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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.

Keywords

SIRT1; SIRT3; sirtuins; mitochondria; redox stress; resveratrol; metabolism

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

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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⁺ [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 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- γ 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 antioxidant 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 proteasomal 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 (~75%) of embryonic lethality. Nevertheless, *in vivo* studies have also 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 being 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 FOXO3a 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 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 (e.g. lipids; see below), by increasing mitochondrial oxidative phosphorylation through deacetylation of various electron transport chain targets, which results in attenuated 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 increases 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, 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.

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Highlights

- > Sirtuin deacetylase enzymes are closely associated with extensions in lifespan.
- > A common phenotype in aging is the dysregulation of biological systems over time.
- > This can be caused by damage accumulation, e.g. from reactive oxygen species (ROS).
- > Recent research has linked the beneficial effects of sirtuins to the control of ROS.
- > We review these links, and examine the mechanisms which regulate sirtuin activity.

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 HIF1α activity; however, SIRT1 has the opposite effect on HIF2α. Finally, SIRT1 deacetylates and activates the eNOS, leading to an increase in intracellular cardioprotective NO.

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.

Table 1

Redox-related Gene	Sirtuin	Localization	Result of Sirtuin Action	Reference(s)
p53	SIRT1, SIRT7	Nucleus	Mediates transcriptional activity depending on sirtuin expression level	33–38,77
FOXO3a	SIRT1, SIRT2	Nucleus, cytoplasm	Activates transcriptional activity	39,40
LBK1	SIRT1	Nucleus	Activates LBK1, leading to AMPK phosphorylation	47,48
PGC-1 α	SIRT1	Nucleus	Activates transcriptional co-activation	49 – 55
eNOS	SIRT1	Nucleus	Activates eNOS activity	59
HIF1 α	SIRT1, SIRT6	Nucleus	Repression of transcriptional activity	62,63
HIF2 α	SIRT1	Nucleus	Activates transcriptional activity	31,61
14-3-3 ζ	SIRT2	Cytoplasm	Upregulates 14-3-3 ζ , prevents anoxia-reoxygenation mediated apoptosis	75
IDH2	SIRT3	Mitochondria	Activates enzyme, reduces ROS	89 – 91
SOD2	SIRT3	Mitochondria	Activates enzyme, reduces ROS	85, 90, 97–99
NDUFA9	SIRT3	Mitochondria	Activates enzyme, upregulates ETC activity	100
SDHB	SIRT3	Mitochondria	Activates enzyme, upregulates ETC activity	101
ATP5A	SIRT3	Mitochondria	Activates enzyme, upregulates ETC activity	86
GDH	SIRT3, SIRT4	Mitochondria	Activates enzyme (SIRT3), represses enzyme by ADP-ribosylation (SIRT4)	81, 102
OTC	SIRT3	Mitochondria	Activates enzyme, reduces ammonia toxicity and ROS	20
CPS1	SIRT5	Mitochondria	Activates enzyme, reduces ammonia toxicity and ROS	104
CypD (PPIF)	SIRT3	Mitochondria	Deactivates enzyme, prevents interaction with mPTP	107
LCAD	SIRT3	Mitochondria	Activates enzyme, promotes lipid processing	20,117
ALDH2	SIRT3	Mitochondria	Deacetylation by SIRT3 allows NAPQI binding to ALDH2, reducing its activity	124