

# Chapter 1

## Biochemistry and Enzymology of Sirtuins

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### 1.1 Biochemistry and Enzymology of Sirtuins

#### 1.1.1 Definition of Sirtuins

Sirtuins are highly conserved NAD<sup>+</sup> 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 sub-specialization of a common catalytic activity. Sirtuins are evolved to recognize the substrate NAD<sup>+</sup>, and react the dinucleotide with lysine-acylated peptide and protein substrates, thereby effecting deacylation. In the canonical sirtuin reaction, sirtuins react as NAD<sup>+</sup>-dependent lysine deacetylases (Sauve et al. 2006). In mammals, sirtuins are found in 7 distinct isoforms, called SIRT1-7. NAD<sup>+</sup>-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-GAGVSVSCGIPDFPRS-RDGIYARLAVDFPDLDPQAMFDIEYFRKDP--RP---FFKFAKEIYP--GQFQPSLCHKFI
SIRT2 1-83-GAGISTPSGIPDFRSPSTGLYSNL--EKYHLPYEAIPEISYFKKHP--EP---FFLAKELYP--GQFKPTICHYFM
SIRT3 1-144-GAGISTPSGIPDFRSPGSGLYSNL--QQYDLPYEAIPELPPFFHNP--KP---FFTFLAKELYP--GNYKPNVTHYFL
SIRT4 1-61-GAGISTESGIPDYRSEKUGLYART--DRRPICQHD-----FVRSAPIRQR--YWARNFVGVQPQFSSHQPNPAHWAL
SIRT5 1-57-GAGVSAESGVPTFR--GAGGYWRKW--QAQDLATPLA-----FAHNP--SRWVEFYHYRREVNV--GSKEPNAGHRAI
SIRT6 1-51-GAGISTAASGIPDFRG-PHG-VVTME-ERG--LAKPDTTP-----ESARPTQTHMAL
SIRT7 1-106-GAGISTAASIPDYRG-PNG-VVTLL--QKGRSVS--AADL-----SEAEPTLTHMSI
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SIRT1 ALSD---KEGKLLRNVTQNIIDTLEQVAGI--QRIIQCHGSPATASC--LIC--K---YKVDCEAVR-----
SIRT2 RLK---DKGLLLRLCYTQNIIDTLERIAGLEQEDLVEAHGCTFYTSHCVSASC--R---HEYPLSMHK-----
SIRT3 RLH---DKGLLLRLTQNIIDGLRLVSGIPASKLVEAHGTFASATC--TVC--Q---RPPGEDIK-----
SIRT4 STNE---KLGKLYLWLTQNIIDVALHTRAGS--RRLTELHGCMDRVLC--LDC--G---EQTPPGVLQERFQVLNPTWSAEAHGL
SIRT5 ACETPLKQGRVVVITQNIIDELHRRKAGT--KNLLEIHGSLPKTRC--TSC--GVVAENYKSPICP-----ALSGKAPPEPTGQ
SIRT6 VQLE---RVGLLRFLVSNQVDGLHVRSQFPRDKLAELHGNMPEEC--AKC--K---TQVVRDTVV---GTMGLKAT-----
SIRT7 TRLH---EQKLVQHVSNQCDGLHRSGLPRTAISELHGNMYIEVC--TSCVNP--REYVR-----VFDVTER-----
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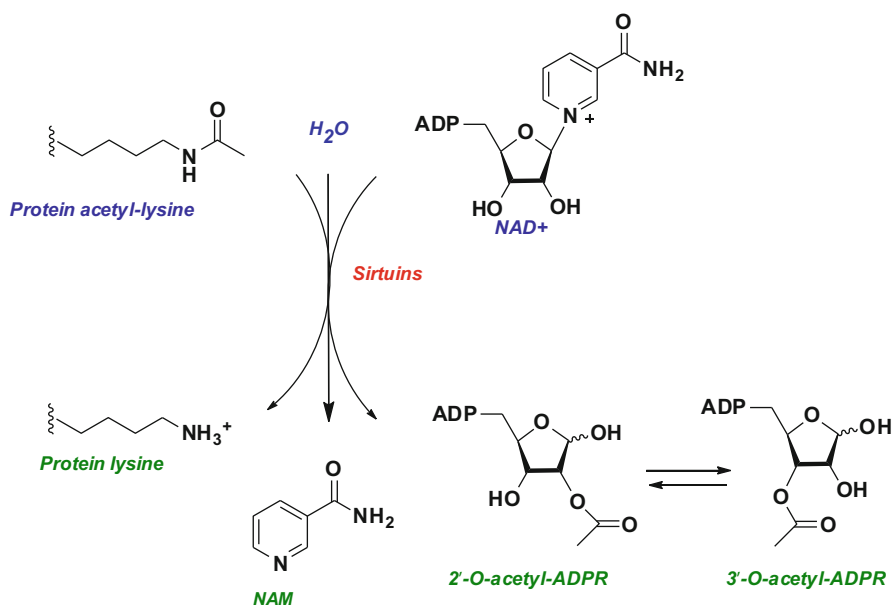
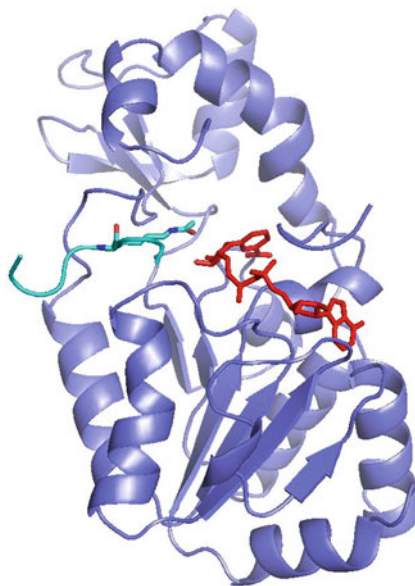
SIRT1 ---GDIFNQ-----VVPRCPR--CPADEFPLAIMKPEIVFFGE--NLPEQ-FHRAMKYDKDEVDLLIVIGSS-443-747
SIRT2 ---EKIPSE-----VTPKCED--CQS-----LVKPDIVFFGE--SLPAR-FFSCMQSDFLKVDLLVMGTS-264-388
SIRT3 ---ADVMAD-----RVPRCFV--CTG-----VVKPDIVFFGE--PLPQR-FLLHV-VDFPMADLLLILGTS-322-399
SIRT4 APDGDVFLSEEQVRSQVPTCVQ--CGG---HLKPDVVFVFGD--TVNPD-KVDFVHKRVKHEADSLLVVGSS-263-314
SIRT5 D--ASIPVE-----KLPRCEEA--CGG-----LLRPHVVVFGE--NLDPV-ILEEVRELAHCDLCLVVGTS-252-310
SIRT6 ---GRLCIV--A---KARGLRA--CRG-----ELRDTILDWED--SLPDR-DLALADEASRNADLSITLGTSS-217-355
SIRT7 ---TALHRH--Q---TGRTCHK--CGT-----QLRDTIVHFGERGTLGQPLNWEAETAASRADTLCLGSS-271-400
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**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  $\text{NAD}^+$ , 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  $\text{NAD}^+$ . 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<sup>+</sup> (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<sup>+</sup>, 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  $\text{NAD}^+$ -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 myristoyl modification found on NF $\kappa$ B (Jiang et al. 2013). Evidence from crystallography suggests that SIRT5 and SIRT6 deacylate these modifications of lysine by analogous mechanisms to  $\text{NAD}^+$ -dependent deacetylation (Zhou et al. 2012).

### 1.1.4 *Sirtuin Reactions and Mechanisms*

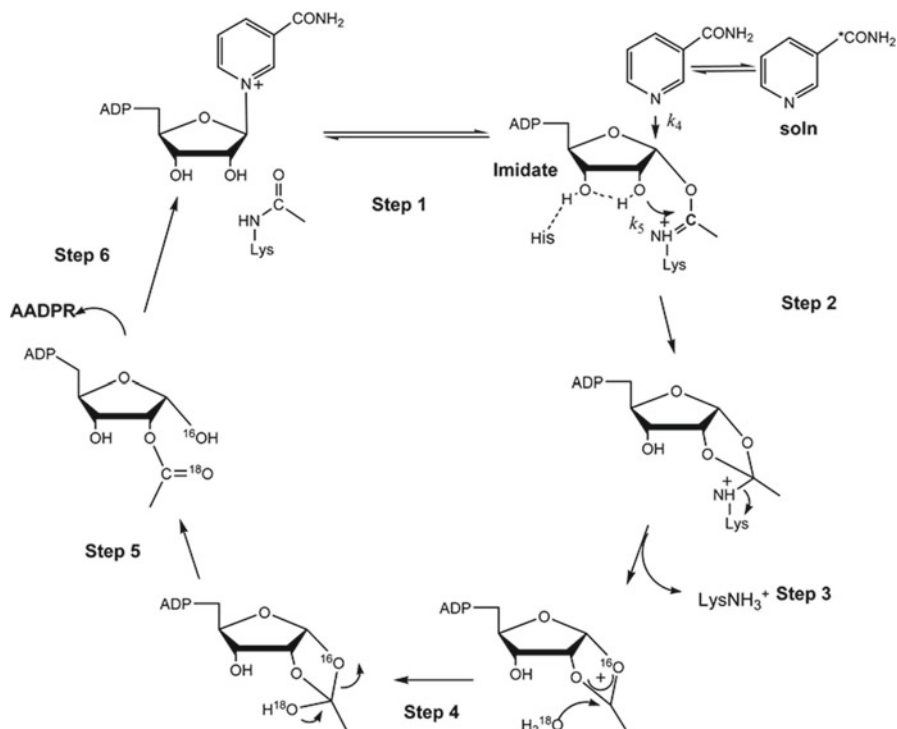
The prototype reaction stoichiometry of sirtuins combines three distinct reactants as substrates:  $\text{NAD}^+$ , water and acetylated peptide or protein substrates. These reactants form products as nicotinamide (NAM), the deacetylated protein substrate and a novel compound called 2'-O-acetyl-ADPR (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  $\text{NAD}^+$ -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  $\text{NAD}^+$  on the alpha face of the  $\text{NAD}^+$  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-κB* 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<sup>+</sup>

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 <sup>14</sup>C-NAM, which resulted in formation of <sup>14</sup>C-NAD<sup>+</sup>, 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<sup>+</sup> (Sauve 2012).

Additional evidence in favor of the imidate mechanism of sirtuin deacetylation has been obtained from X-ray structures of products of ADP-ribosylated 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<sup>+</sup> metabolism, such as NAD<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup>, 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)

Moreover, 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% caloric restriction, expression of SIRT1 increases by 113% in muscle tissue as shown by RT PCR (Civitaese 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

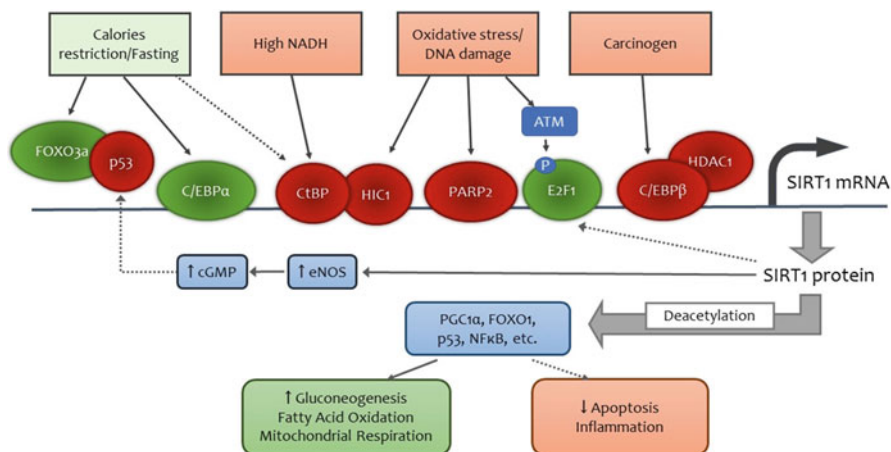


protein (Lombard et al. 2007; Schwer et al. 2009) and SIRT5 activity (Nakagawa et al. 2009) in liver mitochondria of mice. Similarly, exercise elevates SIRT1 mRNA levels in the skeletal muscle of young physically inactive men (Radak et al. 2011) and SIRT3 protein in the triceps of mice. On the contrary, 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<sup>•</sup> 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/EBP $\alpha$ . As a result of SIRT1 activity on substrates such as PGC1 $\alpha$  and FOXO1, gluconeogenesis, 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 NF $\kappa$ B, 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/EBP $\beta$  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  $\alpha$  (C/EBP $\alpha$ ) during adipocyte differentiation (Jin et al. 2010b). C/EBP $\alpha$  can directly bind to the SIRT1 promoter and thereby upregulate SIRT1 transcription. Binding of C/EBP $\alpha$  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/EBP $\beta$  is able to suppress SIRT1 transcription. In a model of diethylnitrosamine-induced liver cancer development, C/EBP $\beta$  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-1 $\alpha$  (PGC-1 $\alpha$ ) plays key roles in regulating energy metabolism through thermogenesis, gluconeogenesis and mitochondrial biogenesis (Liang 2006). In mice myocytes, PGC-1 $\alpha$  co-localizes with estrogen-related receptor  $\alpha$  (ERR $\alpha$ ) and binds to an ERR binding element (ERRE) within the mSIRT3 promoter (Jin et al. 2013b). PGC-1 $\alpha$ -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 1 $\alpha$  (HIF1 $\alpha$ ) 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 (SEN1), 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,  $\alpha$ -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 mechanistic 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  $\alpha$ -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<sup>+</sup> Metabolism

The reaction of sirtuins is initiated by NAD<sup>+</sup> binding to their catalytic sites. Thus, the cellular NAD<sup>+</sup> availability can be posited to be a limiting factor of sirtuin activity (Imai 2009a). In cells, NAD<sup>+</sup> is responsible for transferring electron equivalents for multiple metabolic reactions such as fatty acid  $\beta$ -oxidation, glycolysis and the TCA cycle. In this role, NAD<sup>+</sup> 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<sup>+</sup> levels to sirtuin protein deacetylation activity may have obtained regulatory significance in the course of evolutionary time scales. Importantly, intracellular levels of NAD<sup>+</sup> 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<sup>+</sup> degradative pathways, including the activities of NAD<sup>+</sup> consumers such as PARPs, sirtuins themselves and NAD<sup>+</sup> glycohydrolases, such as CD38. By complex pathways and steady-state perturbations, NAD<sup>+</sup> levels change and are thought to regulate sirtuin activities.

NAD<sup>+</sup> can be synthesized by multiple pathways, but appears to be regulated by a central mechanism. *De novo* biosynthesis of NAD<sup>+</sup> can occur via catabolism of the amino acid tryptophan through the kynurenine pathway. However, the main input for NAD<sup>+</sup> homeostasis appears to be the NAD<sup>+</sup> salvage pathway (Houtkooper et al., 2010). This pathway converts NAM, the by-product of NAD<sup>+</sup> consumption, back to NAD<sup>+</sup>. 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<sup>+</sup> (Canto et al. 2012; Satoh et al. 2011). Disruption of NAD<sup>+</sup> salvage pathways leads to down regulation of NAD<sup>+</sup> 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<sup>+</sup> level (Yang et al. 2007). Overexpression of NAMPT markedly increases NAD<sup>+</sup> levels in neurons and fibroblasts, whereas overexpression of another enzyme in the salvage pathway, NMNAT, does not alter cellular NAD<sup>+</sup> levels (Araki et al. 2004; Revollo et al. 2004). Furthermore, overexpression of NAMPT have been shown to augment cellular levels of NAD<sup>+</sup> 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<sup>+</sup> biosynthesis. Consistently, pharmacologic use of NAMPT inhibitors substantially suppress cellular NAD<sup>+</sup> levels (Bruzzone et al. 2009; Hasmann and Schemainda 2003), cementing the view that NAMPT is essential for homeostatic control of NAD<sup>+</sup> level.

Factors that affect NAD<sup>+</sup> homeostasis are stress-linked. For example, AMP-activated protein kinase (AMPK) is a major sensor of cellular energy status and it regulates cellular NAD<sup>+</sup> level. Activated by nutrient deprivation and exercise, AMPK activation causes increases cellular NAD<sup>+</sup> level, potentiating SIRT1 activity and enabling increased deacetylation of target proteins such as FOXOs and PGC-1 $\alpha$ . These events facilitate increased energy expenditure through fatty acid  $\beta$ -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-1 $\alpha$  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  $\beta$  (CaMKK $\beta$ ) (Iwabu et al. 2010). Subsequently, CaMKK $\beta$  activates AMPK to increase SIRT1 deacetylation activity on PGC-1 $\alpha$ . As a result, mitochondrial biogenesis is enhanced in skeletal muscle by adiponectin (Iwabu et al. 2010).

Cellular NAD<sup>+</sup> level can also be augmented by inhibiting the consumption of NAD<sup>+</sup> by NAD<sup>+</sup> consuming enzymes, such as poly(ADP-ribose) polymerase-1 (PARP-1) (Bai et al. 2011b). PARP-1 is a major consumer of NAD<sup>+</sup> in the cell. Upon recognition of damaged or abnormal DNA, PARP-1 binds to the damaged sequence (Durkacz et al. 1980) and uses NAD<sup>+</sup> 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<sup>+</sup> levels while augmenting SIRT1 activity in vitro. PARP-1<sup>-/-</sup> mice were shown to have higher NAD<sup>+</sup> 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<sup>+</sup> levels in key metabolic tissues.

The effects of NAD<sup>+</sup> level on sirtuin activity, specifically SIRT1 and SIRT3, are consistent with the view that NAD<sup>+</sup> is a positive stimulative regulator of sirtuin activity. For example, Auwerx and co-workers have shown that genetic upregulation of NAD<sup>+</sup> 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<sup>+</sup> 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 PGC1 $\alpha$ , 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<sup>+</sup> 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 PGC1 $\alpha$  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 down-regulate 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

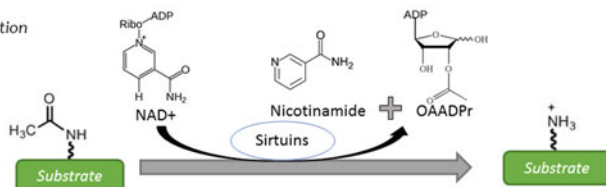
The *Fluor de Lys* fluorescence assay of sirtuin deacetylation activity was introduced by Howitz et al. as a means to screen chemical libraries for activators of SIRT1 (Howitz et al. 2003). The assay is dependent on the SIRT1 substrate comprising a fluorescent peptide called *Fluor de Lys* comprising the amino acid sequence 379–382 of human p53 including N<sup>ε</sup>-acetylated Lys382. Addition of a sirtuin (e.g. SIRT1), in the presence of NAD<sup>+</sup>, 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<sup>+</sup>, 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).

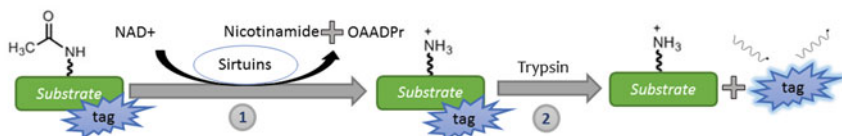
The SIRT1 activating effect of resveratrol was not fully reproducible in later studies using non-fluorophore substrates. For example, Kaerberlein et al. found no significantly increase in the mean or maximum life span of yeast treated with resveratrol, nor was it observed that resveratrol caused effects consistent with Sir2 activation (Kaerberlein et al. 2005). Resveratrol nevertheless activated SIRT1 toward a fluorophore labeled substrate, although the Michaelis constant for NAD<sup>+</sup> was not observed to be decreased. When the fluorophore containing substrate was replaced with a <sup>3</sup>H 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 (Kaerberlein 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 [<sup>3</sup>H]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<sup>+</sup> are added as substrates. Deacetylation activity of sirtuins leads to the breakage of glycosidic bond in NAD<sup>+</sup>, 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, [<sup>14</sup>C] can be used to label NAD<sup>+</sup> and [<sup>14</sup>C] NAM is collected for measurement. Also, [<sup>32</sup>P]NAD<sup>+</sup> or [<sup>3</sup>H] acetylated protein substrate can be used as substrate to monitor the formation of [<sup>32</sup>P]AADPR or [<sup>3</sup>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 *Fluor 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 create 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<sup>+</sup> as substrate. In this assay, [carbonyl-<sup>14</sup>C]NAD<sup>+</sup> 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 [<sup>14</sup>C]NAD<sup>+</sup>, releasing [<sup>14</sup>C]NAM along with the metabolite AADPR. After quenching the reaction, [<sup>14</sup>C]NAD<sup>+</sup> and [<sup>14</sup>C]NAM can be separated and isolated using thin layer chromatography or reversed-phase HPLC. Radioactivity is subsequently quantified by scintillation counter. Alternatively, a [<sup>32</sup>P]NAD<sup>+</sup> 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, [<sup>32</sup>P]NAD<sup>+</sup> and [<sup>32</sup>P]AADPR are separated with thin layer chromatography or reversed-phase HPLC and measured by scintillation counter.

Labeling the acetylated substrate of sirtuin reaction is another way of determining sirtuin activity. In such an assay a [acetyl-<sup>3</sup>H] acetylated peptide or protein is used. With sirtuin deacetylation, [<sup>3</sup>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, [<sup>3</sup>H]acetate can be hydrolyzed away from [<sup>3</sup>H]AADPR. The mixture is then transferred to a charcoal slurry which can bind all the substrates and products except for [<sup>3</sup>H]acetate. The amount of [<sup>3</sup>H]acetate is then quantitated by scintillation counter and the radioactivity corresponds to the [<sup>3</sup>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<sup>+</sup> on sirtuin active sites to form stable thioimide 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 thioimide complex (Cen et al. 2011a). A chemically modified NAD<sup>+</sup>, 6-AMX-NAD<sup>+</sup>, was also designed to form the desired thioimide, but also to chemically react to biotin after thioimide formation. With construction of 6-AMX-NAD<sup>+</sup>, 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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## References

- Adamietz P (1987) Poly(ADP-ribose) synthase is the major endogenous nonhistone acceptor for poly(ADP-ribose) in alkylated rat hepatoma cells. *Eur J Biochem/FEBS* 169:365–372
- Ahuja N, Schwer B, Carobbio S, Waltregny D, North BJ, Castronovo V, Maechler P, Verdin E (2007) Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase. *J Biol Chem* 282:33583–33592
- Ame JC, Rolli V, Schreiber V, Niedergang C, Apiou F, Decker P, Muller S, Hoger T, Menissier-de Murcia J, de Murcia G (1999) PARP-2, A novel mammalian DNA damage-dependent poly(ADP-ribose) polymerase. *J Biol Chem* 274:17860–17868
- Araki T, Sasaki Y, Milbrandt J (2004) Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science* 305:1010–1013

- Bai P, Canto C, Brunyanszki A, Huber A, Szanto M, Cen Y, Yamamoto H, Houten SM, Kiss B, Oudart H et al (2011a) PARP-2 regulates SIRT1 expression and whole-body energy expenditure. *Cell Metab* 13:450–460
- Bai P, Canto C, Oudart H, Brunyanszki A, Cen Y, Thomas C, Yamamoto H, Huber A, Kiss B, Houtkooper RH et al (2011b) PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. *Cell Metab* 13:461–468
- Baur JA (2010) Biochemical effects of SIRT1 activators. *Biochim Biophys Acta* 1804:1626–1634
- Berthiaume M, Boufaied N, Moisan A, Gaudreau L (2006) High levels of oxidative stress globally inhibit gene transcription and histone acetylation. *DNA Cell Biol* 25:124–134
- Bitterman KJ, Anderson RM, Cohen HY, Latorre-Esteves M, Sinclair DA (2002) Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast Sir2 and human SIRT1. *J Biol Chem* 277:45099–45107
- Bode AM, Dong Z (2007) The functional contrariety of JNK. *Mol Carcinog* 46:591–598
- Borra MT, Denu JM (2003) Quantitative assays for characterization of the Sir2 family of NAD<sup>+</sup>-dependent deacetylases. In: Allis CD, Carl W (eds) *Methods in enzymology*. Academic, New York, pp 171–187
- Borra MT, Smith BC, Denu JM (2005) Mechanism of human SIRT1 activation by resveratrol. *J Biol Chem* 280:17187–17195
- Borradaile NM, Pickering JG (2009) Nicotinamide phosphoribosyltransferase imparts human endothelial cells with extended replicative lifespan and enhanced angiogenic capacity in a high glucose environment. *Aging Cell* 8:100–112
- Brown KD, Maqsood S, Huang JY, Pan Y, Harkcom W, Li W, Sauve A, Verdin E, Jaffrey SR (2014) Activation of SIRT3 by the NAD(+) precursor nicotinamide riboside protects from noise-induced hearing loss. *Cell Metab* 20:1059–1068
- Bruzzese S, Fruscione F, Morando S, Ferrando T, Poggi A, Garuti A, D'Urso A, Selmo M, Benvenuto F, Cea M et al (2009) Catastrophic NAD<sup>+</sup> depletion in activated T lymphocytes through Nampt inhibition reduces demyelination and disability in EAE. *PLoS One* 4:e7897
- Burnett C, Valentini S, Cabreiro F, Goss M, Somogyvari M, Piper MD, Hoddinott M, Sutphin GL, Leko V, McElwee JJ et al (2011) Absence of effects of Sir2 overexpression on lifespan in *C. elegans* and *Drosophila*. *Nature* 477:482–485
- Canto C, Jiang LQ, Deshmukh AS, Matakı C, Coste A, Lagouge M, Zierath JR, Auwerx J (2010) Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle. *Cell Metab* 11:213–219
- Canto C, Houtkooper RH, Pirinen E, Youn DY, Oosterveer MH, Cen Y, Fernandez-Marcos PJ, Yamamoto H, Andreux PA, Cettour-Rose P et al (2012) The NAD(+) precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet-induced obesity. *Cell Metab* 15:838–847
- Cen Y, Falco JN, Xu P, Youn DY, Sauve AA (2011a) Mechanism-based affinity capture of sirtuins. *Org Biomol Chem* 9:987–993
- Cen Y, Youn DY, Sauve AA (2011b) Advances in characterization of human sirtuin isoforms: chemistries, targets and therapeutic applications. *Curr Med Chem* 18:1919–1935
- Cen Y, Youn DY, Sauve AA (2011c) Advances in characterization of human sirtuin isoforms: chemistries, targets and therapeutic applications. *Curr Med Chem* 18:1919–1935
- Chalkiadaki A, Guarente L (2012) High-fat diet triggers inflammation-induced cleavage of SIRT1 in adipose tissue to promote metabolic dysfunction. *Cell Metab* 16:180–188
- Chau MD, Gao J, Yang Q, Wu Z, Gromada J (2010) Fibroblast growth factor 21 regulates energy metabolism by activating the AMPK-SIRT1-PGC-1 $\alpha$  pathway. *Proc Natl Acad Sci U S A* 107:12553–12558
- Chen WY, Wang DH, Yen RC, Luo J, Gu W, Baylin SB (2005) Tumor suppressor HIC1 directly regulates SIRT1 to modulate p53-dependent DNA-damage responses. *Cell* 123:437–448
- Cimen H, Han MJ, Yang Y, Tong Q, Koc H, Koc EC (2010) Regulation of succinate dehydrogenase activity by SIRT3 in mammalian mitochondria. *Biochemistry* 49:304–311

- Civitaresse AE, Carling S, Heilbronn LK, Hulver MH, Ukropcova B, Deutsch WA, Smith SR, Ravussin E (2007) Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. *PLoS Med* 4:e76
- Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA (2004) Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305:390–392
- Costford SR, Bajpeyi S, Pasarica M, Albarado DC, Thomas SC, Xie H, Church TS, Jubrias SA, Conley KE, Smith SR (2010) Skeletal muscle NAMPT is induced by exercise in humans. *Am J Physiol Endocrinol Metab* 298:E117–E126
- DeGregori J, Kowalik T, Nevins JR (1995) Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Mol Cell Biol* 15:4215–4224
- Dominy JE Jr, Lee Y, Gerhart-Hines Z, Puigserver P (2010) Nutrient-dependent regulation of PGC-1 $\alpha$ 's acetylation state and metabolic function through the enzymatic activities of Sirt1/GCN5. *Biochim Biophys Acta* 1804:1676–1683
- Duncan JS, Litchfield DW (2008) Too much of a good thing: the role of protein kinase CK2 in tumorigenesis and prospects for therapeutic inhibition of CK2. *Biochim Biophys Acta (BBA) Prot Proteom* 1784:33–47
- Durkacz BW, Omidiji O, Gray DA, Shall S (1980) (ADP-ribose)<sub>n</sub> participates in DNA excision repair. *Nature* 283:593–596
- Fatkins DG, Monnot AD, Zheng W (2006) Nepsilon-thioacetyl-lysine: a multi-facet functional probe for enzymatic protein lysine Nepsilon-deacetylation. *Bioorg Med Chem Lett* 16:3651–3656
- Fjeld CC, Birdsong WT, Goodman RH (2003) Differential binding of NAD<sup>+</sup> and NADH allows the transcriptional corepressor carboxyl-terminal binding protein to serve as a metabolic sensor. *Proc Natl Acad Sci U S A* 100:9202–9207
- Ford E, Voit R, Liszt G, Magin C, Grummt I, Guarente L (2006) Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription. *Genes Dev* 20:1075–1080
- Ford J, Ahmed S, Allison S, Jiang M, Milner J (2008) JNK2-dependent regulation of SIRT1 protein stability. *Cell Cycle* 7:3091–3097
- Fraser M, Chan SL, Chan SS, Fiscus RR, Tsang BK (2006) Regulation of p53 and suppression of apoptosis by the soluble guanylyl cyclase/cGMP pathway in human ovarian cancer cells. *Oncogene* 25:2203–2212
- Fridman JS, Lowe SW (2003) Control of apoptosis by p53. *Oncogene* 22:9030–9040
- Fulco M, Cen Y, Zhao P, Hoffman EP, McBurney MW, Sauve AA, Sartorelli V (2008) Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. *Dev Cell* 14:661–673
- Gao Z, Zhang J, Kheterpal I, Kennedy N, Davis RJ, Ye J (2011) Sirtuin 1 (SIRT1) protein degradation in response to persistent c-Jun N-terminal kinase 1 (JNK1) activation contributes to hepatic steatosis in obesity. *J Biol Chem* 286:22227–22234
- Giralt A, Hondares E, Villena JA, Ribas F, Díaz-Delfín J, Giralt M, Iglesias R, Villarroya F (2011) Peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  controls transcription of the Sirt3 gene, an essential component of the thermogenic brown adipocyte phenotype. *J Biol Chem* 286:16958–16966
- Guarente L, Picard F (2005) Calorie restriction--the SIR2 connection. *Cell* 120:473–482
- Guo X, Williams JG, Schug TT, Li X (2010) DYRK1A and DYRK3 promote cell survival through phosphorylation and activation of SIRT1. *J Biol Chem* 285:13223–13232
- Haigis MC, Sinclair DA (2010) Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol* 5:253–295
- Haigis MC, Mostoslavsky R, Haigis KM, Fahie K, Christodoulou DC, Murphy AJ, Valenzuela DM, Yancopoulos GD, Karow M, Blander G et al (2006) SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. *Cell* 126:941–954
- Hallows WC, Lee S, Denu JM (2006) Sirtuins deacetylate and activate mammalian acetyl-CoA synthetases. *Proc Natl Acad Sci U S A* 103:10230–10235



- Hamaguchi M, Meth JL, von Klitzing C, Wei W, Esposito D, Rodgers L, Walsh T, Welch P, King MC, Wigler MH (2002) DBC2, a candidate for a tumor suppressor gene involved in breast cancer. *Proc Natl Acad Sci U S A* 99:13647–13652
- Han MK, Song EK, Guo Y, Ou X, Mantel C, Broxmeyer HE (2008) SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. *Cell Stem Cell* 2:241–251
- Hasmann M, Schemainda I (2003) FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. *Cancer Res* 63:7436–7442
- Hawse WF, Hoff KG, Fatkins DG, Daines A, Zubkova OV, Schramm VL, Zheng W, Wolberger C (2008) Structural insights into intermediate steps in the Sir2 deacetylation reaction. *Structure* 16:1368–1377
- Hirschev MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, Grueter CA, Harris C, Biddinger S, Ilkayeva OR et al (2010) SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. *Nature* 464:121–125
- Hisahara S, Chiba S, Matsumoto H, Tanno M, Yagi H, Shimohama S, Sato M, Horio Y (2008) Histone deacetylase SIRT1 modulates neuronal differentiation by its nuclear translocation. *Proc Natl Acad Sci U S A* 105:15599–15604
- Hoff KG, Avalos JL, Sens K, Wolberger C (2006) Insights into the sirtuin mechanism from ternary complexes containing NAD<sup>+</sup> and acetylated peptide. *Structure* 14:1231–1240
- Houtkooper RH, Canto C, Wanders RJ, Auwerx J (2010) The secret life of NAD<sup>+</sup>: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* 31:194–223
- Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisilewski A, Zhang L-L et al (2003a) Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* 425:191–196
- Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, Zipkin RE, Chung P, Kisilewski A, Zhang LL et al (2003b) Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* 425:191–196
- Hsu CP, Oka S, Shao D, Hariharan N, Sadoshima J (2009) Nicotinamide phosphoribosyltransferase regulates cell survival through NAD<sup>+</sup> synthesis in cardiac myocytes. *Circ Res* 105:481–491
- Hwang J-W, Yao H, Caito S, Sundar IK, Rahman I (2013) Redox regulation of SIRT1 in inflammation and cellular senescence. *Free Radic Biol Med* 61:95–110
- Imai S (2009a) The NAD World: a new systemic regulatory network for metabolism and aging--Sirt1, systemic NAD biosynthesis, and their importance. *Cell Biochem Biophys* 53:65–74
- Imai S (2009b) Nicotinamide phosphoribosyltransferase (Nampt): a link between NAD biology, metabolism, and diseases. *Curr Pharm Des* 15:20–28
- Imai S, Yoshino J (2013) The importance of NAMPT/NAD/SIRT1 in the systemic regulation of metabolism and ageing. *Diabet Obes Metabol* 15(Suppl 3):26–33
- Iwabu M, Yamauchi T, Okada-Iwabu M, Sato K, Nakagawa T, Funata M, Yamaguchi M, Namiki S, Nakayama R, Tabata M et al (2010) Adiponectin and AdipoR1 regulate PGC-1 $\alpha$  and mitochondria by Ca<sup>2+</sup> and AMPK/SIRT1. *Nature* 464:1313–1319
- Jiang H, Khan S, Wang Y, Charron G, He B, Sebastian C, Du J, Kim R, Ge E, Mostoslavsky R et al (2013) SIRT6 regulates TNF- $\alpha$  secretion through hydrolysis of long-chain fatty acyl lysine. *Nature* 496:110–113
- Jin Q, Yan T, Ge X, Sun C, Shi X, Zhai Q (2007) Cytoplasm-localized SIRT1 enhances apoptosis. *J Cell Physiol* 213:88–97
- Jin Q, Zhang F, Yan T, Liu Z, Wang C, Ge X, Zhai Q (2010a) C/EBP $\alpha$  regulates SIRT1 expression during adipogenesis. *Cell Res* 20:470–479
- Jin Q, Zhang F, Yan T, Liu Z, Wang C, Ge X, Zhai Q (2010b) C/EBP $\alpha$  regulates SIRT1 expression during adipogenesis. *Cell Res* 20:470–479
- Jin J, Iakova P, Jiang Y, Lewis K, Sullivan E, Jawanmardi N, Donehower L, Timchenko L, Timchenko NA (2013a) Transcriptional and translational regulation of C/EBP $\beta$ -HDAC1

- protein complexes controls different levels of p53, SIRT1, and PGC1alpha proteins at the early and late stages of liver cancer. *J Biol Chem* 288:14451–14462
- Jin J, Iakova P, Jiang Y, Lewis K, Sullivan E, Jawanmardi N, Donehower L, Timchenko L, Timchenko NA (2013b) Transcriptional and Translational Regulation of C/EBP $\beta$ -HDAC1 Protein Complexes Controls Different Levels of p53, SIRT1, and PGC1 $\alpha$  Proteins at the Early and Late Stages of Liver Cancer. *J Biol Chem* 288:14451–14462
- Jing E, Gesta S, Kahn CR (2007) SIRT2 regulates adipocyte differentiation through FoxO1 acetylation/deacetylation. *Cell Metab* 6:105–114
- Kaerberlein M, McDonagh T, Heltweg B, Hixon J, Westman EA, Caldwell SD, Napper A, Curtis R, DiStefano PS, Fields S et al (2005) Substrate-specific activation of sirtuins by resveratrol. *J Biol Chem* 280:17038–17045
- Kanfi Y, Naiman S, Amir G, Peshti V, Zinman G, Nahum L, Bar-Joseph Z, Cohen HY (2012) The sirtuin SIRT6 regulates lifespan in male mice. *Nature* 483:218–221
- Kang H, Jung J-W, Kim MK, Chung JH (2009) CK2 Is the regulator of SIRT1 substrate-binding affinity, deacetylase activity and cellular response to DNA-damage. *PLoS One* 4:e6611
- Kawahara TL, Michishita E, Adler AS, Damian M, Berber E, Lin M, McCord RA, Ongaigui KC, Boxer LD, Chang HY et al (2009) SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell* 136:62–74
- Kim E-J, Kho J-H, Kang M-R, Um S-J (2007) Active regulator of SIRT1 cooperates with SIRT1 and facilitates suppression of p53 activity. *Mol Cell* 28:277–290
- Kim J-E, Chen J, Lou Z (2008) DBC1 is a negative regulator of SIRT1. *Nature* 451:583–586
- Kincaid B, Bossy-Wetzell E (2013) Forever young: SIRT3 a shield against mitochondrial meltdown, aging, and neurodegeneration. *Front Aging Neurosci* 5:48
- Knight JR, Allison SJ, Milner J (2013) Active regulator of SIRT1 is required for cancer cell survival but not for SIRT1 activity. *Open Biol* 3:130130
- Kokkola T, Suuronen T, Molnár F, Määttä J, Salminen A, Jarho EM, Lahtela-Kakkonen M (2014) AROS has a context-dependent effect on SIRT1. *FEBS Lett* 588:1523–1528
- Kraus D, Yang Q, Kong D, Banks AS, Zhang L, Rodgers JT, Pirinen E, Puliniikunnil TC, Gong F, Wang YC et al (2014) Nicotinamide N-methyltransferase knockdown protects against diet-induced obesity. *Nature* 508:258–262
- Kumar V, Carlson JE, Ohgi KA, Edwards TA, Rose DW, Escalante CR, Rosenfeld MG, Aggarwal AK (2002) Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase. *Mol Cell* 10:857–869
- Kwon H-S, Ott M (2008) The ups and downs of SIRT1. *Trends Biochem Sci* 33:517–525
- Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, Daussin F, Messadeq N, Milne J, Lambert P, Elliott P et al (2006) Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 127:1109–1122
- Landry J, Sutton A, Tafrov ST, Heller RC, Stebbins J, Pillus L, Sternglanz R (2000) The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc Natl Acad Sci U S A* 97:5807–5811
- Liang HWWF (2006) PGC-1 $\alpha$ : a key regulator of energy metabolism. *Adv Physiol Educ* 30:145–151
- Lombard DB, Alt FW, Cheng HL, Bunkenborg J, Streeper RS, Mostoslavsky R, Kim J, Yancopoulos G, Valenzuela D, Murphy A et al (2007) Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. *Mol Cell Biol* 27:8807–8814
- Maeda N, Toku S, Kenmochi N, Tanaka T (2006) A novel nucleolar protein interacts with ribosomal protein S19. *Biochem Biophys Res Commun* 339:41–46
- Mattagajasingh I, Kim CS, Naqvi A, Yamamori T, Hoffman TA, Jung SB, DeRicco J, Kasuno K, Irani K (2007) SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 104:14855–14860
- Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I (2005) Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol Biol Cell* 16:4623–4635

- Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB et al (2007) Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* 450:712–716
- Mostoslavsky R, Chua KF, Lombard DB, Pang WW, Fischer MR, Gellon L, Liu P, Mostoslavsky G, Franco S, Murphy MM et al (2006) Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* 124:315–329
- Musgrove EA (2006) Cyclins: roles in mitogenic signaling and oncogenic transformation. *Growth Factors* 24:13–19
- Nakae J, Kitamura T, Kitamura Y, Biggs WH 3rd, Arden KC, Accili D (2003) The forkhead transcription factor Foxo1 regulates adipocyte differentiation. *Dev Cell* 4:119–129
- Nakagawa T, Lomb DJ, Haigis MC, Guarente L (2009) SIRT5 Deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. *Cell* 137:560–570
- Nasrin N, Kaushik VK, Fortier E, Wall D, Pearson KJ, de Cabo R, Bordone L (2009) JNK1 phosphorylates SIRT1 and promotes its enzymatic activity. *PLoS One* 4:e8414
- Nemoto S, Fergusson MM, Finkel T (2004) Nutrient availability regulates SIRT1 through a forkhead-dependent pathway. *Science* 306:2105–2108
- Nigg EA (2001) Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol* 2:21–32
- North BJ, Rosenberg MA, Jeganathan KB, Hafner AV, Michan S, Dai J, Baker DJ, Cen Y, Wu LE, Sauve AA et al (2014) SIRT2 induces the checkpoint kinase BubR1 to increase lifespan. *Embo J* 33(13):1438–1453
- Pandithage R, Lilischkis R, Harting K, Wolf A, Jedamzik B, Lüscher-Firzlaff J, Vervoorts J, Lasonder E, Kremmer E, Knöll B et al (2008) The regulation of SIRT2 function by cyclin-dependent kinases affects cell motility. *J Cell Biol* 180:915–929
- Pedersen SB, Olholm J, Paulsen SK, Bennetzen MF, Richelsen B (2008) Low Sirt1 expression, which is upregulated by fasting, in human adipose tissue from obese women. *Int J Obes* 32:1250–1255
- Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, Machado De Oliveira R, Leid M, McBurney MW, Guarente L (2004) Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature* 429:771–776
- Radak Z, Bori Z, Koltai E, Fatouros IG, Jamurtas AZ, Douroudos II, Terzis G, Nikolaidis MG, Chatziz Nikolaou A, Sovatzidis A et al (2011) Age-dependent changes in 8-oxoguanine-DNA glycosylase activity are modulated by adaptive responses to physical exercise in human skeletal muscle. *Free Radic Biol Med* 51:417–423
- Raynes R, Pombier KM, Nguyen K, Brunquell J, Mendez JE, Westerheide SD (2013) The SIRT1 modulators AROS and DBC1 regulate HSF1 activity and the heat shock response. *PLoS One* 8:e54364
- Revollo JR, Grimm AA, Imai S (2004) The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *J Biol Chem* 279:50754–50763
- Rongvaux A, Galli M, Denanglaire S, Van Gool F, Dreze PL, Szpirer C, Bureau F, Andris F, Leo O (2008) Nicotinamide phosphoribosyl transferase/pre-B cell colony-enhancing factor/visfatin is required for lymphocyte development and cellular resistance to genotoxic stress. *J Immunol* 181:4685–4695
- Sasaki T, Maier B, Koclega KD, Chruszcz M, Gluba W, Stukenberg PT, Minor W, Scrabble H (2008) Phosphorylation regulates SIRT1 function. *PLoS One* 3:e4020
- Satoh A, Stein L, Imai S (2011) The role of mammalian sirtuins in the regulation of metabolism, aging, and longevity. *Handb Exp Pharmacol* 206:125–162
- Sauve AA (2012) Sirtuin chemical mechanisms. *Biochim Biophys Acta* 1804:1591–1603
- Sauve AA, Munshi C, Lee HC, Schramm VL (1998) The reaction mechanism for CD38. A single intermediate is responsible for cyclization, hydrolysis, and base-exchange chemistries. *Biochemistry* 37:13239–13249

- Sauve AA, Celic I, Avalos J, Deng H, Boeke JD, Schramm VL (2001) Chemistry of gene silencing: the mechanism of NAD<sup>+</sup>-dependent deacetylation reactions. *Biochemistry* 40:15456–15463
- Sauve AA, Wolberger C, Schramm VL, Boeke JD (2006) The biochemistry of sirtuins. *Annu Rev Biochem* 75:435–465
- Schlicker C, Gertz M, Papatheodorou P, Kachholz B, Becker CF, Steegborn C (2008) Substrates and regulation mechanisms for the human mitochondrial sirtuins Sirt3 and Sirt5. *J Mol Biol* 382:790–801
- Schug TT, Li X (2011) Sirtuin 1 in lipid metabolism and obesity. *Ann Med* 43:198–211
- Schwer B, Bunkenborg J, Verdin RO, Andersen JS, Verdin E (2006) Reversible lysine acetylation controls the activity of the mitochondrial enzyme acetyl-CoA synthetase 2. *Proc Natl Acad Sci U S A* 103:10224–10229
- Schwer B, Eckersdorff M, Li Y, Silva JC, Fermin D, Kurtev MV, Giallourakis C, Comb MJ, Alt FW, Lombard DB (2009) Calorie restriction alters mitochondrial protein acetylation. *Aging Cell* 8:604–606
- Shi T, Wang F, Stieren E, Tong Q (2005) SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. *J Biol Chem* 280:13560–13567
- Sims JL, Berger SJ, Berger NA (1981) Effects of nicotinamide on NAD and poly(ADP-ribose) metabolism in DNA-damaged human lymphocytes. *J Supramol Struct Cell Biochem* 16:281–288
- Sundararajan R, Chen G, Mukherjee C, White E (2005) Caspase-dependent processing activates the proapoptotic activity of deleted in breast cancer-1 during tumor necrosis factor- $\alpha$ -mediated death signaling. *Oncogene* 24:4908–4920
- Tan M, Peng C, Anderson KA, Chhoy P, Xie Z, Dai L, Park J, Chen Y, Huang H, Zhang Y et al (2014) Lysine glutarylation is a protein posttranslational modification regulated by SIRT5. *Cell Metab* 19:605–617
- Tanno M, Sakamoto J, Miura T, Shimamoto K, Horio Y (2007) Nucleocytoplasmic Shuttling of the NAD<sup>+</sup>-dependent Histone Deacetylase SIRT1. *J Biol Chem* 282:6823–6832
- Tissenbaum HA, Guarente L (2001) Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature* 410:227–230
- Vaquero A, Scher M, Lee D, Erdjument-Bromage H, Tempst P, Reinberg D (2004) Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin. *Mol Cell* 16:93–105
- Vaquero A, Scher MB, Lee DH, Sutton A, Cheng HL, Alt FW, Serrano L, Sternglanz R, Reinberg D (2006) SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. *Genes Dev* 20:1256–1261
- Vassilopoulos A, Fritz KS, Petersen DR, Gius D (2011) The human sirtuin family: evolutionary divergences and functions. *Hum Genomics* 5:485–496
- Wang C, Chen L, Hou X, Li Z, Kabra N, Ma Y, Nemoto S, Finkel T, Gu W, Cress WD et al (2006) Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. *Nat Cell Biol* 8:1025–1031
- Wang F, Nguyen M, Qin FX, Tong Q (2007) SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. *Aging Cell* 6:505–514
- Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, Sinclair D (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* 430:686–689
- Yamane K, Kinsella TJ (2005) CK2 Inhibits apoptosis and changes its cellular localization following ionizing radiation. *Cancer Res* 65:4362–4367
- Yang H, Yang T, Baur JA, Perez E, Matsui T, Carmona JJ, Lamming DW, Souza-Pinto NC, Bohr VA, Rosenzweig A et al (2007a) Nutrient-sensitive mitochondrial NAD<sup>+</sup> levels dictate cell survival. *Cell* 130:1095–1107

- Yang Y, Fu W, Chen J, Olashaw N, Zhang X, Nicosia SV, Bhalla K, Bai W (2007b) SIRT1 sumoylation regulates its deacetylase activity and cellular response to genotoxic stress. *Nat Cell Biol* 9:1253–1262
- Yelamos J, Schreiber V, Dantzer F (2008) Toward specific functions of poly(ADP-ribose) polymerase-2. *Trends Mol Med* 14:169–178
- Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW (2004) Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *Embo J* 23:2369–2380
- Yoshino J, Mills KF, Yoon MJ, Imai S (2011) Nicotinamide mononucleotide, a Key NAD(+) intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. *Cell Metab* 14:528–536
- Yu J, Sadhukhan S, Noriega LG, Moullan N, He B, Weiss RS, Lin H, Schoonjans K, Auwerx J (2013) Metabolic characterization of a Sirt5 deficient mouse model. *Sci Rep* 3:2806
- Zhang Q, Wang SY, Fleuruel C, Leprince D, Rocheleau JV, Piston DW, Goodman RH (2007) Metabolic regulation of SIRT1 transcription via a HIC1:CtBP corepressor complex. *Proc Natl Acad Sci U S A* 104:829–833
- Zhang P, Tu B, Wang H, Cao Z, Tang M, Zhang C, Gu B, Li Z, Wang L, Yang Y et al (2014) Tumor suppressor p53 cooperates with SIRT6 to regulate gluconeogenesis by promoting FoxO1 nuclear exclusion. *Proc Natl Acad Sci* 111:10684–10689
- Zhong L, D'Urso A, Toiber D, Sebastian C, Henry RE, Vadysirisack DD, Guimaraes A, Marinelli B, Wikstrom JD, Nir T et al (2010) The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell* 140:280–293
- Zhou Y, Zhang H, He B, Du J, Lin H, Cerione RA, Hao Q (2012) The bicyclic intermediate structure provides insights into the desuccinylation mechanism of human sirtuin 5 (SIRT5). *J Biol Chem* 287:28307–28314