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Tso-Pang Yao
Edward Seto *Editors*

Histone Deacetylases: the Biology and Clinical Implication

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Histone Deacetylases: the Biology and Clinical Implication

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Preface

The discovery of histone deacetylase (HDAC) enzymatic activity was first reported in the late 1960s and, despite early prediction of the potential biological importance, histone deacetylation research was dormant for a long time. It took another 30 years before the first *bona fide* histone deacetylase, HDAC1, was purified and cloned in 1996, spurring an explosion of the HDAC field.

In 2006, a book entitled "Histone Deacetylases: Transcriptional Regulation and Other Cellular Functions" provided an excellent survey of work accomplished in the first 10 years (Verdin 2006). Since 2006, over 7,000 papers have been published on HDACs, affirming an exponential growth of interest in this topic. The purpose of this current book on HDACs is to serve as an update on the progress in this field within a relatively short 5 years. Particular emphasis is placed on discussions of many of the previously unexpected biological functions of HDACs in health and diseases and the tremendous progress in understanding the potential usefulness of HDAC inhibitors in the treatment of diseases.

The book highlights work from many different labs that taught us abnormal HDACs potentially contribute to the development or progression of many human diseases including immune dysfunctions, heart disease, cancer, memory impairment, aging, and metabolic disorders. We hope this book will serve as a reference to catalyze new collaborations between experts within the HDAC field. Equally important, we also hope that this book will entice many who are not working in this area to contribute to the ever-growing HDAC field.

We thank each of the authors for their contributions, and we look forward to continuing interesting discoveries on HDACs for many more years to come.

Durham, NC — Tso-Pang Yao
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K-Acetylation and Its Enzymes: Overview and New Developments

Juliette Adjo Aka, Go-Woon Kim, and Xiang-Jiao Yang

Contents

Abstract Lysine (K) acetylation refers to transfer of the acetyl moiety from acetyl-CoA to the ε-amino group of a lysine residue. This is posttranslational and reversible, with its level dynamically maintained by lysine acetyltransferases (KATs) and deacetylases (KDACs). Traditionally, eukaryotic KDACs have been referred to as HDACs (*h*istone *deac*etylases). Recent proteomic studies have revealed that hundreds of bacterial proteins and thousands of eukaryotic proteins contain acetyllysine (AcK) residues, indicating that K-acetylomes are comparable to phosphoproteomes. The current challenges are to assign enzymes that execute specific acetylation events, to determine the impact of these events, and to relate this modification to other posttranslational modifications, cell signaling networks, and pathophysiology under different cellular and developmental contexts. In this chapter, we provide a brief overview about the acetylomes, KATs, HDACs,

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AcK-recognizing protein domains, and acetylation-modulating therapeutics, and emphasize the latest developments in related areas. The remaining chapters of the book focus on and cover various aspects of HDACs (both the Rpd3/Hda1 and sirtuin families), which shall provide novel insights into how to utilize these enzymes for developing a new generation of HDAC-related therapeutics.

Keywords Bromodomain • HAT • HDAC • Lysine acetylation • Lysine acetyltransferase • Lysine deacetylase • Post-translational modification

1 K-Acetylomes Emerging from Bacteria to Humans

Lysine acetylation yields AcK residues (Fig. 1a). Such modified residues were first identified in histone proteins in the late 1960s (Gershey et al. 1968). Over a decade later, AcK residues were also found to be present in nonhistone proteins, including HMG (*h*igh-*m*obility *g*roup) proteins (Sterner et al. 1979), α-tubulin (Piperno and

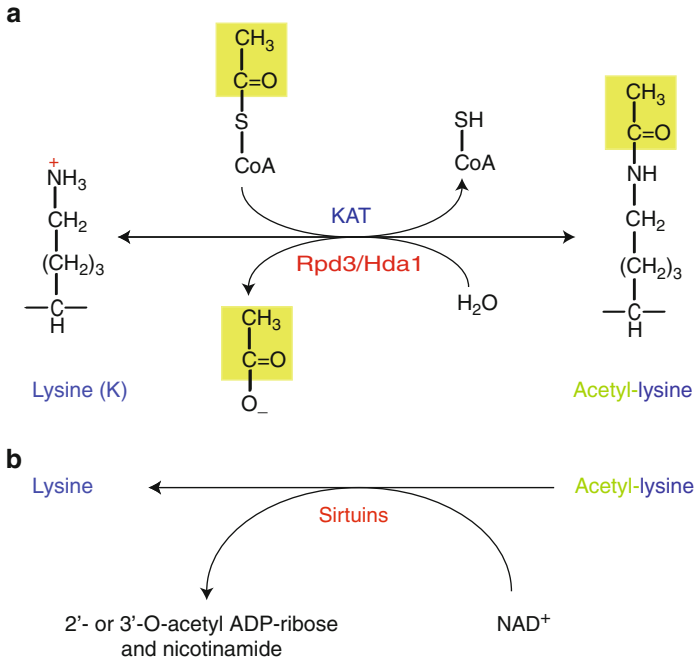

Fig. 1 Cartoon illustration of acetylation and deacetylation at a lysine residue. (**a**) A KAT catalyzes the transfer of the acetyl moiety (*in green*) from acetyl-CoA to the ε-group of a lysine residue, whereas an Rpd3/Hda1 family member removes the acetyl group from an acetyl lysine residue, releasing acetate. The enzymatic activity of the Rpd3/Hda1 family members is Zn^{2+} dependent. (**b**) Deacetylation by sirtuins. Different from the Rpd3/Hda1 family of deacetylases, sirtuins are NAD^+-dependent enzymes and use a catalytic mechanism that is completely different from that employed by the Rpd3/Hda1 family of deacetylases. Adapted from (Kim and Yang 2011)

Fuller 1985), and the bacterial chemotaxis regulator CheY (Barak et al. 1992; Ramakrishnan et al. 1998). In the mid-1990s, the initial KATs were identified and the subsequent candidate-based approach revealed AcKs in over a hundred proteins, many of which are transcription factors (Kouzarides 2000; Yang and Seto 2008a). In the past few years, proteomic studies have identified AcKs in thousands of eukaryotic proteins (Kim et al. 2006; Choudhary et al. 2009; Zhao et al. 2010) and hundreds of bacterial proteins (Yu et al. 2008; Zhang et al. 2009; Wang et al. 2010). It is clear now that K-acetylomes are comparable to phosphoproteomes from bacteria to humans. These proteomic studies reiterate that eukaryotic K-acetylomes are not only vital for regulating chromatin-templated nuclear processes but also play important roles in various cytoplasmic processes, including energy metabolism in mitochondria. In this way, K-acetylation has emerged as a key posttranslational modification with importance potentially comparable to other major posttranslational modifications such as phosphorylation and ubiquitination (Norris et al. 2009). In addition to characterization of individual acetylation events per se, it will be important to investigate how K-acetylation crosstalks with other posttranslational modifications and regulates cellular processes in a fashion coordinated according to spatiotemporal cues.

2 Three Superfamilies of KATs

K-acetylation is reversible and its level is dynamically controlled by KATs and KDACs (Fig. 1). Since the identification of the very first KATs in the mid-1990s, various mammalian proteins have been discovered to possess such an enzymatic activity (Sterner and Berger 2000; Allis et al. 2007; Lee and Workman 2007). These proteins belong to different families, with the best-characterized ones being the GNAT (*G*cn5-related *N*-*a*cetyl*t*ransferases) superfamily, the MYST (*M*OZ, *Y*bf2/Sas3, *S*as2, and *T*ip60) family, the p300 (E1A-associated *p*rotein of 300 kDa)/CBP (*C*REB-*b*inding *p*rotein) family, and the fungal-specific Rtt109 (*r*egulator of *t*y1 *t*ransposition 109) family. A comprehensive list of KATs has been compiled and a systematic nomenclature system has been proposed for these enzymes (Allis et al. 2007), but recent additions such as ATAC2 (*A*DA2A-con*ta*ining *c*omplex subunit 2) (Suganuma et al. 2008) and MEC17 (*mec*hanosensory abnormal 17) (Akella et al. 2010) will need to be incorporated into the system. p300 and CBP were among the first KATs identified in 1996 (Bannister and Kouzarides 1996; Ogryzko et al. 1996). They do not share any sequence similarity to the GNAT and MYST families, and are conserved from the worm to humans (Goodman and Smolik 2000). Rtt109 is fungi specific and is a structural homolog of p300 and CBP (Tang et al. 2008), suggesting that these KATs can be conceptually grouped into one superfamily.

The GNAT (*G*cn5-related *N*-*a*cetyl*t*ransferases) superfamily forms the largest group of KATs (Roth et al. 2001). Family members share several blocks of sequence motifs, one of which is essential for acetyl-CoA binding. Some of the prominent and well-characterized eukaryotic members include HAT1 (*h*istone *a*cetyl*t*ransferase 1), GCN5 (*g*eneral *c*ontrol *n*onderepressible 5), PCAF (*p*300/*C*BP-*a*ssociated *f*actor,

paralog of GCN5), ELP3 (*el*ongation *p*rotein 3), CDY (*c*hromo*d*omain on chromsome *Y*) proteins, Eco1 (*e*stablishment of *co*hesion 1), ESCO1 (*es*tablishment of *co*hesion 1 homolog 1), ESCO2 (paralog of ESCO1), ATAC2, and MEC17 (see below). Moreover, this superfamily contains one bacterial KAT called PAT, thereby widening its range from bacteria to humans (Starai and Escalante-Semerena 2004).

Several recent developments about the GNAT family are noteworthy here. First, ATAC2 was identified as a subunit of a multiprotein Gcn5 complex conserved from fly to humans and plays a role in MAP kinase signaling and mitotic progression (Suganuma et al. 2008, 2010; Wang et al. 2008; Guelman et al. 2009; Orpinell et al. 2010). Second, yeast Eco1 and its mammalian orthologs ESCO1 and ESCO2 play key roles in regulating sister chromatid cohesion (Kim and Yang 2011). Third, two recent reports demonstrated that MEC17 proteins from *C. elegans* and mammals efficiently acetylate α-tubulin at K40 in vitro and in vivo, establishing them as tubulin acetyltransferases (Akella et al. 2010; Shida et al. 2010). Related to this, another report has demonstrated that San acetylates β-tubulin at K252 (Chu et al. 2011). Fourth, in addition to its acetyltransferase domain, ELP3 contains a catalytic domain with a potential role in oxidation, but the function remains to be explored further (Greenwood et al. 2009). Finally, CDY proteins contain a chromodomain and a crotonase-like domain that possesses acetyltransferase activity in vitro (Wu et al. 2009), so it will be interesting to investigate the biological relevance.

The essential acetyl-CoA binding motif conserved in the GNAT superfamily is also present in the MYST family (Lafon et al. 2007). Like the GNAT family, the MYST family has members in all eukaryotes. In humans, there are five members, hMOF (human ortholog of fly mof, MOF, for *m*ales absent *o*n the *f*irst; also known as MYST1), TIP60 (HIV Tat-interactive protein of 60 kDa), HBO1 (HAT bound to Orc1, also named MYST2), MOZ (*mo*nocytic leukemic *z*inc finger protein, or MYST3), and MORF (*MOZ-r*elated *f*actor, or MYST4) (Avvakumov and Côté 2007). Among these, TIP60 is part of a large complex containing over ten proteins, whereas HBO1, MOZ, and MORF are catalytic subunits of tetrameric complexes (Doyon et al. 2006; Avvakumov and Côté 2007; Ullah et al. 2008). Interestingly, subunits of these tetrameric complexes share sequence similarity not only to each other but also to a tetrameric core complex of TIP60 (Avvakumov and Côté 2007). Such similarity is also conserved in yeast MYST proteins (Lafon et al. 2007). By contrast, this similarity is not found in the two complexes containing Mof proteins (Li et al. 2009; Cai et al. 2010; Prestel et al. 2010; Raja et al. 2010), indicating that among members of the MYST family, Mof proteins are different from other members in terms of multisubunit complex formation.

3 Two Superfamilies of HDACs

The very first few HDACs were identified in 1996 (Grozinger and Schreiber 2002). Since then, 18 human proteins have been established as HDACs. As listed in Table 1, known mammalian HDACs are divided into two families based on

sequence similarity to yeast orthologs (Khochbin et al. 2001; Grozinger and Schreiber 2002). The first 11 members belong to the Rpd3/Hda1 (*r*educed *p*otassium *d*ependency *3*/*h*istone *dea*cetylase *1*) family, and the remaining seven are classified as members of sirtuin (*Sir2*-related prote*in*) family (Sauve 2010; Verdin et al. 2010). These two families utilize different catalytic mechanisms: while the Rpd3/Hda1 family members are Zn^{2+}-dependent enzymes (Fig. 1a), sirtuins require NAD^+ as a cofactor (Fig. 1b). Dependent on how similar they are towards yeast Rpd3 and Hda1, members of the Rpd3/Hda1 family have been further grouped into three classes, I, II, and IV, leaving sirtuins also known as class III members. Historically, the 18 deacetylases were initially grouped into three classes, I, II, and III, with HDAC11 being considered a class I member. However, more thorough sequence analysis revealed ambiguity of HDAC11 between classes I and II, so a new class (IV) was created for HDAC11 and its orthologs (Gregoretti et al. 2004).

Due to historical traditional reasons, the acronym "HDAC" has been kept for mammalian members of the Rpd3/Hda1 family even though HDAC6 mainly acts in the cytoplasm to deacetylate nonhistone proteins such as α-tubulin (Hubbert et al. 2002). In addition to HDAC6, other HDACs have unique subcellular localization (Table 1). Class IIa HDACs localize to both the nucleus and cytoplasm, and their

Table 1 Characteristics of 18 human HDACs

Family	Class	Yeast homology	Member	Size (aa)	Catalytic sites	Subcellular localization
Rpd3/Hda1	I	Rpd3-like	HDAC1	483	1	Nuclear
			HDAC2	488	1	Nuclear
			HDAC3	428	1	Cytoplasmic/nuclear
			HDAC8	377	1	Nuclear
	IIa	Hda1-like	HDAC4	1,084	1	Cytoplasmic/nuclear
			HDAC5	1,122	1	Cytoplasmic/nuclear
			HDAC7	991	1	Cytoplasmic/nuclear
			HDAC9	1,069	1	Cytoplasmic/nuclear
	IIb	Hda1-like	HDAC6	1,215	2	Cytoplasmic
			HDAC10	669	1	Cytoplasmic/nuclear
	IV	Rpd3- or Hda1-like	HDAC11	347	1	Cytoplasmic/nuclear
Sirtuin	III	Sir2-like	SIRT1	747	1	Nuclear
			SIRT2	389	1	Nuclear/cytoplasmic
			SIRT3	399	1	Mitochondrial/ nuclear
			SIRT4	314	1	Mitochondrial
			SIRT5	310	1	Mitochondrial
			SIRT6	355	1	Nuclear
			SIRT7	400	1	Nucleolar

For additional information, refer to Grozinger and Schreiber (2002), Michishita et al. (2005), Scher et al. (2007), Yang and Seto (2008b), Verdin et al. (2010). Alternative splicing often generates multiple isoforms, so in majority of cases the longest isoforms are listed here.

cytoplasmic localization functions as an important means of regulation in response to cellular signaling. Sirtuins members, SIRT3, 4, and 5, localize to the mitochondria, whereas SIRT7 is present in nucleoli (Michishita et al. 2005). Consistent with this, the former three sirtuins play a role in mitochondrial metabolism, and while SIRT7 is known to regulate RNA polymerase I-dependent transcription (Verdin et al. 2010). Within the nucleus, HDAC1, 2, and 3 are catalytic subunits of stable multiprotein complexes, and some of the noncatalytic subunits serve to regulate the deacetylase activity (Grozinger and Schreiber 2002; Yang and Seto 2008b). This is also a common theme for many chromatin-modifying enzymes, such as some of the aforementioned acetyltransferases (e.g., HAT1, GCN5, ATAC2, and MYST family members) (Table 1).

4 Bromodomains, PHD Fingers, and Potentially Others for AcK Recognition

Acetylation affects protein function through various mechanisms, with one of them being recognition by protein domains such as the bromodomain. It has been well established that the bromodomain of GCN5, TAF250, or p300 forms an α-helix bundle containing a specific pocket for recognition of a specific AcK residue (Sanchez and Zhou 2009). The binding affinity is relatively weak but provides an opportunity to cooperate with other protein modules. For example, TRIM24 (tripartite motif-containing 24, also known as TIF1α) possesses a bromodomain adjacent to a PHD finger for combinatorial recognition of histone marks (Tsai et al. 2010). While its PHD finger binds to the unmodified N-terminal tail of histone H3, its bromodomain recognizes AcK23 of histone H3, with acetylation increasing the affinity by about 23-fold and the K_d value dropping from 2.3 to 0.1 μM. TRIM24 is a biomarker whose overexpression is associated with breast cancer. In addition to the classical one-to-one interaction mode, a recent study has shown that one bromodomain of BRDT (*br*omo*d*omain-containing *t*estis specific) recognizes histone H4 diacetylated at K5 and K8 (Moriniere et al. 2009), indicating that one bromodomain can recognize two AcK residues. In addition to bromodomains, PHD fingers also possess the ability of AcK recognition. DPF3b (*D*4, zinc and double *P*HD *f*ingers, family 3b) contains tandem PHD fingers (Zeng et al. 2010). The second PHD finger interacts an unmodified histone H3 tail through R2, K4, and K9, whereas the first finger forms a binding pocket for AcK14 and stimulates the affinity for histone H3 by threefold.

The bromodomain and PHD finger proteins are mainly nuclear, so an interesting issue is whether AcK-binding domains are also present in the cytoplasm. This possibility has been raised by others (Norris et al. 2009) and will be a new and potentially exciting avenue for further research.

5 Modulation of K-Acetylation for Drug Development

Owing to their biological significance, KATs and HDACs have been extensively studied as drug targets for treatment of cancer and other diseases, although HDACs have been much more vigorously pursued than KATs. This is in part due to the fact that the first specific inhibitor trichostatin A was found to inhibit both cell proliferation and HDAC activity (Yoshida et al. 1990). Various other compounds modulating HDAC activity have been found subsequently. The potential therapeutic value of several HDAC inhibitors for cancer patients has been evaluated, with some others currently being evaluated in clinical trials with very promising outcome (http://clinicaltrials.gov/) (Mottet and Castronovo 2008; Fischer et al. 2010). The compound suberoxylanilide hydroxamic acid (SAHA), the first HDAC inhibitor available for patients, has been approved by the Food and Drug Administration (FDA) in USA and is now used for the treatment of cutaneous T-cell lymphoma (http://clinicaltrials.gov/) (Mottet and Castronovo 2008; Fischer et al. 2010). HDAC inhibitors induce their effect though the activation or repression of genes following hyperacetylation of histone or nonhistone proteins that are related to acetylation. Their antitumor effects were exerted via the modulation of the expression of genes involved in cancer cell migration, invasion, and metastasis (Mottet and Castronovo 2008). Most of the known HDACi act mainly on the Rpd3/Hda1 HDAC family and can be structurally grouped into four classes. The largest is the hydroxymate class, which includes TSA and SAHA. In addition to cancer treatment, HDACi are also used and/or being studied for the treatment of several other diseases including cognitive and cardiac disorders.

KATs have also been drug targets to develop small molecule inhibitors. One surprising finding was that curcumin, a plant polyphenol used in traditional Indian and Chinese medicine as a therapeutic agent or in cuisines as a dietary spice and/or coloring agent, is a specific inhibitor of p300, CBP, and PCAF (Kang et al. 2006). With curcumin used as the initial lead compound, a water-soluble inhibitor known as CTK7A was developed (Arif et al. 2010). Importantly, CTK7A was shown to inhibit tumor growth in nude mice, suggesting the utility of KAT inhibition as an anticancer approach. In addition, virtual ligand screening has identified three potent inhibitors of p300 at a K_i value ranging from 0.4 to 4.8 μM. All three consist of a linear arrangement of three or four aromatic rings terminating in a benzoic acid (Bowers et al. 2010). Among them, C646 has the lowest K_i of 0.4 μM and is also the most specific to p300. At 10–20 μM, C646 induces hypoacetylation in cultured cells, indicating its potential value as a pharmacologic probe. In addition to p300 and CBP, fungal-specific Rtt109 has been used to develop drugs (Lopes da Rosa et al. 2010; Wurtele et al. 2010). In this case, antifungal agents were developed. This is significant as few effective antifungi means are currently available.

In addition to the AcK enzymatic machinery, AcK-recognizing modules such as bromodomains and PHD fingers can be valuable drug targets (Sanchez and Zhou 2009). Related to this, two recent studies developed small molecule inhibitors targeting the bromodomains (Filippakopoulos et al. 2010). One such study utilized

structure-based rational design and developed a cell-permeable small molecule, JQ1, targeting the bromodomains of BRD4. The *BRD4* gene is rearranged in a recurrent t(15;19) chromosomal translocation associated with an aggressive form of human squamous carcinoma (referred to as NUT midline carcinoma) as such translocations express the tandem N-terminal bromodomains of BRD4 as an in-frame fusion protein with NUT (nuclear protein in testis). JQ1 treatment induces differentiation and growth arrest of NUT midline carcinoma cells and exhibits antitumor activity in related xenograft models. Through gene expression-based screened and subsequent affinity purification, the other study identified I-BET, a selective antagonist against the bromodomains of BET proteins (Nicodeme et al. 2010). This antagonist suppresses inflammation-dependent gene expression in both cell-based and mouse-based models.

6 K-Acetylation Modulation for Cell Reprogramming and Regenerative Medicine

Generation of iPS (*i*nduced *p*luripotent *s*tem) cells by ectopic expression of four transcription factors, Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka 2006), has been rapidly adopted to generate such cells from various cell types in mice, humans, and other mammals (Jaenisch and Young 2008; Stadtfeld and Hochedlinger 2010; Yamanaka and Blau 2010). Because of its great promise and potential for in vitro disease modeling and autologous stem cell therapy, this has generated lots of excitement. One challenge is to increase the efficiency of iPS cell generation. In this regard, HDAC inhibitors have been found to promote iPS clone formation. Among the tested inhibitors, valproic acid (VPA), suberoylanilide hydroxamic acid (SAHA), and trichostatin A (TSA), VPA was shown to be the most potent that could increase iPS clone production by 100-fold (Huangfu et al. 2008a). As a result, VPA enables reprogramming of primary human fibroblasts with only *Oct4* and *Sox2* (Huangfu et al. 2008b). In addition, VPA was found to synergize with vitamin C in promoting iPS clone production (Shi et al. 2010). Interestingly, VPA has also been used in a protein-based reprogramming protocol (Zhou et al. 2009). In addition, butyrate was found to stimulate programming dramatically (Mali et al. 2010). As VPA and butyrate are less specific inhibitors than TSA, it is unclear whether they have activity other than HDAC inhibition accounting for the high efficiency. On the other hand, it is reasonable to assume that HDAC inhibition promotes histone hyperacetylation and opens up chromatin for cell reprogramming.

In addition to iPS clone production, a recent study has demonstrated that HDAC inhibition stimulates direction conversion of germ cells to neurons in *C. elegans* (Tursun et al. 2011), suggesting the utility of HDAC inhibitors in promoting transdifferentiation, another type of cell reprogramming that is also important for regenerative medicine.

7 Conclusion

K-acetylation has emerged as a fundamental regulatory mechanism that occurs in diverse organisms from bacteria to humans. The newly revealed K-acetylomes have provided ample opportunities to elucidate different roles that this modification plays in various cellular processes. The acetyltransferases, deacetylases, and AcK-recognizing protein modules play important roles in these processes and are intimately linked to human health and diseases. As a result, these proteins are important drug targets for developing novel therapeutics, and AcK modulation is also an effective means to improve the generation of cell reprogramming protocols important in individualized medicine.

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The Biology of HDAC in Cancer: The Nuclear and Epigenetic Components

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Contents

Abstract Traditionally, cancer has been regarded to originate from genetic alterations such as mutations, deletions, rearrangements as well as gene amplifications, leading to abnormal expression of tumor suppressor genes and oncogenes. An increasing body of evidence indicates that in addition to changes in DNA sequence, epigenetic alterations contribute to cancer initiation and progression. In contrast to genetic mutations, epigenetic changes are reversible and therefore an attractive target for cancer therapy. Many epi-drugs such as histone deacetylase (HDAC) inhibitors showed anticancer activity in cell culture and animal models of carcinogenesis.

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Recently, the two HDAC inhibitors suberoylanilide hydroxamic acid (SAHA, Vorinostat) and Romidepsin (Depsipeptide, FK228) were FDA approved for the treatment of cutaneous T-cell lymphoma (CTCL). Although HDAC inhibitors are potent anticancer agents, these compounds act against several HDAC family members potentially resulting in numerous side effects. This stems from the fact that HDACs play crucial roles in a variety of biological processes including cell cycle progression, proliferation, differentiation, and development. Consistently, mice deficient in single HDACs mostly exhibit severe phenotypes. Therefore, it is necessary to specify the cancer-relevant HDACs in a given tumor type in order to design selective inhibitors that target only cancer cells without affecting normal cells. In this chapter, we summarize the current state of knowledge of individual nuclear HDAC family members in development and tumorigenesis, their contribution to the hallmarks of cancer, and the involvement of HDAC family members in different types of human malignancies.

Keywords Epigenetic therapy • Hallmarks of cancer • Malignancies • Tumorigenesis

1 The Role of HDAC Family Members in Development and Cancer

To understand the role of individual HDACs in tumorigenesis it is of utmost importance to clarify the function of HDACs in proliferation, differentiation, and development by loss-of-function studies in animal models.

Since HDACs control many essential mechanisms during development and tissue maintenance, it is not surprising that deletion of individual HDACs leads to severe phenotypes in mice. Knockouts of HDAC1, 3, and 7 result in embryonic lethality due to impaired cell cycle (HDAC1 and 3) and endothelial dysfunction (HDAC7) (Bhaskara et al. 2008; Chang et al. 2006; Lagger et al. 2002; Montgomery et al. 2008). Mice lacking HDAC2, 4, and 8 exhibit peri/postnatal lethality due to cardiac abnormalities, ectopic cartilage ossification, and skull instability, respectively (Haberland et al. 2009b; Montgomery et al. 2007; Trivedi et al. 2007; Vega et al. 2004). In contrast, mice lacking HDAC5, 6, and 9 are viable and show only minor defects: deletion of HDAC5 and 9 leads to myocardial hypertrophy after stress, and HDAC6-deficient mice are the only HDAC mutant mice without an obvious phenotype (Chang et al. 2004; Zhang et al. 2002, 2008). Similarly to classical HDACs, loss of particular sirtuins results in developmental defects. Depending on the background, mice lacking SIRT1 exhibit embryonic lethality due to the reduced ability to repair DNA damage, die during the early postnatal period, or show sterility in adulthood (Cheng et al. 2003; McBurney et al. 2003; Wang et al. 2008). The absence of SIRT6 leads to numerous developmental defects and eventually death at about 4 weeks of age (Mostoslavsky et al. 2006), whereas SIRT7 deficiency results in cardiac failures and lifespan reduction (Vakhrusheva et al. 2008).

As discussed later, several members of the HDAC family have been shown to be either aberrantly expressed or mistargeted in different tumors and are consequently potential targets for cancer therapy. Fortunately, HDAC inhibitors are relatively well tolerated by patients and do not lead to as dramatic side effects as the phenotypes of HDAC knockout mice would suggest. The contrasting phenotypes between genetic deletion and pharmacological inhibition might be explained by the following facts: (1) While genetic deletion is permanent, HDAC inhibitors act transiently. (2) Ablation of HDACs leads to their outright inactivity, whereas their pharmacological inhibition might be incomplete. (3) As components of multisubunit complexes, certain deacetylases might have also nonenzymatic functions such as stabilization of corepressor complexes. Genetic deletion of HDACs results in their complete absence annihilating both the catalytic activity and their potential scaffolding function. In contrast, most HDAC inhibitors do not affect the assembly and integrity of HDAC-containing multisubunit complexes [reviewed in Haberland et al. (2009c)]. (4) Loss of HDACs can cause different effects in the developing and the adult organism. For instance, combined ablation of HDAC1 and HDAC2 is lethal in early development, whereas deletion in adult, postmitotic cells is well tolerated (Haberland et al. 2009a; Yamaguchi et al. 2010). This is in line with the particular HDAC inhibitor sensitivity of fast cycling cells such as tumor cells.

There are several mechanisms leading to a deregulated HDAC activity observed in many cancer types. HDACs can be mutated, changed in their expression levels, or aberrantly recruited in tumor cells.

The involvement of HDACs in cancer development has been initially demonstrated for hematological malignancies, where aberrant recruitment of HDAC-containing complexes to specific promoters by fusion proteins resulting from chromosomal translocations leads to abnormalities in differentiation and proliferation of myeloid cells (Mercurio et al. 2010; Ropero and Esteller 2007) (see Sects. 2.2 and 3.1). Structural mutations affecting HDAC expression and/or activity appear to be rare in tumors. To date, the only mutation identified in an HDAC gene is a frameshift in the *HDAC2* gene, leading to the loss of HDAC2 protein and activity in human endometrial and colon cell lines (Ropero et al. 2006).

Strikingly, numerous clinical studies in cancer patients have established that the most prevalent alteration of HDAC function in tumors is overexpression. Increased mRNA as well as protein levels for different HDAC family members have been reported for a wide variety of human malignancies (listed in Sects. 3.1–3.7). Although a huge body of evidence indicates a crucial role of deregulated HDAC expression in cancer development, the mechanisms underlying HDAC overexpression in tumors are still poorly understood.

Only recently, deregulated miRNA expression has been attributed to aberrant HDAC expression in tumors. Noonan et al. have identified a miRNA targeting *HDAC1* (miR-449), which induces cell cycle arrest and apoptosis of prostate cancer cells (Noonan et al. 2009). Interestingly, miR-449 is frequently downregulated in prostate cancer. A similar mechanism of regulation has been described for HDAC4

in hepatocellular carcinoma (Zhang et al. 2010), where decreased levels of miR-22, which targets *HDAC4*, have been correlated with worse prognosis.

In contrast to permanent and irreversible cancer-associated genetic abnormalities such as overexpression of oncogenes or mutation of tumor suppressor genes, the elevated levels of HDAC activity can be in principle modulated due to the dynamic nature of histone modifications. Therefore, inhibiting HDAC activity appears to be an attractive approach in cancer therapy. However, due to the delicate balance of histone acetylation – which controls expression of many genes involved in crucial cellular processes – and the fact that HDACs can affect the function of many nonhistone targets, it is difficult to predict the benefits, risks, and potential side effects of HDAC inhibition.

2 The Hallmarks of Cancer: Revisited

One decade ago, Hanahan and Weinberg determined the six most important factors for cancer development and tumor progression (Hanahan and Weinberg 2000). Blocking of apoptosis and differentiation as well as the stimulation of angiogenesis, proliferation, and metastasis are commonly described as hallmarks of cancer and known to be regulated by epigenetic mechanisms including histone acetylation (Fig. 1). During tumorigenesis, the global pattern of histone acetylation is changed. For instance, cancer cells undergo a loss of acetylation at H4K16, suggesting a crucial role of HDAC activity in establishing the tumor phenotype (Fraga et al. 2005). In this chapter, we try to build a bridge between the previously established features of the multistep process of tumorigenesis and the wide-ranging contribution of HDAC family members to these mechanisms.

2.1 Proliferation and Cell Cycle Progression

Several HDACs including HDAC1, 2, 3, 4, and 6 are implicated in cancer cell proliferation and their loss negatively affects proliferation of tumor cells (Glaser et al. 2003; Haberland et al. 2009a; Lee et al. 2008; Mottet et al. 2009; Senese et al. 2007; Wilson et al. 2008). Mouse HDAC1 was originally identified as growth factor regulated gene with high expression in proliferating and transformed cells (Bartl et al. 1997). HDAC1 knockout mice show proliferation defects, increased levels of the cyclin-dependent kinase inhibitors (CKI) $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, and severe developmental abnormalities, which lead to embryonic lethality before E10.5 (Lagger et al. 2002). In accordance with the role of HDAC1 as positive regulator of proliferation by repressing the CKI $p21^{WAF1/CIP1}$, HDAC1-deficient embryonic stem (ES) cells show reduced proliferation rates. Additional deletion of $p21^{WAF1/CIP1}$ rescues the proliferation phenotype in ES cells, but not the embryonic lethality of HDAC1 knockout mice, suggesting that additional genes and

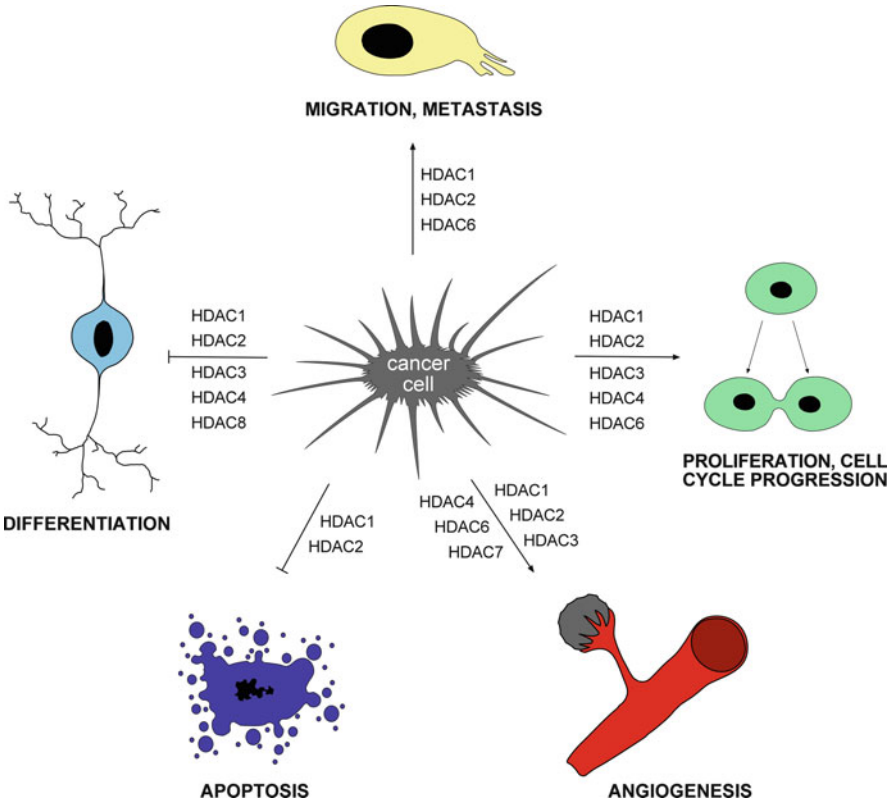

Fig. 1 Histone deacetylases regulate various hallmarks of cancer. By blocking apoptosis and differentiation in addition to inducing proliferation, angiogenesis as well as metastasis, individual HDACs dictate malignant growth

developmental programs are affected in the absence of HDAC1 (Zupkovitz et al. 2010). Accordingly, many genes involved in development and proliferation control have been identified as HDAC1 targets (Zupkovitz et al. 2006). Interestingly, silencing of HDAC1 in ES cells and mouse embryos leads to upregulation of HDAC2, which cannot counterbalance for the loss of HDAC1 during embryonic development.

In contrast to the previously described findings, conditional deletion of HDAC1 in T cells resulted in increased proliferation (Grausenburger et al. 2010) and in a murine teratoma model HDAC1 seemed to be required to attenuate proliferation of epithelial cells (Lagger et al. 2010). This might be explained by the fact that the influence of HDAC1 on proliferation depends on the specific cell type and the corresponding HDAC1 target genes. Furthermore, upregulation of HDAC2 in the absence of HDAC1 might cause additional cell type-specific perturbations of gene expression.

Originating from a gene duplication, the two paralogs HDAC1 and HDAC2 are often found in the same multiprotein complexes and share 82% identity on the amino acid level suggesting a certain redundancy in their functions. However, in the developing mouse embryo HDAC1 and HDAC2 cannot compensate for each other, indicating some nonredundant roles during early development. A remarkable example of specific roles for HDAC1 and HDAC2 is the developing brain, where these two enzymes exhibit different developmental stage- and lineage-specific expression patterns (MacDonald and Roskams 2008). However, mice lacking HDAC1 or HDAC2 in neuronal precursors exhibit no overt neurological phenotype (Montgomery et al. 2009). Also in other differentiated cell types such as fibroblasts, B cells, hematopoietic cells, and cardiomyocytes, HDAC1 and HDAC2 have partially redundant functions, as there are no strong phenotypes for ablation of individual genes observed (Montgomery et al. 2007; Wilting et al. 2010; Yamaguchi et al. 2010). Nevertheless, combined loss of these two class I HDACs leads to dramatic defects in cell cycle progression, survival, and development (Montgomery et al. 2007, 2009; Wilting et al. 2010; Yamaguchi et al. 2010): Fibroblasts lacking both enzymes express increased levels of p21 and p57, display a strong cell cycle block in the G1 phase and fail to proliferate, suggesting a cooperation of HDAC1 and HDAC2 to promote G1-S progression (Yamaguchi et al. 2010). Similarly, lack of HDAC1 and HDAC2 in early B cells inhibits B-cell development and the rare remaining pre-B cells exhibit a block in G1 phase and induction of apoptosis (Yamaguchi et al. 2010). Simultaneous loss of HDAC1 and HDAC2 in the hematopoietic system results in apoptosis of megakaryocytes and thrombocytopenia (Wilting et al. 2010). Knockdown of HDAC1 in a human osteosarcoma cell line leads to $p21^{WAF1/CIP1}$ upregulation and arrest either in G1 phase or at the G2/M transition, resulting in the loss of mitotic cells and an increase in apoptotic cells (Senese et al. 2007). Knockdown of HDAC2 in a human cervical carcinoma cell line results in upregulation of $p21^{WAF1/CIP1}$, increased apoptosis, but no apparent G1 or G2/M cell cycle arrest (Huang et al. 2005). Another study has shown that double deletion of HDAC1/2 in transformed cells leads to nuclear bridging, nuclear fragmentation, and cell death due to mitotic catastrophe (Haberland et al. 2009a). In addition, deletion of HDAC1/2 in tumor cells results in a complete block of tumor growth, when these cells are injected into nude mice, suggesting that pharmacological inhibition of both HDAC1 and HDAC2 might be desirable (Haberland et al. 2009a).

Besides HDAC1 and 2, also HDAC3 has been shown to repress $p21^{WAF1/CIP1}$ in a study using colon cancer cells as silencing of HDAC3 in these cells induced G2/M arrest and apoptosis (Wilson et al. 2006). Consistent with this and other studies in transformed cell lines, cre-recombinase-mediated inactivation of HDAC3 in mouse embryonic fibroblasts (MEFs) causes apoptosis. However, conditional deletion of HDAC3 in primary MEFs does not lead to obvious mitotic defects, but results in delayed S-phase progression and causes DNA damage (Bhaskara et al. 2008).

To sum up, loss of class I HDACs affects the cell cycle in a cell type-specific manner at various stages and can thereby lead to G1 or G2/M cell cycle arrest and a delay in S-phase progression. Likewise, HDAC inhibitors can induce G1

and G2/M cell cycle arrest in normal and transformed cells in a dose- and cell type-dependent manner (Xu et al. 2007).

Furthermore, HDAC4 was shown to affect proliferation and repress $p21^{WAF1/CIP1}$ in human cancer cells. Knockdown of this class II deacetylase stimulates the expression of $p21^{WAF1/CIP1}$ in several human cancer cell lines and inhibits tumor cell proliferation in vitro and tumor growth in vivo (Mottet et al. 2009; Wilson et al. 2008). Depending on the cell type, $p21^{WAF1/CIP1}$ repression might be mediated by HDAC4 associating with the HDAC3-N-CoR/SMRT corepressor complex (Wilson et al. 2008) or an HDAC1-dependent corepressor complex, where HDAC4 might act as a scaffold (Mottet et al. 2009).

Strikingly, in addition to transcriptional regulation of crucial cell cycle regulatory genes, such as $p21^{WAF1/CIP1}$, HDAC activity also affects cell cycle progression via other, less studied, nontranscriptional mechanisms (Li et al. 2006). Since dynamic changes in histone modifications, including acetylation and deacetylation, have been shown to affect chromatin structure during mitosis (Krebs et al. 2000), it is not surprising that inhibition of HDAC activity results in defects in mitotic progression. Several HDAC inhibitors have been demonstrated to induce G2/M arrest in many human cancer cell lines (Cha et al. 2009; Li et al. 2006; Senese et al. 2007; Wilson et al. 2006); however, the mechanism underlying this phenomenon seems to be tumor specific. For instance, in human colon cancer cells, knockdown of HDAC3, but not HDAC1 or HDAC2, resulted in accumulation of cells at G2/M stage (Wilson et al. 2006), whereas in a human osteosarcoma cell line this effect has been observed upon siRNA-mediated depletion of HDAC1 (Senese et al. 2007). The role of HDAC3 in inducing G2/M arrest has been linked to impaired Aurora B activity, since deacetylation of histone H3 by HDAC3 during mitosis is a crucial step for Aurora B-mediated phosphorylation of serine 10 residue at histone H3 (H3S10) (Wilson et al. 2006). Consequently, knockdown of HDAC3 as well as pharmacological inhibition of HDAC3 activity resulted in a decrease in the level of phosphorylated H3S10 and mitotic defects (Wilson et al. 2006). Interestingly, a significant reduction of phosphorylated H3S10 has also been reported for HDAC1-deficient cells (Senese et al. 2007); however, the role of Aurora B in this process has not yet been determined. In addition, G2/M arrest of human renal cancer cells upon pharmacological inhibition of HDAC3 and HDAC6 has been attributed to proteasomal degradation of Aurora A and B (Cha et al. 2009), further supporting the rationale for targeting HDAC activity in cancer therapy.

In addition to classical HDACs also NAD^+-dependent class III HDACs have been implicated in tumor cell proliferation. Several in vitro studies suggested that SIRT1 is a tumor-promoting gene and SIRT1 knockdown as well as inhibitor treatment were shown to stimulate growth arrest and apoptosis in several tumor cell lines including breast, lung, and colon cancer cells (Ford et al. 2005; Heltweg et al. 2006; Lain et al. 2008; Ota et al. 2006). Conversely, recent mouse studies revealed a potential role for SIRT1 as a tumor suppressor: In an $APC^{min/+}$ model of colon cancer, calorie restriction-mediated SIRT1 overexpression and ectopic induction of SIRT1 reduces colon cancer cell growth by suppressing β-catenin-driven cell proliferation (Firestein et al. 2008). Furthermore, SIRT1+/− p53+/− mice tend

to develop more spontaneous tumors in multiple tissues than p53+/− mice, suggesting that an appropriate dose of SIRT1 is crucial for preventing tumorigenesis (Wang et al. 2008). A recent study elucidated one mechanism of SIRT1-mediated tumor suppression and revealed a c-Myc-SIRT1 feedback loop regulating cell growth and transformation (Yuan et al. 2009). The proto-oncogenic transcription factor c-Myc induces the expression of SIRT1, which in turn deacetylates c-Myc. This leads to a decreased c-Myc stability and consequently prevents cellular transformation.

2.2 Differentiation

During cell differentiation, certain genes have to be activated, while others have to be silenced and correct cell lineage decisions have to be made. The establishment of a specific gene expression pattern is coordinated by epigenetic mechanisms including histone acetylation.

HDACs regulate genes involved in the differentiation process in various tissues and their inhibition has been shown to modulate neuronal, epithelial, adipocyte, osteogenic, muscle cell, and hematopoietic differentiation (Cho et al. 2005; Hsieh et al. 2004; Leder et al. 1975; Mal et al. 2001; Tou et al. 2004; Yoo et al. 2006). In most of the systems, HDAC inhibitors induce terminal differentiation. Also the antitumorigenic potential of HDAC inhibitors was first attributed to their ability to induce differentiation of erythroleukemia cells (Leder et al. 1975; Richon et al. 1998; Tanaka et al. 1975). In cancer, the balance between proliferation and differentiation is disturbed: tumor cells exhibit altered or no differentiation, display limitless proliferation and are thus kept in an undifferentiated, immature state. HDAC inhibitors restore the balance and stimulate tumor cells to differentiate and relinquish their proliferative potential.

Acute myeloid leukemia (AML) features a block in myeloid differentiation and is an excellent disease model to examine the regulation of differentiation and cancer progression (Tenen 2003). In normal myeloid cells, retinoic acid (RA) interacts with retinoic receptor-alpha (RARα) to induce differentiation: In the absence of RA, RARα recruits HDACs which results in transcriptional repression of genes responsible for hematopoietic differentiation, but presence of RA leads to dissociation of the RARα-complex and therefore to activation of differentiation-specific genes. In acute promyelocytic leukemia (APL), reciprocal chromosomal translocations of the transcription factor RARα give rise to RAR-fusion proteins including RAR-PML or RAR-PLZF. Binding of these chimeric proteins to retinoic acid responsive elements (RAREs) results in a more stable interaction with HDAC recruiting complexes. HDAC3 as a component of the NCoR complex was found to be recruited by RAR-PML (Atsumi et al. 2006) and HDAC4 was shown to interact with RAR-PLZF (Chauchereau et al. 2004) to repress differentiation-specific transcription. Due to the presence of fusion partners, structural properties of RAR are altered and physiological concentrations of RA are no longer sufficient to trigger the dissociation of HDAC-containing complexes, which leads to the maintenance of transcriptional

silencing of genes governing hematopoietic differentiation (Minucci et al. 2001). Interestingly, a similar mechanism of retinoic acid signaling inhibition in hematopoietic cells has been identified for AML1-ETO fusion proteins expressed in AML (Wang et al. 1998), where AML1-ETO binds to HDAC1, 2, and 3 (Amann et al. 2001). In addition, overexpression of the HDAC2-recruiting transcription factor Bcl6 substantially contributes to the malignant phenotype of large B cell lymphoma (Deltour et al. 1999), providing an example for a role of aberrant recruitment of HDACs in tumorigenesis.

In addition to HDAC inhibitor-induced differentiation in hematological malignancies, various HDAC inhibitors were also shown to induce differentiation in breast cancer cells (Munster et al. 2001), hepatoma cells (Yamashita et al. 2003), endometrial stromal sarcoma (Hrzenjak et al. 2006), small cell lung cancer cells (Platta et al. 2007), gliablastoma cells (Svechnikova et al. 2008), and thyroid carcinoma cells (Yuan et al. 2010).

Most of the data about blocked differentiation as a hallmark of cancer come from studies with HDAC inhibitors, which prevent the activity of several HDAC family members, but little is known about the contribution of individual HDACs. However, in neuroblastoma cells it has been shown that a particular HDAC – namely HDAC8 – is a crucial regulator of tumor cell differentiation (Oehme et al. 2009). In neuroblastomas, high HDAC8 expression correlates with neuroblastoma progression, while the levels of the ten other HDACs are not connected with the disease state. Knockdown of HDAC8 and the use of an HDAC8-selective inhibitor cause induction of neuronal differentiation and inhibition of cell proliferation in neuroblastoma cells, which underlines HDAC8 as a promising target for differentiation therapy. Remarkably, knockdown of HDAC2 causes apoptosis in the same cell system, suggesting that different HDACs regulate distinct cellular programs (Oehme et al. 2009).

2.3 Apoptosis

In tumorigenesis, HDACs act as regulators in the process of apoptosis, which is also known as programmed cell death. The evasion of this pathway is an important factor for the ability of tumor cells to dramatically expand and therefore represents a hallmark for ongoing tumor progression. The acquired resistance to apoptosis is part of the development of all different types of cancer [reviewed in Hanahan and Weinberg (2000)].

There are two different possibilities how programmed cell death is executed in cells – the intrinsic and the extrinsic apoptotic pathway. Both are affected by HDACs and can therefore be induced by HDAC inhibitor treatment [reviewed in Beumer and Tawbi (2010)].

The extrinsic apoptotic pathway functions through binding of death receptors, such as Fas-, TNF (tumor necrosis factor)-, or TRAIL (tumor necrosis factor-related apoptosis inducing ligand)-receptor to their corresponding ligands including FasL,

TNF, or TRAIL. Subsequently, this receptor–ligand interaction leads to release of Caspase-8 and -10, which then results in the activation of effector caspases [reviewed in Ashkenazi (2002)]. HDAC inhibitors trigger an increase in expression levels of death receptors and their ligands in transformed cells in vitro and in vivo, which is not the case in untransformed cells [reviewed in Xu et al. (2007)]. Hydroxamide acid-based hybrid polar compounds such as SAHA and trichostatin A inhibit tumor cell growth selectively and do not affect untransformed cells. Hence these compounds specifically alter the gene expression of cultured tumor cells.

Another effect of HDAC inhibitors on tumor cells is the activation of the intrinsic apoptotic pathway. This form of programmed cell death is mediated by mitochondria that release proteins such as cytochrome c, which activates caspases. The intrinsic apoptotic pathway is guided by pro- and antiapoptotic proteins of the Bcl-family (Wang et al. 2004). HDAC inhibitors cause a decreased expression of antiapoptotic factors (Bcl2, Bcl-XL, XIAP, survivin, Akt, c-FLIP, c-RAF, Mcl1) and elevated expression of proapoptotic Bcl-family members (Bax, Bak, Bim, TP2, Apaf-1, DR4, DR5, Bmf). Thus, tumor cells are sensitized for intrinsic apoptosis when treated with HDAC inhibitors [reviewed in Beumer and Tawbi (2010)].

Chronic lymphocytic leukemia cells exhibit resistance to TRAIL, but susceptibility can be caused by HDAC inhibitor treatment. The usage of different HDAC inhibitors and siRNA for HDAC1, 2, 3, 4, 6, and 8 showed that only HDAC1 and 2 inhibition markedly sensitized cells to TRAIL-induced apoptosis, which suggests HDAC1 and HDAC2 as the major targets for the HDACi-mediated sensitization to TRAIL-induced apoptosis. Consequently, a combination of HDAC inhibitors and TRAIL could be used in the treatment of patients with chronic lymphocytic leukemia and other lymphoid malignancies (Inoue et al. 2006).

In contrast to HDAC1 ablation in most untransformed cell lines, knockdown of HDAC1 in breast adenocarcinoma and osteosarcoma cells leads to activation of multiple proapoptotic genes including Fas, Bcl2-like 1, and members of the TNF family and therefore to increased apoptosis. Furthermore, knockdown of HDAC1 results in the release of Caspase-3, which is related to the intrinsic as well as to the extrinsic apoptotic pathway. Therefore, the absence of HDAC1 and the following expression of Caspase-3 guide initiation of apoptosis (Senese et al. 2007).

Additionally, another study showed that HDAC2 diminishes TRAIL-induced apoptosis of pancreatic cancer cells, whereas HDAC1 is not involved in this process. In general, human primary tumor cells show resistance to TRAIL-induced apoptosis. If HDAC2 is inhibited through the usage of valproic acid – a class I specific HDAC inhibitor – pancreatic ductal adenocarcinoma (PDAC) cells are sensitized towards TRAIL-induced apoptosis (Schuler et al. 2010), suggesting that HDAC2 controls resistance to TRAIL. Therefore, specific degradation of HDAC2 points out a new strategy for the treatment of this type of carcinoma. Additionally, other studies showed high expression of HDAC2 in many solid tumors (Weichert 2009). Moreover, HDAC2 contributes to epithelial to mesenchymal transition, which is important for tumor cells to metastasize and spread throughout the body. Hence HDAC2-specific inhibitors might serve as therapeutic agents with lower toxicity and higher inhibitory capacity compared to currently used HDAC inhibitors (Kramer 2009).

Besides class I histone deacetylases HDAC1 and 2, also class III HDAC SIRT1 is involved in apoptosis. As mentioned earlier, there is a debate on the function of sirtuins in tumorigenesis and it seems that SIRT1 plays a dual role in cell survival, which can be modulated by various stimuli. SIRT1 was found to inhibit p53-dependent apoptosis in response to DNA damage and oxidative stress (Luo et al. 2001) by deacetylating lysine 382 on p53 and thereby reducing its transcriptional activity (Vaziri et al. 2001). In addition, SIRT1 deacetylates the DNA repair factor Ku70 leading to sequestration of the proapoptotic factor Bax from the outer mitochondrial membrane and thereby inhibiting apoptosis (Cohen et al. 2004). Conversely, SIRT1 was shown to play a crucial role in tumor suppression and DNA repair as SIRT1-deficient mice exhibit genome instability, impaired DNA damage response, and an increased rate of tumorigenesis (Wang et al. 2008).

2.4 Metastasis

The ability to metastasize and spread is certainly the most deadly hallmark of cancer cells. Several metastasis repressors such as RECK (reversion-inducing-cysteine-rich protein with Kazal motifs), Kangai 1, RhoB (Ras homologue gene family member B), and TIMP-1 (tissue inhibitor of metallo-proteinases-1) are induced in their expression in response to HDAC inhibitors suggesting a promoting role of HDACs for tumor metastasis (Ma et al. 2009). The class I deacetylase HDAC1 seems to play an important role in this process. For instance, HDAC1 was shown to repress the expression of RECK and RhoB in tumor cells (Lee et al. 2010). Similarly, an HDAC1-containing β-catenin corepressor complex was shown to negatively affect the levels of Kangai 1 in metastatic cells (Kim et al. 2005).

A key element of metastasis is the conversion from adhering epithelial cells to motile mesenchymal cells (termed EMT), which are then able to migrate from the site of the primary tumor. In embryonic development, EMT is an essential process to establish the migration of cells in the course of gastrulation (Moustakas and Heldin 2007). However, during tumorigenesis cancer cells use this program to break down intercellular junctions, travel to other parts of the body, and generate new tumors at distant sites. The cell-adhesion molecule and tumor suppressor E-Cadherin maintain contact between epithelial cells and its function is frequently lost during the development of most epithelial cancers including carcinomas of the breast, colon, prostate, stomach, liver, esophagus, skin, kidney, and lung [reviewed in Christofori and Semb (1999)]. EMT is induced by transcription factors belonging to the SNAIL/SLUG family of transcriptional repressors, which mediate repression of E-Cadherin by the recruitment of the SIN3/HDAC1/HDAC2 corepressor complex and additionally upregulate members of the matrix metalloproteinase family. SNAIL1 interacts with HDAC1 and HDAC2 and requires HDAC activity to repress the E-Cadherin promoter, since treatment with the HDAC inhibitor trichostatin A blocks the repressor effect of SNAIL1 in SNAIL1-expressing epithelial cell lines (Peinado et al. 2007). A recent study confirmed the crucial role of SNAIL1

interacting with HDAC1/HDAC2 in the repression of E-Cadherin in tumor cells (von Burstin et al. 2009). Interestingly, HDAC1 seems to act as a negative regulator of both E-Cadherin and SNAIL1, as HDAC inhibitor treatment of ovarian carcinoma cells results in upregulation of both SNAIL1 and E-Cadherin (Hayashi et al. 2010). In a murine teratoma model, loss of HDAC1 leads to elevated SNAIL1 expression and the formation of partially undifferentiated embryonic carcinomas (Lagger et al. 2010). To our knowledge, this is the first study indicating that loss of HDAC1 is not necessarily beneficial for tumor inhibition. Besides the well-known notion that HDAC1 represses tumor suppressors such as E-Cadherin, the enzyme also controls the EMT-inducer SNAIL1, which – when overexpressed in the absence of HDAC1 activity – might even aggravate the disease.

2.5 *Angiogenesis*

It has been well established that the formation of new blood vessels from a preexisting vasculature (termed angiogenesis) is critical for progression and metastasis of solid tumors (Carmeliet 2005) as well as hematologic malignancies (Moehler et al. 2001). Induction and maintenance of its own vasculature is essential for the tumor to grow beyond 2–3 mm^3 in size, since passive diffusion is no longer sufficient to supply the malignant tissue with oxygen and nutrients as well as efficiently remove waste products of its metabolism (Folkman 1995). Hypoxic microenvironment and nutrient deficiency inhibit cell proliferation and limit tumor growth; therefore acquiring an angiogenic phenotype is considered to be fundamental in cancer progression (Folkman et al. 1989). Under physiological conditions, angiogenesis is tightly regulated by the balance between pro- and antiangiogenic factors (Bergers and Benjamin 2003). An alternation of this equilibrium, called angiogenic switch, results in secretion of proangiogenic molecules by tumor cells which in turn causes invasion, migration, and proliferation of vascular endothelial cells, ultimately leading to new capillary vessel formation (Kerbel and Folkman 2002; Madhusudan and Harris 2002). Primary triggers for this multistep process are hypoxia within the tumor mass and/or genetic mutations, both affecting hypoxia-inducible factor 1α (HIF-1α) – a key regulator of tumor angiogenesis (Ellis et al. 2009). In order to exert its function as a transcription factor, HIF-1α heterodimerizes with another member of the HIF protein family – HIF-1β. In contrast to HIF-1β, which is constitutively expressed, cellular levels of HIF-1α are regulated posttranslationally by oxygen tension. In normoxic conditions, the oxygen-dependent degradation domain (ODDD) of HIF-1α undergoes hydroxylation and acetylation, which leads to rapid degradation via the ubiquitin-proteasome pathway due to interaction with von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex. In hypoxia, the ODDD domain of HIF-1α remains unhydroxylated, which results in stabilization of the protein and transcriptional activation of its target genes, including vascular endothelial growth factor (VEGF) – a key mediator of angiogenesis (Ke and Costa 2006). An additional layer of

regulation of HIF-1α transcriptional activity is provided by class I and II HDACs. In response to hypoxia, increased expression of class I HDACs (HDAC1, HDAC2, and HDAC3) mRNA and protein was observed in vitro in primary as well as in malignant cell lines (Kim et al. 2001). Interestingly, overexpression of HDAC1 results in a decrease in expression of p53 and pVHL, leading to increased levels of both HIF-1α and VEGF. This effect was not observed in the presence of the HDAC inhibitor Trichostatin A (TSA) (Kim et al. 2001). In addition, HDAC1 and HDAC3 have been shown to interact with the ODDD domain of HIF-1α, providing a direct mechanism of regulation of HIF-1α stability and transcriptional activity by HDACs (Kim et al. 2007). HIF-1α functions are furthermore affected by the activities of individual class II HDACs. HDAC4 and HDAC6 associate with HIF-1α, thereby increasing its stability and transcriptional activity in a VHL-independent manner (Qian et al. 2006). HDAC7, another member of the class II HDAC family, was shown to translocate from cytoplasm to the nucleus to form a complex with HIF-1α and p300, which potentiates the transcriptional activity of HIF-1α (Kato et al. 2004). Loss of HDAC7 in human umbilical vein endothelial cells (HUVECs) resulted in morphological changes and impaired endothelial cell migration, accompanied by increased expression of platelet derived growth factor (PDGFβ) and its receptor (PDGFR-β) (Mottet et al. 2007). Importantly, HDACs have been shown to contribute to angiogenesis via other mechanisms, independent of HIF-1α regulation. For instance, HDAC6 is involved in negative regulation of ADAMTS1 transcription by binding to its promoter. ADAMTS1 is a metalloproteinase responsible for sequestration of VEGF and release of angioinhibitory thrombospondin 1/2, therefore constituting a potent inhibitor of angiogenesis (Chou and Chen 2008). Taken together, a huge body of experimental evidence demonstrates crucial roles of individual class I and class II HDACs in regulation of HIF-1α activity as well as migration of vascular endothelial cells. Therefore, class I and class II HDACs represent attractive candidate targets for antiangiogenic therapy.

3 HDAC Involvement in Specific Tumor Development

Deregulated activity of various HDACs contributes to the malignant phenotype of many hematological as well as solid cancers. Numerous oncology studies have demonstrated that the elevated levels of different HDAC family members in malignant tissues are often linked to more aggressive tumor behavior and poor prognosis. In this part, we summarize the most important and recent findings about the involvement of individual HDAC family members in particular tumors.

3.1 Hematological Malignancies

Altered HDAC expression or mistargeted HDAC activity leads to the development of hematological malignancies – such as leukemias, lymphomas, and myelomas.

To date the best results with HDAC inhibitors as anticancer drugs were obtained in the treatment of these hematological malignancies (Mercurio et al. 2010).

Aberrant recruitment of HDACs to promoters through association with oncogenic DNA-binding fusion proteins caused by chromosomal translocations is the main source of these diseases and leads to the onset of tumorigenesis, as already pointed out in Sect. 2.2. This is exemplified by the fusion protein PML-RAR, which stimulates APL through recruitment of HDAC3-containing repressor complexes, leading to a decreased expression of differentiation-specific genes (Atsumi et al. 2006). Similarly, also T-cell lymphomas are known to originate from chromosomal rearrangements resulting in the recruitment of class I HDACs to promoters of genes, which normally limit cell proliferation (Piekarz et al. 2009).

Furthermore, hematological malignancies can be caused by overexpression of repressive transcription factors, which physically interact with HDACs. In non-Hodgkin lymphomas, such as diffuse large B-cell lymphoma (DLBCL), the overexpression of the transcription-factor Bcl6 leads to recruitment of HDAC2, which in turn causes the repression of growth-regulatory target genes (Pasqualucci et al. 2003). In addition, overexpression of HDACs themselves influences the onset of tumorigenesis in these cancer types. Diffuse large B-cell lymphomas (DLBCL) along with peripheral T-cell lymphomas exhibit HDAC1, 2, and 6 overexpression (Marquard et al. 2009) and classical Hodgkin lymphomas display increased HDAC1, 2, and 3 expression levels (Adams et al. 2010).

Another possible correlation between hematological malignancies and HDACs is indicated by the inhibition of HDAC6 through 17-AAG (17-allylamino-demothoxygeldanamycin), which leads to hyperacetylation of Hsp90 and thereby to an abnormal chaperone function of this protein in human leukemia cells, suggesting antileukemia activity of 17-AAG (Mercurio et al. 2010; Rao et al. 2008).

In contrast to lymphomas and leukemias, there are currently no studies demonstrating abnormal expression or activity of HDACs in plasma cell malignancies such as myelomas. However, numerous HDAC inhibitors were shown to act as effective antimyeloma agents in both myeloma cell lines and primary patient cells. HDAC inhibitors such as SAHA were found to upregulate $p21^{WAF1/CIP1}$, induce cell cycle arrest, and cause apoptosis (Mitsiades et al. 2003).

The benefit of HDAC inhibitor treatment in hematological malignancies becomes evident by the fact that the two agents Vorinostat and Romidepsin are already FDA approved and used in the treatment of CTCL.

3.2 Breast Cancer

Several HDACs, particularly HDAC1, 2, 3 as well as HDAC6, have been implicated in modulation of the estrogen signaling pathway, which plays an essential role in growth and progression of human breast cancer (Bicaku et al. 2008; Thomas and Munster 2009). One of the most potent prognostic factors that determine the therapeutic approach in patients suffering from this malignancy is the

expression level of estrogen receptors (ERs), ERα and ERβ, as well as progesterone receptors (PRs), PRA and PRB, since ER- and PR-negative tumors respond poorly to antihormonal therapy (Osborne et al. 1980). Several studies have shown a link between different HDAC family members and specific tumor characteristics. Tissue microarray analysis has demonstrated a significant correlation between the expression of HDAC1 and HDAC3 and estrogen and progesterone receptors (Krusche et al. 2005). Moreover, ER- and PR-positive tumors have been shown to express significantly higher *HDAC1* mRNA levels (Zhang et al. 2005). Importantly, the loss of ER expression has been attributed to aberrations in epigenetic mechanisms, including DNA methylation and histone acetylation (Cameron et al. 1999). In line with those findings, combinatorial treatment of MDA-MB-231 cells with the DNMT inhibitor 5-aza-2′-deoxycytidine and the HDAC inhibitor TSA resulted in reactivation of the ERα promoter (Sharma et al. 2005). Interestingly, in the same cell line, another HDAC inhibitor, LBH589, restored ERα expression without loss of promoter hypermethylation. In addition to transcriptional regulation, HDAC activity influences ERα protein stability. Specific inhibition of HDAC6 results in hyperacetylation of Hsp90 in accordance with Hsp90 in chapter 3.1, which impairs its association with ERα, leading to subsequent ubiquitination and degradation of ERα (Fiskus et al. 2007). In ER-positive cells, inhibition of HDAC activity has been shown to repress ERα expression and sensitize breast cancer cells to tamoxifen (Hirokawa et al. 2005).

3.3 Ovarian Cancer

Ovarian cancers have been found to express significantly higher levels of class I HDACs in comparison to normal ovarian tissue (Jin et al. 2008; Khabele et al. 2007). siRNA-mediated knockdown of the class I HDAC family members HDAC1, 2, and 3 in the ovarian cancer cell line SKOV-3 resulted in reduced cell growth with the most pronounced impact observed for HDAC3 depletion (Khabele et al. 2007).

3.4 Cancers of the Digestive System

Elevated levels of several HDAC family members have been reported in PDAC. Immunohistochemical analysis has revealed the presence of HDAC1 in 56% of tumor samples analyzed (Miyake et al. 2008). In addition, coexpression of HDAC1 and HIF-1α has been shown to correlate with poor prognosis (Miyake et al. 2008). Moreover, HDAC2 overexpression has been identified in a tissue microarray of pancreatic cancer (Fritsche et al. 2009), which has been linked to c-Myc (Marshall et al. 2010). Importantly, it has been shown that HDAC2 mediates the resistance of PDAC cells to the chemotherapeutic drug etoposide due to silencing of the proapoptotic NOXA gene (Fritsche et al. 2009). Furthermore, increased expression of HDAC7 has been reported in pancreatic cancer (Ouaissi et al. 2008).

Elevated expression of two members of class I HDAC family has been demonstrated for gastric cancer. Increased levels of *HDAC1* mRNA have been correlated to reduced expression of gelsolin and retinoic acid receptor β in primary human tumor samples (Kim et al. 2004). Moreover, increased expression of HDAC2 has been observed in advanced stages of gastric cancer (Song et al. 2005).

In a considerable subset of colorectal carcinomas, high expression of class I HDACs has been observed, which correlates with reduced patient survival (Weichert et al. 2008b); however, the contribution of different HDAC family members towards a malignant phenotype seems to be unequal. In the HCT-166 cell line, siRNA-mediated depletion of HDAC1, 2, and 3 resulted in reduction of cell growth with the greatest effect seen for HDAC3 (Wilson et al. 2006). In contrast, in CX-2 cells the strongest growth inhibition was observed upon HDAC2 depletion (Weichert et al. 2008b). Interestingly, in the HT-29 colon carcinoma cell line, which is deficient in the tumor suppressor adenomatosis polyposis coli (APC), c-Myc induces HDAC2 expression (Zhu et al. 2004). Moreover, HDAC2 alone has been shown to be sufficient to prevent apoptosis of colon cancer cells, indicating a crucial role of this HDAC family member in colorectal tumorigenesis (Zhu et al. 2004).

3.5 Neuroblastoma

The analysis of mRNA levels of 11 classical HDAC family members in primary neuroblastomas has revealed a significant role of HDAC8 in neuroblastoma biology (Oehme et al. 2009). High levels of *HDAC8* mRNA correlate with metastasizing and advanced stage disease with poor prognosis (stage 4), whereas in early stage neuroblastomas (stage 1) HDAC8 levels were downregulated. Importantly, stage 4S neuroblastomas associated with spontaneous regression express low levels of HDAC8 (Oehme et al. 2009). Functional studies in neuroblastoma cell lines have demonstrated that selective targeting of HDAC8 by RNAi as well as a small-molecule inhibitor reduces proliferation and induces differentiation of neuroblastoma cells. In contrast, knockdown of HDAC2 results in apoptosis of neuroblastoma cells indicating individual roles of different HDACs in neuroblastoma pathogenesis (Oehme et al. 2009). In addition, increased levels of *HDAC1* mRNA have been found in multidrug-resistant neuroblastoma cell lines and siRNA-mediated knock-down of HDAC1 restores sensitivity of the cells to chemotherapeutic agents (Keshelava et al. 2007). The role of HDAC1 in neuroblastoma has been attributed to N-Myc-mediated gene regulation. For instance, N-Myc has been shown to recruit HDAC1 to Sp1 binding site of transglutaminase 2 (TG2) promoter, which results in transcriptional repression of *TG2* gene (Liu et al. 2007).

3.6 Prostate Cancer

A large cohort study has revealed that HDAC1, 2, and 3 are highly expressed in prostate cancer tissues, which has been correlated with dedifferentiation and

increased tumor proliferation (Weichert et al. 2008a). This study indicates a significant prognostic value of HDAC2 in prostate cancer, since the relapse-free survival time is significantly shorter in patients with increased levels of HDAC2 in tumor tissues. Moreover, HDAC8 levels have been found upregulated in prostate cancer tissue (Nakagawa et al. 2007). In addition to class I HDACs, elevated function of HDAC4 has been reported in prostate cancer: in hormone refractory tumors, HDAC4 localizes in the nucleus (in contrast to the cytoplasmic localization in primary prostate cells), which might influence tumor behavior (Halkidou et al. 2004).

3.7 Lung Cancer

It has been shown that all class I HDACs (HDAC1, 2, 3, and 8) are upregulated in lung cancer tissues in comparison with noncancerous samples (Nakagawa et al. 2007). In vitro studies using HDAC inhibitors have revealed that elevated HDAC activity contributes to the transcriptional repression of the RhoB gene (Mazieres et al. 2007) as well as silencing of Notch1 signaling (Platta et al. 2008) in lung tumor cells. Moreover, the decreased expression of class II HDACs has been correlated with poor prognosis of lung cancer patients (Osada et al. 2004).

4 Summary and Outlook

HDACs have numerous histone and nonhistone substrates involved in crucial cellular processes in normal development and cancer. The amount and the widespread actions of these substrates make it difficult to dissect the epigenetic and the nongenomic components, which are discussed in this and the following chapter. During the multistep process of tumorigenesis, individual HDAC family members contribute to the hallmarks of cancer by blocking differentiation and apoptosis as well as inducing proliferation, angiogenesis, and metastasis. Therefore, it is not surprising that several HDAC family members are frequently overexpressed or aberrantly recruited in various tumor types. Due to the extensive effects of different HDACs on a plethora of substrates, HDACs represent attractive drug targets for cancer treatment. HDAC inhibitor-caused acetylation leads to both changes in gene expression and functional modifications of nonhistone proteins, thereby triggering antitumor pathways. Cancer cells accumulate a variety of defects in proteins that control cell proliferation and survival and it was shown that pan-HDAC inhibitors attack the cancer cell at several levels leading to broad-spectrum antitumor effects. Remarkably, fast cycling tumor cells with multiple defects seem to be more sensitive to HDAC inhibitor treatment than normal cells, which are more likely to compensate for the inhibitory effects.

In the last years, the knowledge about the role of individual HDACs in cancer has increased, new nonhistone targets have been identified, and two pan-HDAC

inhibitors Vorinostat and Romidepsin have been FDA approved for the treatment of CTCL. However, in spite of the vast amount of studies of HDAC knockout mice and inhibitor treatments, there are still questions that need to be clarified. It is particularly important to specify the cancer-relevant HDACs in a given tumor type in order to design selective inhibitors. Concerning selective HDAC inhibitors it needs to be further elucidated if they show the same efficiency as pan-inhibitors and whether it is possible to reduce the side effects occurring as a consequence of pan-HDAC inhibitor treatment. Promising findings come from combination therapies, where HDAC inhibitors together with other agents lead to additive or synergistic antitumor effects, but still more information is needed to learn about the optimal combinations, the ideal schedule, and the underlying mechanisms. Furthermore, the generation of mouse models with catalytically inactive HDACs mimicking a highly specific HDAC inhibitor treatment would be a valuable tool to reveal the particular HDACs involved in a given cancer type.

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Deacetylation of Nonhistone Proteins by HDACs and the Implications in Cancer

Lirong Peng and Edward Seto

Contents

Abstract Acetylation and deacetylation of lysine residues controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, are among the most common posttranslational modifications of proteins. In addition to histones, a large number of nonhistone proteins that can undergo reversible acetylation have been identified. These nonhistone acetylated/deacetylated proteins are involved in a wide range of cellular processes including transcription, translation, DNA repair, metabolism, and cell structure. Aberrant deacetylation of nonhistone proteins is implicated in many human diseases, including cancer. In this chapter, we review and describe the involvement of HDACs in cancer-associated cellular processes via deacetylation of nonhistone proteins, and the possible implications for carcinogenesis and cancer development.

Keywords HDAC • Histone deacetylase • Nonhistone acetylation • Nonhistone deacetylation • Cancer

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1 Introduction

Reversible protein acetylation on the ε-amino group of a lysine residue is a key posttranslational modification (PTM) found in many different organisms (Kouzarides 2000; Glozak et al. 2005; Yang and Seto 2008b). Acetylation and deacetylation of histones play crucial roles in transcription regulation through alteration of chromatin structure, which is an important factor in determining whether a particular gene is expressed. In general, histone acetylation by histone acetyltransferases (HATs) causes an open-chromatin configuration and facilitates transcription activation. Histone deacetylases (HDACs), functioning in opposition to HATs, deacetylate histones by removing acetyl groups from the acetylated lysine residues and induce a closed-chromatin configuration and transcriptional repression (Jenuwein and Allis 2001; Johnstone 2002). To date, 18 human HDACs have been identified, and these can be subdivided into four families according to their sequence similarity and cofactor dependency: Class I HDACs (HDAC1, 2, 3, and 8, similar to yeast Rpd3); Class II HDACs (4, 5, 6, 7, 9, and 10, similar to yeast Hda1); Class III HDACs (SIRT1–7, related to yeast Sir2); and Class IV HDAC (HDAC11, similar to both Class I and II) (Yang and Seto 2008a, b).

HDACs have a wider range of substrates than initially thought. Studies in eukaryotic cells revealed that reversible acetylation occurs in a large number of nonhistone proteins, regardless of their cellular localizations (Kim et al. 2006; Choudhary et al. 2009). Deacetylation of nonhistone proteins allows HDACs to exert direct effects on multiple physiologic processes, including apoptosis, autophagy, and metabolism, without affecting gene expression (Xu et al. 2007). Acetylation/deacetylation of nonhistone proteins is also more conserved than first expected. Even in bacteria, which are devoid of histones, many proteins, particularly those that are crucial in metabolism, are subject to reversible acetylation (Wang et al. 2010a; Yu et al. 2008; Zhang et al. 2009a). In a search for acetylated proteins that are potentially regulated by deacetylation, we treated *Escherichia coli* BL-21 (DE3) with the Class I/II HDAC inhibitor trichostatin A (TSA) and analyzed protein acetylation with LC-MS/MS. We identified 208 acetylated lysine sites in 174 proteins; the functional classifications are shown in Fig. 1. Seventy-nine acetylated proteins were detected in both the control (untreated) and the TSA-treated samples, while 51 acetylated proteins were found only in the control samples and 44 were present only in TSA-treated sample (Fig. 2). Further analysis revealed that acetylated proteins exist in nearly every glucose, fatty acid, amino acid, urea, and nucleotide metabolic pathway (e.g., malate dehydrogenase [MDH], isocitrate dehydrogenase [IDH], citrate synthase [CS], argininosuccinate lyase [ASL], glucose-6-phosphate isomerase [GPI], phosphoglycerate kinase [PGK], 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase [PGM], enolase, and 3-hydroxyacyl-CoA dehydrogenase [HDAH]). HDAC1-like proteins have been reported in *Aquifex aeolicus* (Finnin et al. 1999) and *Thermus caldophilus* (Song et al. 2007), and an Sir2-like protein, CobB, was identified in *Salmonella enterica* (Starai et al. 2002). Because our results showed that some proteins are

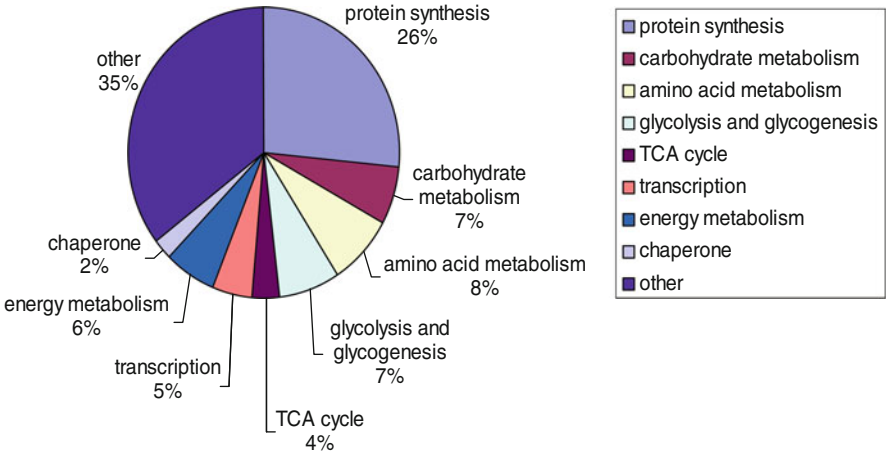

Fig. 1 Functional classification of acetylated proteins from *E. coli* BL-21 (DE3). *E. coli* proteins that were unambiguously identified to be acetylated were assigned to a functional group based on gene ontology molecular functions or biochemical process groups, or previously published literature

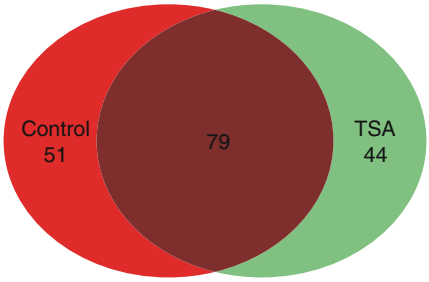

Fig. 2 Comparison of acetylated proteins identified from TSA-treated *E. coli* BL-21 (DE3) with those from nontreated ones (control). *Green* indicates the acetylated proteins found only in TSA-treated samples, *red* indicates those only found in the control sample, and *merged color* indicates those found in both samples

hyperacetylated in TSA-treated relative to untreated samples, we speculate that additional unidentified Class I/II HDAC-like proteins exist in *E. coli*. Thus, reversible acetylation of nonhistone proteins is a very important and conserved PTM mechanism in many organisms ranging from bacteria to humans.

One motivation to study HDACs is to increase our understanding of histone and protein modifications. However, equally important is the desire to understand the relevance of HDACs in health and disease. Results from many studies strongly implicate dysregulation of HDACs in cancer. First, treatment of mammalian cell cultures with HDAC inhibitors causes G1 and G2 arrests, indicating that histone deacetylation is tightly linked to cell cycle and cell growth control (Yoshida et al. 1995; Ogryzko et al. 1996). Second, some HDACs are abnormally expressed in

a variety of cancers, including gastric cancer, prostate cancer, ovarian and breast cancers, colon cancer, and oral squamous cell carcinoma (Choi et al. 2001; Halkidou et al. 2004; Hida et al. 2007; Huffman et al. 2007; Sakuma et al. 2006; Song et al. 2005; Weichert et al. 2008; Wilson et al. 2008; Zhang et al. 2005). Third, several oncogenes and tumor suppressors, with p53 being the most extensively studied, are regulated by HDACs (Luo et al. 2000, 2001; Vaziri et al. 2001). Fourth, a large number of studies have shown that HDAC inhibitors can effectively arrest and revert transformation of some cells and inhibit the growth of cancers in tumor-bearing animals (e.g., Yoshida et al. 1990; Sugita et al. 1992; Yoshida and Sugita 1992; Futamura et al. 1995; Butler et al. 2000; Coffey et al. 2001; Minucci and Pelicci 2006; Dokmanovic et al. 2007).

Cancer occurs as a result of loss in normal cell functions. Many of these cell functions including apoptosis, autophagy, cell motility, and DNA repair are regulated at least in part by HDACs. In this chapter, we discuss the effects of nonhistone deacetylation by HDACs on several important cellular functions, and how alterations in nonhistone protein modification might contribute to the development and progression of cancer.

2 HDACs and Apoptosis

Apoptosis is the process of programmed cell death. Escaping from apoptosis allows damaged cells to proliferate and could lead to tumor development. A number of apoptotic regulators are deacetylated and inhibited by HDACs. Here we select p53 (as well as p73) and Ku70 as two representative nonhistone proteins to illustrate the relationship between HDACs and apoptosis.

The tumor suppressor p53 is the first identified and best characterized nonhistone substrate of HDACs. Upon DNA damage or other stresses, p53 is activated and stabilized through acetylation and phosphorylation, thus triggering cell cycle arrest or apoptosis. It is widely accepted that the reversible acetylation of p53 is closely associated with its activation. The acetylation of p53 by p300/CBP at K305, K370, K372, K373, K381, and K382 or by PCAF at K320 increases its DNA-binding ability, and consequently transcriptional activation of its target genes, thus inducing cell cycle arrest or apoptosis depending on the cell type and nature of the cellular stress (Gu and Roeder 1997; Liu et al. 1999; Luo et al. 2004; Sakaguchi et al. 1998; Wang et al. 2003). The acetylation of p53 by Tip60 at K120 was demonstrated to modulate the decision between cell-cycle arrest and apoptosis, which is required for p53-dependent apoptosis but dispensable for p53-mediated growth arrest (Tang et al. 2006). In addition, acetylation of p53 at K320, K373, and K382 disrupts the Ku70-BAX complex and is required for transcription-independent functions in BAX activation, reactive oxygen species production, and apoptosis in response to histone deacetylase inhibitors (HDACi) suberoylanilide hydroxamic acid and LAQ824 (Yamaguchi et al. 2009). Acetylation also affects the stability of p53 by destabilizing the p53–Mdm2 interaction (Tang et al. 2008).

Interestingly, p53 can be deacetylated by two different HDACs: HDAC1 and SIRT1. The HDAC1 complex specifically interacts with p53 and significantly reduces the steady-state levels of acetylated p53, thus repressing p53-dependent transcription (Luo et al. 2000). MDM2–HDAC1-mediated deacetylation of p53 is required for its degradation (Ito et al. 2002). SIRT1 interacts with p53, deacetylates it at multiple lysine residues, and represses p53-dependent growth arrest and apoptosis in response to cellular damage, resulting in an increased risk of cancer (Luo et al. 2001; Vaziri et al. 2001).

The transcription factor p73, another member of the p53 family, is acetylated by p300 in response to DNA damage, resulting in either cell cycle arrest or apoptosis. Acetylated p73 is required for selective activation of apoptotic target genes, for example, p53AIP1 but not p21 (Costanzo et al. 2002). SIRT1 could interact with p73 in vivo and in vitro, and modulate its biological activity through deacetylation (Dai et al. 2007).

Ku70 has a role in repairing double-strand breaks (DSBs) (Collis et al. 2005). It is acetylated by CBP and PCAF at multiple lysine residues in the C-terminal linker (Cohen et al. 2004a). Acetylation of either K539 or K542 in the C-terminal linker of Ku70 completely blocks its ability to suppress BAX-mediated apoptosis. Three classes of HDACs might be able to deacetylate Ku70 (Subramanian et al. 2005), but SIRT1 is the one found firstly to be responsible for the deacetylation of Ku70 upon DNA damage or other stresses, allowing it to sequester Bax from the mitochondria, ultimately inhibiting apoptosis (Cohen et al. 2004b). SIRT3, another member of Class III HDACs, can deacetylate Ku70 in cardiomyocytes. Like SIRT1, SIRT3 may also modulate Bax-mediated apoptosis through deacetylation of Ku70 and thus promote cell survival under stress conditions (Sundaresan et al. 2008).

3 HDACs and Autophagy

Autophagy is regarded as a form of programmed cell death distinct from apoptosis that involves the degradation of nonfolded proteins, aberrant protein aggregates, and damaged cell components via the lysosomal machinery. Abnormal autophagy is believed to have an important role in tumor development (Mathew et al. 2007). HDACs, especially SIRT1 and HDAC6, are involved in the regulation of autophagy.

Sirt1 has been found to promote autophagy through its deacetylase activity upon starvation. It forms a molecular complex with several essential components of the autophagy machinery, including autophagy proteins (Atg)5, Atg7, and Atg8, and it directly deacetylates these components in an NAD-dependent fashion in vitro. Lack of Sirt1 leads to markedly elevated protein acetylation in both cultured cells and embryonic and neonatal tissues. Moreover, $Sirt1^{-/-}$ mice partially resemble $Atg5^{-/-}$ mice, suggesting that Sirt1 is an important regulator of autophagy (Lee et al. 2008). The SIRT1–PARP-1 axis plays a critical role in the regulation of cigarette smoke (CS)-induced autophagy in lung epithelial cells, fibroblasts, and macrophages as evidenced by the attenuation of CS-induced autophagy by the

SIRT1 activator resveratrol, and the opposite effect of the SIRT1 inhibitor sirtinol (Hwang et al. 2010). Moreover, increased autophagy is induced by CS in the lungs of Sirt1 deficient mice (Hwang et al. 2010). SIRT1 also regulates autophagy via a longevity factor network, which includes the FOXO and p53 pathways (Salminen and Kaarniranta 2009).

HDAC6 is a cytoplasmic HDAC involved in autophagy. Starvation-induced autophagy is usually considered to be a nonselective degradation process, distinct from quality-control (QC) autophagy in its substrate selectivity and machinery (Luzikov 1999). This selective degradation requires an intact microtubule cytoskeleton and HDAC6 (Iwata et al. 2005), and acts as a compensatory degradation system when the ubiquitin proteasome system is impaired (Pandey et al. 2007). A recent study demonstrated that HDAC6 promotes selective autophagy through a cortactin-dependent, actin-remodeling machinery, which assembles an F-actin network that stimulates autophagosome–lysosome fusion and substrate degradation (Lee et al. 2010). Although it is clear that HDAC6 functions in the aggresome pathway and HDAC6 is capable of deacetylating both alpha-tubulin and cortactin (Hubbert et al. 2002; Zhang et al. 2007), whether QC autophagy involves a direct deacetylation of cortactin and tubulin by HDAC6 is not yet understood.

4 HDACs and DNA Damage Repair/Genome Integrity

Proper DNA repair is important for the elimination of gene mutation and maintenance of genome integrity during cell growth. Failure or abnormity of DNA repair may result in genome instability, such as deletion of tumor suppresser genes or amplification of oncogenes, and thus lead to tumorigenesis (Khanna and Jackson 2001; Risinger and Groden 2004). One of the most important functions of HDACs, besides the role of transcriptional repression, is their regulation of DNA damage responses (DDR). HDAC(s) could directly deacetylate some DNA repair proteins and alter their functions.

SIRT1 is the leading HDAC involved in the regulation of DNA repair proteins through deacetylation, suggesting that it plays significant roles in DNA repair and genome integrity. As mentioned before, Ku70 and p53, the two important effectors downstream of the repair signal cascades initiated by double strand break (DSB) damage, are directly deacetylated and inhibited by SIRT1. SIRT1 also deacetylates many other DDR proteins. NBS1 is the regulatory subunit of MRE11–RAD50–NBS1 (MRN), a conserved nuclease complex that exhibits properties of a DNA damage sensor and is critical for the cellular response to DNA DSBs. Phosphorylation of NBS1 by the ATM kinase is essential for its activation in response to DNA damage (Buscemi et al. 2001). Our previous work demonstrated that NBS1 is an acetylated protein and that its acetylation level is tightly regulated by SIRT1. SIRT1 associates with the MRN complex and keeps NBS1 in a hypoacetylated state, which is required for the ionizing radiation-induced phosphorylation of NBS1 at Ser343 and, in turn, activation. This work indicated the presence of crosstalk between two different PTMs

in NBS1 and strongly suggested the key role of deacetylation of NBS1 by SIRT1 in the dynamic regulation of the DDR and maintenance of genomic stability (Yuan et al. 2007).

SIRT1 also regulates WRN (Werner syndrome protein)-mediated cellular responses to DNA damage. WRN is a member of the RecQ DNA helicase family and involved in premature aging and cancer (Opresko et al. 2007). WRN could be acetylated by acetyltransferase CBP/p300 and deacetylated by SIRT1 in vitro and in vivo. SIRT1-mediated deacetylation regulates the cellular localization of WRN, and also its helicase and exonuclease activities (Li et al. 2008). Furthermore, SIRT1 deacetylation was required for SIRT1-mediated homologous recombination (HR) in DSB repair (Uhl et al. 2010). In addition, SIRT1 is capable of deacetylating and interplaying with poly (ADP-ribose) polymerase 1 (PARP-1) in maintaining genome integrity (El Ramy et al. 2009). These results provide sound evidence of a link between SIRT1 and HR with possible implications for genomic stability during aging and tumorigenesis.

Some other DNA repair proteins (e.g., DNA glycosylases, Flap endonuclease 1 [FEN1], and Dna2 endonuclease/helicase [Dna2]) are also acetylated, and acetylation changes their biological activities (Balakrishnan et al. 2010; Bhakat et al. 2004, 2006; Friedrich-Heineken et al. 2003; Hasan et al. 2001). However, the HDAC responsible for the deacetylation of these proteins is unknown.

5 HDACs and Differentiation

Dysregulation of differentiation is a characteristic of cancer. Through histone deacetylation, HDACs regulate cell differentiation via modulation of the transcription of differentiation-associated genes (Brunmeir et al. 2009; Glozak and Seto 2007; Hisahara et al. 2008). However, only a few nonhistone proteins have been shown to be direct deacetylation substrates of HDACs in differentiation.

The myogenic activator, MyoD, is a deacetylation substrate of HDAC1. MyoD plays an important role in muscle cell differentiation. It interacts with and is acetylated by p300/CBP and PCAF. Acetylation of MyoD at the evolutionarily conserved lysine residues enhances its DNA-binding activity and is necessary for the execution of the muscle program (Polesskaya et al. 2000). HDAC1 directly binds and deacetylates MyoD in vitro and, more importantly, represses MyoD-mediated transcription in vivo by preventing MyoD from converting undifferentiated skeletal muscle cells to mature muscle cells in a deacetylase activity-dependent manner (Mal et al. 2001).

The myocyte enhancer factor 2 (MEF2) was originally identified as a muscle differentiation related-transcription factor involved in the regulation of skeletal muscle development and the stress-response of cardiomyocytes (Lilly et al. 1994; Zhang et al. 2002). Interestingly, MEF2-dependent myogenesis may be simultaneously controlled by three HDACs: HDAC3, HDAC4, and SIRT1 (Zhao et al. 2005; Gregoire et al. 2007). SIRT1 and HDAC3, but not HDAC4, are capable of

directly deacetylating MEF2. HDAC4 might act indirectly, and integrate sumoylation and deacetylation signals via its interaction with Ubc9 and SIRT1. Because acetylation of MEF2 is inducible upon muscle cell differentiation and enhances its activity, HDAC4-mediated sumoylation is thought to inhibit MEF2 (Zhao et al. 2005).

SIRT1 is also involved in differentiation by deacetylation of the tumor suppressor retinoblastoma protein (Rb). Rb represses gene transcription by directly binding to the transactivation domain of E2F, promoting recruitment of chromatin-remodeling enzymes, such as HDACs, to the E2F-target gene promoters, and facilitating adipocyte differentiation by inducing cell-cycle arrest (Giacinti and Giordano 2006; Zhu 2005). Transient phosphorylation of Rb by G1-specific CDKs is the main mechanism by which Rb activity is regulated (Mittnacht 1998). Acetylation of Rb by p300 at the C-terminus is an additional mechanism to regulate Rb activity, which results in hypo-phosphorylated Rb and causes cell cycle arrest (Chan et al. 2001). Lysine residues in regions of Rb other than the C-terminus could be acetylated by PCAF. Acetylation of these sites affected Rb-mediated terminal cell cycle exit and the induction of late myogenic gene expression, but not Rb-dependent growth arrest (Nguyen et al. 2004). This evidence suggests a role of acetylation in the regulation of the differentiation-specific function of Rb. SIRT1 can deacetylate Rb in response to contact inhibition and DNA-damage, indicating that SIRT1 can negatively regulate Rb and act as a switch to restart the cell cycle (Wong and Weber 2007).

Runt domain transcription factors (RUNXs) play essential roles in both neoplasias and normal development, such as hematopoiesis, osteogenesis, neurogenesis, and thymopoiesis. Various functions of Runx family are regulated by acetylation and deacetylation (Bae and Lee 2006). Acetylation of Runx1 at K24 and K43 by p300 is required for its transforming ability (Yamaguchi et al. 2009). Runx3 is necessary for T-cell development and serves as a gastric tumor suppressor, and competitive acetylation and deacetylation of Runx3 at three lysine residues modulates its stability. Upon TGF-beta stimulation, p300 bound to Runx3 and acetylated it at K148, K186, and K182. HDAC4 and HDAC5 reduced its acetylation. Acetylation enhanced Runx3 stability by preventing its ubiquitination by Smurf ubiquitin ligases (Jin et al. 2004).

Deacetylation of FOXO1 by SIRT2 and deacetylation of peroxisome proliferator-activated receptor gamma (PPAR-gamma) by HDAC3 may both be involved in the regulation of development and differentiation (Fajas et al. 2002; Jing et al. 2007). SIRT2 modulates adipocyte differentiation through the regulation of FOXO1 acetylation. SIRT2 was found to be downregulated during preadipocyte differentiation in 3T3-L1 cells. Moreover, overexpression of SIRT2 inhibited the differentiation of 3T3-L1, whereas a decrease of SIRT2 promoted adipogenesis. A reduction of SIRT2 affected the acetylation, phosphorylation, and localization of FOXO1 and its function in adipocyte differentiation (Jing et al. 2007). PPAR-gamma, a nuclear receptor pivotal for adipogenesis, promotes adipocyte differentiation more efficiently in the absence of Rb. Both Rb and HDAC3 are reported to attenuate the ability of PPAR-gamma to drive gene expression and adipocyte

differentiation. Inhibition of HDAC3 or disruption of the PPAR-gamma–HDAC3– Rb interaction is predicted to stimulate adipocyte differentiation (Fajas et al. 2002).

6 HDACs and Invasion/Metastasis

Invasion and metastasis are some of the most distinct characteristics of cancers and involve multiple factors and steps including, for example, motility/migration, angiogenesis, and hydrolysis of matrix. HDACs might contribute to tumor invasion and metastasis by regulating these processes.

HDAC6 is microtubule-associated and regulates cell motility via deacetylation of nonhistone proteins, including alpha-tubulin. Reversible acetylation of tubulin has been implicated in the regulation of microtubule stability. Overexpression of HDAC6 increases chemotactic cell migration, suggesting that HDAC6-mediated deacetylation of tubulin promotes cell motility (Hubbert et al. 2002; Wang et al. 2010b). Furthermore, HDAC6-dependent deacetylation of alpha-tubulin is required for the transforming growth factor (TGF)-beta1-mediated epithelial–mesenchymal transition (Shan et al. 2008). HDAC6 also modulates cell motility by deacetylation of cortactin, an F-actin binding protein involved in a variety of cellular processes, including cell motility, invasiveness, and endocytosis (Cosen-Binker and Kapus 2006). Our previous work showed that HDAC6 deacetylates cortactin in vivo and in vitro, and increases the ability of cortactin to bind F-actin, thereby promoting cell motility (Zhang et al. 2007).

HDAC6 also regulates matrix hydrolysis, a necessary step for invasion and metastasis. HDAC6 deacetylates hsp90 and modulates its downstream biological functions (Bali et al. 2005; Kovacs et al. 2005; Scroggins et al. 2007). Seven acetylated residuals were indentified in hsp90 alpha, an isoform of hsp90, which promotes extracellular maturation of matrix metalloproteinase (MMP)-2, a tumor invasion and metastasis-related proteinase. Hsp90 alpha was up-regulated, secreted, and hyperacetylated in breast cancer cells upon serum starvation. Treatment with the HDAC inhibitor panobinostat also increased the acetylation of extracellular hsp90 alpha. Moreover, antiacetylated hsp90α antibody could inhibit in vitro invasion of tumor cells (Yang et al. 2008). These results indicate that HDAC6 may inhibit tumor invasion and metastasis by deacetylating hsp90 alpha and impeding MMP maturation. It is important to note that the role of HDAC6 in cancer may be cell type dependent.

HDACs also regulate angiogenesis. Some HDAC inhibitors (e.g., TSA) have shown antiangiogenesis potential (Kim et al. 2001). Several HDACi (TSA, SB, Apicidin, and VPA) dramatically inhibit hypoxia-inducible factor-1 alpha (HIF-1 alpha) in some tumor cell lines. HIFs are a class of transcription factors which respond to hypoxia, a common status in tumors, and promote the formation of the vascular system (Benizri et al. 2008). HDAC1 and HDAC3 directly bind to the oxygen-dependent degradation domain of HIF-1 alpha and enhance its stability and activity under hypoxic conditions, suggesting important roles for HDAC1 and

HDAC3 in HIF-1-induced tumor angiogenesis (Kim et al. 2007). SIRT1 has been reported to facilitate endothelial angiogenic function during vascular growth through deacetylating and inhibiting FOXO1, a negative regulator of vascular system development (Potente et al. 2007). These findings suggest that SIRT1 and other HDACs might be involved in neovascularization in tumors.

7 HDACs and Tumor Immune Responses

Dysfunction of the immune system is one way for tumor cells to escape attack from immune cells. HDACs may play roles in the regulation of immune system. HDAC inhibition can negatively regulate the functions of dendritic cells, important types of immune cells, by regulating the acetylation of signal transduction and activation of transcription 3 (STAT3), a crucial transcription activator which responds to the stimuli of cytokines, such as interferon, growth factors, and hormones (Sun et al. 2009). STAT3 is implicated in the regulation of immune cells and a variety of autoimmune diseases by regulating immune cell growth and apoptosis (Yang et al. 2007). HDAC1 binding to STAT3 in an interleukin-6 (IL-6)-dependent manner is required for the nucleocytoplasmic distribution of STAT-3, and affects the responsiveness of STAT3 target genes (Ray et al. 2008). A mutant of STAT3 defective in acetylation blocked STAT3-mediated NF-kappaB p100 processing to p52 and also acted as a dominant negative in blocking the production of p52, which could protect cells from apoptosis and facilitate lymphocyte hyperplasia and transformation (Nadiminty et al. 2006).

8 HDACs and Viral Proteins

It is widely accepted that some viruses can cause cancer. Viruses are etiologically linked to approximately 20% of all malignancies worldwide (Blattner 1999), and can cause solid tumors, as in the case of simian virus 40 (SV40) infection (Paracchini et al. 2006), or have oncogenic activities, as in the case of HIV. Recent studies show that some viral proteins (e.g., HIV Tat, SV40 T-Ag, S-HDAg, and L-HDAg) undergo regulated reversible acetylation. The reversible acetylation of viral proteins might have a major impact on virus–host interactions and carcinogenesis. Here we elaborate on HIV Tat and SV40 large T-antigen as examples.

HIV Tat plays a critical role in HIV replication by binding the leader RNA (TAR sequence) of the viral genome. Tat can be acetylated by p300 at K50 and K51 in its RNA-binding region (Ott et al. 1999), and by PCAF at K28 in its activator domain (Kiernan et al. 1999). Acetylation of Tat by p300 releases it from TAR sequences and thereby enhances transcription from the LTR (Kiernan et al. 1999). Deacetylation of Tat by SIRT1 allows its rapid recycling to TAR and facilitates the repeated replication of the viral genome. Therefore, cycles of Tat acetylation by p300

and deacetylation by SIRT1 appear to regulate viral transcription. SIRT1 might act as a coactivator during Tat transactivation (Pagans et al. 2005).

T-Ag, the early product large T antigen of SV40, is considered a proto-oncoprotein, expression of which can transform normal cells (Lanford et al. 1985), and is known to be reversibly acetylated. Acetylation of T-Ag increases upon the treatment with the HDACi TSA, and HDAC1, HDAC3, and SIRT1 are all capable of binding and deacetylating T-Ag. Deacetylation of T-Ag increases its stability and enhances its transforming ability on NIH 3T3 cells (Shimazu et al. 2006). These findings raise the possibility that HDACs might promote tumorigenesis by deacetylating oncogenic viral proteins.

9 Discussion and Perspective

Though many lines of evidence implicate HDACs in the regulation of tumorigenesis by deacetylation of nonhistones, there remains little direct evidence linking HDACs to cancer. Whether HDACs are primarily tumor promoters or tumor suppressors is difficult to discern. For example, SIRT1 deacetylates and deactivates some tumor suppressors, such as p53 and Rb, and thus represses apoptosis, suggesting that SIRT1 acts as a tumor promoter. The fact that SIRT1 is overexpressed in a variety of tumor tissues further argues for a role of SIRT1 as a tumor promoter (Hida et al. 2007; Huffman et al. 2007). However, several lines of evidence suggest SIRT1 is a tumor suppressor. Some of the more convincing evidences are as follows. First, SIRT1 inhibits the functions of tumor-promoting proteins such as survivin and beta-catenin (Firestein et al. 2008; Wang et al. 2008). Second, mice overexpressing SIRT1 show no tumor formation compared to the control (Banks et al. 2008; Pfluger et al. 2008). In fact, overexpression of Sirt1 in colon-cancer-prone $APC^{-/+}$ mice reduced the levels of colon cancer (Firestein et al. 2008). Third, although there is no way to examine the roles of Sirt1 in carcinogenesis in homozygous knockout mice because of the embryonic lethality of $Sirt1^{-/-}$, analysis of the early embryos of $Sirt1^{-/-}$ mice revealed that Sirt1 deficiency results in genome instability and defects in DNA damage repair, suggesting that it has a tumor suppressor function (Wang et al. 2008). Fourth, resveratrol, a grape extract with chemo-preventive effects on some tumors such as leukemia and prostate cancer (Estrov et al. 2003; Hsieh and Wu 2000), was found to be an agonist of SIRT1 (Aziz et al. 2005; Howitz et al. 2003). Finally, SIRT1 could be up-regulated by some tumor suppressors, for example BRCA1. Up-regulation of SIRT1 by BRCA1 in turn inhibits the formation of BRCA1-mutation associated cancer (Wang et al. 2008).

It is not impossible to explain, or even reconcile, these apparent tumor-promoting and tumor-suppressing functions of SIRT1. For example, SIRT1's negative regulation on tumor suppressors, notably p53, might be cell context, temporality, and spatiality dependent. Laboratory cell culture is different from real tumor microenvironments, which are hypoxic and hotspots of inflammation, apoptosis,

and angiogenesis. Therefore, the observation that SIRT1 deacetylates and deactivates some tumor suppressors may not reflect in vivo function. Whether SIRT1 (or the other HDACs) has a primary role of promoting tumor development, or inhibiting it, or some combination of effects, will remain a hot topic for many coming years.

Given that there are multiple steps and factors in the process of tumorigenesis, much more work awaits to clarify the role of HDACs in cancer. Initial identification of nonhistone proteins regulated by HDACs relied heavily on the use of antiacetyl lysine antibodies. However, this method is tedious and has many limitations related to the low affinity and low specificity of the currently available antiacetyl lysine antibodies. The lack of a conserved recognition motif for HDACs also contributes to the problem. Encouragingly, mass spectrometry has revealed that numerous nonhistone proteins undergo acetylation (Kim et al. 2006; Choudhary et al. 2009). As the technology for detection of protein acetylation/deacetylation becomes more advanced, we predict that even more acetylated proteins will be discovered, and reversible acetylation might be one of the most common PTM of proteins. Global acetylation analysis (may be termed "acetylationomics") under physiological or pathological conditions and studies that bridge "acetylationomics" and other "modificationomics" would help to fully elucidate the role of protein deacetylation and HDACs in diverse cellular processes.

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The Biology and Therapeutic Implications of HDACs in the Heart

Timothy A. McKinsey

Contents

Abstract The heart responds to stresses such as chronic hypertension and myocardial infarction by undergoing a remodeling process that is associated with myocyte hypertrophy, myocyte death, inflammation and fibrosis, often resulting in impaired cardiac function and heart failure. Recent studies have revealed key roles for histone deacetylases (HDACs) as both positive and negative regulators of pathological cardiac remodeling, and small molecule HDAC inhibitors have demonstrated efficacy in animal models of heart failure. This chapter reviews the functions of individual HDAC isoforms in the heart and highlights issues that need to be addressed to enable development of novel HDAC-directed therapies for cardiovascular indications.

Keywords Histone deacetylase • Heart failure • Signaling • Small molecule inhibitors

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1 Introduction

Heart failure is typically classified as either systolic, in which there is reduced ventricular pump function, or diastolic, which is characterized by impaired cardiac relaxation and abnormal ventricular filling. At the cellular level, systolic heart failure is associated with myocyte hypertrophy and myocyte death, which often lead to interstitial fibrosis, chamber dilation and ventricular wall thinning. Diastolic heart failure is typified by myocyte hypertrophy and fibrosis without chamber dilation. Heart failure is a major health problem and growing economic burden worldwide. With more than five million heart failure patients in the US alone, treatment of this condition represents an estimated annual cost to the American health care system of over $37 billion. Furthermore, the 5-year mortality rate following first admission for heart failure is 42.3%, highlighting an urgent need for new therapeutic approaches (Lloyd-Jones et al. 2009).

Studies in animal models suggest a link between histone deacetylases (HDACs) and the pathogenesis of heart failure. HDACs catalyze removal of acetyl groups from ε-amino groups of lysine residues in a variety of proteins. In the heart, HDACs control diverse processes, including hypertrophy (Antos et al. 2003; Kook et al. 2003), fibrosis (Iyer et al. 2010; Kee et al. 2006; Kong et al. 2006), contractility (Gupta et al. 2008) and energy metabolism (Montgomery et al. 2008). The 18 HDACs are encoded by distinct genes and are grouped into four classes on the basis of similarity to yeast transcriptional repressors (Fig. 1). Class I HDACs (HDACs 1, 2, 3 and 8) are related to yeast RPD3, class II HDACs (HDACs 4, 5, 6, 7, 9 and 10) to yeast HDA1 and class III HDACs (SirT1–7) to yeast Sir2. Class II HDACs are further divided into two subclasses, IIa (HDACs 4, 5, 7 and 9) and IIb (HDACs

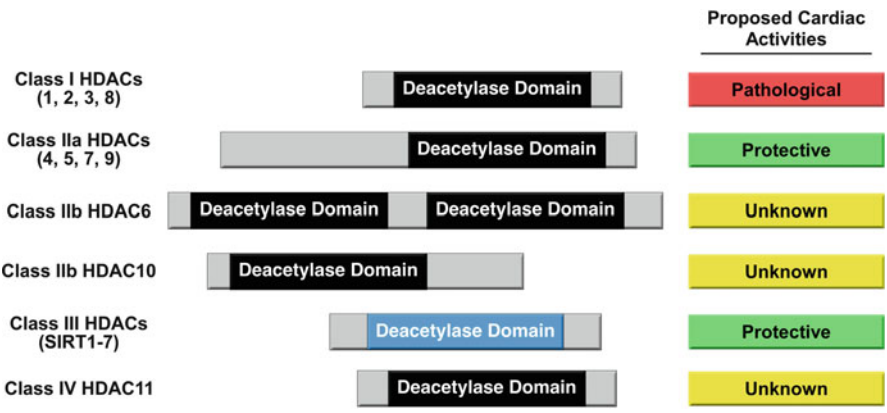

Fig. 1 HDAC classifications. HDACs are categorized into four distinct classes. Class II HDACs are further divided into two subclasses, IIa and IIb. Class III HDACs are also known as sirtuins. Current data suggest that class I HDACs promote pathological cardiac remodeling while class IIa and class III HDACs are protective. There is nothing known about the functions of class IIb and class IV HDACs in the heart

6 and 10). HDAC11 falls into a fourth class (Gregoretti et al. 2004). Over the past 10 years, genetic and pharmacologic gain- and loss-of-function studies have shed light on the roles of distinct HDAC isoforms in the heart. This chapter highlights these studies and discusses the potential for translating basic, mechanistic discoveries related to HDACs in the myocardium into new drugs for the failing heart.

2 Class IIa HDACs in the Heart

Cardiac hypertrophy in response to pathological stimuli has long been viewed as a compensatory mechanism that normalizes wall stress and enhances cardiac performance. However, long-term suppression of cardiac hypertrophy is associated with reduced morbidity and mortality in patients with hypertension, and thus chronic cardiac hypertrophy is now considered maladaptive (Devereux et al. 2004; Gardin and Lauer 2004).

The first connection between HDACs and regulation of pathological cardiac remodeling was provided by the discovery that class IIa HDACs interact with members of the myocyte enhancer factor-2 (MEF2) transcription factor family (McKinsey et al. 2002), which are key regulators on cardiac hypertrophy. The transcriptional activity of MEF2 factors is upregulated in response to pathological stress in the heart (Kolodziejczyk et al. 1999; Lu et al. 2000; Passier et al. 2000; Zhang et al. 2002), and ectopic overexpression of constitutively active forms of MEF2 in mouse heart causes dilated cardiomyopathy (Xu et al. 2006). All class IIa HDACs were found to associate with MEF2 on DNA through a conserved binding domain (Han et al. 2003, 2005), resulting in repression of downstream target genes.

Ectopic overexpression of either HDAC4 (Backs et al. 2006), HDAC5 (Bush et al. 2004; Vega et al. 2004; Zhang et al. 2002) or HDAC9 (Zhang et al. 2002) in cultured rat cardiomyocytes coordinately suppresses MEF2-dependent transcription and agonist-dependent cardiac hypertrophy. In contrast, disruption of the gene encoding *HDAC9* in mice leads to superactivation of cardiac MEF2 activity (Zhang et al. 2002), and mouse knockouts for *HDAC5* (Chang et al. 2004) or *HDAC9* (Zhang et al. 2002) develop exaggerated cardiac hypertrophy in response to pressure overload and spontaneous, pathologic hypertrophy with advancing age. These results support a role for class IIa HDACs as endogenous inhibitors of cardiac hypertrophy.

The ability of class IIa HDACs to block cardiac gene expression appears to be governed not only by direct association with MEF2, but also through indirect interactions with members of the serum response factor (SRF) (Davis et al. 2003; Xing et al. 2006), nuclear factor of activated T cells (NFAT) (Dai et al. 2005) and NK families of transcription factors (Song et al. 2006), all of which have been implicated in the control of pathological cardiac gene expression. In each case, class IIa HDAC binding to the transcription factor is governed by bridging cofactors. HDAC4 is coupled to NFAT by a mammalian relative of DnaJ (Mrj)

(Dai et al. 2005). HDAC5 interacts with SRF via myocardin (Davis et al. 2003; Xing et al. 2006), and CAMTA links HDAC5 to NKX2.5 (Song et al. 2006).

Although knocking out class IIa HDACs in the heart appears to be generally detrimental, in some cases class IIa HDAC deletion is beneficial. Removal of HDAC5 or HDAC9 in female mice results in protection from postmyocardial infarction (MI) remodeling due to enhanced estrogen receptor-mediated transcription of proangiogenic genes in the heart. Interestingly, however, male knockout mice succumb to MI at a higher frequency than male, wild-type littermates (van et al. 2010).

2.1 Extranuclear Roles for Class IIa HDACs in the Heart

Nontranscriptional roles for class IIa HDACs in the heart are also emerging. HDAC4 was shown to associate with cardiac sarcomeres and decrease myofilament calcium sensitivity by promoting deacetylation of muscle LIM protein (MLP) (Gupta et al. 2008). Consistent with this, HDAC inhibitor treatment increased calcium sensitivity of myofilaments from skinned fibers. It is not known whether deacetylation of MLP is governed by HDAC4 or another HDAC. Indeed, for many years it was believed that class IIa HDACs lacked intrinsic catalytic activity, because recombinant forms of the enzymes failed to deacetylate canonical HDAC substrates; catalytic activity of class IIa HDACs was attributed to associated class I HDACs (Fischle et al. 2002). However, subsequent work led to the identification of a synthetic substrate that is efficiently deacetylated by class IIa HDACs (Heltweg et al. 2004). Additional studies revealed that class IIa HDACs are relatively insensitive to commonly used HDAC inhibitors (Bradner et al. 2010), including those used in the contractility studies. As such, further investigation is needed to address the role of HDAC4 and other HDACs in the control of cardiac contractility, as well as the general role of class IIa HDAC catalytic activity in the heart.

2.2 Signaling to Class IIa HDACs in the Heart

Amino-terminal extensions unique to class IIa HDACs possess two conserved serine residues that are phosphorylated in response to stress signaling in the heart. Upon phosphorylation, these sites are bound by the intracellular chaperone protein 14-3-3, resulting in the activation of a cryptic, carboxy-terminal nuclear export sequence conserved in HDACs 4, 5, 7 and 9. 14-3-3 binding also appears to mask an internal nuclear localization signal in the HDACs (Grozinger and Schreiber 2000; Nishino et al. 2008). Phosphorylated class IIa HDACs are thus removed and excluded from cardiomyocyte nuclei, resulting in derepression of pathological downstream target genes (Fig. 2).

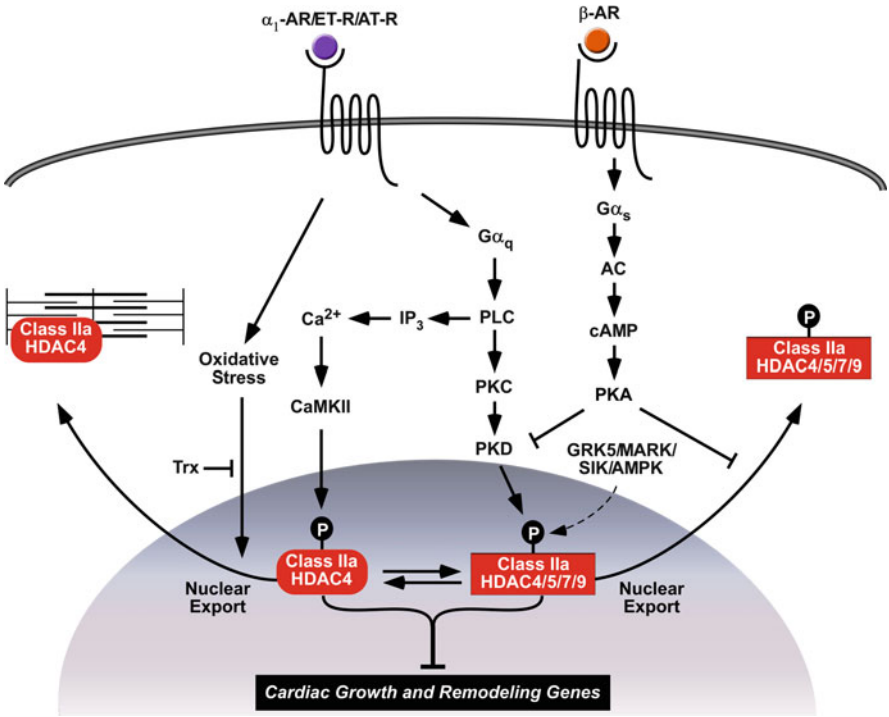

Fig. 2 Signal-dependent regulation of class IIa HDACs in cardiac myocytes. Genes under the control of class IIa HDACs are derepressed by signals that trigger phosphorylation-dependent nuclear export of the HDACs. $G_{\alpha q}$-coupled receptors, including the α_1-adrenergic receptor (α_1-AR), the endothelin receptor (ET-R) and the angiotensin receptor (AT-R) trigger class IIa HDAC phosphorylation by activating PKD. Inositol trisphosphate (IP3) produced by phospholipase C (PLC) can also trigger release of intracellular Ca^{2+}, with subsequent activation of CaMKII (Wu et al. 2006), which selectively phosphorylates HDAC4. Signaling through $G_{\alpha s}$-coupled β-adrenergic receptors (β-ARs) activates adenylyl cyclase (AC) and subsequent production of cAMP, which activates protein kinase A (PKA). PKA inhibits PKD and also phosphorylates unique sites on class IIa HDACs that result in inhibition of nuclear export. HDAC4 can heterodimerize (↔) with other class IIa HDACs and thereby transmit CaMKII signals to HDACs 5, 7 and 9. G protein-coupled receptor kinase-5 (GRK5), microtubule affinity-regulating kinase (MARK), salt-inducible kinase (SIK) and AMP-dependent protein kinase (AMPK) have also been demonstrated to phosphorylate class IIa HDACs on the sites that trigger nuclear export. Oxidative stress can promote phosphorylation-independent nuclear export of class IIa HDACs, and this is antagonized by thioredoxin (Trx). HDAC4 can associate with cardiac sarcomeres and has been implicated in the control of contractility

Signal-dependent nuclear export of class IIa HDACs occurs in cardiomyocytes exposed to diverse hypertrophic agonists that stimulate $G_{\alpha q}/G_{\alpha 11}$ signaling, including endothelin-1, angiotensin II, and the α_1-adrenergic agonist, phenylephrine (Harrison et al. 2004). Increased nuclear export of class IIa HDACs has also been observed in failing human heart explants (Calalb et al. 2009). Blockade of class IIa HDAC nuclear export through substitution of the phospho-acceptor sites with

nonphosphorylatable alanine residues creates a "superrepressor" that potently suppresses cardiac hypertrophy (Zhang et al. 2002), suggesting that it may be possible to manipulate cardiac disease with inhibitors of class IIa HDAC kinases.

2.2.1 PKD Is a Class IIa HDAC Kinase

Protein kinase D (PKD) has been shown to function as class IIa HDAC kinase that promotes pathological cardiac remodeling (McKinsey 2007). Three PKD isoforms (1, 2 and 3) make up a family of related serine/threonine kinases. PKD1 was discovered in 1994 and was also referred to as PKCμ due to the presence of amino-terminal diacylglycerol binding domains resembling those of PKC (Johannes et al. 1994; Valverde et al. 1994). However, the catalytic domain of PKD1 is divergent from those of PKC isoforms and more closely related to that of Ca^{2+}/calmodulin-dependent protein kinase (CaMK). Each PKD isoform is capable of phosphorylating all class IIa HDACs on the serines that are targeted by 14-3-3 (Huynh and McKinsey 2006; Parra et al. 2005; Vega et al. 2004), suggesting the potential for redundant control of class IIa HDACs by PKD family members. The antihypertrophic transcription factor, YY1, appears to suppress hypertrophy by physically associating with HDAC5 and preventing PKD-mediated phosphorylation of the HDAC (Sucharov et al. 2008).

In rodents, cardiac PKD is activated in response to chronic hypertension, pressure overload mediated by aortic constriction and infusion of agonists such as norepinephrine (Avkiran et al. 2008; Harrison et al. 2006; Haworth et al. 2000). Knockdown of endogenous PKD1 expression with siRNA blunts agonist-dependent nuclear export of class IIa HDACs and associated hypertrophy of cultured neonatal rat cardiac myocytes (Harrison et al. 2006). Conversely, ectopic overexpression of constitutively active PKD1 in these cells has been reported to induce hypertrophy (Iwata et al. 2005). In vivo, cardiac-specific expression of constitutively active PKD1 in mice causes a brief phase of cardiac hypertrophy, followed by chamber dilation and impaired systolic function (Harrison et al. 2006). These mice do not have significant fibrosis (Massare et al. 2010). Conversely, mice in which the *PKD1* gene was conditionally deleted in cardiac myocytes showed dramatically reduced cardiac hypertrophy and cardiac fibrosis in response to pressure overload or chronic administration of adrenergic or angiotensin receptor agonists (Fielitz et al. 2008).

In addition to class IIa HDACs, cardiac PKD has also been shown to phosphorylate the CREB transcription factor as well as sarcomeric proteins such as troponin I (Bardswell et al. 2010; Haworth et al. 2004; Ozgen et al. 2009). PKD may also have beneficial effects on the heart by preventing lipoprotein lipase driven accumulation of triglycerides in the diabetic state (Kim et al. 2008, 2009). However, with regard to heart failure, the genetic gain- and loss-of-function data described above suggest a pathological role for PKD signaling. Consistent with this, PKD is hyperactivated in ventricular cardiac myocytes from humans with heart failure (Bossuyt et al. 2008), and efficacy of an HMG-CoA reductase inhibitor in a hypertensive rat model of heart failure was linked to attenuation of PKD signaling

(Geng et al. 2010). Effects of small molecule inhibitors of PKD on cardiac growth have recently been described. PKD inhibition blocked agonist-dependent phosphorylation of class IIa HDACs and hypertrophy of cultured cardiac myocytes (Monovich et al. 2010), but failed to alter pathologic growth of the heart in vivo (Meredith et al. 2010a, b). Additional work with these and chemically distinct PKD inhibitors is required to fully address the potential therapeutic benefit of inhibiting PKD in the context of heart failure.

Studies by the Avkiran Lab recently elucidated a role for protein kinase A (PKA) as a negative regulator of PKD-mediated phosphorylation of class IIa HDACs in cardiac myocytes. β-Adrenergic receptor (β-AR) signaling, which stimulates cAMP production and downstream activation of PKA, blocks PKD activation in cardiomyocytes in response to α-adrenergic receptor signaling (Haworth et al. 2010). PKA also blocks PKD activation in response to endothelin stimulation (Haworth et al. 2007), and inhibition of cAMP-directed phosphodiesterases 3 and 4 is sufficient to antagonize PKD in myocytes (Haworth et al. 2010).

2.2.2 Other Class IIa HDAC Kinases

Additional kinases including salt-inducible kinase (SIK) (Berdeaux et al. 2007), microtubule affinity-regulating kinase (MARK) (Chang et al. 2005; Dequiedt et al. 2006) and AMP-dependent protein kinase (AMPK) (McGee et al. 2008) have been implicated as class IIa HDAC kinases. A connection between CaMKII and class IIa HDACs has also been described (Backs et al. 2006, 2008; Zhang et al. 2007), and G protein-coupled receptor kinase-5 (GRK5) was shown to translocate to the nucleus and phosphorylate HDAC5 in cardiomyocytes in response to $G_{\alpha q}$ signaling (Martini et al. 2008). This novel function of GRK5 is associated with enhanced cardiac remodeling due to pressure overload. All of these redundant pathways converge on the 14-3-3 target phospho-acceptors on class IIa HDACs. In contrast, protein kinase C-related kinase and Dyrk phosphorylate sites in or near the class IIa HDAC nuclear localization signal, and thereby impair nuclear import of the enzymes (Deng et al. 2005; Harrison et al. 2010).

Some signaling pathways appear to enhance the nuclear function of class IIa HDACs by blocking their export from the nucleus. In addition to suppressing PKD activity, PKA was also shown to directly phosphorylate HDAC5, resulting in repression of agonist-dependent nuclear export of the HDAC and suppression of cardiac hypertrophy (Ha et al. 2010). Thus, PKA utilizes dual mechanisms for suppressing class IIa HDAC nuclear export in cardiac myocytes.

A kinase-independent mechanism for regulation of class IIa HDAC nuclear export was also described (Ago et al. 2008; Oka et al. 2009). HDAC4 harbors two cysteine residues that become oxidized in response to hypertrophic stimuli. In the oxidized state, HDAC4 undergoes nuclear export. However, reduction of these cysteines through a thioredoxin-dependent mechanism results in inhibition of HDAC4 nuclear export. These findings suggest alternative therapeutic strategies based on inhibiting class IIa HDAC nuclear export via modulation of redox state.

3 Class IIb HDACs in the Heart

The function of class IIb HDACs in the heart remains largely unknown. HDAC6 is a microtubule deacetylase (Hubbert et al. 2002), and microtubules comprise a major component of the cardiomyocyte cytoskeleton. The accumulation of stable, polymerized microtubules is thought to contribute to myocardial dysfunction in cardiac hypertrophy and heart failure (Hein et al. 2000; Tagawa et al. 1997), and HDAC inhibition in cultured cardiomyoytes decreases the total amount of tubulin associated with polymerized microtubules (Davis et al. 2003).

As highlighted elsewhere in this book, HDAC6 also plays an important role in the regulation of autophagy (Lee et al. 2010b), and autophagy is emerging as an important regulator, both positive and negative, of heart failure (Rothermel and Hill 2008). It will be interesting to determine whether selective inhibition of HDAC6 affects heart failure progression in animal models through effects on tubulin stability, autophagy or other mechanisms.

HDAC10 is the other class IIb HDAC. HDAC10 has not been studied in the heart. However, knockdown of HDAC10 expression in cancer cells induces expression of an endogenous thioredoxin inhibitor, thioredoxin-interacting protein (Lee et al. 2010a). Given the fact that thioredoxin suppresses class IIa HDAC nuclear export (Ago et al. 2008), it is possible that HDAC10 will affect genes involved in cardiac hypertrophy.

4 Class I HDACs in the Heart

Transgenic and knockout mice have been employed to address the functions of class I HDACs in the heart. HDAC1 and HDAC2 appear to serve functionally redundant roles in the heart, as conditional cardiac deletions of either isoform produce no phenotype, while homozygous loss of both genes results in dilated cardiomyopathy, arrhythmia and neonatal lethality (Montgomery et al. 2007). *HDAC1* or *HDAC2* conditional null mice remain susceptible to isoproterenol- or pressure overload-induced cardiac hypertrophy, suggesting that these isoforms may serve redundant functions in the control of cardiac remodeling. Different results were obtained with mice harboring a *lacZ* insertion in the *HDAC2* locus (Trivedi et al. 2007). These mice were found to be viable and resistant to hypertrophic stimuli, perhaps indicating that the *lacZ* insertion produced a hypomorphic *HDAC2* allele rather than a true null. As a result of the discrepancy between data obtained with HDAC2 knockout and knock-in mice, the role of this HDAC isoform in heart failure remains in question.

Transgenic overexpression of HDAC2 in the mouse heart results in pathological cardiac hypertrophy (Trivedi et al. 2007). Studies of HDAC2 in cell culture suggest that it lies downstream of heat shock protein 70 in the control of cardiomyocyte hypertrophy (Kee et al. 2008). Interestingly, transgenic mice that overexpress

HDAC3 in the heart do not develop hypertrophy, but rather develop cardiomyocyte hyperplasia (Trivedi et al. 2008). Likewise, conditional cardiac deletion of HDAC3 results in profound cardiac hypertrophy and metabolic defects, providing additional evidence that this isoform plays a role in the heart that is distinct from HDAC1 and HDAC2 (Montgomery et al. 2008). HDAC8 null mice die shortly after birth due to skull instability (Haberland et al. 2009); the consequences of conditional HDAC8 deletion in the heart have not been described.

Brg1, the ATPase subunit of the BAF chromatin-remodeling complex, was recently shown to associate with HDAC2 on the promoter of the gene for α-myosin heavy chain, resulting in pathological repression of this gene during heart failure (Hang et al. 2010). Brg1 activity appears to be required for cardiac hypertrophy.

5 Class III HDACs in the Heart

Class III HDACs (sirtuins), which are NAD+ dependent, appear to serve protective functions in the heart. SIRT1 overexpression enhances the survival of cultured neonatal rat cardiomyocytes under conditions of serum starvation (Alcendor et al. 2004). In vivo, transgenic overexpression of moderate levels of SIRT1 in mouse heart protects against cardiac apoptosis and hypertrophy in response to aging and oxidative stress (Alcendor et al. 2007). In contrast, suppression of endogenous SIRT1 activity with a small molecule inhibitor of class III HDACs results in exaggerated apoptosis (Alcendor et al. 2004).

The mechanisms whereby class III HDAC activity protects cardiomyocytes from apoptosis appear to involve repression of p53 and increased expression of antioxidants, such as catalase, via induction of the FoxO transcription factor. In addition, class III HDACs target a prohypertrophic histone variant, H2A.z, for degradation by the ubiquitin-proteasome pathway (Chen et al. 2006). SIRT1 has been shown to associate with MEF2 via HDAC4 and deacetylate MEF2, which would be predicted to result in repression of downstream target genes that promote cardiac hypertrophy (Zhao et al. 2005).

SIRT3 and SIRT7 have also been implicated as cardioprotective factors. SIRT3 overexpression protects cultured cardiac myocytes from apoptosis by promoting deacetylation of Ku70 (Sundaresan et al. 2008). Hypo-acetylated Ku70 physically associates with Bax and neutralizes the proapoptotic function of this protein.

The most compelling evidence of a cardioprotective role for class III HDACs came from studies of SIRT7 deficient mice. *SIRT7* knockout mice develop cardiac hypertrophy with severe interstitial fibrosis, which is associated with massive apoptosis of cardiac myocytes (Vakhrusheva et al. 2008). Similar to SIRT1, SIRT7 appears to inhibit apoptosis by deacetylating and thus inhibiting the activity of p53.

Resveratrol is a polyphenol found in the skin of red grapes; it is capable of stimulating class III HDAC activity. Interestingly, resveratrol has been shown to inhibit cardiac hypertrophy (Chan et al. 2008; Cheng et al. 2004; Juric et al. 2007; Liu et al. 2005; Biala et al. 2010; Sulaiman et al. 2010), suggesting an approach to

heart failure therapy involving class III HDAC activation. However, it should be noted that many HDAC-independent functions of resveratrol have been described (Pirola and Frojdo 2008), and the antihypertrophic action of the compound was recently attributed to activation of AMP-activated Protein Kinase (AMPK) (Chan et al. 2008).

6 HDAC Inhibitors

Dysregulation of HDACs is associated with a variety of pathophysiological processes, including cancer, neurodegeneration and inflammation. As such, there is focus in the pharmaceutical industry and in academic labs on the development of novel small molecule inhibitors of HDACs, particularly since the first HDAC inhibitor reached the market in 2006 with the FDA approval of SAHA/vorinostat (Zolinza) for the treatment of cutaneous T cell lymphoma (Marks and Breslow 2007).

Most HDAC inhibitors possess a stereotypical three-part structure consisting of a zinc-binding "warhead" group that docks in the active site, a linker and a surface recognition domain that interacts with residues near the entrance to the active site. This general HDAC inhibitor pharmacophore is represented in at least four chemical classes: hydroxamic acids, short chain fatty acids, benzamides and cyclic peptides. Relative potencies and selectivity profiles differ between and within these classes (Bradner et al. 2010). The strong zinc-chelating properties of the hydroxamic acid warhead produce potent (low nanomolar) pan-HDAC inhibitors. In contrast, the short chain fatty acids are weak (millimolar) HDAC inhibitors, with perhaps modest selectivity towards class I HDACs. Benzamide HDAC inhibitors are generally highly selective for HDACs 1, 2 and 3, as are the cyclic peptides.

Compounds containing aryl substitutions on the benzamide warhead have recently been found to be highly selective for HDAC1 and HDAC2 over all other HDACs (Methot et al. 2008; Moradei et al. 2007; Wilson et al. 2008; Witter et al. 2008). Conversely, other benzamide scaffolds appear to be selective for HDAC3 (Chen et al. 2009). Facilitated by the solution of the human HDAC8 crystal structure (Vannini et al. 2004), selective hydroxamic acid inhibitors of this distinct class I HDAC subfamily member have recently emerged (Balasubramanian et al. 2008; Krennhrubec et al. 2007). Finally, the first known HDAC6/class IIb-selective inhibitor, tubacin, was described in 2003 (Haggarty et al. 2003). More recent compounds, such as tubastatin A (Butler et al. 2010), exhibit greater selectivity for HDAC6 than tubacin.

6.1 Efficacy of HDAC Inhibitors in Preclinical Models of Heart Failure

Since class IIa HDACs function as suppressors of cardiac hypertrophy, HDAC inhibitors were initially expected to promote hypertrophy. However, experiments

with cultured cardiac myocytes revealed that HDAC inhibitors effectively suppress myocyte hypertrophy (Antos et al. 2003). There are at least two explanations for these seemingly paradoxical findings. First, as mentioned above, the class IIa HDAC enzymatic assay revealed that these HDACs are relatively insensitive to standard HDAC inhibitors, including those used in the initial hypertrophy studies (Bradner et al. 2010). Second, it was determined that class IIa HDACs do not require catalytic activity to suppress hypertrophic signaling (Zhang et al. 2002).

Follow-up in vivo studies further validated the protective activity of HDAC inhibitors in the heart (Fig. 3). Treatment with the hydroxamic acid, pan-HDAC

In Vitro Activity (nM); *Ki, #IC_{50}

	TSA*	Scriptaid*	SAHA*	Api-D#	VPA#
Class I					
HDAC1	0.2	1.5	1.3	13	1584000
HDAC2	0.65	2.2	1.6	18	3068000
HDAC3	0.5	4.1	5.0	11	3071000
HDAC8	45	105	480	>1000	7442000
Class IIa					
HDAC4	1400	7500	—	>1000	1500000
HDAC5	260	1000	3600	>1000	1000000
HDAC7	195	2250	—	>1000	13000000
HDAC9	800	8000	—	N/A	>10000
Class IIb					
HDAC6	1	2.5	1.6	680	>20000000
HDAC10	N/A	N/A	N/A	N/A	>20000000
Class IV					
HDAC11	N/A	N/A	N/A	N/A	N/A
Pre-Clinical Cardiac Efficacy Models					
	TAC MI AngII ISO Hop-Tg (all mouse)	mouse TAC mouse MI	rat DOCA	mouse TAC	rat TAC rat MI rat AngII SHR mouse TAC

Fig. 3 In vitro and in vivo activity of HDAC inhibitors used in heart failure models. The indicated compounds have been tested in models of heart failure. The values represent activities of compounds against recombinant HDAC isoforms. Values (nM) for trichostatin A (TSA), scriptaid and SAHA are dissociation constants (K_i) (Bradner et al. 2010), while those for apicidin-derivative (Api-D) (Gallo et al. 2008) and valproic acid (VPA) (Gurvich et al. 2004; Khan et al. 2008) are half maximal inhibitory concentrations (IC_{50}). – no activity; *N/A* not available. The rodent heart failure models in which the compounds have shown efficacy are indicated. *TAC* transverse aortic constriction; *MI* myocardial infarction; *AngII* angiotensin II; *ISO* isoproterenol; *Hop-Tg* homeodomain only protein transgenic; *DOCA* deoxycorticosterone acetate; *SHR* spontaneously hypertensive rat

inhibitor, trichostatin A (TSA) or the short chain fatty acid, valproic acid, for 2 weeks blocked the development of cardiac hypertrophy in transgenic mice that overexpress an HDAC2-dependent SRF inhibitor, Hop (Kook et al. 2003). Similar 2-week regimens of pan-HDAC inhibitor treatment also effectively suppressed cardiac hypertrophy induced by continuous infusion of isoproterenol (Kook et al. 2003) or angiotensin II (Kee et al. 2006), as well as pressure-overload imposed by aortic constriction (Kee et al. 2006). Importantly, TSA treatment was also shown to regress established cardiac hypertrophy in mice subjected to aortic constriction (Kee et al. 2006), suggesting potential therapeutic benefit of HDAC inhibitors for pre-established heart failure. Of note, data obtained with valproic acid should be interpreted cautiously because this compound is a weak HDAC inhibitor (Gurvich et al. 2004; Khan et al. 2008) that is associated with a plethora of other pharmacological activities, including regulation of glycogen synthase kinase-3β, mitogen-activated protein kinases and ion channels (Terbach and Williams 2009).

Subsequent studies confirmed that 3 weeks of treatment with TSA and another pan-HDAC inhibitor, scriptaid, blunted cardiac hypertrophy in a pressure-overload mouse model, reducing cardiomyocyte cross-sectional area and significantly improving ventricular performance (Kong et al. 2006). The reduction in cardiac hypertrophy and functional improvements were maintained at 9 weeks, and TSA appeared to be well tolerated, because chronic administration over the course of the investigation did not adversely impact survival. Pan-HDAC inhibitors have also been shown to reduce maladaptive ventricular remodeling and improve cardiac performance in rodent models of myocardial infarction (Granger et al. 2008; Lee et al. 2007; Zhao et al. 2007), and in the setting of chronic hypertension in rats (Cardinale et al. 2010; Iyer et al. 2010). Valproic acid was recently shown to block right ventricular hypertrophy in response to pulmonary artery banding and monocrotaline-induced lung injury (Cho et al. 2010). However, as mentioned above, since valproic acid has many pharmacological activities, it is difficult to know whether the efficacy observed in these models was a consequence of HDAC inhibition.

It will be essential to determine the HDAC isoform(s) that promote pathological growth of the heart. As described above, although genetic studies suggested a role for HDAC2 in the process (Kee et al. 2008; Trivedi et al. 2007), the findings remain controversial (Montgomery et al. 2007). More definitive answers will likely come from the use of small molecule inhibitors of select HDAC isoforms. SK-7041, a hydroxamic acid HDAC inhibitor that is reportedly specific for class I HDACs, was shown to block hypertrophy in mice in response to aortic constriction and angiotensin II (Kee et al. 2006). However, independent evaluation of SK-7041 in vitro revealed that the compound is a pan-HDAC inhibitor (E.W. Bush and T.A. McKinsey, unpublished observations). More recently, an apicidin derivative, which is predominantly selective for class I HDACs 1, 2 and 3, was shown to effectively suppress hypertrophy and improve cardiac performance in the setting of pressure overload (Gallo et al. 2008). However, this compound appeared to exhibit activity, albeit modest, against HDAC6 in vitro. An essential next step is to extend these findings by testing benzamide HDAC inhibitors that inhibit class I HDACs but are devoid of HDAC6 inhibitory activity, and newer generations of HDAC1/

2-, HDAC3-, HDAC6- and HDAC8-selective compounds in animal models of pathological cardiac remodeling.

6.2 Translating Preclinical Findings with HDAC Inhibitors to the Clinic

Based on preclinical studies in animal models, advancement of an HDAC inhibitior into clinical trials for heart failure is warranted. A small, Phase IIa proof-of-concept trial would be ideal, perhaps with patients suffering from heart failure with preserved ejection fraction (HFpEF). HFpEF is a particularly attractive indication for HDAC inhibitors because it is characterized by myocyte hypertrophy and interstitial fibrosis, two processes that are highly sensitive to HDAC inhibition. Furthermore, there are no FDA-approved drugs for HFpEF, and current standards-of-care for systolic heart failure provide little benefit to patients with this condition (Borlaug 2009).

Nonetheless, the therapeutic benefit of HDAC inhibitors must be carefully weighed against their potential for causing toxicity. In the context of cancer, pan-HDAC inhibitors are currently regarded as effective and generally well-tolerated chemotherapeutics. Beyond nausea and fatigue, hematologic toxicity and QT prolongation have been reported with HDAC inhibitor treatment (Prince et al. 2009). Pan-HDAC inhibition can produce transient thrombocytopenia and in some instances, myelosuppression. While the class I HDAC-selective benzamides, MS-275 and MGCD0103, were notably free from significant hematologic toxicity in Phase I (Garcia-Manero et al. 2008; Gojo et al. 2007; Gore et al. 2008; Hauschild et al. 2008; Ryan et al. 2005; Siu et al. 2008), thrombocytopenia was observed with CI-994, a third class I HDAC-selective HDAC inhibitor in development (Prakash et al. 2001). Importantly, a recent study with mice in which HDAC1 and HDAC2 were conditionally deleted in bone marrow revealed that these HDACs function redundantly to control differentiation of the megakaryocyte–erythrocyte cell lineage; double knockout mice suffered from severe anemia and thrombocytopaenia (Wilting et al. 2010). These results confirm that the hematological toxicity observed with HDAC inhibitors is mechanism based (i.e., controled by HDAC inhibition as opposed to an off-target activity of the compounds).

QT prolongation has been reported as a dose-limiting toxicity in trials with pan-HDAC inhibitors (Badros et al. 2009; Galli et al. 2009; Giles et al. 2006; Piekarz et al. 2006). Though debate continues as to whether QT prolongation can be considered a class effect of pan-HDAC inhibitors, it should be noted that Phase I and Phase II trials of MS-275, MGCD0103 and CI-994 have not reported this toxicity (Garcia-Manero et al. 2008; Gojo et al. 2007; Gore et al. 2008; Hauschild et al. 2008; Nemunaitis et al. 2003; Pauer et al. 2004; Prakash et al. 2001; Ryan et al. 2005; Siu et al. 2008; Undevia et al. 2004).

Overall, emerging clinical data support the concept that isoform-selective HDAC inhibitors are safer than pan-HDAC inhibitors. However, it is important

to note that clinical experience with isoform-selective HDAC inhibitors is quite limited. Thus, safety and efficacy studies with HDAC1/2-, HDAC3-, HDAC6- and HDAC8-selective compounds will be particularly enlightening.

7 Conclusions

The past 10 years of research have significantly advanced our understanding of the functions and modes of regulation of HDACs in the heart. The next wave of discovery will likely focus on translating these basic, mechanistic findings into novel therapeutics for heart failure. This process would be greatly facilitated by the discovery of the pathological HDAC(s) in the heart. Genetic studies have implicated HDAC2 as a positive regulator of heart failure, but the results remain equivocal. Assessing the activity of new generations of isoform-selective HDAC inhibitors in animal models of heart failure will likely provide more definitive answers. Furthermore, these pharmacology studies will establish whether isoform-selective HDAC inhibition widens the therapeutic index to a level that is acceptable for the treatment of a chronic indication such as heart failure.

Once the pathological HDAC(s) have been identified, it will be important to determine how it regulates heart failure. Does it simply repress cardioprotective genes via deacetylation of histones, or does it deacetylate nonhistone substrates to altered cardiac signaling? Finally, it remains to be determined whether the beneficial effects of HDAC inhibitors in the heart are mediated solely by effects on myocytes or involve nonmyocytes as well. In this regard, it seems likely that the profound efficacy of HDAC inhibitors in models of heart failure is due to the ability of the compounds to affect multiple cells types (e.g., myocytes, fibroblasts, epithelial cells, inflammatory cells) and pathological mechanisms (e.g., myocyte hypertrophy, inflammation, apoptosis, autophagy and fibrosis). This next phase of research on HDACs in the heart should set the stage for clinical assessment of HDAC inhibitors in patients with cardiovascular disease.

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HDACs in Skeletal Muscle Remodeling and Neuromuscular Disease

Bryan J. Simmons, Todd J. Cohen, Richard Bedlack, and Tso-Pang Yao

Contents

Abstract Skeletal muscle is made of heterogeneous myofibers with different contractile and metabolic properties. The diverse functionality of myofibers enables skeletal muscle to carry out different tasks from maintaining body posture to performing active movements. In addition to motility, skeletal muscle, which constitutes 40% of body mass, is also a key target of insulin action and performs an essential function in glucose metabolism. Adult skeletal muscle is a highly adaptive organ system and can undergo specific changes in contractile and metabolic properties to meet different functional demands. This plasticity of myofibers reflects a highly

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coordinated change in gene expression program that is controlled by neural activity. The capacity for on-demand remodeling confers skeletal muscle the remarkable adaptability important for animal survival; its dysregulation, however, could contribute to muscle and metabolic diseases. How neural activity dictates transcriptional programming to modify muscle functionality and diversity is a fundamental issue. Recent studies have identified members of class IIa HDACs as important effectors in both physiological and pathological muscle remodeling. By way of modifying myofiber properties, pharmacological manipulation of IIa HDACs activity could have potential therapeutic utility in the treatment of muscle disorders.

Keywords Amyotrophic lateral sclerosis • HDAC • HDAC inhibitor • HDAC4 • Muscle atrophy • Muscle remodeling

1 Skeletal Muscles Are Heterogeneous in Contractile and Metabolic Properties

Skeletal muscle is composed of myofibers and connective tissue with satellite cells scattered along the myofibers. Muscle fibers can be broken down into myofibrils and subsequently myofilaments. Myofilaments, the contractile components of the myofiber, are composed of myosin and actin filaments. Together, actin and myosin form the functional subunit of myofibrils called the sarcomere. During contraction, actin filaments act as scaffolding while myosin filaments equipped with motor subunits crawl along the actin filaments resulting in shortening of the sarcomere and muscle contraction.

Within a given muscle, myofibers differ in the diameter, color, contractile speed, and metabolic profile. Historically, these differences have led to the classification of myofibers into three types: type I (slow-oxidative), type IIa (fast-oxidative), and type IIb (fast-glycolytic). Muscle fiber type is determined at the transcriptional level by the expression of unique sets of contractile proteins and metabolic enzymes. Type I fibers express myosin heavy chain 7 (MYH7), which has a slow velocity of contraction, but is highly resistant to fatigue. These fibers appear small and red due to their high concentrations of mitochondria and myoglobin. This selective enrichment dictates that oxidative metabolism serves as the main source of energy for type I fibers. In contrast, type IIb fibers express myosin heavy chain 4 (MYH4), which have an intrinsically high contractile speed, but are easily fatigued. These fibers are large, white, and rely upon glycolytic metabolism for energy. Intermediate to type I and type IIb, type IIa fibers express myosin heavy chain 2 (MYH2) and have a relatively high oxidative capacity and contractile speed. In addition to contractile properties, myofibers also differ in their role in glucose metabolism with slow-oxidative fibers being more responsive to insulin than fast-glycolytic fibers. On the metabolic end, the differential expression of the transcriptional coactivator PGC1α, which promotes mitochondrial biogenesis, likely plays a critical role (Al-Khalili et al. 2005). PGC1α is expressed at a higher level in mitochondrion-rich slow-oxidative fibers than in fast-glycolytic fibers. Thus, the

composition of myofibers is not only critical for performing different movements but also important for glucose metabolism.

2 Specification of Myofibers

The proper specification of myofibers is essential for body movement, endurance, and metabolic regulation. Myofibers adopt their specific contractile and metabolic properties depending upon the pattern of excitation by motor neurons. These properties are best illustrated by the classical cross-innervation studies where reinnervation of slow-oxidative muscle by motor neurons normally innervating fast-glycolytic fibers are able to induce a slow-oxidative to fast-glycolytic change (Buller et al. 1960). Motor neurons that stimulate myofibers at tonic, low frequencies (10–20 Hz) result in a slow-oxidative phenotype, while those stimulating myofibers at phasic, high frequencies (100–150 Hz) results in a fast-glycolytic phenotype (Chin and Allen 1996; Hennig and Lomo 1985). Thus, the innervating motor neuron determines the pattern of excitation and thus myofiber type.

Importantly, the adult myofiber phenotype is not permanent. The size, contractility, and metabolic properties of myofibers can all undergo changes or remodeling [reviewed in Bassel-Duby and Olson (2006)]. For example, reduced neuromuscular activity caused by inactivity, aging or neuromuscular disease can lead to reduction in muscle size, termed atrophy. Inactivity also causes an increase in fast-glycolytic fibers. Conversely, repetitive use such as aerobic exercise induces muscle hypertrophy and a concomitant increase in slow-oxidative fibers. This plasticity, which is central to the physiological adaptation, is necessary for skeletal muscle to meet changing functional demands and perform different tasks. On the other hand, the pathological remodeling of skeletal muscle could lead to serious health problems, such as a loss of muscle mass, termed muscle atrophy (Fig. 1).

3 Calcium Signaling and Activity-Dependent Muscle Remodeling

The remarkable adaptability of skeletal muscle is achieved by a highly regulated gene transcription program responsive to differential neuromuscular activity (Fig. 1). In muscle, neural activity is converted into unique calcium transients. The nature and oscillations of intracellular Ca^{2+} levels specified by neural inputs then determines myofiber type and size by activating specific transcriptional programs. Two sets of Ca^{2+}-sensitive enzymes, calcium/calmodulin-dependent protein kinase (CaMK) and calcineurin phosphatase (PP2B), play major roles in mediating neural activity-dependent fiber specification and remodeling [reviewed in Bassel-Duby and Olson (2006)]. Slow frequency oscillations in intracellular calcium have been proposed to activate calcineurin. Activated calcineurin then dephosphorylates

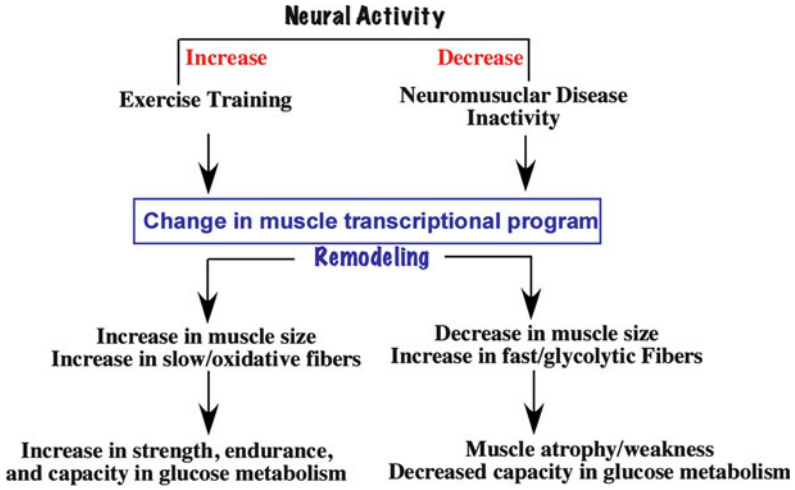

Fig. 1 Skeletal muscle remodeling under physiological and pathological conditions

NFAT transcription factors, leading to transcription of several slow fiber-specific genes (Chin et al. 1998). However, the calcineurin–NFAT axis is clearly not sufficient to specify the entire slow fiber-type specific transcription program [reviewed in Spangenburg and Booth (2003)]. The second transcription pathway involving CaMK and myocyte enhancer factor-2 (MEF2) transcription is the key determinant of fiber specification in response to neural activity.

Members of MEF2 are MADS box-containing proteins initially identified as transcription factors that cooperate with MyoD-related myogenic factors to promote skeletal muscle differentiation (Molkentin et al. 1995). Alone, MEF2 is unable to initiate transcription of myogenic genes (Molkentin et al. 1995). Nonetheless, MEF2 DNA-binding sites are found flanking numerous skeletal muscle-specific genes, supporting a critical role for MEF2 in the muscle transcription program (Black and Olson 1998). In *Drosophila*, a single MEF2 exists and its mutation prevents myoblast differentiation and muscle formation (Lilly et al. 1995). In vertebrates, there are four MEF2 genes (A–D) in skeletal muscle. Studies of transgenic mice carrying a tandem MEF2 binding element-driven LacZ reporter revealed that MEF2 is active in all embryonic muscles, consistent with its role in promoting myogenesis. In contrast, in adult muscle this reporter is selectively active in soleus muscle, which primarily consists of slow-oxidative fibers, and largely inactive in muscle consisting of fast-glycolytic fibers, such as extensor digitorum longus (EDL) (Wu et al. 2000). Interestingly, the MEF2–LacZ reporter can be activated in EDL if muscles are subject to prolonged motor nerve stimulation or exercise training, a regimen that promotes a fast-glycolytic to slow-oxidative fiber transition (Wu et al. 2000, 2001). This elegant reporter study reveals that MEF2 is selectively more active in adult muscle of slow/oxidative fibers and this activity can be regulated by neural inputs. In agreement with this supposition, the soleus-specific expression of slow myosin light chain (MLCslow) requires both

MEF2-binding element in its promoter and proper innervation. Surgical denervation abrogates both MLCslow expression and MEF2 activity in soleus (Esser et al. 1999). These findings suggest an instructive role for MEF2 in specifying myofiber phenotype controlled by neural inputs.

Fiber-type-selective MEF2 activity is established in a posttranslational manner. MEF2 protein levels are similar in different muscle types. The control of fiber-specific MEF2 activity likely involves both calcineurin and CaMK family members, which positively regulate MEF2 transcriptional activity (Wu et al. 2000). In mice, transgenic expression of a constitutively active CaMKIV in skeletal muscle dramatically induces mitochondrial biogenesis consistent with a slow-oxidative fiber phenotype (Wu et al. 2002). While CaMKIV is not normally expressed in skeletal muscle, other CaMK members could play an important role in muscle fiber-type specification. Among them, CaMKII activity is uniquely sensitive to the frequency of calcium oscillation and can be activated by depolarization in response to neural inputs (De Koninck and Schulman 1998). Consistent with its role in relaying neural activity, both *Drosophila* and mammalian CaMKII are present at the neuromuscular junctions (NMJ), the specialized synapses formed between motor neuron axons and myofibers (Koh et al. 1999). In principle, CaMKII would decode differential neural activities, phosphorylate specific substrates, and modify MEF2-dependent muscle gene expression programs. In this context, the CaMKII substrates would be important components that control activity-dependent muscle remodeling.

4 Class IIa Histone Deacetylases Are Potent Regulators of MEF2

Members of the histone deacetylase (HDAC) family have emerged as critical downstream targets of CaMKII in response to neural activity. Specifically, the class IIa HDAC subfamily, which includes HDAC4, HDAC5, HDAC7 and HDAC9, has been intimately linked to MEF2 activity. All class IIa HDACs bind and inhibit MEF2 transcriptional activity (Sparrow et al. 1999; Lu et al. 2000a, b; Miska et al. 1999; Wang et al. 1999; Lemercier et al. 2000). They share a common domain structure: an N-terminus coiled-coil domain and a C-terminus deacetylase domain (Fig. 2). The N-terminus binds MEF2, transcriptional corepressors, and SUMO-conjugating enzyme Ubc9 (Verdin et al. 2003; Zhang et al. 2002a). Most studies regarding class IIa HDACs in skeletal muscle development has revolved around HDAC4 and HDAC5. HDAC4 and HDAC5 are highly homologous, sharing 51% identity and 63% similarity in their amino acid sequence (Grozinger et al. 1999). Both are potent inhibitors of MEF2-dependent transcription. Indeed, overexpression of HDAC4 or -5 prevents cultured myocytes from differentiating into myotubes (Lu et al. 2000a). However, this antidifferentiation activity probably does not reflect the physiological function of HDAC4 or -5 in skeletal muscle.

HDACs have been well characterized as transcriptional corepressors. It is widely accepted that recruitment of HDACs by DNA-binding transcription factors leads to

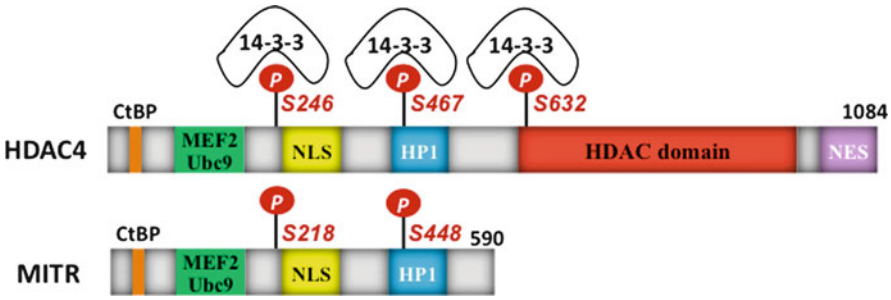

Fig. 2 The domain structure of class IIa HDAC members. Serine residues subject to phosphorylation (P) and 14-3-3 binding are *marked*. The domains responsible for binding CtBP, MEF2, Ubc9, and HP1 are color coded. *NLS* Nuclear localization signal; *NES* Nuclear export signal

histone deacetylation at the promoter region, resulting in transcription repression. This simple model, however, does not apply to class IIa HDACs, as the deacetylase domain is dispensable for repressing MEF2 activity (Zhang et al. 2001a). The conserved N-terminus is all that is required for class IIa HDACs to bind and repress MEF2 activity. In fact, Fischle et al. first reported that HDAC4 lacks intrinsic histone deacetylase activity (Fischle et al. 2002), a conclusion subsequently supported by the structural analysis of the catalytic domain of HDAC4 (Lahm et al. 2007; Bottomley et al. 2008). When ectopically expressed in cells, HDAC4 and -5 were found to associate with a class I deacetylase, HDAC3 (Grozinger et al. 1999). An extensive examination of the HDAC4–HDAC3 interaction has led to a conclusion that HDAC3, and not HDAC4, is responsible for the histone deacetylase activity assigned to purified HDAC4 (Fischle et al. 2002). The detailed nature of the HDAC4–HDAC3 complex remains to be established but it requires N-CoR/SMRT, related transcriptional cofactors that can stimulate HDAC3 deacetylase activity by a chaperone-like activity (Guenther et al. 2001). HDAC3 and N-CoR/SMRT exist in a native multiprotein corepressor complex that is responsible for the transcriptional repressive activity of unliganded retinoic acid receptor and thyroid hormone receptor (Yoon et al. 2003; Wen et al. 2000). Thus, it is possible that HDAC4 interacts with the HDAC3/N-CoR/SMRT complex to repress MEF2 transcription activity. However, it should be noted that endogenous HDAC4 was not reported in the HDAC3/N–CoR/SMRT complex (Yoon et al. 2003; Wen et al. 2000). Conversely, HDAC3 was not found in the native HDAC4 complex (E. Seto, unpublished observation). Whether HDAC3 is an obligatory cofactor for HDAC4 and other class IIa HDAC to repress MEF2 transcriptional activity remains to be established.

Two additional mechanisms could account for the deacetylase-independent activity of class IIa HDACs. In a yeast two hybrid study, HDAC4 and the N-terminus splice variant of HDAC9, MEF2-interacting transcription repressor (MITR), were found to interact with HP1, a heterochromatin-associated protein involved in transcriptional silencing. HP1 binds lysine 9-methylated histone H3, which typically marks transcriptional repressive region of the chromatin (Zhang et al. 2002a). Indeed, histones (H3) in chromatin flanking myogenic genes are highly

methylated at K9 in undifferentiated, proliferating myoblasts, but become demethylated upon myocyte differentiation (Zhang et al. 2002a). The recruitment of HP1 by class IIa HDAC could silence MEF2-dependent transcription.

The conserved N-terminus of HDAC4 also binds Ubc9, an E2-conjugating enzyme for small ubiquitin-like molecule, SUMO (Gregoire and Yang 2005). By simultaneously interacting with Ubc9 and MEF2, HDAC4 promotes MEF2 sumoylation (Gregoire and Yang 2005; Zhao et al. 2005). In a reconstituted in vitro system, recombinant HDAC4 can directly stimulate MEF2 sumoylation (Zhao et al. 2005). HDAC4 therefore acts as SUMO E3 ligase for MEF2, whose sumoylation results in a loss of transcription activity. MEF2 is sumoylated at lysine 424, one of the lysine residues subject to acetylation by the transcriptional coactivator p300. The coupled HDAC4-dependent deacetylation and sumoylation at K424 unravels an efficient mechanism to inhibit MEF2 transcriptional activity. Interestingly, HDAC4 does not deacetylate MEF2 directly (Zhao et al. 2005); rather it recruits SIRT1, a member of the sirtuin deacetylase family, to catalyze MEF2 deacetylation (Zhao et al. 2005). As SIRT1 is regulated by nutrient and metabolic status, this finding suggests that HDAC4 and SIRT1 might work in conjunction to regulate MEF2 activity in skeletal muscle in response to nutrient availability and change in metabolic demands. In addition to SIRT1, Gregoire et al. showed that HDAC3 is able to bind and deacetylate MEF2 (Gregoire et al. 2007). It is also possible that a potential complex of HDAC4 and HDAC3 might work in tandem to control MEF2 acetylation and sumoylation.

5 Class IIa HDACs Are Signal-Responsive Deacetylases

The potent repressive activity of class IIa HDACs on MEF2 dictates that these deacetylases must be subject to strict regulation to protect skeletal muscle. Studies in cultured cells showed that all class IIa HDAC members are regulated by phosphorylation and intracellular trafficking. Phosphorylation of several conserved serine residues in the N-terminus creates docking sites for the 14-3-3 protein (Fig. 2) (Grozinger and Schreiber 2000; Wang et al. 2000). Binding of 14-3-3 to HDAC4 and -5 causes their dissociation from MEF2 and exposes the nuclear export sequence (NES) for CRM1, leading to their nuclear export (Wang and Yang 2001). Cytoplasmic retention of HDAC4 and -5 releases nuclear MEF2 from their inhibitory effect (Miska et al. 1999; Zhao et al. 2001; Grozinger and Schreiber 2000). The most well-characterized kinase pathway that regulates class IIa HDAC phosphorylation is via CaMK. The combined effects of CaMK-mediated phosphorylation on both MEF2 dissociation and HDAC nuclear export explains how CaMK stimulates MEF2 transcriptional activity and reverses the inhibitory effect of class IIa HDACs upon MEF2 (McKinsey et al. 2002; Wu et al. 2000; Naya et al. 1999).

However, not all class IIa HDACs share the same subcellular compartment under the identical cultured condition, revealing that their intracellular trafficking is regulated differently. For example, HDAC4 and -5, despite their extensive

sequence homology, often display different subcellular localization patterns. In undifferentiated myoblasts, HDAC4 localizes predominately to the cytoplasm as a result of phosphorylation and 14-3-3-mediated nuclear export; however upon differentiation into myotubes, significant portions of HDAC4 accumulate in the nucleus (Zhao et al. 2001). The prominent nuclear translocation implies a regulatory function for HDAC4 in differentiated myofibers. In contrast, HDAC5 was reported to reside predominately in the nucleus of undifferentiated myoblasts but was exported to the cytoplasm upon differentiation, an observation consistent with an inhibitory role for HDAC5 in muscle differentiation (McKinsey et al. 2000). It should be noted that HDAC5 remains largely nuclear in terminally differentiated myotubes (Zhao et al. 2001). The biological significance of this differential localization of HDAC4 and HDAC5 is not yet understood but correlates with their differential affinity for CaMKII. HDAC4 but not HDAC5 is preferentially bound and phosphorylated by CaMKII (Backs et al. 2006). The differential affinity could underlie a more prominent activity of HDAC4 in skeletal muscle transcription remodeling (Cohen et al. 2007), a process thought to be regulated by CaMKII.

In addition to CaMKII, other kinases for class IIa HDACs have been identified and likely operate in response to different physiological cues. For example, AMP-activated kinase (AMPK), the critical sensor for intracellular metabolic state, can phosphorylate HDAC5 and lead to its nuclear export (McGee et al. 2009). An increase in AMP (energy deficiency) caused by exercise could activate AMPK, leading to HDAC5 phosphorylation and MEF2 activation. Even the regulation of HDAC4 phosphorylation in actively contracting and resting myofibers involves different kinases (Shen et al. 2006). When stimulated, HDAC4 nuclear efflux falls under the control of CaMKII; however, under resting conditions, CaMKII is unable to account for HDAC4 nuclear export (Shen et al. 2006). Instead, nuclear export under resting conditions is likely controlled by another kinase, such as PKD or PKC isoforms. It should be noted that other kinases have been implicated in phosphorylating class IIa HDACs including Mirk/dyrk1B and salt-inducible kinase (SIK) (Deng et al. 2005; Matthews et al. 2006; van der Linden et al. 2007). The physiological relevance of these phosphorylation events in skeletal muscle, in most cases, remains to be established. It is clear that despite their extensively shared biochemical activities, class IIa HDAC members are subject to differential regulation. The involvement of multiple kinases also indicates that HDAC4 and related deacetylases could integrate multiple signaling events and modify muscle properties accordingly.

6 Class IIa HDAC in Neural Activity-Dependent Muscle Remodeling

A connection of class IIa HDACs to neural activity was established by the observation that HDAC4 mRNA as well as protein is dramatically induced in skeletal muscle subject to surgical denervation (Cohen et al. 2007). To a lesser extent, HDAC5 is also induced whereas MITR expression is modestly repressed (Cohen

et al. 2007; Mejat et al. 2005). While an extreme condition, surgical denervation has served as a useful model to investigate how skeletal muscle responds to a loss of neural input. Accumulation of HDAC4 is clearly observed in the nucleus of denervated muscle (Cohen et al. 2007), which could contribute to a decrease in MEF2 activity upon denervation. This neural activity-dependent transcription and subcellular localization of HDAC4 opens a possibility that members of class IIa HDACs might play an important role in skeletal muscle remodeling controlled by neural activity.

After embryonic development, myofibers retain the ability to change their properties, including shape, size, and metabolic profile. For instance, during aerobic exercise, myofibers transition from type IIb to type IIa, favoring oxidative metabolism to meet functional demands. These myofibers also increase in size, termed hypertrophy. In contrast, chronically reduced neural activity associated with neuromuscular disease, inactivity, or aging can lead to debilitating loss of muscle mass (atrophy) and a switch from oxidative fibers to glycolytic fibers, a condition that could contribute to insulin resistance. Neural input also affects synaptic gene expression and organization of neuromuscular synapses. These changes reflect alterations in gene transcription influenced by neural input. Class IIa HDACs have emerged as the critical link that connects neural signaling to all three key aspects of muscle reprogramming: fiber type specification, synaptic gene expression, and muscle fiber size regulation.

7 HDACs in Fiber Type Specification

Inactivation of HDAC genes in mouse models have been performed for all class I and class IIa HDACs. HDAC4-null mice die shortly after birth with labored breathing due to abnormal ossification of the rib cage (Vega et al. 2004), whereas HDAC5- and HDAC9-null mice are viable, but develop cardiac hypertrophy due to increased responsiveness to calcineurin-mediated MEF2 transcriptional activation (Zhang et al. 2002b; Chang et al. 2004). In HDAC4, HDAC5, and HDAC9-null mouse models, there was no report of any grossly abnormal skeletal muscle phenotype (Potthoff et al. 2007a). However, analysis of class IIa HDAC mutant mice revealed that a combined inactivation of any two class IIa HDACs (HDAC4, -5, or -9) lead to an increase in type I slow-oxidative muscle fiber composition while skeletal muscle development proceeds normally (Potthoff et al. 2007a). These findings reveal that class IIa HDACs play an instructive but redundant role in muscle fiber type specification. The aberrant induction of type I fibers in class IIa HDAC mutant skeletal muscle could explain the observation that MEF2 is more active in slow-oxidative fibers than fast-glycolytic fibers. Potthoff et al. further reported that class IIa HDACs are selectively targeted for degradation in slow-oxidative fibers, resulting in greater MEF2 activity (Potthoff et al. 2007a). However, in a report by Cohen et al., no apparent difference in HDAC4 levels was observed in soleus (oxidative) vs. tibialis anterior (TA) (glycolytic) muscle.

Interestingly, the apparent mobility of HDAC4 is slower in oxidative fibers than in glycolytic fibers, suggesting a differential modification (Cohen et al. 2007). This mobility shift of HDAC4 is associated with hyperphosphorylation on serine 467, a major target of CaMKII (Backs et al. 2006). The latter result indicates that HDAC4 is differentially phosphorylated, possibly by CaMKII, in myofibers of different contractility and metabolic properties. Indeed, in cultured skeletal muscle fibers, repetitive slow fiber-type electrical stimulation, but not fast fiber type stimulation, induced HDAC4 phosphorylation and translocation from the nucleus to the cytoplasm in a CaMK-dependent manner (Liu et al. 2005). Similarly, the exercise-induced myofiber transition from type IIb to type IIa involves a concurrent activation of MEF2-dependent transcription and nuclear export of HDAC4 and HDAC5, a process that could involve both AMPK and CaMKII (McGee et al. 2009). Supporting this conclusion, when a HDAC5 mutant resistant to phosphorylation is expressed in skeletal muscle, exercise-induced fiber-type switching from fast-glycolytic to slow-oxidative fibers is suppressed. Thus, activity-dependent phosphorylation, subcellular localization and/or degradation of class IIa HDAC members could all contribute to myofiber specification and remodeling.

8 HDACs in Synaptic Gene Expression

The NMJ is a specialized synapse between the motor neuron and myofiber. As the motor axon nears the myofiber, it divides into multiple terminal boutons where vesicles filled with neurotransmitter acetylcholine (ACh) are released upon firing of the motor neuron. Across the synaptic cleft, the myofiber increases its surface area by developing elaborate junctional folds with a high concentration of acetylcholine receptors (AChRs). This spatial concentration of AChRs at the NMJ but not other surface areas of the myofiber is key to efficient neural–muscular communication. The expression of AChRs in skeletal muscle is strictly controlled by neural activity. In normally innervated muscle, transcription of AChR and other synaptic genes is actively repressed in all nuclei but those directly underlying the NMJs (subsynaptic nuclei). Spatially restricted expression of AChR contributes to its concentration at the NMJ. This spatially restricted expression is rapidly reversed upon loss of neural input by denervation, resulting in robust induction of AChR and other synaptic genes throughout the muscle by myogenin transcription factor. The signaling cascade that leads to synaptic gene re-expression in denervated muscle is controlled by HDAC4 (Cohen et al. 2007). Upon denervation, the induction of HDAC4 represses the transcription of Dachschund-related transcriptional corepressor 2 (Dach2), an inhibitor of myogenin transcription (Tang and Goldman 2006). Consequently, denervation induces myogenin transcription, which in turn activates synaptic genes. The activity-responsive HDAC4–Dach2–myogenin axis therefore plays a critical role in regulating the transcription of synaptic genes in response to differential neural inputs. It should be noted that siRNA analysis showed that

HDAC4 plays a more dominant role than HDAC5 in the regulation of synaptic gene transcription (Cohen et al. 2007).

The HDAC9 isoform, MITR, has also been linked to synaptic gene expression (Mejat et al. 2005). MITR was initially identified as MEF2-interacting transcriptional repressor (Sparrow et al. 1999). It turns out to be a N-terminus splice variant of HDAC9 lacking the C-terminus deacetylase domain. This finding provides the first evidence that the deacetylase domain of class IIa HDACs is not required for repressing MEF2 activity. Although both MITR and HDAC4 act as potent MEF2 repressors, MITR seems to oppose the role of HDAC4 in synaptic gene expression (Mejat et al. 2005). Whereas HDAC4 induces AChR transcription in denervated muscle (Cohen et al. 2007), MITR represses it (Mejat et al. 2005). It was proposed that MITR repressed AChR expression by recruiting HDAC1 and -3 in order to catalyze histone (H3) deacetylation near myogenic genes. The distinct roles of HDAC4 and MITR/HDAC9 in synaptic gene expression are mirrored by their opposite responses to denervation. As a target gene of MEF2 (Haberland et al. 2007), HDAC9 expression is repressed in denervated muscle (Mejat et al. 2005). Coordinated induction of HDAC4 and repression of MITR would ensure the transcriptional induction of synaptic genes in muscle that lost neural input. How HDAC4 and MITR display opposite activity on synaptic gene transcription is not understood. Given their similar repressive activity on MEF2-dependent transcription, MEF2 is not likely responsible for the transcriptional regulation of synaptic genes. An interesting possibility would be that the catalytic domain of HDAC4, which is lacking in MITR, might play a positive role in instructing the induction of synaptic genes in response to denervation.

HDAC4 is also found concentrated at the NMJ (Cohen et al. 2007). This localization suggests a possibility that HDAC4 might regulate neuromuscular synapses. The most compelling evidence regarding the role of HDAC4 in the motor neuron–myofiber interaction came from the analysis of a micro RNA, miR-206 (Williams et al. 2009). miR-206 is induced in denervated muscle and . In miR-206-deficient mice, reinnervation of the tibialis anterior (TA) muscle following nerve injury is delayed by nearly a week. Based upon computational analysis, HDAC4 is predicted to be a target of miR-206. Indeed, mice carrying a genetic deletion of HDAC4 in skeletal muscle display more rapid reinnervation of myofibers after nerve injury (Williams et al. 2009). It was proposed that HDAC4 affects reinnervation by inhibiting retrograde neurotrophic signaling via fibroblast growth factor 7 (FGF-7), which supports and directs growth of the motor neuron. HDAC4 also regulates the production of large number of cytokines in denervated muscle (TJC, MCC, and TPY, unpublished). It is probable that HDAC4 modulates muscle–axon interactions through multiple cytokines. These findings further underscore the importance of HDAC4 in the communication of neural and muscular compartments. Whether inactivation of HDAC4 could facilitate functional reinnervation in muscles affected by nerve damage would have important therapeutic implication for various neuromuscular disorders. The study of HDAC4 in denervated muscle clearly shows that HDAC4 plays an important role in regulating NMJ through multiple mechanisms.

9 HDACs in Muscle Fiber Size Control and Atrophy

Chronically reduced neural activities associated with neuromuscular disease, aging and denervation can cause muscle atrophy, a condition characterized by excessive reduction in muscle size and strength. In neuromuscular diseases, such as amyotrophic lateral sclerosis (ALS), motor neuron dysfunction leads to severe muscle atrophy in both diaphragm and limb muscles, contributing to breathing and mobility problems, and eventual death. The muscle atrophy program employs two ubiquitin E3 ligases, atrogin-1/MAFbx and MURF1, which are thought to promote muscle structural protein degradation by proteasomes (Bodine et al. 2001). In atrophic muscle arising from various dysfunctions such as denervation and immobilization, both atrogin-1 and MURF1 are transcriptionally induced. Genetic ablation of either atrogin1 or MURF1 partially spares muscle from atrophy (Bodine et al. 2001; Gomes et al. 2001). Recent data reveal that HDAC4 and HDAC5 induced in denervated muscle positively stimulates MURF1 transcription, thereby promoting muscle atrophy. Consistent with these data, inactivation of HDAC4 and HDAC5 by genetic ablation or specific siRNA spares denervated muscle from undergoing atrophy (Moresi et al. 2010). How HDAC4 regulates atrophy and MURF1 transcription is not known but it seems to involve the Dach2–myogenin transcriptional axis, which is also responsible for denervation-dependent synaptic gene induction (Cohen et al. 2007). However, as myogenin knockout mice are only partially resistant to denervation-induced atrophy, additional HDAC4-dependent signaling pathways must participate in muscle atrophy. Interestingly, the HDAC4-dependent muscle atrophy appears to be independent of MEF2 activity, suggesting a complete independent HDAC4 target in the skeletal muscle (TJC, MCC, TPY, unpublished observation).

Sarcopenia is another form of muscle atrophy commonly associated with aging. Interestingly, in a gene expression study that compares muscle from young and old individuals, HDAC4 is one of the genes that are elevated in older individuals, possibly reflecting their reduced physical activity (Welle et al. 2003). If this induction of HDAC4 also contributes to sarcopenia, targeted inhibition of HDAC4 could have an important clinical utility in the geriatric population.

10 Complex Regulation of HDAC4 in Skeletal Muscle

The study of HDAC4 in denervated muscle also reveals a surprising complexity of its regulation. While it is generally thought that phosphorylated HDAC4 resides in the cytosol, in denervated muscles, HDAC4 is phosphorylated but accumulates in the nucleus. Phosphorylation therefore does not necessarily lead to nuclear export. Despite the robust nuclear accumulation of HDAC4 and its potent repressive activity toward MEF2, denervation is not accompanied by a global shutdown of MEF2-target genes, many of which are structural proteins essential for muscle integrity. In other words, MEF2 target genes are partially protected from the

buildup of nuclear HDAC4 induced by denervation. Interestingly, phosphorylation was reported to dissociate HDAC4 from MEF2 independent of HDAC4 nuclear export (McKinsey et al. 2000). It was therefore proposed that phosphorylation of nuclear HDAC4 in denervated muscle might serve to protect muscle from a dramatic loss of MEF2-dependent structural genes (Cohen et al. 2009). A nondiscriminative repression of MEF2 transcription by nuclear HDAC4 and HDAC5 would lead to severe muscle dysfunction. Indeed, in muscle fibers expressing phosphorylation-deficient and nuclear-localized HDAC4-3SA mutant, a dramatic reduction in structural genes accompanied by loss of muscle integrity was observed (Cohen et al. 2009). In fact, the skeletal muscle phenotype induced by the HDAC4-3SA mutant is similar to that observed in MEF2C knockout mice (Potthoff et al. 2007b). These studies reveal that phosphorylation of HDAC4 and HDAC5 serves as a critical regulation that protects muscle integrity in response to loss of neural activity. It should be noted that phosphorylated HDAC4, while less active in repressing MEF2 activity, could engage in other signaling events important for muscle remodeling (TJC, MCC and TPY, unpublished observation). In this context, phosphorylation could play an instructive role to redirect HDAC4 to a MEF2-independent pathway.

The mechanism that elicits the transcriptional induction of HDAC4 by denervation remains poorly understood. Although CaMKII or related kinase-mediated phosphorylation could explain the differential phosphorylation and fiber type-selective activity of HDAC4, it is not clear how the same mechanism could affect HDAC4 gene transcription. In addition to calcium-dependent CaMK signaling, denervation also ceases contraction of the muscle. The mechanical contraction of myofibers is believed to have signaling capacity via a kinase titin, which spans half the length of the sarcomeres from the Z-line to the M-line. By sensing the mechanical force generated during contraction, it is thought that titin could regulate the atrophy-promoting E3 ligases MURF1 and MURF2, which are also associated with the sarcomere. Denervation or disease-associated titin mutation causes the dissociation of MURF2 from the sarcomere and entry to the nuclei, resulting in changes in gene transcription (Lange et al. 2005). Interestingly, HDAC4 was also found to associate with the sarcomere in cultured cardiomyocytes (Gupta et al. 2008). The physical interaction of HDAC4 and atrophy-promoting E3 ligases with the contraction apparatus suggests that mechanical contraction might regulate HDAC4 transcription and the muscle atrophy program. If proven correct, HDAC4 would act as a central effector in response to both calcium flux and mechanical force induced by motor neuron activity.

11 HDACs in Skeletal Muscle Regeneration

Skeletal muscle has the capability to regenerate, but like other tissues, its regenerative capacity is limited. Skeletal muscle regeneration and repair can be divided into three, sometimes overlapping, phases: (1) inflammation, (2) tissue formation,

and (3) tissue remodeling [reviewed in Grefte et al. (2007)]. Upon myofiber injury, damage to the plasma membrane causes a large influx of extracellular calcium, resulting in calcium-dependent proteolysis (Alderton and Steinhardt 2000). Myofiber necrosis ensues and inflammatory cytokines are released from myofibers as well as from nearby inflammatory cells. These cytokines attract neutrophils and later macrophages that engulf and digest cellular debris. Growth factors and cytokines released from the site of injury activate a distinct population of progenitor cells, called satellite cells, which are located between the sarcolemma and the basement membrane of myofibers. Satellite cells proliferate and differentiate into myoblasts. Satellite cell differentiation is similar to embryonic myogenesis in that it requires a specific milieu of transcription factors, most importantly myogenic regulatory factors (MRFs). Eventually, these activated satellite cells fuse to each other or existing myofibers, thus forming new muscle tissue. Interestingly, tissue macrophages are required for satellite cell proliferation and muscle cell regeneration (Camargo et al. 2003). The final stage of muscle regeneration and repair is tissue remodeling, which involves maturation of differentiated satellite cells. Shortly after fusion of the myoblasts, the myofibers are rather small with centrally located nuclei (Hawke and Garry 2001). Maturation involves migration of the nuclei to the periphery and hypertrophy of the myofiber.

A few studies have suggested that HDACs may play a role in muscle regeneration and satellite cell activation. TIS7 is a transcriptional corepressor important to muscle cell regeneration. TIS7-null mice display a delay in injury-induced muscle regeneration and reduced expression of MRFs, MyoD and myogenin, despite normal muscle development (Vadivelu et al. 2004). Apparently, the satellite cells of TIS7-null mice have a decreased capacity for differentiation. TIS7 transcriptional repression is HDAC-dependent, requiring HDAC1 and possibly HDAC4 as well (Vietor et al. 2005). Conversely, HDAC inhibitors (HDACIs) have proven therapeutically useful in treating mouse models of muscular dystrophies by enhancing muscle regeneration (Colussi et al. 2008; Minetti et al. 2006). The beneficial effects of HDACIs in the treatment of muscular dystrophy are derived from their influence upon the follistatin–myostatin pathway (Iezzi et al. 2004). Myostatin belongs to the TGF-β family and negatively regulates myofiber size (Lee and McPherron 2001). Myostatin antagonist, follistatin, is activated upon muscle injury, recruits myoblasts, and promotes myoblast fusion (Iezzi et al. 2004). HDACIs act by upregulating follistatin thereby stimulating myoblast recruitment and fusion, and hence muscle regeneration. Furthermore, in mouse models of Duchenne muscular dystrophy (DMD), skeletal muscle has elevated levels of class I HDACs. Blockade of class I HDACs, specifically HDAC2, successfully abates disease progression (Colussi et al. 2008). Whether class IIa HDACs are involved in skeletal muscle regeneration is not known.

12 Other Signaling Pathways in Skeletal Muscle Remodeling

Peroxisome proliferator-activated receptor delta (PPARδ) and its coactivator peroxisome proliferator-activated receptor coactivator-1 alpha (PGC-1α) are key regulators of genes involved in mitochondria and oxidative metabolism in skeletal muscle. Expression of PGC-1α is higher in type I fibers. One study showed that PGC-1α mRNA levels are ~5-fold higher in soleus (slow-oxidative fibers) than gastrocnemius (mixed) or EDL (fast-glycolytic fibers) muscle. Supporting an instructive role for PGC-1α and PPARδ in oxidative fiber phenotype overexpression of PGC-1α or PPARδ increases the number of slow-oxidative fibers type I myofibers (Koves et al. 2005; Lin et al. 2002; Grimaldi 2003). Transgenic overexpression of PGC-1α also causes fast-glycolytic fibers to become more resistant to atrophy. The PGC1-α promoter contains several MEF2 binding sites and its expression can be repressed by HDAC5 and HDAC4 (Czubryt et al. 2003). The regulation by the HDAC4, -5/MEF2 complex could explain the more abundant expression of PGC1-α in slow-oxidative fibers and after exercise, as well as reduced expression in denervated muscle (Koves et al. 2005; Sandri et al. 2006).

MAPK pathways are also coupled to electrical stimulation, affecting both fiber type and size (Aronson et al. 1997; Murgia et al. 2000). Electrical stimulation transiently increases JNK activity via the MEKK1–MKK4 cascade with JNK activity peaking within 30 min following stimulation (Aronson et al. 1997). JNK activation leads to an increase in c-jun mRNA levels; however, the physiological significance of this pathway remains unclear. Over a longer period of time (3–10 days), low-frequency electrical stimulation results in activation of Ras signaling, which proceeds through a MAPK (ERK) pathway. Interestingly, the Ras-MAPK (ERK) pathway promotes transition to type I myofibers in a muscle regeneration model; however, does *not* greatly affect fiber size (Murgia et al. 2000). In contrast, it was also reported that extracellular signal-regulated kinase (ERK) pathway is preferentially activated in fast-glycolytic fibers and is required for the maintenance of this fiber type (Shi et al. 2008).

The final kinase pathway that deserves mention in muscle remodeling involves protein kinase B (PKB)/Akt and mammalian target of rapamycin (mTOR). Upon loss of neural input, the activity of Akt is greatly diminished (Pallafacchina et al. 2002). As opposed to the Ras-MAPK pathway, activation of Akt increases myofiber size without affecting fiber type. Akt signaling proceeds through mTOR, as rapamycin is able to ablate the effects of activated Akt on muscle fiber size. Interestingly, this pathway is responsible for muscle hypertrophy occurring in response to muscle overload, such as weightlifting. mTOR phosphorylates its targets, $p70^{S6K}$ and PHS-1/4E-BP1, in order to increase protein synthesis and myofiber size. A number of different stimuli can activate this pathway, including growth factors such as insulin-like growth factor-I (IGF-1). Thus, it seems kinase signaling, especially the Ras-MAPK and Akt-mTOR pathways, provide additional mechanisms to fine-tune myofiber size and type. Whether and how class IIa HDACs are linked to Ras-MAPK and mTOR-AKT signaling axis remains to be established.

13 HDAC4 and Amyotrophic Lateral Sclerosis

ALS is a neurodegenerative disease characterized by progressive loss of upper and lower motor neurons (Robbins et al. 2010). With an incidence of 2 per 100,000 per year, this is a rare but devastating disease. Symptom onset is usually in the fifth decade, beginning with decreased fine motor control and coordination. Typically, motor strength and function continues to decline over a 3- to 5-year time course until patients succumb to death due to inability to breathe or swallow. Only 10% of the cases are familial (fALS) with the remaining 90% being sporadic. Of the fALS cases, roughly 20% are associated with a gain-of-function mutation in the superoxide dismutase-1 (SOD-1) gene. Although a number of mutations have been associated with ALS, the underlying pathogenesis remains elusive. Signs and symptoms of ALS result directly from the death of motor neurons, leading to a gradual loss of neural input into myofibers and subsequent neurogenic muscle atrophy. Currently, the only effective treatment for ALS is riluzole, which extends the life span of ALS patients by only a few months (Doble 1996). As the primary pathogenesis is unknown, therapeutic strategies targeting the underlying cause of ALS are limited. Thus, therapy must focus on the

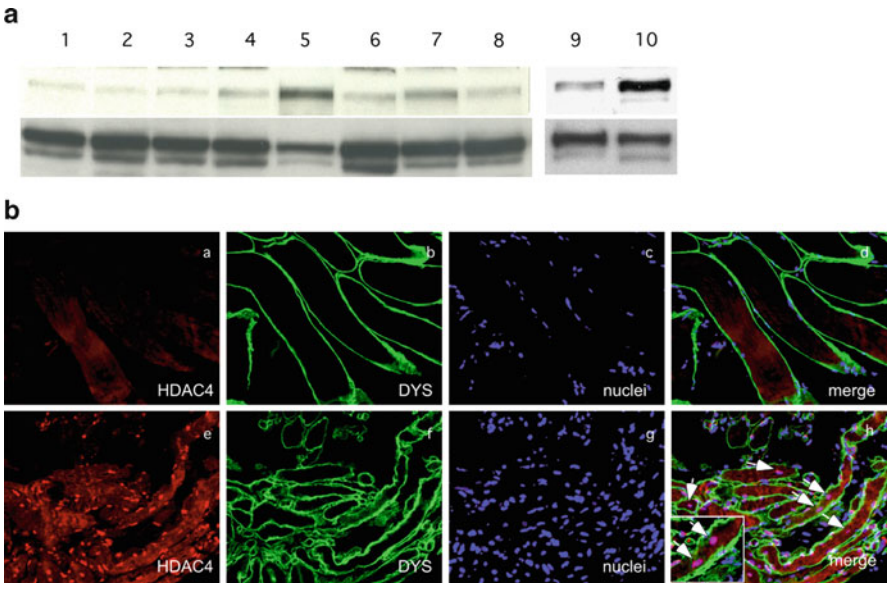

Fig. 3 (**a**) Deltoid muscle biopsies taken from patients at the Duke ALS clinic were homogenized and analyzed for HDAC4 and actin levels by immunoblotting. Healthy control: 1, 6, 9; myopathy patients: 2, 4, 7; ALS patients: 5, 10. (**b**) Muscle biopsy from healthy control 1, and ALS patient 5 were sectioned and processed for immunofluorescence microscopy using HDAC4 (*red*) and dystrophin antibodies (DYS). Hoechst dye was used to identify nuclei. *Arrowheads* in merged images indicate the accumulation of HDAC4 within nuclei of highly atrophic muscle

manifestations of ALS, namely the association of the motor neuron and myofiber as well as neurogenic muscle atrophy.

HDAC4 is a critical regulator of neurogenic muscle atrophy (Moresi et al. 2010), a dominant manifestation of ALS. In human ALS patients, HDAC4 is dramatically induced (Fig. 3a) and accumulates in the nuclei of skeletal muscle (Fig. 3b). A similar deregulation of HDAC4 is observed in an ALS mouse model caused by a human SOD-G93A transgene (Cohen et al. 2007). These observations suggest that HDAC4 might be a potential therapeutic target for ALS and related motor neuron diseases. Given the prominent role of HDAC4 in neurogenic muscle atrophy and synapse regulation, it would be of great clinical importance to investigate whether inhibition of HDAC4 could stimulate the reinnervation of NMJ and suppress muscle atrophy in ALS patients.

14 Class IIa HDACs as Therapeutic Targets

MITR potently inhibits MEF2 transcription activity despite lacking the catalytic deacetylase domain [Fig. 2, Zhang et al. (2001b)]. The deacetylase-independent repression by class IIa HDACs is further bolstered by findings that mutations in conserved residues in the catalytic domain have little effect on the ability of HDAC4 to repress MEF2 (Wang et al. 1999). Therefore, the deacetylase domain of class IIa HDACs is not essential for their transcriptional repressive activity toward MEF2. This begs a critical question: is there a function for the conserved catalytic domain in class IIa HDAC?

Although it is logical to propose that the HDAC4 catalytic domain acts to deacetylate specific substrates, as discussed previously, evidence indicates that deacetylase activity of ectopically expressed HDAC4 was conferred by associated HDAC3 (Fischle et al. 2002). Sequence analysis of the catalytic domain of vertebrate HDAC4 and other class IIa HDACs reveals that a conserved tyrosine in the catalytic center is replaced by histidine (Lahm et al. 2007). The crystal structure of HDAC4 deacetylase domain demonstrates that this substitution results in a loss of critical contact with acetylated substrate for deacetylation (Bottomley et al. 2008). Indeed, conversion of this histidine to tyrosine restores the histone deacetylase activity (Lahm et al. 2007). These findings support the proposition that HDAC4 and related class IIa deacetylases do not possess intrinsic deacetylase activity. This would pose a challenge to inhibit HDAC4 directly via classical HDAC inhibitors. If HDAC4 indeed exerts its deacetylase function via HDAC3, it is possible to inhibit HDAC4 through many available HDAC inhibitors that target HDAC3. It also remains possible that specific protein–protein interactions or protein modifications could activate the cryptic deacetylase domain of IIa HDACs. Alternatively, the deacetylase domain of HDAC4 was proposed to serve as a acetylated lysine binding module, an activity analogous to the BROMO domain (Bradner et al. 2010). One potential clue to the importance of the deacetylase domain came from a mutant mouse strain whose HDAC4 is disrupted by insertional mutagenesis, resulting in

a truncated HDAC4 with intact MEF2 binding domain but lacking the C-terminus catalytic domain. In contrast to the HDAC4 null mutant mice, which show severe skeletal defect and die within a few days after birth (Vega et al. 2004), this HDAC4 mutant (ΔCAT) strain is viable and grossly normal (Rajan et al. 2009). These phenotypes are consistent with findings that dysregulation of MEF2 is responsible for the skeletal defect observed in HDAC4 KO mice (Vega et al. 2004). Nonetheless, these mice show reduced thermal nociception and seizures, suggesting that the catalytic domain of HDAC4, independent of MEF2, regulates pain signaling and neural function. The intact catalytic domain of HDAC4 is also required for the regulation of muscle atrophy caused by denervation (MCC, TJC, TPY, unpublished results). Therefore, the conserved deacetylase domain in HDAC4 has a unique function and might be targeted pharmacologically.

Given the prominent role for HDAC4 in muscle atrophy and reinnervation, the development of inhibitors for HDAC4 could prove to be effective in the treatment of ALS and related motor neuron disease. The utility of broad-spectrum HDACIs has been tested in ALS mouse and rat models. Valproic acid (VPA), an anticonvulsant used in the treatment of epilepsy and mood disorders, is known to have HDACI activity. In one study, preonset treatment of ALS mice with VPA resulted in prolongation of life span, while VPA treatment after disease onset had no effect (Sugai et al. 2004). In a similar study, preonset treatment of ALS mice with VPA displayed a delay on symptom onset, but no effect on life span (Rouaux et al. 2007). VPA has also been shown to enhance motor function and peripheral nerve regeneration following nerve injury in a rat model (Cui et al. 2003). Analogously, treatment with another HDACI, phenylbutyrate (NaBP), yielded similar results. Treatment of ALS mice with NaBP led to prolonged survival (22%) and improved ALS phenotype (Ryu et al. 2005; Petri et al. 2006). Both VPA and NaBP are relatively weak HDAC inhibitors. Vorinostat (SAHA) is a more potent pan-HDAC inhibitor, which has been approved for cutaneous T-cell lymphoma treatment (Marks 2007). Its utility on ALS therapy is not known. Nevertheless, pan HDACI treatment could, in theory, have some adverse effects, as it could affect important processes controlled by other HDAC members. Ideally, the development of HDAC4-selective inhibitors could potentially offer more efficacious agents for treating ALS and other related neuromuscular diseases.

15 Concluding Remark

Class IIa HDAC members have emerged as critical regulators of skeletal muscle remodeling. Although they were initially considered as inhibitors of muscle differentiation by negatively regulating MEF2, it has become clear that HDACs have much more versatile and crucial function in structural and functional adaptation of skeletal muscle. Delineating the signaling events controlled by HDAC4-related deacetylases in skeletal could lead to fundamental understanding of physiological and pathological muscle remodeling and provide new therapeutic strategies for neuromuscular diseases.

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Rationale for HDAC Inhibitor Therapy in Autoimmunity and Transplantation

Wayne W. Hancock

Contents

Abstract While there are currently more than 70 ongoing clinical trials of inhibitors of so-called classical HDACs (HDACi) as anticancer therapies, given their potency as antiproliferative and angiostatic agents, HDACi also have considerable therapeutic potential as anti-inflammatory and immunosuppressive drugs. The utility of HDACi as anti-inflammatory agents is dependent upon their proving safe and effective in experimental models. Current pan-HDACi compounds are not well suited to this role, given the broad distribution of target HDACs and their complex and multifaceted mechanisms of action. In contrast, the development of isoform-selective HDACi may provide important new tools for therapy in autoimmunity and transplantation. This chapter discusses which HDACs are worthwhile targets in inflammation and progress toward their therapeutic inhibition, including the use of HDAC subclass and isoform-selective HDACi to promote the functions of Foxp3+ T regulatory cells.

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1 Overview of HDACi

The fields of HDAC biology and HDAC targeting using small-molecule inhibitors are intimately linked. Thus, the first HDACi compounds were identified by their anticancer effects during drug screens before the existence of HDACs was known (Richon and O'Brien 2002), and the HDACi, trapoxin, was used as a tool to isolate the first identified HDAC, HDAC1, in 1996 (Taunton et al. 1996). These interrelationships continue to prove of utmost value in dissecting the roles of HDACs in cells and disease models, and will be an ongoing theme of this chapter. Given a voluminous literature, we will emphasize data using agents that target the so-called classical HDACs and discuss the effects of agents that target the NAD-dependent sirtuin family of class III HDACs (SIRT1–7). There are three broad classes of classical HDACs. Class I HDACs (HDAC1, -2, -3 and -8) are homologous to yeast Rpd3, class IIa (HDAC4, -5, -7 and -9) and class IIb (HDAC6 and -10) HDACs are homologous to yeast Hda1, and the sole class IV HDAC (HDAC11) has homology to both Rpd3 and Hda1.

Classical HDACs (HDAC1–11) share a cavity containing a catalytic Zn^{2+} ion that is connected to the protein–solvent interface by an 8 Å tunnel. Classical HDACi literally come in various shapes and sizes, but a typical HDACi consists of a moiety that chelates the Zn^{2+} ion, has a variably sized linker to extend out from the tunnel and one or more aryl groups that form a cap and interact with residues near the entrance to the binding pocket. The latter residues have less homology between HDAC isoforms than those of the active site, allowing for different cap modifications to achieve relative HDAC isoform selectivity. Structure–activity relationship (SAR) analysis has typically involved variations in the zinc-binding group and modifications of the cap-binding moiety, with the latter in some cases resulting in HDAC6-selective inhibitors (discussed below). The main classes of HDACi are hydroxamic acids (e.g., trichostatin A [TsA], suberoylanilide hydroxamic acid [SAHA]), benzamides (e.g., MS275), electrophilic ketones (e.g., trapoxin), cyclic tetrapeptides (e.g., depsipeptide [FK228], apicidin), and short-chain fatty acids (e.g., butyrate, valproic acid).

Despite the focus on modification of acetylation of histone tails suggested by the very names of these enzymes, much recent biology points to HDAC regulation of the levels of acetylation of nonhistone proteins. Hence, HDACi can act in the cytoplasm or the nucleus and potentially modulated a large number of proteins. This is of more than of academic interest due to rapid advances in the field, including recognition of an "acetylome" likely encompassing thousands of proteins and responsible for the fine-regulation of functions in many normal cells (Choudhary et al. 2009). Moreover, while all HDACi in clinical trials inhibit proliferation of transformed cells in culture by inducing cell cycle arrest and/or apoptosis, and

inhibit tumor growth in animal models, far less is known of the effects of HDACi on the immune system. HDACi are known to induce lymphocyte cell cycle arrest, differentiation or apoptosis in vitro (Lam et al. 2005; Choi et al. 2005a; Moreira et al. 2003), but in microarray analyses TsA was found to modulate expression of only 2% of genes in TCR-activated CD4+ T cells (Moreira et al. 2003), comparable to studies showing that only 1–2% of genes were modified in non-T cells by HDACi (Van Lint et al. 1996; Richon et al. 2000). The effects of HDACi on protein acetylation and function would not be apparent from such mRNA-based studies, suggesting a prime opportunity for new investigational studies and therapeutic applications.

This chapter will summarize current data as to the therapeutic applications of HDACi in inflammation and autoimmunity, including the key cellular targets of HDACi, associated mechanisms of action, data from clinical studies and animal models of disease, and the rationale for therapeutic development of HDAC isoform-selective agents for use in inflammation and autoimmunity. Additional details are available from recent reviews on this topic (Blanchard and Chipoy 2005; Halili et al. 2009; Glauben et al. 2009; Mai 2007; Adcock 2007; Huang 2006; Wang et al. 2009a; Villagra et al. 2010).

2 Cells and Diseases Targeted by HDACi

Systemic administration of HDACi can potentially affect many, if not all, cells of the body depending upon whether a given agent can cross the blood–brain barrier. Some HDACi, such as valproic acid but typically not the hydroxamates, such as TsA, do cross the blood–brain barrier. Indeed, valproic and certain newer antiepileptic agents may exert their effects by inhibition of HDAC activity and differential effects on excitatory and inhibitory neuronal activities (Hoffmann et al. 2008; Fukuchi et al. 2009; Eyal et al. 2004). However, with regard to HDACi-associated modulation of inflammatory responses, the precise sites of actions, cellular targets and mechanisms of action are not well understood, with a considerable number of in vitro and in vivo studies offering complex, incomplete, and sometimes contradictory data-sets. Resolution of this problem may take a long time, given the divergent approaches of scientists ranging from studies of the effects of an HDACi on cells in vitro (Table 1) to assessment in whole animals and complex disease models (Table 2).

2.1 HDACi and Inflammatory Cells

Early studies showed that hydroxamate-based pan-HDACi compounds, such as TsA, SAHA and similar agents, have inhibitory effects on cytokine production by LPS-treated monocytes cultured in vitro (Table 1) as well as in mice injected

Table 1 Effects of HDACi on cells in vitro

Cells	Stimulus	HDACi	Effect of HDACi
PBMC	LPS	SAHA (Leoni et al. 2002), ITF2357 (Leoni et al. 2005)	Decreased TNF-α, IL-1α, IL-1β, IFN-γ, GM-CSF, NO
MØ	LPS or IFN-γ	Butyrate (Saemann et al. 2000), NVP-LAQ824 (Brogdon et al. 2007), TsA (Han and Lee 2009)	Decreased expression of many cytokines and chemokines, and upregulation of IL-10
PBMC	IL-12 and IL-18	SAHA (Leoni et al. 2002), ITF2357 (Leoni et al. 2005)	Decreased TNF-α, IFN-γ, IL-6
PBMC	CD3/CD28 mAb	SAHA (Leoni et al. 2002), ITF2357 (Leoni et al. 2005) TsA, scriptaid, oxamflatin, butyrate and other HDACi (Edens et al. 2006)	No effect on IL-2, IFN-γ or GM-CSF production by primary T cells, but impaired proliferation and IL-2 and IFN-γ production and increased anergy using Th1 clones
Th1/Th2 clones	Antigen-pulsed DC	NVP-LAQ824 (Brogdon et al. 2007)	Impaired Th1-linked chemokines (CXCR3 ligands) but preserved IL-4 and Th2 chemokines (CCR4 ligands); impaired Th1 but not Th2 proliferation; and impaired IFN-γ but not IL-4 production

Abbreviations: *DC* dendritic cells, *mAb* monoclonal antibody, *MØ* monocyte-derived macrophages ± DC, *PBMC* peripheral blood mononuclear cells

with LPS, acting to dampen monocyte production of TNF-α, IL-1α, and IL-1β (Leoni et al. 2002, 2005). Moreover, hydroxamate compounds typically did not affect CD3 mAb-induced proliferation of primary T cells when used at physiologic doses that do not induce apoptosis (Leoni et al. 2002, 2005). These early data of the effects of HDACi on monokine production and macrophage activation have been confirmed (Han and Lee 2009) and extended to additional areas in subsequent studies. Thus, synovial macrophages and intact synovial tissue samples from patients with rheumatoid arthritis showed decreased cytokine production (IL-6, TNF-α) and increased apoptosis when exposed to TsA in vitro (Grabiec et al. 2010). These data are consistent with the upregulation of HDAC expression by LPS and increased expression of proinflammatory genes, such as COX-2, though which combination of HDACs are upregulated appears to vary by cell type and, more broadly, by proinflammatory stimulus (Aung et al. 2006; Suen et al. 2010). By contrast to their effects on monocytes and dendritic cells, the effects of HDACi on T-cell responses are more nuanced than initially appeared, with evidence of HDACi impairing generation of signals 2 (costimulatory molecules) and 3 (activating cytokines) in antigen-pulsed antigen-presenting cells and associated impairment of the proliferation and chemotaxis of Th1 but not Th2 cells (Brogdon et al. 2007; Jung et al. 2009).

Table 2 Effects of HDACi in vivo

Model	HDACi	Effect of HDACi
Arthritis	Butyrate (Chung et al. 2003), Depsipeptide (Nishida et al. 2004; Nakamura et al. 2005) MS-275 (Lin et al. 2007) SAHA (Lin et al. 2007), TsA (Chung et al. 2003), VPA (Saouaf et al. 2009)	Protective effects in collagen- or antibody-induced arthritis
Asthma	TsA (Choi et al. 2005a)	Decreased airway hyperresponsiveness and inflammation
CD3 mAb	SAHA (Leoni et al. 2002), ITF2357 (Leoni et al. 2005)	No effect on IL-2 or IFN-γ
Colitis	VPA and SAHA (Glauben et al. 2006)	Protective effects in DSS and TNBS models
EAE, EAN	TsA (Camelo et al. 2005; Gray and Dangond 2006), MS-275 (Zhang et al. 2010a)	Reduced disability scores during chronic relapsing EAE, reduced inflammation in autoimmune neuritis model
GVHD	SAHA (Leng et al. 2006; Reddy et al. 2004; Li et al. 2008)	Decreased inflammation and improved donor cell engraftment
Hepatitis	SAHA (Leoni et al. 2002), ITF2357 (Leoni et al. 2005)	Decreased liver injury in Con-A hepatitis
Hypertension	SAHA (Iyer et al. 2010), VPA (Cardinale et al. 2010)	Decreased BP, inflammatory cytokine expression, cardiac hypertrophy and myocardial fibrosis in spontaneously hypertensive or DOCA-salt fed rats
LPS	SAHA (Leoni et al. 2002), ITF2357 (Leoni et al. 2005)	Decreased TNF-α IL-1β, IL-6, IFN-γ
Lupus	TsA and SAHA (Mishra et al. 2003)	Decreased IL-12, IFN-γ, IL-6, IL-10, proteinuria and glomerulo-nephritis but not autoantibody production or IgG or C3 deposition in MRL-lpr/lpr
Sepsis	Butyrate (Zhang et al. 2010b), SAHA (Li et al. 2009; Halili et al. 2010; Finkelstein et al. 2010; Li et al. 2010), TsA (Zhang et al. 2010b; Halili et al. 2010; Alamdari et al. 2010)	Decreased lethality and sepsis-related liver, lung and muscle injury
UUO	TsA or VPA (Marumo et al. 2010)	Decreased renal tubulointerstitial injury after ureteric obstruction

Rodent models unless specified; abbreviations: *EAE* experimental allergic encephalomyelitis, *EAN* experimental allergic neuritis, *GVHD* graft versus host disease, *LPS* lipopolysaccharide, *UUO* unilateral ureteric obstruction

2.2 HDACi and Disease Models

The range of diseases beyond malignancies in which HDACi use has proven therapeutic, experimentally, is considerable, though various negative data may, of course, not be reported. Much work has been directed toward experimental studies

of sepsis and autoimmunity, especially rodent models of arthritis and colitis (Table 2). There are also some less expected areas in which use of HDACi has proven beneficial. For example, studies of the effects of HDACi use in rodents with genetically associated or salt-induced hypertension showed remarkable reduction in expression of proinflammatory cytokines, cardiovascular injury, and stress responses (Iyer et al. 2010; Cardinale et al. 2010). While HDACi administration also lowered blood pressure, the effects noted were not seen by use of a standard antihypertensive agent (hydralazine), and likely include suppression of inflammatory pathways known to be activated by hypertension (Iyer et al. 2010; Cardinale et al. 2010; Bush and McKinsey 2010). Additional developing areas include potential beneficial effects of HDACi therapy on the development of chronic inflammation and epithelial/mesenchymal transformation (EMT) (Shan et al. 2008; Marumo et al. 2010; Noh et al. 2009; Kaimori et al. 2010), and also effects on the inflammatory process associated with the development of atherosclerosis (Choi et al. 2005b; Jung et al. 2010).

3 Mechanisms Action and Caveats

At a first glance, the use of compounds that promote acetylation of histone tails and increase accessibility to the DNA of various transcription factors may not appear likely to have useful anti-inflammatory effects. This assessment is also consistent with at least initial insights concerning one of the main proinflammatory pathways, involving NF-κB activation. Thus, much attention has been directed toward effects of HDACi on the NF-κB pathway, and it appears that HDACi compounds can be either activators of proinflammatory gene or inhibitors based on their molecular target, the proinflammatory mediator used or the cell type (Blanchard and Chipoy 2005). HDACi can downregulate NF-κB activation by inducing expression of IκBα, inhibiting its proteasomal degradation, and blocking the nuclear translocation of NF-κB and its DNA binding, but other different effects can also occur. For example, p300/CBP-dependent acetylation at K310 of p65 prevents its association with IκBα and promotes DNA binding and transactivation (Chen et al. 2001). Deacetylation of this lysine is catalyzed by HDAC3 (Chen et al. 2001; Kiernan et al. 2003), such that selective HDAC3 inhibition might increase NF-κB activation, though this may occur in a gene-specific manner (Gloire et al. 2007). The induction of multiple proinflammatory genes by p65 is also regulated by HDAC1 and HDAC2 (Ashburner et al. 2001).

The question thereby arises as to why does selective targeting of an HDAC such as HDAC3 promote NF-κB activation and induction of many proinflammatory genes, whereas HDACi are under investigation as anti-inflammatory drugs? Beyond all the empirical studies showing that HDACi do have important anti-inflammatory actions, fundamentally, this question remains unanswered, though additional clues are available. At least in some test systems, HDACi can stabilize IκBα expression and prevent its proteasomal degradation (Chakravortty et al. 2000;

Yin et al. 2001), and prevent the nuclear translocation and DNA binding of NF-κB (Rahman et al. 2003). Likewise, class I HDACs appear to be required for STAT-dependent transcriptional activation and pro-inflammatory gene expression (Koyama et al. 2000; Nusinzon and Horvath 2003; Xu et al. 2003; Klampfer et al. 2004). Perhaps most critically, while tyrosine phosphorylation of STAT1 promotes dimerization, nuclear translocation and activation of IFN-γ-responsive genes, the acetylation of STAT1 by CBP destabilizes this enhancesosome through recruitment of the tyrosine phosphatase TCP45, leading to termination of IFN-γ-dependent STAT1 signaling (Kramer and Heinzel 2010; Kramer et al. 2009). Acetylated but dephosphorylated STAT1 exits the nucleus and is deacetylated by HDAC3, and latent STAT1 is now available for reactivation. HDACi that inhibit HDAC3 promote STAT1 acetylation and thereby have potent anti-inflammatory effects. These data demonstrate that important exceptions exist to the concept that act HDACs as transcriptional repressors and HATs act as transcriptional activators, and help explain the anti-inflammatory effects of HDACi in vivo.

In addition to modifying chromatin accessibility or key signaling pathways such as those involving NF-κB and JAK/STAT pathways, there are several further mechanisms that may contribute to the anti-inflammatory actions of HDACi. First, HDACi may induce the apoptosis of cytokine-producing inflammatory cells (Glauben et al. 2006; Kankaanranta et al. 2010), though there is relatively little data as yet for this mechanism of action using normal rather than transformed cells. Second, HDACi may disrupt the functional microtubule network in monocytes and thereby disrupt exocytotic release of cytokines from lysosomes (Carta et al. 2006). The significance of this mechanism is unclear given the data were generated in vitro, with maximal effect on IL-1β release, lesser effect on TNF-α and essentially no effect on IL-8 secretion upon LPS stimulation of cultured human monocytes. In addition, the effects of HDACi effects were reversed as doses of HDACi were increased and the intracellular levels of cytokines were unaffected (Carta et al. 2006). Third, HDACi might affect the development and functions of cells with suppressive functions. There are a number of such cell types, beginning with the Foxp3+ Treg cells that are discussed in detail below, but also including other populations of lymphocytes, including CD8+ suppressor T cells (Vlad et al. 2010; Guillonneau et al. 2010; Van Kaer 2010) and IL-10-producing Tr1 cells (Gregori et al. 2010; Apetoh et al. 2010; Gandhi et al. 2010), and nonlymphoid cells, such as myeloid-derived suppressor cells (MDSC) (Nagaraj et al. 2010; Boros et al. 2010). However, apart from effects on Foxp3+ Tregs, as yet, little is known of the effects of HDACi on these various suppressor cell populations. Also unexplored are the possible effects of HDACi on micro-RNA production and the stability of mRNA encoding inflammatory mediators.

In addition to acknowledging the uncertainties as to how HDACi exert their anti-inflammatory responses, it should be noted that important caveats exist as to their use. For example, at least in vitro, hydroxamates such as TsA and SAHA can potentiate microglial production of proinflammatory mediators, in association with enhanced NF-κB activation (Suuronen et al. 2003), though TsA and comparable hydroxamates use decreased injury in vivo in murine models of experimental

allergic encephalomyelitis (Camelo et al. 2005) and neuroinflammation (Faraco et al. 2009). Theoretically, such agents may also exacerbate acute and chronic respiratory diseases (Marwick et al. 2004; Moodie et al. 2004; Hamalainen et al. 2008; Ito et al. 2005), and increased histone acetylation and proinflammatory gene expression are reported in asthma (Ito et al. 2002a, b). Moreover, deacetylation of the glucocorticoid receptor by HDAC2 appears necessary for optimal responses to steroid therapy in steroid-resistant asthma (Ito et al. 2000). These outcomes appear related to gene induction by acetylation and also by removal of the inhibitory effect of one or more HDACs on expression of proinflammatory genes. A notable example of the latter is the finding that both high glucose and HDACi decrease HDAC1 binding to the TNF-α promoter and increase TNF-α expression by monocytes from patient with type 1 diabetes (Miao et al. 2004). Thus, there are largely in vitro and/or descriptive data suggesting the need for caution in use of HDACi as anti-inflammatory agents for disease of specific organs or throughout the vasculature (Pons et al. 2009), but in each case in vivo experimental data often support this application, illustrating how much needs to be learned before this complex and multifaceted puzzle can be resolved.

4 HDACi and Foxp3+ Tregs

The clinical use of many pan-HDACi is associated with a common adverse effect profile of cardiac QT prolongation, nausea, diarrhea, vomiting, hypokalemia, loss of appetite and thrombocytopenia, plus in many cases, profound and debilitating fatigue. Likewise, their ability to induce cytotoxicity is considered a key and highly desirable action in the context of malignancies, which often overexpress HDAC1 and HDAC2, but the toxicity profile and cytotoxic effects render these agents far less suitable for nononcologic applications. To that end, various groups are seeking to avoid the class-associated side effects of pan-HDACi by trying to design isoform-selective HDACi for use in oncology and inflammation.

Our focus on selective HDACi arose from our findings in testing several HDACi compounds for their effects in murine models of colitis, including dextran sodium sulfate (DSS)-induced colitis and the T-cell-dependent $CD45RB^{hi}$ adoptive transfer model (Tao et al. 2007; de Zoeten et al. 2010). Two pan-HDACi compounds, trichostatin-A (TsA) and suberoylanilide hydroxamic acid (SAHA), but not MS275, a potent and long-acting HDAC class I-specific inhibitor, blocked development of colitis as shown by prevention of weight loss and associated blood in the stool, diarrhea, and histologic injury. Likewise, in T-cell-dependent adoptive transfer models, both pan-HDACi but not MS275 were effective in preventing the development of colitis, and in promoting the resolution of established colitis. The beneficial effects of pan-HDACi were dependent upon the presence of Foxp3+ T regulatory (Treg) cells, since Treg depletion or use of *Scurfy* mice with a mutation in Foxp3 abrogated any therapeutic benefit of HDACi administration (de Zoeten et al. 2010). Foxp3+ Tregs play a key part in limiting autoimmunity

and maintaining peripheral tolerance, and mutations of Foxp3 lead to lethal autoimmunity in humans and mice (Brunkow et al. 2001; Bennett et al. 2001; Hori et al. 2003; Fontenot et al. 2003; Khattri et al. 2003). In wild-type (WT) mice, pan-HDACi but not MS275 use decreased mucosal inflammatory cytokine production, and increased Foxp3 and anti-inflammatory cytokine expression, and enhanced Treg suppressive function (Tao et al. 2007; de Zoeten et al. 2010).

Further in vitro analysis (Wang et al. 2009b) demonstrated that multiple pan-HDACi hydroxamates such as TsA, SAHA, M344 and Scriptaid were effective in low nanomolar levels at enhancing murine Treg function, as well as the suppressive functions of rhesus macaque (Johnson et al. 2008) and human (Akimova et al. 2010) Treg cells. Additional pan-HDACi, such as the short-chain fatty acids, phenylbutyrate and valproic acid, also enhanced murine Treg function, but were only active in the micromolar and millimolar ranges, respectively. Our findings with regard to TsA-induced in vivo expansion of Foxp3+ Treg numbers and function were confirmed by other groups (Reilly et al. 2008; Koenen et al. 2008; Moon et al. 2009; Lei et al. 2010), as was the induction of Foxp3 using other HDACi, such as SAHA (Lucas et al. 2009). In contrast to our data using pan-HDACi, we found that class I-specific HDACi, such as the benzamides, MS275 and MC1293, and the quinolinol, NSC3852, lacked any effect on Treg functions in vitro when used at micromolar or higher levels (de Zoeten et al. 2010; Wang et al. 2009b). Hence, at least when using standard therapeutic dosages, only agents that blocked both class I and class II HDACs were effective at enhancing Treg function, and since class I-selective HDACi compounds were ineffective in the same assays, our data point to a key role for class II HDAC in control of Treg functions.

4.1 Targeting Class IIa HDACs

Compared to the extensive literature on pan-HDACi, the identification of HDAC class- or subclass- or isoform-selective inhibitors is in its infancy. Class I-selective HDACi include MS275 (Hu et al. 2003) and MC1293 (Massa et al. 2001) noted above, as well as 4-phenylimidazole (Jones et al. 2008a), and compounds selective for HDAC1 (Lee et al. 2008) are reported. Class II-selective HDACi include MC1568 and MC1575; these were originally reported as class IIa-selective but are now known to also inhibit the class IIb HDAC, HDAC6 (Mai et al. 2005; Nebbioso et al. 2009). HDAC isoform-specific inhibitors include agents with a high selectivity for HDAC4 (Muraglia et al. 2008), HDAC6 (Haggarty et al. 2003; Suzuki et al. 2006; Schafer et al. 2008; Chen et al. 2008; Kozikowski et al. 2008; Smil et al. 2009; Butler et al. 2010), or HDAC8 (Krennhrubec et al. 2007; Balasubramanian et al. 2008). This section will consider aspects of class IIa HDAC biology and therapeutic targeting.

Class II HDAC lack potent catalytic activity when assayed using conventional acetyl-lysine peptide substrates (Jones et al. 2008b), in large part because of the presence of a tyrosine in the active site, instead of a histidine as occurs in class I and

class IIb HDACs; mutation of this tyrosine to histidine improves HDAC activity against conventional acetyl-lysines substrates by 1,000-fold (Lahm et al. 2007). The development of alternate nonacetyl lysine (trifluoroacetate) substrates has allowed identification of significant catalytic activity of class IIa HDACs (Schuetz et al. 2008), but the physiologic relevance of this activity remains unclear, especially since class I and IIb HDACs are inactive against these alternate substrates (Jones et al. 2008b; Bradner et al. 2010). A developing view is that class IIa HDACs serve as recognition units or receptors for acetylated lysines (Bradner et al. 2010) and function by recruiting class I HDACs, especially HDAC3 (Fischle et al. 2002; Bottomley et al. 2008), so as to thereby provide deacetylating activity. This recruitment involves residues within a zinc-binding subdomain conserved only in class IIa HDACs, such that small molecules that bound to the active site of class IIa HDACs can thereby disrupt interaction with HDAC3/N-CoR repressor complexes and block the associated catalytic activity provided by HDAC3 to class IIa HDACs via protein–protein interactions (Fischle et al. 2002; Bottomley et al. 2008). However, additional recruitment of HDAC3 via N-terminal binding of CtBP may also need to be targeted to effectively block HDAC3/class IIa HDAC interactions (Zhang et al. 2001).

Our interest in class IIa HDACs arose from our finding of prominent expression of HDAC9 in murine (Tao et al. 2007) and human (Akimova et al. 2010) Foxp3+ Tregs, and that gene targeting or siRNA knockdown of HDAC9 enhanced Treg suppressive function in vitro and in vivo (Tao et al. 2007; de Zoeten et al. 2010). Likewise, gene targeting of HDAC7 increases Treg suppressive functions in vitro and in vivo (Tao and Hancock 2008; Wang et al. 2009c). Microarray studies indicated that the effects of HDAC7 and HDAC9 on Treg gene expression were distinct, such that targeting of both might be of therapeutic potential, but how to undertake this is not clear. We are currently undertaking an analysis of the effects of HDAC3 targeting, since many of the actions of class IIa HDACs may actually be attributable to the deacetylase activity of HDAC3 (Fischle et al. 2002; Bottomley et al. 2008). Nevertheless, these class IIa HDAC proteins are known to regulate gene expression through protein/protein interactions (Zhang et al. 2002; Han et al. 2005). Moreover, even in the case of HDAC9, a rather ill-understood and relatively little studied HDAC, genes regulated by HDAC9 as a result of its apparent weak catalytic activity are beginning to be identified (Wong et al. 2009; Yuan et al. 2010). Hence, the development of small molecules that inhibit the low catalytic activity of one or more class IIa HDACs, or disrupt their protein/protein interactions, may eventually show therapeutic potential as novel types of anti-inflammatory HDACi (Wang et al. 2009a).

4.2 Targeting Class IIb HDACs

Localized primarily to the cytoplasm, HDAC6 not only regulates the acetylation of multiple proteins, such as α-tubulin and heat shock protein 90 (HSP90), but also has

deacetylase-independent functions (Grozinger et al. 1999; Hubbert et al. 2002; Kovacs et al. 2005; Bali et al. 2005; Valenzuela-Fernandez et al. 2008). Unique in the field of HDACi, multiple HDAC6 isoform-selective HDACi (HDAC6i) are reported (Haggarty et al. 2003; Suzuki et al. 2006; Schafer et al. 2008; Chen et al. 2008; Kozikowski et al. 2008; Smil et al. 2009; Butler et al. 2010). These considerations led us to explore the effects of HDAC6 targeting on Tregs and, by extension, whether isoform-selective HDAC6i show utility as anti-inflammatory agents (Hancock et al. 2008). We found that HDAC6 was expressed at several-fold higher levels in Tregs versus conventional T cells, and HDAC6 knockout mice thereby provided a gold-standard as to how effective pharmacologic inhibitors of HDAC6 might be expected to be in modulating immune events. HDAC6−/− mice are known to be immunocompetent and not prone to tumorigenesis or chronic infections (Zhang et al. 2008). However, their Tregs were more suppressive in vitro and in vivo than WT Tregs. While HDAC6−/− Tregs express more Foxp3, CTLA4 and IL-10 than their WT counterparts, the basis for this increased suppressive capability may be multifaceted. HDAC6 gene targeting would likely disrupt both the deacetylase-dependent and -independent functions of normal HDAC6. The latter include roles for HDAC6 in regulation of cell migration and proteasomal degradation.

Evidence of the effects of HDAC6 targeting on deacetylase-dependent functions was readily apparent in our studies, including hyperacetylation of HSP90 and the upregulation of many HSF1-regulated genes in HDAC6−/− Tregs, including multiple HSP family members. While many additional genes of importance to Treg biology, but without known regulation via HSF1, were also differentially expressed in HDAC6−/− Tregs, effects on the HSF1/HSP pathway are likely of major importance both mechanistically and therapeutically. We have recently shown that HSP70 forms a complex with Foxp3 in Tregs, that upregulation of HSP70 promotes Treg survival and suppressive functions under conditions of cell stress, and that inhibition of HSP70 impairs Treg survival and suppressive functions (de Zoeten et al. 2010). The current data point to a major role for intracellular heat shock responses in control of Treg functions.

We found in colitis and transplant models that the presence or absence of HDAC6 just within Tregs is a powerful determinant of Treg-dependent resolution of colitis and resistance to allograft rejection (Hancock et al. 2008). These data underscore the importance of HDAC6 as a therapeutic target for modulation of Treg responses. Analysis of the effects of HSP90i in vitro and in vivo in our studies indicated that at least for the models under consideration, targeting of HDAC6 or HSP90 had broadly comparable effects and did not show obvious additional benefits when used together. Such combination might allow for lower doses of each inhibitor to be used, but the broad message from our work so far is that the benefits of targeting the HSF1/HSP pathway appear to be achieved by pharmacologic modulation of HDAC6 or HSP90. Some 14 HSP90i compounds, including 17-AAG, are currently being evaluated in Phase 1 and Phase 2 clinical trials; while data are preliminary, toxicity was rarely observed (Porter et al. 2010; Pacey et al. 2010). Clinical development of HDAC6i is less developed, but

HDAC6 targeting is being considered as a therapy for neurodegenerative conditions (Butler et al. 2010).

Our finding that selective targeting of an individual HDAC isoform can provide comparable effects on Tregs, and associated suppression of T cell-dependent immune responses, to that seen using broadly acting pan-HDACi provides a powerful rationale for the ongoing evaluation of HDAC6i in the regulation of inflammation. Ultimately, selective HDAC6i may provide an alternate, pharmacologic approach to therapies dependent upon Treg expansion and adoptive transfer for the management of autoimmunity and transplant rejection.

4.3 Targeting Class IV HDAC

In the first evidence as to a physiologic function for the sole class IV HDAC, HDAC11, data from gene targeting and siRNA approaches showed that HDAC11 expression suppressed macrophage production of IL-10 (Villagra et al. 2009). HDAC11 has antiproliferative effects (Glozak and Seto 2009; Wong et al. 2010) and is upregulated in at least some cell types, such as pancreatic beta cells, by cytokine simulation (Lundh et al. 2010). As for class IIa HDACs, no specific inhibitors of HDAC11 are reported, as yet. However, HDAC11 may be present in complexes that also contain HDAC6 (Gao et al. 2002; Toropainen et al. 2010), such that studies of the effects of HDAC6i on the biology of HDAC11−/− mice, including in models of inflammation, may be informative.

5 Summary

HDACi act in cancer models by inhibiting the cell cycle, inducing apoptosis and limiting angiogenesis. While HDACi likely exhibit the same effects in models of inflammation, the relative importance of these actions is likely to be markedly different. HDACi exhibit anti-inflammatory effects in a remarkable variety of models and contexts, although their effects on macrophages and DC are such that Th1-dependent responses are most commonly suppressed than Th2-dependent responses, at least in models reported to date. There are also new mechanisms that involve further cell types than the commonly studied APC and T cells. These include clinically important effects on the acetylation of Foxp3 and potentiation of Foxp3+ Treg-dependent immune suppression. Ongoing studies to further dissect and target individual HDAC isoenzymes are underway and may have important advantages over the predominant one-size-fits-all strategy of using pan-HDACi. In particular, targeting of HDAC6 using selective HDAC6i has considerable therapeutic potential inflammation.

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The Role of Mammalian Sirtuins in the Regulation of Metabolism, Aging, and Longevity

Akiko Satoh*, Liana Stein*, and Shin Imai

Contents

Abstract Ever since the discovery of sirtuins a decade ago, interest in this family of NAD-dependent deacetylases has exploded, generating multiple lines of evidence implicating sirtuins as evolutionarily conserved regulators of lifespan. In mammals, it has been established that sirtuins regulate physiological responses to

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metabolism and stress, two key factors that affect the process of aging. Further investigation into the intimate connection among sirtuins, metabolism, and aging has implicated the activation of SIRT1 as both preventative and therapeutic measures against multiple age-associated disorders including type 2 diabetes and Alzheimer's disease. SIRT1 activation has clear potential to not only prevent age-associated diseases but also to extend healthspan and perhaps lifespan. Sirtuin activating compounds and NAD intermediates are two promising ways to achieve these elusive goals.

Keywords Age-associated disorders • Aging • Lifespan • NAD intermediates • NAD world • Nicotinamide adenine dinucleotide • Nicotinamide mononucleotide • SIRT1 • Sirtuins • Sirtuin activating compounds

1 Introduction

The *s*ilent *i*nformation *r*egulator *2* (SIR2) family of proteins, also called sirtuins, are evolutionarily conserved nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases/ADP-ribosyltransferases. Now classified as class III histone deacetylases (HDACs), the founder *SIR2* gene was originally identified as one of the genes that regulate the mating types of budding yeast, *Saccharomyces cerevisiae* (Klar and Fogel 1979). The biochemical function of SIR2 proteins had been a mystery for 20 years when the *Salmonella typhimurium* SIR2-like protein, CobB, was found to catalyze the reaction in the late step of cobalamin biosynthesis, transferring phosphoribose from nicotinic acid mononucleotide to dimethylbenzimidazole (Tsang and Escalante-Semerena 1998). This first important clue for the enzymatic activity of SIR2 proteins suggested that they might catalyze a related pyridine nucleotide transfer reaction. Indeed, both bacterial and mammalian SIR2 proteins were reported to transfer ^{32}P from [^{32}P]NAD to bovine serum albumin (Frye 1999). Subsequently, it was proposed that the ADP-ribosyltransferase activity of SIR2 was essential for gene silencing in budding yeast (Tanny et al. 1999). Furthermore, it was found that acetylated amino-terminal tails of histone H3 or H4 could specifically accept ^{32}P from [^{32}P]NAD in the reactions mediated by recombinant yeast and mammalian SIR2 proteins (Imai et al. 2000). Surprisingly, analysis of the reaction products by mass spectrometry revealed that SIR2 proteins across evolution could specifically deacetylate lysine 16 of H4 in an NAD-dependent manner, strongly indicating that this novel and unique deacetylase activity of SIR2 proteins plays a critical role in establishing silenced chromatin structures in vivo (Imai et al. 2000). The absolute requirement of NAD for the SIR2 deacetylase activity suggested that SIR2 and its closely related homologs function as sensors of the cellular energy status represented by NAD. Following this breakthrough, reports appeared showing that both SIR2 and a yeast SIR2 homolog, HST2, catalyze the NAD-nicotinamide exchange reaction and NAD-dependent deacetylation (Landry et al. 2000; Smith et al. 2000).

Further studies have demonstrated that sirtuins play an important role in the regulation of lifespan. For example, in yeast, an extra copy of the *SIR2* gene increases replicative lifespan up to 30%, while its deletion or mutation shortens lifespan to ~50% (Kaeberlein et al. 1999). In *C. elegans*, increasing the dosage of *sir-2.1*, the ortholog of yeast SIR2, extends lifespan by up to 50% (Tissenbaum and Guarente 2001). An increase in the dosage of *Drosophila Sir2* (*dSir2*) also extends lifespan, whereas a decrease in the dosage of *dSir2* shortens lifespan and blocks the lifespan extension by caloric restriction (CR) (Rogina and Helfand 2004). CR extends lifespan in diverse organisms, from yeast (Lin et al. 2000), worms (Lakowski and Hekimi 1998), and flies (Chapman and Partridge 1996) to rodents (McCay et al. 1989; Weindruch and Walford 1982) and primates (Colman et al. 2009). In certain genetic backgrounds, sirtuins are also required for CR-induced lifespan extension (Anderson et al. 2003; Boily et al. 2008; Lin et al. 2000, 2002; Rogina and Helfand 2004; Wang and Tissenbaum 2006). Collectively, these findings implicate sirtuins as evolutionary conserved regulators of lifespan. In mammals, it has been established that sirtuins regulate metabolic and stress responses, two important components that affect the process of aging. In this chapter, we will focus on the metabolic functions of mammalian sirtuins as well as mechanisms regulating their activity. We will further discuss the potential connections among sirtuin biology, age-associated diseases, and the aging process, and finally introduce the manipulation of sirtuin function as a pharmacological approach to remedy and prevent age-associated pathophysiological changes.

2 The Catalytic Reaction of Sirtuins

The principal factor distinguishing sirtuins from other HDACs is that sirtuins require the cosubstrate NAD to perform their lysine deacetylase reaction (Fig. 1) (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000). In addition to this dependency on NAD, sirtuin activity can be pharmacologically separated from that of other HDACs by their insensitivity to a potent HDAC inhibitor, Trichostatin A (Dali-Youcef et al. 2007). All sirtuin family members contain a highly conserved, 250- to 270-residue catalytic domain consisting of a large domain containing a reverse Rossmann fold and a smaller domain composed of a zinc ribbon and a flexible helical subdomain (Finnin et al. 2001; Min et al. 2001). The cleft between these two domains forms a tunnel lined with hydrophobic residues in which catalysis occurs. The Rossmann fold contains a Gly–X–Gly sequence important for binding of the phosphate of NAD as well as a small pocket and charged residues to bind the two ribose groups of NAD. The zinc ribbon is composed of a three-stranded antiparallel β sheet, an α helix, and a zinc atom bound and stabilized by two pairs of cysteine residues. To commence catalysis, sirtuins require both the acetylated lysine substrate and NAD to respectively bind the cleft and the Rossmann fold, forming a ternary complex (Borra et al. 2005). Substrate binding triggers a conformational change that buries the acetyl-lysine of the substrate in the

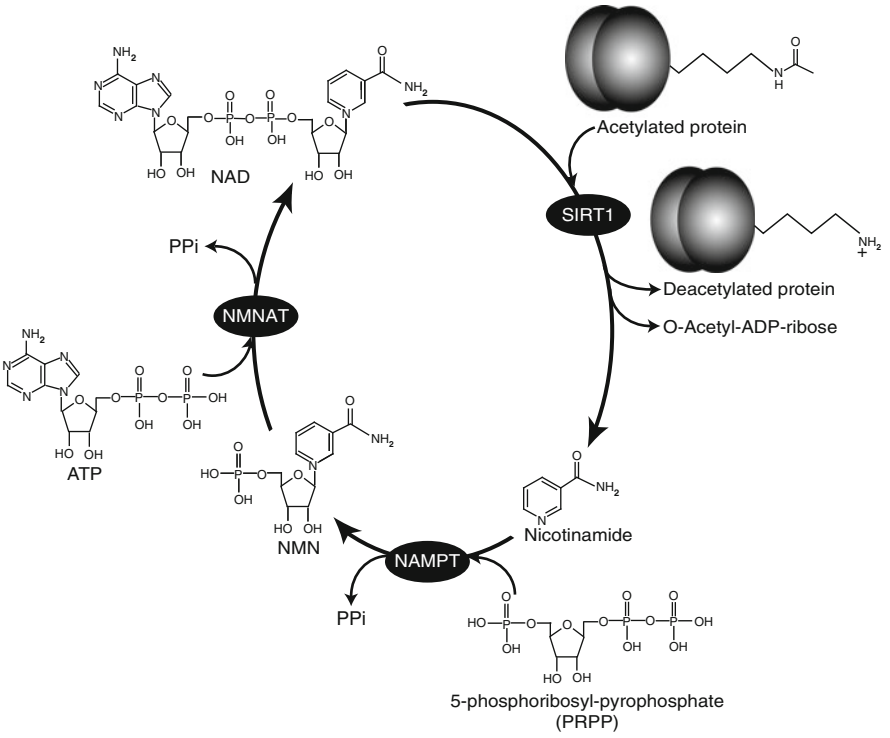

Fig. 1 The deacetylation reaction of sirtuins. Upon the binding of both an acetylated lysine substrate and NAD, sirtuins cleave the glycosidic bond separating NAD into its nicotinamide and ribose moieties, producing nicotinamide, 2′-*O*-acetyl-ADP-ribose, and a deacetylated substrate

hydrophobic tunnel (Avalos et al. 2002). This conformational change also forms an enzyme-substrate β sheet as well as generates a charge destabilization that promotes productive binding of NAD (Avalos et al. 2002, 2004). Subsequent to these events, sirtuins cleave the glycosidic bond separating nicotinamide and ribose moieties of NAD, forming nicotinamide and an enzyme–ADP–ribose intermediate (Landry et al. 2000). Sirtuins then transfer the acetyl group from the acetylated substrate to the ADP–ribose portion of NAD, generating 2′-*O*-acetyl-ADP-ribose (Borra et al. 2005; Zhao et al. 2004). Next, nicotinamide is released, followed by the 2′-*O*-acetyl-ADP-ribose and the deacetylated lysine.

In contrast to the high degree of structural conservation across family members in this catalytic domain (Finnin et al. 2001; Min et al. 2001), the N- and C-terminal regions flanking it are highly divergent (Zhao et al. 2003). Interestingly, the X-ray crystal structure of a yeast sirtuin family member, HST2, provides evidence for the involvement of the N- and C-terminal regions in catalysis, potentially indicating that the sequence divergence in the regions among family members may play a role in their substrate binding differences and/or biological activities. The number of sirtuin family members tends to increase with organismal complexity (except for

Table 1 The enzymatic activity and localization of mammalian sirtuins

	Enzymatic activity		
	Deacetylase	ADP-ribosyltransferase	Localization
Sirt1	✓		Nucleus, cytoplasm
Sirt2	✓		Cytoplasm, nucleus
Sirt3	✓		Mitochondria
Sirt4		✓	Mitochondria
Sirt5	✓		Mitochondria
Sirt6	✓	✓	Nucleus
Sirt7	✓		Nucleolus

budding yeast having five): prokaryotes express one to two family members, fission yeast expresses three, worms express four, flies express five, and mammals express seven (Blander and Guarente 2004). The seven mammalian family members, SIRT1 through SIRT7, have slightly different enzymatic activities, localizations, and functions (Table 1). Among them, all except SIRT4 exhibit deacetylase activity (Imai and Guarente 2010). For SIRT4, only ADP-ribosyltransferase activity has been reported (Imai and Guarente 2010). SIRT1 and SIRT2 can be cytoplasmic or nuclear proteins, while SIRT3, SIRT4, and SIRT5 are localized to the mitochondria, and SIRT6 and SIRT7 reside in the nucleus or nucleolus, respectively (Finkel et al. 2009; Imai and Guarente 2010). Of these seven members, SIRT1 is the ortholog of yeast SIR2 (Finkel et al. 2009), and its function has been characterized most extensively. Therefore, the following sections will mainly focus on SIRT1 function.

3 Metabolic Regulations by Mammalian Sirtuins

Numerous studies now show an intricate connection exists between metabolism and aging (Conti et al. 2006; Hakimi et al. 2007; Harrison et al. 2009; Pawlikowska et al. 2009; Selman et al. 2009; Tatar et al. 2003). It has been well established that mammalian sirtuins regulate metabolic responses to nutritional input in multiple tissues/organs. In this section, we will outline the functions of SIRT1 in the liver, skeletal muscle, adipose tissue, pancreatic β-cells, and hypothalamus (Fig. 2).

3.1 Liver

The liver plays an important role in the regulation of glycolysis, gluconeogenesis, and lipid metabolism, such as fatty acid oxidation. Under nutrient deprivation, gluconeogenesis increases while glycolysis decreases in the liver in order to maintain plasma glucose levels. Meanwhile, fatty acid oxidation is enhanced in

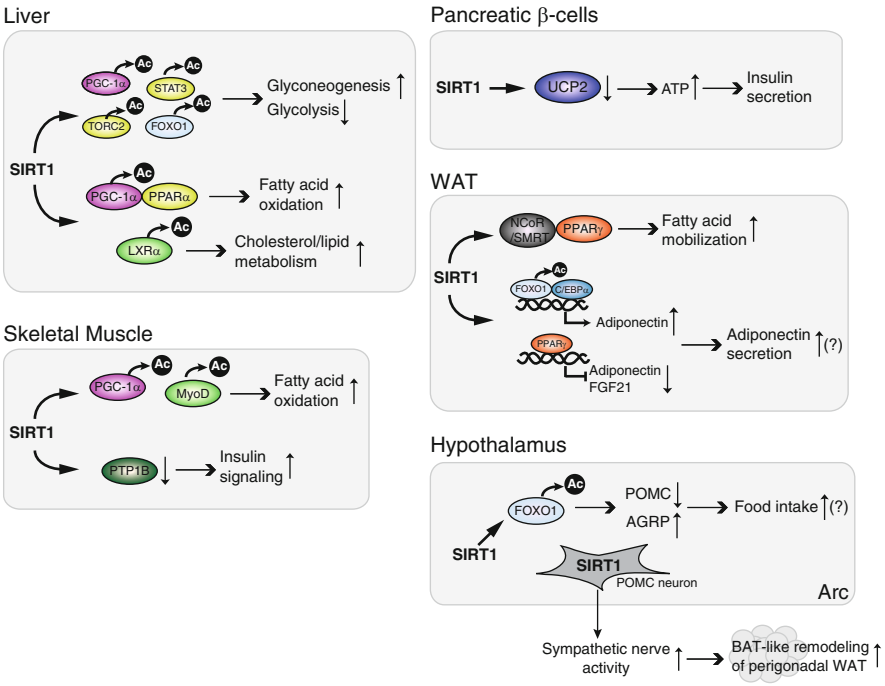

Fig. 2 The metabolic regulation of SIRT1 in the liver, skeletal muscle, white adipose tissue (WAT), and hypothalamus. In the liver, SIRT1 regulates gluconeogenesis by deacetylating PGC-1α, TORC2, FOXO1, and STAT3. SIRT1 promotes fatty acid oxidation by deacetylating PGC-1α, promoting an interaction between SIRT1 and PPARα. SIRT1 also regulates cholesterol homeostasis by positively regulating the function of LXRα. In skeletal muscle, SIRT1 enhances mitochondrial fatty acid oxidation by deacetylating PGC-1α and MyoD. SIRT1 also improves insulin sensitivity by inhibiting the transcription of PTP1B. In WAT, SIRT1 enhances fatty acid mobilization by repressing the transcriptional activation of PPARγ by binding to NCoR/SMRT complex. SIRT1 regulates the production/secretion of adiponectin by deacetylating FOXO1, and possibly by inhibiting PPARγ. In pancreatic β-cells, SIRT1 enhances glucose-stimulated insulin secretion and improves glucose tolerance, at least in part, by repressing the expression of UCP-2. In the hypothalamus, SIRT1 in POMC neurons prevents the pathology of diet-induced obesity by reducing sympathetic nerve activity and BAT-like remodeling of perigonadal WAT. SIRT1 also regulates food intake and feeding behavior by decreasing and increasing the protein levels of AgRP and POMC, respectively

response to pancreatic glucagon and adrenal cortisol, promoting gluconeogenesis (Bhathena 2000; McGarry and Foster 1980). The liver also plays a vital role in the excretion of ammonia through the urea cycle (Haussinger 1990).

SIRT1 is important for the positive regulation of gluconeogenesis and fatty acid oxidation under nutrient deprivation. In the case of gluconeogenesis, SIRT1 deacetylates and activates peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) (Rodgers et al. 2005), stimulating hepatic gluconeogenic gene expression while inhibiting glycolytic genes, as well as multiple targets including CREB-regulated transcription coactivator 2 (CRTC2, also known as TORC2), forkhead

transcription factor O1 (FOXO1), and signal transducer and activator of transcription 3 (STAT3) (Frescas et al. 2005; Liu et al. 2008b; Nie et al. 2009). To up-regulate fatty acid oxidation, SIRT1 deacetylates PGC-1α, promoting an interaction between SIRT1 and peroxisome proliferator-activated receptor α (PPARα). This complex transcriptionally induces PPARα target genes, which in turn enhances fatty acid oxidation (Purushotham et al. 2009).

It has also been reported that SIRT1 positively regulates the function of liver X receptor α (LXRα), a nuclear receptor that functions as cholesterol sensor and regulates cholesterol and lipid homeostasis (Zelcer and Tontonoz 2006). SIRT1 interacts with and deacetylates LXRα, activating the transcription of the LXRα target gene ABCA1, a transporter that mediates HDL synthesis and HDL-mediated reverse cholesterol transport (RCT) (Li et al. 2007). Consistent with this finding, *Sirt1*-deficient mice display lower levels of HDL cholesterol in plasma and accumulate hepatic cholesterol (Li et al. 2007). However, total plasma cholesterol levels are reduced in both *Sirt1*-deficient mice (Li et al. 2007) and *Sirt1*-overexpressing transgenic mice (Bordone et al. 2007). Thus, further study is required to fully understand the function of SIRT1 in cholesterol homeostasis.

Similar to SIRT1, two other sirtuin family members, SIRT5 and SIRT6, also play important roles in metabolic regulation in the liver. SIRT5 regulates urea cycle by deacetylating carbamoyl phosphate synthase1 (CPS1), an enzyme that catalyzes the initial step of the urea cycle for ammonia detoxification and disposal, and up-regulating its activity (Nakagawa et al. 2009; Schapira 2011). On the other hand, SIRT6 down-regulates glycolysis by interacting with and deacetylating/inactivating hypoxia-inducible factor-1α (HIF1α), a transcription factor that modulates multiple genes to activate glycolysis (Zhong et al. 2010; Mahajan et al. 2011). The evidence that liver-specific *Sirt6*-deficient mice results in hepatic steatosis suggests that increasing SIRT6 activity in the liver would, at least in part, prevent the liver dysfunction caused by hepatic steatosis (Kim et al. 2010).

3.2 Skeletal Muscle

In skeletal muscle, mitochondrial fatty acid oxidation is vital for preserving glycogen stores and blood glucose levels upon nutrient deprivation and after exercise. SIRT1 enhances mitochondrial fatty acid oxidation by binding to and deacetylating PGC-1α, which increases its activity on its promoter through an interaction with myogenic determining factor (MyoD) (Amat et al. 2009; Canto et al. 2010). Given that dysregulation of skeletal muscle fatty acid oxidation is associated with insulin resistance and obesity (Kelley et al. 1999; Newgard et al. 2009), SIRT1 might also improve the insulin sensitivity of skeletal muscle. Indeed, in myotube cells, SIRT1 also inhibits the transcription of protein tyrosine phosphatase 1B (PTP1B). PTP1B dephosphorylates the insulin receptor and negatively regulates insulin signaling (Sun et al. 2007). In fact, *Ptp1b*-deficient mice display higher susceptibility to insulin when fed a high-fat diet (HFD) and resistance to diet-induced obesity

(Elchebly et al. 1999). Moreover, a decrease in SIRT1 protein levels in the gastrocnemius muscle under HFD is accompanied by an increase in PTP1B levels (Sun et al. 2007). On the other hand, during fasting, SIRT1 protein levels increase while PTP1B protein levels decrease (Sun et al. 2007). Together, these findings suggest that SIRT1 plays an important role in the maintenance of skeletal muscle insulin sensitivity.

3.3 Adipose Tissue

A primary function of white adipose tissue (WAT) is to produce and store fatty acids as an energy reserve to allow their mobilization into the blood upon nutrient deprivation. WAT also plays an important role as an endocrine organ for adipokines, including leptin, adiponectin, and tumor necrosis factor-α (TNF-α) that modulate insulin resistance, hepatic lipoprotein production, and vascular inflammation (Mohamed-Ali et al. 1998).

SIRT1 can enhance fatty acid mobilization from WAT. Upon fasting, SIRT1 is recruited by PPARγ to DNA-binding sites in the aP2 promoter, promoting adipogenesis and fat storage (Lehrke and Lazar 2005). SIRT1 binds to PPARγ and represses the transcriptional activation of PPARγ by binding to nuclear receptor coreceptor/silencing mediator of retinoid and thyroid hormone receptor (NCoR/SMRT) complex, resulting in the reduction of fat accumulation in WAT and higher level of free fatty acids in blood (Picard et al. 2004).

On the other hand, whether or not SIRT1 regulates adipokine secretion from WAT is still debatable. Whole-body bacterial artificial chromosome (BAC)-driven *Sirt1*-overexpressing transgenic mice have increased levels of plasma adiponectin (Banks et al. 2008). Adiponectin is known as antidiabetic and antiatherogenic adipokine by exerting a potent insulin sensitizing effect (Kadowaki et al. 2006). In contrast, suppression of SIRT1 enhances the expression of endoplasmic reticulum oxidoreductase Ero1-Lα, a membrane-associated oxidoreductase that generates disulfide bonds (Frand and Kaiser 1998), and stimulates adiponectin secretion in adipocytes (Qiang et al. 2007). By binding to and deacetylating FOXO1, SIRT1 enhances the formation of FOXO1-CCAAT/enhancer-binding protein α (C/EBPα) transcription complex in the adipocytes, increasing the transcription of adiponectin (Qiao and Shao 2006). However, it has also been reported that SIRT1 suppresses the expression of adiponectin and fibroblast growth factor 21 (FGF21, an activator of glucose uptake in adipocytes), possibly by inhibiting of PPARγ in adipocytes (Qiang et al. 2007; Wang et al. 2008). Since it has been reported that SIRT1 upregulates the expression of adiponectin in adipocytes (Qiang et al. 2007; Qiao and Shao 2006; Wang et al. 2008), and the direction of the adiponectin secretion is not conclusive between in vivo and in vitro (Banks et al. 2008; Qiang et al. 2007), further work is required to understand adiponectin secretion.

While SIRT1 is predominantly a nuclear protein, SIRT2 is predominantly a cytoplasmic one (Michishita et al. 2005; North et al. 2003). In fact, SIRT2 is the

most abundant sirtuin in adipocytes, where it plays an important role for adipocyte differentiation by deacetylating FOXO1 (Jing et al. 2007). Thus, it is possible that SIRT2 targets FOXO1 in the cytoplasm, while SIRT1 catalyzes FOXO1 deacetylation in the nucleus. It is also possible that SIRT1 and SIRT2 target different molecules, giving them different physiological or pathological functions in WAT. Further in vivo studies using genetically modified SIRT2 mice are required to address this possibility.

Brown adipose tissue (BAT) is a crucial regulator of energy expenditure through mitochondrial uncoupling protein-1 (UCP-1), thus protecting against obesity and diet-induced insulin resistance in mammals (Cederberg et al. 2001; Kopecky et al. 1995; Nedergaard et al. 2007; Seale et al. 2008). Although it has been reported that SIRT3 is highly expressed in BAT and plays an important role in adaptive thermogenesis by activating mitochondrial function (Scher et al. 2007; Schapira 2011), the metabolic regulatory function of SIRT1 in BAT is still unknown. It is reported that SIRT1 might regulate brown adipocyte differentiation based on microarray gene expression study (Timmons et al. 2007). Since adult humans have morphologically distinguishable BAT and increasing brown adipocyte differentiation may offer a new therapeutic treatment for obesity and type 2 diabetes (Cypess et al. 2009; Kajimura et al. 2010; Lidell and Enerback 2010; Virtanen et al. 2009), it will be of great interest to further investigate the role of SIRT1 in the brown adipocyte differentiation.

3.4 Pancreatic β-Cells

Pancreatic β-cells play a major role in the regulation of glucose homeostasis by secreting insulin in response to elevated blood glucose. In pancreatic β-cells, SIRT1 enhances glucose-stimulated insulin secretion and improves glucose tolerance, at least in part, by repressing the expression of uncoupling protein 2 (UCP-2), an inner mitochondrial membrane protein (Bordone et al. 2006; Moynihan et al. 2005). UCP-2 functions as a proton transporter, whose activity has the effect of uncoupling the electron transport chain and ATP biosynthesis. Suppression of UCP-2 by SIRT1 increases ATP production, inducing glucose-stimulated insulin secretion. Indeed, islets isolated from beta cell-specific *Sirt1*-overexpressing (BESTO) transgenic mice exhibit increased ATP production in response to glucose. Interestingly, both pancreata and islets of BESTO mice show the enhancement of insulin secretion not only by glucose, but also by KCl-induced depolarization, suggesting that SIRT1 might also regulate insulin secretion downstream of β-cell depolarization, independently of UCP-2 (Moynihan et al. 2005).

β-cell dysfunction can be caused by a variety of cellular injuries, including glucolipotoxicity (Fontes et al. 2010). SIRT1 is able to prevent β-cell dysfunction against cellular injury. BESTO mice maintain glucose tolerance under a long-term HFD by increasing glucose-stimulated insulin secretion (Ramsey et al. 2008). The finding that BESTO mice exposed to HFD-induced stress display better β-cell

functionality than wildtype littermates suggests that increasing SIRT1 activity in pancreatic β-cells might be able to sustain β-cell function under hyperlipidemic, diabetogenic conditions. Indeed, by deacetylating FOXO1, SIRT1 induces the expression of *NeuroD* and *MafA*, which are both transcriptional regulators of insulin 2 (*Ins2*) gene expression and protect β-cells under conditions that could cause apoptosis and promote β-cell senescence (Kitamura et al. 2005). Furthermore, SIRT1 overexpression completely prevents IL-1β and/or IFNγ-mediated cellular injury by inhibiting NF-κB signaling and subsequently repressing the expression of inducible nitric oxide synthase (iNOS) (Lee et al. 2009). Together, these findings strongly suggest that SIRT1 activity can prevent pancreatic β-cell dysfunction and thus type 2 diabetes.

Contrary to the function of SIRT1 in pancreatic β-cells, SIRT4 suppresses insulin secretion in response to amino acids and glucose (Schapira 2011). SIRT4 ADP-ribosylates and represses the activity of glutamate dehydrogenase (GDH), an enzyme that converts glutamate to α-ketoglutamate in mitochondria, thereby decreasing amino acid-stimulated insulin secretion (Haigis et al. 2006). Furthermore, depletion of SIRT4 increases glucose-stimulated insulin secretion through with the effects on ATP/ADP translocase (ANT), an ADP/ATP carrier protein, and insulin-degrading enzyme (IDE) (Ahuja et al. 2007). It is of great interest to investigate how SIRT1 and SIRT4 work together to regulate insulin secretion under various metabolic conditions such as HFD.

3.5 Hypothalamus

The hypothalamus regulates feeding behaviors in response to metabolic signals from peripheral tissues by way of hormones such as insulin, leptin, and ghrelin (Elmquist 2001). Neurons in diverse nuclei of the hypothalamus including arcuate and paraventricular nuclei (Arc and PVN, respectively) and the ventromedial, dorsomedial, and lateral hypothalamic nuclei (VMH, DMH, and LH, respectively) produce and secrete multiple neuropeptides and neurohormones that play important roles in the regulation of metabolism, body temperature, food intake, arousal, circadian rhythm, and the secretion of pituitary hormones (Cone 2005; Elmquist 2001; Green et al. 2008; Morrison et al. 2008; Morton et al. 2006; Sakurai 2007).

Hypothalamic SIRT1 appears to regulate food intake and feeding behavior, but the direction of regulation currently remains controversial. Intracerebroventricular administration of EX527, a selective inhibitor of SIRT1, or siRNA-mediated knockdown of *Sirt1* in the Arc inhibits feeding behavior in rats through decrease in augouti-related protein (AgRP) and increase in pro-opiomelanocortin (POMC) protein (Cakir et al. 2009). In contrast, it has been reported that intracerebroventricular administration of adenovirus expressing *Sirt1* in the mediobasal hypothalamus suppresses food intake (Sasaki et al. 2010). Since each hypothalamic nucleus has a distinct role in systemic metabolic regulation, neuron-specific or

nuclei-specific modification of *Sirt1* by genetic or stereotactic manipulation is necessary to elucidate the function of SIRT1 in the hypothalamus.

Numerous experimental and clinical findings suggest that hypothalamic dysfunction might be one of the underlying causes of abnormal glucose and lipid metabolism that occurs in type 2 diabetes and diet-induced obesity (Rosmond and Bjorntorp 2000; Schwartz and Porte 2005). Hypothalamic SIRT1 is reported to prevent the pathology of diet-induced obesity. Deletion of SIRT1 in POMC neurons results in weight gain and reduced energy expenditure by reducing sympathetic nerve activity and BAT-like remodeling of perigonadal WAT under HFD (Ramadori et al. 2010). BAT-like remodeling of perigonadal WAT increases mitochondrial content and UCP-1 expression under HFD, resulting in increased energy expenditure against obesity and insulin resistance (Plum et al. 2007). These results suggest that SIRT1 in POMC neurons is required for normal autonomic adaptation to diet-induced obesity and possibly insulin resistance.

4 The Regulation of Sirtuin Function

Given the importance and widespread effects of SIRT1 activity, it is important to understand factors that regulate its activity and expression. These regulatory factors can be broken down into four categories: NAD, protein regulators of SIRT1 activity, nucleocytoplasmic shuttling of SIRT1, and regulators of SIRT1 expression (Fig. 3).

4.1 NAD

Perhaps the most important regulator of sirtuin activity is cellular NAD (Imai 2009). NAD is specifically required for the sirtuin deacetylase reaction and cannot be substituted by NADH, NADP, and NADPH (Imai et al. 2000). However, NAD is required for many vital cellular processes besides the sirtuin reaction. For example, it acts as a cofactor in redox reactions of glycolysis, the trichloroacetic acid cycle, and the catabolism of carbohydrates, fats, proteins, and alcohols and also participates in DNA repair, G-protein coupled signaling, intracellular calcium signaling, and transcription (Bogan and Brenner 2008; Garten et al. 2009). As a result, the pool of intracellular NAD is limiting for sirtuin activity despite its seemingly adequate concentration of 300–400 μM (Bogan and Brenner 2008; Canto et al. 2010; Houtkooper et al. 2010; Koltai et al. 2010; Penberthy and Tsunoda 2009; Rodgers et al. 2005; Sauve 2008; Yang et al. 2007a). In addition to affecting SIRT1 activity, high levels of NAD can augment SIRT1 expression over twofold in multiple tissues (Hayashida et al. 2010; Hwang et al. 2009; Qin et al. 2006; Rodgers et al. 2005), whereas depletion of NAD had the reverse effect (Borradaile and Pickering 2009; de Kreutzenberg et al. 2010; Liu et al. 2008a).

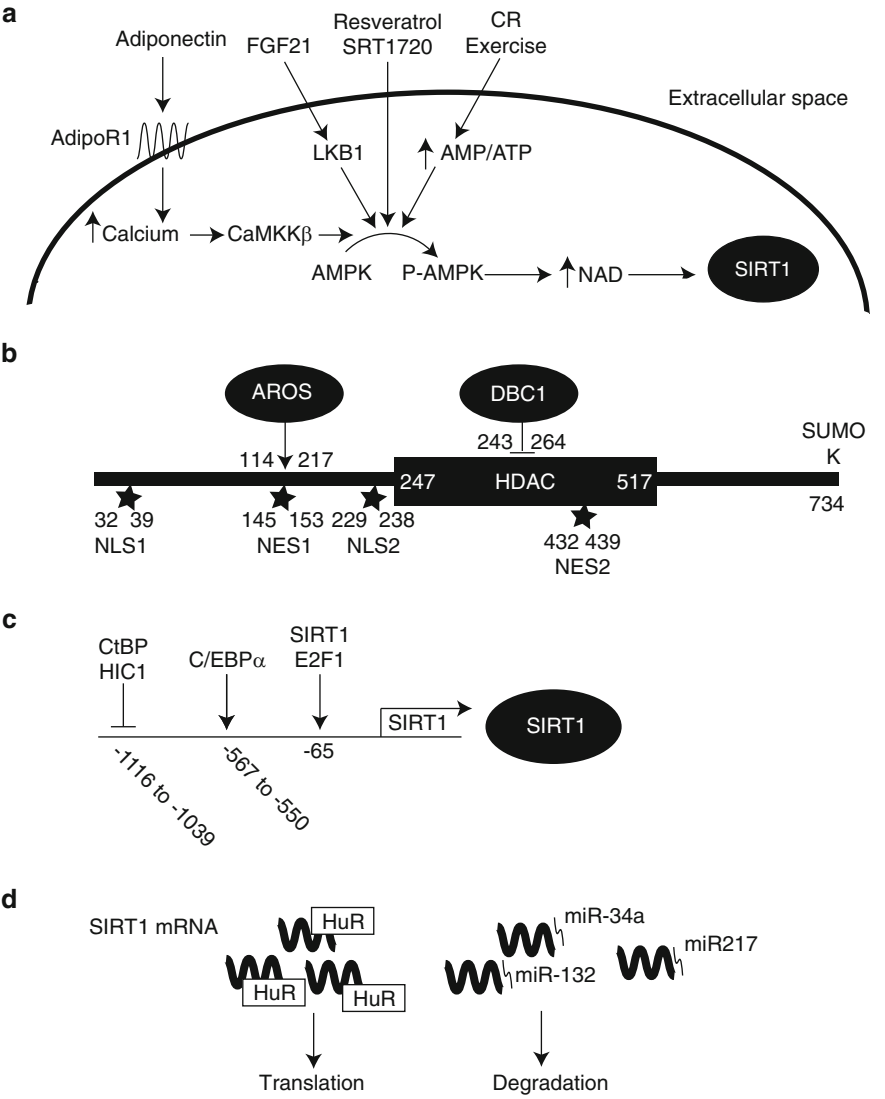

Fig. 3 Regulation of SIRT1. SIRT1 activity is regulated by (A) NAD levels, (B) protein–protein interactions and posttranslational modifications, (C) factors that modulate its transcription, and (D) factors that modulate its translation. (A) SIRT1 activity is primarily regulated by NAD levels. NAD levels are regulated by AMPK-mediated up-regulation of NAMPT expression. AMPK can be activated by FGF21 and adiponectin. FGF21 induces LKB1, one of two major AMPK activators, to phosphorylate AMPK. Adiponectin induces calcium influx through its receptor adiponectin receptor 1 (adipoR1) and this calcium activates the other major AMPK kinase, calcium/calmodulin-dependent protein kinase kinase β (CaMKK β). (B) SIRT1 is activated by direct binding of AROS and sumoylation at Lysine734. Conversely, SIRT1 is inhibited by direct binding of DBC1. (C) p53 and HIC1 repress transcription of SIRT1 while C/EBPα and E2F1 enhance it. (D) Binding of HuR to SIRT1 mRNA increases its half-life. In contrast, binding of miR-34a, miR-132, or miR-217 results in translational repression of SIRT1 mRNA

Since high concentrations of NADH inhibit sirtuin activity, there is some debate as to whether absolute NAD levels or the NAD/NADH ratio is more important to sirtuin function (Bogan and Brenner 2008; Lin et al. 2004). With the IC50 for NADH at 11–28 mM and the amount of NADH in the cytosol less than 1% of total free NAD and NADH, it is unlikely for NADH levels to become high enough to inhibit SIRT1 activity (Schmidt et al. 2004). Thus, NAD levels are expected to be a more critical indicator of SIRT1 activity.

Four different substrates can be used to generate NAD: tryptophan (Trp), nicotinamide (NAM), nicotinic acid (NA), and nicotinamide riboside (NR) (Fig. 4) (Bogan and Brenner 2008). Of these substrates, the pathway starting from nicotinamide is the predominant source of NAD in mammals (Houtkooper et al. 2010). In this pathway, homodimeric nicotinamide phosphoribosyltransferase (NAMPT) converts NAM to nicotinamide mononucleotide (NMN), and nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT) converts NMN to

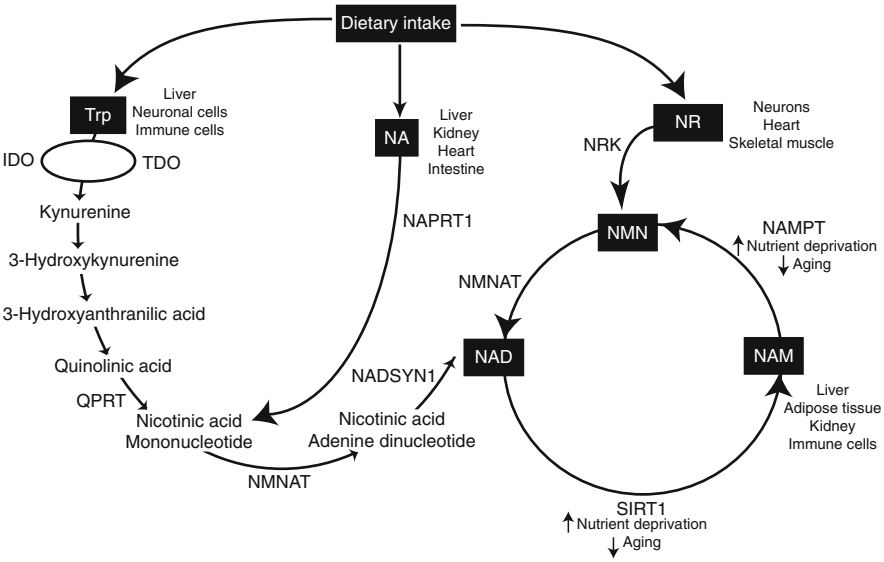

Fig. 4 Pathways of mammalian NAD biosynthesis. Four dietary metabolites can be used to generate NAD: tryptophan (Trp), nicotinamide (NAM), nicotinic acid (NA), and nicotinamide riboside (NR). De novo NAD biosynthesis occurs from Trp via the eight-step Kynurenine pathway. The first and rate-limiting step in this pathway is shared by tryptophan dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO), with TDO acting in the liver and the brain and IDO acting in the immune system. NA generates NAD through the Preiss–Handler pathway (PHP). In this pathway, nicotinic acid phosphoribosyltransferase (NAPRT1) forms nicotinic acid mononucleotide (NaMN), which is converted to NAD by the sequential actions of glutamine-dependent NAD synthetase (NADSYN1) and Nmnat. In the salvage pathway, homodimeric nicotinamide phosphoribosyltransferase (NAMPT) converts NAM to nicotinamide mononucleotide (NMN) and nicotinamide nucleotide adenylyltransferase (Nmnat) converts NMN to NAD. NR generates NAD by way of nicotinamide riboside kinase (Nrk) or purine nucleoside phosphorylase (Pnp) and nicotinamide salvage

NAD (Houtkooper et al. 2010; Revollo et al. 2004, 2007). Because NAMPT functions as the rate-limiting enzyme in this NAD biosynthetic pathway, NAMPT is perhaps the most important component for the regulation of mammalian NAD biosynthesis. Expression levels of NAMPT are varied throughout the body: high in the liver, kidney, and BAT; intermediate in the heart; and low in the skeletal muscle, brain, pancreas, WAT, lung, spleen, and testis in mice (Revollo et al. 2007). Overexpression of NAMPT increases NAD levels, while overexpression of NMNAT has no effect (Araki et al. 2004; Revollo et al. 2004). The fact that intracellular NAD levels increase with NAMPT overexpression in multiple, diverse cell types (Borradaile and Pickering 2009; Fulco et al. 2008; Hsu et al. 2009; Pillai et al. 2005; Revollo et al. 2004; Rongvaux et al. 2008; Song et al. 2008; van der Veer et al. 2005) suggests that NAMPT expression is sufficient to induce NAD biosynthesis. As pharmacological inhibition of NAMPT activity by the highly specific inhibitor FK866 reduces NAD levels 50–95% (Billington et al. 2008; Bruzzone et al. 2009; Hasmann and Schemainda 2003; Revollo et al. 2007; Rongvaux et al. 2008), indicating that NAMPT is necessary for NAD biosynthesis. NAMPT is required for survival, with lack of NAMPT causing embryonic lethality prior to day 10.5 (Revollo et al. 2007). In fact, NAMPT function is so vital that reducing NAMPT activity hampers cellular viability up to 90% (Billington et al. 2008; Dahl et al. 2010; Jia et al. 2004; Rongvaux et al. 2008; van der Veer et al. 2005). Thus, the factors that regulate NAMPT activity and expression should also be potent regulators of mammalian sirtuins.

One of these factors is AMP-activated protein kinase (AMPK). As knockdown of SIRT1 in myocytes impairs the ability of AMPK to regulate the expression of genes related to mitochondrial metabolism and fatty acid oxidation and to increase lipid oxidation-driven O_2 consumption (Canto et al. 2009), SIRT1 is clearly a primary downstream component of AMPK signaling. AMPK activates SIRT1 to deacetylate FOXO1, FOXO3a, and PGC-1α by increasing cellular NAD levels with no effect on SIRT1 protein levels, its protein–protein interactions, or its phosphorylation status (Canto et al. 2009; Chau et al. 2010). Thus, conditions that activate AMPK, such as nutrient deprivation and exercise, are associated with higher NAD levels and SIRT1 activity (Canto et al. 2010; Fulco et al. 2008). While the mechanism by which AMPK increases NAD levels is not fully elucidated, it appears to be, at least in part, by up-regulating expression of NAMPT (Canto et al. 2010; Costford et al. 2010; Fulco et al. 2008). Interestingly, an increase in mitochondrial β-oxidation appears to be required for AMPK to increase NAD levels and PGC-1α deacetylation. FGF21 and adiponectin have been reported to modulate SIRT1 activity by activating AMPK. FGF21 regulates energy homeostasis in adipocytes by inducing LKB1 that phosphorylates AMPK (Chau et al. 2010). Indeed, an increase in levels of phosphorylated AMPK by FGF21 triggers SIRT1 to deacetylate PGC-1α in *ob*/*ob* mice, resulting in enhanced mitochondrial function and reduced body weight (Chau et al. 2010). In myocytes, adiponectin has a similar effect. Adiponectin induces calcium influx through its receptor adiponectin receptor 1 (adipoR1), and this calcium activates the other major AMPK kinase, calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) (Iwabu et al. 2010). CaMKKβ-mediated activation of AMPK increased SIRT1-mediated deacetylation

of PGC-1α and increased mitochondrial biogenesis. Accordingly, skeletal muscle-specific AdipoR1 knockout mice exhibited decreased SIRT1 activity, PGC-1α expression, PGC-1α deacetylation, and mitochondrial biogenesis. Supporting the notions that FGF21 and adiponectin act upstream of AMPK, both factors also increase NAD levels (Chau et al. 2010; Iwabu et al. 2010).

4.2 Protein Regulators of SIRT1 Activity

Two proteins have been identified that regulate SIRT1 activity via a direct interaction: active regulator of SIRT1 (AROS) and deleted in breast cancer 1 (DBC1). Binding of the nuclear protein AROS to SIRT1 enhanced SIRT1-mediated p53 deacetylation, thus inhibiting the transcriptional activity of p53 and preventing p53-induced cell cycle arrest and apoptosis in response to DNA damage (Kim et al. 2007b). Opposite to AROS, binding of DBC1 to the catalytic domain of SIRT1 inhibits its activity, preventing SIRT1 from deacetylating p53 and FOXO3 following cellular stress, with the result of up-regulating p53 and FOXO-mediated apoptosis (Kim et al. 2008, 2009; Zhao et al. 2008). The activity of SIRT1 has been shown to be regulated by posttranslational modifications: phosphorylation and sumoylation (Yang et al. 2007b). At least 14 residues in SIRT1 are phosphorylated in vivo, with phosphorylation increasing the activity of SIRT1 (Guo et al. 2010; Nasrin et al. 2009; Sasaki et al. 2008). The kinases responsible for this phosphorylation are known to include CyclinB/Cdk1, cJun N-terminal kinase (JNK1), and two dual specificity tyrosine phosphorylation-regulated kinases, DYRK1A and DYRK3. It will be interesting to determine what other SIRT1 kinases exist. Sumoylation of human SIRT1 has also been reported to enhance its ability to deacetylate p53 sufficiently to prevent stress-induced apoptosis, although the sumoylation site is not evolutionarily conserved. Thirdly, two proteins have been identified which enhance the activity of SIRT1 by binding SIRT1 substrates. Four-and-a-half LIM2 (FHL2) enhances SIRT1-mediated deacetylation of FOXO1 in prostate cancer cells by binding FOXO1 in a manner that promotes the interaction between SIRT1 and FOXO1 (Yang et al. 2005). Since deacetylation inactivates FOXO1, FHL2 activity decreases the levels of FOXO target genes and FOXO1-induced apoptosis. Similarly, in postmitotic neurons, necdin, a melanoma antigen family protein, interacts with SIRT1 and p53, potentiating SIRT1-mediated deacetylation of p53, and thus, protecting neurons from DNA damage-induced apoptosis (Hasegawa and Yoshikawa 2008).

4.3 Nucleocytoplasmic Shuttling of SIRT1

Finally, nucleocytoplasmic shuttling may modulate SIRT1 activity. Cytoplasmic localization of SIRT1 is higher in adulthood than during embryonic development in

the heart (Tanno et al. 2007) and during postnatal development in brain (Li et al. 2008). The cellular localization of SIRT1 is also affected by differentiation and apoptosis. In myoblasts, SIRT1 is a nuclear protein until differentiation, at which point SIRT1 translocates to the cytoplasm (Tanno et al. 2007). In neural precursor cells, SIRT1 translocates from the cytoplasm into the nucleus for differentiation and then returns to the cytoplasm (Hisahara et al. 2008). SIRT1 also translocates from the nucleus to the cytoplasm during apoptosis (Jin et al. 2007; Ohsawa and Miura 2006). Despite these events, the significance of cytoplasmic SIRT1 remains unclear. As only nuclearly localized SIRT1 can engender direct transcriptional events (Tanno et al. 2007), cytoplasmic shuttling may be a way to down-regulate SIRT1 activity. However, SIRT1 deacetylase activity could still be highly relevant in the cytoplasm, as many substrates of SIRT1, such as p53, FOXOs, and NF-κB, also shuttle between compartments (Kwon and Ott 2008). Some evidence even suggests that cytoplasmically localized SIRT1 may promote apoptosis (Cohen et al. 2004; Jin et al. 2007; Tanno et al. 2007; Zhang 2007).

4.4 Regulators of SIRT1 Expression

Four transcriptional regulators have been reported to modulate of SIRT1 expression: p53, hypermethylated in cancer 1 (HIC1), C/EBPα, and E2F1. The first three may play a role in up-regulating SIRT1 expression under CR. Under basal nutritional conditions, p53 binds the SIRT1 promoter with the effect of repressing transcription of SIRT1 (Nemoto et al. 2004). However, upon starvation, the association of p53 with the SIRT1 promoter is prevented by the binding of FOXO3α to p53, thus up-regulating SIRT1 expression. HIC1 forms a transcriptional repression complex with SIRT1, the transcriptional corepressors CtBP, and class I HDACs that binds to the 5′ end of the SIRT1 promoter CpG island (Chen et al. 2005b). The association of CtBP with HIC1 is reduced upon glycolytic blockade, thus increasing SIRT1 expression in response to nutrient deprivation (Jin et al. 2010; Zhang 2007). SIRT1 expression is also enhanced upon nutrient deprivation by increased binding of C/EBPα to the SIRT1 promoter (Jin et al. 2010). While not linked to metabolic state, the cell-cycle regulator E2F1 may connect SIRT1 expression with replicative aging, as it activates SIRT1 transcription when cells begin to enter S phase (Wang et al. 2006).

Translationally, at least one protein and three microRNAs have been shown to regulate SIRT1 protein levels by binding the 3′ untranslated region of SIRT1's mRNA: Hu antigen R (HuR), miR-34a, miR-132, and miR-217. HuR is an mRNA-binding protein whose binding increases the half-life of SIRT1 mRNA from 1.2 to 8 hours (Abdelmohsen et al. 2007). miR-34a decreases SIRT1 protein levels in multiple, diverse cell types, with the effect of increasing acetylation levels of p53 and FOXO (Lee et al. 2010; Tarantino et al. 2010; Yamakuchi et al. 2008; Zhao et al. 2010). miR-132 decreased SIRT1-mediated deacetylation of p65 in adipocytes, leading to activation of NF-κB (Strum et al. 2009). In endothelial cells, inhibition of SIRT1 by miR-217 induced premature senescence due to high

levels of FOXO1 and nitric oxide synthase acetylation (Menghini et al. 2009). It will be interesting to see future work delineating the relevance of these molecular mechanisms to SIRT1 activity in vivo.

5 Sirtuin in Age-Associated Disorders

To achieve healthy aging, it is important to decrease the incidence and delay the onset of age-associated disorders. Accumulating bodies of evidence indicate that SIRT1 prevents age-associated disorders including type-2 diabetes (T2D) and Alzheimer's disease (AD) (Imai and Guarente 2010; Wang et al. 2010). Therefore, SIRT1 is an important therapeutic target to prevent age-associated diseases and extend healthspan (Fig. 5).

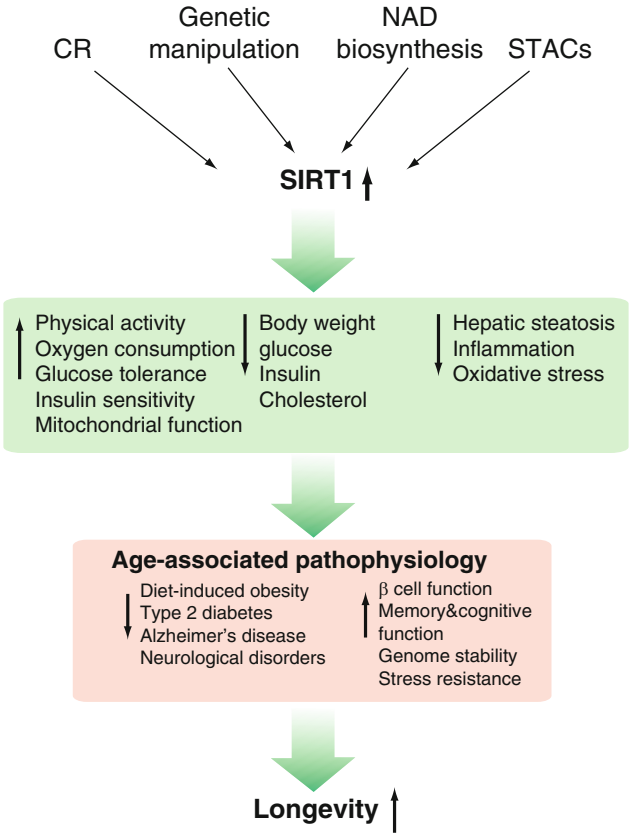

Fig. 5 Manipulation of SIRT1 function. Caloric restriction (CR), genetic manipulation, NAD biosynthesis, and sirtuin activating compounds (STACs) increase the dosage/activity of SIRT1, providing beneficial metabolic responses against numerous age-associated physiology and thereby promoting longevity

5.1 SIRT1 and T2D

T2D has a complex pathology consisting of defects in insulin secretion and action that together result in hyperglycemia (Bell and Polonsky 2001; Cavaghan et al. 2000). SIRT1 plays an important role in promoting insulin secretion by pancreatic β-cells and protecting against insulin resistance in the liver, skeletal muscle, and adipose tissue. These findings support the notion that SIRT1 is important for the maintenance of glucose homeostasis and the prevention of T2D. Indeed, under diabetogenic HFD conditions (Kraegen et al. 1991; Kubota et al. 1999; Surwit et al. 1988), the activity and/or protein expression of SIRT1 are reduced in several tissues (Deng et al. 2007; Escande et al. 2010; Qiao and Shao 2006). Moreover, increasing SIRT1 by genetic manipulation prevents metabolic disorders induced by HFD feeding. It has been reported that whole-body *Sirt1*-overexpressing transgenic mice improves glucose tolerance by decreasing hepatic glucose production without changes in body weight or fat composition in mice on a HFD (Banks et al. 2008). Similarly, another line of whole-body *Sirt1*-overexpressing transgenic mice show improvement of glucose tolerance, reduced lipid-induced inflammation, and protection against HFD-induced hepatic steatosis (Pfluger et al. 2008). In the kidney, which is highly susceptible to diabetic nephropathy, SIRT1 inhibits oxidative stress by inducing cyclooxygenase-2 (COX-2) expression (He et al. 2010b). It has also been reported that kidney-specific overexpression of SIRT1 protects cisplatin-induced acute kidney injury, likely by maintaining peroxisome number, upregulating catalase, and reducing renal reactive oxygen species (Hasegawa et al. 2010). Furthermore, SIRT1 genetic variation influences survival in the subjects with T2D (Zillikens et al. 2009a, b). These findings strongly suggest that SIRT1 could be a potential therapeutic target to improve insulin resistance and to protect against cell damage due to T2D.

Supporting the idea that SIRT1 could be a potential therapeutic target to combat metabolic disorders, SIRT1 also plays an important role in circadian rhythm. SIRT1 regulates CLOCK/BMAL1, the key transcription factor complex that controls the expression of clock genes (Nakahata et al. 2009). It has also been reported that SIRT1 directly regulates PER2 (Asher et al. 2008). The facts that *Clock* mutant mice and *Bmal1* mutant mice display metabolic disorders (Oishi et al. 2006; Turek et al. 2005) and that diet-induced obesity disrupts circadian behavior and circadian clock genes (Kohsaka et al. 2007) suggest that the maintenance of circadian rhythm is vital to prevent metabolic disorders. Interestingly, it has also been demonstrated that CLOCK:BMAL1 complex produces the circadian oscillation of NAMPT and NAD levels in vivo, comprising a novel circadian clock feedback loop involving NAMPT/NAD and SIRT1/CLOCK:BMAL1 (Nakahata et al. 2009; Ramsey et al. 2009). These findings strongly suggest that up-regulation of SIRT1 activity by NAMPT-mediated NAD biosynthesis could maintain proper circadian rhythm and prevent metabolic disorders.

5.2 SIRT1, AD, and Other Age-Associated Neurological Disorders

AD is chronic neurodegenerative disorder leading to synaptic dysfunction and neuronal cell loss (Haass and Selkoe 2007). Increasing lines of evidence suggest that SIRT1 provides protection from AD. Injection of lentivirus carrying *Sirt1* in the CA1 region of the hippocampus protects against neurodegeneration in the *p25* transgenic mouse model of AD (Cruz et al. 2006; Kim et al. 2007a). In an AD mouse model encoding the human APPswe and PSEN1 dE9 alleles, which exhibits strong β-amyloid (Aβ) plaque formation, SIRT1 suppresses the production of Aβ and plaques by deacetylating retinoic acid receptor β (RARβ) and thereby activating the transcription of ADAM10 encoding α-secretase (Donmez et al. 2010). α-secretases cleave amyloid precursor protein (APP), a type I transmembrane glycoprotein and the precursor of Aβ, preventing Aβ formation (He et al. 2010a). As a result, the overexpression of ADAM10 prevents amyloid plaque formation and hippocampal behavioral defects in AD mice (Postina et al. 2004). These findings potentially indicate that increasing SIRT1 dosage can prevent AD. Interestingly, NAD treatment attenuates Aβ generation, by activating SIRT1 in primary hippocampal neuronal cultures (Qin et al. 2006). Epidemiological studies have shown a strong association between diabetes and AD (Biessels and Kappelle 2005; Janson et al. 2004), and risk of AD is increased with development of T2D (Leibson et al. 1997; Ott et al. 1999; Takeda et al. 2010). Although it is still uncertain whether or not SIRT1 has a unifying pathophysiological role in T2D and AD, it is of great interest to investigate whether NAMPT-mediated systemic NAD biosynthesis plays an important role in AD pathophysiology as well as T2D.

SIRT1 has also been shown to be required for basal neural functions such as synaptic plasticity, cognition, and memory. In the hippocampus, SIRT1 modulates synaptic plasticity and memory formation by miR-134, a brain-specific microRNA, through the up-regulation of the expression of cAMP response element binding protein (CREB) and brain-delivered neurotrophic factor (BDNF) (Gao et al. 2010), which have critical roles in synaptic plasticity and synapse formation (Frank and Greenberg 1994; Kang and Schuman 1995). Moreover, *Sirt1*-deficient mice have impaired cognitive abilities such as deficits in immediate memory, classical conditioning, and spatial learning due to defects in synaptic plasticity (Michan et al. 2010). These findings highlight the benefits of increasing SIRT1 activity in preserving brain function and preventing age-associated neurological disorders.

6 Sirtuins in the Aging Process

Although it is still unclear whether sirtuins also play an important role in the regulation of mammalian lifespan, as it does in lower eukaryotes, recent results suggest that SIRT1 is important in the regulation of age-induced physiological changes (Fig. 5). For example, SIRT1 is involved in DNA damage-induced

chromatin reorganization that promotes genome stability and causes the reduction in silencing of SIRT1 target genes in mouse embryonic stem (ES) cells (Oberdoerffer et al. 2008; Oberdoerffer and Sinclair 2007). This damage-induced SIRT1 redistribution requires DNA damage signaling through a mammalian PI3-kinase ATM and histone H2AX, one of the targets of ATM. Importantly, these SIRT1-bound target genes that are derepressed by oxidative stress in mouse ES cells are also derepressed in aged mouse brain, and SIRT1 overexpression can suppress these age-associated changes (Oberdoerffer et al. 2008), suggesting that the damage-induced SIRT1 redistribution might trigger certain age-associated physiological changes in the brain.

The activity and expression of SIRT1 declines with age in several tissues. For example, in the kidney, mRNA and protein levels of SIRT1 significantly decline with age. Aged mice show the reduction of the interaction between SIRT1 and FOXO3, its transcriptional activity is regulated by SIRT1. Thus, SIRT1 activity is decreased in the aged kidney (Kume et al. 2010). In pancreatic β cells, SIRT1 activity appears to be reduced in aged mice due to a decline in systemic NAD biosynthesis. Aged BESTO mice lose the phenotype (see Sect. 3.4) that was shown in young BESTO mice, displays reduced level of *Ucp2* consistent with the lack of a difference in ATP content (Ramsey et al. 2008). Furthermore, the protein levels of SIRT1 are declined in several murine disease or accelerated aging models. (Pallas et al. 2008; Sommer et al. 2006), as well as during replicative senescence in normal human fibroblasts (Michishita et al. 2005). These findings strongly suggest that the maintenance of expression and/or activity of SIRT1 during senescence prevents against age-associated disorders.

Investigation into the intimate connection among sirtuins, metabolism, and aging has implicated that SIRT1 as a key mediator for physiological responses to CR in mammals. CR has been employed in aging research as an antiaging dietary intervention that shows protective effects on age-associated pathology of metabolic disorders and lifespan extension (Bronson and Lipman 1991; Colman et al. 2009; Fontana et al. 2004; Weindruch et al. 1986). Interestingly, CR-induced elevation of physical activity is abrogated in *Sirt1*-deficient mice (Chen et al. 2005a). *Sirt1*-deficient mice also exhibit dramatically reduced oxygen consumption under CR (Boily et al. 2008) and are resistant to CR-mediated improvement in the accumulation of damaged mitochondria under hypoxia in the kidney (Kume et al. 2010). Additionally, *Sirt1*-overexpressing transgenic mice display phenotypes similar to CR mice, including reduced body weight, improved glucose tolerance, reduced blood cholesterol, insulin, and fasted glucose levels, increased oxygen consumption, better performance on a rotarod challenge, and a delay in reproduction (Bordone et al. 2007). Additionally, two other systemic *Sirt1*-overexpressing transgenic mice have delayed the onset of age-associated disorder including obesity and T2D (Banks et al. 2008; Pfluger et al. 2008). Furthermore, SIRT1 functions as a key regulator of cell defenses and survival in response to stress, promoting CR-induced cell survival (Cohen et al. 2004). These findings suggest that manipulation of SIRT1 activity might mimic beneficial effects of CR on reducing age-associated disorders and possibly increasing longevity in mammals.

However, because SIRT1 has a divergent role in the regulation of metabolism in multiple tissues in mammals, it is conceivable that simply increasing SIRT1 activity systemically might not retard aging and show lifespan extension as well as CR. Indeed, it has recently been reported that whole-body SIRT1-overexpressing transgenic mice do not show lifespan extension (Herranz et al. 2010). Additionally, while most tissues show increases in SIRT1 protein levels under CR (Cohen et al. 2004; Qin et al. 2006), some other tissues show decreases or no change (Chen et al. 2008a; Cohen et al. 2009). In the brain, the effects of CR on SIRT1 expression are highly regional (Chen et al. 2008b). Therefore, increasing SIRT1 in a tissue/organ-specific manner by genetic manipulation might be important to retard aging and extend lifespan.

In this regard, the function of SIRT1 in the brain is interesting. In the hypothalamus, SIRT1 protein levels increase in the DMH, LH, and SCN (Satoh et al. 2010) under CR. Increasing SIRT1 in the brain of transgenic mice (brain-specific *Sirt1*-overexpressing transgenic mice: BRASTO mice) enhances neural activity in the DMH and LH, maintains body temperature, and promotes physical activity by increasing the expression of orexin type-2 receptor, a G protein-coupled receptor that binds to the neuropeptide hormone orexin. Moreover, BRASTO mice are more responsive to ghrelin, a gut hormone whose plasma level is increased under CR, than wildtype littermate, suggesting the function of SIRT1 in the hypothalamus, particularly in the DMH and LH, as a key mediator of the central adaptive response to CR (Satoh et al. 2010). These results support the idea that increasing hypothalamic SIRT1 activity mediates neurobehavioral adaptation to CR. Additionally, it has been reported that SIRT1 in the brain is a link between somatotropic signaling and CR in mammals (Cohen et al. 2009). Brain-specific *Sirt1* knockout (BSKO) mice induce dwarfism, reduce somatotropic signaling such as levels of growth hormone (GH) and insulin-like growth factor 1 (IGF-1) in plasma, displaying similar phenotypes to those in long-lived mutant mice (Chen et al. 2010), while BSKO mice do not increase their physical activity in response to CR as well as whole-body *Sirt1*-deficient mice (Chen et al. 2005a) and develop severe glucose intolerance with age (Cohen et al. 2009).

Other sirtuins might also play an important role for the regulation of aging and longevity. For example, SIRT3 has been linked to human longevity (Rose et al. 2003; Schapira 2011). *Sirt6*-deficient mice display the phenotype of premature senescence such as severe lymphopenia, loss of subcutaneous fat, osteopenia, and metabolic disorders, dying at around 4 weeks of age (Mostoslavsky et al. 2006). SIRT6 modulates telomeric chromatin by deacetylating lysine 9 of histone H3, preventing telomere dysfunction and cellular senescence (Michishita et al. 2008). Moreover, SIRT6 attenuates NF-κB signaling by deacetylating lysine 9 of histone H3 at chromatin, inhibiting apoptosis and cellular senescence (Kawahara et al. 2009; Mahajan et al. 2011). These findings provide the idea that increasing SIRT6 might prevent against age-induced physiological changes, thus possibly extend lifespan. It has been recently reported that SIRT1 is involved in maintaining SIRT6 expression under nutrient deprivation in both in vivo and in vitro (Kim et al. 2010). It should be addressed whether SIRT6 and other mammalian sirtuins serve to maintain physiological responses to CR.

7 Therapeutic Manipulation of Sirtuin Function

Considering the physiological effects of SIRT1 activity, SIRT1 activation has clear potential to combat numerous age-associated diseases, including metabolic syndrome, type 2 diabetes, cardiovascular disease, and neurodegenerative diseases (Borradaile and Pickering 2009; Ginsberg and MacCallum 2009; Hwang et al. 2009; Liu et al. 2008b; Milne et al. 2007; Picard et al. 2004; Qin et al. 2006; Ramsey et al. 2008; Sun et al. 2007). Augmentation of SIRT1 activity may also combat functional declines that occur with normal aging (Marton et al. 2010; Revollo et al. 2007). With this degree of potential, small-molecule sirtuin activating compounds (STACs) have been searched for and developed.

The STAC that coined the phrase is the polyphenolic compound, resveratrol, naturally found in grape skins (Howitz et al. 2003). First identified in a screen of small molecule libraries for hits which enhanced the deacetylase activity of human SIRT1 towards a synthetic, fluorophore-conjugated p53 peptide substrate, resveratrol increases deacetylation 2.0- to 2.6-fold, with 35- and 5-fold decreases in the Km of SIRT1 towards its substrate and NAD, respectively (Feige et al. 2008; Howitz et al. 2003; Milne et al. 2007; Pacholec et al. 2010). Supporting these biochemical assays, resveratrol can only convey its effects in cells expressing SIRT1 (Howitz et al. 2003; Lagouge et al. 2006). In mice, resveratrol (22 mg/kg/day) can significantly ameliorate or prevent HFD-induced impairments in insulin sensitivity, glucose tolerance, and motor function (Baur et al. 2006; Lagouge et al. 2006; Smith et al. 2009; Um et al. 2010). At higher doses (400 mg/kg/day), resveratrol can also protect HFD-fed mice against diet-induced weight gain. Furthermore, it has been reported that resveratrol prevents the HFD-induced decrease in lifespan (25–31%) (Baur et al. 2006; Pearson et al. 2008).

However, use of resveratrol is limited by two significant confounding factors. Firstly, resveratrol has many off-target effects (Baur 2010). Besides SIRT1, it can modulate AMPK, the estrogen receptor, the aryl hydrocarbon receptor, a cannabinoid receptor, and quinine reductase 2. Secondly, resveratrol has such low bioavailability that it is difficult to achieve serum levels in vivo high enough to activate SIRT1. For example, long-term administration of resveratrol (200 or 400 mg/kg/day) in rodents only generated nanomolar plasma concentrations (Lagouge et al. 2006). Furthermore, a volunteer trial in healthy humans found that even high doses of resveratrol could not elicit systemic levels high enough to be beneficial (Chaudhary and Pfluger 2009). To find compounds with higher potency and systemic retention, another small molecule screen was conducted, also using deacetylation of the fluorophore-conjugated p53 substrate to assess SIRT1 activity (Milne et al. 2007). The most potent molecule identified by this screen was SRT1720, which activates SIRT1 7.4- to 8.7-fold (Feige et al. 2008; Milne et al. 2007; Pacholec et al. 2010). Similar to resveratrol, treating HFD-fed mice or rodent models of diabetes with SRT1720 (100 mg/kg/day) improved glucose tolerance and insulin sensitivity in the liver, adipose tissue, and skeletal muscle as well as increased mitochondrial biogenesis (Feige et al. 2008; Milne et al. 2007; Smith et al. 2009). A higher dosage of SRT1720 (500 mg/kg/day) prevented diet-induced weight gain and reduced triglyceride and

cholesterol levels in HFD-fed mice (Feige et al. 2008). It also strongly promoted fatty acid oxidation and fat consumption in the skeletal muscle, liver, and BAT. Both molecules appear to induce these physiological effects by stimulating the transcription of genes involved in oxidative phosphorylation and mitochondrial biogenesis while decreasing those involved in inflammatory NF-κB signaling (Baur et al. 2006; Lagouge et al. 2006; Smith et al. 2009).

Even though these molecules were shown to activate SIRT1 in vitro (Baur et al. 2006; Feige et al. 2008; Howitz et al. 2003; Lagouge et al. 2006; Milne et al. 2007), recent work has cast doubt on the notion that resveratrol, SRT1720, and other STACs are direct activators of SIRT1 (Borra et al. 2005; Kaeberlein et al. 2005; Pacholec et al. 2010). The in vitro assays originally used to identify all of these compounds were shown to be dependent upon the fluorophore placed on the substrate peptide. One explanation for the fluorophore dependency of SIRT1 activity is that the fluorophore mimics a hydrophobic residue or pocket found on the native, full-length substrate and/or endogenous regulators that promote a higher affinity for SIRT1. Another potentially reconciling explanation is that these compounds do affect SIRT1 in vitro but indirectly do so through AMPK-mediated increases in cellular NAD levels in vivo. Both resveratrol and SRT1720 have been shown to activate AMPK in multiple organs, and resveratrol almost doubles NAD levels in myotubes in an AMPK-dependent manner (Baur et al. 2006; Dasgupta and Milbrandt 2007; Feige et al. 2008; Um et al. 2010). In fact, resveratrol (400 mg/kg/day) cannot affect any of the aforementioned physiological processes in mice lacking functional AMPK (Um et al. 2010). In other words, while the downstream effects of STACs show clear promise, further enhancement of their specificity and bioavailability are required.

While these STACs currently provide hope for sirtuin-targeted pharmaceutical interventions, several potential problems exist. Firstly, as SIRT1 activity is limited by NAD availability and as NAD levels decline with age, attempts to increase SIRT1 activity long term may prove difficult. Increasing SIRT1 activity without increasing the NAD pool may even be deleterious, as unnaturally high and/or prolonged SIRT1 activity can deplete NAD levels (Liu et al. 2008a). Secondly, attempts to generate highly specific sirtuin activators may prevent concurrent, beneficial activation of other sirtuin family members. Finally, although STACs can protect against or ameliorate disease pathology, they have minimal effects on metabolism and aging in regular chow fed rodents (Barger et al. 2008; Baur et al. 2006; Feige et al. 2008; Milne et al. 2007; Pearson et al. 2008).

One approach that may solve all these problems is to augment NAD levels through dietary supplementation of NAD substrates and intermediates. As these compounds are natural biological entities, they could be taken in the absence of disease pathology, potentially enhancing normal bodily functions throughout life. As evidence for this idea, pharmacologically increasing NADH oxidation in vivo increases SIRT1 activity with the effect of dramatically ameliorating all aspects of metabolic syndrome in a rodent model of the condition (Hwang et al. 2009). Systemic administration of NAD intermediates has further benefits of supplying NAD to the tissues/organs in which NAMPT expression is low, such as pancreatic β-cells and neurons (Imai 2009). These cell types likely depend upon extracellular

NAD intermediates. Indeed, the existence of an extracellular form of NAMPT (eNAMPT) has led us to generate the concept of the NAD World, in which systemic NAD biosynthesis mediated by intra- and extracellular NAMPT intricately links metabolic status throughout the body. Based on this concept, systemic administration of NAD intermediates would result in a coordinated adjustment of a variety of metabolic functions.

Interestingly, multiple studies have shown that SIRT1 activity directly depends upon that of NAMPT. For example, NAMPT activity augments SIRT1-mediated transcriptional effects (Revollo et al. 2004; Zhang et al. 2009) and cellular survival upon insult (Araki et al. 2004; Pillai et al. 2005). NAMPT has also been shown to activate SIRT1 to lengthen replicative lifespan, delay senescence (Ho et al. 2009), promote cellular maturation (van der Veer et al. 2007), and induce differentiation (Skokowa et al. 2009). NAMPT can also stimulate SIRT1 to enhance the ability of smooth muscle cells to develop blood vessels (van der Veer et al. 2005), chrondrocytes to produce cartilage (Dvir-Ginzberg et al. 2008), osteoblasts to produce type I collagen (Xie et al. 2007), THP-1 monocytes to induce matrix metalloproteinase-9 activity, peripheral blood mononuclear cells to relate IL-8 and TNF-α (Dahl et al. 2007), and aortic endothelial cells to form endothelial tube networks (Borradaile and Pickering 2009). Since NAMPT produces NMN, administration of NMN should have similar effects. As increased dosage of NAMPT has similar effects on gene expression profiles as increasing the dosage of SIRT1 (Revollo et al. 2004), it appears that SIRT1 is the main transcription factor downstream of NAMPT, and thus the primary target of NMN. Consistent with this idea, systemic administration of NMN can correct defects in insulin secretion and glucose tolerance caused by aging (Ramsey et al. 2008). NMN may even be able to reduce plasma cholesterol and increase insulin sensitivity in the liver and WAT, effects were seen upon injection of a NAMPT expression vector into HFD rats (Sun et al. 2009). Thus far, no side effects of NMN administration have been reported, making NMN supplementation an ideal way to optimize SIRT1 function through the enhancement of NAD biosynthesis. NR is another therapeutic possibility as it can maintain NAD levels and improves SIR2-dependent functions in yeast (Belenky et al. 2007; Bieganowski and Brenner 2004). In mammals, NR generates NAD by way of nicotinamide riboside kinase (Nrk) or purine nucleoside phosphorylase (Pnp) and nicotinamide salvage (Belenky et al. 2009; Bieganowski and Brenner 2004). However, this newly discovered compound has yet to be applied therapeutically in mammalian studies (Houtkooper et al. 2010; Sauve 2008). Thus, more studies will be required to understand the effects of NR.

8 Conclusions

In the past 10 years, interest in the field of sirtuin biology has exploded, generating countless investigations into the functions of mammalian sirtuins. These findings have clearly established that sirtuins are critical mediators of physiological responses to

nutritional availability and also that SIRT1 activity has beneficial effects against numerous age-associated disorders, especially those accompanied by metabolic complications. As SIRT1 functionality declines in metabolic syndrome, age-associated disorders, and the aging process, we hypothesize that augmentation of SIRT1 activity will positively affect mammalian metabolism, aging, and longevity. This notion also gives clear therapeutic appeal to long-term augmentation of SIRT1 activity. Currently, using STACs and NAD intermediates are two promising ways to achieve this potentially valuable goal. Nevertheless, the path before us is still long. Answering the following critical questions in the near future will greatly further our progress:

1. Which tissue(s)/organ(s) play the most critical roles in, and thus are the most relevant therapeutic targets for sirtuin-mediated physiological responses to metabolic state and aging?
2. How is the activity and/or expression of SIRT1 regulated in each tissue in response to metabolic state and aging?
3. How is NAD biosynthesis regulated in each subcellular compartment, and how does that regulation impact on each sirtuin?
4. Which NAD intermediates most effectively increase SIRT1 activity in each tissue/organ, and what are the pharmacokinetics of each NAD intermediate?
5. Can chronic supplementation of STACs or NAD intermediates augment SIRT1 activity and combat the symptoms of metabolic syndrome and the aging process in humans?

Future work addressing these questions will greatly enrich our understanding of how sirtuin function can be fine-tuned throughout our body and surely provide insights into pharmaceutical and nutriceutical interventions that will promote healthy aging for mankind.

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Mitochondrial Sirtuins in the Regulation of Mitochondrial Activity and Metabolic Adaptation

David B. Lombard, Daniel X. Tishkoff, and Jianjun Bao

Contents

Abstract In eukaryotes, mitochondria carry out numerous functions that are central to cellular and organismal health. How mitochondrial activities are regulated in response to differing environmental conditions, such as variations in diet, remains an important unsolved question in biology. Here, we review emerging evidence suggesting that reversible acetylation of mitochondrial proteins on lysine residues

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represents a key mechanism by which mitochondrial functions are adjusted to meet environmental demands. In mammals, three members of the sirtuin class of NAD^+-dependent deacetylases – SIRT3, SIRT4, and SIRT5 – localize to mitochondria and regulate targets involved in a diverse array of biochemical pathways. The importance of this activity is highlighted by recent studies of SIRT3 indicating that this protein suppresses the emergence of diverse age-related pathologies: hearing loss, cardiac fibrosis, and malignancy. Together, these findings argue that mitochondrial protein acetylation represents a central means by which mammals regulate mitochondrial functions to maintain cellular and organismal homeostasis.

Keywords Acetylation • AceCS2 • Apoptosis • β-Oxidation • Cancer • Cardiac hypertrophy • CPS1 • Electron transport • GDH • Glutathione • Hearing loss • HMGCS2 • IDH2 • Ketone body • LCAD • Metabolism • Mitochondria • Reactive oxygen species • Respiration • Sir2 • SIRT3 • SIRT4 • SIRT5 • SOD2 • Urea cycle

1 Introduction: Mitochondrial Regulation in Health and Disease

Mitochondria are cytoplasmic organelles that carry out numerous functions critical to cellular and organismal homeostasis (Wallace 2005). They generate the majority of cellular ATP via the Krebs cycle, oxidation of fatty acids, and oxidative phosphorylation. These activities make them the principal source of reactive oxygen species (ROS) within the cell. They also carry out amino acid degradation along with a portion of the urea cycle, and are the site of ketone body formation and heme biosynthesis. They represent a major storage site for cellular Ca^{2+}. In brown adipocytes, they function in heat generation. Finally, they are key mediators of apoptosis. Structurally, mitochondria consist of a matrix space enclosed by an impermeable inner mitochondrial membrane (IMM), in turn surrounded by a permeable outer mitochondrial membrane (OMM). Embedded in the IMM are electron transport chain complexes I–IV that extrude protons from the matrix, generating an electrochemical gradient across the IMM. At complex V (ATP synthase), protons flow back into the matrix with this gradient, coupled to ATP synthesis. Mitochondria possess circular genomes encoding 13 electron transport chain subunits plus some tRNA and rRNA genes. Thus, the great majority of proteins required for respiratory function and other mitochondrial activities (~1,500) are encoded in the nucleus. Inherited mutations in the mitochondrial genome cause a variety of syndromes of varied severity and age of onset, whereas acquired mitochondrial dysfunction may contribute to the degenerative manifestations of aging, as well as age-associated diseases such as type 2 diabetes, neurodegeneration, and malignancy (Wallace 2005).

1.1 The Challenge of Mitochondrial Regulation

Coordination of mitochondrial processes with those occurring in other parts of the cell represents a formidable regulatory challenge. This chapter focuses on emerging roles for acetylation of mitochondrial proteins in regulating functions of this organelle, and the involvement of sirtuin family deacetylases in this process. However, it is important to point out that many other pathways play roles in regulating mitochondrial number and function, a broader topic that is the subject of a number of excellent recent reviews (Finley and Haigis 2009; Ryan and Hoogenraad 2007; Scarpulla 2008). Briefly, factors that induce mitochondrial biogenesis – exercise, electrical stimulation, cold challenge, nitric oxide, thyroid hormone, and glucocorticoids – induce a coordinated transcriptional response from the mitochondrial and nuclear genomes (Butow and Avadhani 2004). Nuclear proteins involved in regulating the expression of nuclear-encoded mitochondrial genes include transcription factors (NRF-1 and -2, PPARα and γ, ERRα, and Sp1, among others) and members of the PGC-1 coactivator family (PGC-1α and -1β, and PRC) (Butow and Avadhani 2004; Scarpulla 2008). Other proteins with crucial roles in regulating mitochondrial functions include the deacetylase SIRT1 via its role in activating PGC-1α (Gerhart-Hines et al. 2007; Rodgers et al. 2008), as well as the AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) kinase (Finley and Haigis 2009; Schieke et al. 2006). Mitochondrial dysfunction is signaled to the nucleus by a variety of mechanisms, collectively termed the retrograde response in yeast, that remain poorly understood in mammals. A specialized system involving the nuclear transcription factors CHOP and C/EBPβ exists to indicate the presence of unfolded proteins in mitochondria (Ryan and Hoogenraad 2007).

1.2 Calorie Restriction-Induced Alterations in Mitochondrial Functions

Variations in diet represent a challenge to mitochondrial function. Mitochondrial adaptation to altered diet is thought to be particularly significant in the context of calorie restriction (CR) – i.e., reduced caloric intake without malnutrition – an intervention that robustly extends lifespan in organisms ranging from budding yeast to rodents, and potentially primates as well (Fontana 2009). In rodent models, CR extends lifespan and delays the onset of a host of age-associated pathologies, including type 2 diabetes, cardiovascular disease, renal failure, cancer, and neurodegeneration (Fontana 2009). Many CR-associated health benefits have been observed in studies of nonhuman primates, notably dramatic reductions in cancer, cardiovascular disease, brain atrophy, sarcopenia, and type 2 diabetes

(Colman et al. 2008, 2009). In humans, CR is associated with greatly improved metabolic and cardiovascular function (Fontana 2009). Thus, pharmacologic mimics of CR would likely have far-reaching health benefits in humans.

Mechanisms of longevity extension by CR remain incompletely understood; however, metabolic alterations occurring in the adaptation to CR are likely in part responsible for the beneficial effects of this intervention. These changes implicate alterations in mitochondrial functions as an integral component of the CR response (Anderson et al. 2008b). In budding yeast, increased mitochondrial respiration is required for longevity extension by some (Lin et al. 2002) but not all (Kaeberlein et al. 2005) CR regimens. In *C. elegans*, CR or genetic and pharmacologic CR mimetics also induce increased mitochondrial respiration, which is required for CR-induced longevity (Bishop and Guarente 2007; Houthoofd et al. 2002a, b; Schulz et al. 2007). In mammals, CR induces mitochondrial biogenesis in a tissue-specific manner, at least in part through increased expression and activity of endothelial nitric oxide synthase and PGC-1α (Anderson et al. 2008a; Civitarese et al. 2007; Lambert et al. 2004; Lopez-Lluch et al. 2006; Nisoli et al. 2005). Conflicting data have been published regarding the effects of CR on mitochondrial respiration in mammals (Hunt et al. 2006; Lambert et al. 2004; Nisoli et al. 2005; Weindruch et al. 1980). Numerous reports indicate that CR reduces mitochondrial ROS generation, a source of chronic cellular injury, and concomitantly reduces accumulation of macromolecular oxidative damage (Gredilla and Barja 2005). Several mechanisms may account for this decline in ROS during CR. The recruitment of a larger complement of mitochondria to generate ATP during CR could in and of itself reduce ROS generation (Guarente 2008), as could increased mitochondrial turnover occurring during CR, via removal of damaged mitochondria that would otherwise produce excessive ROS (Miwa et al. 2008). Increased mitochondrial uncoupling observed under CR conditions could also attenuate ROS generation (Brand 2000). As described below, new data suggest that the SIRT3 deacetylase plays a key role in bolstering mitochondrial antioxidant defenses during CR (Qiu et al. 2010; Someya et al. 2010). Overall, CR leads to increased mitochondrial biogenesis and decreased ROS generation in mammals.

In addition to these impacts, CR entails a shift from glucose utilization to the use of alternative sources of energy such as amino acids, ketones, and fatty acids (Spindler and Dhahbi 2007). This transition necessitates the adaptation of numerous mitochondrial metabolic pathways. Indeed, within mitochondria, activities of a wide variety of enzymes have been shown to be altered in response to CR; much of this work has focused on the liver (Dhahbi et al. 2001; Hagopian et al. 2003, 2004, 2005; Tillman et al. 1996). Transcriptional changes occurring during CR have been extensively characterized (Anderson and Weindruch 2007). However, modulation of gene expression does not account for all of these activity changes (Spindler and Dhahbi 2007). This implies that nontranscriptional mechanisms to regulate mitochondrial functions during CR must exist.

2 Protein Acetylation Is a Conserved Mechanism of Metabolic Regulation

Reversible acetylation on the ε-amino group of internal lysine residues (hereinafter referred to as acetylation) has emerged as a posttranslational modification with a crucial role in regulating target protein function, akin to phosphorylation. This modification is distinct, chemically and functionally, from acetylation of the α-amino groups of N-terminal residues; the latter occurs during translation and is irreversible (Polevoda and Sherman 2002). Although lysine acetylation was originally discovered on histones in the context of chromatin regulation, it is now clear that acetylation plays a crucial role in regulating a plethora of nonhistone proteins, including transcription factors and metabolic enzymes (Spange et al. 2009). As discussed in depth below, acetylation/deacetylation of proteins within mitochondria, regulated by sirtuin deacetylases, likely represents one mechanism by which mitochondrial functions are tailored to meet the demands of dietary challenges such as CR and other metabolic perturbations.

2.1 Mass Spectrometry Surveys Reveal That Acetylation of Mitochondrial Proteins Is Widespread

One of the first clues as to the wide-ranging impact of acetylation on diverse cellular functions came from a large-scale proteomic survey to identify acetylated proteins (Kim et al. 2006). Using acetyl-lysine affinity purification coupled with mass spectrometry, this study identified acetylation sites on 195 proteins, including numerous nonhistone proteins. Strikingly, 133 acetylated proteins were identified within the mitochondrion, an organelle where acetylation of only a single protein had previously been documented (Hallows et al. 2006; Schwer et al. 2006). Moreover, this report showed that acetylation in liver mitochondria changed in response to fasting, suggesting that this modification might play a role in mitochondrial response to food deprivation. The widespread nature of mitochondrial protein acetylation was confirmed in several subsequent independent mass spectrometry surveys (Choudhary et al. 2009; Kendrick et al. 2010; Schwer et al. 2009; Zhao et al. 2010). Work by many laboratories suggests a central role for acetylation of mitochondrial proteins in the regulation of individual enzyme activities and overall metabolism (Zhao et al. 2010). Acetylation of proteins involved in virtually every mitochondrial function has been reported. Much of the work examining the role of acetylation in regulating mitochondrial protein activities has been performed in the context of studies of sirtuin deacetylases and is discussed below. This role of acetylation in regulating metabolic functions is ancient and conserved, and exists even in bacteria, where it is controlled by the opposing activities of Pat acetyltransferase and the sirtuin CobB (Starai et al. 2002; Wang et al. 2010; Zhang et al. 2009).

2.2 *Mitochondrial Protein Acetylation Is Altered in Response to Calorie Restriction and Other Dietary Interventions*

Work from our group has implicated alterations in mitochondrial protein acetylation specifically in the response to CR (Schwer et al. 2009). Global mitochondrial acetylation is altered in a tissue-specific manner during CR; these changes are particularly striking in liver and in brown adipose tissue, where global mitochondrial acetylation rises and falls, respectively, in response to this diet. Mass spectrometry analysis revealed that acetylation of at least 72 hepatic mitochondrial proteins increases during CR, involving essentially all mitochondrial metabolic pathways. The functional impact of these acetylation changes at the level of individual enzyme activities has been elucidated for only a very few targets (Ahn et al. 2008; Cimen et al. 2010; Nakagawa et al. 2009). One target of CR-associated acetylation changes identified in this study, the E1α subunit of the pyruvate dehydrogenase complex (PDC) (Schwer et al. 2009), is of particular interest, in that this enzyme performs the rate-limiting, final step in glycolysis: conversion of pyruvate to acetyl-CoA for use in the Krebs cycle or biosynthetic processes. Potential alterations in PDC activity mediated by acetylation could have far-reaching metabolic consequences in the cell. In this regard, in budding yeast, PDC is required for CR-induced lifespan extension, and overexpression of a PDC subunit extends replicative lifespan in this organism (Easlon et al. 2007), suggesting that regulation of PDC function may represent a conserved component of the response of mitochondria to CR. The actual impact of altered acetylation on PDC remains to be elucidated.

Aside from CR, acetylation of many mitochondrial and nonmitochondrial proteins in liver also rises in response to high-fat diet (HFD) and chronic ethanol ingestion, conditions associated with diminished mitochondrial respiratory function (Kendrick et al. 2010; Picklo 2008; Shepard et al. 2010; Shulga and Pastorino 2010). Whether or not the acetylation changes occurring during CR and other dietary stresses represent regulated events, and whether overlapping or distinct sets of protein substrates and individual lysine residues are targeted under different conditions remain unsolved but important questions. Overall, acetylation of mitochondrial proteins has emerged as a widespread modification that plays a crucial role in regulating mitochondrial functions. The critical question is how this modification is regulated at the level of individual substrates in response to varied environmental conditions.

3 Mitochondrial Sirtuins in Metabolic Regulation

Conserved from bacteria to mammals, the sirtuins are a protein family involved in regulating many biological processes, including stress responses, metabolism, development, and longevity (Haigis and Sinclair 2010). Sirtuins modify target

proteins by means of their lysine deacetylase and ADP-ribosyltransferase activities; both require NAD^+ as an obligate cofactor. Since NAD^+ levels rise in response to reduced energy status and/or altered redox, sirtuins provide a means by which cells sense and respond to their environment (Guan and Xiong 2010). Sirtuin activity can be repressed by NADH and by a product of the sirtuin deacetylase reaction, nicotinamide (NAM) (Bitterman et al. 2002; Lin et al. 2004). Mammals possess seven sirtuins, SIRT1–SIRT7; these are a diverse protein family, with varied tissue expression, subcellular localization patterns, activity profiles, and targets (Imai et al. 2011). Three mammalian sirtuins (SIRT3, SIRT4, and SIRT5) are mitochondrial. These sirtuins are thus ideally positioned to regulate mitochondrial functions via modification of proteins within this organelle. Characterization of mitochondrial sirtuin functions has been aided immensely by the availability of mouse strains with targeted mutations in these genes (Haigis et al. 2006; Lombard et al. 2007). SIRT3 is a potent deacetylase with many mitochondrial targets, whereas SIRT5 is a more selective deacetylase; in contrast, the only function characterized for SIRT4 to date is as an ADP-ribosyltransferase (Ahuja et al. 2007; Haigis et al. 2006; Lombard et al. 2007; Nakagawa et al. 2009; North et al. 2003). Although HDAC7, a nonsirtuin deacetylase, has been reported to localize to mitochondria (Bakin and Jung 2004), no mitochondrial substrates have been reported for this enzyme. By contrast, a large amount of data exists supporting a crucial role for mitochondrial sirtuins in the regulation of mitochondrial functions.

4 SIRT3 Is a Master Regulator of Mitochondrial Functions and Suppresses Age-Associated Phenotypes

Among the mitochondrial sirtuins, SIRT3 functions have been characterized in the greatest detail. Initial studies of SIRT3-deficient mice indicated that loss of SIRT3, but not SIRT4 or SIRT5, led to dramatic protein hyperacetylation within mitochondria, suggesting that SIRT3 deacetylates numerous targets in this organelle and is the major mitochondrial deacetylase (Lombard et al. 2007). The mitochondrial localization of SIRT3 has been extensively demonstrated by multiple independent laboratories (Cooper et al. 2009; Lombard et al. 2007; Onyango et al. 2002; Schwer et al. 2002). In humans, full length SIRT3 is a 44-kilodalton (kDa) protein with an N-terminal mitochondrial targeting sequence that is enzymatically inactive in vitro. It is proteolytically processed in mitochondria to a mature 28-kDa, catalytically active deacetylase (Onyango et al. 2002; Schwer et al. 2002). The first mouse SIRT3 cDNA sequence identified encoded a 28-kDa protein lacking the N-terminal mitochondrial targeting sequence (Yang et al. 2000). However, several recent studies have identified a longer isoform of murine SIRT3 encoding a 37-kDa protein that can be imported into mitochondria and processed into the mature 28-kDa protein (Bao et al. 2010a; Cooper et al. 2009; Jin et al. 2009; Yang et al. 2010b).

Whether or not an active fraction of SIRT3 exists outside mitochondria and modifies extramitochondrial proteins is a controversial topic. There is one report that human SIRT3 localizes to nuclei, where it deacetylates histones, and is imported into mitochondria upon cellular stress such as genotoxic insult (Scher et al. 2007). Co-overexpression of SIRT5 along with SIRT3 is reported to drive SIRT3 to the nucleus (Nakamura et al. 2008). Rat SIRT3 was detected not only in mitochondria but also in the nucleus and cytoplasm of cardiomyocytes (Sundaresan et al. 2008). Unfortunately, current data supporting the presence of active extramitochondrial SIRT3 are based on overexpression and/or knockdown approaches rather than on analysis of cells and tissues derived from SIRT3-null mutants. While it is clear that SIRT3 deficiency impacts cellular physiology outside mitochondria, this could occur due to retrograde signaling occurring in the context of mitochondrial dysfunction induced by lack of SIRT3. Conversely, it remains a possibility that an active fraction of SIRT3 might exist outside mitochondria in specific tissues and cell types (e.g., cardiomyocytes) (Sundaresan et al. 2008). The role of SIRT3 in regulating extramitochondrial signaling is reviewed below; we now turn to a discussion of mitochondrial processes targeted by SIRT3.

4.1 SIRT3 Regulates Numerous Metabolic Pathways Within Mitochondria

Expression studies of SIRT3 have shown that SIRT3 levels rise in adipose tissue, skeletal muscle, and liver during CR or fasting (Hirschey et al. 2010; Palacios et al. 2009; Schwer et al. 2009; Shi et al. 2005), and conversely decline in insulin-resistant states (Yechoor et al. 2004) or in response to high-fat feeding (Bao et al. 2010b; Kendrick et al. 2010; Palacios et al. 2009). These expression data suggest that SIRT3 might play a role in the response to caloric deprivation. The first mitochondrial SIRT3 substrate identified was acetyl-CoA synthetase 2 (AceCS2) (Hallows et al. 2006; Schwer et al. 2006; Shimazu et al. 2010b). AceCS2 converts free acetate, produced from endogenous catabolic reactions or absorbed from the gut, into the active metabolite acetyl-CoA for energy production in the Krebs cycle. In mammals, two independent studies showed that SIRT3 interacts with and deacetylates AceCS2 at the active site lysine to promote AceCS2 activity (Hallows et al. 2006; Schwer et al. 2006). Interestingly, in *Salmonella*, the homolog of AceCS2 is also activated by the sirtuin CobB (Starai et al. 2002; Starai and Escalante-Semerena 2004), an activity that is crucial for bacterial growth on acetate. In mammals under fed conditions, the majority of acetyl-CoA is generated through metabolism of pyruvate by PDC and by fatty acid β-oxidation, largely bypassing the need for AceCS2. In this regard, studies of AceCS2-deficient mice revealed that AceCS2 is specifically required for metabolic homeostasis in the context of a low carbohydrate (LC)/HFD; AceCS2-deficient animals are essentially normal on a chow diet but show poor weight gain, hypothermia, hypoglycemia, and

impaired survival on a LC/HFD (Sakakibara et al. 2009). It will be of interest to assess the impact of a LC/HFD on SIRT3-deficient animals to determine whether they show similar defects in vivo. Presumably, the role of SIRT3 in regulating AceCS2 could also be important during fasting, when acetate can be used as a source of energy in extrahepatic tissues (Hirschey et al. 2010). In this context, SIRT3 has recently been shown to deacetylate and activate 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), a mitochondrial enzyme that converts acetyl-CoA into ketone bodies (acetoacetate, β-hydroxybutyrate, and acetone) in the liver under fasting conditions, which can in turn be used as a source of energy in certain tissues such as the brain (Shimazu et al. 2010a). SIRT3-deficient mice are unable to produce normal levels of ketone bodies upon fasting. Thus, SIRT3 promotes multiple aspects of the response to fasting, and, as we shall see, CR.

Emerging data from several laboratories have shown that one major function of SIRT3 is regulation of mitochondrial electron transport chain activity to maintain energy homeostasis. Initial analysis of SIRT3-deficient animals demonstrated that these mice were metabolically unremarkable with respect to overall respiration, fuel utilization, activity, and cold tolerance (Lombard et al. 2007). However, subsequent detailed studies revealed that SIRT3-deficient liver, heart, kidney, and fibroblasts all show reduced basal ATP levels (Ahn et al. 2008). Moreover, SIRT3 interacts with the mitochondrial complex I component NDUFA9, and SIRT3 deficiency is associated with increased complex I acetylation and inhibition of its activity (Ahn et al. 2008; Bao et al. 2010b; Kim et al. 2010). Besides complex I, SIRT3 also regulates other electron transfer chain components, including complex II (Cimen et al. 2010), III (Kendrick et al. 2010; Kim et al. 2010), IV (Kendrick et al. 2010), and V (Bao et al. 2010b). In all of these cases, increased complex acetylation occurring in the context of SIRT3 deficiency or knockdown correlates with decreased complex activity. These findings suggest that SIRT3 regulates many aspects of mitochondrial oxidative phosphorylation. In future studies, it will be of interest to define at a mechanistic level how acetylation on electron transport chain subunits affects ATP generation. For example, acetylation could in principle affect electron transport chain subunit activity, turnover, and/or complex assembly/stability, among other parameters. It is also important to elucidate why it might be desirable under some physiologic conditions to downregulate electron transport chain activity via increased acetylation. As an added wrinkle, SIRT3 negatively regulates translation within mitochondria by deacetylating the ribosomal protein MRPL10, a function proposed to reduce respiration (Yang et al. 2010a).

Another recently identified role for SIRT3 involves its regulation of mitochondrial β-oxidation of fatty acids, the major pathway of fatty acid breakdown in mammals (Hirschey et al. 2010). SIRT3-deficient animals show elevated levels of long-chain fatty acids upon fasting, along with impaired β-oxidation. The authors of this study found that SIRT3 deacetylates and activates the β-oxidation enzyme long-chain specific acyl-CoA dehydrogenase (LCAD). Although young adult (12-week old), fed SIRT3-deficient animals show no cold sensitivity (Lombard et al. 2007), very young (4-week old), fasted SIRT3-deficient mice are cold intolerant, much like mice lacking LCAD (Guerra et al. 1998) and other models

of defective β-oxidation. As mass spectrometry surveys have revealed acetylation of additional β-oxidation enzymes besides LCAD (Schwer et al. 2009), it remains to be seen whether SIRT3 regulates β-oxidation by deacetylation of additional targets. Independently, it was found that SIRT3-deficient hepatocytes are more susceptible to fatty-acid induced cell death, a phenotype suppressed by antioxidants and which the authors attributed to electron transport chain dysfunction leading to elevated ROS production (Bao et al. 2010b). SIRT3 also increases β-oxidation in muscle cells in culture and reduces lipid accumulation in HepG2 cells, effects ascribed to the role of SIRT3 in promoting AMPK activity (Palacios et al. 2009; Shi et al. 2010). Overall, SIRT3 allows the efficient use of fatty acids as fuel with minimal cellular toxic effects through deacetylation of multiple mitochondrial targets.

4.2 SIRT3 Impacts Extramitochondrial Signaling

One important issue that remains to be resolved is how SIRT3 impacts extramitochondrial processes. As mentioned above, PGC-1α is a coactivator protein that plays a critical role in promoting mitochondrial biogenesis, adaptive thermogenesis, fatty acid oxidation, and ROS detoxification, among other metabolic processes (Jeninga et al. 2010). PGC-1α stimulates SIRT3 expression by binding to the SIRT3 promoter together with estrogen-related receptor α (ERRα) (Kong et al. 2010). Strikingly, the reciprocal relationship holds as well; SIRT3 is required for normal PGC-1α expression in brown adipocytes and in skeletal muscle (Palacios et al. 2009; Shi et al. 2005). SIRT3 promotes PGC-1α expression by stimulating phosphorylation and activity of factors known to regulate PGC-1α expression, CREB and AMPK (Palacios et al. 2009; Pillai et al. 2010; Shi et al. 2005). SIRT3 is also required for PGC-1α to induce mitochondrial biogenesis and expression of its target genes, particularly genes involved in ROS detoxification, and for PGC-1α to suppress ROS levels (Kong et al. 2010). How SIRT3 impacts PGC-1α is not completely clear, although one study attributes this effect to a role of (presumably extramitochondrial) SIRT3 in deacetylating and activating LKB1, a kinase upstream of AMPK (Pillai et al. 2010). Since SIRT3 promotes increased ATP production in many tissues (Ahn et al. 2008), and AMPK is phosphorylated and activated in response to an increased AMP:ATP ratio, SIRT3 might be predicted to suppress rather than stimulate AMPK phosphorylation and activity. These effects may represent functions of extramitochondrial SIRT3, or the indirect effects of a retrograde response. In any case, the fact that SIRT3 impacts activities of master metabolic regulators such as PGC-1α and AMPK complicates the interpretation of data generated using SIRT3-deficient mice and cells, since it may be unclear whether a given phenotype results from loss of SIRT3 itself and its role in deacetylating a particular mitochondrial target, or from secondary effects on overall cellular physiology associated with loss of SIRT3.

4.3 SIRT3 and Cell Death

Because mitochondria are central to the intrinsic pathway of cell death, several groups have assessed roles of SIRT3 in modulating cell survival, and have obtained conflicting results. Given that SIRT3 has numerous substrates exerting diverse biological effects (Lombard et al. 2007), it is perhaps not surprising that discrepant results have been obtained in this regard. Experimental differences in the cell type analyzed, the nature of cellular injury, etc., likely explain these disparities. SIRT3 overexpression sensitizes lymphoma cells to kaempferol, a flavonoid that induces ROS and cell death (Marfe et al. 2009). SIRT3-deficient mouse embryonic fibroblasts (MEFs) are resistant to cell death induced by DNA damage (Kim et al. 2010). Similarly, in a variety of tumor cell lines, SIRT3 knockdown confers resistance to cell death induced by depletion of Bcl-2 (Allison and Milner 2007). Conversely, SIRT3 protects cardiomyocytes against genotoxin-induced killing, an effect attributed to a role of extramitochondrial SIRT3 in deacetylating the Ku70 protein to promote its interaction with the proapoptotic protein Bax (Sundaresan et al. 2008). SIRT3 is also required for cell survival in response to the genotoxin methyl methanesulfonate (Yang et al. 2007). Similarly, SIRT3 promotes viability of hepatocytes in response to TNFα exposure via deacetylation and inactivation of cyclophilin D (cypD) (see below) (Shulga and Pastorino 2010). In bladder cancer cells, SIRT3 also allows continued proliferation following induction of the p53 tumor suppressor, which ordinarily induces senescence (permanent growth arrest) (Li et al. 2010). This function of SIRT3 has been ascribed to the ability of SIRT3 to deacetylate p53 within mitochondria. In vivo, SIRT3 plays an important role in promoting long-term survival of cells in the inner ear to preserve hearing during CR (see below) (Someya et al. 2010), suggesting that in this specific context, the prosurvival function of SIRT3 is dominant.

SIRT3 likely modulates cell death through multiple different mechanisms and targets. Several groups have reported that SIRT3 plays a major role in suppression of intracellular ROS levels (Bao et al. 2010b; Kim et al. 2010; Kong et al. 2010; Qiu et al. 2010; Someya et al. 2010). Since ROS are a potent inducer of apoptosis, this activity of SIRT3 provides a potential means by which SIRT3 could promote cellular survival. Recent studies have revealed that another key mechanism by which SIRT3 suppresses cell death is through deacetylation of cypD. CypD is a peptidyl-prolyl isomerase that potentiates the activity of the mitochondrial permeability transition pore (MPTP), a nonselective high-conductance channel promoting cell death, particularly in the contexts of cardiac and neuronal ischemia (Giorgio et al. 2010). SIRT3 was shown to deacetylate and inactivate cypD to promote mitochondrial respiration in the presence of a nonfermentable carbon source (Shulga et al. 2010). Subsequent elegant studies have revealed that this role of SIRT3 is also important in antagonizing cell death (Shulga and Pastorino 2010). Ethanol treatment of cells suppresses SIRT3 function via decreasing the NAD^+: NADH ratio. This in turn promotes hyperacetylation and increased activity of cypD and the MPTP. This effect of ethanol can be prevented by treatment of cells with

either the AMPK activator AICAR or acetoacetate, both of which elevate the NAD^+:NADH ratio and restore SIRT3 activity. As an important control, introduction of a cypD acetylation site mutant that mimics deacetylation prevents ethanol-induced sensitization to TNFα and bypasses the requirement for SIRT3. Interestingly, although cypD promotes cell death in the context of TNFα treatment, it actually suppresses apoptosis in response to other stimuli (Li et al. 2004; Schubert and Grimm 2004). Thus, this single SIRT3 substrate may represent a key target in the context of both pro- and antisurvival roles for SIRT3 described above. Future studies are needed to assess the roles of SIRT3 in regulating cypD in the context of cell death in vivo, particularly in response to medically relevant stressors such as ischemic injury.

4.4 SIRT3 in the Regulation of Lifespan and Age-Associated Phenotypes

In addition to elucidating its roles in regulating specific biochemical pathways in mitochondria, there is great current interest in testing whether SIRT3 might modulate age-associated phenotypes, or indeed lifespan itself. In this regard, some studies have linked polymorphisms in the *SIRT3* genomic locus to human longevity, though others have failed to demonstrate this association (Bellizzi et al. 2005, 2007; Lescai et al. 2009; Rose et al. 2003). A polymorphism associated with decreased SIRT3 mRNA expression was present in cohorts of young but not old men, suggesting that reduced SIRT3 expression may be detrimental to survival in old age (Bellizzi et al. 2005, 2009). In sedentary individuals, SIRT3 protein expression declined with age in skeletal muscle mitochondria, concomitant with a reduction in respiratory function (Lanza et al. 2008).

4.4.1 SIRT3 and Cardiac Hypertrophy/Fibrosis

Emerging data from mouse models suggest that SIRT3 may indeed play an important role in delaying the onset of age-associated disorders (Fig. 1). Cardiac hypertrophy is a common age-associated pathology in Western societies, where it is most frequently caused by hypertension. While initially an adaptive response, cardiac hypertrophy can lead to various downstream sequelae such as arrhythmias and ischemia. New data suggest a role for SIRT3 in antagonizing the onset of this disease. SIRT3-deficient mice show mild cardiac hypertrophy and fibrosis at baseline, and greatly exaggerated hypertrophy response to pharmacologic stimuli; conversely, a SIRT3 overexpressor is protected (Sundaresan et al. 2009). The authors find that SIRT3 negatively modulates intracellular signaling pathways known to promote hypertrophy. The authors attribute this effect to an extramitochondrial role of SIRT3 in suppressing ROS levels via deacetylation of the FoxO3A

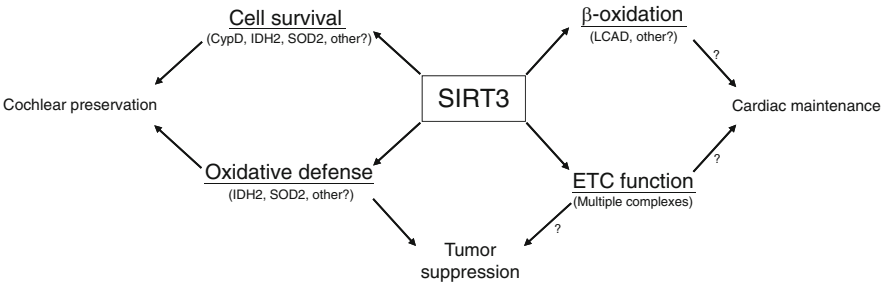

Fig. 1 SIRT3 suppresses the onset of diverse age-associated pathologies. Mitochondrial and cellular impacts of SIRT3 are *underlined*, along with proposed mitochondrial substrates in *parentheses*. Some postulated substrate–phenotype relationships represent speculation by the authors; these are designated with *question marks*. Note that some roles of SIRT3 (e.g., promoting ketone body synthesis and suppressing mitochondrial translation) are omitted for clarity's sake. See text for details

forkhead transcription factor, inducing FoxO3A nuclear localization and increased expression of the oxidative defense proteins catalase and superoxide dismutase 2 (SOD2). FoxO3A activity is required for SIRT3 to induce its antihypertrophic effects in a tissue culture model system. The same group has found that exogenous NAD^+ can block cardiomyocyte hypertrophy in vivo and in tissue culture in a SIRT3-dependent manner. The authors attribute this role of SIRT3 both to its aforementioned role in suppressing ROS levels, and to its role in deacetylating and inactivating the LKB1 kinase (Pillai et al. 2010). This work reveals important roles for SIRT3 in preventing age-associated cardiac pathology; given the many mitochondrial substrates for SIRT3, it is difficult to know whether extramitochondrial FoxO3A and/or LKB1 represents the relevant substrates for SIRT3 in this process in vivo. In particular, some human patients with mitochondrial genome mutations develop cardiac hypertrophy, suggesting that impaired respiratory function occurring in SIRT3 deficiency per se could conceivably contribute to this phenotype (Vydt et al. 2007). Significantly, mice deficient in LCAD, a known SIRT3 substrate, also develop cardiac hypertrophy (Cox et al. 2009; Kurtz et al. 1998); thus, SIRT3 could promote cardiac health by multiple mechanisms.

4.4.2 SIRT3 and Mammary Cancer

In most mammals, an increased incidence of malignancy is a prominent feature of aging (Lombard et al. 2005). A recent ground-breaking study has revealed SIRT3 to be a tumor suppressor (Kim et al. 2010). SIRT3-deficient MEFs show higher levels of ROS in response to various forms of stress, aberrations in nuclear chromosome number, and decreased mitochondrial genome integrity, which is also observed in the livers of SIRT3-deficient animals with age. Moreover, SIRT3-deficient MEFs are more easily transformed, and resist apoptosis in response to DNA damage. Many of these cellular phenotypes associated with SIRT3 deficiency can be rescued

by expression of exogenous SOD2, suggesting that mitochondrial superoxide plays a causative role in these defects. Most strikingly, a significant fraction of SIRT3-deficient mice develop mammary carcinoma after 1 year of age. Expression of SIRT3 is decreased in human breast cancer, suggesting that SIRT3 may play a similar tumor suppressor role in humans.

This work reveals a critical novel aspect of SIRT3 function and raises important mechanistic questions regarding how SIRT3 suppresses mammary tumorigenesis. The authors suggest that SIRT3 might promote SOD2 expression via regulation of FoxO3A activity. However, while SIRT3-deficient mice develop mammary tumors, mice with reduced SOD2 levels develop primarily lymphomas (Van Remmen et al. 2003). Such a discrepancy could in principle result from differences in mouse strain background used in the two studies; alternatively, regulation of SOD2 by SIRT3 could be crucial in mammary epithelium but less important in the hematopoietic system. The aneuploidy observed in SIRT3-deficient cells is also notable, in that oxidative stress is most closely associated with chromatid and chromosome breaks leading to genomic rearrangements, rather than ploidy alterations. It would be of interest to test whether SIRT3 deficiency leads to these types of DNA lesions, in addition to altered chromosomal number. In this regard, elevated ROS levels could promote growth of malignant cells by mechanisms other than increased genomic instability. In particular, many phosphatases that negatively regulate growth factor signaling can be inactivated by oxidative modification (Finkel 2003), suggesting that chronically increased ROS in SIRT3 deficiency might provide a trophic stimulus to developing tumors. The authors note that SIRT3-deficient cells generate a greater fraction of their ATP via glycolysis (Kim et al. 2010). This is a phenotype observed in many cancer cells (aerobic glycolysis; the Warburg effect) that promotes aspects of the malignant phenotype (Hsu and Sabatini 2008). Thus, lack of SIRT3 might promote oncogenesis through impaired respiratory function, in turn permitting Warburg-like metabolism. Similarly, germline mutations in genes encoding enzymes of the Krebs cycle, succinate dehydrogenase (SDH) and fumarate hydratase (FH), lead to tumor susceptibility in humans (King et al. 2006). SIRT3 deacetylates SDH to promote its activity in the context of electron transport; FH is also acetylated, although no role of SIRT3 in regulating FH has been identified (Cimen et al. 2010; Kim et al. 2006; Schwer et al. 2009; Zhao et al. 2010). Thus, SIRT3 could conceivably suppress tumorigenesis via effects on the Krebs cycle, in addition to the roles discussed above.

4.4.3 SIRT3 and Age-Related Hearing Loss

Age-related hearing loss (ARHL), or presbycusis, is a common and vexing problem in elderly people, occurring secondary to cell loss and other degenerative changes in the cochlea (Liu and Yan 2007). A recent elegant study has firmly established a role for SIRT3 in antagonizing ARHL (Someya et al. 2010). Previously, it was known that CR or suppression of oxidative damage prevents cochlear cell loss and ARHL (Someya et al. 2007, 2009). The protective effects of CR on ARHL are

SIRT3 dependent (Someya et al. 2010). One mechanism by which SIRT3 mediates this effect is via deacetylation of isocitrate dehydrogenase 2 (IDH2) (Schlicker et al. 2008; Someya et al. 2010), which converts isocitrate to α-ketoglutarate concomitant with reduction of $NADP^+$. NADPH in turn allows regeneration of reduced glutathione to promote mitochondrial oxidative defense. In response to CR, wild-type mice, but not SIRT3-deficient animals, show increased NADPH levels, increased reduced glutathione in mitochondria, and decreased DNA damage in the cochlea and in other tissues. In tissue culture cells, overexpression of SIRT3 or IDH2 is protective against oxidative stress-induced cell death, and the two proteins together have a synergistic prosurvival effect. These results do not rule out the possibility that SIRT3 might modify other substrates to prevent AHRL during CR aside from IDH2. Similarly, Qiu and colleagues reported that SIRT3-deficient mice fail to suppress ROS levels and macromolecular damage during CR (Qiu et al. 2010). They find that SIRT3 directly deacetylates SOD2 to increase its activity during CR, whereas SIRT3-deficient mice do not show SOD2 deacetylation in response to this diet (Qiu et al. 2010). Overall, these papers point to a crucial role of SIRT3 in suppressing oxidative damage and its negative sequelae during CR. It remains to be seen how SIRT3, or the other mitochondrial sirtuins, might impact other phenotypes of aging and/or effects of CR. In this context, the reduction of serum insulin and triglycerides normally occurring during CR is not observed in SIRT3-deficient mice (Someya et al. 2010), implying that SIRT3 plays additional, uncharacterized roles in the adaptation to this dietary regimen.

5 SIRT4 Regulates the Urea Cycle, Insulin Secretion, Fatty Acid Oxidation, and Respiration

In contrast to numerous substrates and pathways regulated by SIRT3, comparatively little is known regarding the other two mitochondrial sirtuins, SIRT4 and SIRT5 (Fig. 2). SIRT4 is localized in the mitochondrial matrix in both mouse and human cells (Ahuja et al. 2007; Haigis et al. 2006; Michishita et al. 2005) and is broadly expressed, with high SIRT4 levels present in kidney, heart, brain, liver, and pancreatic β-cells (Ahuja et al. 2007; Haigis et al. 2006). In liver, SIRT4 expression

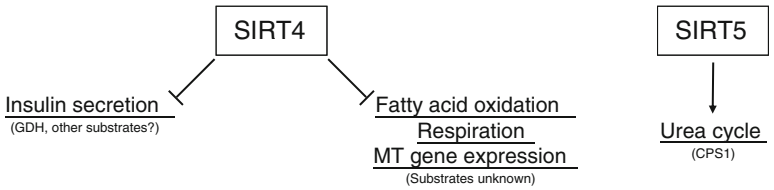

Fig. 2 Metabolic functions of SIRT4 and SIRT5. Mitochondrial and cellular processes are *underlined* and the relevant substrate is indicated in *parentheses*. *MT* mitochondrial. See text for details

declines slightly during CR and increases in genetic models of diabetes (Haigis et al. 2006; Nasrin et al. 2010; Schwer et al. 2009). Thus far, no deacetylase function of SIRT4 has been detected, although it is possible that SIRT4 may possess very specific deacetylase activity on as-yet unidentified substrates (Ahuja et al. 2007; Black et al. 2008; Haigis et al. 2006; North et al. 2003). Instead, using SIRT4-deficient mice, it was shown that SIRT4 ADP-ribosylates and inactivates glutamate dehydrogenase (GDH) (Haigis et al. 2006). GDH converts glutamate to α-ketoglutarate in mitochondria, and its inhibition by SIRT4 results in repression of amino acid-stimulated insulin secretion in pancreatic β-cells (Haigis et al. 2006). Reduced SIRT4 activity allows coupling of insulin secretion to serum amino acid levels during CR, when amino acids serve as an important carbon source. It is of interest that SIRT3 deacetylates and activates GDH (Lombard et al. 2007; Schlicker et al. 2008), implying possible coordinated control of GDH by SIRT3 and SIRT4. Indeed, since SIRT5 regulates an additional urea cycle enzyme (CPS1) (Nakagawa et al. 2009), this pathway is the target of regulation by all three mitochondrial sirtuins. In an independent study, SIRT4 was shown to repress insulin secretion in response to glucose in insulinoma cells and to interact with insulin degrading enzyme (IDE) and the ADP/ATP carrier proteins ANT2 and ANT3; the impact of these interactions is not known (Ahuja et al. 2007).

A recent study investigating additional functions of SIRT4 showed that SIRT4 knockdown in tissue culture cells or in mouse liver in vivo results in increased expression of mitochondrial and fatty acid metabolism enzymes (Nasrin et al. 2010), as well as of SIRT1 and SIRT3. SIRT4 knockdown also leads to increased fatty acid oxidation, respiration, and AMPK phosphorylation (Nasrin et al. 2010). The effects of SIRT4 knockdown on fatty acid oxidation require SIRT1. Many of these phenotypes are the opposite of those observed in SIRT3 deficiency, suggesting once again that SIRT3 and SIRT4 activities may antagonize one another in some contexts. Whether SIRT4 mediates these effects via ADP-ribosylation or deacetylation of mitochondrial targets, or alternatively whether an extramitochondrial fraction of SIRT4 might play a role in direct regulation of nuclear gene expression are important unanswered questions raised by this work.

6 SIRT5 Regulates the Urea Cycle

SIRT5 is localized to the mitochondrial matrix and is broadly expressed, with highest levels in brain, heart, liver, and kidney (Michishita et al. 2005; Nakagawa et al. 2009; Schlicker et al. 2008). Unlike SIRT3 or SIRT4, hepatic SIRT5 levels are unchanged during CR (Nakagawa et al. 2009; Schwer et al. 2009). SIRT5-deficient mice do not show any gross phenotypes and do not display the global increase in hepatic mitochondrial acetylation observed in SIRT3-deficient animals (Lombard et al. 2007). Also unlike SIRT3, SIRT5 has minimal deacetylase activity on canonical sirtuin substrates such as histones (Black et al. 2008; North et al. 2003; Scher et al. 2007; Schlicker et al. 2008). In vitro, SIRT5 deacetylates purified

cytochrome c, although the biological significance of this activity has not yet been determined (Schlicker et al. 2008). More recently, SIRT5 was shown to interact with and deacetylate carbamoyl phosphate synthetase I (CPS1), which catalyzes the first, rate-limiting step of the urea cycle for ammonia detoxification and disposal (Nakagawa et al. 2009). Deacetylation of CPS1 by SIRT5 results in increased enzyme activity, and SIRT5-deficient mice fail to upregulate CPS1 activity and show elevated blood ammonia during a prolonged fast. In the same study, it was shown that CPS1 is deacetylated during CR and that CPS1 activity increases on this diet, although the impact of CR on SIRT5-deficient mice was not directly tested (Nakagawa et al. 2009). The ability of SIRT5 to deacetylate and activate CPS1 was independently verified in transgenic mice overexpressing SIRT5 (Ogura et al. 2010). However, we identified CPS1 as a hepatic protein *increasing* in acetylation during CR (Schwer et al. 2009). The resolution to this discrepancy is not clear, but it may be that CPS1 acetylation and activity are regulated by proteins other than SIRT5 during CR. Alternatively, different techniques of assessing CPS1 acetylation might preferentially uncover different acetylation sites, which could be regulated in opposing ways during CR. In this regard, CPS1 possesses over 20 acetylation sites, implying that acetylation-mediated regulation of CPS1 activity may be quite complex (Schwer et al. 2009).

It is likely that other SIRT5 substrates and functions remain to be identified. In this context, a recent study reported that the presence of a SIRT5 promoter polymorphism correlates with reduced SIRT5 expression and an "older" pattern of gene expression in the human brain (Glorioso et al. 2010). Many of the transcripts altered in the presence of this polymorphism encode mitochondrial proteins, including Parkinson's disease genes; the authors suggest that SIRT5 polymorphisms may represent a risk factor for diseases related to mitochondrial dysfunction. It will be of great interest to test this hypothesis in human cohorts as well as use cells and mice with altered levels of SIRT5.

7 Mitochondrial Protein Acetylation and Sirtuins: Unresolved Questions

Acetylation of mitochondrial proteins plays a major role in regulating functions of this organelle. Despite the rapid progress in this area, there are still many outstanding questions that will no doubt provide fruitful avenues for research for years to come. In particular, how mitochondrial proteins are acetylated in the first place is currently unknown. The identity of putative mitochondrial acetyltransferases remains elusive; identification of such proteins would represent a major step forward in this field. Alternatively or in addition to enzymatic acetylation within mitochondria, mitochondrial proteins could in principle be acetylated outside this organelle, prior to or concomitant with mitochondrial import, or even be acetylated nonenzymatically. These latter models would not permit rapid cycles of

acetylation/deacetylation of mitochondrial proteins to regulate target protein function in response to varied environmental challenges. Instead, following deacetylation, restoration of acetylation status would require new protein synthesis. Such models could be distinguished through pulse-chase experiments assessing acetylation of newly synthesized mitochondrial proteins prior to and following mitochondrial import. The fact that a mitochondrially encoded subunit of complex V is acetylated means that some mechanism of acetylating proteins within this organelle must exist (Huang et al. 2010).

Similarly, how sirtuin activity is regulated in the mitochondria is incompletely understood. Sirtuin require NAD^+, and therefore mitochondrial NAD^+ levels play a critically important role in governing mitochondrial sirtuin function. Increased NADH generation from NAD^+ in the context of HFD leading to reduced sirtuin function may explain the increased global mitochondrial protein acetylation observed during this diet, as could increased acetyl-CoA levels, the substrate for acetyltransferases (Kendrick et al. 2010). It has been reported that NAD^+ levels in mouse liver mitochondria rise during CR, which would be predicted to increase sirtuin activity (Nakagawa et al. 2009), and moreover SIRT3 protein levels rise in this tissue in response to this diet (Schwer et al. 2009). However, these observations are at odds with the observation that global mitochondrial acetylation rises dramatically in liver during CR (Schwer et al. 2009). This overall increased acetylation may represent the net effect of increased acetyltransferase activity superimposed upon elevated sirtuin function; alternatively, some protein species hyperacetylated during CR or other conditions may not be substrates for mitochondrial sirtuins. The activity of SIRT3 and other mitochondrial sirtuins might be governed by other influences besides NAD^+ levels, such as posttranslational modification or interactions with regulatory proteins. It is now clear that SIRT1 activity is tightly regulated by both mechanisms. Surprisingly, no comprehensive quantitative assessment of mitochondrial NAD^+ and its metabolites has yet been performed under varied dietary/environmental conditions in different tissues.

While several proteomic studies have provided a detailed snapshot of the suite of acetylated mitochondrial proteins, how this modification changes at individual lysines on various targets in response to different environmental conditions is a topic that is only beginning to be explored. This effort will require the use of mass spectrometry-based approaches that allow quantitative comparison of acetylation on a given peptide between different samples, such as label-free quantitation (Schwer et al. 2009), stable labeling by amino acids in culture (SILAC) (Choudhary et al. 2009), isobaric tagging for relative and absolute quantification (iTRAQ) (Meany et al. 2007), or stable isotope dimethyl labeling (Boersema et al. 2009). It is currently unclear whether interventions that impact acetylation of many mitochondrial proteins – SIRT3 deficiency, CR, HFD, and chronic ethanol ingestion – lead to modification of common sets of proteins on the same lysine sites, or whether this response is tailored to different environmental perturbations. Similarly, it remains unclear whether the mitochondrial sirtuins share targets and/or functions in common. This question could be addressed in mice or cells with compound mitochondrial sirtuin deficiencies or knockdowns. As noted above, whereas SIRT3

deacetylates GDH to stimulate its activity modestly (Lombard et al. 2007; Schlicker et al. 2008), SIRT4 ADP-ribosylates this protein to suppress function (Haigis et al. 2006). This observation raises the possibility that other proteins might be common substrates for multiple sirtuins. Given that SIRT3 deacetylates many proteins in mitochondria as well as suppresses some age-associated phenotypes, it will be of interest to test whether acetylation of mitochondrial proteins changes with age, either individually or globally, and whether prevention of this effect might have a beneficial effect on healthspan or even lifespan.

In addition, whereas the functional impact of acetylation on a few individual protein targets is clear, a global understanding of how sirtuins impact the activity of metabolic pathways at the level of mitochondria, cells, tissues, and the organism overall is still lacking. Metabolic flux analysis, which has already been successfully applied to bacterial cells with altered protein acetylation, can be used to address this question (Wang et al. 2010). The answers to these and related questions regarding sirtuins and mitochondrial protein acetylation will no doubt reveal novel aspects of mitochondrial biology, and perhaps ultimately provide the basis for novel therapeutic strategies for a variety of disorders.

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Characterization of Nuclear Sirtuins: Molecular Mechanisms and Physiological Relevance

Debra Toiber, Carlos Sebastian, and Raul Mostoslavsky

Contents

Abstract Sirtuins are protein deacetylases/mono-ADP-ribosyltransferases found in organisms ranging from bacteria to humans. This group of enzymes relies on nicotinamide adenine dinucleotide (NAD^+) as a cofactor linking their activity to the cellular metabolic status. Originally found in yeast, Sir2 was discovered as a silencing

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factor and has been shown to mediate the effects of calorie restriction on lifespan extension. In mammals seven homologs (SIRT1−7) exist which evolved to have specific biological outcomes depending on the particular cellular context, their interacting proteins, and the genomic loci to where they are actively targeted. Sirtuins biological roles are highlighted in the early lethal phenotypes observed in the deficient murine models. In this chapter, we summarize current concepts on nonmetabolic functions for sirtuins, depicting this broad family from yeast to mammals.

Keywords Angiogenesis • α-synuclein • *Caenorhabditis elegans* • Caloric restriction • Cellular senescence • Chronological life span • Drosophila • Fragile X syndrome • Glucose metabolism • *HML* and *HMR* • Hst proteins • Hypoxia • Nucleotide • Parkinson's disease • Senescence • Sir2

1 Introduction

Sirtuins are protein deacetylases/mono-ADP-ribosyltransferases found in organisms ranging from bacteria to humans. They comprise a unique group of enzymes, relying on nicotinamide adenine dinucleotide (NAD^+) as a cofactor, in this way linking their activity to the cellular metabolic status. Originally found in yeast, Sir2 was discovered as a silencing factor (*s*ilencing *i*nformation *r*egulator) and has been shown to mediate the effects of calorie restriction on lifespan extension. In this regard, high levels of Sir2 activity promote longevity. In addition, Sir2 participates in repression of transcriptional activity and in DNA double-strand break (DSB) repair, and it has been associated to stress responses, cell fate, and many other cellular reactions. There are seven mammalian homologs (SIRT1–7). Metabolic functions of sirtuins have been explored in previous chapters. In this section, we will first focus on nonmetabolic roles of sirtuins in lower organisms and then cover mammalian sirtuins and their pleiotropic roles as deacetylases.

2 Yeast Sirtuins

The *Saccharomyces cerevisiae* Sir2 protein is the founding member of the sirtuin family of histone deacetylases. It was first described to be a major contributor of transcriptional silencing of mating-type loci in budding yeast (Klar et al. 1981; Rine and Herskowitz 1987). Subsequent studies showed that Sir2 is also crucial for the establishment of silent chromatin at yeast telomeres and in the ribosomal DNA (rDNA) (Bryk et al. 1997; Smith and Boeke 1997). In addition to this founding member, budding yeast have four *SIR2* paralogs, *HST1–HST4*. However, the fission yeast *Saccharomyces pombe* encodes only three Sir2-like proteins: Sir2, Hst4, and Hst2. Besides their role in transcriptional silencing, yeast sirtuins have been involved in regulating genomic stability, cell cycle progression, life span extension, and oxidative stress (Brachmann et al. 1995). Importantly, Sir2 orthologs in higher organisms, such as *Caenorhabditis elegans*, *Drosophila*, and mammals, control

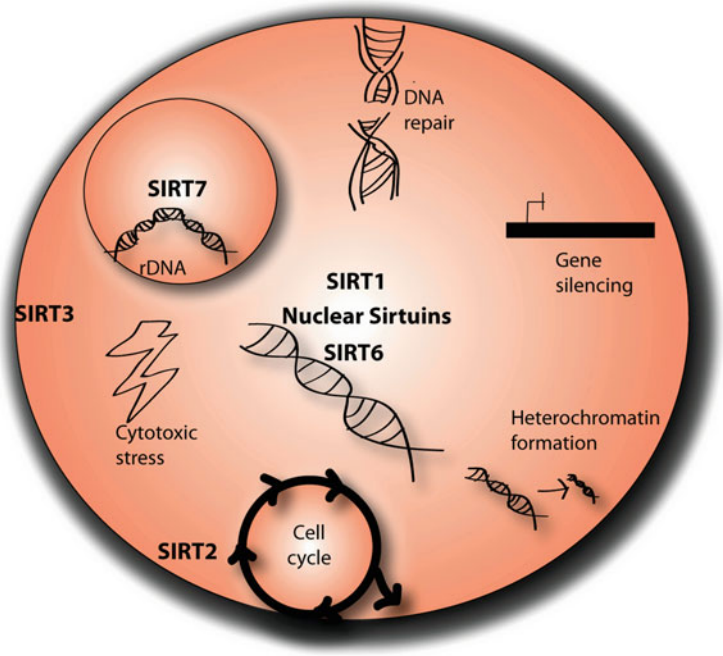

Fig. 1 Molecular functions for the different nuclear sirtuins. For a detailed list of specific targets, please see Table 1

many of these functions as well, denoting remarkable evolutionary conservation for sirtuins, and highlighting the importance of this family of proteins in organism's health and survival (Fig. 1).

2.1 Gene Silencing

The mating-type loci in *S. cerevisiae*, *HML* and *HMR*, were the first examples of "silent" chromatin (Kayne et al. 1988; Loo and Rine 1995). Genes clustered in this loci are repressed as a result of the particular "compacted" chromatin structure they are packaged in. Other examples of silent chromatin are the budding yeast telomeres, yeast rDNA genes, and telomeric and centromeric heterochromatin (Bryk et al. 1997; Fritze et al. 1997; Smith and Boeke 1997). In all of these cases, Sir2 is required to achieve silencing (Fritze et al. 1997; Imai et al. 2000) and mutations that abolish the activity of Sir2 result in an impaired silencing in vivo (Imai et al. 2000; Tanny et al. 1999; Tanny and Moazed 2001). Sir2 carries out its silencing function in a complex with other proteins. Sir2, together with Net1 and Cdc14, form the *re*gulator of *n*ucleolar silencing and *t*elophase exit (RENT)

Table 1 Substrates of nuclear Sirtuins and physiological relevance

	SIRT1	SIRT2	SIRT6	SIRT7
Genomic stability	DNA repair: Ku70, NBS1, WRN, XPA, H3K56	Mitosis: tubulin*, H4K16* DNA repair: H3K56*	DNA repair: H3K56*, DNA-PK, CtIP	Unknown
Cell fate and stress responses	Apoptosis: FOXO3, p53, p73, E2F, Ku70, Smad7 Oxidative stress: FOXO3, FOXO4 Angiogenesis: FOXO1 Hypoxia: Hif-1α, Hif-2α	Oxidative stress: FOXO3	Apoptosis: NF-κB Hypoxia: Hif-1α	Oxidative stress: p53
Cell differentiation	Adipocytes: NCoR Myoblasts: MyoD	Adipocytes: FOXO1	Unknown	Unknown
Cancer	Protumorigenic: p53, H4K16 Antitumorigenic: β-catenin, c-Myc, H3K56	Antitumorigenic: tubulin*, H4K16*, H3K56*	Antitumorigenic: Hif-1α*, GCIP, H3K56*	Unknown
Inflammation and immune system	Inflammation: NF-κB Viral response: HIV-Tat T-cell tolerance: AP1	Unknown	Inflammation: NF-κB*	Unknown
Nervous system	AD: p53, RAR, NF-κB Fragile X syndrome: H3K9, H4K16 (in FMR1 gene) Oxidative stress: IRS-2 Brain development: miR-134	Parkinson disease: tubulin*	Unknown	Unknown
Cardiovascular system	Angiogenesis: FOXO1, Hey2 Atherosclerosis: LXR, eNOS	Unknown	Unknown	Apoptosis: p53

*Physiological relevance unclear

complex required for rDNA silencing (Shou et al. 1999; Straight et al. 1999). At the mating-type/telomeric regions, Sir2 forms a complex with Sir3 and Sir4 (Hoppe et al. 2002; Moazed et al. 1997). The establishment of the silent chromatin at these loci occurs in a stepwise fashion involving three steps: initiation, nucleation, and spreading (Hoppe et al. 2002; Luo et al. 2002; Rusche et al. 2002). In the first step, the Sir2/4 complex is recruited to the silencer elements. Sir3 also becomes associated with the silencer region through interaction with Sir4. Sir2 has H4K16 deacetylase activity (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000) and Sir3 and Sir4 bind to deacetylated histone H3 and H4 N-termini (Hecht et al. 1995). Thus, Sir2 provides a deacetylated nucleosome platform to where Sir3 and Sir4

bind. In the next step, iterative cycles of deacetylation coupled to association of many copies of the Sir complex across several kilobases lead to the spreading of silencing (Hoppe et al. 2002). Finally, NAD^+-dependent deacetylation by Sir2 generates *O*-acetyl-ADP-ribose that is thought to act together with H4 N terminus to promote the association of multiple copies of Sir3 with Sir2/4 (Liou et al. 2005). In addition to Sir2, Hst proteins are also involved in gene silencing. It has been shown that *HST1* overexpression restores transcriptional silencing to a *SIR2* mutant and that *HST3 HST4* double mutants are defective in telomeric silencing (Brachmann et al. 1995), suggesting that some of these proteins perform in overlapping functions.

In fission yeast, Sir2 also acts at silent regions by deacetylating both H3K9 and H4K16 (Shankaranarayana et al. 2003). In *S. pombe*, Hst4 regulates telomeric and centromeric silencing (Freeman-Cook et al. 1999), repressing genes involved in amino acid metabolism. Furthermore, it is critical for Tf2 retrotransposon silencing and 5′ mRNA processing, whereas Hst2 directly represses genes involved in transport and membrane function (Durand-Dubief et al. 2007).

2.2 Life Span Regulation

Yeast life span can be measured in two ways. *S. cerevisiae* division is asymmetrical and one mother cell gives rise by budding to a smaller daughter cell. The number of buds produced by an individual mother cell defines its replicative life span and is independent of calendar time (Mortimer and Johnston 1959). In contrast, the chronological life span is a measure of the viability of yeast cells in stationary phase and, therefore, models aging in the natural environment (Longo et al. 1997). Sir2 might regulate both types of yeast aging. However, whether this is the case still remains under controversial debate (described below) (Longo and Kennedy 2006).

2.2.1 Replicative Life Span

One cause of yeast replicative aging is the accumulation in the nucleolus of extrachromosomal rDNA circles (ERCs) that are generated during cell division by homologous recombination within rDNA repeats (Sinclair and Guarente 1997). Sir2 avoids the accumulation of ERCs by inhibiting rDNA recombination. Deletion of *SIR2* results in hyper-recombination within the rDNA and elevated levels of ERCs, leading to a reduction in life span, whereas overexpression of Sir2 increases replicative life span (Kaeberlein et al. 1999). However, ERCs have not been detected in higher organisms in association with aging, and thus, it is an unlikely mechanism to explain the aging process in these organisms. More recently, Berger and colleagues explored chromatin-related changes at Sir2-regulated loci outside the nucleoli (Dang et al. 2009). They have found an age-related decrease in Sir2

protein levels accompanied by increased acetylation of H4K16 and loss of histones at specific subtelomeric regions in replicatively old yeast cells. This, in turn, results in compromised transcriptional silencing at these loci. Since these telomeric regions are also present in higher organisms, this model may represent a conserved function of sirtuins in regulation of replicative aging. In addition to ERCs and gene silencing, progressive accumulation of oxidized proteins in mother cells is also involved in yeast replicative aging. Recently, another mechanism has also been proposed to be regulated by Sir2 in yeast. During asymmetrical division, mother cells retain the most part of carbonylated proteins, allowing daughter cells to grow free of oxidatively damaged proteins. This process is Sir2 dependent since mother cells of yeast strain lacking *SIR2* are unable to retain oxidatively damaged proteins during cytokinesis, suggesting that Sir2 controls the asymmetrical inheritance of oxidized proteins (Aguilaniu et al. 2003; Erjavec et al. 2007; Liu et al. 2010).

Another connection of Sir2 with aging came from the observation that caloric restriction (CR) extends yeast replicative life span. Caloric restriction, defined as a reduction in energy intake, has been shown to enhance longevity of organisms ranging from yeast to mammals. However, the involvement of Sir2 in this process has been controversial and is still under debate. Initially, it was reported that Sir2 was required for life span extension by calorie restriction in yeast. This was supported by the fact that short-lived strains lacking *SIR2* did not exhibit life span extension under CR conditions. Two models have been proposed to explain this finding. It was first proposed that CR causes a metabolic switch from fermentation to respiration, leading to an increased NAD:NADH ratio and concomitant increase in Sir2 activity (Lin et al. 2000, 2002, 2004). According to the second model, CR does not alter NAD:NADH ratio, but rather activates Pnc1, which degrades the endogenous Sir2 inhibitor nicotinamide, thus increasing Sir2 activity (Anderson et al. 2003). In both models, the activation of Sir2 leads to a decreased formation of ERCs, thereby delaying aging (Sinclair and Guarente 1997).

Contrasting these results, other studies reported that CR might extend life span even in the absence of Sir2 in some yeast strains, by mechanisms involving the Tor and Shc9 kinase signaling pathways (Jiang et al. 2002; Kaeberlein et al. 2004). The debate currently rests on the conditions used to achieve calorie restriction. Some authors consider that reducing glucose concentration from 2 to 0.5% is optimum since it has minimal effect on yeast growth rate and may mimic the levels of CR employed in other organisms (Lamming et al. 2005; Lin and Guarente 2006), whereas other authors use 0.05% of glucose because it maximizes life span extension (Kaeberlein et al. 2004). In addition, a recent work has shown that CR reduces modestly the formation of ERCs, although in a Sir2-independent manner (Smith et al. 2009). In the same work, the authors show that Sir2 is not activated during CR, suggesting that Sir2-independent pathways may function in life span regulation (Smith et al. 2009). In agreement with this, Riesen et al. have recently found that suppression of rDNA recombination in CR-mediated life span extension is not related to rDNA silencing, and thus it is Sir2 independent (Riesen and Morgan 2009).

2.2.2 Chronological Life Span

The role of Sir2 in the chronological survival of nondividing yeast cells appears to be quite different from its role in replicative aging. Although Sir2 can regulate stress resistance, changes in Sir2 levels have minimal effect in the chronological life span of yeast. However, mutations in *SIR2* do extend the chronological life span under severe CR or when combined with mutations that reduce the activity of the Ras and Shc9 pathways (Fabrizio et al. 2005). Moreover, overexpression of Sir2 inhibits life span extension in mutants lacking *SCH9* (Fabrizio et al. 2005). These results suggest that the effect of Sir2 on chronological life span appears to be opposite to its role in replicative life span. In a contrasting study, it has been reported that neither Sir2 nor Hst1–4 are required for the chronological life span extension caused by CR, suggesting a sirtuin-independent mechanism in the chronological aging system (Smith et al. 2007).

2.3 Genomic Stability

Several sirtuins have been implicated in DNA damage and genomic stability. Besides its role in homologous recombination within rDNA loci, Sir2, together with Sir3 and Sir4, is required for efficient repair of DSBs by nonhomologous end joining (NHEJ) (Lee et al. 1999; Tsukamoto et al. 1997). Moreover, it has been described that Sir proteins dissociate from telomeres and are recruited at sites of damage by interaction with Hdf1 (a yeast homolog of Ku protein) in a Mec1-dependent manner (Martin et al. 1999; Mills et al. 1999; Tsukamoto et al. 1997). According to this, it has been proposed that Sir proteins could alter chromatin at the sites of damage, facilitating the rejoining of DNA ends. However, further studies suggested that Sir2 enhances DNA repair via an indirect mechanism (Lee et al. 1999).

In addition to Sir2, Hst proteins are also involved in genomic stability. Hst1 is recruited to an induced DSB, suggesting that it may be important for efficient DNA repair (Tamburini and Tyler 2005). Furthermore, Hst3 and Hst4 are required for genome integrity since *HST3 HST4*-deficient cells show low viability, altered cell cycle distribution, and increased chromosome loss and recombination (Brachmann et al. 1995). More recently, it has been shown that Hst3 and Hst4 regulate the acetylation of lysine 56 of histone H3 (H3K56Ac), which is required for surviving DNA damage (Celic et al. 2006; Maas et al. 2006; Masumoto et al. 2005). In this context, inactivation of Hst3 and Hst4 leads to spontaneous DNA damage, chromosome loss, thermosensitivity, and acute sensitivity to genotoxic agents. During S phase, all newly synthesized histone H3 are acetylated at K56 and deposited into nucleosomes during DNA replication, becoming deacetylated at G2. However, after DNA damage, this modification persists at the sites of damage in a checkpoint-dependent manner (Masumoto et al. 2005). Interestingly, both transcriptional and posttranslational mechanisms have been described to mediate a decrease in Hst3 protein levels after DNA damage in a Mec1-dependent fashion, suggesting a

regulated mechanism to keep H3K56 acetylated (Maas et al. 2006; Masumoto et al. 2005; Thaminy et al. 2007). It has been proposed that acetylated H3K56 retained at sites of damage could facilitate the recruitment of proteins involved in DNA repair or in the DNA damage response (Thaminy et al. 2007). In the absence of this regulated acetylation, this modification could be distributed throughout chromatin precluding the use of this mark to direct DNA damage response proteins to sites of damage. However, another scenario has been recently proposed. According to Chen et al., acetylation of H3K56 is required for chromatin reassembly after DNA repair, signaling for deactivation of the DNA damage response (Chen et al. 2008). Thus, the inactivation of Hst3 after DNA damage would retain H3K56Ac at sites of breaks, thereby leading to the reassembly of chromatin following repair and the deactivation of the DNA damage checkpoint.

3 *Caenorhabditis elegans*

Caenorhabditis elegans has four genes with shared similarity to the yeast Sir2. The most homologous and best characterized is called Sir2.1, which exhibits conserved deacetylase activity. A Sir2.1 chimera protein fused to GFP localizes primarily to the nucleus and its expression was most prominent in head and tail neurons from embryos to adults (Wang and Tissenbaum 2006). Sir-2.1-GFP is also seen in many pharyngeal cells in the hypodermis. It was first seen at the threefold stage of embryogenesis, and it is weakened by late L3 and expressed at low levels in young adults (Wang and Tissenbaum 2006).

3.1 Gene Silencing

Sir2.1 is found in both cytoplasm and nucleus, but its concentrations are higher in chromatin, especially in the telomeric regions cTEL3X and cTEL4X (Wirth et al. 2009). This pattern is similar to the linker histone HIS-24. In *C. elegans*, there are eight linker histone variants, but only HIS-24 is able to promote germ line development, influencing histone H3 methylation in the germ line (Wirth et al. 2009). HIS-24 localization to chromatin depends on Polycomb group proteins and also on Sir2.1, since lack of Sir2.1 enzymatic activity induces hyperacetylation of telomeric regions (specifically H3K9Ac) while decreasing His-24 binding to these regions (Wirth et al. 2009). However, no direct interaction was found for these two proteins, suggesting that binding of HIS24 depends on the acetylated condition of the chromatin (Wirth et al. 2009). In addition, HIS-24 was able to bind to H3K27 unmodified or methylated peptides. Both Sir2.1 and HIS-24 depletion caused reduction of H3K27me3 in germ line, indicating a role of Sir2.1 and HIS-24 in regulating this modification (Wirth et al. 2009). Overall, it appears that linker histones and Sir2.1 act in a coordinated fashion to modulate chromatin condensation and gene silencing (Wirth et al. 2009).

3.2 Life Span Regulation

In *C. elegans*, most of the Sir2-related research has been related to life span studies. Worms with an extra copy or overexpression of Sir2.1 exhibit a 50% increase in life span (Tissenbaum and Guarente 2001). This effect depends on the insulin-like receptor pathway, since mutation of DAF16 (the Foxo homolog) in the context of Sir2 overexpression abolished life span extension (Wang and Tissenbaum 2006). When Sir2.1 overexpressing worms were crossed with the long-lived DAF2 (Insulin Receptor homolog) mutant, the effect was not additive, further indicating that Sir2.1 and DAF2 function in the same pathway (Tissenbaum and Guarente 2001). However, a full mechanistic picture remains unclear. Silencing of the 14-3-3 chaperone homologs par-5 and geIn3 abolished the life span extension observed in the Sir2.1 or DAF16 overexpressors. In this regard, it is possible that 14-3-3 proteins, known to have many effects in stress responses, help export DAF16 from the nucleus (Berdichevsky et al. 2006; Wang et al. 2006).

Another set of studies have shown that mutating Sir2.1 has a minor effect on life span (Wang and Tissenbaum 2006; van der Horst and Burgering 2007). However, in the context of the *eat* mutants, which mimic calorie restriction treatments, Sir2.1 mutation abolished the longevity phenotype, indicating that CR-life span extension is dependent on Sir2.1 activity (Wang et al. 2006). In contrast, glucose deprivation, which induces longevity by influencing mitochondrial respiration, did not require Sir2.1 activity (Schulz et al. 2007). Similar to yeast, these differences might arise from the different methods utilized to attain nutrient deprivation, or else differences in the knockdown methods used.

3.3 Sir2 and Genotoxic Stressors

Sir2.1 mutants in worms are also more sensitive to various stressors such as H_2O_2, heat shock, and UV irradiation, indicating that lack of Sir2.1 increases sensitivity to damaging agents. When Sir2.1 was mutated and crossed with worms expressing a mutant PrP but not the WT PrP (known to induce a prion-like phenotype in *C. elegans*), they showed enhanced neuronal dysfunction, as shown by touch response in the tail. These effects were observed without changes in PrP expression or proteinase resistance (a known test for aggregate formation and stability). Consistently, overexpression of Sir2.1 reversed the neuronal dysfunction induced by mutant PrP (Bizat et al. 2010). Overall, these results suggest that Sir2.1 and PrP might work in a coordinated fashion. However, the molecular mechanism behind this protective effect for Sir2.1 against genotoxic and neurotoxic stress remains unclear. In this context, recent studies in mammal models of neurodegeneration describe multiple protective functions for SIRT1, as described in detail below (Fig. 2).

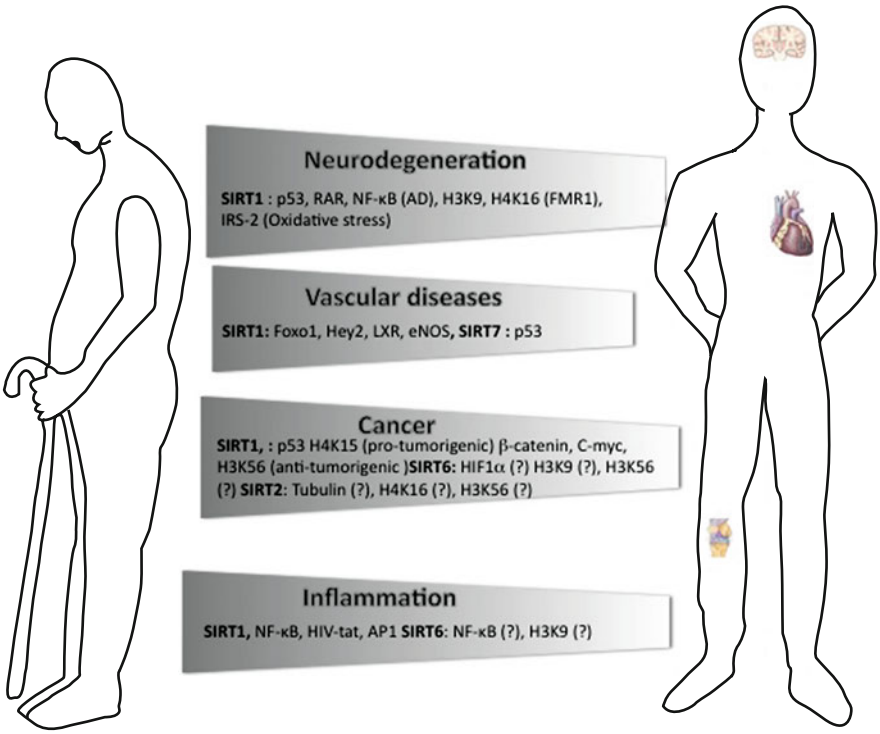

Fig. 2 Summary of the roles of nuclear sirtuins in disease, (?) denotes unclear physiological relevance

4 Drosophila

Five sirtuins have been found in Drosophila, but most of the studies thus far have focused on dSir2, who shares the highest degree of similarity with the yeast Sir2. dSir2 conserves the deacetylase and the ADP ribosylase domains (Parsons et al. 2003) and is able to deacetylate a wide range of histone lysine residues in H3, H4 and H2B. It also exhibits auto ADP-ribosylation activity (Furuyama et al. 2004), but this activity is considered a side reaction and might not be relevant in vivo. dSir2 can repress transcription in the presence of hyperacetylated histones, but had no effect on nonacetylated histones or naked DNA (Parsons et al. 2003).

4.1 Gene Silencing

Using a Drosophila model of position effect variegation (PEV) for the white *w* + phenotype in drosophila's eyes, it was shown that dSir2 has a role in heterochromatin silencing, but not in the telomeric region, differing from yeast Sir2 (Rosenberg and

Parkhurst 2002). However, another group using the same system found only a minor effect in PEV (Astrom et al. 2003); therefore, it remains unclear whether dSir2 plays a role in PEV. On the contrary, dSir2 coimmunoprecipitates and colocalizes with the PcG proteins E(Z), RPD3, and p55 in larval stages (Furuyama et al. 2004). Although dSir2 mutants do not exhibit PcG-like phenotypes – such as anterior transformations – when mutated alone (Campbell et al. 1995), they aggravated these phenotypes when combined with PcG mutants (Furuyama et al. 2004). dSir2 also interacts with the Hairy transcription factor Hes. Drosophila HES proteins are key repressors in the developmental processes of segmentation, neurogenesis, and sex determination. Studies by Rosenberg et al. showed that dSir2 was an essential gene whose loss of function results in both segmentation defects and skewed sex ratios, associated with reduced activities of the Hairy and Deadpan bHLH repressors (Rosenberg and Parkhurst 2002).

4.2 Life Span Regulation

As previously indicated, dSir2 loss of function is embryonic lethal, and heterozygous dSir2 flies resulted in segmentation defects and sex ratio defects (Rosenberg and Parkhurst 2002). However, this essential role of dSir2 has been challenged, since others did not find any effect on viability, developmental rate, or sex ratio (Astrom et al. 2003). The evidence for a role of dSir2 in longevity is controversial as well. While some studies suggest that its overexpression induces longevity in many different fly lines (Frankel et al. 2010), others found that its overexpression leads to premature death during development through a JNK- and Forkhead box class O (FOXO)-dependent pathway, partially through transcription of proapoptotic genes such as reaper, grim, and hid (Griswold et al. 2008).

Overall, it appears that dSir2 has a conserved role as a histone deacetylase, and an established role in chromatin silencing, through chromatin interaction with PcG proteins and transcription factors; nevertheless, its involvement in longevity and viability is still under debate and further studies will be required to determine its precise function and mechanism of action.

5 Mammalian Sirtuins

In mammals, there are seven Sir2 homologs divided into four subclasses according to their phylogenetic analysis (Frye 2000). They also differ in their subcellular location and the effectiveness of their deacetylase activity. SIRT1–3 have a very robust activity, whereas SIRT4 appears to work mainly as an ADP-ribosyl transferase (Finkel et al. 2009). SIRT1, 6, and 7 are mostly nuclear sirtuins; SIRT2 is mostly cytoplasmic, although it can be found in the nucleus during the cell cycle; and SIRT3, 4, and 5 are mitochondrial sirtuins. Thus, their location, activities,

patterns of expression, and the differences in their nonconserved domains allow the different sirtuins to deacetylate/ADP rybosylate a wide range of substrates during different stimuli, developmental stages, and subcellular compartments. Although most of its metabolic functions have been covered in previous chapters, we will summarize here our current knowledge on the pleiotropic functions of this remarkably unique family of proteins.

5.1 DNA Repair and Genomic Stability

A role of sirtuins in maintaining genomic integrity was suggested by an early work in model organisms (described above). Recently, these observations have been extended to mammalian sirtuins. The first example came with the analysis of SIRT6-deficient cells and mice (Mostoslavsky et al. 2006). Both mouse embryonic fibroblasts (MEFs) and ES cells lacking SIRT6 grow more slowly compared to WT controls due to a reduced proliferative capacity, suggesting an alteration in the normal cell cycle. When metaphase spreads were analyzed, SIRT6-deficient cells showed increased chromosomal aberrations including fragmented chromosomes, detached centromeres, gaps, and translocations. Furthermore, depletion of SIRT6 results in increased sensitivity to genotoxic damage. Together, these results indicate that SIRT6 plays a general role in maintaining genome integrity. Genomic instability can result from ineffective DNA repair, impaired cell cycle checkpoints, or increased load of genotoxic insults. SIRT6 KO cells show normal cell cycle checkpoints. However, SIRT6 deficiency compromises the BER pathway, which is responsible for repairing lesions induced by alkylating agents and oxidative damage. In this context, it has been proposed that SIRT6 could promote BER by increasing the accessibility of BER factors to the broken DNA by modulating chromatin structure (Mostoslavsky et al. 2006), a possibility that has also been proposed for sirtuins in lower organisms (described above). However, it is also possible that SIRT6 influences genomic stability indirectly by regulating metabolism (Lombard et al. 2008). In this context, increased genomic instability would be due to an overload in DNA damage rather than a defect in DNA repair. SIRT6-deficient animals show prominent metabolic defects, including hypoglycemia and low levels of IGF1 and insulin (Mostoslavsky et al. 2006; Zhong et al. 2010). In this context, SIRT6 appears to regulate glucose metabolism, affecting mitochondrial metabolism and indirectly influencing genomic stability (Zhong et al. 2010). In this regard, metabolic perturbations or altered IIS can lead to DNA damage accumulation and defects in DNA repair (Levine et al. 2006). According to this second model, the metabolic defect conferred by SIRT6 deficiency could promote an increase in reactive oxygen species (ROS) levels due to elevated metabolic activity, leading to the accumulation of DNA damage.

However, further studies propose a direct role of SIRT6 in DNA repair. Histone acetylation and deacetylation have been widely demonstrated to be important in DNA repair processes. Deacetylation of H3K9 by SIRT6 plays a crucial role in

maintaining telomere integrity, and its depletion leads to telomere dysfunction with end-to-end chromosome fusion and premature senescence (Michishita et al. 2008). Interestingly, another substrate for SIRT6 is H3K56 (Michishita et al. 2009; Yang et al. 2009). This modification is involved in DNA damage and repair in yeast and it is modulated by the sirtuins Hst3 and Hst4 (described above). Recently, it has been shown that H3K56Ac is also present in mammalian cells and that it might also influence DNA damage (Das et al. 2009; Yuan et al. 2009b). SIRT6 directly deacetylates H3K56 to regulate global and telomere-associated levels of this histone modification in vivo, suggesting a role of SIRT6 in regulating telomere biology and DNA repair (Michishita et al. 2009; Yang et al. 2009). In this context, SIRT6 has also been shown to be associated to DNA breaks in a DNA-PK-dependent manner (McCord et al. 2009). Furthermore, a recent study found that SIRT6 promotes end-resection by deacetylating the DSB-processing protein CtIP (Kaidi et al. 2010), suggesting a yet another mechanism through which SIRT6 might regulate DNA repair. Overall, it is clear that SIRT6 plays multiple roles in maintaining genomic integrity; however, the precise functions of SIRT6 and H3K56 acetylation in mammalian DNA repair remain to be fully explored, and will likely be the subject of intense future research.

Mammalian SIRT1 also appears to influence genomic stability. First, SIRT1 can deacetylate multiple DNA repair factors. Upon exposure to ionizing radiation (IR), SIRT1 interacts with and deacetylates Ku70, increasing DNA repair capacity (Jeong et al. 2007). In addition, SIRT1 can also regulate the acetylation of NBS1, modulating the repair of DSBs. NBS1 associates with MRE11 and RAD50 to form the MRN complex, which is involved in detection, signaling, and repair of DNA damage. Upon IR, NBS1 is phosphorylated by ATM (Lim et al. 2000), and this phosphorylation is required for the proper function of NBS1 (Gatei et al. 2000; Lim et al. 2000; Zhao et al. 2000). SIRT1 binds to and deacetylates NBS1 and, importantly, maintains NBS1 in a hypoacetylated state that is required for IR-induced NBS1 phosphorylation (Yuan et al. 2007). Another repair protein that was found to be a substrate of SIRT1 is WRN (Li et al. 2008a). CBP/p300 acetylates WRN, inhibiting its helicase and exonuclease activities. After DNA damage, SIRT1 deacetylates WRN, promoting its activation and nuclear distribution to facilitate DNA repair. Finally, it has been recently shown that SIRT1 facilitates nucleotide excision repair (NER) following UV-induced DNA damage through XPA deacetylation (Fan and Luo 2010). UV irradiation stimulates SIRT1–XPA interaction, leading to the deacetylation of XPA, which is required for binding of RPA32 at sites of damage.

The role of SIRT1 in genomic integrity is supported by the increased chromosomal aberrations and impaired DNA repair in *SIRT1*$^{-/-}$ embryos (Wang et al. 2008). SIRT1-deficient MEFs exhibit impaired DNA damage response and decreased ability to repair DNA damage induced by both IR and UV irradiation. This defect correlates with a decrease in γ-H2AX foci formation in the absence of SIRT1. Furthermore, in response to oxidative stress and IR, SIRT1 dissociates from repetitive DNA sequences and genes, relocalizing to DNA breaks to promote DNA repair (Oberdoerffer et al. 2008). This dissociation is accompanied by the

derepression of previously silenced genes. Finally, as in the case of SIRT6, it has been reported that SIRT1 can deacetylate H3K56Ac in vitro, promoting genomic stability (Das et al. 2009; Yuan et al. 2009b). Whether such an activity is physiologically relevant and whether H3K56 represents a *bona fide* target of SIRT1 have not been confirmed in vivo yet.

SIRT2 is also implicated in genomic stability. Although it was originally reported to be a cytoplasmic protein (Frye 1999; North et al. 2003; Perrod et al. 2001), SIRT2 can localize to the nucleus where it plays a role in mitosis (Inoue et al. 2007; Dryden et al. 2003). Indeed, SIRT2 levels increase at the G2/M transition and its overexpression leads to a prolongation of the mitotic phase of the cell cycle. These results suggest that a decrease in SIRT2 protein levels, or its inactivation, is important for mitotic exit. In addition, these authors demonstrated that the cell cycle-dependent abundance of SIRT2 is regulated by phosphorylation, which stabilizes it, protecting SIRT2 from proteasomal degradation (Dryden et al. 2003). Thus, SIRT2 appears to maintain genomic integrity during mitosis by blocking chromosome condensation and subsequent hyperploid cell formation in response to mitotic stress. Several mechanisms have been proposed to explain how SIRT2 regulates mitotic exit, although none of them have yet been proven. First, SIRT2 could modulate mitosis through its tubulin deacetylase activity, controlling in this way mitotic spindles formation. Second, SIRT2 could regulate facultative heterochromatin formation during mitosis via deacetylation of H4K16 (Vaquero et al. 2006), which may facilitate the generation of condensed chromatin. Finally, SIRT2 has also been demonstrated to have an H3K56Ac-specific deacetylase activity in vitro (Das et al. 2009), suggesting that this sirtuin may regulate this histone mark in the context of DNA repair.

5.2 Cell Fate and Stress Responses

Mammalian sirtuins have also a prominent role in regulating cell fate and differentiation, and how cells respond to several stresses. In part, they do so by modulating the activity of key proteins involved in stress responses. Among these, the FOXO family of transcription factors appears to be major players in regulating both energy status and stress resistance. In this context, several studies have shown that SIRT1 regulates FOXO-mediated transcription by deacetylating FOXO1, 3, and 4 (Brunet et al. 2004; Daitoku et al. 2004; Frescas et al. 2005; Motta et al. 2004; van der Horst et al. 2004). In response to oxidative stress, SIRT1 forms a complex with FOXO3 that contributes to deacetylation of this protein (Brunet et al. 2004). The consequences of this interaction are dual: SIRT1 increases the ability of FOXO3 to induce cell cycle arrest and resistance to oxidative stress, but inhibits FOXO3-dependent induction of cell death. Interestingly, deacetylation of FOXO4 by SIRT1 also controls oxidative stress resistance (van der Horst et al. 2004). SIRT1-dependent deacetylation of FOXO1 also promotes vascular growth by reducing the antiangiogenic actions of FOXO1 (Potente et al. 2007). Similar to SIRT1, SIRT2

can also regulate FOXO activity under oxidative stress. This type of stress results in increased SIRT2 expression, promoting the binding of SIRT2 to FOXO3. As a result, FOXO3 is deacetylated, thereby resulting in a reduction in ROS levels (Wang et al. 2007).

In addition to FOXOs, p53 also represents a key molecule in regulating cell fate in the setting of exogenous stress. p53 is activated in response to DNA damage by several posttranslational modifications including phosphorylation and acetylation, among others. This transcription factor was one of the first nonhistone substrates identified for SIRT1 (Luo et al. 2001; Vaziri et al. 2001). SIRT1 deacetylates several lysines of p53, repressing its activity (Cheng et al. 2003). SIRT1-mediated deacetylation inhibits p53-dependent apoptosis in response to DNA damage and oxidative stress (Cheng et al. 2003; Vaziri et al. 2001). Furthermore, a connection between SIRT1, FOXOs, and p53 has been proposed. FOXO proteins and p53 can directly interact under stress conditions (Brunet et al. 2004; Nemoto et al. 2004), and p53 can regulate SIRT1 expression through various means, including p53-dependent microRNAs expression (Chen et al. 2005b; Nemoto et al. 2004; Yamakuchi et al. 2008). Besides FOXO and p53, SIRT1 also exerts antiapoptotic functions by deacetylating and modulating the activity of p73, E2F, Ku70, and Smad7 (Michan and Sinclair 2007). In addition, a recent report suggested that SIRT1 may also protect cells against stress by regulating the heat shock response (Westerheide et al. 2009). Finally, a role in hypoxic stress resistance has been recently demonstrated for SIRT1 (Dioum et al. 2009; Lim et al. 2010). The cellular adaptation to hypoxia requires the activation of the hypoxia-inducible factors (HIF)-1α and HIF-2α. SIRT1 positively regulates HIF-2 signaling by deacetylating HIF-2α (Dioum et al. 2009). However, SIRT1 is inhibited during hypoxia, allowing HIF-1α to be acetylated and activated (Lim et al. 2010). Thus, these studies show that HIF-1α and HIF-2α are oppositely regulated by SIRT1 during hypoxia. Because these two transcription factors have distinct roles during the hypoxic response (Lofstedt et al. 2007; Wang et al. 2005), it has been proposed that SIRT1 may function as a switch that determines whether cells choose HIF-1α or HIF-2α in response to hypoxia. Overall, it appears that SIRT1 acts as a master regulator of stress responses, modulating the activity of multiple factors in a coordinated fashion, in this way ensuring efficient adaptation against stress.

Regarding the other sirtuins, little is known about their implication in stress resistance and apoptosis. SIRT6 has been shown to act as a transcriptional corepressor of NF-κB by deacetylating H3K9 at target promoters. In SIRT6-deficient cells, NF-κB-dependent gene expression is increased, leading to apoptotic resistance and induction of senescence (Kawahara et al. 2009). In addition, recent work from our laboratory has demonstrated that SIRT6 regulates glucose homeostasis by inhibiting HIF-1α transcriptional activity (Zhong et al. 2010). Moreover, in the absence of SIRT6, HIF-1α is stabilized leading to an increased protection from hypoxia-induced apoptosis (Zhong et al. 2010), suggesting that SIRT6 may have a role in controlling hypoxic responses. SIRT7 has also been postulated to modulate resistance to genotoxic and oxidative stress (Vakhrusheva et al. 2008b). SIRT7 deficiency leads to a degenerative cardiac hypertrophy and inflammatory

cardiomyopathy in mice. This phenotype appears to be in part due to p53 hyperacetylation and decreased stress resistance (Vakhrusheva et al. 2008b). Finally, although SIRT3 appears to be localized to the mitochondria, it has been reported that a small fraction of SIRT3 is present in the nucleus where it deacetylates H4K16. Upon cellular stress, nuclear SIRT3 is transported to the mitochondria (Scher et al. 2007). However, the functional implication of this transport in stress resistance remains unknown.

Besides their role in modulating cell death, sirtuins are also important in regulating some cellular differentiation programs. In this regard, SIRT1 has been shown to inhibit adipocyte differentiation and adiponectin secretion by promoting transcriptional repression by the NCoR corepressor (Picard et al. 2004; Qiang et al. 2007). SIRT2 is also involved in adipocyte differentiation but it exerts its actions by modulating FOXO1 acetylation (Jing et al. 2007). In addition, sirtuins have a physiological role in regulating gene expression and muscle differentiation by sensing changes in NAD/NADH ratio. In this setting, it has been reported that SIRT1 suppresses myoblast differentiation by deacetylating and inhibiting the transcription factor MyoD (Tanno et al. 2007). Similarly, SIRT1 also affects cell fate decisions of neural progenitor cells (NPCs), sensing the redox state of the cell (Prozorovski et al. 2008). Under oxidizing conditions, SIRT1 and Hes1, a negative regulator of the neuron-specific transcription program, form a complex that binds to and deacetylates histones at target promoters, in turn blocking neuronal differentiation and promoting astrocytes generation (Prozorovski et al. 2008). Moreover, recent studies in mouse embryonic stem cells also suggest a role of SIRT1 in regulating stem cell homeostasis and differentiation (Han et al. 2008). All together, these results from mouse models indicate that SIRT1 is a central regulator of embryonic and somatic stem cell function. A recent report further extended these findings by showing that SIRT1 may be an important regulator of human ES cell differentiation as well (Calvanese et al. 2010).

5.3 Cancer

Because of its role in regulating cellular stress responses, genome stability, and metabolism, sirtuins represent excellent candidates to control tumorigenesis. Most of the data regarding the role of sirtuins in cancer come from studies with SIRT1. However, it remains controversial whether SIRT1 promotes or suppresses tumor formation. Initially, SIRT1 was proposed to be an oncogene due to its ability to modulate p53 activity. Deacetylation of Lys382 on p53 by SIRT1 reduces p53 transactivation, allowing cells to bypass p53-mediated apoptosis (Luo et al. 2001; Vaziri et al. 2001). Consistent with this, overexpression of SIRT1 leads to decreased levels of acetylated p53 in response to DNA damage and increased resistance to p53-dependent cell cycle arrest and apoptosis. Furthermore, expression of a catalytically inactive SIRT1 or repression of SIRT1 by siRNA increases p53 activity, rendering cells more sensitive to stress. In addition, SIRT1 inhibits the

expression or activity of many other factors involved in stress responses and DNA repair (see above). Similarly, SIRT1 may promote cancer development by deacetylating histones. In this context, loss of H4K16 acetylation, a substrate for SIRT1 (Vaquero et al. 2004), is a hallmark of human tumors (Fraga et al. 2005). Furthermore, inhibition of SIRT1 caused reexpression of multiple tumor-suppressor genes in cancer cells, rendering these cells with less tumorigenic potential (Pruitt et al. 2006).

Several tumor suppressors regulate the expression and activity of SIRT1. Hypermethylated in cancer 1 (HIC1) is a zinc-finger/BTB domain transcriptional repressor that cooperates with p53 to suppress age-dependent development of cancer in mice (Chen et al. 2003). HIC1 and SIRT1 form a complex that binds to the *SIRT1* promoter inhibiting *SIRT1* expression. Inactivation of HIC1 upregulates *SIRT1* transcription, leading to decreased p53 activity, thereby rendering cells resistant to DNA damage-induced apoptosis. Moreover, p53 also regulates the expression of HIC1, suggesting that a regulatory loop among HIC1, SIRT1, and p53 may exist to control cell cycle arrest and apoptosis in response to DNA damage (Chen et al. 2005b). Another tumor suppressor that inhibits SIRT1 is deleted in breast cancer-1 (DBC1) (Anantharaman and Aravind 2008; Kim et al. 2008; Zhao et al. 2008). DBC1 binds to and inactivates SIRT1 deacetylase activity. In this context, knockdown of DBC1 promotes SIRT1 activation, p53 deacetylation, and, therefore, cell survival after genotoxic stress. Thus, these results indicate that DBC1 may promote breast cancer by activating SIRT1. In addition, *SIRT1* expression is significantly increased in many types of cancer, including prostate, colon, skin, and breast cancer, and leukemia (Bradbury et al. 2005; Hida et al. 2007; Huffman et al. 2007; Stunkel et al. 2007).

The high expression of SIRT1 in many human tumors together with the protumorigenic effects described above argues for an oncogenic function of SIRT1. However, recent studies have demonstrated that SIRT1 levels are reduced in some other types of cancer. By analyzing a public database, Wang et al. found that the expression of SIRT1 is downregulated in glioblastoma, bladder carcinoma, prostate carcinoma, and ovarian cancers (Wang et al. 2008). They further analyzed samples from breast cancer and hepatic carcinoma and found that SIRT1 levels are also lower compared with that in normal tissues. These data suggest that SIRT1 may act as a tumor suppressor. This concept is reinforced by some results obtained in vivo from several mouse models. It has been reported that SIRT1 suppresses intestinal tumorigenesis in the $APC^{min/+}$ mouse model and inhibits colon cancer growth (Firestein et al. 2008). The $APC^{min/+}$ mouse contains a germ line mutation in the adenomatous polyposis coli (APC) tumor suppressor gene. Overexpression of SIRT1 in the intestine and colon reduces both the size and the number of adenomas. This effect is explained by the ability of SIRT1 to deacetylate β-catenin, promoting its cytoplasmic localization, thereby inhibiting cell proliferation. Furthermore, the authors found an inverse correlation between the presence of nuclear SIRT1 and the oncogenic form of β-catenin in 81 human colon tumor samples, further confirming the role of SIRT1 in suppressing this type of cancer.

Another important piece of data involving SIRT1 in tumor suppression comes from the study of SIRT1 activation/inactivation in the context of p53 heterozygosity. In one study, resveratrol treatment decreased the incidence of lymphomas in $p53^{+/-}$ mice, an effect that was attributed to SIRT1 activation by this compound (Oberdoerffer et al. 2008). In agreement with this, overexpressing SIRT1 in a $p53^{+/-}$ background has a protective effect, decreasing the frequency of thymic lymphomas in these animals (Oberdoerffer et al. 2008). In a complementary study, *SIRT1* KO mice were crossed to $p53^{+/-}$ animals (Wang et al. 2008). In this setting, SIRT1 deficiency results in accelerated tumorigenesis. Analysis of the tumors reveals aneuploidy and genomic instability in the absence of SIRT1, including translocations, chromosome breaks, deletions, end fusions, and dicentric chromosomes. Finally, Herranz et al. recently generated a mouse model that moderately overexpresses SIRT1 (Herranz et al. 2010). They found that old transgenic mice have lower levels of DNA damage and fewer spontaneous carcinomas and sarcomas.

Besides the in vivo data, some experiments in cells have also highlighted the relevance of SIRT1 in tumor suppression. First, as previously described, SIRT1 can promote apoptosis (see above). In addition, it has been reported that c-Myc positively controls the expression of SIRT1, but SIRT1 then interacts with and deacetylates c-Myc, resulting in decreased c-Myc stability. This feedback loop could prevent cellular transformation by inhibiting the activity of this proto-oncogenic transcription factor (Yuan et al. 2009a). Finally, a recent report shows that the acetylation of H3K56, a target of SIRT1/SIRT2, is increased in multiple types of cancer in a manner that is proportional to tumor grade (Das et al. 2009). Although this increase seems to correlate positively with ASF1 levels, it is tempting to speculate that decreased levels of this sirtuin could also promote tumorigenesis by increasing the levels of acetylated H3K56. All together, the above studies clearly indicate that SIRT1 could influence tumorigenesis. However, the ability of SIRT1 to function as an oncogene or a tumor suppressor gene will likely depend on the specific tumor type, cellular context, and signaling pathway affected.

Similar to other biological processes, little is known about the other nuclear sirtuins in cancer progression. Due to its role in mitotic progression (see above), SIRT2 has been proposed to act as a tumor suppressor by preventing chromosomal instability during mitosis. Further supporting this idea, SIRT2 expression is downregulated in glioma and glioma-derived cell lines (Hiratsuka et al. 2003). Similarly, SIRT7 mRNA expression inversely correlates with tumorigenic potential in several murine cell lines (Vakhrusheva et al. 2008a). Regarding SIRT6, all the available data suggest a role of this sirtuin in tumor suppression. First, SIRT6 is required for DNA repair, genomic stability, and telomere maintenance (Michishita et al. 2008; Mostoslavsky et al. 2006; Kaidi et al. 2010). In addition, SIRT6 represses glycolysis, acting as a corepressor of HIF-1α, and SIRT6 deficiency shifts cellular metabolism from oxidative respiration to aerobic glycolysis (Zhong et al. 2010). Interestingly, this pathway, described long ago by Otto Warburg (1956) (Warburg effect), is a hallmark of most cancer and highly proliferative cells, thereby suggesting that SIRT6 deficiency could provide an advantage for tumorigenic growth. Finally, it has been reported that SIRT6 interacts with GCIP (or

CCNDBP1/DIP/HHM), a potential tumor suppressor on chromosome 15 that is downregulated in colon, breast, and prostate cancers (Ma et al. 2007). Thus, these data reinforce the idea that SIRT6 may act as a tumor suppressor.

5.4 Inflammation and Immune System

In the last years, increasing amount of data has implicated nuclear sirtuins in the regulation of the immune system. SIRT1 was the first sirtuin shown to be involved in inflammatory processes. Numerous studies have shown that SIRT1 can suppress inflammation in multiple tissues by regulating the acetylation of NF-κB (Csiszar et al. 2006, 2008; Nayagam et al. 2006; Pfluger et al. 2008; Shen et al. 2009; Yang et al. 2007; Yoshizaki et al. 2009). The regulation of NF-κB involves the phosphorylation and subsequent degradation of IκB, which retains NF-κB in the cytoplasm, allowing NF-κB to migrate to the nucleus and activate the expression of many proinflammatory genes. SIRT1 physically interacts with the p65/RelA protein and deacetylates the lysine 310 of p65, inhibiting the transcriptional activity of NF-κB (Yeung et al. 2004). In addition to NF-κB, SIRT1 also targets the JNK and IKK inflammatory pathways in macrophages (Yoshizaki et al. 2010). In this context, SIRT1 overexpression downregulates the expression of proinflammatory genes in vitro and in vivo (Pfluger et al. 2008; Yeung et al. 2004), whereas knockdown of SIRT1 in immune cells leads to increased expression of proinflammatory cytokines (Schug et al. 2010; Yoshizaki et al. 2009). According to this, it has been shown that SIRT1 activation has a beneficial effect on metabolic disorders due to its ability to suppress inflammation in adipocytes and macrophages (Pfluger et al. 2008; Schug et al. 2010). Thus, besides its role as a regulator of metabolism, SIRT1 is involved in metabolic disorders by modulating inflammatory processes.

In addition to inflammatory processes, SIRT1 has been involved in viral infection. For instance, SIRT1 appears to modulate HIV-1 infection at several levels. First, HIV-1 Tat protein, which is required for the transcriptional activation of HIV, can bind to SIRT1, inhibiting its deacetylase activity, thereby promoting NF-κB activity (Kwon et al. 2008). This leads to a hyperactive immune response that facilitates HIV-1 infection and replication. In addition, SIRT1 can also bind to Tat and synergistically activate the HIV promoter (Kwon et al. 2008; Pagans et al. 2005). Therefore, while SIRT1 has a positive effect on HIV infection, SIRT1-dependent deacetylation of NF-κB has to be blocked by Tat in order to achieve maximum infection.

SIRT1 is also implicated in the differentiation and activation of immune cells. SIRT1 is involved in G-CSF-induced myeloid differentiation. G-CSF treatment induces the activation of NAMPT, which produces NAD^+, thus leading to SIRT1 activation and the subsequent induction of C/EBPα and C/EBPβ (Skokowa et al. 2009). These transcription factors upregulate G-CSF synthesis and G-CSF receptor expression, leading to the establishment of a G-CSF-NAD-SIRT1-C/EBP positive feedback loop. Moreover, SIRT1 is also essential for the maintenance of T-cell

tolerance in mice. This sirtuin deacetylates the transcription factor AP1, suppressing its transcriptional activity, thereby controlling T-cell activation and induction of energy (Zhang et al. 2009). In a different study, van Loosdregt et al. demonstrated that SIRT1 regulates the degradation of Foxp3 and, therefore, controls the number of regulatory T cells, a subset of lymphocytes that is critical for the maintenance of self-tolerance (van Loosdregt et al. 2010).

SIRT6 has also been reported to modulate immune responses. First, the characterization of *SIRT6* KO mice showed that these animals have a severe lymphopenia associated with massive lymphocyte apoptosis as a result of a non-cell-autonomous mechanism (Mostoslavsky et al. 2006). Next, SIRT6 represses NF-κB-dependent gene expression by binding to its target promoters and deacetylating H3K9 (Kawahara et al. 2009). In line with this, expression of some NF-κB target genes is elevated in SIRT6-deficient animals. However, two independent studies have shown that SIRT6 upregulates the levels of some proinflammatory cytokines (Bruzzone et al. 2009; Van Gool et al. 2009). Upon stimulation of immune cells, NAMPT levels are upregulated, leading to an increase in NAD^+ levels that activate SIRT6, which is required for IFN-γ and TNF-α production. Thus, SIRT6 appears to be critical in immune responses. However, due to the early death of SIRT6 null animals, the precise in vivo role of this sirtuin in inflammation and immunity remains to be elucidated.

5.5 *Sirtuins and the Central Nervous System*

Although neurodegenerative diseases may have a hazardous genetic compound, the risk of developing a neurodegenerative disease increases with age, ranging to 1 in 3 for Alzheimer's disease for people over the age of 80 years. Neurodegenerative diseases are associated with neuron loss, which can be caused by oxidative damage, DNA damage, defects in repair mechanism, disruption of calcium homeostasis, and accumulation of unfolded proteins. Since sirtuins play an important role in aging, DNA repair, ER stress, and oxidative damage, it is not surprising to find that sirtuins have an effect in neurological processes in higher organisms.

SIRT1 exhibits a protective effect in two models of neurodegeneration. First, overexpression of p25, an activator of cdk5, which induces hyperphosphorylation of Tau, enhances the amyloidogenic process (Wen et al. 2008; Ahlijanian et al. 2000) resembling Alzheimer's disease. Second, mutation of SOD1 causes severe, progressive motor neuron disease (Wong et al. 1995), representing a murine model for amyotrophic lateral sclerosis (ALS). In each of these models, overexpression of SIRT1 in the primary neurons showed a protective effect against neuronal loss (Kim et al. 2007). The protective effect could be due to p53 deacetylation, since silencing of p53 also reduced p25 cytotoxicity, and p53 is a known SIRT1 target. Furthermore, p53 acetylation levels were reduced in resveratrol-treated hippocampal tissue, suggesting a link between cytotoxicity in neurons and SIRT1 activity (Kim et al. 2007).

In a different model for AD (where the APP Swedish and PSEN1dE9 mutations are expressed), SIRT1 overexpression protected the brain from amyloid beta (AB) plaque formation, behavioral changes, and neuronal loss (Donmez et al. 2010). In this context, brain-specific deletion of SIRT1 resulted in severe worsening of the symptoms in AD-mutant mice, causing early lethality, and strongly implicating SIRT1 in the pathogenesis of AD (Donmez et al. 2010). The molecular mechanism leading to neuroprotection involves ADAM10, an α-secretase enzyme that is involved in the cleavage of APP (Lammich et al. 1999). SIRT1 can deacetylate and activate the retinoic acid receptor (RAR), which in turn enhances the expression of ADAM10. SIRT1 deficiency would lead to reduced ADAM10 levels and consequently accumulation of AB plaques. Since ADAM10 has been implicated in Notch signaling as well, the effect of SIRT1 on AD might also be secondary to Notch-dependent neuroprotection (Donmez et al. 2010). Past studies have shown that AB production stimulates NF-κB signaling, further increasing neurological damage in AD models (Akama et al. 1998). In this context, a different study has demonstrated that SIRT1 overexpression caused reduction of NF-κB signaling, in turn protecting against AD (Chen et al. 2005a).

Notably, SIRT1 inhibition could be beneficial in the context of other neurodegenerative diseases. Fragile X syndrome is the leading cause of mental retardation in males. In these patients, the gene FMR1 has an increase in the number of CGG–CCG repeats in the 5′UTR of the gene, leading to gene silencing and methylation. The gene FMR1 normally produces a neuronal protein that binds mRNA and regulates synthesis of many proteins. The use of HDAC inhibitors, and more specifically the sirtuin inhibitor Splitomicin, derepresses FMR1 expression. Moreover, a dominant negative form of SIRT1 has the same effect, whereas wild-type SIRT1 decreases FMR1 expression in normal cells (Biacsi et al. 2008). Notably, SIRT1 binding to chromatin is increased in the FMR1 mutant allele, causing local H3K9 and H4K16 hypoacetylation with concomitant silencing of the gene. It remains unclear what targets SIRT1 specifically to the mutant allele (Biacsi et al. 2008).

SIRT1 inhibition was also beneficial for neurons under oxidative damage. On one hand, SIRT1 silencing increased acetylation of the IRS2 protein, in turn decreasing ERK1/2 activity. Inhibition of this kinase resulted in protection against oxidative stress (Li et al. 2008b). In addition, conditions of oxidative stress can promote differentiation of NPCs into astrocytes, leading to astrogliosis and inhibition of neurogenesis. Several studies showed that oxidative stress increased SIRT1 binding to the promoter of the Mash1 gene, preventing its expression. Mash1 is a proneuronal transcription factor, and therefore silencing of SIRT1 caused Mash1 derepression, promoting neuronal differentiation under conditions of stress (Prozorovski et al. 2008). In contrast, a different study showed that SIRT1 could promote neuronal differentiation through inhibition of the Hes1 and Hes5 corepressors (Hisahara et al. 2008). Thus, SIRT1 effect on neuronal differentiation might lead to contrasting phenotypes, depending on multiple factors, including the proteins and gene promoters it interacts with.

The role of other sirtuins has been less investigated. Parkinson's disease (PD) is caused by the loss of dopaminergic neurons and formation of Lewis bodies

(aggregates of α-synuclein) in the *substantia nigra*. Mutations in α-synuclein have been linked to PD (Bonifati 2005). In a cellular model of PD, where α-synuclein transfection induces cytotoxicity, the silencing of SIRT2 but not SIRT3 could rescue the phenotype, suggesting that SIRT2 inhibition could have a beneficial effect in PD (Outeiro et al. 2007). In addition, the SIRT2-specific inhibitor AKG2 showed protective effects in primary midbrain cultures infected with the cytotoxic α-synA53T, and in a Drosophila model for PD. Although the mechanism remains unclear, it is possible that increased stabilization of α-tubulin (through increased tubulin acetylation) might lead to stronger interactions with α-synuclein, stabilizing the formation of larger protein aggregates that are less toxic (Outeiro et al. 2007).

In a cellular system for neurotoxicity, overexpression of the different sirtuins brought about quite dissimilar effects. Whereas SIRT1 protected from low potassium levels, SIRT2, 3, 5, and 6 induced apoptosis (Pfister et al. 2008). The protective effects of SIRT1 were neither suppressed by the SIRT1 inhibitor sirtinol or by nicotinamide, nor abolished when overexpressing a catalytic mutant protein, suggesting a noncatalytic role of SIRT1 in this phenotype (Pfister et al. 2008).

In addition to its role in neuropathological conditions, SIRT1 is critical for normal brain development. A brain-specific SIRT1 mutant exhibits synaptic plasticity defects and various memory and learning task defects (Gao et al. 2010). Notably, one of the roles of SIRT1 in the brain is to modulate the expression of miR-134, which inhibits the expression of CREB and BDNF, two critical proteins in memory formation. Lack of SIRT1 leads to overexpression of miR-134, and antisense oligos against miR-134 rescued the phenotype of the brain-specific SIRT1-deleted animals (Gao et al. 2010). Lastly, brain expression of sirtuins can also modulate metabolism. In a recent study, Coppari and colleagues show that depletion of SIRT1 specifically in pro-opiomelanocortin (POMC) neurons caused increased obesity and hyperleptinemia secondary to decreased energy expenditure at the brown-like adipocytes in perigonadal fat (Ramadori et al. 2010).

5.6 Cardiovascular System

Cardiovascular diseases are the principal cause of death in developed countries. The risk factors involved in these diseases are related to unhealthy food, sedentary life style, and smoking (Lim et al. 2007). Cardiovascular diseases develop over a period of decades, in some cases starting as early as adolescence, and the risk to develop fatal conditions increases with age (Lim et al. 2007). Risk factors include defects in cholesterol biosynthesis, autoimmunity, excess of oxidative damage, and loss of vascular endothelial functions. Endothelial cells are the inner surface of the vasculature and are essential for angiogenic blood vessel growth; they control vascular tone as well as blood coagulation, and are important mediators of inflammation (Potente and Dimmeler 2008). Deterioration of endothelial function promotes vascular pathogenesis. Early studies demonstrated that CR represents a robust nonpharmacological approach to reduce arterial blood pressure;

furthermore, it also improves endothelium-dependent vascular relaxation in obese and overweight patients with hypertension. Based on the link between CR and sirtuins, multiple laboratories explored the role of sirtuins in cardiovascular disease.

In a three-dimensional spheroid assay (which measures endothelial differentiation and angiogenesis), specific inhibition of SIRT1, but not SIRT2, 3, or 5, impaired sprout formation (Potente et al. 2007). SIRT1 silencing also prevented the formation of vasculature-like network and blocked endothelial cell migration, whereas its overexpression increased sprouting formation and migratory activity (Potente et al. 2007). In this context, specific deletion of SIRT1 in endothelial cells sensitized to ischemia-induced neovascularization, with increased incidence of foot and toe necrosis in response to ischemia (Potente et al. 2007). Similar results were obtained using a zebrafish model, suggesting that this protective role of SIRT1 in angiogenesis might be evolutionary conserved. The molecular mechanism could involve SIRT1 deacetylation and inhibition of Foxo1, a known inhibitor of angiogenesis (Potente et al. 2007). In addition, SIRT1 was shown to interact with Hey2. Hey2 is involved in cardiovascular development, and mice deficient in this protein are embryonic lethal due to defects in vascular development, implying that SIRT1 regulation of Hey2 activity might be critical for vascular development (Takata and Ishikawa 2003).

Apolipoprotein E (apoE)-deficient mice have the propensity to develop atherosclerotic lesions spontaneously on a standard chow diet (Piedrahita et al. 1992; Plump et al. 1992). Remarkably, overexpression of SIRT1 in these animals caused less atherosclerotic lesions (Zhang et al. 2008); however, the mechanism behind this effect remains unclear. In this context, SIRT1 also regulates the nuclear receptor LXR, which activates transcription of genes involved in lipid metabolism and the efflux of cholesterol, reducing the levels of triglycerides (Li et al. 2007). This effect could explain the beneficial effects of SIRT1 against vascular atherosclerosis. In addition, Nitric oxide (NO) is a critical factor regulating vascular homeostasis, and it is produced by the enzyme eNOS. eNOS was shown to be deacetylated by SIRT1 in lysine residues 496 and 506, stimulating its activity and increasing NO. Inhibition of SIRT1 expression decreases NO bioavailability, inhibiting endothelium-dependent vasorelaxation and inducing premature senescence of endothelial cells (Mattagajasingh et al. 2007). In addition, human endothelial cells treated with oxidized LDL or H2O2 exhibit increased levels of SIRT1. Treatment with oxLDL usually induces apoptosis; however, cells overexpressing SIRT1 showed reduced apoptosis. In this context, SIRT1 overexpression also protected against the detrimental effects of high-fat diet, which is known to impair endothelium-dependent vasorelaxation (Zhang et al. 2008). Although the precise mechanism behind the protective effect of SIRT1 in these models remains unclear, one possibility could be linked to SIRT1 regulation of eNOS, as indicated above (Zhang et al. 2008).

Separate studies demonstrated that CR is helpful to reduce blood pressure. In this context, CR mice had higher levels of deacetylated eNOS, suggesting a link between SIRT1-induced CR activation and eNOS activation. CR also improves endothelial function and attenuates oxidative stress in aged rat arteries, enhancing

acetylcholine-dependent relaxation of vascular tissue (Csiszar et al. 2009); however, it remains unclear whether this effect depends on the eNOS-SIRT1 pathway as well. In addition, CR reduces oxidative damage in the heart through a mechanism that involves reduction of the common variant of IGF-1 and induction of the autocrine variant mIGF-1 (Vinciguerra et al. 2009). Apparently, mIGF-1 increases the levels of SIRT1, which in turn prevents activation of the hypertrophic agonist AngII, in this way reducing ROS in the heart (Vinciguerra et al. 2009).

Overall, SIRT1 appears to exert several protective effects in the cardiovascular system. However, excessive SIRT1 overexpression can be detrimental, leading to apoptosis, hypertrophy, and decreased cardiac function (Alcendor et al. 2007). This narrow window in which SIRT1 might benefit cardiac function puts a word of caution and has to be carefully considered when planning therapeutic uses for SIRT1 activators.

Although less studied, other sirtuins might regulate the vascular system as well. SIRT7-deficient mice develop progressive heart hypertrophy, enlargement of the cardiomyocytes with concomitant apoptosis, increased fibrosis in the heart, and re-expression of α-actin in these cells. They also have an increase in lipofuscin inclusions (deposits that aggregate in the heart of aged mice), already present by 9 months in the SIRT7-deficient animals (Vakhrusheva et al. 2008b). Inflammation might also play a role in the defective hearts of SIRT7-deficient mice. Nevertheless, it is difficult to determine the contribution of inflammation, as it is not clear whether these changes are the cause of or reaction to the damage (Vakhrusheva et al. 2008b). The molecular mechanism behind the effect of SIRT7 in the heart may involve p53 deacetylation, which is a substrate for SIRT7 in cardiomyocytes and is hyperacetylated in SIRT7-deficient cells (Vakhrusheva et al. 2008b).

5.7 Sirtuins and Aging

Since the discovery of long-lived mutants in Yeast and *C. elegans*, there has been a strong interest in determining whether sirtuins are linked to longevity in mammals as well. Mice that overexpress SIRT1 display some phenotypes similar to mice on a calorie restricted diet: they are leaner than littermate controls; are more metabolically active; and display lower blood cholesterol, adipokines, insulin, and fasted glucose. However, they do not live longer (Bordone et al. 2007). Thus, although SIRT1 does not influence maximum life span, it appears that it does provide certain health benefits under particular conditions. Rats under calorie restriction expressed 50% more SIRT1 than rats fed ad libitum. Furthermore, cells grown in media containing the serum of these rats exhibit reduced Bax-induced apoptosis, similar to the effect observed upon SIRT1 overexpression. In this context, SIRT1 was shown to deacetylate Ku70, which sequesters Bax, preventing its proapoptotic effect. The authors claim that increased resistance to apoptosis promotes the long-term survival of irreplaceable cells, an effect dependent on SIRT1 (Cohen et al. 2004).

Another sirtuin, SIRT6, also appears to play a role in organismal life span. SIRT6-deficient animals exhibit an acute degenerative phenotype, resembling progeroid syndromes. SIRT6 knockout mice are born relatively normal, but rapidly develop severe metabolic defects, dying from fatal hypoglycemia before 4 weeks of age. These animals also show lordokyphosis secondary to low mineralization in bones (reminiscent of osteoporosis), loss of subcutaneous fat, and lymphocyte attrition (Mostoslavsky et al. 2006). These phenotypes are likely due to the critical role of SIRT6 in glucose homeostasis, as described before (Zhong et al. 2010). Nevertheless, a second study proposed a role for the transcription factor NF-κB in this phenotype. NF-κB target gene activities increase with age in many mammalian tissues and in stem cells. NF-κB is also implicated in age-dependent induction of cellular senescence in epithelial and hematopoietic progenitor cells (Adler et al. 2007); (Chambers et al. 2007). In this context, recent work by Chua and colleagues showed that SIRT6 interacts with the NF-κB subunit RELA, which in turn recruits SIRT6 to NF-κB target genes, where it represses their expression (Kawahara et al. 2009). Furthermore, heterozygous deletion of RelA partially rescued the lethality observed in SIRT6-deficient mice; however, the glucose phenotype was not affected. It is likely that both the severe glucose phenotype and the derepression of NF-κB target genes contribute to the phenotype observed in the absence of this sirtuin.

Overall, final proof for the role of these sirtuins on organismal life span would rely on the development of transgenic animals overexpressing these proteins. Few studies in this regard failed to demonstrate increased life span for SIRT1 and SIRT6 transgenics, although a somewhat beneficial effect was observed under conditions of stress, such as high caloric diet (Bordone et al. 2007; Kanfi et al. 2010). Only future studies will clarify the precise role of these proteins in life span.

Due to the natural complexity of aging in multicellular organisms, much of the studies in this field have been done in simpler cellular systems. Cellular senescence is a process in which primary cells in culture lose their ability to divide, and is characterized by specific morphological changes, appearance of senescence-associated β-galactosidase activity, and proliferation arrest (Funayama and Ishikawa 2007). In SIRT1-deficient MEFs, replicative senescence was reduced, and the cells continue to proliferate unrestrained due to reduced levels of the checkpoint factor p19 (Chua et al. 2005). In contrast, human diploid fibroblasts that overexpress SIRT1 exhibit reduced levels of β-galactosidase activity, senescence-associated heterochromatin foci, and increased proliferation, likely through activation of the map kinase ERK (Huang et al. 2008). Such contrasting effects between these studies might reflect species differences. For instance, human fibroblasts senesce from shortening of their telomeres, while mouse fibroblasts, which carry much longer telomeres, senesce mainly due to chronic accumulation of oxidative damage (Serrano and Blasco 2001). In embryonic stem cells, genotoxic stress induces the relocation of SIRT1 from repetitive sequences and promoters to sites of DNA damage. SIRT1 redistribution induces changes in gene expression that resemble the derepression seen during aging in mouse brains (Oberdoerffer et al. 2008). Concomitantly, brain-specific overexpression of SIRT1 can rescue some of the

changes observed in aged brains, indicating a link between aging, oxidative damage, and SIRT1 (Oberdoerffer et al. 2008).

In human fibroblasts, knock down of SIRT6 reduced their replicative life span. These cells undergo premature cellular senescence with increased levels of senescence-associated β-galactosidase (Michishita et al. 2008). Furthermore, their telomeres were dysfunctional, exhibiting increased end-to-end chromosomal fusions. In addition, ectopic expression of telomerase (hTERT) reversed the premature senescence observed in these cells. As a mechanism, the study describes that SIRT6 deacetylates H3K9 at telomeres, which in turn influences the binding of WRN protein to these sites (Michishita et al. 2008). Since this phenotype is not observed in mouse fibroblasts – due to the large size of telomeres in this species – it remains unclear whether this represents an evolutionary conserved function for SIRT6. Furthermore, it suggests that SIRT6 might have evolved to control different mechanisms even in species as close as mice and humans.

6 Summary and Perspective

Discovered less than 20 years ago, nuclear sirtuins have diverged to play such broad roles that multiple biological functions have been described for them in literally every cell, every organ, and every tissue. These particular enzymes appear to have evolved akin to other broadly functioning enzymes, such as phosphatases, where the specific biological outcome likely depends on a particular cellular context, their interacting proteins, and the genomic loci to where they are actively targeted. Sirtuins' unique and fundamental biological roles are clearly highlighted in the early lethal phenotypes observed in murine models of sirtuin deficiency, as described before. Their targets and functions cover a vast and yet growing network of biological pathways, and likely many more remains to be discovered.

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Structural Biology of Human Metal-Dependent Histone Deacetylases

Matthieu Schapira

Contents

Abstract Class I, II, and IV histone deacetylases (HDACs) are metal-dependent enzymes involved in a broad and partly unexplored array of biological mechanisms that include epigenetic control of gene expression. The catalytic domain of human classes I and IIa enzymes has been solved in complex with a substrate peptide and inhibitors, which revealed a conserved architecture, uncovered the catalytic mechanism of deacetylation, and outlined a chemical framework for inhibitor design. We will review the different structural elements of metal-dependent HDACs and their contributions to substrate recognition, catalysis, and inhibitor specificity.

Keywords Binding pocket • Catalysis • Class IIa HDAC • Electrostatics • HDAC • HDAC4 • HDAC7 • Hydroxamate • Inhibitors • Metal • Pharmacophore • Selectivity • Structure • Substrate • Trifluoroacetyl-lysine

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1 Introduction

Histone deacetylases (HDACs) are important regulators of cellular mechanisms, generally operating within large multiprotein complexes, and remove acetyl marks from lysine side chains of target proteins. Deacetylation of histone proteins by HDACs mediates the epigenetic control of gene expression and cellular differentiation (Grozinger and Schreiber 2002). Additionally, it is now clear that the regulatory role of protein acetylation extends vastly beyond epigenetic mechanisms (Choudhary et al. 2009) and so is the expected scope of HDAC biology. Some HDAC inhibitors are approved anticancer agents, and applications in other therapeutic areas are investigated, as more compounds with diverse selectivity profiles are being developed (Bradner et al. 2010; Marks 2010; Xu et al. 2007).

Human HDACs are divided into five evolutionarily related classes based on phylogenetic analysis (Gregoretti et al. 2004) (Fig. 1): HDAC1, 2, 3, 8 (class I), HDAC4, 5, 7, 9 (class IIa), HDAC6, 10 (class IIb), the sirtuins SIRT1–7 (class III), and HDAC11 (class IV). Classes I, II, and IV enzymes require a divalent metal ion for catalysis. Sirtuins are NAD^+-dependent enzymes structurally and biochemically unrelated to other classes, and will not be discussed in this review (Sauve et al. 2006). The first structure of an HDAC catalytic domain was that of a bacterial protein sharing 35% sequence identity with human HDAC1, and revealed a topology similar to arginase (Finnin et al. 1999; Kanyo et al. 1996). Structures of human HDAC2 (Bressi et al. 2010), HDAC4 (Bottomley et al. 2008), HDAC7 (Schuetz et al. 2008), and HDAC8 (Dowling et al. 2008, 2010; Somoza et al. 2004; Vannini et al. 2004, 2007) solved since confirmed the general architecture revealed by the seminal work on the bacterial enzyme, and define a set of canonical features (Fig. 2): (1) A funnel-shaped lysine-binding channel located at the center of an overall α/β-fold, (2) a combination of conserved catalytic residues organized around the catalytic Zn ion at the bottom of the channel, and (3) a set of loops with variable length and flexibility at the rim of the channel, forming protein interaction interfaces. These features play different roles in substrate recognition, catalytic mechanism, and inhibitor specificity, which will be reviewed in the following sections.

2 Substrate Recognition

Insertion of the acetylated lysine side chain into the central channel of HDACs implies that the rim of the channel is in direct contact with protein substrates. This rim is mainly composed of a set of four loops (named L1–L4) with variable sizes and conformations between different HDAC isoforms, and between different structures of a same isoform for L1 and L2 (Fig. 3a–e). Importantly, these loops are often found at the interface of crystallographic dimers in HDAC structures, which probably reflects their propensity to act as protein interaction interface.

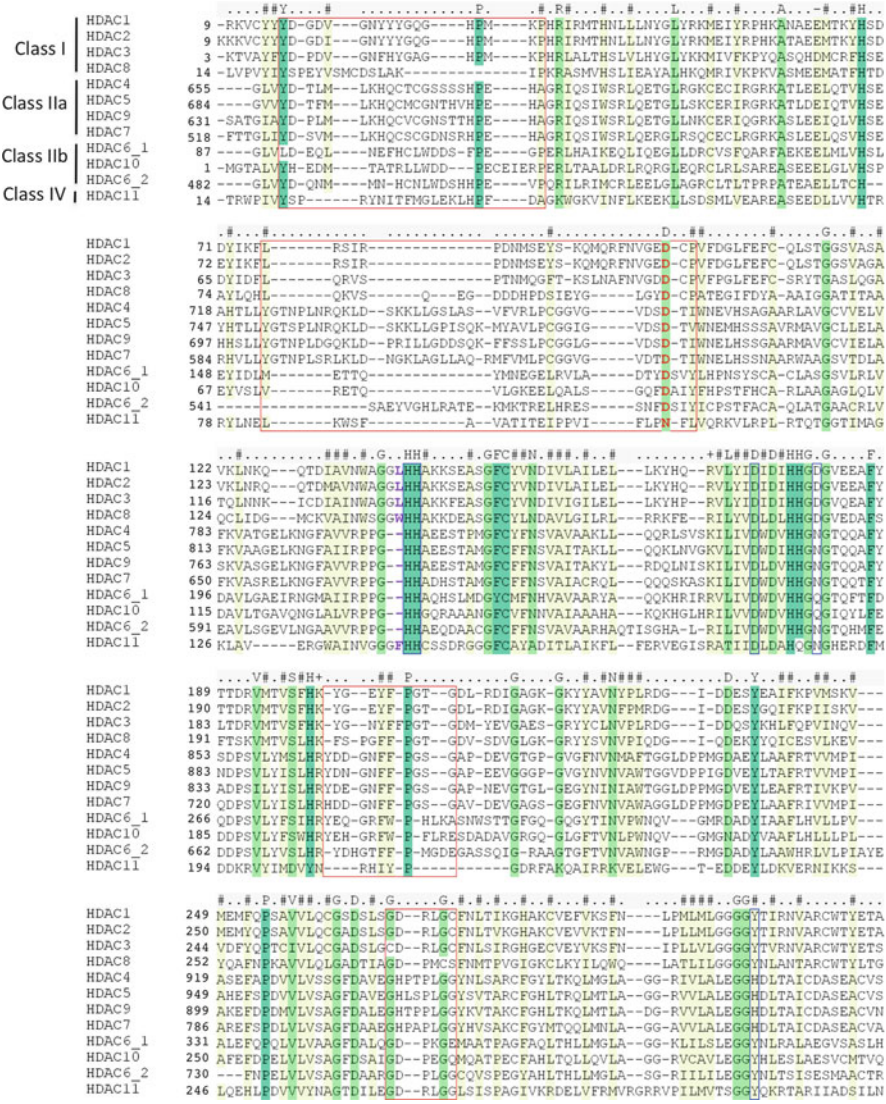

Fig. 1 Sequence alignment of the catalytic domain of human metal-dependent HDACs. Variable loops L1–L4 are delimited by *red* boxes. Catalytic residues: *blue* boxes. The conserved aspartate at the rim is highlighted in *red*. The residue opening or closing the foot pocket in class I enzymes is in *magenta*. The alignment was generated with ICM (Molsoft LLC) from a seed alignment of 157 sequences downloaded from the PFAM database (domain PF00850). Variable regions were manually edited based on available structures. Both catalytic domains of HDAC6 are shown

A structure of HDAC8 in complex with an acetylated tetrapeptide shows that residues from L2 and L4 are in direct contact with the acetylated lysine. Asp101 of loop L2 makes two hydrogen bonds with the backbone nitrogen atoms flanking the

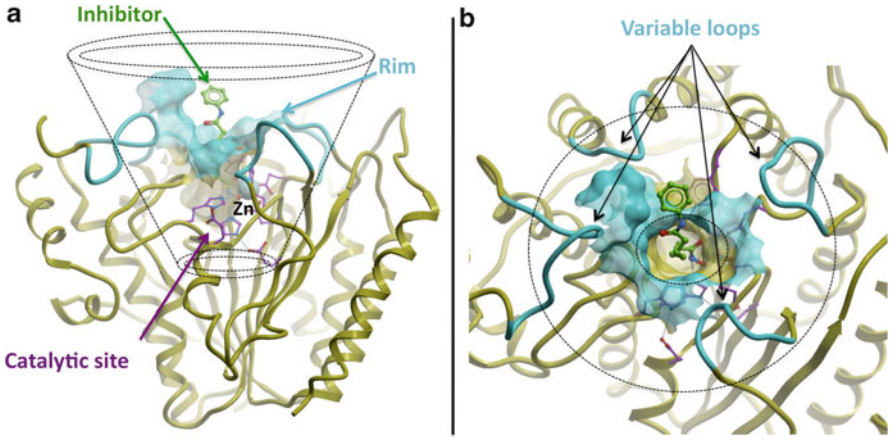

Fig. 2 Overall structure of HDAC catalytic domains. Side (**a**) and top (**b**) views of HDAC8 in complex with the inhibitor SAHA [pdb code: 1t69] (Somoza et al. 2004) show that the catalytic domain is composed of (1) a structurally conserved core (*mustard*), (2) a rim made of variable loops (*cyan*) where substrate recognition is expected to occur, and (3) a lysine-binding channel leading to the catalytic site (*magenta*), organized around a zinc ion (*gray* ball). All cocrystallized inhibitors to date occupy the central channel of HDACs and participate in the coordination of the zinc, as illustrated here by the bound conformation of SAHA (*green*). Oxygen and nitrogen atoms are colored *red* and *blue*, respectively

substrate lysine, and constrains the peptide in a *cis*-conformation (Vannini et al. 2007) (Fig. 3F). Asp 101 is the only residue from the L2 loop that is absolutely conserved across all HDACs (except for HDAC11) (Fig. 1), and is positioned at the entrance of the lysine channel in structures of all human HDACs solved to date (Fig. 3a, b, d, e). D101 mutations result in loss of HDAC8 activity on peptide and purified histone substrates (Dowling et al. 2008; Vannini et al. 2007); HDAC4 activity is similarly antagonized by mutation of the corresponding D759 to Ala (Bottomley et al. 2008). These observations suggest that the interaction between D101 and substrates is critical in positioning HDAC8 substrates and that this mechanism is conserved in other HDACs.

Class IIa HDACs are characterized by short and large inserts in their L1 and L2 loops, respectively. Two Cys and one His from L1 and one Cys from L2 coordinate a Zn ion in these enzymes, which is believed to stabilize the large and flexible loops. These Zn-coordinating cysteine residues can be oxidized and reduced in cells, which affects nuclear localization of HDAC4, and may be linked to cardiac hypertrophy in vivo (Ago et al. 2008). Their mutation abrogates interaction with the corepressor complex N-CoR-HDAC3 (Bottomley et al. 2008). The structural Zn places Asp626 of HDAC7 (the equivalent Asp101 in HDAC8) at the entrance of the lysine channel, in its putative catalytically competent conformation (Fig. 3d). The same conformation is observed in the apo structure of the gain-of-function H976Y HDAC4 mutant (Fig. 3b). Surprisingly, in the structure of wild-type HDAC4 in complex with a trifluoromethyl ketone inhibitor, L1 and L2 loops adopt very

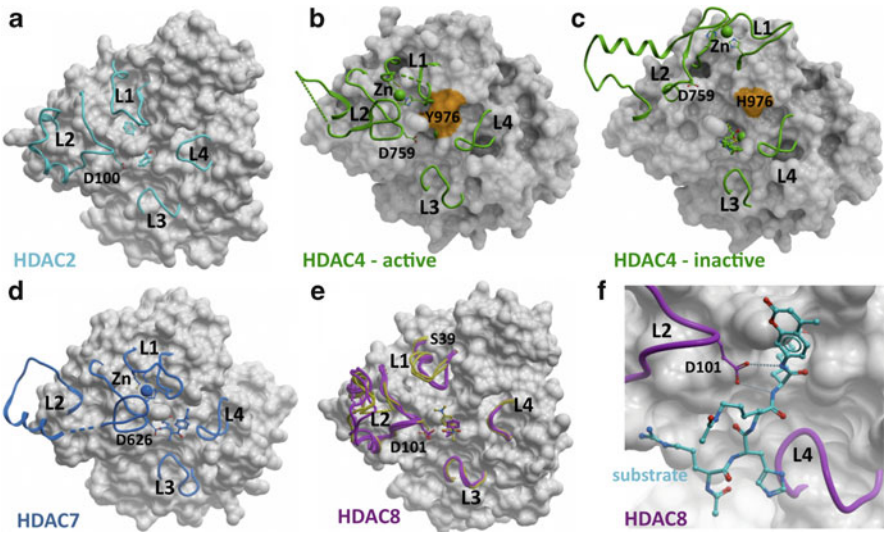

Fig. 3 Variability and flexibility of loops forming the rim. While the core structure of the catalytic domain is well conserved (symbolized here by a *white molecular surface* representation where loops have been truncated – orientation as in Fig. 1b), the length and conformation of the loops at the rim of the lysine-binding channel are extremely variable, which probably contributes to substrate specificity. (**a**) HDAC2 bound to an *N*-(2-amino phenyl) benzamide inhibitor ([pdb code: 3max], (Bressi et al. 2010)). (**b**) Inhibitor-free, gain-of-function HDAC4 mutant (H976Y – *orange*) ([2vqw], (Bottomley et al. 2008)). (**c**) HDAC4 bound to a trifluoromethyl ketone inhibitor ([2vqj], (Bottomley et al. 2008)). (**d**) HDAC7 bound to TSA ([3c10], (Schuetz et al. 2008)). (**e**) HDAC8. *Magenta*: loops from HDAC8 bound to a peptide substrate ([2v5w], (Vannini et al. 2007)), and inhibitors MS-344 ([1t67], (Somoza et al. 2004)), SAHA ([1t69], (Somoza et al. 2004)) (shown), and APHA ([3f07], (Dowling et al. 2008)). *Yellow*: loops from HDAC8 bound to inhibitors TSA ([1t64], (Somoza et al. 2004)) (shown) and CRA-19156 ([1vkg], (Somoza et al. 2004)). (**f**) HDAC8 bound to a tetrapeptide substrate (*cyan*) ([2v5w], (Vannini et al. 2007))

different conformations, the structural Zn is coordinated by a different set of residues, and the conserved L2 aspartate (D759) is positioned far from the lysine channel (Fig. 3c). Superimposition of the two HDAC4 structures shows that the inhibitor occupying the lysine channel in the wild-type structure clashes with the conformation of the conserved L2 D759 observed in the active structure. It is unclear whether small structural adjustments would be sufficient to resolve this clash, but this observation reveals a possible allosteric mechanism of inhibition whereby inhibitors would not necessarily occupy the catalytic site, but would prevent positioning of the L2 aspartate at the entrance of the lysine channel.

Structural variations are also observed between different structures of class I enzymes. For instance, different HDAC8 structures reveal alternate conformations of the L1 and L2 loops. L1 seems to adopt one of two possible conformations, while L2, larger and more flexible, is not always structured (Fig. 3e). Ser39 is a phosphorylation site in HDAC8 only 5A away from Lys36 of the L1 loop; this posttranslational modification is accompanied by decreased activity, which could

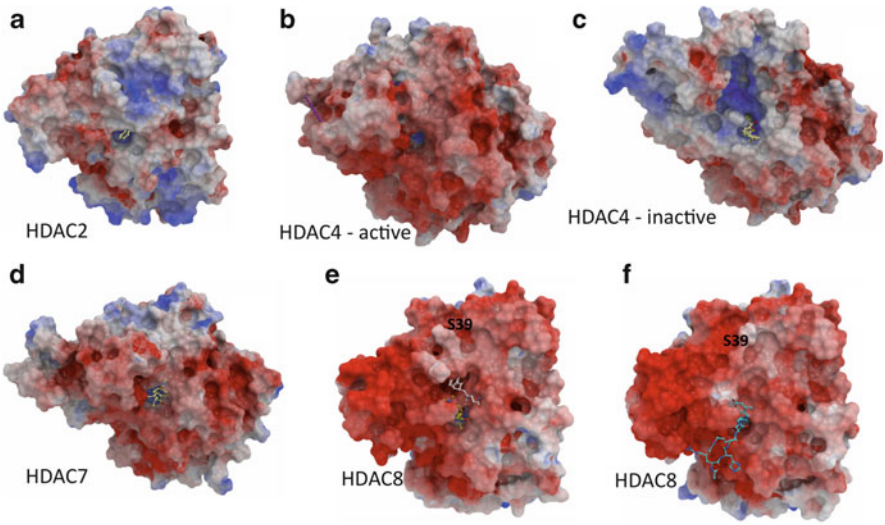

Fig. 4 Electrostatics of HDACs. Coloring the molecular surface according to the electrostatic potential (*red*: electronegative, *blue*: electropositive) reveals a significant diversity in charge distribution among HDACs that is expected to contribute to substrate specificity. Structure orientations are the same as in Fig. 3. Alternate conformations of loops L1 and L2 captured in different structures of HDAC4 (**b**, **c**) or HDAC8 (**e**, **f**) induce dramatic variations in shape and electrostatics that alter protein interaction interfaces. A large electropositive pocket in HDAC4 (**c**) and a small cleft occupied by a second TSA molecule (*white sticks*) in HDAC8 (**e**) may be exploited to develop inhibitors that would lock the protein in a specific conformation. (**a**) HDAC2 bound to an *N*-(2-amino phenyl) benzamide inhibitor ([3max], (Bressi et al. 2010)). (**b**) Inhibitor-free, gain-of-function HDAC4 mutant (H976Y – *orange*) ([2vqw], (Bottomley et al. 2008)). (**c**) HDAC4 bound to a trifluoromethyl ketone inhibitor ([2vqj], (Bottomley et al. 2008)). (**d**) HDAC7 bound to TSA ([3c10], (Schuetz et al. 2008)). (**e**) HDAC8 bound to TSA ([1t64], (Somoza et al. 2004)). (**f**) HDAC8 bound to a tetrapeptide substrate ([2v5w], (Vannini et al. 2007))

be mediated by the destabilization of an active L1 conformation (Lee et al. 2004; Somoza et al. 2004).

The diversity in size and conformation of the L1 and L2 loops translates in structurally and electrostatically diverse molecular surfaces (Fig. 4), and probably protein interaction interfaces. While the electrostatic potential around the rim is generally electronegative, which would favor electropositive substrates (such as histone tails) or interaction partners, this property appears much less pronounced in the HDAC2 structure, which may indicate different substrate specificity. In the HDAC8–Trichostatin A (TSA) complex, L1 is more distant from the lysine channel, probably because the dimethylaniline group of TSA would clash with the L1 conformation observed in the substrate-bound structure. This uncovers a secondary pocket, next to, but distinct from the lysine channel, occupied by another TSA molecule in the structure (Fig. 4e). This pocket is absent from the substrate-bound conformation (Fig. 4f) and may be exploited to design selective inhibitors. Similarly, in the inactive structure of wild-type HDAC4, the "open" conformation of L1 and L2 defines a large electropositive cavity (Fig. 4c) that is absent from the active

conformation (Fig. 4b). The two structures capture protein interaction interfaces that vary drastically in shape and electrostatics. This raises the possibility that different binding partners may stabilize the protein in different activation states.

Importantly, a conserved tyrosine residue located at the entrance of the lysine channel is mutated to histidine in class IIa HDACs (H976 in HDAC4, H843 in HDAC7 – Fig. 1). This variation is associated with a 1,000-fold loss in catalytic activity, which can be rescued by His to Tyr gain-of-function mutation, as shown in HDAC4, 5, and 7 (Lahm et al. 2007; Schuetz et al. 2008). While a tyrosine side chain at this position invariably points toward the catalytic site (and participates in catalysis, as discussed later), the histidine side chain is flipped away from the catalytic site and projects toward the solvent. It is tempting to speculate that the conformation of this surface-exposed His at the rim of class IIa HDACs (Fig. 3b, c) is linked to catalytic activity (as discussed later in greater details) and may be controlled by protein interaction events.

In conclusion, most HDACs are found within large multiprotein complexes that regulate substrate specificity and function (Nicolas et al. 2007; Verdin et al. 2003; Yang and Seto 2003). The genetic diversity and the flexibility of loops at the rim of the substrate lysine channel underlie a diverse and malleable molecular surface that can accommodate combinations of substrates and binding partners with diverse shapes and electrostatics. Specific conformational states of the loops captured experimentally revealed noncanonical binding pockets that may be exploited toward allosteric inhibition, but the chemical tractability of these putative binding sites has not yet been validated.

3 Catalytic Mechanism

The catalytic mechanism of metal-dependent HDACs was originally deduced from the crystal structure of a bacterial HDAC-like protein, and later confirmed by the structure of HDAC8 in complex with a p53 tetrapeptide substrate (Dowling et al. 2008; Finnin et al. 1999; Vannini et al. 2007) (Fig. 5a). A catalytic zinc ion polarizes the carbonyl group of the departing acetate and increases the electrophilicity of its carbonyl carbon. Additionally, the Zn orientates a catalytic water molecule located next to the scissile bond. A pair of conserved histidine residues (H142 and H143 in HDAC8) forms hydrogen bonds with the water molecule and enhances its nucleophilic property. The basicity of H142 is itself increased by a conserved aspartic acid (D176), completing a charge-relay system. The aspartate is additionally polarized by a neighboring potassium ion. Nucleophilic attack of the substrate's carbonyl carbon by the water molecule is followed by hydrolysis where the hydroxyl group of a neighboring tyrosine (Y306 in HDAC8), initially engaged in a hydrogen bond with the acetyl carbonyl oxygen, would donate a proton to the amine.

A water-filled channel linking the active site to the outer molecular surface can be observed in some HDAC structures, including HDAC8, and was proposed to act

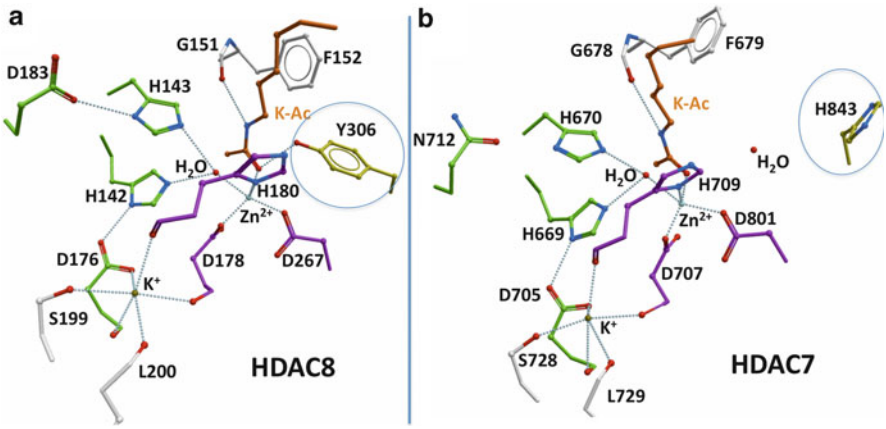

Fig. 5 HDAC catalytic mechanism. (**a**) Crystal structure of HDAC8 in complex with an acetylated peptide substrate (*orange*) ([2v5w] (Vannini et al. 2007), [3ewf] (Dowling et al. 2008)). D176 and D183 polarize H142 and H143 in a charge-relay system (*green*) that enhances the nucleophilicity of a catalytic water molecule. The latter attacks the carbonyl carbon of the substrate, which is particularly susceptible through its contribution to the coordination system (*magenta*) of a neighboring zinc ion. The hydroxyl moiety of Y306 forms a hydrogen bond with the substrate's carbonyl oxygen and protonates the amino group of the reaction product. (A wild-type tyrosine is shown at position 306, as seen in [3ewf], and a wild-type histidine is shown at position 143, as seen in [2v5w], though one or the other residue was mutated in the two structures to capture the complex with a catalytically dead form of the enzyme.) (**b**) The catalytic site of class IIa HDACs, such as apo-HDAC7 shown here ([3c0y] (Schuetz et al. 2008)) with substrate from superimposed HDAC8 [2v5w], differs in one important way (*circled*): the catalytic tyrosine is replaced by a histidine (H843 in HDAC7). Oxygen: *red*. Nitrogen: *blue*

as a way of evacuation for the acetate byproduct (Finnin et al. 1999; Nielsen et al. 2005; Vannini et al. 2004) (Fig. 6). A conserved arginine is located at the entrance of the tunnel and may attract the departing acetate through long-range electrostatics. In HDAC8, Ser39 is positioned at the exit of the evacuation channel, and phosphorylation of this residue by PKA inhibits deacetylase activity (Lee et al. 2004), raising the possibility that structural rearrangements induced by the phosphorylation event affect the release of the reaction by-product, thereby inhibiting turn over and catalysis. Whether HDAC exit channels are chemically tractable remains an open question.

While it is clear that nonclass III HDACs are metal-dependent enzymes, the identity of the metal in vivo remains uncertain. HDAC8 activity increases when Zn^{2+} is substituted by Fe^{2+} (Gantt et al. 2006). And, while affinity for Zn^{2+} is about five orders of magnitude higher than that for Fe^{2+}, cellular levels of Fe^{2+} are three to five orders of magnitude higher than that of Zn^{2+} (Dowling et al. 2010). It was also reported that recombinant HDAC8 purified from *E. coli* contains eightfold more iron than zinc before dialysis (Gantt et al. 2006). The nature of the metal should nevertheless not affect the catalytic mechanism derived from zinc-bound structures.

Most residues participating in this mechanism are conserved across HDACs, with one important exception: in class IIa HDACs, the catalytic tyrosine, which is essential

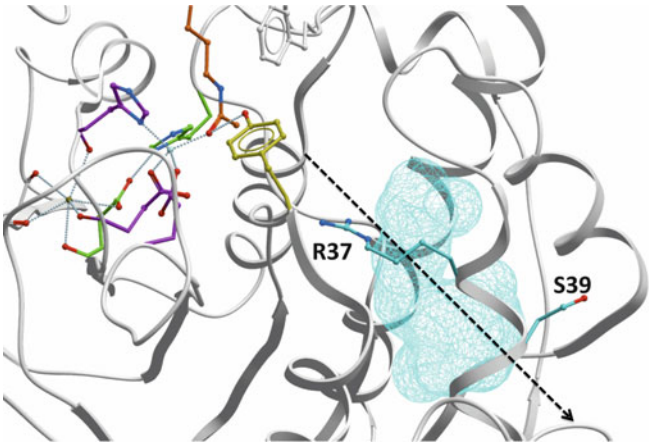

Fig. 6 Exit channel. A water-filled channel (*cyan*) can be observed in some HDAC structures (HDAC8 shown here [2v5w], (Vannini et al. 2007)). The channel links the catalytic site to the outer surface of the protein and may be used to evacuate the departing acetate. A conserved arginine in the vicinity of the catalytic tyrosine (R37 here) may guide the enzymatic by-product to the entrance of the channel after cleavage of the scissile bond. Phosphorylation events reported at S39 affect the structure at the channel output and may regulate release of the acetate

for HDAC8 to deacetylate peptidic substrates and purified histones (Vannini et al. 2007), is replaced by a histidine (Y306 in HDAC8, H843 in HDAC7, and H976 in HDAC4 – Figs. 1 and 5). This does not seem to affect significantly binding of acetylated peptides to HDAC7, but dramatically reduces catalytic activity of class IIa enzymes (Bottomley et al. 2008; Lahm et al. 2007; Schuetz et al. 2008). Conversely, the H843Y HDAC7 mutant has a similar affinity than wild type for peptide substrates, but is over 5,000 times more active (Schuetz et al. 2008).

In HDAC7, H843A and H843F mutants are 60 times less active than H843Y but 80 times more active than the wild-type enzyme, indicating that the loss of the tyrosine's hydroxyl group that engages in a hydrogen bond with the substrate's carbonyl and protonates the reaction product in nonclass IIa HDACs accounts only in part for the negative impact of a histidine at this position (Schuetz et al. 2008). This raises the possibility that forcing the histidine side chain to project toward the catalytic site rather than the protein surface would rescue in part catalytic activity to levels observed in the H843A or H843F mutants. The nitrogen of the scissile bond could possibly accept a proton from a second water molecule observed in the HDAC7 and HDAC4 structures [3c0y, 2vqq] (Fig. 5b), or from the second conserved histidine (H670 in HDAC7), as initially proposed for the bacterial HDAC-like protein (Finnin et al. 1999). The latter, however, seems less likely since the basicity of this second histidine is enhanced by a neighboring aspartic acid present only in class I enzymes (D183 in HDAC8, N712 in HDAC7 – Fig. 1).

It is not clear whether the dramatic loss in enzymatic activity observed biochemically for class IIa HDACs equally applies in vivo. The observation that H843A and H843F HDAC7 mutants are 80 times more active than wild type suggests that

conformational rearrangement of the histidine, possibly induced by protein interaction events, may rescue limited but significant activity in specific cellular contexts (Schuetz et al. 2008). It is also possible that class IIa HDACs are specific for other, not yet identified posttranslational modifications. Alternatively, they may act as binders of acetyl marks in the nucleus or cytoplasm where they shuttle once phosphorylated, a model supported by two observations: HDAC7 has an affinity comparable to that of other HDACs for acetylated peptides (Hildmann et al. 2006; Schuetz et al. 2008), and an acetylated lysine peptide potently inhibits class IIa enzymatic activity against a more labile trifluoroacetylated substrate (Bradner et al. 2010). Finally, class IIa HDACs are part of multiprotein complexes and may simply act as docking platforms for other proteins, such as transcription factors and more active HDACs, as suggested by the observation that they only regulate transcription by recruiting SMRT/N-CoR HDAC3, regardless of their enzymatic activity (Fischle et al. 2001, 2002).

4 Inhibitor Specificity

A pharmacophore model of HDAC inhibitors previously proposed is composed of a cap that sits at the rim of the substrate channel, a chelator that occupies the bottom of the channel and interacts with the Zn ion, and a linker that bridges the cap and the chelator (Miller et al. 2003). This model still holds, but, based on recent structures, should probably be complemented with a fourth optional element that we name the foot, and occupies the so-called foot pocket present in some isoforms (Fig. 7a). We will now review the structural chemistry of these different elements in the light of available complex structures of human HDACs (Fig. 8).

The cap of HDAC inhibitors varies in its chemical nature and orientation from one isoform to another, and within single isoforms. For instance, TSA, APHA, and *N*-hydroxy-4-{methyl[(5-pyridin-2-yl-2-thienyl)sulfonyl]amino}benzamide (referred to as PTSB-hydroxamate) all occupy different areas of the rim in HDAC8, formed by the flexible loops discussed above: TSA, mainly contacts loop L2, APHA loop L3, and PTSB-hydroxamate loop L1 (Fig. 7b–d). It should be noted that the conformations observed may also be influenced by intermolecular contacts with crystallographic dimers. Not surprisingly, smaller capping elements, such as the phenyl ring of SAHA, make fewer interactions with the rim, are more flexible – as revealed by the absence of electronic density in the complex structure with HDAC7 ((Schuetz et al. 2008) [3c0z]), and contribute less to binding, while the larger cap of a substrate-inspired HDAC8 inhibitor contributes significantly to binding enthalpy through its methoxy indole ring and a pair of amide nitrogens engaged in multiple hydrogen bonds with the conserved Asp101 (figure not shown (Vannini et al. 2007) [2v5x]). A potent cap-less HDAC2 inhibitor was recently crystallized entirely buried in the lysine channel, in a confirmation that extends deeply into a foot pocket, showing that the capping moiety is an optional feature,

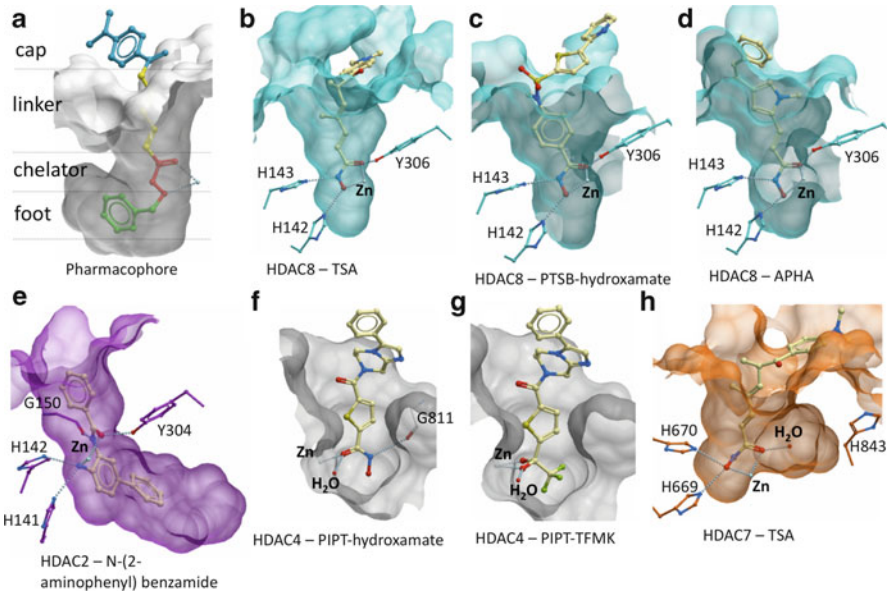

Fig. 7 Structural chemistry of HDAC inhibitors. (**a**) Pharmacophoric scheme: HDAC inhibitors are composed of a capping end, a linker, and a zinc chelating moiety. An additional group can in some cases extend beyond the catalytic center, into a foot pocket. (**b–d**): Compounds with diverse linkers were cocrystallized with HDAC8. (**b**) TSA ([1t64], (Somoza et al. 2004)). (**c**) PTSB-hydroxamate ([1w22], (Vannini et al. 2004)). (**d**) APHA ([3f07], (Dowling et al. 2008)). (**e**) HDAC2 was solved in complex with a cap-less inhibitor that features a nonhydroxamate chelating group and a foot that occupies a cavity at the entrance of the by-product exit channel ([3max], (Bressi et al. 2010)). (**f**, **g**) The HDAC4 channel can adopt a partly open conformation that could accommodate larger linkers. (**f**) The protonated hydroxamic acid adopts an unfavorable monodentate chelation mode ([2vqm], (Bottomley et al. 2008)); (**g**) the hydrated trifluoromethyl ketone is a better chelating agent ([2vqj], (Bottomley et al. 2008)). (**h**) The HDAC7–TSA complex features a foot pocket that may be exploited to achieve selectivity ([3c10], (Schuetz et al. 2008))

as long as sufficient interactions are provided by other elements (Bressi et al. 2010) (Fig. 7e).

Varying uniquely the capping moiety in a library of *para*-substituted cinnamic hydroxamic acids resulted in IC50 values ranging from 20 nM to over 50 μM against HDAC5, which suggests that the capping feature can be a used as a driver of specificity (Bradner et al. 2010). However, such strategy is probably unreliable, as it is resting on interactions with a very malleable surface composed of flexible loops. The latter may adopt alternate conformations to accommodate inhibitor binding, as observed in HDAC4, where the capping element of the inhibitor is suspected to contribute to major conformational rearrangement of loops L1 and L2, which may alter functional protein interaction events (Fig. 3c).

The linker element needs to accommodate the geometry of the lysine channel, while maintaining an acceptable pose at the capping end and complying with the very strict structural constraints imposed by the orientation of the chelator, as detailed later.

Ligand Name	Gene	PDB Code	Reference
APHA	HDAC8	3f07	1
CRA-19156	HDAC8	1vkg	2
IPIO-hydroxamate	HDAC8	2v5x	3
MS-344	HDAC8	1t67, 3mz3/4/6/7, 3ew8, 3f06, 3ezp/t	1,2,4
N-(2-aminophenyl) benzamide	HDAC2	3max	5
PIPT-hydroxamate	HDAC4	2vqm/v	6
PIPT-TFMK	HDAC4	2vqj/o/q	6
PTSB-hydroxamate	HDAC8	1w22	7
SAHA	HDAC7	3c0z	8
	HDAC8	1t69	2
TSA	HDAC7	3c10	8
	HDAC8	1t64, 3f0r	1,2

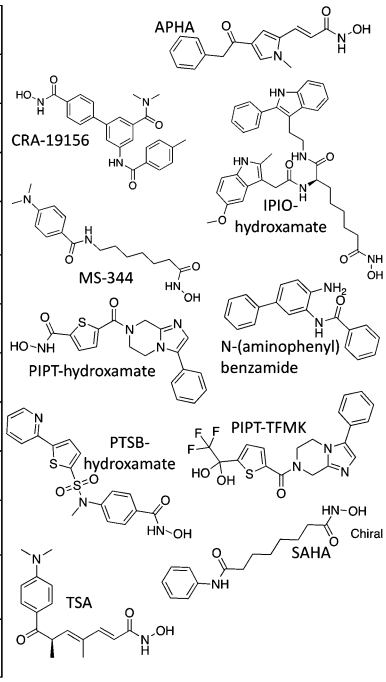

Fig. 8 Publically available structures of human HDACs in complex with inhibitors. References: 1. Dowling et al. (2008), 2. Somoza et al. (2004), 3. Vannini et al. (2007), 4. Dowling et al. (2010), 5. Bressi et al. (2010), 6. Bottomley et al. (2008), 7. Vannini et al. (2004), 8. Schuetz et al. (2008)

Structures of inhibitors with different linkers in complex with HDAC8 reveal how diverse scaffolds can occupy the same lysine channel, while keeping the orientation of the hydroxamate chelator absolutely unchanged (Fig. 7b–d). Linkers that better occupy the lysine channel while maintaining favorable orientations at the capping and chelating ends are expected to better contribute to binding. The width of the channel is not absolutely conserved between the different HDAC isoforms, which may open some opportunity to engineer specificity: a bulkier linker may still fit in the wider channel of HDAC7, but not that of HDAC2 (Fig. 7e, h). The large conformational rearrangement of HDAC4 loops induced by the cap of phenyl-imidazo-pyrazin-thiophenyl (PIPT) inhibitors results in a partial opening of the lysine channel (Fig. 8f, g). This surprising mechanism seems particularly fitted to design HDAC4-selective compounds. The observation that HDAC7 is more susceptible to cinnamic hydroxamic acids harboring a *para*- versus *meta*-substituted linker, while no distinction is observed for HDAC2, HDAC3, and HDAC7, confirms that, at least in some cases, linker chemistry can be exploited to achieve selectivity (Bradner et al. 2010).

The canonical chelating group of HDAC inhibitors is hydroxamic acid. The mechanism of binding has conserved features with zinc metalloprotease hydroxamate inhibitors (Grams et al. 1995). Typically, both carbonyl and hydroxyl oxygen atoms participate in zinc coordination in a bidentate manner (Fig. 7b–d).

Two conserved catalytic histidines are engaged in hydrogen bonds with the oxygen and nitrogen of the hydroxylamine. Another hydrogen bond is formed between the carbonyl oxygen and the catalytic tyrosine. It is speculated that this network of hydrogen bonds would lower the pK_a and facilitate deprotonation of the chelator, which would result in tighter binding (Bradner et al. 2010; Wang et al. 2007). This argument is supported by the observation that hydroxamic acid-based inhibitors are less potent against class IIa HDACs, where the catalytic tyrosine is absent (Bradner et al. 2010). Structures of HDAC4 and HDAC7 in complex with hydroxamic inhibitors reveal that a water molecule is hydrogen bonded to the chelating carbonyl in place of the catalytic tyrosine, while interactions with the histidine are lost in HDAC4 and exclusively directed at the hydroxyl oxygen in HDAC7 (Fig. 7f, h). Consequently, a monodentate chelating mode is observed in HDAC4, while a suboptimal bidentate geometry may take place in the HDAC7 complex where the distinction between mono- and bidentate orientation is probably not within resolution limits. The altered network of interaction, geometry, and suspected protonation state in class IIa HDACs is in agreement with the observation that hydroxamic acids are 10- to 100-fold more potent against gain-of function HDAC4 or HDAC7 mutants where the catalytic tyrosine has been restored (Bottomley et al. 2008; Schuetz et al. 2008).

The mode of binding of nonhydroxamate chelators has recently been elucidated in complex with classes I and IIa enzymes. The crystal structure of HDAC2 bound to a potent *o*-amino-anilide-based inhibitor revealed that the amine nitrogen and amide carbonyl could recapitulate bidentate chelation of the zinc ion as well as hydrogen bonding with the catalytic histidine and tyrosine residues. The amide nitrogen makes an additional bond with the carbonyl oxygen of G150 (Fig. 7e). A hydrated trifluoromethyl ketone bound to HDAC4 also chelates the catalytic zinc in a bidentate geometry via its two oxygen atoms (Fig. 7f). Interestingly, the same compound with a hydroxamic acid chelating group is about three times less potent, but has an activity increased 30-fold against the H976Y gain-of-function mutant, while the activity of the trifluoromethyl ketone remains unchanged at about 300 nM IC50 (Bottomley et al. 2008). This, along with recent observations that hydroxamic acid compounds are suboptimal against class IIa HDACs (Bradner et al. 2010), suggests that hydrated trifluoromethyl ketones may be better suited toward this class of enzymes.

The recent structure of HDAC2 in complex with an *o*-amino-anilide inhibitor revealed a large foot pocket occupied by a diphenyl moiety (Bressi et al. 2010) (Fig. 7e). The pocket is located at the entrance of the narrow exit channel for the acetate by-product discussed above. The cavity is occupied by a proline residue in class IIa structures (P667 in HDAC7), due to different protein backbone conformation. The protein backbone is conserved between HDAC2 and HDAC8, but in the latter, the side chain of Trp 141 partially obstructs the foot pocket. The corresponding residue is a leucine in HDAC1, 2, and 3 (Fig. 1), suggesting that this foot pocket is conserved in some isoforms. The selectivity profile of compounds occupying this cavity remains to be documented.

The crystal structure of HDAC7 in complex with inhibitors also revealed the presence of a foot pocket with a geometry distinct from that observed in HDAC2 (Schuetz et al. 2008) (Fig. 7h). This time, the pocket is generated by the conformation of the class IIa-specific His 843 that is flipped away from the active site. In class I enzymes, this cavity is occupied by the catalytic tyrosine that forms a hydrogen bond with the substrate or the chelating group of inhibitors. This pocket is expected to be conserved in class IIa enzymes, even though it was not observed in available wild-type HDAC4 structures due to the large opening of the lysine channel induced by the cocrystallized inhibitors.

Limited protein availability and lack of adequate substrates have seriously challenged efforts to characterize and rationalize the selectivity profile of HDAC inhibitors systematically. Recent progress in assay development (Bradner et al. 2010), and novel structural data are now crystallizing into a formalized understanding of HDAC inhibition, which should accelerate the development of compounds with diverse selectivity profiles, help dissect the cellular biology of protein deacetylation, and contribute to the discovery of better targeted clinical candidates.

5 Conclusion

The structural mechanism of classes I and IIa HDACs is now well understood and relies on interconnected elements involved in substrate recognition and catalysis. The chemistry of competitive inhibitors articulates around a well-established conceptual framework derived from available complex structures. This understanding will allow the development of class- and isoform-specific compounds with novel chemotypes and should result in a chemogenomic coverage of metal-dependent HDACs with clinical impact.

The biological reality that HDACs generally operate within large protein complexes remains, however, structurally unexplored. What are the conformational rearrangements induced by diverse functional binding partners, and what are their consequences on enzymatic activity? What are the contributions of structural elements outside the catalytic domain to substrate recognition and HDAC biology? Are class IIa HDACs activated within specific multiprotein complexes? Or are they, as bromodomains, simply readers of acetyl marks, or sensors of other posttranslational modifications? Are novel allosteric sites present within multisubunit complexes, and are these druggable? The structural biology of metal-dependent HDACs remains in many ways a mystery.

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Sirtuin Modulators

Sumit S. Mahajan, Vid Leko, Julian A. Simon, and Antonio Bedalov

Contents

Abstract Members of the sirtuin family including the founding protein Sir2 in *Saccharomyces cerevisiae* have been linked to lifespan extension in simple organisms. This finding prompted evaluation of the role of Sir2 orthologues in many aging-associated conditions including neurodegeneration, type II diabetes and cancer. These studies have demonstrated that genetic and pharmacologic manipulation of sirtuin activity have beneficial effects in a surprisingly broad spectrum of aging-associated conditions suggesting that the Sir2-family of enzymes presents an attractive target for the development of pharmacological agents. While the initial model favored pharmacological activators of sirtuins as calorie restriction mimetics, it now appears that either activation or inhibition of sirtuins may be desirable for ameliorating disease depending on the pathological condition and the target tissue. In this chapter we review the development of pharmacological small molecule activators and inhibitors of the sirtuin family of enzymes.

Keywords Sirtuins • NAD • Sirtuin inhibitors

Extension of human life span through direct intervention has been a goal since the ancient Greeks and probably longer. The explorer Ponce de Leon was just one of

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many seekers for the magical formula that would extend human life. The latest chapter in this saga is based on the observation that calorie restriction promotes longevity in organisms ranging from yeast to primates. This finding raises the expectation that molecular mechanisms mediating life span extension may also be shared between species. Pharmacological modulation of these mechanisms could potentially yield a fountain of youth. In *Saccharomyces cerevisiae*, Sir2, the founding member of the sirtuin family of nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases, has been proposed to be a link between metabolism, epigenetic silencing, genome stability and lifespan control (Guarente 2000). In yeast, the unequal division of "mother" and "daughter" cells makes it amenable to analysis of replicative lifespan (RLS), the number of daughter cells that a mother cell can produce before it dies (Mortimer and Johnston 1959). One of the events that limits mothers' lifespan is accumulation of rDNA circles that are excised during recombination at the rDNA locus and are preferentially retained in mother cells during cell division (Sinclair and Guarente 1997). Although the exact mechanism by which accumulation of rDNA circles decreases lifespan is still an active area of research, the inverse correlation between the amount of rDNA circles and longevity has fueled the search for regulators of rDNA recombination. Increased SIR2 dosage was found to extend the lifespan of mother cells by reducing recombination at the rDNA locus (Sinclair and Guarente 1997). This observation led to studies in multicellular eukaryotes such as nematodes (Tissenbaum and Guarente 2001) and fruit flies (Rogina and Helfand 2004), which showed that increased gene dosage of the Sir2 orthologues also extends lifespan. Because nematodes and flies do not show accumulation of rDNA circles during aging, and yet Sir2 orthologues promote longevity in these organisms, it has been proposed that the longevity control role of sirtuins has been retained during evolution despite differences in the specific degenerative processes that occur during aging in different species. Since both calorie restriction and increased dosage of Sir2 extend lifespan in the different species, a hypothesis was put forward that the beneficial effects of calorie restriction during aging are mediated through a conserved molecular pathway that involves calorie-restriction-induced increase in sirtuin activity (Bordone and Guarente 2005). Despite ongoing controversy whether calorie-restriction mediated lifespan extension requires Sir2 (Kaeberlein et al. 2004), this hypothesis generated a high level of enthusiasm for studies from many laboratories that evaluated the role of Sir2 orthologues in various aspects of aging (e.g., neurodegeneration, type II diabetes) in metazoan organisms including mice. Genetic and pharmacologic manipulations of sirtuin activity have shown beneficial effects in a surprisingly broad spectrum of aging-associated conditions and diseases suggesting that the Sir2-family of enzymes presents an attractive pharmacological target.

Over the past 10 years, studies in mice have shown that either activation or inhibition of sirtuin activity, depending on the pathological state and the tissue, may be desirable for ameliorating disease state (Imai and Guarente 2010). Accordingly, efforts have been undertaken to identify compounds that can either activate or inhibit specific sirtuins and serve as leads for the development of human therapeutics. Most of these efforts have been focused on developing modulators of major

nuclear sirtuin, SIRT1, however as our understanding of the cellular roles of other sirtuins expands, other sirtuins are likely to be targeted as well in the future.

1 Endogenous Modulators of Sirtuin Activity

Acetylation of lysine residues in proteins by acetyltransferases and deacetylation by deacetylases serves a regulatory function analogous to the way kinases and phosphatases modulate the ionic charge of serine, threonine and tyrosine residues and as a result regulate protein/protein interactions or enzyme function. Acetylation and deacetylation of lysines play similar roles as an ionic switch that regulates protein function. The molecular mechanism of sirtuin activity is well understood. During the sirtuin-mediated deacetylation reaction, cleavage of the glycosidic bond between nicotinamide and ribose of NAD+ is coupled to transfer of an acetyl group from the acetylated lysine residue in the target protein to the ribose moiety of ADP-ribose. The reaction yields deacetylated lysine, *O*-acetyl-ADP-ribose and nicotinamide as products (Tanner et al. 2000). Cellular sirtuin activity can be modulated by enzyme abundance, availability of NAD+ as well as the local presence of the nicotinamide, a deacetylation byproduct that has been shown to inhibit sirtuin activity. Furthermore, SIRT1 has been found in different complexes with other proteins which can either directly activate (Kim et al. 2007) or inhibit (Zhao et al. 2008) SIRT1 activity.

The concentration of cellular NAD+ is maintained by balancing NAD+ biosynthesis with cellular NAD+ utilization. In humans, the dietary sources used for synthesis of NAD+ include tryptophan, nicotinic acid, nicotinamide and a newly discovered precursor, nicotinamide ribose [reviewed in Bogan and Brenner (2008)]. De novo NAD+ biosynthesis from tryptophan through the kynurenin pathway requires eight enzymatic steps that are highly conserved in evolution. In yeast, expression of the enzymes in the kynurenine pathway is controlled by a sirtuin, Hst1, which functions as a sensor for cellular NAD+ level that represses the expression of de novo biosynthesis enzymes according to the availability of NAD+ (Bedalov et al. 2003). SIRT1-dependent control of the genes in a salvage pathway has been recently shown in mammals (Ramsey et al. 2009; Nakahata et al. 2009) (see below). Besides de novo biosynthesis, NAD+ can be synthesized from its breakdown product, nicotinamide, through a salvage pathway. The biggest consumers of cellular NAD+ are mono-ADP ribose and poly-ADP ribose transferases. These enzymes cleave the glycosidic bond in NAD+ and transfer or polymerize ADP onto other proteins. DNA double strand breaks activate poly-ADP ribose polymerase (PARP) and may result in catastrophic depletion of cellular NAD+ (Ha and Snyder 1999). In addition to regenerating NAD+, the salvage pathway also reutilizes and thus removes the deacetylase inhibitor, nicotinamide. Both of these outcomes can promote sirtuin activity (Anderson et al. 2002, 2003; Revollo et al. 2004). The key enzyme in the NAD salvage pathway is nicotinamide phosphoribosyl transferase (NAMPT) whose activity has been shown to modulate NAD+

levels and SIRT1 cellular activity (Anderson et al. 2002, 2003; Revollo et al. 2004). The expression of NAMPT was recently shown to be a target of the circadian transcription factors that induce diurnal oscillations in NAD+ levels and thus sirtuin activity (Ramsey et al. 2009; Nakahata et al. 2009).

Besides reactions that consume NAD+ such as those involving PARPs and sirtuins, NAD+ acts a cofactor for hydrogen transfer enzymes and results in interconversion of NAD+ and NADH. The redox state in cells and concomitant alterations of NAD+/NADH ratio have been implicated in the regulation of cellular sirtuin activity and control of several processes including calorie-restriction-induced life span extension in yeast (Lin et al. 2004), muscle differentiation (Fulco et al. 2003) and neurogenesis (Prozorovski et al. 2008). The NAD+/NADH ratio has been proposed to influence sirtuin activity in two ways. A decreased NAD+/NADH ratio may result from a decreased amount of NAD+, a sirtuin activator, or conversely from an increase in cellular NADH, a sirtuin inhibitor. Although this model is appealing for a variety of reasons, a detailed characterization of coenzyme specificity of Sir2-proteins failed to generate biochemical support for the role of cellular NAD+/NADH alterations as cellular regulators of sirtuin activity (Schmidt et al. 2004). Two key observations undermine this hypothesis. First, given that only a fraction of total cellular NAD+ and NADH pool is in the reduced state (NADH) (i.e., NAD+/NADH ratio is estimated to be very high) even if the total pool were converted to NAD+, the result would be only a minor increase in the available NAD+. Second, NADH has been shown to be extremely inefficient sirtuin inhibitor (IC_{50} 15 mM) (Schmidt et al. 2004). Because cellular NADH levels are at least two orders of magnitude lower than the measured IC_{50} of NADH it is unlikely that cellular NADH level can increase sufficiently to have a significant direct influence on sirtuin enzymatic activity.

While the alterations in NAD+/NADH ratio are unlikely to directly influence sirtuin enzymatic activity, there is evidence that cellular redox state regulates SIRT1 protein level at the transcriptional and posttranscriptional level. Cellular NADH levels and thus NAD+/NADH ratio have been proposed to control SIRT1 transcription though a regulatory circuit that involves redox sensor CtBP and HIC1, an inhibitor of SIRT1 transcription (Zhang et al. 2007). Furthermore, a separate study suggested that glucose deprivation and cellular pyruvate control SIRT1 protein even though the level of SIRT1 mRNA does not change (Rodgers et al. 2005). These studies suggest that overall cellular SIRT1 activity may be influenced by NAD+/NADH ratio through alterations of SIRT1 protein level rather than through direct control of SIRT1 enzymatic activity.

1.1 Sirtuin Inhibitors

Since the discovery of sirtuin's enzymatic activity 10 years ago several compounds that inhibit this class of enzymes have been described. Both whole cells and biochemical screens have been employed for identification of these inhibitors.

More recently, crystal structures of human sirtuins and homology models have allowed for structure-based design of more potent and selective sirtuin inhibitors. Among the seven human homologs, SIRT1 and SIRT2 have been exploited the most for drug discovery due to recognition of their therapeutic potential in diabetes and in neurodegenerative diseases. The inhibitors can be classified into categories based on their pharmacophore.

1.1.1 β-Napthols

The β-naphtol nucleus is a key group for several sirtuin inhibitors. Both sirtinol (**1**) and splitomicin (**2**) (Bedalov et al. 2001; Grozinger et al. 2001) (Fig. 1) were identified through cell-based screens in yeast *S. cerevisiae* for compounds that abrogate telomeric silencing (see Fig. 1). We observed limited activity of sirtinol in vivo against SIRT1 and SIRT2 enzymes judged by acetylation of the known SIRT1 and SIRT2 cellular targets. Structure activity relationship (SAR) studies on sirtinol resulted in improved analogs such as salermide (**3**), which has been shown to induce apoptosis in cancer cells (Lara et al. 2009).

The lactone in splitomicin was found to be essential for activity but at the same time conferred instability at physiological pH (Posakony et al. 2004). Replacement of the lactone with a lactam resulted in analogs with improved pH stability but decrease efficacy. Further studies on splitomicin led to identification of β-phenylsplitomicins

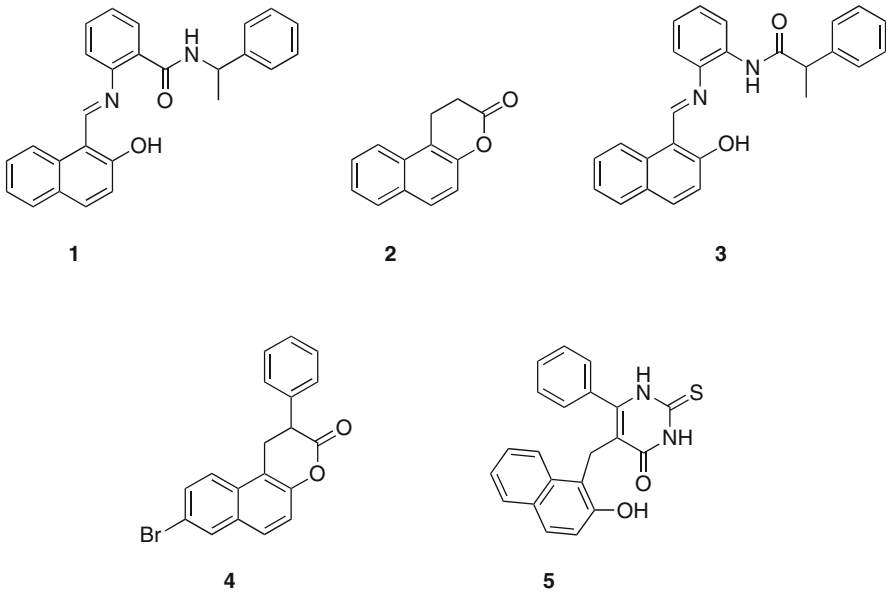


Fig. 1 Sirtuin inhibitors with β-napthol pharmacophore: **1**. Sirtinol, **2**. Splitomicin, **3**. Salermide, **4**. β-phenylsplitomicin, **5**. Cambinol

(**4**) with low micromolar inhibition against SIRT1 (Neugebauer et al. 2008). Phenyl splitomicins with substitutions on the 8-position of naphthalene ring were found to have increased selectivity for SIRT2 over SIRT1. Point mutations within the small helical domain close to substrate-binding site resulted in loss of splitomicin Sir2 inhibitory activity in cell-based assays identifying a potential binding pocket close to the substrate-binding site (Bedalov et al. 2001; Hirao et al. 2003).

Cambinol (**5**), a β-naphthol derivative with a substituted thiouracil ring, represents the most promising sirtuin inhibitor in this class of compounds. It is a nonselective SIRT1 (56 μM) and SIRT2 (59 μM) inhibitor but shows no inhibitory activity against other human sirtuins and HDACs. In contrast to sirtinol and splitomicin, cambinol is stable and highly effective in vivo. It has shown to induce hyperacetylation of SIRT1 and SIRT2 substrates such as p53, BCL6, α-tubulin in cells and inhibited growth of lymphoma xenograft in mice (Heltweg et al. 2006). Several analogs of cambinol have been developed with low micromolar activity and improved selectivity for SIRT1 over SIRT2 (Medda et al. 2009). Cambinol shows competitive inhibition with the acetyl-peptide, suggesting that it binds close to the substrate-binding site similar to splitomicin. The fact that β-naphthol class of compounds bind to a site other than the NAD+ binding site make them potentially less toxic due to off-target interactions.

1.1.2 Indoles

Similar to the β-naphthol scaffold, the indole ring has been exploited in the development of potent sirtuin inhibitors. The most notable in this class is EX-527 (**6**) (Fig. 2), which was identified by high throughput screening (Napper et al. 2005). The compound was initially reported to inhibit SIRT1 in the low nanomolar range (60–100 nM); however, higher IC_{50} values have been reported depending on the assay used for analysis. The compound has been proven to be highly useful in understanding the role of SIRT1 in cell survival and its interaction with p53. Docking studies and nicotinamide release-based assays suggest that indoles such as EX-527 bind to NAD+ binding site unlike the β-napthols (Neugebauer et al. 2008). A distinct set of inhibitors containing an indole nucleus but potentially binding to adenine part of the ATP binding pocket have been identified. These compounds, referred as bis(indolyl)maleimides (BIMs, **7**) resemble kinase inhibitors and are selective SIRT2 inhibitors in the low micromolar range (e.g., Ro31-8220) (Trapp et al. 2006). Recently, additional compounds that resemble ATP-competitive kinase inhibitors have been identified. An interesting compound belonging to this indole class is the oxyindole (**8**) which is a hybrid consisting of structural features of a kinase inhibitor and an antiproliferative natural product bauerine C. The compound has good selectivity for SIRT2 in vitro and was shown to inhibit deacetylation of α-tubulin in MCF-7 cells (Huber et al. 2010).

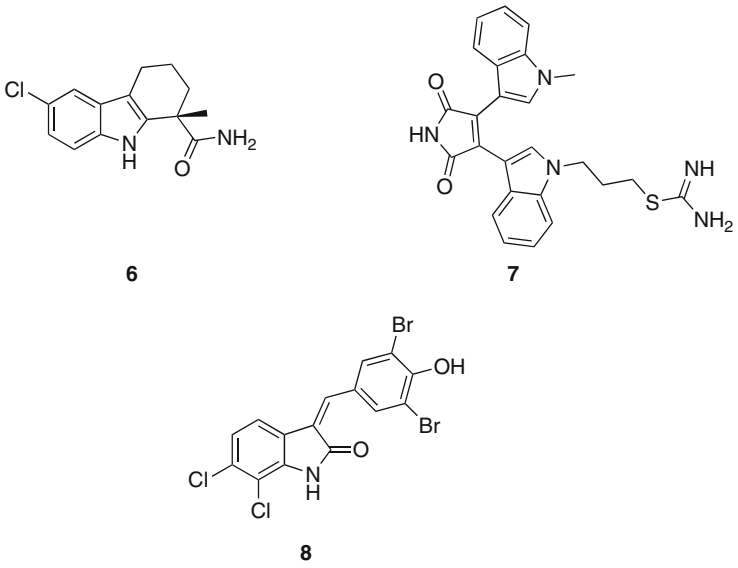


Fig. 2 Sirtuin inhibitors based on indole: **6**. EX-527, **7**. Bis(indolyl)maleimides, Ro31-8220, **8**. Oxyindole

1.1.3 Ureas and Thioureas

The compounds in this class were primarily identified during screening for different targets and disease states. Subsequent studies revealed that they exhibit sirtuin inhibition. One such compound is suramin (**9**) (Fig. 3), a polyanionic urea derivative, which is used in treatment of trypanosomiasis and has antiviral and anticancer activity (Voogd et al. 1993). It was later found to be a potent sirtuin inhibitor with IC_{50} of 297 nM and 1,150 nM for SIRT1 and SIRT2, respectively (Trapp et al. 2007). Synthesis of suramin analogs has resulted in more selective SIRT1 inhibitors. The compounds show noncompetitive inhibition with both NAD+ and acetylated peptide substrate suggesting that their binding site spans these two sites. Binding mode analysis based on suramin cocrystallized with SIRT5 and docking studies using SIRT2 model also suggest that the suramin binding site exist between NAD+ and the peptide substrate (Schuetz et al. 2007). Similar to suramin-related compounds, the thiourea-based tenovins (**10**) were initially identified in a cell-based screen for p53 activators (Lain et al. 2008). Further studies revealed that tenovins are low micromolar inhibitors of SIRT1 and SIRT2. Their high hydrophobicity precluded any in vivo use even though they showed decreased tumor growth in all the major tumor cell lines. Tenovin-6 a more water soluble analog has shown to be effective in reducing tumor growth in mouse model of melanoma thereby showing promise for this class of compounds.

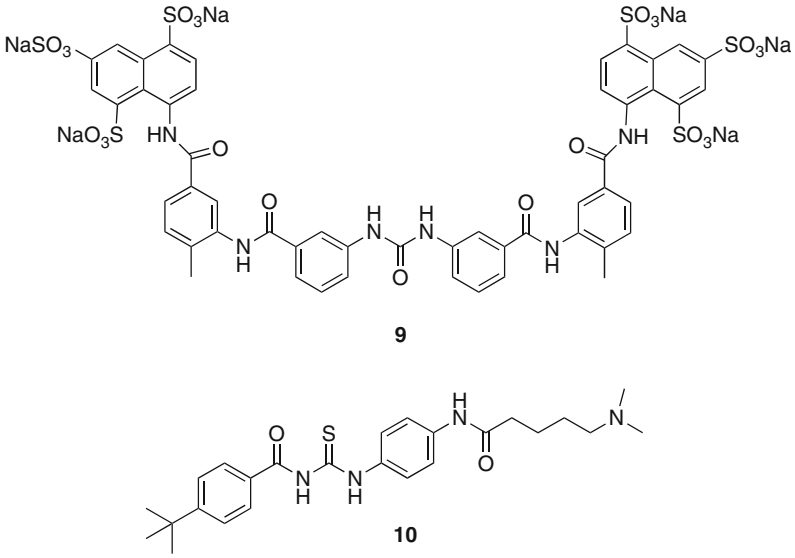


Fig. 3 Sirtuin inhibitors based on urea and thiourea moiety: **9**. Suramin, **10**. Tenovin

1.1.4 Miscellaneous

The inhibitors discussed here do not fall into a particular structural class but hold promise as additional, potent sirtuin inhibitors. The 1,4 dihydropyrdines (**11**) (Fig. 4) are interesting as a slight variation in substituent pattern results in sirtuin activation rather than inhibition (Mai et al. 2009). Compound (**12**) is novel as it is a mechanism-based inhibitor of sirtuin. The enolate at the lysine mimetic group attacks NAD+ at the active site of sirtuins forming a stable inhibitor-ADP-ribose conjugate that inhibits the enzyme in low micromolar range (Asaba et al. 2009). Tanikolide dimers (**13**) and AC-93253 (**14**) are selective and potent SIRT2 inhibitors (3–6 μM) (Gutierrez et al. 2009; Zhang et al. 2009). Polyphenols such as the chalcone (**15**) have also shown to inhibit sirtuins both in vitro and in vivo (Kahyo et al. 2008).

It should be noted that the in vitro IC_{50} reported for some of the inhibitors discussed above was measured solely using assays that employ fluorophore-containing substrates that do not always faithfully reproduce deacetylation of the native substrates (Kaeberlein et al. 2005; Borra et al. 2005). While the discrepancy between deacetylation of native and fluorophore-containing substrates has been particularly evident for putative sirtuin activators (see below), it is not clear yet if this is a problem for sirtuin inhibitors. It has therefore become imperative to confirm the IC_{50} using multiple methods such as the radio-labeled, HPLC or mass spectrometry assays and followed by validation in cell-based assays using acetyl p53 (for SIRT1) or tubulin acetylation (for SIRT2) as a readout. A major drawback is that these alternative methods have limited high throughput capabilities and has therefore thwarted their implementation routinely.

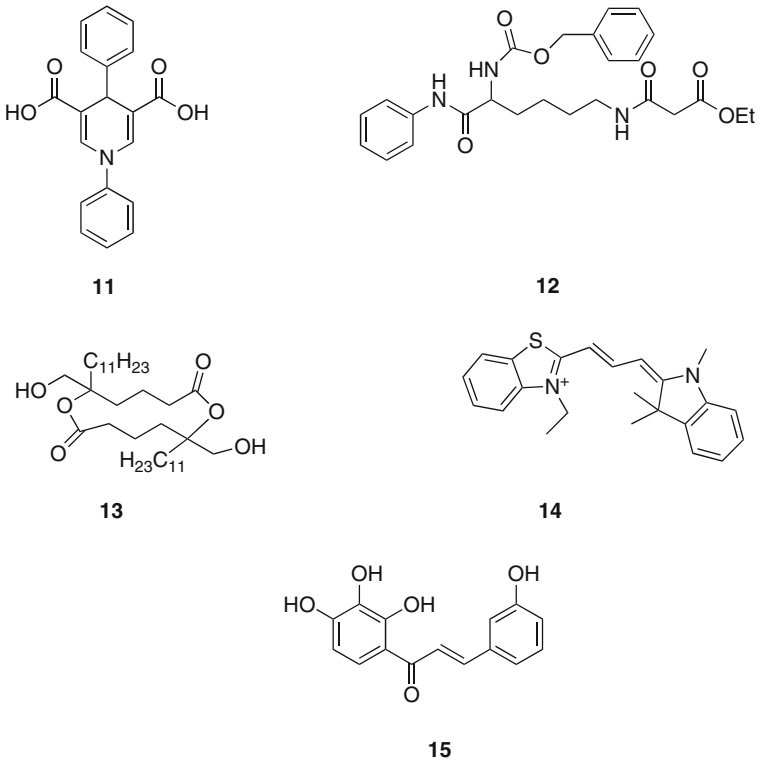


Fig. 4 Miscellaneous Sirtuin inhibitors: **11**. Dihydropyridine, **12**. Lysine-mimetic mechanism-based inhibitor, **13**. Tankolide dimer, **14**. AC-93253, **15**. Chalcone

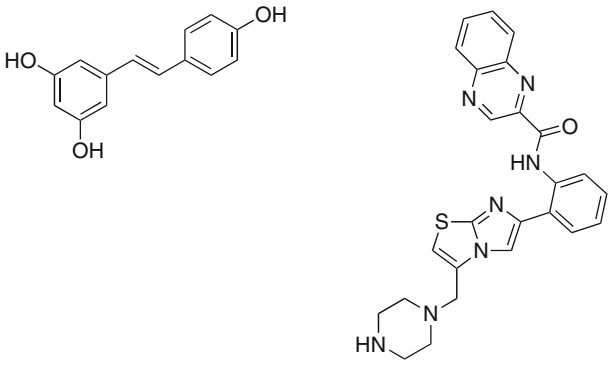
Despite the progress there is still a great need to develop both more potent nonselective as well a selective sirtuin inhibitors. While different sirtuins and the members of class I and II deacetylases (HDACs) have distinct substrates that define their cellular roles, the division of labor among these enzymes is not absolute. Several acetylated proteins have been found to be deacetylated by more than one member within a family or by the enzymes belonging to different families. For example, lysine 16 of the histone H4 has been shown to be deacetylated both with SIRT1 and SIRT2 (Vaquero et al. 2004, 2006), whereas tubulin is deacetylated by both SIRT2 and HDAC6 (Hubbert et al. 2002; North et al. 2003). The functional and substrate overlap among different deacetylases has important implications in designing and using small molecule inhibitors of these enzymes for therapeutic purposes and for dissecting biology as the inhibitors that are highly specific may not fully ablate specific cellular roles of a given deacetylase. Therefore, less selective inhibitors may be advantageous for altering cellular function and bringing therapeutic benefit. By the same token, cancer therapies may involve the combination of inhibitors of different deacetylase classes (e.g., sirtuin and HDACs). In contrast, highly selective inhibitors may be advantageous over nonselective ones as their side

effects are expected to be lessened by the relatively unperturbed function of the backup systems. Therefore, it will be important to develop both highly selective as well broadly active inhibitors for evaluating sirtuins as therapeutic targets.

1.2 Sirtuin Activators

First activators of SIRT1 were identified by high throughput biochemical screening using a commercially available fluorescent deacetylation assay by Howitz et al. (2003). The most potent activator identified from this screen was resveratrol (**16**) (Fig. 5), a polyphenol commonly found in red wine and associated with beneficial effects of wine in general. Resveratrol was shown to decrease the K_m of the peptide substrate and also NAD+ without any significant effect on V_{max} suggesting that it is an allosteric effector of SIRT1. In the initial report resveratrol was also shown to increase yeast replicative life span in a Sir2-dependent fashion. This work resulted in tremendous excitement and led to many studies that evaluated a resveratrol as an antiaging drug and calorie-restriction mimetic (Wood et al. 2004; Lagouge et al. 2006; Baur et al. 2006).

Two independent reports in 2005 raised the possibility that the in vitro effects of resveratrol on SIRT1-catalyzed deacetylation may be related to the substrate that was used in the initial screen fluorophore (Kaeberlein et al. 2005; Borra et al. 2005). The screen that led to identification of activators employed as a substrate peptide containing acetyl-lysine and a coumarin fluorophore (Howitz et al. 2003). SIRT1 deacetylation of the peptide enabled trypsin to cleave the peptide releasing the fluorophore, which was then capable of light emission. The two reports demonstrated that this substrate peptide has significantly lower affinity for SIRT1 compared to the same peptide without a fluorophore (Kaeberlein et al. 2005; Borra et al. 2005). Resveratrol specifically increases the affinity and deacetylation of the



16 **17**

Fig. 5 Sirtuin activators: **16**. Resveratrol, **17**. SRT1720

fluorophore-containing peptide while having no influence on the same peptide without the fluorophore. Furthermore, increased deacetylation of the fluorophore-containing substrate appeared to be specific to SIRT1 as neither human SIRT2 nor yeast Sir2 was activated by resveratrol even when the fluorophore-containing peptides was used. Consistent with the lack of effect on yeast Sir2 [regardless of the presence or absence of fluorophore and in contrast to the initial report (Howitz et al. 2003), in a study by Kaeberlein et al. (2005)], resveratrol did not extend yeast RLS. These findings raised the possibility that the activation of SIRT1 by resveratrol may be an artifact of the biochemical screen used for their identification.

In 2007, synthetic compounds structurally unrelated to resveratrol were also reported to activate SIRT1 (e.g., SRT1720, SRT2183) (Milne et al. 2007). These compounds were collectively named STACs for SIRT1 activating compounds. STACs were shown to activate SIRT1 at submicromolar concentrations and increase SIRT1 catalytic activity several hundredfold (Milne et al. 2007). Furthermore, these compounds were orally bioavailable and were reported to lead to improvement of several metabolic parameters in mice fed a high fat diet and other diabetes model systems. Based on these encouraging results, human clinical trials with several synthetic activators as well as with reseveratrol had been initiated. Similar to resveratrol, these compounds were proposed to act as allosteric enzyme activators by increasing binding of the peptide substrates and mapping studies using truncated forms of enzyme revealed that small helical domain of SIRT1 was required for their activity.

However, because STACs were also identified using a fluorophore-containing substrate via fluorescence polarization assay and characterized by mass spectrometry with the same substrate, there was a possibility that these compounds may suffer from the same shortcomings as resveratrol. Indeed, in 2010 a detailed characterization of these compounds using biochemical assays was published by Pacholec et al. (2010), and revealed that apparent activation of SIRT1 enzymatic activity was completely dependent on the presence of the fluorophore in the substrate. While extremely effective in deacetylating acetyl-TAMRA-p53 peptide, SRT1720 (**17**) and related compounds failed to activate deacetylation of any substrates that did not contain a fluorophore including native acetylated p53 peptide, full length acetylated p53 protein or acetyl-CoA synthetase 1. In the same report, the authors went on to demonstrate, using NMR and Surface Plasmon Resonance, that SRT1720 binds directly to TAMRA peptide (but not to native peptides) and that binding occurs in the absence of SIRT1. These findings argue against a model that putative SIRT1 activators bind directly to SIRT1 and act as allosteric activators that are specific for deacetylation of fluorophore-containing substrates (an increase in deacetylation of native substrates has never been demonstrated). Rather, the most plausible model, for which the study by Pacholec et al. provides solid experimental support, is that STATs bind to the substrate in a fluorophore-dependent fashion, which promotes substrate deacetylation. The fluorophore-dependent binding of the STATs to the substrate provides a more straightforward explanation for the substrate selectivity than the allostery model in which binding of the putative activators would specifically promote deacetylation of the fluorophore but not the native substrates.

Regardless of the model, it appears that apparent SIRT1 activation by small molecule ligands including resveratrol is a screening artifact.

If resveratrol and STATs do not affect sirtuin activity, what accounts for the reported in vivo effects of these agents and resveratrol? *First*, as discussed above, the initial in vivo result that resveratrol extends yeast lifespan has been called into question (Kaeberlein et al. 2005). Likewise, the reexamination of STATs activity in mouse diabetes models by Pacholec et al. did not reproduce glucose-lowering activity or improvements in mitochondrial capacity (Pacholec et al. 2010) that were shown in the initial report (Milne et al. 2007). At the present time, the reasons for the discrepancy for the in vivo results are not clear. However, it is an important issue that will need to be resolved, particularly in light of clinical trials that have been initiated with this class of compounds. *Second*, resveratrol has many other proposed cellular targets that could account for the various metabolic benefits observed with this compound (Harikumar and Aggarwal 2008). While it has been proposed that some of these effects (e.g., stimulation of AMP-activated protein kinase) could indirectly promote sirtuin activity (Feige et al. 2008), the multitude of cellular targets makes it difficult to determine which of these is mediating the desired biological effects.

Beside resveratrol and other compounds that were thought to activate SIRT1 by promoting peptide binding, a different mechanism has been described for isonicotinamide (**11**), which was shown to activate yeast Sir2 through relief of nicotinamide inhibition (Sauve et al. 2005). As discussed previously nicotinamide is an endogenous sirtuin inhibitor that promotes the chemical reversal of the covalent reaction intermediates and generation of NAD+ and acetyl-lysine resulting in nicotinamide exchange. Isonicotinamide is competitive with nicotinamide in the exchange reaction thus promoting deacetylation both in vitro and in vivo.

The multitude of the protein targets and physiological roles that SIRT1 has in different tissues as well as complex regulation of SIRT1 protein level by nutritional cues makes it difficult to predict the net benefit of promoting or inhibiting SIRT1 activity on metabolism in diabetes. It should be noted, however, that, while extremely valuable, rodent models have only limited value in prediction of the utility of drug targets in metabolic diseases in humans. The role of SIRT1 inhibitors and activators in the treatment of diabetes and obesity associated metabolic syndrome will ultimately be decided through clinical trials.

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