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# Sirtuin Regulation of Mitochondria - Energy Production, Apoptosis, and Signaling

**Eric Verdin**[1,2,*], **Matthew D. Hirschey**[1,2], **Lydia W.S. Finley**[3], and **Marcia C. Haigis**[3,*]

[1]Gladstone Institute of Virology and Immunology, San Francisco, CA

[2]Department of Medicine, University of California, San Francisco, CA

[3]Department of Pathology, The Paul F. Glenn Labs for the Biological Mechanisms of Aging, Harvard Medical School, Boston, MA

## Abstract

Sirtuins are a highly conserved family of proteins whose activity can extend lifespan in model organisms such as yeast, worms, and flies. Mammals contain seven sirtuins (SIRT1-7) that modulate distinct metabolic and stress response pathways. Three sirtuins, SIRT3, SIRT4 and SIRT5, are located in the mitochondrion, a dynamic organelle that functions as the primary site of oxidative metabolism and plays critical roles in apoptosis and intracellular signaling. Recent findings have shed light on how the mitochondrial sirtuins function in the control of basic mitochondrial biology, including energy production, metabolism, apoptosis, and intracellular signaling.

## Mitochondrial biology

Mitochondria play critical roles in energy production, metabolism, apoptosis, and intracellular signaling [1-3]. These highly dynamic organelles have the ability to change their function, morphology and number in response to physiological conditions and stressors such as diet, exercise, temperature, and hormones [4]. Proper mitochondrial function is crucial for maintenance of metabolic homeostasis and activation of appropriate stress responses. Not surprisingly, changes in mitochondrial number and activity are implicated in aging and age-related diseases, including diabetes, neurodegenerative diseases, and cancer [1]. Despite the important link between mitochondrial dysfunction and human diseases, in most cases, the molecular causes for dysfunction have not been identified and remain poorly understood.

One of the principal bioenergetic functions of mitochondria is to generate ATP through the process of oxidative phosphorylation (OXPHOS), which occurs in the inner-mitochondrial membrane. Mitochondria are unique bi-membrane organelles that contain their own circular genome (mtDNA) encoding 13 protein subunits involved in electron transport. The remainder of the estimated 1000-1500 mitochondrial proteins are encoded by the nuclear genome and imported into mitochondria from the cytoplasm [5,6]. These imported proteins can be found either in the matrix, associated with inner or outer mitochondrial membranes or in the inner membrane space (Figure 1). Dozens of nuclear-encoded protein subunits form

*Correspondence: marcia_haigis@hms.harvard.edu, everdin@gladstone.ucsf.edu.

complexes with the mtDNA-encoded subunits to form electron transport complexes I-IV and ATP synthase, again highlighting the need for precise coordination between these two genomes. The transcriptional coactivator PGC-1α, a master regulator of mitochondrial biogenesis and function, is responsive to a variety of metabolic stresses, ensuring that the number and capacity of mitochondria keeps pace with the energetic demands of tissues [7].

As high-energy electrons derived from glucose, amino acids or fatty acids fuels are passed through a series of protein complexes (I-IV), their energy is used to pump protons from the mitochondrial matrix through the inner membrane into the inner-membrane space, generating a proton gradient known as the mitochondrial membrane potential ($D\psi_m$) (Figure 1). Ultimately, the electrons reduce oxygen to form water, and the protons flow down their gradient through ATP synthase, driving the formation of ATP from ADP. Protons can also flow through uncoupling proteins (UCPs), dissipating their potential energy as heat. Reactive oxygen species (ROS) are a normal side-product of the respiration process [1,8]. In addition, an increase in $D\psi_m$, whether caused by impaired OXPHOS or by an overabundance of nutrients relative to ADP, will result in aberrant electron migration in the electron transport chain and elevated ROS production [1]. ROS react with lipids, protein and DNA, generating oxidative damage. Consequently, cells have evolved robust mechanisms to guard against an increase in oxidative stress accompanying ROS production [9].

Mitochondria are the primary site of ROS production within the cell, and increased oxidative stress is proposed to be one of the causes of mammalian aging [1,2,10]. Major mitochondrial age-related changes are observed in multiple tissues and include decreased $D\psi_m$, increased ROS production and an increase in oxidative damage to mtDNA, proteins, and lipids [11-14]. As a result, mitochondrial bioenergetic changes that occur with aging have been extensively reviewed [15-17].

## Sirtuins in the mitochondria

Silent information regulator (SIR) 2 protein and its orthologs in other species, termed sirtuins, promote an increased lifespan in model organisms such as yeast, worms and flies. Mammals contain seven sirtuins (SIRT1–7) that are characterized by an evolutionary conserved sirtuin core domain [18,19]. This domain contains the catalytic activity and invariant amino acid residues involved in binding $NAD^+$, a metabolic co-substrate. All sirtuins exhibit two major enzymatic activities *in vitro*: $NAD^+$-dependent protein deacetylase activity and ADP-ribosyltransferase activity. Except for SIRT4, well-defined acetylated substrates have been identified for the other sirtuins. So far, only ADP-ribosyltransferase activity has been described for SIRT4 [20,21]. Thus, these enzymes couple their biochemical and biological functions to an organism's energetic state via their dependency on $NAD^+$. A decade of research, largely focused on SIRT1, has revealed that mammalian sirtuins regulate metabolism and cellular survival. In brief, SIRT1–7 target distinct acetylated protein substrates and are localized in distinct subcellular compartments. SIRT1, SIRT6 and SIRT7 are found in nucleus, SIRT2 is primarily cytosolic and SIRT3, 4 and 5 are found in the mitochondria. The mitochondrial-only localization of SIRT3 is controversial and other groups have reported non-mitochondrial localization of this sirtuin [22,23]. The biology and biochemistry of the seven mammalian sirtuins have been extensively discussed in the literature [24-26] and is not the topic of this review. Instead, we focus on the mitochondrial sirtuins, their substrates, and their impact on mitochondrial biology.

The mitochondrial sirtuins, SIRT3–5 [21,27-29], participate in the regulation of ATP production, metabolism, apoptosis and cell signaling. Unlike SIRT1, a 100 kDa protein, the mitochondrial sirtuins are small, ranging from 30–40 kDa. Thus, their amino acid sequence

consists mostly of an N-terminal mitochondrial targeting sequence and the sirtuin core domain, with small flanking regions. Whereas, SIRT3 and SIRT5 function as $NAD^+$-dependent deacetylases on well defined substrates, SIRT4 has no identified acetylated substrate and only shows ADP-ribosyltransferase activity. It is likely, however, that SIRT4 possesses substrate-specific $NAD^+$-dependent deacetylase activity, as has been demonstrated for SIRT6 [30,31]. The three-dimensional structures for the core domains of human SIRT3 and human SIRT5 have been solved and reveal remarkable structural conservation with other sirtuins, such as the ancestral yeast protein and human SIRT2 (Figure 2) [32-34]. Given its sequence conservation with the other sirtuins [18], it is likely that SIRT4 adopts a similar three-dimensional conformation.

Although mitochondrial sirtuins have not been as extensively studied as yeast Sir2 or its ortholog SIRT1, recent reports illustrate the importance of SIRT3, 4 and 5 in basic mitochondrial biology. Furthermore, a growing body of mass spectrometry data demonstrates that numerous mitochondrial proteins involved in metabolism and stress are acetylated [35-37], hinting that sirtuins might regulate a broad range of mitochondrial biology, including energy production, signaling, and apoptosis.

## Role of mitochondrial sirtuins in metabolism and energy production

The $NAD^+$ dependence of sirtuins provided the first clue that these enzymes function as metabolic sensors. For instance, sirtuin activity can increase when $NAD^+$ levels are abundant, such as times of nutrient deprivation. In line with this model, mass spectrometry studies have revealed that metabolic proteins, such as tricarboxylic acid (TCA) cycle enzymes, fatty acid oxidation enzymes and subunits of oxidative phosphorylation complexes are acetylated in response to metabolic stress [35-37].

### Fatty acid oxidation

Consistent with the hypothesis that nutrient stress alters sirtuin activity, a recent report identified significant metabolic abnormalities in *Sirt3*$^{-/-}$ mice during fasting [38]. In this study, hepatic SIRT3 protein expression increased during fasting, suggesting that both its levels and enzymatic activity are elevated during nutrient deprivation. SIRT3 activates hepatic lipid catabolism via deacetylation of long-chain acyl-CoA dehydrogenase (LCAD), a central enzyme in the fatty acid oxidation pathway. *Sirt3*$^{-/-}$ mice have diminished fatty acid oxidation, develop fatty liver, have low ATP production, and show a defect in thermogenesis and hypoglycemia during a cold test [38].

Surprisingly, many of the phenotypes observed in *Sirt3*$^{-/-}$ mice were also observed in mice lacking acetyl-CoA synthetase 2 (AceCS2), a previously identified substrate of SIRT3 [39,40]. For example, fasting ATP levels were reduced by 50% in skeletal muscle of *AceCS2*$^{-/-}$ mice, in comparison to wild type (WT) mice. As a result, fasted *AceCS2*$^{-/-}$ mice were hypothermic and had reduced capacity for exercise. By converting acetate into acetyl CoA, AceCS2 provides an alternate energy source during times of metabolic challenges, such as thermogenesis or fasting. Interestingly, *Acadl*-deficient mice (*Acadl* encodes LCAD) also show cold intolerance, reduced ATP, and hypoglycemia under fasting conditions [41]. These overlapping phenotypes between *Sirt3*$^{-/-}$, *AceCS2*$^{-/-}$ and *Acadl*$^{-/-}$ mice indicate that the regulation of LCAD and AceCS2 acetylation by SIRT3 represents an important adaptive signal during the fasting response (Figure 2).

### Electron transport chain

Of all mitochondrial proteins, oxidative phosphorylation complexes are among the most heavily acetylated. One study reported that 511 lysine residues in complexes I-IV and ATP synthase are modified by acetylation [37], hinting that a mitochondrial sirtuin might

deacetylate these residues. Indeed, SIRT3 interacts with and deacetylates complex I subunits (including NDUFA9) [42], succinate dehydrogenase (complex II) [43]. SIRT3 has also been shown to bind ATP synthase in a proteomic analysis [44]. SIRT3 also regulates mitochondrial translation, a process which can impact electron transport [45]. Mice lacking SIRT3 demonstrate reduced ATP levels in many tissues [42 46]; however, additional work is required to determine if reduced ATP levels in *Sirt3*$^{-/-}$ mice is a direct result of OX PHOS hyperacetylation or an indirect effect, via decreased fatty acid oxidation, or a combination of both effects.

Less is known about the roles of SIRT4 and SIRT5 in electron transport. SIRT4 binds adenine nucleotide translocator (ANT), which transports ATP into the cytosol and ADP into the mitochondrial matrix, thereby providing a substrate for ATP synthase [20]. SIRT5 physically interacts with cytochrome C. The biological significance of these interactions, however, remains unknown [21].

## TCA cycle

Enzymes for the TCA cycle (also called the Kreb's cycle) are located in the mitochondrial matrix; this compartmentalization provides a way for cells to utilize metabolites from carbohydrates, fats and proteins. Numerous TCA cycle enzymes are modified by acetylation, although the functional consequences of acetylation have been examined for only a few of these proteins. SIRT3 interacts with several TCA cycle enzymes, including succinate dehydrogenase (SDH, see above [43]) and isocitrate dehydrogenase 2 (ICDH2) [33]. ICDH2 catalyzes the irreversible oxidative decarboxylation of isocitrate to form alpha-ketoglutarate and $CO_2$, while converting $NAD^+$ to NADH. Although the biological significance of these interactions is not yet known, it seems possible that SIRT3 might regulate flux through the TCA cycle.

SIRT3 also influences the TCA cycle indirectly via its deacetylation and activation of AceCS2 [39,40] and glutamate dehydrogenase (GDH) [33,47]. GDH interconverts glutamate and alpha-ketoglutarate and is a pivotal enzyme in regulating amino acid entry into the TCA cycle. AceCS2 generates acetyl-CoA from acetate and ATP in extra-hepatic tissues. Thus, SIRT3 activity could provide a general mechanism to increase carbon entry into the TCA cycle during fasting by increasing acetyl-CoA and amino acid utilization.

SIRT4 also regulates the TCA cycle indirectly via interaction with GDH. However, unlike SIRT3, SIRT4 is inhibitory to GDH function and suppresses its activity via ADP-ribosylation [21]. Extracts from pancreatic islets or livers from *Sirt4*$^{-/-}$ mice show higher GDH activity [21,48]. Moreover *Sirt4* loss results in increased insulin secretion in response to glucose and amino acids [21], whereas SIRT4 overexpression in insulinoma cells suppresses insulin secretion [20]. Future studies will be needed to elucidate how SIRT4 regulates fuel utilization and mitochondrial respiration.

## Other roles

SIRT5 regulates carbamoyl phosphate synthetase (CPS1) [48,49], the rate-limiting and first step of the urea cycle. CPS1 is required for removing ammonia generated by amino acid catabolism. SIRT5 binds and deacetylates CPS1, stimulating its enzymatic activity [48]. Mice lacking SIRT5 have elevated ammonia levels after a prolonged fast. Additionally, mice overexpressing SIRT5 show increased CPS1 activity. These observations suggest that SIRT5 coordinates the detoxification of hepatic by-products of amino acid catabolism by activating the urea cycle [48]. Although the initial characterization of *Sirt5*$^{-/-}$ mice found no obvious defects in basal glucose, insulin, or lipid homeostasis [47,48], further work could

identify other SIRT5 targets and illuminate additional roles in maintaining energy production.

## Role of mitochondrial sirtuins in signaling

During cellular stress or damage, mitochondria release a variety of signals to the cytosol and the nucleus to alert the cell of changes in mitochondrial function. In response, the nucleus generates transcriptional changes to activate a stress response or repair the damage. For example, mitochondrial biogenesis requires a sophisticated transcriptional program capable of responding to the energetic demands of the cell by coordinating expression of both nuclear and mitochondrial encoded genes [4]. Unlike anterograde transcriptional control of mitochondria from nuclear transcription regulators such as PGC-1α, the retrograde signaling pathway, from the mitochondria to the nucleus is poorly understood in mammals. Although there is no evidence directly linking sirtuins to a mammalian retrograde signaling pathway, changes in mitochondrial sirtuin activity could influence signals transmitted from the mitochondria. Interestingly, the nuclear sirtuin SIRT1 deacetylates and activates PGC-1α, a key factor in the transcriptional regulation of genes involved in fatty acid oxidation and oxidative phosphorylation (Figure 3) [50,51]. Thus, mitochondrial and nuclear sirtuins might exist in a signaling communication loop to control metabolism.

Numerous signaling pathways are activated by changes in mitochondrial release of metabolites and molecules, such as $Ca^{2+}$, ATP, $NAD^+$, NADH, nitric oxide (NO), and ROS (Figure 3). Of these, $Ca^{2+}$ is the best studied as a mitochondrial messenger. Mitochondria are important regulators of $Ca^{2+}$ storage and homeostasis, and mitochondrial $Ca^{2+}$ uptake is directly tied to the membrane potential of the organelle. Membrane potential serves as a gauge of mitochondrial function: disruption of OXPHOS, interruption in the supply or catabolism of nutrients or loss of structural integrity generally result in a fall in membrane potential, and, in turn, decreased mitochondrial $Ca^{2+}$ uptake. Subsequent increases in cytosolic free $Ca^{2+}$ will activate calcineurin and several $Ca^{2+}$-dependent kinases [52] and affect a wide variety of transcription factors to produce appropriate cell-specific transcriptional responses [53]. Through regulation of nutrient oxidation and electron transport or yet to be identified target(s), mitochondrial sirtuins could influence membrane potential and $Ca^{2+}$ uptake.

Although the effect of sirtuins on intracellular calcium signaling has not been studied directly, sirtuin effects on ATP production have been shown. ANT facilitates the exchange of mitochondrial ATP with cytosolic ADP. As a result the cytosolic ATP:ADP ratio reflects changes in mitochondrial energy production. A fall in ATP production activates AMP-activated protein kinase (AMPK), which directly stimulates mitochondrial energy production, inhibits protein synthesis through regulation of mammalian target of rapamycin (mTOR), and influences mitochondrial transcriptional programs [54]. SIRT3 regulates ATP levels in a variety of tissues, suggesting that its activity could have an important role in ATP-mediated retrograde signaling [46,55]. Indeed, recent studies have shown that SIRT3 regulates AMPK activation [56-58]. Furthermore, SIRT4 interacts with ANT [20], raising the possibility that SIRT4 activity also influences the ATP:ADP ratio or membrane potential and modulates important mitochondrial signals.

$NAD^+$ and NADH levels are intimately connected with mitochondrial energy production and regulate mitochondrial sirtuin activity. Unlike $NAD^+$, however, NADH is not a sirtuin co-substrate. Indeed, changes in the $NAD^+$:NADH ratio can change the redox state of the cell and alter the activity of enzymes such as poly-ADP-ribose polymerases and sirtuins, with subsequent effects on signaling cascades and gene expression [59-61]. Changes in mitochondrial sirtuin activity could change the balance of these metabolites within the

mitochondria. For example, fatty acid oxidation reduces $NAD^+$ to NADH, which is oxidized back to $NAD^+$ by OXPHOS. However, it is unclear whether changes in $NAD^+$/NADH can be transmitted outside the organelle. The inner mitochondrial membrane is impermeable to $NAD^+$ and NADH; however, the mitochondrial malate-aspartate shuttle could transfer reducing equivalents across the mitochondrial membranes.

ROS and NO are well-established signaling molecules that exert pleitropic effects in a large part by modifying the cellular redox state. Changes in SIRT3 expression are associated with aberrant ROS production or scavenging [62]. It will be important for future studies to elucidate how sirtuin-mediated changes in mitochondrial metabolism influence the redox status of the cell as well as the resulting transcriptional consequences.

## Mitochondrial sirtuin control of apoptosis

Apoptosis is a cellular process of programmed cell death. Mitochondria play an important role in apoptosis by the activation of mitochondrial outer membrane permeabilization, which represents the irrevocable point of no return in committing a cell to death. Outer membrane permeabilization leads to the release of caspase-activating molecules, caspase-independent death effectors, and disruption of ATP production. Despite the central role for mitochondria in the control of apoptosis, surprisingly little is known about how mitochondrial sirtuins participate in apoptotic programs. SIRT3 plays a pro-apoptotic role in both BCL2-53- and JNK-regulated apoptosis [63]. Additionally, cells lacking SIRT3 show decreased stress-induced apoptosis, lending further support for a pro-apoptotic role for SIRT3 [62]. Furthermore, recent work points to a tumor suppressive role for SIRT3: SIRT3 levels are decreased in human breast cancers and *Sirt3* null mice develop mammary tumors after 12 months [62]. The mechanism for the tumor suppressive function of SIRT3 is incompletely understood, but involves repression of ROS and protection against DNA damage [62]. In conflicting studies, SIRT3 has been shown to be anti-apoptotic. For example, in the cellular response to DNA damage when mitochondrial $NAD^+$ levels fall below critical levels, SIRT3 and SIRT4 display anti-apoptotic activity, protecting cells from death [64]. SIRT3 has also been shown to be cardioprotective, in part by activation of ROS clearance genes [65]. In future studies, it will be important to elucidate the balance achieved by SIRT3 between stress resistance (anti-apoptosis) and tumor suppression (pro-apoptosis). Additionally, the role of SIRT4 and SIRT5 in regulating metabolism suggests that these mitochondrial sirtuins could also contribute to apoptosis in tumor suppressive or stress resistant manners.

## Concluding remarks

An elegant coordination of metabolism by mitochondrial sirtuins is emerging where SIRT3, SIRT4 and SIRT5 serve at critical junctions in mitochondrial metabolism by acting as switches to facilitate energy production during nutrient adaptation and stress. Rather than satisfy, these studies lead to more questions. How important are changes in global mitochondrial acetylation to mitochondrial biology and is acetylation status a readout for sirtuin activity? What are other substrates for SIRT4 and SIRT5? What molecular factors dictate substrate specificity for mitochondrial sirtuins? Moreover, further studies will provide insight into the therapeutic applications for targeting mitochondrial sirtuins to treat human diseases. It is clear that many discoveries have yet to be made in this exciting area of biology.

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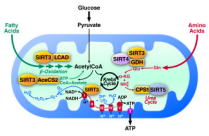


**Figure 1. Network of mitochondrial sirtuins**

Mitochondria can metabolize fuels, such as fatty acids, amino acids, and pyruvate, derived from glucose. Electrons pass through electron transport complexes (I-IV; red) generating a proton gradient, which is used to drive ATP synthase (AS; red) to generate ATP. SIRT3 (gold) binds complexes I and II, regulating cellular energy levels in the cell [43,55]. Moreover, SIRT3 binds and deacetylates acetyl-CoA synthetase 2 (AceCS2) [39,40] and glutamate dehydrogenase (GDH) [33,47], thereby activating their enzymatic activities. SIRT3 also binds and activates long-chain acyl-CoA dehydrogenase (LCAD) [46]. SIRT4 (light purple) binds and represses GDH activity via ADP-ribosylation [21]. In the rate-limiting step of the urea cycle, SIRT5 (light blue) deacetylates and activates carbamoyl phosphate synthetase 1 (CPS1) [48,49].

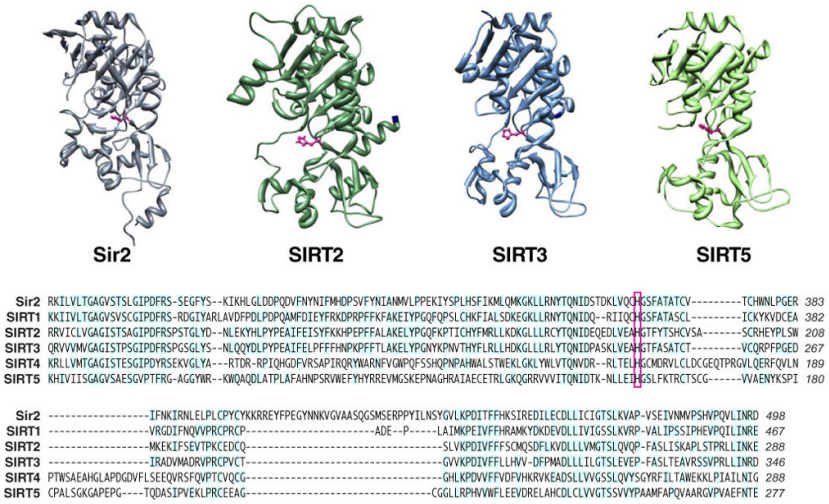


**Figure 2. Structure and alignment of sirtuins**

Three dimensional structures of the sirtuin core domains from yeast Sir2 (DOI: 10.2210/pdb2hjh/pdb), mammalian SIRT2 [66], SIRT3 [32] and SIRT5 [34] were generated using UCSF Chimera [67]. In each structure, the catalytic histidine residue is highlighted in pink, and the N and C termini are shown in navy. Reference sequences for yeast Sir2 and mammalian SIRT1-5 were aligned by ClustalW and the region surrounding the catalytic core is shown with highly conserved amino acid residues shaded in blue and the catalytic histidine residue boxed in pink.

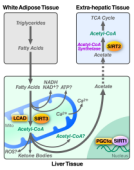


**Figure 3. Mitochondria at nexus of cellular signaling**

Mitochondria and mitochondrial sirtuins play a central role in intra- and extra-cellular signaling. Circulating fatty acids and acetate provide whole body energy homeostasis. The mitochondrial metabolites $NAD^+$, NADH, ATP, $Ca^{2+}$, ROS, ketone bodies, and acetyl-CoA participate in intracellular signaling.