time of its initial description, two distinct gene products were identified by Northern Blot analysis, however the authors concluded that the shorter form was the correct transcript from this gene (18). Interrogation of GenBank shows that the longer form has now been cloned in *Homo sapiens* (accession number NM_001487), while the shorter form has been identified in

both *H. sapiens* and *Mus musculus* (accession numbers BC130640 and BC034662, respectively). The human sequence, identifying the two in-frame start codons, is shown in Figure 1A. Using an antibody directed to a shared region of both isoforms, we determined the relative protein expression of these transcripts, in concert with ascertaining their tissue distribution. GCN5L1 appeared to be ubiquitously expressed as a \approx 15 kDa protein in mouse, while a larger (\approx 18 kDa) form is evident at much lower levels in a few tissues, such as liver and kidney (Fig. 1B). The molecular weights of these peptides corresponds closely to those calculated from the deduced amino acid sequences of both the short (125 aa; 14.3 kDa) and long (153 aa; 17.3 kDa) forms of GCN5L1 found in humans. To determine whether both isoforms are present in mitochondria, we fractionated liver tissue into subcellular compartments using differential centrifugation. While both isoforms were found in the whole-cell preparation, the longer isoform is not evident in the mitochondrial-enriched fractions (Fig. 1C). This suggests that the shorter isoform is the mitochondrially-active version, and we therefore investigated this further. To determine whether the 15kDa mitochondrial enriched protein has a classical mitochondrial localization sequence, FLAG-tagged deletion constructs were generated to remove the amino-, carboxy- or both termini (as schematized in Fig. 1D). These various constructs were transfected into human liver HepG2 cells, and cellular localization were determined by subcellular fractionation, followed by immunoblot assay for FLAG expression. Interestingly, GCN5L1 does not appear to harbor a classic mitochondrial localization sequence, as deletion of either or both termini did not alter its mitochondrial

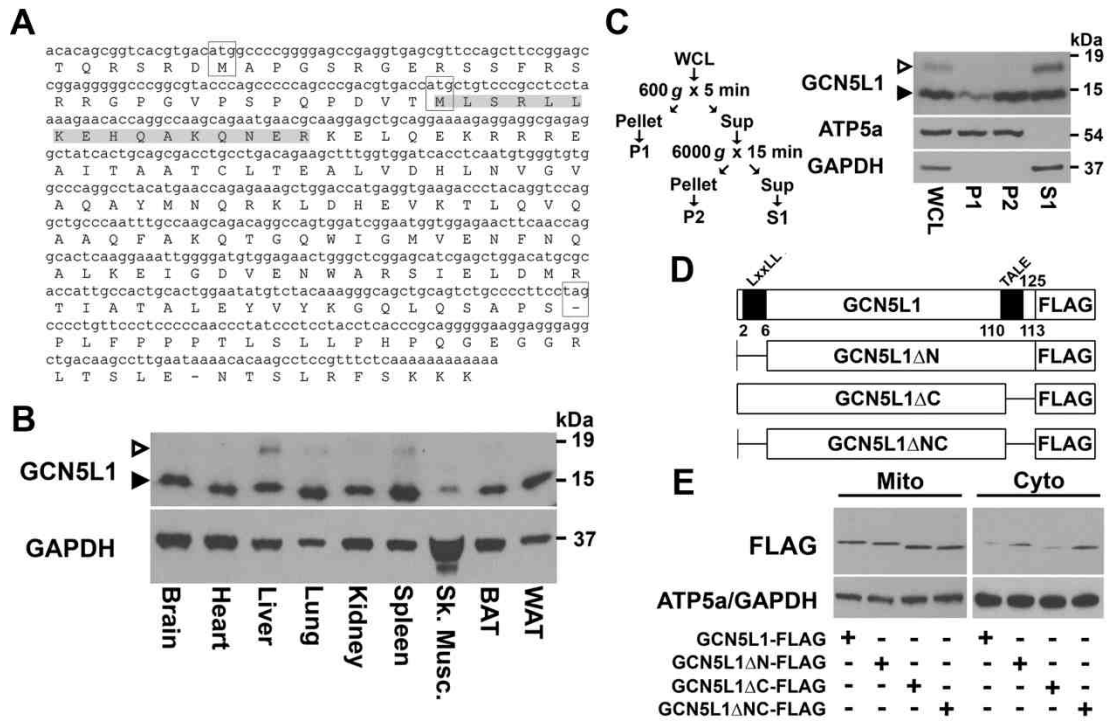


Figure 1. The Short Isoform of GCN5L1 is Enriched in Mitochondria. A. Nucleotide and deduced amino acid sequences of both GCN5L1 isoforms in human. The two in-frame start codons, along with the stop codon, are marked by boxes. The epitope of the GCN5L1 antibody is highlighted. B. Tissue distribution of the short (black triangle) and long (open triangle) isoforms of GCN5L1 in mouse. C. Subcellular fractionation of mouse liver tissue to identify localization of the short (black triangle) and long (open triangle) isoforms of GCN5L1. D. Human GCN5L1-FLAG expression constructs used in this study. E. Subcellular localization of human GCN5L1-FLAG constructs following transient expression in human HepG2 liver cells.

enrichment (Fig. 1E). This suggests that GCN5L1 enters the mitochondria through another import process, perhaps via an internal, non-cleavable targeting sequence (22).

The xenobiotic acetyltransferase region of GCN5L1 is phylogenetically preserved.

As the initial description of GCN5L1 as a component of the mitochondrial acetyltransferase machinery noted its similarity to prokaryotic proteins (16), we compared the sequence of GCN5L1 to the *Burkholderia thailandensis* streptogramin xenobiotic acetyltransferase (BtXAT; accession number ZP_02389502). BtXAT is a member of the Left-handed parallel beta-Helix (LbetaH; cd00208) class of proteins characterized by a tandem hexapeptide repeat motif (x-[STAV]-x-[LIV]-[GAED]-x). Proteins in this class typically display acyltransferase activity, and are most commonly found in microbes (23, 24). We found that the GCN5L1 XAT-containing region has 53% sequence similarity to the BtXAT substrate- and acetyl-coA-binding domains, although the similarity is mainly restricted to the former region (Fig. 2A). BtXAT has four XAT hexapeptide repeat motifs that largely bracket its two binding domains, suggesting that these regions are either involved directly in substrate binding, or aid the formation of the correct tertiary structure required for this process.

GCN5L1 has three XAT hexapeptide repeat motifs, with two of these regions separated by a single amino acid, and the third separated by 61 amino acids (Fig. 2B). We determined whether this architecture, and the fidelity of these XAT repeat regions, is conserved across species. We find that this hexapeptide repeat architecture and sequence similarity is highly conserved in GCN5L1 orthologues from fruit flies to humans, with 100% sequence fidelity in the XAT repeat regions throughout members of the Chordata

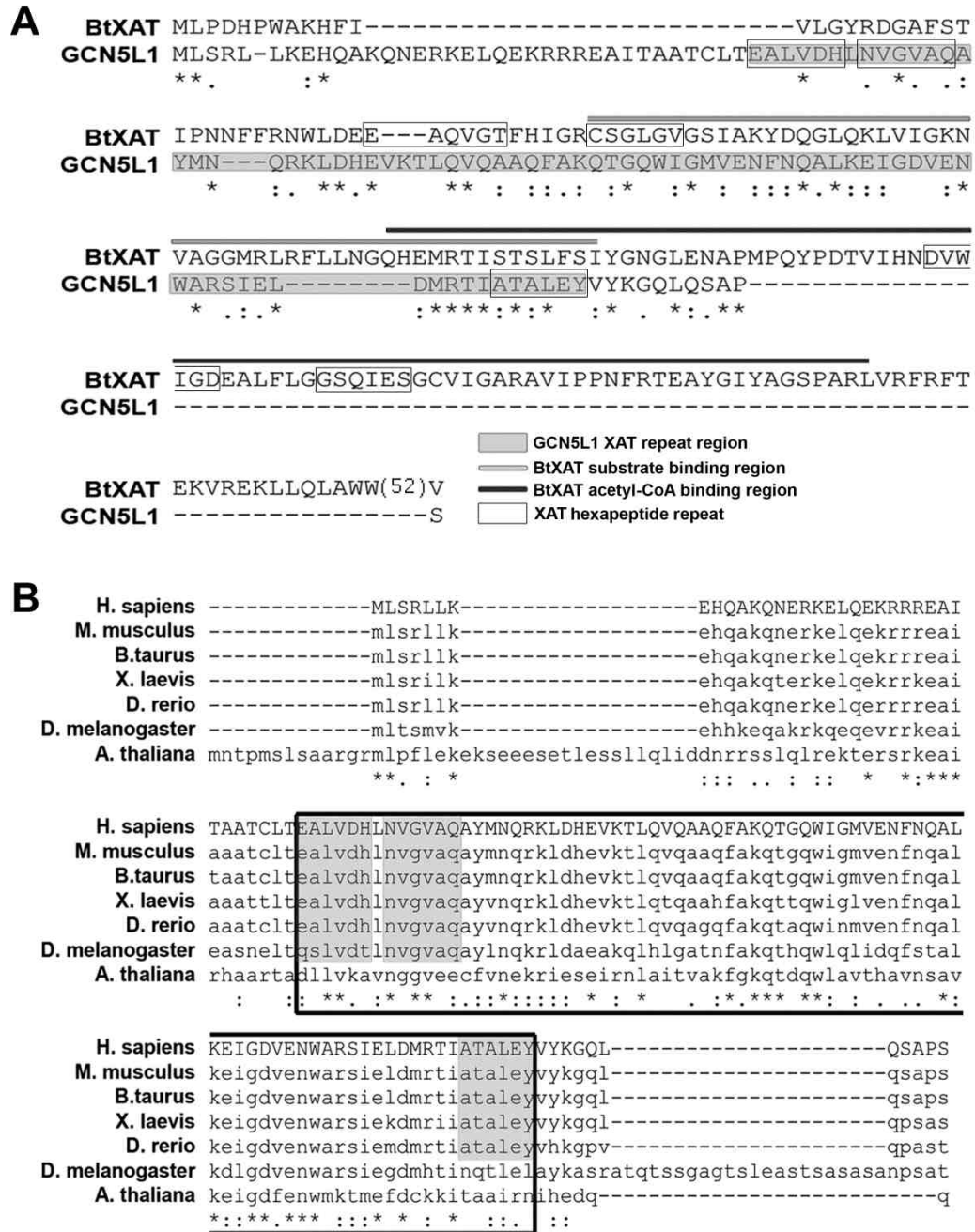


Figure 2. The Prokaryotic Xenobiotic Acetyltransferase (XAT) Architecture of GCN5L1 is Highly Conserved. A. Alignment of the streptogramin acetyltransferase from *Burkholderia thailandensis* (BtXAT) and the short isoform of GCN5L1. The XAT-containing region of GCN5L1 (grey highlight), BtXAT substrate-binding (grey line), BtXAT acetyl-CoA-binding (black line) and XAT repeats (open box) are marked. B. Sequence alignment of GCN5L1 proteins from *Homo sapiens*, *Mus musculus*, *Bos taurus*, *Xenopus laevis*, *Danio rerio*, *Drosophila melanogaster* and *Arabidopsis thaliana*. The full XAT-containing regions of the proteins (open box) and each individual XAT repeat (grey highlight) are marked.

phylum (Fig. 2B). Interestingly, while maintaining much similarity to the animal sequences throughout its length, the *Arabidopsis thaliana* deduced protein sequence is devoid of XAT hexapeptide motifs (Fig. 2B).

Phylogenetic conservation of the XAT region across known acetyltransferases.

Given the well-characterized presence of XAT hexapeptide repeats in prokaryotic acetyltransferases, and their presence in the sequence of GCN5L1, we then reasoned that these features may be conserved within other mammalian lysine acetyltransferase proteins. Using a MOTIF search, we looked for acetyltransferase proteins harboring multiple tandem repeats of the XAT repeat signature in the KEGG human protein database (25). Surprisingly, several members from the three major lysine acetyltransferase families – GCN5, p300 and MYST – were found to contain multiple XAT regions in addition to GCN5L1 (Table 1). Of these proteins, 82% contained at least one XAT repeat within their acetyltransferase domain. We then investigated the position of these XAT repeats in HAT1, a GCN5 family acetyltransferase whose yeast crystal structure has been reported previously (20). The acetyltransferase domain of human HAT1 contains two XAT repeats, which bracket motif A of its conserved GCN5 catalytic core (Fig. 3A). This motif, which contains the conserved [QR]-x-x-G-x-G sequence necessary for acetyltransferase activity, is thought to be the main area of acetyl-CoA contact (20). It would appear, therefore, that there is a large degree of similarity between XAT repeat placement conserved between human and prokaryotic acetyltransferases.

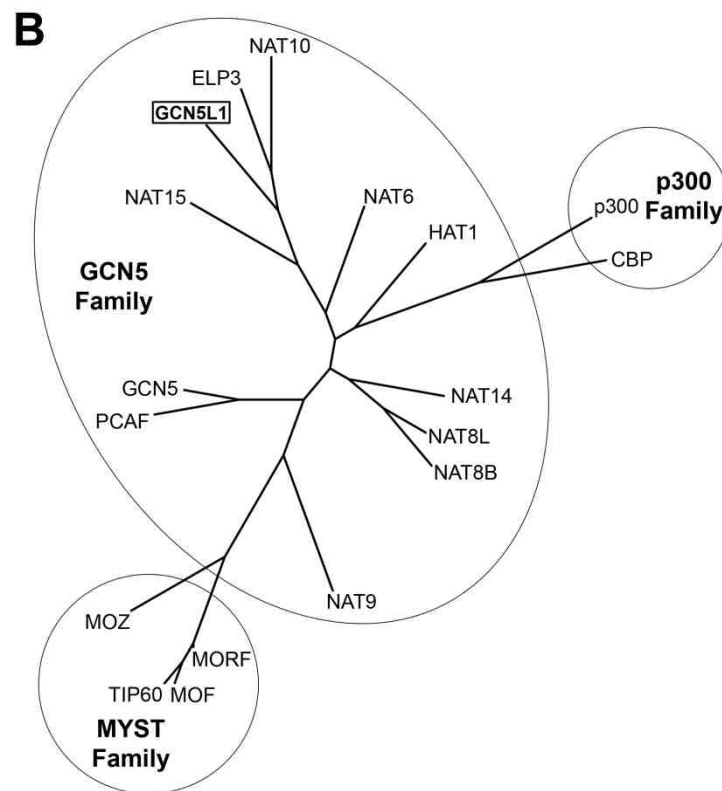
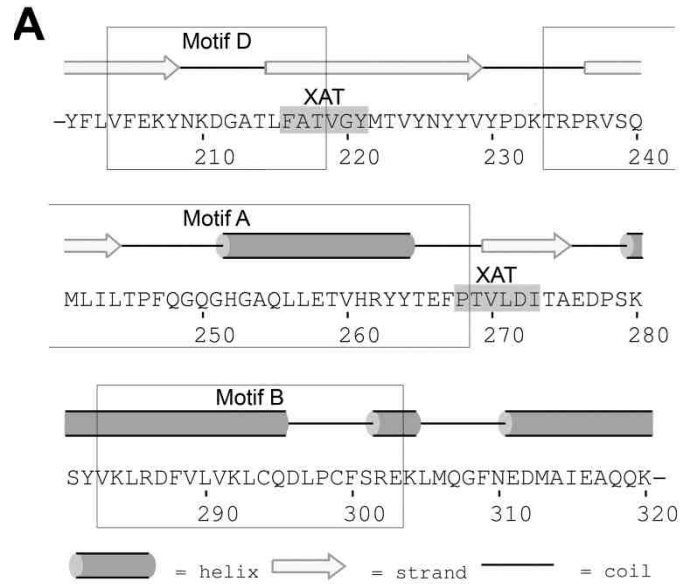


Figure 3. The XAT Motif from Prokaryotic Acetyltransferases is Retained in Human Lysine Acetyltransferases. **A.** The human HAT1 acetyltransferase core domain contains two XAT repeats (grey highlight), which bracket the conserved motif A. The three motifs of the HAT1 core domain are identified by boxes. **B.** Phylogenetic sorting of the XAT repeat regions from human lysine acetyltransferases matches their overall placement into GCN5, p300 and MYST family groupings.

To see if there are connections between the XAT repeat regions and overall homology within acetyltransferase families, we analyzed the phylogenetic relationships of this sequence region in human proteins. We extracted the deduced amino acid sequence of each protein between two bordering XAT repeats (Suppl. Fig. 1), and then separated these peptides into clades using PhyML. Interestingly, there was 100% concordance between the sequences of human XAT repeat regions and their known acetyltransferase family (Fig. 3B).

The XAT region is required for the acetyltransferase functioning of GCN5L1.

The evolutionary conservation of the XAT architecture and sequence suggests an important functional role of this region. To test this, we deleted the first two hexapeptide repeats of GCN5L1 using overlap PCR, and designated this construct Δ XAT-FLAG (Fig. 4A). Overexpression of both GCN5L1-FLAG and Δ XAT-FLAG in HepG2 led to similar mitochondrial enrichment of the FLAG constructs (Fig. 4B). We then compared the capacity of control (empty FLAG vector), GCN5L1 and Δ XAT constructs to acetylate mitochondrial proteins. In the presence of acetyl-CoA, mitochondria extracted from HepG2 cells overexpressing GCN5L1 displayed a greatly-increased capacity to acetylate mitochondrial proteins, relative to those expressing control or Δ XAT-FLAG plasmids (Fig. 4C). To further explore the functional effects of the XAT region, we then measured mitochondrial respiration in these HepG2 cells. We have previously shown that the genetic knockdown of GCN5L1 increases mitochondrial respiration (16). Here we show

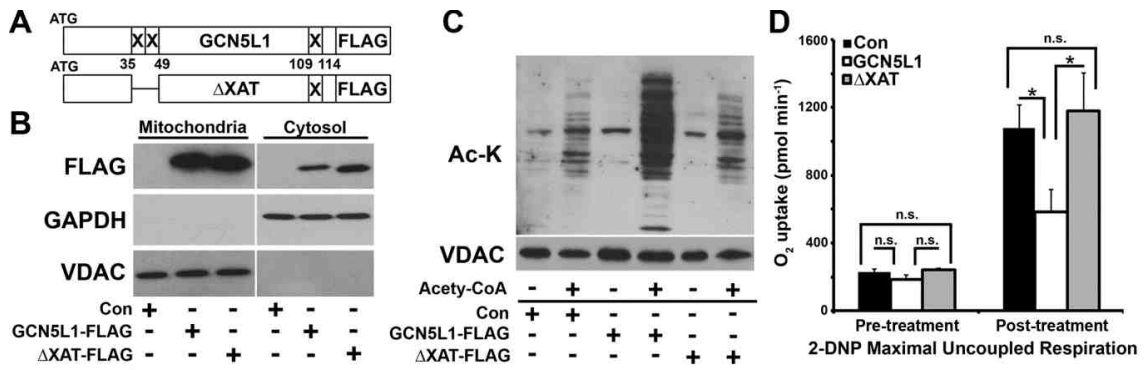


Figure 4. The XAT Repeat Region of GCN5L1 is Required for Acetyltransferase Activity in Mitochondria. A. GCN5L1 constructs used in this study. B. Subcellular localization of human GCN5L1-FLAG constructs following transient expression in human HepG2 liver cells. C. An *in vitro* acetylation assay of mitochondrial proteins from cells expressing control, GCN5L1-FLAG and ΔXAT-FLAG constructs, in the presence or absence of exogenous acetyl-CoA. D. Maximal uncoupled oxygen consumption in HepG2 cells expressing control, GCN5L1-FLAG and ΔXAT-FLAG constructs, following treatment of with 2-DNP.

that the overexpression of GCN5L1 suppresses maximal uncoupled respiration (Fig. 4D) compared to the control and Δ XAT-FLAG expression plasmids.

DISCUSSION

To enhance our understanding of mitochondrial acetyltransferase function, it is necessary to characterize the proteins operational in this program (16). We recently identified GCN5L1, a mitochondrial protein displaying homology to prokaryotic acetyltransferases, as the first member of this regulatory program. In this study we examined the function of GCN5L1 in light of its secondary structure, and show that its prokaryotic-derived features are crucial to its acetyltransferase activity. We also show that these same prokaryotic features are evident in other human lysine acetyltransferases, hinting at a previously-unrecognized link between the corresponding enzymes in these two phylogenetic groups.

Although the structure-function of prokaryotic BtXAT protein has not been characterized, two closely related bacterial XAT proteins have been studied in terms of tertiary structure (24, 26). In common with other XAT-type proteins Vat(D), a virginiamycin acetyltransferase from the human pathogen *Enterococcus faecium*, exhibits a homotrimeric structure, with active sites formed between the surfaces of two apposed subunits. The imperfect tandem hexapeptide XAT repeats of Vat(D) create a series of β -sheets and turns, which fold to form a triangular prism that is characteristic of LbetaH proteins (26). These prisms form part of the acetyl-CoA- and substrate-binding surfaces between two subunits of the trimer. As such, the hexapeptide repeat is crucial to the

correct confirmation of XAT acetyltransferase proteins. Removal of two XAT repeats from GCN5L1 blocked its acetyltransferase activity *in vitro*, with a subsequent loss of function in regulating mitochondrial respiration *in vivo* (Fig. 4). It is therefore tempting to speculate that this mutation may have altered the conformation of GCN5L1, preventing it from binding either acetyl-CoA or its protein substrates.

While examining the conservation of XAT repeat architecture in GCN5L1, we were interested to discover that these repeats are common in other human lysine acetyltransferase proteins (Table 1). While these motifs are not restricted to acetyltransferases *per se*, it was also interesting to note that there was a close correlation between the position of human XAT repeats and the acetyltransferase core domain. This is exemplified in the GCN5-family protein HAT1, where there are two XAT repeats bordering the highly conserved motif A (Fig. 3A). This region has been shown to be the acetyl-CoA-binding region of yeast HAT1 (Dutnall et al, 1998), and disruption in this area can reduce the acetyltransferase activity of GCN5 family proteins by > 90% (27). We therefore would speculate that the folding generated by the XAT repeat may aid in the binding of acetyl-CoA, and that this feature has therefore evolved, or been retained, in acetyltransferases in many phylogenetic groups. Further work on the importance of the XAT repeat in acetyltransferase function is therefore warranted.

Acetyltransferase Family	Gene Name	NCBI Gene ID	Protein Length (aa)	XAT Repeat Coordinates	XAT Repeat In Acetyltransferase Domain?
GCN5	HAT1	8520	419	15-273	Yes
	ELP3	55140	547	240-380	No
	GCN5	2648	837	158-248	No
	PCAF	8850	832	55-249 350-489	No
	NAT14	57106	206	69-173	Yes
	NAT15	79903	242	61-93	Yes
	NAT9	26151	207	95-196	Yes
	NAT8B	51471	227	15-177	Yes
	NAT6	21412	286	118-269	Yes
	NAT8L	339983	302	29-255	Yes
p300	NAT10	55226	953	138-384 506-611	Yes
	GCN5L1	2647	125	36-114	-
MYST	p300	2033	2414	432-473 1212-1400 2373-2413	Yes
	CBP	1387	2404	1140-1398	Yes
MOF	MOZ	7994	2004	683-771 1349-1555 1788-1884	Yes
	TIP60	10524	494	373-406	Yes
	MOF	84148	458	288-372	Yes
	MORF	23522	2073	894-913 1402-1462 1789-1919	Yes

Table 1. Human Lysine Acetyltransferase Proteins Containing XAT Repeat Regions. List of human lysine acetyltransferase proteins identified by the MOTIF search, along with the positions of their XAT repeat regions.

The architecture of GCN5L1 is closely conserved in its orthologs throughout eukaryotes, with particularly striking sequence homology in the XAT hexapeptide repeat regions in vertebrate animals (Fig. 2B). Apart from its identification as a part of the mitochondrial acetyltransferase machinery in humans, the only other functional characterization of a GCN5L1 protein has been in the flowering plant *Arabidopsis thaliana* (28). Here, it was shown that GCN5L1 interacted with proteins that mediated endosomal-vacuolar transport, with the authors speculating that loss of GCN5L1 led to an inhibition of protein degradation in the vacuole (28). While there have been suggestions of a role for human GCN5L1 in protein degradation through analogous autophagic pathways (29, 30), it is interesting to note that the *Arabidopsis* GCN5L1 does not contain any XAT repeats, and therefore may have evolved to perform a very different, non-related role in plants.

Our findings show that the XAT hexapeptide repeat region plays an important function role in GCN5L1 in mitochondrial protein acetylation and that these repeat regions are a common feature across mammalian acetyltransferase enzymes. The next task is to evaluate whether this region functions in protein-protein interaction as a component of the mitochondrial acetyltransferase machinery and/or whether it functions to incorporate acetyl-CoA into this acetylation reaction.

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Appendix 2

The role of sirtuins in modulating redox stressors

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ABSTRACT

For much of the time since their discovery, the sirtuin family of deacetylase enzymes has been associated with extensions in lifespan. This longevity promoting capacity in numerous model systems has enabled the sirtuins to gain ‘celebrity status’ in the field of aging research. However, the mechanisms underpinning these changes remain incompletely defined. A general phenotype long associated with aging is the dysregulation of biological systems, which partly occurs via the accumulation of damage over time. One of the major sources of this damage is oxidative stress, which can harm both biological structures, and the mechanisms with which they are repaired. It is now becoming clear that the beneficial lifespan effects of sirtuins, along with many of their other functions, are closely linked to their ability to regulate systems that control the redox environment. Here we investigate the links between sirtuins and their oxidative/redox environment, and review the control mechanisms which are regulated by the activity of sirtuin deacetylase proteins.

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 Additional nuclear and cytosolic sirtuins and redox biology

Mitochondrial sirtuins

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 Biological effects of mitochondrial Sirtuins

 Oncogenesis

 Lipotoxicity

 Cardiac hypertrophy

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SIRT3

Conclusions and future directions

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Introduction to sirtuin biology

The free radical hypothesis governing the pathophysiology of disease advocates that excess oxidative stress, and/or the diminished capacity to control reactive species, plays a role in disease initiation and progression [1-4]. In parallel, members of the NAD-dependent sirtuin deacetylase family are increasingly recognized as pivotal mediators in the regulation of nuclear, cytosolic and mitochondrial quality control programs – mechanisms which enhance cellular homeostasis and diminish degenerative disease susceptibility. Taken together, these concepts suggest that the control of redox stress, and the biological effects of sirtuin-dependent protein deacetylation, may be intertwined. To date, the majority of studies have suggested caloric-dependent factors in the control of sirtuin biology (recently reviewed [5, 6]). However, evidence is emerging that several sirtuin isoforms are activated during redox stress and may modulate crucial responses, including the adaptation to hypoxia and ameliorating reactive oxygen species-induced pathologies.

As background, the Sir2 (silence information regulator 2) or sirtuin family of class III deacetylases differ from class I and II histone deacetylases (HDACs) by their protein sequences and in that they are NAD⁺- rather than Zn-dependent enzymes [7]. Seven mammalian homologues of Sir2 have been identified, and are designated as SIRT1 through SIRT7 [8]. SIRT1, SIRT2 and SIRT3 are classified as subclass I enzymes, which show closest homology to yeast Sir2, and exhibit the most robust deacetylase activity. SIRT4 and SIRT5 are assigned to subclasses II and III, respectively; while SIRT6 and SIRT7 are classified as subclass IV enzymes [8]. Sirtuins appear to also catalyze ADP-

ribosyl transferase activity, although this biology has been less well studied [9]. There is also recent work to suggest that Sirt5 functions as a ‘desuccinylase’ and/or a ‘demalonylase’ rather than a deacetylase, which may explain a lack of functional targets for Sirt5 compared to Sirt3 [10]. The mammalian sirtuins are found in distinct subcellular compartments, and emerging evidence shows that they regulate specific biological functions, via the deacetylation of target proteins, within these restricted subcellular locations. The biological effects of sirtuin-mediated lysine residue deacetylation include the direct modulation of transcription, and substrate-specific diverse effects on cell growth, aging, stress-tolerance and metabolism [11, 12]. This review will focus on how redox signaling controls sirtuin abundance and activity and, conversely, how sirtuins regulate the response to changes in redox potential and oxygen concentration through substrate-specific deacetylation events.

Nuclear and Cytosolic Sirtuins

SIRT1 is the most widely characterized protein in the sirtuin family, and as such a great deal of information has been amassed regarding its regulation over the last decade. The potential role of SIRT1 in a number of vital physiological pathways has made it a prime target for pharmacological intervention (see [13, 14]). One major area of SIRT1 research has been its role in caloric restriction (CR), which has long been known to increase lifespan in a variety of organisms. CR was first shown to have an effect on sirtuin activity in yeast and worms, where loss or gain of the SIRT1 homologue led to a reduction or increase, respectively, in lifespan [15, 16]. These effects were mediated in a large part by the availability of the sirtuin activator, NAD⁺, which was increased during

CR and is required for the function of the sirtuin protein [17]. Later work demonstrated that CR may affect sirtuin function through several NAD⁺-linked redox pathways in yeast: CR may increase nicotinamide levels, a specific inhibitor of SIRTs [18]; or reduce the levels of cellular NADH, which acts as a competitive inhibitor of sirtuin activity [19]. The extent to which NAD⁺ is involved in the overall control of SIRT function in mammals is, however, less well established. Finally, CR has been shown to increase the protein expression of a number of SIRTs, including SIRT1, SIRT3 and SIRT5 [20-22], which may further enhance their overall deacetylase activity.

The ability of SIRT1 homologues to increase lifespan in various organisms led to the search for novel pharmacological activators of sirtuins. One of the best characterized is resveratrol, a weak polyphenolic antioxidant found in red wine. Resveratrol improves the enzyme kinetics of SIRT1 by increasing its ability to bind and deacetylate substrates, such as p53, in an NAD⁺-dependent manner [13]. Addition of resveratrol to yeast activated the SIRT1 homologue, Sir2, leading to increased DNA stability and lengthening lifespan by 70% [13]. Further work by the same group extended these findings to metazoans, showing that resveratrol increased lifespan in fruit flies and worms by 20-30% in a sirtuin-dependent manner [23]. More recent studies have identified a group of small-molecule activators of SIRT1, which have a 1000-fold increase in potency compared to resveratrol. Treatment of mice and rat diabetes-model animals with these compounds, which include SRT1720, SRT1460 and SRT2183, led to an improvement in overall metabolic function and a reduction in diabetic progression [14]. While the authors related these results to an upregulation of mitochondrial function, based on the ability of SIRT1 to deacetylate and activate PGC-1 α (see below; [14]), there has been some

controversy regarding whether these compounds are in fact direct activators of SIRT1, and therefore what mechanism is behind this improvement [24]. While the role of SIRT1 in increasing lifespan in higher animals remains an open question [25], it would appear from knockout and over-expression studies (see below) that SIRT1 may be involved in ameliorating some of the phenotypes linked to aging diseases, such as diabetes, obesity and cancer (reviewed in detail here:[26]).

SIRT2, SIRT6 and SIRT7 are the other sirtuins that reside in the nucleus and cytosol [11]. SIRT2 is the only isoform predominantly localized in cytoplasm, although it does transiently reside in the nucleus during phases of mitosis [27]. Multiple target proteins that are deacetylated by SIRT2 have been characterized [12]. SIRT6 appears to possess nuclear ADP-ribosyltransferase activity, and it functions as a classical histone deacetylase [28, 29]. SIRT7 associates with the nucleolus, and only a limited number of substrates for this deacetylase have been identified [11]. The biology relevant to redox and hypoxic stress associated with these sirtuins will be discussed later in this review.

The role of redox signaling and oxygen in the regulation of SIRT1

In contrast to the emerging characterization of substrates responsive to SIRT1 deacetylation, our knowledge pertaining to the molecular control of SIRT1 itself, and especially in response to redox stress and differing oxygen levels, is less well established. The gene transcript encoding SIRT1 is induced in response to mild oxidative stress [30], although the transcriptional regulatory program controlling this regulation has not been delineated. Furthermore, in response to hypoxia the SIRT1 promoter is directly trans-activated by HIF transcription factors [31]. Emerging data also shows that the SIRT1

protein is modulated by numerous regulatory events in response to altered redox states. At pathological levels, H₂O₂ and redox stress associated with environmental toxins diminish SIRT1 protein levels, via increased proteosomal degradation [32]. The activity of SIRT1 is additionally modulated by post-translational modifications that alter deacetylase activity. For example, oxidative stress has been shown to promote the desumoylation and inactivation of SIRT1, resulting in increased susceptibility to apoptosis [33].

Redox Targets of SIRT1

A large number of sirtuin deacetylation substrates have now been identified in the human proteome, and several of these are related to maintaining redox homeostasis within the cell. One of the first major targets of SIRT1 to be identified was the tumor suppressor p53. As a transcription factor, p53 is involved in activating a suite of pro- and anti-oxidant genes, such as sestrins, mitochondrial manganese superoxide dismutase and glutathione peroxidase 1 (reviewed in [34]). SIRT1 binds and deacetylates p53 at Lys382, which attenuates its transcriptional activity [35, 36]. The cellular localization of p53 in response to reactive oxygen species (ROS) has been shown to be dependent on SIRT1 deacetylation, and may help to regulate the switch from antioxidant protection to apoptosis. Over-expression of SIRT1 attenuated the transcriptional activity of p53 in response to exogenous hydrogen peroxide treatment, which prevented the induction of a p53-mediated apoptosis program [37]. At lower ROS levels, the SIRT1-p53 interaction appears to be responsible for regulating the induction of an antioxidant program. In wild-type mouse embryonic stem cells, the removal of culture medium antioxidants led to the

translocation of p53 to mitochondria and subsequent apoptosis. However in SIRT1^{-/-} cells, this endogenous increase in ROS led to the nuclear translocation of p53, which the authors speculated would result in the induction of an antioxidant response [38].

A second pathway that relies on sirtuins to activate an antioxidant response involves the forkhead box O (FOXO) transcription factor family. Both SIRT1 and SIRT2 have been shown to deacetylate and activate FOXO3a in response to oxidative stress [39, 40]. This is significant, as FOXO3a is a transcriptional activator of the *SOD2* gene which encodes the mitochondrial-localized MnSOD antioxidant protein [41]. A second target of SIRT1-FOXO3a is catalase, an important enzyme that protects against damage caused by excess hydrogen peroxide. Again, the action of SIRT1 appears to be bi-directional, with low levels of hydrogen peroxide leading to the upregulation of FOXO3a-induced catalase, and higher levels leading to the switch to FOXO3a-mediated apoptosis [42]. In the heart, pressure overload and related oxidative stress leads to an upregulation in SIRT1, and mimicking this increase using transgenic over-expression of SIRT1 in mice leads to the induction of protective mechanisms, such as increased catalase expression [43]. However, high levels of SIRT1 led to increased oxidative stress, apoptosis and cardiac hypertrophy. As such, these sirtuins appear to act as ROS sensors, with the ability to induce protective mechanisms in response to low-level stresses, and signaling for apoptosis when the stress becomes too great.

Given the requirement for NAD⁺ in sirtuin function, mechanisms which regulate the intracellular NAD⁺:NADH ratio have a powerful role in SIRT1 regulation. One of the most closely studied is the interaction between cellular NAD⁺ levels (particularly the

NAD⁺ salvage pathway) and AMP-activated kinase (AMPK), a key energy homeostasis enzyme. One of the first studies to link AMPK function and SIRT1 activity looked at glucose metabolism during muscle development, and found that reduced levels of glucose led to the activation of SIRT1 [44]. Reducing the glucose available to myoblasts led to the activation of AMPK and, consequently, the induction of NAD⁺ salvage pathway enzyme Nampt, which increased intracellular NAD⁺ levels and activated SIRT1 [44]. These results were further extended by studies demonstrating that AMPK, acting as a metabolic fuel sensor, could stimulate transcriptional activity downstream of SIRT1 [45]. A reduction in cellular energy stores leads to the phosphorylation and activation of AMPK, which leads to an increase in available NAD⁺ within the cell. This stimulates SIRT1 to activate several transcriptional activators, such as FOXO proteins and the peroxisome proliferator-activator receptor- α coactivator 1 α (PGC-1 α), leading to an upregulation of genes involved in catabolism and mitochondrial biogenesis [46]. The activation of AMPK in this manner can also occur through SIRT1, using positive feedback mechanisms. LBK1, an upstream kinase that phosphorylates and activates AMPK under nutrient stress conditions, has been shown to be acetylated on multiple lysine residues. Stimulation or over-expression of SIRT1 leads (either directly or indirectly) to the deacetylation of LBK1, which promotes its translocation from the nucleus to the cytoplasm, allowing it to phosphorylate AMPK [47], [48]. As such, there appears to be multiple levels of metabolic regulation occurring through the AMPK-SIRT1 axis, and many of these steps require further elucidation.

As noted above, SIRT1 has the ability to interact with and deacetylate PGC-1 α , a major transcriptional coactivator involved in cellular metabolism and mitochondrial

biogenesis. The functional role of this deacetylation, however, appears to depend greatly upon the tissue type and metabolic conditions in which it takes place. It has been demonstrated that in the liver, SIRT1 is activated in response to fasting, which leads to the deacetylation of PGC-1 α [49]. Acting through this pathway, PGC-1 α deacetylation can both inhibit glycolytic genes and induce the expression of those involved with gluconeogenesis [49]. A contemporary study showed that in the adrenal PC12 cell line, SIRT1 could directly interact with and deacetylate PGC-1 α , and that this led to a decrease in both PGC-1 α transcriptional activity and related mitochondrial oxidative metabolism [50]. Conversely, induction of SIRT1 activity using resveratrol, and subsequent PGC-1 α deacetylation, led to an increase in mitochondrial function in numerous oxidative tissue types (including muscle and brown adipose tissue), indicating that deacetylation was responsible for an increase in PGC-1 α transcriptional activity [51]. In skeletal muscle, fasting induced SIRT1 activity and PGC-1 α deacetylation, which led to an increase in mitochondrial fatty-acid oxidation [52]. More recent work has indicated that SIRT1-dependent deacetylation of PGC-1 α is required for the induction of mitochondrial biogenesis and activity in response to adiponectin in muscles [53] and Fibroblast Growth Factor 21 in adipocytes [54], and therefore is crucial to the function of these anti-diabetic metabolic regulators. Loss of PGC-1 α activity has been linked to a down-regulation of mitochondrial anti-oxidant proteins, such as MnSOD, indicating that SIRT1 may have a direct effect on cellular redox states through this pathway [55].

Following the discovery of resveratrol, and its effect on health and lifespan, there were several avenues of research that opened up to elucidate the mechanisms involved. One pathway that was of interest to the cardiovascular field was the effect of resveratrol

on the endothelial nitric oxide synthase gene, eNOS, which when uncoupled leads to ROS production and quenching of nitric oxide (NO). Previous work had shown that resveratrol was an agonist of the estrogen receptor [56], and that estrogens led to the upregulation of the eNOS gene involved in the synthesis of cardioprotective NO (reviewed in [57]). Addition of resveratrol to endothelial HUVEC cells led to a time- and concentration-dependent increase in eNOS expression and cellular NO levels [57], indicating a direct effect on the NO pathway. Similar research showed that calorie restriction, another known SIRT1 activator, was capable of enhancing eNOS expression and increasing mitochondrial biogenesis through transcription factors such as PGC-1 α [58]. To square the circle, further research demonstrated that SIRT1 interacted with and deacetylated eNOS *in vivo*, leading to an upregulation of eNOS activity and intracellular NO levels [59]. As such, it would appear that SIRT1 has an important cardioprotective effect on vascular tone in an eNOS-dependent manner.

SIRT1 in hypoxia

As with other aspects of redox stress, non-mitochondrial sirtuins play a prominent role in the response to hypoxia. The transcriptional response to hypoxia is regulated, in a large part, by the Hypoxia Inducible Factor (HIF) family of proteins, the best characterized of which are HIF1 α and HIF2 α (reviewed in [60]). HIF proteins are constitutively expressed, however under normoxic conditions they are kept at low levels by continual hydroxylation and proteosomal degradation. A change in the cellular environment from normoxia to hypoxia attenuates this proteolysis, stabilizing the HIF subunits and allowing them to transcriptionally upregulate key hypoxia-response genes.

Both HIF1 α and HIF2 α have been shown to be targets of SIRT1 deacetylation, however this interaction leads to very different outcomes for these two subunits. HIF2 α forms a complex with SIRT1 under hypoxic conditions, and is deacetylated at three lysine residues (K385, K685, and K741) in the carboxy terminus [61]. This deacetylation upregulates HIF2 α transcriptional activity, and leads to an increase in the abundance of HIF2 α -dependent hypoxia-related proteins such as erythropoietin [61]. Recent work by the same group has shown that SIRT1 protein levels are increased at the initiation of hypoxia, and that this induction is regulated by HIF2 α transcriptional activity [31].

In contrast to HIF2 α , SIRT1 has been shown to act as a repressor of HIF1 α activity. Under normal conditions SIRT1 can bind to, and deacetylate, HIF1 α . This deacetylation prevents HIF1 α from interacting with the transcriptional coactivator p300, thereby blocking its transcriptional activity [62]. However, under hypoxic conditions, the repression of HIF1 α by SIRT1 is lifted indirectly through changes in the metabolic environment. The reduction in oxygen levels during hypoxia leads to a shift in the NAD⁺:NADH ratio, and results in a decrease in the NAD⁺ available to stimulate SIRT1. This NAD⁺-dependent loss of SIRT1 activity allows HIF1 α to remain acetylated, thereby facilitating its hypoxic transcriptional activity [62]. Interestingly, HIF1 α activity has also been shown to be regulated by SIRT6. HIF1 α transcriptionally regulates a number of genes involved in the shift to glycolysis under low oxygen conditions. These genes, along with HIF1 α itself, are upregulated in SIRT6-deficient cells, leading to an increase in glucose uptake and glycolysis [63]. In wild-type cells, SIRT6 acts as a H3K9 histone deacetylase, blocking the HIF1 α -dependent transcription of multiple glycolytic genes, and co-repressing HIF1 α in a deacetylase-dependent manner [63]. As such, it would

appear that while SIRT1 can regulate the activity of HIF subunits in general, SIRT6 may act as a glucose metabolism-specific regulator of hypoxia-related genes.

The redox phenotype in SIRT1 knockout and transgenic mice

The composite of data discussed would suggest that SIRT1 should play an adaptive role in protection against redox stressors. A major limit to testing this hypothesis has been the production of SIRT1^{-/-} mice, as this deletion leads to high levels of embryonic lethality. Nevertheless, *in vivo* studies have been performed with either heterozygous SIRT1^{+/-} mice, tissue-specific knockout mice, or following over-expression of SIRT1 using transgenic technology. Here mice have been studied in the context of degenerative and redox stress diseases. Heterozygous SIRT1 knockout mice show increased susceptibility to oxidative stress in the kidney [64] and the combined SIRT1^{+/-} p53^{+/-} mice show greater tumor susceptibility compared to p53 haploinsufficiency alone [65]. Tissue-specific deletion of SIRT1, including in the liver and brain, has been shown to negatively alter fat metabolism and increase susceptibility to diet-induced obesity [66, 67]. Limited copy number over-expression of SIRT1 has been shown to protect against redox stress in the brain, heart and kidneys, to ameliorate hepatic hepatosteatosis that results from diet induced obesity, and to ameliorate colon cancer [43, 68-70]. SIRT1 is also implicated, as necessary, to enable the innate adaptive reprogramming to resist ischemia-reperfusion injury, as a component of the ischemic preconditioning program [71].

Additional nuclear and cytosolic sirtuins and redox biology

Although the role of SIRT2 has not been as well characterized as SIRT1 or SIRT3,

it does play a regulatory role in modulating redox stress tolerance. SIRT2 is mainly localized in the cytosol, however it can undergo translocation to the nucleus during certain events, such as mitosis [72]. Indeed, other sirtuins such as SIRT3 have been shown to translocate under different cellular conditions [73], although this may be the result of using an over-expression vector with an incomplete murine SIRT3 [74]. Redox stress has been shown to result in the upregulation of both SIRT2 transcript and protein levels [40, 75]. Functional characterization of this process suggests that redox-stress upregulation of SIRT2 is associated with increased cell death, in parallel with the induction of the pro-apoptotic protein Bim [40], and that overexpression of SIRT2 similarly promotes neurodegeneration [76]. In contrast, basal levels of SIRT2 under low-stress conditions upregulate mitochondrial MnSOD via FOX3a deacetylation, with the subsequent attenuation of reactive oxygen species levels [40]. Genetic knockdown of SIRT2 is associated with upregulation of the cytosolic chaperone 14-3-3 ζ , which in turn sequesters the pro-apoptotic mitochondrial protein BAD in the cytosol and augments tolerance against anoxia-reoxygenation induced cell death [75]. A ‘dose-dependent’ function of SIRT2 under redox stress conditions is further supported by data showing that a SIRT2 inhibitor rescues α -synuclein-mediated toxicity in a cellular model of Parkinson’s disease [77], and that SIRT2 knockdown is protective in neuronal and cardiac cells [75, 77].

The role of SIRT6 in biological control under redox and hypoxic stress has not been well characterized, although mice lacking SIRT6 are predisposed to accelerated senescence. This includes the development of degenerative features, telomerase shortening and a lifespan limited to approximately 4 weeks [78]. As noted above, SIRT6

additionally modulates hypoxic signaling through corepression of HIF1 α during the control of glucose homeostasis [63]. SIRT7 is the least-well studied sirtuin, although the genetic knockout of this gene does have a robust phenotype, with a 50% reduction in lifespan and the development of degenerative features and an inflammatory cardiomyopathy [79]. In the absence of SIRT7, p53 exhibits robust acetylation, which is postulated to diminish resistance to genotoxic and oxidative stress [79]. The role of SIRT7 in ameliorating oxidative stress is also evident in that it blunts cell proliferation under oxidative stress conditions [80]. A summary of the role played by non-mitochondrial sirtuins in redox biology can be found in Figure 1.

Mitochondrial sirtuins

The role of redox signaling and oxygen in the regulation of SIRT3

Of the three family members found in the mitochondria, SIRT3 functions as the major sirtuin deacetylase [81]. An initial controversy regarding the localization of the murine SIRT3 [82] has now been resolved, with the identification and functional characterization of its mitochondrial localization sequence [74, 83, 84]. The number of SIRT3 substrates identified is increasing at a prodigious pace, and their role in numerous biological programs is still being explored. Here we focus on those substrates that modulate redox-stress directly, and discuss their role in redox-stress associated pathology. SIRT4 and SIRT5 are also enriched in the mitochondria, although fewer substrates of these two sirtuins have been described. As mentioned previously Sirt5 may function to remove succinyl and/or malonyl, rather than acetyl, groups from mitochondrial targets [10]. However, where they are relevant to the role of sirtuin biology in redox stress, these data have been included. As numerous SIRT3 mitochondrial targets

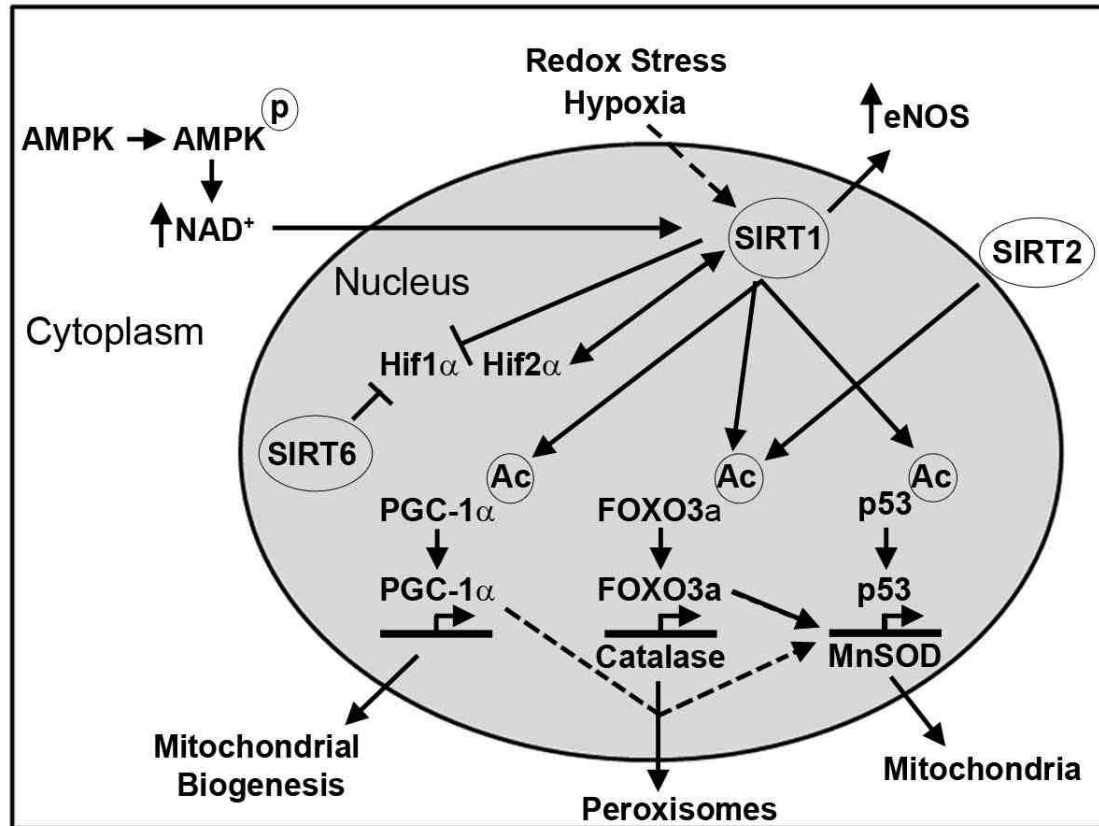


Figure 1 Of the non-mitochondrial sirtuins, SIRT1 is the most widely characterized deacetylase. In common with the other sirtuins, SIRT1 is activated by NAD⁺; levels of which can be increased following AMPK activation. Redox stress and hypoxia may, directly or indirectly, increase SIRT1 levels or activate its deacetylase activity (broken arrow). In terms of oxidative stress, one of the main roles of SIRT1 is to deacetylate and activate transcription factors involved in upregulating antioxidant proteins, such as MnSOD and catalase. In addition, SIRT2 has also been shown to activate the transcription factor FOXO3a through deacetylation. The activity of SIRT1, along with SIRT6, can inhibit HIF1 α activity; however, SIRT1 has the opposite effect on HIF2 α . Finally, SIRT1 deacetylates and activates the eNOS, leading to an increase in intracellular cardioprotective NO.

play a role in the control of ROS generation and amelioration (see below), studies have begun to explore whether ROS levels themselves regulate SIRT3. Both SIRT3 transcript and protein levels are induced by the generation of mitochondrial ROS [85]. Whether SIRT3 is modulated at the post-translational level, in response to redox signaling, has not been explored. However, prolonged high fat feeding results in evidence of hepatic redox stress, and an associated reduction in SIRT3 protein levels [86]. SIRT3 levels are also induced in parallel with mitochondrial biogenesis, where the transcriptional co-activator PGC-1 α and transcription factor ERR α , respectively, transactivate the SIRT3 gene promoter [87].

Redox Targets of SIRT3

Mitochondria are the major source of cellular oxidative stress, with ROS formation occurring during normal function of the electron transport chain (ETC). Any reduction in mitochondrial function, through processes such as metabolic disease progression or aging, can augment oxidative stress [88]. Mitochondria have therefore developed numerous biological programs to combat this innate oxidative stress and maintain functional homeostasis. One such program utilizes reduced glutathione, via the generation of NADPH by isocitrate dehydrogenase 2 (IDH2). IDH2 uses NADP⁺ as a substrate in the TCA cycle, during the decarboxylation of isocitrate to α -ketoglutarate, which yields NADPH. This, in turn, is used for the regeneration of reduced glutathione to confer antioxidant properties [89]. IDH2 was initially shown to be differentially acetylated in a nutrient dependent manner in a proteomics screen by Kim *et al.* [90]. A role for SIRT3 in this process was identified by Schlicker *et al.*, who showed that in the

presence of NAD⁺ purified SIRT3, but not SIRT5, deacetylated IDH2 and increased its activity [91]. The sites of SIRT3 deacetylation (K211 and K212) found by Schlicker *et al.* were different to those identified in the initial proteomics screen (K75 and K241), and it is unclear how the latter two residues are regulated [90, 91]. Recently, this work has been linked to age-related hearing loss (AHL). It was previously shown that CR attenuates AHL by slowing neuronal loss in the cochlea, which correlated with increased SIRT3 expression levels [89]. Further work demonstrated that CR prevented AHL in 12 month old mice and attenuated spiral ganglion neuronal loss. However, these benefits were completely abolished in SIRT3 knockout animals, suggesting that the process was SIRT3-dependent [89]. CR also ameliorated oxidative damage to DNA in various organs, including the brain and liver, and promoted hair cell survival in the cochlea of aged mice. To provide a mechanism for these SIRT3-dependent benefits, Someya *et al.* showed that GSH/GSSG ratios were increased, and that NADPH levels were elevated in liver, brain and the inner ear following CR. CR also decreased the acetylation levels of IDH2, increasing its activity, in a SIRT3-dependent manner [89]. Confirming previous results, they further demonstrated that SIRT3 directly deacetylates and augments the activity of IDH2, and that overexpression of SIRT3 or IDH2 was sufficient to increase NADPH levels and attenuate oxidative stress-mediated cell death [89]. Linking CR, SIRT3 and IDH2 supports the concept that oxidative stress is a major component of aging, and that nutrient status can regulate the cellular response to degenerative pathologies.

Another major mitochondrial defense against oxidative stress is superoxide dismutase (SOD). Humans express three forms of SOD, which converts superoxide

anion (O_2^-) to hydrogen peroxide (H_2O_2), limiting ROS damage to nucleic acids, proteins and lipids. SOD1 and SOD3 are localized in the cytosol and extracellular space, using copper and zinc as prosthetic groups, respectively. SOD2 contains manganese (MnSOD) and localizes to the mitochondria [92]. MnSOD, like IDH2, has been linked to age-related disorders such as cancer and cardiovascular disease [93-95]. SIRT3 was first shown to regulate SOD2 by modulating FOXO3a activity in the mitochondria, which led to subsequent FOXO3a-mediated SOD2 expression [95, 96]. It is not clear if these results were due to an over-expression artifact leading to the nuclear localization of SIRT3 [74]. Nevertheless, more recent evidence has directly tied SIRT3 to MnSOD function. Qiu *et al.* showed that caloric restriction lowers oxidative stress in a SIRT3 dependent manner [97]. SIRT3 overexpression in mouse embryonic fibroblasts (MEFs) lowered ROS levels in an SOD2-dependent manner, suggesting that acetylation status regulates SOD2 activity. They further showed that SIRT3 interacts with SOD2, and that SIRT3-mediated deacetylation of SOD2 increased its activity, which provided protection against oxidative stress [97]. SOD2 was previously identified as an acetylated protein at K68 and K130 [90, 98]. However, there is some uncertainty regarding which acetylated lysine residue regulates SOD2 activity. Qui *et al.* showed that mutating K68 and K130 did not alter total acetylation levels of SOD2. However acetylation of K53 and K89, two lysine residues near the active site of SOD2, regulated its activity [97]. While a second study by Tao *et al.* confirmed that SIRT3 regulates SOD2 activity, and subsequent protection against ionizing radiation (see below), this group identified K122 as the crucial lysine [99]. Fasting wild-type mice for 36 hours promoted deacetylation of SOD2, which was blocked in SIRT3 knockout animals. Hyperacetylation of SOD2 in SIRT3-deficient mice

led to lower enzymatic activity and elevated ROS levels [99]. Further complicating the post-translational regulation of SOD2, Chen *et al.* recently showed that acetylation of K68 does indeed regulate SOD2 activity, and that SIRT3 deacetylates SOD2 at this site [85]. The differences observed in the site-specific regulation of SOD2 by SIRT3 may reflect cell type, species or stress conditions, or may reflect a higher-order regulation that requires changes in several lysine residues. As Chen *et al.* state, it is interesting to note that the lysine to arginine mutation (which mimics deacetylation) increased SOD2 activity for K53R, K89R and K122R mutants, while in the K68R mutant it does not [85]. Although the precise site(s) of regulation remains unclear, it is clear ROS levels are tightly controlled by SIRT3 by increasing the activity of two major ROS regulating enzymes, IDH2 and SOD2.

Indirect SIRT3 and SIRT5 targets for regulation of mitochondrial oxidative stress

MnSOD and IDH2 represent SIRT3 targets that directly regulate mitochondrial ROS. However, other targets of SIRT3 and SIRT5 have indirect effects on oxidative stress. As mentioned previously, the electron transport chain generates ROS, and SIRT3 has been shown to deacetylate complexes I, II, V and most recently III [86, 100, 101]. SIRT5 is also implicated in the deacetylation of cytochrome c, although this has not been functionally characterized [91]. Secondly glutamate dehydrogenase (GDH), which can regulate glutamate oxidative stress and generate NADPH, is deacetylated by SIRT3 [81] (and inhibited by SIRT4 mediated ADP-ribosylation) [102]. A final example of indirect regulation of ROS levels by mitochondrial sirtuins occurs during fasting, when protein catabolism is augmented to provide TCA cycle intermediates. Protein catabolism leads

to the generation of ammonium, which can result in oxidative stress [103]. To prevent damage, ammonium is converted to urea for subsequent elimination in the urea cycle. SIRT3 and SIRT5 regulate the mitochondrial localized reactions of the urea cycle, with SIRT5 acting on carbamoyl-phosphate synthase 1 (CPS-1), and SIRT3 deacetylating ornithine transcarbamoylase (OTC) [20, 104]. Both deacetylation reactions promote urea cycle function, leading to the elimination of the oxidative stress-promoting ammonium.

Biological roles of SIRT3 in redox-associated pathology

Oncogenesis

Numerous studies have highlighted a pivotal role for SIRT3 in cancer, even going as far as referring to it as a tumor suppressor. However, the ability of SIRT3 to act as a tumor suppressor is directly related to its role in regulating ROS levels. SIRT3 knockout cells display a propensity towards carcinogenesis, with increased ROS, nuclear and mitochondrial genomic instability and diminished contact inhibition [93]. Combining the expression of oncogenes with deletion of SIRT3 further promotes a cancer phenotype, which can be rescued by overexpression of SOD2. SIRT3 knockout mice also have a higher incidence of breast cancer, while human breast cancer samples exhibit lower levels of SIRT3 [93].

Cancer cells display a characteristic metabolic profile, and SIRT3 appears to play a role in preventing the switch to this phenotype. In cancer cells, hexokinase II activity is greatly upregulated, which promotes increased glycolysis and attenuates oxidative phosphorylation – classic indicators of the Warburg effect (reviewed in [105]).

Hexokinase II binds to VDAC in the outer mitochondrial membrane, and utilizes ATP to generate glucose-6-phosphate (G6P). G6P is shuttled through glycolysis, generating pyruvate and lactic acid; or through the pentose phosphate pathway to generate NADPH, which is used to drive anabolic reactions. Some of the G6P is also used to generate citrate in the mitochondria, which enters the cytosol to allow phospholipid and cholesterol synthesis. SIRT3 knockout MEFs show elevated levels of glycolytic and pentose phosphate pathway metabolites, increased glucose uptake and lactate production, and lower levels of TCA cycle intermediates – all suggesting a metabolic profile similar to cancer cells [106]. SIRT3 has been shown to deacetylate cyclophilin D (CypD), which limits its interaction with the mitochondrial permeability transition pore (MPTP). Importantly, this deacetylation also prevents hexokinase II from binding to VDAC and the mPTP, limiting its ability to use ATP and augment glycolysis [107]. Shulga *et al.* speculate that this would limit the transition to a Warburg phenotype, thereby attenuating tumor growth [107]. Other studies have also found that SIRT3 drives oxidative phosphorylation and augments ATP generation [74, 100].

Metabolic reprogramming of cancer cells relies on HIF-1 α , and the loss of SIRT3 leads both to its stabilization, and increased levels of HIF-1 α gene targets, including hexokinase II [106]. As noted above, SIRT1 can deacetylate HIF-1 α . Although SIRT3 does not directly interact with HIF-1 α , HIF-1 α is stabilized in SIRT3 knockouts via an indirect mechanism. The increased ROS levels found in SIRT3 knockouts increase HIF-1 α stabilization promoting cancer cell metabolism [106]. Bell *et al.* also showed the effect of SIRT3 on HIF-1 α stability, along with the ability of SIRT3 to attenuate tumor growth using xenograft models [108]. SIRT3 also appears to regulate p53 activity, by

altering growth arrest and apoptosis in a complex manner involving a similar mechanism to HIF-1 α , namely elevated ROS levels affecting p53 activity [109-111]. Although the majority of data shows that SIRT3 can act as a tumor suppressor, SIRT3 may promote tumorigenesis in some forms of cancer. SIRT3 expression levels were elevated in oral squamous cell carcinoma (OSCC); while inhibition and down regulation of SIRT3 slowed growth, promoted apoptosis and increased susceptibility to radiation and chemotherapy [112]. These conflicting roles of SIRT3 as tumor suppressor or promoter may be due to its role in regulating p53, with SIRT3 inhibiting the ability of p53 to arrest growth [110]. Overall it appears that Sirt3 can regulate cellular energetics, and possibly substrate selection such as lipids (see below), by increasing mitochondrial oxidative phosphorylation through deacetylation of various electron transport chain targets including complexes I, II and V (sources) and cyclophilin D attenuating ROS levels and glycolytic metabolism.

Lipotoxicity

Obesity and over-nutrition can lead to diabetes, the metabolic syndrome and non-alcoholic fatty liver disease (NAFLD), along with an increase in circulating free fatty acids, i.e. lipotoxicity. Lipotoxicity generates a pro-inflammatory state with increased oxidative stress, promoting insulin resistance systemically and diminishing insulin secretion by the pancreas (reviewed in [113]). Concurrent with this pro-inflammatory state, mitochondrial function and biogenesis, through defects in PGC-1 α , also diminish with obesity and diabetes – further promoting ROS generation [3]. Interestingly,

expression of SIRT3 leads to an upregulation of PGC-1 α and consequently the transcription of its biogenesis gene targets [87, 114, 115].

Nutritional status greatly alters mitochondrial protein acetylation levels, with fasting and high fat diets leading to the hypo- and hyper-acetylation of mitochondrial targets, respectively [90, 116]. Hirschey *et al.* showed that fasting increases SIRT3 expression and lowers acetylation levels in mouse livers. Although phenotypically normal, SIRT3 knockout animals had higher levels of triglycerides and β -oxidation intermediates, suggesting a defect in lipid processing. They showed that long chain acyl-CoA dehydrogenase (LCAD) is hyperacetylated at K42 in SIRT3 knockout mice, leading to lower activity, and that SIRT3 deacetylates LCAD directly [117]. Hallows *et al.* also found LCAD to be a SIRT3 target [20]. It has also been shown that SIRT3 prevents lipid accumulation in HepG2 cells by acting alongside AMPK, which phosphorylates acetyl-CoA carboxylase (ACC) and inhibits lipid synthesis. SIRT3 appears to regulate AMPK activity, however the mechanism of the cross-talk between these two nutrient sensing proteins is currently unknown [118]. On the opposite end of the nutritional spectrum, six weeks of high fat diet (HFD) led to hyperacetylation of numerous protein targets in mouse livers involved in metabolic pathways [116]. Under these conditions, SIRT3 activity was diminished, while oxidative stress was elevated, further supporting a role of SIRT3 in regulating oxidative stress [116]. SIRT3 knockout animals exposed to HFD showed even higher levels of acetylation and diminished mitochondrial respiration [116]. The absence of SIRT3 similarly increased susceptibility to lipotoxicity in primary hepatocytes, and this phenotype was ameliorated by either the reconstitution of SIRT3, or via the administration of the antioxidant N-acetylcysteine [86]. It appears that SIRT3

may alleviate the pro-inflammatory state induced by lipotoxicity through a variety of mechanisms, including altering mitochondrial biogenesis, aiding oxidative stress systems and regulating lipid accumulation. SIRT3 may therefore represent a potential new target to combat lipotoxicity and its concurrent disease states.

Cardiac hypertrophy

Sirtuins and protein deacetylation have also been linked to cardiovascular health [119]. The renin-angiotensin system (RAS) and Angiotensin II (Ang II) are common targets in the treatment of cardiovascular disease, including hypertension, renal disease, hypertrophy and Marfan syndrome. Ang II is pro-inflammatory and increases ROS generation [120]. Similar to CR, knockout of Ang II Receptor (AT1R) in mice increased lifespan by 26%, and AT1R knockouts have lower levels of cardiac fibrosis, hypertrophy and attenuated aortic damage [121]. AT1R deficient mice also showed lower levels of oxidative stress, and increased mitochondrial density when compared to controls. As a possible mechanism explaining this anti-aging phenotype, it was shown that AT1R knockouts had higher levels of both SIRT3 and the NAD⁺ generating enzyme nicotinamide phosphoribosyltransferase, Nampt. Conversely, AngII treatment lowered the expression levels of Nampt and SIRT3, with these effects being blocked by candesartan, an AT1R blocker [121]. Further linking the sirtuins to promoting cardiovascular health, Miyazaki et al. showed that Sirt1 attenuates AT1R expression levels [122].

To directly investigate the role of SIRT3 in cardiac hypertrophy, Sundaresan *et al.* induced cardiac hypertrophy chemically and physically; both of which enhanced SIRT3

expression [94, 95]. SIRT3 knockout animals subjected to the same stimuli displayed an exacerbated hypertrophic response, suggesting that the augmented SIRT3 level seen in wild-type animals is a protective response used to combat the hypertrophic program. Indeed, overexpression of SIRT3 attenuated the hypertrophic response both *in vivo* and *in vitro*, preserving cardiac function [95]. Increasing exogenous levels of the SIRT3 substrate, NAD⁺, attenuated the hypertrophic response through SIRT3 deacetylation and activation LKB1 and AMPK. Hsu *et al.* also showed that elevated NAD⁺, as mediated by Nampt, induces a cardio-protective phenotype [123]. As mentioned previously, it is not clear if the SIRT3-mediated effect on nuclear and cytosolic targets, i.e. FOXO3a and LKB1, is a by-product of over-expression of the short form of SIRT3 that fails to localize to the mitochondria, or if SIRT3 acts on targets outside the mitochondria through unspecified mechanisms. An overview of the role of the mitochondrial sirtuins on redox stress biology is schematized in Figure 2.

ALDH2 and acetaminophen - an exception to the redox-stress ameliorative role of SIRT3

Unlike the protective role of SIRT3 in modulating redox-stress described above [86, 89, 99], it has recently been found that SIRT3-mediated mitochondrial protein deacetylation exacerbated oxidative stress associated with acetaminophen-induced liver injury (AILI) [124]. The level of the lipid peroxidation adduct, trans-4-hydroxy-2-nonenal (4-HNE) was lower in SIRT3^{-/-} mice, compared to SIRT3^{+/+}, in response to a toxic dose of acetaminophen, in parallel with less AILI in the SIRT3^{-/-} mice. The resistance of SIRT3^{-/-} mice to AILI was partly due to the acetylation of mitochondrial aldehyde dehydrogenase 2 (ALDH2), a dehydrogenase which functions to oxidize and detoxify aldehydes,

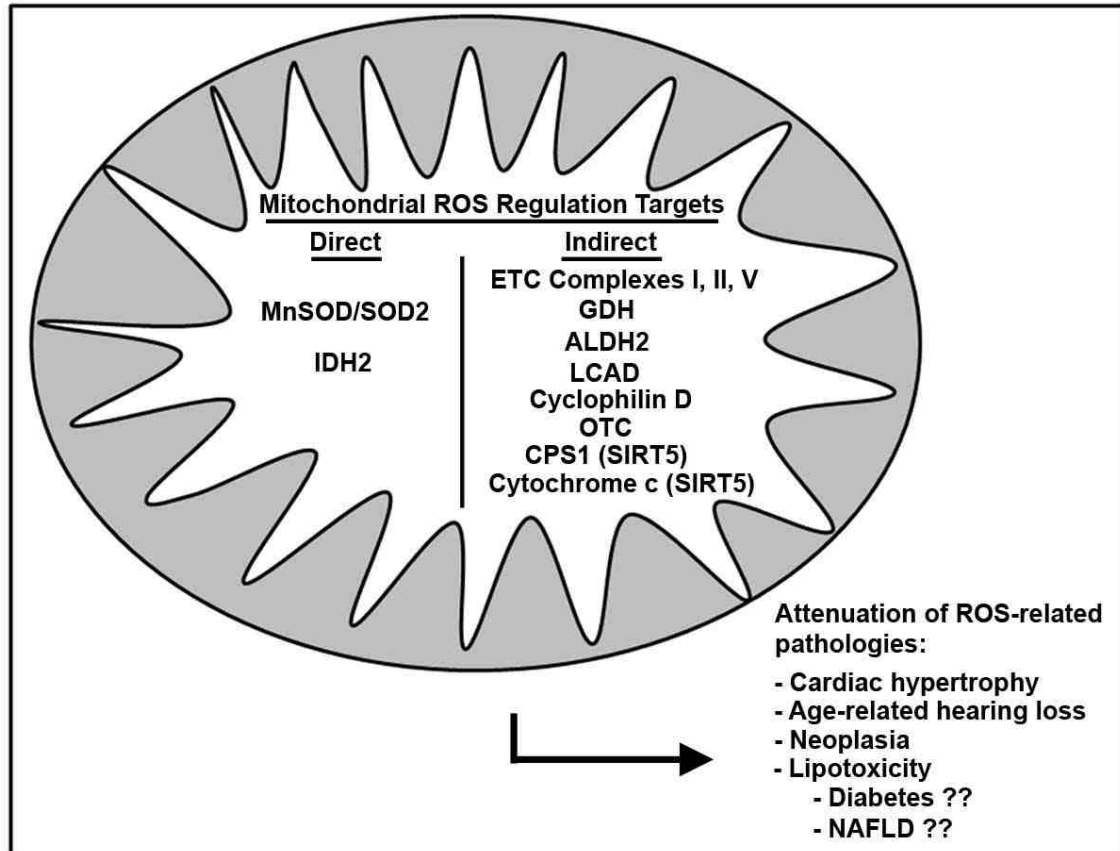


Figure 2 Mitochondrial localized SIRT3 and SIRT5 regulate oxidative stress utilizing both direct and indirect mechanisms. Deacetylating manganese superoxide dismutase (MnSOD/SOD2) and isocitrate dehydrogenase 2 (IDH2) augments their activity, providing a direct mechanism to regulate ROS. The deacetylation of various other mitochondrial enzymes indirectly attenuates oxidative stress, which includes regulating mitochondrial function, namely through deacetylation of cytochrome c and complexes of the electron transport chain (ETC I, II and V). Another indirect mechanism is through controlling ROS generating metabolic intermediate production by deacetylating aldehyde dehydrogenase (ALDH2), glutamate dehydrogenase (GDH), long chain acyl-dehydrogenase (LCAD), carbamoyl phosphate synthetase I (CPS-1) and ornithine transcarbamylase (OTC). Finally SIRT3 can regulate apoptosis and glucose metabolism through its actions on cyclophilin D. The cumulative effect of this ROS regulation is to attenuate various age-related pathologies including oncogenesis, cardiac hypertrophy, lipotoxicity and age-related hearing loss.

including the lipid peroxidation product 4-HNE [125]. In contrast to the known anti-oxidant effect of SIRT3, via the deacetylation and activation of redox scavenger substrates, SIRT3-mediated deacetylation of ALDH2 does not change its enzyme activity directly. Rather, the deacetylation of ALDH2 facilitates the binding of the acetaminophen toxic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI), directly to ALDH2, with a concomitant blunting of dehydrogenase activity [124]. Taken together, this finding suggests that the acetylation of mitochondrial proteins can also function as an allosteric inhibitor to the binding of reactive metabolites. This mechanism is proposed to function in the enhanced susceptibility to acetaminophen-induced injury under fasting and caloric restricted conditions [126, 127], nutrient states known to promote the activation of sirtuin enzymes.

Conclusions and future directions

The majority of the work carried out thus far on mammalian sirtuin proteins has focused on SIRT1 and SIRT3. SIRT1, as the major nuclear deacetylase, plays a pivotal role in the acetylation-based regulation of numerous proteins, including many that form the transcriptional response to changes in redox conditions. SIRT3, as the major mitochondrial deacetylase, acts as the *in situ* regulator of proteins, which ameliorate damage in one of the major ROS-producing organelles in the cell. Our knowledge of the functional activity of these proteins has grown exponentially over the last few years, and will likely continue to uncover new pathways regulating the cellular response to oxidative stress. Table 1 summarizes all the ROS modulatory proteins that are substrates of the sirtuins and describes the functional consequences of sirtuin activation. One of the major

fundamental areas to be tackled in this field is how intracellular redox conditions affect the transcriptional control of SIRT1 and SIRT3 protein levels. Work in this area will help to determine the level of feedback control inherent in this system, and will establish how finely balanced these mechanisms are. Additionally, both in the redox field in particular, and the sirtuin field in general, future research effort will likely turn to the other sirtuins. While SIRTs 2,4,5,6 and 7 may have a more limited functional scope than SIRT1 and SIRT3, it is likely that their substrate-specific activity will be crucial in the regulation of many important pathways. As such, while they appear to be the forgotten members currently, the future may yield much more insight into their function within the cell.

Acknowledgements

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Redox-related Gene	Sirtuin	Localization	Result of Sirtuin Action	Reference(s)
p53	SIRT1, SIRT7	Nucleus	Mediates transcriptional activity depending on sirtuin expression level	33,34,77
FOXO3a	SIRT1, SIRT2	Nucleus, cytoplasm	Activates transcriptional activity	37,38
LBK1	SIRT1	Nucleus	Activates LBK1, leading to AMPK phosphorylation	45,46
PGC-1 α	SIRT1	Nucleus	Activates transcriptional co-activation	47-53
eNOS	SIRT1	Nucleus	Activates eNOS activity	57
HIF1 α	SIRT1, SIRT6	Nucleus	Repression of transcriptional activity	60,61
HIF2 α	SIRT1	Nucleus	Activates transcriptional activity	29, 59
14-3-3 ζ	SIRT2	Cytoplasm	Upregulates 14-3-3 ζ prevents anoxia-reoxygenation mediated apoptosis	73
IDH2	SIRT3	Mitochondria	Activates enzyme, reduces ROS	87-89
SOD2	SIRT3	Mitochondria	Activates enzyme, reduces ROS	83, 95-97
NDUFA9	SIRT3	Mitochondria	Activates enzyme, upregulates ETC activity	98
SDHB	SIRT3	Mitochondria	Activates enzyme, upregulates ETC activity	99
ATP5A	SIRT3	Mitochondria	Activates enzyme, upregulates ETC activity	84
GDH	SIRT3, SIRT4	Mitochondria	Activates enzyme (SIRT3), represses enzyme by ADP-ribosylation (SIRT4)	79,100
OTC	SIRT3	Mitochondria	Activates enzyme, reduces ammonia toxicity and ROS	19
CPS1	SIRT5	Mitochondria	Activates enzyme, reduces ammonia toxicity and ROS	102
CypD (PPIF)	SIRT3	Mitochondria	Deactivates enzyme, prevents interaction with mPTP	105
LCAD	SIRT3	Mitochondria	Activates enzyme, promotes lipid processing	19,115
ALDH2	SIRT3	Mitochondria	Deacetylation by SIRT3 allows NAPQI binding to ALDH2, reducing its activity	122

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Appendix 3

The emerging characterization of lysine residue deacetylation on the modulation of mitochondrial function and cardiovascular biology

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ABSTRACT

There is emerging recognition of a novel fuel and redox sensing regulatory program that controls cellular adaptation via non-histone protein lysine-residue acetyl post-translation modifications. This program functions in tissues with high energy demand and oxidative capacity and is highly enriched in the heart. Deacetylation is regulated by NAD⁺-dependent activation of the sirtuin family of proteins while acetyltransferase modifications are controlled by less clearly delineated acetyltransferases. Subcellular localization specific protein targets of lysine-acetyl modification have been identified in the nucleus, cytoplasm and mitochondria. Despite distinct subcellular localizations, these modifications appear, in large part, to modify mitochondrial properties including respiration, energy production, apoptosis and anti-oxidant defenses. These mitochondrial regulatory programs are important in cardiovascular biology, although how protein acetyl modifications effects cardiovascular pathophysiology has not been extensively explored. This review will introduce the role of non-histone protein lysine-residue acetyl modifications, discuss their regulation and biochemistry and present the direct and indirect data implicating their involvement in the heart and vasculature.

Non-Standard Abbreviations and Acronyms:

AceCS2 – acetyl-coA synthetase 2; ADP – adenosine diphosphate; AMP – adenosine monophosphate; AMPK – AMP-kinase; CREB – cyclic AMP response element binding protein; CPS1 – carbamoyl phosphate synthetase 1, eNOS – endothelial nitric oxide synthase; ETC – electron transfer chain; FAO – fatty acid oxidation; Foxo – forkhead box O transcription factors; GDH – glutamate dehydrogenase; GNAT – Gcn5-related N-acetyltransferase; HAT – histone acetyltransferase; HDAC – histone deacetylase; HIF-2 α – hypoxia inducible factor alpha; ISDH2 – isocitrate dehydrogenase 2; MnSOD – manganese superoxide dismutase; NAD – nicotinamide adenine dinucleotide; NAMPT – nicotinamide phosphoribosyltransferase; NMN – nicotinamide mononucleotide; NMNAT – NMN adenylyltransferase; LKB1 – serine-threonine liver kinase B1; OAADPr – O-acetyl-ADP-ribose; PTM – post translational modification; PPAR – peroxisome proliferator activated receptor; PGC-1 – PPAR gamma coactivator 1; ROS – reactive oxygen species; TCA – tricarboxylic acid; TIP60 – Tat-interactive protein 60.

In the year of the bicentennial celebration of Darwin's birth, the pursuit of regulatory programs that underpin 'adaptations to confer survival' remains an important area of scientific investigation. Adaptation to acute stressors, as opposed to evolutionary 'pressures', would require rapidly inducible 'stress-sensing systems' that could initiate biological modifications, enabling survival advantage. As mitochondria are central to important cellular functions, including essential pathways for energy production, reactive oxygen species (ROS) signaling, calcium homeostasis and apoptosis, it would not be surprising if mitochondrial adaptations are instrumental in this ameliorative reprogramming. The heart has a high density of mitochondria with robust energetic demands and the concept of mitochondrial adaptation in the cardiovascular system to resist biomechanical stressors is well recognized.¹⁻⁴ To date, the most extensively explored sensing program delineated in the heart is controlled by AMP protein kinase and this signaling network is responsive to energy depletion and rising AMP levels.⁵ A more recently identified nutrient and redox sensing regulatory program, exemplified by protein lysine-residue acetyl modification, is beginning to be investigated as an additional homeostatic control program.

Lysine-residue acetylation is a reversible post-translational modification (PTM) orchestrated by a diverse family of structurally unrelated enzymes collectively known as acetyltransferases. This modification usually involves the covalent transfer of an acetyl group from acetyl-coenzyme A to a ϵ -amino group on lysine. The reverse reaction is driven by deacetylase enzymes. These lysine-modifications have been most extensively explored in the regulation of histones to fine-tune gene transcription, and these regulatory programs are dynamically controlled by histone acetyltransferases (HATs) and histone

deacetylases (HDACs). The role of acetylation/deacetylation in the modification of histones has recently been well described⁶ and is not the focus of this review. Initially identified in 1997, the first non-histone protein to exhibit acetyl-lysine modification was p53.⁷ A steady increase in the number of non-histone lysine acetylation modifications are being discovered and may play a diverse role in diffuse biological processes.⁸ Coupled to this, the enzymes controlling these PTMs are not confined to HATs and HDACs but include an expanding array of lysine acetyltransferases and deacetylases.

The modulation of mitochondrial biology and function via lysine acetylation involves proteins residing in the nucleus (mitochondrial regulatory proteins), cytoplasm (chaperones) and within mitochondria themselves. Together, these PTMs of target proteins contribute towards alterations in numerous mitochondrial functions including the regulation of mitochondrial biogenesis, apoptosis, thermogenesis, metabolism and possibly in the contribution of mitochondrial function to longevity.⁹ Although this field was initially identified and characterized in non-vertebrate eukaryotes, we focus on mammalian biology. In this review we explore: (1) the enzymes that orchestrate these modifications; (2) identify the substrates of these enzymes; (3) investigate the regulation of lysine acetylation; (4) describe the cardiovascular functional consequences of these regulatory events and (5) highlight potential areas of importance that need to be investigated to expand our understanding of the role of non-histone lysine acetylation and deacetylation on mitochondrial biology and cardiovascular homeostasis.

Non-Histone Lysine Acetyltransferases

Acetylation, as a process to regulate the availability of DNA for transcription, has been described for over forty years.¹⁰ HATs remove the positive charge of lysine in the histone tail, altering its interaction with DNA and allowing access to other DNA-associated proteins.^{11,12} Since the discovery of non-histone lysine-acetylation,⁷ the number of candidate proteins documented has grown steadily, with a recent proteomics study identifying 195 new acetylated proteins in mammalian tissue.¹³ Strikingly, in this study, 133 proteins with acetylated lysine residues were concentrated in mitochondria.¹³ These data suggest that ~20% of the mitochondrial proteome may be targeted for acetyl modification. This concept is further supported by the finding that protein acetylation is similarly abundant in prokaryotes.¹⁴ The functional characterization of the role of lysine-residue acetylation in the vast majority of these proteins has yet to be determined.

The non-histone lysine acetyltransferases can be loosely grouped into three main families, though there are several other identified acetyltransferases that fall outside these defined categories.^{15,16} These include the Gcn5-related N-acetyltransferase (GNAT), the MYST and the p300/CBP families.

The Gcn5-related N-acetyltransferase (GNAT) family contains HAT1, the first identified histone acetyltransferase,¹⁷ along with Gcn5/PCAF and multiple N-acetyltransferase (NAT) proteins. The GNAT family share 3-4 motifs involved in acetyl-CoA binding and catalysis, with nuclear-localized proteins and a bromodomain to facilitate DNA binding.^{18,19} A role of Gcn5 relating to mitochondrial function is inferred by the acetylation and inactivation of peroxisome proliferator gamma coactivator 1 (PGC-1) family members, which are known to be master regulators in mitochondrial biogenesis and mediators of mitochondrial metabolism.²⁰ Gcn5, activated by the steroid

receptor coactivator SRC-3, acetylates the PGC1 α and β , which inhibits their regulatory control of mitochondrial content and metabolic functioning.²¹⁻²³

The MYST family, named after the original members MOZ, YBF2, SAS2 and TIP60, share both a ~240 amino acid core acetyl-CoA-binding domain and a C2HC zinc-finger domain.²⁴ MYST proteins are found throughout the Eukaryota and are predominantly involved in histone acetylation, although some members are also involved in the regulation of transcription factors such as p53.²⁵ Tat-interactive protein 60 (TIP60) is transiently expressed in heart tissues during early embryonic development, suggesting that acetylation is involved in regulating cardiac myocyte differentiation.²⁶

Finally, there is the p300/CBP (CREB binding protein) family, which are large nuclear proteins that function as transcriptional co-regulators which have intrinsic histone acetyltransferase activity.^{27,28} It has subsequently been shown that these large multi-domain proteins additionally have non-histone acetyltransferase activity and are capable of PGC-1 α acetylation.²⁹ The presence of p300 is required for the correct formation of several mouse tissues, with a single mutant allele being sufficient to produce major defects in heart structure and coronary vascularization.³⁰ p300 also acetylates the early embryonic transcription factor GATA-4 which functions in differentiating embryonic stem cells into cardiac myocytes and in the development of cardiac hypertrophy.^{31,32} Collectively, it appears that the lysine-acetyltransferases are crucial for several steps in cardiac development, and may play a role in mitochondrial biology via this regulation of PGC-1. Interestingly, despite the prevalence of acetylated lysine residues on numerous mitochondrial proteins, to date mitochondrial enriched lysine-acetyltransferases have not been identified and are therefore not discussed further in this review.

Lysine Deacetylase Enzymes

The mammalian deacetylases are grouped according to phylogenetic analysis and sequence homology. The mammalian class I and II enzymes are nuclear and cytosolic-nuclear localized enzymes respectively that predominantly function as HDACs. To date, one HDAC has sequence similarity to both class I and II enzymes and has been designated as a class IV enzyme. Class I, II and IV enzymes have zinc-dependent deacetylase activity.³³ The sirtuins are designated as class III deacetylases and are NAD⁺-dependent enzymes. The founding member of these enzymes is yeast Sir2, which silences chromatin via deacetylation of histones.³⁴ Sir2 enzymes have been shown to mediate lifespan extension in yeast, worms and flies and are postulated to function, in part, via the modulation of mitochondrial function.⁹ Mammals have 7 sirtuin enzymes designated as SIRT1 through SIRT7. These have distinct tissue distributions and subcellular localizations which together probably contribute to their distinct biological functions³⁵. The mammalian sirtuins are further phylogenetically divided into five subclasses based on the homology of their 250 amino acid core domain.³⁶ The mitochondrial enriched SIRT3 clusters with SIRT1 and SIRT2 in subclass I. These three enzymes show closest homology to yeast Sir2 and exhibit the most robust deacetylase activity. The additional mitochondrial enriched sirtuins SIRT4 and SIRT5 are assigned to subclasses II and III, and exhibit predominant ADP-ribosyltransferase and deacetylase activity respectively.³⁷

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NAD⁺ Biochemistry

As sirtuin activity is dependent on NAD^+ , it has now been established that sirtuin activation is directly linked to the energetic and redox status of the cell as measured by the ratio of $\text{NAD}^+:\text{NADH}$, by the absolute levels of NAD^+ , NADH , and by the NAD^+ catabolite nicotinamide.³⁹⁻⁴¹ Interestingly, nicotinamide itself inhibits sirtuin activity and nicotinamide-depletion during NAD biosynthesis inversely activates sirtuins.⁴²

The NAD biosynthetic pathways are distinct in prokaryotes and invertebrates compared to vertebrates (reviewed⁴³). We only briefly review vertebrate biochemistry here. *De novo* biosynthesis using tryptophan and nicotinic acid as precursors is the minor pathway for NAD generation. However, this pathway is induced by exercise and following the administration of peroxisome proliferator activated receptor alpha ($\text{PPAR}\alpha$) agonists.^{44,45} The predominant pathway to generate NAD involves the salvage of NAD using nicotinamide as the precursor. In mammals there are two intermediary steps in NAD generation, initiated by the conversion of nicotinamide to nicotinamide mononucleotide (NMN) via the nicotinamide phosphoribosyltransferase (NAMPT) enzyme. Nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT) then converts NMN to NAD . These biochemical pathways are most well characterized in the nucleus, and are pivotal for the activity of SIRT1.⁴⁶ Moreover, NAMPT has been identified as the rate-controlling step in NAD biosynthesis in that overexpression of Nampt but not Nmnat increased cellular NAD levels.⁴⁶

The investigation into the biology of NAD in the mitochondria has begun to be explored, and the identification of a mitochondrial-enriched NMNAT isoform supports the concept of subcellular compartment specific functioning of NAD biosynthesis.⁴⁷ Moreover, mitochondrial NAD^+ levels can now be measured by mass spectroscopy.⁴⁸

Using this novel technique, the metabolic stress of fasting has been shown to increase mitochondrial NAMPT and to concomitantly induce mitochondrial NAD⁺ levels.⁴⁸

Sirtuin Biochemistry

Sirtuins are known to deacetylate lysine residues on histone and non-histone proteins.^{49,50} This deacetylation of target residues, coupled to the cleavage of NAD⁺ results in the generation of nicotinamide and the metabolite O-acetyl-ADP-ribose (OAADPr). The deacetylation of lysine residues on non-histone proteins modulates their cognate target protein function, as evidenced by the activation of Peroxisome proliferator activator receptor Gamma Coactivator - 1 alpha (PGC-1 α) following its deacetylation by SIRT1.^{29,51} Two potential biological consequences of lysine deacetylation include the unmasking of lysine to facilitate other PTMs, and a putative biological role following the generation of OAADPr.

In terms of PTMs, the lysine residue is highly promiscuous, with the potential to undergo, for example, acetylation, methylation, ubiquitinylation or SUMOylation on their ϵ -amino group (reviewed¹⁶). This multi-potential capacity for individual residue modification, the potential competition between these distinct PTMs at the same site and the emerging data to support cross-talk between multiple protein residue modifications highlights the emerging complexity of intramolecular signaling that may well govern biological processes.¹⁶ The role of lysine acetylation and/or deacetylation in this complex interplay is a challenging concept to be explored.⁵²

Interestingly the deacetylation metabolite OAADPr itself may directly facilitate post-translational modulatory effects following the enzymatic transfer of ADP-ribosyl groups

to proteins.⁵³ Although mono-ADP-ribosylation enzymes have been characterized in prokaryotes, and in extracellular compartments in eukaryotes, identification of intracellular enzymes in eukaryotes has been elusive.⁵⁴ Sirtuins may possess the dual functionality, as both enzymatic deacetylases, and as mono-ADP ribosyltransferases using OAADPr as substrate for this PTM. To date, the mitochondrial- and nuclear-enriched SIRT4 and SIRT6 proteins respectively, exhibit ADP-ribosyltransferase activities.^{37,55} However, the rate-constant of sirtuin ADP-ribosyltransferase activity is 5000 fold lower than classical bacterial enzymatic rates, and 500 times weaker than their deacetylase activities.⁵⁶ These data question the physiological role of sirtuins in mono-ADP-ribosylation, however, the development of tools to investigate this biochemistry and to identify the target proteome⁵⁷ should facilitate major advances in our understanding of this component of NAD and sirtuin biology in the near future. A diagram depicting NAD biosynthesis pathways are shown as the top half of figure 1.

Sirtuin Subcellular Localization

The subcellular localization of the sirtuins is probably a pivotal feature in dictating their biological targets. Of the sirtuins known to modulate mitochondrial biology, SIRT1 has been established to predominantly reside and function in the nucleus, SIRT2, in the cytoplasm, SIRT4 in the mitochondria matrix and SIRT5 in the inner mitochondrial membrane⁵⁸ or matrix.³⁸ These locations are not exclusive and may be dynamic under specific conditions. For example, SIRT1 is exclusively nuclear during cardiac embryogenesis and then displays both nuclear and cytoplasm postnatal localization.⁵⁹ Similarly, the subcellular localization of SIRT3 is predominantly in the mitochondrial matrix,⁶⁰⁻⁶² although some studies suggest that SIRT3 is exclusively mitochondrial⁶³

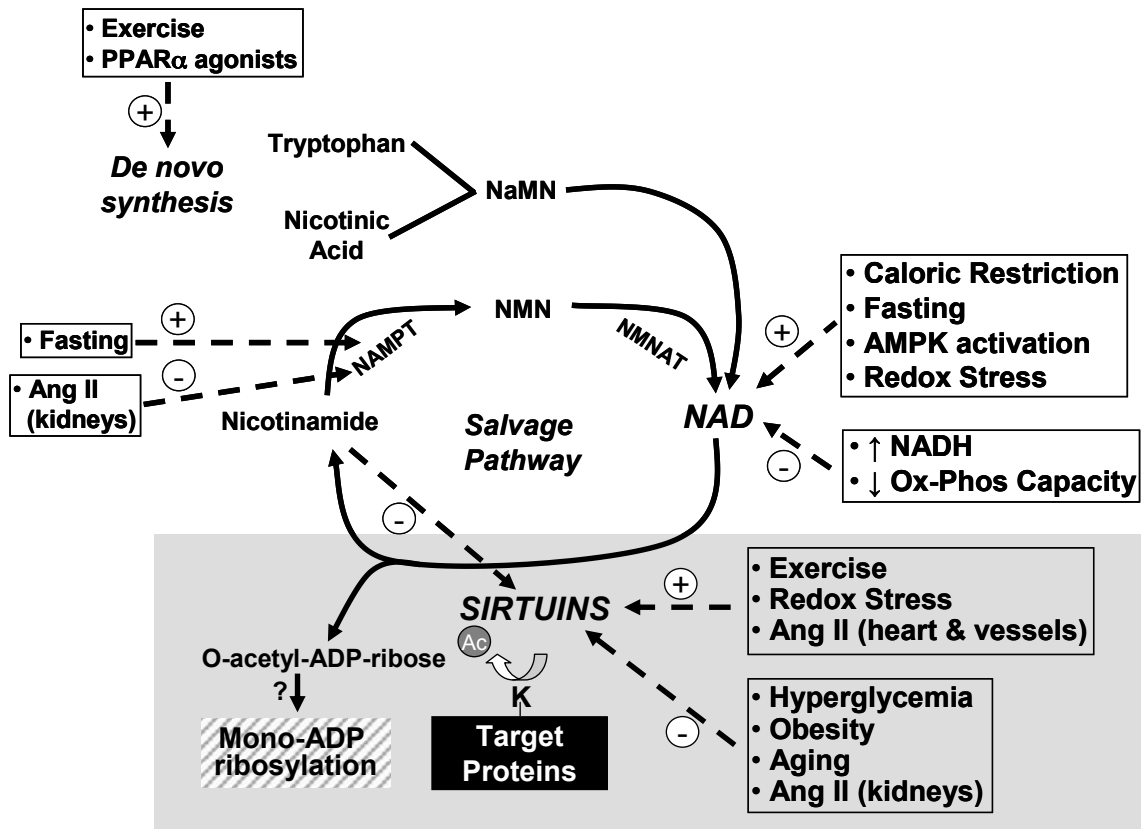


Figure 1. Biochemistry of NAD biosynthesis and sirtuin activity. The *de novo* pathway is the minor pathway and the salvage pathway the major pathway. The solid lines depict the biochemical pathways. The white boxes with their hatched lines represent biochemical and physiologic mediators of the NAD biosynthesis and sirtuin activation pathways. The black box shows the consequences of sirtuin mediated deacetylation. The hatched box represents a hypothetical modification that has not been robustly characterized. Abbreviations: NaMN – nicotinic acid mononucleotide; NMN – nicotinamide mononucleotide; NAMPT – nicotinamide phosphoribosyltransferase; NMNAT – nicotinamide/nicotinic acid mononucleotide adenylyltransferase.

while others show nuclear and cytosolic locations in whole tissue preparations⁶⁴ and following overexpression studies.⁶⁵⁻⁶⁷ Whether changes in the subcellular localization of SIRT3 are associated with biological stressors, are tissue specific^{62, 64} or result from the genetic manipulation studies is not completely resolved. Nevertheless, the capacity of SIRT3 to alter its subcellular localization with compartment distinct functions is an intriguing concept that warrants further investigation.

Biological Triggers Orchestrating Sirtuin Activity

The biochemical pathways operational in sirtuin regulation suggest that metabolic cues are integral to their activity. Moreover, as the ratio of NAD:NADH is an important regulator of the cellular redox state, oxidative stress signaling may similarly be implicated in the regulation of sirtuin activity. More recently, the AMP-activated protein kinase (AMPK), the prototypic fuel sensing signaling kinase has also been found to modulate sirtuin activity.

Caloric restriction, which promotes cell survival and longevity functions, in part, through increasing NAD⁺ levels. As such, the modulation of sirtuin levels and activity has been investigated in response to this nutrient deficit. SIRT1, SIRT2 and SIRT3 levels and activity are induced in multiple organs in response to caloric restriction.⁶⁷⁻⁶⁹ However, the uniformity of this response has been questioned with the demonstration that both the ratio of NAD:NADH and SIRT1 levels are decreased in the liver in response to caloric restriction.⁷⁰ Similarly, at least in pancreatic b-cells, SIRT4 activity is diminished in response to caloric restriction.³⁷

Other modifications of nutrient exposure are also implicated in the regulation of sirtuins. In contrast to chronic caloric restriction, fasting acutely increases NAD:NADH ratio in the liver, activating SIRT1.⁷¹ A consequence of fasting induced SIRT1 induction is the deacetylation and activation of PGC-1 α and PGC-1 β leading to the activation of mitochondrial metabolism.²² Conversely, elevated glucose has been shown to downregulate SIRT1 in skeletal muscle and in hepatocytes,⁷¹ while insulin, with or without elevated glucose, similarly downregulates SIRT1.^{68,72} Although nutrient mediated modulation of the mitochondrial enriched sirtuins has not been extensively studied, SIRT3 is downregulated in skeletal muscle in mice with streptozotocin induced severe hyperglycemia⁷³ and in brown adipose tissue in various murine genetic models of obesity.⁶⁷ In human subjects SIRT3 levels have been shown to be diminished in skeletal muscle of sedentary older individuals, while endurance training ameliorates this effect.⁷⁴

By virtue of the fact that sirtuins are activated by changes in the cellular redox state as reflected by their induction with higher levels of oxidized NAD⁺ or a change in the ratio of NAD⁺ to its reduced NADH form, imply these enzymes as redox sensitive. To date, all three subclass I sirtuins i.e. SIRT1, SIRT2 and SIRT3 have been shown to be induced by oxidative stressors.^{64,69,75,76} Interestingly, with respect to cardiac biomechanical stressors, pressure overload and angiotensin II have been shown to increase SIRT1 and SIRT3 levels in the heart and cardiomyocytes respectively.^{64,75}

A more global role of AMPK in modulating the sirtuins has not been established. However, AMPK activates SIRT1 by increasing intracellular NAD⁺ levels⁷⁷ and conversely SIRT1 deacetylates and activates the AMPK kinase LKB1.⁷⁸ These observations suggest integrated biological effects of these two major nutrient and redox

stress sensors in the cell. How these collectively modulate mitochondrial deacetylases and organelle homeostasis, however, has not been well characterized. The biochemistry of Sirtuin activation and the biological triggers modulating this program are schematized in the lower half of figure 1.

Modulation of mitochondrial function via nuclear protein lysine-acetylation

The control of mitochondrial function is not restricted to regulatory events within the mitochondria itself as exemplified by inter-genomic regulation between the nuclear and mitochondrial genomes and due to nuclear regulation of the intrinsic mitochondrial apoptotic program. Thus, it stands to reason that acetyl modification of non-histone lysine residues of nuclear proteins may be operational in the control of mitochondrial integrity and function.

The nuclear enriched SIRT1 has been shown to deacetylate numerous transcription factors and transcriptional co-activators that are known to control mitochondrial function. This includes the deacetylation and activation of PGC-1 α . . Activation of this transcriptional co-activator is known to upregulate mitochondrial biogenesis and increase mitochondrial metabolism with tissue specific preference.^{20, 79, 80} Furthermore, SIRT1-mediated deacetylation of FoxO proteins has been linked to the preferential activation of stress resistant targets^{81, 82} including the induction of mitochondrial antioxidant defenses. The deacetylation of p53 inactivates this transcription factor, thereby attenuating its pro-apoptotic action. Finally, the nuclear encoded mitochondrial transcription factor A (TFAM) has been shown to undergo acetyl

PTM, however, the regulatory enzymes controlling this reaction and its functional significance, remains to be shown.⁸³ In adipose tissue SIRT3 overexpression has also been shown to upregulate PGC-1 α and promote mitochondrial uncoupling.⁶⁷ However, the veracity of this data has been questioned due to the lack of alteration in thermogenesis in SIRT3 knockout mice exposed to hypothermic stress.⁶²

Known Mitochondrial Targets of Lysine Acetylation/Deacetylation

The suggestion that mitochondria contain acetyltransferase and deacetylase enzymes was recognized in 1962, with the demonstration that isolated mitochondria could reversibly acetylate carnitine.⁸⁴ The biology and multiple enzymes involved in carnitine ester formation has now been firmly established.⁸⁵ However, the biochemistry and functional role of mitochondrial protein lysine acetylation is less well characterized and has become a recent focus of study in the context of the identification of mitochondrial enriched lysine-deacetylases.

Whether the mitochondrial enriched sirtuins (SIRT3, 4 and 5) function as deacetylases has recently been explored, using genetic deletion of each of these three genes to investigate global hepatic mitochondrial protein lysine acetylation.⁶² While the SIRT4 and SIRT5 knockouts showed little change relative to the control mice, the SIRT3 knockout show enhanced mitochondrial protein acetylation, suggesting that SIRT3 is a major mitochondrial deacetylase.⁶² Despite these post-translational changes, no obvious basal phenotype is evident in the SIRT3^{-/-} mice. However, using affinity purification and mass spectroscopic analysis multiple SIRT3 interaction proteins have begun to be

identified and a majority of these putative interacting proteins reside and function in the mitochondria.⁸⁶

The functional characterization of individual target proteins is now being actively investigated. The mitochondrial enzyme acetyl-CoA synthetase 2 (AceCS2) was the first target of SIRT3 to be identified and partially characterized.^{87,88} ACS2 is inactivated following acetylation and rapidly reactivated by SIRT3-mediated deacetylation.^{87,88} Interestingly, AceCS2 is abundant in the murine heart and skeletal muscle and has been shown to be induced during ketosis.⁸⁹ This implies that AceCS2 is involved in acetate conversion for energy production under ketogenic conditions. The functional characterization of the modulation of AceCS2 by SIRT3 in the heart has yet to be explored. Two additional mitochondrial matrix proteins have been identified as substrates of SIRT3 and in both instances lysine deacetylation results in increased enzyme activity. These include glutamate dehydrogenase (GDH) which facilitates the oxidative deamination of glutamate to alpha-ketoglutarate, and the citric acid cycle enzyme isocitrate dehydrogenase 2 (ICDH2).^{58,62} Interestingly GDH is also a substrate for SIRT4 and this interaction results in the ADP-ribosylation and inactivation of GDH.³⁷ The functional significance of the opposing effect of SIRT3 and SIRT4, on GDH activity, requires further characterization. The most recent functional characterization of a mitochondrial matrix metabolic target of the sirtuins is the interaction with, deacetylation of, and activation of the urea cycle enzyme carbamoyl phosphate synthetase 1 (CPS1) by SIRT5.³⁸ This enzymes functions to detoxify and dispose of ammonia and in the SIRT5 knockout mouse, fasting results in elevated serum ammonia levels.³⁸

SIRT3 has also been shown to physically interact with NDUF9A, a subunit of complex I of the electron transfer chain.⁹⁰ The interaction of SIRT3 with this inner mitochondrial membrane protein results in its deacetylation and activation and the genetic depletion of SIRT3 accordingly compromises complex I activity and ultimately mitochondrial oxygen consumption and ATP production.⁹⁰ SIRT5 has also been shown to associate with an electron transfer chain protein, i.e. cytochrome c, although the functional consequences of this interaction has not to date been established.⁵⁸ Figure 2 schematizes the currently identified nuclear, cytosolic and mitochondrial proteins that are targets of sirtuin deacetylation and PTM.

Although a role of sirtuins in modulating outer mitochondrial membrane proteins has not been functionally characterized, it has recently been shown that long chain acyl CoA synthetase isolated from the outer membrane, also undergoes lysine-residue acetylation.⁹¹

Roles of Mitochondrial Protein Lysine Acetylation in the Heart

To date, the interrogation of the function of sirtuins in the heart has been limited, especially with respect to mitochondrial enriched sirtuins. However, prior studies linking sirtuins to metabolism, apoptosis, autophagy and aging suggest that altering sirtuin activity may modulate cardiovascular function and the responses to pathophysiological stressors. This inference is further supported by the induction of NAMPT in rat cardiomyocytes in response to both hypoxia and serum deprivation.⁴⁸ In that study, NAMPT mediated protection against cell death via mitochondrial NAD⁺ salvage was dependent on the presence of SIRT3 and SIRT4.⁴⁸

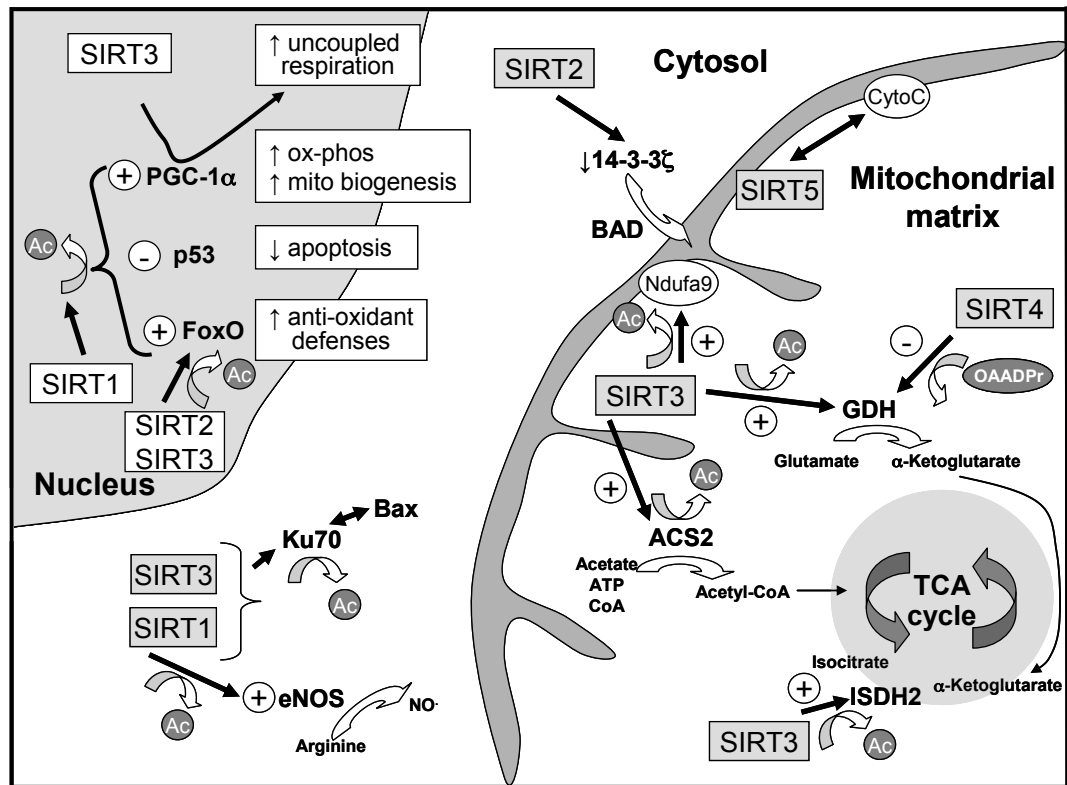


Figure 2. Schematic of sirtuin targets that are proposed to be operational in the modulation of mitochondrial function in the cardiovascular system. Note the SIRT5 target carbamoyl phosphate synthetase 1 is not shown in this schematic as the detoxification of ammonia is not thought to be operational in the cardiovascular system. A single sided arrow indicates a functional interaction. A double sided arrows represents a protein:protein interaction.

Cardiac SIRT1 levels are induced by pressure overload, in response to the systemic administration of the oxidative stressor paraquat, with heart failure and during aging.^{75,92} Together, these changes suggest that SIRT1 is modulated in response to cardiac biomechanical stressors. The first study to functionally characterize sirtuin function in the heart employed SIRT1 transgenic mice.⁷⁵ Low to moderate-overexpression attenuates age-associated cardiac fibrosis, apoptosis, hypertrophy and cardiac dysfunction. Furthermore, modest SIRT1 induction protects against cardiac oxidative stress. The mitochondrial salutary effects associated with this SIRT1 induction include: increased FoxO dependent catalase expression; elevated cellular ATP levels and increased mitochondrial citrate synthase activity.⁷⁵ A gene-dose effect of SIRT1 is evident in that transient overexpression of SIRT1 in cardiomyocytes prevents apoptosis via deacetylation and inactivation of p53,⁹² but robust SIRT1 induction in transgenic mice results in increased cardiac apoptosis, fibrosis and hypertrophy.⁷⁵

Anoxia-reoxygenation stress in cardiac derived H9c2 cells results in the induction of the cytoplasm enriched sirtuin, SIRT2.⁷⁶ Conversely, we have previously observed, in unpublished data, that cardiac SIRT2 gene expression is diminished by the cardioprotective program induced by delayed ischemic preconditioning.⁹³ To characterize the stress-response to SIRT2 downregulation we used siRNA to genetically deplete SIRT2 in H9c2 myoblasts.⁷⁶ SIRT2 depletion upregulates the cytosolic chaperone 14-3-3 ζ , which in turn sequesters the pro-apoptotic mitochondrial protein BAD in the cytosol and augments tolerance against anoxia-reoxygenation induced cell death.⁷⁶ Interestingly, the biology of SIRT2 in cellular homeostasis is not exclusively determined by levels of this deacetylase but also appears to have opposing functions under basal and

stress conditions.⁶⁹ These divergent phenotypes are illustrated by SIRT2-mediated induction of mitochondrial manganese superoxide dismutase via FOX3a deacetylation with the subsequent attenuation of reactive oxygen species levels at baseline. In contrast, under conditions of increased oxidative stress, SIRT2 promotes cell death in parallel with the induction of the pro-apoptotic protein Bim.⁶⁹ Collectively these studies show that despite its extra-mitochondrial localization, SIRT2 modulates mitochondrial function via the modulation of mitochondrial pro-apoptotic proteins and via mitochondrial antioxidant enzyme regulation.

In rat neonatal cardiomyocytes SIRT3 levels are increased in response to H₂O₂, the alkylating agent MNNG, serum-starvation, and in response to phenylephrine and Angiotensin II.⁶⁴ This regulation shows similarity to SIRT1 and suggests stress-responsive functioning. In parallel, SIRT3 overexpression in cardiomyocytes enhanced resilience to genotoxic and oxidative stress.⁶⁴ An identified target for this ameliorative function is the deacetylation of Ku70. Ku70 is predominantly localized to the nucleus, although it is evident as a smaller cytoplasmic pool.⁹⁴ The cytoplasmic pool is proposed to sequester the pro-apoptotic Bax protein and prevent its translocation to the mitochondria.⁹⁴ During stress Ku70 is acetylated, which facilitates the release of Bax to promote mitochondrial-mediated apoptosis.⁹⁵ Conversely, SIRT3 and SIRT1 both deacetylate Ku70, sequestering Bax in the cytosol and reducing genotoxic cell death.⁶⁴ A recent study shows that SIRT3 knockout mice, display increased cardiac hypertrophy and interstitial fibrosis with an increased susceptibility to the development of angiotensin II-induced hypertrophy.⁹⁶ In parallel, the cardiac restricted transgene overexpression an SIRT3 isoform ameliorates this phenotype. The mechanism of SIRT3 mediated

protection here is shown to be via Foxo3a mediated induction of anti-oxidant defense enzymes suggesting, in part, a nuclear regulatory role of SIRT3 in cardiac stress-mediated adaptation.⁹⁶

For completeness sake, we note that a cardiac phenotype is also evident in SIRT7 knockout mouse although whether this is associated with modulation in mitochondrial biology or function has not been investigated.⁹⁷ SIRT7 resides in the nucleoli and following its genetic depletion mice develop and die from cardiac hypertrophy and an inflammatory cardiomyopathy.⁹⁷ The absence of SIRT7 in primary murine cardiomyocytes enhances p53 acetylation, additionally leading to increased apoptosis and increased susceptibility to oxidative and genotoxic stressors.⁹⁷

Potential Roles of Mitochondrial Protein Lysine Acetylation in Vascular Biology

Sirtuin function in the vasculature has not been directly explored; however, sirtuin biology is beginning to be investigated in biological programs that in part modulate vascular function, including nitric oxide biology, angiotensin signaling and hypoxia responsiveness.

Nitric oxide (NO) is a multifunction signaling molecule with diverse vascular functions which include the modulating of blood vessel tone, leukocyte adhesion, platelet activation and the development of atherosclerosis.⁹⁸ Interestingly, NO also regulates mitochondrial biology through the modulation of the mitochondrial biogenesis regulatory program and via the regulation of electron transfer chain flux.⁹⁹⁻¹⁰¹ The salutary effects of calorie restriction on mitochondrial biogenesis and metabolism has been shown to be dependent on NO, and this molecule is furthermore implicated in the transcriptional

induction of SIRT1.¹⁰² SIRT1 itself deacetylates and activates endothelial NOS (eNOS), which via the generation of NO promotes endothelial dependent vasodilation.¹⁰³

Together these regulatory events align with the known blood pressure lowering effect associated with caloric restriction,¹⁰⁴ although whether mitochondrial biology *per se* and/or NO are important in these biological effects is yet to be determined.

Renin-angiotensin system (RAS) activation contributes to cardiovascular and renal disease resulting in significant morbidity and mortality. Deletion of the Ang-II type 1a receptor (AT1R) in mice extends their lifespan in association with blood pressure reduction and decreased cardiac hypertrophy and fibrosis.^{105,106} Interestingly AT1R deletion leads to the upregulation of genes encoding for SIRT3 and NAMPT in the kidney.¹⁰⁵ Consistent with this but in contrast to the heart, angiotensin II administration downregulates Sirt3 gene expression in cultured proximal tubule epithelial cells.¹⁰⁵ As the kidney is central in angiotensin II induced hypertension,¹⁰⁷ whether the modulation of SIRT3 and by inference mitochondrial biology plays a role in this pathophysiology is unknown. Interestingly and of relevance to the modulation of blood pressure, the overexpression of SIRT1 in vascular smooth muscle cells results in the downregulation of AT1R.¹⁰⁸ In parallel, the SIRT1 activator resveratrol represses AT1R gene transcription and this pharmacologic compound blunts angiotensin II-induced hypertension in mice.¹⁰⁸ Taken together, these data reveal a complex interaction between SIRT1 and SIRT3 and the RAS system, with apparent target organ specific effects. Although intriguing, the associations between sirtuins and hypertension-target organ biology are observational, and mechanistic studies need to be performed to understand the role of deacetylases and the mitochondria in this disease process.

Hypoxia is a potent trigger for the modulation of vascular tone and to promote angiogenesis. The latter program is orchestrated by the induction of hypoxia inducible factors (HIFs). HIFs are transcription factors that, under hypoxic conditions, regulate the shift in cell metabolism to glycolysis; augment cell survival through induction of anti-oxidant systems such as Heme-oxygenase-1 and superoxide dismutase (SOD); and initiate the angiogenesis regulatory program. This leads to improved mitochondrial function and cell survival.¹⁰⁹ Hypoxia-induced redox stress stimulates SIRT1 activity, which in turn deacetylates and activates HIF-2 α .⁴¹ In keeping with the role of HIF activation, this SIRT1 mediated deacetylation results in the upregulation of, for example, the mitochondrial anti-oxidant enzyme SOD2, and the angiogenesis regulatory factor VEGF.⁴¹ These data suggest that through its redox-sensing capacity, SIRT1 can modulate vascular biology via the activation of HIF-2 α .

Taken together, these studies suggest that SIRT1 and SIRT3 have modulatory effects on numerous integrated biological programs governing vascular health and adaptation. The study into the mechanisms orchestrating these effects, and the determination of the physiologic role of the sirtuin program in vascular pathophysiology appears to be a promising area for future investigation. Table 1 summarizes all of the currently identified sirtuin protein targets and their function in the modulation of mitochondrial biology.

Conclusions/Future Directions

In this review we discuss how the nutrient and redox sensing sirtuins modulate mitochondrial function via predominant nuclear (SIRT1), cytosolic (SIRT2) and mitochondrial (SIRT3) mediated effects. Their roles in cardiac biology have been limited

to date, showing that: (1) the induction of SIRT1 mediated mitochondrial antioxidant and anti-apoptotic effects, (2) anti-apoptotic effects of SIRT2 deletion in cardiac derived cell lines and (3) SIRT3 mediated anti-apoptotic effects in response to oxidative and genotoxic stress in primary cardiomyocytes. A direct role of sirtuins in the vasculature has not been established but the augmentation of SIRT1 is associated with: (1) the downregulation of angiotensin receptor type 1, (2) lowering of blood pressure and (3) with the induction of HIF-2 α mediated angiogenesis signaling and the upregulation of mitochondrial anti-oxidant enzymes. In addition, in the long-lived ATR1 knockout mice, which have low blood pressure, the genes encoding for SIRT3 and NAMPT are upregulated in the kidney. Collectively these data suggest a functional role of sirtuin biology in the modulation of cardiovascular pathophysiologic adaptations to stressors. Further and more direct in-vivo studies to investigate this biology are warranted.

The recognition that a large number of proteins that reside in the mitochondria undergo lysine-acetylation PTMs,¹³ and that their target proteins include numerous proteins controlling fatty acid oxidation (FAO), the tricarboxylic acid cycle (TCA) and the electron transfer chain (ETC) question whether these PTMs play an important role in cardiac substrate preference and utilization. Substrate preference and utilization and the efficient integration between metabolic pathways, for example coordination between the TCA cycle with the ETC,¹¹⁰ and the modulation of anaplerosis¹¹¹ have begun to be recognized as important modulators of cardiac ischemia tolerance,^{112,113} cardiac hypertrophy and the transition to heart failure.^{114,115} Whether lysine acetyl PTMs are important and operational in the modulation and integration of these metabolic fluxes are important questions that have not been addressed.

Table 1. Summary of subcellular targets of sirtuin deacetylation or ribosylation* and proposed functional consequences

Sirtuin	Target	Function
Nuclear Targets		
SIRT1	PGC-1 α	Promotes mitochondrial biogenesis
	Foxo proteins	Upregulation of anti-oxidant defense programs
	eNOS	? vasodilation and/or mitochondrial biogenesis effects
	p53	<i>Inactivated</i> by deacetylation – diminishes apoptosis
	HIF-2 α	Upregulates anti-oxidant enzymes and VEGF
SIRT2	Foxo3a	Activates anti-oxidant defense programs
	Foxo3a	Activates Bim to promote apoptosis under redox stress
	14-3-3 ζ	Downregulation - Bad translocation - mitochondrial apoptosis
SIRT3	Foxo3a	Upregulates anti-oxidant enzymes
	PGC-1 α	Upregulates genes encoding mitochondrial proteins

Cytosolic

Targets

SIRT1	Ku70	Sequestered Bax in cytosol to inhibit mitochondrial apoptosis
	LKB1	Activates AMP kinase
SIRT3	Ku70	Sequestered Bax in cytosol to inhibit mitochondrial apoptosis

Mitochondrial

Targets

SIRT3	AceCS-2	Acetate conversion to acetyl-CoA
	GDH	<i>Activation</i> - deaminates glutamate to α -KG for TCA cycle
	NDUF9a	Activation of complex I of the ETC
	ISDH2	Facilitates TCA cycling
SIRT4	GDH	<i>Inactivation</i> by ADP-ribosylation
SIRT5	Cytochrome c	? Promotes electron flux through ETC
	CPS1	Detoxification of ammonia via urea cycle

An additional area of research that has yet to be functionally characterized, but may be important, is the role of sirtuin biology in type 2 diabetes mellitus and obesity associated cardiovascular perturbations. Diabetes and obesity both associate with nutrient excess, mitochondrial dysfunction, and a predisposition to cardiovascular pathology.^{116,117} Gene expression analysis shows that SIRT3 levels are diminished with obesity and hyperglycemia. Moreover, our understanding of how nutrient excess modulates biological function was recently enhanced with the recognition that elevated glucose could promote acetyl modification of proteins.¹¹⁸ Here, increasing concentrations of glucose promote histone acetylation through ATP citrate lyase mediated conversion of citrate to the acetylation precursor acetyl CoA.¹¹⁸ Whether excess nutrients modulates non-histone protein lysine acetylation via increasing acetyl CoA in distinct subcellular distributions remains to be determined¹¹⁹ and specifically to be evaluated in the cardiovascular system. Figure 3, schematizes both the identified pathways modified by sirtuin biology in the cardiovascular system and highlights areas of interest that have yet to be explored.

The pharmacologic manipulation of the sirtuins as a potential therapeutic strategy is being investigated. The first compound explored was the plant-derived polyphenol resveratrol (3,5,4'-trihydroxystilbene) which has been shown to upregulate both SIRT1 and AMPK.¹²⁰⁻¹²² and to induce mitochondrial biogenesis.¹²² Consistent with its known pleiotropic effects resveratrol administration confers protection against cardiac ischemia-reperfusion injury.^{120,123} Recently SIRT1-specific small molecule activators have been identified and are shown to promote fatty acid oxidation and mitochondrial function.¹²⁴ Whether these specific SIRT1 activators can directly ameliorate cardiac stress-tolerance

Sirtuin effects on the heart	Sirtuin effects on vascular pathways	PTMs that can potentially modulate cardiac function
<ul style="list-style-type: none"> • Sequestration of mitochondrial apoptotic proteins in the cytoplasm • ↓ p53 mediated apoptosis • ↑ mitochondrial anti-oxidant enzymes 	<ul style="list-style-type: none"> • ↑ HIF2α <ul style="list-style-type: none"> - ↑ glucose metabolism - promote angiogenesis - ↑ mitochondrial anti-oxidant defenses • Activation of eNOS <ul style="list-style-type: none"> - ? role in mitochondrial respiration 	<ul style="list-style-type: none"> • Fuel substrate selection <ul style="list-style-type: none"> - PTM of FAO enzymes • TCA to ETC coupling <ul style="list-style-type: none"> - PTM of TCA and ETC proteins • Modification in response to fuel overload e.g. with diabetes obesity – increased acetyl-CoA and/or modulation of NAD:NADH

Figure 3. Potential role of sirtuin mediated modulation of mitochondrial biology in the cardiovascular system. The direct and indirect effects of mitochondrial function that have been characterized are shown under the heart and vascular pathways columns. The potential effects of mitochondrial metabolic protein modifications are shown in the column to the right. How these may affect fuel substrate use and selection and the adaptations to direct cardiac stressors and to metabolic stresses on the heart and vasculature have yet to be ascertained. The speculative functions of acetyl-lysine PTM's is highlighted by their grey background.

and whether these effects include the modulation in mitochondrial function need to be investigated. No specific pharmacologic modulators of SIRT3 have been described, however numerous SIRT2 inhibitors have been identified.¹²⁵⁻¹²⁷ One of these inhibitors has been shown to prevent the development of Parkinson's-like disease in neuronal cells¹²⁶ and it would be intriguing to investigate this compound in the context of cardiac redox stress.

Although, we have discussed the actions of sirtuins slanted towards the modulation of mitochondrial function, this family of proteins has a myriad of additional functions including for example roles in gene transcription,¹²⁸ autophagy¹²⁹ and the circadian rhythm.¹³⁰ Hence, although our understanding of this important non-histone lysine-residue PTM should develop with ongoing investigations, we must be cognizant of the fact that the complexity and hierarchy of this biology is putatively more complex than the mitochondrial-centric focus elaborated on in this review.

In conclusion, fuel and redox sensing are important cellular commodities required to adapt to biomechanical and metabolic stressors. The PTM of non-histone lysine residues by acetylation/deacetylation appears to contribute to these sensing programs in the orchestration of important nuclear, cytosolic and mitochondrial responses to augment tolerance to injury. The investigation into this field is in its early stages and although many gaps exist in our knowledge, initial studies suggest that the modulation of this regulatory program may be important in controlling cardiovascular stress adaptation. Additionally, the emerging data supporting integration between sirtuin and AMPK adaptive programs may shed insight into the complexity of intracellular metabolic sensing and responses to nutrient and redox stress. Overall, these findings advance the

observations of Charles Darwin from more than 150 years ago, which were initially directed at whole organism adaptations over the long-term, to the modern interrogation of subcellular adaptive reprogramming to acute-stressors. The recent identification and ongoing characterization of this acetylation-dependent stress-adaptive programming should further enlighten us to the myriad of innate programs exploited by nature to advance survival whether acutely and/or for the propagation and survival of species.

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Disclosures

None.

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Appendix 4

PHOSPHOINOSITIDE-DEPENDENT KINASE-1 AND PROTEIN KINASE C δ CONTRIBUTE TO ENDOTHELIN-1 CONSTRICTION AND ELEVATED BLOOD PRESSURE IN INTERMITTENT HYPOXIA

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Nonstandard Abbreviations:

cPKC: classical protein kinase C

DAG: diacyl glycerol

eNOS: endothelial nitric oxide synthase

ET-1: endothelin-1

ET_AR: endothelin-1 A receptor

IH: intermittent hypoxia

NO: nitric oxide

OSA: obstructive sleep apnea

PDBu: phorbol 12,13-dibutyrate

PDK-1: phosphoinositide-dependent kinase-1

PH: pleckstrin homology

PI3K: phosphoinositide 3 kinase

PIP₃: phosphoinositol 3,4,5-tris-phosphate

PKC: protein kinase C

PSS: physiological salt solution

ROS: reactive oxygen species

Recommended Section Assignment: Cardiovascular

ABSTRACT

Obstructive sleep apnea (OSA) is associated with cardiovascular complications including hypertension. Previous findings from our laboratory indicate that exposure to intermittent hypoxia (IH), to mimic sleep apnea, increases blood pressure in rats. IH also increases endothelin-1 (ET-1) constrictor sensitivity in a protein kinase C (PKC) δ -dependent manner in mesenteric arteries. Because phosphoinositide-dependent kinase-1 (PDK-1) regulates PKC δ activity, we hypothesized that PDK-1 contributes to the augmented ET-1 constrictor sensitivity and elevated blood pressure following IH. Male Sprague-Dawley rats were exposed to either Sham or IH (cycles between 21%O₂/0%CO₂ and 5%O₂/5%CO₂) conditions for 7 hours/day for 14 or 21 days. The contribution of PKC δ and PDK-1 to ET-1-mediated vasoconstriction was assessed in mesenteric arteries using pharmacological inhibitors. Constrictor sensitivity to ET-1 was enhanced in arteries from IH rats. Inhibition of PKC δ or PDK-1 blunted ET-1 constriction in arteries from IH but not Sham rats. Western analysis revealed similar levels of total and phosphorylated PDK-1 in arteries from Sham and IH rats but decreased protein:protein interaction between PKC δ and PDK-1 in arteries from IH rats compared to those from Sham, consistent with observations that increased PKC δ -phosphorylation causes dissociation from PDK-1. Blood pressure was increased in rats exposed to IH and treatment with the PDK-1 inhibitor OSU-03012 (33 mg/day) lowered blood pressure in IH but not Sham rats. Our results suggest that exposure to IH unmasks a role for PDK-1 in regulating ET-1 constrictor sensitivity and blood pressure that is not present under normal conditions. These novel findings suggest PDK-1 may be a uniquely effective antihypertensive therapy for OSA patients.

Key Words: ET-1, obstructive sleep apnea, OSU-03012

INTRODUCTION

Obstructive sleep apnea (OSA) occurs due to a cyclical loss of pharyngeal muscle tone during sleep, resulting in airway collapse and subsequent arousal with disruption of sleep and restoration of airflow. Upon air flow restoration, sleep resumes and the cycle repeats whereas central sleep apnea is caused by loss of the central drive to breathe. In both cases, the repeated cycles of apneic events lead to intermittent hypoxia (IH) and are associated with increased risk for hypertension, stroke, coronary artery disease, arrhythmias, and heart failure. It is estimated that 24% of men and 9% of women experience at least 5 apneic episodes per hour with an estimated 80% undiagnosed for OSA (Bradley and Floras, 2009). Studies in rodent models demonstrate that chronic exposure to IH to mimic the apneic episodes of sleep apnea causes vascular dysfunction that might underlie the increased cardiovascular complications observed in OSA patients (Dematteis et al., 2008).

Previous studies from our laboratory have shown that 14 days of exposure to IH increases mean arterial blood pressure in rats (Kanagy et al., 2001). This augmented blood pressure is dependent on activation of the endothelin-1 (ET-1) A receptor (ET_AR) and generation of reactive oxygen species (ROS); moreover, ROS mediate increased levels of plasma ET-1 in IH-exposed rats (Troncoso Brindeiro et al., 2007). Along with increased levels of circulating ET-1, IH rats show augmented constrictor responses in mesenteric arteries to ET-1 but not to phenylephrine or depolarizing concentrations of KCl (Allahdadi et al., 2005). This increased constrictor response is dependent on greater calcium sensitivity as opposed to greater increases in intracellular calcium levels. Arteries

from IH rats also have increased expression of the ET_AR, and the increased vasoconstrictor sensitivity to ET-1 in IH rats can be attenuated by pharmacologically blocking ET_AR (Allahdadi et al., 2005; Allahdadi et al., 2008a). We subsequently reported that IH-augmented vasoconstriction is mediated by the δ -isoform of protein kinase C (PKC δ) in a calcium-independent manner and that ET-1 increases PKC δ autophosphorylation levels (Allahdadi et al., 2008b). However, it is not clear what increases ET-1 activation of PKC δ -dependent contraction in vascular smooth muscle from IH-exposed rats.

Similar to all members of the protein kinase A, B, and C families, PKC δ has multiple phosphorylation sites that differentially regulate function. PKC isoforms in particular are phosphorylated as part of the “maturation” process. Specifically, phosphoinositide-dependent kinase-1 (PDK-1), which activates at least 23 members of the AGC family of protein kinases, including PKC isoforms (Le Good et al., 1998) and Akt (Bayascas et al., 2008), phosphorylates the activation loop domain in PKC, “priming” PKC for DAG binding, autophosphorylation, and activation (Reviewed in (Newton, 2009)). For PKC δ , Thr⁵⁰⁵ is the PDK-1 activation site, whereas Ser⁶⁴³ and Ser⁶⁶² are the turn motif and hydrophobic domain autophosphorylation sites, respectively (Kikkawa et al., 2002).

Regulation of PDK-1 activity is not as well defined as that of its downstream targets. Of interest, ROS and insulin are two known activators of PDK-1 phosphorylation and activity. Specifically, H₂O₂ increases serine and tyrosine phosphorylation of PDK-1 in cultured cells (Prasad et al., 2000), whereas Zou *et al.* (Zou et al., 2003) observed

increased phosphorylation of Ser²⁴¹ in bovine aortic endothelial cells following exposure to peroxynitrite. In light of the previously reported increase in ROS levels following IH exposure, we hypothesized that increased PDK-1 activity mediates the increased ET-1 constrictor sensitivity and elevation in blood pressure following IH exposure. To test this hypothesis, we examined the effect of PDK-1 inhibition on ET-1-dependent vasoconstriction in mesenteric arteries from control and IH-exposed rats and used the orally active PDK-1 inhibitor OSU-03012 to investigate a role for PDK-1 in the IH-induced increase in blood pressure.

METHODS

Rodent Model of Sleep Apnea

Male Sprague-Dawley rats (250-275 g) were exposed to either Sham or IH conditions as described previously (Allahdadi et al., 2005; Troncoso Brindeiro et al., 2007). Briefly, all rats were housed in enclosed Plexiglas boxes with free access to food and water. IH exposure consisted of cycling between room air (21% O₂/0% CO₂) and hypoxia (5% O₂/5% CO₂), whereas Sham conditions consisted of exposure to room air only in identical boxes that subjected control rats to sounds and airflow similar to that produced by the hypoxia cycler. Rats were exposed to 20 hypoxic episodes/hour for 7 hours/day during their sleep period and were maintained on a 12:12-hour light:dark cycle. We have previously shown that this IH exposure drops arterial P_{O₂} levels from ~70 mmHg to 35 mmHg while P_{CO₂} levels remain unchanged (Snow et al., 2008). The Institutional Animal Care and Use Committee of the University of New Mexico School of Medicine reviewed and approved all animal protocols. All protocols conform to the National Institutes of Health guidelines for animal use.

Isolated Mesenteric Artery Preparation

At the end of the Sham or IH exposure period, rats were euthanized with sodium pentobarbital (150 mg/kg, i.p.). Mesenteric arterial arcades were isolated and placed in physiological salt solution (PSS; in mM: 129.8 NaCl, 5.4 KCl, 0.44 NaH₂PO₄, 0.83 MgSO₄, 19.0 NaCO₃, 1.8 CaCl₂, and 5.5 glucose) at 4°C until used for contractile studies or protein analysis. Third-order mesenteric arteries with a pressurized inner diameter of 150-250 μm were dissected from the mesenteric arterial cascades and cleared of adipose and connective tissue. An artery segment was placed in a vessel chamber (Living Systems Instrumentation; Burlington, VT) and secured with silk thread on glass micropipettes. Unless noted, experiments were conducted in endothelium-intact arteries. Endothelial disruption was performed by physically rubbing the endothelium with moose mane in a subset of experiments. Vessels were pressurized to 60 mmHg with a peristaltic pump (Living Systems Instrumentation) and superfused with PSS (bubbled with 21% O₂/6% CO₂/balance N₂ and heated to 37 °C) at a rate of 5 mL/minute.

Constrictor Studies

ET-1 constriction was assessed in isolated mesenteric arteries in the absence or presence of the following inhibitors: the nonselective PKC inhibitor Go6983 (1 μM; 3-[1-[3-(Dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione;

Biomol International; Farmingdale, NY), the classical PKC (cPKC) inhibitor Go6976 (1 μM; 5,6,7,13-Tetrahydro-13-methyl-5-oxo-12*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-12-propanenitrile; Biomol International), the PKCδ inhibitor rottlerin (3 μM; 3'-[(8-

Cinnamoyl-5,7-dihydroxy-2,2-dimethyl-2*H*-1-benzopyran-6-yl)methyl]-2',4',6'-trihydroxy-5'-methylacetophenone; Biomol International), the PDK-1 inhibitor OSU-03012 (10 μ M; 2-Amino-N-[4-[5-(2-phenanthrenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]phenyl]acetamide; Cayman Chemical; Ann Arbor, MI), and the phosphoinositide 3 kinase (PI3K) inhibitor LY294002 (10 μ M, 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; Cayman Chemical). The concentration used for each inhibitor was based on previous studies that established inhibition parameters for PKC isoform(s) (Martiny-Baron et al., 1993; Gschwendt et al., 1994; Gschwendt et al., 1996; Zhu et al., 2004). All inhibitors were added to the PSS superfusate for 30 minutes prior to initiation of ET-1 concentration-response curves and remained in the superfusate throughout the contractile studies. For vehicle studies an equal volume of the appropriate vehicle was added to the superfusate. Baseline diameter was recorded and then vessels were exposed to increasing concentrations of ET-1 (American Peptide; Sunnyvale, CA) from 10^{-10} to $10^{-7.5}$ M. The presence of active tone was verified by comparison with the maximal passive diameter in Ca^{2+} -free PSS (in mM: 129.8 NaCl, 5.4 KCl, 0.44 NaH_2PO_4 , 0.83 MgSO_4 , 19.0 NaCO_3 , 5.5 glucose, and 4.5 EGTA). Constriction to the PKC activator phorbol 12,13-dibutyrate (PDBu; Sigma-Aldrich; St. Louis, MO) in the absence or presence of OSU-03012 was also assessed in arteries from Sham rats to determine if the effects of OSU-03012 were mediated by non-specific effects on PKC. Additionally, constriction to PDBu was assessed in the presence of Go6983 and rottlerin to compare the effects of OSU-03012 with general PKC and PKC δ inhibition, respectively. All data are expressed as percent constriction compared to baseline diameter.

PDK-1 Phosphorylation

Phosphorylation of PDK-1 at the Ser²⁴¹ residue was assessed in mesenteric arteries from Sham and IH rats. Arteries were isolated and placed in ice-cold lysis buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo Scientific; Waltham, MA) containing 1X Halt phosphatase inhibitor cocktail (Thermo Scientific), 2.5 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich), and 1X Complete protease inhibitor cocktail (Santa Cruz Biotechnology; Santa Cruz, CA). Tissues were homogenized and centrifuged 10 minutes at 1,500 × g at 4°C. Supernatants containing equal protein concentrations (15 µg; as assessed by a Pierce BCA Protein Assay Kit; Thermo Scientific) were added to 5X sample buffer, heated to 95°C for 5 minutes, separated on a 4-15% gradient gel (BioRad Laboratories; Hercules, CA), and then transferred to a polyvinylidene fluoride membrane. Phosphorylated and total PDK-1 protein (58-68 kDa) were analyzed following overnight incubation with phospho-PDK-1 Ser²⁴¹ (1:250; Cell Signaling Technology; Danvers, MA) and PDK-1 (1:250; Cell Signaling Technology) primary antibodies. Proteins were visualized using a LI-COR Odyssey Infrared Imaging System (LI-COR; Lincoln, NE). Phosphorylated PDK-1 is expressed as a ratio of total PDK-1. Total PDK-1 levels were normalized to coomassie blue staining.

PKCδ/PDK-1 Immunoprecipitation

Association of PKCδ and PDK-1 was evaluated using a Pierce Co-Immunoprecipitation Kit (Thermo Scientific). Mesenteric arterial arcades were isolated from Sham- and IH-exposed rats, placed into 150 µL lysis buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1% NP-40; Thermo Scientific), homogenized, and centrifuged as described above. Following centrifugation, lysates (100 µg) were incubated overnight at 4°C in an

immunoprecipitation column prepared with a PKC δ antibody (AbCam; Cambridge, MA) according to the manufacturer's protocol. Samples were eluted and prepared for Western blotting as described above using 50 μ g of protein for each sample. Blots were incubated overnight with a PKC δ (1:250; Cell Signaling Technology) or PDK-1 (1:250; AbCam) primary antibody at 4°C. Data are expressed as the ratio of PDK-1 (58-68 kDa) to PKC δ (78 kDa). Total PKC δ levels were normalized to coomassie blue staining.

Blood Pressure Measurements

Blood pressure was measured by radiotelemetry in a second group of rats exposed to Sham or IH conditions. Telemetry transmitters (Data Sciences International; St. Paul, MN) were surgically implanted under isoflurane anesthesia with the catheter placed in the abdominal aorta via the femoral artery; rats were allowed to recover for at least 7 days. Baseline blood pressure was recorded for 3 days after the recovery period and then Sham or IH exposure was initiated as described above. The IH exposure was extended to 21 days to allow for pressure to reach a plateau, as previously described (Kanagy et al., 2001). After 21 days of Sham or IH cycling, rats received either vehicle (transgenic dough diet; Bio-Serv; Frenchtown, NJ) or the PDK-1 inhibitor OSU-03012 (33 mg/day in dough diet [based on a previous study (Gao et al., 2008)]; Cayman Chemical) for 3 days with continued measurement of blood pressure and continued exposure to Sham or IH conditions.

Statistical analysis

Concentration-response curves were analyzed by two-way repeated-measures ANOVA and Student-Newman Keuls post hoc analysis was used to detect differences between

groups. Western blot densitometry levels and blood pressure changes were compared by student's T-tests. The blood pressure response over time was compared between Sham and IH by two-way ANOVA. Data are expressed as mean \pm SEM. A *p*-value of <0.05 was considered statistically significant.

RESULTS

ET-1-mediated Constriction in Mesenteric Arteries

A comparison of ET-1 constriction under various conditions in mesenteric arteries from Sham and IH rats is shown in Table 1. ET-1-mediated vasoconstriction was greater in endothelium-intact mesenteric arteries from IH-exposed rats compared to arteries from Sham rats (Figure 1). Pan-PKC inhibition with the non-isoform-selective inhibitor Go6983 had no effect on ET-1-mediated vasoconstriction in mesenteric arteries from control rats (Figure 2A) but attenuated constriction in arteries from IH rats (Figure 2B). In contrast, Go6976, a selective cPKC inhibitor, had no effect on ET-1-induced constriction in arteries from either Sham or IH rats (Figure 2C and 2D). These results confirm that PKC activation contributes to ET-1-mediated vasoconstriction following IH exposure and that the PKC isoform(s) involved is not a cPKC isoform. Much like the non-selective PKC inhibitor Go6983, the PKC δ inhibitor rottlerin attenuated ET-1-mediated constriction in arteries from IH-exposed rats but was without effect in arteries from Sham rats (Figure 3). It has been demonstrated that rottlerin can inhibit PKC ϵ , but ET-1-mediated constriction was unaffected by the PKC ϵ inhibitor V1-2myr (data not shown). Taken together, these results suggest that PKC δ is involved in the heightened constriction to ET-1 following IH exposure.

Effects of PDK-1 Inhibition on ET-1-mediated Constriction

Table 1. Characteristics of Concentration-response Curves.

Group	-LogEC₅₀	E_{max}
Sham	8.64 ± 0.09	70.1 ± 1.1
IH	8.70 ± 0.05	77.4 ± 0.7 *
Sham + Go6976	8.88 ± 0.10	69.2 ± 1.0
IH + Go6976	8.68 ± 0.05	74.4 ± 2.2
Sham + Go6983	8.80 ± 0.07	69.0 ± 2.2
IH + Go6983	8.70 ± 0.11	49.1 ± 3.7 *#
Sham + Rottlerin	8.64 ± 0.08	65.7 ± 2.0
IH + Rottlerin	8.45 ± 0.06	43.2 ± 4.6 *#
Sham + OSU-03012	8.41 ± 0.05	71.7 ± 1.72
IH + OSU-03012	8.41 ± 0.15 #	57.2 ± 2.0 *#

* $P < 0.05$ vs. respective Sham treatment, # $P < 0.05$ vs. IH.

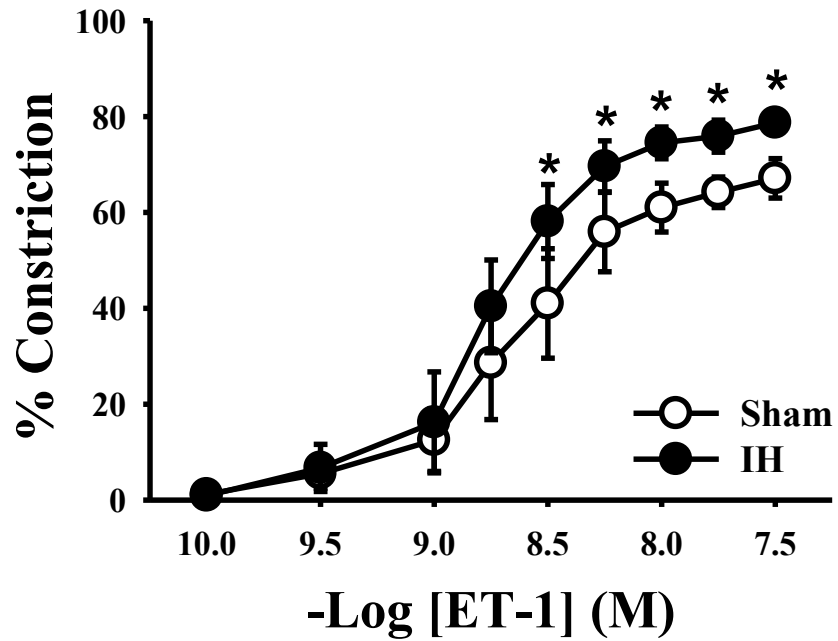


Figure 1: Exposing rats to two weeks of intermittent hypoxia (IH) leads to increased constrictor sensitivity to endothelin-1 (ET-1) in endothelium-intact mesenteric arteries when compared to arteries from Sham controls. Values are expressed as percentage of constriction as compared to baseline lumen diameter. * denotes $p < 0.05$ vs. Sham; $n=10/\text{group}$.

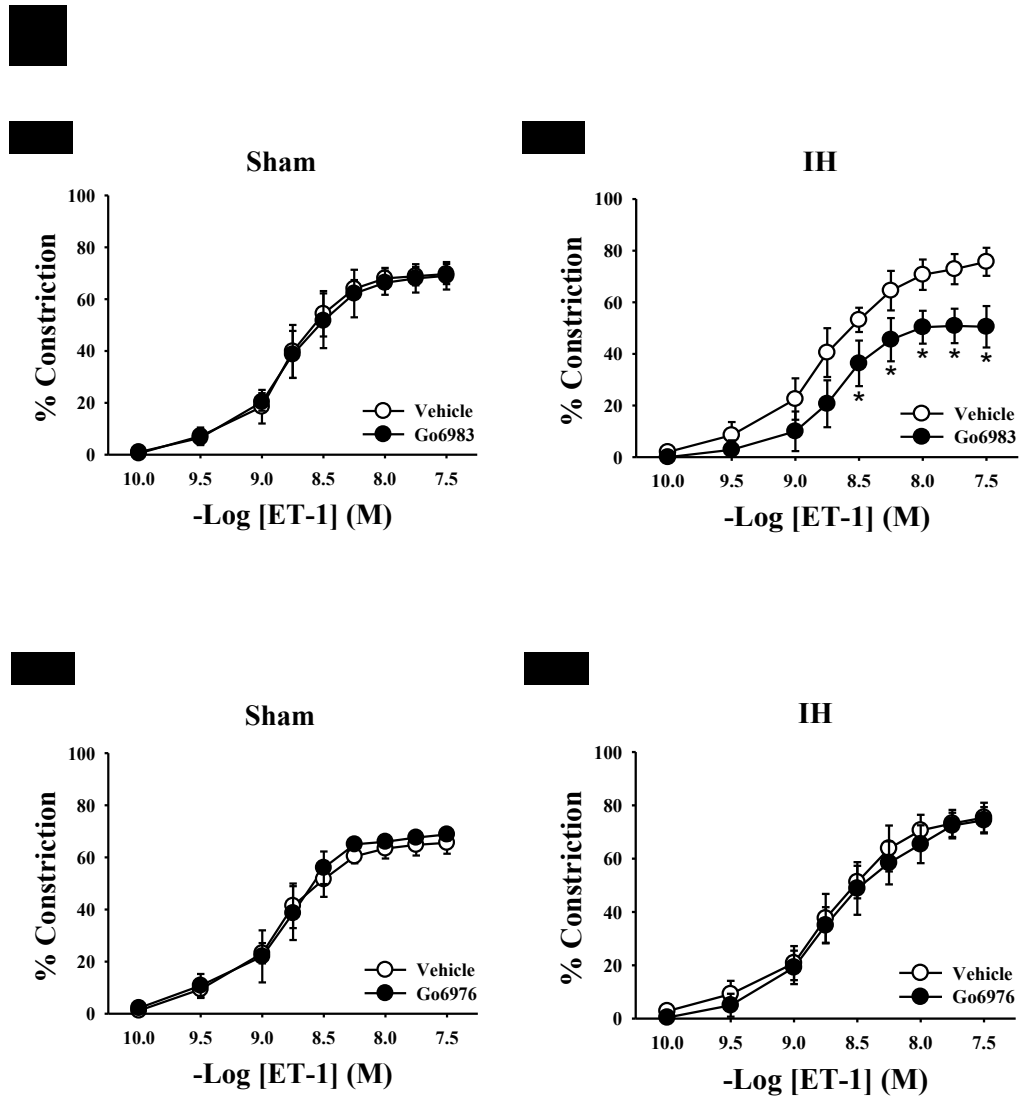


Figure 2: Pan-PKC inhibition with Go6983 (1 μ M) had no effect on ET-1-mediated vasoconstriction in mesenteric arteries from Sham rats (A) but attenuated constriction in arteries from IH rats (B). Go6976 (1 μ M), a selective classical PKC inhibitor, did not affect ET-1-induced constriction in arteries from either Sham (C) or IH (D) rats. * signifies $p < 0.05$ vs. respective vehicle treatment; $n=5-6$ /group.

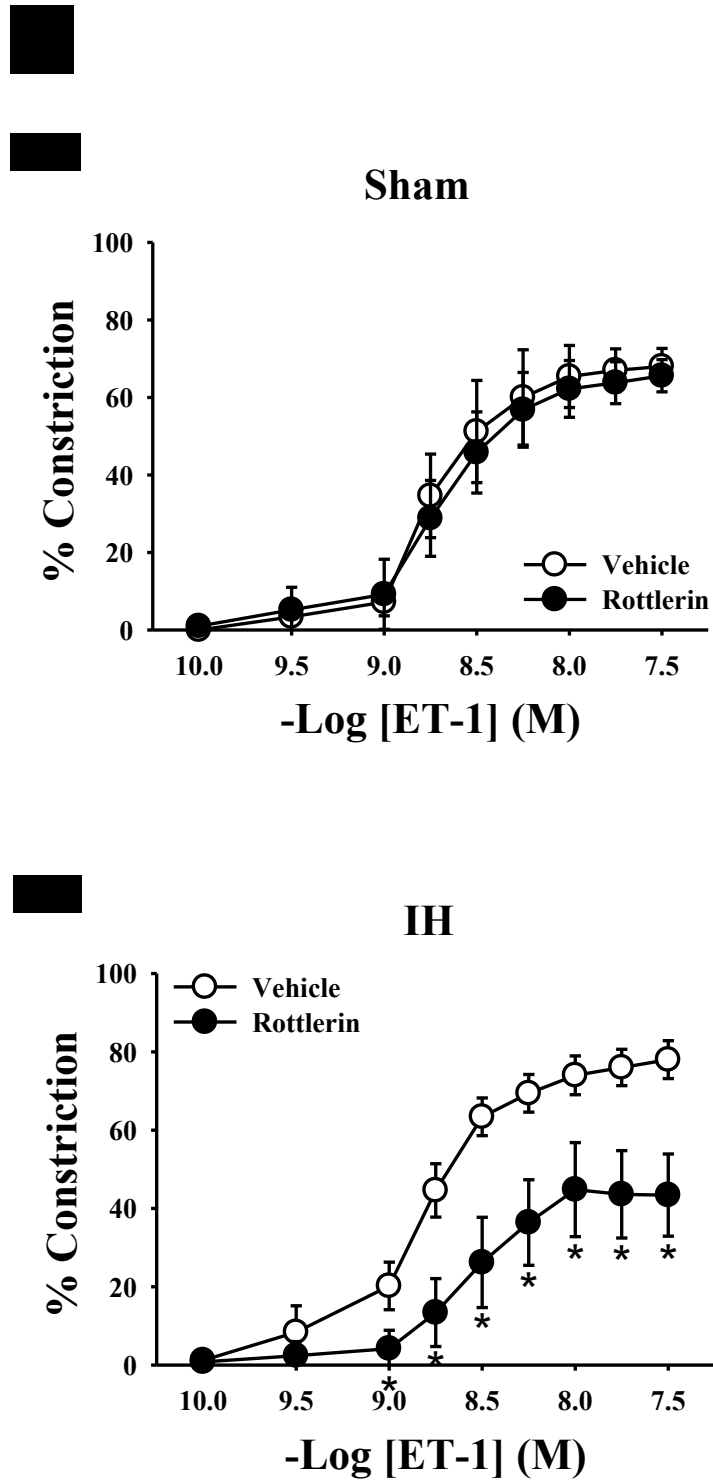


Figure 3: The PKC δ inhibitor rottlerin (3 μ M) was without effect on the ET-1 constrictor response in arteries from Sham rats (A) but attenuated constriction in response to ET-1 in mesenteric arteries from IH-exposed rats (B). * $p < 0.05$ vs. vehicle treatment; $n=5$ /group.

Similar to the effect of the PKC inhibitors rottlerin and Go6983, ET-1-mediated constriction in mesenteric arteries from IH rats was greatly reduced after exposure to the PDK-1 inhibitor OSU-03012 (Figure 4). In contrast, OSU-03012 had no effect on ET-1-mediated constriction in arteries from Sham rats, suggesting that ET-1 does not normally signal through PDK-1 to constrict mesenteric arteries. PKC-mediated vasoconstriction was measured in the absence and presence of Go6983, rottlerin, and OSU-03012 using the PKC activator PDBu in arteries from Sham rats to verify that PKC δ can contribute to constriction in control arteries if activated and that the effects of OSU-03012 were not due to non-specific inhibition of PKC. As shown in Figure 4C, OSU-03012 had no effect on PDBu-mediated vasoconstriction. Conversely, inhibition of PKC δ (rottlerin) greatly blunted PDBu-mediated constriction while the non-selective PKC inhibitor (Go6983) completely blocked constriction. These results suggest that OSU-03012 does not attenuate ET-1 constriction through inhibition of PKC δ and that PKC δ can mediate vasoconstriction in mesenteric arteries from control rats if it is activated.

It is not clear whether augmented PDK-1/PKC δ signaling in arteries from IH rats is unique to ET-1 signaling. However, constriction to the alpha adrenergic receptor agonist phenylephrine was similar in arteries from Sham and IH rats and was not differentially affected by administration of OSU-03012, rottlerin or Go6983, inhibitors of PDK-1 and PKC, respectively (data not shown).

Effects of PI3K Inhibition on ET-1-mediated Constriction

PI3K is a potential upstream regulator of PDK-1 activity; thus, the effect of PI3K inhibition on ET-1 vasoconstriction was assessed. The PI3K inhibitor LY294002 had no effect on ET-1-mediated constriction in mesenteric arteries from IH rats (Figure 5B);

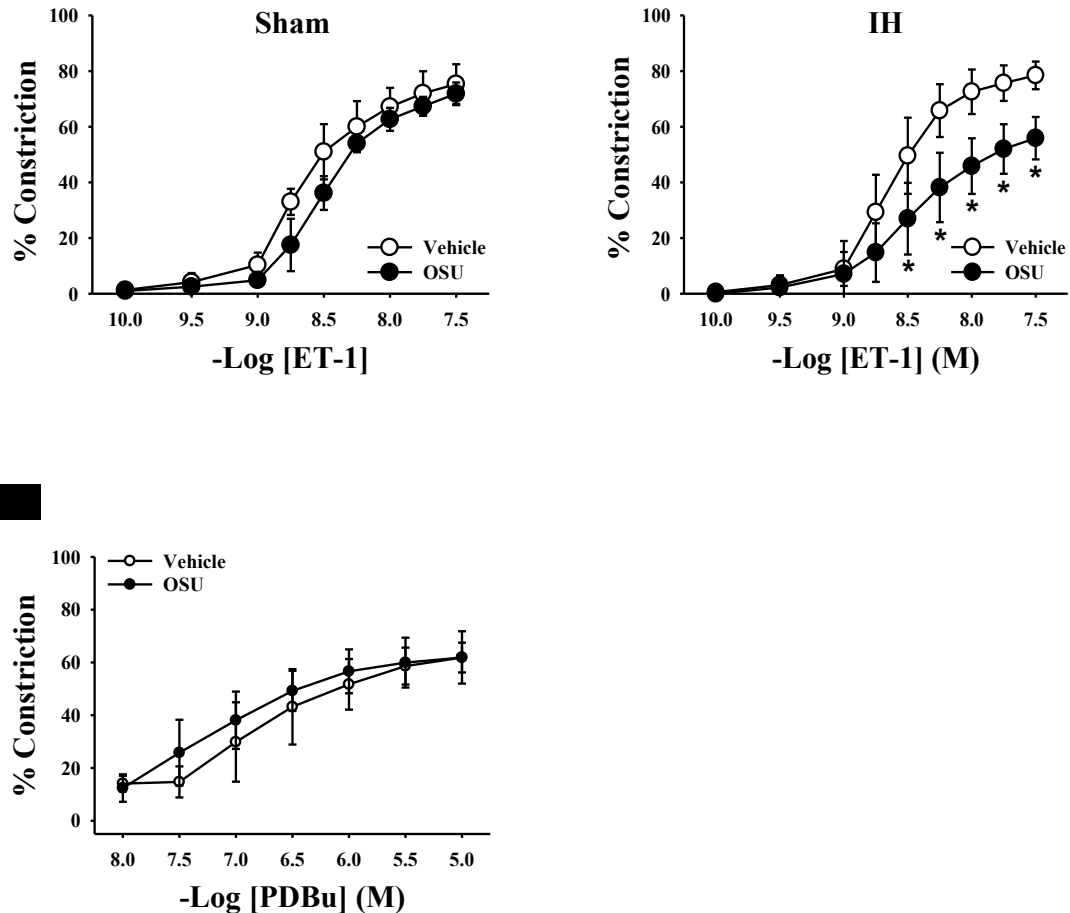


Figure 4: Mesenteric arteries were incubated with the phosphoinositide-dependent protein kinase 1 (PKC-1) inhibitor OSU-03012 (OSU; 10 μ M), and constriction to ET-1 was assessed. As with PKC δ inhibition, ET-1-mediated vasoconstriction was unaffected by PDK-1 inhibition in arteries from Sham rats (A), but constriction was attenuated in arteries from IH-exposed rats (B). (C) Constriction to the PKC activator phorbol 12,13-dibutyrate (PDBu) in arteries from Sham rats was similar in the absence or presence of PDK-1 inhibition but was diminished by non-selective PKC inhibition with Go6983 (1 μ M) or inhibition of PKC δ with rottlerin (3 μ M). * denotes $p < 0.05$ vs. vehicle treatment; n=6-10/group.

however, PI3K inhibition augmented ET-1-mediated constriction in arteries from Sham rats (Figure 5A). To determine whether the augmented ET-1 constriction in the presence of PI3K inhibition in arteries from Sham rats was due to an effect on some endothelial factor, ET-1 constriction was evaluated in the presence of LY294002 in endothelium-disrupted arteries. The augmented ET-1 response in arteries from Sham rats was largely abolished by endothelial disruption, with only a slight, although significant, difference between vehicle- and LY294002-treated arteries (Figure 5C), suggesting that PI3K may activate both an endothelial pathway and have an additional inhibitory effect in vascular smooth muscle. PI3K inhibition had no effect in endothelium-disrupted arteries from IH rats (Figure 5D). Overall these data suggest that the contribution of PDK-1 to augmented ET-1 constrictor sensitivity in IH is independent of PI3K activity.

PDK-1 Phosphorylation and Interaction with PKC δ

Phosphorylation of PDK-1 at the Ser²⁴¹ residue was measured as one indication of PDK-1 activity. There were no detectable differences in phosphorylation levels between mesenteric arteries from Sham and IH rats (Figure 6A-B), indicating that phosphorylation of this residue is not differentially regulated by exposure to IH. ET-1 stimulation of arteries did not affect PDK-1 phosphorylation (data not shown). Total levels of PDK-1 were not altered by IH exposure (Figure 6C).

Interactions between PKC δ and PDK-1 in mesenteric arteries from Sham and IH rats were assessed by measuring co-immunoprecipitation of these proteins (Figure 7) because the degree of their interaction can be used as an indirect indication of PKC δ activation—PKC δ phosphorylation by PDK-1 decreases its association with PDK-1 (Gao

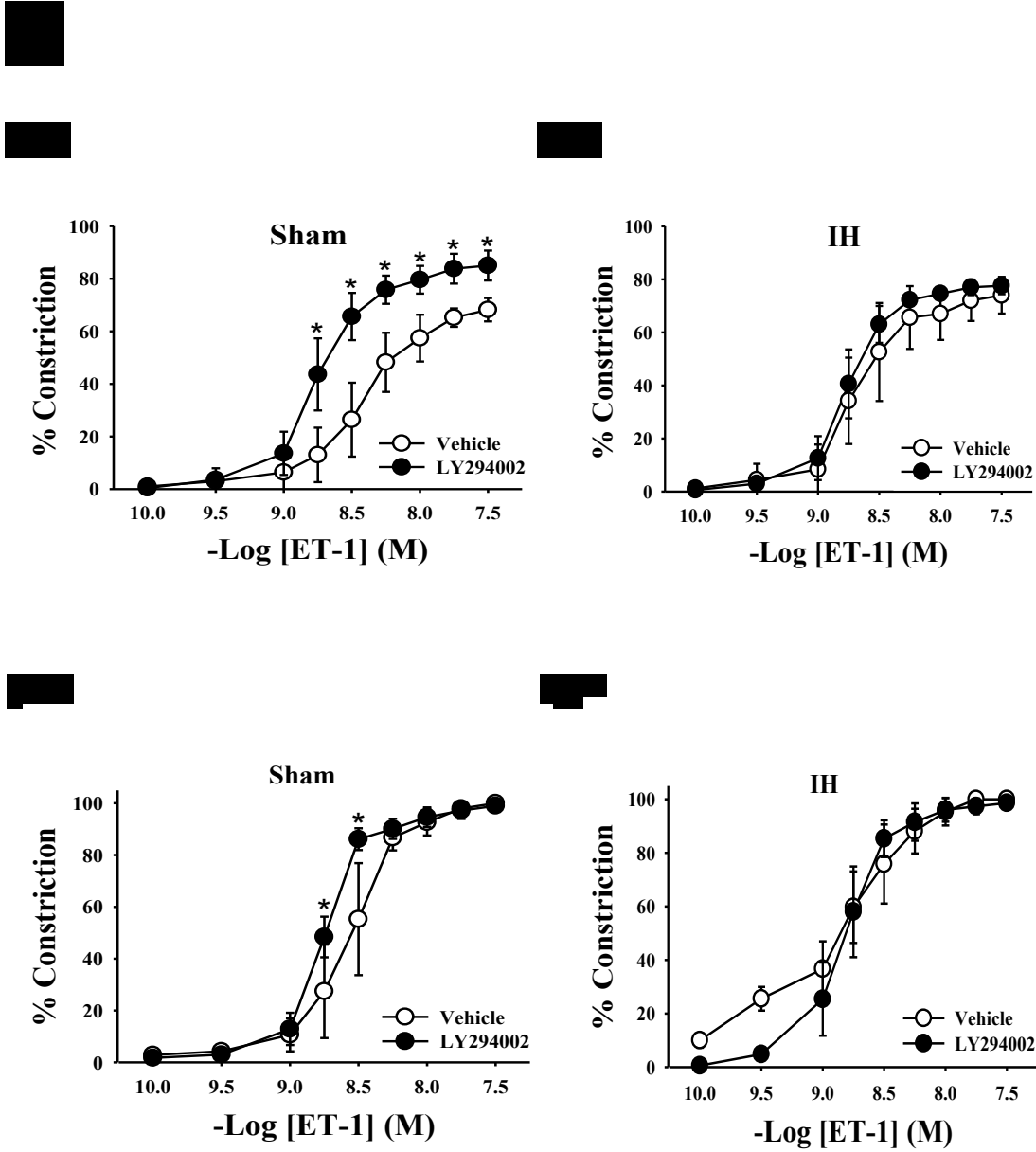


Figure 5: LY294002 (10 μ M), an inhibitor of phosphoinositide 3 kinase, augmented vasoconstriction in endothelium-intact mesenteric arteries from Sham rats (**A**) but had no effect in arteries from IH rats (**B**). Treatment with LY294002 in endothelium-disrupted arteries diminished the augmented constrictor response in Sham arteries (**C**) and had no inhibitory effect in IH arteries (**D**). * $p < 0.05$ vs. respective vehicle group; $n=5$ /group.

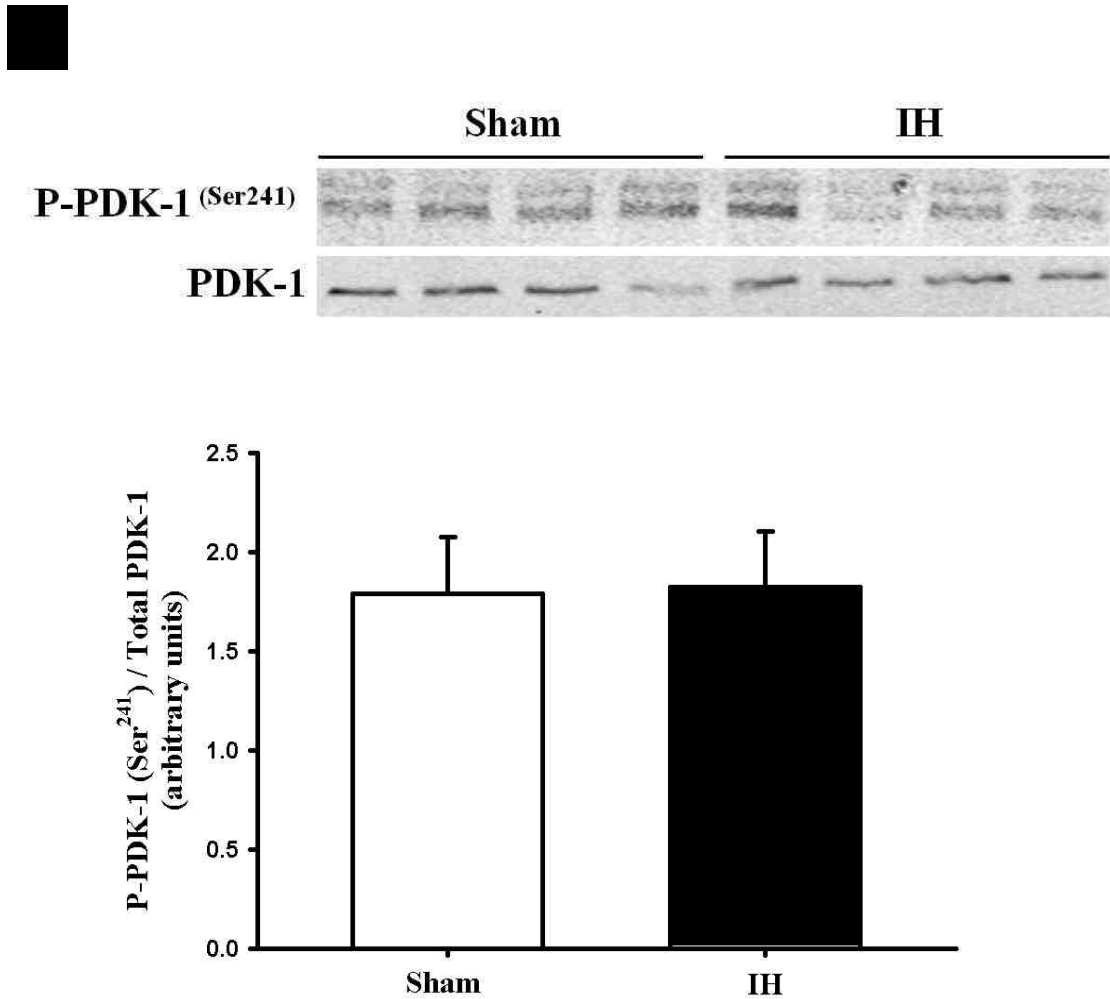


Figure 6: Total and phosphorylated PDK-1 (58-68 kDa) was measured in mesenteric artery homogenates from Sham and IH rats. Representative images are shown in (A) and the average ratios of densitometry values of phosphorylated to total PDK-1 are shown in (B). When expressed as a ratio of total PDK-1 protein, PDK-1 phosphorylation at Ser²⁴¹ was similar between Sham and IH. Total PDK-1 was normalized to coomassie blue staining and was not altered by IH exposure (C). The images shown include tissue from 4 rats/group and are representative of 3 replicates. (A.U. is arbitrary units.)

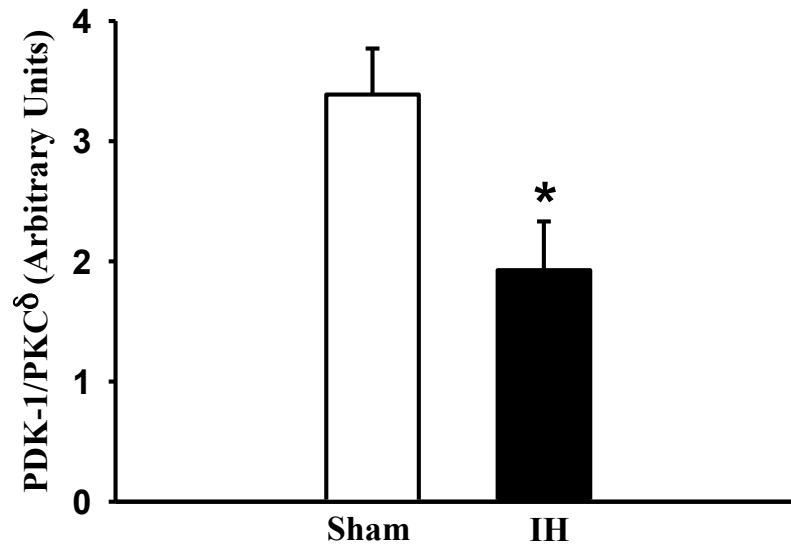
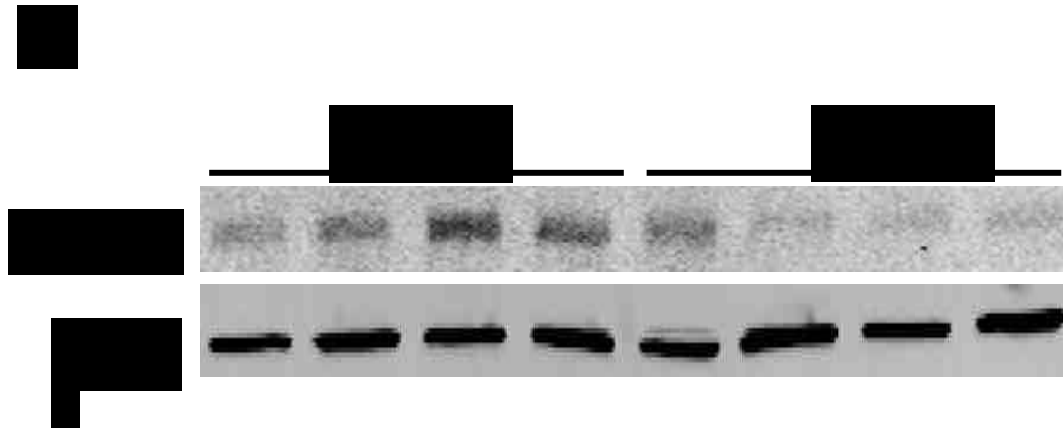


Figure 7: Mesenteric artery homogenates from Sham and IH rats were immunoprecipitated with PKC δ (78 kDa) antibody and probed for PDK-1 (58-68 kDa). There was diminished interaction between PDK-1 and PKC δ in arteries from IH rats compared to those from Sham rats. Total PKC δ levels were normalized to coomassie blue staining and were not affected by exposure to IH. * $p < 0.05$ vs. Sham. The images shown include tissue from 4 rats/group and are representative of 3 replicates. (A.U. is arbitrary units.)

et al., 2001). PKC δ was immunoprecipitated from mesenteric artery lysates and levels of PKC δ -bound PDK-1 were assessed. The ratio of PDK-1 to PKC δ was lower in arteries from IH rats compared to those from Sham rats (Figure 7A-B), demonstrating that IH treatment results in decreased protein:protein interaction between PDK-1 and PKC δ and suggesting enhanced PDK-1 phosphorylation of PKC δ . Total PKC δ levels were similar between arteries from Sham and IH rats (Figure 7C).

PDK-1 Contribution to Blood Pressure

Blood pressure was measured continuously at baseline and throughout the Sham or IH exposure period. Blood pressure was elevated after exposure to IH was initiated, but Sham exposure had no effect on blood pressure (Figure 8). Three days of treatment with the PDK-1 inhibitor OSU-03012 significantly decreased blood pressure in IH rats compared to pressure prior to beginning treatment with OSU-03012; however, PDK-1 inhibition did not affect blood pressure in Sham rats. Treatment with OSU-03012 did not affect plasma concentrations of ET-1 in Sham or IH rats. These results provide evidence of a role for PDK-1 in the IH-induced increase in blood pressure.

DISCUSSION

The findings of this study demonstrate that PDK-1 and PKC δ contribute to the observed increase in ET-1 vasoconstrictor sensitivity in a rodent model of sleep apnea. Furthermore, PDK-1 contributes to the elevated blood pressure observed following chronic exposure to IH to mimic sleep apnea. These results provide the first evidence of a novel role for PDK-1 in blood pressure regulation and ET-1 constrictor sensitivity that is not present under normal conditions but is unmasked after exposure to IH.

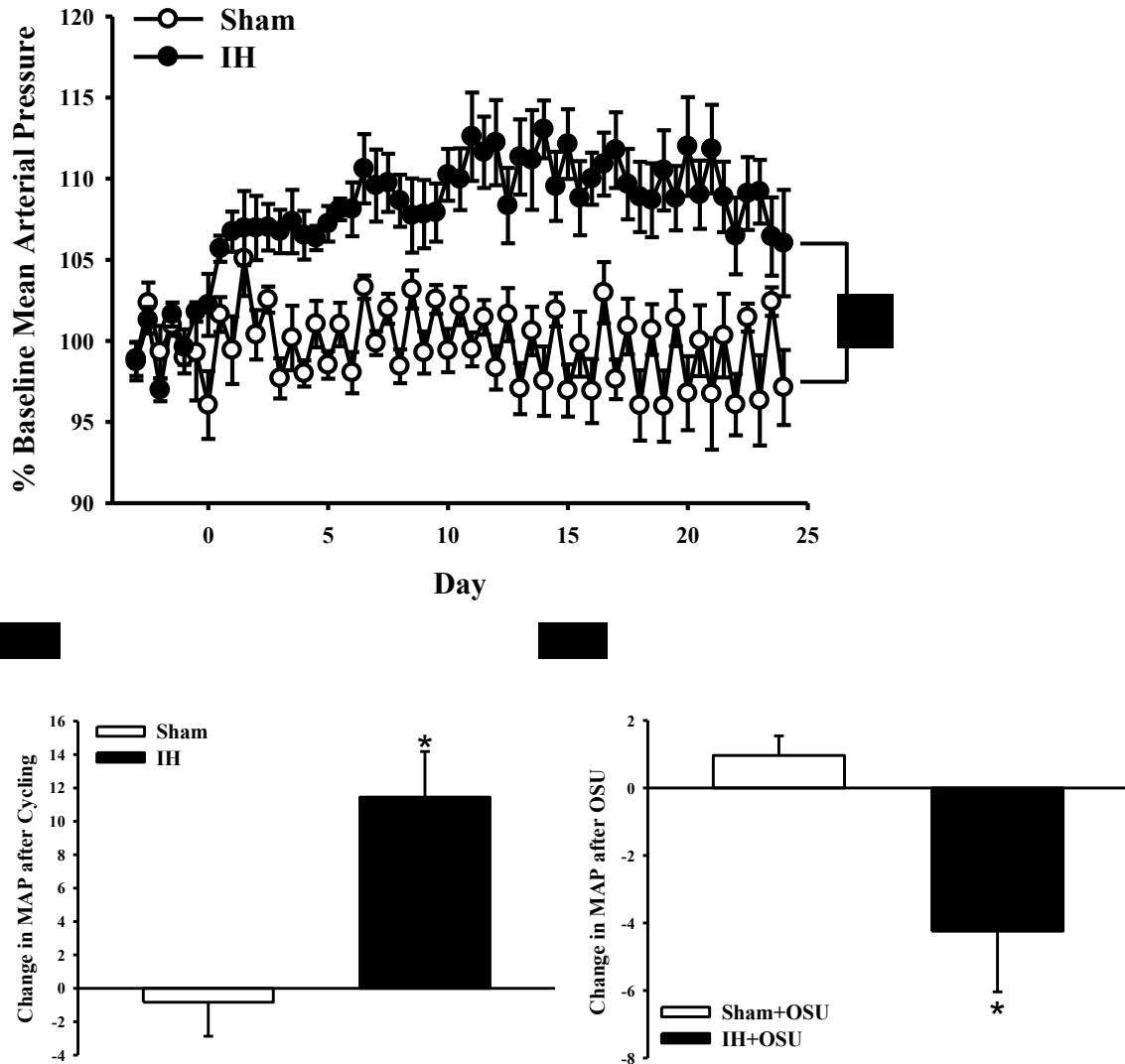


Figure 8: Blood pressure was monitored by telemetry at baseline and throughout Sham and IH exposure protocols (A). The gray box indicates the period of vehicle or OSU-03012 (OSU; 33 mg/day) treatment. The change in mean arterial pressure compared to baseline is shown in (B). Exposure to IH caused an increase in blood pressure, but Sham conditions did not. (C) Treatment with the PDK-1 inhibitor OSU caused a decrease in blood pressure in IH but not Sham rats. * $p < 0.05$ vs. Sham; $n = 4-6$ /group.

In agreement with previous findings using endothelium-disrupted mesenteric arteries (Allahdadi et al., 2008b), this study demonstrates that augmented ET-1-mediated vasoconstriction is dependent on PKC δ in endothelium-intact mesenteric arteries. This PKC δ -dependent mechanism does not appear to be present in all vascular beds since isolated aortic segments from Sham- and IH-exposed rats both show attenuated vasoconstriction in response to pan-PKC and cPKC inhibitors but PKC δ inhibition has no effect (Allahdadi et al., 2008b). Furthermore, inhibition of PDK-1, a kinase activator of PKC δ , with OSU-03012 attenuates ET-1-mediated vasoconstriction in arteries from IH rats, not in those from Sham rats.

Previous studies from our laboratory showed that 2-week exposure to IH increased blood pressure with a corresponding augmentation of vasoconstrictor reactivity to ET-1 (Kanagy et al., 2001; Allahdadi et al., 2005). Circulating concentrations of ET-1 are increased following exposure to IH, and both increased ET-1 expression and increased blood pressure were attenuated by *in vivo* treatment with the antioxidant tempol, suggesting that the IH-induced increase in blood pressure is oxidative stress dependent (Troncoso Brindeiro et al., 2007). We also reported that the IH-induced increase in blood pressure is prevented or reversed by ET_AR antagonists (Kanagy et al., 2001; Allahdadi et al., 2008a).

Our results indicate that PKC inhibitors that block PKC δ activity inhibit ET-1-mediated constriction in mesenteric arteries from IH rats, but those that do not inhibit this isoform are without effect. Therefore, even though rottlerin has non-PKC δ targets (Soltoff, 2007) the selective attenuation of ET-1 constriction in arteries from IH rats, suggests that one or more targets of rottlerin —PKC δ or others—is activated by ET-1

only in IH arteries. Taken together with the finding that OSU-03012 attenuates ET-1 constrictor sensitivity only in arteries from IH rats, our results suggest that exposure to IH unmasks a novel ET-1 constrictor pathway that includes both PDK-1 and PKC δ that is not present under normal conditions.

Results of the current study highlight a novel role for PDK-1 in regulating cardiovascular function following exposure to IH. The majority of studies using the PDK-1 inhibitor OSU-03012 have focused on cancer therapy and the drug's ability to block Akt signaling rather than using it as a tool to inhibit PKC signaling (Zhu et al., 2004; Gao et al., 2008). Thus, the current finding that PDK-1 can regulate constrictor sensitivity to ET-1 and blood pressure following exposure to IH is the first suggestion that this kinase may play a role in blood pressure regulation under some conditions.

The increase in blood pressure following IH exposure likely involves multiple pathways rather than simply being a result of increased PDK-1/PKC δ activity. Previous studies from our laboratory have demonstrated that inhibiting the ET_AR (Allahdadi et al., 2008a) or scavenging of ROS (Troncoso Brindeiro et al., 2007) completely prevent or reverse the IH-induced increase in blood pressure, suggesting that pathways contributing to elevated blood pressure are downstream of these mediators. Additionally, PDK-1 has multiple targets, including Akt. It is possible that a 3-day treatment with the PDK-1 inhibitor OSU-03012 impairs Akt and NO signaling to mitigate blood pressure lowering in the Sham rats.

As described above, PDK-1 can be activated by binding to phosphoinositides, such as phosphoinositol 3,4,5-tris-phosphate (PIP₃) generated by PI3K. However, PI3K

inhibition with LY294002 did not affect ET-1-induced constriction in mesenteric arteries from IH rats even though PI3K inhibition augmented vasoreactivity in endothelium-intact arteries from Sham rats. This augmented constriction in the Sham group could be due to inhibition of PI3K/PDK-1/Akt activation of endothelial nitric oxide synthase (eNOS) (Dimmeler et al., 1999; Zeng et al., 2000; Xiao-Yun et al., 2009). This is supported by the observation that disrupting the endothelium greatly attenuated the effect of PI3K inhibition in arteries from Sham rats but has not been confirmed by direct measurement of nitric oxide (NO) production or eNOS activity. The lack of effect of LY294002 in the arteries from IH rats suggests that these arteries have lower basal NO production or some other form of endothelial dysfunction that may contribute to the hypercontractile state mediated by ET-1 and PKC δ as shown in Figure 3. This potential loss of a PI3K-dependent dilator effect following IH warrants further investigation.

PDK-1 can be activated by conformational changes following binding of lipid products such as PIP₃ at the pleckstrin homology (PH) domain. However the lack of effect on ET-1 vasoreactivity by LY294002 in IH vessels suggests that the augmented PDK-1/PKC δ contribution to ET-1-induced constriction apparent after IH exposure is not caused by increased PIP₃ production. Indeed, other studies demonstrated that lipid binding at the PH domain does not affect PKC phosphorylation levels (Bayascas et al., 2008), and PDK-1 phosphorylates PKC even when the PH domain is mutated (Sonnenburg et al., 2001). Our results are thus more consistent with PDK-1 relying on PI3K-generated PIP₃ to activate Akt and eNOS but phosphorylating PKC independently of PIP₃ binding to its PH domain.

Interestingly, our data suggest that IH exposure diminishes the physical association of PKC δ and PDK-1. Gao and colleagues (Gao et al., 2001) demonstrated that PDK-1 preferentially binds unphosphorylated PKC β II over the phosphorylated form and concluded that PDK-1 not only phosphorylates the activation loop of PKC β II and upregulates PKC β II autophosphorylation, but it is the release from PDK-1 that primes PKC β II for autophosphorylation. Therefore, the decreased association of PDK-1 and PKC δ observed in mesenteric arteries from IH-exposed rats is consistent with the previous observation of increased PKC δ autophosphorylation (Allahdadi et al. H920-H927), and thus increased PKC δ activation.

The mechanism by which IH exposure leads to dysregulation of PDK-1 and its downstream target PKC δ is not yet defined. We have previously shown that arteries from IH rats have increased levels of ROS (Troncoso Brindeiro et al., 2007) and ROS have been shown to activate PDK-1 (Prasad et al., 2000; Zou et al., 2003), raising the possibility that IH-induced elevations in ROS may contribute to the aberrant PDK-1 signaling in mesenteric arteries from IH rats. Block *et al.* (Block et al., 2008) showed that increased ROS in mesangial cells activate the tyrosine kinase Src, which phosphorylates PDK-1, increasing its activity. Tyrosine kinases such as Src have also been shown to activate PKC δ (Rybin et al., 2007) and may contribute to IH-mediated increases in PDK-1 and PKC δ activity, and future studies examining the effects of ROS on PDK-1 activity and phosphorylation of its target PKC δ may provide insight into the mechanism of IH-induced dysregulation of PDK-1 signaling. Of interest, constriction to phenylephrine and PDBu is similar in arteries from Sham and IH rats and vascular expression of PDK-1 and PKC δ is not increased by IH. Thus it appears that IH augments ET-1 constriction by

facilitating coupling of the ET_AR to PKC δ in a PDK-1-dependent manner and that the blood pressure lowering effect of OSU-03012 in IH but not Sham rats, similar to our earlier findings with ET_AR antagonists, is consistent with a selective effect of IH on ET-1 constriction.

In conclusion, our results indicate that exposure to IH increases constrictor sensitivity to ET-1 in a PDK-1- and PKC δ -dependent manner. This pathway is not active under normal conditions, as evidenced by the lack of effect of PDK-1 and PKC δ inhibitors in control conditions, but is unmasked by IH exposure. Although PDK-1 does not appear to regulate blood pressure under normal conditions, our results provide novel evidence that PDK-1 contributes to the IH-induced elevation in blood pressure. The intriguing role of PDK-1 in various pathologies such as cancer, diabetes, and insulin resistance, and now OSA-induced hypertension, suggests that this kinase plays a pivotal role in vascular smooth muscle cell biology. With a PDK-1 inhibitor already in clinical trials, PDK-1 may provide a novel therapeutic target for blood pressure regulation in OSA patients.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Webster, Osmond, Walker, Kanagy

Conducted experiments: Webster, Osmond, Paredes, DeLeon, Jackson-Weaver

Performed data analysis: Webster, Osmond, Paredes, DeLeon, Jackson-Weaver

Wrote or contributed to the writing of the manuscript: Webster, Osmond, Kanagy

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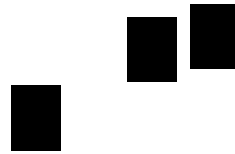
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FOOTNOTES

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