Proteins and Cell Regulation 10

Riekelt H. Houtkooper *Editor*

Sirtuins

Proteins and Cell Regulation

Volume 10

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Aims and Scope

Our knowledge of the ways in which a cell communicates with its environment and how it responds to information received has reached a level of almost bewildering complexity. The large diagrams of cells to be found on the walls of many a biologist's offi ce are usually adorned with parallel and interconnecting pathways linking the multitude of components and suggest a clear logic and understanding of the role played by each protein. Of course this two-dimensional, albeit often colourful representation takes no account of the three-dimensional structure of a cell, the nature of the external and internal milieu, the dynamics of changes in protein levels and interactions, or the variations between cells in different tissues.

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Sirtuins

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Introduction

Sirtuins are a conserved protein family with pleiotropic effects on the regulation of cellular function. Initially identified as the *silent information regulator 2* (Sir2) in yeast, which regulates transcriptional silencing of mating-type loci, telomeres, and ribosomal DNA, the sirtuin field received a big boost after the discovery of its life-span-enhancing effect. Indeed, not only in yeast, but also in other simple organisms like flies and worms, sirtuin activation leads to longer life similar to caloric restriction, the only physiological way to extend life-span. This opened up a hunt for the mechanisms underlying the sirtuin effect. Sir2 was described as an NAD-dependent histone deacetylase, suggesting that sirtuins integrate metabolic cues into transcriptional adaptations. In the years to follow, it became apparent that sirtuins indeed serve as metabolic sensors, but their functional output is not restricted to histones and includes a variety of metabolic regulatory proteins.

Considering their role in metabolic regulation, the sirtuins were particularly studied in the context of metabolic disease. The prime focus was on mammalian SIRT1, which is most homologous of Sir2 and epitomizes the seven mammalian sirtuin family members. The development of SIRT1 activators spurred this research area even further, as these compounds prevent the negative effects of diet-induced obesity. Several nutraceutical or pharmaceutical sirtuin activators proved successful in mitigating the effects of such Westernized diets.

More recently, the other mammalian sirtuins started to receive attention, particularly because of the development of specific knockout mouse models. This led to an expanded view of sirtuin biology. First, these different sirtuins integrate metabolic signals such as NAD levels in different compartments and, hence, lead to specific adaptive responses. Second, they regulate various downstream targets, not only representing transcriptional regulators but also single enzymes. Third, novel protein modifications were identified that are subject to sirtuin-mediated deacylation. Finally, the expanded view on sirtuin mode of action and downstream consequences also led to a plethora of disease states that are potentially affected by sirtuin activity, for instance, in cancer and neurodegeneration. With these points in mind, the picture emerged that sirtuins constitute a family of stress response proteins, integrating environmental conditions into adaptive actions.

In this book, leaders in the sirtuin field present their view on various aspects of sirtuin biology. The first part of the book (Chaps. 1, 2, and 3) focuses on the biochemical aspects of sirtuin function, including enzymology (Chap. 1), the sirtuin substrate NAD (Chap. 2), and the role of sirtuins in the expanding array of protein deacylation reactions (Chap. 3). Chapters 4, 5, 6, and 7 discuss the functional importance of several individual sirtuins. The best characterized sirtuins, SIRT1 and, SIRT3 are extensively discussed in Chaps 4 and 5, respectively. Other sirtuins that started to receive more attention in recent years—SIRT5 and SIRT6—are covered in Chaps. 6 and 7. The last part of the book (Chaps. 8, 9, 10, 11, and 12) encompasses the main physiological processes in which sirtuins play a role, such as cancer (Chap. 8), muscle function (Chap. 9), aging (Chap. 10), and circadian rhythm (Chap. 11). Chapter 12 reports on the pharmacological ways by which sirtuin activity can be tuned.

Finally, Chap. 13 offers an outlook into the future. While the road for sirtuin research was occasionally bumpy, there are many options to move forward and use sirtuins to improve metabolic health.

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Chapter 1
Biochemistry and Enzymology of Sirtuins

Yue Yang and Anthony A. Sauve

1.1 Biochemistry and Enzymology of Sirtuins

1.1.1 Definition of Sirtuins

Sirtuins are highly conserved NAD^+ dependent deacylase enzymes found in all phyla of life. Genes encoding sirtuins are distributed in practically all unicellular organisms, even as single genes, with well characterized examples found in mycobacteria, eubacteria, and archaea. Sirtuin genes are also encoded in genomes of unicellular eukaryotes including yeast and protozoans. In eukaryotes, sirtuins are generally encoded by multiple genes, leading to distinctive isoforms, so that differentiated cellular functions have been developed for the isoforms, based upon subspecialization of a common catalytic activity. Sirtuins are evolved to recognize the substrate NAD^+, and react the dinucleotide with lysine-acylated peptide and protein substrates, thereby effecting deacylation. In the canonical sirtuin reaction, sirtuins react as NAD^+-dependent lysine deacetylases (Sauve et al. 2006). In mammals, sirtuins are found in 7 distinct isoforms, called SIRT1-7. NAD^+-dependent deacetylation activity has been determined to occur for SIRT1-3, and more weakly for SIRT5-7. Human sirtuin diversification has been accompanied by organelle compartmentalization (Vassilopoulos et al. 2011). Specifically, sirtuins SIRT3, SIRT4 and SIRT5 preferentially locate to the mitochondrial compartment, whereas SIRT1, SIRT6 and SIRT7 are predominantly nuclear (Michishita et al. 2005). SIRT2 is typically found in the cytoplasmic compartment. This compartmentalization accounts for different substrate preferences and different biological roles (Cen et al. 2011c).

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SIRT1  1-260-GAGVSVSCGIPDFRS-RDGIYARLAVDFPDLPDPQAMFDIEYFRKDP--RP---FFKFAKEIYP--GQFQPSLCHKFI
SIRT2  1--83-GAGISTSAGIPDFRSPSTGLYDNL--EKYHLPYPEAIFEISYFKKHP--EP---FFALAKELYP--GQFKPTICHYFM
SIRT3  1-144-GAGISTPSGIPDFRSPGSGLYSNL--QQYDLPYPEAIFELPFFFHNP--KP---FFTLAKELYP--GNYKPNVTHYFL
SIRT4  1--61-GAGISTESGIPDYRSEKVGLYART--DRRPIQHGD------FVRSAPIRQR---YWARNFVGWPQFSSHQPNPAHWAL
SIRT5  1--57-GAGVSAESGVPTFR--GAGGYWRKW--QAQDLATPLA-----FAHNP--SRVWEFYHYRREVM---GSKEPNAGHRAI
SIRT6  1--51-GAGISTASGIPDFRG-PHG-VWTME-ERG--LAPKFDTTF-------------------------ESARPTQTHMAL
SIRT7  1-106-GAGISTAASIPDYRG-PNG-VWTLL--QKGRSVS-AADL--------------------------SEAEPTLTHMSI
             ******  ******    * *                             *                        *    *

SIRT1 ALSD----KEGKLLRNYTQNIDTLEQVAGI--QRIIQCHGSFATASC--LIC--K---YKVDCEAVR---------------
SIRT2 RLLK----DKGLLLRCYTQNIDTLERIAGLEQEDLVEAHGTFYTSHCVSASC--R---HEYPLSWMK---------------
SIRT3 RLLH----DKGLLLRLYTQNIDGLERVSGIPASKLVEAHGTFASATC--TVC--Q---RPFPGEDIR---------------
SIRT4 STWE----KLGKLYWLVTQNVDALHTKAGS--RRLTELHGCMDRVLC--LDC--G---EQTPRGVLQERFQVLNPTWSAEAHGL
SIRT5 AECETRLGKQGRRVVVITQNIDELHRKAGT--KNLLEIHGSLFKTRC--TSC--GVVAENYKSPICP----ALSGKGAPEPGTQ
SIRT6 VQLE----RVGLLRFLVSQNVDGLHVRSGFPRDKLAELHGNMFVEEC--AKC--K---TQYVRDTVV---GTMGLKAT------
SIRT7 TRLH----EQKLVQHVVSQNCDGLHLRSGLPRTAISELHGNMYIEVC--TSCVPN---REYVR--------VFDVTER------
          * *    *** * *    *     * * **       *    *

SIRT1 ---GDIFNQ-------VVPRCPR--CPADEPLAIMKPEIVFFGE--NLPEQ-FHRAMKYDKDEVDLLIVIGSS-443-747
SIRT2 ---EKIFSE-------VTPKCED--CQS-----LVKPDIVFFGE--SLPAR-FFSCMQSDFLKVDLLLVMGTS-264-388
SIRT3 ---ADVMAD-------RVPRCPV--CTG-----VVKPDIVFFGE--PLPQR-FLLHV-VDFPMADLLLILGTS-322-399
SIRT4 APDGDVFLSEEQVRSFQVPTCVQ--CGG-----HLKPDVVFFGD--TVNPD-KVDFVHKRVKEADSLLVVGSS-263-314
SIRT5 D--ASIPVE-------KLPRCEEAGCGG-----LLRPHVVWFGE--NLDPA-ILEEVDRELAHCDLCLVVGTS-252-310
SIRT6 ---GRLCTV--A----KARGLRA--CRG-----ELRDTILDWED--SLPDR-DLALADEASRNADLSITLGTS-217-355
SIRT7 ---TALHRH--Q----TGRTCHK--CGT-----QLRDTIVHFGERGTLGQPLNWEAATEAASRADTILCLGSS-271-400
                         * *   *       * ** ***   *                **  *   * *
```

Fig. 1.1 Pileups of human sirtuins SIRT1-7. The alignment of the seven human sirtuins polypeptides from the first to the last identical amino acid. Sequence homology are highlighted; asterisks indicates an identical amino acid in five or more sirtuins. Adopted from Lavu et al. (2008), *Nature Reviews Drug Discovery* **7**, 841–853

1.1.2 Sirtuin Reactivity

Sequence comparisons as well as functional studies of sirtuins from phylogenetically diverse species confirm that sirtuins have universally conserved biochemical functions as deacylases. The most conserved part of sirtuin proteins is their catalytic machinery, which is embedded into the center of most known sequences, with flanking C and N-terminal sequences involved in targeting, trafficking and protein-protein interactions (See Fig. 1.1 for pileups of human sirtuins SIRT1-7). The catalytic domain includes a binding pocket to enable recognition of NAD^+, and is comprised of a folded domain called a Rossman fold (See Fig. 1.2 for Michaelis complex of a sirtuin complex) (Hoff et al. 2006). The domain also incorporates a hydrophobic channel that can accommodate a lysine residue. The active site is designed to bring together at a reactive geometry the carbonyl oxygen of an acylated lysine substrate and the alpha face of the anomeric carbon of the nicotinamide riboside portion of NAD^+. This binding proximity accelerates the chemical reaction of the acyl-oxygen with the anomeric carbon, thereby leading to nicotinamide bond cleavage, ADP-ribosyl-transfer to the acyl-oxygen and eventual deacylation. The standard stoichiometry for sirtuin chemistry is depicted in Fig. 1.3. Although some interesting exceptions to this general chemistry have been observed, such as ADP-ribosyl-transfer to protein substrates, and ADP-ribosylation of small nucleophiles, this chemistry has been documented to occur on sirtuins derived from diverse species, including human, archaea, protozoan and mycobacterial organisms.

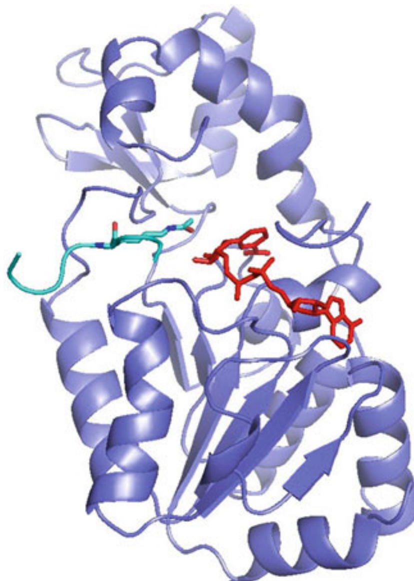


Fig. 1.2 Michaelis complex of a sirtuin complex. The sirtuin (purple) consists of a Zinc-binding domain on top, a cofactor binding loop and a Rossmann-fold domain on the bottom. The cleft at the interface of the two domains contains both the cofactor NAD+ (red) and the acetylated-lysine containing substrate (teal) (Adapted from Reference 5)

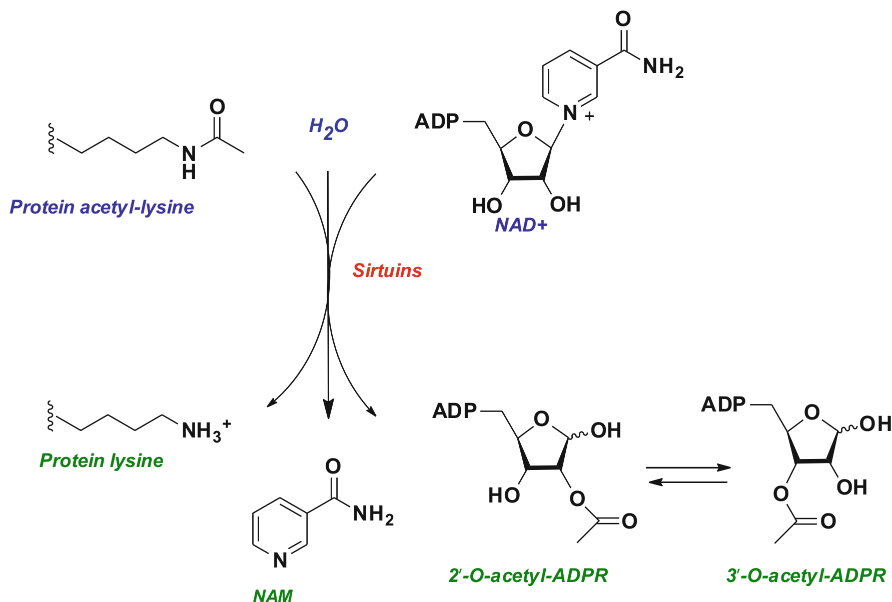


Fig. 1.3 Standard stoichiometry for sirtuin chemistry. Sirtuins use three substrates including NAD^+, water and acetyl-lysine containing protein to generate nicotinamide (NAM), the deacetylated protein substrate and 2′-*O*-acetyl-ADPR. A spontaneous non-enzymatic equilibration between 2′-*O*-acetyl-ADPR and 3′-*O*-acetyl-ADPR occurs in solution

1.1.3 Sirtuin Substrates

Extended surveys and discussions of the full scope of human sirtuin substrates are beyond the scope of this chapter, but several examples are listed in Table 1.1. These target lists indicate that sirtuins interact with a variety of different kinds of protein substrates. Additional reviews that have surveyed sirtuin substrates can be found elsewhere (Baur 2010; Cen et al. 2011c; Haigis and Sinclair 2010). Among notable substrate targets of sirtuins are histones, which are modified extensively in chromatin by acylation and deacylation cycles. This initially led to classification of sirtuins as histone deacetylases, or HDACs. However, it is clearly apparent from many years of work that the majority of sirtuin substrates are in fact not histones, but rather proteins such as transcriptional regulators and enzymatic proteins that are responsible for adaptive changes in cellular biology. The observation that some sirtuins are largely confined to cellular compartments where no histones are present, has led to a complete reconsideration of this nomenclature. More accurately, sirtuins are lysine deacylases or KDACs. It is still true that the most characterized reaction of sirtuin enzymes is NAD^+-dependent deacetylation. However, several examples of non-acetyl substrates are known. For example, SIRT5 is capable of removing succinyl and glutaryl modifications of protein lysines (Tan et al. 2014; Yu et al. 2013). SIRT6 reportedly is able to deacylate long chain fatty acids, such as a myristol modification found on NFκB(Jiang et al. 2013). Evidence from crystallography suggests that SIRT5 and SIRT6 deacylate these modifications of lysine by analogous mechanisms to NAD^+-dependent deacetylation (Zhou et al. 2012).

1.1.4 Sirtuin Reactions and Mechanisms

The prototype reaction stoichiometry of sirtuins combines three distinct reactants as substrates: NAD^+, water and acetylated peptide or protein substrates. These reactants form products as nicotinamide (NAM), the deacetylated protein substrate and a novel compound called 2′-*O*-acetyl-ADPR (AADPR). AADPR has no known cellular function to date, although it appears to provide a diffusible acetate and ADPR equivalent, and may be a second messenger. It spontaneously isomerizes to 3′-O-acetyl-ADPR under physiological conditions (Fig. 1.3) However, its functions have remained largely mysterious ever since it was first fully described in 2001 (Sauve et al. 2001).

The mechanism by which sirtuin catalyzed NAD^+-dependent deacetylation occurs was first proposed by Sauve and Schramm in 2001 (Sauve et al. 2001). These workers proposed that the formation of the AADPR must derive from a reaction of the acetylated substrate interacting with NAD^+ on the alpha face of the NAD^+ substrate. These authors proposed an unprecedented reaction in which the acyl-oxygen would be directly ADP-ribosylated by an electrophilic ADPR species to release NAM and an active site species called an alpha-peptidyl-imidate (originally called

Table 1.1 Substrates for the sirtuins

	Localization	Substrate	Function	References
SIRT1	Nuclear and cytoplasmic	p53	Inhibit apoptosis	Han et al. (2008)
		NF-κB	Reduce inflammatory response	Sauve et al. (1998) and Yeung et al. (2004)
		PPARγ	Repress adipocyte differentiation	Picard et al. (2004)
		PGC1α	Stimulate gluconeogenesis, increase fatty acid oxidation, enhance mitochondrial biogenesis	Dominy et al. (2010), Lagouge et al. (2006), and Yang et al. (2007)
SIRT2	Cytoplasmic and nuclear	α-tubulin, H4K16	Delay cell cycle progression through mitosis	North et al. (2014) and Vaquero et al. (2006)
		FOXO1	Inhibit adipocyte differentiation	Jing et al. (2007) and Nakae et al. (2003)
		FOXO3a	Decrease oxidative stress and promote apoptosis.	Wang et al. (2007)
SIRT3	Mitochondrial	PGC1α	Enhance adaptive thermogenesis and reduce oxidative stress	Kraus et al. (2014) and Shi et al. (2005)
		AceCS2	Accelerate acetyl-CoA conversion	Hallows et al. (2006) and Schwer et al. (2006)
		GDH, ICDH2, SdhA	Facilitate TCA cycle and mitochondrial energy production	Cimen et al. (2010), Kraus et al. (2014), and Schlicker et al. (2008)
		ACAD	Enhance fatty acid oxidation	Hirschey et al. (2010)
SIRT4	Mitochondrial	GDH	Repress amino acid-stimulated insulin secretion	Ahuja et al. (2007) and Haigis et al. (2006)
SIRT5	Mitochondrial	CPS1	Enhance urea cycle	Nakagawa et al. (2009)
SIRT6	Nuclear	HIF-1α	Promote apoptosis, reduce glycolysis and increase mitochondrial respiration	Zhong et al. (2010)
		NF-κB	Reduce inflammatory response	Kawahara et al. (2009)
		DNA pol β	Facilitate DNA damage repair and prevent against aging related disorders	Mostoslavsky et al. (2006)
SIRT7	Nucleolus	RNA pol I	Increase rDNA transcription	Ford et al. (2006)

Abbreviations: *NF-kB* nuclear factor kappa B, *PPARγ* peroxisome proliferator-activated receptor gamma, *PGC1α* PPARγ coactivator 1-alpha, *FOXO* Forkhead box O, *AceCS2* acetyl-CoA synthetase 2, *GDH* glutamate dehydrogenase, *ICDH2* isocitrate dehydrogenase, *SdhA* succinate dehydrogenase flavoprotein, *ACAD* acyl CoA dehydrogenase, *GDH* glutamate dehydrogenase, *CPS1* carbamoyl phosphate synthetase 1, *HIF-1α* hypoxia-inducible factor 1-alpha, *DNA pol β* DNA polymerase β, *RNA pol I* RNA polymerase I

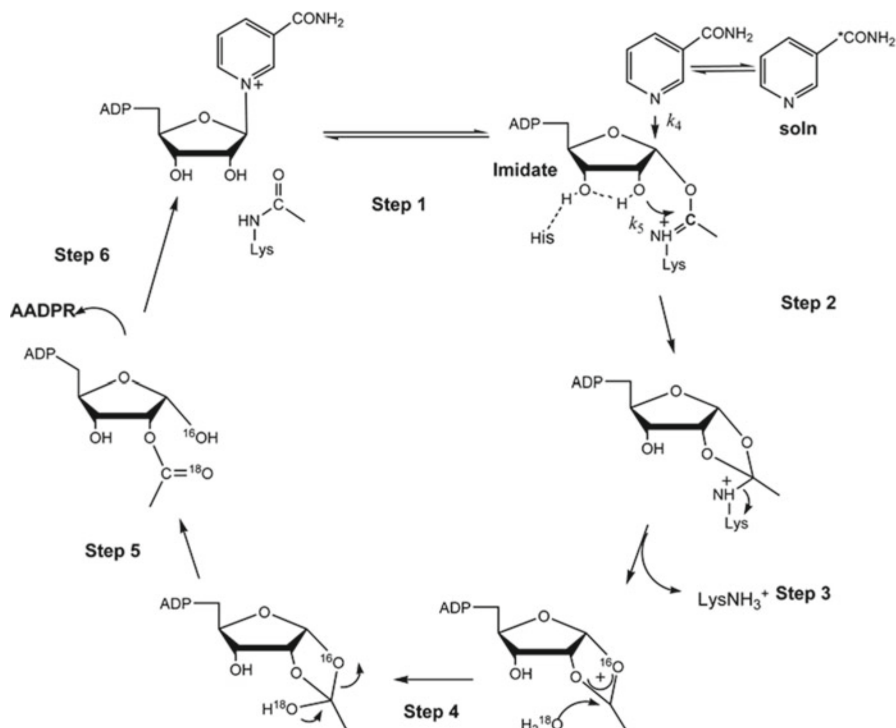


Fig. 1.4 Mechanism of sirtuin catalyzed deacetylation featuring imidate intermediate. The first step in the sirtuin deacetylation reaction generates NAM and an intermediate called a peptidyl-imidate, which has special properties. The imidate is a reactive intermediate that is subjected to intramolecular attack by 2′-OH to produce 2′-*O*-acetyl-ADPR (AADPR). The asterisk indicates radioactively labeled NAM that can be used to monitor the reversal of imidate to NAD^+

an alkylamidate (Sauve et al. 2001)). The putative active site ADP-ribosyl-transfer intermediate was postulated to be somewhat stable and formed reversibly (See Fig. 1.4). These properties were considered to provide a means to enable the reaction mechanism to be sensitive to intracellular NAM concentrations. Supportive mechanistic evidence for this mechanistic proposal was obtained by performing incubations in the presence of ^{14}C-NAM, which resulted in formation of ^{14}C-NAD^+, as well as concomitant inhibition of deacetylation activity. Schramm and Denu laboratories independently established that NAM inhibition of sirtuins, which is important for their biological regulation, relies on this imidate reaction with NAM, which reverses sirtuin chemistry back to NAD^+ (Sauve 2012).

Additional evidence in favor of the imidate mechanism of sirtuin deacylation has been obtained from X-ray structures of products of ADPribosylated thioacetyl and thiosuccinyl substrates on sirtuin active sites. The thio derivatives, having sulfur atoms substituted for oxygen in the acyl position, produce stabilized ADPR-thioimidates (Hawse et al. 2008). In one example, a bicyclic compound predicted by

the Sauve deacetylation mechanism (Fig. 1.4) was found to be formed, a byproduct of reaction of the imidate with the 2-hydroxyl of the ADPR (Zhou et al. 2012). These X-ray structures form analogous structures to typical endogenous substrates, but are stabilized by the sulfur substitution, thereby persisting on the active sites. These structures have provided key evidence to support the original Sauve and Schramm mechanism. Indeed, thioacetyl and thioacyl substrates provide a means to inhibit sirtuins via their cognate catalytic mechanism, and have developed as valuable biological tools.

The complexity of sirtuin chemistry suggests that the enzyme integrates complex realtime metabolic information from NAD^+ metabolism, such as NAD^+ and NAM concentrations, as a means to regulate protein acylation status. Over time, the sirtuins evolved complex regulatory roles in adapting physiology to nutrient status. Work on yeast and human cells indicate that NAM as well as NAD^+ levels are important sirtuin regulators and that these metabolic inputs are highly sensitive to nutrient conditions. This issue is discussed extensively in the next section.

1.1.5 Mechanisms of SIRT1-7 Regulation

1.1.5.1 Transcriptional Regulation of Sirtuins

Caloric or nutrient restriction is known to extend the lifespan of several organisms (Guarente and Picard 2005). The involvement of sirtuins in regulation of nutrient sensing pathways and the coupling of sirtuin activity to a central metabolite, NAD^+, have provided hints that sirtuins might extend lifespan. Interestingly, the prototype sirtuin, silent information regulator (Sir2) from yeast, was identified to extend replicative lifespan in this microbe (Howitz et al. 2003b). In addition, overexpression of Sir2 homologues extends lifespan in experimental organisms *Caenorhabditis elegans* (Tissenbaum and Guarente 2001) and *Drosophila melanogaster* (Wood et al. 2004). Although some controversy has developed on the view that sirtuins are longevity genes (Burnett et al. 2011), the simple idea that increased activity of sirtuin homologues confers lifespan increase has solidified. Overexpression of mammalian sirtuin homologues is associated with health benefits in multiple studies. For example overexpression of SIRT6 causes extension of mouse lifespan. (Kanfi et al. 2012)

Moveover, the expression of mammalian sirtuins is altered by nutrient availability. A 40 % caloric restriction diet induces increased SIRT1 protein level in multiple tissues including in brain, adipose, kidney, and liver in rats (Cohen et al. 2004). In humans subject to 25 % calorie restriction, expression of SIRT1 increases by 113 % in muscle tissue as shown by RT PCR (Civitarese et al. 2007). In 12.5 % reduced calorie intake in combination with 12.5 % increased energy expenditure from exercise, SIRT1 levels were increased by 61 %. These human studies indicate a clear coupling of SIRT1 expression with perturbation in nutritional abundance, linking SIRT1 transcription to this stress. Caloric restriction also causes increases in SIRT3

protein (Lombard et al. 2007; Schwer et al. 2009) and SIRT5 activity (Nakagawa et al. 2009) in liver mitochondria of mice. Similarly, exercise elevates SIRT1 mRNA levels in the skeletal muscle of young physically inactive men (Radak et al. 2011) and SIRT3 protein in the triceps of mice. On the contrary, excessive nutrient intake such as a high-fat diet reduces both mRNA and protein levels of SIRT1 in the adipose of mice (Chalkiadaki and Guarente 2012) whereas obesity diminishes SIRT1 mRNA expression in subcutaneous adipose tissue in humans (Pedersen et al. 2008). In light of emerging evidence for different sirtuin isoforms regulating various metabolic processes, it is crucial to understand the transcriptional regulatory mechanisms that act upstream of sirtuins.

SIRT1 is the most studied sirtuin isoform, and SIRT1 transcription is subject to diverse transcriptional regulatory inputs. The transcriptional regulation of SIRT1 is illustrated in Fig. 1.5. Multiple transcriptional binding sites have been identified on the promoter sequence of SIRT1 and many of the transcription factors are involved in apoptosis (Chen et al. 2005; Fridman and Lowe 2003) and cell cycle regulation (Wang et al. 2006). For example, SIRT1 transcription is regulated by oxidative stress and DNA damage. In the regulatory region of the SIRT1 promoter, two functional p53-binding sites have been identified (Nemoto et al. 2004). p53 is a tumor suppressor and a stress-responsive transcription factor (Fridman and Lowe 2003). Under normal energy status, p53 is activated, exposing its DNA binding domain. The activated p53 is recruited to two p53-binding sites present within the SIRT1 promoter, leading to repression of SIRT1 gene expression. When energy is deprived, forkhead box O transcription factor 3a (FOXO3a) is translocated into the nucleus, where it complexes and removes p53 from the SIRT1 promoter, thus promoting SIRT1 gene transcription (Nemoto et al. 2004). Interestingly, p53 is a direct target for SIRT1 deacetylation. Deacetylated p53 is destabilized and inactivated, which further promotes SIRT1 transcription (Sauve et al. 1998). The p53 activity is affected by a SIRT1 substrate target, namely endothelial NOS (eNOS). SIRT1 deacetylates and activates eNOS (Mattagajasingh et al. 2007). In endothelial cells, eNOS produces $NO^{\bullet}$ and promotes the production of cGMP from GTP. High cGMP content diminishes p53 level and permits SIRT1 transcription (Fraser et al. 2006).

SIRT1 expression is also regulated by hypermethylated in cancer 1 (HIC1), a transcriptional repressor. Upon DNA damage induced by the topoisomerase II inhibitor etoposide, HIC1 forms a transcriptional repression complex with SIRT1. This complex directly binds to the SIRT1 promoter and represses SIRT1 transcription. Knockdown of HIC1 significantly augments SIRT1 expression, which in turn leads to p53 deacetylation and inactivation, allowing cells to survive under the influence of DNA damage (Chen et al. 2005). HIC1 can also repress SIRT1 transcription by forming a complex with the carboxy terminal of E1A-binding protein (CtBP) (Zhang et al. 2007). As a redox sensor, CtBP is activated by elevated NADH levels. Activated CtBP is dimerized and has high affinity towards HIC1 (Fjeld et al. 2003; Kumar et al. 2002). Changes in cellular redox status can alter the recruitment of CtBP to SIRT1 promoter and hence regulate SIRT1 expression (Zhang et al. 2007). Specifically, caloric restriction reduces the association of CtBP with HIC1 thereby promoting SIRT1 transcription (Zhang et al. 2007).

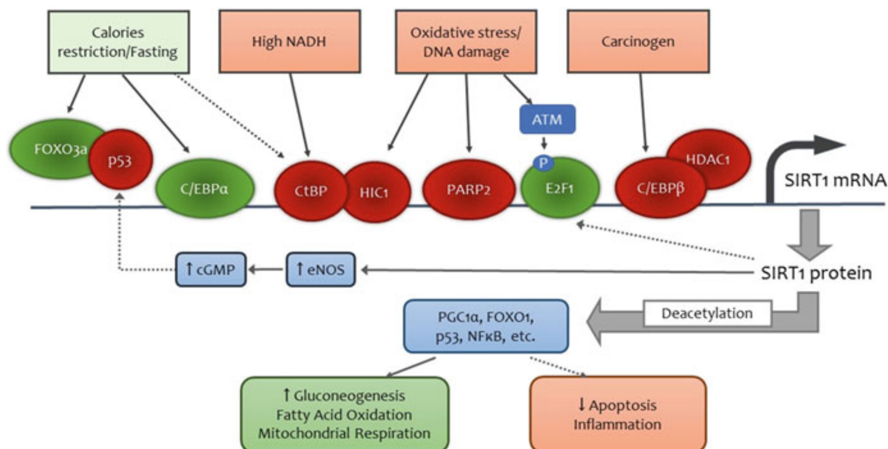


Fig. 1.5 Transcriptional regulation of SIRT1. Multiple transcriptional binding sites have been identified on the promoter sequence of SIRT1. The green circles show the activators of SIRT1 transcription whereas the red circles indicate repressors. The solid arrows suggest positive relationship and the dotted arrows indicate negative regulation. Calories restriction or fasting induces SIRT1 transcription through FOXO3a, which removes the repressor p53 binding to the promoter of SIRT1 under normal energy status, and C/EBPα. As a result of SIRT1 activity on substrates such as PGC1α and FOXO1, gluoconeogenesis, fatty acid oxidation and mitochondrial respiration are enhanced for energy yielding. SIRT1 also activates eNOS and increases cGMP, which diminishes p53 and promotes SIRT1 transcription. Moreover, oxidative stress and DNA damage can either enhance or reduce SIRT1 transcription. Upon DNA damage, ATM is activated to phosphorylate E2F1, which allows it activate SIRT1 transcription. Increased production of SIRT1 protein provides a negative feedback loop by deacetylating and inhibiting the transcriptional activity of E2F1. In addition, oxidative stress induced DNA damage activates HIC1 and PARP2, both are negative regulators of the SIRT1 transcription. Repression of SIRT1 level leads to reduced deacetylation on substrates such as p53 and NFkB, therefore diminishing stress-induced apoptosis or inflammation. Furthermore, high NADH level stimulates the redox sensor CtBP to form a repressor complex with HIC1 to inhibit SIRT1 transcription. The association of CtBP with HIC1 can be reduced by calories restriction to promote SIRT1 mRNA production. Lastly, in liver treated with the carcinogen diethylnitrosamine, C/EBPβ complexes with HDAC1 to repress promoter activity of SIRT1, eventually leading to increased cancer cell proliferation

The transcription factor E2F1 regulates SIRT1 expression. E2F1 is a transcription factor inducing cell cycle progression from G1 to S phase in response to cellular stress (DeGregori et al. 1995). In cells treated with etoposide, E2F1 directly binds to SIRT1 promoter. When cells are under DNA damage, the stress-responsive kinase ataxia telangiectasia mutated (ATM) phosphorylates E2F1 and allow it to bind to SIRT1 promoter in order to activate SIRT1 transcription. In addition, SIRT1 largely regulates its own transcription by a feedback inhibitory mechanism (Sauve et al. 1998). Elevated SIRT1 protein level is able to inhibit the transcriptional activity of E2F1 by deacetylating E2F1, which concomitantly inhibits pro-apoptotic activities of E2F1 as well (Wang et al. 2006).

SIRT1 plays important roles in the regulation of metabolic pathways such as lipid metabolism. Activation of SIRT1 has been shown to protect against obesity

and obesity-associated diseases (Schug and Li 2011). Consistent with a strong interplay between SIRT1 and these pathways, transcription factors involved in lipid metabolism have been demonstrated to modulate SIRT1 transcription. In adipocytes, both SIRT1 mRNA and protein levels were increased along with CCAAT/enhancer-binding protein α (C/EBPα) during adipocyte differentiation (Jin et al. 2010b). C/EBPα can directly bind to the SIRT1 promoter and thereby upregulate SIRT1 transcription. Binding of C/EBPα to the SIRT1 promoter is enhanced upon fasting, and attenuated by feeding (Jin et al. 2010a). In addition, another member of the C/EBP family, C/EBPβ is able to suppress SIRT1 transcription. In a model of diethylnitrosamine-induced liver cancer development, C/EBPβ forms complexes with HDAC1 to repress promoter activity of SIRT1. SIRT1-dependent downstream pathways are reduced, eventually leading to increased liver proliferation and cancer progression (Jin et al. 2013a).

Poly(ADP-ribose) polymerase-2 (PARP-2) has emerged as an additional transcriptional repressor of SIRT1 (Bai et al. 2011a). PARP-2 is a 66.2 kDa nuclear protein capable of binding to damaged DNA (Ame et al. 1999). DNA binding of PARP-2 results in catalytic activation of PARP activity, and PARP-2 subsequently catalyzes the formation of poly(ADP-ribose) polymers (PAR) onto itself and to different acceptors (Ame et al. 1999; Yelamos et al. 2008). In myotubes with PARP-2 knockdown, expression of SIRT1 mRNA is augmented. PARP-2 binds directly to a highly conserved proximal region within the SIRT1 promoter in both mice and human cells. These findings suggest that PARP-2 acts as a direct negative regulator of the SIRT1 promoter (Bai et al. 2011a).

Although studies have also been conducted to address the transcription regulation of other members of sirtuin family, current information is still preliminary. Of important sirtuins, SIRT3 is a mitochondrial enzyme with prominent roles in energy metabolism (Kincaid and Bossy-Wetzel 2013). Peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) plays key roles in regulating energy metabolism through thermogenesis, gluconeogenesis and mitochondrial biogenesis (Liang 2006). In mice myocytes, PGC-1α co-localizes with estrogen-related receptor α (ERRα) and binds to an ERR binding element (ERRE) within the mSIRT3 promoter (Jin et al. 2013b). PGC-1α-induced SIRT3 gene expression is essential for brown adipocyte differentiation (Giralt et al. 2011). Another sirtuin, SIRT6, functions as a corepressor of the transcription factor Hypoxia-inducible factor 1α (HIF1α) to suppress glucose uptake and glycolysis. Thereby, SIRT6 plays crucial roles in glucose and lipid metabolism. In human and mouse cells, p53 binds to the SIRT6 promoter, thus activating the expression of SIRT6. Elevated SIRT6 then interacts with FOXO1 to promote deacetylation and nuclear export, which consequently abolishes FOXO1-induced gluconeogenesis (Zhang et al. 2014).

1.1.5.2 Regulation by Posttranslational Modification

The enzymatic activities of sirtuins can be affected by post-transcriptional modifications. Specifically, phosphorylation is one of the most common mechanisms by which sirtuin function is regulated. SIRT1 is known to have 15 phosphorylation sites (Hwang et al. 2013). The phosphorylation and dephosphorylation status of SIRT1 not only affects the activity of the enzyme, but it also regulates SIRT1 protein levels through proteasome dependent or independent degradation processes (Hwang et al. 2013). Seven of the phosphorylation sites of SIRT1 localize to the N-terminus whereas eight sites are in the C-terminus (Sasaki et al. 2008). Various protein kinases are known to regulate the phosphorylation of these sites including cJun N-terminal kinases (JNKs), casein kinase 2 (CK2), CyclinB/Cdk1, and the dual specificity tyrosine phosphorylation-regulated kinases (DYRKs).

JNKs belong to the mitogen-activated protein kinases (MAPKs) family and are responsive to cellular stresses such as heat shock, radiation and oxidative stress (Bode and Dong 2007). JNK1, when phosphorylated and activated, phosphorylates human SIRT1 on three sites: Ser27, Ser47, and Thr530, whereas the phosphorylation of SIRT1 increases its nuclear localization and enzymatic activity, increasing activity toward a specific deacetylation substrate histone H3 over p53 (Nasrin et al. 2009). Decreases in histone H3 and H4 acetylation are associated with global gene transcription inhibition due to high oxidative stress (Berthiaume et al. 2006). Histone deacetylation potentially increases DNA integrity and reduces DNA damage mediated by free radicals, which could be a protective mechanism to promote cell survival (Berthiaume et al. 2006). Of note, mouse SIRT1 does not contain the aforementioned Ser27 and Thr530 phosphorylation sites, however, JNK1 can also effectively phosphorylate an N-terminal fragment of mSIRT1 (Nasrin et al. 2009). Another group showed that in response to insulin or glucose treatment, JNK1 is activated, leading to phosphorylation of Ser46 in mouse SIRT1, which is equivalent to Ser47 in human. This phosphorylation event on Ser46 leads to SIRT1 ubiquitination which is followed by proteasome-mediated protein degradation, eventually reducing SIRT1 protein level (Gao et al. 2011). In addition, JNK2 is reported to mediate SIRT1 phosphorylation at Ser27, Depletion of JNK2 in human colon cancer cells inhibits phosphorylation of newly expressed SIRT1 protein at Ser27 and reduces the abnormally high accumulation of SIRT1 protein in these cancer cells. (Ford et al. 2008).

CK2 is a ubiquitously expressed and conserved serine/threonine kinase essential for cell viability (Yamane and Kinsella 2005) and it can promote tumor development (Duncan and Litchfield 2008). Upon exposure of HEK293T cells to ionizing radiation, CK2 is recruited to SIRT1 and phosphorylates conserved residues Ser154, Ser649, Ser651, and Ser683 in the N- and C-terminal domains of mouse SIRT1. Phosphorylated SIRT1 has increased deacetylation activity and higher substrate-binding affinity. As a consequence, CK2-mediated phosphorylation increases the ability of SIRT1 to deacetylate p53 and protect HEK293T cells from apoptosis after DNA damage (Kang et al. 2009).

In somatic cells, cyclinB/Cdk1 complex is activated upon passing the G2/M checkpoint and inactivated upon entry into anaphase (Nigg 2001). CyclinB/Cdk1 can phosphorylate human SIRT1 on two conserved sites, Thr530 and Ser540. This phosphorylation is necessary for the entry into G2 phase and progression through the cell cycle. Cell proliferation is impaired when CyclinB/Cdk1 phosphorylation of SIRT1 is blocked or when SIRT1 is absent (Sasaki et al. 2008).

DYRKs are highly conserved protein kinases that catalyze the auto-phosphorylation on tyrosine residues and the phosphorylation of serine/threonine residues on substrates. DYRKs are associated with the regulation of cell proliferation and apoptosis. DYRK1A and DYRK3 can directly interact with and phosphorylate mouse SIRT1 on Thr522 (Thr530 of human SIRT1), which activates SIRT1, promoting cell survival by deacetylating p53 upon cytotoxic drug treatment or heat-shock (Guo et al. 2010).

In addition to phosphorylation, SIRT1 is also subject to sumoylation (Yang et al. 2007b). Small ubiquitin-related modifiers (SUMOs) are covalently linked to lysine residues of target proteins. Under basal conditions, human SIRT1 is sumoylated at Lys734 and is active, deacetylating and inactivating proapoptotic substrates such as p53. In response to hydrogen peroxide induced oxidative stress and UV radiation induced DNA damage, SIRT1 associates with sentrin-specific protease 1 (SENP1), which desumoylates SIRT1. As a consequence, p53 acetylation accumulates and actively induces cell death (Yang et al. 2007b). Although the Lys734 sumoylation motif is evolutionarily conserved from rat to human, the sumoylation residue is replaced by an arginine residue in mouse SIRT1 (Yang et al. 2007b).

Similar to SIRT1, SIRT2 is also subject to phosphorylation modifications. Modifiers of SIRT2 include Cyclin E/Cdk2; an important regulator of the G1/S phase transition known to be deregulated in human tumors (Musgrove 2006). A single Cdk2 phosphorylation site, Ser331, has been identified within the C-terminal of the catalytic domain of human SIRT2. This site is conserved in mouse SIRT2. Phosphorylation at Ser331 inhibits the enzymatic activity of SIRT2 on two substrates, α-tubulin and histone H4, leading to inhibited cell cycle progression and arrested cells in G1 phase (Pandithage et al. 2008).

1.1.5.3 Regulation by Protein Protein Interactions

Two proteins are known to directly bind SIRT1 and regulate its activity. These are active regulator of SIRT1 (AROS) and deleted in breast cancer 1 (DBC1).

AROS is a 142 amino acid protein found exclusively in the nucleus. It was originally identified to play a role in ribosomal function (Maeda et al. 2006). When DNA is damaged with the use of etoposide and TSA, AROS interacts with SIRT1 to facilitate SIRT1 deacetylase function, leading to p53 deactivation which can prevent cell cycle arrest and apoptosis (Kim et al. 2007). The exact molecular mechanism of AROS-mediated activation of SIRT1 remains elusive; however, it has been suggested that AROS binding to SIRT1 is abolished when the N-terminus of SIRT1 is deleted. The N-terminus is reported to mediate recruitment of the substrate histone

H1 (Vaquero et al. 2004) and increase SIRT1 catalytic activity. It has also been speculated that AROS binding replaces a SIRT1 repressor such as DBC1 (Raynes et al. 2013), or recruits an unknown activator which can induce conformational changes in SIRT1 structure. However, later studies questioned the necessity of AROS binding in SIRT1 activation. Under basal condition, knocking down of AROS using RNAi did not increase acetylated or total p53 levels compared with control treatment, suggesting that SIRT1 is capable of suppressing p53 even when AROS levels are low (Knight et al. 2013). Use of recombinant human AROS protein also failed to stimulate SIRT1 activity although cofactors or interacting proteins required for binding and regulating SIRT1 could have been missing from assays (Knight et al. 2013). Another study suggested that the use of recombinant AROS had no effect on SIRT1 deacetylase activity (Kokkola et al. 2014). More mechanisitic studies are needed to clarify the role for AROS in regulating SIRT1 functions.

In contrast to AROS, DBC1 has been implicated as a suppressor of SIRT1 activity. DBC1 was originally identified to be homozygously deleted in human breast cancer specimen (Hamaguchi et al. 2002), and downregulated in some lung and colon cancer cell lines (Hamaguchi et al. 2002). A previous study suggested that DBC1 may play a role in tumor necrosis factor α-mediated apoptosis (Sundararajan et al. 2005). However, the function of DBC1 in tumor cells is still unclear (Kim et al. 2008). A DBC1 binding region was identified within the SIRT1 catalytic domain. When cells were treated with etoposide, DBC1 directly associates with SIRT1, suggesting that DBC1 may inhibit SIRT1 activity in response to stress and cause acetylation of SIRT1 substrates after stress stimuli (Kim et al. 2008). The presence of DBC1 specifically blocks interactions between SIRT1 and p53, which results in upregulation of p53-mediated apoptotic responses (Fulco et al. 2008). Consequently, the current view is that DBC1 inhibition of SIRT1 facilitates the progression of cell death pathways initiated by stress stimuli. In this view, DBC1 may function as a tumor suppressor, and loss of DBC1 can result in the inhibition of cell death and thereby promote tumorigenesis (Kim et al. 2008).

1.1.5.4 Regulation by Alterations in NAD^+ Metabolism

The reaction of sirtuins is initiated by NAD^+ binding to their catalytic sites. Thus, the cellular NAD^+ availability can be posited to be a limiting factor of sirtuin activity (Imai 2009a). In cells, NAD^+ is responsible for transferring electron equivalents for multiple metabolic reactions such as fatty acid β-oxidation, glycolysis and the TCA cycle. In this role, NAD^+ and its reduced form NADH, systematically regulate and coordinate metabolic reactions by direct integration into metabolic pathways, with additional inputs available from various nutritional and environmental signals (Imai 2009). Due to its importance in metabolic reactions, the coupling of NAD^+ levels to sirtuin protein deacetylation activity may have obtained regulatory significance in the course of evolutionary time scales. Importantly, intracellular levels of NAD^+ are dynamic and subject to multiple biosynthetic influences including *de*

novo and salvage pathways, along with nutritional inputs. Dynamics also are coupled to several major NAD^+ degradative pathways, including the activities of NAD^+ consumers such as PARPs, sirtuins themselves and NAD^+ glycohydrolases, such as CD38. By complex pathways and steady-state perturbations, NAD^+ levels change and are thought to regulate sirtuin activities.

NAD^+ can be synthesized by multiple pathways, but appears to be regulated by a central mechanism. *De novo* biosynthesis of NAD^+ can occur via catabolism of the amino acid tryptophan through the kynurenine pathway. However, the main input for NAD^+ homeostasis appears to be the NAD^+ salvage pathway (Houtkooper et al., 2010). This pathway converts NAM, the by-product of NAD^+ consumption, back to NAD^+. The synthesis of nicotinamide mononucleotide (NMN) from NAM is effected by the homodimeric enzyme nicotinamide phosphoribosyltransferase (NAMPT). NMN is subsequently adenylated by the nicotinamide/nicotinic acid mononucleotide adenylyltransferases (NMNAT1-3) to regenerate NAD^+ (Canto et al. 2012; Satoh et al. 2011). Disruption of NAD^+ salvage pathways leads to down regulation of NAD^+ level, and precipitates decline in sirtuin activities (Araki et al. 2004). NAMPT is the rate-limiting enzyme in this pathway, and levels of NAMPT activity are crucial for setting the NAD^+ level (Yang et al. 2007). Overexpression of NAMPT markedly increases NAD^+ levels in neurons and fibroblasts, whereas overexpression of another enzyme in the salvage pathway, NMNAT, does not alter cellular NAD^+ levels (Araki et al. 2004; Revollo et al. 2004). Furthermore, overexpression of NAMPT have been shown to augment cellular levels of NAD^+ in cell models such as endothelial cells, lymphocytes and cardiomyocytes (Borradaile and Pickering 2009; Hsu et al. 2009; Revollo et al. 2004; Rongvaux et al. 2008; Satoh et al. 2011), suggesting high NAMPT level alone can induce NAD^+ biosynthesis. Consistently, pharmacologic use of NAMPT inhibitors substantially suppress cellular NAD^+ levels (Bruzzone et al. 2009; Hasmann and Schemainda 2003), cementing the view that NAMPT is essential for homeostatic control of NAD^+ level.

Factors that affect NAD^+ homeostasis are stress-linked. For example, AMP-activated protein kinase (AMPK) is a major sensor of cellular energy status and it regulates cellular NAD^+ level. Activated by nutrient deprivation and exercise, AMPK activation causes increases cellular NAD^+ level, potentiating SIRT1 activity and enabling increased deacetylation of target proteins such as FOXOs and PGC-1α. These events facilitate increased energy expenditure through fatty acid β-oxidation and mitochondrial biogenesis (Canto et al. 2010; Fulco et al. 2008). Although the complete mechanism of AMPK action on NAD levels is still under investigation, it is partially achieved by up regulating NAMPT expression (Canto et al. 2010; Costford et al. 2010; Fulco et al. 2008). Interestingly, signaling molecules that activate AMPK converge on SIRT1 activity. For example, in adipocytes, fibroblast growth factor 21 (FGF21) activates AMPK in a serine/threonine kinase 11 (STK11/LKB1)-dependent manner and in turn induces SIRT1 activities (Chau et al. 2010). Administration of recombinant FGF21 in *ob/ob* mice increases phosphorylated AMPK level in white adipose tissue. Concomitantly enhanced SIRT1 deacetylation of PGC-1α causes enhanced mitochondrial oxidative capacity and reduced body weight in these mice (Chau et al. 2010). Adiponectin has a similar

effect in myocytes. Binding of adiponectin to its receptor can induce calcium influx which stimulates calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) (Iwabu et al. 2010). Subsequently, CaMKKβ activates AMPK to increase SIRT1 deacetylation activity on PGC-1α. As a result, mitochondrial biogenesis is enhanced in skeletal muscle by adiponectin (Iwabu et al. 2010).

Cellular NAD^+ level can also be augmented by inhibiting the consumption of NAD^+ by NAD^+ consuming enzymes, such as poly(ADP-ribose) polymerase-1 (PARP-1) (Bai et al. 2011b). PARP-1 is a major consumer of NAD^+ in the cell. Upon recognition of damaged or abnormal DNA, PARP-1 binds to the damaged sequence (Durkacz et al. 1980) and uses NAD^+ as a substrate to transfer poly(ADP-ribose) onto acceptors including itself (Adamietz 1987; Sims et al. 1981). High fat diet feeding in mice is shown to robustly increase PARP-1 protein levels and activity in brown adipose tissue and muscle, whereas fasting lowered PARP-1 enzyme activity in these tissues (Bai et al. 2011b). Knockdown or pharmacological inhibition of PARP-1 has been shown to increase intracellular NAD^+ levels while augmenting SIRT1 activity in vitro. PARP-$1^{-/-}$ mice were shown to have higher NAD^+ content and SIRT1 activity in their skeletal muscle and brown adipose tissue (BAT). The mitochondrial activities were also improved in these tissues (Bai et al. 2011b). Therefore, inhibition of PARP-1 activity can be potentially developed as a therapeutic strategy to improve NAD^+ levels in key metabolic tissues.

The effects of NAD^+ level on sirtuin activity, specifically SIRT1 and SIRT3, are consistent with the view that NAD^+ is a positive stimulative regulator of sirtuin activity. For example, Auwerx and co-workers have shown that genetic upregulation of NAD^+ level in PARP1 knockouts increases SIRT1 activity (Bai et al. 2011b). This genetic manipulation increases mitochondrial biogenesis, resistance to toxicity caused by high fat diet and it provides insulin sensitization. In a complementary study, Auwerx and co-workers used the compound nicotinamide riboside (NR) to increase tissue NAD^+ levels. This led to improvements in insulin sensitivity, improved exercise endurance and improvements in resistance to high fat diet toxicity (Canto et al. 2012). Protein blots of tissues showed decreases in acetylation of FOXO1 and PGC1α, consistent with activation of SIRT1. Similarly, SIRT3 activity increases were inferred by decreased acetylation of SOD2. Most recently, Brown and co-workers showed that NR supplementation increases protection from noise induced hearing loss, by activation of SIRT3 (Brown et al. 2014).

Other laboratories have also found that upregulated NAD^+ synthesis leads to sirtuin activation. The laboratory of Shin Imai, has shown that either NAMPT or its product NMN can provide sirtuin activation and treatment of metabolic syndrome (Imai 2009b; Imai and Yoshino 2013; Yoshino et al. 2011).

1.1.5.5 Regulation by Compartmentalization

The members of the sirtuin family have distinct features and roles in mammalian cells and tissues. In terms of cellular compartmentalization, SIRT1 can be shuttled between the nucleus and the cytoplasm, SIRT2 resides in the cytoplasm, and SIRT3,

SIRT4 and SIRT5 localize to the mitochondria. SIRT6 and SIRT7 are found predominantly in the nucleus. These localizations determine a significant part of their activities and biological functions.

The subcellular localization of SIRT1 varies in different tissues in mice at different developmental stages. For example, SIRT1 is primarily found in the cytoplasm of neurons, and exclusively in the nucleus of spermatocytes. Furthermore, SIRT1 is present in both nucleus and cytoplasm of ependymal cells, specialized neuronal cells within the neuroectoderm (Tanno et al. 2007). SIRT1 compartmentalization is also affected by development in mice. In embryonic cardiomyocytes, SIRT1 is found only in the nucleus, but it is expressed in both nucleus and cytoplasm in the adult cardiomyocytes (Tanno et al. 2007). Similar findings have been obtained in undifferentiated and differentiated C2C12 myoblast cells. In postnatal brains, SIRT1 is localized in both nuclear and cytoplasm, and in adult brain, it predominantly resides in the cytoplasm (Fulco et al. 2008). In neural precursor cells, SIRT1 translocates into the nucleus with a differentiation stimulus, and gradually return to the cytoplasm upon neuron differentiation (Hisahara et al. 2008).

Mouse SIRT1 has two nuclear localization sequences (NLSs) and two nuclear export sequences (NESs) (Jin et al. 2007; Tanno et al. 2007). NLSs and NESs sequences are subject to oxidative stress-induced modifications, which alter the subcellular localization of SIRT1. Oxidative stress can activate redox-sensitive kinases such as phosphoinositide 3-kinase (PI3K). PI3K has been suggested to facilitate nuclear localization of SIRT1. Indeed, a pharmacological inhibitor of PI3K inhibits the nuclear localization of SIRT1 in undifferentiated C2C12 cells. The nuclear export of SIRT1 is likely to be achieved through chromosome region maintenance 1 (CRM-1), a member of the exportin family which mediates the exportation of proteins containing an NES. When a CRM-1 inhibitor is used, the nucleocytoplasmic shuttling of SIRT1 is inhibited and SIRT1 remains in the nucleus as determined by a heterokaryon shuttling assay (Tanno et al. 2007).

Nuclear localization of SIRT1 allows it to interact with target transcription regulators, such as p53 and PGC1α to modulate cellular activities. Overexpression of nuclear SIRT1 significantly augments deacetylation of histone H3, as well as markedly inhibits apoptosis induced by reactive oxygen species in C2C12 cells (Tanno et al. 2007). Shuttling of SIRT1 into the cytoplasm is potentially a way to downregulate SIRT1 activity. The cytoplasmic function of SIRT1 is not well understood. SIRT1 localized in cytoplasm can enhance apoptosis. As increased apoptosis is also observed in mutant SIRT1 without deacetylase activity, the pro-apoptotic effect of SIRT1 is not associated with its deacetylase activity. Moreover, use of pan-caspase inhibitor blocked the SIRT1 effect and it is likely that the SIRT1-enhanced apoptosis is caspase-dependent (Jin et al. 2007). In addition, SIRT1 likely functions as deacetylase in the cytoplasm, as many of its substrates, such as p53, FOXOs, are also known to translocate between both nuclear and cytoplasmic compartments (Kwon and Ott 2008).

1.1.6 Assays of Sirtuins

The successful discovery of small molecule sirtuin modulators is largely dependent on the ability to accurately determine sirtuin activities by rapid and reliable assays. For example, resveratrol, along with other sirtuin-activating compounds (STACs), have been discovered by rapid sirtuin activity assays. However, the reliability of some assays for sirtuins have been debated due to their idiosyncratic qualities, which are further discussed below. Knowledge of the caveats associated with sirtuin assays are clearly important to appreciate for researchers working in the sirtuin field.

1.1.6.1 Fluor de Lys Fluorescence Assay

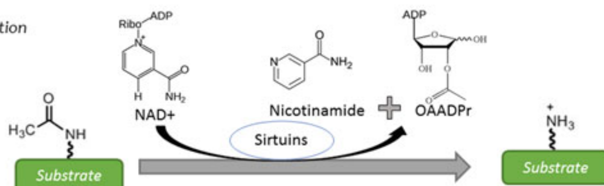
The *Fluor de Lys* fluorescence assay of sirtuin deacetylation activity was introduced by Howitz et al. as a means to screen chemical libraries for activators of SIRT1 (Howitz et al. 2003). The assay is dependent on the SIRT1 substrate comprising a fluorescent peptide called *Fluor de Lys* comprising the amino acid sequence 379–382 of human p53 including N^{ε}-acetylated Lys382. Addition of a sirtuin (e.g. SIRT1), in the presence of NAD^+, leads to deacetylation of Lys382 of p53. Exposure of the deacetylated peptide to trypsin releases a C-terminal fluorophore, which can be detected with excitation of 360 nm light and emission of 460 nm light (Bitterman et al. 2002; Howitz et al. 2003a). The mechanism of this sirtuin assay is shown in Fig. 1.6.

Using this *Fluor de Lys* assay, Howitz et al. discovered that resveratrol is a potent activator of SIRT1. Resveratrol reportedly lowered the Michaelis constant of SIRT1 for both Lys382 of p53 and NAD^+, and resveratrol promoted cell survival in U2OS and HEK 293 cells after exposure to ionizing radiation. The authors also reported that resveratrol was also able to stimulate yeast Sir2 activity and increase the average and maximum lifespan in yeast (Howitz et al. 2003a).

The SIRT1 activating effect of resveratrol was not fully reproducible in later studies using non-fluorophore substrates. For example, Kaeberlein et al. found no significantly increase in the mean or maximum life span of yeast treated with resveratrol, nor was it observed that resveratrol caused effects consistent with Sir2 activation (Kaeberlein et al. 2005). Resveratrol nevertheless activated SIRT1 toward a fluorophore labeled substrate, although the Michaelis constant for NAD^+ was not observed to be decreased. When the fluorophore containing substrate was replaced with a ^3H labeled acetylated histone H3 peptide, resveratrol failed to activate SIRT1. The Michaelis constant of the p53 substrate with *Fluor de Lys* was about 9-fold higher than the native p53 peptide without the *Fluor de Lys* group, suggesting activation restored cognate recognition of the underivatized sequence (Kaeberlein et al. 2005). The result was similar to findings obtained by an independent group. Borra et al. showing that resveratrol had no effect on the enzymatic activity of SIRT1 when the [^3H]acetylated histone H3 was used as substrate (Borra et al. 2005).

Sirtuin Assay

A. Sirtuins reaction



B. Fluorophore containing sirtuins assay

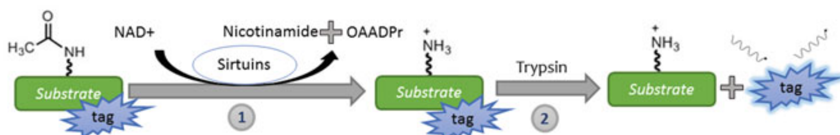


Fig. 1.6 Sirtuin Assays A) In sirtuin activity assays, acetylated proteins such as p53 and NAD+ are added as substrates. Deacetylation activity of sirtuins leads to the breakage of glycosidic bond in NAD^+, releasing NAM and the metabolite AADPR along with the deacetylated protein. In radioisotopic assays, substrates are labeled with different radioisotopes. After quenching the reaction, radiolabeled products are separated and quantified by scintillation counter. For instance, [^{14}C] can be used to label NAD^+ and [^{14}C] NAM is collected for measurement. Also, [^{32}P]NAD+ or [^{3}H] acetylated protein substrate can be used as substrate to monitor the formation of [^{32}P]AADPR or [^{3}H]AADPR. B) In fluorophore containing sirtuin assays, the substrate is an amino acid peptide derived from the sequence of known substrate protein with N-terminus and/or C-terminus modified with a fluorescent tag. For instance, the Fluor de Lys fluorescence assay for SIRT1 utilizes the SIRT1 substrate comprising a fluorescent peptide Fluor de Lys and the amino acids 379–382 of human p53 with Lys382 acetylated. In the first step of the reaction, addition of SIRT1 deacetylates Lys382, which sensitizes the peptide to become a substrate for trypsin. Secondly, proteolytical cleavage of *Fleur de Lys* releases fluorophore which can be detected with excitation of 360 nm light and emission of 460 nm light

Therefore resveratrol does not activate SIRT1 per se, instead, the effect was found to be specific for an artificial substrate which does not exist under physiological conditions. Borra et al. speculated that resveratrol binding potentially induces conformational changes in SIRT1, to creat a binding pocket to allow better accommodate the fluorophore of the substrate (Borra et al. 2005).

Although the use of *Fluor de Lys* has significant defects as an assay for sirtuins, it also revealed a new aspect of SIRT1 behavior toward substrates. The idea that selected SIRT1 substrates in cells might have the features as exemplified by the *Fluor de Lys* substrate is currently in debate. However, an in depth discussion of these issues is beyond the scope of this chapter.

1.1.6.2 SIRT1 Fluorescence Polarization Assay

With the aforementioned caveats still in place, pharmaceutical discovery programs designed to identify SIRT1 activators or inhibitors were conducted. In order to screen a large collection of small molecules, it was crucial to have a high-throughput but sensitive assay. Milne et al. developed an *in vitro* fluorescence polarization assay and successfully used it to identify putative small molecule activators of SIRT1 (Milne et al. 2007). The substrate used in the assay was a 20 amino acid peptide derived from the sequence of human p53 with biotin linked to the N-terminus, whereas the C-terminus was modified with a MR121 or TAMRA fluorescent tag. After SIRT deacetylation, trypsin can cleave the newly exposed lysine residue and release fluorophore. Fluorescent polarization was determined at 650 nm excitation wavelength and 680 nm emission wavelengths. Using this assay, it was possible to screen 290,000 compounds with 127 confirmed hits (Cen et al. 2011b; Milne et al. 2007).

However, the fidelity of this assay for discovery of true activators of sirtuins remains to be fully evaluated. Interestingly, the reported affinity of SIRT1 activators does not correlate to their activation effect (Cen et al. 2011b). For example, one activator required 8 times higher concentration than another activator to reach 1.5 times SIRT1 activity of untreated control. On the other hand, the maximal activation of the first activator is 1.5 times stronger than second activator. Yet, these effects are also substrate dependent. It is possible that similar as the *Fluor de Lys* assay, the presence of fluorophore in the substrate alters the affinity of SIRT1 to the specific substrate and therefore confounds the effect of the compounds on native substrates.

1.1.6.3 Radioisotopic Assays

A radioisotope assay was first described by Landry et al. for measuring Sir2 activity and is specific for sirtuin reactions (Landry et al. 2000). Sirtuins require NAD^+ as substrate. In this assay, [carbonyl-^{14}C]NAD^+ and acetylated substrates, e.g. the amino acid 368–386 of human p53 with acetylated Lys382 for SIRT1 reaction (Borra et al. 2005), were used (Borra and Denu 2003). The deacetylation activity of sirtuins leads to the breakage of glycosidic bond in [^{14}C]NAD^+, releasing [^{14}C] NAM along with the metabolite AADPR. After quenching the reaction, [^{14}C]NAD^+ and [^{14}C]NAM can be separated and isolated using thin layer chromatography or reversed-phase HPLC. Radioactivity is subsequently quantified by scintillation counter. Alternatively, a [^{32}P]NAD^+ can be used in which the label is located in one of the phosphate group in order to monitor the formation of AADPR. After quenching the reaction, [^{32}P]NAD^+ and [^{32}P] AADPR are separated with thin layer chromatography or reversed-phase HPLC and measured by scintillation countering.

Labeling the acetylated substrate of sirtuin reaction reaction is another way of determining sirtuin activity. In such an assay a [acetyl-^{3}H] acetylated peptide or protein is used. With sirtuin deacetylation, [^{3}H] is transferred to the acetate in

AADPR. After reaction is quenched, a separation method called charcoal-binding can be used. By heating up the reaction mixture at 95°C, [^{3}H]acetate can be hydrolyzed away from [^{3}H]AADPR. The mixture is then transferred to a charcoal slurry which can bind all the substrates and products except for [^{3}H]acetate. The amount of [^{3}H]acetate is then quantitated by scintillation counter and the radioactivity corresponds to the [^{3}H]AADPR produced (Borra and Denu 2003). Schematic views of these assays and commentary are shown in Fig. 1.6.

1.1.6.4 Mechanism-Based Affinity Capture of Sirtuins

Thioacetylated peptides can react with NAD^+ on sirtuin active sites to form stable thioimidate complexes (Fatkins et al. 2006). Based on this reaction, Cen et al. develop a sirtuin capturing assay with a thioacetylpeptide identified to react with sirtuins to form a stable thioimidate complex (Cen et al. 2011a). A chemically modified NAD^+, 6-AMX-NAD^+, was also designed to form the desired thioimidate, but also to chemically reacted to biotin after thioimidate formation. With construction of 6-AMX-NAD^+, sirtuins can be captured by conjugation to biotin, which can be later retrieved on streptavidin beads. SDS-PAGE methods allow the detection, capture and molecular weight determination of sirtuins. The authors have shown that this assay is capable of capturing a variety of human sirtuin isoforms including SIRT1, SIRT2, SIRT3, SIRT5, SIRT6 and can react with microbial derived sirtuins as well. Although further improvement, such as quantitation of the captured sirtuins and efficiency of the conjugation strategy, this assay can increase the flexibility in understanding of sirtuin activities in biological systems and could provide insights into enzymatic activity (Cen et al. 2011b).

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Chapter 2
NAD$^+$ as a Pharmacological Tool to Boost Sirtuin Activity

Riekelt H. Houtkooper

2.1 Introduction

The sirtuins constitute a family of proteins with homology to the yeast protein *silent information regulator 2* (Sir2p), a protein involved in yeast mating type regulation (Ivy et al. 1986; Rine and Herskowitz 1987). The sirtuin field was boosted by the discovery of the anti-aging effect of the yeast Sir2p. Overexpression of *sir2* in yeast extends lifespan while deletion of the gene results in short lifespan (Kaeberlein et al. 1999). These results were soon extended to other species, demonstrating involvement of the Sir2p ortologues in *C. elegans* (*sir-2.1*) and *D. melanogaster* (Sir2) in the aging process (Rogina and Helfand 2004; Tissenbaum and Guarente 2001). Even though these studies are now contested (Burnett et al. 2011), it is well accepted that sirtuins play a major role in metabolic control and are a central target for the treatment of metabolic diseases (reviewed in (Houtkooper et al. 2012)).

A second major discovery that catapulted the field forward is the clarification of the enzymological characteristics of sirtuin enzymes. Yeast Sir2 was shown to possess deacetylase activity, for which it is dependent on nicotinamide adenine dinucleotide (NAD), in particular its oxidized form NAD$^+$ (Imai et al. 2000). Considering that NAD is a classical cofactor in enzymatic redox reactions (Houtkooper et al. 2010), it was tempting to speculate that sirtuins could act as metabolic sensors. Indeed, when yeast or flies were calorie restricted (CR), lifespan increased in a Sir2-dependent fashion (Lin et al. 2000; Rogina and Helfand 2004). These results led to the hypothesis that regulation of NAD$^+$ levels could modulate sirtuin activity and thereby mimic the beneficial effects of CR.

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In this chapter, I will discuss the pathways that are involved in NAD+ homeostasis and how this balance can be tipped into a favorable direction to prevent metabolic decline.

2.2 NAD Biosynthesis

Initial work on the biosynthesis of NAD stems from research into the disease pellagra. Pellagra is clinically characterized by the 4 D's—diarrhea, dermatitis, dementia, and ultimately death—and is the result of poor nutritional supply of NAD precursors. Supplementation studies, particularly in a dog model of pellagra, led to the discovery of several NAD precursors that were able to treat pellagra (Elvehjem et al. 1937), while follow-up studies exposed the underlying enzymology (for more information on this topic, I refer the reader to (Houtkooper et al. 2010; Magni et al. 2004)).

2.2.1 Primary Biosynthesis from Tryptophan

The amino acid tryptophan serves as a precursor for primary biosynthesis of NAD (Fig. 2.1). Synthesis through this route is only limited and tryptophan also serves as a precursor for other biologically active molecules such as serotonin (Houtkooper et al. 2010). Nevertheless, in patients suffering from pellagra, supplementation of tryptophan is sufficient as a treatment, suggesting that enough NAD is produced (Elvehjem et al. 1937). In five enzymatic reactions, tryptophan is converted to α-amino-β-carboxymuconate-ε-semialdehyde (ACMS; Fig. 2.1), the first branch point in the pathway. For NAD biosynthesis, ACMS undergoes spontaneous cyclization, after which its product quinolinic acid enters the Preiss-Handler pathway, and is enzymatically converted to NAD through two additional enzymatic reactions (Fig. 2.1). In parallel, ACMS can also be metabolized to glutaryl-CoA, which can be converted to acetyl-CoA and subsequently enter the TCA cycle (Houtkooper et al. 2010).

Although the localization of NAD biosynthesis, and cycling of its intermediates or products is still subject of intense investigation, tryptophan metabolism to NAD is likely confined to the liver and kidney, as the rate-limiting enzyme QPRT is strictly localized to these tissues (Houtkooper et al. 2010). The more downstream enzymes, which converge with the salvage and Preiss-Handler pathways (see below), are more ubiquitously expressed, indicating that intermediates of these pathways may serve as cycling precursors of NAD in other tissues.

It is unclear whether switches to the alternative tryptophan metabolism pathways, i.e. serotonin or the branch towards glutaryl-CoA, play a role in the modulation of NAD levels. Since the primary NAD biosynthesis pathway contributes only a limited supply to cellular NAD levels, however, this seems unlikely, and the control of NAD levels rather involves other parts of NAD metabolic pathways.

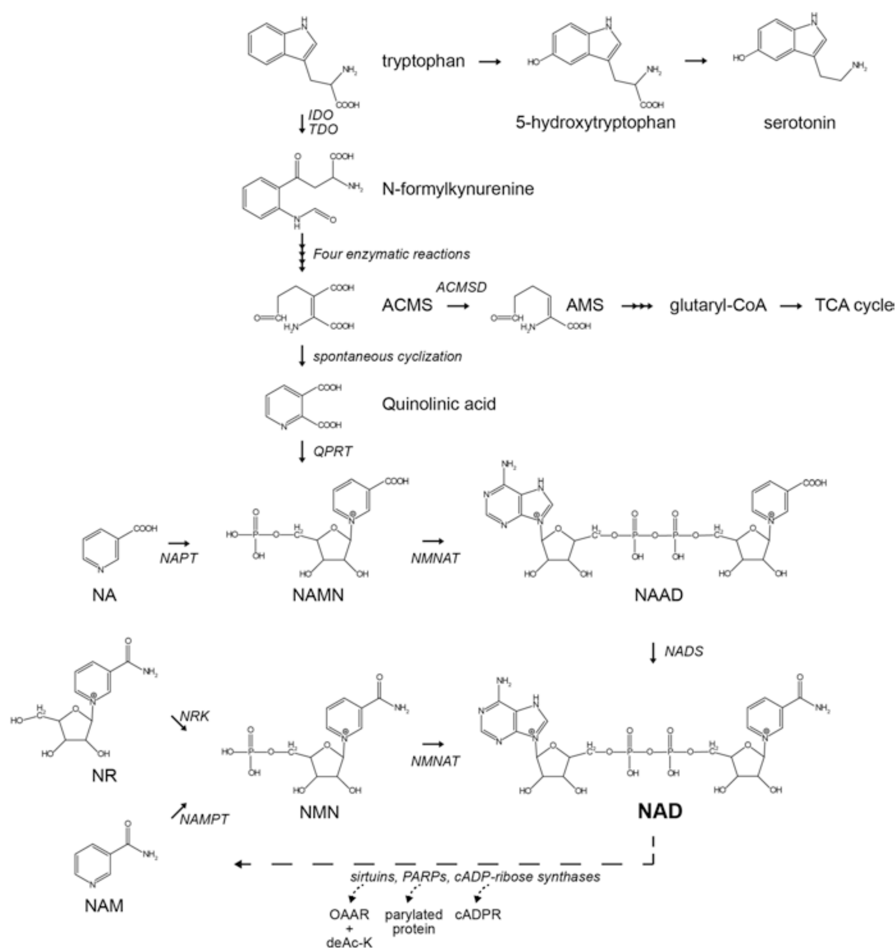

Fig. 2.1 Biochemistry of NAD metabolism. NAD can be synthesized *de novo*, through the Preiss-Handler pathway, or from the salvage pathway. *De novo* biosynthesis starts from the precursor tryptophan, while salvage is initiated from the precursors NA, NAM, or NR

2.2.2 Preiss-Handler Pathway and NAD Salvage

The main pathways to synthesize NAD involve the precursors nicotinic acid (NA) and nicotinamide (NAM) (Fig. 2.1). Both precursors have a rapid effect when supplemented in dogs suffering from pellagra (Elvehjem et al. 1937), suggesting that these can be efficiently taken up and converted. NA enters the Preiss-Handler pathway for NAD biosynthesis. Following the initial phosphoribosyltransferase step, this pathway converges with the biosynthetic pathway from tryptophan at the level of NA mononucleotide for further synthesis of NAD^+ (Fig. 2.1). The adenylyltransferase activity is catalyzed by one of three NMNAT enzymes, which are localized to

different subcellular compartments (Houtkooper et al. 2010). The final enzymatic step in the Preiss-Handler pathway, however, is confined to the cytosol (Hara et al. 2003), suggesting that the conversion of NA to NAD is in fact purely cytosolic.

One of the products of NAD utilization is NAM. This NAM enters the so-called salvage pathway for the resynthesis of NAD. In line with the various subcellular compartments where NAD is being consumed, the salvage of NAD is similarly distributed around the cell. While lower organisms have an enzymatic pathway to convert NAM to NA, mammalian cells initialize the salvage through the rate-limiting enzyme NAMPT, which has been found in the cytosol, nucleus and mitochondria and produces NMN (Houtkooper et al. 2010). The central role of NAMPT in the synthesis of NAD as well as the regulation of NAM levels suggest that NAMPT could dictate the activity of NAD-dependent enzymes. The second step in the resynthesis of NAD from NAM, catalyzed by NMNAT enzymes, also takes place in various subcellular compartments (Houtkooper et al. 2010). This conversion is even more relevant since NMN can be transported into the cell and between organelles. Using a reporter system based on the NAD-dependent poly (ADP ribose) polymerase PARP1, the Ziegler group demonstrated that NMN enters mitochondria and is converted to NAD^+ (Nikiforov et al. 2011). How NAD^+ levels are sustained or increased in other organelles is subject of future investigation. Similarly, which transporters are involved in the channeling of NAD precursors is an open question.

2.2.3 Nicotinamide Riboside

In 2004, nicotinamide riboside (NR) was discovered as a bona-fide NAD^+ precursor in humans (Bieganowski and Brenner 2004). NR was long known as a bacterial substrate for NAD biosynthesis, but the Brenner group identified the human genes coding for the first enzymatic step in NR conversion to NAD^+, the NR kinases *NRK1* and *NRK2* (Bieganowski and Brenner 2004) (Fig. 2.1). The resulting product, NMN, converges with the salvage pathway for a one-step resynthesis of NAD^+.

2.3 NAD Utilization

Besides the enzymes that use NAD^+ for redox-coupled reactions there are three key classes of enzymes that utilize NAD^+ as a substrate for enzyme activity, (1) sirtuins, (2) poly(ADP-ribose)polymerases (PARPs), and (3) cyclic ADP-ribose synthases. Because of their NAD^+-dependence these enzymes can be regulated by the levels of NAD^+. Along a different line, as these enzymes compete for the cellular pool of NAD^+, inhibiting one of them could increase the NAD availability for the other NAD^+ consumers.

2.3.1 Sirtuins

The mammalian sirtuin family of proteins consists of seven proteins with varying subcellular localization (Guarente 2013; Houtkooper et al. 2012). The sirtuins are named after the yeast *silent regulator 2* (Sir2) (Ivy et al. 1986; Rine and Herskowitz 1987). The sirtuins were originally classified as histone deacetylases (Imai et al. 2000) but over the years many non-histone targets have been described. In addition, sirtuins are not only deacetylase enzymes, but also perform other posttranslational modifications, such as ADP-ribosylation by SIRT4 (Haigis et al. 2006), demalonylation, desuccinylation, and deglutarylation by SIRT5 (Du et al. 2011; Peng et al. 2011; Tan et al. 2014), and long-chain deaceylation by SIRT6 (Jiang et al. 2013). In addition to their specific enzymatic reactions, sirtuin activity is also regulated by their subcellular distribution. SIRT1, SIRT6 and SIRT7 are considered nuclear, SIRT2 is cytosolic, and SIRT3, SIRT4 and SIRT5 are in mitochondria (Pirinen et al. 2012). For its enzymatic activity, sirtuins require NAD^+ and typically produce NAM (Fig. 2.1) (Imai et al. 2000). The dependence on a central metabolite optimally places sirtuins as metabolic sensors, adapting protein activity and gene expression according to the metabolic needs of the cell. Indeed, yeast Sir2 is a critical protein for the lifespan extension that is observed in response to caloric restriction (Howitz et al. 2003; Lin et al. 2002).

For more information on specific sirtuins and their role in mammalian physiology I refer the reader to the other chapters in this book, or to extensive reviews on the topic (Blander and Guarente 2004; Chalkiadaki and Guarente 2012; Haigis and Sinclair 2010; Houtkooper et al. 2012; Menzies et al. 2016).

2.3.2 Poly(ADP-Ribose)Polymerases

The poly(ADP-ribose)polymerase enzymes (PARPs) are best known for their role in DNA damage. A family of 17 proteins in humans, the PARPs utilize NAD^+ as a substrate for PARylation, a post-translational modification that typically changes the conformation of target proteins and thereby modulates their activity (Bai et al. 2015). For instance, in response to DNA breaks, PARylation leads to the recruitment and activation of DNA repair proteins. Considering that tumors often accumulate DNA mutations, PARP inhibitors have been developed as treatment for various types of cancer (Curtin and Szabo 2013). Interestingly, similar to sirtuins, PARP enzymes require NAD^+ as a substrate (Figs. 2.1 and 2.2), but the Km for NAD^+ is much lower—in the low micromolar range—suggesting that the regulation of PARP activity is not so much at the level of NAD^+ but rather by DNA binding (D'Amours et al. 1999). Upon DNA damage PARPs become activated, in particular PARP1 and PARP2, and deplete a large portion of the cellular NAD^+ pool (Houtkooper et al. 2010). Considering that PARPs are such major NAD^+ consumers, blocking its activity could mobilize a pool of NAD^+ that can serve as a substrate for other enzymes

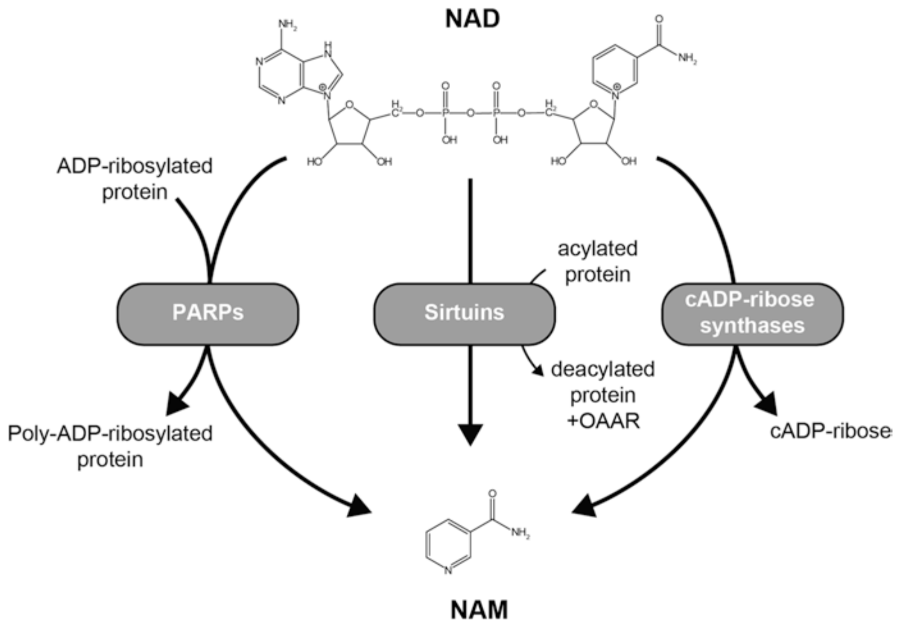

Fig. 2.2 Enzymatic activities of NAD consuming enzymes. Sirtuins, PARPs and cADP-ribose synthases are major classes of NAD-consuming enzymes. The three enzyme classes display a different activity but all form NAM as a product

such as the sirtuins. Indeed, deletion of *Parp1*—the major PARP enzyme—in mice leads to a marked accumulation of NAD^+ levels in various tissues (Bai et al. 2011b). This increase in NAD^+ levels is accompanied by activation of nuclear SIRT1, while cytosolic SIRT2 and mitochondrial SIRT3 are not activated, suggesting that NAD^+ levels only increase locally. As a consequence, PGC-1α is deacetylated leading to mitochondrial biogenesis in brown adipose tissue and skeletal muscle. Phenotypically, *Parp1*$^{-/-}$ mice display reduced body weight, improved glucose homeostasis, cold tolerance, and resistance to high fat diet (Bai et al. 2011b). Deletion of Parp2 also improved metabolic parameters, but rather than increasing NAD^+ levels these mice exhibited markedly increased SIRT1 expression (Bai et al. 2011a). Importantly, SIRT1 was not only activated in genetic models of PARP deficiency, but also in mice that were treated with PARP inhibitors (Bai et al. 2011b). Along the same line, *C. elegans* that were treated with RNAi against the main PARP *pme-1* or with the PARP inhibitor olaparib lived longer than controls, in a *sir-2.1*-dependent manner (Mouchiroud et al. 2013). Together, these studies identify PARP enzymes as regulators for NAD^+ levels that are tractable for pharmacological intervention.

2.3.3 Cyclic ADP-Ribose Synthases

A third family of NAD consuming enzymes is the cyclic ADP-ribose (cADPR) synthases. The cADPR synthases—including their most prominent family members CD38 and CD157—regulate processes such as calcium homeostasis and cell cycle, through the production of the second messenger cADPR (Malavasi et al. 2008). Both are highly conserved ectoenzymes that are ubiquitously expressed (Lee 2006). In addition to its role as a receptor on many different cell types, CD38 and CD157 also have enzymatic activity that occurs both intra- and extracellularly (Jackson and Bell 1990; Lee 2012; Zhao et al. 2012). This pH dependent activity utilizes NAD^+ as a substrate for the cyclization reaction that leads to the formation of cADPR and the concurrent release of NAM. As such, CD38 and CD157 could regulate NAD^+ levels similar to PARPs. In line with this idea, tissues from CD38 knockout mice displayed 2-10x higher NAD^+ levels that resulted in SIRT1 activation and increased mitochondrial biogenesis (Aksoy et al. 2006a; Aksoy et al. 2006b; Barbosa et al. 2007). Phenotypically, these mice have enhanced energy expenditure and are resistant to high-fat diet induced obesity (Barbosa et al. 2007). These phenotypes are reminiscent of SIRT1 activation with resveratrol (Baur et al. 2006; Lagouge et al. 2006), and both the biochemical and clinical phenotypes of the CD38 knockout mice can be attenuated by treating the mice with the sirtuin inhibitor sirtinol (Barbosa et al. 2007). Even though these experiments suggest a marked similarity in the mode of action, and a favorable clinical outcome in the mice, more work is needed to establish CD38 and/or CD157 as pharmacological targets for the treatment of metabolic diseases. First, the biochemical routing of NAD^+ metabolites upon CD38/CD157 enzymatic action needs to be determined. Second, it is currently unclear whether inhibition of CD38/CD157 is strictly beneficial or may have adverse effects related to the high consumption rates of NAD^+.

2.4 Pharmacological Opportunities to Activate Sirtuins

After the identification of NAD^+ as an essential substrate for sirtuin enzymatic activity, the idea emerged that this could serve as a target for therapeutic intervention. Indeed, supplementation of NAD^+ precursors NA or NAM alleviated the clinical symptoms associated with pellagra (Elvehjem et al. 1937). Furthermore, NA is effective in the treatment of hyperlipidemia, despite causing undesired flushing as an adverse effect (Houtkooper et al. 2010). Developments over the last couple of years have established a potent role for other NAD precursors such as NMN and NR. Indeed, supplementing these compounds to the diet of mice leads to an overall improved metabolic profile. NR supplementation attenuated the body weight gain on a high-fat diet, and thereby prevents the development of insulin resistance (Canto et al. 2012). Furthermore, these mice are resistant to cold and perform better on a treadmill (Canto et al. 2012). Consistent with the molecular events that are expected

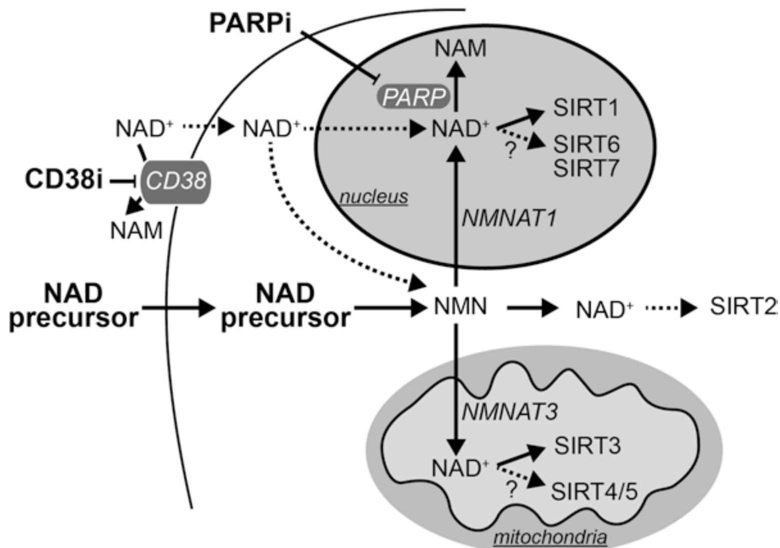

Fig. 2.3 Subcellular regulation of NAD metabolism and sirtuin activity. Little is known about the subcellular distribution of NAD synthesis, and which are the NAD intermediates that are transported between these subcellular compartments. Considering the ubiquitous localization of NMNAT enzymes it is likely that supplementation of NAD precursors increases NAD levels and sirtuin activity in all compartments. In contrast, PARP inhibitors might only increase nuclear NAD and sirtuins considering the nuclear localization of the PARPs

to arise from NR supplementation, prominent metabolic tissues such as muscle and brown adipose tissue displayed a marked activation of both nuclear SIRT1 and mitochondrial SIRT3, which was accompanied by increased mitochondrial biogenesis (Canto et al. 2012). This is in line with the biochemical routing of these NAD precursors, which are distributed along the various subcellular compartments and lead to NAD synthesis throughout the cell (Fig. 2.3). Similar to NR, supplementation of NMN improves glucose homeostasis in diabetic mice (Yoshino et al. 2011). In addition to multifactorial, complex diseases such as diabetes, NAD precursors may also serve to treat inherited metabolic diseases in which mitochondrial function is compromised (Andreux et al. 2013). Indeed, treating the Deletor mice—a model for mitochondrial myopathy caused by Twinkle mutations—with NR rescued important aspects of their pathophysiology (Khan et al. 2014). Similarly, NR limited disease progression in the *Sco2$^{KO/KI}$* model for mitochondrial myopathy (Cerutti et al. 2014). These mice suffer from OXPHOS deficiency, which is rescued upon NR supplementation. In addition, four weeks of supplementation fully restores the exercise capacity that is severely impaired in the *Sco2$^{KO/KI}$* mice (Cerutti et al. 2014). Although data from NAD precursor supplementation in humans are scarce and often limited to pellagra and hyperlipidemia, a recent study demonstrated that supplementation of acipimox—a more stable version of niacin—improves glucose

homeostasis in diabetic patients (van de Weijer et al. 2015). Further human intervention studies with other NAD precursors are eagerly awaited.

Rather than increasing the supply of NAD precursors, inhibiting its consumption has also proven successful in increasing NAD levels and activating sirtuins. In cultured C2C12 muscle cells, the PARP inhibitor PJ34 dose-dependently increased NAD levels leading to SIRT1-dependent PGC-1α deacetylation, which was accompanied by increased mitochondrial gene expression and function (Bai et al. 2011b). At the organismal level, PARP inhibitors extended lifespan of *C. elegans* in a similar way to NR and other NAD precursors (Mouchiroud et al. 2013). Indeed, the PARP inhibitor olaparib increased NAD^+ levels, and the lifespan extension was mediated through *sir-2.1*-dependent activation of mitochondrial stress response pathways (Mouchiroud et al. 2013). Little is known about the potential applicability of PARP inhibitors in mammalian models of metabolic disease. PARP inhibitor treatment in mice led to a marked attenuation of high-fat diet induced obesity, accompanied by improved exercise capacity (Pirinen et al. 2014). Along a similar line, the flavonoid apigenin was shown to inhibit the other NAD-consuming enzyme CD38, and apigenin treatment of obese mice improved glucose tolerance, even after just a few days of treatment (Escande et al. 2013).

Even though more research is needed to validate the potential of PARP and CD38 inhibitors for the treatment of metabolic diseases it is important to mention that PARP inhibitors are being developed for the treatment of various types of cancer (Rouleau et al. 2010), and underwent extensive clinical testing, even up to phase III. As a consequence, the route to clinical development for metabolic diseases may be shorter, especially in the case of severe rare inherited metabolic diseases (Bleeker and Houtkooper 2016).

2.5 Conclusion

After the discovery that sirtuins are NAD dependent metabolic regulators a lot of research has refocused on the elucidation of NAD metabolism, which was long considered an old-fashioned metabolite. As the intracellular NAD levels are around the Km of sirtuins changing the NAD concentrations is a meaningful strategy to increase sirtuin activity. In this chapter, I outlined several of these strategies, including (1) enhanced NAD synthesis from various precursors, (2) reducing the activity of competitive NAD consumers, rendering this NAD pool available for sirtuins. Indeed, both strategies proved successful as treatment with NAD precursors—e.g. NR or NMN—and PARP or CD38 inhibitors led to increased NAD levels and improved cellular or organismal metabolic state. Nevertheless, more work is needed to fully understand the dynamics of this response, as the enzymology, subcellular distribution, and downstream consequences on the various sirtuins are still incompletely understood. Despite these gaps in our knowledge, it is evident that the NAD^+/sirtuin axis is a promising treatment avenue for rare inherited and common acquired diseases. The success stories with NR in the treatment of mice with

high-fat diet induced obesity or mitochondrial myopathy nicely illustrate this point. More recently, NR treatment also proved effective in maintaining a healthy muscle and neural stem cell pools in aged mice, extending mouse lifespan even though the treatment was only started when mice were already 700 days old (Zhang et al. 2016). Despite the immense progress showing beneficial effects of NR on simple organisms and mouse metabolism and healthspan, the translatability of these treatments needs to be further established. Such human studies are expected soon and might firmly establish these compounds as potent sirtuin boosters with human clinical relevance.

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Chapter 3
Protein Lysine Acylation: Abundance, Dynamics and Function

Olga Pougovkina and Vincent C.J. de Boer

3.1 Introduction

Protein acylation is the modification of one or more amino acid residues of a protein with an acyl chemical moiety. Although N-acylation of lysines is the most abundant acylation modification, O-acylation modifications also play a role in cellular regulation. Ghrelin, a circulating peptide hormone that regulates food intake in humans, is O-acylated on a serine residue (Kojima et al. 1999). Ghrelin is non-functional when the O-acylation is not present. Also Wnt is O-acylated on a serine residue, which is essential for its proper trafficking and activity (Takada et al. 2006). Ghrelin is acylated mainly by an acyl moiety derived from octanoate (C8:0) (Kojima et al. 1999) and Wnt is acylated by a palmitoleic acid moiety, which is a monounsaturated fatty acid (C16:1) (Takada et al. 2006). N-acylation can occur either co-translationally or post-translationallly. Co-translational acetyl group transfer occurs primarily on N-alpha-terminal amino acids. Functionally, N-alpha-acetylation of protein terminal chains was shown to affect protein stability and protein subcellular targeting, but the function of n-alpha-terminal acetylation still remains incompletely understood (Starheim et al. 2012). Post translational modification (PTM) of proteins by N-acylation occurs on internal lysines. Lysine is an amphipathic residue with a hydrophobic side chain and a positively charged ε-amino group at physiological pH. Due to these chemical properties, lysine acylation plays an important role in

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protein structure and interactions. Lysine residues often form salt bridges, hydrogen bonds and are frequently present in active or binding sites of the protein. Underlining its importance in protein function, lysine undergoes a wide variety of PTMs including methylation, ubiquitination and acylation, which play a significant role in the regulation of protein properties. In this chapter, we focus on the post-translation N-acylation of proteins on internal lysines. The most studied lysine acylation modification is acetylation, however new advances in proteomic studies identifying novel acylation modifications on proteins have extended the group to formylation, propionylation, buyrylation, crotonylation, malonylation, succinylation and glutarylation (Fig. 3.1).

3.2 Lysine Acetylation

Lysine acetylation was first discovered on histones in the 1960s, where it was shown to be involved in regulating gene expression (Allfrey et al. 1964). Along with other modifications, such as methylation, acetylation forms the 'histone code' which is comprised of a combination of modifications that induces specific downstream effects (Strahl and Allis 2000). By neutralizing the positive charge of the lysine, histone acetylation weakens histone interaction with DNA and it also regulates the binding and exclusion of bromo-domain containing proteins to and from chromatin (Lee and Workman 2007). Therefore, targeted acetylation of histone lysine residues is associated with regulation of virtually all processes involving DNA, including replication, transcription, repair and heterochromatin formation (Kouzarides 2007).

For a long time acetylation was considered to be involved primarily in epigenetic control. However, since acetylation was first identified on a non-histone protein p53 (Gu and Roeder 1997), a significant effort has been made to identify the full potential and abundance of lysine acetylation modifications in cellular networks. Especially the discovery of the sirtuin family of NAD^+ dependent deacetylases by Guarente and co-workers generated a large boost in protein acetylation research (Imai et al. 2000; Kaeberlein et al. 1999). See Table 3.1 for an overview of sirtuin target proteins.

3.2.1 Function of Protein Acetylation

Many studies addressed the function of protein acetylation by mapping the acetylated lysines and mutating the specific lysines to arginine, yielding a constitutively positively charged amino acid residue. From these studies it can be derived that acetylation impacts protein stability, protein-protein interaction, protein activity and protein subcellular localization. All of these properties are also part of the functional toolbox of PTMs in general. For example, methylation of a specific lysine in the RORα orphan nuclear receptor by the methyltransferase Ezh2 allows for the

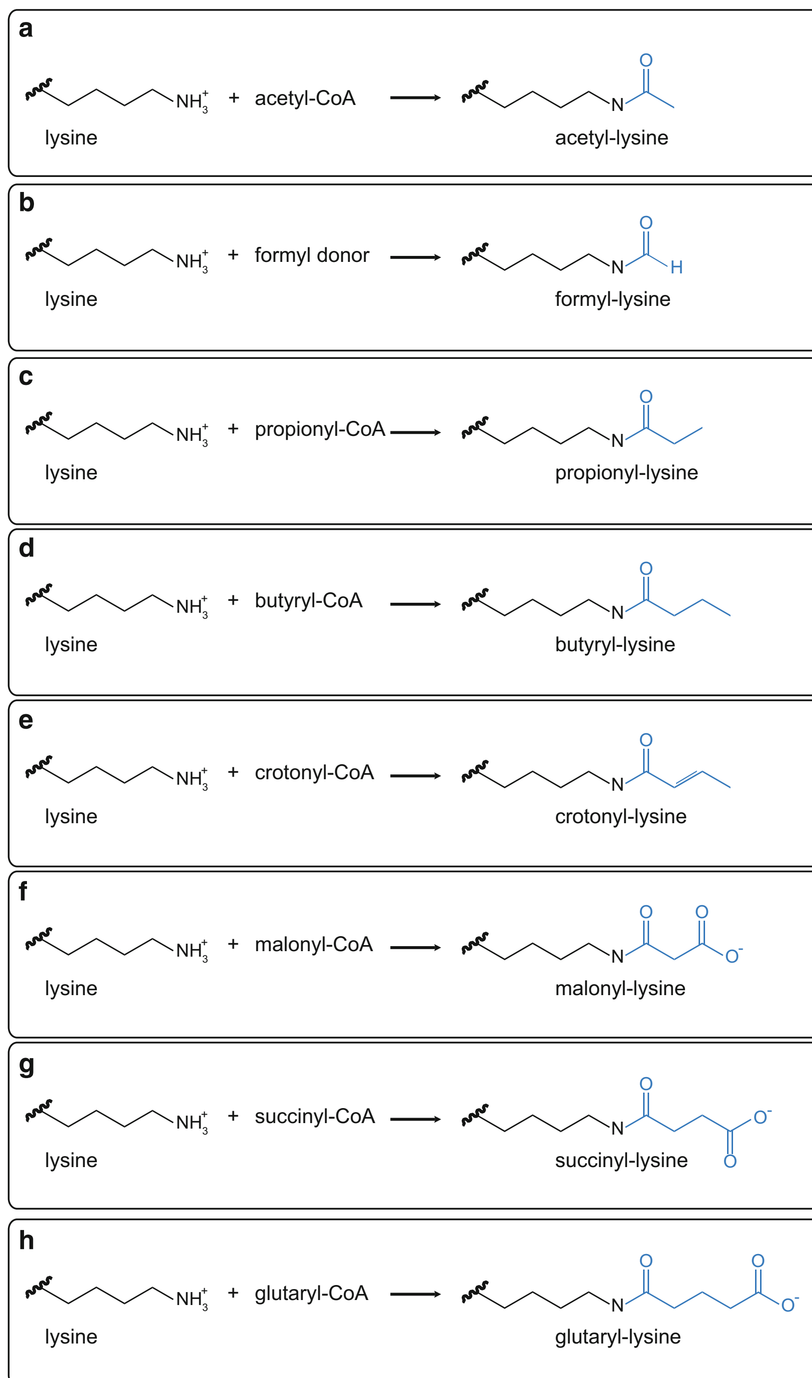

Fig. 3.1 Chemical structures of post-translational lysine acylation modifications.

Table 3.1 Protein targets of sirtuins that have been demonstrated to be deacylated by sirtuins (SIRT1-7)

Protein	References
SIRT1 deacetylation targets:	
P53	Luo et al. (2001) and Vaziri et al. (2001)
FOXO1, 3, 4	Brunet et al. (2004), Motta et al. (2004), van der Horst et al. (2004), and Yang et al. (2005)
Ku70	Cohen et al. (2004)
RELA/p65	Yeung et al. (2004)
PGC1α	Rodgers et al. (2005)
P300	Bouras et al. (2005)
MEF2	Zhao et al. (2005)
E2F1	Wang et al. (2006)
ACS1	Hallows et al. (2011)
AR	Fu et al. (2006)
eNOS	Mattagajasingh et al. (2007)
Rb	Wong and Weber (2007)
LXR	Li et al. (2007)
NBS1	Yuan et al. (2007)
IRS2	Li et al. (2008b) and Zhang (2007)
P73	Dai et al. (2007)
SUV39H1	Vaquero et al. (2007)
CRTC	Liu et al. (2008)
BMAL1	Nakahata et al. (2008)
PER2	Asher et al. (2008)
ATG	Lee et al. (2008)
WRN	Li et al. (2008a)
LKB1	Lan et al. (2008)
STAT3	Nie et al. (2009)
PARP	Rajamohan et al. (2009)
MYC	Yuan et al. (2009)
NoRC	Zhou et al. (2009)
FXR	Kemper et al. (2009)
PCAF	Pediconi et al. (2009)
Cortactin	Zhang et al. (2009b)
ER	Elangovan et al. (2011)
ACC	Law et al. (2009)
FOXP3	van Loosdregt et al. (2010)
HSF1	Ahn et al. (2008)
HNF4	Yang et al. (2009)
HIF2alpha	Dioum et al. (2009)
c-FOS	Zhang et al. (2010)
c-JUN	Zhang et al. (2010)
SREBP	Ponugoti et al. (2010) and Walker et al. (2010)

(continued)

Table 3.1 (continued)

Protein	References
XPA	Fan and Luo (2010)
APEX1	Yamamori et al. (2010)
TIP60	Peng et al. (2012) and Wang and Chen (2010)
Tau	Min et al. (2010)
NRF2	Kawai et al. (2011)
AKT	Sundaresan et al. (2011)
PDK1	Sundaresan et al. (2011)
CIITA	Wu et al. (2011)
DNMT1	Peng et al. (2011b)
NICD	Guarani et al. (2011)
TORC1	Jeong et al. (2012)
EVI1	Pradhan et al. (2011)
NHLH2	Libert et al. (2011)
HMGCS1	Hirschey et al. (2011a)
PPARg	Qiang et al. (2012)
hMOF	Peng et al. (2012)
USP22	Armour et al. (2013)
HIPK2	(Hwang et al. 2013)
FOXA2	van Gent et al. (2014)
EGR1	Vedantham et al. (2014)
PML	Guan et al. (2014)
SIRT2 deacetylation targets:	
a-tubulin	North et al. (2003)
FOXO3	Wang et al. (2007)
P300	Black et al. (2008)
P65	Rothgiesser et al. (2010)
FOXO1	Zhao et al. (2010b)
PEPCK	Jiang et al. (2011)
PAR-3	Beirowski et al. (2011)
CDC20	Kim et al. (2011)
CDH1	Kim et al. (2011)
EIF5A	Ishfaq et al. (2012)
CDK9	(Zhang et al. 2013)
ACLY	(Lin et al. 2013a)
BUBR1	North et al. (2014) and Suematsu et al. (2014)
PGAM	Xu et al. (2014)
G6PD	Wang et al. (2014)
ALDH1A1	Zhao et al. (2014)
SIRT3 deacetylation targets:	
ACS2	Schwer et al. (2006)
GDH	Lombard et al. (2007)
Ku70	Sundaresan et al. (2008)

(continued)

Table 3.1 (continued)

Protein	References
NDUFA9	Ahn et al. (2008)
FOXO3A	Sundaresan et al. (2009)
LCAD	Hirschey et al. (2010)
HMGCS2	Shimazu et al. (2010)
IDH2	Someya et al. (2010)
SDH	Cimen et al. (2010) and Finley et al. (2011)
MRPL10	Yang et al. (2010)
CYPD	Hafner et al. (2010)
SOD2	Chen et al. (2011), Qiu et al. 2010, and Tao et al. (2010)
OTC	Hallows et al. (2011)
ALDH2	Lu et al. (2011)
PDH	(Sol et al. 2012)
SKP2	Inuzuka et al. (2012)
NMNAT2	Li et al. (2013)
ACAT1	Still et al. (2013)
MDH2	Hebert et al. (2013)
F0F1-atpase	Wu et al. (2013)
LKB1	Pillai et al. (2010)
PDHA1	Fan et al. 2014 and Ozden et al. (2014)
LON	Gibellini et al. (2014)
ATP synthase	Rahman et al. (2014)
LRP130	Liu et al. (2014)
OPA1	Samant et al. (2014)
OSCP	Vassilopoulos et al. (2014)
SIRT4 ADP ribosylation, deacetylation, delipoylation targets:	
GDH	Ahuja et al. (2007) and Haigis et al. (2006)
MCD	Laurent et al. (2013)
PDH	Mathias et al. (2014)
SIRT5 demalonylation, desuccinylation, deglutarylation targets:	
CPS	Nakagawa et al. (2009)
Urate oxidase	Nakamura et al. (2012)
SDH	Park et al. (2013)
PDH	Park et al. (2013)
Glutaminase	Polletta et al. (2015)
SIRT6 ADP ribosylation, deacetylation, demyristoylation targets:	
CTIP	Kaidi et al. (2010)
PARP1	Mao et al. (2011)
USP10	Lin et al. (2013b)
G3P1	Simeoni et al. (2013)
TNF/relA	Jiang et al. (2013)
NPM1	Lee et al. (2014)

(continued)

Table 3.1 (continued)

Protein	References
SIRT7 deacetylation targets:	
P53	Vakhrusheva et al. (2008)
PAF53	Chen et al. (2013)
NPM1	Lee et al. (2014)
GABPβ1	Ryu et al. (2014)

Only sirtuin targets that were confirmed with other techniques than large-scale proteomics were included in this table

recognition of the ubiquitin proteosomal system, targeting the receptor for degradation and thus determining its stability (Lee et al. 2012). Also, phosphorylation of the pyruvate dehydrogenase (PDH) complex by PDH kinase is an established mechanism by which PDH activity is inhibited (Behal et al. 1993), thus regulating its activity. Acetylation affects structural properties of the target protein or is involved in a PTM crosstalk. Structural changes induced by acetylation can either impact active site confirmation so that substrate binding is affected or alter interaction with other proteins or DNA. Also for PTM crosstalk two different modes of action can be envisioned. Either acetylation blocks a residue so that another PTM cannot bind to that specific residue or acetylation affects the binding of nearby PTMs. Examples of these functional properties of acetylation are discussed below.

Interestingly, acetylation can have both positive and negative effect on protein activity. Manganese superoxide dismutase (MnSOD) is a mitochondrial superoxide scavenger which is activated by the action of the SIRT3 deacetylase (Fig. 3.2a) (Chen et al. 2011; Qiu et al. 2010; Tao et al. 2010). On the other hand, aldehyde dehydrogenase 2 (Aldh2) has been shown to be inactivated upon deacetylation by SIRT3 (Lu et al. 2011). Also, protein-protein interaction can be regulated positively or negatively by acetylation (Fig. 3.2b).

Protein stability is amongst others determined by ubiquitin dependent breakdown via the proteasome. It is clear from many studies that communication between lysine acetylation and lysine ubiquitination provides the cell with a sophisticated mechanism for fine-tuning protein stability. SREBP1, a master transcription factor that controls lipogenesis, is acetylated by acetyltransferases which renders the protein more stable by blocking the binding of ubiquitin (Giandomenico et al. 2003) (Fig. 3.2c). Phosphoenolpyruvate carboxykinase (PEPCK) was recently shown to be more susceptible for degradation when a lysine residue nearby ubiquitin targeted lysines was acetylated (Jiang et al. 2011). In this case acetylation allowed for the recruitment of an E3 ubiquitin ligase, targeting PEPCK for degradation (Fig. 3.2c).

Subcellular localization of proteins can be controlled by PTMs. Especially nuclear-cytoplasmic shuttling of proteins has been shown to be affected by acetylation (Sadoul et al. 2011). For example, acetylated elF5a accumulates in the nucleus, whereas its deacetylated form resides in the cytosol with its acetylation status being regulated by SIRT2 and HDAC6 (Ishfaq et al. 2012) (Fig. 3.2d). Acetylation of p53 impacts its function on many different levels, of which one of them is the nuclear-cytoplasmic trafficking. P53 was shown to accumulate in the cytosol where it was acetylated by the p300 acetyltransferase (Kawaguchi et al. 2006) (Fig. 3.2d).

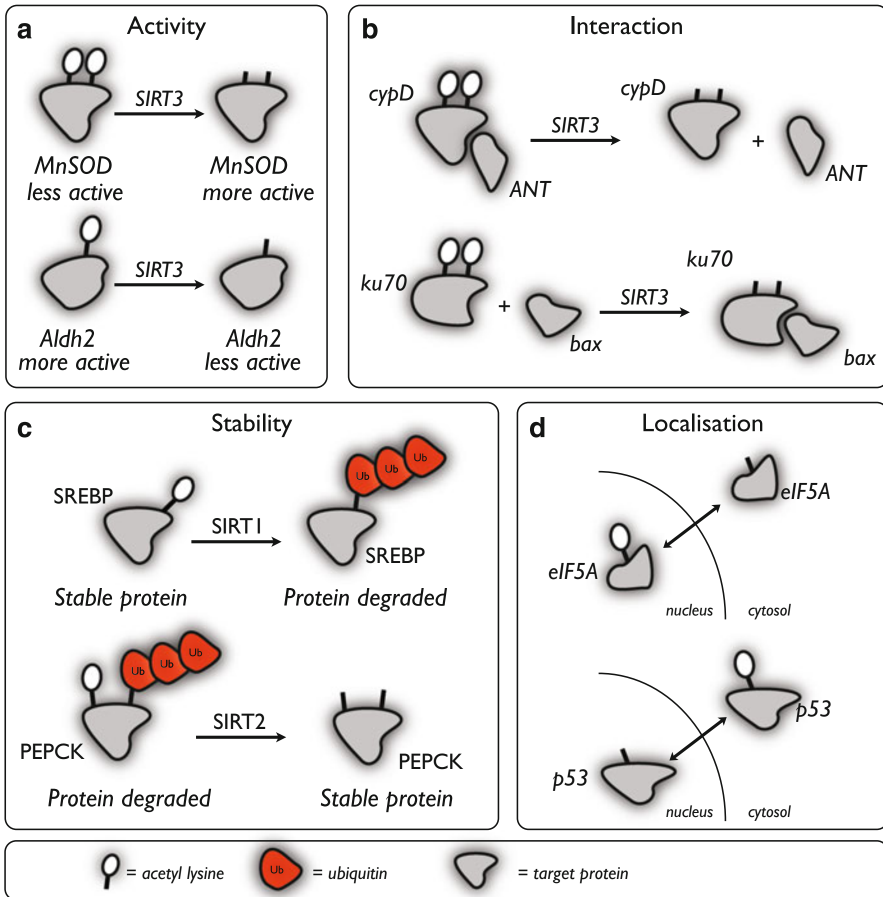

Fig. 3.2 Protein acetylation affects protein function by altering protein activity (**a**) protein-protein interaction (**b**), protein stability (**c**), and localization (**d**). (**a**) Mitochondrial manganese superoxide disomutase (MnSOD) was shown to be activated by the deacetylase activity of SIRT3 (Qiu et al. 2010). Aldehyde dehydrogenase 2 (Aldh2) was inactivated upon deacetylation by SIRT3 (Lu et al. 2011). (**b**) Acetylation promotes the interaction between cyclophilin D (cypD) and adenine nucleotide transporter (ANT) (Hafner et al. 2010), whereas acetylation disables the interaction between Ku70 and Bax (Sundaresan et al. 2008). (**c**) Acetylation of SREBP1 increases the stability of the protein by disabling the ubiquitination of the same lysine (Giandomenico et al. 2003). PEPCK acetylation promotes the binding of an E3 ubiquitin ligase, resulting in decreased stability upon acetylation of PEPCK. (**d**) Acetylation of eukaryotic initiation factor 5A (eIF5A) results in accumulation of eIF5A in the nucleus (Ishfaq et al. 2012). Acetylated p53 is retained in the cytosol (Kawaguchi et al. 2006)

Interestingly, acetylation prevented the induction of an oligomeric state which unmasked a nuclear export signal, leading to trafficking of p53 to the cytosol (Kawaguchi et al. 2006). Thus, by affecting the structural properties of the p53 protein, acetylation directed the trafficking of the p53 protein (Kawaguchi et al. 2006).

In a similar fashion, acetylation also affected the trafficking of pyruvate kinase M2 (PKM2) (Lv et al. 2011). Acetylation of PKM2 allowed for the interaction of PKM2 with a chaperone that targets proteins for autophagy to the lysomes. Acetylation of PKM2 resulted in alteration of protein-protein interaction, loss of protein stability and change in protein subcellular localization (Lv et al. 2011).

3.3 Lysine Formylation

Lysine formylation was first discovered on linker histone H1 in a proteomic study which aimed to map H1 modification in two human cell lines and nine mouse tissues (Wisniewski et al. 2007). Formylation has the same nominal mass as dimethylation of 28 Da, with a difference of 0.036385 Da and therefore formylation was discovered accidently when some dimethylated peptides displayed unusually low mass accuracies (Wisniewski et al. 2007). In subsequent proteomic analysis lysine formylation in nuclear proteins was analyzed (Wisniewski et al. 2008). This study identified 48 formylation sites in 10 different proteins, with H1 being most frequently modified among histones. Interestingly some sites that were modified were also targeted by acetylation and methylation, which are important for histone-DNA interactions. Formylation can thus be part of or interfering with the histone code (Wisniewski et al. 2008).

The biological source of formylation remains undefined. It has been hypothesized that formylation could be catalyzed from formyltetrafolate or formaldehyde released from demethylation of lysine (Wisniewski et al. 2007), however a recent study has demonstrated that histone formylation is not fueled by lysine demethylation but exposure to formaldehyde does result in dose-dependent increase in protein formylation (Edrissi et al. 2013). Jiang et al. demonstrated that lysine formylation can be driven by products of DNA oxidation in cells (Jiang et al. 2007). Treating the cells with DNA damaging reagent neocarzinostatin resulted in increased formylation due to production of formyl phosphate (Jiang et al. 2007).

The physiological role of formylation and its regulation are unknown. It is structurally similar to acetylation and methylation and therefore may be regulated in a similar way or compete for lysines with these modifications. The fact that formylation is enhanced by oxidative stress suggests that it could be a deleterious by-product caused by cellular stress. As histones have a slow turnover rate (Ozbal et al. 1994), lysine formylation could be a result of accumulating damage and a cause for deregulation of epigenetic control (Jiang et al. 2007).

3.4 Lysine Propionylation and Butyrylation

Recent mass spectrometry studies by Zhao and co-workers have identified propionylation and butyrylation on histones and non-histone proteins in eukaryotic cells (Chen et al. 2007; Cheng et al. 2009; Zhang et al. 2009a). The first analysis was

carried out in HeLa cells, where 3 propionylation sites (K5, 8 and 12) and 2 butyrylation sites (K5 and 12) were discovered on H4 (Chen et al. 2007). All of these sites are known to be targeted by acetylation as well and H4K12 can also undergo methylation, indicating the involvement of these lysine residues in gene regulation. A subsequent study by Zhang et al. has further investigated these modifications in yeast histones identifying propionylation and butyrylation sites in H3 and H4, and 1 butyrylation site in H2B (Zhang et al. 2009a). The abundance of these modifications seemed to be lower than that of acetylation, as for example the MS signal intensities of acetylated H3K56 was 85 times higher than of propionylated H3K56 (Zhang et al. 2009a). These two novel lysine modifications are not restricted to histones, as one propionylation and three butyrylation sites were also detected on p53 in H1299 cells co-transfected with p300/CBP (Cheng et al. 2009).

Enzymes dedicated specifically to the regulation of butyrylation and propionylation have not been identified. Chen et al have tested five acetyltransferases, from which CBP and p300 effectively propionylated and butyrylated H4 and p53 in vitro (Chen et al. 2007). P300 and CBP were also capable of autopropionylation and autobutyrylation (Chen et al. 2007). Butyrylation and propionylation of H3 and H4 were enhanced after treating yeast cells with HDAC inhibitors (Zhang et al. 2009a). Transfecting 293 T cells with p300 and treating them with HDAC inhibitors lead to enhancement of propionylation. Co-transfecting these cells with Sirt1 resulted in depropionylation of p53 and p300 (Cheng et al. 2009). The fact that there are means to regulate propionylation within the cell suggests that this modification could be physiologically functional. In fact, propionylation of propionyl-CoA synthetase (PrpE) in Salmonella enterica results in ~70 % loss of its specific activity (Garrity et al. 2007) and it was shown to be regulated by acetyltransferase Pat and sirtuin deacetylase CobB.

3.5 Lysine Malonylation, Succinylation and Glutarylation

Lysine malonylation, succinylation and glutarylation change the charge of lysine from positive to negative due to their negatively charged carboxylate groups. It is likely that this influences protein properties in a different way than acetylation which merely neutralizes the lysine residue. Widespread occurrence of malonylation and succinylation in the cell was discovered by two groups using mass spectrometric analysis (Peng et al. 2011a; Zhang et al. 2011) and through functional studies with SIRT5 (Du et al. 2011). A more recent study had discovered lysine glutarylation, which was also shown to be regulated by SIRT5.

3.5.1 Malonylation and Succinylation

Zhao and colleagues developed several approaches to identify malonylation and succinylation (Peng et al. 2011a; Zhang et al. 2011). They screened for succinylation in *Escherichia coli* (*E. coli*) and for malonylation in both *E. coli* and HeLa

cells. Succinylation sites on 14 proteins, including isocitrate dehydrogenase (IDH), serine hydroxyl-methyltransferase (SHMT) and glyceraldehyde-3-phosphate dehydrogenase A (GAPDH) were detected in E. coli. Lysine malonylation sites on 17 proteins in HeLa cells and 3 proteins in E. coli. Among the proteins identified in HeLa cells were GAPDH, MDH2, and several other metabolic enzymes (Peng et al. 2011a).

Lysine succinylation and malonylation were also reported to be present on histone lysines suggesting their potential role in epigenetic regulation (Xie et al. 2012). The study was carried out in purified histones from *S. cerevisiae*, *Drosophila*, HeLa cells and mouse, where 7, 10, 13 and 7 succinylation sites were identified, respectively (Xie et al. 2012). Lysine malonylation was also detected but with a lower abundance: 2 sites in *S. cerevisiae* and 1 in HeLa cells. To uncover physiological relevance of these modifications functional analysis was performed in yeast by site-specific mutations of lysines to alanine and arginine in order to prevent succinylation or glutamine to mimic constitutively modified lysine. From the 6 residues analyzed, only substitution of H4K31 with glutamine resulted in significantly reduced cell viability. A similar analysis was performed with malonylated sites, however no effect on cell viability was observed (Xie et al. 2012).

Hening Lin and co-workers succeeded in the elucidation of a crystal structure of one of the mitochondrial sirtuins, SIRT5, only when they realized that a negatively charged side group of a CHES buffer molecule was co-crystalized with SIRT5 and a thioacetyl lysine peptide substrate (Du et al. 2011). On the basis of this finding, they deducted that a peptide containing a negatively charged succinyl or malonyl group would be a better substrate for the SIRT5 than an acetyl lysine peptide (Du et al. 2011). Indeed SIRT5 proved to be a robust desuccinylase and demalonylase.

Furthermore, Du et al. also identified succinylation sites on three metabolic enzymes, which co-immunoprecipitated with flag tagged human SIRT5 using bovine liver mitochondrial lysates. These were 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase 2, thiosulfate sulfurtransferase and aspartate aminotransferase. Furthermore, succinylation sites were detected on commercial glutamate dehydrogenase (GDH), malate dehydrogenase (MDH) and citrate synthase (CS), which were obtained from mammalian sources giving additional evidence that succinylation is a naturally occurring modification. Malonylation sites were also detected on GDH and MDH. SIRT5 was shown to regulate CPS1 activity in vivo. Succinylation and acetylation was detected on K44, K287 and K1291 of CPS1. Succinylation levels of K1291 showed a substantial increase in SIRT5 KO mice, whereas the acetylation levels remained unchanged. This was accompanied by reduced CPS1 activity in the SIRT5 KO mice livers (Du et al. 2011).

3.5.2 Glutarylation

A recent study has identified that SIRT5 can also catalyze the removal of glutaryl groups from lysines (Tan et al. 2014). Glutarylation was shown to be conserved in eukaryotic and prokaryotic cells as it was detected in *E. coli*, *S. cerevisiae*,

Drosophila melanogaster, MEFs and HeLa cells. Similar to acetylation, glutarylation was enriched on metabolic enzymes and mitochondrial proteins. CPS1 was highly glutarylated in the SIRT5 KO mice after 48 h starvation and also had reduced enzymatic activity (Tan et al. 2014). In total eight sites were identified on CPS1 which could be targeted by SIRT5 for deglutarylation (Tan et al. 2014). It is therefore possible that SIRT5 regulated the activity of CPS1 through desuccinylation and deglutarylation.

3.6 Lysine Crotonylation

Lysine crotonylation was first discovered on histones by Tan et al., in a study which conducted an MS analysis to profile novel PTMs in histones (Tan et al. 2011). Crotonylation was detected on 28 sites on histones in HeLa cells. In the subsequent analysis the occurrence of crotonylation was confirmed in cells from other eukaryotes cells, including *S. cerevisiae*, *C. elegans*, *Drosophila melanogaster* (S2) cells, and mouse embryonic fibroblasts. The physiological relevance of lysine crotonylation was then investigated in human somatic cells and in mouse spermatids. It had a high prevalence in human fetal lung fibroblast and most of the crotonylation peaks were associated with promoter, transcription starting site (TSS) and predicted enhancer regions, suggesting that crotonylation is involved in gene regulation. Additionally, there was a strong overlap between crotonylation and acetylation sites (Tan et al. 2011). Crotonylation dynamics were similar in mouse spermatids, where crotonylation was prevalent at the TSS of genes. The accumulation of crotonylation on the sex chromosomes in mouse postmeiotic round spermatids, suggested that it may play a role in the escape of sex-linked genes from transcriptional inactivation after completion of meiosis. Because multiple identified lysine crotonylation sites were not acetylated on these specific lysines, crotonylation may indeed have an independent function in these cells (Tan et al. 2011).

The regulation mechanisms of crotonylation are apparently distinct from that of acetylation as overexpression of two HATs, CBP and p300 in 293T cells did lead to enhanced acetylation, but had no significant effect on crotonylation levels in histones. CBP and p300 are not specialized in acetylation as they have also shown to have potent butyrylation and propionylation activities (Chen et al. 2007; Cheng et al. 2009). Histone crotonylation is fueled by crotonyl-CoA as exposing HeLa cells to propionate resulted in enhanced histone crotonylation (Tan et al. 2011) and therefore it is likely that there is an enzyme with crotonyltransferase activity. It has been recently reported that HDAC3-NCoR1 exhibits in vitro decrotonylase activity, however the rate was significantly lower than that that of deacetylation (Madsen and Olsen 2012). Tan et al. have also reported no or extremely weak decrotonylase activities for HDACs 1, 2, 3 and 6 (Tan et al. 2011). The crotonyl group has a more rigid three dimensional structure than the acetyl group and this is likely to influence its suitability as a substrate for HATs and HDACs (Tan et al. 2011).

3.7 Regulation of Lysine Acylation

The mechanisms of enzymatic regulation of acetylation have been thoroughly studied however there is still little knowledge about enzymatic regulation of other acylation modifications. Acetylation is regulated by two groups of enzymes with opposing modes of action: namely lysine acetyltransferases (KATs), which transfer the acetyl group from acetyl-CoA to a lysine and histone deacetylases (HDACs), which remove the acetyl group.

3.7.1 Acetyltransferases

KATs were first referred to as histone acetyltransferases (HATs) as they were initially known to acetylate histones. However, with the discovery of numerous non-histone targets, they have been given a more general name (Allis et al. 2007). KATs are classified into five families based on their sequence and function. These families include the Gcn5-related acetyltransferases (GNATs) , the MYST (Moz, Ybf2/Sas3, Sas2 and Tip60)-related acetyltransferases, the general transcription factor (including TFIID subunit TAF250) and the nuclear hormone-related acetyltransferases (SRC and ACTR) (Carrozza et al. 2003). Most of KATs are characterized as being nuclear, but some of them are also localized in the cytosol (Sadoul et al. 2011). The subcellular localization of known acetyltransferases provokes further questions, as it has been established that protein acetylation is abundant in mitochondria (Kim et al. 2006), however no KAT with mitochondrial localization has been identified.

The discovery of longer chain acyl-modifications has led to investigation on whether known acetyltransferases can use longer chain acyl-CoAs as substrates. There is evidence that some acetyltransferases can catalyze propionylation and butyrylation (Berndsen et al. 2007; Cheng et al. 2009; Leemhuis et al. 2008; Zhang et al. 2009a). This suggests that the known acetyltransferases may also catalyze other acylation modifications apart from acetylation.

3.7.2 Histone Deacetylases

There are two families of histone deacetylases (HDACs). The first is the conventional HDAC family, members of which use Zn^{2+} as cofactor. It is composed of 11 members that are divided into class I, II and IV (de Ruijter et al. 2003). These HDACs are located in the nucleus and cytosol (de Ruijter et al. 2003). Apart from regulating histone acetylation, they also regulate the acetylation of non-histone proteins like transcription factors, chaperones and signaling molecules (Glozak et al. 2005).

The second, more recently discovered family is class III HDACs, which is also known as sirtuins. These HDACs do not require Zn^{2+} as they have NAD^{+} dependent deacetylase activity (Blander and Guarente 2004). The sirtuins are a conserved family of proteins present in all domains of life (Frye 2000). The first sirtuin discovered, Silent Information regulator 2 (SIR2) was shown to promote longevity in budding yeast (Kaeberlein et al. 1999) through NAD^{+} dependent deacetylation of histones H3 and H4 (Imai et al. 2000). Although initially named HDACs, sirtuins regulate acetylation throughout the entire cell and their activity is by far not limited to histones. The seven mammalian sirtuins (SIRT1-7) have distinct functions, subcellular localization and tissue specificity. Sirtuins SIRT 1, 6, and 7 are localized in the nucleus, SIRT 3, 4 and 5 in the mitochondria and SIRT2 in the cytoplasm (Haigis and Sinclair 2010). Depending on their cellular localization, sirtuins enable the cells to adapt to its environment either through regulating gene transcription or by directly regulating specific cellular processes by deacetylating their targets. From the seven sirtuins only SIRT1-3 were shown to possess strong deacetylase activity (North et al. 2005).

The finding that SIRT5 with weak deacetylase activity possesses potent demalonylase and desuccinylase activities (Du et al. 2011) stimulated further research to determine whether other sirtuins with weak deacetylase activity have preference for longer chain acyl-substrates. Latter studies have also established that SIRT5 can deglutarylate proteins (Tan et al. 2014). Recently, SIRT6 was shown to hydrolyze long-chain acyl groups more efficiently than acetyl group (Feldman et al. 2013; Jiang et al. 2013). In particular, SIRT6 showed high affinity for removing myristoyl group and demyristoylation of TNF-α was shown to regulate its secretion (Jiang et al. 2013). Furthermore, an *in vitro* study with recombinant sirtuins and acylated peptides has demonstrated that sirtuins with strong deacetylase activity are also capable of catalyzing other deacylation reactions. For instance, the sirtuins SIRT1-3 demonstrated affinity for propionyl and butyryl groups, though to a lesser extent than their usual substrate the acetyl group. Also SIRT1, 2, 3 and 5 were able to hydrolyze long-chain acyl groups, like decanoyl and dodecanoyl (Feldman et al. 2013). Thus it appears that the role of sirtuins in regulation of cellular processes is not limited to deacetylation, but additionally they control protein acylation.

3.7.3 Non-enzymatic Lysine Acylation

Interestingly, lysine acylation is abundant in mitochondria however enzymatic regulation of acylation in this organelle remains obscure. The fact that there are high levels of acyl-CoAs in mitochondria generated by intermediary metabolism, has led to speculations that lysine acylation can occur non-enzymatically. This is also favored by the chemical environment of mitochondria as intramitochondrial pH was shown to be optimal for non-enzymatic protein acylation reactions *in vitro* including acetylation, butyrylation, propionylation and malonylation (Pougovkina et al. 2014a; Wagner and Payne 2013).

The evidence that mitochondrial acetylation can be non-enzymatic is in its distribution and dynamics. First of all, acetylation is not evenly distributed throughout the mitochondria as it was found to be concentrated on pathways consuming or generating acetyl-CoA (Rardin et al. 2013b). Furthermore, acetylation is a dynamic modification as its levels fluctuate in response to metabolic changes. For instance, mice subjected to caloric restriction, fasting and high fat diet have increased protein acetylation (Hebert et al. 2013; Hirschey et al. 2011b; Schwer et al. 2009). Indeed, it has been demonstrated that acetyl-CoA produced by mitochondrial fatty acid oxidation serves as substrate for mitochondrial protein acetylation (Pougovkina et al. 2014a). Consistently cells with deficient fatty acid oxidation have low acetylation levels (Pougovkina et al. 2014a).

The concept that acyl-CoA levels can be determining for the extent of protein acylation also holds for other acylations apart from acetylation. Cells that have altered flux of specific metabolic pathway due to a metabolic defect can also present with aberrant protein acylation. For instance short-chain acyl-CoA dehydrogenase cells that accumulate butyryl-CoA have elevated protein butyrylation levels (Pougovkina et al. 2014b). This is also the case for malonyl-CoA decarboxylase, propionyl-CoA decarboxylase and glutaryl-CoA dehydrogenase deficiencies, which result in increased malonylation, propionylation and glutarylation, respectively (Pougovkina et al. 2014b; Tan et al. 2014).

3.8 Proteomic Studies: Mapping the Cellular Acylome

3.8.1 Acetylation

Proteomic studies identifying acetylation sites on proteins have contributed significantly to our understanding of protein acetylation function and dynamics. The current view of protein acetylation is that protein acetylation is not restricted to DNA regulatory processes in the nucleus but is in fact a major cellular PTM with prevalence that is comparable to phosphorylation. The proteomic studies aimed to identify acetylated proteins, but also to gain insight into the dynamics of this modification. These studies were performed in a variety of models ranging from bacteria to humans, thereby giving a good overview of acetylation in different organisms and emphasizing its evolutionary conservation.

Kim et al. were the first to perform a large scale proteomic study to map the lysine acetylome, demonstrating that acetylation is not restricted to the nucleus but is also abundant in other cellular compartments and primarily in the mitochondria (Kim et al. 2006). The analysis was carried out in nuclear and cytosolic fractions from HeLa cells and mitochondria from fed and fasted mice, using acetyl lysine immunoprecipitation of tryptic digests of cell or tissue lysates. They identified 388 lysine acetylation sites in 195 proteins, from which 37 were cytosolic, 38 nuclear and 133 mitochondrial (Kim et al. 2006). Acetylation was found on proteins

involved in a wide variety of cellular processes to which it has not been associated previously, implying that this modification has applications beyond epigenetic regulation. Among these are proteins involved in RNA splicing and translation factors, chaperones, chromatin and transcription regulators, signaling and structural proteins (Kim et al. 2006). This study indicated that at least one fifth of mitochondrial protein was acetylated, most of which were metabolic enzymes involved in the TCA cycle, oxidative phosphorylation, beta-oxidation, lipid metabolism, amino acid metabolism, nucleotide metabolism and the urea cycle. Apart from mitochondrial metabolic enzymes they also identified five cytosolic metabolic proteins being acetylated. Interestingly, they found that 14 % of the acetylated proteins were specific for fed mice and 24 % were specific for fasted mice, indicating that acetylation is possibly a dynamic modification influenced by the metabolic state (Kim et al. 2006).

The acetylome studies by Choudhary et al. in human cell lines demonstrated the prevalence of acetylation as a PTM in the cell (Choudhary et al. 2009). They have further emphasized that the size of cellular acetylome, 3500 acetylation sites on 1750 proteins, is close to the scale of phosphoproteome (Pearlman et al. 2011). A large number of acetylated proteins in macromolecular complexes involved in all major nuclear processes such as chromatin remodeling and DNA replication was identified. They also revealed that acetylation contributes to control of many cytosolic functions as for example it is likely to be involved in the regulation of cytoskeleton reorganization and cell motility, since most of the proteins involved in these processes were acetylated (Choudhary et al. 2009).

Two studies carried out in liver cells and *Salmonella* again connected protein acetylation to cellular metabolic processes. Zhao et al. performed qualitative proteomic analyses of nuclear, cytosolic and mitochondrial fractions of human liver tissue identifying a total of 1047 acetylated proteins (Zhao et al. 2010a). Lysine acetylation was detected on almost every enzyme in central metabolic pathways including glycolysis, gluconeogenesis, the TCA cycle, the urea cycle, fatty acid metabolism and glycogen metabolism (Zhao et al. 2010a). Wang et al. investigated acetylation dynamics in *Salmonella* grown in media with different carbon sources, namely glucose vs. citrate (Wang et al. 2010). They found that 90 % of enzymes involved in major metabolic pathways were acetylated. Interestingly, they observed higher global acetylation levels in cells grown in glucose medium. Using acetyltransferase (PAT) and NAD^+ dependent deacetylase (CobB) deletion mutants they established that increased acetylation favors growth on glucose medium. They further investigated the effect of acetylation on the activity of individual enzymes involved in glycolysis/gluconeogenesis flux regulation. Indeed, increased acetylation of these enzymes directed the metabolic flux toward glycolysis (Wang et al. 2010).

In a recent resource study published by Jesper Olsen and co-workers the lysine acetylome was profiled in 16 different rat tissues. With 15474 acetylation sites on 4541 proteins, many new sites and acetylated proteins were identified by this study, giving indications for previously unknown physiological functions of protein acetylation (Lundby et al. 2012). For instance, most of the proteins involved in contraction of striated muscle were found to be acetylated, suggesting a regulatory role of

acetylation in muscle contraction. Interestingly, this was also confirmed in human muscle biopsies. Acetylation sites and cellular distribution were tissue specific and there was a correlation between acetylation of specific sites and tissue function. For example, mitochondrial acetylation was abundant in general, with a high average number of acetylated sites per protein, however mitochondrial acetylation was particularly high in energy generating tissues. More than 50 % of acetylation sites in brown adipose tissue were found in mitochondria and the highest prevalence of acetylation in the nuclear fractions was in the lymphoid containing organs which have high mitotic activity (Lundby et al. 2012). The degree of acetylation in the cell proved to be comparable to phosphorylation although the two modifications had distinct subcellular targets. Acetylation seemed to be more prevalent in the mitochondria, whereas phosphorylation was prominent at the plasma membrane (Lundby et al. 2012).

Mitochondrial protein acetylation is controlled by the NAD^+ dependent deacetylase SIRT3 (Hirschey et al. 2010; Lombard et al. 2007). Three proteomic studies have mapped the acetylome in mouse and cell models where SIRT3 was genetically modified (Hebert et al. 2013; Rardin et al. 2013b; Sol et al. 2012). Quantitative acetylation proteomics using SILAC methods in human and mouse cell lines with genetic manipulation of SIRT3, revealed a large number of endogenous metabolic SIRT3 substrates in mammalian cells (Sol et al. 2012). Label-free quantitative proteomics by Rardin et al have investigated acetylome in SIRT3 KO mouse livers. They have established that a large fraction of mitochondrial proteins is acetylated with acetylation sites detected on 483 of mitochondrial proteins. Absence of SIRT3 resulted in selective hypeacetylation of lysines on 136 of these proteins, many of which were metabolic enzymes (Rardin et al. 2013b). Hebert et al. analyzed changes in specific acetylation sites induced by caloric restriction (CR) and genetic loss of SIRT3 in mouse liver mitochondria using a quantitative isobaric tagging acetyl-proteomic approach (Hebert et al. 2013). This study has demonstrated that CR greatly affects mitochondrial protein acetylation in two ways: a large number of mitochondrial acetylation sites were significantly enhanced by more than two-fold in CR livers as compared to control fed livers, whereas another group of sites followed an opposite trend. Genetic loss of SIRT3 also greatly altered mitochondrial acetylome both under control diet and CR. As in the previously discussed studies, the sites regulated by SIRT3 were identified primarily on enzymes involved in mitochondrial metabolic pathways (Hebert et al. 2013) demonstrating the involvement of SIRT3 mediated deacetylation in metabolic adaption. Interestingly, all of the acetylation sites identified by this study could be clustered into three classes: the sites controlled by SIRT3 with decreased acetylation during CR, sites not controlled by SIRT3 with increased acetylation during CR and sites that are hardly influenced by CR or SIRT3 loss (Hebert et al. 2013).

The studies by Rardin et al. and Hebert et al. both emphasize the fact that SIRT3 does not globally regulate mitochondrial acetylation but instead targets specific sites with regulatory function (Hebert et al. 2013; Rardin et al. 2013b). This underlines the distinction between specific and global acetylation. Specific acetylation is targeted by SIRT3 and based on a number of functional studies has a regulatory role

in cellular metabolism. However, a substantial fraction of mitochondrial acetylation is not regulated by SIRT3 and is referred to as global acetylation. The physiological consequences and regulation of global untargeted acetylation have not been determined. These aspects are also difficult to address due to the lack of information about regulation of global acetylation in mitochondria.

3.8.2 Succinylation

Several proteomic studies have been performed to provide a global overview of cellular succinylome (Park et al. 2013; Rardin et al. 2013a; Weinert et al. 2013). They have looked at the prevalence of succinylation throughout the cell, the overlap of succinylation sites with acetylation sites and functional role of succinylation in metabolic pathways.

Two studies by Park et al. and Rardin et al. focused on the regulation of succinylome by SIRT5 by analyzing succinylation in the SIRT5 KO mice (Park et al. 2013; Rardin et al. 2013a). Park et al. have investigated succinylation dynamics in WT and SIRT5 KO mouse livers and MEFs detecting 2565 sites on 779 proteins (Park et al. 2013). Using stable isotope labeling of amino acids in cell culture (SILAC) they quantified the differences in succinylation levels of specific sites between WT and SIRT5 KO MEFs. The majority of quantified sites in MEFs had increased succinylation state in the SIRT5 KO cells. This study has also established a link between SIRT5 and mitochondrial respiration as desuccinylation of pyruvate dehydrogenase complex (PDC) and succinate dehydrogenase (SDH) by SIRT5 repressed their activities (Park et al. 2013).

The study by Rardin et al. used label free quantitative proteomics to determine potential SIRT5 targets in the mouse liver (Rardin et al. 2013a). 386 sites across 140 proteins were identified to be more succinylated in the livers of SIRT5 KO mice, among which were the enzymes in β-oxidation and ketogenesis pathways indicating that SIRT5 regulates succinylation of these enzymes and possibly the flux of these pathways. The latter is supported by metabolic analysis, which detected accumulation of medium-chained and long-chained acylcarnitines along with decreased ketogenesis in SIRT5 KO mice.

A study by Weinert et al. has looked at succinylation in prokaryotes and eukaryotes (Weinert et al. 2013). They have mapped succinylation sites in *E. coli*, *S. cerevisiae*, HeLa cells and mouse liver tissue. In HeLa cells and mouse liver the most succinylation was found in the mitochondria. They found that more than 50 % of succinylation sites overlapped with acetylation sites in *E. coli*, *S. cerevisiae* and mouse. In mouse liver they found that many metabolic pathways in mitochondria were enriched in succinylation.

To gain more understanding in the function of succinylation, the overlap between succinylation and acetylation sites was analyzed. Two of the three studies have

detected a substantial overlap of lysine succinylome and acetylome with many enzymes having the same succinylation and acetylation sites (Rardin et al. 2013a; Weinert et al. 2013). However, only a fraction of succinylation sites in MEFs overlapped with acetylation sites indicating that these modifications target distinct sites (Park et al. 2013). Interestingly, succinylation demonstrated a similar metabolic dynamic to acetylation with observed increase of succinylation levels in kidney and liver of fasted mice (Park et al. 2013). Further analysis is required to investigate the possibility of a crosstalk between these two modifications.

3.8.3 Glutarylation

Tan et al. have performed proteome-wide analysis in the livers of SIRT5 KO mice to analyze protein glutarylation (Tan et al. 2014). This study has detected 683 glutarylation sites on 191 proteins. A large portion of the glutarylated proteins contained one or two glutarylation sites, however some proteins were found to be heavily glutarylated, including carbamoyl-phosphate synthase 1 (CPS1, 33 sites), long-chain enoyl-CoA hydratase (HADHA, 15 sites) and aspartate aminotransferase (GOT2, 14 sites). More than three quarters of the proteins were localized in mitochondria and gene ontology enrichment analysis showed that glutarylation was enriched in many cellular processes including oxygen reduction, generation of precursor metabolites and energy, fatty acid metabolism and aerobic respiration.

3.9 Conclusion

Numerous lysine acetylation studies have demonstrated that lysine acetylation is abundant and a dynamic modification which plays an important role in regulation of protein function. Much less is known about the novel acylation modifications, which have only been discovered. Nevertheless, the studies so far reveal that there are many overlapping functional properties between the newly discovered modifications and acetylation: First, they are identified on similar targets such as histones and metabolic enzymes. Second, all protein acylation PTMs seem to be dynamically regulated. Third, they are evolutionary conserved. Fourth, there has been evidence of their physiological relevance. All in all there is a strong indication that lysine acylation has a significant impact on the cellular proteome. Future studies will provide novel insights to gain full understanding on the abundance, dynamics and function of lysine acylation modifications.

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Chapter 4
SIRT1 in Metabolic Health and Disease

Marie Boutant and Carles Cantó

4.1 Introduction

The story of SIRT1, and sirtuins in general, began more than three decades ago, with the identification of an enzyme, Sir2, participating in gene silencing at specific genomic regions (Ivy et al. 1986; Shore et al. 1984). However, it was not until fifteen years later that we began to truly grasp the significance of Sir2 activity. A key finding in the story of sirtuins was the identification of Sir2 as an NAD^+-depedent deacetylase enzyme (Imai et al. 2000a). This meant that Sir2, and all its homologs along the evolutionary scale, used NAD^+ as a cosubstrate to catalyze deacetylation of a substrate, rendering nicotinamide and 2'-O-acetyl-ADP-ribose as concomitant reaction products. The engagement of sirtuins with NAD^+ as a cosubstrate is unique compared to all other deacetylase enzyme families known to date. In addition, this fundamental feature suggests that sirtuins might respond directly to changes in intracellular metabolism by "sensing" NAD^+ levels (Canto and Auwerx 2012; Imai et al. 2000b). Since reversible acetylation has putative regulatory properties on the activity and function of a constellation of proteins, including histones, sirtuins were proposed to act as enzymes coupling metabolic cues to transcriptional outputs.

Among all sirtuins, SIRT1 is the most well-studied mammalian homolog of the yeast enzyme Sir2. The initial interest in this protein rapidly spread due to its possible role in eukaryote lifespan regulation (Berdichevsky et al. 2006; Canto and Auwerx 2009; Kaeberlein et al. 1999; Viswanathan and Guarente 2011; Viswanathan et al. 2005). While this is still a matter of debate (Burnett et al. 2011; Lombard et al. 2011), an overwhelming amount of evidence in animal models suggests that SIRT1 might play key role in metabolic regulation and adaptation (Canto and Auwerx 2012). This, in turn, impinges on the sensibility of organisms to develop metabolic

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and age-related diseases, including insulin resistance, cancer and diverse neurodegenerative pathologies. Here, we aim to review the most recent advances on SIRT1 functions, with strong emphasis on the knowledge obtained using transgenic animal models.

4.2 SIRT1 Biology

4.2.1 Basic Structure and Localization

The human SIRT1 protein spans for 747 amino acids. The structure of the protein is immaculately conserved within mammals and contains the defining conserved catalytic core of sirtuins flanked by both N- and C-terminal extensions (Michan and Sinclair 2007). These extensions span each around 240 amino acids and serve as docking and interactive platforms for regulatory proteins and substrates (Canto and Auwerx 2012; Michan and Sinclair 2007).

The expression of *Sirt1* is rather ubiquitous, and can be detected at the protein level in most, if not all, mammalian tissues. However, as described later, *Sirt1* expression is very plastic, and can dramatically change in response to metabolic stress in an organ, tissue and cell-autonomous fashion. In addition, the SIRT1 protein can be found in the nuclei and/or the cytosolic compartment (Michishita et al. 2005; Tanno et al. 2007). SIRT1 contains two nuclear localization signals, that drive nuclear import, and two nuclear exportation signals, that drive the export (Tanno et al. 2007). The balanced function of these different signals determines SIRT1 localization in diverse tissues. This way, SIRT1 can display a very predominant nuclear localization in some tissues and cells, such as COS-7 cells or mouse embryonic fibroblasts (McBurney et al. 2003; Sakamoto et al. 2004). In turn, SIRT1 is predominantly cytosolic in pancreatic β-cells, myotubes and cardiomyocytes (Moynihan et al. 2005; Tanno et al. 2007). Furthermore, SIRT1 can actively shuttle between these compartments in response to environmental cues. For example, inhibition of the phosphatidylinositol 3-kinase (PI3K) pathway rapidly promotes nuclear exclusion of SIRT1 (Tanno et al. 2007). These observations further support the possible interrelation between SIRT1 activity and the cellular metabolic state.

4.2.2 SIRT1 Regulation

4.2.2.1 Regulation at the Expression Level

Intracellular SIRT1 activity can be modulated at the transcriptional level. The overexpression of *Sirt1* in cultured cells, tissues and organisms is enough to increase global SIRT1 activity (Banks et al. 2008; Pfluger et al. 2008; Rodgers et al. 2005; Rodgers and Puigserver 2007). At the endogenous level, the expression of *Sirt1* is

generally upregulated in situations of low nutrient availability and energy stress (Canto and Auwerx 2012; Nemoto et al. 2004; Noriega et al. 2011). Hence, it was not surprising to find out how many transcriptional effectors of key metabolic signals directly regulate the activity of the *Sirt1* promoter.

Initial studies trying to elucidate how the expression of the *Sirt1* gene increased upon glucose deprivation found out that the *Sirt1* promoter is critically regulated by members of the Forkhead-O-box (FOXO) family of transcription factors (Nemoto et al. 2004). FOXOs are transcription factors whose activity is negatively modulated by the PI3K pathway via direct Akt-mediated phosphorylation (Brunet et al. 1999). Therefore, insulin and other PI3K-activating growth factors, inhibit FOXO activity (Calnan and Brunet 2008). Elegant studies from the Finkel lab demonstrated that FOXO3a is a key regulator of the activity of the *Sirt1* promoter (Nemoto et al. 2004). FOXO3a modulated Sirt1 gene expression via its interaction with p53 (Nemoto et al. 2004). When nutrients are abundant, FOXO3a is exported to the cytosol and p53 is bound to the proximal region of the *Sirt1* promoter, repressing its transcriptional activity. However, upon nutrient depletion, FOXO3a is no longer sequestered to the cytosol via phosphorylation, hence shuttling to the nucleus where it directly interacts with p53 at the *Sirt1* promoter (Nemoto et al. 2004). The interaction between FOXO3a and p53 relieves the inhibition of *Sirt1* transcription, probably by modulating the accessibility of coactivator/corepressor complexes. It was exciting to find that FOXOs can also regulate *Sirt1* transcription through direct binding to the *Sirt1* promoter. Indeed, a number of FOXO binding sites have been identified in more distal regions (up to 1.5 kbp) of the rat *Sirt1* promoter (Xiong et al. 2011). To date, however, only FOXO1 has been shown to bind to them and positively influence SIRT1 transcription (Xiong et al. 2011), and the conservation of these binding sites has to be yet fully analyzed. While organismal confirmation of these findings needs still to be solidified, FOXOs provide an excellent link on how nutrient deprivation enhances *Sirt1* transcription. Similarly, the nuclear exclusion of FOXO constitutes a beautiful mechanism to integrate hormonal signals linked to nutrient abundance and the repression of the *Sirt1* promoter.

Additional mechanism by which feeding/fasting cycles might influence *Sirt1* expression in diverse tissues was unraveled recently in animal models. In the fed state, the *Sirt1* promoter is repressed via the direct binding of the carbohydrate response element-binding protein (ChREBP) (Noriega et al. 2011). Upon fasting, ChREBP is translocated to the cytosol and its binding region is liberated. This goes together with the activation of CREB by fasting-derived cAMP signals triggered for example, by glucagon. CREB and ChREBP can bind to similar response elements (Noriega et al. 2011). Therefore, during the fasting state, the activated CREB can bind to the regions liberated by ChREBP and activates *Sirt1* transcription.

Further supporting the metabolic regulation of *Sirt1* gene expression, the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors also has key roles on *Sirt1* transcriptional regulation. PPARs are directly activated by different lipid species and critically control lipid anabolism and catabolism (Michalik et al. 2006). The distal region of the *Sirt1* promoter contains PPAR response elements (PPREs) (Han et al. 2010; Hayashida et al. 2010), even though a thorough

analysis of their mapping and conservation is still lacking. This way, it was demonstrated that PPARγ can directly bind to and repress the *Sirt1* promoter (Han et al. 2010). This could explain how situations of nutrient overload, when PPARγ is activated, are generally correlated with Sirt1 downregulation, both in mice (Coste et al. 2008) and humans (Costa Cdos et al. 2010). Interestingly, the existence of PPREs also opens the door for other PPARs to regulate the *Sirt1* gene. Indeed, the activation of PPARα and PPARβ can also regulate *Sirt1* expression, both in a positive manner (Hayashida et al. 2010; Kim et al. 2012; Okazaki et al. 2010). However, whether this regulation occurs via the same PPREs described is far from clear. In the case of PPARβ, it has been reported that such positive regulation could rely on an alternative mechanism, where PPARβ would enhance the positive action of p21 on the *Sirt1* promoter (Okazaki et al. 2010). While the possible regulation of *Sirt1* expression by PPARs provides a beautiful link between lipid metabolism and SIRT1 transcriptional activity, the mechanisms remain still poorly defined and even conflictive observations have been reported on the role of PPARγ on *Sirt1* gene regulation (Chiang et al. 2013). Further work in vivo will be key to fully grasp the relevance of PPARs on *Sirt1* transcription.

Other transcriptional regulators have been described on the *Sirt1* promoter, but with either poor understanding of their in vivo relevance or their regulatory mechanisms. For example, the poly(ADP-ribose) polymerase (PARP)-2 protein has recently been described as a powerful repressor of *Sirt1* transcription by directly binding to the proximal promoter region (Bai et al. 2011a). The downregulation or genetic ablation of *PARP-2* is enough to enhance basal *Sirt1* expression and protein levels (Bai et al. 2011a). However, the mechanism by with PARP-2 represses the *Sirt1* promoter is still nebulous, and so is the possible physiological modulation of PARP-2 inhibitory action. Another mechanism for *Sirt1* transcriptional repression that deserves attention is that constituted by the hypermethylated in cancer 1 (HIC1) protein. HIC1 naturally forms a corepressor complex on the SIRT1 promoter (Chen et al. 2005). The stability of this complex is critically modulated by the presence of the C-terminal binding protein (CtBP), which depends on NADH (Zhang et al. 2007). In situations of low NADH levels, such as in cells treated with 2-deoxyglucose – mimicking low glucose availability – the complex is destabilized and the inhibitory effect is relieved, allowing enhanced *Sirt1* transcription rates, which, in turn, optimizes the adaptation to low nutrient availability (Zhang et al. 2007). In this case the molecular regulation of the HIC1:CtBP complex on the *Sirt1* promoter is very well defined, but support of such findings in animal models will be required.

Another level of regulation of *Sirt1* mRNA occurs through microRNAs (miRNAs), which promote the cleavage of specific mRNAs or inhibit their translation (Neilson and Sharp 2008). To date, more than 16 miRNAs have been described to regulate *Sirt1* expression and activity (Yamakuchi 2012). Among them, miR-34a has been the most widely studied. Briefly, miR-34a binds to the 3'-untranslated region of the *Sirt1* mRNA in a partial complementary manner and represses its translation (Lee et al. 2010; Yamakuchi et al. 2008). Several interventions have

demonstrated a strong negative correlation with *Sirt1* levels in physiological situations (Lee et al. 2010; Yamakuchi 2012; Yamakuchi et al. 2008). We kindly refer the reader to other recent reviews in order to gain more insight into the fascinating new level of complexity on the regulation of *Sirt1* introduced by miRNAs (Yamakuchi 2012).

4.2.2.2 Regulation by NAD$^+$ and NAM

The fact that SIRT1 activity requires NAD$^+$ as a mandatory cosubstrate raised the hypothesis that SIRT1 could act as an NAD$^+$ sensor in the cell, coupling the metabolic and redox status of the cell to transcriptional outputs (Imai et al. 2000b). However, to act as a true metabolic sensor, the activity of SIRT1 should be rate-limited by NAD$^+$ availability. But, is this really the case?

Most studies to date suggest that the Km of SIRT1 for NAD$^+$ is around 150-200 μM (Houtkooper et al. 2010). Whether this is the true range of NAD$^+$ availability in the cell is difficult to confirm. First, most estimates indicate that basal intracellular NAD$^+$ levels in most cells and tissues fall in a range between 0.2 and 0.5 μM (Houtkooper et al. 2010). This originally led to think that NAD$^+$ might not be rate-limiting for SIRT1. However, these values hardly take into account NAD$^+$ intracellular compartmentalization. In fact, some estimates have indicated that nuclear NAD$^+$ concentrations might be around 70 μM (Fjeld et al. 2003), which would be critically rate-limiting for SIRT1 activity. Similarly, the most commonly used techniques to measure intracellular NAD$^+$ fail to distinguish between free (available) and protein-bound NAD$^+$. Therefore, our current knowledge is still too preliminary to unequivocally determine NAD$^+$ bioavailability. However, given the above considerations, it seems plausible that NAD$^+$ could truly be rate-limiting for SIRT1 activity. Supporting this possibility, most – if not all – experimental strategies aimed to alter intracellular NAD$^+$ levels have consistently been shown to influence SIRT1 activity (Canto and Auwerx 2012).

A number of in vivo strategies have demonstrated how increases in NAD$^+$ levels translate into SIRT1 activation. One strategy relied in the deletion of alternative NAD$^+$ consumers, such as PARP-1 or the cADP-ribose synthase CD38. Both of these enzymes are avid NAD$^+$ consumers and, therefore, their deletion should allow higher NAD$^+$ bioavailability for SIRT1. This way, if NAD$^+$ was rate-limiting for SIRT1, these models should display enhanced SIRT1 activity. In line with this hypothesis, the deletion of either PARP-1 or CD38 has consistently shown a correlative increase in NAD$^+$ levels and SIRT1 activity in most tissues examined (Aksoy et al. 2006; Bai et al. 2011b). A second strategy has relied in enhancing NAD$^+$ biosynthesis by providing NAD$^+$ precursors or manipulating the expression of NAD$^+$ biosynthetic enzymes. The efforts from the Imai lab have consistently demonstrated how intraperitoneal (ip) injection of nicotinamide mononucleoside (NMN) is enough to raise NAD$^+$ levels and robutstly increase SIRT1 activity (Yoshino et al. 2011). Parallel experiments demonstrated that ip injections of NMN

prevented fructose rich diet-induced islet dysfunction, likely through SIRT1 activation (Caton et al. 2011). Similarly, the Auwerx lab has demonstrated how food supplementation with the natural NAD^+ precursor nicotinamide riboside (NR) also leads to an elevation in NAD^+ levels and enhanced SIRT1 activity in mouse tissues (Canto et al. 2012). The overexpression of NAD^+ biosynthetic enzymes, such as the nicotinamide phosphoribosyltransferase (Nampt) or the nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1), provides an alternative way to boost NAD^+ availability, which generally leads to enhanced SIRT1 activity (Araki et al. 2004; Revollo et al. 2004; Wu et al. 2011; Zhang et al. 2009). In fact, it has been reported that SIRT1 might directly interact with NMNAT1 (Zhang et al. 2009). Such a complex could potentially channel NAD^+ production to fuel SIRT1 enzymatic catalysis, creating a microdomain for the regulation of SIRT1 activity. Altogether, these strategies support that boosting NAD^+ availability enhances SIRT1 activity.

Many physiological challenges also promote fluctuations in NAD^+ levels. In most cases, these fluctuations rarely increase NAD^+ levels beyond 2-fold (Canto et al. 2009; Chen et al. 2008; Fulco et al. 2008; Rodgers et al. 2005), very much in line with the range expected to affect SIRT1 activity. In general, NAD^+ levels and SIRT1 activity increase in most tissues in response to nutrient deprivation or energy stress, such as exercise (Canto et al. 2009; Costford et al. 2010), fasting (Canto et al. 2010; Rodgers et al. 2005) or calorie restriction (Chen et al. 2008). The degree of the response, however, depends on the baseline NAD^+ levels and SIRT1 activity of each tissue. For example, the exercise-induced enhancement in SIRT1 activity was evident in glycolytic muscle, but not in oxidative muscle, where SIRT1 activity was already high on the basal state (Canto et al. 2009).

SIRT1 activity can also be modulated by other NAD^+ metabolites. For example, it has been shown that NADH can compete with NAD^+ binding to SIRT1 and inhibit SIRT1 activity (Lin et al. 2004; Schmidt et al. 2004). While this could prompt the hypothesis that SIRT1 could also act as an NADH sensor, the levels of NADH required to promote competitive inhibition are in the millimolar range, which are unlikely to be met physiologically (Schmidt et al. 2004). Therefore, NADH levels should rarely determine SIRT1 activity in most common physiological situations, even if it cannot be fully ruled out that such cases might exist.

Another prominent natural inhibitor of SIRT1 activity is nicotinamide (NAM), which is a product of the sirtuin reaction. NAM exerts end-product inhibition of SIRT1 in a non-competitive fashion with NAD^+ (Anderson et al. 2003; Bitterman et al. 2002). While it is known that NAM can inhibit SIRT1 at concentrations around 200 µM or lower, the true intracellular content of NAM content is far from clear. In addition, NAM can diffuse across membranes (van Roermund et al. 1995), which further complicates compartmentalization studies. An important point is that, at low levels, NAM actually prompts activation of SIRT1, due to its property as an NAD^+ precursor (Houtkooper et al. 2010; Revollo et al. 2004). Therefore, NAM at lower micromolar range might be beneficial for SIRT1 activity, while its accumulation is deleterious.

4.2.2.3 Regulation by Post-translational Modifications

The enzymatic activity of SIRT1 can also be influenced through post-translational modifications. Initial evidence for this came from a large mass spectrometry-based screening, which identified SIRT1 as a phosphorylated protein (Beausoleil et al. 2004). Two phosphoresidues, Ser^{27} and Ser^{47}, on the human SIRT1 were identified, but their respective function and modulation is not yet clear. A later SIRT1-centered study revealed up to 13 phosphorylable residues, mostly located on the N- and C-terminal expansions flanking the conserved catalytic domain (Sasaki et al. 2008). Among them, the authors identified Thr^{530} and Ser^{540} as residues directly phosphorylated by cyclinB/cdk1 (Sasaki et al. 2008). The phosphorylation of these residues resulted in higher SIRT1 activity (Sasaki et al. 2008). Very much in the same line, it was reported how Ser^{27}, Ser^{47} and Thr^{530} could be phosphorylated by JNK1, resulting in nuclear translocation and enhanced SIRT1 activation (Nasrin et al. 2009). Interestingly, the phosphorylation by JNK1 targetted SIRT1 activity to specific substrates, as it triggered the deacetylation of histone H3 but not that of p53, which is another well-established SIRT1 substrate (Nasrin et al. 2009). This is actually a key concept, as it is often overlooked that SIRT1 affects many different targets and cellular processes, sometimes with opposite effects. Therefore, SIRT1 action must be targeted to some degree. In this sense, post-translational modifications might be an attractive mechanism by which SIRT1 could be channeled to specific subsets of targets.

A constellation of additional kinases have been described to phosphorylate one or more residues of SIRT1 (see (Canto and Auwerx 2012) for review). A critical point, however, is that most of the residues identified – or their flanking sequences – are very poorly conserved, making it difficult to argue that they are key to the primordial metabolic functions described for SIRT1 orthologs across evolution. Similarly, the physiological modulation of these possible phosphorylation events is far from understood. A major change in the field was hence provided by the Puigserver lab, when they identified mouse Ser^{434} (human Ser^{442}) as a phosphorylation substrate for PKA (Gerhart-Hines et al. 2011). First, because Ser^{442} is located in the catalytic core domain of SIRT1 and is highly conserved across species. Second, because the authors elegantly demonstrated how the phosphorylation of this residue is modulated by hormonal inputs and physiological stimuli in mice. Third, and most importantly, because the phosphorylation of this residue allowed SIRT1 to decrease the Km for NAD^+ (Gerhart-Hines et al. 2011). The enhanced affinity for NAD^+ via phosphorylation can explain how SIRT1 activity can change even in the absence of changes in NAD^+.

SIRT1 activity can also be influenced by the addition of small ubiquitin-like modifier (SUMO). SIRT1 is SUMOylated at Lys^{734} upon irradiation or treatment with toxic concentrations of hydrogen peroxide (Yang et al. 2007). Upon SUMOylation SIRT1 increases its intrinsic deacetylase activity and enhances the ability of the cell to survive to the above mentioned damaging agents (Yang et al. 2007). A key caveat of the described SUMOylation event is the poor conservation

of the Lys^{734} residue, even within mammals. While this does not rule out the potential contribution of SUMOylation in some species, including humans, it is unlikely that SUMOylation is a major contributor of the conserved key metabolic actions of SIRT1. Moreover, further studies will have to underscore the physiological implications and regulation of SIRT1 SUMOylation.

While we are just beginning to grasp the complex interplay of post-translational modifications that occur on SIRT1, it is tempting to hypothesize that they do not act independently. Rather these modifications might interact to control substrate accessibility, modify the kinetic properties or serve as interaction platforms for SIRT1. Understanding these specific mechanisms will be essential to design future strategies to selectively channel SIRT1 into specific functions.

4.2.2.4 Regulation by Protein Interactions

Another layer of regulation is provided by the association of SIRT1 with different proteins. As described previously, SIRT1 was originally described as a transcriptional silencing enzyme. Therefore, it was not surprising that SIRT1 was found in mammalian cells and tissues forming part of corepressor complexes with the Nuclear Receptor Corepressor 1 (NCoR1) (Picard et al. 2004). In there, SIRT1 participated in silencing of the transcriptional activitiy of nuclear receptors, such as PPARγ. However, the coregulator properties of SIRT1 are not limited to silencing. For example, when associated to the PPAR coactivator 1α (PGC-1α), it participates in the transcriptional activation of mitochondrial and fatty acid oxidation genes (Gerhart-Hines et al. 2007; Rodgers et al. 2005). Therefore, the association with different partners confers SIRT1 the ability to act both as a corepressor and as a coactivator.

A key finding in the SIRT1 field came with the simultaneous identification by two different labs of a nuclear protein, deleted in breast cancer 1 (DBC1), as a protein forming a stable complex with SIRT1 (Kim et al. 2008; Zhao et al. 2008). DBC1 binds to the catalytic domain of SIRT1 and inhibits SIRT1 activity both in vivo and in vitro (Kim et al. 2008; Zhao et al. 2008). This way, reductions in DBC-1 prompted an increase in SIRT1 activity in cultured cells (Kim et al. 2008; Zhao et al. 2008). Mice with a germline deletion of DBC1 display a 2- to 4-fold increase in endogenous SIRT1 activity in a wide range of tissues (Escande et al. 2010). Under normal circumstances, it was estimated that at least 50 % of the total SIRT1 in liver is associated with DBC1, and that this interaction was nearly absent upon starvation, when SIRT1 activity is higher (Escande et al. 2010). In contrast, the interaction was more prominent after chronic high-fat feeding, when SIRT1 activity is decreased (Escande et al. 2010). While these evidences illustrate that the interaction of DBC1 and SIRT1 can be modulated, the nature of this plasticity has been elusive for a long time. However, it has been recently reported that this interaction can be modulated by phosphorylation events. For example, the interaction between DBC1 and SIRT1 increases following

DNA damage and oxidative stress (Yuan et al. 2012). This is due to the phosphorylation of DBC1 at Thr^{454} by the ATM (ataxia telangiectasia-mutated) and ATR (ataxia telangiectasia and Rad3-related) kinases, which create a second binding site for SIRT1 (Yuan et al. 2012; Zannini et al. 2012). In contrast, it has been found that the activation of the AMP-activated protein kinase (AMPK) pathway leads to the dissociation of DBC1-SIRT1 complexes (Nin et al. 2012), even though the underlying mechanism is not fully understood.

Almost simultaneous to the identification of DBC1 as a negative regulator of SIRT1, Kim and colleagues identified the active regulator of SIRT1 (AROS) as an activator of SIRT1 activity (Kim et al. 2007). The interaction of AROS with SIRT1, presumably in its catalytic domain, enhances SIRT1 activity by 2-fold (Kim et al. 2007). However, the exact nature of the action of AROS on SIRT1 and its physiological relevance has been barely explored, still constituting promising area for research.

4.2.3 SIRT1 Functions

While initially described as a transcriptional silencing enzyme, the actions of SIRT1 have unfolded as a rich universe that expands far beyond histone modifications. In fact, a very large number of non-histone protein targets have been identified. There are a number of reviews that extensively recapitulate SIRT1 targets, and we kindly refer the reader to them for further information (Canto and Auwerx 2012; Houtkooper et al. 2012).

In general, the physiological activation of SIRT1 orchestrates cellular and organismal adaptations aimed to favor survival in situations of nutrient scarcity. This way, SIRT1 activation potentiates the extraction of energy from non-carbohydrate sources, mostly through mitochondrial respiration. Given the dual localization of SIRT1, either in the cytoplasm and/or the nucleus, it is not surprising that SIRT1 targets have also been identified in both compartments (Canto and Auwerx 2012). The cytosolic targets of SIRT1 generally potentiate short-term adaptations, while the nuclear ones are transcriptional regulators by which SIRT1 controls mitochondrial and fatty acid oxidation gene expression to allow chronic adaptations (Canto and Auwerx 2012). The metabolic adaptations prompted by SIRT1 do not only impact directly on metabolic homeostasis, but also indirectly on cellular proliferation, inflammation and survival (Canto and Auwerx 2012; Houtkooper et al. 2012). Therefore, it is not surprising that SIRT1 activation has not only been linked to beneficial effects on metabolic health, but also on cancer and cognitive function, among others.

In the next sections we will analyze SIRT1 functions based on the evidences gathered on animal models, so as to favor a physiological integrative point of view (Table 4.1 and 4.2).

Table 4.1 Prominent energy metabolism phenotypes observed in SIRT1 gain-of-function models through genetic mechanisms

Targeted tissue	Genetic strategy	Prominent phenotypes observed	References
Whole body	Overexpression (moderate)	Protection against dietary and age-related metabolic damage	Pfluger et al. (2008)
			Banks et al. (2008)
		Similar lifespan as WT mice	Herranz et al. (2010)
	Overexpression (moderate)	Calorie-restriction like behavior	Bordone et al. (2007)
	Overexpression (moderate)	Higher susceptibility to atherosclerotic lesions when fed a atherogenic diet	Quiang et al. (2012)
	Overexpression (high)	Higher muscle mitochondrial content	Price et al. (2012)
Liver	Overexpression (adenoviral delivery)	Positive regulation of hepatic glucose production and inhibition of lipid anabolism	Rodgers et al. (2007)
	Overexpression (adenoviral delivery)	Attenuation of hepatic glucose prediction and insulin resistance in ob/ob mice	Wang et al. (2010)
Muscle	Overexpression	Similar aspect, insulin sensitivity and adaptation to calorie restriction as in wild-type mice	White et al. (2013)
	MCK-Cre		
Adipose tissue	Overexpression	Prevention against age-induced deterioration of insulin sensitivity and ectopic lipid distribution. Reduction of whole body fat mass and enhanced locomotor activity	Xu et al. (2013)
	Ap2-Cre		
Pancreas	Overexpression	Enhanced glucose-induced insulin secretion	Moynihan et al (2005)
	SIRT1 insertion under the human insulin promoter		
Brain	Whole brain overexpression	Enhanced foraging behavior upon calorie restriction	Satoh et al. (2010) and (2013)
	SIRT1 insertion under the mouse PrP promoter	Lifespan exetension	

Table 4.2 SIRT1 loss-of-function models through genetic mechanisms

Targeted tissue	Genetic strategy	Prominent phenotypes observed	References
Whole body	Knock-out	High embryonic lethality Numerous developmental defects	McBurney et al. (2003)
	Knock-out	Numerous developmental defects Infrequent postnatal survival	Cheng et al. (2003)
	Knock-out (outbred stocks)	Metabolic inefficiency and defective adaptation to nutrient stress	Boily et al. (2008)
	Knock-out (Adulthood deletion)	Defective mitochondrial function	Price et al. (2012)
	Hemizygosis	Hepatic steatosis	Purushotham et al. (2010) Xu et al. (2012)
Liver	Deletion Alb-Cre; SIRT1 fl/fl (exon 4)	Protection from physiological decline when fed a high-fat diet	Chen et al. (2008)
	Deletion Alb-Cre; SIRT1 fl/fl (exon 4)	Higher susceptibility for the development of hepatosteatosis	Purushotham et al. (2009)
	Deletion Alb-Cre; SIRT1 fl/fl (exon 5–6)	Hepatic steatosis even on chow diet and chronic hyperglycemia	Wang et al. (2010) and (2011)
Muscle	Deletion MCK-Cre; SIRT1 fl/fl (exon 4)	Normal adaptation to exercise, but not to calorie restriction	Schenk et al. (2011) Philp et al. (2011)
	Deletion MLC1f-Cre; SIRT1 fl/fl (exon 4)	Defective mitochondrial function No synergism between resveratrol and exercise on mitochondrial biogenesis	Menzies et al. (2013)
Adipose tissue	Deletion FABP4-Cre; SIRT1 fl/fl (exon 4)	Increased inflammation of white adipose tissue, increased adiposity and higher susceptibility to obesity and insulin resistance	Gillum et al. (2011) Chalkiadaki et al. (2012)
Pancreas	Adulthood deletion Pdx1-ERCre; SIRT1 fl/fl (exon 4)	Disrupted glucose-stimulated insulin secretion	Moynihan et al. (2005)
Brain	Whole brain deletion Nestin-Cre; SIRT1 fl/fl (exon 4)	Altered behavioral response to caloric restriction Defective control of pituitary hormones Increased glucose intolerance with aging	Cohen et al. (2009)

(continued)

Table 4.2 (continued)

Targeted tissue	Genetic strategy	Prominent phenotypes observed	References
	Deletion in AgRP neurons	Decreased food intake and body weight	Dietrich et al. (2010)
	Agrp-Cre; SIRT1 fl/fl (exon 4)		
	Deletion in POMC neurons	Hypersensitivity to HFD-induced obesity	Ramadori et al. (2010)
	POMC-Cre; SIRT1 fl/fl (exon 4)		

4.3 What Animal Models Have Taught Us

4.3.1 SIRT1 and Whole Body Metabolism

SIRT1 orthologs in lower eukaryotes have been proposed to be determinants of lifespan (Canto and Auwerx 2009). Despite the abstract notion of what lifespan determination really means and the controversial results on whether SIRT1 overexpression truly enhances longevity in lower eukaryotes (Burnett et al. 2011), it seems clear that SIRT1 activation does not enhance lifespan in mice under normal food regimes (Baur et al. 2006; Herranz et al. 2010; Pearson et al. 2008). However, SIRT1 transgenic mice are protected against the metabolic damage induced by high-fat diets (Banks et al. 2008; Pfluger et al. 2008), and SIRT1 activation prevents the curving of lifespan induced by high-fat feeding (Baur et al. 2006).

The first SIRT1 gain-of function model reported displayed several features resembling calorie restriction: they were leaner, metabolically more active, and had increased glucose tolerance (Bordone et al. 2007). Two additional SIRT1 transgenic lines were later generated, both of them concluded that mild overexpression of SIRT1 prevented against high-fat diet induced hyperglycemia, insulin resitance and fatty liver, despite no significant differences in body weight (Banks et al. 2008; Pfluger et al. 2008). Posterior efforts have also certified that lines with a higher SIRT1 overexpression have enhanced mitochondrial content and display greater mitochondrial function (Price et al. 2012). While this feature has not been deeply analyzed in the previous lines, it could provide an interesting mechanism to explain the phenotypes observed. The major caveat of this approach is that higher SIRT1 levels do not necessarily have to correlate with SIRT1 activity. This has been demonstrated recently in aging models, where reduced NAD^+ availability compromises SIRT1 activity, despite higher SIRT1 content (Braidy et al. 2011).

A number of compounds have been used as SIRT1 activating compounds (STACS), even though their specificity has been long debated (Canto and Auwerx 2012). A recent publication elegantly demonstrates that STACS directly influence

the activity of SIRT1 through a specific residue, Glu^{230} for human SIRT1 (Hubbard et al. 2013). This mechanism for direct activation of SIRT1, however, would only affect a subset of substrates with specific structural requirements (Hubbard et al. 2013) and would not explain the effects of STACs on lower eukaryotes, where the human Glu^{230} residue is not conserved. While further in vivo consolidation of these observations is required, they provide an interesting conciliatory explanation on why STACs have been reported to trigger SIRT1 activation through both direct and indirect mechanism. Among all STACS, resveratrol has probably been the one receiving more attention. Indeed, mice fed with resveratrol show a number of features in common with the SIRT1 transgenic mice. Most notably, they are protected against high-fat diet induced metabolic damage and display enhanced mitochondrial function (Baur et al. 2006; Lagouge et al. 2006). The effects, however, were more marked than those observed on the transgenic, including prevention against high-fat diet induced obesity (Lagouge et al. 2006). A possible explanation for this relies on the ability of resveratrol to activate also other pathways (Canto and Auwerx 2012; Park et al. 2012). In fact, many studies suggest that the activation of SIRT1 by resveratrol, at least at the doses most commonly used in animal studies, could be an indirect consequence of AMPK activation (Canto et al. 2010; Um et al. 2010), which leads to increased NAD^+ levels (Canto et al. 2009) and promotes Nampt expression (Canto et al. 2009; Fulco et al. 2008). A more recent screening for other possible small molecular SIRT1 agonists led to the identification of a second batch of compounds, among which the best characterized is SRT1720 (Milne et al. 2007). Similar to resveratrol, the treatment of mice with SRT1720 ameliorated the diabetic phenotype of obese mice (Milne et al. 2007) and prevented high-fat diet induced insulin resistance (Feige et al. 2008). Furthermore, SRT1720-fed mice displayed enhanced longevity (Minor et al. 2011). All this correlated with enhanced SIRT1 activity in the tissues of SRT1720 treated mice (Feige et al. 2008; Minor et al. 2011). However, similar to resveratrol, several questions have been raised regarding the specificity of SRT1720 on SIRT1 (Pacholec et al. 2010). In addition to that, the in vitro assays indicated that SRT1720 was a more potent activator of SIRT1 than resveratrol (Milne et al. 2007). This, however was not clearly translated in vivo, indicating either poor bioavailability or that the actions of SRT1720 in vivo on SIRT1 largely rely on an indirect activation.

There are also a number of transgenic models that indirectly affecting SIRT1 activity. For example, mice lacking DBC1, the inhibitory endogenous interacting protein, display many features similar to SIRT1 transgenic mice, such as protection against high-fat diet-induced hepatic steatosis and inflammation (Escande et al. 2010). However, DBC1 deficient mice still developed diabetes under high-fat feeding (Escande et al. 2010), indicating that SIRT1 function is not solely controlled by DBC1. Another strategy aimed to boost SIRT1 activity has been the knockout of alternative cellular NAD^+ consumers. PARP-1 is considered to be a major NAD^+ consumer in the cell, and its activity can deplete intracellular NAD^+ by 70 % (Bai and Canto 2012). The deletion of PARP-1 is enough to increase basal NAD^+ availavility and SIRT1 activity (Bai et al. 2011b). PARP-1 deficient mice also show a number of phenotypes resembling SIRT1 transgenesis, such as enhanced energy expenditure and protection against high-fat diet-induced diabetes (Bai et al. 2011b).

A similar case could be made for CD38, another alternative cellular NAD^+ consumer (Aksoy et al. 2006). Mice defective for CD38 have increased SIRT1 activity in most tissues, probably due to increased NAD^+ availability, and this confers protection against many of the metabolic complications induced by high caloric diets (Barbosa et al. 2007). However, similar to the PARP-1 model, this protective phenotype is markedly more pronounced than that observed in SIRT1 transgenic. This could be mainly due to two reasons: first, that these alternative NAD^+ consumers affect many other processes other than SIRT1 activity and, second, that SIRT1 transgenesis might not reach similar SIRT1 activity levels as in the PARP-1 or CD38 models, due to NAD^+ availability limitations.

A final strategy to enhance NAD^+ availability consists in dosing with NAD^+ precursors. Intraperitoneal injections of NMN for 7 days were enough to ameliorate age and high-fat diet-induced glucose intolerance, coupled to higher SIRT1 activity (Yoshino et al. 2011). Similarly, dietary supplementation of mice with NR was enough to increase SIRT1 activity in diverse tissues (Canto et al. 2012). This was coupled to a marked enhancement of insulin sensitivity, in both chow and high-fat fed animals, as well as increased oxidative capacity and global energy expenditure (Canto et al. 2012). Altogether, these strategies illustrate how a number of strategies aimed to enhance SIRT1 activity converge into higher glucose tolerance and increased capacity for oxidative metabolism.

All the above observations raise an obvious interest in understanding how the deletion of SIRT1 could impact global metabolic homeostasis. This has proven not to be an easy task. The whole-body deletion of SIRT1 leads to elevated prenatal death rates in inbred mice (Cheng et al. 2003; McBurney et al. 2003). The very few pups that were born displayed marked cardiac and neurological problems, leading to death very early in the postnatal period (Cheng et al. 2003; McBurney et al. 2003). In order to bypass this situation, SIRT1 deletion was performed in outbred mice. These mice were viable and displayed a marked metabolic inefficiency, which impaired their ability to metabolically adapt to calorie restriction (Boily et al. 2008). Outbred mouse stocks, however, are not ideal for metabolic studies due to their heterogeneity. Recently, an inducible model has been developed in order to genetically ablate SIRT1 exclusively in adulthood (Price et al. 2012). The induction of SIRT1 knockout in adult mice did not result in any overt phenotype. Similarly, there were no obvious differences between SIRT1 KO and WT mice on most metabolic parameters, although weight gain was slightly lower in the knockouts when placed on a high-caloric diet (Price et al. 2012). Another model worth discussing is the SIRT1 heterozygous mice. The heterozygous SIRT1-KO ($SIRT1^{+/-}$) mice were normal in body weight, fat content, and lean body mass relative to their WT littermates (Purushotham et al. 2012a). Similarly, they did not display any remarkable difference in a series of histologic and gene expression analyses. However, when placed in high fat diets these mice were more prone to develop hepatic steatosis and metabolic damage (Purushotham et al. 2012a; Xu et al. 2010). Overall, these models provide conclusive evidence that SIRT1 deletion leads to inefficient metabolism. While this does not dramatically manifest into an overt phenotype when fed regular

diets, it renders them more prone to metabolic complications upon dietary challenges.

In order to gain knowledge on the role of SIRT1 in particular tissues and how this could contribute to metabolic impairment, several tissue-specific SIRT1 deficient mouse models have been generated. In the sections below we will discuss the hypothesized roles for SIRT1 in different tissues and how the different tissue-specific models have validated or not these possible actions.

4.3.2 SIRT1 Functions in the Liver

4.3.2.1 SIRT1 and Hepatic Glucose Production

Liver is the major gluconeogenic organ in mammalian organisms. Formation of glucose from noncarbohydrate sources such as lactate, glycerol or amino acids is called gluconeogenesis. Initial hypothesis suggested that SIRT1 could potentiate gluconeogenesis by directly deacetylating and enhancing the transcriptional activity of PGC-1α or FoxO1, which are considered key positive controllers of the gluconeogenic transcriptional program (Brunet et al. 2004; Erion et al. 2009; Frescas et al. 2005; Rodgers et al. 2005; Rodgers and Puigserver 2007). Experiments using tail-vein injection of adenoviruses demonstrated that the activation of PGC1α by SIRT1 overexpression induced gluconeogenic genes expression, potentiated glucose production and repressed glycolytic genes mRNA levels (Rodgers and Puigserver 2007). Conversely, shRNA mediated inhibition of SIRT1 in liver was enough to decrease glycemia and improve both glucose and pyruvate tolerance (Rodgers and Puigserver 2007). Moreover, gluconeogenic capacity was almost completely defective upon SIRT1 reduction. Accordingly, the injection of antisense oligonucleotides against SIRT1 induces an increase of FoxO1 and PGC-1α acetylation, leading to decreased glycemia and hepatic glucose production through the down regulation of gluconeogenic gene expression (Erion et al. 2009).

Given the above observations, it would be expected that the genetic ablation of SIRT1 in the liver would lead to compromised gluconeogenic function and decreased basal glycemia. To date, a number of independent liver-specific SIRT1 knockout mice have been generated. Surprisingly, none of them displayed reduced basal glycemia (Chen et al. 2008; Purushotham et al. 2009; Wang et al. 2011; Wang et al. 2010). In fact, one of them had a tendency to have higher glucose levels even on chow diet and displayed enhanced glucose production upon fasting (Wang et al. 2011). Conversely, the initial work using adenoviral vectors suggested that higher SIRT1 levels in the liver should promote hyperglycemia (Rodgers and Puigserver 2007). These results, however, have been challenged recently by findings indicating that liver overexpression of SIRT1 actually attenuates hyperglycemia in insulin resistant mouse models (Li et al. 2011). In line with the latter observation, mice with a whole body overexpression of SIRT1 do not show signs of hyperglycemia and are protected against glucose intolerance (Banks et al. 2008; Pfluger et al. 2008).

The above results indicate that there are certain discrepancies between the results obtained in the initial experiments using adenoviral vectors and the transgenic mouse models. Therefore, either the adenoviral shRNA delivery is causing additional effects or SIRT1 transgenic models suffer from some sort of compensation. To shed some light onto these discrepancies it might be worth also considering a number of molecular and physiological observations. First of all SIRT1 activation in the liver does not seem to take place in the initial phases of gluconeogenesis (Liu et al. 2008). Hepatic glucose production is controlled during the early fasting stages by the cAMP response element binding protein (CREB) regulated transcription coactivator 2 (CRTC2). Detailed time-course analyses revealed that SIRT1 activation occurs during later phases, leading to the deacetylation and degradation of CRTC2, which attenuates the gluconeogenic rate (Liu et al. 2008). Similarly pharmacological activation of SIRT1 by resveratrol does not lead to enhanced hepatic glucose production (Baur et al. 2006; Lagouge et al. 2006), despite leading to a marked deacetylation of SIRT1 targets (Baur et al. 2006). Instead, it normalizes glycemia in insulin resistant mice (Lagouge et al. 2006). Altogether, these observations indicate that SIRT1 activation is not per se linked to enhanced hepatic glucose production, even if in some scenarios this might be the case. Rather, SIRT1 activation generally leads to an attenuation of the gluconeogenic rate, which constitutes a valuable therapeutic strategy in situations of insulin resistance and type 2 diabetes.

4.3.2.2 SIRT1 and Hepatic Lipid Metabolism

As described next, most studies to date agree that SIRT1 activation enhances oxidative metabolism in liver. The knock-down or genetic ablation of SIRT1 in liver induces hepatic lipid accumulation by upregulating the expression of lipogenic genes and reducing fatty acid oxidation capacity (Purushotham et al. 2009; Rodgers and Puigserver 2007; Wang et al. 2010; Xu et al. 2010). This renders SIRT1 deficient livers more sensitive to high-fat diet-induced hepatic steatosis (Purushotham et al. 2009). Conversely, SIRT1 overexpressing mice are protected against hepatic lipid accumulation and inflammation when fed a western diet (Li et al. 2011). Strikingly, one of the liver-specific knock-out models displayed the unusual feature of being protected against hepatic steatosis (Chen et al. 2008). The reasons for such a discordant observation in this particular model are not yet clear

So, how does SIRT1 enhance oxidative metabolism and prevent hepatic lipid accumulation? Rodgers et al., observed an increase in fatty acid oxidation genes and of cholesterol transport and the decrease in lipogenic gene expression induced by SIRT1 was dependent of PGC1α (Rodgers and Puigserver 2007). This is not surprising, as PGC-1α is a key downstream deacetylation target of SIRT1 in the regulation of mitochondrial and fatty acid oxidation gene expression. Complementarily, Purushotham et al. suggested that SIRT1 positively controls fatty acid oxidation though peroxisome proliferator-activated receptor α (PPARα) activation (Purushotham et al. 2009). The nuclear receptor PPARα regulates lipid metabolism, and, more particularly, gene expression implicated in β-oxidation. In SIRT1 deficient

livers, no activation of PPARα target genes expression in presence of PPARα synthethic ligands was observed (Purushotham et al. 2009). Mechanistically, the authors elegantly demonstrated that SIRT1 binds to the ligand binding domain (LBD) and DNA binding domain (DBD) of PPARα. Consistently, SIRT1 was found to be present on the promoter of PPARα-target genes (Purushotham et al. 2009). In turn, SIRT1 allows proper deacetylation of PGC-1α, which then can coactivate PPARα. In the absence of SIRT1, PGC-1α remains associated in a constitutively hyperacetylated state, which dampens PGC-1α coativating activity (Rodgers et al. 2005) and, hence, blunts PPARα transcriptional activation.

SIRT1 inhibits lipogenic gene expression, most likely by acting as a negative regulator of Sterol Regulatory Element Binding Protein (SREBP)-1c (Ponugoti et al. 2010; Rodgers and Puigserver 2007; Walker et al. 2010). SREBP-1c is a transcription factor activated in situations of nutrient abundance. It promotes the expression of lipogenic and cholesterogenic genes in order to facilitate fat storage. Walker et al. have demonstrated that deacetylation of SREBP-1c by SIRT1 makes the protein prone to ubiquitin-mediated degradation (Walker et al. 2010). Hence, SIRT1 activation leads to decreased SREBP-1c protein levels. This is manifested in a decreased abundance of SREBP-1c on the promoter of lipogenic target genes upon SIRT1 activation (Ponugoti et al. 2010; Walker et al. 2010).

4.3.2.3 SIRT1 and Cholesterol Metabolism

Several observations, such as decreased expression of genes involved in cholesterol transport in SIRT1 liver-specific KO mice or the reduction of blood cholesterol levels in SIRT1 overexpressing mice, suggest that SIRT1 could also modulate cholesterol metabolism (Bordone et al. 2007; Rodgers and Puigserver 2007). Indeed, SIRT1 have been shown to modulate cholesterol metabolism though a positive control of Liver X Receptors (LRX), LXRα and LXRβ. To do so, SIRT1 deacetylates LXRα on Lys^{432} and LXRβ on Lys^{433}, promoting their activation (Li et al. 2007). In mouse embryonic fibroblasts (MEFs) from SIRT1 knockout mice, LXR target genes expression is decreased (Li et al. 2007). Similarly, LXR targets are not fully activated in SIRT1 liver-specific knockout mice mice fed with high-caloric diet, a condition where LXRs are highly active (Chen et al. 2008). Upon ligand binding, LXRs interact with SIRT1, which then mediates its deacetylation and activation. This deacetylation event, however, also makes LXRs more susceptible to ubiquitination and degradation (Li et al. 2007). The knock-down of SIRT1 in the liver also leads to decreased expression of CYP7A1, a key LXR target, even though whether this happens in a LXR dependent fashion is unclear (Rodgers and Puigserver 2007). It is worth mentioning that LXRs are also a potent inducer of lipid anabolism by increasing SREBP-1c activity (Kalaany and Mangelsdorf 2006). However, SIRT1 can deacetylate SREBP-1c and lead it to proteasomal degradatation (Walker et al. 2010). Therefore, SIRT1 activation might prompt the beneficial effects of LXR activity on cholesterol homeostasis while preventing the detrimental effects on lipid anabolism by deacetylating SREBP-1c. This scenario is perfectly aligned with the

data obtained in mouse models, where SIRT1 transgenesis improves cholesterol metabolism and prevents hepatic steatosis, while SIRT1 deletion in the liver favours lipid accumulation.

The bile acid sensing Farnesoid X-receptor (FXR) is another key player in the regulation of cholesterol and lipid metabolism that can be directly deacetylated on Lys^{157} and Lys^{217} by SIRT1 (Kemper et al. 2009). Down regulation of hepatic SIRT1 increases FXR acetylation, which inhibits its heterodimerization with RXRα (Kemper et al. 2009). Indeed, high FXR acetylation levels are observed in a mouse model of metabolic disease (Kemper et al. 2009). In line with these observations, SIRT1 deletion in the liver is enough to deregulate FXR transcriptional program and lead to the formation of cholesterol gallstones (Purushotham et al. 2012b). Activation of SIRT1 might, hence, become an attractive strategy to activate FXR and induce its target genes expression, which could contribute to the better cholesterol homeostasis.

4.3.3 SIRT1 Functions in Skeletal Muscle

Sketetal muscle is a key player in whole body metabolic homeostasis. A major feature of skeletal muscle is its plasticity. It can dramatically increase glucose uptake upon insulin stimulation or contraction and accounts for up to 80 % of total postpandrial glucose disposal (Hawley et al. 2006). Similarly it can regulate oxidative metabolism and mitochondrial biogenesis in response to a number of stimuli. For example, skeletal muscle switches from glucose to fatty acid utilization during fasting so to spare glucose for other tissues. Therefore, muscle exquisitely fine tunes fuel utilization to environmental cues.

Initial hints on the possible roles of SIRT1 in skeletal muscle were obtained when SIRT1 was identified as a negative myogenic regulator. Overexpression of SIRT1 impairs myotube formation (Fulco et al. 2003). Conversely, decreased SIRT1 triggers premature differentiation (Fulco et al. 2003). Mechanistically this effect could be explained because SIRT1 represses the muscle transcriptional regulator MyoD, which acts as a critical determinant of skeletal muscle differentiation (Fulco et al. 2003). SIRT1 is also a key mediator by which nutrient restriction impairs muscle differentiation. Upon glucose depletion, AMPK is activated. In turn, AMPK enhanced Nampt expression, which increased the NAD^+ availability SIRT1 activity (Fulco et al. 2008).

As mentioned above, muscle is a very plastic tissue, and has the ability to regulate oxidative metabolism and mitochondrial function in response to contraction or changes in nutrient availability. PGC-1α is a key master regulator of mitochondrial biogenesis in mammals. PGC-1α overexpression is enough to trigger mitochondrial biogenesis and oxidative metabolism in skeletal muscle (Lin et al. 2002). SIRT1 deacetylates PGC-1α in skeletal muscle during fasting and this deacetylation is required for PGC-1α-mediated induction of mitochondrial and fatty acid oxidation gene expression (Gerhart-Hines et al. 2007). Similarly, PGC-1α becomes deacetylated after an exercise

bout (Canto et al. 2009). AMPK seems to play a key role in triggering SIRT1 activity during energy stress. Indeed, various labs have demonstrated that the activation of SIRT1 in response to nutrient or energy deprivation depends on AMPK activation (Canto et al. 2010; Fulco et al. 2008). The link between AMPK and SIRT1 activities can be explained by, at least, two non-exclusive mechanisms. The first link proposed consists in the modulation of NAD^+ bioavailabily. The shift from glucose to fat oxidation induced by AMPK allows an increase in NAD^+ that is enough to activate SIRT1 in a relatively short time frame (Canto et al. 2009). Additionally, AMPK triggers Nampt expression, which helps maintaining a more protracted increase in NAD^+ (Canto et al. 2009; Fulco et al. 2008). As an alternative possibility, it has been recently proposed that AMPK could phosphorylate SIRT1 and abrogate this way its interaction with DBC1 (Nin et al. 2012). However, the phosphorylation of SIRT1 by AMPK has not been observed previously by other labs (Canto et al. 2009; Greer et al. 2007), and the residues reported are far from conserved or from matching AMPK target consensus sequences. At this level, it is also interesting to note that resveratrol, which is generally considered as a SIRT1 activator, also requires AMPK in vivo to activate SIRT1 and achieve its beneficial metabolic effects (Canto et al. 2010; Um et al. 2010). However, it has been recently noticed that this might only be the case when resveratrol is used at high doses (Price et al. 2012).

Considering the above data, one would expect that SIRT1 transgenesis would increase mitochondrial biogenesis in skeletal muscle. In line with this speculation, global SIRT1 transgenic mice have been shown to display higher mitochondrial content (Price et al. 2012). Conversely, deletion of SIRT1 in adulthood led to impaired mitochondrial function (Price et al. 2012). Similarly, muscle-specific deletion of SIRT1 leads to a slight impairment in mitochondrial function (Menzies et al. 2013), even though this was not clearly observed in another study in a similar mouse model (Philp et al. 2011). The reason for this discrepancy might derive from the different Cre lines used to ablate the *Sirt1* gene in muscle. In both mouse models, however, the deletion of SIRT1 in skeletal muscle, however, did not seem to have a major effect on metabolic homeostasis of mice on regular diet (Menzies et al. 2013; Schenk et al. 2011).

A second extrapolation derived from above mentioned the cell-based assays would be that mice deficient in SIRT1 would display impaired adaptation to nutrient and energy stress. In line with this, muscle-specific SIRT1 knock-out mice failed to become more insulin sensitive upon caloric restriction (CR) (Schenk et al. 2011). CR increases SIRT1 deacetylase activity in skeletal muscle, in parallel with enhanced insulin-stimulated PI3K signaling and glucose uptake (Schenk et al. 2011). These adaptations in skeletal muscle insulin action triggered by CR were completely abrogated in mice lacking SIRT1 deacetylase activity in muscle (Schenk et al. 2011). This could be mechanistically explained by various reasons. Firstly, SIRT1 was found to be required for the deacetylation and inactivation of the transcription factor Stat3 during CR, which resulted in decreased gene and protein expression of the p55α/p50α subunits of PI3K, thereby promoting more efficient PI3K signaling during insulin stimulation (Schenk et al. 2011). Alternatively, SIRT1 has also been demonstrated to be a repressor of PTP1b, a major tyrosine phospha-

tase for the insulin receptor and the insulin receptor substrate proteins, IRS1 and IRS2 (Sun et al. 2007). Therefore, it is likely that SIRT1 deficient muscles also display higher PTP1b activity, which would also prevent the enhancement of insulin signaling in response to calorie restriction. These results clearly support the notion that SIRT1 is key for metabolic adaptations triggered by nutrient deprivation in skeletal muscle. Given this premise, it was surprising to see that mice lacking SIRT1 in skeletal muscle could perfectly adapt and enhance oxidative metabolism in response to exercise training (Philp et al. 2011). However, exercise is a complex stimuli, affecting multiple pathways with likely redundant functions. Strikingly, PGC-1α was normally deacetylated in response to exercise, despite the lack of SIRT1 (Philp et al. 2011). To solve this paradox, it was proposed that muscle contraction decreases the interaction of PGC-1α with the acetyltransferase enzyme, GCN5 (Philp et al. 2011). This way, PGC-1α deacetylation upon exercise would not be a consequence of enhanced deacetylation but of decreased acetylation rates. Importantly, it was recently found that resveratrol had synergistic effects with exercise on muscle mitochondrial biogenesis (Menzies et al. 2013). While the effect of exercise on mitochondrial biogenesis was independent of SIRT1, the synergy of resveratrol and exercise was lost on SIRT1 muscle-specific knock-out mice (Menzies et al. 2013).

Altogether, these results indicate that SIRT1 activation can improve mitochondrial function in mice and influence insulin sensitivity. However, they also demonstrate that many physiological stimuli prompting enhanced oxidative metabolism in muscle may rely in additional complementary effectors to induce such adaptations.

4.3.4 SIRT1 Functions in Adipose Tissues

4.3.4.1 White Adipose Tissue

White adipose tissue (WAT) is an important tissue for the regulation of metabolic homeostasis. WAT is the major site for fat storage in mammalian organisms. Fat storages are dynamically regulated in WAT, and lipolytic or lipogenic processes can be activated in response to nutrients and hormones. In obesity and type 2 diabetes (T2D), circulating free fatty acid (FFA) levels are high, generally correlating with insulin resistance in both conditions. Importantly, the WAT also has critical actions as an endocrine tissue, by secreting hormones and cytokines, such as leptin, adiponectin or TNFα, that affect insulin sensitivity, inflammation and, therefore, have major consequences on metabolic homeostasis (Rosen and Spiegelman 2006).

One of the critical regulators of fat storage in WAT is the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), whose activity promotes adipocyte differentiation and lipid anabolism (Rosen and Spiegelman 2006). A possible role of SIRT1 in WAT homeostasis was evidenced when it was found that SIRT1 could act as a PPARγ repressor (Picard et al. 2004). During fasting SIRT1 associated

with PPARγ and promoted the binding of the corepressor NCoR1 (Picard et al. 2004). This favored fat mobilization instead of storage. A complementary study demonstrated that SIRT1 could repress PPARγ transcriptional activity on target lipogenic genes through a direct inhibitory-deacetylation (Qiang et al. 2012). The ablation of SIRT1 in adipose tissue promotes body weight gain, mostly due to an increase in fat mass. The size of adipocyte was bigger than in control mice, even on chow diet (Chalkiadaki and Guarente 2012). Altogether, this renders the adipocyte-specific SIRT1 KO mice prone to develop insulin resistance. Importantly, it has been described that obesity results in decreased SIRT1 in rodent and human adipose tissues (Chalkiadaki and Guarente 2012; Costa Cdos et al. 2010; Gillum et al. 2011). The reason for this decrease might rely on the fact that obesity triggers the cleavage of SIRT1 via a caspase 1 dependent mechanism (Chalkiadaki and Guarente 2012). This cleavage renders SIRT1 prone to degradation, therefore, decreasing global SIRT1 activity.

Two models of SIRT1 overexpression in mice have shown to be protected against HFD-induced insulin resistance (Banks et al. 2008; Pfluger et al. 2008). However, none of them observed decreased fat mass. Interestingly, a third model of global SIRT1 overexpression displayed a decrease in fat mass (Bordone et al. 2007), in line with the observations derived from the use of pharmacological activators of SIRT1, such as resveratrol or SRT1720 (Feige et al. 2008; Lagouge et al. 2006).

Adipose tissue inflammation is believed to be a hallmark of whole body insulin resistance. All animal models examined to date suggest a protective role of SIRT1 in adipose tissue inflammation (Chalkiadaki and Guarente 2012; Gillum et al. 2011). SIRT1 represses the expression of genes implicated in inflammation in adipocytes (Yoshizaki et al. 2009). Adipose-specific SIRT1 knockout mice displayed an increase of macrophage recruitment to adipose tissue (Gillum et al. 2011). In line with these studies, overexpression of SIRT1 in mice (SirBACO mice) prevents against adipose tissue macrophage accumulation caused by HFD (Gillum et al. 2011). Importantly, it has also been shown in humans how SIRT1 mRNA levels are inversely related to adipose tissue macrophage infiltration in human sub-cutaneous fat (Gillum et al. 2011).

4.3.4.2 Brow Adipose Tissue

Brown adipose tissue (BAT) has a remarkable abundance of mitochondria and contributes positively to energy expenditure, at least in mice. BAT is characterized by the expression of the mitochondrial uncoupling protein UCP1, which allows dissipation of energy as heat for thermogenesis (Rosen and Spiegelman 2006). In response to adrenergic stimulation or cold exposure, white adipocytes can also obtain brown adipocyte-like characteristics (Orci et al. 2004; Puigserver et al. 1998; Rosen and Spiegelman 2006; Tiraby et al. 2003; Wu et al. 2012a). The binding of PGC1α to PPARγ promotes brown adipocyte-like features in white adipocytes though an up-regulation of brown-adipocyte specific genes, such as UCP1, and a down-regulation of white-adipocyte specific genes (Puigserver et al. 1998). Adipose

tissue specific SIRT1 knockout mice display both enhanced WAT and BAT mass, due to enhanced fat accumulation (Chalkiadaki and Guarente 2012). Given the ability of SIRT1 to increase PGC-1α activity and lipid oxidation, SIRT1 activation might prevent excessive accumulation of lipids in BAT by boosting fat consumption and enhancing thermogenic function. Studies on indirect SIRT1 activation models would support this hypothesis. First, deletion of PARP-1 in mice increases NAD^+ content and SIRT1 activity in BAT. PARP-$1^{-/-}$ mice can retain body temperature much better upon cold exposure, testifying for enhanced thermogenic function (Bai et al. 2011b). The BAT of these mice display higher mitochondrial content and a transcriptional up-regulation of genes implicated in mitochondrial respiration and fatty acid oxidation, as well as UCP1 (Bai et al. 2011b). Second, the enhancement of SIRT1 activity promoted by nicotinamide riboside also results in a better ability to maintain their body temperature during cold exposure (Canto et al. 2012). These two studies illustrate that activation of SIRT1 could benefit BAT function.

A recent study has demonstrated a role for SIRT1 in the “browning” of WAT. Overexpression of SIRT1 enhances a down-regulation of WAT specific genes in a white adipose depots and up-regulates BAT characteristics, while SIRT1 deletion has the opposite effect (Qiang et al. 2012). To do so, SIRT1 deacetylates PPARγ by SIRT1. The deacetylation of PPARγ favors the recruitment of PRDM16, a transcriptional coregulator that drives the BAT adipogenic program (Seale et al. 2007). This way, mice overexpressing SIRT1 have a more potent induction of a BAT-like phenotype of the subcutaneous WAT upon cold exposure (Qiang et al. 2012).

Altogether, while we are just beginning to understand the influence of SIRT1 on adipose tissue homeostasis, it seems clear that SIRT1 promotes lipid mobilization and enhances BAT-like characteristics. Therefore, SIRT1 activation could protect against metabolic diseases by enhancing energy expenditure and favoring thermogenic function.

4.3.5 SIRT1 Functions in the Pancreas

Pancreatic β-cells play a central position in the regulation of glucose homeostasis by secreting insulin in response to elevated glucose levels. Initial data from transgenic models proposed that SIRT1 positively controls glucose stimulated insulin secretion (GSIS). The overexpression of SIRT1 specifically in pancreatic β-cells is enough to improve glucose tolerance and insulin secretion in response to glucose or KCl stimulation (Moynihan et al. 2005). In line with this model, GSIS is blunted in islets from SIRT1 knockout mice or in β-cells where SIRT1 has been knocked down by siRNAs (Bordone et al. 2006). Both studies converge proposing that SIRT1 improves GSIS though a negative control of the mitochondrial uncoupling protein UCP2. This would favor ATP production in response to higher glucose levels. Along this line, β-cell specific overexpression of SIRT1 suffices to prevent glucose intolerance upon high-fat feeding. The beneficial effects of SIRT1 on insulin secretion, however, are lost upon aging (Ramsey et al. 2008), to be completely lost at 18 -24

months of life. This was explained by a decrease of NAD^+ availability in aged tissues, which could potentially limit the activation of SIRT1. Supporting this hypothesis, increasing NAD^+ by NMN supplementation is enough to recover the benefits of β-cell SIRT1 overexpression in aged mice. Highlighting the relevance of NAD^+ availability for SIRT1 activity and pancreatic function, the Slow Wallerian Degeneration (Wld^S) spontaneous mutant mice, overexpressing a chimeric protein that contains the full length NMNAT1 protein, display enhanced NAD^+ availability and are protected against streptozotocin- and dietary-induced glucose intolerance in a SIRT1 dependent manner (Wu et al. 2011). Further supporting the beneficial effects of SIRT1 on pancreatic function, Wu et al. have shown that SIRT1 activation could be a promising strategy to prevent the deleterious effects of palmitate on insulin secretion and β-cell function (Wu et al. 2012b).

Pancreatic β-cell function can be also influenced by controlling β-cell mass. β-cell mass is determined by the balance between apoptotic, proliferative and neogenic processes. Pancreatic β-cell mass and β cell area are unchanged in β-cell specific SIRT1 overexpressing mice as well as in heterozygous and homozygous SIRT1 knockout mice (Moynihan et al. 2005) (Bordone et al. 2006). The lack of effect of SIRT1 on pancreatic β-cell mass, however, has been challenged recently by a few observations. First, the deletion of the *PARP-2* gene leads to a constitutive increase in SIRT1 expression in many tissues, including pancreas (Bai et al. 2011a). *PARP-2*$^{-/-}$ mice display marked glucose intolerance despite being more insulin sensitive (Bai et al. 2011a). Explaining this, GSIS is dramatically impaired in *PARP-2* deficient mice. Upon close examination β-cell mass was significantly reduced in *PARP-2*$^{-/-}$ mice, and failed to proliferate upon high-fat feeding, further magnifying HFD-induced glucose intolerance (Bai et al. 2011a). Mechanistically, it was proposed that higher SIRT1 activity led to a constitutive deacetylation and activation of FOXO1, a negative regulator of β-cell proliferation (Kitamura et al. 2002). In line with this, the expression of *pdx1*, a FOXO1 target and a critical regulator of β-cell proliferation and differentiation (Kitamura et al. 2002), was dramatically reduced in PARP-2 deficient mice, as well as that of PDX1 target genes (Bai et al. 2011a). In line with this, GLP-1 positively influences β-cell proliferation by disrupting the association between FoxO1 and SIRT1 (Bastien-Dionne et al. 2011). This promotes FoxO1 hyperacetylation and nuclear exclusion, therefore relieving the repression of pancreatic β-cell proliferation. This constitutes another example where SIRT1 is also regarded as a negative regulator of β-cell mass and where SIRT1 inhibition might actually be positive to enhance β-cell function.

While seemingly opposite, these dichotomy of effects might have an explanation. While chronic activation of SIRT1 could be deleterious, SIRT1 might be implicated in the protection and adaptation of β-cells to oxidative stress and inflammation. In line with this, SIRT1 have been shown to be protective against cytokine induced β-cell toxicity (Lee et al. 2009). Several mechanisms might contribute to do so. First, SIRT1 negatively controls the pro-inflammatory NF-kB signaling pathway though nuclear deacetylation of the subnit p65 which prevent the DNA binding and, consequently, the transcriptional activity of NF-kB (Lee et al. 2009; Yeung et al. 2004). Similarly, the control of FoxO1 activity by SIRT1 might critical

for the protection against oxidative stress in many cell types and tissues, including β-cells (Brunet et al. 2004; Hughes et al. 2011; Kitamura et al. 2005). This way, SIRT1 might have both protective and detrimental roles on β-cell function, depending on the timing and flexibility of its activation. Therefore, therapeutic approaches aimed to increase SIRT1 activity in β-cells should take into account this delicate balance.

4.3.6 SIRT1 and Food Intake Behaviour

The hypothalamus has a key role in the control of food intake, glucose homeostasis and energy balance. Hypothalamic neurons are able to detect changes in circulating hormones and nutrients and to respond to these changes by secreting several hunger/satiety hormones such as a-melanocyte-stimulating hormone (α-MSH) or agouti-related protein (AgRP). The proopiomelanocortin (POMC) expressing neurons and the AgRP expressing neurons in the hypothalamus constitute central nodes in the regulation of feeding behaviour and energy expenditure. POMC neurons negatively controls food intake principally through the release of the α-MSH, which is a ligand for melanocortin-4 receptor (MC4R) neurons. This way, α-MSH acts as an agonist of the MC4R to promote satiety. Conversely, the AgRP is an antagonist of MC4R and has the opposite effect, that is promoting food intake in response to fasting or caloric restriction (Gao and Horvath 2008).

SIRT1 is highly expressed in the arcuate nucleus (ARC), where we find the AgRP and POMC neurons, and in the vendromedial nuclei (VMN), where we find the MC4R neurons (Ramadori et al. 2008; Satoh et al. 2010). Intracerebroventicular (ICV) injection of Ex527, a SIRT1 inhibitor, or SIRT1 siRNAs leads to reduced food intake in rodents (Cakir et al. 2009; Dietrich et al. 2010). In fact, the selective deletion of SIRT1 in AgRP neurons is enough to decrease food intake due to impaired MC4R antagonism from this neurons (Dietrich et al. 2010). This result suggests that SIRT1 might be required to increase food intake in situations of nutrient deprivation. Strikingly, no effects on food intake where observed when SIRT1 gene was deleted in POMC neurons (Ramadori et al. 2010). Interestingly, mice lacking SIRT1 in POMC neurons were prone to obesity upon high-fat feeding. This effect, however, does not seem to stem from the control of food intake behaviour but, rather by indirectly decreasing the metabolic rate of peripheral tissues (Ramadori et al. 2010).

Whole brain overexpression of SIRT1 (BRASTO mice) promotes physical activity in response to CR (Satoh et al. 2010). This observation suggests SIRT1 could play a role in the control of pituitary hormones and metabolic peripherical effects in response to different dietetary. Again, this points out how SIRT1 might be activated upon nutrient scarcity and promotes adaptations aimed to enhance food foraging behavior. In apparent discrepancy, whole-body overexpression or deletion of SIRT1 does not seem to lead to major changes in food intake (Banks et al. 2008; Pfluger et al. 2008; Price et al. 2012). However, these mice undergo very significant metabolic changes, which

might feedback and interfere with the natural regulation and effects of SIRT1. Indeed, the regulation of endogenous SIRT1 activity in the hypothalamus is still nebulous. While it seems clear that SIRT1 expression and activity might be modulated by food intake, a couple of studies have led to apparently opposite conclusions. On one hand, it has been shown that fasting increases SIRT1 activity, inducing the deacetylation of FoxO. This, in turn, represses POMC neurons and enhances AgRP expression, therefore promoting food intake (Cakir et al. 2009). Another study, however, demonstrates that SIRT1 protein level decreases during fasting in hypothalamus (Sasaki et al. 2010). In this case, shockingly, the authors argue that SIRT1 inhibits FoxO1-dependent expression of AgRP and consequently leads to the cessation of feeding (Sasaki et al. 2010). While these two studies illustrate the relevant nature of the SIRT1-FoxO1 axis for the regulation of food intake, the intricacy of the system is still far from unraveled.

Other studies have tried to clarify SIRT1 actions during feeding cycles by analyzing a possible role of SIRT1 in circadian food intake behavior. Indeed, SIRT1 has been found to act as a key regulator of the core circadian clock molecular machinery (Asher et al. 2008; Nakahata et al. 2008). The regulation of NAD^+ bioavailability might constitute an attractive mechanism tying the circadian fluctuations of SIRT1 activity. Essentially, the expression levels of Nampt, the critical rate limiting enzyme in the mammalian NAD^+ salvaging pathway, display a robust diurnal oscillation, with a peak around the beginning of the dark period in mice, in line with the maximal peak for the circadian fluctuation of SIRT1 activity (Nakahata et al. 2009; Ramsey et al. 2009). SIRT1 negatively regulates CLOCK:BMAL-1 transcriptional activity, which is a key positive controller of Nampt expression (Nakahata et al. 2009; Ramsey et al. 2009). Hence, the activation of SIRT1 shuts down Nampt expression. This will likely promote a decrease in NAD^+ levels low enough to limit SIRT1. Once SIRT1 activity is low enough, CLOCK:BMAL-1 activity will be increased, and Nampt expression will be slowly recovered, reaching full circle. This way, SIRT1 constitutes an attractive mechanism by which metabolism can be tightly interconnected with circandian food intake behavior.

4.4 Conclusions and Future Perspectives

In this chapter we have provided an overview on the myriad of functions that SIRT1 exerts on metabolic regulation, based on the lessons learnt from transgenic mouse models (Fig. 4.1). Most data support the notion that SIRT1 contributes to an efficient adaptation to couple cellular and organismal metabolism to the nutritional and energy status. The activation of SIRT1 can be intimately linked to cellular metabolism by the rate-limitation imposed by NAD+ bioavailability. In general, most animal models demonstrate that SIRT1 activation is generally linked to a more efficient use of lipid energy sources and respiratory metabolism.

Despite the initial claims of SIRT1 as a “longevity” gene are debatable, SIRT1 can certainly impact on health and age-related decline in a more pleiotropic manner.

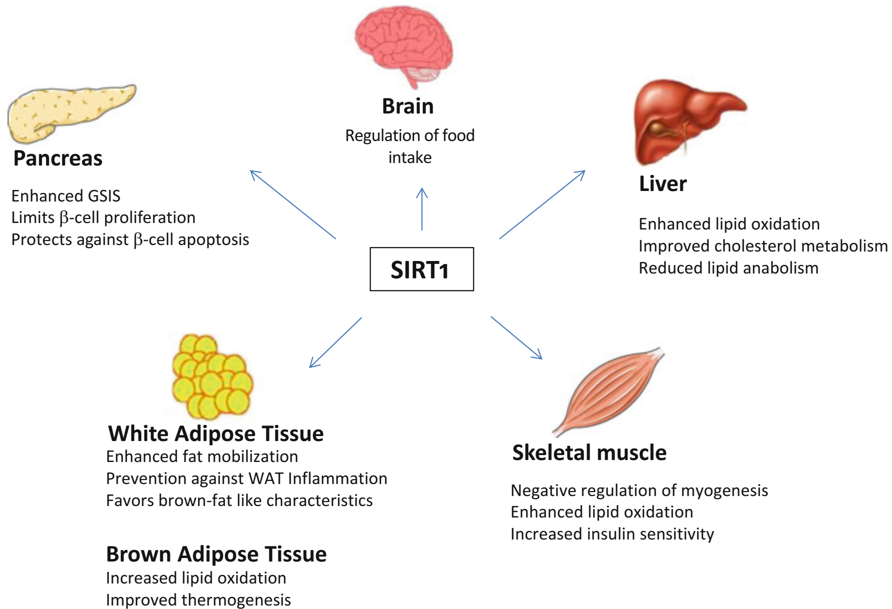

Fig. 4.1 Roles of SIRT1 in tissues implicated in metabolism regulation

In this sense, the complexity of SIRT1 physiology, largely conserved throughout evolution, involves an intricate network of downstream substrates, whose activation is only partially controlled via SIRT1. This can explain why initial findings on cultured cell models of SIRT1 overexpression or downregulation have sometimes not clearly mirrored into mouse physiology, where changes of SIRT1 activity might be more subtle or temporarily controlled. Another caveat on our understanding of SIRT1 comes from the knowledge inferred through the use of resveratrol or other so-called small molecule SIRT1 activators, whose specificity of action is far from clear.

Altogether, the collection of mouse models generated to date, have largely clarified the true role and limitations of SIRT1 actions. First, they established SIRT1 as a key gene for proper early organismal development. Tissue or temporatilly-controlled transgenic models all converge in the key role of SIRT1 for metabolic efficiency. Therefore, SIRT1 constitutes an extremely attractive target to improve oxidative metabolism and mitochondrial function, generally impaired in insulin resistant and aged population. However, a fine-tuned SIRT1 activity might be key to fully provide metabolic advantages. This is exemplified on the pancreatic regulation of SIRT1 activity, where constitutive activation of SIRT1 has been reported to be detrimental for global glucose tolerance. Similarly, enhanced SIRT1 activity in the heart can lead to cardiac failure by promoting dilated cardiomyopathy (Oka et al. 2011). Indeed, too much of a good thing might not always be that desirable and critical balances between metabolic benefits and side-effects might need to be balanced upon pharmacological approaches aimed to increase SIRT1 activity.

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Chapter 5
Deacetylation by SIRT3 Relieves Inhibition of Mitochondrial Protein Function

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5.1 Introduction

Lysine acetylation is an abundant post-translational modification (PTM) that plays an important role in protein regulation. Lysine acetylation was first identified as a modification on histone proteins that regulates gene expression (Allfrey et al. 1964; Isenberg 1979; Strahl and Allis 2000). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) catalyze lysine acetylation and deacetylation reactions, respectively, on histone proteins. Additionally, non-histone protein acetylation is recognized as an important modification that regulates various aspects of cellular function, including cell cycle, gene and protein expression, survival, and cell death (Choudhary et al. 2009; Yi et al. 2011; Verma et al. 2012).

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In addition to canonical HDACs, the sirtuins are a family of nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacylases that regulate the acylation status of several non-histone proteins (Hirschey et al. 2011b; Shimazu et al. 2010; Haigis and Sinclair 2010; Haigis and Yankner 2010; Hirschey et al. 2011a; Finkel et al. 2009). In mammals, the sirtuins have seven homologous proteins that occupy different subcellular compartments, including the cytoplasm (SIRT1, 2), the nucleus (SIRT1, 2, 3, 6, 7), and mitochondria (SIRT3, 4, 5). Of the mitochondrial sirtuins, SIRT3 appears to be the primary protein deacetylase (Hirschey et al. 2011a; Lombard et al. 2007; Haigis et al. 2006; Du et al. 2011; Peng et al. 2011b). SIRT4 has no detectable deacetylase activity (Verdin et al. 2004), but instead exhibits ADP-ribosyltransferase activity (Haigis et al. 2006). SIRT5 exhibits weak deacetylase activity *in vitro* (Verdin et al. 2004); however, it exhibits robust demalonylase, desuccinylase, and deglutarylase activities *in vitro* and *in vivo* (Du et al. 2011; Tan et al. 2014; Peng et al. 2011b), indicating this is likely the primary biological activity of SIRT5 (Hirschey 2011). Together, these findings suggest protein acetylation in the mitochondria is largely regulated by the sirtuin SIRT3.

Of all known non-histone proteins that are acetylated, metabolic proteins are over-represented, which positions SIRT3 to be a crucial regulator of energy production and overall metabolic homeostasis. In 2007, a large proteomic study in mouse liver identified lysine acetylation as an abundant post-translational modification in mitochondria (Kim et al. 2006). This study estimated that as many as 20 % of all mitochondrial proteins have at least one site of acetylation. More recent proteomic acetylation surveys showed that a significant portion of proteins in major metabolic pathways are acetylated, including the urea cycle, fatty acid oxidation, gluconeogenesis, and glycolysis (Choudhary et al. 2009; Wang et al. 2010; Zhao et al. 2010; Sol et al. 2012; Hebert et al. 2013; Rardin et al. 2013), which suggested that lysine acetylation may serve as a general regulatory mechanism for metabolism (Wang et al. 2010; Zhao et al. 2010). Based on these proteomic surveys, the number of mitochondrial proteins modified by reversible acetylation is now estimated to be

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between one-third and two-thirds of all mitochondrial proteins (Anderson and Hirschey 2012; Hebert et al. 2013), which suggests that 350–750 mitochondrial proteins have at least one site of acetylation (Pagliarini et al. 2008).

With an ever-increasing inventory of acetylated proteins, the current challenge is to understand the biological role of acetylation and its regulation by the sirtuins. SIRT3 has been reported to regulate several key mitochondrial pathways, including the tricarboxylic acid (TCA) cycle (Finley et al. 2011), fatty acid oxidation (Hirschey et al. 2010), ATP production (Ahn et al. 2008), and the urea cycle (Hallows et al. 2011). In each of these examples, protein acetylation consistently suppresses enzymatic activity, and reduces overall metabolic pathway activity. Thus, in this review, we put forward the hypothesis that acetylation is generally suppressive on enzymatic activity, and that SIRT3 serves to relieve the suppression, thereby increasing enzymatic function. We discuss the possible mechanisms of suppression, and the future studies needed to fully understand the biological role of acetylation and its regulation by the sirtuins.

5.2 Acetylation Abrogates Lysine Charge

To begin to explore this model, we considered how acetylation would influence lysine side chains and affect the overall protein. Abrogating the charge of lysine is one of the most dramatic changes induced by acetylation. The protonation state of a lysine is determined by the pK_a and the pH of the surrounding environment. The pK_a of a completely solvated, non-perturbed lysine is 10.4 and the mitochondrial pH is estimated to be 7.9 (Hsiao et al. 2011), which results in lysine residues in the mitochondria mostly in the protonated state (+1 charge) (Lund-Katz et al. 2000). However, determination of the lysine charge state in specific microenvironments is difficult. For example, within the hydrophobic interior of proteins, the pK_a of lysine residues can be depressed as low as 5.3 (Lund-Katz et al. 2000; Hsiao et al. 2011), which would result in lysines in the deprotonated state (0 charge). Generally, however, solvent-exposed lysines of mitochondrial proteins are protonated and positively charged (Fig. 5.1a). Covalent modification of the ε-amino group of exposed lysines by acetylation abrogates the positive charge on the amino acid (+1) and results in a net neutral charge (0) (Fig. 5.1b). As a result, lysine acetylation changes the hydrogen-bond donor potential of lysine to become a hydrogen-bond acceptor (Fig. 5.1c).

Thus, changing the protonation state (+1) of the ε-amino group of lysine residues could disrupt several biochemical processes, and is one possible mechanism by which lysine acetylation could suppress enzymatic activity. For example, many known roles of histones are contingent upon the lysine protonation state and the ability to covalently modify lysine residues. These include interactions between histone tails and chromatin remodeling enzymes (Shogren-Knaak et al. 2006; Lachner et al. 2001), electrostatic interactions between histone dimers (Placek and Gloss 2002), formation of histone tail secondary structure (Zhang et al. 1998), binding

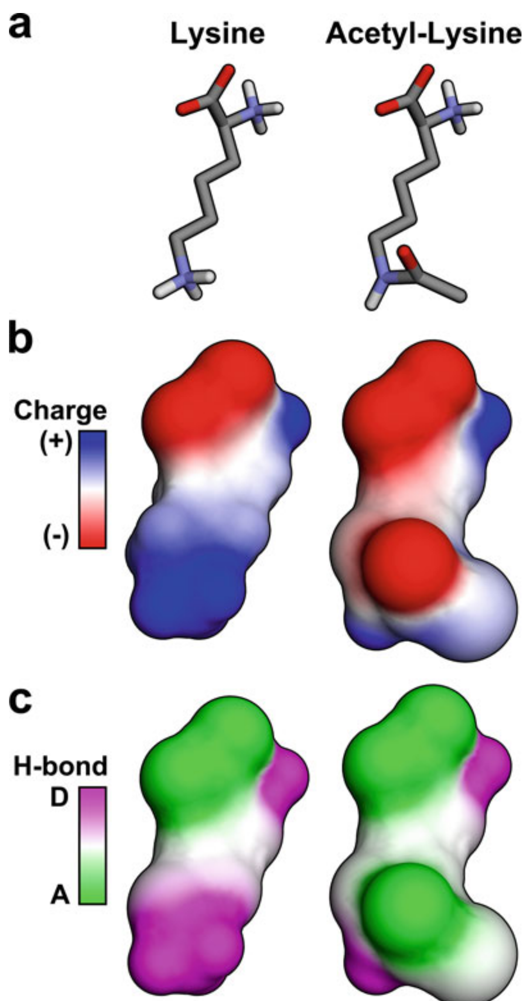

Fig. 5.1 Comparison between lysine and acetyl-lysine. Acetylation is an important post-translational modification that alters protein structure and function. A comparison between lysine (*left*) and acetyl-lysine (*right*) reveals differences in structure (**a**), surface charge (**b**), and hydrogen bond donor/acceptor capacity (**c**)

of DNA (Dou and Gorovsky 2000), and compaction of chromatin (Shogren-Knaak et al. 2006). Based on the well-established literature of histone acetylation, lysine charge and its neutralization by acetylation can influence several aspects of histone function.

Therefore, similar to lysine acetylation of histones, acetylation of non-histone proteins will also influence charge and could affect overall protein function. In particular, several properties of proteins could be influenced by acetylation, including structural features [protein-protein interaction, protein-subunit interaction (quaternary structure), protein structure (secondary or tertiary)], substrate/cofactor features [cofactor binding, substrate binding], or allosteric activation/inhibition. Furthermore, protein stability could be influenced by acetylation. Based on this idea, we put forward

the model that mitochondrial protein acetylation suppress enzymatic function by abrogating the lysine charge required for full enzymatic activity. In this model, SIRT3 restores the charge state, and thereby relieves the suppression of enzymatic activity.

5.3 Strategy

To explore this hypothesis, we considered the possible mechanisms by which lysine acetylation could suppress mitochondrial enzyme function. Specifically, we looked for example proteins known to be regulated by SIRT3, where acetylation could influence structural features, substrate/cofactor binding, allosteric regulation, and other possible mechanisms. We identified specific examples of lysines targeted by SIRT3 (see below), which when acetylated could reduce enzymatic activity by disrupting the features described above. We recognize that several substrates of SIRT3 have been identified that could fall into these categories, and therefore we chose examples of proteins which were most strongly supported by published data.

Next, to make a prediction of how acetylation regulates mitochondrial protein function, we first identified candidate sites of acetylation on mitochondrial proteins that could be regulated by SIRT3. We queried previously published proteomic studies, including several large-scale qualitative proteomics (Choudhary et al. 2009; Kim et al. 2006; Wang et al. 2010; Zhao et al. 2010; Schwer et al. 2009; Hornbeck et al. 2011), as well as recent quantitative proteomic studies describing SIRT3-targeted acetylation sites (Hebert et al. 2013; Sol et al. 2012; Rardin et al. 2013) in both human cell line and mouse model systems. A meta-analysis of these studies identified the landscape of acetylation and provided strong candidates for acetylated lysines targeted by SIRT3.

Based on this information, we used the rationale that reversible acetylation of these sites would also be important in the regulation of protein activity. To strengthen these predictions, when possible, we integrated published studies that validated proteomic findings and explored in detail the potential roles of acetylation on protein function. Finally, we prioritized candidate sites of acetylation based on conservation of lysines through evolution, which is a common strategy used to prioritize regulatory sites of phosphorylation (Covian and Balaban 2012). Indeed, some candidate sites of acetylation from murine proteomic studies were excluded, which identified sites that were not conserved in humans.

Based on this workflow, we identified several candidate sites of acetylation on key mitochondrial metabolic proteins that potentially regulate protein function. To gain insight into possible mechanisms of regulation, we modeled sites of acetylation on enzyme structures, or, in the absence of solved structures, we built homology models and/or looked to protein family (PFAM) domains and regions where acetylation was found. Together, this strategy allowed us to explore the proposed model that mitochondrial protein acetylation affects key features of proteins required for activity. We also discuss possible experiments required to validate these hypotheses.

5.4 Structural Regulation

5.4.1 MDH2

Malate Dehydrogenase 2 (MDH2) catalyzes the NAD^+/NADH-dependent interconversion of the substrates malate and oxaloacetate as a part of the TCA cycle within the mitochondrial matrix. Cytosolic MDH1 plays an important role in the malate-aspartate shuttle, and primarily reduces oxaloacetate to form malate. In contrast, mitochondrial MDH2 exists with other TCA cycle enzymes in a multi-enzyme complex that generates oxaloacetate, which is transferred to citrate synthase (Beeckmans and Kanarek 1981; Robinson et al. 1987).

The functional MDH2 enzyme is a homodimer comprised of 35 kDa protein subunits. In the crystal structure of MDH2 (Fig. 5.2a), the NAD^+ cofactor- and malate substrate-binding domains occupy the amino- and carboxyl-terminal halves of the protein, respectively (Fig. 5.2a). Single molecules of NAD^+ and malate substrate are overlaid (Fig. 5.2a, magenta and orange, respectively). The active site of MDH2 is in the cleft formed by the amino- and carboxy-terminal domains. Although each subunit functions independently in terms of catalysis with no evidence of cooperativity between catalytic sites, several studies indicate that dimeric structure is critical for activity (Breiter et al. 1994; Bleile et al. 1977; Wood et al. 1981). The dimer interface consists of interacting alpha helices that fit compactly together and amino acid residues critical for dimeric structural integrity have been identified, including D45 and S226 (Breiter et al. 1994).

Proteomic surveys have collectively identified over a dozen MDH2 acetylated lysine residues (Fig. 5.2a). In one study with human cell lines, the acetylation state of four of these, K185, K301, K307, and K314 (Fig. 5.2a, yellow) was increased by treatment of the cells with the sirtuin inhibitor nicotinamide (NAM), suggesting each was regulated by a sirtuin. Increase in acetylation of these sites was correlated with an increase in MDH2 activity in the direction of malate formation (Zhao et al. 2010). However, this study did not identify if any of these sites could be mediating the change in activity. In a second study, a combination of calorie restriction and deletion of SIRT3 in mice increased acetylation of MDH2 K239 (Fig. 5.2a, red) in liver by 83-fold (Hebert et al. 2013). Follow-up work in this study showed that acetylation, or acetylation mimetics, reduced enzymatic activity and malate formation in mammalian cell culture. While the discrepancy between the MDH2 activities in these two studies is difficult to reconcile, both studies measured enzymatic activity by NADH consumption, which is indicative of malate formation. Because mitochondrial MDH2 consumes malate which is directly passed to citrate synthase, measuring the activity of MDH2 in the context of the mitochondrion might be important to understand the effect of acetylation of mitochondrial MDH2 activity.

To clarify the sites of acetylation and possible effects of acetylation on MDH, we modeled all known sites of acetylation on the structure of MDH2 (Fig. 5.2a). Of the 26 lysines in MDH, our meta-analysis identified 23 lysines that have been reported as acetylated (Table 5.1). The original studies of MDH2 acetylation identified K185,

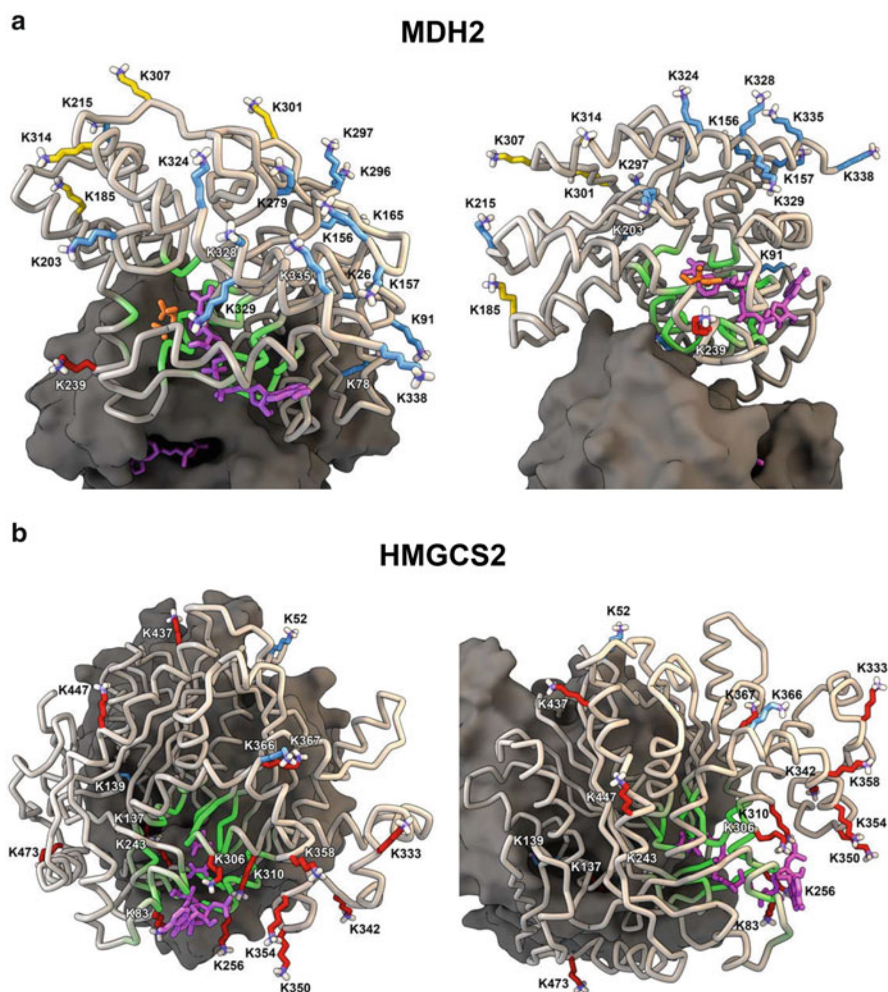

Fig. 5.2 Structural Regulation. Lysine residues known to be subject to post-translational acetylation are depicted as sticks with their specific identities indicated. Carbon atoms of the lysine residues known to be regulated or are candidates to be regulated by SIRT3 are colored *red*; previously described SIRT3-regulated sites lacking quantitative proteomic data evidence are colored *yellow*; while those that are acetylated but not candidates to be regulated are colored *blue*. (**a**) Two views of the crystal structure of dimeric human mitochondrial MDH (PDB ID: 2DFD) with one monomer represented as a protein backbone and the other as a solvent-accessible surface. The co-crystallized NAD^+ cofactor is colored *magenta* and the malate substrate colored in *orange*, with their binding sites highlighted in green; (**b**) Two views of the crystal structure of dimeric human mitochondrial HMGCS2 (PDB ID: 2WYA; (Shafqat et al. 2010)) with one monomer represented as a protein backbone and the other as a solvent-accessible surface. The co-crystallized HMG-CoA product is colored *magenta* with the active site highlighted in *green*

Table 5.1 Summary of acetylation sites from qualitative, quantitative, and targeted proteomic studies [1–14]

Protein name	AA ID	Acetylated	Candidate SIRT3 target	Candidate regulatory site	SIRT3KO/WT ratio
LCAD	K42	X	X	[4]	~20 [4]
	K43				
	K62				
	K66	X	X		
	K81	X	X	[14, 12]	2.24 [14], 1.83 [12]
	K92	X	X		
	K95				
	K151	MOUSE ONLY			
	K155				
	K156	1AA Shift			
	K165				
	K185				
	K189	X	X		
	K190				
	K201				
	K240	X	X		
	K244				
	K247				
	K250				
	K254	X	X		
	K279	X	X		
	K286				
	K315	X	X		
	K318	X	X		
	K322	X	X	[14]	1.92 [14]
	K333				
	K338				
	K358	X	X	[14]	1.29 [14]
	K369				
	K400				
	K419				
	K430				
SOD2	K25				
	K53	X	X	[6]	Not quantified
	K68	X	X	[10]	~2 [10]
	K75				
	K89	X	X	[6]	Not quantified
	K108	MOUSE ONLY		[13]	1.777 [13]
	K114	X	X	[14]	1.14 [14]
	K122	X	X	[9]	~2.2 [9]
	K130	X	X		
	K132	X	X		

(continued)

Table 5.1 (continued)

Protein name	AA ID	Acetylated	Candidate SIRT3 target	Candidate regulatory site	SIRT3KO/WT ratio
	K134				
	K154				
	K194				
	K202				
	K221	X	X	[13]	1.647 [13]
	K222	X	X		
GDH	K68				
	K84	X	X	[12]	
	K98				
	K110	X	X		
	K147				
	K162	X	X		
	K171				
	K183	X	X		
	K187				
	K191				
	K200				
	K211	X	X		
	K212				
	K258				
	K302				
	K326				
	K346	X	X	[12]	0.74 [12]
	K352	X	X		
	K363	X	X		
	K365	X	X		
	K373				
	K375				
	K386	X	X		
	K390	X	X		
	K397	X	X		
	K399	X	X		
	K415	X	X	[12]	0.75 [12]
	K444				
	K457	X	X		
	K477				
	K480	X	X	[13]	7.31 [13]
	K503	X	X	[12]	0.71 [12]
	K527	X	X	[12]	0.82 [12]
	K545	X	X		
	K548				

(continued)

Table 5.1 (continued)

Protein name	AA ID	Acetylated	Candidate SIRT3 target	Candidate regulatory site	SIRT3KO/WT ratio
SDHA	K20				
	K41				
	K46				
	K92				
	K128				
	K167	X	X		
	K179	X	X	[3, 13, 12, 14]	5.66 [14]; 2.918 [12]; 17.63 [13]
	K182	X	X		
	K250	X	X		
	K335	X	X		
	K361	X	X		
	K397				
	K418				
	K423	MOUSE ONLY			
	K480	X	X		
	K485	X	X	[3]	Not quantified
	K498	X	X	[3]	Not quantified
	K517	X	X		
	K538	X	X	[3]	Not quantified
	K541	HUMAN ONLY			
	K547	X	X		
	K550	X	X		
	K586				
	K598	X	X		
	K608	X	X		
	K615				
	K616				
	K624	X	X		
	K633	MOUSE ONLY			
	K636	X	X		
	K647	X	X		

(continued)

Table 5.1 (continued)

Protein name	AA ID	Acetylated	Candidate SIRT3 target	Candidate regulatory site	SIRT3KO/WT ratio
MDH2	K26	X	X		
	K45				
	K74	HUMAN ONLY			
	K78	X	X		
	K91	X	X		
	K105				
	K156	X	X		
	K157	X	X		
	K165	X	X		
	K185	X	X	[5]	Not quantified
	K203	X	X		
	K215	X	X		
	K239	X	X	[13, 14]	27.93 [14]; 65.799 [13]
	K241				
	K269				
	K279	HUMAN ONLY			
	K281	MOUSE ONLY			
	K296	X	X		
	K297	X	X		
	K301	X	X	[5]	Not quantified
	K307	X	X	[5]	Not quantified
	K314	X	X	[5]	Not quantified
	K324	X	X		
	K328	X	X		
	K329	X	X		
	K335	X	X		
	K338	X	X		

(continued)

Table 5.1 (continued)

Protein name	AA ID	Acetylated	Candidate SIRT3 target	Candidate regulatory site	SIRT3KO/WT ratio
HMGCS2	K9				
	K46	X	X	[14]	6.290 [14]
	K52	X			
	K75				
	K83	X	X	[14, 13]	3.31 [14]; 9.318 [13]
	K118	MOUSE ONLY		[14]	1.330 [14]
	K137	X	X	[14, 13]	2.010 [14]; 4.659 [13]
	K139	X			
	K142				
	K221				
	K243	X	X	[13, 14]	1.79 [14]; 2.43 [13]
	K256	X	X	[14]	2.59 [14]
	K275				
	K276				
	K282				
	K306	X	X	[13, 14]	1.35 [14]; 3.655 [13]
	K310	X	X	[8, 14, 13]	1.82 [14]; 3.864 [13]; 80 [8]
	K327	MOUSE ONLY		[13, 14]	3.35 [14]; 7.160 [13]
	K333	X	X	[14, 13]	3.96 [14]; 9.254 [13]
	K342	X	X	[13, 14]	1.78 [14]; 3.56 [13]
	K350	X	X	[8, 14]	1.45 [14]; 10 [8]
	K354	X	X	[8, 14]	1.05 [14]; 10 [8]
	K358	X	X	[13, 14]	2.010 [14]; 3.784 [13]
	K366	X			
	K367	X	X		
	K369				
	K427	MOUSE ONLY		[14]	1.090 [14]
	K437	X	X	[13, 14]	1.260 [14]; 1.74 [13]
	K447	X	X	[8, 13, 14]	1.92 [14]; 2.888 [13]; 90 [8]
	K453				
	K473	X	X	[8, 13, 14]	1.910 [14]; 9.254 [13]; 35 [8]
	K502				

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(continued)

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K301, K307, and K314, which are located within the c-terminal half of MDH2. Because these lysines are located at the surface of the protein, we predict they are unlikely to directly influence NAD^+, malate binding, or overall catalysis (Fig. 5.2a, yellow). However, the large number of acetylated lysines in close proximity could shift structure of the enzyme in a manner that is conveyed to the active site. Alternatively, these surface lysines may participate in orienting MDH2 within the TCA cycle multi-enzyme complex which could promote efficient functioning

of the enzyme. In this setting, acetylation of these lysines would affect MDH2 activity by disrupting the multi-enzyme complex. However, these lysines are not candidates for regulation by SIRT3.

The acetylation of liver MDH2 K239, and its regulation by SIRT3, was confirmed in a second line of *Sirt3*$^{-/-}$ mice that exhibited a 28-fold increase over wild-type control mice (Rardin et al. 2013). Therefore, K239 is a strong candidate for regulation by SIRT3, and may coordinate overall enzymatic activity. K239 is located in the alpha-helix that directly interacts in the MDH2 subunit-subunit interface where acetylation of K239 might disrupt MDH2 subunit interaction, which is critical for enzyme activity (Breiter et al. 1994). Together, we predict that acetylation of K239 suppresses MDH2 activity by disrupting the homodimer structural complex, which can be reversed by SIRT3-mediated deacetylation, as has been predicted previously (Hebert et al. 2013). Alternatively, because K239 lies along a small channel in the protein, acetylation of this site could influence substrate or product entry/exit from the enzyme complex (Fig. 5.2a). Further studies on complex stability and on oxidative enzymatic activity are required to test these hypotheses.

5.4.2 HMGCS2

3-Hydroxy-3-methylglutary coenzyme A (CoA) synthase 2 (HMGCS2) is a mitochondrial enzyme positioned to sense and mediate important responses to nutrient availability. It is responsible for the condensation of acetyl-CoA and acetoacetyl-CoA that yields 3-hydroxy-3-methylglutary CoA (HMG-CoA) (Hegardt 1999). This reaction represents the rate-limiting step in ketogenesis, which functions as an important glucose-sparing mechanism in response to fasting (Hegardt 1999). HMGCS2 functions as a homodimer (Shafqat et al. 2010) and is post-translationally regulated by phosphorylation, palmitoylation, and succinylation (Grimsrud et al. 2012; Kostiuk et al. 2010; Du et al. 2011). Recently, acetylation has emerged as an important regulatory mechanism for HMGCS2 (Shimazu et al. 2010).

Acetylation was first identified on HMGCS2 in a large-scale proteomic survey of hepatic mitochondrial proteins in mice (Kim et al. 2006). These early studies found three lysines (K83, K427, K437) were acetylated, with the acetylation of K427 increasing during fasting. Subsequent studies identified a functional role for acetylation in the regulation of HMGCS2, which is targeted for deacetylation by SIRT3 (Shimazu et al. 2010). In the fasted state, elevated expression of hepatic SIRT3 is correlated with a decrease in acetylation of HMGCS2, implicating HMGCS2 as a substrate of SIRT3 (Hirschey et al. 2010; Shimazu et al. 2010). This initial observation was validated *in vitro* by SIRT3-mediated deacetylation of HMGCS2 using recombinant protein (Shimazu et al. 2010). Further, the same study demonstrated an increase in HMGCS2 activity in the presence of SIRT3 via steady-state kinetic analysis, supporting the model that acetylation is inhibitory and SIRT3 effectively releases this inhibition (Shimazu et al. 2010). This study was the first to provide

evidence of a mechanism by which acetylation could regulate enzymatic activity. Molecular dynamic simulations of acetylated and deacetylated HMGCS2 identified conformational and electrostatic perturbations in the acetylated state, supporting the findings that acetylated HMGCS2 exhibited reduced activity. Specifically, acetylation of K310, K447, and K473, shown to be targeted by SIRT3, positions the substrate binding region of HMGCS2 further from the acetyl-CoA substrate, weakening protein-substrate interactions. Thus, in this model, deacetylation of these three residues by SIRT3 allows for greater proximity of the substrate to the binding pocket and permits ion-pair formation between the positively charged K310 and the 3'-phosphate of acetyl-CoA (Shimazu et al. 2010). Two loops (L1 and L2) far from the catalytic pocket were also affected. Despite their distance, molecular dynamic simulations showed acetylation of residues in these loops propagated conformational changes to the acetyl-CoA active site (Shimazu et al. 2010). Together, the *in vivo*, *in vitro*, and *in silico* data demonstrated one mechanism by which SIRT3 regulates HMGCS2 activity.

The increased resolution of recent quantitative proteomic studies enabled us to re-examine the sites of acetylation targeted by SIRT3 and their possible effects on enzymatic activity. Out of 29 (30) lysines on human (mouse) HMGCS2, we identified 18 (19) lysines in human (mouse) that have been previously reported as acetylated (Fig. 5.2b, red and blue), representing over one-half of all lysines on HMGCS2 (Table 5.1). While analysis of MDH2 identified only one site of acetylation that is a strong candidate for regulation by SIRT3, the picture for HMGCS2 is much more complex. Different lysines on HMGCS2 show different levels of hyperacetylation in the absence of SIRT3 as measured by quantitative proteomics (Sol et al. 2012; Hebert et al. 2013; Rardin et al. 2013). Collectively, these analyses identified 15 sites of acetylation on lysines that were homologous in human HMGCS2 that increase by at least 20 % in the absence of SIRT3, and 14 of which increase by 2-fold or more (Fig. 5.2b, red). Remarkably, the majority of acetylated lysines on HMGCS2 are candidates for regulation by SIRT3. To gain insight into the possible mechanisms of regulation that these lysines exhibit over HMGCS2, we modeled them on the crystal structure (Fig. 5.2b). These lysines occupy different regions on the protein, and may therefore confer regulation of the enzyme via different mechanisms. First, K83, 306, and K310 are involved in direct hydrogen bond interactions with the HMG-CoA product. Therefore, abrogating the charge of these lysines with an acetyl group would likely inactive the enzyme, or severely reduce its activity. In this case, SIRT3 regulation would serve to remove the acetyl and enhance enzymatic activity. Additionally, K333, K350, K354, and K358 are grouped around the binding site, potentially contributing to alterations in charge and/or the accessibility of the binding pocket, thereby affecting the capacity of the substrate to bind. Second, lysine acetylation may affect HMGCS2 activity through interactions with known catalytic residues H301 and C166 (Shafqat et al. 2010). K137 and K243 lie close to C166, which is the site of the acyl-bound enzymatic intermediate, and may therefore influence its binding (Fig. 5.2b). Interestingly, these three lysines are buried in the interaction interface, which in the dimeric form of HMGCS2 would not be readily accessible by SIRT3 for deacetylation. Therefore, these sites may be exposed in

the HMGCS2 monomer, and may also play a role in the formation of the functional homodimer. Similarly, given its proximity to the dimeric interface, K437 may also play a role in the formation of the functional dimer. Together, we predict that acetylation of lysines on HMGCS2 suppresses its activity, and SIRT3-mediated deacetylation coordinates activation by optimizing the overall structure and permitting substrate binding or product release. Further enzymatic studies beyond molecular modeling are required to test these hypotheses.

5.5 Substrate/Cofactor Regulation

5.5.1 SDH

Succinate dehydrogenase (SDH), also known as Complex II or succinate-coenzyme Q reductase, is composed of four subunits and uniquely participates in both the TCA cycle and the electron transport chain (ETC). Of the four subunits, succinate dehydrogenase flavoprotein (SHD) catalyzes the oxidation of succinate to fumarate with the subsequent reduction of ubiquinone to ubiquinol. The covalent binding of the cofactor flavin adenine dinucleotide (FAD) to SDH subunit A (SDHA) is required for Complex II activity and transfer of electrons to the ETC (Hederstedt and Rutberg 1981; Kim and Winge 2013). While SDH has been studied for decades, the mechanism of FAD transfer and binding to SDHA is not well understood (Kim and Winge 2013).

Acetylation of SDHA was first identified in a large-scale proteomics screen (Kim et al. 2006) and has since been verified in a number of studies (Choudhary et al. 2009; Zhao et al. 2010; Hebert et al. 2013; Finley et al. 2011; Schwer et al. 2009; Sol et al. 2012; Rardin et al. 2013; Cimen et al. 2010). SDHA was first described as a novel SIRT3 substrate based on observations that SDHA is hyperacetylated in *Sirt3*$^{-/-}$ mice and the activity of Complex II is reduced in *Sirt3*$^{-/-}$ mice compared to wild-type mice (Cimen et al. 2010). SDHA was further established as a substrate of SIRT3 by showing that SIRT3 physically interacts with SDHA and that purified SDHA is deacetylated *in vitro* by SIRT3, but not a catalytically inactive mutant (Finley et al. 2011). The acetylated residues discovered in these studies were initially identified as candidate sites of regulation by SIRT3 (K179, K485, K498, and K538; Fig. 5.3a, yellow and red), but proteomic technologies at the time limited the identification of specific sites of SIRT3 regulation. However, since these sites are found on the surface of SDHA (Cimen et al. 2010; Finley et al. 2011), it was initially suggested that acetylation modifications serve to modify the surface of SDHA (Finley et al. 2011). Recently, one study identified acetylation of K179 as enriched in the skeletal muscle of *Sirt3*$^{-/-}$ mice (Schilling et al. 2012). Another quantitative proteomics study reported a 22-fold increase in acetylation of lysine 179 of SDHA in the livers of *Sirt3*$^{-/-}$ mice during caloric restriction (Hebert et al. 2013). A third proteomics study identified K179 as a target of SIRT3 deacetylation by comparing

lysine acetylation of wild-type and *Sirt3*$^{-/-}$ mice using mass spectrometry (Rardin et al. 2013). These observations further agree with data generated from a quantitative proteomics screen (Choudhary et al. 2009).

To gain insight into how acetylation could influence SDHA activity, we performed a meta-analysis and modeled all known sites of acetylation on the crystal structure (Fig. 5.3a). A survey of literature describing SDHA lysine acetylation suggests that of the 27 lysines in the processed mitochondrial SDHA human protein, as many as 19 of these lysines are acetylated (Fig. 5.3a, Table 5.1). Of the four original sites identified (Fig. 5.3a, yellow and red), and 15 additional sites from subsequent proteomic studies, all quantitative proteomic studies provide convincing evidence that K179 is the single candidate site of regulation by SIRT3 (Fig. 5.3a, red). Sequence analysis identifies that the bulk of the A subunit of SDH is composed an FAD-binding protein family domain. Lysine 179 is located in this domain, and it has been suggested that deacetylation of K179 by SIRT3 may serve to regulate FAD binding (Hebert et al. 2013). Since acetylated K179 lies far from residues directly involved with FAD, it is unlikely that K179 reversible acetylation perturbs FAD binding directly. However, we observed that a cleft is formed near K179 on the surface of SDHA that is close to the FAD-binding site. An alternative explanation could be that acetylation of K179 inhibits FAD loading (i.e. flavination) of SDHA. A recent model of SDHA flavination has proposed that SDH subunit E (SDHE) is required to transfer FAD to SDHA (McNeil and Fineran 2013; Hao et al. 2009). Therefore, acetylation of a residue near a subunit binding site could disrupt protein-protein interaction and overall protein activity. In this model, we predict that acetylation of K179 in SDHA suppresses activity by reducing flavination of the protein, which can be reversed by SIRT3-mediated deacetylation. As another possibility, K179 lies on a flexible loop and therefore acetylation of K179 may regulate the entry of succinate or exit of fumarate from SDHA. Further studies on SDHE, the content of FAD in SDHA as a function of acetylation, as well as enzymatic activity assays, together will identify the mechanism by which acetylation suppresses SDHA function.

5.5.2 LCAD

Long chain acyl-CoA dehydrogenase (LCAD) is a member of the acyl-CoA dehydrogenase (ACAD) family of enzymes. Four members of the ACAD family [short chain acyl-CoA dehydrogenase (SCAD), medium chain acyl-CoA dehydrogenase (MCAD), LCAD, and very long chain acyl-CoA dehydrogenase (VLCAD)] can catalyze the first dehydrogenation step of fatty acid oxidation whereby a fatty acyl-CoA is dehydrogenated at the α and β carbons in a FAD-dependent manner. Following this, electrons are transferred from the ACAD to non-covalently bound electron transferring flavoprotein (ETF) and then on to the electron transport chain. Each of the ACADs catalyzes the same reaction but differ in their substrate specificity. LCAD has been reported to dehydrogenate both saturated and unsaturated fatty

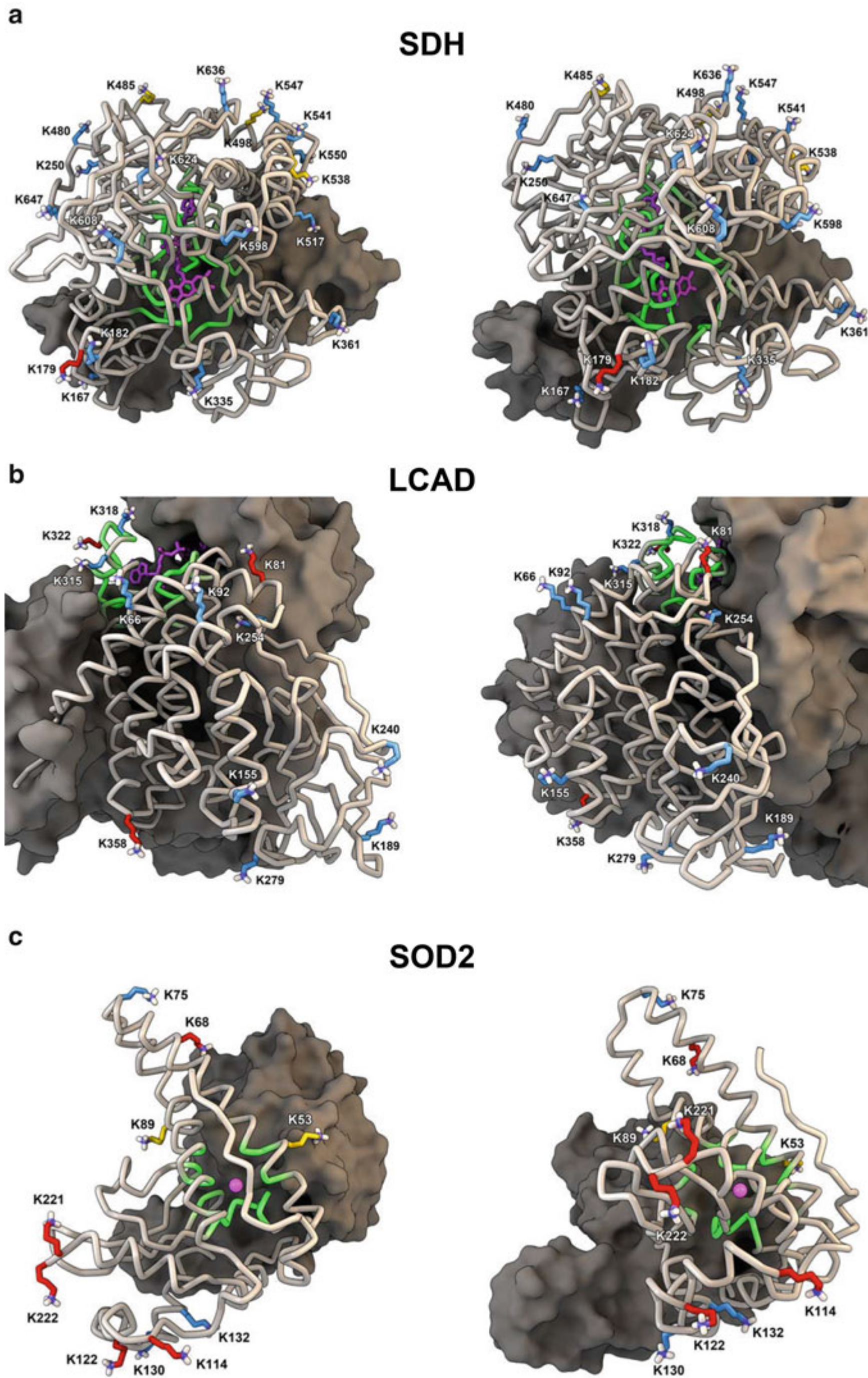

Fig. 5.3 Substrate/Cofactor regulation. Lysine residues known to be subject to post-translational acetylation are depicted as sticks with their specific identities indicated. Carbon atoms of the lysine residues known to be regulated or are candidates to be regulated by SIRT3 are colored *red*;

acyl-CoAs (Lea et al. 2000) with a preference for chain lengths of 10–14 carbons (Eder et al. 1997) and double bonds at the 4,5 or 5,6 position (Lea et al. 2000). In mice, LCAD deficiency results in elevated hepatic and cardiac lipid accumulation, hypoglycemia, and elevated serum and hepatic C_{10}-C_{14} fatty acids during fasting- a phenotype reminiscent of human deficiency of VLCAD (Kurtz et al. 1998). Despite many documented cases of humans deficient in SCAD, MCAD, and VLCAD, humans with LCAD deficiency have not been reported. Interestingly, LCAD knock-out mice display frequent gestational loss (Kurtz et al. 1998) and it has been suggested that LCAD may be involved in embryonic development (Berger and Wood 2004). Thus, the lack of LCAD-deficient humans is thought to be due to the likelihood that LCAD deficiency is pre-natally lethal.

Similar to SCAD and MCAD, active LCAD exists as a homotetramer and its activity is dependent on FAD as a cofactor (Nagao and Tanaka 1992). Although the crystal structure of LCAD has not been solved, molecular modeling and site-specific mutagenesis suggests that E260 is the catalytic residue (Djordjevic et al. 1994). A cysteine near the FAD binding site, but not affecting FAD binding, has also been reported to affect LCAD activity (Okamura-Ikeda et al. 1985). Another mechanism of LCAD regulation is reversible acetylation of lysine residues, supported by findings that lack of SIRT3 results in hyperacetylation of LCAD and reduced LCAD activity (Hirschey et al. 2010). While SIRT3 has been shown to regulate LCAD activity, the exact mechanism is still being elucidated.

Several studies have shown that LCAD is acetylated, with a total of 13 acetylated lysine residues being identified out of 32 total (Zhao et al. 2010; Hebert et al. 2013; Hirschey et al. 2010; Schwer et al. 2009; Sol et al. 2012; Simon et al. 2012) (Fig. 5.3b, Table 5.1). Using proteomic approaches, four of these acetylated sites (K42, K81, K322, K358) were identified as candidates for being regulated by SIRT3 (Hebert et al. 2013; Hirschey et al. 2010; Sol et al. 2012) (Fig. 5.3b, red). Without the crystal structure of LCAD solved, it is difficult to predict precisely how lysine deacetylation of LCAD by SIRT3 might affect LCAD activity. However, examina-

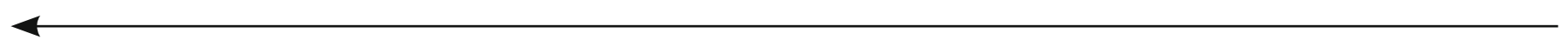

Fig. 5.3 (continued) previously described SIRT3-regulated sites lacking quantitative proteomic data evidence are colored *yellow*; while those that are acetylated but not candidates to be regulated are colored *blue*. (**a**) Two views of a protein homology model of human SDH generated using the crystal structure of porcine SDH (PDB ID: 1ZOY; 96 % identity, 98 % similarity to the human sequence) as a template (Sun et al. 2005). The FAD binding domain (SDHA) is depicted as a protein backbone with the neighboring iron-sulfur binding domain (SDHB) represented as the solvent-accessible surface. The bound FAD cofactor is colored *magenta* with the FAD binding site highlighted in green; (**b**) Two views of a protein homology model of the human LCAD tetrameric complex generated using the crystal structure of human MCAD (PDB ID: 1EGD; 32 % identity, 55 % similarity to the human sequence) as a template (Lee et al. 1996). One LCAD monomer is represented as a protein backbone with the remaining three monomers represented as the solvent-accessible surface. The bound FAD cofactor is colored *magenta* with the FAD binding site highlighted in *green*; (**c**) Two views of the crystal structure of dimeric human MnSOD (PDB ID: 1MSD) with one monomer represented as a protein backbone and the other as the solvent-accessible surface (Wagner et al. 1993). A Mn^{2+} ion (magenta sphere) is contained within the active site (highlighted in *green*)

tion of a protein homology model of human LCAD based on the crystal structure of MCAD (Lee et al. 1996) (Fig. 5.3b) reveals that at least seven acetylated lysine residues lie in or near the active site, including K81 and K322, both of which are candidates for SIRT3 regulation. Steric inhibition of the catalytic residue or the substrate-binding domain may occur in the hyperacetylated state, which could be de-repressed by SIRT3 deacetylation of these lysines. Interestingly, the FAD-binding domain is adjacent to the active site and includes residues of two adjacent LCAD monomers. The SIRT3-regulated K322 is located in the FAD-binding pocket and therefore, acetylated K322 could regulate both FAD binding and LCAD subunit interaction. Indeed, acetylation of K318 and K322 was recently shown to regulate LCAD activity by modulating the hydride transfer from the fatty acyl substrate to FAD (Bharathi et al. 2013). Acetylation of these lysine residues was predicted to alter the conformation of the active site thereby hindering the binding and function of FAD (Bharathi et al. 2013).

The possibility for SIRT3 to regulate subunit interaction is highlighted by the fact that another SIRT3-regulated lysine, K81, is at the interaction interface of two LCAD monomers. K81 on one LCAD monomer is adjacent to two potential H-bond donors, H249 and R246, on its neighboring monomer. Therefore, acetylation of K81 could introduce H-bonds that are not present in the non-acetylated state. Interestingly, K81 is located at a turn between two alpha-helices at the N-terminal region of LCAD, which based on homology to MCAD, contains the ETF binding domain (Toogood et al. 2004). Thus, deacetylation of K81 could impact the conformation or flexibility of the N-terminal alpha-helices and in turn affect ETF binding. Another N-terminal lysine regulated by SIRT3, K42, also has the potential to affect ETF binding, but we were unable to model this lysine residue since the region containing its homologous residue on MCAD is absent in the crystal structure. Further, while we were able to identify K358 as a candidate for being SIRT3 regulated, it is not apparent from our model how acetylation of K358 might regulate LCAD function directly. However, because this site is located on the surface of the protein, acetylation of K358 could influence protein-protein interactions between LCAD and its binding partners. Together, we propose a model where SIRT3-mediated deacetylation of lysine residues enhances overall LCAD activity by permitting ETF binding, FAD binding, and substrate binding. However, more detailed studies are needed to test these hypotheses and determine exactly how SIRT3 affects LCAD structure and ultimately regulates LCAD activity.

5.5.3 SOD2

Manganese superoxide dismutase (SOD2) is a tetrameric mitochondrial enzyme that serves to protect against reactive oxygen species (ROS)-induced oxidative damage by converting superoxide into oxygen and hydrogen peroxide, which is then further neutralized to O_2 and H_2O by the enzyme catalase (Zhu et al. 2012). As such, SOD2 plays a crucial role in managing ROS produced in mitochondria as a

result of respiration. SOD2 is a protein encoded by the nuclear genome and localizes to mitochondria to form a homotetrameric complex (Weisiger and Fridovich 1973; Borgstahl et al. 1992). SOD2 is the sole mitochondrial superoxide dismutase and its activity is dependent on binding to the co-factor, manganese, in contrast to non-mitochondrial Fe-, Cu- and Zn-dependent superoxide dismutases (Borgstahl et al. 1992). While SOD2 transcription is regulated under certain conditions, its enzymatic activity is regulated post-translationally via the balance between acetylation and deacetylation by SIRT3 (Tao et al. 2010; Qiu et al. 2010; Kim et al. 2010; Chen et al. 2011). Loss of SIRT3 leads to increased levels of ROS both *in vitro* and *in vivo*, suggesting SIRT3 protects the cell from oxidative stress (Tao et al. 2010). Two early studies found that SIRT3 deacetylates SOD2, which leads to an increase in SOD2 activity and an overall decrease in cellular ROS (Qiu et al. 2010; Kim et al. 2010). An additional study later validated several of these findings (Chen et al. 2011).

Collectively, studies on the acetylation of SOD2 have demonstrated a role for SIRT3 in its regulation; however, the specific lysines on SOD2 that are regulated by SIRT3 remain unclear. The first study showing SOD2 acetylation identified that K68 as a residue acetylated in fed and fasted mouse liver (Kim et al. 2006). In later studies, K68 was identified as a target of SIRT3 by showing that over expression of SIRT3 lead to ~20 % decrease in K68 acetylation of SOD2, while knockdown of SIRT3 lead to ~30 % increase in K68 acetylation (Chen et al. 2011). Additionally, an inhibitory mutation of K68 reduced SOD2 activity by more than 50 %, demonstrating this lysine could be important for SOD2 activity (Chen et al. 2011). Later studies identified K53 and K89 as sites regulated by SIRT3 (Qiu et al. 2010), or identified K122 as the most significant SIRT3-regulated lysine (Tao et al. 2010). Thus, we turned to the recent quantitative proteomic studies to identify any lysines that are potentially regulated by SIRT3. To our surprise, no significant ($p < 0.05$) or marked changes (2-fold or greater) in the ratio of acetyl occupancy in *Sirt3*$^{-/-}$ mice compared to wild type at any of the sites of acetylation identified on SOD2 (Rardin et al. 2013) (Table 5.1). One site, K114, emerged as the strongest candidate from this list (1.14 SIRT3KO/WT, $p = 0.28$), but is weak compared to candidate sites for SIRT3 regulation on other proteins in this study. Consistent with these data, an additional study showed no statistically significant increases in SOD2 acetylation in the absence of SIRT3 (Hebert et al. 2013). Remarkably, both quantitative proteomic studies failed to identify enrichment of acetylation at sites that have been previously recognized as SIRT3-regulated on SOD2 (Fig. 5.3c, Table 5.1). Therefore, in this example, previously published quantitative proteomic studies cannot be used to identify candidate sites of acetylation for SOD2.

Based on qualitative proteomic data, we surmise that K122, K68, K221-K222, and K114 are sites of acetylation that could be regulated by SIRT3, in descending order of likelihood, with K122 having the strongest evidence, followed by K68 (Table 5.1). Furthermore, K221-222 and K114 are repeatedly identified in proteomic acetylation studies (Hornbeck et al. 2011). Insight into how acetylation regulates SOD2 activity was recently proposed to be an electrostatic facilitation mechanism, first described by Irwin Fridovich (Benovic et al. 1983). In the electrostatic funnel

model, the removal of an acetyl group exposes positive charges at specific lysine residues on SOD2 and guides superoxide through the SOD2 complex towards the manganese active site of SOD2 (Zhu et al. 2012). In this paper, K122, along with K114 and K221, were described to line the entrance of superoxide into the active site of SOD2. Analysis of the structure of SOD2 and the location of potential SIRT3-regulated lysines support this model (Fig. 5.3c). Additionally, K222 lies in the same region. We observed the entrance site proposed above might be too far for the superoxide to travel in order to reach the catalytic manganese atom (>30 angstroms), and might not provide a clear path through the protein to the active site. However, the crystal structure might not accurately reflect the conformation and flexibility of the active enzyme. K68, the remaining candidate SIRT3-regulated site, faces the neighboring monomer, suggesting that homotetramer complex formation could be disrupted if K68 were acetylated.

Together, studies on the regulation of SOD2 by acetylation provide convincing evidence that SIRT3 regulates its activity; however, uncertainty remains in the field about which lysine residues are regulated. While no candidates emerged from our quantitative proteomic meta-analyses, five conserved acetylated lysines on SOD2 remain candidates for regulation by SIRT3 in both mice and humans (Fig. 5.3c). One possible explanation for lack of candidate lysines emerging in the proteomics studies could be that neither the basal condition nor the calorie-restricted condition are sufficient stressors to induce SIRT3-mediated SOD2 deacetylation compared to other mitochondrial metabolic proteins. Thus, more work is required to definitively identify the specific SIRT3-regulated lysines on SOD2.

5.6 Allosteric Regulation

5.6.1 GDH

Glutamate Dehydrogenase (GDH) is an important metabolic protein found in all living organisms. Using NAD(P) + as a cofactor, it converts glutamate into alpha-ketoglutarate for subsequent oxidation in the TCA cycle, and plays a key role in nitrogen and carbon metabolism (Hudson and Daniel 1993). Mammalian GDH exists as a homohexamer, made up of two homotrimers (Banerjee et al. 2003; Smith et al. 2002; Smith et al. 2001). GDH is regulated by phosphorylation (Bell and Storey 2010), ADP-ribosylation (Herrero-Yraola et al. 2001), as well as allosteric regulation, which together coordinate metabolic homeostasis (Li et al. 2011; Smith and Stanley 2008). Allosteric regulation is primarily controlled by GTP and ADP via competition for binding regions on a regulatory alpha-helix called the pivot helix. The position of these two metabolites on the pivot helix allows fine-tuned regulation of separate substrate and $NAD(P)^{+}$-binding domains (Hudson and Daniel 1993). In an energy-replete state, GTP occupies a pocket on one side of the pivot helix, which results in a closed conformation that inhibits the release of product from the active site and decreases catalytic turnover. In an energy-depleted state,

ADP occupies another side of the pivot helix, which opens the active site and promotes release of the product and increased catalytic turnover (Li et al. 2011; Smith and Stanley 2008).

GDH has a long history of being known as acetylated, and more recently as an enzyme regulated by the mitochondrial sirtuins. Early studies on GDH showed that low levels of chemical acetylation slow catalytic activity of the active GDH homohexmer and render it unresponsive to allosteric effectors, whereas high levels can promote dissociation of the active complex (Colman and Frieden 1966; Freedman and Radda 1969; Holbrook and Jeckel 1969). More recently, GDH was identified as acetylated under physiological conditions in the liver of fasted mice and hyperacetylated in *Sirt3*$^{-/-}$ mice (Lombard et al. 2007; Kim et al. 2006), in the pancreatic islets of mice (Kim et al. 2012), and in the ovarian granulosa and cumulus cells of women with reduced ovarian reserve (Pacella-Ince et al. 2014). These findings overall suggest a role for SIRT3 in regulating acetylated GDH. Indeed, repressed catalytic activity of acetylated GDH can be relieved by SIRT3 deacetylation (Lombard et al. 2007; Kim et al. 2012; Schlicker et al. 2008), which co-localizes with GDH in the mitochondrial matrix. (Haigis et al. 2006; Onyango et al. 2002; Schwer et al. 2002) The molecular details of SIRT3 enzymatic regulation of GDH, however, remain unclear.

Our quantitative proteomic meta-analysis revealed 35 lysines on GDH, of which 19 have been described as acetylated (Table 5.1). However, we identified only six of these acetylated lysine residues as candidates for regulation by SIRT3 (Du et al. 2011; Hebert et al. 2013; Sol et al. 2012; Rardin et al. 2013). Interestingly, three of these six sites are found within the ADP and GTP binding regions of GDH (Fig. 5.4). Of particular interest is the highly conserved K527, which is found on the same face of the pivot helix that contains R463 – a residue essential for activation of GDH by ADP (Banerjee et al. 2003). Deacetylation of K527 by SIRT3 could permit binding of ADP and consequent activation of GDH. Furthermore, residues K346 and K503 lie nearby the GTP binding pocket, which negatively regulates GDH activity. However, neither K346 nor K503 have been shown to participate in GTP binding (Li et al. 2011; Smith and Stanley 2008). Because these two lysines are located on the outer edge of the pocket, we predict that acetylation could prevent GTP dissociation thereby suppressing GDH activity (Fig. 5.4). In this model, SIRT3 would deacetylate these residues, and relieve inhibition of GDH by GTP. Of the three remaining proposed SIRT3 regulated sites, human K480 is found at the tip of a unique 50-residue "antenna" region that is essential for catalytic activity by controlling movement of the mammalian GDH complex into its active conformation (Li et al. 2011; Smith and Stanley 2008). Mutations in this region may disrupt inter-subunit coordination of the active GDH homohexamer complex (Li et al. 2011), underscoring an important role for regulation of complex stability and formation through residue modification on the antenna (Fig. 5.4). Together, these six sites suggest an overall mechanism for SIRT3 regulation of GDH in which deacetylation promotes ADP activation of GDH, de-represses GTP inhibition of GDH, and/or promotes homohexamer stability. Future experiments directed at these mechanisms will determine how SIRT3 regulates GDH activity.

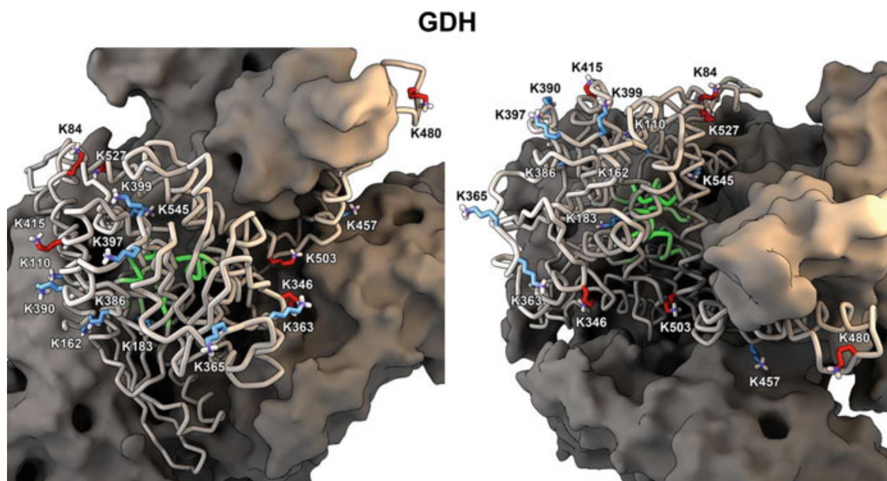

Fig. 5.4 Allosteric Regulation. Two views of the crystal structure of a hexameric complex of human mitochondrial GDH (PDB ID: 1L1F; (Smith et al. 2002)) with a single monomer represented as a protein backbone and the remaining subunits as a solvent-accessible surface. Lysine residues known to be acetylated are depicted as sticks with their specific identities indicated. Carbon atoms of the residues known to be regulated or are candidates to be regulated by SIRT3 are colored *red*, and those that are acetylated but not candidates to be regulated are colored *blue*. The glutamate substrate binding site is highlighted in *green*

5.7 Other Mechanisms for Future Studies

Presented above are examples of mitochondrial proteins with strong evidence for regulation by SIRT3-mediated deacetylation, candidate sites of regulation, and predicted mechanisms of regulation based on structural information. In addition to the mechanisms described above, we anticipate that future studies could identify how acetylation regulates other aspects of mitochondrial protein function.

Several metabolic enzymes have lysines required for enzymatic function, and therefore blocking these important lysines may reduce activity. For example, protein kinases, which are enzymes that phosphorylate cellular proteins by catalyzing the exchange of a phosphate group of adenosine triphosphate (ATP) to proteins, have a conserved lysine required for catalysis (Iyer et al. 2005). In the binding pocket, a single lysine coordinates ATP, which is required for the phosphotransfer reaction (Carrera et al. 1993). In the absence of this lysine, kinases lose catalytic activity, and absence of this lysine is a common characteristic of pseudo-kinases (Manning et al. 2002). In principle, acetylation of this lysine would prevent ATP binding to the protein kinase and subsequent phosphotransfer.

Transaminases are enzymes that participate in amino acid metabolism and require a lysine for their enzymatic function, providing another example of regulation by targeted lysine modification. The exchange of the amine group on an amino acid and the keto group in an α-keto acid is catalyzed by transaminases in order to

produce various amino acids. Transaminases use the cofactor pyridoxal phosphate to catalyze their enzymatic activity. In the active site, the aldehyde group on the pyridoxal phosphate forms a temporary Schiff base linkage to a lysine on the transaminase. The Schiff base is displaced when a substrate amino acid interacts with transaminase to allow for the amine-keto exchange and the free lysine is regenerated (Hayashi et al. 2003). Again, if the lysine were acetylated, this amine-keto exchange would theoretically be inhibited. Indeed, this idea is further supported by studies demonstrating that chemical acetylation of a transaminase inhibits enzymatic activity via this mechanism (Turano et al. 1967).

Biotin-dependent carboxylases use biotin as a carboxyl carrier, which is covalently bound to the ε-amino group of a lysine side chain on the carboxylases (Chapman-Smith and Cronan 1999). In the reaction, a carboxybiotin intermediate bound to the carboxylase transfers the carboxyl group to the substrate. If this essential lysine within the biotin-carboxylase complex were acetylated, the cofactor-enzyme interaction could be abolished and thus, block carboxylase activity.

Ubiquitination (a.k.a. ubiquitylation) is a process by which the small regulatory protein ubiquitin is attached to lysine residues, and directs proteins to compartments in the cell, including the proteasome for their degradation. Since both acetylation and ubiquitination occur on lysines, an acetylated lysine would prevent subsequent ubiquitination, and could reduce protein turnover or increase protein stability.

Lysine acetylation could influence non-enzymatic processes, such as transport through mitochondrial membrane channels. For example, the mitochondrial permeability transition pore or its regulator cyclophilin D (Shulga et al. 2010; Hafner et al. 2010), are thought to be modulated by acetylation and SIRT3-mediated deacetylation. Lysine acetylation could also influence protein localization, which is a common mechanism for regulation of nuclear import and export [for a review, see (Sadoul et al. 2011)].

Together, these examples illustrate other lysines on metabolic enzymes that are important for activity, which could be acetylated and affect enzymatic function. While no biological evidence exists for acetylation to regulate protein function by these mechanisms, future studies could be directed at testing these examples and overall represent a rich area for discovery.

5.8 Challenges

High-resolution mass spectrometry and sophisticated molecular biology tools have facilitated rapid advancement of this field. However, several challenges remain. In particular, the high abundance of acetylated proteins in mitochondria makes determining the biologically relevant sites of acetylation difficult. Additionally, identifying the mechanisms by which acetylation influence enzymatic activity and overall biology remains a challenge.

Even though quantitative proteomics studies have provided important insight into the complex landscape of acetylation, antibody-based tools required for qualitative

or quantitative proteomic studies can be biased. Antibodies are used to enrich samples for mass spectrometry-based proteomic analyses. However, antibodies against acetyl-lysine only recognize some sites of acetylation on proteins, and may not bind to other acetylated proteins or peptides. Indeed, different *pan*-anti-acetyl lysine antibodies have led to discrepancies in how acetylation levels of some proteins change in response to metabolic challenges (Schwer et al. 2009; Nakagawa et al. 2009). In order to address this challenge, better tools are needed for detecting sites of acetylation. One approach that might be useful is to leverage chemical biology strategies. For example, the field of protein glycosylation, which was originally hindered by lack of specific antibodies against this modification, has developed several bio-orthogonal reagents that have facilitated their study (Prescher and Bertozzi 2005). Recently, an alkynyl-acetyl-CoA analog was developed as a bio-orthogonal chemical reporter for monitoring protein acetylation (Yang et al. 2010). This emerging area might produce tools to overcome some of the challenges associated with antibody bias.

Current evidence indicates that mitochondrial protein acetylation levels are regulated by the sirtuin SIRT3. For example, as described above, SIRT3 regulates the acetylation state of the proteins. However, alternations in metabolism have also been correlated with changes in protein acetylation. In one example, high-fat diet feeding increases hepatic mitochondrial protein acetylation in mice, which can be attributed to reduced SIRT3 protein levels and activity (Hirschey et al. 2011a; Kendrick et al. 2011; Bao et al. 2010). In another example, calorie-restricted feeding increases mitochondrial protein acetylation, and paradoxically, SIRT3 protein expression and activity is increased (Schwer et al. 2009; Qiu et al. 2010). In this setting, the change in global protein acetylation cannot be attributed directly to less SIRT3, and therefore must be occurring through alternative mechanisms.

One possible explanation for this disconnect between acetylation levels and SIRT3 activity could be due to changes in the levels or activity of a mitochondrial protein acetyltransferase. However, the mechanisms leading to protein acetylation in the mitochondria remain largely unexplored. While histones have histone acetyltransferases (HATs) that enzymatically catalyze the acetylation of histone tails in the nucleus, mitochondrial acetyltransferases are much less characterized (Scott et al. 2012). Alternatively, non-enzymatic mitochondrial protein acetylation has been postulated (Newman et al. 2012). Recently, Brenner and co-workers have proposed that the lysine pKa of mitochondrial enzymes has been under evolutionary selection in order to attract acetylation to particular residues (Ghanta et al. 2013). Knowing little about the mechanism of acetylation is a major gap in our understanding of how acetylation regulates mitochondrial protein function.

Furthermore, the discovery of other mitochondrial protein modifications, such as propionylation (capable of being removed by SIRT3, (Garrity et al. 2007)) and negatively charged acyl modifications [removed by SIRT5, (Du et al. 2011; Tan et al. 2014; Peng et al. 2011a)], add to the complexity of how post-translational modifications may affect enzymatic function and structure in the mitochondria. We anticipate several of these modifications will be found on the same sites, and thus the

study of their crosstalk will become important for understanding mitochondrial control by post-translational modifications.

Together, the complex nature of the acetylation and acylation landscape, in addition to its sheer abundance, makes determining the specific sites of acetylation on mitochondrial proteins and understanding their function a scientific challenge. Therefore, taking a protein-by-protein approach to validate proteomic data and to gain insight into the mechanisms of regulation of acetylation on mitochondrial proteins is necessary to understand the role of mitochondrial protein acetylation in the regulation of metabolism. Indeed, the effect of acetylation cannot be generalized to all mitochondrial proteins; however, the strategy described above serves as first steps towards understanding the global role of acetylation in the mitochondria.

5.9 Conclusions

In this review, we have discussed sites of acetylation regulated by SIRT3 and hypothesized possible mechanisms of control that reversible acetylation has on these targets. In some examples, multiple sites of acetylation on a single protein appear to be regulated by SIRT3, and are deacetylated in a concerted manner to coordinate enzymatic activity, such as HMGCS2, LCAD, and GDH. In other examples, few sites, or just one site, appears to be regulated by SIRT3 to activate the protein, such as MDH, SDH, and SOD2. Remarkably, in either of these cases, relatively few sites of overall acetylation are targeted by SIRT3 compared to the highly abundant global acetylation state of the protein. One interpretation could be that some sites that have been reported as acetylated are low stoichiometry sites, sites that are regulated by a not-yet-identified mitochondrial deacetylase, or are sites that are not reversibly deacetylated and therefore would not be considered regulatory.

Current evidence suggests that acetylation suppresses the activity of mitochondrial proteins and overall mitochondrial function; SIRT3 functions to relieve this suppression on the mitochondria, serving as an activator of mitochondrial enzymatic activity. Future studies may find examples where acetylation activates mitochondrial proteins and in turn, SIRT3 deacetylase activity suppresses mitochondrial protein enzymatic activity. This could be particularly relevant for anabolic proteins whose activity would be suppressed by SIRT3 in response to metabolic stress. Continued work in this emerging field will address these complexities, and lead to a deeper understanding of how mitochondrial protein acetylation regulates mitochondrial function.

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Chapter 6
SIRT5 Reveals Novel Enzymatic Activities of Sirtuins

Bin He and Hening Lin

The human SIRT5 gene was first identified in 1999 using the sequence of yeast SIR2 protein (Frye 1999). Sequence analysis suggests that it is more similar to prokaryotic sirtuins (Frye 1999, 2000). Among the seven mammalian sirtuins, SIRT5 played a special role in our understanding of sirtuin enzymatic activity as it was the first sirtuin that was found to have a novel enzymatic activity other than the well-known NAD-dependent deacetylase activity (Imai et al. 2000; Tanner et al. 2000), the hydrolysis of succinyl and malonyl lysine on proteins. This finding suggested that other sirtuins (e.g. SIRT4, SIRT6, and SIRT7) with weak deacetylase activity may also prefer other acyl lysine modifications, which was confirmed later with several sirtuins. In this chapter, we will summarize what is known about SIRT5, with a focus on the discovery of its new enzymatic activity and how the new activity finding influences the understanding of SIRT5 function, inhibitor development, and the study of other sirtuins.

6.1 Cellular and Tissue Expression of SIRT5

SIRT5 was found to be localized in mitochondria based on several methods. First, transiently expressed C-terminal GFP-SIRT5 was found to co-localize with the mitochondrial marker MitoFluor in normal human fibroblasts (NHF) (Michishita et al. 2005). Subcellular fractionation of NHF and HeLa cells transiently expressing C-terminal V5-tagged SIRT5 also revealed that SIRT5 was in the mitochondrial fraction (Michishita et al. 2005). SIRT5 with the HA tag at the N-terminus could not be detected, suggesting that it has an N-terminal mitochondrial-targeting sequence (first 36 amino acids) that is cleaved off in the mitochondria (Nakagawa et al. 2009;

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Schwer et al. 2002). PCR gene expression analysis showed that SIRT5 was broadly expressed in normal fetal and adult tissues, with relatively high level in the heart, muscle, brain, liver and kidney (Frye 1999; Michishita et al. 2005). The wide distribution of SIRT5 indicates that it may play an important role in regulating protein function.

6.2 Enzymatic Activity of SIRT5

Among seven human sirtuins, only SIRT1-3 have robust deacetylase activity while SIRT5 has detectable but much weaker deacetylase activity on an acetylated histone H4 peptide (North et al. 2005), chemically acetylated histones and bovine serum albumin (Schuetz et al. 2007). We were interested to understand why SIRT5 had a weak deacetylase activity. Because some sirtuins were thought to be ADP-ribosyltransferases (Frye 1999; Haigis et al. 2006; Liszt et al. 2005), we initially investigated the ADP-ribosyltransferase activity of several sirtuins with synthetic NAD analogs and ^{32}P-labeled NAD and found that the ADP-ribosyltransferase activity of sirtuins was very weak (Du et al. 2009). Compared to the bacterial ADP-ribosyltransferase diphtheria toxin, the observed ADP-ribosylation rate constants for sirtuins, including SIRT4 and SIRT6, were more than 5000-fold lower (Ref 12 and our unpublished result). This is even lower than the weak deacetylase activity of SIRT5. Screening a panel of acetyl peptides with different sequences also failed to identify an acetyl peptide that could be efficiently hydrolyzed by SIRT5 (Du et al. 2011). A breakthrough was made when the crystal structure of human SIRT5 was obtained in complex with a thioacetyl lysine peptide and an *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer molecule (Du et al. 2011). The negatively charged sulfonate group of CHES was bound by Tyr102 and Arg105 of SIRT5 in the structure (Fig. 6.1a). The sulfonate group of CHES was close to the bound thioacetyl lysine. These structure features led to the hypothesis that SIRT5 might prefer to recognize negatively charged acyl lysine modifications, such as malonyl and succinyl lysine (Fig. 6.1a). This was proved to be true by in vitro enzymology studies. The catalytic efficiency of desuccinylation and demalonylation was 29–1000 folds higher than that of deacetylation depending on the sequence of the peptides (Du et al. 2011). A crystal structure of SIRT5 with succinyl peptide and NAD bound was also obtained. In this structure, the carboxylate of succinyl indeed interacted with Tyr102 and Arg105, as predicted from the structure of SIRT5 with CHES bound (Fig. 6.1b). Mutating these two residues significantly decreased the catalytic efficiency on succinyl peptides, but not on acetyl peptides, confirming that the two residues are important for recognizing negatively charged acyl groups (Du et al. 2011). These two residues are conserved in most Class III sirtuins (Frye 2000), suggesting that these Class III sirtuins may also have desuccinylase or demalonylase activity (Du et al. 2011).

When we discovered the more efficient desuccinylase/demalonylase activity of SIRT5, it was not known whether lysine succinylation and malonylation were common protein posttranslational modifications (PTMs) like acetylation. However,

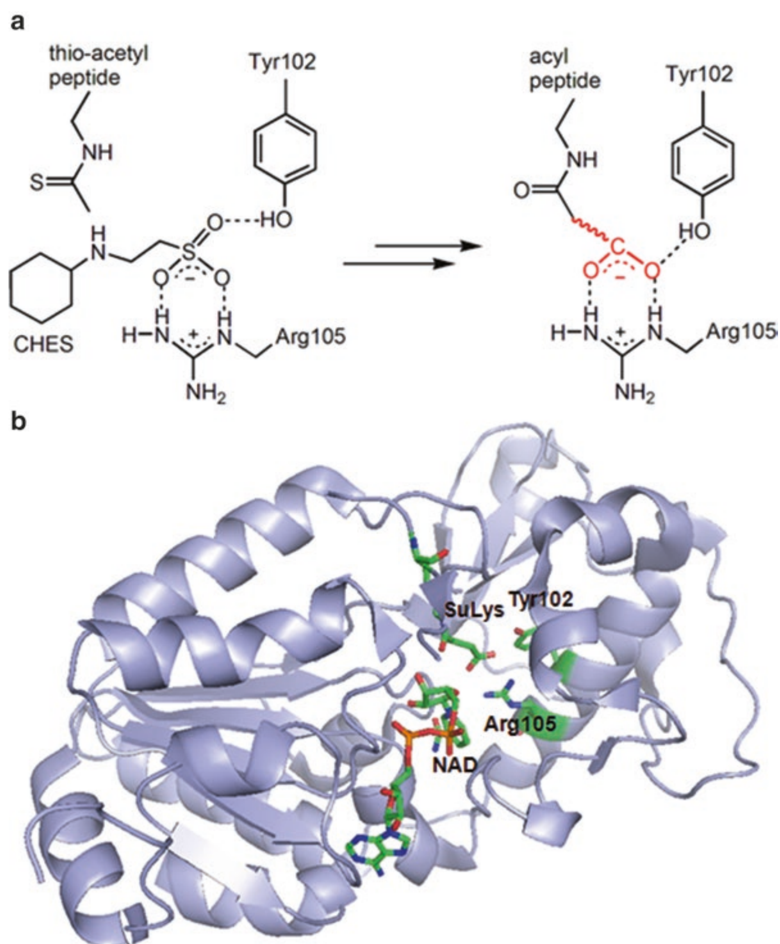

Fig. 6.1 Sirt5's desuccinylase and demalonylase activity was revealed by the structure of SIRT5 in complex with a thioacetyl lysine peptide and the CHES buffer molecule. (**a**) a schematic representation showing that the sulfonate of CHES interacts with Tyr102 and Arg105 of SIRT5 and the sulfonate is close to the acyl group of the peptide. This led to the hypothesis that SIRT5 may recognize peptides modified with negatively charged succinyl or malonyl groups. (**b**) The structure of SIRT5 in complex with a succinyl peptide and NAD (PDB 3RIY). The carboxylate of succinyl interacts with Tyr102 and Arg105 of SIRT5, similar to how the sulfonate of CHES interacts with SIRT5

several isolated examples did provide some indirect support for the possible existence of protein succinylation (Acker et al. 2009; Kawai et al. 2006; Rosen et al. 2004). We used both biochemical and mass spectrometry methods to confirm the existence of lysine succinylation and malonylation in mammalian cells (Du et al. 2011). Lysine succinylation were found in many mammalian mitochondrial metabolic enzymes on multiple lysine residues (Du et al. 2011). At the same time, by MS method, Zhao and co-workers also identified lysine succinylation and malonylation

as new PTMs (Peng et al. 2011; Zhang et al. 2011). They further identified several lysine succinylation sites on histones in Hela cells, mouse embryonic fibroblasts, Drosophila S2 cells, and yeast cells (Xie et al. 2012).

6.3 Substrate Proteins and Biological Function of SIRT5

Since SIRT5 is mainly in the mitochondria and many metabolic enzymes have been found to be succinylated or malonylated, we suspect that SIRT5 may regulate the activity of metabolic enzymes in mitochondrial, similar to the regulation of metabolic enzymes by SIRT3 via deacetylation (Kim et al. 2006; Wang et al. 2010; Zhao et al. 2010). However, relatively few *in vivo* substrates have been identified for SIRT5. This is in part because previously most people were focusing on the identification of deacetylase substrates of SIRT5, which is difficult because SIRT5 has only weak deacetylase activity. The discovery of the more efficient desuccinylase/demalonylase activity should permit the identification of more SIRT5 substrate proteins provided that good succinyl/malonyl lysine antibodies are broadly available.

The first confirmed *in vivo* substrate protein of SIRT5 is carbamoyl phosphate synthetase 1 (CPS1) (Du et al. 2011; Nakagawa et al. 2009), the enzyme catalyzing the first step of the urea cycle, which plays a critical role in the ammonia detoxification and disposal (Morris 2002), Guarente and co-workers showed that SIRT5 promoted CPS1 activity and urea formation under nutrient deprivation conditions by deacetylating CPS1 (Nakagawa et al. 2009). Interestingly, another report showed that CPS1 had increased acetylation level during calorie restriction (Schwer et al. 2009). By MS, we found that SIRT5 regulated the succinylation level of CPS1 on Lys1291, but not the acetylation level on this residue (Du et al. 2011). Two other lysine residues were also modified by acetylation and succinylation, but the levels of acetylation and succinylation were not affected by SIRT5 knockout (Du et al. 2011). Our data confirmed that SIRT5 can regulate CPS1 activity, and most likely by desuccinylation instead of deacetylation (Du et al. 2011).

Urate oxidase, an enzyme involved in purine metabolism, was reported to be a substrate protein of SIRT5 in mice (Nakamura et al. 2012). The acetylation level of urate oxidase, measured using a pan-specific acetyl lysine antibody, was decreased in a SIRT5 over-expression transgenic mouse strain (Nakamura et al. 2012). It was not clear whether the succinylation level was affected or not by SIRT5 overexpression. Cytochrome c, a protein of the mitochondrial intermembrane space with a central function in oxidative metabolism and apoptosis initiation was also reported to be a SIRT5 deacetylase substrate *in vitro* (Schlicker et al. 2008). However, no in vivo data was presented and this should only be considered as a putative substrate (Schlicker et al. 2008).

There is no doubt that more SIRT5 substrates will be discovered after the novel activity of SIRT5 was known. Several metabolic enzymes are modified by succinylation (Du et al. 2011; Zhang et al. 2011), including isocitrate dehydrogenase 2 (IDH2), serine hydroxymethyltransferase (SHMT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamate dehydrogenase 1 (GDH), malate

dehydrogenase 2 (MDH2), citrate synthase (CS), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), thiosulfate sulfurtransferase (TST), and aspartate aminotransferase (AST). Some of them, such as IDH2 (Someya et al. 2010; Yu et al. 2012), GDH (Lombard et al. 2007) and HMGCS2 (Shimazu et al. 2010) have been reported to be regulated by the deacetylation activity of SIRT3. Because acetylation, malonylation, and succinylation tend to occur on the same lysine residues (Du et al. 2011), it is very likely that these metabolic enzymes can also be regulated by SIRT5's desuccinylase activity. This hypothesis needs to be tested in future research.

The above discussion has mainly focused on metabolic enzymes in mitochondria because SIRT5 is localized mainly in the mitochondria. However, it is possible that SIRT5 could regulate other biological pathways. Zhao and co-workers reported that non-mitochondrial proteins were succinylated (Xie et al. 2012) and these proteins could be potential SIRT5 substrates. Localization information in the literature has to be taken with caution. SIRT5 is mainly in the mitochondria does not necessarily mean that it is not present in other cellular compartments. For example, SIRT6 was reported to be mainly in the nucleus, but we recently find that it is also associated with ER (Jiang et al. 2013). SIRT1 was reported to be mainly in the nucleus, but it was recently reported to be mainly in the cytosol in cancer cells (Andersen et al. 2011).

Although SIRT5 knock-out mice exhibit no obvious phenotype except for the defect in the urea cycle (Nakagawa et al. 2009), some indirect data show SIRT5 may have important roles in cancer cells. The SIRT5 gene was found to be localized to a chromosomal region associated with malignancies[8]. Strikingly, the analysis of copy number changes at the SIRT5 locus revealed significantly focal amplifications across 14 types of cancer (Beroukhim et al. 2010; Finley et al. 2011). In contrast, SIRT3 was focally deleted in many cancer cells like p53, a tumor suppressor known to be frequently deleted in many human cancers (Finley et al. 2011). Identifying the pathways that SIRT5 may regulate will be needed to further confirm the importance of SIRT5 in cancer cells.

6.4 SIRT5 Inhibitors

The discovery of SIRT5's more efficient desuccinylase/demalonylase activity also facilitated the development of SIRT5 inhibitors. SIRT5-specific inhibitors can be useful tools for studying the biological function of SIRT5 and for understanding the function of protein lysine succinylation/malonylation. Given the potential involvement of SIRT5 in cancer, SIRT5 specific inhibitors may also have therapeutic value.

Before SIRT5's more efficient activity was discovered, there were only a few inhibitors of SIRT5 reported, with one of them being suramin (Schuetz et al. 2007). This is at least in part due to the lack of robust deacetylation activity assay for SIRT5. The more efficient desuccinylation activity now provides a reliable assay for the development of SIRT5 inhibitors. In particular, this allowed the development of high-throughput assays in our own lab (unpublished) and in two other labs (Madsen and Olsen 2012; Peng et al. 2011). These high-throughput assays are all similar to

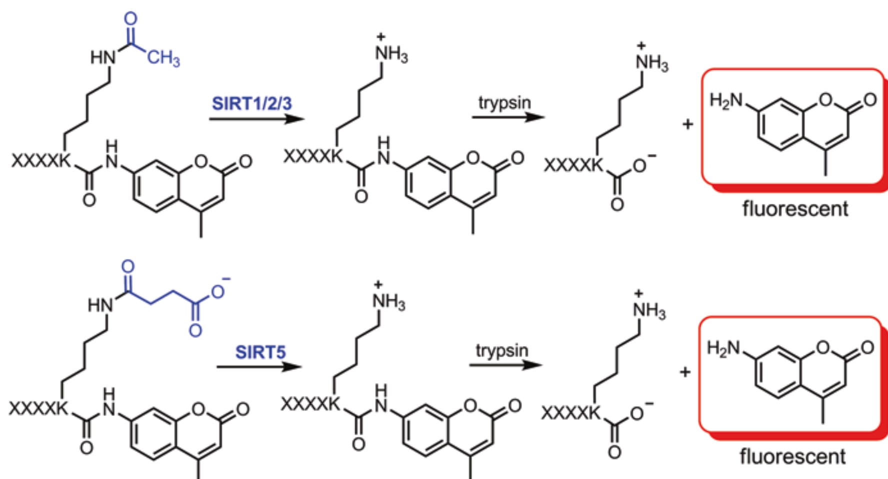

Fig. 6.2 The fluorogenic assay for the deacetylases (SIRT1, 2, and 3) using coumarin-acetyl peptides and the fluorogenic assay for SIRT5 using coumarin-succinyl peptides. X stands for variable amino acids

the reported fluorogenic assays for lysine deacetylases (Wegener et al. 2003), but with the acetyl group being replaced by succinyl. These assays should allow the screening of compound libraries for the discovery of SIRT5 inhibitors (Fig. 6.2).

SIRT5's unique acyl group preference also facilitated the rational design of SIRT5-specific inhibitors. Among the seven human sirtuins, SIRT5 is the only one that prefers negatively charged acyl groups (Du et al. 2011). We took advantage of this and developed thiosuccinyl peptides (H3K9TSu) as SIRT5-specific inhibitors (He et al. 2012). In vitro assays showed that H3k9TSu is the most potent and most selective SIRT5 inhibitor among all the known sirtuin inhibitors, including suramin. Kinetics and crystallography study showed H3K9TSu is indeed a mechanism-based and competitive inhibitor for SIRT5 (He et al. 2012; Zhou et al. 2012). Recently, several other SIRT5 inhibitors have been reported (Fig. 6.3), but none of them can match the selectivity of H3K9TSu for SIRT5 (Maurer et al. 2012; Suenkel et al. 2013). None of the reported SIRT5 inhibitors have been shown to be able to inhibit SIRT5 in cells, in part due to the unavailability of high quality succinyl lysine antibody and due to cell permeability issues (our H3K9TSu is not cell permeable). Therefore, more efforts are needed to develop SIRT5 inhibitors.

6.5 The Influence of SIRT5's New Activity on the Study of Other Sirtuins

Among the seven mammalian sirtuins, SIRT4, SIRT6, and SIRT7 also have weak deacetylase activity. The discovery of SIRT5's desuccinylase/demalonylase activity suggested that other sirtuins with weak deacetylase activity might also have more

	H3K9TSu	suramin
IC_{50} for Sirt5:	5 μM	25 μM
IC_{50} for other enzymes:	noinhibition for Sir1/2/3 at 100 μM	5 μM for Sirt1

	thiobarbiturate derivatives	GW5074
IC_{50} for Sirt5:	2.3 μM	20 μM
IC_{50} for other enzymes:	5.3 μM for Sirt1 9.7 μM for Sirt2	nM inhibitor for c-Raf μM for Sirt2

Fig. 6.3 Structures and IC_{50} values of reported SIRT5 inhibitors (He et al. 2012; Maurer et al. 2012; Schuetz et al. 2007; Suenkel et al. 2013)

efficient novel activities. Guided by this, we made peptides with various acyl lysine modifications and tested whether any other sirtuins could hydrolyze them efficiently. Using this approach, we found that one of the sirtuins in malaria parasite, Plasmodium falciparum, PfSir2A, can hydrolyze long chain fatty acyl groups more efficiently than acetyl (Zhu et al. 2012). Similarly, human SIRT6 also showed a preference for long chain fatty acyl groups (Jiang et al. 2013). Structures of Sirt6 and PfSir2A in complex with a myristoyl peptide showed that both sirtuins have a big hydrophobic pocket that can accommodate the long chain fatty acyl groups. Interestingly, we found that SIRT6 controls the fatty acylation of tumor necrosis factor α (TNFα) and regulates its secretion (Jiang et al. 2013). It is very likely that SIRT4 and SIRT7 also prefer other acyl lysine modifications as substrates and the study of their activities may reveal more new protein posttranslational modifications. In this sense, SIRT5 is an important sirtuin that have taught us a lot about the activity of sirtuins.

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Chapter 7
Diverse Roles for SIRT6 in Mammalian Healthspan and Longevity

Bernadette M.M. Zwaans*, William Giblin*, and David B. Lombard

7.1 Introduction

Aging is a conserved but poorly understood biological phenomenon. In diverse invertebrate and mammalian model organisms, advancing age is associated with functional decline and impaired stress resistance. In humans, most common afflictions – type 2 diabetes (T2D), cardiovascular disease, cancer, and neurodegeneration, among many others – are strongly associated with advancing age. Conversely, genetic and environmental interventions that promote increased longevity typically also delay or even prevent many age-associated pathologies. Therefore, an understanding of molecular mechanisms of aging offers the possibility of improved treatments for many common diseases. This realization has led to a hunt for pathways that regulate health- and lifespan, at least some of which function in a conserved manner across different phyla.

Sirtuins are a conserved family of NAD^+-dependent deacetylases/deacylases/ADP-ribosyltransferases that promote longevity in budding yeast, mammalian healthspan, and, in the case of at least two sirtuins (SIRT1 and SIRT6), extended lifespan in mice. The founding member of this family is Silent Information Regulator 2 (Sir2), a budding yeast protein that promotes chromatin silencing to regulate

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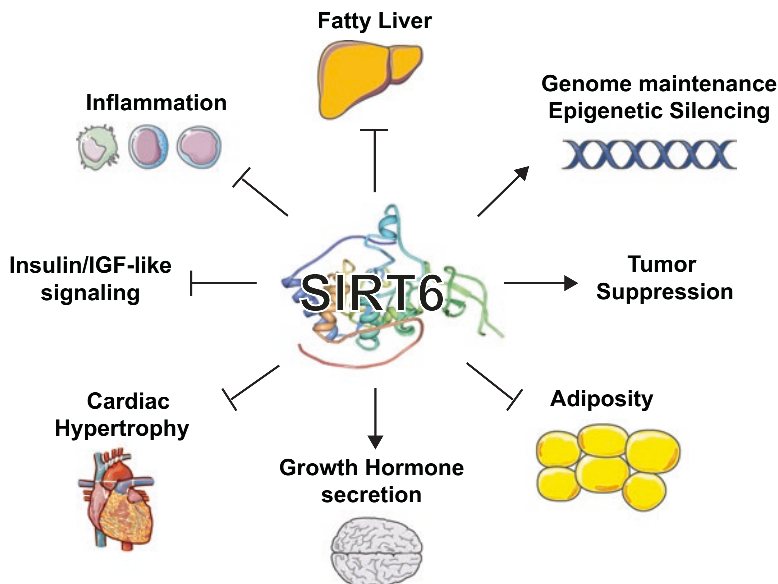

Fig. 7.1 Suppression of disease and promotion of healthspan by SIRT6. SIRT6 improves overall healthspan by impacting diverse physiological processes. SIRT6 crystal structure was obtained from RCSB Protein Data Bank (Pan et al. 2011) (Figure was produced using images from Servier Medical Art (www.servier.com))

genomic stability and increase replicative lifespan (Longo et al. 2012). Mammals possess seven sirtuins, termed SIRT1-SIRT7 (Frye 2000). Mammalian sirtuins share a conserved central catalytic domain, but differ at their N- and C-termini, domains that help to confer upon these proteins divergent biological properties. In this chapter, we focus on SIRT6, a mammalian sirtuin with a wide spectrum of biological functions, and its roles in suppressing diseases of aging (summarized in Fig. 7.1).

SIRT6 is a predominately nuclear protein that associates with heterochromatin (Liszt et al. 2005; Michishita et al. 2005; Mostoslavsky et al. 2006; Xiao et al. 2010). However, SIRT6 and the invertebrate homologue SIR-2.4 has been observed in the cytoplasm, where they are critical for formation of cytoplasmic stress granules (Jedrusik-Bode et al. 2013; Michishita et al. 2005; Simeoni et al. 2013). Biochemically, SIRT6 functions as a lysine deacetylase, a mono-ADP-ribosyltransferase, and a long-chain fatty acid deacylase (Jiang et al. 2013; Liszt et al. 2005; Mao et al. 2011; Michishita et al. 2008). *In vitro* studies suggest that SIRT6 possesses very modest deacetylase activity (Pan et al. 2011). However, *in vivo* SIRT6 functionally deacetylates at least four targets: two histone sites (H3K9 and H3K56) (Michishita et al. 2008; Michishita et al. 2009; Yang et al. 2009), and two non-histone proteins (CtIP and GCN5) (Dominy et al. 2012; Kaidi et al. 2010). SIRT6 mono-ADP ribosylates PARP1 (poly (ADP-ribose) polymerase 1) to promote DNA repair (Mao et al. 2011), and the nuclear corepressor protein,

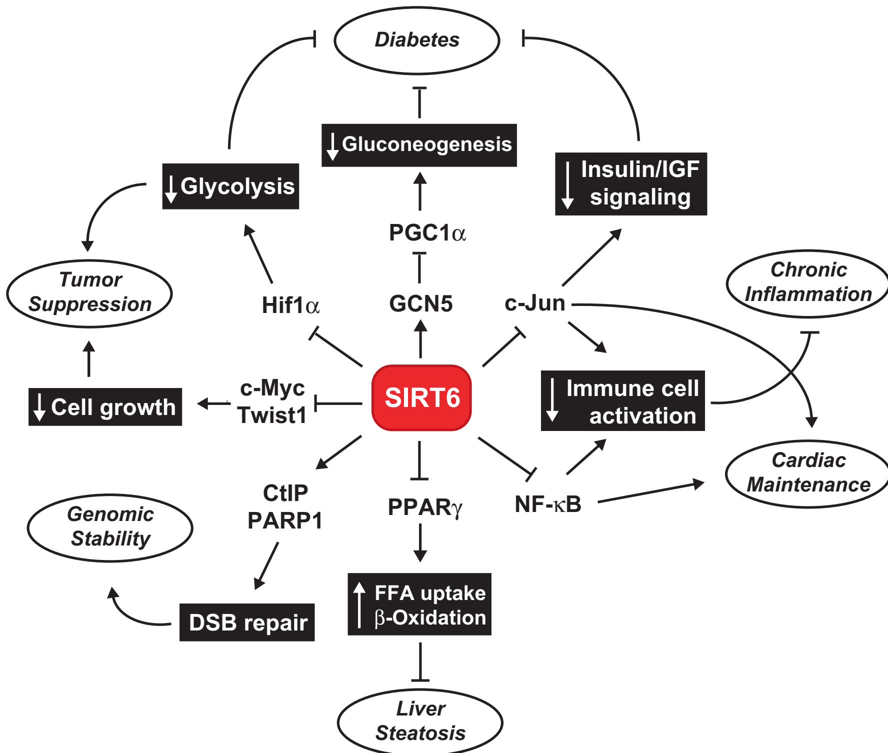

Fig. 7.2 Schematic overview of SIRT6 functions. Through its deacetylase, mono-ADP-ribosyltransferase, and deacylase activities, SIRT6 affects activities of transcription factors and other proteins, causing alterations in key cellular processes (*black boxes*). Through these activities, SIRT6 suppresses multiple metabolic and age-associated pathologies (*ovals*)

KAP1 (KRAB-associated protein 1), facilitating the interaction of KAP1 with HP1α to maintain the silent heterochromatic state of repetitive DNA (Van Meter et al. 2014). SIRT6 promotes secretion of the pro-inflammatory cytokine TNFα, via removal of long-chain fatty acyl groups in this protein (Jiang et al. 2013).

Initial insights into SIRT6 function derived from studies of SIRT6-deficient cells and mice (Mostoslavsky et al. 2006). SIRT6-deficient fibroblasts and ES cells show poor growth and genomic instability. *Sirt6* knockout (KO) mice appear normal at birth but soon manifest growth retardation, perhaps in part due to very low serum IGF1 levels. These animals display a premature aging-like phenotype and show severe pleiotropic defects, indicating that SIRT6 impacts multiple processes important for organismal health (summarized in Fig. 7.2). They are frail, with a hunched posture (lordokyphosis) and lose most of their white adipose tissue (WAT), including subcutaneous fat. At approximately two weeks of age, blood glucose levels begin to decline precipitously in *Sirt6* KO animals. These mice also show rapid depletion of lymphocytes via a massive wave of apoptosis. The latter phenotype represents a systemic rather than a cell-autonomous defect, as SIRT6-deficient bone

marrow cells are able to efficiently reconstitute the lymphocyte compartment of irradiated wild-type recipient mice (Mostoslavsky et al. 2006). Clearly, SIRT6 plays crucial roles in cellular and organismal homeostasis.

As described below, there has been significant recent progress in elucidating molecular functions of SIRT6. A common theme that has emerged is that SIRT6 negatively regulates the transcriptional output of key cellular signaling pathways by deacetylating histones at their target promoters. This is in contrast to SIRT1, the most well-studied sirtuin family member. Though SIRT1 can also deacetylate histones, it exerts many of its functions by directly deacetylating transcription factors and other non-histone targets themselves (Guarente 2011). Much of the more recent functional analysis of SIRT6 has been carried out using tissue-specific *Sirt6* KO animals, avoiding the early postnatal lethality associated with global SIRT6 deficiency, and permitting a finer dissection of SIRT6's roles. In this chapter we review various functions of SIRT6, and potential impacts of this protein on human disease.

7.2 SIRT6 Is a Key Metabolic Regulator

As noted above, SIRT6 is required for maintenance of glucose homeostasis, a role critical for organismal survival. Two different *Sirt6* germline KO mouse strains have been described (Mostoslavsky et al. 2006; Xiao et al. 2010). Both show severe hypoglycemia and greatly reduced serum IGF1 levels. On a pure 129SvJ strain background, under standard husbandry conditions, SIRT6 deficiency results in completely penetrant postnatal lethality by one month of age (Mostoslavsky et al. 2006). However, in an outbred background, a minority of SIRT6-deficient mice survives this hypoglycemia and lives into adulthood (Xiao et al. 2010). Glucose supplementation of these SIRT6-deficient mice further improves their survival, proving that hypoglycemia is a major cause of death in the absence of SIRT6 during postnatal development.

Recent work has elucidated multiple roles for SIRT6 in glucose homeostasis. SIRT6 controls blood glucose levels by regulating at least three distinct pathways: HIF-1α signaling, insulin/IGF-like signaling (IIS), and gluconeogenesis (Dominy et al. 2012; Xiao et al. 2010; Xiong et al. 2013; Zhong et al. 2010). HIF-1α is a major metabolic regulator; under low oxygen or glucose conditions, HIF-1α promotes a shift from oxidative metabolism to glycolysis (Koh and Powis 2012). HIF-1α promotes expression of multiple genes encoding proteins in the glycolytic cascade; conversely, HIF-1α drives increased expression of PDK1, in turn inhibiting carbon flow into mitochondria. The glucose transporter GLUT1 is another key HIF-1α target. SIRT6 functions as a repressor of HIF-1α transcriptional output by deacetylating H3K9 at the promoters of HIF-1α target genes, and also by reducing overall HIF-1α levels (the latter through mechanisms that remain obscure). Hence, in the absence of SIRT6, elevated HIF-1α activity results in increased glycolysis and glucose uptake, most evident in skeletal muscle and brown adipose tissue, culminating in hypoglycemia (Zhong et al. 2010). Increased glycolytic gene

expression has also been identified in the livers of hepatic-specific *Sirt6* KOs (Kim et al. 2010).

In addition to GLUT1, SIRT6-deficient mice show increased abundance of the glucose transporter GLUT4 at the cell membrane (Xiao et al. 2010). Unlike GLUT1, GLUT4 translocation to the plasma membrane is promoted by IIS. Despite lower serum insulin and IGF1 levels in *Sirt6* mutant mice, IIS is actually much more active in these animals (Sundaresan et al. 2012; Xiao et al. 2010). This may reflect the role for SIRT6 as a co-repressor of c-JUN, since c-JUN promotes expression of many genes involved in IIS (Sundaresan et al. 2012). c-JUN is a component of the activator protein 1 (AP-1) transcription factor, which is involved in diverse processes such as apoptosis, cell proliferation and development (Dunn et al. 2002).

SIRT6 also suppresses hepatic gluconeogenesis by deacetylating the acetyltransferase GCN5, activating it to acetylate PGC-1α (Dominy et al. 2012). PGC-1α is a transcriptional co-activator that functions as a regulator of mitochondrial biogenesis and other metabolic processes (Puigserver and Spiegelman 2003). In the liver, PGC-1α promotes gluconeogenesis by co-activation of *FoxO1* and *Hnf4* (Puigserver et al. 2003; Rhee et al. 2003). Acetylation of PGC-1α inhibits PGC-1α transcriptional activity (Rodgers et al. 2005). Hence, via activation of GCN5 (Lerin et al. 2006), SIRT6 inhibits hepatic glucose production (Dominy et al. 2012). The tumor suppressor p53 may also serve as an important regulator of gluconeogenesis, via regulation of a SIRT6/FOXO1 axis. P53 induces hepatic SIRT6 expression. SIRT6, in turn, promotes nuclear exclusion of the FOXO1 transcription factor, and subsequent downregulation of genes involved in gluconeogenesis. Knockdown of hepatic *Sirt6* expression renders mice resistant to the hypoglycemic effects of p53 (Zhang et al. 2014). The twin roles of SIRT6 in suppressing glycolysis while simultaneously inhibiting hepatic glucose output might superficially seem at odds with one another. These functions might be rationalized as a means to avoid futile cycling of glucose production and breakdown (Dominy et al. 2012).

The relationship between SIRT1 and SIRT6 in the context of PGC-1α acetylation is also intriguing. SIRT1 deacetylates PGC-1α to activate its transcriptional function, thus opposing the activity of SIRT6/GCN5 (Rodgers et al. 2005). Consistent with antagonistic functions of these sirtuins, protein kinase A (PKA) suppresses SIRT6 activity while stimulating SIRT1 function (Dominy et al. 2010; Gerhart-Hines et al. 2007; Nin et al. 2012). However, these findings seem inconsistent with the published role of SIRT1 in stimulating SIRT6 expression (Kim et al. 2010). Further studies are required to elucidate the precise interplay between SIRT1 and SIRT6 in response to varied dietary conditions.

In addition to this role in glucose homeostasis, SIRT6 also controls hepatic fatty acid metabolism by regulating expression of genes involved in this process, via H3K9 deacetylation at target promoters. SIRT6 suppresses accumulation of triglycerides in hepatocytes by inhibiting fatty acid uptake and synthesis, while promoting their breakdown via β-oxidation (Kim et al. 2010). A number of genes involved in these processes are regulated by the nuclear receptor PPARγ. PPARγ induces expression of genes that regulate lipid metabolism and adipocyte differentiation (Zhang et al. 2013a), including angiopoietin-like protein 4 (ANGPTL4) and

adipocyte fatty acid binding protein (A-FABP). ANGPTL4 negatively regulates lipoprotein lipase, which hydrolyzes serum triglycerides into free fatty acids, and thus mediates triglyceride clearance from the blood (Kim et al. 2010). A-FABP is a chaperone for cytosolic fatty acids, elevated levels of which are associated with obesity and metabolic syndrome (Xu et al. 2006). Furthermore, SIRT6 binds to the promoter of DGAT1, a key enzyme in triglyceride synthesis, repressing its expression (Kim et al. 2010). Through this mechanism, SIRT6 protects against fatty liver formation in response to a high fat diet (HFD). SIRT6 also inhibits pancreatic inflammation under these dietary conditions (Kanfi et al. 2010). Human fatty livers exhibit lower levels of SIRT6, and liver specific *Sirt6* knockout mice develop fatty liver and hypercholesterolemia, in particular LDL cholesterol (Tao et al. 2013a; Wu et al. 2014). Conversely, overexpression of SIRT6 protects the liver from excessive lipid accumulation (Kanfi et al. 2010) and lowers serum LDL cholesterol in response to a HFD by recruiting FOXO3 and repressing transcription of *Pcks9* and *Srebf1/2*, major regulators of cholesterol homeostasis (Elhanati et al. 2013; Tao et al. 2013b). The SREBPs (sterol regulatory element binding proteins) are transcription factors that bind the sterol regulatory element DNA sequence, and are required for cholesterol and fatty acid biosynthesis (Tao et al. 2013b). In addition to suppressing their expression, SIRT6 indirectly suppresses SREBP1/2 cleavage into their active forms, and SIRT6 inactivates SREBP1 through enhancing its phosphorylation (Elhanati et al. 2013). SIRT6 directly interacts with the circadian proteins CLOCK (circadian locomotor output cycles kaput) and ARNTL (aryl hydrocarbon receptor nuclear translocator-like, or BMAL) to modulate their recruitment to chromatin, thereby controlling circadian-dependent metabolism, including fatty acid synthesis and β-oxidation through inhibition of SREBP-1-mediated transcription of target genes (Masri et al. 2014).

Given the roles of SIRT6 in glucose and lipid homeostasis, it is perhaps not surprising that it plays protective roles against obesity and T2D, both common age-associated pathologies (Dominy et al. 2012; Schwer et al. 2010). Brain-specific *Sirt6* KO mice become obese in adulthood, associated with reduced levels of pituitary growth hormone and the hypothalamic factors proopiomelanocortin (POMC), single-minded homolog 1 (SIM1) and brain-derived neurotrophic factor (BDNF). These factors have all been linked to obesity in humans. SIRT6 deficiency in the brain causes hyperacetylation of H3K9 and H3K56, possibly leading to dysregulation of these and likely many other genes (Schwer et al. 2010). Furthermore, ectopic expression of SIRT6 in a mouse model of diabetes reduces hepatic glucose output and normalizes serum glucose levels (Dominy et al. 2012). Thus, roles of SIRT6 in regulating obesity-associated gene expression and glucose and lipid metabolism might conceivably be exploited therapeutically. It will be of great interest to assess roles for SIRT6 in other metabolically active tissues such as skeletal muscle, adipose tissue, and pancreatic β-cells. Similarly, it remains an outstanding question whether the depletion of WAT observed in global *Sirt6* KOs indicates a primary role for SIRT6 in maintaining WAT, or a secondary consequence of hypoglycemia and overall disordered metabolism in these animals.

7.3 Regulation of SIRT6

Despite its central role in maintenance of metabolic homeostasis, relatively little is known regarding regulation of SIRT6 expression and enzymatic activity. Like other sirtuins, SIRT6 requires the metabolic cofactor NAD^+ for activity. In response to fasting or long term calorie restriction, SIRT6 protein levels are elevated in brain, heart and WAT (Kanfi et al. 2008; Kim et al. 2010), promoting a metabolic switch from glycolysis to oxidative phosphorylation (Dominy et al. 2012; Kim et al. 2010; Xiao et al. 2010; Zhong et al. 2010). However, there is conflicting evidence regarding the underlying mechanism of altered *Sirt6* expression. SIRT6 levels are decreased in livers of obese and diabetic mice (Dominy et al. 2012). Conversely, *Sirt6* mRNA levels rise in liver and subcutaneous fat in response to severe weight loss; possibly due to decreased inflammation as TNFα can suppress *Sirt6* expression (Moschen et al. 2013). Likewise, SIRT6 protein levels increase in response to caloric restriction. Kanfi and colleagues reported that SIRT6 protein levels, but not mRNA levels, rise in response to fasting, due to stabilization of the SIRT6 protein (Kanfi et al. 2008). However, Kim and coworkers found that induction of *Sirt6* during fasting occurs transcriptionally and requires SIRT1 (Kim et al. 2010). They found that SIRT1 deacetylates FOXO3A to allow FOXO3A to form a complex with NRF1 and induce *Sirt6* gene expression (Kim et al. 2010). As noted above, PKA also inhibits SIRT6 expression, while simultaneously increasing SIRT1 levels (Dominy et al. 2010; Gerhart-Hines et al. 2007; Nin et al. 2012). The ubiquitin ligase CHIP (carboxyl terminus of Hsp70-interacting protein) ubiquitinates and consequently stabilizes SIRT6, by preventing SIRT6's interaction with other ubiquitin ligases (Ronnebaum et al. 2013). Likewise, SIRT6 is protected from proteasomal degradation by the ubiquitin-specific peptidase USP10 (Lin et al. 2013).

SIRT1 is regulated by a complex network of interactors and post-translational modifications (Revollo and Li 2013); analogously SIRT6 activity is governed by means other than regulation of expression levels. Physiological concentrations of free fatty acids increase SIRT6's catalytic activity *in vitro* (Feldman et al. 2013). SIRT6 is post-translationally modified in response to both oxidative and nitrosative stress, and the catalytic activity of recombinant SIRT6 is stimulated upon nitration of tyrosine 257 (Hu et al. 2015). A recent study suggested that SIRT6 is more active when interacting with nucleosomes than on isolated histones (Gil et al. 2013).

Other studies report alterations of SIRT6 levels and/or activity under various pathological conditions; however it remains unclear for the most part how this occurs mechanistically. In one case, recent data (discussed below) suggest that altered c-JUN and c-FOS signaling inhibit *Sirt6* expression during induction of hepatocellular carcinoma (Min et al. 2012). SIRT6 associates with nuclear chromatin and upon stress induction, *e.g.* TNFα treatment, SIRT6 relocalizes dynamically to different promoters (Kawahara et al. 2011). Similarly, SIRT6 relocalizes to sites of DNA damage, perhaps via interaction with DNA repair machinery (McCord et al. 2009). The interaction of SIRT6 with chromatin appears to be mediated at least in part by transcription factors. Kawahara and coworkers demonstrated that SIRT6

and RELA bind to a large panel of common promoter sites of genes involved in processes such as cell cycle progression, immune system development, suppression of apoptosis, and glycolysis. At a large fraction of these promoter sites, binding of SIRT6 was dependent on RELA (Kawahara et al. 2011). Also, SIRT6-bound promoters were enriched for several transcription factor motifs, notably SP1, STAT1/3 and FOXO1/4 (Kawahara et al. 2011) and thus it is reasonable to hypothesize that those transcription factors may also be necessary to recruit SIRT6 to these specific promoters in response to stimuli. Conversely, numerous post-translational modifications present on SIRT6 that have been identified by mass spectrometry (*cf.* www.phosphosite.org), whose functions have not yet been elucidated, could regulate the interaction of SIRT6 with specific transcriptional activators and/or repressors.

7.4 SIRT6 Regulates Inflammation

Increased sterile inflammation is a common feature of aging in many mammalian tissue types (Agrawal et al. 2009; Agrawal et al. 2010). Evidence from global and tissue-specific *Sirt6* KOs suggests that SIRT6 has important roles in limiting the inflammatory response. As part of their overall degenerative syndrome, SIRT6-deficient mice develop severe colitis with erosion of the intestinal mucosa (Mostoslavsky et al. 2006). In outbred SIRT6-deficient mice that survive hypoglycemic crisis, inflammation develops in the liver, where it eventually leads to fibrosis, as well as, to a lesser extent, in the kidneys, pancreas and lung (Xiao et al. 2012). In the context of hepatic inflammation, using tissue-specific knockouts, it was shown that SIRT6 in lymphocytes and macrophages, but not in hepatocytes, is required to suppress this phenotype. Liver inflammation coincides with increased expression of numerous pro-inflammatory genes, including *Mcp-1* and *IL-6*, in Kuppfer cells and T-cells. Mechanistically, SIRT6 binds the transcription factor c-JUN at the promoters of these pro-inflammatory genes, where it deacetylates acH3K9 and inhibits c-JUN transcriptional output (Xiao et al. 2012).

In addition to c-JUN, SIRT6 also inhibits the transcriptional output of NF-kB signaling resulting in decreased expression of genes involved in aging (Kawahara et al. 2009). NF-kB is a family of transcription factors implicated in multiple processes such as inflammation, cell death, proliferation, and development. The NF-kB protein family consists of five members: RELA (p65), RELB, c-REL, p50 and p52, among which RELA interacts with SIRT6. Under basal conditions, they are retained in the cytoplasm by an inhibitor of NF-kB (IkB) family member. In response to diverse stimuli, such as the inflammatory cytokine TNFα, NF-kB is released and consequently translocates to the nucleus where it can activate expression of its target genes. The transcriptional output of NF-kB is dependent on various co-regulators and chromatin modulators, including SIRT6 (Wan and Lenardo 2010). SIRT6 attenuates NF-kB mediated gene expression by deacetylating histone H3 lysine K9 (H3K9) at the promoters of NF-kB target genes (Kawahara et al. 2009). Reduced NF-kB signaling can partially rescue the lethality of SIRT6 deficiency (Kawahara et al. 2009).

Therefore, factors other then hypoglycemia may contribute to the lethality of SIRT6 deficiency.

Overexpression of SIRT6 can reduce arthritis in a collagen-induced arthritis mouse model by blocking NF-kB transcriptional output and consequently diminishes secretion of pro-inflammatory cytokines (Lee et al. 2013). SIRT6 may also mediate the inflammatory response through the pro-inflammatory cytokine TNFα, however their interplay is somewhat complex. Xiao and coworkers showed that TNFα protein levels are elevated in SIRT6-deficient macrophages under both basal and lipopolysaccharide stimulated conditions (Xiao et al. 2012). Conversely, Van Gool and colleagues reported that SIRT6 increases TNFα protein levels at a post-transcriptional level, indicating that under some conditions, SIRT6 may actually promote secretion of pro-inflammatory cytokines (Van Gool et al. 2009; Xiao et al. 2012). A recent study revealed that SIRT6 stimulates TNFα secretion by removing long-chain fatty acyl groups of lysine 19 and 20 in this protein (Jiang et al. 2013). Treatment of HeLa cells with TNFα increases SIRT6 translocation to NF-kB/RELA target promoters (Kawahara et al. 2009). Further studies are needed to clarify interactions between TNFα and SIRT6; it is possible that a negative feedback loop exists in which SIRT6 inhibits TNFα from stimulating NF-kB mediated transcription of pro-inflammatory genes.

In addition to roles for SIRT6 in modulating c-JUN and NF-kB function, the function of SIRT6 in metabolism may be relevant in its suppression of inflammation. Both lymphocytes and macrophages shift their metabolism from respiration to aerobic glycolysis upon activation (Ardawi and Newsholme 1982; Garedew and Moncada 2008). Conversely, cells that limit inflammation, such as regulatory T-cells, show relatively low levels of glycolysis (O'Neill and Hardie 2013). Therefore, it is possible that increased glycolysis occurring in the absence of SIRT6 preferentially drives activation of pro-inflammatory cells.

Overall, most studies have identified roles for SIRT6 in suppressing inflammation. Based on known functions of SIRT6, it is possible that SIRT6 activators might be useful in treating age-associated chronic inflammatory diseases such as diabetes, and cardiovascular and autoimmune diseases.

7.5 SIRT6 Promotes Genomic Stability via Diverse Mechanisms

Initial studies of SIRT6-deficient cells revealed that SIRT6 plays a major role in genome integrity (Mostoslavsky et al. 2006). *Sirt6* KO cells show reduced proliferation, an elevated incidence of chromosomal abnormalities, and increased sensitivity to DNA damaging agents. Originally it was hypothesized that SIRT6 might play a role in base excision repair (BER), pathways that repair small DNA lesions, including those induced by oxidative insult (Parsons and Dianov 2013). This hypothesis was based on the spectrum of sensitivities of SIRT6-deficient cells, as well as the

ability of the catalytic domain of Polβ, the major polymerase involved in BER, to rescue the genotoxin sensitivity associated with SIRT6 deficiency (Mostoslavsky et al. 2006). In support of an involvement of SIRT6 in BER, overexpression of SIRT6 can suppress oxidative DNA damage in porcine fetal fibroblasts, possibly by enhancing BER (Xie et al. 2012). SIRT6 protein levels dramatically decrease in human fibroblasts from older donors. SIRT6 is able to rescue the acquired BER defect in human fibroblasts from aged donors in a plasmid-reporter assay of BER efficiency. Rescue of the BER defect is dependent upon PARP1, and requires both the deacetylase and the ADP-ribosyltransferase activities of SIRT6 (Xu et al. 2015).

In contrast to the limited mechanistic insight into how SIRT6 facilitates BER, there has been significant progress made in understanding how SIRT6 promotes DNA double strand break (DSB) repair. DNA DSBs represent a severe threat to cell viability. They are repaired via three major pathways: classical non-homologous end-joining (C-NHEJ), homologous recombination (HR), and alternative end-joining (A-EJ) (Boboila et al. 2012). Overexpression of SIRT6 increases clearance of γH2AX foci and accelerates overall DSB repair, via multiple mechanisms (Mao et al. 2011). A recent study showed that, in response to DNA insult, SIRT6 is recruited to the breakage site where it interacts with the helicase SNF2H. This complex remodels chromatin around the DSB and recruits various DNA repair factors such as BRCA1 and 53BP1 (Toiber et al. 2013). SIRT6 stimulates both C-NHEJ and HR by activating PARP1 (Mao et al. 2011). PARP1 binds and stabilizes broken DNA ends, and mediates the recruitment of other DNA repair factors. In this context, ectopically-expressed SIRT6 can rescue the decline of HR capacity associated with replicative exhaustion (Mao et al. 2012). SIRT6 is required for optimal recruitment of the C-NHEJ factor DNA-PKcs to DNA DSB breaks, an effect potentially occurring via modulation of local chromatin structure by SIRT6 (McCord et al. 2009). SIRT6 promotes HR by deacetylating and activating CtIP, a factor required for DNA end resection to generate ssDNA for initiation of HR (Kaidi et al. 2010).

SIRT6 also deacetylates acH3K56 (Michishita et al. 2009; Yang et al. 2009). H3K56 acetylation levels are normally very low in mammalian cells, but increase dramatically in the setting of SIRT6 deficiency (Yang et al. 2009). Improper regulation of H3K56 acetylation in mammalian cells leads to impaired cell cycle progression, sensitivity to genotoxins and spontaneous DNA damage (Michishita et al. 2009; Yang et al. 2009; Yuan et al. 2009), phenotypes reminiscent of SIRT6 deficiency. The exact mechanism(s) through which deacetylation of acH3K56 by SIRT6 might repress DNA damage accumulation are not fully understood. In wild-type cells, SIRT6 is among the initial factors recruited to the damage site, where it rapidly deacetylates H3K56 (Toiber et al. 2013). It has been hypothesized that H3K56 acetylation is asymmetric around the replication fork during S-phase: acetylated behind the fork on newly synthesized DNA, and non-acetylated ahead of the replication fork. Thus, H3K56 acetylation may allow newly-replicated DNA to be distinguished from template DNA for proper targeting of HR during S-phase (Munoz-Galvan et al. 2013). H3K56 acetylation is also required for chromatin reassembly following DSB repair (Chen et al. 2008) and for recovery from post-repair checkpoint arrest following UV irradiation (Battu et al. 2011). In SIRT6-deficient

cells, which show dramatically elevated levels of H3K56 acetylation, it is possible that any or all of these processes may be perturbed. It should be noted however that SIRT6-deficient cells are not hypersensitive to UV, despite their dramatic H3K56 hyperacetylation (Mostoslavsky et al. 2006).

In addition to these roles for SIRT6 in promoting global genome stability, SIRT6 in human cells plays a role in stabilizing telomeres specifically. In normal human cells, SIRT6 deacetylates H3K9 and H3K56 at telomeres to promote telomeric heterochromatinization and association of telomeric binding proteins (Michishita et al. 2009; Tennen et al. 2011). Hyperacetylation of telomeric chromatin in the absence of SIRT6 disrupts the interaction of telomeric regions with WRN, a protein involved in telomere maintenance, which is mutated in the premature aging-like disorder Werner Syndrome. Telomeric attrition is a major cause of replicative senescence in human cells; indeed SIRT6 depletion in human fibroblasts causes premature cellular senescence (Michishita et al. 2009; Tennen et al. 2011). Furthermore, SIRT6 is essential for maintaining telomere position effect (TPE) (Tennen et al. 2011), a phenomenon by which telomere-proximal genes are silenced. TPE is lost with replicative aging in yeast. This role of SIRT6 is reminiscent of the function of yeast Sir2 in promoting heterochromatinization of the rDNA array and telomeric regions to suppress recombination and promote increased replicative lifespan (Dang et al. 2009; Longo and Kennedy 2006). In contrast, despite the fact that SIRT6 also deacetylates H3K9 at telomeres in mouse cells, telomeres are much longer in laboratory mice than humans, and thus do not apparently display dysfunction upon SIRT6 deletion (Michishita et al. 2008). It will be of interest to determine whether SIRT6 has roles in stabilizing other non-telomeric heterochromatic loci in mammalian cells. Overall, SIRT6 plays many roles through which it can maintain the genomic integrity of the cell.

7.6 SIRT6 Extends Mammalian Lifespan

Interest in the sirtuin protein family in the context of the biology of aging began with the observation that Sir2 overexpression in budding yeast extends longevity in this organism (Kaeberlein et al. 1999). Therefore, the finding that SIRT6 overexpression increases median and maximal lifespan in male (but not female) mice on an C57BL6/J-BALB/cOlaHsd mixed background represents an extremely significant milestone in sirtuin biology (Kanfi et al. 2012). The mechanisms underlying this effect are not entirely clear. Sahin and colleagues observed an age-dependent increase in methylation of the human *SIRT6* promoter, suggesting that *SIRT6* expression may decrease with age (a hypothesis that has not yet been directly tested) (Sahin et al. 2014). In this regard, an extra *Sirt6* gene copy might conceivably compensate for a normal age-associated reduction in SIRT6 levels. Reduced IGF1 levels are observed in male SIRT6 overexpressors; reductions in IIS are associated with increased lifespan in mice as well as invertebrates (Kenyon 2010; Lombard and Miller 2014). However, this effect is typically more pronounced in female animals,

whereas the impact of SIRT6 overexpression on lifespan is seen in male mice only. It is likely that other functions of SIRT6 may be relevant for its pro-longevity role. A higher incidence of spontaneous tumors is observed in male mice compared to females (Kanfi et al. 2012); thus, a tumor suppressor role of SIRT6 might explain why lifespan extension is only observed in male SIRT6-overexpressors (Lombard and Miller 2012).

Other roles of SIRT6 may also be relevant for its pro-longevity effects. The functions of SIRT6 in DNA repair, maintenance of genomic integrity and epigenetic silencing could contribute to increased longevity of SIRT6-overexpressing male mice. As described above, through its histone deacetylase activity, SIRT6 attenuates activities of HIF-1α and NF-kB. Both of these factors have been implicated in regulating aging. Data on HIF-1α and longevity are somewhat controversial. Deletion of HIF-1α can extend lifespan in *C. elegans* by inhibiting IIS (Zhang et al. 2009). However, others have reported that overexpression of HIF-1α causes lifespan extension, possibly by reducing mitochondrial respiration and thus ROS production, and/or by acting as a stress response factor (Mehta et al. 2009). Hence, both deletion and overexpression of HIF-1α may have beneficial, context-dependent effects, and it is possible that increased levels of SIRT6 could cause lifespan extension by inhibiting HIF-1α. Moreover, pharmacological inhibition of NF-kB signaling can extend lifespan in both male and female *Drosophila* (Moskalev and Shaposhnikov 2011). NF-kB activity increases with age in mammals, promoting increased tissue inflammation (Baker et al. 2011). Blocking the age-associated increase in NF-kB levels in the skin of aged mice reverts the gene expression profile to that observed in young animals (Adler et al. 2007). Inhibition of NF-kB signaling in the mammalian hypothalamus increases mouse lifespan (Zhang et al. 2013b). Therefore it is possible that SIRT6 overexpression might attenuate age-associated NF-kB-mediated inflammation, helping to preserve tissue function and/or centrally-regulated organismal metabolic homeostasis.

7.7 SIRT6 Suppresses Cardiac Hypertrophy and Promotes Cardiac Stress Resistance

We now turn to a discussion of SIRT6's roles in modulating age-associated disease. Cardiac hypertrophy is a condition characterized by cardiac enlargement, occurring either physiologically in response to normal stimuli such as pregnancy or exercise, or as a consequence of disease states (the latter referred to as pathological hypertrophy). Even in the absence of overt stress stimuli, thickening of the ventricular wall occurs with age. In humans, hypertension is a frequent cause of pathological cardiac hypertrophy. Age-associated cardiac hypertrophy is characterized by loss of cardiomyocytes, interstitial fibrosis, and hypertrophy of the remaining cells. Pathological cardiac hypertrophy and consequent ventricular dysfunction is thought to be a mostly irreversible process, which can eventually result in cardiac failure (Dai et al. 2012; Olivetti et al. 2000).

Mice induced to develop cardiac hypertrophy have elevated NF-kB activity in cardiomyocytes, and inhibition of RELA in these mice can prevent this phenotype (Gupta et al. 2008). Likewise, overexpression of SIRT6 ameliorates hypertrophy *in vitro* and inhibits the increase in hypertrophic marker genes in cardiomyocytes by repressing NF-kB gene expression (Yu et al. 2013). SIRT6 expression in rat cardiac fibroblasts is upregulated in response to pro-fibrotic stimuli: pressure overload or angiotensin II (Ang II) treatment. Cells treated with *Sirt6* siRNA display hallmarks of myofibroblast differentiation, such as increased cell proliferation, extracellular matrix deposition and higher expression α-smooth muscle actin (α-SMA). SIRT6 overexpression reverses α-SMA expression in *Sirt6* KD or Ang II–treated cardiac fibroblasts. NF-kB DNA binding and transcriptional activities are enhanced upon SIRT6 depletion. Pharmaceutical or siRNA-mediated inhibition of NF-kB reversed the expression of myofibroblast markers of differentiation, indicating that SIRT6 inhibits NF-kB signaling to block differentiation of cardiac fibroblasts (Tian et al. 2014). Sundaresan and colleagues showed that a large reduction in SIRT6 levels occurs in cardiac hypertrophy in both human and mouse hearts (Sundaresan et al. 2012). In contrast, Yu and coworkers reported that SIRT6 levels are elevated in cardiac hypertrophy in rats, but that this coincides with decreased SIRT6 activity due to a concomitant reduction in NAD^+ levels (Yu et al. 2013). Whole-body *Sirt6* KO and cardiomyocyte-specific *Sirt6* KO mice spontaneously develop cardiac hypertrophy as early as two months post-partum, characterized by increased cardiomyocyte size, degenerative cellular changes, and increased expression of aging-associated cytoskeletal proteins, as well as fibrotic and apoptotic markers. Conversely, SIRT6 overexpression protects animals against induction of cardiac hypertrophy (Sundaresan et al. 2012). In the absence of SIRT6, both c-JUN and NF-kB are hyperactive, and silencing either of these transcription factors can prevent hypertrophy *in vitro* (Gupta et al. 2008; Sundaresan et al. 2012; Yu et al. 2013).

The underlying mechanism(s) through which SIRT6 promotes cardiac health are not fully understood. Sundaresan and colleagues reported that SIRT6 protects against cardiac hypertrophy by inhibiting IIS, and *Sirt6* KO hearts showed elevated expression of proteins involved in this pathway. Furthermore, pharmacological inhibition of IGF1 signaling was able to protect *Sirt6* KO mice from development of cardiac hypertrophy (Sundaresan et al. 2012). Conversely, SIRT6 overexpression was able to decrease the expression of these proteins *in vivo*. Likewise, both mouse and human hypertrophic hearts showed increased levels of phosphorylated AKT and IGFR in comparison to controls, indicative of hyperactivity of the IIS pathway. Indeed, suppression of IIS in *Drosophila* can prevent the age-associated decline in cardiac performance. However, the role of IIS in cardiac health may be at odds with this mechanism, as previous studies have reported that age-dependent heart failure is associated with low serum IGF1 levels in elderly with no history of heart disease (Dai et al. 2012), and treatment of cardiomyocytes with a locally-produced IGF1 isoform can protect these cells from hypertrophy in a SIRT1 dependent manner (Vinciguerra et al. 2010). Therefore, the hypertrophy observed in *Sirt6* KO mice might not mimic age-related cardiac hypertrophy, and possibly other age-associated changes in elderly hearts could contribute to the opposing effects of IIS on cardiac health.

7.8 SIRT6 Is an Intestinal Tumor Suppressor

An increased incidence of neoplasia is a major feature of aging in mammals. SIRT6's functions in regulating glucose homeostasis, genomic stability and cellular senescence have prompted multiple groups to assess roles for SIRT6 in cancer. Recent work has revealed that SIRT6 functions as a tumor suppressor, at least in part by modulating cellular metabolism and cellular proliferation (Sebastian et al. 2012). SIRT6-deficient mouse embryonic fibroblast cell lines, immortalized by serial passage or p53 knockdown, show increased proliferation relative to controls. In contrast to controls, these cells are able to form colonies *in vitro* and tumors *in vivo*. These results are consistent with a cell-autonomous tumor suppressor role for SIRT6. Furthermore, conditional deletion of *Sirt6* in intestinal epithelial cells in the *Apc^{min}* adenomatosis model results in a 3-fold increase in the number of tumors, which are larger and of higher grade than lesions in littermate controls. *SIRT6* mRNA levels are reduced in human colorectal cancers (CRCs) relative to normal tissue, and the *SIRT6* gene is deleted in a substantial proportion of human cancer cell lines. These data support a tumor suppressor role for SIRT6 in mice, and potentially in humans as well (Sebastian et al. 2012).

Molecularly, SIRT6 deficiency leads to increased levels of glycolytic gene expression. Ribosomal biogenesis and glutaminase expression are also elevated in the absence of SIRT6; these are regulated by the proto-oncogene c-MYC. Mechanistically, SIRT6 interacts with c-MYC and deacetylates H3K56 at the promoters of c-MYC target genes, attenuating their expression. Inhibition of c-MYC in the absence of SIRT6 reduces cellular proliferation and inhibits tumor growth, indicating that SIRT6 acts as a tumor suppressor at least in part by inhibiting c-MYC activity (Sebastian et al. 2012).

In addition to targeting c-MYC, SIRT6 inhibits HIF-1α transcriptional activity resulting in decreased glycolysis (Sebastian et al. 2012; Zhong et al. 2010). Cancer cells, and other rapidly dividing cell types, shift their energy production from mitochondrial respiration to glycolysis and lactate production (Warburg effect) (Warburg 1956). Upon SIRT6 ablation, increased HIF-1α activity causes reprogramming of cellular metabolism by enhancing glucose uptake and glycolysis, conferring tumorigenic potential upon SIRT6-deficient cells (Sebastian et al. 2012). Conversion of pyruvate to lactate is the rate-limiting step in glycolysis, and blocking this step enhances mitochondrial respiration, reduces proliferation and inhibits colony formation of SIRT6-deficient cells. Treatment of adenoma-prone SIRT6-deficient mice with dichloroacetate (DCA), a small molecule that promotes mitochondrial respiration, reverts increased tumorigenesis of these mice *in vivo* (Sebastian et al. 2012). Finally, lower SIRT6 protein expression in locally metastatic CRCs is associated with increased propensity for relapse. Consistent with this finding, reduced SIRT6 expression has been reported in hepatocellular carcinomas (HCC), and low levels of SIRT6 are associated with more rapid recurrence in patients with this disease. Furthermore, liver-specific *Sirt6* KO mice show elevated expression of HCC biomarkers (*e.g.* AFP, IGF2 and H19) and re-expression of SIRT6 in HCC cell lines

sensitizes these cells to chemotherapeutic drugs resulting in increased apoptosis (Marquardt et al. 2013). This indicates that decreased SIRT6 expression in some tumors is correlated with more aggressive clinical behavior.

SIRT6 plays tumor suppressor functions in other contexts as well. Overexpression of SIRT6 has been reported to induce apoptosis specifically in cancer cells via activation of p53 or p73 (Van Meter et al. 2011). The tumor suppressor p53 is mutated or inactivated in most human cancers. Both p53 and its homolog p73 are involved in cell cycle regulation and induction of apoptosis (Murray-Zmijewski et al. 2006). Additionally, SIRT6 can prevent tumor formation in liver cells by blocking RELA-mediated expression of survivin to promote cell survival (Min et al. 2012). Survivin is a member of the inhibitor of apoptosis (IAP) family and inhibits cell death. It is mainly expressed during embryogenesis and, with the exception of a few cell types, is not normally expressed in adult tissue (Church and Talbot 2012). In human dysplastic liver nodules, c-JUN interferes with c-FOS transcriptional output. As *Sirt6* is a target of c-FOS, c-JUN thereby inhibits *Sirt6* expression. Decreased SIRT6 levels correlate with increased acH3K9 levels at the survivin promoter, allowing for increased NF-kB-driven expression of this gene (Min et al. 2012). Survivin is only upregulated during the initiation phase of liver cancer, as survivin levels were not altered in normal livers or in advanced hepatic carcinomas (Min et al. 2012). Furthermore, a recent study showed that *SIRT6* mRNA levels are decreased in human non-small cell lung cancer (NSCLC), and that overexpression of SIRT6 in lung cancer cell lines decreased cellular proliferation by inhibiting the expression of the oncogenic transcription factor TWIST1 (Han et al. 2014). Finally, in breast cancer cell lines, SIRT6 is phosphorylated by AKT and consequently degraded by MDM2. Inhibition of MDM2-mediated degradation of SIRT6 suppresses cellular proliferation, and low levels of phosphorylated SIRT6 (Ser 338) are positively correlated with breast cancer patient survival (Thirumurthi et al. 2014). These findings indicate that SIRT6 functions as a tumor suppressor in multiple tissues, and suggest that SIRT6 activators could be useful therapeutic tools for cancer treatment, potentially in both early-stage and advanced lesions.

In contrast to the tumor suppressor function of SIRT6 in colorectal and hepatic carcinomas (Khongkow et al. 2013; Marquardt et al. 2013; Sebastian et al. 2012) elevated SIRT6 levels are associated with chemotherapeutic drug resistance in MCF-7 breast cancer cells (Khongkow et al. 2013). While SIRT6 expression is elevated in several human tumor types (Bauer et al. 2012; Khongkow et al. 2013; Liu et al. 2013), *SIRT6* knockdown specifically in prostate cancer cells sensitizes them to chemotherapeutic agents and decreases cellular survival (Liu et al. 2013). Deacetylation of the tumor suppressor proteins FOXO3A and p53 by SIRT6, resulting in reduced cell cycle arrest and apoptosis in response to chemotherapeutic treatment, has been proposed to explain this effect. However, importantly there currently is no direct evidence *in vitro* that SIRT6 can deacetylate these proteins. Khongkow and colleagues reported that high nuclear levels of SIRT6 correlated with a poor prognosis in breast cancer patients (Khongkow et al. 2013). This group also identified a fraction of SIRT6 in the cytoplasm; in contrast to nuclear SIRT6, cytoplasmic SIRT6 levels correlate with a better clinical outcome in breast cancer. It will be of

interest to determine the identities of the cytoplasmic proteins that SIRT6 targets in breast carcinoma cells – for example, whether SIRT6 regulates stress granule formation in this context – or alternatively whether SIRT6 localization in this compartment represents simply a means to attenuate nuclear SIRT6 function.

SIRT6 also appears to play an oncogenic role in the skin. In squamous cell carcinoma (SCC), SIRT6 expression is negatively regulated by mIR-34a; mIR-34a promotes differentiation of benign and malignant keratinocytes, whereas SIRT6 is highly expressed in SCC and contributes to maintenance of the undifferentiated state (Lefort et al. 2013). Similarly, another study found that SIRT6 suppresses AMPK signaling and enhances COX-2 expression in keratinocytes in response to UV irradiation, thereby promoting inflammation, survival, and oncogenesis (Ming et al. 2014). Overall, the tumor suppressor and oncogenic functions of SIRT6 likely reflect the diverse substrates and roles of this protein. Given the underlying complexity of SIRT6 function, it is perhaps not surprising that the roles of SIRT6 in neoplasia are context- and tissue-specific, a phenomenon which has also been described for the sirtuin proteins SIRT1 and SIRT3 (Morris 2013).

7.9 Conclusion

SIRT6 is a multi-faceted protein that maintains organismal healthspan via diverse molecular roles in mammals. Current data point to mammalian SIRT6 as a functional ortholog of the yeast Sir2 protein; like yeast Sir2, SIRT6 promotes heterochromatin stability and increased longevity. SIRT6 attenuates the transcriptional output of key transcription factors such as HIF-1α, c-JUN, and c-MYC to regulate cellular processes such as glucose and adipose tissue metabolism, cellular senescence, and inflammation. SIRT6 also promotes genome integrity through multiple mechanisms, potentially contributing to lifespan extension. Loss of SIRT6 exerts severe pathological consequences: cardiac hypertrophy, diabetes, liver steatosis, chronic inflammation and cancer.

Since sirtuins require NAD^+ for their enzymatic activity, an age-associated decline in NAD^+ levels would be predicted to impair SIRT6 function and potentially recapitulate the pathological effects of SIRT6 deficiency or knockdown. Measurements of NAD^+ levels have been reported in several studies; NAD^+ decreases with age in multiple tissues in worms, rodents, and humans (Braidy et al. 2011; Braidy et al. 2014; Massudi et al. 2012; Mouchiroud et al. 2013; North et al. 2014; Ramsey et al. 2008). An emerging literature suggests that SIRT6 overexpression or restoration of NAD^+ may be beneficial in mouse models of metabolic disease and other pathological states (Canto et al. 2012; Gomes et al. 2013; North et al. 2014; Stein and Imai 2014). Current research is focused on the discovery and characterization of putative SIRT6 activators with the goal of mitigating age-associated disease processes and perhaps promote longevity. Small molecules that supplement cellular NAD^+ levels extend lifespan in *C. elegans*, and could play beneficial roles in mammals via activation of SIRT6 and other sirtuins.

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Chapter 8
Sirtuins in Cancer – Emerging Role as Modulators of Metabolic Reprogramming

Jaewon J. Lee, Karina N. Gonzalez Herrera, and Marcia C. Haigis

8.1 Introduction

With a global increase in life expectancy, the incidence and prevalence of age-related diseases have also increased. As such, cancer is no longer a disease of developed countries but rather a global entity, and it has become one of the leading causes of death around the world. This increase, however, is not only as a result of the aging global population (resulting from decreased mortality in youth and increased survival of the elderly) in developing countries, but also from the adoption of western lifestyles such as smoking, physical inactivity, dietary excess, and changes in reproductive timetables. For instance, in 2008, cancer incidence was 12.7 million with 5.6 million new cases (44 %) arising from developed countries and 7.1 million new cases (56 %) from developing countries. Disparity in cancer mortality was even greater, with 2.8 million deaths (37 %) of 7.6 million total deaths occurring in developed countries and 4.8 million deaths (63 %) in developing countries (American Cancer Society 2011).

These statistics highlight the importance of continuing research on cancer not only to identify causative mechanisms but also to develop treatment options that are efficient and readily available. Hence, cancer research has become a multibillion-euro industry with special focus on understanding the disease etiology as well as identifying new modes of detection, prevention, and treatment (Eckhouse et al. 2008). An emerging area of research focuses on the reprogramming of metabolism in cancer cells, first noted by Otto Warburg in the 1920s (Warburg et al. 1927). Warburg observed that cancer cells have elevated glucose consumption even with ample oxygen, which would normally allow for oxidative mitochondrial metabolism, a finding that has been verified and substantiated in a wide range of cancers.

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From this initial observation, an incredible amount of research has continuously supported the active role of metabolic alterations in cancer.

Sirtuins are important cellular nutrient/metabolic sensors given that their deacylase/ADP-ribosyltransferase activities are dependent on nicotinamide adenine dinucleotide (NAD). As such, sirtuins exist in a constant feedback loop with input from various metabolic pathways leading to altered levels of activity and an output in modulating metabolic homeostasis. Mammalian cells contain seven sirtuin proteins, SIRT1-7, which reside in different subcellular compartments and have unique biological functions (Haigis and Guarente 2006). Recently, sirtuins have been discovered to regulate cancer cell metabolism, both by direct modulation of metabolic enzymes and indirect regulation of metabolic pathways, i.e., expression of metabolic genes. In this chapter, we will discuss the role of sirtuins in tumor cell metabolism.

8.2 Cancer Cell Metabolism: Understanding the Biology

8.2.1 Glucose Metabolism

Normal cells metabolize glucose efficiently: a single molecule of glucose is broken down into two molecules of pyruvate through glycolysis, producing two molecules of adenosine-5'-triphosphate (ATP) – the main form of energy within the cell – then the pyruvate molecules undergo oxidative metabolism in the mitochondria to produce 32–36 additional molecules of ATP. Mitochondrial metabolism of carbohydrates is highly dependent on the presence of oxygen as the molecular oxygen (O_2) is the final electron acceptor from the electron transport chain (ETC), and it is the coupling of the proton gradient established by the ETC and oxidative phosphorylation via F_0F_1-ATP synthase (complex V) that is responsible for the majority of energy produced within the mitochondria. ATP molecules in the resting cells are used for maintenance of cellular homeostasis and survival, such as protein turnover and preservation of the membrane potential.

Importantly, cellular metabolism is flexible, and cells must be able to maintain energy production even during times of stress. For instance, when oxygen supply to the tissue becomes limited, the capacity of oxidative metabolism is reduced. If mitochondrial metabolism of pyruvate were to continue, the excess electrons would wreak havoc in the cells. To maintain adequate energy levels with limited O_2, cells shift metabolism toward glycolysis by activating hypoxia inducible factor-1 (HIF1), a major transcriptional regulator of the hypoxic response (Kaelin and Ratcliffe 2008; Semenza 2010). The labile α subunit of HIF1 is normally stabilized with low molecular oxygen levels: under normoxia, HIF1α is hydroxylated by prolyl hydroxylases (PHDs), ubiquitinated by the von Hippel-Lindau (VHL) protein and targeted for rapid degradation by proteasomes (Kaelin and Ratcliffe 2008; Semenza 2010). However, HIF1α can be aberrantly stabilized under normoxia in certain diseases,

such as from VHL inactivating mutations in von Hippel-Lindau syndrome. PHDs can also be inactivated by the accumulation of (proto)oncometabolites fumarate and succinate, as well as reactive oxygen species (ROS) and ferric iron (replacing the ferrous iron required for PHD catalytic activity), thereby stabilizing HIF1α and upregulating HIF1 transcriptional activity even under normoxia (Kaelin and Ratcliffe 2008; Semenza 2010). Once activated, HIF1 concomitantly upregulates the expression of glycolytic genes (e.g. *GLUT1*, *HK2*, and *PFK1*) and suppresses the entry of pyruvate into the mitochondria (via *LDHA* and *PDK1*), thereby repressing oxidative mitochondrial metabolism and minimizing the potential damage. Therefore, physiologic HIF1 plays a pro-survival role by appropriately rewiring cellular metabolism based on external cues to maintain energetic homeostasis (Kaelin and Ratcliffe 2008; Semenza 2010). It is important to note, however, that the cellular response to hypoxia is not sustainable and prolonged depletion of oxygen can lead to cell death (Gatenby and Gillies 2004).

Intriguingly, some of the metabolic changes observed during hypoxia mirror the metabolic reprogramming in many cancer cells. Otto Heinrich Warburg (1883–1970) first observed in the 1920s that cancer cells consume more glucose and secrete more lactate compared to normal cells even in the presence of oxygen, something he incorrectly hypothesized was due to mitochondrial defects in cancer cells (Vander Heiden et al. 2009; Warburg et al. 1927). This aerobic glycolysis, now termed the Warburg effect, at first glance appeared counterintuitive and wasteful given that the cells are not utilizing the available nutrients to their maximal capacity for energy production. However, proliferative cancer cells still rely on mitochondrial function to produce most of their ATP. In fact, proliferation itself is an ATP-dependent process and biosynthetic pathways require energy, more so than when the cells are quiescent (Cantor and Sabatini 2012; Ward and Thompson 2012).

Why do cancer cells increase their glycolytic flux to such an extent? Current evidence suggests that it may be less about efficient energy production and more about creating biomass (Lunt and Vander Heiden 2011). Enhanced glycolysis drives proliferation, as glycolytic intermediates can be shuttled towards biosynthetic pathways, e.g., pentose phosphate pathway, which is important for nucleotide synthesis. The pentose phosphate pathway also produces reduced nicotinamide adenine dinucleotide phosphate (NADPH), a critical cofactor required for biosynthetic pathways due to its reducing potential, as well as for maintaining redox balance within the cell (important metabolic changes in glycolysis, as well as in glutamine metabolism and fatty acid metabolism, are summarized in Fig. 8.1). Hence, while increasing the glycolytic flux and secreting the excess lactate appears wasteful, metabolic rewiring is valuable to proliferative cancer cells, especially when nutrients are readily available (Lunt and Vander Heiden 2011).

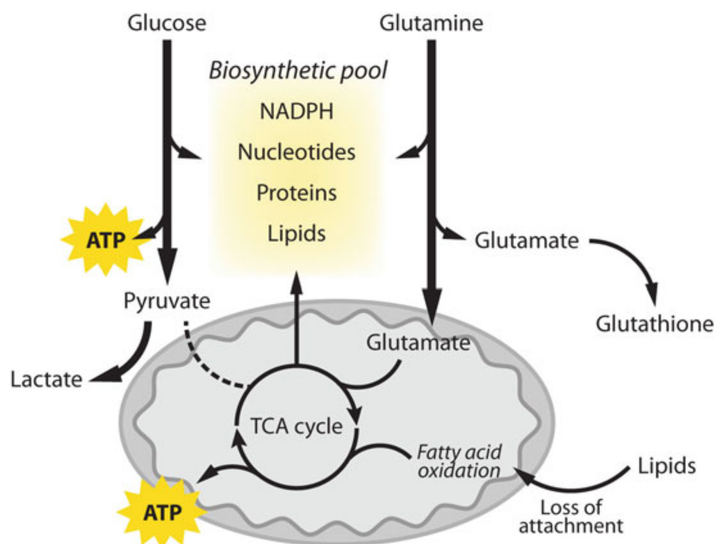

Fig. 8.1 Reprogramming of cellular metabolism commonly observed in cancer cells. In many cancers, metabolism of glucose via glycolysis is upregulated, and the excess pyruvate is converted to lactate, which is secreted from the cells. Glutamine uptake and glutaminolysis (breakdown of glutamine into TCA cycle intermediates) are also commonly increased. The extra glutamine can be used in glutamine anaplerosis or for glutathione synthesis to maintain the intracellular redox state. Intermediates of glycolysis and glutamine itself can be diverted into the biosynthetic pool, thereby promoting the syntheses of NADPH, nucleotides, proteins, and lipids. Under certain metabolic stress conditions, such as during loss of attachment, fatty acid oxidation is increased to maintain ATP production in the mitochondria to promote cell survival

8.2.2 Glutamine Metabolism

Upregulation of aerobic glycolysis is not the only metabolic reprogramming important to tumor cell proliferation. Another crucial metabolic shift in cancer is the contribution of mitochondrial tricarboxylic acid (TCA) cycle intermediates into biosynthetic pathways. Given that these metabolites may be shuttled out of the mitochondria, yet cancer cells still depend on mitochondrial metabolism for generation of ATP, the mitochondrial intermediates must be replenished in a process called anaplerosis, in which TCA cycle metabolites are produced from glucose or glutamine (Cantor and Sabatini 2012; Lunt and Vander Heiden 2011). In the mitochondria, glutamine is converted to glutamate then to α-ketoglutarate, an intermediate in the TCA cycle, by glutaminase and glutamate dehydrogenase, respectively. Alternatively, glutamate can be converted to α-ketoglutarate via alanine transaminase or aspartate transaminase to replenish TCA cycle intermediates. Under certain circumstances (e.g. hypoxia), α-ketoglutarate can undergo reductive carboxylation to form citrate, which is normally formed by the combination of glucose-derived acetyl-CoA and oxaloacetate (Wise et al. 2011). Citrate can then be shuttled out of

the mitochondria and into the cytosol, where ATP citrate lyase catalyzes its breakdown into oxaloacetate and acetyl-CoA, the latter of which is a precursor for *de novo* lipid synthesis (Finley et al. 2013).

Of note, glutamine, which is normally a nonessential amino acid, becomes essential in many proliferative cells. It is the most abundant amino acid found in the plasma, and it supports biosynthesis in conjunction with glucose by providing not only the TCA cycle intermediates, as discussed above, but also by directly contributing its carbon backbone and nitrogen atoms (glucose and other hexoses do not contain nitrogen atoms) for synthesis of macromolecules to support cellular growth and proliferation. Hence, increased glutamine uptake and glutaminolysis are key features observed as part of the metabolic reprogramming in cancer cells. Glutamine enters the cell via high-affinity glutamine transporters, such as ASCT2 (*SLC1A5*) and SN2 (*SLC38A5*), and their increased expression is evident across various types of tumors (Daye and Wellen 2012; Matés et al. 2009). Once inside the cell, glutamine can donate its nitrogen atoms for several biosynthetic pathways including those for hexosamines, nucleic acids, and other amino acids. Hexosamines, such as uridine diphosphate *N*-acetylglucosamine, contribute to glycosylation of cell surface receptors and promote further glutamine uptake to support cellular proliferation. Nucleotide synthesis is of course required for proliferation as the entire genome of the cell needs to be duplicated and RNA transcription for protein synthesis needs to be upregulated. Nitrogen atoms from glutamine are used in five steps of nucleotide synthesis – two steps in pyrimidine synthesis and three in purine synthesis. Like nucleotides, the demand for protein synthesis in proliferating cells is also increased, and glutamine contributes to the formation of asparagine and other amino acids required for translation. In this manner, glutamine is essential to maintain the pool of nucleotides and nonessential amino acids for DNA/RNA and protein syntheses, respectively, which are necessary in larger quantities during growth and proliferation (Daye and Wellen 2012; Matés et al. 2009).

In addition to its biosynthetic roles, glutamine can regulate cellular redox homeostasis in several ways. While it can upregulate mitochondrial ROS formation via oxidative decarboxylation, glutamine contributes to the production of glutamate, cysteine, and glycine, which are the three amino acids required for the synthesis of glutathione tripeptide, a critical cellular ROS detoxification molecule (Lu 2013). Not only is glutamate used for glutathione synthesis itself, but it is also exchanged for the import of cysteine, the rate-limiting amino acid of glutathione synthesis, by the cysteine/glutamate exchanger xCT (*SLC7A11*). Inside the cell, glutathione exists in a reduced (GSH) or oxidized (GSSG) form. Glutathione becomes oxidized as it reduces ROS, and the restoration of reduced GSH is catalyzed by glutathione reductase, a NADPH-dependent enzyme. The conversion of malate to pyruvate by malic enzyme, via glutaminolysis, serves as an additional source of NADPH (the other major source being the pentose phosphate pathway as previously mentioned). Thus, glutamine regulates redox state by contributing to glutathione levels and producing NADPH for glutathione reduction (Daye and Wellen 2012; Matés et al. 2009).

8.2.3 Lipid Catabolism

The least well-understood aspect of cancer metabolism is the contribution of fatty acid oxidation (FAO) to tumorigenesis. In normal cells, the balance between FAO and fatty acid synthesis (FAS) is tightly controlled by the metabolite malonyl-CoA, which stimulates lipogenesis while suppressing β-oxidation (Saggerson 2008), to meet the different cellular needs for fat synthesis and oxidation during fed and fasted states, respectively. While FAS has been well studied in conjunction with increased glycolysis and glutaminolysis in cancer cells, the role of FAO in cancer is only beginning to emerge. Intriguingly, FAO is indeed important for cancers, most often in times of metabolic stress (Carracedo et al. 2013). For instance, during loss of attachment, a critical aspect of transformation (anchorage-independent growth), cells do not take up any glucose, leading to decreased ATP and NADPH levels, as well as increased levels of ROS (Schafer et al. 2009). In such cases, energetic needs must be met by other sources or the cell undergoes an energetic crisis and ultimately dies. The increased ROS production during loss of attachment inhibits FAO, which could be the alternate fuel source. Therefore, increased oxidant scavenging rescues the FAO defect and prevents anoikis (programmed cell death after matrix detachment), thereby promoting tumorigenesis (Schafer et al. 2009). In addition, promyelocytic leukemia (PML) protein promotes breast cancer formation, also by upregulating FAO and preventing anoikis, further supporting the importance of FAO during loss of attachment (Carracedo et al. 2012). Another potential role of FAO, especially in cancers that do not require matrix attachment such as leukemia, is promoting cell survival, possibly through BAX- and BAK-dependent intrinsic programmed cell death (Samudio et al. 2010). Finally, FAO can contribute to the production of NADPH via production of acetyl-CoA (end product of β-oxidation in the mitochondria), which is converted to citrate and utilized in metabolic reactions that produce NADPH (Jeon et al. 2012). Taken together, current evidence suggests that FAO becomes especially critical when normal cells transition into a cancerous state, representing a transient reprogramming of metabolism.

Additional evidence suggests that some cancer cells may depend on external sources of lipids for growth. For instance, certain oncogene-driven cancers (e.g. H-Ras^{V12G}) and hypoxic cells may rely on lipid scavenging for proliferation rather than relying on *de novo* synthesis (Kamphorst et al. 2013). Furthermore, FAO may not be the only pathway in which cancer cells utilize lipids for energy production. In K-Ras^{G12D}-driven p53-null lung cancer, lipid catabolism becomes dependent on autophagy, a process that degrades and recycles intracellular components, as mitochondrial function is impaired in these cells. Blocking autophagy by deleting the necessary gene (*atg7*) causes the cancer cells to accumulate lipid-filled vesicles as the lipids cannot be broken down (Guo et al. 2013). This area of cancer metabolism is only beginning to be explored in depth, and future research will definitively elucidate the relevant contributions of FAO and FAS to tumorigenesis and cancer progression.

8.3 Sirtuins and Cancer Metabolism

Sirtuins function as critical modulators of posttranslational modification based on metabolic input as their catalytic activities depend on the presence of NAD. While the traditional role of sirtuins in cancer has been attributed to the direct posttranslational modifications of oncogenes and tumor suppressors, their deacylase activities also regulate proteins involved in global metabolic pathways crucial for cancer cell proliferation. In the following sections, we will discuss the contributions of mitochondrial and extramitochondrial sirtuins to the regulation of metabolic reprogramming in cancers.

8.3.1 Mitochondrial Sirtuins and Cancer Metabolism

Mitochondria, primarily known for being the powerhouses of the cell, play a key role in cancer through their ability to produce ATP and contribute to biosynthetic pathways. Three of the seven mammalian sirtuins, SIRT3, SIRT4, and SIRT5, reside in the mitochondria where they regulate metabolism via posttranslational modification of various proteins, including metabolic enzymes. SIRT3 remains the best-characterized mitochondrial sirtuin, and its loss drives a Warburg-like metabolic reprogramming (Fig. 8.2a). In addition, SIRT4 plays an important role in preventing a metabolic shift towards tumorigenesis via a mechanism distinct from SIRT3

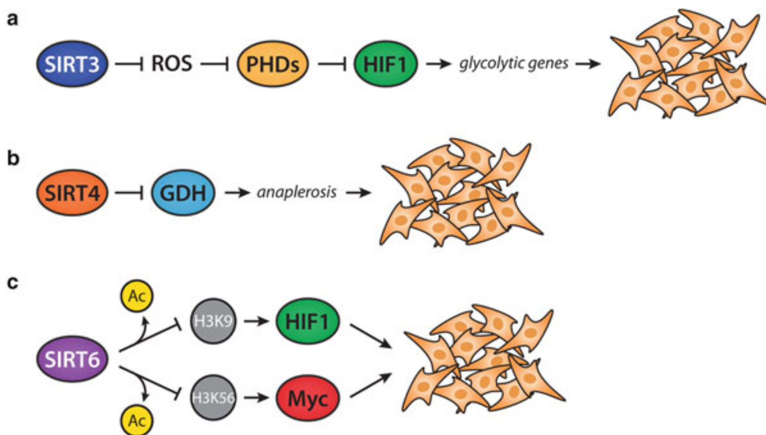

Fig. 8.2 Confirmed involvement of mammalian sirtuins in regulating cancer cell metabolism. (**a**) SIRT3, by decreasing mitochondrial and cellular ROS, destabilizes HIF1α and represses aerobic glycolysis. (**b**) By inhibiting GDH, SIRT4 decreases glutamine anaplerosis and tumorigenesis. (**c**) SIRT6 deacetylates H3K9 and H3K56 in the promoter regions of HIF1- and Myc-target genes, respectively, resulting in decreased activities of these cancer-associated transcription factors

(Fig. 8.2b). Of the mitochondrial sirtuins, SIRT5 biology remains the least studied and has not been reported to regulate cancer cell metabolism.

8.3.2 SIRT3 Regulates HIF1α Stability

As a major mitochondrial deacetylase, SIRT3 targets and activates a variety of mitochondrial proteins including the components of the electron transport chain, other metabolic enzymes, and proteins contributing to the maintenance of ROS and the redox state of the cell. Collectively, SIRT3 substrates promote efficient fuel usage by oxidative metabolism from a variety of metabolic sources including fatty acids, amino acids, and glucose (Finley and Haigis 2012).

In addition to activating oxidative mitochondrial metabolism, SIRT3 serves to decrease ROS levels through several mechanisms. SIRT3 plays an important role in mitochondrial ROS scavenging pathway, as it activates manganese superoxide dismutase (SOD2) as well as isocitrate dehydrogenase 2 (IDH2), which is responsible for the production of mitochondrial NADPH (Qiu et al. 2010; Someya et al. 2010; Tao et al. 2010). By activating electron transport chain proteins, SIRT3 also decreases ROS production during oxidative phosphorylation (Bause and Haigis 2013; Finley and Haigis 2012; Sena and Chandel 2012).

Given the pro-tumorigenic potential of ROS, SIRT3 and its antioxidant functions have been implicated in the suppression of tumorigenesis (Bell et al. 2011; Finley et al. 2011; Kim et al. 2010). SIRT3-null mouse embryonic fibroblasts (MEFs) proliferate faster than wild type MEFs, and while SIRT3$^{-/-}$ mice are viable and healthy at birth and throughout life, there is an increased incidence of age-related mammary tumors in these mice compared to their wild type littermates (Kim et al. 2010). The tumors from SIRT3$^{-/-}$ mice accumulate more protein damage, indicating that the oxidative stress caused by the loss of SIRT3 contributes to tumorigenesis (Kim et al. 2010).

While SIRT3 was initially shown to suppress cancer by preventing ROS-mediated genotoxic stress, further studies have revealed that SIRT3 also inhibits the metabolic rewiring commonly seen in cancer cells (Bell et al. 2011; Finley et al. 2011). In MEFs, the loss of SIRT3 leads to increased glucose consumption and lactate secretion. While SIRT3$^{-/-}$ MEFs have lower complex I and complex III activities, the increase in aerobic glycolysis occurs independent of reduced mitochondrial activity (Finley et al. 2011; Kim et al. 2010). This Warburg-like metabolism is also evident in the tissues of SIRT3-null mice as demonstrated by FDG-PET and validated in a separate nuclear magnetic resonance (NMR)-metabolomics analysis of SIRT3 MEFs (Finley et al. 2011; Hebert et al. 2013). In addition, steady-state metabolomics analysis revealed that the loss of SIRT3 increases the levels of many glycolytic metabolites, as well as intermediates in the pentose phosphate pathway (Finley et al. 2011). These results, in combination with a decrease in several TCA cycle intermediates, comprise a global metabolic shift that could support cellular proliferation with the loss of SIRT3.

The above metabolic reprogramming – increased glycolysis with concomitant reduction in mitochondrial metabolism – is similar to the changes seen in hypoxia. As mentioned previously, hypoxia leads to a reprogramming of cellular metabolism through stabilization of HIF1α, but HIF1α can also be stabilized under normoxia by accumulation of oncometabolites, ferric iron, and ROS (Kaelin and Ratcliffe 2008; Semenza 2010). It is through this ROS-PHDs-HIF1α axis that SIRT3 modulates metabolic reprogramming in cancer cells. Accordingly, SIRT3$^{-/-}$ cells and tissues have abnormal stabilization of HIF1α as well as increased HIF1-target gene expression (e.g. *GLUT1*, *HK2*, *LDHA*) (Finley et al. 2011). Furthermore, microarray analysis in tissues from wild type and SIRT3-null mice show an enrichment of hypoxia signatures, indicating elevated HIF1 transcriptional activity *in vivo* (Finley et al. 2011).

Analysis of human cancers highlights the clinical importance of SIRT3 as a tumor suppressor. Various types of cancers contain loss of at least one copy of SIRT3, with an increased propensity of gene deletion in breast and ovarian cancers (Finley et al. 2011). Gene expression data from clinical samples and immunohistochemical analyses of breast cancer samples at a U.S. Cancer Center revealed a decreased SIRT3 expression in these cancers as well as an inverse correlation between SIRT3 and HIF1 target gene expression (Finley et al. 2011). Furthermore, overexpression of SIRT3 in breast cancer cell lines represses the Warburg effect by inhibiting HIF1α stabilization (Finley et al. 2011). SIRT3 also represses the growth of pancreatic cancer cells by downregulating iron metabolism in a HIF1-dependent manner (Jeong et al. 2014a). Therefore, current evidence supports the tumor suppressor function of SIRT3 by modulating the metabolic reprogramming observed in breast and pancreatic cancers. SIRT3 levels could provide a diagnostic marker for tumors dependent on glycolytic metabolism and potentially elucidate the best clinical treatments for such pathophysiologies.

8.3.3 SIRT4, a Regulator of Glutamine Metabolism

SIRT4 plays a pivotal role in regulating fuel utilization within the mitochondria. Compared with SIRT3, SIRT4 biochemistry is less well-understood; it has been shown to possess weak NAD-dependent ADP-ribosyltransferase and deacetylase activities (Haigis et al. 2006; Laurent et al. 2013). One of the first studies on SIRT4 revealed its inhibition of glutamate dehydrogenase (GDH) via ADP-ribosylation. In the pancreas, the loss of SIRT4 activates GDH and promotes amino acid-stimulated insulin secretion (Haigis et al. 2006). SIRT4 loss also promotes insulin secretion in response to glucose (Ahuja et al. 2007).

GDH catalyzes the conversion of glutamine-derived glutamate into α-ketoglutarate, an intermediate in the TCA cycle (Matés et al. 2009). In tumors, this step may be important for refueling the mitochondrial metabolites during proliferation when certain components of the TCA cycle are depleted for biosynthesis. In line with this hypothesis, SIRT4 exerts tumor suppressive function by inhibiting

GDH and glutamine anaplerosis (Jeong et al. 2013). Intriguingly, SIRT4 expression is regulated in the context of tumorigenesis and cancer cell metabolism; so far, both DNA damage and mammalian target of rapamycin (mTOR) pathway inhibition have been shown to upregulate SIRT4 expression (Jeong et al. 2013; Csibi et al. 2013).

Induction of genotoxic stress by treatment with camptothecin, a topoisomerase I inhibitor, or ultraviolet light causes cells to decrease their glutamine consumption and ammonia production, while glucose uptake and lactate secretion do not change (Jeong et al. 2013). These observations suggest that glutaminolysis is suppressed upon DNA damage, although the reason behind this change remains unclear. Moreover, metabolic flux analysis revealed a decreased contribution of glutamine to TCA cycle intermediates (Jeong et al. 2013). Additionally, analysis of sirtuin gene expression showed a marked elevation in SIRT4 expression in response to DNA damage, suggesting that SIRT4 plays a crucial role in repressing glutamine metabolism after DNA damage. In the absence of SIRT4, cells take up more glutamine and have higher levels of TCA cycle intermediates, and they fail to downregulate glutamine uptake and proliferation after DNA damage (Jeong et al. 2013). Inhibiting glutaminase or GDH, enzymes involved in glutamine anaplerosis, reverses this phenotype (Jeong et al. 2013). SIRT4$^{-/-}$ cells have increased double-stranded breaks and chromosome number per cell, indicating genomic instability in the absence of SIRT4 and its regulation of glutamine metabolism (Jeong et al. 2013).

Another relevant pathway in cancer metabolism that regulates SIRT4 expression is the mTOR signaling cascade, which is known to promote cellular growth and proliferation by inducing transcription and protein translation based on the nutrient state of the cell (DeBerardinis et al. 2008). In addition, mTOR has been shown to stimulate many metabolic pathways, including glycolysis, pentose phosphate pathway, lipid synthesis, and nucleotide synthesis – essential processes required for growth and proliferation of cancer cells (Yecies and Manning 2011). In fact, hyperactive mTOR signaling is observed across various types of cancer, especially in the context of PTEN and PI3K signaling alterations (Guertin and Sabatini 2005). Not only does mTOR regulate glucose metabolism, but it also stimulates glutamine anaplerosis (Csibi et al. 2013). Inhibition of this pathway leads to a decreased conversion of glutamine-derived glutamate to α-ketoglutarate as well as an increase in SIRT4 expression, suggesting that mTOR stimulates GDH activity via downregulation of SIRT4 (Csibi et al. 2013). Tumors formed by rat leiomyoma cells with hyperactive mTOR signaling have low levels of SIRT4, which is reversed with rapamycin (mTOR inhibitor) treatment (Csibi et al. 2013). Expression of SIRT4 is necessary for repression of GDH activity upon mTOR inhibition. The repression of SIRT4 expression occurs via destabilization of cAMP-responsive element binding-2 (CREB2) by mTOR signaling (Csibi et al. 2013). This link between pro-oncogenic signaling pathway and the effects of SIRT4 on mitochondrial glutamine metabolism poses an interesting therapeutic avenue for certain cancers, especially those dependent on upregulated glutamine metabolism.

The pro-tumorigenic effect of SIRT4 loss is observed *in vivo* when transformed MEFs are injected into mice (Jeong et al. 2013). More importantly, many types of human cancers including lung cancer and leukemia have lower expression of SIRT4 compared to counterpart normal tissues, and lower levels of SIRT4 correlate with decreased survival in lung cancer patients (Jeong et al. 2013). In addition, SIRT4-null mice develop many types of tumors with a high incidence of lung cancers. Addition of SIRT4 to cells derived from SIRT4-null tumors downregulates glutamine metabolism, recapitulating the inhibition of glutamine anaplerosis by SIRT4 as an important source of its tumor suppressive activity (Jeong et al. 2013). In addition, SIRT4 represses glutamine metabolism in Myc-activated Burkitt lymphoma Ramos and Raji cell lines as well as Myc-driven lymphoma mouse model (Jeong et al. 2014b).

SIRT4 also mediates lipid metabolism in skeletal muscle and white adipose tissue by deacetylating and inhibiting malonyl-CoA decarboxylase (MCD), which converts malonyl-CoA into acetyl-CoA and promotes lipogenesis while repressing fat oxidation (Laurent et al. 2013). Therefore, SIRT4-null mice are resistant to diet-induced obesity (Laurent et al. 2013), which raises interesting prospects for SIRT4 in maintaining whole-body metabolic homeostasis with another potential link to cancer. For instance, the link between obesity, diabetes, and cancer is becoming more appreciated (Patterson et al. 2013). Furthermore, as the role of FAS and FAO in cancer becomes better understood, SIRT4 may also be elucidated to regulate cancer pathophysiology via lipid metabolism.

8.3.4 Potential Regulation of Cancer Metabolism by SIRT5

SIRT5 was initially found to deacetylate carbamoyl phosphate synthase-1 (CPS1) in the liver to promote the urea cycle for clearance of ammonia produced from catabolism of amino acids (Nakagawa et al. 2009). New catalytic activities have been identified for SIRT5, such as lysine desuccinylation and demalonylation (Du et al. 2011). In addition, SIRT5-null mice display hypersuccinylation and hypermalonylation of various proteins (Park et al. 2013). Although SIRT5 has not been well studied in cancer, it has the potential to regulate cancer cell metabolism. Approximately 80 % of the enzymes involved in the TCA cycle and 60 % of enzymes involved in fatty acid metabolism are succinylated in the absence of SIRT5 (Park et al. 2013). Moreover, SIRT5 represses succinate dehydrogenase and pyruvate dehydrogenase activities (Park et al. 2013), which could result in stabilization of tumorigenic pathways dependent on oncometabolites. SIRT5 may have the ability to regulate cancer cell metabolism by altering the activity of TCA cycle enzymes via desuccinylation or demalonylation. Furthermore, SIRT5 promotes ROS detoxification by desuccinylating and activating Cu/Zn superoxide dismutase (Lin et al. 2013), which may repress tumorigenesis in a similar mechanism as SIRT3.

8.3.5 Extramitochondrial Sirtuins and Cancer Metabolism

While mitochondrial sirtuins can affect cancer metabolism by direct regulation of mitochondrial metabolic proteins and signaling pathways, extramitochondrial sirtuins have been shown to impact cancer metabolism primarily through transcriptional reprogramming that regulates cellular metabolism. To date, among the extramitochondrial sirtuins, SIRT6 has been best studied as a regulator of cancer metabolism (Fig. 8.2c).

8.3.6 SIRT6, a Modulator of HIF1 and c-Myc Transcriptional Activities

SIRT6 is a chromatin-bound sirtuin that possesses tumor suppressive activity through at least two mechanisms: 1) by promoting genome stability and 2) via control of cellular fuel utilization. Originally, SIRT6 was described as a chromatin-bound protein that mediates base excision repair (BER) and maintains genomic stability, and its loss is linked to progeroid (premature aging) degenerative phenotype in mice (Mostoslavsky et al. 2006). And while the loss of SIRT6 causes genomic instability as characterized by the increase in chromosomal fragments/breaks and translocations (among other abnormalities), SIRT6$^{-/-}$ cells possess intact cell-cycle checkpoints, double-strand break repair mechanisms, and BER factors (Mostoslavsky et al. 2006). Thus, how the loss of SIRT6 causes defects in BER remains unknown. In addition, SIRT6 delays senescence through maintenance of telomere structure by deacetylating lysine 9 of histone H3 (H3K9), as well as by corepressing NF-kB transcriptional activity via deacetylation of H3K9 in NF-kB target genes, further accounting for its anti-aging functions (Michishita et al. 2008).

Among the various abnormalities in SIRT6-null mice, one notable phenotype is a significant reduction in serum glucose levels, which was later revealed to be through HIF1. SIRT6$^{-/-}$ cells consume more glucose, secrete more lactate, and use less oxygen. Accordingly, these cells express higher levels of glycolytic genes that promote Warburg-like metabolism, which are HIF1 target genes (Zhong et al. 2010). At the promoters of these genes, SIRT6 deacetylates H3K9 and represses HIF1 transcriptional activity. Additionally, hypoxia or nutrient stress may inactivate SIRT6, leading to hyperacetylated H3K9 in the promoter regions of glycolytic genes and promoting the expression of HIF1 target genes (Zhong et al. 2010). The mechanism by which SIRT6 is inactivated under these conditions remains to be uncovered. Furthermore, the loss of SIRT6 leads to increased synthesis and stability of HIF1α, the labile subunit of HIF1, although how this occurs is unknown (Zhong et al. 2010).

Given that the loss of SIRT6 leads to metabolic reprogramming commonly observed in cancer cells, one would expect that SIRT6 is a tumor suppressor. This is indeed the case, as SIRT6$^{-/-}$ cells are tumorigenic. SIRT6$^{-/-}$ cells can form colonies

in soft agar, indicating a loss of anoikis – a key hallmark of transformation – and they form tumors when injected into mice (Sebastian et al. 2012). Interestingly, the transformation (i.e. immortalized SIRT6$^{-/-}$ MEFs are able to form colonies in soft agar and tumors in mice) seen with SIRT6 loss is independent of activation in any oncogenic pathways, suggesting a purely metabolic contribution to tumorigenesis in the absence of SIRT6 (Sebastian et al. 2012). And while the stabilization of HIF1α can account for the increased glycolysis and tumorigenic phenotypes, SIRT6 also represses cancer formation through inhibition of Myc via deacetylation of H3K56, another putative target for SIRT6 activity, at the promoters of Myc target genes (Sebastian et al. 2012).

The regulation of Myc transcriptional activity by SIRT6 is important in cancer metabolism, as Myc activation traditionally has been linked with upregulation of both glucose and glutamine metabolism (Gordan et al. 2007). As mentioned before, glutamine is an important source of carbon atoms for refueling metabolic intermediates drained by proliferation as well as of nitrogen atoms for nucleotide and protein syntheses that are required for cell growth and proliferation. Surprisingly, repression of Myc transcriptional activity by adding back SIRT6 into null cells does not lead to a reversal of metabolic programming as it would be expected. Instead, SIRT6 keeps proliferation in check by suppressing Myc-dependent expression of ribosomal proteins via deacetylation of H3K56 sites in the promoters of those genes (Sebastian et al. 2012). Silencing Myc in the context of SIRT6 loss represses tumor growth and ribosomal protein expression without affecting metabolism (Sebastian et al. 2012). Importantly, SIRT6 is expressed at lower levels in pancreatic and colon cancers. In an animal model of colon cancer (mutation in *Apc*, or adenomatosis polyposis coli tumor suppressor gene), tissue-specific deletion of SIRT6 in the intestines leads to an increased formation of adenomas, especially of the high and invasive grades (Sebastian et al. 2012). To conclude, SIRT6 exerts its tumor suppressive functions at least in part by repressing the metabolic phenotypes associated with cancer, and this is achieved by modulating the activities of key transcription factors that mediate changes in cellular metabolism towards a more anabolic state.

8.3.7 SIRT1 in Cancer and Potential Metabolic Implications

SIRT1 is the most studied and the best characterized sirtuin and targets many proteins to control cellular response to stress and metabolic adaptation. Relevant to cancer, SIRT1 affects many proteins involved in programmed cell death, including pro-apoptotic factor p53, DNA repair factor Ku70, and transcription factors FOXO1, FOXO3, FOXO4, and NF-kB (Brunet et al. 2004; Cohen et al. 2004; Luo et al. 2001; Motta et al. 2004; Vaziri et al. 2001; Yeung et al. 2004). Still, the role of SIRT1 in cancer remains controversial. Of note, its first substrate to be identified – the p53 tumor suppressor – is inhibited by SIRT1-mediated deacetylation (Luo et al. 2001; Vaziri et al. 2001), which initially led to the speculations that SIRT1 could be an oncogene. Indeed, some studies have shown that SIRT1 may promote tumorigenesis.

For instance, hypermethylated in cancer-1 (HIC1), a transcriptional repressor that suppresses tumorigenesis, represses the transcription of the *SIRT1* gene (Chen et al. 2005). Therefore, the loss of HIC1 activity promotes the expression of SIRT1, which then may promote cancer via repression of p53 and inhibition of programmed cell death. In addition, deleted in breast cancer-1 (DBC1) directly binds and represses SIRT1 activity, again supporting the protumorigenic role of SIRT1 by inhibiting programmed cell death of abnormal or damaged cells (Kim et al. 2008; Zhao et al. 2008). Other studies, however, have demonstrated that SIRT1 is a tumor suppressor, consistent with the observation that calorie restriction, which activates SIRT1, prevents cancer (Firestein et al. 2008). SIRT1 transgenic (overexpressing) mice have decreased spontaneous cancer formation, which represents a reduction in both carcinomas (cancers arising from epithelial tissues) and sarcomas (cancers arising from mesenchymal tissues) but not in lymphomas (Herranz et al. 2010). Molecularly, SIRT1 may promote tumorigenesis by inhibiting appropriate programmed cell death pathways and activating stress response, or it may suppress cancer by triggering DNA repair before the cells are committed to death. In addition, the role of SIRT1 in cancer may be context-dependent, such as the mutational background, the species, and the tissue type of the cancer being studied (Wilking and Ahmad 2015).

Although SIRT1 substrates are involved in cancer metabolism, SIRT1 itself has not yet been well studied in this regard. For instance, p53 has been shown to downregulate glycolysis and promote mitochondrial metabolism via activating TP53-induced glycolysis and apoptosis regulator (TIGAR) and synthesis of cytochrome *c* oxidase-2 (SCO2) while inhibiting phosphoglycerate mutase (PGM) (Bensaad and Vousden 2007). Therefore, SIRT1 may support tumorigenesis by opposing the role of p53 in maintaining proper cellular metabolism. On the other hand, SIRT1 also interacts with and deacetylates HIF1α at lysine 674, inhibiting its transcriptional activity by blocking p300 recruitment (Lim et al. 2010). Physiologically, hypoxia decreases NAD levels and leads to a negative transcriptional regulation of SIRT1 by C-terminal binding protein (CtBP) – although low NAD levels would decrease SIRT1 catalytic activity as well. Decreased SIRT1 levels during hypoxia lead to acetylation of HIF1α and activation of HIF1-mediated transcription (Lim et al. 2010). If SIRT1 activity is maintained under hypoxia (e.g. inhibition of glycolysis and maintenance of NAD levels), then HIF1 transcriptional activity is inhibited via deacetylation of HIF1α (Lim et al. 2010). Accordingly, human fibrosarcoma cells overexpressing SIRT1 form smaller, less-vascularized tumors when injected into mice, which could at least partially result from the inhibition of metabolic reprogramming in these tumor cells (Lim et al. 2010). SIRT1 also binds and deacetylates Myc at lysine 323, which destabilizes it independently of F-box protein FBW7 that normally targets Myc for degradation (Yuan et al. 2009). How the SIRT1-mediated deacetylation of K323 regulates Myc stability remains to be elucidated. Therefore, SIRT1 inhibits Myc-induced colony formation and anchorage-independent growth (Yuan et al. 2009). Given the role of Myc in regulating glucose and glutamine metabolism, it is also possible that SIRT1 inhibits metabolic reprogramming in cancers.

8.3.8 Potential Link Between SIRT2 and Cancer Metabolism via K-Ras

SIRT2, a cytosolic sirtuin, inhibits tumorigenesis via mitotic checkpoint regulation through deacetylation of not only tubulin but also anaphase-promoting complex/cyclosome (APC/C) activators/adaptors CDH1 and CDC20 (Kim et al. 2011). Interestingly, SIRT2 was later shown to deacetylate K-Ras at lysine 104 and inhibit its transforming capability (Yang et al. 2013). K-Ras is commonly aberrantly activated through point mutations in many cancer types including lung, colon, and pancreatic cancers (Prior et al. 2012; Pylayeva-Gupta et al. 2011). Of relevance, activation of K-Ras drives downstream signaling events that control cell metabolism such as the MAPK and PI3K/Akt/mTOR pathways (Pylayeva-Gupta et al. 2011). By upregulating these signaling pathways, mutated K-Ras (e.g. K-Ras^{G12D}) promotes glucose uptake and pentose phosphate pathway for nucleotide synthesis as well as production of NADPH to mediate cellular redox and contribute to biosynthesis. Therefore, it is possible that the tumor suppressive function of SIRT2 is partly a result of the involvement of K-Ras mutants in cancer metabolism.

8.3.9 SIRT7 and Transcriptional Repression in Transformation, Metabolic Implications Through Myc

Lastly, SIRT7 is a nuclear sirtuin that promotes oncogenic transformation by deacetylating H3K18 and mediating transcriptional repression of tumor suppressor genes such as *NME1* and *COPS2* (Barber et al. 2012). This pro-tumorigenic activity of SIRT7 is synergistic with ELK4, a member of the ETS transcription factor family, which recruits SIRT7 to the promoters of these tumor suppressors (Barber et al. 2012). Interestingly, SIRT7 ameliorates endoplasmic reticulum stress (unfolded protein response) and prevents fatty liver disease by Myc-mediated recruitment to the promoters of ribosomal genes and subsequent repression of their expression (Shin et al. 2013), suggesting that SIRT7 may repress the expression of other Myc target genes. This observation that SIRT7 opposes Myc transcriptional activity appears contradictory to the previous findings that SIRT7 may promote tumorigenesis, but nonetheless SIRT7 may be involved in cancer cell metabolism. Furthermore, Myc has many effects inside the cell as a transcriptional regulator – both as an activator and a repressor – of many genes (Dang 2012), and the interplay between SIRT7 and Myc may be more context-specific.

8.4 Concluding Remarks

At its root, cancer is characterized by uncontrolled cell proliferation arising from diverse tissue types. How a normal (e.g. terminally differentiated, quiescent) cell gains the ability to undergo unregulated growth is a topic of intense investigation. It has become clear that a diverse set of genetic and epigenetic alterations lead to the rewiring of normal cellular program to promote a malignant phenotype. Furthermore, cancer cells intentionally alter their nutrient usage, which has garnered significant interest as a potential therapeutic avenue, given these metabolic changes could be specific to highly proliferative cancer cells. This altered metabolism is so ubiquitous in cancer that it has been characterized as an emerging hallmark of cancer (Hanahan and Weinberg 2011), and a new focus in cancer biology is the identification of molecular regulators of metabolic reprogramming in cancers. As reviewed in this chapter, many of the biological functions of sirtuins have been tied to the rewiring of cellular metabolism in cancers. These observations raise promising possibilities that sirtuins may be good therapeutic targets in modulating cancer metabolism to affect disease prognosis. To that end, efforts to identify small-molecule activators/ inhibitors of sirtuins have not yet yielded promising results but this area is still under active investigation. The current state of sirtuin research therefore leaves room for more discoveries to be made, and they continue to be potential therapeutic targets in cancer as well as other age-related diseases.

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Chapter 9
Sirtuins as Metabolic Modulators of Muscle Plasticity

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9.1 Sirtuins as Metabolic Modulators of Skeletal Muscle

Skeletal muscle holds great adaptive potential in response to both physiological and pathophysiological stressors. In accordance with physiological demands, higher organisms are capable of modulating muscle metabolism, function and mass. As skeletal muscle represents the largest organ in the human body, these adaptive changes impact whole body energy homeostasis. Two of the most important examples of muscle plasticity include triggered changes in mitochondrial function and content, and vascular remodeling after exercise. Exercise is an energetic stressor that forces muscle fibers to produce sufficient levels of ATP necessary for contraction/relaxation while maintaining an adequate redox potential. It is the promotion of mitochondrial homeostasis for aerobic ATP production and the maintenance of physiological redox conditions that assures the adequate function of healthy muscle. This, in turn, can prevent imbalance in whole body energy status that can otherwise lead to obesity, diabetes and cardiovascular disease and contribute to accelerated aging (Joseph et al. 2012; Patti et al. 2003).

Despite the vast number of studies on muscle metabolic adaptations following exercise, many of the underlying cellular pathways involved remain to be identified. Over the last decade, a conserved family of proteins named sirtuins (SIRT1-7) was shown to be part of a complex network of energy sensing molecules, that regulate each other along with a cascade of other metabolic targets, all responsible for fine-tuning cellular adaptations to energetic challenges. Sirtuins have emerged as impor-

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tant sensors of energy abundance. Accumulating evidence indicates that nuclear and mitochondrial sirtuins play important roles in the induction of mitochondrial biogenesis and muscle vascularization following metabolic stress, such as exercise training. In light of muscle activity and exercise being the most powerful inducers of cardiovascular and metabolic health, sirtuins could represent interesting targets for the prevention of metabolic and age-related diseases.

SIRT1 (Silent mating type information regulator 2 homolog 1) is the most studied sirtuin protein and initially received considerable attention for its potential role in the determination of longevity and healthspan in lower organisms [reviewed in (Guarente 2012; Houtkooper et al. 2012)]. Further work identified the SIRT1 homolog in mammals as a metabolically regulated protein, capable of sensing cellular redox and energy status and subsequently triggering changes in key metabolic pathways including mitochondrial energy production and defense against reactive oxygen species (ROS) generation (Cantó et al. 2012). SIRT1 achieves these effects by impacting on the biogenesis of mitochondria, the clearance of damaged organelles and the expression of proteins involved in antioxidant responses.

Besides SIRT1, research on other sirtuins has also yielded information on their potential roles in muscle physiology. For instance, SIRT2, a sirtuin predominantly found in the cytoplasm, may regulate autophagy (Zhao et al. 2010) and differentiation of adipocytes (Jing et al. 2007) in a FOXO1 dependent manner, but so far has not been studied in skeletal muscle. Some recent papers have linked SIRT2 with inflammation and necrosis in cardiac tissue with a postulated role in cardiac protection from ischemic injury that is still to be confirmed (Narayan et al. 2012). SIRT3, SIRT4 and SIRT5 are all located within the mitochondria, modulate the mitochondrial protein acetylome, and hence could play important roles in muscle metabolism and stress responses (Pirinen et al. 2012). The last two sirtuins, SIRT6 and SIRT7, are preferentially chromatin bound. SIRT6 protein has been shown to increase with exercise in muscle and to be important for the regulation of glucose metabolism (Koltai et al. 2010; Xiao et al. 2010). SIRT7, however, does not appear to be highly expressed in skeletal muscle tissue, but may have relevance in cardiac muscle function and plasticity (Ford 2006). Elucidation of the pathways involved in sirtuin-mediated muscle homeostasis will undoubtedly lead to a better understanding of the mechanisms underlying the beneficial effects of exercise and perhaps help in the design of nutra- and pharmaceuticals for the treatment of degenerative processes that are associated with metabolic and mitochondrial health in muscle.

The main purpose of this chapter is to provide an overview of how sirtuins are, or may be, involved in adaptive skeletal muscle responses to energetic stress, including triggered changes in mitochondrial function and biogenesis, intermediary metabolism and vascular remodeling.

9.2 Skeletal Muscle Function and Plasticity

Skeletal muscle is not only responsible for locomotion but through its use of energetic substrates, and its metabolic interaction with other organs, participates in the regulation of whole body metabolic homeostasis. The mechanisms of

muscle contraction requires a very complex molecular machinery composed of ionic channels, electro-mechanical couplers, structural proteins, contractile apparati, ionic pumps, and the synthesis of ATP from intermediary substrates to fuel energy consuming processes. Skeletal muscle can utilize different intermediary substrates for ATP synthesis depending on physiological factors that include the availability of different energy stores within the body and the relative intensity of muscle activity. In general, as the relative exercise intensity increases, there is a decrease in the proportion of energy derived from fat oxidation and an increase in carbohydrate oxidation [reviewed in (Philp et al. 2012; Sahlin and Harris 2008)]. Indeed, the proportion of energy from fatty acid oxidation is higher during prolonged exercise at low and moderate intensities, while strenuous intense exercise relies heavily on carbohydrate sources for energy. Fatty acids, which can be directly oxidized by mitochondria in the muscle, are provided by the lipolysis of adipose tissue and intramuscular triacylglycerols. Carbohydrates can be derived from the circulation in the post-prandial phase or following hepatic gluconeogenesis or glycogenolysis. Alternatively, they can also be generated by intramuscular glycogenolysis.

In addition, to this innate muscle substrate plasticity, repetitive exercise training results in an adaptation to metabolic stress. This triggers longer-term adaptive reactions that modify muscle function and are capable of inducing structural remodelling to efficiently cope with the nature of the metabolic challenges, and to favour particular substrate sources. In particular, endurance exercise training results in a sparing of carbohydrate use during exercise, with an increase in the use of fat oxidation for the production of energy. This adaptation can result in a dramatic reduction in the probability of an individual to develop a metabolic disease such as obesity and diabetes. In the next paragraph we will deal with the mechanisms in skeletal muscle that are essential for the detection and adaptation to metabolic stress.

9.3 SIRT1 Activation Is an Important Node for Energy Sensing Pathways and Adaptive Responses During Oxidative Metabolism

During both aerobic exercise training and CR, it has become increasingly apparent that skeletal muscle bioenergetics are tightly coordinated by metabolic signaling mechanisms that sense changes in available primary energetic substrates such as NADH and ATP stores. These energy sensing proteins include the AMP-activated protein kinase (AMPK) and the NAD^+-dependent deacetylase SIRT1, which are activated upon changes in the abundance of the corresponding metabolites AMP and NAD^+ [reviewed in (Cantó and Auwerx 2012; Hardie et al. 2012)] (Fig. 9.1). Evidence summarized here demonstrates how AMPK and SIRT1 are nodal components of a complex metabolic sensing network that regulates muscle mitochondrial biogenesis, substrate utilization and vascular remodeling.

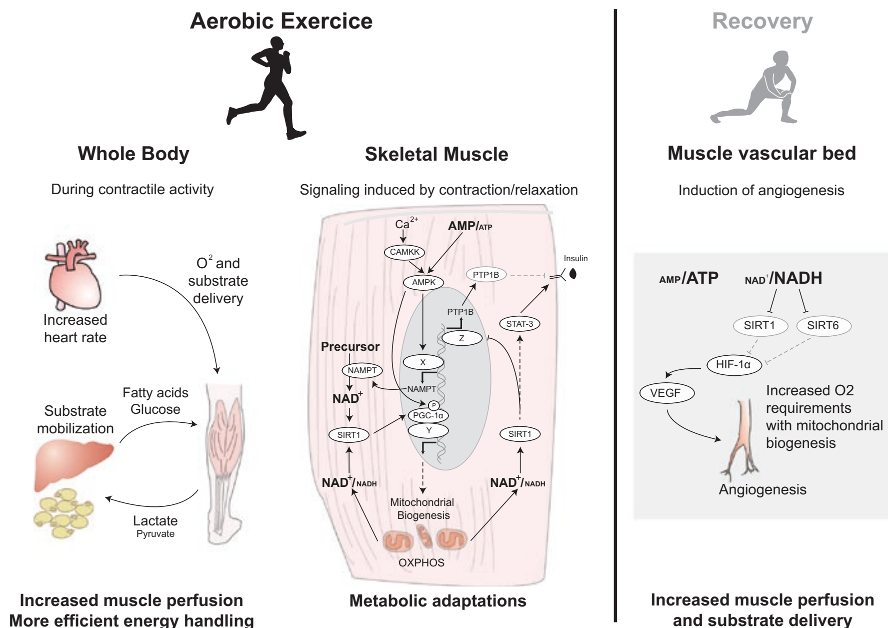

Fig. 9.1 This schematic demonstrates the balance between SIRT1 signals in the body and muscle during exercise and recovery. Skeletal muscle participates in the regulation of whole body fat and glucose metabolism (*left panel*). With aerobic physical activity, fatty acids and glucose act as substrates for metabolism and ATP production. The activation of signalling mechanisms leading to skeletal muscle adaptations originate with changes in AMPK and SIRT1 activation. These adaptations include increases in mitochondrial biogenesis and an improvement in glucose homeostasis and insulin sensitivity (see figure text for full description). Following the increase in mitochondrial biogenesis, the development of a hypoxic environment increases HIF-1α stability and activity to induce angiogenesis through the induction of VEGF (*right panel*). Inhibitory signals are indicated by *gray dashed lines*

During exercise, muscle contraction is triggered by the depolarization of the muscle surface membrane, the sarcolemma, inducing excitation-contraction coupling through the release of Ca^{2+} from the sarcoplasmic reticulum. Muscle relaxation requires abundant ATP to terminate the filament interactions that underlie muscle contraction and to pump Ca^{2+} out of the sarcoplasma and back into the extracellular space and sarcoplasmic reticulum. These processes cause ATP depletion. As a result, AMPK activation occurs through increased AMP abundance arising from ATP utilization (Xiao et al. 2007), and its phosphorylation by activated calcium calmodulin dependent kinase kinase β (CaMKKβ) and the constitutively activate liver kinase B1 (LKB1) (Sakamoto et al. 2004; Steinberg et al. 2010). Additionally, in aerobic metabolism NADH is extensively used to transfer electron loads from intermediary substrates to the electron transport chain to synthesize ATP. Consequently, the relative levels of NAD^+ increases and can then act as a co-substrate for SIRT1 activation (Cantó et al. 2009; Koltai et al. 2010; Price et al. 2012). Since AMPK activity is under control of the AMP/ATP ratio and SIRT1

activity depends on the NAD^+/NADH ratio, these proteins are responsible for sensing independent energy availability measures and together activate energy-producing processes such as mitochondrial biogenesis and the oxidation of fatty acids (Cantó and Auwerx 2012; Carling 2004; Hardie 2004). Both AMPK and SIRT1 possess overlapping qualities, as both proteins seem to be key players amongst a larger network of metabolic sensors and regulators. In *C. elegans* for example, the overexpression of the AMPKα subunit, of the AMPK hetero-trimeric complex, induces longevity (Apfeld et al. 2004) similar to what early Sir2p (the yeast SIRT1 homolog) longevity studies suggest (Kaeberlein et al. 2005; Lin et al. 2004; Tissenbaum and Guarente 2001). As a Ser/Thr kinase, AMPK is responsible for the phosphorylation of metabolic enzymes, such as acetyl-CoA carboxylase (ACC) for fatty acid oxidation, or the phosphorylation of proteins that regulate mitochondrial biogenesis and gene expression, such as the peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) (Jager et al. 2007; Winder et al. 2000). In a similar regulatory manner, SIRT1 deacetylates and modulates PGC-1α activity (Nemoto 2005; Rodgers et al. 2005) (Fig. 9.1). In pharmacological experiments AICAR, an activator of AMPK, induces SIRT1 activity further revealing the interconnection of these two energy sensors (Cantó et al. 2009; Gurd et al. 2011). Likewise CR has been demonstrated to increase mitochondrial biogenesis in muscle via the activation of both AMPK and SIRT1 (Cantó et al. 2009; Chen et al. 2008; Fulco et al. 2008; Zong et al. 2002). In contrast to CR, when nutrients are readily available, the biological functions of PGC-1α are inhibited by the acetyltransferase GCN5 (Lerin et al. 2006). GCN5 expression in skeletal muscle is furthermore positively controlled by the transcriptional coactivator SRC-3 (Coste et al. 2008). In line with this, germline SRC-3 knock-out mice display increased mitochondrial function, and are protected against obesity (Coste et al. 2008). *In vitro* experiments show that glucose restriction (and therefore nutrient deprivation) in primary skeletal myoblasts results in AMPK activation leading to the induction of nicotinamide phosphoribosyltransferase (*Nampt* transcription (Fulco et al. 2008), the rate-limiting enzyme that converts NAM to NAD^+ (Revollo et al. 2004). The elevation in NAMPT expression increases the NAD^+/NADH ratio, thereby activating SIRT1, and thus providing a clear mechanistic link between the activation of AMPK and an increase in SIRT1 activity (Fig. 9.1). *In vivo*, animals lacking the AMPKγ3 subunit show less NAD^+, NAMPT and deacetylated PGC-1α following exercise (Cantó et al. 2010). Therefore AMPK controls the activity of SIRT1; however, in a reciprocal manner some evidence has demonstrated that SIRT1 can also control the activity of AMPK. Human cultured myotubes that carry a SIRT1 protein point mutation exhibit reduced AMPK activation (Biason-Lauber et al. 2013). Therefore, it appears that these two pathways can be independently activated but may posses overlapping and potentiating properties on each other.

As has been discussed briefly, increased oxidative metabolism and the subsequent relative enrichment of NAD^+ activates SIRT1 during fasting or exercise. Upon activation, SIRT1 deacetylates numerous target proteins beyond PGC-1α, such as p53 (Luo et al. 2001; Shah et al. 2012) and FOXO (Brunet et al. 2004; Xiong et al. 2011) proteins and thus modifies their transcriptional activity. In addition, SIRT1

has been shown to deacetylate the myogenic regulatory factor MYOD, inhibiting it from inducing satellite cell differentiation and myogenesis (Fulco et al. 2008). Thus, muscle growth could putatively be delayed by SIRT1 activity when glucose stores are low as a mechanism to maintain energy balance. Concurrently, this pathway compensates for a low glucose environment by inducing mitochondrial biogenesis and fatty acid oxidation through the deacetylation and activation of PGC-1α (Cantó et al. 2010). In a positive feedforward loop, PGC-1α enhances its own transcription via the formation of a ternary complex, that includes MYOD, SIRT1 and PGC-1α, on a region of the PGC-1α promoter that contains E2 boxes (Amat et al. 2009). Skeletal muscle can then increasingly rely on β-oxidation of fatty acids as a source of energy. In addition, SIRT1 represses the mitochondrial uncoupling protein UCP3, a protein that is known to dissipate energy in the mitochondria (Amat et al. 2007). This is corroborated by other observations that UCP3 increases significantly during C2C12 muscle cell differentiation (Kim et al. 2007), when SIRT1 is known to be inactive (Fulco et al. 2008; Rathbone et al. 2009).

All these data suggest a model for a complex metabolic network where AMPK and SIRT1 mediate the plasticity of muscle in oxidative conditions and modulate the use of energy sources through their activation by AMP and NAD^+ respectively.

9.4 Changes in the NAD^+/NADH Ratio Influences Both Energy Homeostasis and Metabolic Adaptations

Besides directly affecting sirtuin activity, NAD^+ is required for a variety of processes in the cell. The NAD^+/NADH ratio influences redox reactions during glycolysis and oxidative phosphorylation, along with the catabolism of fats, proteins, carbohydrates, and alcohols. NAD^+ has been shown to impact on various fundamental regulatory functions, such as transcription, DNA repair, G-protein coupled signaling and intracellular calcium signaling (Bogan and Brenner 2008; Garten et al. 2009). As a result of these NAD^+-consuming pathways, the availability of NAD^+ for sirtuin activity is limited despite seemingly high intracellular concentrations ranging from 300-400 μM (Bogan and Brenner 2008; Cantó et al. 2010; Hayashida et al. 2010; Koltai et al. 2010; Mouchiroud et al. 2013; Rodgers et al. 2005). Aside from activation by NAD^+, SIRT1 can be inhibited by both NADH (Lin et al. 2004) and nicotinamide (NAM), a by-product of NAD^+ metabolism (Bitterman 2002). The inhibitory nature of NAM therefore stresses the importance of the NAD^+ salvage pathway, which cycles NAM back to NAD^+ for its replenishment. In line with the importance of NAD^+ as a rate-limiting co-substrate for sirtuins, increasing the supply of NAD^+ precursors has been shown to impact sirtuin activity. Nicotinamide riboside, a natural NAD^+ precursor, increases NAD^+ levels and activates SIRT1 and SIRT3 culminating in enhanced skeletal muscle mitochondrial function (Cantó et al. 2012). The supply of NAD^+ has also been associated with longevity (Mouchiroud et al. 2013). The yeast ortholog of mammalian NAMPT,

PNC1, was shown to be essential for SIRT1-dependent lifespan extension during CR (Anderson et al. 2003). Therefore, it has been proposed that NAMPT may play a role in the regulation of longevity in mammals, or at least play an important role in metabolism. Similar to SIRT1, NAMPT expression has been shown to increase in cells that are either deprived of serum (Yang et al. 2007) or glucose (Fulco et al. 2008), and in skeletal muscles of fasted mice (Cantó et al. 2009; Fulco et al. 2008). In addition, elevated NAMPT expression correlates well with the oxidative capacity of a given muscle. In support of this, NAMPT expression is greatest in postural muscles, such as the highly oxidative soleus muscle. Beyond this, there is also evidence that NAMPT increases in skeletal muscle following aerobic exercise training of both rats and humans (Costford et al. 2010; Koltai et al. 2010). These observations provide further evidence for the activation or inactivation of SIRT1, and perhaps other sirtuins, in various exercise or sedentary models since NAMPT expression controls the overall level of the sirtuin co-substrate NAD^+.

9.5 The Effect of Exercise, CR and SIRT1-Agonists on SIRT1 and AMPK Signaling in Skeletal Muscle

Downstream of the activation of both SIRT1 and AMPK, activated AMPK has been found to increase mitochondrial biogenesis in a PGC-1α and NRF-1-dependent manner (Bergeron et al. 2001; Zong et al. 2002). In conjunction with the activation of AMPK, SIRT1 is needed for the deacetylation and synchronized activation of PGC-1α following exercise in skeletal muscle (Gerhart-Hines et al. 2007). Similarly, treatment of mice with SIRT1 agonists, such as SRT1720 (Feige et al. 2008; Smith et al. 2009) and resveratrol (RSV) (Lagouge et al. 2006), results in increased skeletal muscle mitochondria via the deacetylation and activation of PGC-1α. In combination with exercise, RSV induces a synergistic effect on mitochondrial biogenesis that is reliant on SIRT1 expression, despite a modest SIRT1-independent effect of RSV on sedentary mice (Menzies et al. 2013). Major factors that affect the oxidative capacity of muscle revolve around mitochondrial content and function. In humans, there is a larger volume of mitochondria in slow-twitch red (STR), followed by fast-twitch red (FTR) then fast-twitch white (FTW) fibers (Howald et al. 1985). There are however, differences in the distribution of mitochondria in fiber types amongst different animal species. For example, in rodents FTR fibers have a higher mitochondrial volume than STR fibers (Dudley et al. 1982; Kirkwood et al. 1986). Evidence shows that with the activation of PGC-1α there is a coordinated nuclear and mitochondrial gene expression wave that harmonizes the mitochondrial oxidative capacity with the fiber-type distribution in skeletal muscle (Puigserver 2003; Rasbach et al. 2010). Similarly, evidence from muscle-specific SIRT1-KO mice show a coordination between the reduction in oxidative capacity (Menzies et al. 2013; Price et al. 2012) and expression of FTW fibers in skeletal muscle (Price et al. 2012). However, there exists some contention as to whether oxidative capacity

and fiber-type changes are truly linearly associated. The uncertainty in this relationship was demonstrated in mice with both germline and muscle-specific PGC-1α-KOs that did not exhibit a change in the fiber-type distribution of skeletal muscle (Arany et al. 2005; Geng et al. 2010; Zechner et al. 2010) despite exhibiting a shift from oxidative toward glycolytic muscle fibers (Handschin et al. 2007). Hence, the main muscle-specific alterations that occur with higher oxidative capacity, and that are not contentious, include a higher percentage of mitochondrial, capillary and myoglobin content (Baldwin et al. 1972; Dudley et al. 1982; Pette and Staron 1990; Schiaffino and Serrano 2002; Spangenburg and Booth 2003).

However, some experimental evidence about the role of SIRT1 in oxidative muscle plasticity remains inconclusive and/or shows that we still do not understand the complexities of these regulations. The signaling mechanisms responsible for increases in ATP production, following CR or exercise, have been hypothesized to rely on SIRT1, or possibly a combination of sirtuin proteins. But SIRT1 expression is not proportional to mitochondrial content in several different tissues or muscles under steady state conditions. As an example, the soleus muscle exhibits higher SIRT1 expression than the heart, yet the heart is more oxidative and contains considerably more mitochondria than the soleus (Chabi et al. 2009). In addition, following chronic exercise (multiple bouts of prolonged exercise) in rats, only nuclear SIRT1 activity was positively correlated with PGC-1α protein expression and mitochondrial content, while unexpectedly, total SIRT1 protein expression seemed to have a negative correlation to exercise and PGC-1α expression (Gurd et al. 2011). In contrast to this observation, several studies have demonstrated an increase in total SIRT1 expression following exercise in old (Pauli et al. 2010) or adult (Suwa et al. 2008) rat skeletal muscle. In addition, both an acute bout of sprint exercise (Guerra et al. 2010) or high intensity training for 2 weeks (Little et al. 2010) resulted in elevated SIRT1 protein content in humans. However, despite these observations, muscle-specific SIRT1-KO mice exhibited only modest reductions (Menzies et al. 2013) or no reductions in skeletal muscle mitochondrial content (Philp et al. 2011). This suggests that SIRT1 expression may play a less dramatic role in the maintenance of basal mitochondrial content than once thought. Several groups have demonstrated a more important role for SIRT1 on mitochondrial function, as opposed to biogenesis, in sedentary animals (Cantó et al. 2012; Menzies et al. 2013; Price et al. 2012). Moreover, muscle-specific SIRT1-KO models have shown that exercise-induced increases in mitochondrial biogenesis and function are independent of SIRT1 expression (Menzies et al. 2013; Philp et al. 2011). Whether or not SIRT1 expression or activity is proportional to mitochondrial oxidative capacity, and whether exercise can provoke an increase in SIRT1 levels or activation in WT skeletal muscle, may depend on the type of model being used. It would appear that more severe exercise or energy storage crisis models induce a SIRT1-dependent response, while submaximal exercise protocols in animals fed ad-libitum chow diets exhibit SIRT1-independent mitochondrial adaptations. In line with this, chronic electrical stimulation of rat skeletal muscle results in increased SIRT1 activity, while no effect is observed with voluntary running (Chabi et al. 2009). Similarly, voluntary running distance did not differ between WT and muscle-specific SIRT1-KO mice, yet

fatigue occurs earlier in KO animals during a maximal electrically-stimulated ex-vivo muscle fatigue test (Menzies et al. 2013). These results may indicate that SIRT1 plays a role in muscle metabolism following chronic bouts of strenuous or extreme endurance stress, and possibly more so if combined with energy deprivation. This hypothesis would also tend to agree with the observation that insulin sensitivity in muscle-specific SIRT1-KO mice only exhibits an enhancement when the animals are CR and not while on a regular chow diet (Schenk et al. 2011). It also remains to be determined if SIRT1 overexpression in mice may facilitate a stronger metabolic response to extended treadmill training or CR.

In coordination with the hypothesis that SIRT1 plays a role in longevity and muscle metabolism, SIRT1 expression decreased with age in rat gastrocnemius muscle, however, exercise was shown to attenuate this loss (Pauli et al. 2010). In addition, a reduction of rat gastrocnemius SIRT1 activity occurs with aging, that was also attenuated with exercise (Koltai et al. 2010). However, despite SIRT1 activity being modulated by the phosphorylation of its catalytic domain via protein kinase A, there is still some ambiguity in these *in vitro* test-tube results of SIRT1 activity in muscle enzyme extracts. Specifically, these measurements do not take into account the cellular stores of the co-substrate NAD^+ or the presence of inhibitory NAM or NADH metabolites. Therefore more clear evidence is still required to understand the relationship between SIRT1 expression/activity, and exercise-induced mitochondrial biogenesis. For example, future work could focus on the enzymatic activity of this protein, as reflected by *in situ* protein deacetylation. Since active SIRT1 is responsible for the deacetylation of PGC-1α, FOXO1, MYOD and others, these measured protein acetylation changes may provide a more accurate picture of the involvement of SIRT1 in muscle than measurements of SIRT1 expression or activity.

9.6 Muscle Metabolism and Whole Body Energy Homeostasis: A Role for SIRT1 in Overall Health Maintenance?

The importance of muscle and exercise in whole body homeostasis is evidenced by solid experimental data. Although the beneficial effects of exercise have been known for decades, if not centuries, the first epidemiological studies that linked exercise to cardiovascular health were performed in the late 1950s (Morris and Crawford 1958; Morris et al. 1953). These studies led to the findings that skeletal muscle also participates in the regulation of whole body fat and glucose metabolism. Under exhaustive physical workloads, fatty acids and glucose act as substrates for aerobic metabolism and ATP production. However, muscles can also use glucose as a substrate for anaerobic energy production, resulting in the production of lactate (Fig. 9.1). Indeed, following a meal and the release of insulin, skeletal

muscle accounts for approximately 80 % of the total blood glucose uptake, and about half of this glucose stays in the muscle as insulin stimulates glycogen synthesis.

Interestingly, cultured human myotubes from individuals that have point mutations in SIRT1 exhibit reduced glucose uptake and insulin sensitivity (Biason-Lauber et al. 2013). Additionally, in diet-induced-obese mice SIRT1 agonists improved insulin resistance (Smith et al. 2009). This provides experimental evidence that SIRT1 may be an active target for the prevention of diabetes in humans through insulin sensitization effects. The mechanism(s) behind the ability of SIRT1 to positively effect insulin sensitivity may be in part related to its ability to regulate mitochondrial biogenesis and thus stimulate oxidative energy expenditure. The ability of skeletal muscle to protect against diet-induced obesity relies on the capacity of muscle to perform β-oxidation of lipids for the maintenance of energy homeostasis through fat metabolism. Moreover this SIRT1-mediated effect on insulin resistance may also occur through the downregulation of PTP1B at the chromatin level (Sun et al. 2007). PTP1B acts as a key insulin receptor phosphatase and is therefore a negative regulator of the insulin signal transduction cascade. In addition, SIRT1 positively mediates caloric restriction (CR)-induced insulin sensitivity by deacetylating STAT-3, enhancing PI3K signaling and thereby increasing glucose uptake (Schenk et al. 2011) (Fig. 9.1). SIRT1 can therefore increase β-oxidation of lipids and insulin sensitivity to protect the body from lipid-induced insulin resistance.

9.7 Adaptive Vascular Remodeling: A Role for Sirtuins?

Skeletal muscle is a highly metabolic tissue and must therefore regulate the supply of oxygen to match the demand needed to perform work. Exercise training is a well-known stimulus for increasing capillarization, which ultimately contributes to an improved aerobic capacity. Various angiogenic factors have been recognized for the induction of capillary growth. One of the most important factors, vascular endothelial growth factor (VEGF), has been shown to increase in response to acute exercise (Amaral et al. 2001; Breen et al. 1996). This proangiogenic factor is expressed following the activation of the transcription factor hypoxia inducible factor-1α (HIF-1α). VEGF can also be activated by low partial pressure of oxygen, high oxidative stress, inflammatory cytokines, and energy balance-related changes in AMPK activity [reviewed in (Breen et al. 2008)]. This oxygen-sensitive protein can facilitate ATP generation following mitochondrial biogenesis by inducing an angiogenic response that includes the elevation of VEGF to match the new demand of oxygen as a substrate for oxidative respiration (O'Hagan et al. 2009). HIF-1α induction of VEGF-A expression occurs in skeletal muscle during exercise and with ischemic conditions in the heart (Ameln et al. 2005; Gustafsson et al. 1999). VEGF-A reaches its highest levels following 4 days of chronic muscle electrical stimulation, while the capillary-to-muscle fiber ratio continually increased from 7- to 28-day days of muscle stimulation (Dawson and Hudlicka 1989; Hang et al. 1995; Hudlicka and

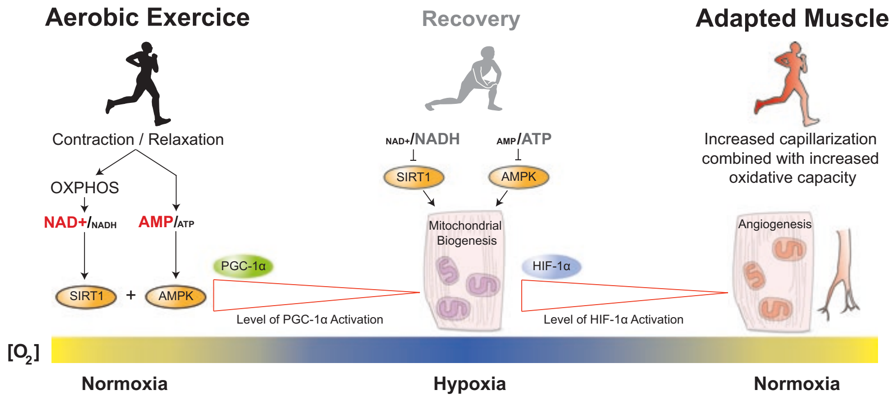

Fig. 9.2 With aerobic exercise there is an increase in oxidative phosphorylation and an elevation in both the NAD^+/NADH and AMP/ATP ratio. This results in the activation of SIRT1 and AMPK, which together activate PGC-1α to induce mitochondrial biogenesis. Increases in mitochondrial biogenesis then lead to intracellular hypoxia and activation of HIF-1α. SIRT1, a negative regulator of HIF-1α, demonstrates reduced activity in hypoxic environments due to the reduction in OXPHOS activity and NAD^+ availability. With SIRT1 inhibition, the elevation in HIF-1α activity activates angiogenesis to help supply the increased demand for oxygen by the additional mitochondrial content

Price 1990). Interestingly, PGC-1α overexpression leads to the induction of mitochondrial biogenesis in primary human skeletal muscle cells, followed by an increase in oxygen consumption, leading to intracellular hypoxia, and the stabilization of HIF-1α (O'Hagan et al. 2009).

Although not yet demonstrated in skeletal muscle, SIRT1 was found to negatively regulate HIF-1α via deacetylation, while PCAF was responsible for acetylating and activating the oxygen-sensitive protein (Lim et al. 2010). Mice and cells exposed to hypoxic conditions exhibit activation of glycolysis, inhibition of the tricarboxylic acid cycle and decreases in NAD^+, thereby inactivating SIRT1 and the inhibition of HIF-1α. During normoxic conditions, NAD^+ concentrations are higher and therefore activate SIRT1-mediated deacetylation and inactivation of HIF-1α (Lim et al. 2010). This may also indicate an inactivation of HIF-1α in muscle with exercise, as exercise has also been shown increase the NAD^+/NADH ratio and SIRT1 activity (Cantó et al. 2009; 2010; Costford et al. 2010; Graham and Saltin 1989) (Fig. 9.2). Based on these findings the induction of mitochondrial biogenesis following exercise would lead to intracellular hypoxia and a reduction in OXPHOS activity, NAD^+ availability, and the deactivation of SIRT1 and activation of HIF-1α. Therefore, it is possible that the induction of mitochondrial biogenesis with exercise results in a temporary hypoxic environment, which would activate angiogenesis to supply the renewed demand for oxygen resulting from increased mitochondrial content. In addition, with the increases in mitochondrial content and the partial pressure of oxygen comes an elevated potential for ROS generation. However, with training there is an increase in the expression of the mitochondrial uncoupling protein UCP3

that restricts the production of mitochondrially generated ROS (MacLellan et al. 2005). Interestingly, SIRT1 was shown to inhibit the expression of UCP3, thus corroborating the hypothesis that SIRT1 is only active during certain windows of metabolic change that are dictated by the intracellular NAD^+/NADH ratio (Amat et al. 2007). Therefore, following exercise-induced increases in muscle mitochondrial biogenesis, reductions in the NAD^+/NADH ratio and SIRT1 activity would allow for the increased expression of UCP3, which would balance the heightened potential for ROS generation.

To further emphasize the negative relationship between SIRT1 and HIF-1α, HIF-1α is also involved in cellular pathways related to glycolysis, such as glucose uptake and enzymes that regulate carbohydrate catabolism. This includes the transcriptional activation of glucose transporter 1 in muscle and the elevated expression of hexokinase 1 and 2, lactate dehydrogenase, pyruvate kinase and other enzymes involved in glucose metabolism [reviewed in (Semenza 2001)]. There is also some evidence that in muscle of aged rats SIRT1 activity is reduced, due to low NAD^+ levels, thereby releasing inhibition of HIF-1α and supporting the increasing reliance of aged muscle on glycolysis for energy production (Koltai et al. 2010). Specifically, both liver and muscle exhibit an accumulation of glycogen intermediates, which would suggest altered glycogen metabolism in aged mice, while increases in lactate and reduced glycolytic intermediates suggest elevated anaerobic glycolysis (Houtkooper et al. 2011). Interestingly, SIRT6, as described in the following section, also inhibits HIF-1α along with glucose uptake in muscle. Consequently, it is possible that SIRT1 and SIRT6 coordinate their functions to regulate HIF-1α, glucose uptake and glycolysis in muscle (Fig. 9.2). A similar concept has been demonstrated in macrophage precursors during an acute inflammatory response where the NAD^+-dependent activation of SIRT1 and SIRT6 were responsible for the activation of PGC-1α and the silencing of HIF-1α transcription, respectively, leading to a metabolic switch from glucose metabolism to fatty acid oxidation (Liu et al. 2012). Future research will help delineate the potential role of SIRT1 in coordinating a metabolic substrate switch during changes in oxygen availability and redox and energetic states in skeletal muscle.

In contrast to what occurs within the muscle cell, SIRT1 plays a more active role during hypoxic conditions in peripheral muscle satellite cells as a regulator of satellite cell proliferation (Rathbone et al. 2009). In satellite cells hypoxic conditions induced a SIRT1-mediated increase in cell cycle progression through the modulation of cyclin-dependent kinase inhibitors, key regulators of G_1-S phase transitions. Therefore, by activating cell-cycle progression in satellite cells during hypoxia, SIRT1 acts to protect the surrounding muscle tissue by preparing the satellite cells for muscle regeneration. Concurrently, SIRT1 inhibits the differentiation of muscle satellite cells indicating that a muscle under duress will prepare for regeneration but will not initiate this program until the muscle is once again is in a normoxic condition.

9.8 Evidence for the Involvement of Other Sirtuins in Muscle Function

Other sirtuin proteins have also demonstrated potential roles for the maintenance of muscle homeostasis. SIRT2 binds and deacetylates FOXO1, a regulator of autophagy and muscle wasting, in mammalian cells. During oxidative stress or serum starvation FOXO1 is released by SIRT2, allowing for the acetylation of FOXO1 and the induction of an autophagic response (Zhao et al. 2010). Therefore, since FOXO proteins regulate key pathways during sarcopenia and muscle disuse (Sandri et al. 2004; Senf et al. 2011), SIRT2 may prove to be an interesting new avenue to examine autophagy and atrophy signaling in skeletal muscle.

SIRT3, 4 and 5 are localized to the mitochondria and may therefore play key roles in skeletal muscle metabolism [reviewed in (Pirinen et al. 2012)]. The involvement of SIRT3 in muscle metabolism has proven to be controversial, as evidenced by the various physiological observations of SIRT3-KO models. Whole body SIRT3-KO mice exhibit reduced fatty acid oxidation by approximately 50 %, due to the hyperacetylation of long-chain acyl-CoA dehydrogenase, yet show no reductions in complexes I, III, and V of the electron transport chain (Hirschey et al. 2010). However, muscle-specific SIRT3-KO mice, despite also demonstrating reductions in mitochondrial protein hyperacetylation, do not exhibit changes in global metabolic homeostasis (Fernandez-Marcos et al. 2012). Interestingly, SIRT3 expression increases with both treadmill training and chronic contractile activity in rodent skeletal muscle (Gurd et al. 2012; Hokari et al. 2010; Palacios et al. 2009), but not following acute bouts of exercise. In addition, increases in SIRT3 expression occur with CR, another chronic energy stress (Palacios et al. 2009). Furthermore, oxidative muscles from resting animals, such as the soleus, were found to have the greatest expression level of SIRT3 (Hokari et al. 2010; Palacios et al. 2009). In contrast, with hindlimb immobilization, SIRT3 expression is reduced along with mitochondrial biogenesis (Hokari et al. 2010). There is also a correlation between reduced levels of SIRT3 expression in streptozotocin-induced diabetic mice and chronic high-fat treatment (Jing et al. 2011; Palacios et al. 2009). However, surprisingly, in aging mice SIRT3 expression remains stable in mice from 3- to 24-months of age (Jing et al. 2011). Therefore, it would appear that SIRT3 expression may coordinate with the metabolic environment in some studies, but certainly more evidence is needed to fully understand what role SIRT3 plays in the cell and if it in fact plays a role at all on mitochondrial biogenesis and metabolic homeostasis in skeletal muscle.

SIRT4 is the only sirtuin that has been described to have potentially physiologically relevant NAD^+-dependent ADP-ribosyltransferase activity. Specifically, SIRT4 was demonstrated to ADP-ribosylate and downregulate glutamate dehydrogenase activity in pancreatic cells thus inhibiting insulin secretion (Haigis et al. 2006). Glutamate dehydrogenase catabolizes glutamine to the TCA cycle intermediate α-ketoglutarate, a major step in anaplerosis, which is crucial for the production of biomass building blocks such as nucleotides, amino acids and lipids. Notably, the

mammalian target of rapamycin complex 1 (mTORC1), a complex that regulates nutrient uptake and anabolism, inhibits SIRT4 transcription thereby releasing the inhibition of glutamate dehydrogenase activity and leading to enhanced cell proliferation (Csibi et al. 2013). Despite there being very little research examining SIRT4 function in muscle, the knockdown of SIRT4 in muscle primary cells increases AMPK phosphorylation, fatty acid oxidation and oxygen consumption (Nasrin et al. 2010) and therefore could also prove to be an important sirtuin for muscle metabolism.

Although SiRT5 was previously shown to be part of the skeletal muscle mitochondrial proteome, its role in the mitochondria has not yet been demonstrated (Lefort et al. 2009). SIRT6, however, plays an important role in glucose homeostasis. Skeletal muscle deficiency of the SIRT6 protein leads to severe hypoglycemia (Xiao et al. 2010). Under normal conditions, SIRT6 inhibits HIF-1α leading to decreased glycolysis and activation of oxidative phosphorylation (Zhong et al. 2010) (Fig. 9.2). Cells that are deficient in SIRT6 exhibit elevated levels of HIF-1α activity and glucose uptake along with reduced mitochondrial respiration (Zhong et al. 2010). Interestingly, SIRT6 expression increases with age as does HIF-1α, while both proteins are reduced in these animals following exercise (Koltai et al. 2010). SIRT6 may therefore act as a compensation mechanism during old age to limit HIF-1α overactivity, which can be reversed with exercise.

It is apparent that the function of many of these sirtuins has only begun to be elucidated and thus warrants additional work to further define their roles in skeletal muscle and metabolism. In particular, nuclear and cytoplasmic sirtuins may play more transcriptional roles while mitochondrial sirtuins may prove to have more immediate adaptive properties to changes in energy homeostasis. Future work on sirtuins may therefore reveal potential targets for the manipulation of mitochondrial function and muscle homeostasis during disease.

9.9 Conclusions

In muscle, mitochondrial biogenesis and capillarization is required to maintain an active and healthy cellular state that synchronizes energy status and blood flow to changes in local muscle metabolism. These processes, involved in the maintenance of muscle tissue health, have become increasingly complex. With the current rise in the elderly population there is an urgent need to understand the basic mechanisms of muscle homeostasis and to tackle, in a rational fashion, medical problems such as age-related loss of muscle function or glucose homeostasis. In addition, much like what occurs with aging, pathologies such as muscular dystrophies, amyotrophic lateral sclerosis, diabetes and cancer have also been defined by reductions in muscle mass, mitochondrial content and function, and the associated reduced capacity for adaptations to metabolic stress.

From our discussion, it should now be clear that the tight regulation and coordination of SIRT1, AMPK, NAMPT and PGC-1α positively regulate mitochondrial biogenesis and function, while also coordinating HIF-1α and VEGF controlled increases in blood flow and supply in response to muscle adaptations. A better understanding of the involvement of sirtuins in mechanisms that underlie the pathogenesis of metabolic dysregulation has been the goal of many studies and is an important step for the development of new therapeutic approaches. It would appear that in many cases sirtuins orchestrate a protective mechanism to attenuate the development of age related diseases. Pharmacological therapies or lifestyle interventions (i.e. exercise) may offer promising approaches that can modulate the activity of this integrated system, leading to the maintenance of mitochondrial content and function, energy and oxygen supply and ultimately skeletal muscle health.

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Chapter 10
Sirtuins and Aging

Carles Cantó and Riekelt H. Houtkooper

10.1 Introduction

Aging was long considered a passive decline in physiological function that was ultimately followed by death. Nevertheless, in western societies mean lifespan increased from about 60 to more than 80 years in the last century. Most of this increase was simply attributed to healthier environments, e.g. less infectious diseases due to cleaner drinking water. The accumulating knowledge on the pathophysiology of aging suggested, however, that aging and age-related diseases could be averted. Indeed, tissues of aged individuals undergo various stresses with which to cope, including but not limited to DNA damage, deficient nutrient sensing, protein imbalance, and mitochondrial dysfunction (Lopez-Otin et al. 2013).

Caloric restriction (CR) is a physiological condition defined by a 20–50 % reduced caloric intake while maintaining proper micronutrient balance. Strikingly, CR improved many conditions related to poor health and extended lifespan in many organisms including worms, flies, and rodents (reviewed in (Fontana et al. 2010)). CR was also tested in non-human primates, and although the effect on lifespan was inconclusive, likely because of differences in experimental setup, the older monkeys on CR were generally in better health (Colman et al. 2009; Mattison et al. 2012). Although the mechanisms by which CR achieves these better profiles are still under intense investigation, several metabolic signaling pathways, including sirtuins, have been implicated (Houtkooper et al. 2010b).

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With the identification of the *C. elegans* gene *daf-2* (homolog of insulin/IGF-1 receptor) as an aging gene in the early 1990s (Kenyon et al. 1993), more research has focused on the molecular underpinning of the age-related physiological decline. Indeed, extensive molecular networks were identified that impinge on the aging process (Houtkooper et al. 2010b). Activation of caloric excess pathways, such as insulin/IGF-1 or mTOR, limits lifespan (Johnson et al. 2013; Kenyon 2010). Conversely, activation of energy deprivation-related pathways, such as AMPK, sirtuins or FOXO, extends lifespan (Canto and Auwerx 2011; Eijkelenboom and Burgering 2013; Houtkooper et al. 2012).

10.2 Sirtuins as Aging Genes in Lower Organisms

The sirtuin field was first linked to aging following the discovery that a complex consisting of yeast *Sir2p*, *Sir3p*, and *Sir4p* regulates lifespan (Kaeberlein et al. 1999). Deletion of any of these genes led to shortened lifespan. In the case of *sir3* and *sir4*, this was due to the fact that both mating types were expressed, leading to sterility. For *sir2*, however, this was not true. Irrespective of mating type, *sir2*-deficient yeast cells lived shorter than controls, which was associated with the accumulation of extrachromosomal rDNA circles, a cause of replicative lifespan in yeast (Kaeberlein et al. 1999). Most convincingly, however, ectopic expression of *sir2* increased lifespan by about 25 % (Kaeberlein et al. 1999). In addition, the yeast *SIR2* gene proved critical for the lifespan extension brought about by CR (Lin et al. 2000).

Following these initial discoveries, the role of sirtuin genes in longevity was translated to other species. The *C. elegans* genome contains four sirtuin genes, *sir-2.1–sir2.4. sir-2.1* is most homologous to yeast *Sir2p*, and similar to the latter its overexpression extended nematode lifespan (Tissenbaum and Guarente 2001). The fly *D. melanogaster* has five sirtuin homologs, and dSir2 also extends lifespan when overexpressed (Rogina and Helfand 2004). However, these findings were challenged and ascribed to poorly controlled genetic background (Burnett et al. 2011). Indeed, when the transgenic worms and flies were backcrossed to identical backgrounds, the lifespan extension was lost (Burnett et al. 2011), although a small but significant effect may still be attributed to *sir-2.1* overexpression in *C. elegans* (Mouchiroud et al. 2013; Viswanathan and Guarente 2011).

Even though the role of sirtuin genes in extending lifespan is under debate, there is considerable evidence that sirtuins are involved in managing normal health or protecting against stress conditions. Most strikingly, *SIR2/sir-2.1* was identified as a critical regulator of the nutritional stress induced by CR. Indeed, when *SIR2* was deleted, yeast did not display the beneficial effects of this dietary intervention (Lin et al. 2000), and similar effects occurred in *Sir2*-deficient flies (Rogina and Helfand 2004). Worms lacking *sir-2.1* did not live longer upon CR, and displayed shorter lifespan when exposed to hydrogen peroxide, heat stress, or UV radiation (Wang and Tissenbaum 2006). Additionally, and as discussed in more detail below,

activation of sirtuins using pharmacological compounds, e.g. resveratrol or NAD^+ precursors, protects both mice and worms against different types of stress, and extends worm lifespan.

After *SIR2/sir-2.1* was identified as a metabolic regulator of CR and lifespan, several groups embarked on the quest for pharmacological activators. The most famous example is resveratrol, a polyphenol that is found in various foods such as mulberries, peanuts and grapes (Howitz et al. 2003). Resveratrol, but also other polyphenols such as fisetin and butein, increased SIRT1 deacetylase activity towards a fluorescently-labeled target peptide, and extended yeast lifespan by 30–70 % (Howitz et al. 2003). Similarly, resveratrol extended lifespan in *C. elegans* and *D. melanogaster* in a *sir-2.1/Sir2*-dependent fashion (Wood et al. 2004). As for sirtuin overexpression, however, the effect of resveratrol on longevity was questioned in later work (Bass et al. 2007). Additionally, several groups reported evidence that resveratrol might not directly activate SIRT1 in vivo, but rather through AMPK (Beher et al. 2009; Canto et al. 2010; Pacholec et al. 2010; Um et al. 2010) or the cAMP-degrading phosphodiesterase PDE4 (Park et al. 2012), although a direct allosteric effect on SIRT1 cannot be excluded (Hubbard et al. 2013). Therefore, small-molecule activators of SIRT1 were developed, leading to a range of so-called SIRT1 activating compounds (STACs), most notably SRT1720 (Milne et al. 2007). While these compounds were far more potent than resveratrol with respect to in vitro activation of SIRT1 (Milne et al. 2007), and some positive health effects were reported in mice (see below), no lifespan extension was observed after treatment in simple organisms (Zarse et al. 2010). A more recent strategy to activate sirtuins is based on the premise that these enzymes require NAD^+ as an enzymatic cosubstrate and that increasing the bioavailability of NAD^+ dictates sirtuin activity (Houtkooper et al. 2010a). Two strategies were developed to achieve this, either increasing the load of NAD^+ precursors or blocking the utilization of NAD^+ by competing enzymes such as PARPs (poly(ADP-ribose) polymerases). Indeed, when worms were treated with the NAD^+ precursor nicotinamide riboside (NR), or when NAD^+ consumption was blocked using PARP inhibitors, these worms lived 25 % longer, an effect that was completely *sir-2.1*-dependent (Mouchiroud et al. 2013).

10.3 Sirtuins and Mammalian Aging

The evidence obtained on lower eukaryote organisms raised the possibility that sirtuins might also influence mammalian lifespan. The first complication to test such hypothesis is that mammalians host seven sirtuin enzymes (SIRT1-7). Their initial characterization already pointed out possible individual non-overlapping roles, as sirtuins can differ in subcellular compartmentalization, catalytic activity and substrate specificity. Therefore, most studies have focused on the possible role of individual sirtuins on aging. In this sense, only solid and conclusive links have been established between SIRT1, SIRT3, SIRT6 and aging. Hence, we will place our main focus on these three sirtuins.

10.3.1 SIRT1

Being the closest homolog to the yeast *Sir2* and worm *sir2.1*, SIRT1 was early highlighted as a mammalian sirtuin that could influence lifespan. Consistent with this, diverse experimental lines have demonstrated how SIRT1 might be an attractive target for the protection against many hallmarks of aging. Mice overexpressing SIRT1 are protected against age-related insulin resistance, osteoporosis, impaired wound healing and hepatic steatosis (Herranz et al. 2010). However, SIRT1 transgenic mice do not live longer than wild-type mice (Herranz et al. 2010). This demonstrates that whole-body SIRT1 overexpression *per se* is not enough to enhance mice lifespan, even though it delays the apparition of age-related symptoms. At this point on would wonder why enhanced healthspan does not translate into longer lifespan. While this is currently not clearly understood, one possibility is that SIRT1 overexpression loses linearity with SIRT1 activity in the late aging phases, therefore not supporting healthspan in an indefinite manner. In line with this possibility, NAD^+ levels, a rate limiting co-substrate for SIRT1, dramatically decrease upon aging (Braidy et al. 2011; Massudi et al. 2012; Ramsey et al. 2008). In fact, SIRT1 protein levels are higher in tissues from old rats than in young ones, but its activity is lower, due to the rate-limitation exerted by the decrease in NAD^+ (Braidy et al. 2011). Therefore, NAD^+ levels might also rate-limit the ability of SIRT1 transgenesis to maintain metabolic benefits in the late aging phases. Alternatively, SIRT1 might protect against some metabolic-related diseases, but might affect the susceptibility of the animal to develop other primordia deathly diseases, such as lymphomas (Herranz et al. 2010), or even potentiate the incidence of cancers driven by PTEN mutations (Herranz et al. 2013).

Further evidence on the role of SIRT1 during aging comes from the use of SIRT1 activating compounds (STACs). While it is still controversial whether resveratrol directly activates SIRT1 in cellular contexts (Hubbard et al. 2013; Pacholec et al. 2010), it seems clear SIRT1 activity is required for resveratrol to promote many of its metabolic and health benefits in mammalian cells an organisms (Price et al. 2012). Mice treated with resveratrol do not live longer when fed regular chow diets (Pearson et al. 2008). However, as observed in SIRT1 transgenic mice, resveratrol supplementation led to a marked reduction in a myriad of age-related symptoms, including preserved motor coordination, higher bone mineral density and reduced inflammation (Pearson et al. 2008). Importantly, despite the similar lifespan on chow diet, mice fed with resveratrol were protected against the reduction in lifespan induced by high caloric diets (Baur et al. 2006). As with resveratrol, treatment with SRT1720, a second generation STAC (Milne et al. 2007), also had protective effects against the metabolic damage induced by high-fat feeding and improved insulin sensitivity, preventing the lifespan curbing associated with high caloric diets (Minor et al. 2011). The astonishing similarities between resveratrol and SRT1720 effects are, however, also clouded by comparable doubts on their mechanism of action (Pacholec et al. 2010). In addition, both compounds show substrate-specific effects on SIRT1 activity (Hubbard et al. 2013; Lakshminarasimhan et al. 2013). While this

might be somehow discouraging to truly extrapolate the effects of STACs exclusively to SIRT1 activation, it might, however, help identifying SIRT1 subsets of targets that could be key in healthspan regulation.

One key point on the use of STACs or whole-body SIRT1 transgenic mice is that they target activation in multiple tissues. This, however, might not be the best strategy. For example, calorie restriction has divergent effects in muscle and liver SIRT1 regulation (Chen et al. 2008). Therefore, targeted tissue-specific activation of SIRT1 might be required so that lifespan effects are not blunted by compensatory effects in other tissues where SIRT1 should be naturally repressed during the aging process. In line with this concept, it has recently been demonstrated that brain-specific SIRT1 overexpression is enough to extend lifespan and delay aging in mice (Satoh et al. 2013). The observation that a second brain-specific transgenic line did not display similar lifespan effects led the authors to hypothesize that the effect of brain SIRT1 on lifespan depends on an exquisite balance between SIRT1 expression levels in the dorsomedial and lateral hypothalamic nuclei versus other brain regions (Satoh et al. 2013). To date, this is the only SIRT1 gain-of-function mammalian model that displays enhanced lifespan.

Additional information on how SIRT1 can influence mammalian lifespan can be retrieved from models of defective SIRT1 activity. The total deletion of SIRT1 severely compromises embryonic development and postnatal life in inbred mice (Cheng et al. 2003; McBurney et al. 2003). When outbred, SIRT1 defective mice are viable, but metabolically inefficient, and many fail to survive the first year following birth (Boily et al. 2008). While CR generally prolongs lifespan in mice, preliminary data suggest that it rather exacerbates mortality in SIRT1-defective mice (Boily et al. 2008). A recent study has certified that the absence of SIRT1 in outbred mice impedes the ability of CR to extend lifespan (Mercken et al. 2014). Interestingly, SIRT1 heterozygous mice showed similar lifespan extension by CR than wild-type mice. This indicates that endogenous SIRT1 levels are enough to maximize the lifespan effects triggered by CR.

Altogether, the critical role SIRT1 on development and early postnatal life complicates the evaluation of the contribution of SIRT1 on adult lifespan. Therefore, the use of temporally or spatially controlled models is required to demonstrate the relevance of SIRT1 on adult mammalian lifespan. To overcome this issue, the Sinclair lab has recently generated a tamoxifen-induced mouse model that allows a temporally controlled deletion of the SIRT1 gene (Price et al. 2012). The use of genetically engineered mouse models to delete the SIRT1 gene in a tissue-specific fashion has also provided relevant data indicating that SIRT1 is a key contributor in mammalian healthspan. Deletion of SIRT1 in liver has been generally linked with hepatic steatosis, hyperlipidemia and other complications associated with dyslipidemia, such as cholesterol gallstones (Purushotham et al. 2009; Purushotham et al. 2012; Wang et al. 2010, 2011). Similarly, the deletion of SIRT1 in adipose tissue leads to increased adiposity and prompts mice to develop obesity and insulin resistance (Chalkiadaki and Guarente 2012; Gillum et al. 2011). In this respect, it is interesting to note that SIRT1 deletion in muscle, a key tissue contributing to metabolic homeostasis, did not show any overt phenotype (Menzies et al. 2013; Philp et al. 2011;

Schenk et al. 2011). Altogether, while lifespan has not been closely examined in these models, most information to date points out that SIRT1 is required for the maintenance of metabolic homeostasis and that defective SIRT1 activity leads to metabolic complications that might negatively influence lifespan.

Further evidence on whether SIRT1 can contribute to mammalian lifespan can be inferred from genetic linkage studies on human populations. The results to date, however, have not highlighted an obvious correlation of SIRT1 with human longevity. Pioneering studies on German centenarians found no noteworthy influence of five known single nucleotide polymorphisms across the entire SIRT1 gene on exceptional human longevity (Flachsbart et al. 2006). Similar observations were reported shortly after on a Japanese population (Willcox et al. 2008) and two additional European based studies (Kuningas et al. 2007; Zillikens et al. 2009). In none case SIRT1 could be associated with higher lifespan prevalence. This said, however, SIRT1 targets, such as FOXO3a (Brunet et al. 2004) have been closely associated with enhanced human longevity (Willcox et al. 2008). This might indicate that many of the observed effects of SIRT1 on mammalian lifespan might be secondary to protection against age-related diseases or through its influence on the activity of lifespan effectors, such as FOXO3a.

10.3.2 SIRT3

SIRT3 was the first to provide a tangible link between mammalian aging and sirtuins. Even before the elucidation of its biological actions, human genetic studies indicated a link between SIRT3 polymorphisms and exceptional aging (Rose et al. 2003). Concomitant efforts highlighted how a polymorphism linked to an enhancer region of the SIRT3 gene could be clearly segregated in aged individuals, to the point that the allele lacking enhancer activity was virtually absent in males older than 90 years (Bellizzi et al. 2005). While further confirmation of such findings will be required, they suggest that reduced SIRT3 expression might negatively impinge on longevity. During the last five years a constellation of works have demonstrated how SIRT3 is a major mitochondrial deacetylase enzyme with key roles in the maintenance of mitochondrial function (Ahn et al. 2008; Hirschey et al. 2011a; Hirschey et al. 2011b; Lombard et al. 2007). Given the close link between mitochondrial function and many age-related pathologies, it should not come as a surprise that SIRT3 might influence lifespan. While SIRT3 gain-of-function models have not yet been reported, the use of SIRT3 deficient mice has demonstrated that SIRT3 is critical for some of the anti-aging effects of caloric restriction, such as protection against hearing loss (Someya et al. 2010). Furthermore, SIRT3 deficient mice are prone the development of insulin resistance and to exacerbate the metabolic damage caused by high-fat diets (Hirschey et al. 2011b). Strikingly, deletion of SIRT3 exclusively in liver or in muscle did not lead to any major phenotype, despite a dramatic hyperacetylation of mitochondrial proteins in the respective tissues (Fernandez-Marcos et al. 2012). This indicates (1) that mitochondrial hyperacetylation does not

necessarily mean mitochondrial dysfunction, and (2) that the health complications derived from global SIRT3 deletion cannot be explained just by defective SIRT3 activity on the liver or muscle tissue. Most of the protective effects of SIRT3 have been linked to the ability of SIRT3 to modulate reactive oxygen species (ROS) species production and detoxification (Chen et al. 2011; Qiu et al. 2010; Tao et al. 2010). Consequently, it would be expected that SIRT3 actions might have broader implications on age-related pathologies that are critically affected by ROS metabolism. In this line, the links between SIRT3, ROS and aging were further consolidated when SIRT3 activity was also linked to the protection of oncogenic processes (Bell et al. 2011; Finley et al. 2011; Kim et al. 2010).

10.3.3 SIRT6

While type I sirtuins (SIRT1-3) historically received most scientific attention, recent reports are highlighting how SIRT6 might actually take center spot in the relation between sirtuins and mammalian aging. SIRT6 deficient mice appear relatively normal at birth, but in a few weeks they develop an acute degenerative phenotype, reminiscent of the progeroid syndrome (Mostoslavsky et al. 2006). This was accompanied by a severe hypoglycemia, which leads to the death of SIRT6 null mice by one month of life (Mostoslavsky et al. 2006). Later efforts identified SIRT6 as a repressor for the Hypoxia Inducible Factor 1α (*Hif1α*) (Zhong et al. 2010). In essence, SIRT6 maintains deacetylation of H3K9 residues in the promoters of *Hif1α* and glycolytic genes, maintaining them in a repressed state (Zhong et al. 2010). Upon SIRT6 deletion, *Hif1α* is transcriptionally upregulated and generates an abnormal potentiation of glucose uptake, leading to severe hypoglycemia (Zhong et al. 2010). Overall, this would highlight that SIRT6 would work as a critical metabolic regulator.

The impact of SIRT6 in mammalian lifespan has recently been further supported by the observation that transgenic overexpression of SIRT6 enhances longevity in male mice (Kanfi et al. 2012). The lifespan extension promoted by SIRT6 overexpression is somehow modest, around 15 %, and could not be attributed to prevention against any specific sort of age-related incidence (Kanfi et al. 2012). Rather, SIRT6 overexpression simply delayed the aging process, with animals dying from the same disease spectrum as wild-type mice. This observation was the culmination of several indirect evidences indicating that SIRT6 gain-of-function could impact on mammalian lifespan. First, SIRT6 overexpression was shown to prevent the metabolic damage and inflammation induced by high-fat diet (Kanfi et al. 2010).. Second, SIRT6 has been intimately linked to the maintenance of genomic stability (Mostoslavsky et al. 2006). Finally, SIRT6 has recently been shown to act as a tumor suppressor (Sebastian et al. 2012). Therefore, enhanced SIRT6 expression during the aging process might be protective to slow down physiological decline.

10.3.4 Other Sirtuins

Unfortunately very little efforts have been reported to date on the roles of other sirtuins in the aging process. Any possible link can only be extrapolated by very indirect evidence. For example, no study has been done on how SIRT2 gain-of-function influences lifespan, and SIRT2 defective mice do not show any overt phenotype (Bobrowska et al. 2012). However, several (Luthi-Carter et al. 2010; Outeiro et al. 2007; Taylor et al. 2011), but not all (Bobrowska et al. 2012), studies have proposed that inhibition of SIRT2 might be beneficial in the battle against neurodegenerative diseases, by diminishing cholesterol levels in neurons (Luthi-Carter et al. 2010). In contrast, SIRT2 has been proposed to be protective against cancer, as SIRT2 is downregulated in gliomas and gastric sarcomas (Peters et al. 2010), and inactivating mutations have been found in melanomas (Lennerz et al. 2005). Therefore, it would be tempting to speculate about a possible role for SIRT2 in tumor suppression. Altogether, these observations suggest that, while the physiological influence of SIRT2 on lifespan remains largely unexplored, the activity of SIRT2 might be preventive against some aging comorbidities.

SIRT4 and SIRT5 deficient mice also lack any overt phenotype and are indistinguishable from wild-type mice in basal conditions (Haigis et al. 2006; Yu et al. 2013), When challenged with a high-fat diet, SIRT5 mice still remain phenotypically indistinguishable from wild-type mice (Yu et al. 2013), but SIRT4 are protected from body weight gain due to increased fat catabolism (Laurent et al. 2013). So far, there are no human genetic reports highlighting any linkage between the SIRT4 or SIRT5 genes with aging. However, the potential roles of SIRT4 and SIRT5 regulating mitochondrial metabolism makes it difficult to completely rule out potential implications of these mitochondrial sirtuins in age-related disorders. SIRT4, for example, has been shown to coordinate the balances between lipid synthesis and oxidation. SIRT4 represses fatty acid oxidation, while promoting lipid anabolism (Laurent et al. 2013; Nasrin et al. 2010), probably through the deacetylation and repression of malonyl-CoA decarboxylase (MCD) (Laurent et al. 2013). This, in turn, would increase the intracellular amount of malonyl-CoA, which allosterically inhibits CPT1 activity, and, hence, lipid intake into the mitochondria for oxidation, while, at the same time, constitutes a major backbone for lipid anabolism. In addition, SIRT4 has recently been reported as a gatekeeper for glutamine metabolism, influencing this way tumor development (Csibi et al. 2013; Jeong et al. 2013). Altogether, the complex metabolic actions of SIRT4 make it difficult to predict any possible impact on lifespan. In the case of SIRT5, very little is known on its physiological actions other than an involvement in urea metabolism upon prolonged fasting periods (Nakagawa et al. 2009).

10.4 Molecular Mechanisms of Longevity by Sirtuins

Despite the fact that the lifespan regulation by sirtuins was already recognized in the 1990s, the exact mechanisms by which they exert these effects are still nebulous. The pleiotropic nature of SIRT1 as a central metabolic stress regulator suggests that multiple protective pathways are activated simultaneously (Houtkooper et al. 2012). Many of the beneficial effects involve mitochondrial function. One of the key players in this process is PGC-1α, a master regulator of mitochondrial biogenesis in mammals, although no homolog has been described in *C. elegans* (Canto and Auwerx 2009). PGC-1α is deacetylated and thereby activated by SIRT1, driving this way mitochondrial metabolism. Hence, by coupling metabolic sensing through NAD^+ and mitochondrial biogenesis, SIRT1 activation ensures the fine-tuning energy expenditure, metabolic flexibility and stress response for long-term protection (Canto and Auwerx 2009). Several groups identified the FOXO transcription factor (*daf-16* in *C. elegans*) as a deacetylation target of SIRT1 (Brunet et al. 2004; Motta et al. 2004; van der Horst et al. 2004). Since FOXO regulates the expression antioxidant proteins and controls metabolic flexibility (Eijkelenboom and Burgering 2013), it was hypothesized that this was a critical component for lifespan extension. Indeed, when *daf-16* was knocked out, *sir-2.1* overexpression could not induce longevity (Berdichevsky et al. 2006; Tissenbaum and Guarente 2001). The same is true for pharmacological activation of sirtuins, as *daf-16* mutant worms did not live long after supplementation with the NAD^+ precursor NR or PARP inhibitors (Mouchiroud et al. 2013).

Besides ROS defense, a second mitochondrial stress pathway was identified in the defense against aging, the mitochondrial unfolded protein response (UPR^{mt}) (Andreux et al. 2013; Haynes and Ron 2010). UPR^{mt} was identified as a mitochondrial protein folding stress pathway (Zhao et al. 2002), but pioneering work from the Dillin lab established the functional link between mitochondrial function, UPR^{mt}, and aging (Durieux et al. 2011). When primary OXPHOS function was disturbed in worms, for instance by silencing the complex IV gene *cco-1*, this induced UPR^{mt}, which in turn delays age-related decline and extends lifespan (Durieux et al. 2011). Similarly, interfering with mitochondrial translation induces UPR^{mt} through a state called mitonuclear protein imbalance (Houtkooper et al. 2013). Mitonuclear protein imbalance refers to a dyssynchrony between OXPHOS subunits encoded by mtDNA and nuclear DNA. If the expression of OXPHOS subunits from one of these genomes is not matched by expression of the other genome, this may lead to accumulation of misfolded protein and activation of UPR^{mt} (Houtkooper et al. 2013). Since mitochondrial biogenesis induced by sirtuin activation may lead to an increased flux of proteins towards the mitochondrial import machinery and increased burden on the protein folding capacity, it was hypothesized that UPR^{mt} is central to the lifespan extension imposed by sirtuin activators. Indeed, overexpression of *sir-2.1* in worms induced UPR^{mt}, and deletion of the UPR^{mt} regulator *ubl-5* was essential for its longevity induction (Mouchiroud et al. 2013). Along the same line, pharmacological sirtuin activation using resveratrol, NAD^+ precursors, or PARP

inhibitors extended lifespan in a UPR^{mt}-dependent fashion (Houtkooper et al. 2013; Mouchiroud et al. 2013). Interestingly, while UPR^{mt} is an early phase protective response, ROS defense trails behind and is only activated in a later phase (Mouchiroud et al. 2013). Though it is evident that ROS defense and UPR^{mt} are linked under these conditions, more work is necessary to establish the chain of events and the molecular links.

10.5 Perspectives

The initial excitement following the identification of sirtuins as aging genes and metabolic regulators boosted a wealth of studies into the mechanisms underlying these processes. Several compounds activating SIRT1 lead to improved metabolic health and prevent the development of age-related diseases. Although the lifespan effects of SIRT1 activation are under debate, both in simple organisms and primates, the beneficial effects on metabolic health are uncontested. Additionally, SIRT1 may extend lifespan under stressed conditions. Indeed, resveratrol extends lifespan of mice on high-fat diet, while not affecting lifespan on regular diet (Pearson et al. 2008). This is reminiscent of the CR-fed monkeys. While not extending lifespan compared to healthily fed monkeys, CR extended lifespan when compared to monkeys on ad libitum diet (Colman et al. 2009; Mattison et al. 2012).

In recent years, evidence surfaced for a role of other sirtuins, notably SIRT3 and SIRT6, in the aging process. The role of SIRT3 in the protective effects of CR on age-related hearing loss seems convincing, and the gain- and loss-of-function studies of SIRT6 in mice suggest a strong involvement of this protein in certain aspects of metabolic aging. Nevertheless, more research is needed to establish the full role of these sirtuins in the physiology of normal aging.

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Chapter 11
Sirtuins and the Circadian Clock: Epigenetic and Metabolic Crosstalk

Selma Masri, Marina Maria Bellet, and Paolo Sassone-Corsi

11.1 Introduction to Circadian Rhythms:

Circadian rhythms are governed by a central clock system that establishes inherent biological timekeeping to regulate physiological processes with a periodicity of 24-h. In mammals, biological rhythms are established and maintained by a central clock pacemaker found in the suprachiasmatic nucleus (SCN)(Reppert and Weaver 2001). The SCN clock is entrained by light, the most powerful *zeitgeber* (time-giver). Subsequently, the central clock directs rhythms in a number of peripheral tissues using several output cues, including numerous secreted paracrine signals (Bass and Takahashi 2010; Masri and Sassone-Corsi 2010), and thereby helps to synchronize biological rhythms systemically. Therefore, the circadian clock can be considered a hierarchical network of biological pacemakers operating in all tissues and required to maintain proper rhythms in endocrine and metabolic pathways, thus critically contributing to organism homeostasis (Green et al. 2008; Schibler and Sassone-Corsi 2002).

Circadian rhythms are directed by the core transcription factors, CLOCK and BMAL1, which positively drive the oscillatory expression of clock-controlled genes (CCG)(Ripperger and Schibler 2006). Among these transcribed clock genes are *Period* (*Per*) and *Cryptochome* (*Cry*) family members, which encode proteins that comprise the negative regulatory arm of the circadian clock system (Shearman et al. 1997; Thresher et al. 1998). The clock machinery is tightly regulated by

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transcriptional/translational events (Sahar and Sassone-Corsi 2009) as well as post-translational modifications of key circadian proteins (Gallego and Virshup 2007), including phosphorylation (Akashi et al. 2002; Lamia et al. 2009; Sanada et al. 2002; Shim et al. 2007), acetylation (Asher et al. 2008; Hirayama et al. 2007; Nader et al. 2009; Nakahata et al. 2008), SUMOylation (Cardone et al. 2005) and ubiquitylation (Busino et al. 2007; Godinho et al. 2007; Siepka et al. 2007). In addition, enzymatic feedback loops exist that directly link oscillating metabolites such as NAD^+ with enzymes involved in circadian function and ultimately regulation of CCG expression (Nakahata et al. 2009; Ramsey et al. 2009). These multiple 'checkpoints' of regulation ensure that the circadian timekeeping system is properly tuned, while maintaining the ability to receive physiological signals that render the clock adaptable to its environment.

A number of genetic mouse models revealed that disruptions in the circadian machinery have important metabolic consequences (Marcheva et al. 2013). The clock mutant (*ClockΔ19*) mice are obese compared to their WT littermates and display hyperlipidemia, hepatic steatosis, hyperglycemia, and hypoinsulinemia (Turek et al. 2005). The liver-specific *Bmal1*$^{-/-}$ mouse model displays hypoglycemia and disruption in rhythmicity of hepatic glucose regulatory genes (Lamia et al. 2008). The conditional knockout of the pancreatic clock results in diabetes mellitus (Marcheva et al. 2010). Genetic disruption of *Cry1/2* in mice results in glucose intolerance and altered glucocorticoid signaling in the liver (Lamia et al. 2011). NR1D1 (nuclear receptor subfamily 1, group D, member 1) also known as Rev-Erbα, a repressor of *Bmal1* transcription, is reported to be critical for lipid and cholesterol metabolism through the circadian regulation of the sterol regulatory element-binding protein (SREBP) pathway (Le Martelot et al. 2009). The extensive control that the circadian clock exerts on metabolism is beginning to be appreciated and will be discussed in later sections. Yet, what remains to be fully elucidated is the influence of metabolic state on the circadian clock; the concept of a food-entrainable oscillator in the hypothalamus that can sense systemic metabolic fluctuation is a compelling notion (Masri and Sassone-Corsi 2013; Mieda et al. 2006).

11.2 Sirtuins and the Circadian Clock

The mammalian sirtuin family is comprised of seven members that are homologs of yeast silent information regulator 2 (Sir2) and have been implicated in a variety of physiological functions including aging, maintenance of genome integrity, stress and nutrient challenge, metabolism and cancer (Finkel et al. 2009). Though all sirtuins have been postulated to require nicotinamide adenine dinucleotide (NAD^+) as a cofactor for enzymatic activity, not all mammalian sirtuins perform the same function (Haigis and Sinclair 2010). Debate in the field has focused around the HDAC activity of the sirtuins, of which SIRT1, SIRT2 and SIRT3 are powerful deacetylases with a number of histone and non-histone targets. Other sirtuins, such as SIRT6 and SIRT7, have been reported to have deacetylase activity, which may be

weak or on different histone lysine residues than initially expected (Barber et al. 2012; Michishita et al. 2008). Aside from deacetylation, the sirtuins have been implicated in ADP-ribosyl transferase, desuccinylase, demalonylase, demyristoylase and depalmitoylase activities. As an example, SIRT5 is reported to have weak deacetylase activity but robust desuccinylase and demalonylase activities (Du et al. 2011). Quite remarkably, the subcellular localization of the mammalian sirtuins is quite different and could be a reason for the diverse set of physiological functions these proteins are involved in. SIRT1 and SIRT2 shuttle between the nucleus and cytoplasm, SIRT6 is nuclear and chromatin bound, SIRT7 is found in the nucleolus and SIRT3, SIRT4 and SIRT5 are mitochondrial (Finkel et al. 2009; Masri and Sassone-Corsi 2014).

SIRT1 has been implicated in the regulation of circadian rhythms by multiple modes of action (Fig. 11.1). SIRT1 has been reported to target and deacetylate the circadian repressor PER2, which is important for protein degradation (Asher et al. 2008). Asher and colleagues reported that the expression of SIRT1 itself was oscillatory and bound the CLOCK:BMAL1 transcriptional complex in a circadian manner, resulting in modulation of circadian gene expression. In addition, SIRT1 has been demonstrated to deacetylate BMAL1 and is recruited with the CLOCK/BMAL1 transcriptional complex to circadian gene promoters, resulting in histone deacetylation and regulation of gene expression (Nakahata et al. 2008). Nakahata and colleagues reported that the expression of SIRT1 was not circadian, though its HDAC activity was, suggesting other factors implicated in regulating SIRT1 activity. This is due to the circadian oscillation of NAD^+ and will be discussed further in a later section. These differences in mechanism of action of SIRT1 on the circadian machinery are not exclusive and could both be functioning simultaneously. Yet, more recent data using sirtuin activating compounds (STACs) has provided further evidence for the role of SIRT1 as an HDAC in modulating circadian gene expression (see section on STACs).

More recently, the circadian role of SIRT1 has been described in the brain and linked with aging. The brain-specific *Sirt1*$^{-/-}$ (BSKO) mice showed marked dampening in circadian gene expression in the anterior hypothalamus, a region of the brain that contains the SCN, suggesting that SIRT1 positively regulates CCG expression (Chang and Guarente 2013). While this mouse model is not ideal as many other brain regions are targeted by the deletion in addition to the SCN, the changes in gene expression also mirrored altered circadian function, as the BSKO mice exhibited a lengthened circadian period. In 22-month old aged mice, it was observed that the expression of SIRT1, BMAL1 and PER2 was decreased compared to 5-month old animals (Chang and Guarente 2013). At 5 months, the BSKO mice exhibited the 'aged' phenotype observed in 22-month old animals, with dampened expression of SIRT1, BMAL1 and PER2, suggesting that a SIRT1-driven alteration of the circadian clock plays an important role in aging (Chang and Guarente 2013).

The question that remains is what other mammalian sirtuins are involved in clock regulation, especially given that multiple sirtuins localize to the nucleus and may be involved in modulating circadian transcription? Specifically, SIRT6 is an attractive candidate as it is chromatin-bound (Mostoslavsky et al. 2006; Tennen et al. 2010)

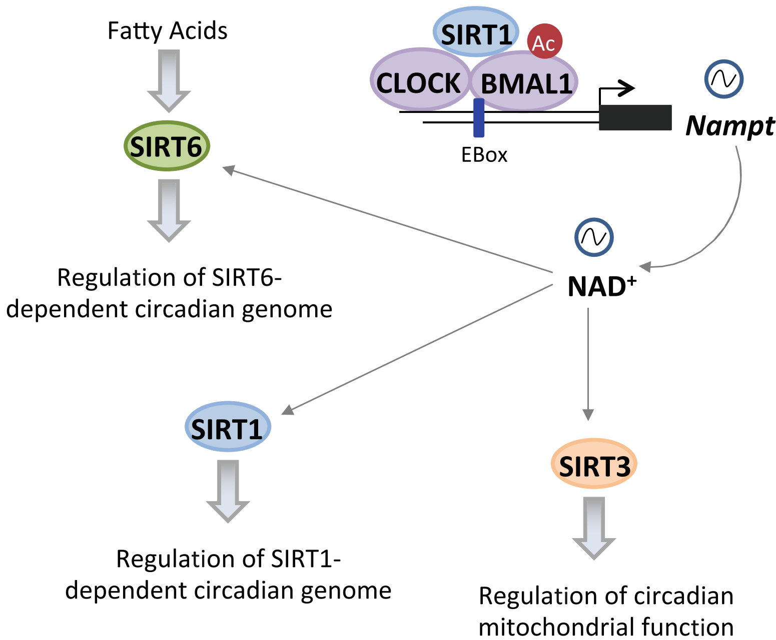

Fig. 11.1 Circadian clock-dependent changes in metabolism influence the epigenome and mitochondrial function. The circadian clock machinery controls the cyclic synthesis of NAD^+ through control of the NAD^+ salvage pathway (Nakahata et al. 2009; Ramsey et al. 2009). The gene encoding the enzyme NAMPT, the rate-limiting step in the NAD^+ salvage pathway, is controlled by the CLOCK/BMAL1 transcriptional complex and therefore is one of the 10 % of CCGs that oscillate in a circadian manner. A crucial step in the NAD^+ salvage pathway is controlled by SIRT1, which also contributes to the regulation of the *Nampt* promoter by associating with the CLOCK/BMAL1 complex. Oscillating levels of NAD^+ also regulate chromatin remodelling events through SIRT1 and SIRT6 and ultimately connect clock-dependent transcriptional control with cellular metabolic pathways in the liver (Asher et al. 2008; Masri et al. 2014; Nakahata et al. 2008). A role for SIRT3 was shown in the mitochondria to control fatty acid oxidation and glucose oxidation rates, in an NAD^+-dependent manner (Peek et al. 2013). [NAD^+, nicotinamide adenine dinucleotide; ~, indicates oscillation; Ac, acetylation]

and its genome-wide occupancy co-localizes with sites enriched for Serine 5 phosphorylated RNA polymerase II, a marker of actively transcribed genomic loci (Ram et al. 2011). Strikingly, microarray analysis of liver-specific *Sirt6*- and *Sirt1*-deficient mice at circadian time-points showed that SIRT6 regulated hepatic CCG expression and that genes regulated by SIRT6 are largely exclusive from those regulated by SIRT1 (Masri et al. 2014). Moreover, SIRT6 is necessary for the recruitment of circadian transcription factors to target promoters as loss of SIRT6 increased recruitment of BMAL1 and acetylation of histone H3 lysine 9 at target CCG promoters (Masri et al. 2014). In addition to recruitment of the core clock transcription machinery, SIRT6 also modulated the circadian recruitment of the transcription factor SREBP-1 to its target gene promoters, such as *Fasn* (Masri et al. 2014). Unlike SIRT6, loss of SIRT1 does not affect circadian regulation of transcription factor

recruitment. Thus, the nuclear functions of SIRT6 and SIRT1 are distinct; SIRT1 functions as a HDAC targeting histone H3 and non-histone circadian proteins, whereas SIRT6 organizes circadian chromatin recruitment of transcriptional machinery. In addition to regulation of circadian gene expression, SIRT6 is also involved in regulation of fatty acid metabolism, largely through the SREBP pathway. The circadian hepatic metabolome identified that loss of SIRT6 resulted in deregulated fatty acid metabolism (synthesis and breakdown by beta-oxidation) and disrupted circadian metabolites involved in fatty acid storage into triglycerides and cellular membrane lipids (Masri et al. 2014). This data further expands on the concept of crosstalk between chromatin modifying enzymes and metabolic control as loss of SIRT6, a chromatin-enriched nuclear HDAC, results in a disruption of circadian fatty acid metabolism and related pathways.

Expanding on the connection with metabolism, the clock and mitochondrial function are closely tied, which could implicate the mitochondrial sirtuins in modulation of protein acetylation. Importantly, analysis of the hepatic circadian acetylome has recently revealed a large number of mitochondrial protein targets. Using the *Clock*$^{-/-}$ mouse model, clock-dependent lysine acetylation was significantly enriched in proteins involved in amino acid metabolism, fatty acid elongation and metabolism, oxidative phosphorylation, citric acid cycle and glycolysis/gluconeogenesis (Masri et al. 2013). Of the seven mammalian sirtuins, three are localized to the mitochondria (SIRT3-5) and could be implicated in circadian clock-dependent protein acetylation state. One interesting link between mitochondrial function, sirtuins and the clock could be calorie restriction (CR). CR is believed to enhance mitochondrial function and decrease ROS formation (Lopez-Lluch et al. 2006; Martin-Montalvo and Cabo 2013), and has been linked to aging. Also, CR is able to reset the circadian clock in terms of CCG expression, feeding time and body temperature (Froy et al. 2006). A master regulator of mitochondrial biogenesis during CR is Peroxisome Proliferator-Activated Receptor Gamma-Coactivator-1 α (PGC-1α) which is believed to control transcription of a number of factors involved in mitochondrial respiration, such as Peroxisome Proliferator-Activated Receptors (PPARs), nuclear respiratory factors 1 and 2 (NRFs), hepatic nuclear factor-4α (HNF4α) and liver X receptor (LXR) (Dominy et al. 2010; Martin-Montalvo and Cabo 2013). The critical aspect of PGC-1α activity is its acetylation state, which is nutrient dependent, and has been linked to SIRT1 HDAC activity (Rodgers et al. 2005) (Fig. 11.2). Intriguingly, SIRT3 has been reported as a downstream target of PGC-1α, and SIRT3 is implicated in PGC-1α-dependent suppression of ROS production and mitochondrial biogenesis (Kong et al. 2011). Moreover, CR has been reported to modulate mitochondrial protein acetylation (Hebert et al. 2013; Schwer et al. 2009) and a recent acetylome study has linked the effects of CR and protein acetylation in the mitochondria to SIRT3 (Hebert et al. 2013).

A recent report on SIRT3 has indeed confirmed the role of this sirtuin in circadian clock-dependent regulation in the mitochondria (Fig. 11.1). It was reported that defects in oxygen consumption rates (OCR) were observed in *Bmal1*$^{-/-}$ liver mitochondria (Peek et al. 2013). This was due to lower rates of fatty acid oxidation as well as decreased glucose oxidation in *Bmal1*$^{-/-}$ liver and mouse embryonic

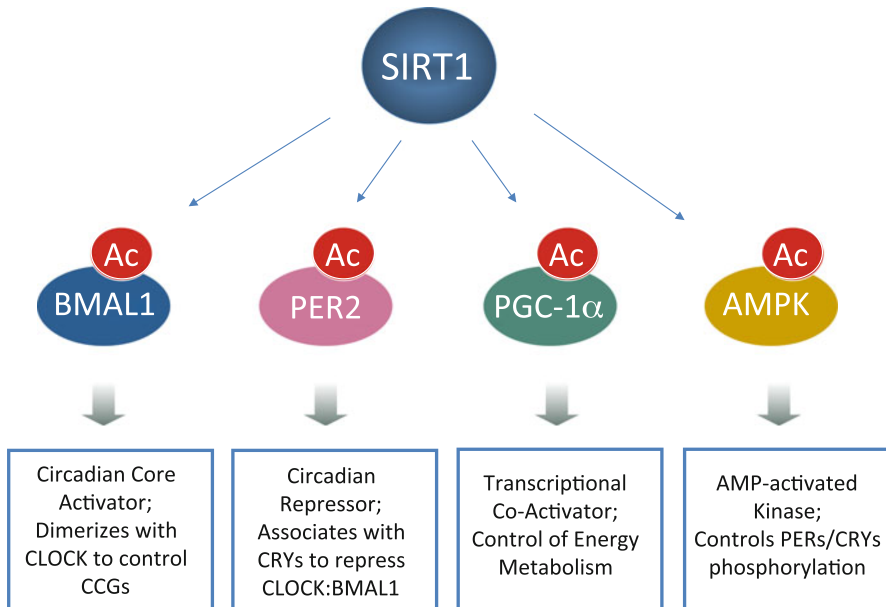

Fig. 11.2 The role of SIRT1 in controlling circadian clockwork and energy metabolism. The NAD⁺-dependent HDAC SIRT1 has been reported to deacetylate various histone and non-histone targets. As indicated, SIRT1 deacetylates the core circadian transcription factor BMAL1 (Nakahata et al. 2008) and core repressor PER2 (Asher et al. 2008) to modulate clock function and expression of CCGs. Also, the nutrient-dependent activation of SIRT1 has been implicated in energy metabolism by targeting PGC-1α (Rodgers et al. 2005). The metabolic sensor, AMPK, similar to SIRT1, is involved in regulation of NAD⁺ (Canto et al. 2009) and has been linked to clock function by targeting CRY and PER proteins (Lamia et al. 2009; Um et al. 2007)

fibroblasts (MEFs), while the opposite trend was seen in *Cry1/2*$^{-/-}$ MEFs. This impaired mitochondrial function was attributed to NAD⁺-dependent modulation of SIRT3 activity, which was dampened, subsequently leading to altered protein acetylation state and activity of medium and long-chain acyl-CoA dehydrogenases (MCAD and LCAD) and isocitrate dehydrogenase 2 (IDH2) acetylation (Peek et al. 2013). While these results shed new light on the clock-dependent activity of SIRT3 in mitochondria, some important questions remain unanswered. For example, given the NAD⁺-dependency of the mammalian sirtuins, is SIRT3 the only sirtuin in the mitochondria implicated in clock-dependent regulation of oxidative pathways?

11.3 Linking the Clock to NAD⁺ Metabolism

Two major groups of chromatin regulatory enzymes require NAD⁺ as cofactor: sirtuins and poly-ADP ribose polymerases (PARPs). Members of both families (SIRT1 and PARP1) have been shown to modulate the circadian machinery (Asher et al. 2008;

Asher et al. 2010; Nakahata et al. 2008). This suggests that NAD^+ could have a central role in modulating circadian rhythms. It has been reported that the DNA-binding activity to E-boxes of CLOCK (and its homologue NPAS2) changes depending on NAD^+/NADH redox state, binding is increased in the presence of high NADH and NADPH levels (Rutter et al. 2001). The *in vivo* significance of this observation is still unclear. However, it is noteworthy that *in vivo* NAD^+ intracellular levels oscillate with a 24-h rhythm (Nakahata et al. 2009; Ramsey et al. 2009). This is possible because of the circadian transcriptional control of the expression of nicotinamide (NAM) phospho-ribosyltransferase (NAMPT), the rate-limiting enzyme in the molecular pathway regulating intracellular NAD^+ levels (the NAD^+-salvage biosynthetic pathway). The *Nampt* gene promoter contains E-boxes to which SIRT1, together with CLOCK and BMAL1, is recruited in a circadian manner (Fig. 11.1). Remarkably, this clock-controlled regulation leads to the oscillatory expression of NAMPT, driving rhythms of NAD^+, which in turn contributes to the reported circadian SIRT1 activity. In *Clock mutant* mice and cells, the oscillatory expression of *Nampt* and consequently that of NAD^+ is abolished. Moreover, the amount of NAD^+ measured in these cells is extremely low (Nakahata et al. 2009), thus confirming the circadian control on NAD^+ production through the salvage pathway. Similarly, pharmacological inhibition of the NAMPT enzyme depletes NAD^+ levels in cells and impairs SIRT1 activity. This event leads to hyperacetylation of BMAL1 and disruption of circadian gene expression (Nakahata et al. 2009). Conversely, mice deficient for the NAD^+ hydrolase CD38, in which the endogenous levels of NAD^+ are chronically high, display disturbances in circadian behavior and metabolism (Sahar et al. 2011).

Absence of SIRT1 affects circadian rhythmicity of CCGs and metabolites. $SIRT1^{-/-}$ MEFs and liver-specific $Sirt1^{\Delta ex4}$ mice (SIRT1 LKO) display increased CCG expression, including *Nampt* (Bellet et al. 2013; Nakahata et al. 2008). As a direct consequence, NAD^+ levels are higher in mice and cells in which SIRT1 is ablated, as compared to WT counterparts, thus reinforcing the concept that SIRT1 is directly involved in controlling the levels of its own coenzyme NAD^+ through the circadian system.

Interestingly, another sensor of the energy state of the cell, the AMP-activated protein kinase (AMPK), similarly to SIRT1, appears to have an important role in the circadian system (Fig. 11.2). AMPK has a circadian oscillatory activity and it was shown to phosphorylate both PER2 (Um et al. 2007) and CRY1 (Lamia et al. 2009), leading to their destabilization and to derepression of circadian transcription. An important crosstalk exists between SIRT1 and AMPK, which explains many of the convergent biological effects on energy metabolism (Canto et al. 2009). In particular, AMPK enhances SIRT1 activity by increasing NAD^+/NADH ratio. Specifically, this happens through the transcriptional regulation of *Nampt* by AMPK. Upon AMPK activation by reduced glucose availability (increased AMP/ATP ratio), levels of NAMPT increase, more NAD^+ is produced from NAM through the NAD^+ salvage pathway and SIRT1 deacetylase activity is enhanced. These intriguing findings do not address whether the role of AMPK on clock function is SIRT1-dependent or not.

The circadian oscillation of intracellular NAD^+ levels begs the questions as to what other metabolites are under control of the circadian clock. Recent studies have reported that in human (Dallmann et al. 2012) and mouse plasma (Minami et al. 2009) as well as in mouse liver (Eckel-Mahan et al. 2012), a number of metabolites are circadian. In human plasma or saliva, about 15 % of identified metabolites followed a rhythmic pattern, including circulating fatty acid levels in plasma and amino acids in saliva (Dallmann et al. 2012). In mouse plasma, amino acids as well as metabolites of the urea cycle were reported to follow a circadian oscillation (Minami et al. 2009). In mouse liver, interesting differences in metabolite peaks were observed over the circadian cycle, including nucleotide, carbohydrate and lipid metabolite peaks at zeitgeber time (ZT) 9 versus amino acid and xenobiotic metabolite peaks at ZT 15–21 (Eckel-Mahan et al. 2012). These metabolomics studies were critical in revealing the large number of metabolites under circadian control. How do these metabolic rhythms feedback and modulate the circadian clock? This notion again highlights the important effects of feeding and nutrition on the clock. Moreover, it should be stressed that several enzymes are regulated in a circadian manner at the level of their catalytic activity rather than abundance, leading to control of cellular metabolic state.

Considering that a number of enzymes (including histone modifiers) utilize varying cofactors and metabolites, an important connection exists between metabolism and the circadian epigenome (Katada et al. 2012). One important example that has been discussed is the circadian oscillation of NAD^+ which modulates the activity of SIRT1 (Nakahata et al. 2009; Ramsey et al. 2009), resulting in direct regulation of the clock machinery and histone acetylation state (Asher et al. 2008; Nakahata et al. 2008). Another critical example is the circadian oscillation of adenosine-5'-triphosphate (ATP) (Womac et al. 2009). We could envision that this oscillation in intracellular ATP levels can modulate the activity of a host of proteins (such as AMPK). Also, chromatin remodelers, such as the SWI/SNF family, possess ATPase activity and are responsible for nucleosome architecture along DNA, allowing for transcription machinery accessibility. Interestingly, at least in the fungus Neurospora the implication of ATP-dependent chromatin remodeling has been linked to clock function (Belden et al. 2007). Considering that the CLOCK:BMAL1 transcription complex would require chromatin remodelers, the circadian oscillation in ATP levels could work harmoniously with the clock machinery to create a permissive state for CCG expression. What about the level of *S*-adenosyl methionine (SAM), a methyl group donor for a host of methyltransferases, is there circadian oscillation? Importantly, levels of histone H3 lysine 4 tri-methylation have been reported to oscillate at specific clock-controlled gene promoters (Katada and Sassone-Corsi 2010), as well as at circadian gene promoters on a genome-wide scale (Koike et al. 2012). This begs the question as to how these methyltransferases are regulated in a circadian fashion and is there a coordinated role of demethylases in maintaining histone methylation marks. Specifically, it would be important to elucidate the interplay between remodelers and various metabolic pathways. For example, exploring whether sirtuin-controlled deacetylation could regulate the enzymatic

activity of methyltransferases and subsequently alter clock function would reveal an important relationship. These concepts suggest that an important crosstalk exists between the epigenome and cellular metabolic state, and the circadian clock potentially dictates multiple aspects of control.

11.4 Pharmacological Modulation of Circadian Rhythm by Small Molecule Activators of SIRT1 (STACs)

In the last 10 years, SIRT1 has drawn attention as a highly promising pharmacological target for the treatment of age-related metabolic diseases, given its role as a regulator of metabolic processes such as lipolysis, fatty acid oxidation, mitochondrial activity and gluconeogenesis (Chalkiadaki and Guarente 2012). Moreover, as SIRT1 is activated by CR, the possibility to pharmacologically induce its activity and thereby prevent metabolic dysfunction is promising. After the initial identification of a group of polyphenolic compounds from plant metabolites, including resveratrol, a large variety of synthetic compounds, structurally unrelated to resveratrol and with improved selectivity, potency and efficacy, have been developed as direct sirtuin activating compounds (STACs). Small molecules were identified (SRT1720, SRT2183) and tested both *in vitro* and *in vivo* for their effects on increasing SIRT1 function, including protection from diet-induced obesity, insulin resistance and type II diabetes (Feige et al. 2008; Milne et al. 2007). Some controversies exist about the mechanism of action and the specificity of these compounds. The observation that resveratrol can activate both SIRT1 and AMPK raised the question of efficacy: do these compounds directly target SIRT1? Also STACs have been challenged because of their apparent property of increasing SIRT1 activity toward fluorophore-tagged substrates but not toward corresponding non-tagged native peptides, thus raising the suspicion that they may activate SIRT1 indirectly (Dai et al. 2010; Pacholec et al. 2010). A recent publication has helped to shed light on the subject as it identified a mechanism of direct allosteric activation of SIRT1, common to chemically different STACs, that explains at least some of the physiological effects (Hubbard et al. 2013).

Given the role of SIRT1 as a component of the clock machinery, it can be assumed that SIRT1 pharmacological activation may also cause important changes in circadian gene expression. The effect of STACs on circadian transcription was recently investigated (Bellet et al. 2013). When used both *in vitro* in luciferase-based assays, and *in vivo*, using synchronized cells or 12-h light-dark cycle entrained mice, different generations of STACs always showed a repressive effect on clock gene expression, as compared with chemically-related inactive compounds (Bellet et al. 2013). Interestingly, the mechanism of how STACs modulate CCGs expression appears to be associated to the HDAC function of SIRT1. In MEFs and livers from mice treated with various STACs, reduced histone H3K9 acetylation was associated to decreased CLOCK/BMAL1 binding to chromatin at CCG promoters (Bellet et al. 2013). These results suggest that the enzymatic activity of SIRT1 might

somehow regulate the nuclear physical association between CLOCK, BMAL1 and SIRT1. This event occurs in a time-specific manner, as there is a more pronounced activation during circadian times when SIRT1 activity is physiologically low, and vice versa. Furthermore, STAC treatment was not associated with changes in NAD^+ rhythmic expression, or with behavioral changes in WT mice. While it is unclear whether some of the metabolic effects of STACs are directly or indirectly mediated by SIRT1, STAC modulation of circadian rhythms was demonstrated to be SIRT1-dependent. When STACs were used in SIRT1-deficient cells and mice, the effects observed at the chromatin level were completely abolished (Bellet et al. 2013). These observations suggest that SIRT1 could be efficiently targeted to alter circadian physiology and that the use of STACs might be a viable therapeutic intervention strategy for circadian rhythm dysfunction. Moreover, since circadian function appears to decline with age because of the loss of SIRT1, it could be speculated that STACs might be useful to reverse the effects of clock-dependent aging phenotypes (Chang and Guarente 2013). To date at least two different pathways can be proposed on how SIRT1 influences the circadian machinery. As previously discussed, the effect of SIRT1 on the amplitude of the central circadian clock has been shown to be positive (Chang and Guarente 2013), as earlier observed in peripheral tissues by Asher and colleagues (Asher et al. 2008). In contrast, the role of SIRT1 as negative regulator of circadian transcription by virtue of its HDAC activity (Nakahata et al. 2008) has been confirmed by the use of STACs on circadian expression in the liver (Bellet et al. 2013). These notions highlight the differences that exist between central and peripheral circadian control that still need to be deciphered, especially given the complexity that tissue specificity adds to the scenario of circadian regulation in mammals.

11.5 Conclusions

In this chapter, we have focused our attention on the central role of sirtuins as the molecular link connecting chromatin remodeling to circadian and metabolic processes. We have discussed the importance of circadian regulation of the epigenome and the influence of cell physiology and metabolism. Central to this crosstalk between the circadian epigenome and the metabolome are the sirtuins, especially SIRT1. The circadian regulation of sirtuin activity is due to the clock-driven oscillation of NAD^+. Recent metabolomics studies revealed that, in addition to NAD^+, a large number of metabolites are controlled by the circadian clock. These studies suggest that circadian control of cellular metabolism is extensive and the biological consequences of these regulatory events are not fully explored.

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Chapter 12
Sirtuin Activation by Small Molecules

Hassina Massudi, Lindsay E. Wu, and David A. Sinclair

12.1 Introduction

Over the last two decades, biogerontologists have gained enough understanding of the biological process of ageing that many believe it is now possible to slow down human ageing and prevent multiple age related diseases. Scientists have moved from merely describing ageing to manipulation of these systems to influence lifespan. Advances in the scientific understanding in the areas of genetic, cellular, nutritional, physiological and behavioural mechanisms have made it possible to slow down ageing in laboratory organisms so that they maintain physical and cognitive function well beyond the usual age at which they begin to decline. The recent discovery of genes and small molecules that can extend lifespan in model organisms such as yeast, worms, flies and mice suggest that interventions which delay ageing in humans may be achievable (Baur et al. 2006; Harrison et al. 2009; Howitz et al. 2003; Wood et al. 2004). Multiple genes that control longevity are now known including components of the insulin/IGF-1 pathway, TOR (target of rapamycin) signalling, AMP-activated kinase (AMPK) and Sir2 homologs, collectively known as the sirtuins. This review will discuss recent advances and opportunities regarding the pharmacological activation of sirtuins, which has increasingly become a focus of biologists and drug developers alike.

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12.2 The Sirtuin Enzymatic Reaction

A class of enzyme known as the sirtuins, first identified in yeast as the gene information regulator (SIR2), mediate their biological functions primarily via deacylase activity. In this reaction, nicotinamide adenine dinucleotide (NAD^+) acts as a co-substrate to remove an acetyl group from a lysine of a target protein creating nicotinamide and O-acetyl-ADP-ribose (OAADPr) as by products (Imai et al. 2000; Sauve et al. 2001; Tanner et al. 2000). SIRT1, a well-studied member of the sirtuin family, is involved in regulating various adaptive pathways that regulate stress resistance, metabolism, genomic stability by targeting some of the key histone residues involved in the regulation of transcription such as H3-K9, H4-K16, and H1-K26, and non-histone protein targets including p53, FOXO1/3, PGC-1α, and NF-κB (Morris 2013). Although the literature on other sirtuins is not as extensive as SIRT1, they seem to play important roles in regulation of antioxidant defences, energy metabolism, DNA damage, inflammation and glucose/insulin homeostasis (Hirschey et al. 2010; Morris 2013; Someya et al. 2010). SIRT3 plays a key role in the regulation of metabolism by deacetylating many enzymes such as acetyl CoA synthase 2 (ACS2) and long chain acyl-CoA dehydogenase (LCAD) which are involved in the process of oxidative catabolism of amino acids and fatty acids during situations of limited energy availability (Hirschey et al. 2010). In addition, the urea cycle enzyme, ornithine transcarbamoylase, which is involved in the disposal of ammonia produced during amino acid catabolism, is also activated via SIRT3 mediated deacetylation (Shimazu et al. 2010). These metabolic changes promoted by SIRT3 cause a shift away from carbohydrate anabolism towards energy production. In mice, whole body over-expression of SIRT6 (Kanfi et al. 2012) and brain specific over-expression of SIRT1 (Satoh et al. 2013) has been shown to extend overall lifespan.

Since their initial discovery as NAD+ dependent deacetylases, sirtuins are now know to possess multiple enzymatic activities (Feldman et al. 2013). SIRT4-7 display extremely low deacetylase activity and recent observations suggests that these sirtuins may possess diverse activities by catalysing other different post-translational modifications, such as lysine desuccinalyation and demalonylation (Feldman et al. 2013). In line with this idea, SIRT6 can hydrolyse long-chain fatty acyl groups, including myristoyl and palmitoyl groups from lysine groups (Jiang et al. 2013). Similarly, SIRT5 can catalyze lysine demalonylation and desuccinylation reactions, in peptide and protein substrates (Du et al. 2011; Peng et al. 2011). SIRT5 interacts with carbamoyl phosphate synthase 1 (CPS1), which is required for clearing ammonia generated by amino acid metabolism (Nakagawa et al. 2009). SIRT5 deacetylates CPS1, thereby regulating ammonia entry into the urea cycle, and activating ammonia detoxification. Du et al. (2011) reported that SIRT5 is a dessucinalyse of CPS1, suggesting that different post-translational modifications by SIRT5 on the CPS1 may coexist (Hallows et al. 2006). Furthermore, SIRT4 modifies protein activity through ADP ribosylation (Haigis and Guarente 2006; Haigis and Sinclair 2010; Imai et al. 2000; Landry et al. 2000). One target of SIRT4 is glutamate dehydrogenase (GDH), an enzyme that regulates the usage of amino acids into energy

production (Haigis et al. 2006). SIRT4 mediated ADP-ribosylation of GDH results in suppression of the activity of this enzyme, which in turn limits metabolism of glutamate and glutamine to generate ATP. Laurent et al. (2013) showed that SIRT4 represses catabolism by inhibiting malonyl CoA decarboxylase through a newly discovered deacetylase activity for this sirtuin (Laurent et al. 2013). Furthermore, some activities of SIRT1, including survival during embryonic development and tumor suppression, do not require catalytic activity, arguing that protein-protein interactions are also critically important for some of the actions of SIRT1 (Clark-Knowles et al. 2013). Together, these studies expand our insight into the diverse actions and function of sirtuins. As the list of pathologies that can be delayed or treated by increasing sirtuin activity grows, there is growing interest in identifying sirtuin activating compounds (STACs) for the treatment of age-related diseases (Blum et al. 2011). To date, sirtuin activators exist for only a single member of the family, SIRT1.

12.3 SIRT1 Activating Compounds (STACs)

In 2003, a high-throughput screen to identify small molecule SIRT1 activators was developed (Howitz et al. 2003) (Fig. 12.1). This assay involves a synthetic peptide containing a single acetylated lysine, derived from a previously identified SIRT1 deacetylation site in p53, with a C-terminal aminomethylcoumarin (AMC) fluorophore. Cleavage of the deacetylated peptide by trypsin, and subsequent release of a c-terminal fluorophore enables detection of SIRT1 activity. From a screen of ~18,000 compounds, more than 20 sirtuin activating compounds (STACs) were identified (Howitz et al. 2003) that included various natural compounds including flavones, stilbenes, chalcones and anthocyanidins. Interestingly, they were all activated by a direct allosteric mechanism that lowers the K_M for the substrate (Howitz et al. 2003).

Of all the natural SIRT1 activators discovered to date, resveratrol (3,5,4′-trihydroxystilbene), remains the most potent. This phytoalexin was first identified in the 1940 from the dried root of white hellebore Veratrum grandiflorum (Baur and Sinclair 2006). Interest in resveratrol research was sparked in the 1990s when it was discovered as an active ingredient in red wine that might explain the so-called “French paradox,” the unusually low rates of mortality from chronic heart disease (CHD) in regions with high red wine consumption (Frankel et al. 1993; Renaud and de Lorgeril 1992; Richard 1987).

Unfortunately, resveratrol as a drug is not likely to succeed given its insolubility, its poor bioavailability, and rapid half-life. In plasma, resveratrol has a half-life of 12–15 min (Asensi et al. 2002; Marier et al. 2002) and even gram amounts of resveratrol administered orally only reach concentrations in the low micromolar range (Subramanian et al. 2010). Furthermore, resveratrol is rapidly metabolised to its glucurinaded and sulfated forms (Asensi et al. 2002; Marier et al. 2002). Interestingly, the half-life of resveratrol conjugates have a much longer half-life (~9 h) compared

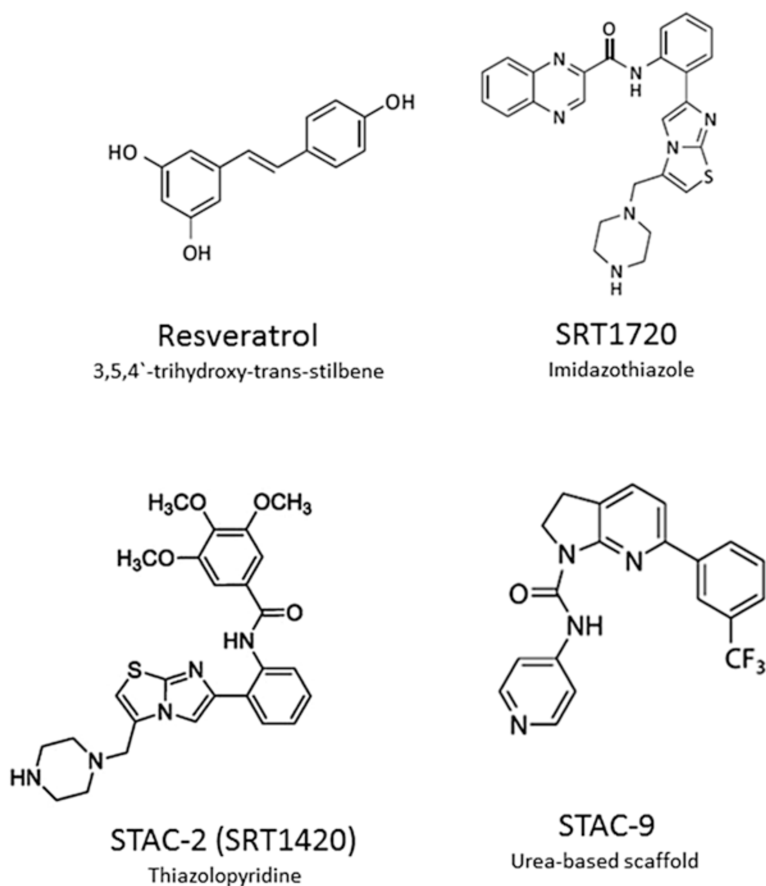

Fig. 12.1 Sirtuin activating compounds (STACs). The search for small molecule sirtuin activators as a therapeutic target for age related diseases have advanced in recent years. These molecules include the polyphenols such as resveratrol (**a**) (Howitz et al. 2003) and more potent imidazothiazoles such as SRT1720 (**b**), thiazolopyridines such as SRT1420 (**c**) and urea based scaffolds such as STAC-9 (**d**) (Dai et al. 2010; Hubbard et al. 2013; Milne et al. 2007)

to its native form, raising the possibility that these conjugates could be used in vivo to obtain superior pharmacokinetic outcomes than first thought (Walle et al. 2004), but whether the conjugates activate SIRT1 in vivo remains to be seen. Recent work by Patel et al. (2013) indicates that resveratrol administered in a stable sulfate-conjugated form may be a better approach as it allows delivery of the conjugated forms to the target tissues where they can be gradually regenerated into resveratrol, resulting in a sustained exposure to the parent compound (Patel et al. 2013).

Clinical trials of resveratrol have explored the health impacts on individuals with obesity, diabetes and cardiovascular diseases (Timmers et al. 2011; Baur and Sinclair 2006). In many, but not all cases, clinical trials data are consistent with the improvements shown in the animal models upon resveratrol treatment, including

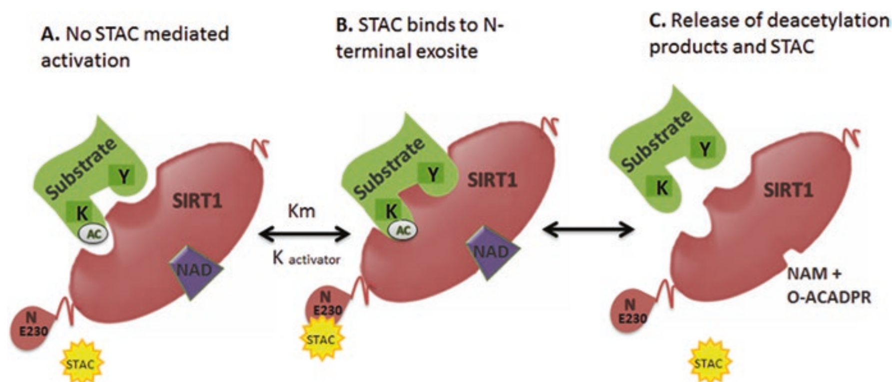

Fig. 12.2 Proposed mechanism of assisted allosteric activation (AAA) of SIRT1 by STACs. NAD^+ is used as a co substrate to mediate deacetylation of the target substrate (*green*) to generate a deacetylated product, O-acetyl-ADP ribose (OAADPR) and nicotinamide (NAM). STACs bind adjacent to a glutamic acid residue (Glu230) in the N-terminal activation domain. Mutations of glutamate to lysine or alanine (E230K/A) in the N terminus prevent activation by resveratrol and synthetic STACs, arguing for a common mechanism of activation (Hubbard et al. 2013). Hydrophobic amino acids (Y, F, W) in the substrate at positions +1 and +6 (relative to the acetylated lysine (K-Ac) assist with activation, a fact discovered because early SIRT1 assays serendipitously utilized hydrophobic fluorophores attached to the substrate in the same or similar positions

improved insulin sensitivity, improved neuro-cognition and amelioration of cardiovascular disease (Baur et al. 2006; Pearson et al. 2008). So far, the limited number clinical trials of resveratrol conducted suggest resveratrol is safe with no apparent toxicity or signs of severe adverse effects. A study assessing the safety of a single 5 g dose of oral resveratrol in subjects found no serious adverse effects (Boocock et al. 2007). Similarly in rats, daily oral administration of *trans*-resveratrol at doses up to 300 mg/kg of body weight for four weeks was well tolerated and considered safe (Crowell et al. 2004; Juan et al. 2002). The most commonly reported side effect of resveratrol is mild cases of gastrointestinal disorders such as nausea, diarrhoea and abdominal discomfort (Brown et al. 2010; Yoshino et al. 2012).

The initial discovery of SIRT1 activators such as resveratrol set off intense interest in identifying more potent and efficacious STACs with improved bioavailability (Fig. 12.2). Sinclair and colleagues synthesized a variety of resveratrol analogs with substitutions on the 3,5, and 4′ positions. Some of these derivatives were more potent than resveratrol and in the case of the tri-acetyl-stilbene, was considerably more effective at extending lifespan (Yang et al. 2007). A new assay was developed in 2007 that places the fluorphore TAMRA C-terminal to the acetylated lysine. Detection methods included fluorescent polarization and mass spectrometry (Milne et al. 2007). Screening efforts with this new assay identified STACs that were structurally unrelated to resveratrol and orders of magnitude more potent (Milne et al. 2007). In all, more than 500,000 compounds were screened.

The first class of synthetic STACs was based on an imidazothiazole scaffold (e.g. SRT1460, SRT1720, and SRT2183) (Milne et al. 2007). As with resveratrol, these

molecules activated SIRT1 through a similar mechanism by lowering the Michaelis-Menten constant (K_m) of SIRT1 for its substrates (Milne et al. 2007). A second class of STACs, based on benzimidazole and urea-based scaffolds (such as SRT1720 and SRT1460) exceeded the potency of resveratrol and other natural STACs by a 1000-fold (Dai et al. 2010; Hubbard et al. 2013; Hubbard and Sinclair 2014). Several of these molecules have been reported to have beneficial effects in many models of chronic diseases and a number of these drugs have been translated to clinical trials, as described below (Baur et al. 2012; Hall et al. 2013).

12.4 STAC Mechanism of Action

Though it is now generally accepted that SIRT1 can be activated by small molecules (Hubbard and Sinclair 2014), the mechanism by which resveratrol and other STACs activate SIRT1 has been the subject of great controversy. In 2005, two studies reported that removal of the AMC fluorophore from the peptide substrate (Ac-Arg-His-Lys-LysAc-AMC) abolished the ability of resveratrol to stimulate SIRT1 activity (Borra et al. 2005; Kaeberlein et al. 2005). Even though SIRT1 activating compounds extend lifespan and promote deacetylation of SIRT1 substrates in organisms ranging from yeast to mice (Howitz et al. 2003; Morris 2013; Smith et al. 2009), Borra and colleagues concluded that STACs were not direct activators of SIRT1, based on the fact that there is no endogenous AMC (Borra et al. 2004). Two subsequent studies came to the same conclusion (Beher et al. 2009; Pacholec et al. 2010). To their credit, Denu and colleagues left open the possibility that the results were indicative of substrate selectivity *in vivo* (Borra et al. 2004).

A key clue to reconciling these disparate findings was the recognition that certain amino acid sequences were conserved in naturally occurring SIRT1 substrates, such as FOXO3a and PGC-1α (Hubbard and Sinclair 2014). In these substrates, there are bulky hydrophobic amino acid residues that lie six residues C-terminal to the site of SIRT1 deacetylation. Strikingly, both AMC and TAMRA fluorophores used in the SIRT1 activator assays are bulky, hydrophobic moieties and both were located C-terminal to the site of deacetylation. In 2013, Sinclair and colleagues showed that substitution of the fluorophore with hydrophobic amino acids (tryptophan, phenylalanine or tyrosine) restored the ability of STACs to activate (Hubbard and Sinclair 2014). These and other data indicated that the AMC fluorescent moiety used in the initial screen to identify SIRT1 activators unintentionally mimicked a preferred consensus site for SIRT1 mediated deacetylation. Consistent with this, a screen consisting of native SIRT1 substrates including PGC-1α and FOXO3 found that activation by STACs only occurred when substrates possessed bulky hydrophobic amino acid sequences adjacent to the acetyl-lysine (Hubbard and Sinclair 2014; Lakshminarasimhan et al. 2013). These studies suggest that there are structural and positional requirements in native SIRT1 substrates that promote activation and explain why some substrates were not responsive to the STACs (Borra et al. 2005; Kaeberlein et al. 2005).

Another challenge to the field of sirtuin activating compounds have been the suggestions that STACs such as resveratrol do not directly interact with SIRT1 and may in fact exert the biological effects through off-target binding to other proteins. Effective doses for resveratrol vary considerably *in vivo* (Table 12.1), (Agarwal and Baur 2011; Baur and Sinclair 2006). For instance, at higher doses of 100 μM, resveratrol can block the electron transport chain and mitochondrial oxidative phosphorylation, decreasing ATP levels and increasing AMP, leading to AMPK activation (Baur and Sinclair 2006; Hawley et al. 2010; Hubbard and Sinclair 2014). AMPK may also be activated by resveratrol through the direct inhibition cyclic-AMP specific phosphodiesterases (PDE) through a multistep mechanism leading to cAMP and AMPK activation (Park et al. 2012).

To test whether resveratrol acts via SIRT1, an inducible SIRT1 knockout mouse was generated, transcending previous difficulties in generating SIRT1 knockout mice due to embryonic lethality or developmental defects (Price et al. 2012). In wildtype strains, resveratrol increases muscle mitochondrial biogenesis, mitochondrial function and AMPK activation, at moderate dose of 25-30 mg/kg but in the absence of SIRT1, none of these effects were seen. At a higher dose of resveratrol (215–230 mg/kg) AMPK was induced even in the SIRT1 knockout, although its effects on mitochondrial biogenesis were still SIRT1- dependent (Price et al. 2012). Together these findings demonstrated that dose is a critical factor when elucidating a mechanism and that it is best to use the lowest efficacious dose to avoid potential off-target activities.

Further support for the direct activation of SIRT1 through a direct allosteric mechanism comes from the identification of a conserved STAC binding domain in the N-terminus. A screen for non-responsive mutants of SIRT1 identified an amino acid critical for the activation of SIRT1 by diverse, structurally unrelated STACs. This amino acid residue, glutamine 230 (Glu230), forms part of a putative substrate-induced binding site on SIRT1 required to enhance substrate binding (Hubbard et al. 2013). Mutation of Glu230 to alanine or glutamate (E230K/A) completely blocked activation of SIRT1 by all STACs tested from many distinct chemical classes. The metabolic effects of STACs were consistently blocked in primary cells reconstituted with the STAC-insensitive SIRT1-E230K mutant, arguing for a common allosteric mechanism of activation by all chemically diverse STACs (Hubbard et al. 2013). Interestingly, the residue is conserved from humans to flies indicating there is an endogenous activator of SIRT1 but whether it exists is not known. The E230 residue is not present in the N-terminal domains of worm or yeast SIRT1 homologs (ySir2 and Sir-2.1), suggesting that other residues may perform substitute for its function or that the these homologs are activated via different dynamics (Hubbard and Sinclair 2014). The physiological effects of the STACs are discussed below.

Table 12.1 Pharmacokinetics of STACs, resveratrol and its metabolites

Model	Dose (mg/kg)*	Maximum plasma concentration(Cmax)		Time taken to reach Cmax (Tmax)(hours)	References
		Original form	Metabolites		
Resveratrol					
Mice	20	~593 ng/ml		0.04	Asensi, Medina et al. (2002)
Mice	20	Trace amount	~5 μM (sulfate) ~1 μM (glucuronide)	–	Yu, Shin et al. (2002)
Rat	50	1499 ng/ml	105.21 μM (glucuronide)	0.29	Marier, Vachon et al. (2002)
Rat	2	20.5 ng/ml	1.2 μM (Total)	4	Meng, Maliakal et al. (2004)
Rat	5	25 ng/ml	1.3 μM (total)	1. 5	Meng, Maliakal et al. (2004)
Rat	20	273.9 ng/ml		0.08	Asensi, Medina et al. (2002)
Rabbit	20	251 ng/ml		0.04	Asensi, Medina et al. (2002)
Human	25 mg/P	<5 ng/ml	500 ng/ml (total)	–	Walle, Hsieh et al. (2004)
Human	0.5 g/P	68 ng/ml	1135 ng/ml (sulfate) 404.6 ng/ml (glucuronide)	0.833	Boocock, Faust et al. (2007)
Human	1 g/P	548 ng/ml	2102 ng/ml (sulfate) 473.6 ng/ml (glucuronide)	0.759	Boocock, Faust et al. (2007)
Human	5 g/P	539 ng/ml	4294 ng/ml (sulfate) 1285 ng/ml (glucuronide)	1.5	Howells, berry et al. (2011)
SRT501 (formulated resveratrol)					
Human	5 g/p	1942 ng/ml		2.8	Howells, berry et al. (2011)
SRT2104					
Human	30 mg/p	~20 ng/ml		2	Hoffmann, Wald et al. (2013)
Human	3 g/p	~220 ng/ml		4	Hoffmann, Wald et al. (2013)
Human	0.5 g/p	343 ng/ml		3	Libri, Brown et al. (2012)
Human	2 g /p	477.6 ng/ml		3	Libri, Brown et al. (2012)

(continued)

Table 12.1 (continued)

Model	Dose (mg/kg)*	Maximum plasma concentration(Cmax) Original form	Metabolites	Time taken to reach Cmax (Tmax)(hours)	References
SRT1720					
Mice	30	300 ng/ml		2	Milne, Lambert et al. (2007)
Rat	3	<1 ng/ml		–	Hopner, Delahaut et al. (2013)

Resveratrol and STACs as CR Mimetics

Modulation of SIRT1 activity by naturally occurring STACs such as resveratrol extend health and lifespan in a variety of organisms including *S. cerevisiae, C. elegans, D. melanogaster* in a Sir2-dependent manner (Howitz et al. 2003; Wood et al. 2004), though these findings have been disputed (Bass et al. 2007; Kaeberlein et al. 2005) (Fig. 12.3). Valenzano and colleagues showed that resveratrol was able to extend lifespan in this species in a dose dependent manner with maximum lifespan of 59 % was achieved at a higher dose of up to 600 μg/g of food (Valenzano et al. 2006). Resveratrol also extends lifespan of mice on a high fat diet (Baur et al. 2006) but not on a chow diet unless animals are subjected to every other day feeding (Pearson et al. 2008). Resveratrol treatment of mice on a high calorie diet for 6 weeks displayed increased survival with lower levels of markers for metabolic dysfunction such as glucose and insulin levels, and decreased markers of shorter lifespan including lower levels of insulin, glucose and IGF-1 (Baur et al. 2006). Resveratrol also induced gene expression patterns that mimic calorie restriction, showing distinct reductions in signs of age related phenotypes, including decreased inflammation levels, increased motor coordination, reduced cataract formation and preserved bone mineral density (Baur et al. 2006). In line with this, a recent study of high fat diet fed rhesus monkeys showed that a 2 year resveratrol supplementation exhibited similar protective effects on metabolic and functional outcomes with improved insulin sensitivity, reduced inflammation and decreased adipocyte size (Jimenez-Gomez et al. 2013). Furthermore, administration of SRT1720, a synthetic STAC possessing improved selectivity, potency and efficacy, from 56 weeks of age, was also reported to increase lifespan in a dose dependent manner with multiple beneficial effects on the metabolism of high fat fed mice, similar to the effects observed during resveratrol treatment (Minor et al. 2011). The benefits of resveratrol on lifespan seemed to be restricted to high fat fed animals, as no lifespan extension was observed in chow fed animals unless they were fed every other day (Pearson et al. 2008; Strong et al. 2013). This suggested that resveratrol may provide protection against the negative consequences of obesity and in conditions of biological stress. Synthetic STACs, such as SRT1720 and SRT3025 produce similar

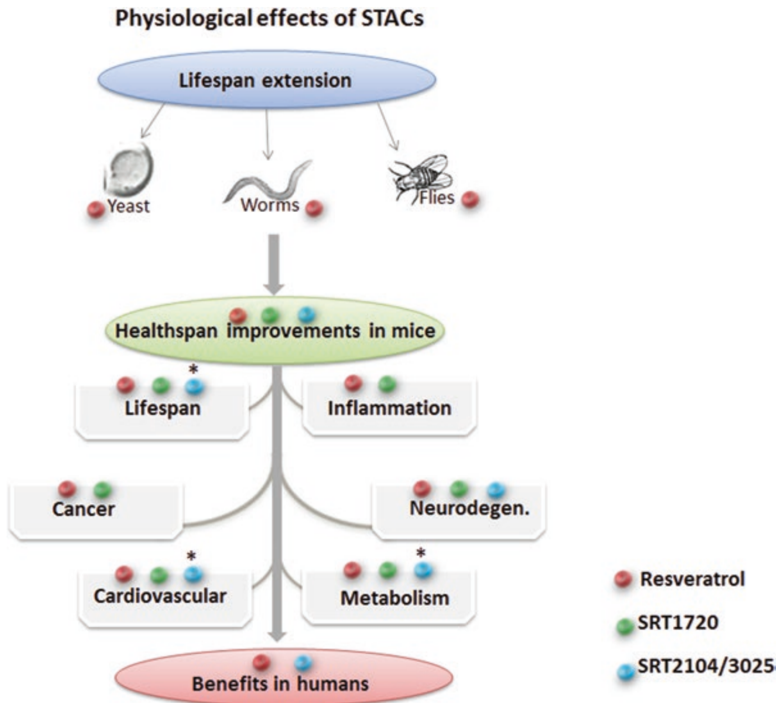

Fig. 12.3 Effects of STACs on ageing and age-related diseases. STACs ameliorate a number of age-related diseases. The STACs and their involvement with health and lifespan are denoted by coloured pegs. *Asterisks* indicate studies in progress or completed

effects as resveratrol in mice, including improvements in glucose metabolism (Milne et al. 2007), cardiovascular protection (Miranda et al. 2015), increased endurance (Feige et al. 2008), improved lipid profile and lifespan extension on a high fat diet (Minor et al. 2011).

Recently, SRT1720 and SRT2104 were reported to extend mouse lifespan not only on a high fat diet, but on a standard chow diet (Mercken et al. 2014; Mitchell et al. 2014). Mice treated with a 100 mg/kg of SIRT1720 and SRT2104, supplementation of which began at 28 weeks of age, displayed an increase in lifespan of 10 % and 14 % respectively, and were protected against age related functional and metabolic decline including reduced inflammation, decreased incidence of cataracts, decreased lipoprotein levels and an increased glucose disposal rate (Mercken et al. 2014; Mitchell et al. 2014). Mice treated with SRT2014 also displayed improvements in health parameters including improved motor skills and coordination, preserved trabecular bone volume and mineral density, improved insulin sensitivity and decreased oxidative stress and inflammation (Mercken et al. 2014). SRT1720 and SRT2104 are the first effective, synthetic molecules that slow aging, and suggest that aspects of aging and age-related diseases can be treated by rationally designed pharmaceuticals.

12.4.1 STACs in Neurodegeneration and Brain Damage

Resveratrol has been shown to provide protection against ischemia reperfusion injury and neurodegenerative diseases including Alzheimer's disease and Huntington's disease (Jiang et al. 2012; Karuppagounder et al. 2009; Lofrumento et al. 2013). In a Wistar rat model of cerebral ischemia, daily injection of 20 mg/kg resveratrol for three weeks showed significant protection against oxidative stress markers and blocked motor impairment as well as a reduction of the volume of infarction as compared to non-resveratrol treated control rats (Sinha et al. 2002). In a similar study, mice treated with the same dose of resveratrol for 3 days prior to occlusion of cerebral artery displayed a 36 % reduction in total infarct volume (Inoue et al. 2003). Furthermore, in gerbils, injection of 30 mg/kg resveratrol either during or after cerebral occlusion significantly lowered neuronal cell death and glial activation (Wang et al. 2002). Interestingly, the protective effects of resveratrol against ischemia required both SIRT1 and PPARα (Della-Morte et al. 2009; Inoue et al. 2003). Several other studies have also reported protective roles of resveratrol in reducing cerebral oedema (Wang et al. 2003), cognitive impairments (Sharma and Gupta 2002), and kainite-induced epilepsy (Wu et al. 2009). These data suggest that resveratrol is able to cross the blood brain barrier where it can impart neuroprotection.

In Alzheimer's disease (AD), both the overexpression of SIRT1 and treatment with resveratrol can reduce the toxic amyloid-β production in cell based models (Albani et al. 2010). Low SIRT1 mRNA levels have been reported in the parietal cortex of AD patience and this correlates with the increase in paired helical tau filament accumulation (Julien et al. 2009), and promotes degradation of both intracellular and secreted amyloid-beta in mouse model of Alzheimer's disease (Marambaud et al. 2005). In line with this, a recent study showed that one week of intraperitoneal injections of resveratrol in aged mice improved cognition and long term memory (Zhao et al. 2013) and these effects were diminished in SIRT1 mutant mice. Additionally, in an inducible p25 transgenic mouse model for AD and axonopathies, resveratrol reduced neurodegeneration and prevented a decline in learning and memory. This was associated with decreased acetylation of PGC1α and p53, targets of SIRT1, indicating that SIRT1 may play a role in mediating some of these beneficial effects. This was further supported by lentiviral-mediated overexpression of SIRT1 in the hippocampus of these mice, which also exerted significant protection against neurodegeneration (Kim et al. 2007). More recently, the synthetic SIRT1 activator, SRT3657, a structurally unrelated molecule, was shown to prevent synaptic and neuronal loss, and protect against memory impairment in the p25 model of neurodegeneration (Graff et al. 2013).

SIRT1 overexpression is also protective against Hungtington's disease (Albani et al. 2009; Jeong et al. 2012) with substrates of SIRT1, CREB (cAMP-responsive element binding protein) and CREB regulated transcription coactivator-1 (CRTC1), as likely downstream modulators (Jeong et al. 2012). STACs have also been found to protect in animal models of multiple sclerosis (Shindler et al. 2010). Oral

administration of SRT501 (a formulation of resveratrol) and SRT1720 prevented neuronal loss, suppressed neurological dysfunction, and preserved axonal density in the spinal cords of these mice (Shindler et al. 2010). In humans, resveratrol supplementation (200 mg/day for 26 weeks) improved glucose metabolism and subsequent neuronal function and cognitive performance in elderly overweight healthy individuals. These subjects performed better in memory retention and had increased synchronization patterns of functional connectivity of the hippocampus networks, a key region implicated in memory performance. A significant decrease in the marker for long term glucose control, glycated hemoglobin (HbA1c), and body fat and an increase in leptin levels were also observed compared to placebo treated individuals. Furthermore, the reductions in HbA1c with increases in hippocampal functional connectivity correlated strongly with memory function in the resveratrol supplemented individuals further highlighting resveratrol's potential to improve the integrity and functionality of the hippocampus (Witte et al. 2014).

12.4.2 STACs for the Prevention and Treatment of Cancer

The first demonstration that resveratrol can prevent cancer was in 1997. In this study, Jang and colleagues demonstrated that topical skin application of resveratrol reduced the incidence of skin cancer by up to 98 % (Jang et al. 1997), an effect later shown to be mediated, in part, by SIRT1 (Boily et al. 2009). The anti-proliferative properties of resveratrol have since been demonstrated in a number of cancer models including multiple myeloma, breast, prostate, colon, melanoma and cervical cancer (Aggarwal et al. 2004; Baur and Sinclair 2006; Estrov et al. 2003; Kang et al. 2003), though ineffective against spontaneous tumours in aged mice (Luo et al. 2001; Pearson et al. 2008). The mechanism of resveratrol's actions are likely to be multimodal, with targets including p53 (Wang et al. 2008), Bax (Lu et al. 2001), down regulation of survivin (Hsieh et al. 1999), caspases (Nicolini et al. 2001), beta catenin (Firestein et al. 2008) and transcription factors such as NF-kB (Gao et al. 2006). Genetic studies in mice indicate that SIRT1 is involved in at least some of these effects. Overexpression of SIRT1 suppresses tumor formation in mouse models of colon cancer (Firestein et al. 2008), leukemia (Oberdoerffer et al. 2008) and liver cancer (Herranz et al. 2010).

Several independent studies have reported that inhibition of SIRT1 can also be tumor protective. The SIRT1 inhibitors NAM and Sirtinol block cell growth and increase the sensitivity of prostate cancer cells chemotherapy (Jung-Hynes et al. 2009; Kojima et al. 2008). Other STACs in cancer models have generated mixed results. While SRT1720 can induce apoptosis in cell based model of multiple myeloma (Chauhan et al. 2011), it has also been reported to promote breast cancer mediated lung metastasis (Suzuki et al. 2012). Whether SIRT1 activation or inhibition is the best approach to test in humans will likely depend on the type and genetics of the cancer being treated.

Thus far, there is very limited clinical trial data for the efficacy of resveratrol in cancer treatment and prevention in humans. The first study looking at the effects of resveratrol in patients with cancer was published in 2009 (Nguyen et al. 2009). An analysis of normal and cancerous mucosa from these patients revealed higher target genes of the Wnt signalling pathway, which is associated with the formation of colon cancer, in cancerous compared with normal mucosa. A 14 day daily resveratrol treatment and grape powder supplementation had reduced Wnt signalling gene expression in normal mucosa but not the cancerous mucosa, suggesting that resveratrol may reduce the risk of colon cancer but may not be able to cure it (Nguyen et al. 2009). Other studies have concentrated on resveratrol's ability to reduce and prevent cancer risk factors in healthy individuals. A study by Brown et al. (2010) found that 2.5 g daily dose of resveratrol decreased both plasma IGF1 and IGFB3 levels, circulating proteins of which are highly associated with tumour formation and metastasis, further supporting resveratrol's role as a chemopreventive agent (Brown et al. 2010). There are several other ongoing clinical trials studying the effects of resveratrol on patients with colon cancer or testing the chemo-preventive capability of resveratrol in healthy subjects. Results of these trials may provide the basis for setting up future large-scale clinical trials to determine the full chemo-preventive and chemotherapeutic efficacy of resveratrol and its consideration as a viable option for chemotherapy and chemoprevention.

12.4.3 STACs in Metabolism and Obesity

The most robust and reproducible effect of resveratrol and the synthetic STACs is an improvement in glucose homeostasis and insulin sensitivity in old or obese mice. Resveratrol, SRT1720 and SRT2104 induce physiological and gene expression changes that resemble the effects of calorie restriction, with effects such as decreased insulin resistance, increased mitochondrial content and extensions in lifespan (Baur et al. 2006; Howitz et al. 2003; Lagouge et al. 2006; Milne et al. 2007; Smith et al. 2009). An increase in mitochondrial function has been consistently reported in several tissues such as liver, skeletal muscle and brown fat following resveratrol supplementation (Baur et al. 2006; Lagouge et al. 2006). This increase in mitochondrial capacity in resveratrol treated mice is primarily thought to be caused by deacetylation and a subsequent increase in the activity of PGC-1α, which is known to be a key regulator of mitochondrial biogenesis (Baur et al. 2006; Lagouge et al. 2006). Indeed, the deacetylation of PGC-1α and an increase in its transcriptional levels has been reported in many tissues of mice treated with resveratrol (Lagouge et al. 2006). Activation of the LKB1-AMPK pathway and suppression of the accumulation of hypoxia-inducible factor 1 (HIF-1α) at older ages may also play a key role in the maintenance of mitochondrial function and glucose homeostasis (Gomes et al. 2013; Lan et al. 2008). Interestingly, overexpression of SIRT1 alone is able to mimic many effects of resveratrol on activation of AMPK and mitochondria *in vivo* (Price et al. 2012). Although lower doses of resveratrol mediate beneficial changes in

metabolism through allosteric activation of SIRT1, at higher doses, resveratrol can activate AMPK mediated pathways to trigger beneficial effects of resveratrol in a SIRT1 independent manner (Price et al. 2012). One possibility is that high concentrations of resveratrol may directly inhibit electron transport in the mitochondria, raising intracellular AMP levels and subsequently activating AMPK (Canto and Auwerx 2012).

SRT1720 has similar metabolic effects as resveratrol on obese mice, with overall improvements in whole body glucose homeostasis and insulin sensitivity as well as a decrease in oxidative stress markers (Yamazaki et al. 2009) Mitchell et al. 2014). SRT1720 extend lifespan of mice both on a high fat diet as well on a standard chow diet (Minor et al. 2011; Mitchell et al. 2014) and another STAC, SRT2104, improves the lipid profile of healthy cigarette smokers (Venkatasubramanian et al. 2013).

Although it is still early days, NAD^+-boosting molecules have proven remarkably effective in treating metabolic dysfunction in mice. The NAD^+ precursors NR and NMN have both been shown to increase mitochondrial function and improve glucose homeostasis in obese mice, in a SIRT1-dependent manner (Canto et al. 2012; Gomes et al. 2013; Yoshino et al. 2011). Remarkably, the effects occur within 24 h of treatment (Yoshino et al. 2011) possibly though a central mechanism involving the hypothalamus (Satoh et al. 2010). A recent study by one of us (Gomes et al. 2013) found that NMN reversed key aspects of aging in mouse muscle within only one week of treatment, including muscle type switching, insulin sensitivity, oxidative phosphorylation, and gene expression (Gomes et al. 2013). No studies in humans have been conducted.

The limited number of clinical studies that have been conducted with resveratrol and STACs have focused primarily on testing their effects on glucose levels and lipids. Type 2 diabetic patients treated with resveratrol for four weeks had improvements in insulin sensitivity and decreased oxidative stress compared to non-resveratrol treated patients, though the dose was about 50 times lower than the equivalent mouse dose (Brasnyo et al. 2011). Obese men receiving a 150 mg/day dose of resveratrol (a dose comparable to the mice in the Baur et al. study (2006) showed improved insulin sensitivity, glucose tolerance, increased SIRT1 and PGC-1α levels, improved muscle mitochondrial respiration and decreased circulating levels of triglycerides and lipid contents compared to their non-resveratrol treated counterparts (Timmers et al. 2011). Similarly, a 28 day supplementation of SRT2104 in type 2 diabetic patients aged between 30 and 70, resulted in improvements in lipid profiles by lowering LDL, total cholesterol and increasing HDL in these patients. There was however no improvement in glucose and insulin homeostasis (Baksi et al. 2014). Not all studies have been positive. A 75 mg/day supplementation of resveratrol for 12 weeks did not improve metabolic function in non-obese women with a normal glucose tolerance (Yoshino et al. 2012), suggesting that resveratrol and possibly other STACs may have the greatest effects in individuals whose metabolism is no longer in homeostasis, which would be consistent with observations in mice.

12.4.4 Cardiovascular Effects of STACs

Ever since it was proposed as a cause of the French Paradox, the cardio-protective properties of resveratrol have been keenly studied (Baur and Sinclair 2006). Resveratrol exerts its cardio-protective effects through several pathways, some of which require the presence of SIRT1 (North and Sinclair 2012). For instance, resveratrol can reduce oxidative stress by increasing the expression of superoxide dismutase in cardiac myocytes, in a manner that is dependent on SIRT1 (Tanno et al. 2010). Likewise, resveratrol has cardio-protective effects ostensibly by increasing sarcoplasmic calcium ATPase expression (Sulaiman et al. 2010) and by modulating the endothelial and inducible nitric oxide synthases eNOS and iNOS in vascular smooth muscle cells (VSMC) (Baur and Sinclair 2006). Similar effects of resveratrol on VSMCs have also been observed in monkeys (Csiszar et al. 2012). Resveratrol reduces the risk factor for cardiovascular diseases by preventing arterial wall inflammation in high fat, high sucrose fed rhesus monkeys. Resveratrol supplementation for 2 years in these non-human primates, protected them against inflammation associated central arterial wall stiffening and the associated increases in the aortic pulse wave velocity (Mattison et al. 2014). Similar to resveratrol, SRT1720 exhibits many beneficial cardiovascular effects including reduction of myocardial infarction (Tong et al. 2013), a decrease in the whole body fat mass and increasing high density lipoprotein levels in mice (Minor et al. 2011). Recently, pharmacological Sirt1 activation using SRT3025, is reported to be effective in lowering plasma LDL-cholesterol levels and the accompanying atherosclerotic plaques in a mouse model of atherosclerosis. The decrease LDL levels were a consequence of SIRT1 mediated increases in hepatic low density lipoprotein receptor (LDLR) protein expression (a key determinant of atherogenesis that plays a critical role in the regulation of plasma LDL-cholesterol levels) through a reduction in the serine protease, Pcsk9 (Miranda et al. 2015). Consistent with a key role for SIRT1 in acute cardio protection, increasing NAD^+ levels by treating mice with NMN greatly reduced the size of the infarction and loss of cardiac function in a mouse model of cardiac ischemia (Yamamoto et al. 2014).

12.4.5 Inflammation

SIRT1 exerts anti-inflammatory actions largely through deacetylation and inhibition of NF-ƙB as well as the pro-inflammatory cyclooxygenase enzymes 1 COX1 and COX2 (Morris 2013; Yeung et al. 2004; Baur and Sinclair 2006). Resveratrol is also effective in animal models of disease. These include arthritis in rabbits (Elmali et al. 2007), chronic obstructive pulmonary disease (COPD) (Zhou et al. 2008), and Crohn's disease (Rahal et al. 2012). Furthermore, resveratrol has been recently shown to reduce inflammatory effects by decreasing NF-ƙB in visceral white adipose tissue of rhesus monkeys (Jimenez-Gomez et al. 2013). Like resveratrol,

SRT1720 provide anti-inflammatory effects in several inflammatory disease models including COPD (Yao et al. 2012) and asthma (Ichikawa et al. 2013).

12.5 Perspective

The sirtuins are some of the most interesting and important molecules in biology. The breadth of their reach into almost every process in cell biology makes them exciting drug targets. This reach, however, is precisely why we should proceed with caution to ensure that unexpected effects are not encountered. Clearly, more work is required to determine the long-term effects of activating sirtuins throughout the body. Some solace can be found in the fact that SIRT1 activity rises and falls naturally depending on the time of day, what and when we eat, and even how much we exercise. If STACs can specifically restore youthful sirtuin activity, there may be minimal adverse effects. Over the next few years, many types of sirtuin activator are expected to enter clinical trials, including more potent, soluble STACs. Only then will we know if sirtuin activation has a chance of living up to its promise.

As an alternative to directly activating SIRT1, a fruitful approach has been to elevate intracellular NAD^+ levels. This approach has the advantage that it theoretically activates all sirtuins. The first evidence sirtuins could be activated by raising NAD^+*in vivo* came from genetic experiments in yeast showing that overexpression of NAD salvage pathway genes increased Sir2-dependent silencing and extended lifespan (Anderson et al. 2002; Anderson et al. 2003; Bitterman et al. 2002). Indeed, CR and other stresses extend yeast lifespan by inducing expression of the nicotinamidase gene *PNC1* (Anderson et al. 2002).

In mammals, NAD^+ levels are maintained by balancing biosynthesis and salvage on one side, and breakdown on the other (Imai 2011). NAD^+ can be synthesized from the amino acid tryptophan, but its main precursors include nicotinic acid (NA) and nicotinamide (NAM), nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR). The main NAD^+ breakdown pathways involve three enzyme classes, i.e. sirtuins, poly(ADP-ribose) polymerases (PARPs) and cyclic ADP-ribose synthases (CD38) (Imai 2011). As in yeast, NAD^+levels are modulated in response to physiological processes and the cellular environment, such as energy stress via AMPK activation and DNA damage via PARP activation. During aging, NAD^+ levels naturally decline for reasons that are not yet clear.

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Chapter 13
Sirtuins: A Future Perspective

Brian K. Kennedy

When it comes to biomedical research, Sirtuins have undergone a meteoric rise in popularity and are among the most prominent proteins linked to aging, as well as chronic disease states and metabolism. Interventions targeting human aging and disease are in or on their way to the clinic. Perhaps the most surprising aspect of this is that it all started with yeast aging. Where will it end? One thing seems clear. Our knowledge of mammalian biology has already been enriched from the identification and characterization of Sirtuins, and it seems like we have only scratched the surface.

13.1 The Past

One thing you can say about Sirtuins is that they have never been dull. The initial fame of the prototype member of the family, the yeast Sir2 protein, came from its role as a member of the SIR complex (with Sir3 and Sir4) as a transcriptional silencing factor of yeast mating type genes (Lee and Haber 2015). This was later extended to telomeres, where the SIR complex both silences transcription and maintains telomere length (Aparicio et al. 1991). In addition, Sir2 in the absence of Sir3 and Sir4 prevents rDNA recombination in the nucleolus (Gottlieb and Esposito 1989). Together, these were some of the most elegant studies in yeast genetics and set the stage for understanding how transcriptional silencing could be targeted to and spread along genomic regions.

Working with Nicanor Austriaco as a Ph.D. student in Leonard Guarente's lab, we established a seemingly unbiased screen for yeast with longer replicative lifespan and were very excited when the initial genetic mutant we identified was in the

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SIR4 gene as our thoughts immediately turned to telomeres, which are linked to aging and senescence. The hypothesis was that the SIR4 mutant would promote longer telomeres leading to enhanced replicative longevity, but we were quickly dispelled of that notion. The mutation was much more complex, causing a redistribution of silencing complexes away from telomeres (and silent mating type loci) to an undisclosed location (Kennedy et al. 1995), later determined to be the rDNA (Kennedy et al. 1997). Soon after, Kaeberlein et al. linked the longevity functions of the SIR complex directly to Sir2 function as a suppressor of extrachromosomal rDNA circle production (Kaeberlein et al. 1999), which upon expansion in mother cells can drive aging (Sinclair and Guarente 1997). Although ERCs play a major role, more recent studies extend beyond ERCs and link lifespan extension by Sir2 to other activities, including suppression of ARS replication firing in the rDNA (Kwan et al. 2013) and the promotion of asymmetric inheritance of damaged proteins to mother cell, leaving daughters relatively free of damage (Erjavec et al. 2007; Erjavec and Nystrom 2007). Although I will focus mostly on mammals in the rest of this chapter, it is worth noting that *SIR2* orthologs have been linked to aging in the two main invertebrate models, worms and flies, although again findings remain to an extent controversial (Burnett et al. 2011; Viswanathan and Guarente 2011; Whitaker et al. 2013) (Chap. 10).

Since these early studies linking yeast Sir2 to aging, research in this field has exploded and the findings have come fast and. Also continuing on the early findings, there have been a large number of surprises and plots twists along the way. I list just a few, many of which are thoroughly covered in other chapters. For instance, it was discovered that (1) Sir2 and mammalian Sirtuins (based on homology to the archaea Sirtuin, CobB) are NAD-dependent deacetylases with accompanying ADP-Ribsose activity (see Chap. 1), (2) Sirtuin substrates extend well beyond histones (see Chap. 1), (3) there are many Sirtuins in eukaryotes with overlapping and distinct functions (seven in humans), (4) the Sirtuin enzymes collectively localize to many cellular compartments including mitochondria (see Chaps. 5 and 6), (5) Sirtuins are really protein acylases and not just acetyltransferases (see Chap. 3), and (6) they are involved in a wide range of cellular functions that connect them to development, metabolism, a wide range of diseases and aging (see Chaps. 4, 7, 8, 9, and 10).

Starting with resveratrol, Sirtuin activating compounds have also been identified and are being characterized in the context of a range of disease indications (see Chap. 12). Moreover, supplements are being developed to increase levels of the Sirtuin substrate, NAD+, and show promise for age-related conditions (See Chap. 2). With respect to aging, not only SIRT1 but SIRT3 and SIRT6 have been linked either to longevity (Kanfi et al. 2012; Satoh et al. 2013), the response to calorie restriction (Boily et al. 2008; Mercken et al. 2014; Someya et al. 2010a, b; Zhang et al. 2016), or progeroid phenotypes in mammalian models (Mostoslavsky et al. 2006). How their specific downstream substrates influence aging mechanisms is still hotly debated. In all, Sirtuin research has made impressive strides, making it a good time to evaluate where we are and speculate about future research.

13.2 Present Questions

While much has been learned about the biochemistry of Sirtuins and the physiologic consequences of their perturbation, many of the big questions remain unanswered, highlighting the need for continued studies. Here, I highlight a few of the key present day issues. I will highlight those around aging, although many of the same issues are in play in the contexts of metabolism and disease states.

13.2.1 Sirtuins and Aging: Lifespan vs. Healthspan

Which Sirtuins affect aging? Do they impact lifespan or healthspan, preventing disease and enhancing function with age? At present, we have only partial answers. Nevertheless, it is fair to say that the study of Sirtuins represent one of the most fertile areas of aging research.

To date, studies with mouse models overexpressing Sirtuins have produced only modest effects on longevity. One possible explanation is that SIRT1 activity declines during mouse aging, likely due to reduced NAD+ levels (Braidy et al. 2011; Massudi et al. 2012; Ramsey et al. 2008). Therefore overexpression of a Sirtuin may not lead to enhanced activity in a substrate-limited environment. With respect to SIRT1, whole-body overexpression fails to affect lifespan (Herranz et al. 2010); however, increased expression in specific regions of the hypothalamus does confer modest extension (Satoh et al. 2013). Further studies are needed to determine whether specific expression of SIRT1 in other tissues also affect lifespan. An alternative possibility is that SIRT1 impacts molecular events driving aspects of aging linked to pathologies that largely do not affect survival. Ample evidence exists for SIRT1 overexpression and amelioration of specific aging pathologies (see Chap. 10). This concept of aging, described by David Gems in a recent review, is consistent with the general view in the aging field that at least a handful of different mechanisms drive aging and that each mechanism is linked to specific subsets of aging pathology (Gems 2015). Those that affect lifespan limiting pathologies would enhance longevity; those that affect largely non-lifespan limiting pathologies would have limited effects on overall survival. It may be that SIRT1 targets the non-lifespan limiting pathologies.

Overexpression of yeast SIR2 unambiguously extends replicative lifespan and, although SIRT1 shares the most homology to SIR2, SIRT6 may share the most similarity at the functional level. For instance, SIRT6 mediates telomere position effect in mammals and maintains genome stability (Michishita et al. 2008; Mostoslavsky et al. 2006; Tennen et al. 2011). Moreover, loss of SIRT6 leads to a progeroid phenotype and overexpression of SIRT6 leads to lifespan extension in male mice (Kanfi et al. 2012; Mostoslavsky et al. 2006). Future studies of SIRT6 are needed to understand the mechanisms by which this less-studied Sirtuin slows aging.

13.2.2 Sirtuins as Therapeutic Targets

The discovery of resveratrol as a SIRT1 activator led to another long, circuitous and controversial research path. One challenge is that resveratrol is a relatively non-specific molecule, but further screening led to a range of more specific Sirtuin activating compounds (STACs) (see Chap. 12). It remains debated to what extent these molecules activate SIRT1, but recent reports indicate that they stimulate acetylation of a small subset of SIRT1 targets (Hubbard and Sinclair 2014). A number of these compounds are in clinical studies (see Chap. 12), whose outcome will dictate the ultimate utility of these molecules for therapeutic purposes. For aging, preclinical studies indicate that at least one STAC can modestly extend mouse lifespan (Mitchell et al. 2014).

Based on findings of an age-related reduction in NAD+ levels, recent focus has been directed at strategies to restore NAD+, with a focus on two NAD+ precursors, nicotinamide riboside and nicotinamide mononucleotide (Verdin 2015). An advantage of this approach is that these precursors are normal metabolites and can be provided as supplements. The strategy is sound, but limited data exists to date to suggest whether delivery of NAD+ precursors can extend lifespan or prevent age-related diseases. With ongoing mouse and human studies either planned or underway, the therapeutic value of supplementation with these molecules may be clarified in the near future. Targeting activation of SIRT1, as well as SIRT3 and SIRT6, is one of the most promising strategies to develop human aging interventions and an exciting area of future research, although further plot twists always possible.

13.2.3 Sirtuins and the Mitochondria

The finding that SIRT3, 4 and 5 are localized to the mitochondria was surprising and has sparked a whole new direction in Sirtuin research. SIRT3 appears to be the primary deacetylase and, given that acetylation seems to suppress enzyme activity in the mitochondria, SIRT3 may serve to stimulate energy production and coordinate metabolic homeostasis (see Chap. 5). This activity may be intimately linked to aging and several studies point to a role for SIRT3 in the caloric restriction response (Someya et al. 2010a, b). While it is yet to be determined whether overexpression of SIRT3 leads to lifespan extension, it seems increasing likely that SIRT3 may be a key factor coordinating mitochondrial respiration and aging.

SIRT4 and SIRT5 have proven more enigmatic (see Chaps. 1 and 6). Recent studies indicate SIRT5 primary catalyzes desuccinylation and demalonylation reactions, although the physiological significance of this remains largely unknown and SIRT5 knockout mice have little in the way of phenotypes. SIRT4 is even more of a mystery. Nevertheless, understanding the role of these three enzymes in the control of mitochondrial functions seems a fruitful avenue of future endeavor with the potential for major new discovery in how mitochondrial proteins are regulated through post-translational modification.

13.3 The Future: Deconstruction/Reconstruction

Medical research over the last two decades has followed a familiar path, one that applies to many of the most promising aging proteins including, mTOR, insulin/IGF, p53, FOXO and Sirtuins. Once a protein becomes of interest, investigators study it in the context of their favorite experimental paradigms and, while these studies are certainly of interest, the outcome of this approach has been a bewildering array of potential interacting proteins and/or substrates, as well as and possible physiologic roles, This deconstructionist approach has generated (at the time of writing) 5318 thousand papers on Sirtuins, 1556 linked to aging. A recent review highlight mechanisms of aging identified in a NIA-sponsored geroscience meeting identified seven potential aging mechanisms (Kennedy et al. 2014). Pubmed searches indicate at least XX papers linking Sirtuins to each pathway (Table 13.1).

One can argue that proteins with the potential to modulate human aging may have to be very pleiotropic given the complex nature of the aging process. Nevertheless, there is the feeling that we need strategies to move beyond what proteins "can do" to comprehend what they really do that is relevant to aging. Is it possible to take the mountains of data and reconstruct a holistic understanding of Sirtuins and aging? Can systems biology and network modeling provide insights? Interestingly, while the aging field has made impressive progress identifying interventions that affect longevity, we still don't have much agreement as to the molecu-

Table 13.1 The Sirtuin research explosion

Pillars of aging	Sirtuin references
Total references	5318
General aging	1556
Adaptation to Stress	1285
Epigenetics	589
Inflammation	473
Macromolecular Damage	489
Metabolism	4804
Proteostasis	490
Stem cells and regeneration	295

Numbers were generated by a simple Pubmed search of Sirtuin or Sir2 and each pillar of aging (Kennedy et al. 2014). These numbers are meant simply to indicate the depth of research in the Sirtuin field and to raise the question of whether the current research approach will ultimately lead to an integrated understanding of the role of Sirtuins in mammalian physiology and/or aging. Searches were Sirtuins+Aging, Stress, Chromatin, Inflammation, Cellular Damage, Metabolism, Protein Homeostasis, or Stem Cells

lar events that cause aging. How do interventions mitigate the fundamental molecular changes with aging that drive chronic diseases? If strategies can be developed to integrate knowledge of proteins like Sirtuins, it might reveal key insights that lead unequivocally back to the molecular pathways driving the aging process and it will certainly refine human interventional strategies. The solution to reconstruction problem remains unclear, but a true molecular understanding of the aging process may await insight in this arena.

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