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SIRTUINS IN BIOLOGY AND DISEASE

Topic Editors
Tiago F. Outeiro and Aleksey G. Kazantsev

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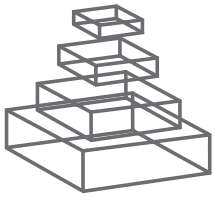

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SIRTUINS IN BIOLOGY AND DISEASE

Topic Editors:
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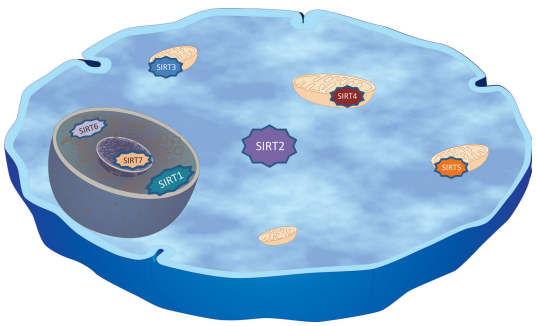

Cellular distribution of mammalian sirtuins. See de Oliveira et al. (2012). SIRT2 as a therapeutic target for age-related disorders. *Front. Pharmacol.* 3:82. doi: 10.3389/fphar.2012.00082 for further details.

Sirtuins comprise a family of NAD+-dependent enzymes that have been shown to impact longevity in a number of eukaryotic organisms. Sir2 (Silent Information Regulator 2) was the first sirtuin protein discovered. The discovery that Sir2 requires NAD+ for its activity suggested a link between Sir2 activity and the phenomenon of caloric restriction in prolonging longevity. This link was strengthened by the observation that lifespan extension by caloric restriction requires Sir2 protein. Under conditions of caloric restriction, NAD+ levels are high, Sir2 is activated, and the rate of aging is decreased. These effects have been replicated in invertebrate organisms, where a close structural and functional homologue of Sir2 was found in C. elegans and Drosophila. The sirtuin-dependent effects on metabolism and ageing, observed in lower organisms, have ignited intensive investigation of their biological and therapeutic roles in mammals.

There are seven known mammalian sirtuins, SIRTs 1-7, the most studied of which is SIRT1, a close structural and functional homologue of yeast Sir2. Enhancement of organismal longevity and other health-promoting effects of mammalian SIRT1 have frequently been attributed to the regulation of metabolism. A recognized molecular link between metabolism and aging stimulated a firestorm of investigations, aiming to combat metabolic and age-dependent human diseases. It has become clear, however, that the sirtuin family of proteins regulates a diverse repertoire of cellular functions in mammals. Mounting evidence implicating SIRT1 in important clinical indications, such as diabetes, cancer, cardiovascular dysfunction and neurodegenerative disease, suggest that modality as attractive therapeutic

target. Subsequently, drug discovery and development, targeting sirtuin activation, has been intensified in the recent years.

Despite rapid progress and accumulation of new data, the biological roles of other mammalian sirtuins have been less studied and remain poorly understood. There are several important questions that remain to be addressed. What are the functions of sirtuins in different cell types and tissues? Are all sirtuins involved in the regulation of metabolism and aging? What is the functional relationship between different sirtuins? What are the mechanisms of regulation of sirtuin activities? What is the role of sirtuins in disease and therapy? This issue aims to address these and other critical questions, relevant to Research Topic on sirtuin biology and therapeutics. To that end the issue solicits expert opinions of sirtuin research on structural biology, biochemistry, cell biology, animal genetics, pharmacology, medicinal chemistry and drug discovery, and on areas of investigation studying human conditions, like diabetes, cancer, cardio-vascular, and neutodegeneration. Of particular interest are the new methods and assays to study sirtuins in various organisms and developing sirtuin-based therapeutics. Furthermore, we propose to encourage contributors to discuss new concepts and paradigms, and to express their perspectives on the future development of the sirtuin research field.

Altogether, we believe this issue provides a unique opportunity for comprehensive and diverse coverage of the topic, and will be of broad interest for the journal's readership.

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EDITORIAL
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Editorial on special topic: sirtuins in metabolism, aging, and disease

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The sirtuin family of NAD^+-dependent enzymes has received much of attention in recent years due to their diverse physiological functions in metabolism, aging, and age-related human diseases. The mammalian sirtuins (SIRT1-7) act as NAD^+-dependent protein deacetylases and weak mono-ADP-ribosyl transferases on a variety of targets, including histones, transcription factors, and apoptotic modulators. The sirtuins appear to be the key sensors for available energy stores, which function as molecular switch between protein acetylation and metabolism. Furthermore, it has been shown in a broad range of experimental disease models, from yeast to mouse models, that modulation of sirtuin activities, particularly that of the most studied SIRT1 protein, suppresses or ameliorates pathological states, and thus sirtuins constitute attractive novel therapeutic targets for many age-related disorders, for most metabolic disorders such as diabetes and obesity.

The major goal of this Special Issue is to discuss and evaluate the current knowledge on sirtuin biology for a rational approach toward the development sirtuin-based therapeutics.

In the article "Sirtuins as regulators of the yeast metabolic network" the authors discuss a network complexity of yeast SIRT1 homolog. The archetypal sirtuin, yeast *silent information regulator2* (*SIR2*), is an NAD^+ dependent protein deacetylase that interacts with metabolic enzymes glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase, as well as enzymes involved in NAD(H) synthesis, that provide or deprive NAD^+ in its close proximity. This influences sirtuin activity, and facilitates a dynamic response of the metabolic network to changes in metabolism with effects on physiology and aging. The molecular network downstream Sir2, however, is complex, and tertiary interactions of Sir2, relevant to metabolic regulation, involve half of the yeast proteome.

The SIRT1 network is further discussed in the manuscript "Interactomic and pharmacological insights on human SIRT1." The article presents studies on human SIRT1, which illuminate functional relationships of gene–protein interactions, controlling major metabolic pathways. This article also shows the mechanism of binding of small molecule activators and discusses critical structural determinants and physico-chemical features important for rational design of SIRT1 effectors.

Rational drug design is also the topic of the article "Structures, substrates, and regulators of mammalian Sirtuins – opportunities and challenges for drug development." The authors recapitulate advances in structural and mechanistic studies on substrate recognition and deacetylation by sirtuins, which provides a basis for the identification and characterization of small molecule sirtuin activators and inhibitors as well as evaluation the molecular mechanisms regulating their activities.

The importance of posttranslational modifications for sirtuin activities is discussed in the article "Regulation of sirtuin function by posttranslational modifications." Here the authors review the posttranslational regulation mechanisms of mammalian sirtuins and discuss their relevance regarding the physiological processes. Based on available data they suggest that the N- and C-termini are the targets of posttranslational modifications, which in turn determines differential interaction with catalytic core domain. The authors emphasize the key regulatory roles of the N- and C-termini in controlling different signaling pathways.

The article "Role and therapeutic potential of the pro-longevity factor FOXO and its regulators in neurodegenerative disease" is focused on downstream sirtuin targets, critical for aging, and age-related neurodegeneration. The author discusses FOXO network of transcription factors, which is most notable for its association with longevity and modulation by upstream regulators such as sirtuins. He emphasizes that the FOXO network is a complex stress response system and thus development of disease-modifying strategies requires careful examination. C. Neri argues that although the FOXO network contains druggable genes such as sirtuins, whether they should be activated or inhibited and whether protection against the early or late phases of neuronal cell decline might require opposite therapeutic strategies remains unclear.

Next, the complexity of therapeutic targeting of sirtuins is discussed in the article "SIRT2 as a therapeutic target for age-related disorders." The authors discuss the second member of sirtuin family, SIRT2, its physiological role(s) in various cellular environments and tissue-specific functions. This article summarizes the main scientific advances on SIRT2 protein biology and explores its potential as a therapeutic target for treatment of age-related disorders.

In the article "Inhibition of sirtuin 2 with sulfobenzoic acid derivative AK1 is non-toxic and potentially neuroprotective in a mouse model of frontotemporal dementia" the authors provide experimental evidence on the efficacy of SIRT2 inhibitor in mouse brain. The rTg4510 mouse model, which expresses a mutant form of the tau protein associated with FTD with Parkinsonism, undergoes dramatic hippocampal and cortical neuronal loss. In present study, the SIRT2 inhibitor was directly delivered to the hippocampus with an osmotic minipump. The treatment was found to be safe in wild-type and transgenic mice and, furthermore, it provided neuroprotection in the rTg4510 hippocampal circuitry. This study provides proof-of-concept for therapeutic benefits of SIRT2 inhibitors in both tau-associated FTD and Alzheimer's disease.

An apparent link between sirtuin modalities and cancer has emerged and is discussed in the two articles, "Sirtuins and disease: the road ahead" and "Sirtuins, Metabolism, and Cancer." In the first article the authors discuss experimental evidences suggesting a critical role for SIRT1 in tumor initiation and progression as well as drug resistance by blocking senescence and apoptosis, and by promoting cell growth and angiogenesis. The present article highlights sirtuin mechanism(s) of action and deregulation in cancer, and focuses on the therapeutic potential of sirtuin modulators both in cancer prevention and treatment. In the second paper authors specifically review recent progress on the role of sirtuins in DNA repair and energy metabolism, and further address the sirtuin role(s) in the biology of cancer.

Overall, the present special issue "Sirtuins in Biology and Disease" provides an overview and insight on therapeutic potentials of sirtuin-based therapeutics and discusses the evident complexity of drug-targeting these modalities for human indications.

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Sirtuins and disease: the road ahead

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Sirtuins represent a promising new class of conserved histone deacetylases, originally identified in yeast. The activity of the sirtuin (SirT) family – made up of seven members (SirT1-7) – is NAD^+ dependent. Sirtuins target a wide range of cellular proteins in nucleus, cytoplasm, and mitochondria for post-translational modification by acetylation (SirT1, 2, 3, and 5) or ADP-ribosylation (SirT4 and 6). Sirtuins regulate responses to stress and ensure that damaged DNA is not propagated, thus contrasting the accumulation of mutations. To date, sirtuins have emerged as potential therapeutic targets for treatment of human pathologies such as metabolic, cardiovascular and neurodegenerative diseases, and cancer. SirT1 is the founding member of this class of enzymes and is currently the best known of the group. SirT1 acts in various cellular processes, deacetylating both chromatin and non-histone proteins, and its role in cancer and aging has been extensively studied. SirT1 may play a critical role in tumor initiation and progression as well as drug resistance by blocking senescence and apoptosis, and by promoting cell growth and angiogenesis. Recently, growing interest in sirtuin modulation has led to the discovery and characterization of small molecules able to modify sirtuin activity. The present review highlights SirT mechanism(s) of action and deregulation in cancer, focusing on the therapeutic potential of SirT modulators both in cancer prevention and treatment.

Keywords: sirtuins, SirT inhibitors, cancer, human diseases, epigenetics

INTRODUCTION

Epigenetic modifications play an important role in regulating gene expression, lifespan, and tumorigenesis. In particular, acetylation regulates a wide variety of cellular functions. Class III histone deacetylases (HDACs), also known as sirtuins, are the silent information regulator 2 (Sir2) family of proteins. Unlike other HDACs, sirtuins are NAD^+-dependent deacetylases, not modulated by inhibitors of class I, II, and IV HDACs. The deacetylase activity of sirtuins is controlled by the cellular $[NAD^+]/[NADH]$ ratio, where NAD^+ works as an activator, while nicotinamide and NADH act as inhibitors. Expressed from bacteria to humans (Vaquero, 2009), sirtuins seem to preferentially target non-histone proteins, although little is currently known about target specificity and selectivity. Two reactions may be catalyzed by sirtuins: deacetylation and ADP-ribosylation. In both reactions, the cleavage of NAD^+ is the initial chemical step. Sirtuins (SirT1, SirT2, SirT3, SirT5, and SirT7) catalyze a deacetylation reaction on lysine residues of target proteins, using NAD^+ as cofactor and releasing nicotinamide with the production of 2′-*O*-acetyl-ADP ribose. In contrast, SirT4 and SirT6 catalyze an ADP-ribosylation reaction, in which ADP-ribosyl moiety is transferred to the substrate (Yamamoto et al., 2007).

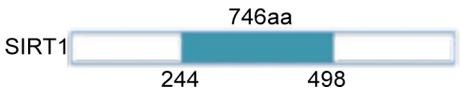
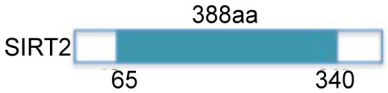
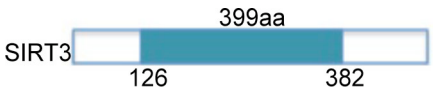
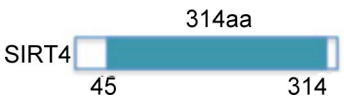
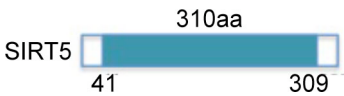
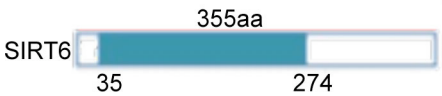
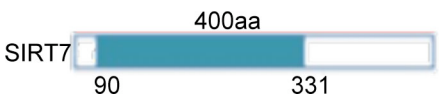
CATALYTIC DOMAIN OF SIRTUINS

Each member of the sirtuin family contains a highly conserved core domain consisting of an NAD^+ binding site and a catalytic domain (Alcaín and Villalba, 2009). Human SirT2 consists of two globular domains: one large and one small. In the large domain, with a Rossmann fold, six β-strands form a parallel β-sheet and six α-helices. The small domain is composed of a helical and a zinc-binding module. The active site is located at the interface between the large and small domains, with a binding site for NAD^+. The NAD^+-binding pocket can be divided into three spatially distinct regions: the A site, with affinity for adenine–ribose, the B site, with affinity for nicotinamide–ribose, and the C site, which binds NAD^+. In the presence of an acetyl-lysine substrate, the NAD^+-bound B site can undergo a conformational change, bringing nicotinamide in proximity to the C site, where it can be cleaved. The ADP ribose product returns to the B site, allowing deacetylation. The C site is the binding site for free nicotinamide. At high concentrations it may occupy the site and block the conformational change of NAD^+ (North and Verdin, 2004).

SIRTUIN FAMILY

The mammalian sirtuin family comprises seven proteins (SirT1-7). These proteins differ in cellular localization, activity, and function, and are subdivided into four classes (Alcaín and Villalba, 2009; **Table 1**). SirT1, the closest to yeast Sir2 in terms of sequence, is a proto-member of the family. SirT1 mediates heterochromatin formation through deacetylation of K26 on histone H1, K9 on histone H3, and K16 on histone H4. SirT1 is also involved in the deacetylation of non-histone proteins, which can be divided into three groups: (i) transcription factors (p53, FOXO, E2F1, BCL6, p73, Rb, and others); (ii) DNA repair proteins; (iii) signaling factors. Due to its ability to deacetylate a variety of substrates, SirT1 is involved in a broad range of physiological functions, including control of gene expression, metabolism, tumorigenesis, and aging.

Table 1 | Diversity of human sirtuins.

Classes		Enzymatic activity	Targets and Substrate	Localization	Function	Involvement in cancer
		Deacetylase	p53, FOXO, MyoD, Ku70, PPARγ NFκB, PCAF, H3K9, H3KI4,	Nuclear/ cytoplasmatic	Glucose metabolism, differentiation, neuroprotection, insulin secretion	Acute myeloid leukemia, colon, bladder, prostate, glioma, nonmalig-nant skin, ovarian
I		Deacetylase	α-Tubulin FOXO	Nuclear/ cytoplasmatic	Cell-cycle control, tubulin deacetylation	Glioma
		Deacetylase	GHD complex 1, AceCS2	Mitochondrial	ATP-production, regulation of mitochondrial proteins deacetylation, fatty-acid oxidation	Breast cancer
II		ADP-ribosyl-transferase	GHD, ANT	Mitochondrial	Insulin secretion	Breast cancer
III		Deacetylase	CPSI	Mitochondrial	Urea cycle	Pancreatic, breast cancer
IV		Deacetylase ADP-ribosyl-transferase	Hif1α, helicase NFκB, DNA polimerasi β	Nuclear	Telomeres and telomeric functions, DNA repair	Colon, breast cancer
		Deacetylase	RNA polymerase type 1, EIA SMAD6	Nuclear	RNA pol I transcription	Breast cancer

Human SirT2 co-localizes with microtubules in cytoplasm. Thus, knockdown of SirT2 results in tubulin hyperacetylation. SirT2 is also able to bind FOXO3a, reducing its acetylation levels.

SirT3 is localized in mitochondrial inner membrane, and is activated by caloric restriction. SirT4 is also mitochondrial, but no NAD^+-dependent deacetylase activity has so far been detected for this sirtuin. SirT4 uses NAD^+ for ADP-ribosylation of glutamate dehydrogenase, resulting in inhibition of insulin secretion and enzymatic activity of β-cells. The third mitochondrial sirtuin, SirT5, is localized in the inter-membrane space and can deacetylate cytochrome c. The last two sirtuins, SirT6 and SirT7, have recently been characterized. SirT6 exerts an NAD^+-dependent histone H3 K9 deacetylase action able to modulate telomeric chromatin functions (Alcaín and Villalba, 2009). SirT6 has a role in genome stability. In mammalian cells subjected to oxidative stress, SirT6 is recruited on DNA double-strand breaks (DSB) and stimulates repair through both homologous and non-homologous

end-joining recombination. Moreover, some results indicate that SirT6 is physically associated with poly [adenosine diphosphate (ADP)-ribose] polymerase 1 (PARP1). The amount of SirT6–PARP1 complexes increase after DNA damage. PARP1 is mono-ADP-ribosylated on lysine residue K521, thus increasing PARP1 activity and enhancing DSB repair under oxidative stress (Mao et al., 2011). SirT7 is a predominantly nucleolar protein associated with active rRNA genes (rDNA), where it interacts with RNA polymerase I and histones (Alcaín and Villalba, 2009). SirT7 does not seem to have any NAD^+-dependent activity. Other functional characteristics of SirT6 and SirT7 remain to be further elucidated.

Some sirtuins may relocalize depending on cell or tissue type, developmental stage, stress condition, and metabolic status, suggesting that their localization is important for regulating function (McGuinness et al., 2011).

SIRTUINS AND GENOME STABILITY

Some sirtuins (SirT1, 2, 3, 6) are linked to chromatin regulation, as they are responsible for the regulation of two histone post-translational modifications crucial for chromatin structure: acetylation of K16 and K9 on histone H4 and H3 respectively (McGuinness et al., 2011).

SirT1 is responsible for heterochromatin formation by a deacetylation process. Histone deacetylation can in turn facilitate histone methylation, thus enhancing global transcriptional repression. SirT1 has no effect on H3K4 methylation (a marker of open chromatin) and promotes H3K9 tri-methylation through its involvement with the histone methyltransferase Suv39H1 (Liu et al., 2009; Bosch-Presegue and Vaquero, 2011).

SirT2 and SirT3 are responsible for regulation of H4K16 acetylation. Deacetylation occurs specifically during cell-cycle in G2/M transition, in particular when SirT2 is shuttled to the nucleus (Shogren-Knaak et al., 2006). The mechanism of SirT3 is more complex. Although SirT3 is localized in mitochondria, its feeble nuclear expression is probably responsible for deacetylation of H4K16ac and H3K9ac in some settings (McGuinness et al., 2011). The finding that SirT3 knockdown does not lead to global H4K16 hyperacetylation suggests that its action occurs in limited regions of the genome.

Recently, SirT6 has been attributed with deacetylase activity (McGuinness et al., 2011), since it modulates acetylation of K9 and K56 on histone H3.

SIRTUINS AND CANCER

Interest in sirtuins has grown in the last decade, mainly because of their critical role in different biological processes, such as regulation of gene expression, control of metabolic processes, apoptosis and cell survival, DNA repair, development, neuro-protection, and inflammation. Sirtuins control many vital functions and are involved in several pathologies such as metabolic diseases, neurodegenerative disorders, and cancer.

SirT1 has been shown to be significantly up-regulated in different types of cancer including acute myeloid leukemia (AML), prostate, colon, and skin cancers.

Given that only SirT1 and SirT2 seem to control levels of H4K16 acetylation in many settings, and that only SirT1 is proven to be altered in certain cancers, H4K16ac loss in cancer has been correlated with SirT1 deregulated activity.

Nevertheless, SirT1 seems to play contradictory roles, both as tumor suppressor or tumor promoter (Deng, 2009; Bosch-Presegue and Vaquero, 2011). The initial evidence that SirT1 acts as tumor promoter derives from its repressive effect on tumor suppressor p53 (Deng, 2009). By interacting physically with p53, SirT1 blocks its functions through deacetylation at the C-terminal K382 residue (Vaziri et al., 2001). Moreover, SirT1 overexpression in cancer represses p53-dependent cell-cycle arrest and apoptosis in response to DNA damage and oxidative stress. DNA damage-induced acetylation of p53 leads to its activation. In response to damage, SirT1 binds to and deacetylates p53, thus reducing its functional and transcriptional activities. Overexpression of SirT1 disrupts p53-dependent pathways, resulting in a significant reduction in the cell's ability to respond to stress and DNA damage. In contrast, the inhibition of SirT1 potentiates p53-dependent apoptosis (Deng, 2009; Rahman and Islam, 2011). These findings underline the involvement of SirT1 in tumorigenesis, given that SirT1 overexpression may increase the risk of cancer in mammals by inhibiting p53 and potentially other tumor suppressor genes, including FOXO family members, p73, Rb, and several others.

In contrast, several studies have suggested that SirT1 also has a tumor suppressor role. Decreased SirT1 levels have been reported for glioma, bladder, prostate, and ovarian cancers. Furthermore, some studies have suggested that SirT1 overexpression in $APC^{-/+}$ mice reduces rather than increases colon cancer formation (Firestein et al., 2008). This action seems to be caused by SirT1 deacetylation of β-catenin, which promotes cytoplasmic localization of the nuclear-localized oncogenic form of β-catenin. In addition, in SirT1-deficient mice, embryos die at middle gestation stages, displaying increased acetylation of H3K9 and H4K16, reduced chromosome condensation and aberrant mitosis (Firestein et al., 2008; Deng, 2009). SirT1 deficiency also causes reduced DNA double-strand break repair and radiation sensitivity (Wang et al., 2008). It has also been suggested that SirT1 plays a role as tumor suppressor by regulating c-Myc. c-Myc binds to and induces SirT1. This interaction results in decreased c-Myc stability, compromising its transformational capability. These data provide strong evidence for a tumor suppressor role of SirT1 in these settings. Importantly, it still seems possible that the expression level and activity of SirT1 may modulate a delicate balance between suppression and promotion of oncogenesis, thus displaying dual dependence on its spatial and temporal distribution and stage of tumorigenesis (Bosch-Presegue and Vaquero, 2011). A potential tumor suppressor role has also been proposed for the other human sirtuins (McGuinness et al., 2011). This hypothesis is supported by several findings such as the reduction of SirT2 in a large number of human brain tumor cell lines, and its involvement in cell-cycle progression. SirT3 is the only mitochondrial sirtuin implicated in tumorigenesis. Its reduction in several cancers leads to an increase in ROS (reactive oxygen species) production, which results in enhanced tumor growth (Kim et al., 2010). SirT5 overexpression has been found in a study of pancreatic cancer (Ouaïssi et al., 2008). Recently, a role for SirT6 and SirT7 in tumorigenesis has also

been proposed. SirT6 might be involved as a result of its control of the NFκB pathway and DNA double-strand repair. SirT7, whose expression inversely correlates with the tumorigenic potential in several murine cell lines (Vakhrusheva et al., 2008), displays increased expression levels in breast cancer (Ashraf et al., 2006).

Following these rationals, tumors with deregulated expression or function of sirtuins may benefit from an approach based on the use of SirT inhibitors.

SIRTUIN AND AGING DISEASES

Sirtuins are believed to be involved in metabolic diseases, neurodegeneration, and aging.

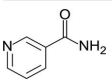
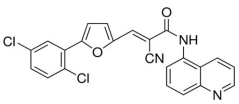
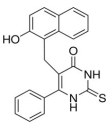
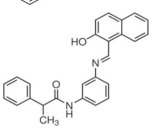
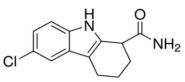
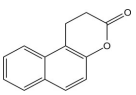
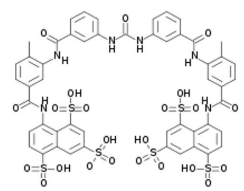
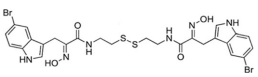
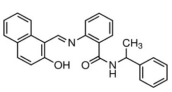
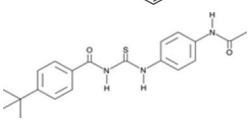
It is well known that overexpression of Sir2 (or its orthologs) can extend organism lifespan in a wide range of lower eukaryotes (Bosch-Presegue and Vaquero, 2011). Sir2 function is often correlated to caloric restriction. The link between the role of sirtuins, caloric restriction, and longevity was first described in *S. cerevisiae*. In yeast, caloric restriction leads to increased replicative lifespan. Lifespan extension has not been observed in yeast lacking Sir2 (Lin et al., 2000). Currently, the role of sirtuins in the regulation of mammalian lifespan is still unclear. However, considering that sirtuins are an evolutionary conserved family of proteins, it is fair to speculate on their role in the modulation of aging-related processes in higher organisms (Westphal et al., 2007; Brooks and How, 2009). In humans, the aging process is associated with telomere erosion. SirT1 and SirT6 have a role in both the maintenance of telomeres and their function, as well as the aging process. Recent studies have demonstrated that reduction or removal of SirT6 results in telomere dysfunction and end-to-end chromosomal fusions. Symptoms displayed in the absence of SirT6 are similar to those caused by a premature aging disease, known as Werner's syndrome. To date, little is known about other sirtuins, but no evidence suggests their involvement with telomere function, formation, and stability (Westphal et al., 2007; McGuinness et al., 2011). Recently, SirT1 has been shown to promote replicative senescence in response to chronic oxidative stress, by upregulation of the $p19^{ARF}$ senescence regulator, which in turn positively regulates p53 via inhibition of MDM2, a protein mediating p53 degradation (Chua et al., 2005). This effect seems to be in contrast to Sir2 function in yeast, which extends replicative lifespan.

Many studies have demonstrated that the pathogenesis of neurodegenerative diseases includes broad changes and involvement of multiple biochemical pathways. Sirtuins play a critical role in several models of neurodegenerative diseases. Many reports support the role of SirT1 in axonal protection from damage in animal models of Wallerian degenerative disease (Parkinson's disease; Araki et al., 2004; McGuinness et al., 2011; Rahman and Islam, 2011). SirT1 overexpression also protects against Alzheimer's and Huntington's disease as well as amyotrophic lateral sclerosis. In models of Huntington's disease, SirT1 activation reduces cell death by inhibition of NFκB signaling. Alzheimer's disease is also related to sirtuin action given that SirT1 overexpression in the brain of mice reduces β-Amyloid production and the formation of plaques. The β-Amyloid peptide generates protein aggregates (plaques) in the brain of patients (Outiero et al., 2008). Recent studies also suggest that SirT1 may benefit learning and memory by activating the gene for brain-derived neurotrophic factor (Gao et al., 2010).

SIRTUIN MODULATORS

Sirtuins play a key role in several pathologies. Recently, a great deal of research interest has been focused on the identification of small chemical compounds that modulate these proteins. To date, many inhibitors of sirtuins have been proposed for therapy against neurodegenerative diseases and cancer (Alcaín and Villalba, 2009). In recent years a number of inhibitors have been discovered and characterized. In addition to nicotinamide, the physiologic sirtuin inhibitor, some specific inhibitors have been proposed, including splitomicin and its analogs, tenovins, AGK2, sirtinol, suramin, the indole derivative EX-257, cambinol, salermide, and UVI5008 (**Table 2**).

Table 2 | Sirtuin modulators.

Structure	Name	Target
	Nicotinamide	SirT1-2
	AGK2	SirT2
	Cambinol	SirT1-2
	Salermide	SirT1-2
	EX-527	SirT1
	Splitomicin	Sir2
	Suramin	SirT1-2-5
	UVI5008	SirT1-2
	Sirtinol	SirT2
	Tenovins	SirT1

Splitomicin and its derivatives were discovered in a screening for inhibitors of yeast Sir2. While splitomicin did not act very efficiently against human sirtuins, a series of its analogs with a different orientation of the β-phenyl group were developed and characterized for their activity on SirT2 (Bedalv et al., 2001; Neugebauer et al., 2008). Sirtinol is mainly an inhibitor of the yeast Sir2 and human SirT2 (Grozinger et al., 2001). Its inhibitory effect on growth has been described in human breast, lung, and prostate cancer cells, though using a high micromolar range.

Another potent and selective inhibitor of SirT2 is AGK2 with IC_{50} of 3.5 μM. It was demonstrated that the inhibition of SirT2 protects against dopaminergic cell death in a *Drosophila* model of Parkinson's disease (Outeiro et al., 2007).

Suramin is a potent inhibitor of many sirtuins: SirT1 with IC_{50} of 297 nM, SirT2 with IC_{50} of 1.15 μM, and SirT5 with IC_{50} of 22 μM. However, its reported neurotoxicity limits therapeutic use (Schuetz et al., 2007; Trapp et al., 2007).

Cambinol and salermide (Heltweg et al., 2006; Lara et al., 2009) are reported as inhibitors of SirT1 and SirT2. Cambinol shows the most potent activity against Burkitt lymphoma cell lines by a mechanism involving BCL6 acetylation. Salermide induces apoptosis in cancer cells in a p53-independent manner. The indole derivative, EX-527, is a cell-permeable, selective inhibitor of SirT1 ($IC_{50} = 98$ nM). Treatment with EX-527 dramatically increases acetylation at K382 of p53 after DNA damage induction in primary human mammary epithelial cells and several cell lines (Solomon et al., 2006). The direct interaction between SirT1 and p53 highlights potential SirT1 involvement in p53 functions, including differentiation, DNA repair, and aging. Currently, EX-527 is in phase 1 clinical trials for the treatment of Huntington's disease. In preclinical studies this inhibitor was shown to reduce neuronal death.

The only SirT inhibitors that have been tested in cancer mice models are cambinol, tenovins, and UVI5008. Tenovins, a family of small-molecule inhibitors, are able to inhibit SirT1 at single-digit micromolar concentration and prevent tumor growth *in vivo* in a p53-dependent manner as single agents. Tenovins have a potential therapeutic interest for the development of new drugs that act against tumor cells overexpressing SirT1 (Lain et al., 2008).

UVI5008 has recently been reported as a novel epigenetic modifier able to inhibit simultaneously histone deacetylases, sirtuins, and DNA methyltransferases. This compound induces cancer cell-selective death in several human models. Importantly, UVI5008 action is independent of p53, Bcl-2-modifying factor (BMF), and TNF-related apoptosis-inducing ligand (TRAIL), affecting the growth of tumor cells deficient or mutated for these factors. This finding potentiates its application in therapy by overcoming potential drug resistance limits. The simultaneous modulation of three classes of epigenetic enzymes offers a promising strategy for applying a single drug exerting multiple-interventions against cancer (Nebbioso et al., 2011).

Phenol derivates, including quercetin, piceatannol, and resveratrol, were shown to have SirT1-activating properties. The most potent activator of the three is resveratrol, a polyphenol found in grapes, and grape products. However, its activity is still debated. Given that the activation of SirT1 by resveratrol requires the use of peptides conjugated with a non-physiological fluorophore and that no activation is observed when peptides lacking this fluorophore are used, other technical approaches are necessary to establish its effective modulation of SirT1 (Borra et al., 2005).

CONCLUSION AND PERSPECTIVES

Sirtuins influence several cellular processes. Growing evidence has recently underlined their involvement in many diseases. Although SirT1 has been extensively researched a better understanding of its involvement in pathogenesis is required. Many questions remain to be addressed, such as the role of the other sirtuins and their function. Further research is needed to provide clearer perspectives. The development of potent sirtuin modulators may revert the disease process and possibly extend healthy human lifespan.

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Structures, substrates, and regulators of mammalian Sirtuins – opportunities and challenges for drug development

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Sirtuins are NAD^+-dependent protein deacetylases regulating metabolism, stress responses, and aging processes. Mammalia have seven Sirtuin isoforms, Sirt1–7, which differ in their substrate specificities and subcellular localizations. The physiological functions of Sirtuins make them interesting therapeutic targets, which has stimulated extensive efforts on development of small molecule Sirtuin modulators. Yet, most Sirtuin inhibitors show limited potency and/or isoform specificity, and the mechanism of Sirtuin activation by small molecules remains obscure. Accumulating information on Sirtuin substrates, structures, and regulation mechanisms offer new opportunities for the challenging task to develop potent and specific small molecule modulators for mammalian Sirtuins for *in vivo* studies and therapeutic applications. We therefore recapitulate advances in structural and mechanistic studies on substrate recognition and deacetylation by Sirtuins, and in the characterization of compounds and molecular mechanisms regulating their activity. We then discuss challenges and opportunities from these findings for Sirtuin-targeted drug development efforts.

Keywords: Sirtuin, structure, mechanism, inhibitor, activator

THE SIRTUIN FAMILY OF NAD^+-DEPENDENT PROTEIN DEACETYLASES

Reversible lysine (de)acetylation was long assumed to be a post-translational protein modification mainly found in histones. It is now established to be widespread, however, with more than 6800 known acetylation sites in mammalian proteins, and thus rivaling phosphorylation in prevalence and importance (Norvell and McMahon, 2010). In fact, acetylation appears to be more ancient and acetylated lysines more conserved than phosphorylation sites, and dominant in some processes such as regulation of mitochondrial metabolism (Weinert et al., 2011). The enzymes catalyzing attachment and removal of this modification, protein acetyl transferases (PAT), and protein deacetylases (PDAC), are thus emerging as drug targets for various indications. Among the four PDAC classes (Xu et al., 2007), the Sirtuin family (class III) is unique in using NAD^+ as a co-substrate, rendering Sirtuins metabolic sensors. Sirtuins were found to regulate metabolic pathways and stress responses (Bell and Guarente, 2011; Cen et al., 2011), and they contribute to some effects of calorie restriction (CR), in particular lifespan extension (Guarente and Picard, 2005). Pharmacological Sirtuin modulation has thus been identified as an attractive therapeutic approach, e.g., for supporting treatment of diabetes and prevention of malnutrition effects (Lavu et al., 2008; Haigis and Sinclair, 2010).

Mammalia have seven Sirtuin isoforms, Sirt1–7, which differ in their substrate specificities and subcellular localizations (Michan and Sinclair, 2007). Sirt1, 6, and 7 are nuclear enzymes and modify, for example, substrates contributing to chromosome stability and transcription regulation (Michan and Sinclair, 2007; Haigis and Sinclair, 2010). Sirt3, 4, and 5 are located in mitochondria – they are in fact the only deacetylases known in this organelle – and appear to regulate metabolic enzymes and stress response mechanisms (Gertz and Steegborn, 2010; Bell and Guarente, 2011). Sirt2 is mainly cytosolic and was reported to deacetylate tubulin and p300 (Black et al., 2008). Despite these examples, the relevant Sirtuin substrates are unknown for many Sirtuin-regulated processes and no or few substrate sites have been described for most mammalian isoforms. Likewise, few selective and potent inhibitors for mammalian Sirtuin isoforms are available, and their mode of action is largely unknown (Cen, 2010). Even more so, activation of Sirtuins using small molecules has been reported, but the mechanism remains enigmatic, hampering efforts to develop improved activators. We propose that Sirtuin features such as different substrates/substrate specificities and their complex catalytic mechanism offer unique opportunities for drug development, and molecular and mechanistic insights into substrate recognition, catalysis, and pharmacological modulation will be essential steps toward exploiting this potential.

ARCHITECTURE OF SIRTUINS AND CATALYTIC MECHANISM

All Sirtuins share a conserved catalytic core of ∼275 amino acids that is flanked by N- and C-terminal extensions. The extensions are variable in length and sequence, and they have been reported to play various roles such as ensuring a proper cellular localization, regulating the oligomerization state, and/or exerting autoregulation mechanisms (Schwer et al., 2002; Zhao et al., 2003; Tennen et al., 2010).

Whereas other PDAC families activate a water molecule for the hydrolysis reaction by using a zinc cofactor, the unique Sirtuin mechanism is based on the use of NAD^+ as a co-substrate. High-resolution structures of several Sirtuins, including human Sirt2, 3, 5, and 6, in apo- or (co)substrate/inhibitor-bound forms have been reported (for a review see Sanders et al., 2010) and have helped to reveal their catalytic mechanism (Cen et al., 2011). The catalytic core adopts an oval-shaped fold composed of two globular subdomains linked by four loops, which contribute to the active site cleft between the subdomains (**Figure 1A**). The larger domain consists of a Rossmann-fold typical for NAD^+ binding proteins and the smaller domain is formed by association of two modules inserted in the Rossmann-fold domain: a zinc-binding motif bearing the consensus sequence Cys-$X_{2–4}$-Cys-$X_{15–40}$-Cys-$X_{2–4}$-Cys, and an α-helical region showing the highest diversity among family members. The relative orientations of the small and the Rossmann-fold domains vary in known Sirtuin structures, but these differences seem to be due to the presence or absence of substrates. Both the acetylated lysine-containing polypeptide substrates and the NAD^+ co-substrate bind to the cleft at the interface of the two domains (**Figure 1A**). The so-called "cofactor binding loop" shows high flexibility and its conformation was shown to evolve in close relation to the catalytic events (Sanders et al., 2010), which comprise formation of a covalent 1′-*O*-alkylamidate intermediate between the two substrates under nicotinamide release. Subsequently, the intermediate is hydrolyzed to yield deacetylated polypeptide and 2′-*O*-acetyl-ADP-ribose (Cen et al., 2011). Studies on different Sirtuin substrate/substrate analog complexes indicate that the cofactor binding loop gets ordered upon substrate binding and changes to a more closed conformation upon acetyl transfer (see, e.g., Chang et al., 2002; Hoff et al., 2006), with a suggested role of this loop-relocation in expelling the first reaction product, nicotinamide. Binding of the acetyl-lysine substrates was also shown to induce a significant reorientation of the two domains relative to each other and to induce a closure of the cleft as well as the correct positioning of conserved residues for formation of the acetyl-lysine binding tunnel (Cosgrove et al., 2006). These rearrangements highlight the dynamic structure of this enzyme class. Furthermore, individual Sirtuins show specific sequence preferences due to differences in the details of their peptide binding groove shape and electrostatics (Cosgrove et al., 2006; Sanders et al., 2010). Also, they recognize in general a wide variety of substrate sequences, again indicating a high adaptability of this enzyme class to its substrates. It is thus essential to obtain structures of different isoforms and enzyme states (such as different ligand complexes) for obtaining a reliable picture of the dynamics and preferred conformations of their binding pockets for drug development.

PHYSIOLOGICAL AND PHARMACOLOGICAL REGULATION OF SIRTUINS

Sirtuins are considered attractive therapeutic targets for metabolic and aging-related diseases, which has stimulated extensive efforts for development of Sirtuin-modulating small molecules (Lavu

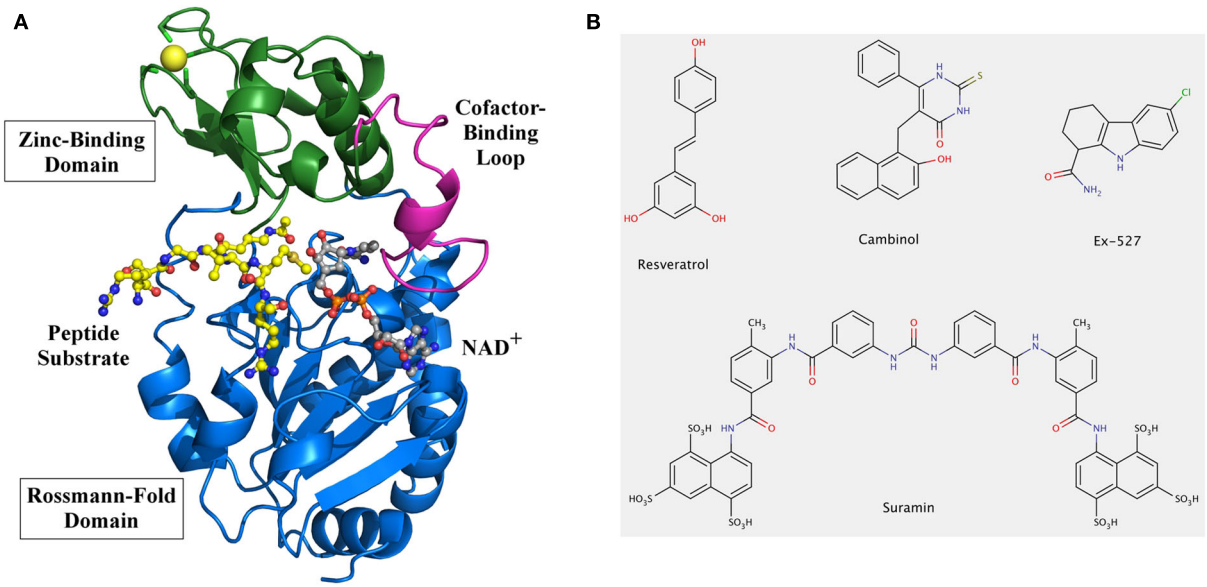

FIGURE 1 | Overall structure and modulators of Sirtuins. (A) Structure of human Sirt3 with bound AceCS2 (acetylCoA synthetase 2) peptide and NAD^+ analog. The structure of Sirt3 (PDB entry 3glr) is shown as a cartoon model with Rossmann-fold domain and Zinc-binding domain colored in blue and green, respectively. The cofactor binding loop (magenta) is in a closed conformation and binds a carba-NAD molecule (gray), which was added to the model based on a superposition with the structure of an Hst2/carba-NAD structure (1szc). The active site cleft also contains the peptide substrate AceCS2 (yellow) with the acetylated lysine directly pointing to the active site. **(B)** Chemical structures of known Sirtuin modulators. Compound screenings identified the polyphenol *resveratrol* as an activator of human Sirt1 activity against suitable substrates, and *Ex527* as one of the most potent and selective Sirt1 inhibitors. The naphtol compound *cambinol* was obtained from structure–activity relationship studies on Sirtinol, one of the first Sirtuin inhibitors identified. *Suramin*, a symmetric diarylurea containing multiple anionic groups, is an inhibitor for several human Sirtuin isoforms. It is the only inhibitor, besides substrate analogs, whose complex with a Sirtuin has been described.

et al., 2008; Haigis and Sinclair, 2010). Sirtuins are also special targets because they are amenable to stimulation, besides inhibition, through small molecules. Sirtuin activating compounds, initially resveratrol-related polyphenols (**Figure 1B**) and subsequently other compound classes (Howitz et al., 2003; Milne et al., 2007), can promote survival of human cells, extend lifespan in various species, and protect against insulin resistance (Guarente and Picard, 2005). However, their mode of action remains enigmatic and little is known about their effects on most Sirtuin isoforms. Furthermore, their physiological effects might in part or mainly rely on effects on other cellular targets, such as receptors or transport proteins (Pacholec et al., 2010). A mechanistically understood Sirtuin activator, in contrast, is isonicotinamide. Nicotinamide is the first product of the Sirtuin catalyzed reaction, released from NAD^+ during formation of the alkyl imidate intermediate (see above). Rebinding of nicotinamide to the Sirtuin/intermediate complex can promote the reverse reaction to reform the substrates, and thus inhibits the deacetylation reaction (Sauve, 2010). This non-competitive mechanism is assumed to inhibit all Sirtuins, with K_i values typically reported around 0.05–0.2 mM (Sauve, 2010). Sirtuins thus appear to be affected by physiological nicotinamide concentrations, assumed to be up to 0.1 mM, and a role of nicotinamide as endogenous Sirtuin regulator is supported by *in vivo* studies in yeast, flies, and mammalian cells (Anderson et al., 2003; Sauve, 2010). Isonicotinamide can compete with nicotinamide for binding but cannot initiate the reverse reaction, thereby leading to apparent activation through relief of nicotinamide inhibition (Sauve et al., 2005; Cen et al., 2011). Assuming that all Sirtuins are equally inhibited by nicotinamide, isonicotinamide would be a general Sirtuin "activator." However, data from our lab suggest that some Sirtuins show nicotinamide-insensitive deacetylase activity (Fischer et al., unpublished), indicating that nicotinamide and isonicotinamide employ isoform discriminating binding sites or modulation mechanisms. Structural and further biochemical studies on these compounds and mechanisms might enable the development of isoform selective modulators.

Various pharmacological Sirtuin inhibitors have been described, but few of them show high potencies, isoform selectivity, and favorable pharmacological properties (Cen, 2010). In fact, for most compounds effects on only few Sirtuin isoforms have been reported, and little is known about their inhibition mechanisms. For example, cambinol (**Figure 1B**) inhibits Sirt1 and Sirt2 with IC_{50} values of 50–60 μM, but has no significant effects on Sirt3 and Sirt5 (Heltweg et al., 2006). Docking studies suggest that it occupies parts of both substrate binding pockets, the one for NAD^+ and the one for the polypeptide (Neugebauer et al., 2008). Such a blocking of binding site areas for both substrates was crystallographically shown for suramin (**Figure 1B**), a huge naphthylurea compound with antiproliferative and antiviral activity that inhibits Sirt1, Sirt2, and Sirt5 – and possibly other, not yet tested isoforms – with low micromolar potency (Schuetz et al., 2007; Trapp et al., 2007). Despite this lack of specificity, the crystal structure of a Sirt5/suramin complex (Schuetz et al., 2007) allows insights into the binding details helpful for drug development efforts, and it was used to rationalize structure–activity relationships for suramin derivatives with improved potency (Trapp et al., 2007). However, the Sirt5/suramin complex is the only published crystal structure of a Sirtuin complex with an inhibitor other than peptide or NAD^+ derivatives co-crystallized for mechanistic insights, and even kinetic data to identify potential competition with one of the Sirtuin substrates is lacking in most cases (Cen, 2010). Thus, to better understand how available compounds interact with Sirtuins and how improved compounds can be obtained, mechanistic data and structural information on their complexes with Sirtuins are of paramount importance.

OPPORTUNITIES FOR DRUG DEVELOPMENT FROM NEW INSIGHTS INTO SIRTUIN SUBSTRATES AND REGULATION MECHANISMS

The large body of biochemical and structural work on Sirtuins has provided us with exciting insights in how Sirtuins recognize their substrates and how they catalyze lysine deacetylation (Sanders et al., 2010; Sauve, 2010). The differences between Sirtuin isoforms in details of structure, physiological targets, and regulators should enable identification of highly specific inhibitors, and possibly also activators. An obvious requirement toward this goal is progress in the identification of Sirtuin substrates, so that the proper Sirtuin isoform(s) can be targeted for modulating a specific cell function. Furthermore, physiological Sirtuin substrates are needed for meaningful modulation tests, as can be learned from the studies on Sirtuin activation by resveratrol, which showed that effects can be substrate-specific and thus that non-physiological substrates can lead to artificial results (Kaeberlein et al., 2005; Cen et al., 2011). These findings have led to heated discussions on the general possibility of Sirtuin activation against physiological substrates (Cen et al., 2011), but rather should stimulate studies on the molecular reasons for seemingly contradicting observations, which promise outstanding opportunities for drug development. Understanding the substrate-specific resveratrol effects offers the exiting possibility to develop modulators not only specific for one Sirtuin isoform, but maybe even affecting only deacetylation of one or few of the substrates of this isoform.

A general challenge for understanding Sirtuin interactions and mechanisms lies in their complexity, with two substrates, one of them a polypeptide that can vary in sequence and the second one releasing the product nicotinamide, which also acts as a non-competitive inhibitor. Some Sirtuins have even been proposed to catalyze physiologically other reactions than deacetylation, such as hydrolytic release of other organic acids or ADP-ribosylation (Haigis et al., 2006; Zhu et al., 2012), but it remains to be seen whether these activities are their dominant physiological functions. However, the sequence variability of the polypeptide substrate poses a challenge, because it means that many Sirtuin/substrate complexes with small differences in conformation exist, whereas only few structures of Sirtuin complexes with physiological substrate sequences have been solved. Thus, exploiting the peptide binding pockets for inhibition so far only yielded peptide mimetics and other lead compounds not yet suitable as drugs (Huhtiniemi et al., 2011; Schlicker et al., 2011), and further studies on peptide binding site differences and dynamics will be required for efficient compound improvement. A major obstacle for fully understanding Sirtuin/ligand interactions is the limited number of Sirtuin complex structures, and that the available structures

were solved with several different Sirtuins, packed through various crystal lattice interactions. Comparing such complexes often does not allow assigning differences in conformation to the different Sirtuin or to the different ligand, or even the different crystal packing. Despite these limitations, using the available structural snapshots for rational drug discovery and for rationalizing results from screening has revealed a number of promising Sirtuin inhibitors (Cen, 2010). Further mechanistic studies on these compounds promise to boost Sirtuin inhibitor development by enabling more sophisticated rational approaches. An especially interesting compound should be Ex527 (**Figure 1B**), a potent Sirt1 inhibitor (IC_{50} ~0.1 μM) with two orders of magnitude lower potency against Sirt2 and Sirt3 (Napper et al., 2005), and no effect on Sirt5 and class I/II HDACs (Solomon et al., 2006). Kinetic data suggest that it binds after nicotinamide release and prevents product release, indicating that an interaction with a reaction intermediate or product complex mediates specificity for Sirtuins. Structural insights on Ex527 inhibition will thus support development of Sirtuin-specific compounds and should reveal the molecular basis of its isoform selectivity, enabling development of similar compounds specific for other isoforms.

Molecular studies on enzyme inhibition have revealed many attractive drug development approaches, such as selective zinc-binding groups for carboanhydrase inhibition (Schlicker et al., 2008b) or non-competitive chelators for adenylyl cyclase inhibition (Steegborn et al., 2005; Schlicker et al., 2008b), and such studies on Sirtuin modulators promise similar progress for these exiting drug targets. Mechanistic studies on Sirtuins beyond the basics of substrate recognition promise additional opportunities for drug development. Many Sirtuins have regulatory regions outside the catalytic domain (Michan and Sinclair, 2007; Schlicker et al., 2008a; Tennen et al., 2010), yet structural studies so far mainly focused on the catalytic cores (Sanders et al., 2010). Also, posttranslational modifications regulating Sirtuins are just emerging (Guo et al., 2010), and their mode of action is largely unknown. Thus, although the accumulated knowledge on Sirtuins already offers approaches for their modulation, further mechanistic studies on Sirtuin modulators and on physiological regulation mechanisms promise exciting insights into Sirtuin function and in Sirtuin features exploitable for highly specific intervention with drugs.

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Role and therapeutic potential of the pro-longevity factor FOXO and its regulators in neurodegenerative disease

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Studies in simple model organisms have yielded crucial insights into the genetic and molecular aspects of longevity. FOXO, which is most notable for its association with longevity, and its upstream regulators such as sirtuins have received particular attention in translational research because these genes modulate cell survival in several models of neurodegenerative diseases. There is a large amount of knowledge on the pathways that regulate FOXO activity and genes that may be regulated by FOXO. However, for the same reason that the FOXO network is a complex stress response system, its therapeutic potential to develop disease-modifying strategies requires further examination. Although the FOXO network contains druggable genes such as sirtuins and AMPK, whether they should be activated or inhibited and whether protection against the early or late phases of neuronal cell decline might require opposite therapeutic strategies remains unclear. Additionally, the mode of action of small compound molecules believed to act on FOXO network targets was questioned. This review recapitulates essential facts and questions about the promises of FOXO and its interactors in neurodegenerative disease.

Keywords: Foxo, longevity, disease

INTRODUCTION

A major challenge in neurodegenerative disease research is to identify disease-modifying strategies. Given the complexity of sporadic disease such as Alzheimer's disease (AD) or Parkinson's disease (PD), the study of inherited neurodegenerative disease is expected to shed light on the mechanisms that underlie neurodegeneration. In this respect, Huntington's disease (HD), a dominantly inherited disease with CAG expansion in the huntingtin (htt) gene and expanded polyglutamine (polyQ) tracts in the htt protein causing striatal and cortical degeneration (Walker, 2007) has become a "model neurodegenerative disease." The large number of models for HD, the knowledge on HD mechanisms (Zuccato et al., 2010) and the availability of well-characterized cohorts of HD subjects indeed provide a unique framework in which to investigate the therapeutic potential of target genes and pathways. Among the many genes that could be targeted in HD (Zuccato et al., 2010), longevity modulators, and more largely, genes which are essential to cellular homeostasis are the subject of intense research because they may directly regulate important cell survival mechanisms such as for example oxidative stress response and protein quality control. While several genes may modulate lifespan (Narasimhan et al., 2009), a limited number of genes may function to promote longevity. This is notably true for the FOXO proteins, a family of forkhead transcription factors that is conserved from *Caenorhabditis Elegans* (*C. elegans*) to mammals. FOXO factors indeed increase longevity in invertebrates, and in mammals they have a wide range of functions as further detailed below. The purpose of this review is to discuss the role of FOXO proteins and members of the FOXO network as neurodegeneration modulators.

THE FOXO NETWORK

Several signaling pathways such as the PI3K–AKT pathway, stress-activated pathways such as the MST-1 and JNK pathways, and the SIR-2 and AMPK pathways converge onto FOXO proteins to regulate a variety of biological processes which are important to development, metabolism, tumor suppression, and longevity (Calnan and Brunet, 2008). A large number of studies have indicated that, in response to external stimuli including growth factors such as insulin and insulin-like growth factor (IGF-1), nutrients, cytokines, and oxidative stress stimuli, these pathways are able to regulate the subcellular localization, DNA binding activity, transcriptional activity, and stability of these FOXO proteins. These studies have been thoroughly reviewed in excellent articles to which to refer for more details (Greer and Brunet, 2005; Calnan and Brunet, 2008; Landis and Murphy, 2010; Yen et al., 2011); a finely tuned regulatory network is emphasized in which, through a series of context-dependent post-translational modifications and interactions with co-regulators, FOXO proteins are able to integrate several types of external stimuli to regulate gene expression and modulate cell cycle arrest, apoptosis, autophagy, angiogenesis, differentiation, stress resistance, stem cell maintenance, glucogenesis, and food intake. Nutrient-sensing pathways are important upstream components of this regulatory network. Nutrient-sensing factors such as insulin/insulin-like growth factor (IGF) signaling (IIS), NAD+-dependent histone deacetylases (sirtuins/SIR-2/SIRTs), the target of rapamycin (TOR) kinase, and ribosomal S6 Kinase (TOR/S6K) pathway and the AMPK pathway may be targeted by dietary restriction to slow aging (Cabreiro and Gems, 2010; Chiacchiera and Simone, 2010). Additionally, nutrient-sensing pathways are also key regulators of FOXO activity.

It is for example well-established that IIS modifies FOXO proteins at specific phosphorylation sites, with reduction of IIS activating FOXO, which may translate into lifespan extension in invertebrates (Calnan and Brunet, 2008). Besides IIS and modulation of FOXO phosphorylation, the SIRT1 nutrient-sensing pathway and modulation of FOXO acetylation is another important FOXO regulator. Increased *sir-2.1*/SIRT1 dosage in high-copy *sir-2.1* transgene containing strains was indeed found to strongly increase lifespan in *C. elegans*, an effect that requires *daf-16*/FOXO (Tissenbaum and Guarente, 2001), suggesting that sirtuins could play important roles in lifespan extension. This notion has been recently challenged by additional studies in *C. elegans* and *Drosophila melanogaster* (Burnett et al., 2011; Rizki et al., 2011). These studies revealed that lifespan extension may be partially attributable to background mutations producing a dye-filling defective ("Dyf") phenotype in high-copy *sir-2.1* overexpressor strains (Burnett et al., 2011; Rizki et al., 2011). While this raised the possibility that sirtuins might not be longevity-promoting factors to that extent, two independent studies indicated that lifespan extension (10–15%) may also be achieved in low-copy *sir-2.1* overexpressor strains (Rizki et al., 2011; Viswanathan and Guarente, 2011). Importantly, one of theses studies (Rizki et al., 2011) was consistent with the notion that *sir-2.1* interacts with *daf-16*/FOXO and its co-factors such as 14-3-3 proteins in modulating lifespan (Berdichevsky et al., 2006). Furthermore, background mutations were not responsible for the strong level of neuroprotection achieved against mutant polyQs by *sir-2.1* overexpression (Burnett et al., 2011). Thus, while additional investigation will be needed to determine to which extent sirtuins may promote lifespan extension, sirtuins stand as important longevity factors that are relevant to neuronal protection in neurodegenerative disease and other protective effects in metabolic disease (Kim et al., 2007; Baur et al., 2010; Burgess, 2011; Jeong et al., 2011; Jiang et al., 2011). One may wonder why mutations producing a Dyf phenotype were found in *sir-2.1* overexpressor strains despite the care made by the *C. elegans* community with distributing mutants after multiple outcrosses. The answer might come from studies made with the search for mutants resistant to avermectin (Rand and Johnson, 1995), an antiparasitic drug. Many low-level ivermectin resistance mutations in *che*, *osm*, and *dyf* genes may produce defects in the amphid sensilla, low-level ivermectin resistance is common in *C. elegans* (1 in 5000 eggs in the absence of mutagenesis) and the ivermectin resistance allele frequency in wild nematode populations may be as high as 1% (C. D. Johnson, personal communication). Additionally, mutations that cause defects in sensory cilia genes may also extend longevity (Apfeld and Kenyon, 1999). The *dyf* mutations may thus occur at relatively high rates, which calls for the regular assessment of *C. elegans* mutants for dye-filling defects in studies of longevity.

In addition to be regulated by kinases and deacetylases, FOXO activity is regulated by ubiquitin complex members such as Skp2, a protein that promotes FOXO1 degradation (Huang et al., 2005), and by co-factors such as β-catenin, a protein that binds to FOXO3a and regulates its transcriptional activity in response to oxidative stress (Essers et al., 2005). The FOXO proteins may compete with the canonical Wnt effector T cell factor (TCF) for binding to β-catenin (Hoogeboom et al., 2008), which further highlighted the importance of β-catenin in the regulation of FOXO activity. While FOXO proteins may act together with other transcription factors to regulate gene expression (Landis and Murphy, 2010), they stand at the center of a large network of upstream regulators, co-factors, and downstream targets (**Figure 1**) which are important for cell homeostasis. Interestingly, studies based on chromatin immunoprecipitation (Oh et al., 2006; Schuster et al., 2010), gene expression profiling in *C. elegans* (McElwee et al., 2003; Murphy et al., 2003) and mouse neural stem cells (NSCs; Paik et al., 2009; Renault et al., 2009), and other approaches (Lee et al., 2003; Yu et al., 2008) highlighted thousands of putative FOXO-regulated genes (Jensen et al., 2006; Murphy, 2006). These results open the avenue to a better understanding of FOXO activity in regulating downstream mechanisms in specific contexts. Given that FOXO proteins act in specific cells and tissues to execute different outputs (Landis and Murphy, 2010), it is anticipated that specific transcriptional targets may be engaged into different FOXO-mediated responses. Gene expression studies performed in either *C. elegans* nematodes (McElwee et al., 2003; Murphy et al., 2003) or mouse NSCs (Lee et al., 2003; Yu et al., 2008) emphasized a small number of FOXO-regulated genes across species and biological conditions (**Table 1**). Although poor overlap may be partially attributable to differences in the species and/or methods used for gene expression profiling, these studies suggest that FOXO proteins indeed regulate specific targets in different contexts. In addition to being important for stem cell homeostasis and adult longevity, FOXO factors are involved in development and cell differentiation (Kitamura et al., 2007; Demontis and Perrimon, 2009; Yuan et al., 2009; de la Torre-Ubieta et al., 2010; Kerdiles et al., 2010; Schuff et al., 2010), and they may be important to promote adult neurogenesis as suggested by studies in *Drosophila melanogaster* (Siegrist et al., 2010). Thus, FOXO factors may be essential to cell response homeostasis during the entire lifetime of a living organism. As such, it is not surprising that FOXO proteins and their main regulators such as sirtuins were implicated in the protection against age-associated diseases such as cancer, diabetes, cardiovascular disease, and neurodegeneration (Dansen and Burgering, 2008; Chiacchiera and Simone, 2010; Herranz and Serrano, 2010).

THE SIR-2/FOXO PATHWAY AND ITS ROLE IN DEGENERATIVE DISEASE

For the same reason that pathways converging onto FOXO are important to cell survival and longevity, they are of particular interest to explore how to promote neuron or muscle cell survival in degenerative disease. Over the past years, accumulating evidence on the therapeutic potential of FOXO and its interactors for age-related diseases has been obtained. Perhaps the first observation suggesting that longevity-promoting factors might be able to modulate the toxicity of degenerative disease proteins was obtained in the nematode *C. elegans*. In transgenic nematodes expressing polyQ tracts in body wall muscle cells, reducing IIS by use of *age-1*/PI3K loss-of-function (LOF) mutants decreased the toxicity associated to expanded polyQs, and this effect was dependent on *daf-16*/FOXO (Morley et al., 2002). Direct evidence for longevity-promoting factors such as SIR-2 and FOXO to protect diseased neurons at an early stage of cellular decline was obtained by using a *C. elegans* model of HD pathogenesis

Table 1 | Putative FOXO targets emphasized by microarray analysis in either *C. elegans* or mouse NSCs, or both.

Mouse gene name	Worm ORF ID	*C. elegans*		Mouse NSCs	
		Murphy et al. (2003)	McElwee et al. (2003)*	Renault et al. (2009)	Paik et al. (2009)
Cav1	T13F2.8	U	–	D	–
Mxd1	R03E9.1	D	–	D	D
Sod2	C08A9.1	D	U	–	–
Crisp	F49E11.9, H10D18.2, ZK384.1, ZK384.2	D	U (F49E11.9)	–	–
RIKEN cDNA 6230409E13	C54D10.1	D	U	–	–
Nxnl2	F17B5.1	D	U	–	–
Dgat2	K07B1.4	D	U	–	–
Birc5	C50B8.2	–	U	–	U
Myo1b	F29D10.4	–	U	–	U
Slc25a5	W02D3.6	–	U	–	U
B3galt1	E03H4.11	U	–	–	U
Chrna7	R02E12.8	U	–	–	U
Gmeb1	C44F1.2	U	–	–	U
Gspt1	H19N07.1	U	–	–	U
Jag1	R107.8	U	–	–	U
Lta4h	ZC416.6	U	–	–	U
Mapk8	B0478.1	U	–	–	U
Pcna	W03D2.4	U	–	–	U
Psmc5	Y49E10.1	U	–	–	U
Bphl	K01A2.5	U	–	–	D
Dpyd	C25F6.3	U	–	–	D
Slc15a2	K04E7.2	U	–	–	D
Comtd1	Y40B10A.2, Y40B10A.6	D	–	–	U
Gnb5	F52A8.2	D	–	–	U
Icmt	F21F3.3	D	–	–	U
Rexo1	Y56A3A.33	D	–	–	U
Abhd4	C37H5.2	D	–	–	D
Acsl3	C46F4.2	D	–	–	D
Alkbh4	F09F7.7	D	–	–	D
Cat	Y54G11A.6	D	–	–	D
Cyb5	W02D3.1	D	–	–	D
Fads2	W08D2.4	D	–	–	D
Ppap2c	T28D9.3	D	–	–	D
Syt11	T23H2.2	D	–	–	D

*U, up-regulated; D, down-regulated. *The promoter of these dysregulated genes also contain FOXO binding sites (McElwee et al., 2003). –, Not dysregulated. This table is based on the simple comparison of four studies performed in either C. elegans (McElwee et al., 2003; Murphy et al., 2003) or mouse NSCs (Lee et al., 2003; Yu et al., 2008).*

in which expression of polyQ-expanded N-terminal huntingtin in touch receptor neurons induced neuronal dysfunction (lossglu of response to light touch) in the absence of cell death (Parker et al., 2001). In these animals, increased *sir-2.1*/SIRT1 dosage protects from expanded polyQs, with reduction of axonal dystrophy, whereas *sir-2.1* LOF aggravates expanded polyQ toxicity (Parker et al., 2005). This neuroprotective effect is not due to unrelated mutations (i.e., mutations producing a Dyf phenotype) in the high-copy *sir-2.1* transgene containing strains as neuroprotection was observed to the same extent in non-Dyf, *sir-2.1* overexpressors (Burnett et al., 2011). Additionally, *sir-2.1* neuroprotection in this model requires *daf-16*/FOXO (Parker et al., 2005), emphasizing a role for the SIR-2.1/DAF-16 pathway. This suggested that raising the activity of the SIRT1 pathway may protect from mutant huntingtin in mammalian models of HD. This notion was supported by other *C. elegans*-based studies (Bates et al., 2006), and consistent with the protective effects of sirtuins in common neurological disorders. However, studies in *Drosophila melanogaster* suggested that Sir-2 knockdown protects from neurodegeneration as induced by N-terminal htt expression in the compound eye, with no effect of overexpressing Sir-2 (Pallos et al., 2008). These results suggested that the role of Sir-2 in

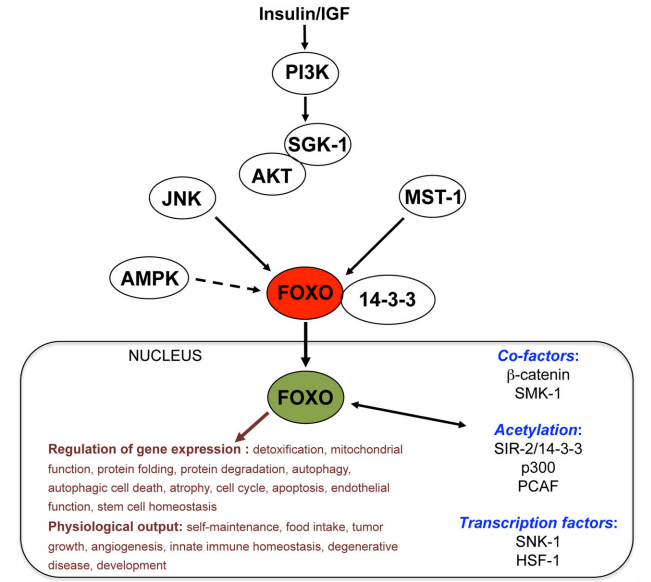

FIGURE 1 | Simplified view of the FOXO network. This diagram shows signaling pathways well-established (straight arrows) or hypothesized (dashed arrow) to converge onto FOXO. The list of target gene classes and physiological outputs is not exhaustive.

mutant polyglutamine neurons may be complex. Nematodes with expanded polyQ expression in touch receptor neurons recapitulate neuron dysfunction before cell death and pointed to the FOXO-dependent effect of homozygous loss of Sir-2 (Parker et al., 2005). Flies with expanded polyQ expression in the eye show neurodegeneration and pointed to an effect of heterozygous loss of Sir-2 that may be unrelated to longevity (Pallos et al., 2008). While these differences illustrated the importance of parameters such as the severity of pathology, type of gene dosage and identity of downstream effectors when pursuing therapeutic targets, two independent studies recently reported that SIRT1 is neuroprotective in several mouse models of HD (Jeong et al., 2011;Jiang et al., 2011). Interestingly, FOXO3a may be involved in the neuroprotective effects of SIRT1 and the reduction of SIRT1 exacerbates mutant huntingtin toxicity (Jiang et al., 2011), which should help settle the question. Besides SIRT1 manipulation, SIRT2 inhibition may also be neuroprotective in diseases such as HD (Luthi-Carter et al., 2010) and PD (Outeiro et al., 2007). As suggested by the function of SIRT2, a microtubule deacetylase, and by the effects of SIRT2 inhibitors in models of HD (Luthi-Carter et al., 2010), the mechanisms for neuroprotection by SIRT2 inhibition may differ from those elicited by SIRT1 manipulation. However, FOXO proteins are deacetylated by SIRT2 (Zhao et al., 2010; Wang et al., 2011), raising the possibility that neuroprotection by SIRT2 inhibition might involve FOXO activity.

Consistent with the protection of mutant polyQ neurons by SIR-2/SIRT1, neuroprotection by *sir-2.1* overexpression was also demonstrated in *C. elegans* nematodes expressing octa-repeat expanded PrP in mechanosensory neurons (Bizat et al., 2010). In these animals, the neuroprotective effect of *sir-2.1* was unrelated to an effect on the conformation of mutant PrP since *sir-2.1* overexpression did not reduce proteinase K-resistant PrP levels (Bizat et al., 2010). Several studies have further extended the neuroprotective activity of SIR-2/SIRT1 and FOXO in models of neurodegenerative diseases. Sirtuin SIRT1 is up-regulated in mouse models of AD and amyotrophic lateral sclerosis (ALS), and SIRT1 is protective in cell-based models of ALS and AD/tauopathies (Kim et al., 2007), providing evidence for SIRT1 to protect diseased neurons. Neurite outgrowth, cell viability and tolerance to degeneration induced by Aβ(1–42) oligomers are promoted in primary neurons from mice with neuron-specific SIRT1 overexpression (Guo et al., 2011), further supporting the notion that SIRT1 is able to protect diseased neurons. In *C. elegans* nematodes expressing Aβ(1–42) in body wall muscle cells (Link et al., 2003), *daf-16*/FOXO and heat shock factor 1 (*hsf-1*) cooperate to regulate a two-step cell survival response involving disaggregation/aggregation (Cohen et al., 2006). Consistently, in a mouse model of AD (APPswe/Presenilin1-ΔE9 mice), genetically reducing IGF signaling protects from Alzheimer's-like disease symptoms (Cohen et al., 2009). Finally, it is notable that allelic variation in FOXO3A was associated with the ability to be long-lived as suggested by several association studies in populations of centenarians (Willcox et al., 2008; Anselmi et al., 2009; Flachsbart et al., 2009; Li et al., 2009; Soerensen et al., 2010; Kleindorp et al., 2011).

While the SIR-2.1/DAF-16 pathway may protect mutant polyQ neurons, it has opposite effects in a *C. elegans* model of oculopharyngeal muscular dystrophy (OPMD), a late-onset disease caused by expanded polyalanines in the PABPN1 protein (Catoire et al., 2008). In *C. elegans* transgenics expressing human PABPN1 in body wall muscle cells, *sir-2.1* overexpression indeed aggravates muscle cell decline and abnormal motility caused by mutant PABPN1 expression, whereas *sir-2.1* LOF is protective in a *daf-16*/FOXO-dependent manner (Catoire et al., 2008). These observations suggested that the status of FOXO activity in mutant polyQ neurons is different from that of mutant polyAla muscle cells. These results also suggested that SIRT1 inhibitors have therapeutic potential in OPMD as further illustrated by pharmacological studies in PABPN1 nematodes (Pasco et al., 2010).

MODULATION OF NEURODEGENERATIVE DISEASE TARGETS IN THE FOXO NETWORK

Over the past years, studies of SIRT1/FOXO activity in models of disease have the search for SIRT1 activators and inhibitors with clinical potential. While the aim of this review is not to cover drug discovery and clinical development in the field of sirtuin research, it is notable that the mechanisms of action of SIRT1-activating compounds (STACs) have been the subject of debate. This applies to resveratrol, a plant polyphenol that has a broad range of beneficial activities on health span (Wood et al., 2004; Baur et al., 2006; Fulda and Debatin, 2006; Lagouge et al., 2006), is neuroprotective in several models of neurodegenerative diseases (Parker et al., 2005; Kim et al., 2007; Bizat et al., 2010; Richard et al., 2011) and has attracted a lot of attention as a potential drug (Patel et al., 2011). Resveratrol was initially thought to primarily activate SIRT1 (Howitz et al., 2003). It is now thought that resveratrol has multiple mechanisms of action

(Harikumar and Aggarwal, 2008). Resveratrol might not always require SIRT1 for activity against expanded polyQs (Parker et al., 2005; Pallos et al., 2008), and it could activate AMPK (Dasgupta and Milbrandt, 2007) and the peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1alpha; Lagouge et al., 2006) and inhibits PI3K (Frojdo et al., 2007). This promoted the search for selective SIRT1 activators (Milne et al., 2007), which led to find that they may act as caloric restriction mimetics (Smith et al., 2009). Although STACs hold promises for the treatment of age-associated diseases, their mechanisms of action and therapeutic potential was controversial. A positive aspect of this controversy was to emphasize the need for more robust acetylation assays and in-depth assessment of the promiscuity of STACs. Studies in the field indeed raised the possibility that complex formation between STACs and fluorophore-labeled substrate peptides plays a role in the activation of SIRT1 (Pacholec et al., 2010), whereas other studies subsequently showed that STACs are able to activate unlabelled peptides, suggesting that STACs may interact directly with SIRT1 and activate SIRT1-catalyzed deacetylation through an allosteric mechanism (Dai et al., 2010). While the debate regarding STAC activities might continue, progress appears to be made with harnessing the therapeutical potential of STACs in metabolic disorder. The development of SRT501 (a formulation of resveratrol) for diabetes was terminated whereas new STACs structurally distinct from resveratrol such as SRT2104 are being evaluated (Huber and Superti-Furga, 2011). Further developments are highly awaited to know if these molecules may provide clinically safe drugs for neurodegenerative disease. The situation has been less controversial in regard to sirtuin inhibition as SIRT inhibitors with good selectivity for either SIRT1 (e.g., EX-527) or SIRT2 (e.g., AGK2) have been identified (Mai et al., 2009; Luthi-Carter et al., 2010; Pasco et al., 2010; Taylor et al., 2011). Compared to the mechanisms for neuroprotection by SIRT1 activation, the biology of neuroprotection by SIRT inhibition is not understood to that extent. In any case, progress with identifying the neuronal function of sirtuins (Dietrich et al., 2010; Ramadori et al., 2010, 2011; Guo et al., 2011; Maxwell et al., 2011) and targets modulated by these enzymes (Wang et al., 2007, 2011; Li et al., 2008; Zhao et al., 2010) will help define the therapeutic potential of SIRT inhibitors in neurodegenerative disease.

Besides sirtuins, another neurodegenerative disease target of interest in the FOXO network is AMPK, a key regulator of cellular metabolism that is activated by a decrease in energy levels (Steinberg and Kemp, 2009). Interestingly, an AMPK–FOXO3a pathway might be involved in shifting the cell from energy consumption to energy production in cancer-specific metabolism (Chiacchiera and Simone, 2009, 2010; Chiacchiera et al., 2009), a mechanism also emphasized by the ability of an AMPK–FOXO pathway to mediate longevity in response to dietary restriction in *C. elegans* (Greer et al., 2007). Since there is multiple evidence for mitochondrial and metabolic defects in neurodegenerative diseases such as HD (Zuccato et al., 2010), it is tempting to speculate that one important mechanism to compensate for metabolic defect in HD could be the use of an AMPK–FOXO pathway. If true, then AMPK activation might protect against the early phases of the pathogenic process in neurodegenerative disease. However, the AMPK–FOXO pathway may end up activating autophagic cell death upon prolonged metabolic stress in cancer cells (Chiacchiera and Simone, 2009, 2010; Chiacchiera et al., 2009), raising the possibility that AMPK–FOXO might contribute to neurodegeneration, which might be particularly harmful during the late phases of the pathogenic process in neurodegenerative disease. Studies in models of neurodegeneration reflect the complexity of the AMPK-mediated regulation of cell survival. Although the AMPK activator metformin protects primary cortical neurons from etoposide-induced cell death (El-Mir et al., 2008) and ameliorates survival and motor defects in R6/2 transgenic mice expressing mutant N-terminal huntingtin (Ma et al., 2007), other studies suggested that metformin may increase the generation of Aβ peptides via BACE1 upregulation in cellular models (Chen et al., 2009). In regard to AMPK activity in neurons, conflicting observations have been made. Because neurons do not efficiently store nutrients, AMPK may be essential to neuron activity and survival, suggesting that AMPK activation might protect neurons from metabolic stress in neurodegenerative disease. However, AMPK appears to be abnormally activated in pre-tangle and tangle neurons in AD and other tauopathies (Vingtdeux et al., 2011). Additionally, there is nuclear translocation of the AMPK-alpha1 subunit in the striatum of post-mortem HD brains and mice with a fast-developing HD pathology (Ju et al., 2011). While this suggests that AMPK might have a pathological role in HD, studies of AMPK in additional models of HD are warranted considering the pro-survival role of AMPK in neurons and diversity of pathology in HD mice. Collectively, these data highlight the need for more studies of the modulation of AMPK activity toward specific targets in models that recapitulate the early/mild versus late/severe components of the pathogenic process in neurodegenerative disease. This will allow the potential of AMPK-targeted therapy in neurodegenerative disease treatment to be clarified. It will also be important to identify the small compound molecules that might be used in this respect (Steinberg and Kemp, 2009).

PERSPECTIVES

There is accumulating evidence on the protective role of FOXO proteins and their interactors in response to cellular stress in age-related diseases. One of the current challenges is to identify the downstream mechanisms by which FOXO factors are essential to neuron survival in neurodegenerative disease. Given the complex regulation of gene expression by FOXO proteins and their interactors, it will be important to investigate how FOXO transcriptional targets may be involved in the regulation of survival responses in specific neurodegenerative diseases and to evaluate whether this may be relevant to other neurodegenerative diseases. Another challenge is to assess whether the pathways that regulate FOXO transcriptional activity should be activated or inhibited to modify the pathogenic process in neurodegenerative disease, and whether the same approach should be used against the early/mild versus late/severe phases of the pathology. Sustaining neuron survival at an early phase of the pathology and reducing neuronal cell death in late disease might require the same FOXO activity to be modulated in opposing fashions. This possibility is supported by the ability of FOXO proteins and their interactors to orchestrate two-step responses in which the net outcome may be cell survival (Cohen et al., 2006) or cell demise (Chiacchiera and Simone,

2010) in response to prolonged stress. Thus, while several questions remain unanswered, future and exciting developments are expected to shed light on the importance and therapeutic potential of the FOXO network in neurodegenerative disease.

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Sirtuins, metabolism, and cancer

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More than a decade ago, sirtuins were discovered as a highly conserved family of NAD^+-dependent enzymes that extend lifespan in lower organisms. In mammals, sirtuins are key regulators of stress responses and metabolism, influencing a range of diseases, including diabetes, neurodegeneration, and cancer. In recent years, new functions of sirtuins have been characterized, uncovering the underlying mechanisms of their multifaceted role in metabolism. Here, we specifically review recent progress on the role of sirtuins in DNA repair and energy metabolism, further discussing the implication of sirtuins in the biology of cancer.

Keywords: SIRT1, SIRT6, SIRT3, Warburg effect, cancer metabolism, DNA repair, genomic instability, sirtuin biology

INTRODUCTION

Cancer can be defined as an uncontrolled proliferation of cells in the body. Cells become malignant by the acquisition of a succession of capabilities that allows them to evade strict regulatory circuits governing cell proliferation and homeostasis (Hanahan and Weinberg, 2011). On their way to tumorigenesis, genomic instability fuels the acquisition of these tumoral capabilities by generating a genomic environment highly propitious to mutations. Among the hallmarks of cancer cells, metabolic reprogramming stands high, allowing these cells to face up to new energetic requirements. While under normal conditions cells rely on mitochondrial oxidative phosphorylation to produce energy from glucose, cancer cells exhibit enhanced glycolysis. Simply put, pyruvate generated from glucose is directed toward production of lactate in the cytosol instead of entering the TCA cycle in the mitochondria. Because oxygen is limiting during oxidation of pyruvate to CO_2 in the mitochondria, anaerobic glycolysis is usually activated in normal cells under low oxygen conditions. However, cancer cells switch their metabolism to glycolysis even under normoxic conditions, in a process that only generates 2 mol of ATP per mol of glucose whereas oxidative phosphorylation generates up to 36 mol of ATP per mol of glucose. This process is known as the *Warburg effect* after Otto Warburg, who observed in the 1920s that cancer cells metabolize tenfold more glucose to lactate than normal cells, even in the presence of sufficient oxygen to support oxidative phosphorylation (Warburg et al., 1924). Although aerobic glycolysis is highly inefficient for ATP production, it is thought to be an anaplerotic reaction that supplies critical intermediate metabolites for biomass production (amino acids, lipids, and nucleotides), imperative in highly proliferating cells (Vander Heiden et al., 2009). In addition, such metabolic shift may also confer a survival advantage to tumor cells, given their characteristic hypoxic environment.

Cells in an organism are subjected to stress arising from nutrient fluctuations as well as from genotoxic damage that menace genome integrity. A proper cellular response to stress is essential to avoid transformation. Studies over the last decade strongly indicate that sirtuins may play a fundamental role in this process. Sirtuins are a highly conserved family of proteins, homologs of the *Saccharomyces cerevisiae* protein Sir2 (Finkel et al., 2009). They are nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylases and/or mono-[ADP-ribosyl] transferases. Mammals contain seven sirtuins (SIRT1–7) with different subcellular localization; SIRT1, SIRT6, and SIRT7 are nuclear, SIRT2 is mainly cytoplasmic and SIRT3, SIRT4, and SIRT5 are located in the mitochondria (Finkel et al., 2009). Nowadays, sirtuins are recognized as crucial regulators of energy metabolism, working as stress adaptors (oxidative, genotoxic and metabolic stress) likely through sensing changes in levels of intracellular NAD^+, an obligated cofactor for their enzymatic activity. As the best-studied sirtuin, SIRT1 has been implicated in a large variety of metabolic processes, in particular in the regulation of glucose and lipid metabolism during fasting and caloric restriction. Several lines of evidence also point to a fundamental role of SIRT3 and SIRT6 in the regulation of glucose utilization (Zhong and Mostoslavsky, 2011). These three sirtuins have also been shown to protect from genomic instability upon genotoxic and oxidative stress, protecting the genome from mutations that can drive tumorigenesis. In this review we will summarize current advances in the biology of these sirtuins.

SIRTUINS IN GLUCOSE METABOLISM AND DNA DAMAGE

SIRT1

During fasting, an increase in pyruvate and NAD^+ levels induces expression and activity of SIRT1 in hepatocytes promoting the deacetylation of PGC-1α, a transcriptional co-activator of a large number of genes (**Figure 1**). Deacetylation of PGC-1α results in the induction of gluconeogenic genes and repression of glycolytic genes (Rodgers et al., 2005). In parallel, SIRT1 activates the transcription factor FOXO1, which induces the same switch in

transcription, reinforcing gluconeogenesis (Frescas et al., 2005) (**Figure 1**). In muscle, like in liver, SIRT1-mediated activation of PGC-1α during fasting activates peroxisome proliferators-activated receptor α (PPARα)-mediated transcription of fatty-acid catabolic genes (Gerhart-Hines et al., 2007; Purushotham et al., 2009). Concretely, the activation of PGC-1α in muscle induces the transcription of genes involved in mitochondrial oxidative phosphorylation and electron transport, coupling fatty-acid oxidation with energy production. In this manner, activation of SIRT1 in muscle coordinates a shift toward preservation of glucose in the cell, while lipids are used to obtain energy. In white adipose tissue, SIRT1 represses PPARγ during fasting, in turn inducing lipolysis and reducing fat storage (Picard et al., 2004). Lastly, SIRT1 also protects from the negative effects of high fat diet (HFD), stimulating secretion of insulin by pancreatic β-cells through PPARγ-mediated transcriptional repression of uncoupling protein 2 (UCP2), improving in this way glucose tolerance (Moynihan et al., 2005; Bordone et al., 2006) (**Figure 1**).

Although SIRT1 null mice die perinatally due to developmental defects, absence of SIRT1 provokes a large amount of chromosome abnormalities in embryos, impairing progression of cells through mitosis (Wang et al., 2008). A defective signaling of DNA double-strand breaks (DSBs) seems to be the cause behind this genomic instability. SIRT1 associates with and deacetylates the repair factor NBS1, a modification required for its subsequent phosphorylation by the Ataxia Telangiectasia Mutated (ATM) kinase in the first steps of the DNA damage response (Yuan et al., 2007) (**Figure 1**). In addition, SIRT1 is also recruited to DNA DSBs in an ATM-dependent manner. The recruitment of SIRT1 at the breaks is important for the accumulation of proteins involved in signaling and repair, and SIRT1-deficient MEFs (mouse embryonic fibroblasts) exhibit a marked reduction of Rad51-, NBS1-, and BRCA1-foci following γ-irradiation (Oberdoerffer et al., 2008; Wang et al., 2008) (**Figure 1**). SIRT1 appears to be involved in multiple DNA-repair pathways. When a DSB occurs during S or G2 phases of the cell cycle, it will be preferentially repaired by homologous recombination (HR) using the sister chromatid as a template for DNA repair. SIRT1 modulates homologous recombination (HR) by deacetylating WRN –the gene mutated in the progeroid Werner Syndrome- and promoting its relocalization to DNA lesions (Li et al., 2008), where it repairs DNA strand breaks that arise from replication arrest. In this context, transgenic mice expressing increased levels of SIRT1 exhibit longer telomeres and higher frequency of sister chromatid HR events throughout the entire genome (Palacios et al., 2010). In addition to the regulation of DSB repair, SIRT1 is involved in the repair of DNA single strand breaks as well. SIRT1 null MEFs are hyper-sensitive to UV damage, which is mainly repaired by the nucleotide excision repair (NER) pathway. Recent studies demonstrated that SIRT1 deacetylates two components of NER: XPA and XPC (Xeroderma Pigmentosum A and C) upon UV damage. This deacetylation is essential for the recognition of DNA lesions by XPA and XPC, affecting the recruitment of downstream NER factors (Fan and Luo, 2010; Ming et al., 2010). Overall, it appears that SIRT1 has evolved to coordinate both proper genomic integrity and adequate metabolic adaptation, in this way allowing cells to adapt against stress (**Figure 1**). Failure on such mechanisms could clearly lead to both metabolic diseases and tumorigenesis, as described in further detail below.

SIRT6

The role of sirtuins in metabolism and maintenance of genomic integrity is not restricted to SIRT1. SIRT6 deficient mice appear relatively normal at birth, but beginning at 2 weeks of age they develop an acute degenerative phenotype, reminiscent of a progeroid syndrome (Mostoslavsky et al., 2006). The most striking phenotype observed in SIRT6 knockout mice is a severe hypoglycemia that causes death before 1 month of age (Mostoslavsky et al., 2006). Deletion of SIRT6 in mice triggers an increase in glucose uptake that is preferentially used for glycolysis instead of mitochondrial respiration, a metabolic switch similar to the *Warburg effect* in cancer cells. Indeed, SIRT6 maintains deacetylation of H3K9 in promoters of glycolytic genes, co-repressing Hif1α on these promoters (**Figure 1**). Under conditions of nutrient scarcity, SIRT6 is likely inactivated, causing activation of Hif1α and transcription of glycolytic genes (Zhong et al., 2010). These results support a model whereby SIRT6 works as a sensor of nutrient availability and as a critical modulator of glucose homeostasis.

In addition to its metabolic functions, SIRT6 knockout cells exhibit hypersensitivity to DNA-damaging agents and genomic instability. According to the selective sensitivity among the spectrum of DNA damage agents tested, SIRT6 was proposed to be an important regulator of base excision repair (BER), a mechanism that repairs the damage of single bases in the DNA (Mostoslavsky et al., 2006). Interestingly, in human cells, SIRT6 appears to regulate repair of DNA DSBs as well. Together with ATM and ATR, DNA-dependent protein kinase (DNA-PK) is a sensor of DSBs, promoting DNA repair by NHEJ. SIRT6 stabilizes DNA-PK at DSBs promoting repair (McCord et al., 2009). Notably, HR is prompted by the resection of a DSB through the action of different proteins like CtIP and PARP1, in turn generating single-stranded DNA that will invade the homologous strand for repair. SIRT6 has been shown to bind to DSBs and deacetylate CtIP. Although the mechanism remains unclear, deacetylation of CtIP would enhance the resection of the DSB, promoting HR (Kaidi et al., 2010). In human cancer cell lines, SIRT6 deacetylation of H3K9 at telomeres promotes the stable association of WRN protein at these regions, critical for the processing of telomeres during S phase. Thus, SIRT6 downregulation leads to telomere dysfunction and premature senescence, as in Werner Syndrome cells (Michishita et al., 2008). Finally, recent studies have demonstrated that upon oxidative stress, SIRT6 associates with PARP1 at the DSBs and stimulates its activity through ADP-ribosylation. PARP1 activity will promote NHEJ as well as HR repair (Mao et al., 2011). This evidence suggests an additional mechanism for SIRT6-mediated genome protection during stress. While studies *in vitro* have implicated SIRT6 in several DNA repair pathways (**Figure 1**), a protective role for SIRT6 *in vivo* remains as yet poorly understood, and future genetic studies in mice will be needed to obtain a clearer picture.

SIRT3

SIRT3 works as the major protein deacetylase within the mitochondrial matrix (Lombard et al., 2007) (**Figure 1**). In low nutrient conditions, SIRT3 stimulates alternative pathways for

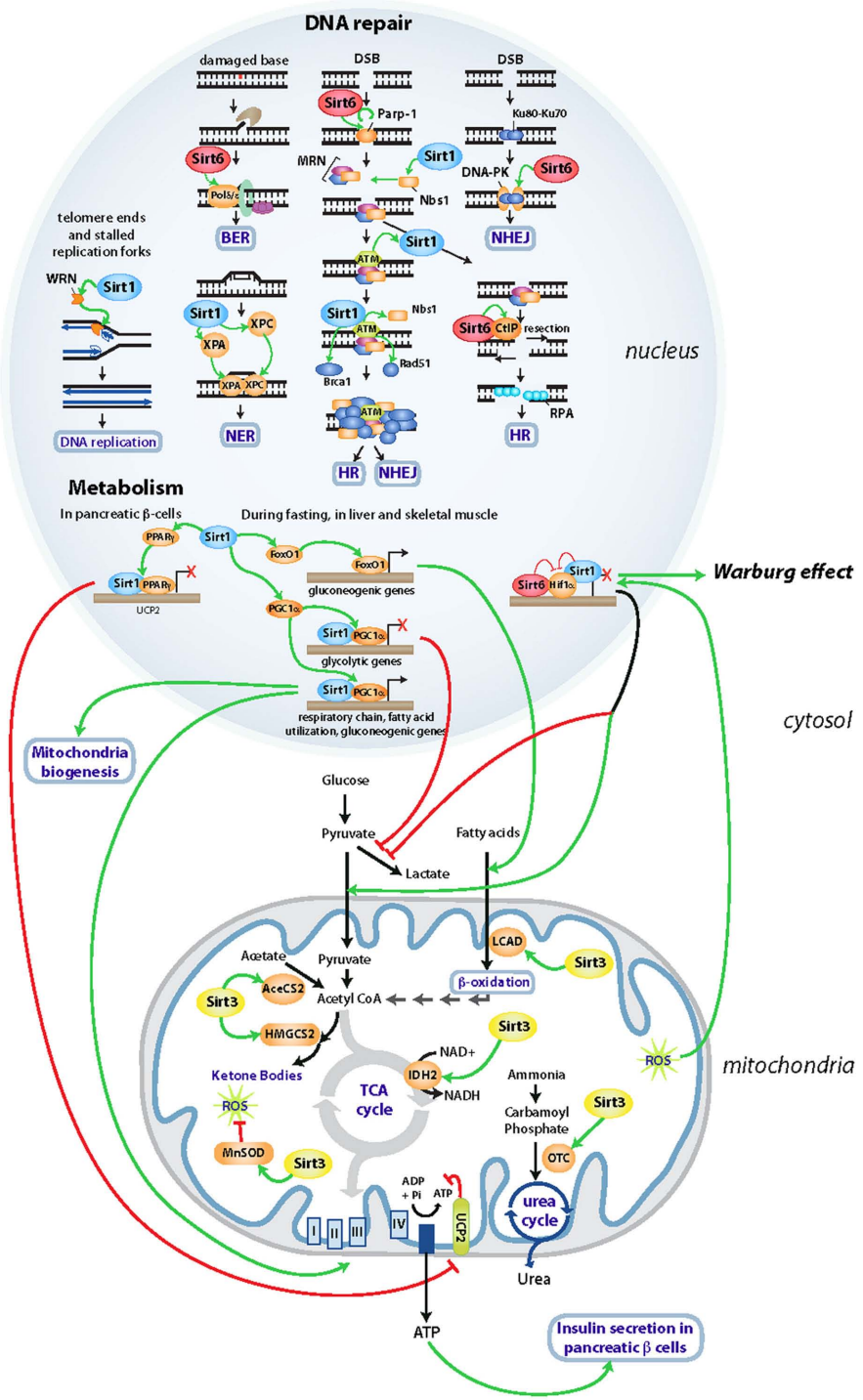

FIGURE 1 | Sirtuins' regulation of DNA repair and metabolism. Diagram indicating the main DNA repair and metabolic pathways regulated by SIRT1, SIRT3, and SIRT6 in the nucleus and in the mitochondria. Activating and inhibitory functions are depicted in green and red, respectively.

energy production in the mitochondria. On one hand, SIRT3 functions as a positive modulator of fatty-acid oxidation through deacetylation of long-chain acyl coenzyme A dehydrogenase (LCAD; Hirschey et al., 2010). In parallel, SIRT3 helps to detoxify the ammonia generated during amino acid catabolism promoting the urea cycle through the deacetylation of ornithine transcarbamoylase (OTC), the enzyme that catalyzes the second step in the urea cycle (Hallows et al., 2011). Finally, it promotes acetate

recycling by activating acetyl-CoA synthetase 2 (AceCS2), the enzyme that converts acetate into acetyl-CoA, that can now enter the TCA cycle (Hallows et al., 2006; Schwer et al., 2006). Under fasting, low-carbohydrate diet as well as in diabetic patients, ketone bodies produced in the liver are used by the brain and skeletal muscle as main energy sources. SIRT3 promotes ketone body production from fatty-acid oxidation by deacetylating 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) (Shimazu et al., 2010) (**Figure 1**).

Although SIRT3 is mainly mitochondrial, and therefore no role in protection of the genome could be presumed for this sirtuin, SIRT3-deficient MEFs exhibit increased cellular superoxide levels and chromosomal instability when stressed (Kim et al., 2010). In fact, metabolic functions of SIRT3 are closely coupled with a protective role against oxidative damage. SIRT3 decreases mitochondrial reactive oxygen species (ROS) generated upon cellular stress by regulating the expression and activity of MnSOD, the main scavenger of superoxide in the mitochondria (Kim et al., 2010; Qiu et al., 2010; Tao et al., 2010; Chen et al., 2011) (**Figure 1**). This seems to play a key role in the cellular response against calorie restriction, where SIRT3 reduces oxidative stress by enhancing glutathione antioxidant defense mechanisms through deacetylation of Idh2 (Someya et al., 2010). Moreover, SIRT3 regulates the electron transport chain Complex III, that can direct ROS out of the mitochondria and into both the matrix and the cytoplasm (Kim et al., 2010). In this manner, SIRT3 management of ROS from mitochondria at different levels might protect the cell from oxidative damage, in turn inhibiting genomic instability.

The aforementioned roles of sirtuins in DNA repair, together with their ability to regulate both glucose homeostasis and ROS levels suggest the possibility that these sirtuins may act as putative tumor suppressors, as discussed in detail below.

SIRTUINS IN CANCER

The first evidences linking SIRT1 and tumorigenesis came from two cell-based studies, which uncovered the ability of SIRT1 to deacetylate and inhibit the tumor suppressor p53 (Luo et al., 2001; Vaziri et al., 2001). In this manner, SIRT1 promotes cell survival under stress conditions by specifically repressing p53 dependent apoptosis. However, as we already mentioned, an increase in SIRT1 associates with enhanced DNA repair, so this phenomena could also be a protecting mechanism to give priority to repair over apoptosis. SIRT1 regulation of p53 was confirmed *in vivo* in SIRT1 knockout mice that exhibit hyperacetylation of p53 and increased apoptosis of thymocytes after DNA damage (Cheng et al., 2003). In this context, it would be interesting to explore if the enhancement of p53 activity could as well be secondary to increased DNA damage in the absence of SIRT1. In addition, SIRT1 knockout mice did not show enhanced p21 induction or sensitivity upon DNA damage, suggesting that only the apoptotic function of p53 is regulated by SIRT1 (Cheng et al., 2003). Although SIRT1-mediated deacetylation of p53 has been confirmed by other groups, the biological relevance is a matter of debate, since alterations in p53 biological activities have not been observed in the absence of SIRT1 *in vivo* (Kamel et al., 2006). Thus, the role for SIRT1 on p53-mediated tumor suppression still remains to be elucidated. In humans, SIRT1 expression is higher in several types of cancer, including acute myeloid leukemia, primary colon cancer, prostate cancers, and non-melanoma skin cancers (reviewed in Deng, 2009), suggesting again a potential role of SIRT1 as an oncogene. This result is also true for several cell lines. Depletion of SIRT1 induces growth arrest and reduces drug resistance of cancer cells *in vitro* (Ota et al., 2006; Liang et al., 2008) and increases tumor cell death with no toxic effects in normal cells in culture (Ford et al., 2005) proposing SIRT1 as a potential target in cancer therapy. Also, overexpression or activation of SIRT1 promotes cellular proliferation, impairs cellular senescence and increases growth rate via ERK/S6K1 signaling pathway (Huang et al., 2008). SIRT1 overexpression also represses epigenetically the activity or expression of tumor suppressor and DNA repair genes including FOXO family members (FOXO1, FOXO3a, and FOXO4) (Motta et al., 2004), p73 (Dai et al., 2007), Rb (Wong and Weber, 2007), MLH1 (Pruitt et al., 2006), and Ku70 (Cohen et al., 2004). Reciprocally, the activity or expression of SIRT1 is inhibited by at least two tumor suppressor genes; the transcriptional repressors HIC1 (hypermethylated in cancer-1) and DBC1 (deleted in breast cancer-1; Chen et al., 2005; Kim et al., 2008; Zhao et al., 2008).

The current data available for SIRT1 expression in cancer contrasts with previous hypothesis pointing toward a role for SIRT1 as a tumor suppressor. Although a variety of cancer cell lines show SIRT1 overexpression, SIRT1 expression is reduced in different human cancers, like glioblastoma, bladder carcinoma, prostate carcinoma, and ovarian cancer (Wang et al., 2008). Moreover, an increase in SIRT1 levels has been shown to inhibit growth of BRCA mutant tumors *in vivo*. Thus far, the data from cell-based studies do not clear up whether SIRT1 acts as an oncogene or a tumor suppressor. Only the generation of mice with genetically modified SIRT1 is finally shedding some light on this matter.

The studies of cancer in mouse models of SIRT1 support the idea that SIRT1 has, indeed, a tumor suppressor activity. In cancer prone mouse models, like $p53^{+/-}$ and $APC^{+/min}$ mice, SIRT1 expression protects from tumor development. Increased expression of SIRT1 has been shown to protect from ionizing radiation-induced cancer in $p53^{+/-}$ mice (Oberdoerffer et al., 2008) whereas $SIRT1^{+/-}$ $p53^{+/-}$ mice show an earlier onset of tumor development in comparison to $SIRT1^{+/+}$ $p53^{+/-}$ littermates (Oberdoerffer et al., 2008). It has been proposed that SIRT1 relocalization to DNA breaks represses transcription of these regions and promotes repair (Oberdoerffer et al., 2008), protecting from genome instability. A different mechanism underlies SIRT1 protection from intestinal tumors in the $APC^{+/min}$ mouse, where SIRT1 expression promotes the cytoplasmic relocalization of the oncogenic form of β-catenin inhibiting its ability to activate transcription and drive cell proliferation (Firestein et al., 2008). Herranz and colleagues demonstrated that SIRT1 overexpression provides strong protection against metabolic syndrome-associated liver cancer by reducing DNA damage and by preventing inflammation and fatty liver. In addition, they demonstrated that a mild increase in SIRT1 levels protects mice from spontaneous and aging-associated cancers (Herranz et al., 2010). Additionally, a recent work demonstrated that overexpression of SIRT1 reduces growth and angiogenesis of xenograft tumors through the interaction and repression of Hif1α (Lim et al., 2010).

Despite the important role of SIRT6 in maintaining genome stability and energy metabolism, it remains as yet unexplored whether SIRT6 plays a role in tumorigenesis. There are no studies

Table 1 | Sirtuins in cancer.

Sirtuin	Cell-based studies and xenografts	Mouse models	Human tumors
SIRT1	• Deacetylates p53 and inhibits apoptosis (Luo et al., 2001; Vaziri et al., 2001) • Overexpression or activation of SIRT1 promotes proliferation, bypasses cellular senescence and increases growth rate via ERK/S6K1 (Huang et al., 2008) • Two tumor suppressors regulate negatively SIRT1: HIC1 and DBC1 (Chen et al., 2005; Zhao et al., 2008; Kim et al., 2008) • Expression of SIRT1 reduces growth and angiogenesis of xenograft tumors (Lim et al., 2010)	• SIRT1-transgenic mouse have reduced incidence of spontaneous and aging related cancers (sarcomas and carcinomas; Herranz et al., 2010) • SIRT1 expression in the intestine in $APC^{+/min}$ mice protects from intestinal tumor formation (Firestein et al., 2008) • $SIRT1^{+/-}$ $p53^{+/-}$ mice show earlier development of sarcomas and lymphomas associated with p53 deficiency (Oberdoerffer et al., 2008)	• Overexpressed in acute myeloid leukemia, colon cancer, prostate cancer, non-melanoma skin cancer (revised in Deng, 2009). • Reduced expression in glioblastoma, bladder carcinoma, prostate carcinoma, and ovarian cancer (Wang et al., 2008)
SIRT3	• Absence of SIRT3 promotes transformation of Ras-infected MEFs (Kim et al., 2010) • Decreases cellular ROS levels by regulating MnSOD (Qiu et al., 2010; Tao et al., 2010)	• SIRT3 knockout mice show higher incidence of spontaneous mammary tumors (Kim et al., 2010)	• Overexpressed in breast cancer and oral squamous cell carcinoma (Ashraf et al., 2006) • Reduced expression in breast cancer (Finley et al., 2011)
SIRT6	• Unknown	• Unknown	• SIRT6 deletions in acute myeloid leukemias (Mahlknecht et al., 2006)

about SIRT6 expression in human cancer either, besides the observation that SIRT6 chromosomal locus is a region prone to chromosomal breaks in human acute myeloid leukemia (Mahlknecht et al., 2006). It has been recently shown that overexpression of SIRT6 induces apoptosis in cancer cell lines but not in non-transformed cells through its ADP-ribosyltransferase activity, suggesting that SIRT6 could be a target for overexpression in cancer therapy (Van Meter et al., 2011).

Increased levels of SIRT3 associate with node-positive breast cancer versus non-malignant breast tissue (Ashraf et al., 2006) as well as with oral squamous cell carcinoma, suggesting that SIRT3 could function as a tumor promoter. However, studies in genetically modified mouse models of SIRT3 question this hypothesis. SIRT3-deficient MEFs are transformed by the expression of a single oncogene (Ras) whereas wildtype MEFs also require inactivation of a tumor-suppressor gene. In addition, loss of SIRT3 *in vivo* results in a mild tumor proneness; higher incidence of spontaneous mammary tumors in 24 months old mice (Kim et al., 2010). How SIRT3 protects from tumorigenesis is a matter of debate. A current hypothesis poses that SIRT3 tumor suppressive role comes from its ability to regulate cellular ROS levels, an idea that fits in the free radical theory that proposes the accumulation of oxidative stress as a major cause of ageing and cancer. Such a model is clearly supported by the above mentioned role for SIRT3 in regulating MnSOD (Kim et al., 2010; Qiu et al., 2010; Tao et al., 2010; Chen et al., 2011). However, the recent observation that SIRT3-deficient cells become highly glycolytic, suggests another possibility (Bell et al., 2011; Finley et al., 2011). SIRT3 loss generates an increase in cellular ROS that would stabilize Hif1α. Stabilization of Hif1α, as it occurs in SIRT6-null cells, will lead to a metabolic reprogramming where cells will prioritize glycolysis over oxidative phosphorylation, a shift that confers metabolic advantages in highly proliferative cells. Therefore, it has been suggested that SIRT3 works as a tumor suppressor by opposing reprogramming of cancer cell metabolism (Bell et al., 2011; Finley et al., 2011), in other words, repressing the *Warburg effect*. According to a tumor suppressive role for this sirtuin, expression of SIRT3 was found significantly reduced in human breast cancer samples (Finley et al., 2011). It remains to be determined whether oxidative stress or glycolytic metabolism plays a dominant role in this phenotype.

SIRTUINS: TUMOR SUPPRESSORS OR ONCOGENES

The above-discussed results suggest that SIRT1 and SIRT3 are tumor suppressor genes. In the multistep development of cancer, loss of SIRT1 or SIRT3 might be a late phenomenon in carcinogenesis allowing the growing tumor to evade apoptosis or acquire different metabolic requirements. Thus, SIRT1 and SIRT3 might be lost in late stages of tumor progression, conferring proliferative advantage to cells with already multiple genetic alterations. In addition, the analysis of updated databases (such as Oncomine and Tumorscape), as well as upcoming results from our laboratory, appears to suggest that SIRT6 is downregulated in tumors, therefore functioning as a tumor suppressor as well (**Table 1**). Much work will be needed to pinpoint the precise molecular mechanisms governing sirtuins' functions in cancer. But one thing is sure: these proteins clearly link DNA repair and metabolism, two hallmarks of cancer. As such, it is tempting to envision that modulators of sirtuin activity could provide future beneficial alternatives against this devastating disease.

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Regulation of sirtuin function by posttranslational modifications

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Sirtuins are homologs of the yeast silencing information regulator 2 protein, an NAD^+-dependent (histone) deacetylase. In mammals seven different sirtuins, SIRT1–7, have been identified, which share a common catalytic core domain but possess distinct N- and C-terminal extensions. This core domain elicits NAD^+-dependent deacetylase and in some cases also ADP-ribosyltransferase, demalonylase, and desuccinylase activities. Sirtuins have been implicated in key cellular processes, including cell survival, autophagy, apoptosis, gene transcription, DNA repair, stress response, and genome stability. In addition some sirtuins are associated with disease, including cancer and neurodegeneration. These findings suggest strongly that sirtuins are tightly controlled and potentially responsive to different signal transduction pathways. Here, we review the posttranslational regulation mechanisms of mammalian sirtuins and discuss their relevance regarding the physiological processes, with which the different sirtuins are associated. The available data suggest that the N- and C-terminal extensions are the targets of posttranslational modifications (PTM) that can affect the functions of sirtuins. Mechanistically this can be explained by the interaction of these extensions with the catalytic core domain, which appears to be controlled by PTM at least in some cases. In contrast little is known about PTM and regulation of the catalytic domain itself. Together these findings point to key regulatory roles of the N- and C-terminal extensions in controlling sirtuin functions, thus connecting these regulators to different signaling pathways.

Keywords: phosphorylation, acetylation, proteolytic cleavage, NAD^+-dependent deacetylation, ADP-ribosylation, sumoylation, methylation

INTRODUCTION

The first paper describing acetylation of histones at lysine residues was published almost 50 years ago (Allfrey and Mirsky, 1964). Although for many years the physiological role of lysine acetylation (Kac) was undefined, the last decade has witnessed a large increase in our knowledge about the consequences of this modification. Acetylation results in the loss of the positive charge at lysines under physiological conditions and affects the chemical appearance of proteins, resulting in altered functional properties (**Figure 1**). These include effects on protein–protein interaction and on the catalytic activity of enzymes among others (reviewed in Kim and Yang, 2011). Parallel to these findings enzymes were discovered that are able to transfer acetyl groups from acetyl-CoA to substrates (K-acetyltransferases, KATs) and enzymes that are able to remove acetyl groups, thus providing evidence that acetylation of lysines is a reversible posttranslational modification (PTM). Besides acetylation, lysines are the target of additional PTMs, including methylation, sumoylation, and ubiquitination, which can compete with each other for one given lysine (**Figure 1**). Besides histones many other proteins have been recognized to be acetylated. These appear to be distributed throughout all cellular compartments. A recent survey using mass spectrometry has identified more than 1700 acetylated proteins in mammals (Kim et al., 2006; Choudhary et al., 2009). Furthermore comparative analyses revealed that acetylation sites are significantly higher conserved than phosphorylation sites (Choudhary et al., 2011). Proteins modified by lysine acetylation control diverse cellular processes such as chromatin remodeling, protein synthesis, cell cycle, nuclear transport, actin nucleation, and mitochondrial metabolism (Choudhary et al., 2011). Therefore it's not surprising that acetylation has been linked to different diseases, including cancer and neurodegeneration. Indeed inhibitors of deacetylases have entered clinics as therapeutic drugs (Marks, 2010). Thus these as well as many other findings demonstrate that acetylation is an important PTM, which participates in controlling key physiological processes in cells.

Based on structural and functional similarities, mammalian deacetylases can be divided into four major classes. Class I HDACs are similar to yeast Rpd3 and contain HDAC1, -2, -3, and -8, whereas class II HDACs are homologs of yeast Hda1 and include HDAC4, -5, -6, -7, -9, and -10. Sirtuins which are related to yeast silent information regulator 2 (Sir2) are also termed class III HDACs, while HDAC11 forms the class IV on its own (reviewed in De Ruijter et al., 2003; Haigis and Sinclair, 2010). The sirtuins are the subject of this review. The sirtuin protein family was founded by the yeast Sir2 (Brachmann et al., 1995). The initial findings from genetic studies suggested that Sir2 controls chromatin and gene expression. In particular Sir2 was identified to participate in silencing of homothallic mating (HM) loci and telomeric

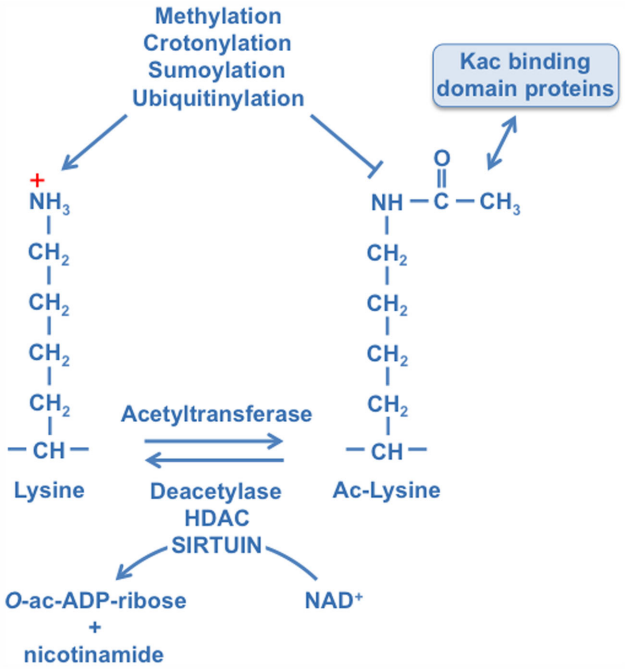

FIGURE 1 | Lysines are targeted by multiple posttranslational modifications. Acetylation is a reversible PTM that is controlled by acetyltransferases and deacetylases. These enzymes transfer acetyl groups from acetyl-CoA to lysine residues with loss of the positive charge. Acetylated lysine residues provide docking sites for proteins that possess a Kac interaction domain, e.g., bromodomains. Lysines can also be modified by a number of additional PTMs as indicated. These modifications compete with each other, thus acetylation can potentially interfere with these other PTMs. The removal of acetyl groups is catalyzed by HDAC and sirtuin deacetylases. Sirtuins are NAD^+-dependent enzymes that transfer the acetyl group onto ADP-ribose under release of nicotinamide. This results in the generation of *O*-acetyl-ADP-ribose, a molecule with second messenger properties.

chromosomal regions and to interfere with rDNA recombination (Gottlieb and Esposito, 1989; Braunstein et al., 1993). These functions have obtained wide interest because they have been linked to lifespan regulation. Indeed overexpression of Sir2 was shown to increase lifespan in yeast (Kaeberlein et al., 1999). Although similar effects were reported for *Drosophila melanogaster* and *Caenorhabditis elegans* (Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004), more recent findings suggest that Sir2 does not affect longevity in these organisms (Burnett et al., 2011). While for many years the molecular base of the observations made in yeast was undefined, a break-through finding was the description of Sir2 as a nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase (Imai et al., 2000). Sir2 and Sir2-like proteins hydrolyze one NAD^+ for each acetyl group removed from a substrate, with release of the nicotinamide moiety (**Figure 1**; Landry et al., 2000). The acetyl group is transferred to ADP-ribose to form a novel *O*-acetyl-ADP-ribose product (Tanner et al., 2000), which has been suggested to function as a second messenger (Tong and Denu, 2010). Very recently it was shown that SIRT5 can also remove acyl-groups from malonylated or succinylated substrate peptides, thereby forming *O*-malonyl-ADP-ribose or *O*-succinyl-ADP-ribose, respectively (Du et al., 2011; Peng et al., 2011). Additionally, distinct sirtuins (SIRT4 and SIRT6) were reported to catalyze the transfer of ADP-ribose from NAD^+ to substrate proteins (Liszt et al., 2005; Ahuja et al., 2007). These observations provide evidence that some sirtuins may be able to perform more than one biochemical reaction.

Sirtuins are conserved from prokaryotes to mammals and they all share a common core domain comprising approximately 200–275 amino acids. A phylogenetic analysis of the catalytic domains allows subdividing the sirtuins into five classes, i.e., I–IV and U, with the latter only found in Gram-positive bacteria (Frye, 2000). Besides the founding member Sir2, the yeast *Saccharomyces cerevisiae* expresses four additional sirtuins, which are termed "homologs of sir two" (Hst1–4). Seven human sirtuins have been identified so far, which can be grouped into four of the phylogenetic classes: SIRT1, SIRT2, and SIRT3 belong to class I, SIRT4 to class II, SIRT5 to class III, and SIRT6 and SIRT7 to class IV (Frye, 2000). Of these, SIRT1 shares the highest sequence similarity with yeast Sir2 and Hst1, and SIRT2 and SIRT3 with Hst2 (North and Verdin, 2004). SIRT4 to SIRT7 are more closely related to prokaryotic sirtuins or sirtuins of *D. melanogaster* and *C. elegans*.

The mammalian sirtuins are localized in different subcellular compartments. While SIRT1, SIRT6, and SIRT7 are predominantly found in the nucleus, albeit with different subnuclear distributions, SIRT3, SIRT4, and SIRT5 are mitochondrial. SIRT2 is the only human sirtuin, which is primarily localized in the cytoplasm (Michishita et al., 2005).

The findings summarized above, including the observations on longevity in yeast, the role of NAD^+ as cofactor, and the localization of the different sirtuins to distinct subcellular compartments, notably the mitochondria, suggested early on that sirtuins might have fundamental roles in metabolism. Indeed Sir2 is mediating at least in part the effects elicited by caloric restriction (reviewed in Lu and Lin, 2010). Of note is also that sirtuins in higher organisms have been suggested to contribute to longevity (reviewed in Guarente, 2011). Moreover the findings that sirtuins carry out NAD^+-dependent reactions suggest an involvement of these enzymes in mammalian metabolic control and offer the possibility for modulation of their activity by small molecules. The involvement of sirtuins in many physiological processes (see also below) suggests that these enzymes themselves are most likely controlled by different signaling pathways in response to both extracellular and intracellular cues. The use of NAD^+ must be controlled because of its central function in metabolic pathways, suggesting that enzymes that consume NAD^+ will most likely be part of feedback control mechanisms of such pathways. In addition the enzymatic processes of deacetylation and of ADP-ribosylation need to be regulated to adjust for optimal, functionally relevant levels of substrate acetylation and ADP-ribosylation. Despite the many reasons for posttranslational control of sirtuin function, we know relatively little about such mechanisms. The available evidence suggests that the N- and C-terminal extensions relative to the catalytic core domains of the seven mammalian sirtuins are targets of PTMs, while hardly any information is available on how the catalytic domain itself is controlled (**Figure 2**). Here we summarize

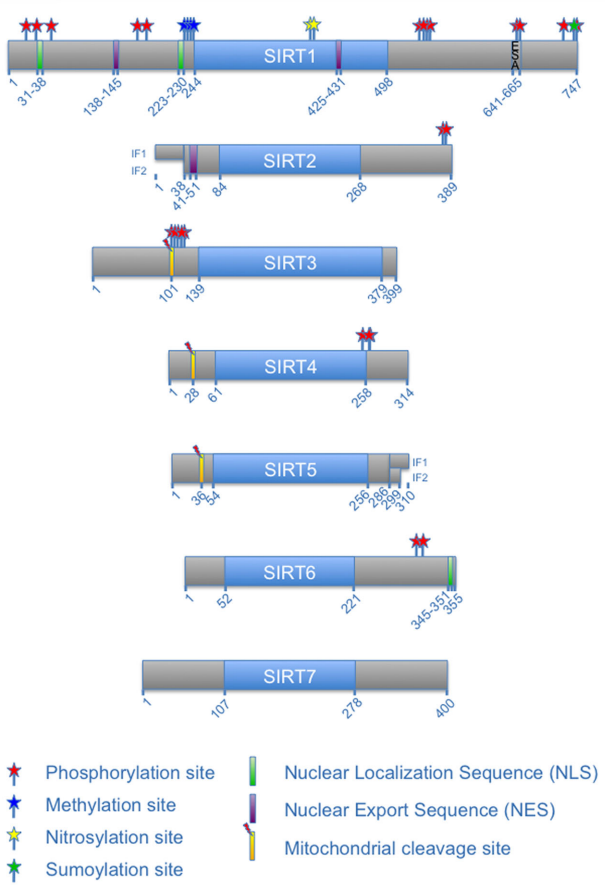

FIGURE 2 | Schematic overview of human sirtuins and their PTMs. The seven mammalian sirtuins are schematically indicated with the blue boxes depicting the sirtuin-typic catalytic core domain. The catalytic domains are flanked by distinct N- and C-terminal extensions (gray boxes). The numbers below indicate amino acid numbers for orientation. Two isoforms (IF) are shown for SIRT2 and SIRT5, respectively. The ESA ("essential for SIRT1 activity") sequence of SIRT1 (see below) is indicated. PTMs, nuclear localization sequences, nuclear export sequences, and proteolytic cleavage sites are indicated. The precise amino acids modified by the different PTMs are given in **Table 1**.

what has been learned about the regulation of mammalian sirtuins by PTMs.

SIRTUINS AND THEIR REGULATION BY PTMs

All sirtuins share a common catalytic domain, which binds NAD^+. In contrast to this conserved core domain, the enzymes differ in sequence and length of their C- and N-terminal extensions. These are well suited to participate in the regulation of sirtuins and indeed most PTMs that have been identified to date target these extensions (**Figure 2**). Several scenarios can be imagined. The extensions may communicate with the catalytic domain, thereby controlling the activity of sirtuins. For example the C-terminal extension of yeast Hst2 interacts with the NAD^+-binding region, while the N-terminal region of Hst2 engages with the Kac substrate binding site, suggesting different modes of autoregulation (Zhao et al., 2003). Furthermore the N-terminal extension has been suggested to function in trimer formation, which might influence enzymatic activity. Together these findings indicate a more general role of these C- and N-terminal sequences in the regulation of sirtuin function.

SIRT1

Among the seven human sirtuins, SIRT1 shares the highest sequence homology with yeast Sir2 (Voelter-Mahlknecht and Mahlknecht, 2006). In addition SIRT1, similar to its ancestor Sir2, is primarily localized in the nucleus and involved in chromatin remodeling as it deacetylates several lysine residues of histones, including acetylated lysines 9 of histones H3 (H3K9ac), H3K14ac, H4K16ac, and H1K26ac (Vaquero et al., 2004). Moreover SIRT1 targets also non-histone proteins and its activity can be regulated by its ability to shuttle between nuclear and cytoplasmic compartments (Tanno et al., 2007; Hisahara et al., 2008). The increasing number of known SIRT1 substrates includes the transcription factor and tumor suppressor p53 as well as several other transcriptional regulators and cofactors, among them NF-κB, members of the forkhead family (FOXOs), peroxisome proliferator-activated receptors (PPAR), and p300 (reviewed in Rahman and Islam, 2011). Molecular studies revealed that SIRT1 is involved in the regulation of diverse cellular processes ranging from lipid and glucose metabolism to aging and stress response. Of particular relevance for many of these processes is the AMP-activated protein kinase (AMPK)-SIRT1 signaling axis. AMPK is activated in response to increasing amount of AMP and thus functions as an energy sensor that responds to cellular metabolic stress, including calorie restriction (reviewed in Fulco and Sartorelli, 2008). Maybe not surprising then is the finding that *Sirt1* knockout mice have a high prenatal or early postnatal death rate (Cheng et al., 2003; McBurney et al., 2003).

With the identification of the tumor suppressor p53 as a SIRT1 substrate, a role of this enzyme in tumor formation was postulated (Luo et al., 2001; Vaziri et al., 2001; Langley et al., 2002). Upon deacetylation by SIRT1, the activity of p53 is reduced and thus SIRT1 appears to function as an oncoprotein (Chen et al., 2005; Kim et al., 2007; Yuan et al., 2011). However, there are also reports that describe SIRT1 as a tumor suppressor (Yi and Luo, 2010). These alternative activities are possibly the result of cell-type specific effects and/or a consequence of distinct regulation of SIRT1 that might differentially affect the activities of substrates.

SIRT1 is by far the largest human sirtuin with 747 amino acids due to its extensive N- and C-terminal extensions (**Figure 1**). The N-terminal extension of SIRT1 contains two functional nuclear localization sequences (NLS) and two nuclear export sequences (NES). These are responsible for the nucleo-cytoplasmic shuttling of SIRT1 (Tanno et al., 2007), which determines at least in part the enzyme's ability to interact with distinct substrates (Hisahara et al., 2008). Furthermore the nuclear-cytoplasmic distribution of SIRT1 is regulated by signals, for example during differentiation (Tanno et al., 2007). While SIRT1 is nuclear in proliferating C2C12 myoblasts, it is cytoplasmic in differentiated cells. Moreover inhibition of PI3K prevents the nuclear localization of SIRT1 in proliferating cells, suggesting that PI3K-dependent signaling controls the shuttling. Whether the PI3K signaling cascade targets directly SIRT1 or some accessory factor or factors is

not known. One kinase that might be involved in this process is JNK, although this kinase is not typically activated downstream of PI3K. JNK interacts with SIRT1 upon oxidative stress, phosphorylates SIRT1 at Ser27, Ser47, and Thr530, thereby enhancing its nuclear localization (Nasrin et al., 2009). Furthermore these phosphorylations increase the enzymatic activity of SIRT1 in a substrate-specific manner with histone H3, but not p53, becoming a better substrate. In contrast to the findings with JNK, mTOR-dependent phosphorylation of Ser47 alone results in inhibition of SIRT1 deacetylase activity (Back et al., 2011). Thus combinatorial effects of different phosphorylations appear to control SIRT1 function.

Using a mass spectrometry approach, 13 phosphorylation sites were identified in SIRT1 (Sasaki et al., 2008). Seven of these sites are located in the N-terminal region, including Ser27 and Ser47, and six in the C-terminal region, including Thr530 (**Figure 2**). Two of the identified sites, Thr530 and Ser540, are potential substrates of cyclin B/cyclin-dependent kinase 1 (CDK) complexes. The functional analysis suggests that these two phosphorylation sites are required for normal cell cycle progression. For example, while wild-type SIRT1 rescues the growth defect of cells lacking endogenous Sirt1, a mutant, in which Thr530 and Ser540 are substituted by alanines, is unable to rescue the knockout cells (Sasaki et al., 2008).

In addition to the sites mentioned above, four protein kinase CK2 phosphorylation sites have been identified in murine Sirt1. These are Ser154, Ser649, Ser651, and Ser683 in the N- and C-terminal extensions (Kang et al., 2009). Two of these sites have been described in human SIRT1 at the corresponding amino acids Ser659 and Ser661 (Zschoernig and Mahlknecht, 2009). It has been suggested that phosphorylation by CK2 stimulates catalytic activity of Sirt1 and its ability to interact with p53, one of its substrates (Kang et al., 2009). Whether all four CK2 sites are required for the observed effects remains to be determined. Of note is that Ser659 and Ser661 lie within a region of SIRT1 that is referred to as the ESA (essential for SIRT1 activity) motif (**Figure 3**). This spans a small region from amino acids 641–665 in human SIRT1 (Kang et al., 2011). The ESA interacts with the catalytic domain, activates the catalytic activity, and increases the affinity for substrates. Moreover the binding site for ESA in the catalytic domain is also the interaction site of DBC1, an endogenous SIRT1 inhibitor (Kim et al., 2008). The two CK2 phosphorylation sites flank one of the two identified key residues within ESA that are important to control catalytic activity (**Figure 3**). Thus it is well possible that these phosphorylation sites modulate the interaction of the C-terminal region with the catalytic domain and therefore are potentially of considerable functional relevance. One possible explanation is that the phosphorylation of the two sites within ESA regulates

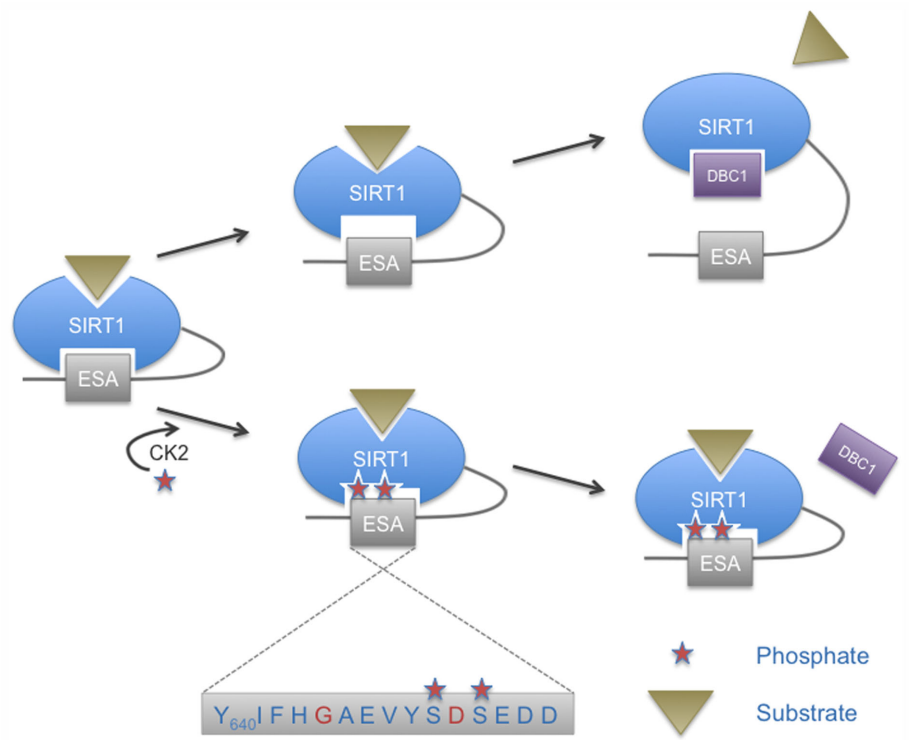

FIGURE 3 | Control of SIRT2 catalytic activity by the ESA motif and regulation by phosphorylation. Two CK2 phosphorylation sites lie within the essential for SIRT1 activity (ESA) sequence motif found in the C-terminal extension of SIRT1. These phosphorylation sites flank one of the two key residues of the ESA motif (indicated in red). CK2-mediated phosphorylation is proposed to enhance the interaction of ESA with the catalytic core, thereby increasing SIRT1 affinity for substrates and enhancing catalytic activity. The ESA motif competes with DBC1 binding, a negative regulator of SIRT1. Enhanced interaction of ESA with the core domain of SIRT2 in response to CK2 phosphorylation would prevent binding of DBC1 and thus abrogate its inhibitory effect.

SIRT1 activity and substrate recognition by modulating the interaction of the ESA with the catalytic domain. This might affect the catalytic center and substrate binding through an allosteric mechanism. Additionally it might affect binding of DCB1, which is a SIRT1 inhibitor. Thus the control of SIRT1 function by its own C-terminal domain and the regulation of this interaction by CK2, although molecularly not fully explored yet, may represent an important regulatory mechanism (**Figure 3**).

Besides phosphorylation, SIRT1 is modified by additional PTMs, including sumoylation. SUMO, a small ubiquitin-related modifier, can be attached *in vitro* close to the C-terminal end of SIRT1 at Lys734, which lies within a sumoylation consensus sequence (ΨKXE). This modification increases catalytic activity as measured by p53 deacetylation (Yang et al., 2007). Upon stress SIRT1 associates with the nuclear desumoylase SENP1, which reduces the catalytic activity of SIRT1 and consequently allows efficient activation of p53. How modification by SUMO stimulates the catalytic activity of SIRT1 is not known. However the recent findings that the C-terminal region is key to enhance SIRT1 activity suggests that sumoylation may participate in this regulation. A possibility is that sumoylation enhances the interaction of the ESA motif with the catalytic domain or modifies CK2 phosphorylation (**Figure 3**). But clearly other explanations are also possible, including the subnuclear relocalization of sumoylated SIRT1, which might affect accessibility to substrates, or allosteric effects of the sumoylation (reviewed in Wilkinson and Henley, 2010). Thus it appears that sumoylation of SIRT1 is relevant for stress control in cells.

Furthermore, SIRT1 is also targeted by methylation. The methyltransferase Set7/9 interacts with and methylates SIRT1 at Lys233, Lys235, Lys236, and Lys238 of the N-terminal extension. Although it is unclear whether methylation affects directly SIRT1 deacetylase activity, the interaction of Set7/9 with SIRT1 disrupts the binding of SIRT1 with p53. Consequently p53 acetylation and transactivating activity is enhanced (Liu et al., 2011). Despite the lack of information about direct consequences of lysine methylation, it is worth remembering that lysines can be modified by multiple PTMs and thus methylation may compete with acetylation and ubiquitination (**Figure 1**; reviewed in Yang and Seto, 2008).

Recently it was reported that nuclear SIRT1 is transnitrosylated by nitrosylated GAPDH (Kornberg et al., 2010). As a consequence acetylation of PGC-1α, a SIRT1 substrate, increases in cells, suggesting that SIRT1 deacetylation activity is inhibited by nitrosylation. Mutational analysis implies that two cysteines, Cys387 and Cys390, within the catalytic core of SIRT1 are targeted by nitrosylation. These cysteines are of special interest because they participate in the coordination of a structurally relevant Zn^{2+} ion and nitrosylation might result in protein misfolding (Kornberg et al., 2010). It will be of interest to define whether nitrosylation is a general regulatory mechanism of sirtuins.

Similar to yeast Hst2, purified endogenous SIRT1 can exist as a homotrimer (Zhao et al., 2003; Vaquero et al., 2004). Because SIRT2 was also purified as a homotrimer (Vaquero et al., 2006), this structural organization may be characteristic for sirtuins. Structural analysis of the Hst2 trimer suggests that the N-terminal region is involved in trimer formation. Whether this is also true for SIRT1 and SIRT2 remains to be determined. Because of the many PTMs that have been mapped in the N-terminal extension of SIRT1 (**Table 1**), it is well possible that trimer formation is regulated by signaling, an aspect that needs further exploration.

SIRT2

SIRT2 is ubiquitously expressed and the only sirtuin, which is predominantly localized in the cytoplasm (Afshar and Murnane, 1999; Michishita et al., 2005; Voelter-Mahlknecht et al., 2005). SIRT2 was purified as a homotrimer out of cell extracts similar to SIRT1 and Hst2 (see above; Vaquero et al., 2006). It is not known how trimer formation is regulated and what the consequences are for SIRT2 function. Human SIRT2 is expressed in at least two isoforms. The longer SIRT2 variant 1 consists of 389 amino acids whereas variant 2 lacks the first 37 N-terminal amino acids, thus being comprised of 352 amino acids (**Figure 2**). The catalytic domain of SIRT2 variant 1 and 2 is located between amino acids 84–268 and 47–231, respectively (Voelter-Mahlknecht et al., 2005). The predominant cytoplasmic localization of SIRT2 is dictated by an NES in its N-terminal extension (amino acids 41–51 and 4–14 of the long and short protein variants, respectively; Wilson et al., 2006; North and Verdin, 2007a). In the cytoplasm, SIRT2 colocalizes at least in part with the microtubule network. Consistent with this finding is that Lys40 of α-tubulin is a SIRT2 substrate (North et al., 2003). Moreover, SIRT2 can also translocate into the nucleus and a predominant nuclear or chromatin-associated SIRT2 localization is detected during G2/M transition and in mitosis of the cell cycle (Vaquero et al., 2006; North and Verdin, 2007a). It is unclear how SIRT2 translocates into the nucleus because the protein lacks any obvious NLS. Nuclear substrates of SIRT2 include H4K16ac and H3K56ac, modifications that are implicated in DNA damage response and cancer (Vaquero et al., 2006; Vempati et al., 2010). Also it remains to be defined how the cell cycle-dependent nuclear localization is regulated. One possibility is that signals in late G2 control the activity of the NES. Indeed in chemically synchronized cells SIRT2 is hyperphosphorylated during the G2/M transition and in M phase, which is paralleled by a mobility shift in SDS-PAGE (Dryden et al., 2003; North and Verdin, 2007b). This correlates with the nuclear translocation of SIRT2 and suggests a regulatory role for Cyclin B/CDK1 and other mitosis-specific kinases (reviewed in Morgan, 2008). But alternative mechanisms may also be in place, such as stimulation of nuclear uptake and/or tight interaction with nuclear structures in late G2 or mitosis.

Particularly high SIRT2 protein expression in the brain is found in myelin-forming oligodendrocytes, correlating with the expression profiles of the differentiation markers CNPase (2′,3′-cyclic nucleotide 3′-phosphodiesterase) and MBP (myelin basic protein; Li et al., 2007; Southwood et al., 2007; Werner et al., 2007). Interestingly only the shorter variant 2 is present in the myelin-enriched fraction of adult mouse brain or in the cytoplasm of murine cerebellar granule cells (Suzuki and Koike, 2007; Werner et al., 2007), suggesting that the N-terminal region is involved in controlling the subcytoplasmic localization.

SIRT2 positively regulates the transcription factor sterol response element binding protein 2 (SREBP-2) thereby promoting cholesterol biosynthesis in neurons (Luthi-Carter et al., 2010). Cholesterol influences membrane thickness and fluidity

Table 1 | Summary of posttranslational modification sites of human sirtuins.

Sirtuin	Target site	Type of modification	Modifier	Source
SIRT1	Ser14	P		Sasaki et al. (2008)
	Ser26	P		Sasaki et al. (2008)
	Ser27	P	JNK	Nasrin et al. (2009), Sasaki et al. (2008)
	Ser47	P	JNK, mTOR	Nasrin et al. (2009), Back et al. (2011), Sasaki et al. (2008)
	Ser172	P		Sasaki et al. (2008)
	Ser173	P		Sasaki et al. (2008)
	Lys233	Me	Set7/9	Liu et al. (2011)
	Lys235	Me	Set7/9	Liu et al. (2011)
	Lys236	Me	Set7/9	Liu et al. (2011)
	Lys238	Me	Set7/9	Liu et al. (2011)
	Cys387	NO	GAPDH	Kornberg et al. (2010)
	Cys390	NO	GAPDH	Kornberg et al. (2010)
	Thr530	P	JNK, CycB/CDK1	Nasrin et al. (2009), Sasaki et al. (2008)
	Ser540	P	CycB/CDK1	Sasaki et al. (2008)
	Ser659	P	CK2	Zschoernig and Mahlknecht (2009)
	Ser661	P	CK2	Zschoernig and Mahlknecht (2009)
	Ser719	P		Sasaki et al. (2008)
	Lys734	Sumo	SENP1	Yang et al. (2007)
	Ser747	P		Sasaki et al. (2008)
SIRT2	Ser368	P	CycB/CDK1, CycE/CDK2, CycA/CDK2, CycD3/CDK4, p35/CDK5	North and Verdin (2007b); Pandithage et al. (2008)
	Ser372	P		Nahhas et al. (2007)
		Ac	p300	Han et al. (2008)
SIRT3	Ser101	P		Olsen et al. (2010)
	Ser103	P		Olsen et al. (2010)
	Ser105	P		Olsen et al. (2010)
	Ser114	P		Olsen et al. (2010)
	Ser117	P		Olsen et al. (2010)
	Ser118	P		Olsen et al. (2010)
SIRT4	Ser255	P		Yu et al. (2007)
	Ser261	P		Yu et al. (2007)
	Ser262	P		Yu et al. (2007)
SIRT6		ADPr	SIRT6	Liszt et al. (2005)
	Tyr294	P		Dephoure et al. (2008)
	Ser303	P		Dephoure et al. (2008)

*For an overview of the localization of the different modifications relative to other recognizable elements and domains of human sirtuins see **Figure 2**. Ac, acetylation; ADPr, ADP-ribosylation; Me, methylation; NO, nitrosylation; P, phosphorylation; Sumo, sumoylation; JNK, JUN N-terminal kinase; Cyc, cyclin; CDK, cyclin-dependent kinase. Note that amino acid numbers for SIRT2 refer to the longer isoform 1 (IF1 in **Figure 2**).*

and is essential for myelin membrane growth (Saher et al., 2005). However, cholesterol is also reported to have a detrimental effect in neurons and presents a risk factor in neurodegenerative diseases like Alzheimer's (AD) and Parkinson's diseases (PD; reviewed in Stefani and Liguri, 2009; Huang et al., 2011). Consistent with these findings, SIRT2 inhibition reduces toxicity of mutant huntingtin by decreasing sterol biosynthesis (Luthi-Carter et al., 2010). Similarly SIRT2 knockdown or SIRT2 inhibition decreases α-synuclein toxicity, a protein frequently mutated in and associated with PD (Outeiro et al., 2007). Together these findings implicate SIRT2 in the control of toxicity resulting from aggregation-prone proteins in both neurons and oligodendrocytes.

Beside SREBP-2, SIRT2 is also involved in the regulation of other transcription factors, including NF-κB (Rothgiesser et al., 2010), FOXO1 (Jing et al., 2007; Zhao et al., 2010), and FOXO3 (Wang et al., 2007). Thus, through modulating these transcriptional regulators, SIRT2 affects most likely multiple cellular processes, such as signaling, gene expression, and autophagy.

To date two phosphorylation sites have been identified in SIRT2 (**Figure 2** and **Table 1**). They are located in the C-terminal extension in close proximity to each other at Ser368/331 and Ser372/335 (numbering according to the two translational variants). The phosphorylation of each site results in a mobility shift of the protein on SDS-PAGE, which results in a characteristic triple band

pattern (Nahhas et al., 2007; Pandithage et al., 2008). Ser368/331 is part of a Cyclin/CDK consensus motif and has been demonstrated to be a substrate of Cyclin B/CDK1, Cyclin E/CDK2, Cyclin A/CDK2, Cyclin D3/CDK4, and p35/CDK5 (North and Verdin, 2007b; Pandithage et al., 2008). Consistent with these *in vitro* studies is the finding that Ser368/331 is phosphorylated when cells enter S phase, suggesting that this phosphorylation is not the signal for nuclear accumulation of SIRT2, which begins in late G2 (Pandithage et al., 2008). Phosphorylation of SIRT2 at Ser368/331 reduces its enzymatic activity as measured by deacetylation of core histones and of α-tubulin (Pandithage et al., 2008). Moreover SIRT2 interferes with neurite outgrowth in primary neurons, correlating with α-tubulin deacetylation, a process that is antagonized by phosphorylating Ser368/331 providing evidence that the C-terminal extension of SIRT2 controls activity in cells (Pandithage et al., 2008). So far, nothing is known about a kinase responsible for phosphorylation of Ser372/335 or about its influence on SIRT2 function.

Phosphorylation of SIRT2 by p35/CDK5 is of special interest because similar to SIRT2, CDK5 is highly expressed in the brain and its protein levels are upregulated in differentiating cells, i.e., oligodendrocytes (He et al., 2011). Moreover CDK5 is capable to interfere with SIRT2 function by phosphorylating Ser368/331 in primary neurons, as mentioned above (Pandithage et al., 2008). Furthermore CDK5 is an important cell cycle suppressor in postmitotic neurons (Cicero and Herrup, 2005). This activity requires nuclear localization of CDK5, which is mediated by an interaction with p27 (Zhang et al., 2008). Upon stress, e.g., induced by β-amyloid expression in an AD model, association between p27 and CDK5 is disrupted resulting in reduced nuclear CDK5 levels (Zhang et al., 2010). An increasing number of publications provide evidence that CDK5 also plays a role in many non-neuronal tissues (reviewed in Lalioti et al., 2010). For example CDK5 is required for the DNA damage response, suggesting that this kinase participates in stress signaling (Turner et al., 2008). Together with the above-summarized studies implicating SIRT2 in stress response in cells of the nervous system the findings suggest that the interaction with and regulation by CDK5 may be part of a stress signaling network.

Additionally SIRT2 is acetylated by the KAT p300. This acetylation, although the site of modification has not been mapped, interferes with the catalytic activity of SIRT2 (Han et al., 2008). Predictions of acetylation sites indicate that the C-terminal extension provides multiple target lysine residues (Li et al., 2006), further supporting the concept that the N- and C-terminal regions are particularly relevant to control catalytic activities of sirtuins.

SIRT3

Three sirtuins are located in mitochondria. Of these SIRT3 is the best studied. It is broadly expressed including brown but not white adipose tissue (Shi et al., 2005). Indeed, SIRT3 is required for PGC-1α-mediated differentiation of brown adipose tissue in an estrogen-related receptor α (ERRα)-dependent manner (Kong et al., 2010; Giralt et al., 2011). The transcriptional coactivator PGC-1α regulates genes involved in energy metabolism, suggesting that SIRT3 participates in this process (Shi et al., 2005). Moreover SIRT3 regulates the cellular response to oxidative stress and calorie restriction. Thus, upon cellular stress, e.g., increase in reactive oxygen species (ROS) or nutrient deprivation, human *SIRT3* transcription is stimulated (Shi et al., 2005; Chen et al., 2011), and the protein translocates to the mitochondrial inner membrane (IMS; Michishita et al., 2005; Scher et al., 2007). There it deacetylates and thereby activates the enzymes isocitrate dehydrogenase 2 (Idh2) and superoxide dismutase 2 (SOD2), which are involved in reducing cellular oxidants, including oxidized glutathione (GSSG) and reactive oxygen species (ROS) (Schlicker et al., 2008; Qiu et al., 2010; Someya et al., 2010; Chen et al., 2011). Recently, it has been discovered that SIRT3 acts as a tumor suppressor. *Sirt3*-deficient mice show increased genomic instability as a result of enhanced superoxide levels. Mouse embryonic fibroblasts (MEFs) of such animals are transformed by a single oncoprotein, i.e., with MYC or RAS (Kim et al., 2010). In addition, SIRT3 activates several key enzymes associated with fatty-acid oxidation (3-hydroxy-3-methylglutaryl-CoA synthase/HMGCS2, Long-chain acyl-CoA dehydrogenase/LCAD, Acetyl-CoA synthetase 2/AceCS2) and the urea cycle (ornithine transcarbamoylase/OTC) (Hallows et al., 2006, 2011; Hirschey et al., 2010; Shimazu et al., 2010). Together these findings suggest that upon caloric restriction SIRT3, in addition to SIRT1, plays a key role in modulating mitochondrial activities and stimulating the use of alternative energy sources by promoting β-oxidation and amino acid catabolism.

The N-terminal extension of SIRT3 contains a mitochondrial targeting signal peptide (**Figure 2**). During import of SIRT3 into the mitochondrial matrix, the protein is proteolytically cleaved at position 101 and thus enzymatically activated (Schwer et al., 2002). It has been postulated that the proteolytically shortened N-terminal region and the C-terminal extension form a module that might regulate the access of substrate proteins to the active site (Schlicker et al., 2008).

Presently we know very little about the regulation of SIRT3 function. The biological significance, as summarized briefly above, would suggest strongly that SIRT3 is regulated by signaling. Indeed, six phosphorylated serine residues (out of a total of eight possible sites) between positions 101 and 118 have been identified in a high-resolution mass spectrometry-based phosphoproteome analysis (**Table 1**) (Olsen et al., 2010). But their biological relevance or influence on SIRT3 function has not been analyzed yet. These phosphorylation sites are close to the mitochondrial cleavage site in the N-terminal extension. Therefore it is possible that phosphorylation modulates the enzymatic activity of SIRT3 in mitochondria either by regulating the proteolytic cleavage, by influencing the interaction between the N- and C-terminal extension, or by regulating the interaction of the N-terminal region with the catalytic domain.

SIRT4

SIRT4 is an additional mitochondrial sirtuin (Michishita et al., 2005; Haigis et al., 2006). It resides as a soluble protein in the mitochondrial matrix (Ahuja et al., 2007; Nakamura et al., 2008). Similar to the other sirtuins, SIRT4 is ubiquitously expressed (Michishita et al., 2005; Haigis et al., 2006; Ahuja et al., 2007). *Sirt4* knockout mice are viable and fertile and did not display apparent phenotypic abnormalities. However these mice exhibit increased insulin levels when compared to wild-type littermates

(Haigis et al., 2006). This anomaly points to a function of SIRT4 in the insulin producing β-cells of the pancreatic islets. Indeed, SIRT4 negatively regulates glutamate dehydrogenase (GDH) via ADP-ribosylation. GDH is a mitochondrial enzyme, which catalyzes the conversion of glutamate to α-ketoglutarate in the tricarboxylic acid (TCA) cycle and induces insulin secretion (Haigis et al., 2006). A second possible explanation for the increased insulin levels in *Sirt4*-deficient mice is that IDE (insulin-degrading enzyme) interacts with SIRT4 (Ahuja et al., 2007). IDE regulates insulin levels and SIRT4 appears to be a negative regulator of this enzyme. Whether this occurs through direct interaction or by ADP-ribosylation has not been determined. It is worth pointing out that so far no deacetylase activity of SIRT4 has been identified. It remains to be determined whether this enzyme is indeed deficient of deacetylase activity or whether this is a reflection of the lack of appropriate substrates.

Similar to SIRT3, SIRT4 possesses a mitochondrial targeting signal in the N-terminal region and is proteolytically cleaved within the N-terminal extension upon entry into the mitochondrial matrix resulting in a 28 amino acids shortened protein (Ahuja et al., 2007; **Figure 2**). It is not known whether the proteolytic cleavage of SIRT4 influences its enzymatic activity, as was reported for SIRT3 (Schwer et al., 2002).

Three phosphorylation sites have been identified in SIRT4 at Ser255, Ser261, and Ser262 in a proteomics approach (Yu et al., 2007). These sites are unique, as far as deduced from the currently available analysis of PTMs of sirtuins, in that they are located within or in close proximity to the catalytic domain (**Figure 2** and **Table 1**). Whether these are functionally relevant has not been determined.

SIRT5

SIRT5, the third mitochondrial sirtuin, is ubiquitously expressed (Michishita et al., 2005; Nakagawa et al., 2009). Very little is known about SIRT5 function. *Sirt5* knockout mice develop inconspicuously until at least 18 months of age (Lombard et al., 2007). However, they exhibit significantly elevated blood ammonia levels compared to wild-type animals after caloric restriction or fasting, which is presumably caused by a deregulated urea cycle. In support SIRT5 can deacetylate and activate the carbamoyl phosphate synthetase 1 (CPS1), a mitochondrial enzyme of the urea cycle (Nakagawa et al., 2009). It has been suggested that elevated mitochondrial NAD^+ levels in response to starvation activate SIRT5 and in turn CPS1 is stimulated and initiates the detoxification of excess ammonia under physiological conditions. In addition to its deacetylase activity SIRT5 was very recently reported to elicit also NAD^+-dependent demalonylase and desuccinylase activities (Du et al., 2011; Peng et al., 2011). In line with this observation CPS1 succinylation at Lys1291 is strongly increased in Sirt5 knockout mice compared to wild-type littermates. Furthermore SIRT5 can *in vitro* deacetylate the mitochondrial IMS protein cytochrome c, which is involved in oxidative metabolism and apoptosis (Schlicker et al., 2008). Up to now the functional relevance of this observation has not been clarified.

SIRT5 is expressed as two distinct transcriptional variants due to alternative splicing, encoding proteins with distinct C-terminal regions (**Figure 2**). Both isoforms can be cleaved after the first 36 amino acids at a consensus sequence for the mitochondrial processing peptidase upon entry into the mitochondrial matrix (Michishita et al., 2005). Similar to SIRT4 no data are available about a relationship between the N-terminal truncation of SIRT5 and its enzymatic activity (Schwer et al., 2002).

Both SIRT5 isoforms display mitochondrial localization. SIRT5 can enter the IMS and the mitochondrial matrix (Schlicker et al., 2008). In contrast to the cleaved isoform two (IF2, derived from the shorter splice variant), which seems to reside exclusively in the mitochondria, cleaved IF1 is found additionally in the cytoplasm. It appears that the different C-termini of the two SIRT5 isoforms are responsible for their distinct subcellular distribution. The C-terminal extension of IF2 is rich in hydrophobic amino acids and functions as a mitochondrial membrane insertion signal (Matsushita et al., 2011). Presently no PTM of SIRT5 are described besides the proteolytic cleavage and thus nothing is known about the role of this protein in signaling processes.

SIRT6

SIRT6 is expressed in most tissues (Liszt et al., 2005; Mostoslavsky et al., 2006). It is, similar to SIRT1 and SIRT7, predominantly localized in the nucleus (Liszt et al., 2005; Michishita et al., 2005), where it associates with chromatin (Mostoslavsky et al., 2006). A nuclear localization signal was discovered between amino acids 345 and 351 in the distal region of the C-terminal extension of SIRT6 (**Figure 2**). This signal is necessary and sufficient for proper nuclear localization of the protein (Tennen et al., 2010). In comparison to other sirtuin knockout mice, *Sirt6*-deficient mice display a severe phenotype. Despite normal development for several weeks after birth, these mice die at about 1 month of age due to degenerative processes of multiple organs. These processes include loss of subcutaneous fat and metabolic defects displayed by dramatic drops of serum glucose and insulin-like growth factor 1 (IGF1) levels. Additional symptoms are lordokyphosis, colitis, and a severe lymphopenia (Mostoslavsky et al., 2006). One suggestion is that this phenotype is the consequence of a loss of Sirt6-mediated inhibition of NF-κB target gene expression (Kawahara et al., 2009, 2011). The absence of SIRT6-dependent repression of HIF1α might also account for the phenotype (Zhong et al., 2010). Under physiologic conditions SIRT6 interacts with these transcription factors, i.e., NF-κB and HIF1α, and is transported to their target gene promoters where it deacetylates H3K9ac or H3K56ac (Michishita et al., 2005; Kawahara et al., 2009; Yang et al., 2009). In both cases, the binding of the respective transcription factor to its target gene promoters is enhanced in *Sirt6*-deficient cells due to locally elevated acetylation levels of H3K9. Further investigations revealed that upon TNF-α signaling, SIRT6 binds to many promoters, which are highly enriched for NF-κB, SP1, STAT1/3, ELK1, E2F1, and FOXO1/4 binding motifs (Kawahara et al., 2011). Thus SIRT6 appears to have widespread activities as a regulator of transcription, in particular of genes whose products are involved in glucose and lipid metabolisms.

Moreover SIRT6 seems to be involved in DNA repair and thus in the maintenance of genomic integrity. Indeed MEFs derived from *Sirt6*-deficient mice are more sensitive to irradiation and display multiple chromosomal aberrations (Mostoslavsky et al., 2006). SIRT6 associates with chromatin in response to DNA damage and

stabilizes the DNA-dependent protein kinase (DNA-PK) at DNA double-strand breaks (DSBs; McCord et al., 2009). Also SIRT6 deacetylates CtIP [C-terminal binding protein (CtBP) interacting protein] in response to DNA damage, which promotes the ability of CtIP to mediate DSB repair by homologous recombination (Kaidi et al., 2010). Finally SIRT6 is required for telomere maintenance (Tennen and Chua, 2011). Together these findings provide strong evidence for a role of SIRT6 in controlling genomic stability.

The conserved core domain of SIRT6 is not sufficient to deacetylate H3K9ac or H3K56ac (Tennen et al., 2010). For SIRT6 the available evidence suggests that the N-terminal region is essential for deacetylase activity. This is reminiscent of the findings with other sirtuins, which require either N- or C-terminal regions to activate catalytic function as described above.

In addition to its deacetylase activity, SIRT6 has been reported to be able to mono-ADP-ribosylate substrates. One substrate identified is PARP1/ARTD1, which is activated by SIRT6 (Mao et al., 2011). This provides an additional link to genomic stability because ARTD1 is a DNA damage sensor and upon activation synthesizes ADP-ribose polymers that are docking sites for repair enzymes (reviewed in Kleine and Luscher, 2009). SIRT6 can also auto-ADP-ribosylate but the site of modification and the functional relevance are unclear (Liszt et al., 2005). It remains to be elucidated whether the N-terminal extension is essential for the ADP-ribosylation activity of SIRT6 as it is for the deacetylase activity. Of note is that besides ADP-ribosylation, two C-terminal phosphorylation sites at Tyr294 and Ser303 have been discovered in a proteomic approach (Dephoure et al., 2008; **Figure 2** and **Table 1**). It remains to be seen whether these phosphorylations influence SIRT6 function.

SIRT7

Out of the seven human sirtuins, SIRT7 is the least studied. It is a nuclear protein that is concentrated in the nucleoli where it interacts with components of the rDNA transcription machinery, like RNA polymerase I (Pol I) and the rDNA transcription factor UBF (Michishita et al., 2005). SIRT7 positively regulates rDNA transcription (Ford et al., 2006; Grob et al., 2009). Knockdown of SIRT7 in human cancer cell lines blocks cell proliferation and causes apoptosis. This drastic effect implies that SIRT7 is required for cancer cell viability (Ford et al., 2006). Further evidence supporting this hypothesis is provided by enhanced SIRT7 expression levels in breast carcinoma biopsies compared to normal tissue (Ashraf et al., 2006). The tumor suppressor p53 is a substrate of SIRT7 and thus this sirtuin appears to interfere with p53 function, similar to SIRT1 (Vakhrusheva et al., 2008). *Sirt7* knockout mice are viable but suffer from progressive heart hypertrophy, accompanied by inflammation and decreased stress resistance, possibly a consequence of altered p53 activity. Indirect evidence suggests that SIRT7 is phosphorylated during mitosis by a CDK complex, but no sites have been mapped nor functional consequences defined (Grob et al., 2009).

CONCLUSION

Sirtuins have been identified as key regulators of multiple cellular processes, mainly by functioning as NAD^+-dependent deacetylases but also as demalonylases, desuccinylases, and ADP-ribosyltransferases. Despite the many processes that are governed, at least in part, by sirtuins, relatively little is known about how these regulators are controlled. This is somewhat surprising because sirtuins are implicated in many signaling processes. But obviously their regulation has not been evaluated in great detail.

Sirtuins share a conserved catalytic domain, but differ in their N- and C-terminal extensions. Apparent from the available data is that the N- and C-terminal regions of the different sirtuins that extend beyond the catalytic domains mediate regulation. Multiple PTMs target these extensions, including phosphorylation, methylation, sumoylation, proteolytic cleavage, and possibly others. Most of the relevant enzymes are not known presently and thus the pathways that target sirtuins are poorly understood. Nevertheless the emerging theme suggests that the N- and C-terminal regions function as signal receivers that transmit information from signaling pathways to the catalytic domain. In addition initial findings suggest that these signals, which target the extensions of sirtuins, may also control properties of the different sirtuins beyond catalytic activity. Although the evidence is not complete, trimerization, substrate specificity, and subcellular localization are likely to be controlled by these extensions. To understand sirtuin biology more thoroughly, it will be necessary to account for all PTMs that control the N- and C-terminal extensions and to integrate and connect these findings with distinct signaling pathways to associate sirtuins with physiological processes. This will need to be combined with more definitive studies addressing the functions of the extensions both for intra- as well as intermolecular interactions (**Figure 4**).

While the available evidence, although still incomplete, suggests that the N- and C-terminal extensions are important to control sirtuin function at multiple levels, considerably less is known about posttranslational regulation that directly targets the catalytic domain of either of the seven sirtuins. It is presently not clear whether this reflects a true lack of direct regulation or whether we simply have not identified the relevant processes yet. Since many other enzymes are controlled by directly modulating

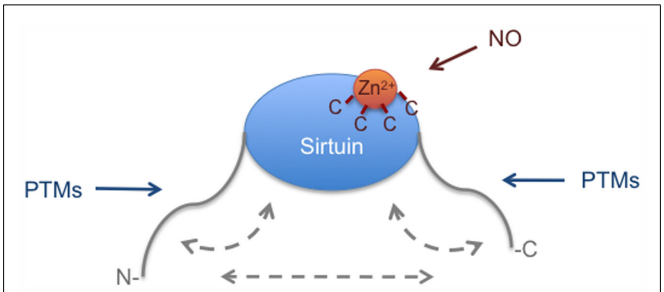

FIGURE 4 | Summary of the regulation of the catalytic activity of Sirtuins. Most of the currently known PTMs that target sirtuins are directed to the N- and C-terminal extensions. These extensions may control sirtuin function by interacting with the catalytic core domain or with each other. Additionally the extensions may control intermolecular interactions (not indicated). Different PTMs target the N- and C-terminal extensions, thereby possibly controlling either intra- or intermolecular interactions. The regulation of Zn^{2+} binding by nitrosylation is potentially a common regulatory mechanism of sirtuins. For more details see the text.

the catalytic domain, it seems more likely that the latter explanation is correct. The findings on nitrosylation of SIRT1 indicate that the catalytic domain of at least this sirtuin is indeed regulated. Nitrosylation is potentially controlling all sirtuins because this modification targets cysteines that are important to coordinate Zn^{2+} binding. Sirtuins have been recognized as targets for clinical intervention. Understanding the repertoire of control mechanisms that target sirtuins will likely provide additional targets worth considering. Thus unraveling the function and regulation of sirtuins is important not only to understand basic cellular processes but also for clinical applications.

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Sirtuins as regulators of the yeast metabolic network

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There is growing evidence that the metabolic network is an integral regulator of cellular physiology. Dynamic changes in metabolite concentrations, metabolic flux, or network topology act as reporters of biological or environmental signals, and are required for the cell to trigger an appropriate biological reaction. Changes in the metabolic network are recognized by specific sensory macromolecules and translated into a transcriptional or translational response. The protein family of sirtuins, discovered more than 30 years ago as regulators of silent chromatin, seems to fulfill the role of a metabolic sensor during aging and conditions of caloric restriction. The archetypal sirtuin, yeast *silent information regulator2* (*SIR2*), is an NAD^+ dependent protein deacetylase that interacts with metabolic enzymes glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase, as well as enzymes involved in NAD(H) synthesis, that provide or deprive NAD^+ in its close proximity. This influences sirtuin activity, and facilitates a dynamic response of the metabolic network to changes in metabolism with effects on physiology and aging. The molecular network downstream Sir2, however, is complex. In just two orders, Sir2's metabolism related interactions span half of the yeast proteome, and are connected with virtually every physiological process. Thus, although it is fundamental to analyze single molecular mechanisms, it is at the same time crucial to consider this genome-scale complexity when correlating single molecular events with complex phenotypes such as aging, cell growth, or stress resistance.

Keywords: sirtuins, metabolic network, glycolysis, caloric restriction, pentose phosphate pathway, aging, redox state, nicotinamide

ROBUSTNESS AND SENSING OF THE METABOLIC NETWORK

Metabolite concentrations are driving forces for enzymatic reactions (Bruice, 2002), which implies that there is evolutionary pressure to keep them in a narrow, controlled range. Indeed, intracellular metabolite concentrations are extraordinarily robust against external as well as genetic perturbations (Blank et al., 2005; Ishii et al., 2007). Important for this robustness is a modular structure of the metabolic network. Modularity of substructures grants network stability, as perturbations may affect the module but not necessarily the flux of the entire system (Parter et al., 2007; Kreimer et al., 2008). Indeed, system-wide consequences on metabolite concentration levels are virtually limited to perturbations that affect common, network-interconnecting metabolic co-factors (i.e., ATP, SAM, or NADH; Blank et al., 2005; Kuepfer et al., 2005).

Maintaining metabolite concentrations requires molecular sensing and monitoring of the network, which occurs both at the level of (a) concentration and (b) metabolic flux (Grüning et al., 2010; Heinemann and Sauer, 2010). An illustrative example for network regulation at the concentration level is purine biosynthesis. Two intermediates, 5′-phosphoribosyl-5-amino-4-imidazole carboxamide (AICAR) and succinyl-AICAR (S-AICAR), bind to the transcription factors Pho2, Pho4, and Bas1. An increase in their concentration induces dimerization of these transcription factors and triggers expression of genes involved in their own biosynthesis (Pinson et al., 2009). This feed-forward mechanism may be required to react appropriately to energy shortage; it may allow the cell to decipher if rapid proliferation or starvation is the reason for the energy shortage.

AICAR and S-AICAR represent so called reporter metabolites, metabolites whose concentration controls a transcriptional response, or in the original definition, metabolic intermediates that are surrounded by transcriptional changes (Patil and Nielsen, 2005; Cakir et al., 2006) The TOR pathway also contains an example of the second considerably less understood type of metabolite control, that of flux-based monitoring of the metabolic network. In mammalian cells the mTOR interactor Rheb is bound to the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Under low glucose conditions, GAPDH prevents Rheb from binding to mTOR and thereby inhibits mTORC1 signaling. High glycolytic flux suppresses the interaction between GAPDH and Rheb and thus allows Rheb to activate TOR signaling (Lee et al., 2009).

THE METABOLIC NETWORK AND THE AGING PROCESS

These systems of metabolome regulation play important roles during the aging process. Metabolism is regarded as a cause of aging, as it is responsible for the increase in molecular and cellular damage observed in senescent cells. Although it is clear that the damage on macromolecules contributes to cell death and apoptosis and is

age dependent, it is subject to ongoing debate if this damage is indeed the basis, or rather a consequence of the aging process, or both (Muller et al., 2007; Blagosklonny, 2008; Gruber et al., 2008; Blagosklonny and Hall, 2009).

Yeast aging research distinguishes between replicative and chronological aging. The first measure, also called mother cell-specific aging, describes how many cell cycles a yeast cell completes before senescence, or in other words, how many daughters bud from each mother (Mortimer and Johnston, 1959). The second, termed "chronological aging," defines how long a yeast culture endures at 30˚C in a non-dividing state (Fabrizio and Longo, 2003), or in its special case of "hibernating lifespan," at 4˚C (Postma et al., 2009). Although it is in general assumed that replicative aging shares more features with the aging process in humans (Laun et al., 2006), both measures depend on the metabolic network. The accumulation of oxidatively damaged proteins during both chronological and replicative aging is undisputed, however, at the same time there exist several yeast mutants which are strongly oxidant resistant, but show massively shortened lifespans [such as peroxiredoxin *tsa1-B7*; Timmermann et al., 2010, or triosephosphate isomerase (*tpi1*) mutants; Ralser et al., 2007). This indicated that the intense relationship between oxidative damage and aging is rather complex, as it is the function of sirtuins, as discussed below.

Among oxidizing molecules that originate in biosynthetic mechanisms, research predominantly focused on reactive oxygen species (ROS). A major source of ROS in the cell is superoxide ($O_2^{\cdot -}$), which leaks from complex III and complex I (in yeast only complex III, as it lacks complex I) of the mitochondrial respiratory chain (Cadenas and Davies, 2000; Breitenbach et al., 2012). In fact, superoxide production is quantitatively quite significant, as 1–2% of consumed oxygen is interconverted into this agent (Cadenas and Davies, 2000). However, there are other important sources of ROS, such as the mitochondrial external NADH dehydrogenases Nde1, Nde2 and the mitochondrial internal NADH dehydrogenase Ndi1, which feed the respiratory chain without proton pumping (Luttik et al., 1998; Li et al., 2006), or the degradation of fatty acids (Martin et al., 2007). Moreover, other oxidizing agents, such as reactive nitrogen species (RNS) also contribute significantly to macromolecular damage (Novo and Parola, 2008). Although RNS are less well studied in yeast than in plants or mammals, they seem to play a role in the yeast aging process, at least it was reported that they accumulate in senescent *RAS* mutants (Wilhelm et al., 2006).

Production of these oxidizing agents is a natural, inevitable consequence of metabolic activity. In young and healthy cells, natural ROS production is unproblematic as all living cells are evolutionarily adapted and are able to clear this ROS through a series of enzymatic and non-enzymatic antioxidant systems (Sies, 1997; Cadenas and Davies, 2000). These stabilize ROS levels; for instance, respiring and non-respiring yeast cells possess the same levels of superoxide and hydrogen peroxide (Gruning et al., 2011). The in quantitative terms most important (but not the only) redox buffer is the glutathione system (Meister and Anderson, 1983; Grant et al., 1996). Glutathione, a cysteine containing tri-peptide of non-ribosomal origin, clears ROS by being oxidized. The oxidized form is then recycled by glutathione reductase, an enzyme which restores GSH under NADPH consumption (Massey and Williams, 1965). Regulation of the NADPH supply is critical for this process. This metabolite is consumed in large amounts under oxidative stress conditions, but cannot be provided in large excess, as this would shift the redox balance toward reduction ("reductive stress"), which is another pathogenic situation (Rajasekaran et al., 2007; Brandes et al., 2009).

The dynamic increase in $NADP^+$ reduction during oxidative stress has largely been attributed to increased activity of the pentose phosphate pathway (PPP), a catabolic carbohydrate pathway alternative to glycolysis (Wamelink et al., 2008). Although other cellular reactions are capable of maintaining a normal NADPH/$NADP^+$ ratio under anaerobic growth conditions, they fail to do so upon contact with an oxidant or when they respire at high rates. A H_2O_2 treatment of *zwf1*Δ yeast – lacking the first $NADP^+$ reducing enzyme of the PPP (glucose 6 phosphate dehydrogenase Zwf1) – causes an immediate collapse of the NADPH/$NADP^+$ ratio resulting in massive cellular sensitivity to oxidants (Castegna et al., 2011).

REGULATION OF DYNAMIC PPP ACTIVATION

The dynamic activation of the PPP is a vivid example for the adaptation of the metabolic network to changing environmental conditions. Upon the addition of an oxidant, PPP activity is increased in temporally discrete steps. This regulation involves both the transcriptome and metabolome level, beginning with the latter (Larochelle et al., 2006; Chechik et al., 2008; Ralser et al., 2009a). In addition, glycolysis and the PPP are self-adapting to prevent oxidative stress when cells switch from anaerobic to oxidative metabolism. Low activity of yeast pyruvate kinase [*PYK*; an enzyme which catalyzes the last glycolytic step converting phosphoenol-pyruvate (PEP) to pyruvate] in respiring cells causes accumulation of its substrate PEP. PEP in turn acts as feedback inhibitor of triosephosphate isomerase (Tpi1). The resultant block in glycolysis drives flux into the PPP (Gruning et al., 2011). This mechanism helps to balance the increased ROS production during oxidative metabolism, and keeps the ROS level similar to that of non-respiring cells. However, interruption of this feedback circuit makes cells vulnerable to oxidative stress: when respiration is induced in *zwf1*Δ yeast, ROS accumulate, and damage macromolecules and organelles by oxidation (Gruning et al., 2011). Recently, it has become clear that this mechanism is conserved in mammalian cells, and might explain central features of the Warburg effect, which is a decrease in oxidative respiration during cancer formation (Warburg, 1956). Also cancer cells suffer from oxidative damage, and redirect the metabolic flux via *PYK* for inducing ROS clearance (Anastasiou et al., 2011; Grüning and Ralser, 2011).

THE DISCOVERY OF YEAST SIRTUINS

The metabolic shift from glycolysis to the PPP is one of the best studied examples, where a metabolic re-configuration induces adaptation of the transcriptome, and the glycolysis/PPP transition functions as a "metabolic signaling cascade" induced under oxidative stress (Kruger et al., 2011). The response in the transcriptome points to the existence of regulating macromolecules that detect these changes in metabolic activity and translate this information into a transcriptional response. Sirtuins are such proteins, they react to changes in metabolic activity and are closely connected with metabolites and enzymes of glycolysis.

The papers dealing with the sirtuin protein family recently exceeded 2200. The authors who first discovered the *Saccharomyces cerevisiae SIR2* gene, from which the name, sirtuin is derived, certainly could not have dreamed that their discovery would within just a few decades lead to such an enormous "gene rush" in the scientific community around the world. For the most part, this development is caused by the association of sirtuins in the aging process. However, the exact nature of this connection is still unclear, and the debate about whether or not the sirtuins are part of a *public mechanism of aging* (term meaning cross-species aging mechanism; Martin et al., 1996) is still ongoing. Prominently, the involvement of sirtuins in caloric restriction (also called "calorie restriction" or "dietary restriction") intervention in the aging process is central to this debate, as it is the mechanism of action of resveratrol, a plant stilbene which was believed to extend *S. cerevisiae* replicative lifespan though activating *Sir2* (Lin et al., 2000; not further discussed in this review).

The *SIR* (*silent information regulator*) mutants and genes were discovered by Rine et al. (1979). Four different complementation groups of unlinked recessive yeast mutations were found that all led to the same phenotype: the mutants were extragenic suppressors of the *matα1-5* mutation. The suppressor mutation caused cells to regain functional *MAT*α genetic information and to mate again normally. This effect was completely dependent on the presence of *MAT*α genetic information at the distant *HML* locus near the left telomere of chromosome III. Viewed from 2012, it is not surprising that the *sir* mutations were shown to influence not only mating, but also sporulation efficiency. Herskowitz and many others have established that mating of alpha strains needs the functional gene product Matα1 and sporulation requires the functional gene products Mata1 and Matα2. In most laboratory strains, the *HML* locus near the left telomere of chromosome III contains functional but silent copies of the *alpha1* and *alpha2* genes, while the *HMR* locus located near the right telomer of chromosome III, contains functional but silent copies of the *a1* and *a2* genes. The first identified mutant *sir* allele (*sir1-1*) suppressed all known mating deficient and sporulation-deficient mutations located in the mating type locus. All four *SIR* genes are needed independently for transcriptional repression of *HML* and *HMR*. Importantly, it was later shown (Moazed et al., 1997; Liou et al., 2005) that three of the four Sir proteins (Sir2/Sir3/Sir4) form a tight heteromeric complex *in vitro;* Sir1 is not part of this complex. (Please see Huang, 2002; for a Review of proteins that are needed for silencing and for the pleiotropic functions of Sir2).

We know that the *sir* mutations relieve the transcriptional repression of genetic information at *HML* and *HMR*, and from work in the ensuing decades that the Sir gene products bind to controlling segments in the DNA of *HML* and *HMR*, and are part of the chromatin at those loci. In addition, the Sir proteins are found at telomeres of all chromosomes and at the rDNA locus on chromosome XII (Strahl-Bolsinger et al., 1997; Mekhail and Moazed, 2010). The mutations were found at the time to have no other phenotypes apart form silent mating type information expression. Herskowitz and Oshima (1981) wrote that "it is clear that sir mutants are not grossly pleiotropic." This statement is no longer true, as will be discussed below.

It was shown that Sir proteins interact genetically with histone H4, indicating that transcriptional silencing includes H4 modification and that co-operation of H4 with Sir2 is needed for silencing (Imai et al., 2000). The first hint of a special role for Sir2 that differs from the other three Sir proteins came from the work of Easton-Esposito and her colleagues (Gottlieb and Esposito, 1989; Fritze et al., 1997). They showed that Sir2 (but not Sir 1, Sir3, or Sir4) suppresses non-allelic recombination between the tandem copies of rDNA on chromosome XII. The authors concluded that Sir2 is a limiting component required for chromatin modeling at the rDNA locus. This was a very important step toward our present understanding of Sir2. Guarente (1999) reviewed the role of the Sir proteins in silencing, chromatin organization at telomers, silent mating type loci, the NOR (nucleolar organizer), in repair of double strand breaks, recombination, cell cycle regulation, and mother cell-specific aging.

THE DISCOVERY OF Sir2'S ROLE IN AGING AND THE METABOLIC NETWORK

Among other groups, the lab of Guarente (1999) started work on yeast mother cell-specific aging in the 1990s. While it is to the present day, for technical reasons, not possible to directly select for replicatively long-lived yeast mutants, several indirect protocols for mutation isolation have been used. After conventional chemical mutagenesis of haploid yeast cells and applying starvation stress, replicatively long-lived mutants were isolated among the survivors of extended periods of starvation (Kennedy et al., 1995). One of them was located within *SIR4* and was shown to be a "semi-dominant" missense mutation. This led to the possibility that the Sir complex could have a special role in replicative aging. Consequently, all components of this complex were tested for aging phenotypes (Kaeberlein et al., 1999). It was shown that yeast replicative lifespan depended on the presence of *SIR2*, *SIR3*, and *SIR4*, but not on *SIR1*. Again, *SIR2* was shown to have a special role that is slightly different from that of *SIR3* and *SIR4*. Comparing the deletion mutants of the three genes (*sir2*Δ, *sir3*Δ, and *sir4*Δ) it appears that *sir2*Δ had the largest effect (−50%). Conversely, overexpression constructs increased lifespan, again with Sir2 overexpression having the largest effect. It appeared that the effects of *sir3*Δ and *sir4*Δ could be fully compensated by deleting *HML*α in the used haploid *MAT*a strain, while the same effect was not seen in *sir2*Δ yeast (Kaeberlein et al., 1999). The authors explained these results with Sir2-triggered repression of minicircles (ERCs), formed by non-allelic recombination of the tandem repeated copies of rDNA in the NOR on chromosome XII. These minicircles behave like non-centromeric plasmids, therefore accumulate in mother cells but are very inefficiently transmitted to daughter cells. In the mother cells, they can become so abundant with replicative age that either the sheer mass of the circular DNA may have an adverse effect on the cells (Sinclair and Guarente, 1997) or they may titrate away the Sir2 which is then limiting for the other important functions like silencing of *HML* and *HMR*. This most likely contributes to the observed mating defect of old haploid mother cells. Other functions compromised in these cells are binding and silencing to telomeres and adjacent chromosomal regions, affecting among other things unwanted non-homologous end-joining (NHEJ), perhaps leading to replication problems and

genome instability in the old mother cells (Gottschling, 2000; McMurray and Gottschling, 2004). FOrk Blocking less 1 (Fob1) is a non-essential recombination factor needed for keeping the sequences of the rDNA repeat units constant. Preventing the formation of minicircles by deleting *fob1*, increases the replicative lifespan of yeast by about 6 generations (Defossez et al., 1999). However, the SIR mutations (in particular *sir2*Δ) have effects on lifespan even in the absence of minicircles, and the gain in lifespan in *fob1*Δ strains is relatively small (Defossez et al., 1998; Borghouts et al., 2004; Heeren et al., 2009). This underlines the fact that replicative aging of yeast and aging in general is a very multicausal process and depends not only on the genetic makeup of the cell but also on many external conditions. Thus, there is no single and "most important" mechanism of aging.

Minicircles have been described only in the yeast *S. cerevisiae* and its close relatives, but not in the other eukaryotic model systems of aging, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Mus musculus*. We therefore call minicircles a "private mechanism of aging" (the term meaning species-specific going back to Martin, 1997). However, in the non-yeast model systems of aging, the *SIR2* gene and its protein family does exist and is even split into a number of paralogs (i.e., seven in humans) some of which are essential for life in mammals (McBurney et al., 2003; Chen et al., 2005).

Despite the absence of minicircles, lifespan extending properties for Sir2 homologs have been reported for *C. elegans* and *D. melanogaster*. However, recently, these studies received a serious setback as these lifespan phenotypes were lost by out-crossing the Sir2 overexpression alleles (Burnett et al., 2011), or at least, were overestimated in one original report (Tissenbaum and Guarente, 2001) due to co-segregation of an unlinked second mutation which alters lifespan as well (Viswanathan and Guarente, 2011). We think that the question of sirtuins involvement in lifespan extension in non-yeast species is undecided at present. The careful genetic crosses of Burnett et al. above everything show one fact: due to the extreme (and not fully analyzed) pleiotropy and the large number of target genes of the deacetylase Sir2, it is extremely important to investigate co-segregation of the aging phenotype with the overexpression mutation to determine hidden factors in the genetic background.

The situation is also different for the involvement of Sir2 in the second measure of yeast aging, chronological lifespan. This measure is quite different to replicative aging as it describes the survival of a stationary yeast population rather then the survival of an individual cell. Indeed, gene sets which extend replicative, chronological, and also hibernating lifespan are only marginally overlapping (Laun et al., 2006; Postma et al., 2009). Nonetheless, systematic analysis of chronological aging led to the discovery of aging factors conserved from yeast to mammals, including the TOR pathway (Fabrizio and Longo, 2003; Powers et al., 2006). Sir2 has however shortening effects on this measure: Lack of Sir2 along with calorie restriction and/or mutations in the yeast AKT homolog, Sch9, or Ras pathways caused a dramatic chronological lifespan extension (Fabrizio et al., 2005).

Irrespective of this debate, studies on Sir2's involvement in the aging process stimulated a massive amount of research on Sirtuins and improved our understanding of their regulatory potential in humans. In animal species SIRT1 shows the highest degree of sequence similarity with yeast Sir2, and displayed aging-related physiological effects in spite of the absence of ERCs (Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004; Chen et al., 2005; Herranz and Serrano, 2010). These effects were explained by the high number and great variety of protein targets of Sir2 deacetylase activity (Westphal et al., 2007). In mammals, considering the targets that have so far been discovered, they connect sirtuins activity to metabolic regulation, stress resistance and diseases of old age like diabetes and cancer.

ASYMMETRIC SEGREGATION, CALORIC RESTRICTION AND Sir2

The literature on Sir2 homologs and aging is closely interrelated with (i) asymmetric segregation, and (ii) caloric restriction. These seem to be unrelated processes from the mechanistic point of view, but both protect cells from oxidative damage.

Asymmetric segregation in yeast *S. cerevisiae* was found in aged mother cells that retain oxidatively damaged proteins; daughter cells are formed from a fresh set of proteins. This is Sir2 dependent; *sir2*Δ mothers are unable to protect their daughters from the transmission of damaged proteins during cytokinesis (Aguilaniu et al., 2003). Thus, *sir2*Δ daughters suffer much earlier from molecular damage as do wild-type cells.

It is not yet clear if Sir2 homologs play a similar role in other species. Thus, it cannot be excluded that Sir2's modulation of asymmetric segregation is again a yeast's private mechanism of aging. In contrast, however, the second treatment associated with Sir2's anti aging effects, caloric restriction, has been found to increase lifespan and improve health in all tested species from yeast to humans (Sohal and Weindruch, 1996). Deleting or downregulating the respective Sir2 homologous gene in the aforementioned species abrogates effects of caloric restriction on lifespan and on the preventive effect of caloric restriction on diseases of aging, like diabetes (Chen et al., 2005; Lamming et al., 2005; Qiu et al., 2010a). However, the question of whether the yeast *SIR2* gene is needed for the increase the replicative lifespan by a reduction of glucose is an open one till the present day. One laboratory maintains that the effect of this mild CR is effective independently of Sir2, and is in the absence of Sir2 dependent on Hst2, a Sir2 paralog (Lamming et al., 2004, 2005; Smith et al., 2007). The *HST2* gene is a member of a small family of four yeast genes which is highly homologous and functionally related to Sir2 (Derbyshire et al., 1996). However, another laboratory insists that life span extension by CR is strictly dependent on the presence of the *SIR2* gene (Lin et al., 2000).

METABOLIC EFFECTS OF CALORIC RESTRICTION IN YEAST

This lifespan extension in regard to Sir2 has been studied by reducing glucose concentration from the usual 2% to the lower 0.5% in *S. cerevisiae* media (Lin et al., 2000). Although this treatment is referred as caloric restriction, it is – if any – a mild form of it, and it is worth speculating that 0.5% glucose may closer resemble the nutrient availability in the yeast's natural environment. Interestingly, it was described that an induction of respiration is required for the lifespan extending effects of a growth on 0.5% glucose, and that Sir2 is involved in this process (Lin et al., 2002). Importantly, *S. cerevisiae* is a "Crabtree" positive yeast: high glucose

concentrations (but not other carbon sources such as galactose, raffinose or glycerol) repress *S. cerevisiae* respiration. Analyzing chronological lifespan, it was shown that in contrast to glucose, inducing CR by reducing the content of galactose, raffinose, or glycerol plus ethanol did not enhance lifespan. Moreover, the lifespan extending effects of diminished glucose concentration were not observed in the Crabtree negative yeast *Kluyveromyces lactis* (Oliveira et al., 2008). In this context, it is worth to mention that yeast strains with extended hibernating lifespan (mutants that survived 5 years in the cold room) contain a high number of strains which cannot survive without respiratory activity (Postma et al., 2009). Moreover, when respiration was induced by *PYK* or by shifting to galactose, yeast cells developed a strong increase in resistance to different oxidants (Gruning et al., 2011), but in the Crabtree negative *K. lactis*, limitation of glucose supply did not protect against ROS as it does in *S. cerevisiae* (Oliveira et al., 2008). Recently, also nitric oxide (NO) production was associated with these lifespan extending effects of low glucose levels. A reduction of glucose concentration induces *S. cerevisiae* NO production; and NO donors such as GSNO (*S*-nitrosoglutathione) were sufficient to extend lifespan (Li et al., 2011). Thus, it is conceivable that the lifespan extending effects of reducing glucose to 0.5% result from circumventing glucose repression causing an activation of mitochondrial metabolism, respiration, and the cellular antioxidant machinery.

Additionally, the nutrient-responsive signaling kinase TOR (target of rapamycin), and the concentrations of NAD^+ play a role in this process. Although it was reported that *tor1* mutants have an increased lifespan independently from Sir2 activity (Kaeberlein et al., 2005), other results supported the notion that the TOR mediated lifespan extension is a direct result of enhanced NAD^+ salvage pathway activity and Sir2 targeting (Silva et al., 2009). It was shown that rapamycin and nitrogen starvation leads to enhanced Sir2 association with rDNA via the inhibition of TOR complex 1 (TORC1; Ha and Huh, 2011). To undergo these reactions, Sir2 required the salvage pathway Nicotinamidase Pnc1 and Net1, the core subunit of the RENT complex, indicating that TORC1 activity results in increased silencing of RNA polymerase genes at rDNA loci, enhanced histone deacetylation and reduced homologous recombination at rDNA repeats. This allowed to conclude on a lowered number of ERCs as basis for this lifespan extension (Ha and Huh, 2011). Indeed, NAD^+ and nicotinamide are directly involved in the functional activity of Sir2 in relation to the metabolic network.

THE ENZYMATIC FUNCTION OF Sir2 AND LINKS TO THE METABOLIC NETWORK

Sir2 has deacetylase and ADP-ribosyl-transferase activity, both of which are required for deacetylating proteins. Sir2 catalyzes a NAD^+-dependent ε-*N*-acetyl-lysine deacetylation from proteins (including histones, class III HDACs), resulting in deacetylated protein, nicotinamide, and the molecule 2′*O*-acetyl-ADP-ribose (OAADPR; Moazed, 2001; Jackson and Denu, 2002). It is an open field of research if Sir2 can for this reason sense the NAD^+/NADH ratio (Yu and Auwerx, 2009; Yu et al., 2009). First Sir2 activity may be directly regulated by changes in the cellular redox potential. This notion is perhaps naïve, as the main determinant of the redox potential of the cell is the glutathione redox couple according to the Nernst equation. The measured changes in NAD^+/NADH ratio during CR have only minor influence on this measure (Schafer and Buettner, 2001). In this line, Anderson et al. (2003) showed that reducing the glucose intake decreases nuclear NAD^+ levels *in vivo*, but that yeast Sir2 as well as its mammalian homolog SirT1 are not affected by this alteration in NAD^+/NADH ratios.

These assumptions however require a more differentiated elaboration as the overall redox state measure does not necessarily reflect the cofactor presence in the protein's microenvironment. A significant part of NAD^+ is protein bound, as it is presumably Sir2, and therefore protein complexes may feed Sir2 directly with its cofactor. Recently, Sir2 was the basis for several genome-scale screens to identify genetic and physical interactors. These include a genome-wide screen to identify anti-silencer factors (Raisner and Madhani, 2008), and proteomic analyses that use methods of label-free protein quantification (Rye et al., 2011). Together with other genes identified in earlier targeted and untargeted screens, they form a large network which is indicative of the global function of Sir2. Based on the information stored in the BioGrid database as of December 2011 (Stark et al., 2006), we generated a metabolism-centric protein interaction network for Sir2 (**Figure 1**). This network contains all direct genetic and physical interaction partners of the enzyme, and interactors that are associated with the gene ontology (GO) term "metabolism" or "carbohydrate metabolism" are highlighted. These two GO terms have been assigned to 40 genes out of the total 84 annotated Sir2 interactors (**Figure 1A**). To illustrate the large interconnectivity that comes with these 40 genes, the network was expanded with their direct interactors, which yielded a "metabolism-centric" Sir2 interactions network (**Figure 1B**). The inclusion of the second order Sir2 interactors considerably increased the network and its interconnectivity; the network is formed of 2888 vertices interconnected by 5566 edges. Thus, in just two steps, the metabolic interaction network of Sir2 interconnects half of the yeast proteome.

Sir2 INTERACTING ENZYMES WHICH GENERATE NAD^+ IN CLOSE PROXIMITY MODULATE ITS BIOLOGICAL FUNCTION

In **Table A1** in Appendix we list the genes of the direct (first order) interactors of Sir2, and their associated GO terms. Interestingly, this list contains three proteins that are among the major NADH/NAD^+ consumers of the cell. Two of them – alcohol dehydrogenase Adh1 and the predominant yeast GAPDH Tdh3 – are found in physical complex with Sir2 (Gavin et al., 2002). In both cases, low-scale genetic interaction studies confirmed that they influence the biological activity of Sir2 (**Figure 2**). The first protein, the predominant yeast GAPDH Tdh3, is a central enzyme of glycolysis and reduces NAD^+ to NADH in its catabolic role, but oxidizes NADH in its gluconeogenetic function (Tristan et al., 2011). A genetic screen conducted in the lab of Scott Holmes revealed that overexpression of Tdh3 rescues lethality caused by *GAL1*-promoter driven overexpression of Sir2, indicating a modulation of Sir2 activity by yeast GAPDH (Matecic et al., 2002). Later, our investigations revealed that overexpression of Tdh3 and *E. coli* GAPDH increases the rate of mitotic recombination in a Sir2 dependent manner (Ralser et al., 2009b). This phenotype was dependent on the catalytic activity of Sir2, and, interestingly, a

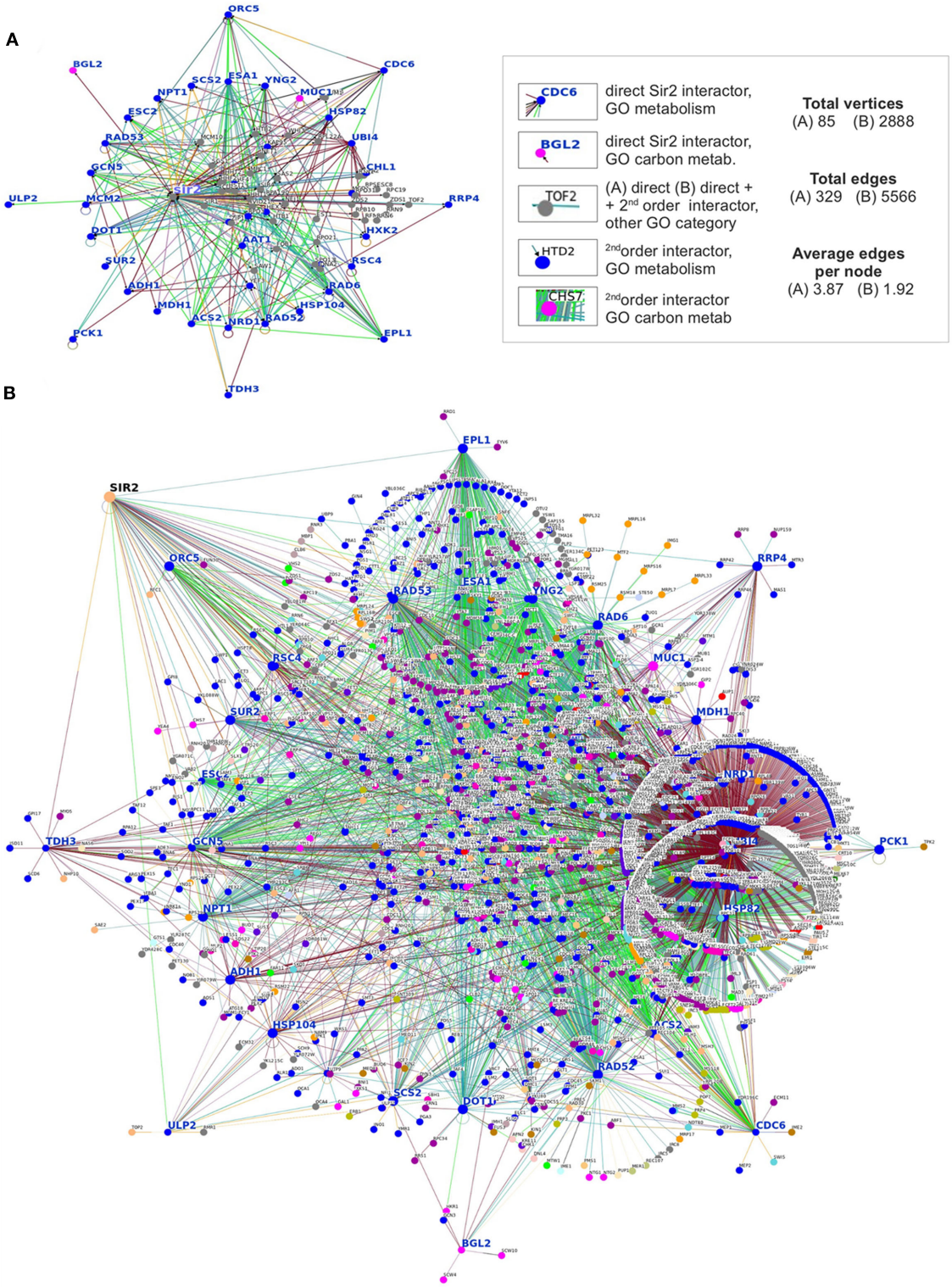

FIGURE 1 | A metabolism-centric interaction network for Sir2. (A) Network of 84 direct interactors of Sir2; the 40 genes associated with the GO term "metabolism" and/or "carbohydrate metabolism" are highlighted in blue or purple, respectively. (See **Table A1** in Appendix for GO terms associated with these Sir2 interactors). **(B)** The network **(A)** expanded with the direct interactors of the 40 genes with annotated metabolic function. This network containing first and second order metabolism related Sir2 interactors interconnects with 2888 vertices half of the yeast proteome. We apologize that due to this complexity, not all genes names may be readable. **(A,B)** Physical- and genetic-interactors for Sir2 were obtained from the BioGrid database as of December 2011 and illustrated with OSPrey (Stark et al., 2006).

function of metabolic activity: The recombination frequency in yeast overexpressing Tdh3 increased from 3 times over wild-type to more than 10 times in exponential batch cultures, the condition that exhibits the highest glycolytic flux (Ralser et al., 2009b).

Similar observations were made with alcohol dehydrogenase *ADH1*, another component of the Sir2 protein complex (Gavin et al., 2002; **Figure 2B**). Adh1 catalyzes the conversion of acetaldehyde to ethanol, regenerating NAD^+. Overexpression of the Adh1 enzyme increased both the NAD^+/NADH ratio and the activity of Sir2. Moreover, this treatment caused a 30% extension in yeast replicative life span (Reverter-Branchat et al., 2007). Thus, it is conceivable that in the close proximity within a protein complex, metabolic enzymes GAPDH (Tdh3) and alcohol dehydrogenase (Adh1) provide or deprive NAD^+ that is used by Sir2. As the catalytic reactions catalyzed by Tdh3 and Adh1 are reversible, this catabolic function does not only rely on the overall metabolic flux, but also on the reaction equilibrium in the microenvironment: these enzyme may provide or metabolize NAD^+ dependent on their localization.

The situation of the third metabolic enzyme, malate dehydrogenase Mdh1 is different, as it localizes to mitochondria, were, to our knowledge, yeast Sir2 is not localized (Blander and Guarente, 2004) Mdh1p, is involved in the citrate cycle and component of the malate-aspartate NADH shuttle. However, it has been reported that Mdh1 is over-expressed under conditions of calorie restriction (Lee and Lee, 2008), and that ectopic overexpression of Mdh1 causes extension of replicative lifespan. Remarkably, this lifespan extension was Sir2 dependent (Easlon et al., 2008). Although these proteins do not co-localize, they are part of a heavily interconnected interaction sub-network involving proteins Sir2, Mdh1, Rad53, Aat1, Fob1, and Hst1 (**Figure 2C**). Thus, although physical association of the two proteins may be unlikely as they predominantly localize to different compartments, they are involved in a network of close genetic interactions, and their interplay is involved in yeast replicative lifespan. Nonetheless, it remains possible that either Sir2 or Mdh1 do co-localize under conditions which have not yet been defined.

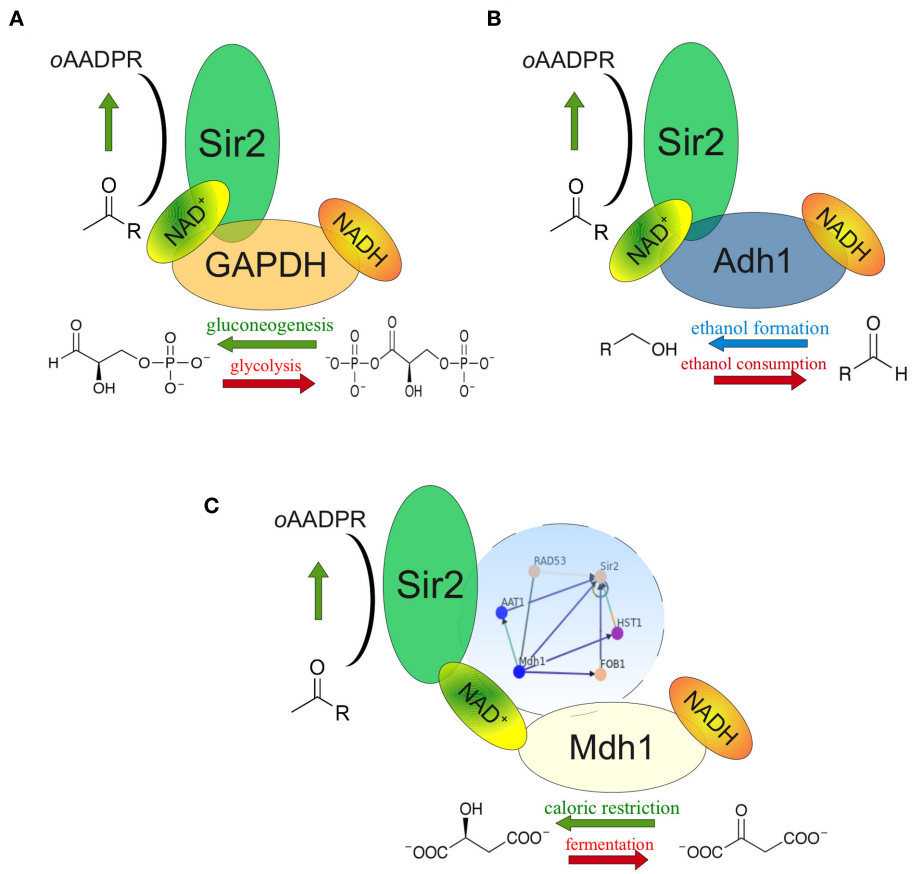

FIGURE 2 | NAD^+ feeding or depletion in the Sir2 microenvironment. **(A)** The NAD(H) metabolizing glycolytic enzyme GAPDH (Tdh3) is found in the same protein complex as Sir2. At high glycolytic flux, GAPDH deprives NAD^+ in close proximity to Sir2; during gluconeogenesis it generates NAD^+. Genetic interplay of Tdh3 and Sir2 influences the rate of mitotic recombination. **(B)** The predominant cytoplasmic alcohol dehydrogenase (Adh11) also complexes with Sir2. Adh1 overexpression increased the NAD^+/NADH ratio and Sir2 activity as well as prolonged yeast replicative lifespan. **(C)** Mitochondrial malate dehydrogenase Mdh1 is up-regulated during caloric restriction. Its overexpression increases NADH oxidation and prolongs replicative lifespan in a Sir2 dependent manner. Complex formation between Sir2 and Mdh1 has not been reported, but both proteins are interconnected through a dense genetic interaction network involving Rad53, Aat1, Fob1, and Hst1 (blue circle).

Metabolic regulation by Sir2 has been associated with NAD(H) biosynthesis and salvage pathways. These differ in some, but important, enzymatic steps among the eukaryotic lineage. While mammals prevalently use nicotinamide as the main NAD^+ source, single-cellular eukaryotes like *S. cerevisiae* have focused on utilizing tryptophan and nicotinic acid as precursors for NAD(H) biosynthesis (Rongvaux et al., 2003). Due to the lack of a homolog to the mammalian Nicotinamide phosphoribosyltransferase (NAMPT) enzyme, the budding yeast is unable to synthesize NAD(H) directly from the precursor nicotinamide. Hence, nicotinamide gets converted to nicotinic acid, a substrate used by Npt1 (nicotinic acid phosphoribosyltransferase), to produce nicotinic acid mononucleotide (NaMN). This metabolite is further converted to NAD^+ by two additional enzymatic steps involving Nma1/Nma2 that generate deamido-NAD, and Qns1, which generates NAD^+ from deamido-NAD (Rongvaux et al., 2003). Although *S. cerevisiae* does not possess NAMPT or nicotinamide mononucleotide (NMN), it was shown that components of the NAD^+ biosynthesis and salvage pathways interact with Sir2 (Gallo et al., 2004).

Interestingly, also the quite recently discovered nicotinamide riboside salvage pathway is involved in the regulation of Sir2, and affects replicative lifespan. The eukaryotic nicotinamide riboside kinase (Nrk) converts nicotinamide riboside to NAD^+ by phosphorylation and adenylylation (Tempel et al., 2007). This pathway promoted Sir2 dependent repression of recombination, improved gene silencing, and extended lifespan without calorie restriction, but was dependent on the rate of NAD^+ synthesis (Belenky et al., 2007).

In general, this close association with central metabolic pathways seem to be specific for Sir2. In **Figure 3**, we illustrate the interaction network of *SIR2* and its homologous genes *HST1-4*. Although they are closely interconnected, the common interactors with a GO annotation metabolism have been associated with assembly or disassembly of the chromatin and, rather then with energy or intermediate-yielding metabolic pathways.

Sir2 CATALYZED CLEAVAGE OF NAD^+ AS REGULATORY METABOLIC REACTION

Another interesting, but still in its entire magnitude elusive mechanism in metabolic regulation concerns the putative signaling function of the cleavage products of NAD^+ (Lu and Lin, 2010; Qiu et al., 2010b). These are formed when Sir2 is active as a protein deacetylase. At low efficiency, the ADP ribose moiety can be transferred to the protein substrates like histone H4, but the majority yields nicotinamide and a unique metabolite, 2′*O*-acetyl-ADP-ribose (OAADPR; Imai et al., 2000; Tanner et al., 2000; Moazed, 2001; Tanny and Moazed, 2001; Bitterman et al., 2002; Jackson and Denu, 2002). There is experimental evidence that OAADPR represents a reporter metabolite with signaling function (Borra et al.,

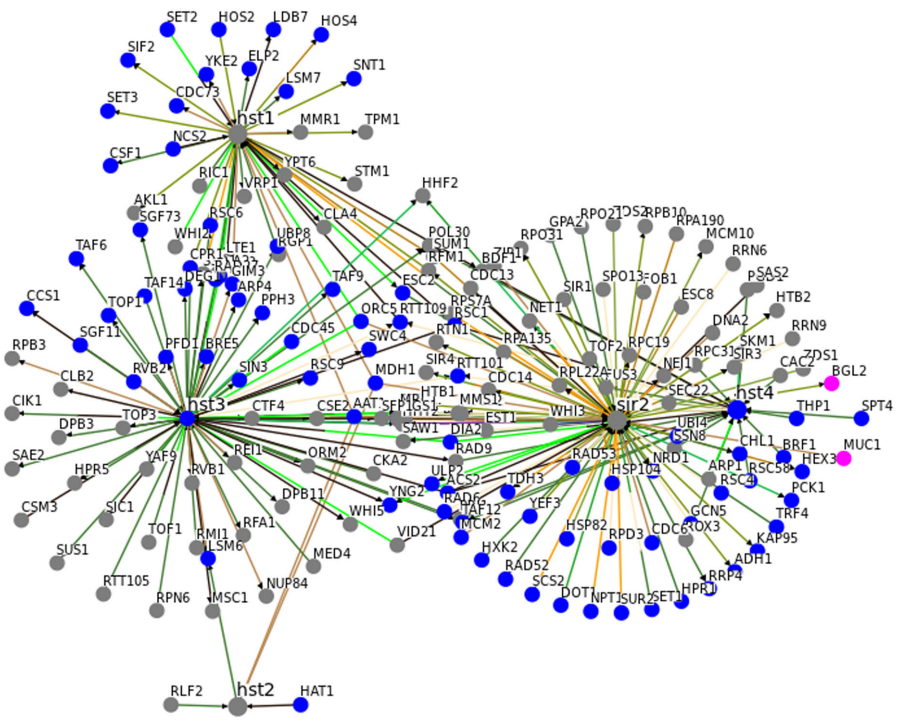

FIGURE 3 | An interaction network for *SIR2* and *HST1-4* indicates metabolism-specificity for *SIR2*. Genetic and physical interaction data for *SIR2 and HST1*, *HST2*, *HST3*, and *HST4* was obtained from yeast BioGrid database (01/2012; Stark et al., 2006). Genes associated with GO terms metabolism are highlighted in blue, those for carbon metabolism in pink. Sir2 has the highest number of metabolism related interactors. Moreover, metabolism related *HST1-4* interactors predominantly contain proteins involved in assembly, disassembly and repair of chromatin and DNA, rather then enzymes of primary and intermediary metabolism. (See **Table A1** in Appendix for GO terms associated with Sir2 interactors).

2002). In yeast, where NAD^+ is concentrated is the millimolar range, OAADPR levels of 0.5 μM have been detected (Lee et al., 2008). Furthermore, Lee et al. (2008) provided strong evidence that *SIR2* and its paralogous genes are the only source of this molecule in yeast, and that the Nudix hydrolase Ysa1 is involved in its degradation *in vivo*.

OAADPR has biological activity, although its exact molecular function remains elusive. Microinjection of OAADPR blocked oocyte maturation and cell division in *Asterina miniata* (Borra et al., 2002). Additional *in vitro* studies demonstrated that OAADPR binds to the cation channel TRPM2 (Grubisha et al., 2006) and the histone variant histone macroH2A1.1 (Kustatscher et al., 2005; Tong and Denu, 2010). Moreover, OAADPR promotes the association of multiple copies of Sir3 with Sir2/Sir4 and induces a structural rearrangement in the SIR complex (Liou et al., 2005). These studies suggest OAADPR functions as a signaling molecule and second messenger; however, there is a paucity of information that directly links cellular OAADPR with sirtuin functions.

More details are known about the second molecule, nicotinamide, which is a precursor of nicotinic acid (niacin/vitamin B3). Nicotinamide acts as inhibitor of Sir2. First, it had been reported that yeast silencing, rDNA recombination, and replicative lifespan in *sir2*Δ yeast depend on nicotinamide concentration (Bitterman et al., 2002). Then, it was demonstrated that the endogenous level of nicotinamide limits *Sir2* activity in wild-type yeast cells, and that this property can be antagonized by isonicotinamide, which caused an increase in Sir2 deacetylation activity (Sauve et al., 2005). This property may be explained by a Sir2 catalyzed transglycosidation reaction, were nicotinamide intercepts an ADP-ribosyl-enzyme-acetyl peptide intermediate regenerating NAD^+ (Jackson et al., 2003). Thus, high deacetylase activity in the Sir2 microenvironment is likely prevented by efficient product inhibition of Sir2.

Taken together, the Sir2's catalytic activity interferes in multiple ways with cellular metabolism, and the other way around, changes in metabolism modulate Sir2 activity. The action of Sir2 through its deacetylase activity is better understood, but also the indirect action of Sir2 through its catabolic products OAADPR and nicotinamide seems of high biological relevance. The analysis of the latter under *in vivo* conditions is very challenging, as it induces a very complex biological response, which demands for further intensive research both at the molecular/biochemical as well as at the systems biology level.

SUMMARY AND CONCLUSION

Stability and dynamics of metabolic networks require the interaction of the small molecule world with the genome and proteome. Sirtuins, at the forefront its founding member Sir2, belong to proteins which fulfill this function in a broad context. They interact with metabolites, proteins and nucleic acids, and spread metabolic signals through these molecular universes. However the complexity of these networks and their interconnectivity makes it difficult to associate unique molecular mechanisms with complex phenotypes such as an extended lifespan. Therefore, there remain several questions to be answered how Sir2 in yeast and the sirtuins in mammals regulate metabolism and thereby increase lifespan and cardiovascular health. Mechanistically, the function of Sir2 in increasing yeast replicative (but not chronological) lifespan is largely overlapping with caloric restriction, and is related to the activity and the stability of the metabolic network. Moreover, Sir2 is present in protein complexes containing major NAD^+ producing enzymes in the cell, indicating that this enzyme is supplied or deprived from NAD^+ in its microenvironment, dependent on the metabolic activity and flux. While these considerations are intuitively convincing, what is still needed and in most cases still missing, is a detailed chain of events based on biochemistry, which would explain the function of Sir2 and sirtuins in metabolic regulation and consequently in regulation of redox state, the aging process, and the diseases of aging like cancer, cardiovascular disease, and neurodegenerative disease.

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APPENDIX

Table A1 | Data analyzed by GO slim mapper, http://www.yeastgenome.org/, 02/02/2012.

GOID	GO term	Frequency	Genome frequency	Gene(s)
RESULTS FOR THE MAPPING OF 39 GENES TO THE YEAST GO-SLIM FUNCTION				
3677	DNA binding	5 out of 39 genes, 12.8%	369 of 6311 genes, 5.8%	MCM2, CDC6, RAD52, ORC5, RAD53
42393	Histone binding	4 out of 39 genes, 10.3%	39 of 6311 genes, 0.6%	DOT1, GCN5, YNG2, RSC4
16491	Oxidoreductase activity	4 out of 39 genes, 10.3%	276 of 6311 genes, 4.4%	SUR2, TDH3, MDH1, ADH1
16746	Transferase activity, transferring acyl groups	4 out of 39 genes, 10.3%	116 of 6311 genes, 1.8%	EPL1, GCN5, YNG2, ESA1
3723	RNA binding	4 out of 39 genes, 10.3%	753 of 6311 genes, 11.9%	SET1, MDH1, YEF3, NRD1
16887	ATPase activity	4 out of 39 genes, 10.3%	230 of 6311 genes, 3.6%	CDC6, RSC4, YEF3, HSP82
16874	Ligase activity	3 out of 39 genes, 7.7%	181 of 6311 genes, 2.9%	SLX5, RAD6, ACS2
3674	Molecular function unknown	3 out of 39 genes, 7.7%	1990 of 6311 genes, 31.5%	ESC2, RRP4, MUC1
8168	Methyltransferase activity	2 out of 39 genes, 5.1%	90 of 6311 genes, 1.4%	DOT1, SET1
16829	Lyase activity	2 out of 39 genes, 5.1%	84 of 6311 genes, 1.3%	PCK1, PAP2
16301	Kinase activity	2 out of 39 genes, 5.1%	199 of 6311 genes, 3.2%	HXK2, RAD53
988	Protein binding transcription factor activity	2 out of 39 genes, 5.1%	127 of 6311 genes, 2.0%	GCN5, RPD3
8289	Lipid binding	1 out of 39 genes, 2.6%	92 of 6311 genes, 1.5%	SCS2
16810	Hydrolase activity, acting on carbon–nitrogen (but not peptide) bonds	1 out of 39 genes, 2.6%	61 of 6311 genes, 1%	RPD3
8135	Translation factor activity, nucleic acid binding	1 out of 39 genes, 2.6%	44 of 6311 genes, 0.7%	YEF3
3729	mRNA binding	1 out of 39 genes, 2.6%	68 of 6311 genes, 1.1%	MDH1
16757	Transferase activity, transferring glycosyl groups	1 out of 39 genes, 2.6%	100 of 6311 genes, 1.6%	NPT1
16779	Nucleotidyltransferase activity	1 out of 39 genes, 2.6%	113 of 6311 genes, 1.8%	PAP2
5198	Structural molecule activity	1 out of 39 genes, 2.6%	356 of 6311 genes, 5.6%	HPR1
16798	Hydrolase activity, acting on glycosyl bonds	1 out of 39 genes, 2.6%	47 of 6311 genes, 0.7%	BGL2
8233	Peptidase activity	1 out of 39 genes, 2.6%	137 of 6311 genes, 2.2%	ULP2
51082	Unfolded protein binding	1 out of 39 genes, 2.6%	66 of 6311 genes, 1.0%	HSP82
4386	Helicase activity	1 out of 39 genes, 2.6%	80 of 6311 genes, 1.3%	CHL1
3924	GTPase activity	1 out of 39 genes, 2.6%	58 of 6311 genes, 0.9%	CDC6
30234	Enzyme regulator activity	1 out of 39 genes, 2.6%	219 of 6311 genes, 3.5%	KAP95
8565	Protein transporter activity	1 out of 39 genes, 2.6%	52 of 6311 genes, 0.8%	KAP95
32182	Small conjugating protein binding	1 out of 39 genes, 2.6%	43 of 6311 genes, 0.7%	SLX5
Other	Other	3 out of 39 genes, 7.7%		MCD1, AAT1, UBI4
RESULTS FOR THE MAPPING OF 39 GENES TO THE YEAST GO-SLIM PROCESS				
6974	Response to DNA damage stimulus	13 out of 39 genes, 33.3%	281 of 6311 genes, 4.5%	MCM2, MCD1, SLX5, HPR1, ESC2, DOT1, EPL1, RAD6, YNG2, RAD52, PAP2, ESA1, RAD53
6281	DNA repair	12 out of 39 genes, 30.8%	232 of 6311 genes, 3.7%	MCM2, MCD1, HPR1, ESC2, DOT1, EPL1, RAD6, YNG2, RAD52, PAP2, ESA1, RAD53
6325	Chromatin organization	10 out of 39 genes, 25.6%	223 of 6311 genes, 3.5%	DOT1, EPL1, RAD6, GCN5, YNG2, SET1, RSC4, ACS2, RPD3, ESA1
6366	Transcription from RNA polymerase II promoter	9 out of 39 genes, 23.1%	438 of 6311 genes, 6.9%	HPR1, EPL1, RAD6, GCN5, SET1, RSC4, NRD1, RPD3, ESA1

(Continued)

Table A1 | Continued

GOID	GO term	Frequency	Genome frequency	Gene(s)
278	Mitotic cell cycle	9 out of 39 genes, 23.1%	288 of 6311 genes, 4.6%	MCM2, MCD1, ESC2, RAD6, ULP2, CDC6, RSC4, RPD3, CHL1
16570	Histone modification	9 out of 39 genes, 23.1%	98 of 6311 genes, 1.6%	DOT1, EPL1, RAD6, GCN5, YNG2, SET1, ACS2, RPD3, ESA1
6310	DNA recombination	8 out of 39 genes, 20.5%	149 of 6311 genes, 2.4%	MCM2, HPR1, ESC2, DOT1, RAD6, RAD52, RPD3, CHL1
51726	Regulation of cell cycle	7 out of 39 genes, 17.9%	183 of 6311 genes, 2.9%	ESC2, DOT1, RAD6, ULP2, RPD3, ESA1, RAD53
6260	DNA replication	7 out of 39 genes, 17.9%	140 of 6311 genes, 2.2%	MCM2, CDC6, ORC5, RPD3, CHL1, RAD53, HSP82
43543	Protein acylation	6 out of 39 genes, 15.4%	64 of 6311 genes, 1.0%	EPL1, GCN5, YNG2, SET1, ACS2, ESA1
18193	Peptidyl-amino acid modification	6 out of 39 genes, 15.4%	83 of 6311 genes, 1.3%	EPL1, GCN5, YNG2, SET1, ACS2, ESA1
6354	Transcription elongation, DNA-dependent	5 out of 39 genes, 12.8%	72 of 6311 genes, 1.1%	HPR1, GCN5, RSC4, RPD3, ESA1
70647	Protein modification by small protein conjugation or removal	5 out of 39 genes, 12.8%	149 of 6311 genes, 2.4%	SLX5, RAD6, ULP2, UBI4, KAP95
5975	Carbohydrate metabolic process	4 out of 39 genes, 10.3%	275 of 6311 genes, 4.4%	HXK2, TDH3, PCK1, ADH1
6091	Generation of precursor metabolites and energy	4 out of 39 genes, 10.3%	163 of 6311 genes, 2.6%	HXK2, TDH3, MDH1, ADH1
51052	Regulation of DNA metabolic process	4 out of 39 genes, 10.3%	71 of 6311 genes, 1.1%	CDC6, RPD3, CHL1, HSP82
51186	Cofactor metabolic process	4 out of 39 genes, 10.3%	164 of 6311 genes, 2.6%	MDH1, ACS2, ADH1, NPT1
48285	Organelle fission	4 out of 39 genes, 10.3%	124 of 6311 genes, 2%	MCD1, ESC2, ULP2, CHL1
51321	Meiotic cell cycle	4 out of 39 genes, 10.3%	162 of 6311 genes, 2.6%	DOT1, RAD6, RAD52, RPD3
32200	Telomere organization	4 out of 39 genes, 10.3%	67 of 6311 genes, 1.1%	SLX5, SET1, RAD52, HSP82
7059	Chromosome segregation	3 out of 39 genes, 7.7%	131 of 6311 genes, 2.1%	MCD1, ESC2, CHL1
33043	Regulation of organelle organization	3 out of 39 genes, 7.7%	143 of 6311 genes, 2.3%	SET1, ULP2, HSP82
6605	Protein targeting	3 out of 39 genes, 7.7%	266 of 6311 genes, 4.2%	SCS2, KAP95, HSP82
6629	Lipid metabolic process	3 out of 39 genes, 7.7%	262 of 6311 genes, 4.2%	SUR2, SCS2, KAP95
55086	Nucleobase-containing small molecule metabolic process	3 out of 39 genes, 7.7%	183 of 6311 genes, 2.9%	ADH1, NPT1, RAD53
51169	Nuclear transport	3 out of 39 genes, 7.7%	163 of 6311 genes, 2.6%	HPR1, SCS2, KAP95
8213	Protein alkylation	2 out of 39 genes, 5.1%	41 of 6311 genes, 0.6%	DOT1, SET1
43934	Sporulation	2 out of 39 genes, 5.1%	126 of 6311 genes, 2%	SET1, UBI4
31399	Regulation of protein modification process	2 out of 39 genes, 5.1%	73 of 6311 genes, 1.2%	SET1, KAP95
51049	Regulation of transport	2 out of 39 genes, 5.1%	58 of 6311 genes, 0.9%	SCS2, RAD6
6520	Cellular amino acid metabolic process	2 out of 39 genes, 5.1%	240 of 6311 genes, 3.8%	AAT1, ADH1
6401	RNA catabolic process	2 out of 39 genes, 5.1%	106 of 6311 genes, 1.7%	RRP4, PAP2
70271	Protein complex biogenesis	2 out of 39 genes, 5.1%	222 of 6311 genes, 3.5%	KAP95, HSP82
43144	snoRNA processing	2 out of 39 genes, 5.1%	37 of 6311 genes, 0.6%	RRP4, PAP2
71554	Cell wall organization or biogenesis	1 out of 39 genes, 2.6%	211 of 6311 genes, 3.3%	BGL2
6397	mRNA processing	1 out of 39 genes, 2.6%	185 of 6311 genes, 2.9%	HPR1
6360	Transcription from RNA polymerase I promoter	1 out of 39 genes, 2.6%	63 of 6311 genes, 1%	RPD3
45333	Cellular respiration	1 out of 39 genes, 2.6%	90 of 6311 genes, 1.4%	MDH1
6457	Protein folding	1 out of 39 genes, 2.6%	88 of 6311 genes, 1.4%	HSP82
42221	Response to chemical stimulus	1 out of 39 genes, 2.6%	351 of 6311 genes, 5.6%	HXK2
6414	Translational elongation	1 out of 39 genes, 2.6%	332 of 6311 genes, 5.3%	YEF3

(Continued)

Table A1 | Continued

GOID	GO term	Frequency	Genome frequency	Gene(s)
7005	Mitochondrion organization	1 out of 39 genes, 2.6%	333 of 6311 genes, 5.3%	HSP82
6353	Transcription termination, DNA-dependent	1 out of 39 genes, 2.6%	34 of 6311 genes, 0.5%	NRD1
15931	Nucleobase-containing compound transport	1 out of 39 genes, 2.6%	116 of 6311 genes, 1.8%	HPR1
6869	Lipid transport	1 out of 39 genes, 2.6%	49 of 6311 genes, 0.8%	SCS2
55085	Transmembrane transport	1 out of 39 genes, 2.6%	186 of 6311 genes, 2.9%	SCS2
51603	Proteolysis involved in cellular protein catabolic process	1 out of 39 genes, 2.6%	192 of 6311 genes, 3.0%	RAD6
6997	Nucleus organization	1 out of 39 genes, 2.6%	54 of 6311 genes, 0.9%	KAP95
7124	Pseudohyphal growth	1 out of 39 genes, 2.6%	65 of 6311 genes, 1.0%	MUC1
8643	Carbohydrate transport	1 out of 39 genes, 2.6%	38 of 6311 genes, 0.6%	HXK2
6364	rRNA processing	1 out of 39 genes, 2.6%	294 of 6311 genes, 4.7%	RRP4
1403	Invasive growth in response to glucose limitation	1 out of 39 genes, 2.6%	52 of 6311 genes, 0.8%	MUC1
9408	Response to heat	1 out of 39 genes, 2.6%	47 of 6311 genes, 0.7%	RPD3
48308	Organelle inheritance	1 out of 39 genes, 2.6%	51 of 6311 genes, 0.8%	SCS2
6970	Response to osmotic stress	1 out of 39 genes, 2.6%	75 of 6311 genes, 1.2%	HSP82

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Inhibition of sirtuin 2 with sulfobenzoic acid derivative AK1 is non-toxic and potentially neuroprotective in a mouse model of frontotemporal dementia

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Tauopathies including tau-associated Frontotemporal dementia (FTD) and Alzheimer's disease are characterized pathologically by the formation of tau-containing neurofibrillary aggregates and neuronal loss, which contribute to cognitive decline. There are currently no effective treatments to prevent or slow this neural systems failure. The rTg4510 mouse model, which expresses a mutant form of the tau protein associated with FTD with Parkinsonism-17, undergoes dramatic hippocampal and cortical neuronal loss making it an ideal model to study treatments for FTD-related neuronal loss. Sirtuins are a family of proteins involved in cell survival that have the potential to modulate neuronal loss in neurodegenerative disorders. Here we tested the hypothesis that sirtuin 2 (SIRT2) inhibition would be non-toxic and prevent neurodegeneration in rTg4510 brain. In this study we delivered SIRT2 inhibitor AK1 directly to the hippocampus with an osmotic minipump and confirmed that it reached the target region both with histological assessment of delivery of a dye and with a pharmacodynamic marker, ABCA1 transcription, which was upregulated with AK1 treatment. AK1 treatment was found to be safe in wild-type mice and in the rTg4510 mouse model, and further, it provided some neuroprotection in the rTg4510 hippocampal circuitry. This study provides proof-of-concept for therapeutic benefits of SIRT2 inhibitors in both tau-associated FTD and Alzheimer's disease, and suggests that development of potent, brain permeable SIRT2 inhibitors is warranted.

Keywords: tauopathy, frontotemporal dementia, Alzheimer, sirtuin, neuroprotection

INTRODUCTION

Tauopathies are devastating conditions involving dramatic neuronal loss and resulting circuit dysfunction for which there are currently no effective treatments (Roberson, 2011). Investigation of the neuroprotective effects of sirtuin modulation may pave the way for future therapeutic studies in tauopathies and other neurodegenerative disorders which share similar mechanisms of degeneration (Spires and Hannan, 2007). Sirtuins are a family of unique NAD^+-dependent enzymes, which regulate diverse cellular pathways, but are primarily involved in metabolic regulation (Guarente and Picard, 2005; Michan and Sinclair, 2007). Microtubule deacetylase sirtuin 2 (SIRT2) is a highly abundant protein in CNS, which accumulates in the aging brain (Maxwell et al., 2011). This is particularly interesting in tauopathies since tau is a microtubule binding protein and microtubule-dependent processes such as axonal transport are disrupted (Zhang et al., 2005).

In vitro evidence indicates that sirtuin modulation may be protective against amyloid toxicity in Alzheimer's disease, particularly that activating SIRT1 can be protective (Chen et al., 2005; Qin et al., 2006). Small molecule inhibitors of SIRT2 have also been found to ameliorate neurodegeneration in model systems (Outeiro et al., 2008). For example, structurally diverse selective SIRT2 inhibitors AGK2 ($IC_{50} = 3.5\,\mu M$) and AK1 ($IC_{50} = 12.5\,\mu M$) prevent alpha-synuclein toxicity in cell-based, and *Drosophila* models of Parkinson's (Outeiro et al., 2007). In Huntington's disease, both inhibitors, AGK2 and AK1, were protective against mutant polyglutamine toxicity in *Drosophila* and *C. elegans* animal models and in primary striatal neurons (Luthi-Carter et al., 2010). In the latter model, the protective effects of SIRT2 inhibition were associated with reduced total cholesterol levels due to facilitated cytoplasmic retention of sterol regulatory element binding protein-2 (SREBP-2), a key transcriptional regulator of cholesterol biosynthesis genes in nuclei (Zhang et al., 2005; Luthi-Carter et al., 2010; Taylor et al., 2011). However, SIRT2 inhibition caused broad changes in transcriptional expression of metabolic genes. Cholesterol processing has also been implicated in both Alzheimer's disease (largely linked to amyloid processing) and in other tauopathies. Loss of function of the NPC1 or NPC2 genes, which are involved in trafficking cholesterol from lysosomes, has been linked to Niemann–Pick disease type C disease which includes tau pathology (Klunemann et al., 2002; Yu et al., 2005). Further, in the THY-Tau22 mouse model of tauopathy, long-term voluntary exercise prevented memory impairment and reduced

hippocampal tau pathology associated with the upregulation of the NPC1 and NPC2 genes (Belarbi et al., 2011).

The promising results in efficacy of SIRT2 inhibition in two neurodegenerative diseases, the mechanistic link between the SIRT2 induced-downregulation of cholesterol biosynthesis, and the association of cholesterol dysregulation in tauopathy led to the hypothesis that SIRT2 inhibition may be neuroprotective in tauopathy. Here we assess the safety of brain delivery of the SIRT2 inhibitor AK1 in wild-type mice and in the rTg4510 transgenic model of tauopathy. The rTg4510 transgenic mouse model expresses a human tau gene with a mutation associated with familial frontotemporal dementia (FTD). These mice exhibit age-related cognitive decline paralleled by the loss of neurons and the formation of tau-containing neurofibrillary tangles similar to those seen in Alzheimer's disease and FTD (SantaCruz et al., 2005; Spires et al., 2006; Spires-Jones et al., 2008).

In this study, we sought to test effects of SIRT2 inhibition *in vivo* and evaluate the safety of such an approach. Since neither of the published high efficiency SIRT2 inhibitors AGK2 nor AK1 are brain permeable, we selected a direct route for drug administration to mouse brain. Despite higher potency of SIRT2 inhibition, AGK2 has poor water solubility (cLogP = 5.9) in contrast to AK1 (cLogP = 4.0), making AK1 the better choice for direct administration to the brain. The high water solubility of sulfobenzoic acid derivative AK1 also permits robust reduction of cholesterol by this compound in striatal neurons (Luthi-Carter et al., 2010; Taylor et al., 2011).

We find that infusion of AK1 with an osmotic minipump directly into the hippocampus of mice for 5 weeks does not cause any neuronal loss in the Cornu Ammonis 1 (CA1) or dentate gyrus (DG) regions of the hippocampus compared to the untreated hemispheres or vehicle control treatment, even in the context of a tau transgenic mouse which is susceptible to neuronal loss. Furthermore, we observe a modest prevention of neuronal loss in the DG of rTg4510 mice, indicating that inhibition of SIRT2 is a safe and promising neuroprotective agent.

MATERIALS AND METHODS

ANIMALS AND SURGERY

Animals used in this study were mixed gender rTg4510 mice expressing both human mutant P301L tau downstream of a tetracycline-operon-responsive element tau-P301L and an activator tetracycline-off transgene controlled by Ca^{2+}-calmodulin kinase II promoter elements and littermate "wild-type" controls (which do not have a phenotype) expressing only the activator transgene (without tau; SantaCruz et al., 2005). **Table 1** shows the numbers of animals used at each age. Each animal had an osmotic minipump (Alzet 2006, DURECT Corporation, Cupertino, CA, USA) implanted, which pumped drug or vehicle for 5 weeks before sacrifice. A further six wild-type animals were used for 1 week studies: three had a pump with 0.05% trypan blue in 5% DMSO implanted for 1 week to verify cannula implantation coordinates and the spread of pumped solution through the hippocampus (**Figure 1E**) and three more received AK1 treatment for 1 week followed by gene expression analysis using qPCR. Before implantation, pumps with cannulas were assembled, filled with 50 μM AK1 [3-(1-azepanylsulfonyl)-N-(3-nitrophenyl)benzamide from ChemBridge, diluted 1:200 in PBS from 10 mM stock in DMSO], or vehicle (1:200 DMSO:PBS) and incubated at 37°C for 60 h to prime the pumps. To asses safety, we used the maximum concentration of AK1 (50 μM) that kept the DMSO levels to a maximum of 5%, above which DMSO can become neurotoxic (Cavas et al., 2005). Animals were anesthetized with ketamine/xylazine anesthesia (100 mg/kg ketamine, 10 mg/kg xylazine, i.p.), the pump body inserted subcutaneously over the mid-scapular area, and the cannula was implanted into the right hippocampal formation through a burr hole at coordinates −2.0 posterior to Bregma and 1.5 mm to the right of the midline, 1.8 mm deep (**Figure 1C**). Five weeks after pump implantation, animals were sacrificed with carbon dioxide, and brains removed and fixed in 4% paraformaldehyde for 48 h followed by paraffin embedding. Each cannula was tested to ensure it was not blocked and the pumps examined to ensure the compound was not remaining in the pump. Due to pump failures, group sizes are not equal. **Table 1** reflects numbers of animals in each group that were analyzed (pumps worked). All animal work conforms to institutional and NIH guidelines.

Table 1 | Animals used in the study.

Genotype	Age at sacrifice (month)	Treatment	*n*
Wild-type	5.5	AK1	3
Wild-type	5.5	vehicle	3
Wild-type	7	AK1	5
Wild-type	7	vehicle	5
rTg4510	5.5	AK1	4
rTg4510	5.5	vehicle	5
rTg4510	7	AK1	7
rTg4510	7	Vehicle	5

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Paraffin embedded brain hemispheres were cut in sagittal orientation in 16 μm sections. Every 30th section throughout the hippocampal formation was deparaffinized and stained with anti PHF-1 tau primary antibody (1:200 in 1% milk, courtesy Dr Peter Davies), and anti mouse IgG secondary antibody conjugated to Cy3 (1:500, Jackson ImmunoResearch) and nuclei counterstained with DAPI (4′,6-diamidino-2-phenylindole). Sections were observed on an upright Olympus BX51 fluorescence microscope with a DP70 camera and a stereology image analysis system (CAST; Olympus, Denmark). Neuronal nuclei were identified based on morphology as described previously (Spires et al., 2006; Fox et al., 2011).

Neuron densities and PHF1 positive neuron densities in the CA1 pyramidal cell layer and DG granular layer were estimated using the optical fractionator method, volumes of these regions estimated using the Cavalieri method, and total neuron number in CA1 and DG calculated as previously described (Spires et al., 2006).

CELL CULTURE

Primary cortical neurons were prepared from mouse embryos at embryonic day 15 and cultured in neurobasal media supplemented with 2% B-27 and 1% penicillin/streptomycin. At 7 days *in vitro*,

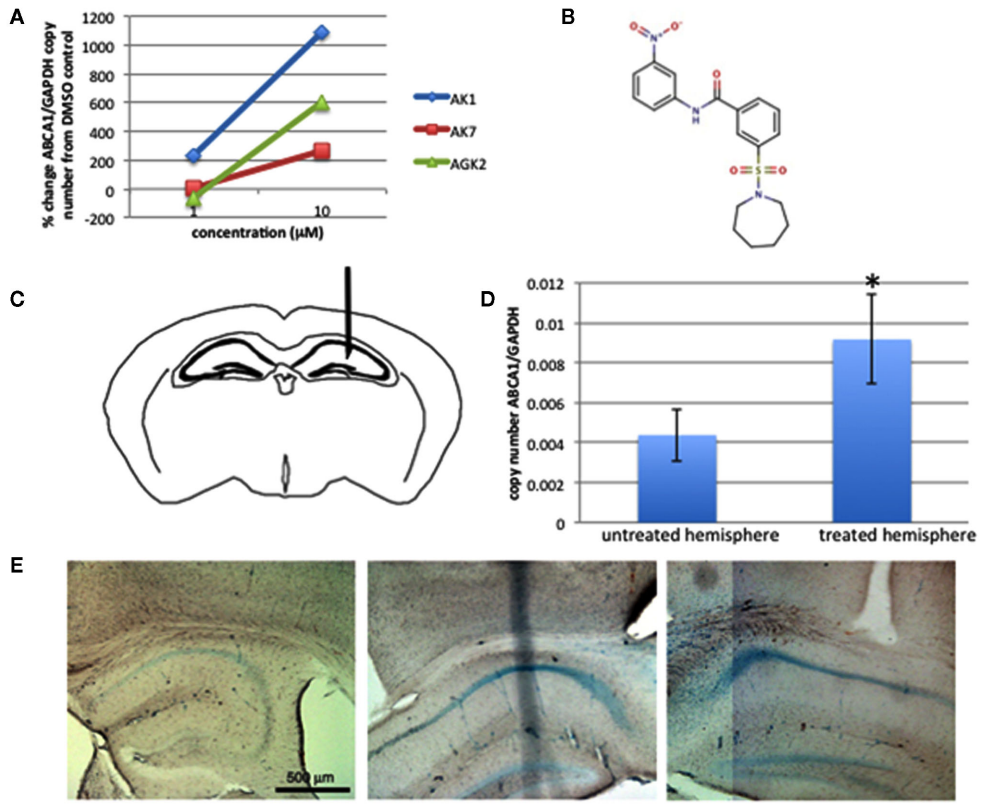

FIGURE 1 | AK1 affects ABCA1 transcription *in vitro* and *in vivo*. Treatment of cultured primary cortical neurons with SIRT2 inhibitors AK1, AK7, and AGK2 caused an increase in ABCA1 mRNA levels **(A)**. AK1 [structure shown in **(B)**] was pumped into the hippocampus of mice [site shown in **(C)**]. As seen in cultured neurons, 1 week of treatment in wild-type mice increases ABCA1 expression in the treated hemisphere compared to the untreated hemisphere [**(D)**, $p < 0.05$], indicating the drug reached the brain. Trypan blue pumped for 7 days further confirms the cannula placement in the hippocampus and shows spread of pumped material through the entire hippocampal formation **(E)**.

neurons were treated for 24 h with 1 μM or 10 μM AK7, AGK2, or AK1 (from ChemBridge, San Diego, CA, USA). Neurons were then harvested and homogenized for RNA extraction as described below.

QUANTITATIVE PCR

Three wild-type mice were treated with AK1 administration to the right hippocampus for 1 week. At the end of the week, mice were sacrificed, brains removed and the hemispheres frozen separately. Tissue was homogenized in RNase free lysis buffer containing 50 mM tris buffer, 175 mM NaCl, 5 mM EDTA, RNase inhibitors (Ambion AM2694), and proteinase inhibitor cocktail (Roche Complete Mini). RNA was isolated with the Stratagene Absolutely RNA Miniprep kit and cDNA generated using SuperScript reverse Transcriptase (Invitrogen). Quantitative PCR was run on an iCycler (Bio-Rad, Hercules, CA, USA) using GAPDH as a standard housekeeping gene and ABCA1 with the following primers: *left*, gctcaatgtcatccgtcttc; *right*, ctggaaacacactttccttatc.

STATISTICS

Statistical tests were run using JMP software (SAS Institute, Cary, NC, USA). Normality of data was assessed using a Shapiro–Wilk test. Non-normal data (including stereological measures) were analyzed with non-parametric tests (Kruskal–Wallis for multiple comparisons, *post hoc* Wilcoxon tests to compare groups). Normal data (ABCA1 expression) were analyzed with parametric statistics (*t*-tests for two groups, ANOVA for multiple groups). $p < 0.05$ is considered significant. Data are presented as mean ± standard deviation from the mean.

RESULTS

AK1 ADMINISTRATION TO THE BRAIN IS NOT NEUROTOXIC

Previous work has shown that SIRT2 inhibition with compounds AGK2, AK1, and AK7 affects transcription of genes involved in cholesterol pathways in striatal neuronal cultures (Luthi-Carter et al., 2010; Taylor et al., 2011). We verified this in primary cortical neuronal cultures (**Figure 1A**), where we observed an increase in mRNA levels of ATP-binding cassette transporter ABCA1 (a known transporter of cholesterol) with SIRT2 inhibition. AK1 showed the highest efficiency in this test in our cortical cultures, and it is known to be protective *in vitro* against toxicity of mutant huntingtin and alpha-synuclein (Outeiro et al., 2007; Luthi-Carter et al., 2010), thus we moved forward to test the safety and efficacy of AK1 in treating tauopathy model mice. To test the safety of administering the SIRT2 inhibitor AK1 (structure shown in **Figure 1B**) directly to the brain, we filled osmotic

minipumps with a solution of 50 μM AK1 in PBS (with 0.5% DMSO) or vehicle and pumped the solution into the hippocampus for 5 weeks (**Figure 1C**). In three wild-type mice treated with AK1 for 1 week then sacrificed for molecular analysis of the brain, we confirmed that AK1 increased expression of ABCA1 in the treated versus untreated hemisphere (146.9% higher in treated versus untreated hemisphere, **Figure 1D**). This indicates that AK1 may act through modulating cholesterol homeostasis as previously reported (Zhang et al., 2005; Luthi-Carter et al., 2010; Taylor et al., 2011). Further, this is a pharmacodynamic marker reporting compound activity in the brain, indicating the AK1 successfully reached the brain tissue. Tests pumping 0.05% trypan blue for 1 week confirmed the delivery of the blue dye throughout the hippocampus (**Figure 1E**), further confirming the delivery of compound through the entire region of interest that we want to treat in models of tau-induced neurodegeneration.

After these preliminary studies pumping the drug into the brain for 1 week confirmed delivery of the drug to the brain and showed a pharmacodynamic response, we treated mice for 5 weeks, which is a therapeutic window long enough to observe neuronal death in mouse models of neurodegeneration. After pumping AK1 or vehicle for 5 weeks (from approximately 4 to 5.5 months of age), neuron numbers in the CA1 and DG regions of the hippocampus were estimated using stereology. In wild-type mice, we observed no neuronal loss after pumping AK1 compared to the vehicle control (**Figure 2A**). Comparing the treated versus untreated hemispheres in individual animals also revealed no loss in the treated side, indicating the placement of the cannula itself does not cause detectable neuronal loss (**Figure 2C**).

rTg4510 mice undergo neuronal loss in the hippocampus, thus we tested whether implantation of the cannula or the AK1 compound itself cause any neuronal loss in this model which is susceptible to neurodegeneration. As seen in the wild-type littermates, 5 weeks of vehicle or AK1 treatment from 4 to 5.5 months of age did not cause any neuronal loss in the CA1 or DG of rTg4510 mice (**Figures 2B,D**).

AK1 DOES NOT AFFECT NEUROFIBRILLARY TANGLE FORMATION

In cellular models of Parkinson's disease, SIRT2 inhibition with AK1 prevented alpha-synuclein mediated cell death and also interestingly promoted alpha-synuclein aggregate formation (Outeiro et al., 2007). Thus we tested whether AK1 treatment from 4 to 5.5 months of age changed the number of neurofibrillary tangles in the brains of treated rTg4510 mice. In previous studies, we have shown that PHF1 staining in rTg4510 mice corresponds exactly to Bielchowski positive neurofibrillary tangles at these ages (Spires et al., 2006), thus PHF1 immunostaining was used to characterize neurofibrillary pathology. Stereological counts of PHF1 positive neurons show no effect of AK1 treatment on tangle numbers either when compared to vehicle treatment or when compared to the contralateral hemisphere (**Figure 3**), indicating that SIRT2 inhibition *in vivo* does not affect neurofibrillary tangle formation or stability.

POTENTIAL NEUROPROTECTIVE EFFECTS OF AK1 TREATMENT

rTg4510 mice undergo neuronal loss in the hippocampus and neocortex that is associated with behavioral deficits (SantaCruz et al., 2005; Spires et al., 2006), and neuronal loss correlates with dementia in FTD and Alzheimer's disease (DeKosky and Scheff, 1990; DeKosky et al., 1996). In control animals, there is no effect of age or treatment on the number of neurons in the DG granular cell layer ($1.57 \times 10^5 \pm 0.85 \times 10^5$ neurons per hemisphere, $p > 0.05$ with treatment and age as independent variables, split by genotype). We observe a 42% loss of DG neurons between 5.5 and 7 months in vehicle treated rTg4510 mice from $1.6 \times 10^5 \pm 0.3 \times 10^5$ neurons/hemisphere to

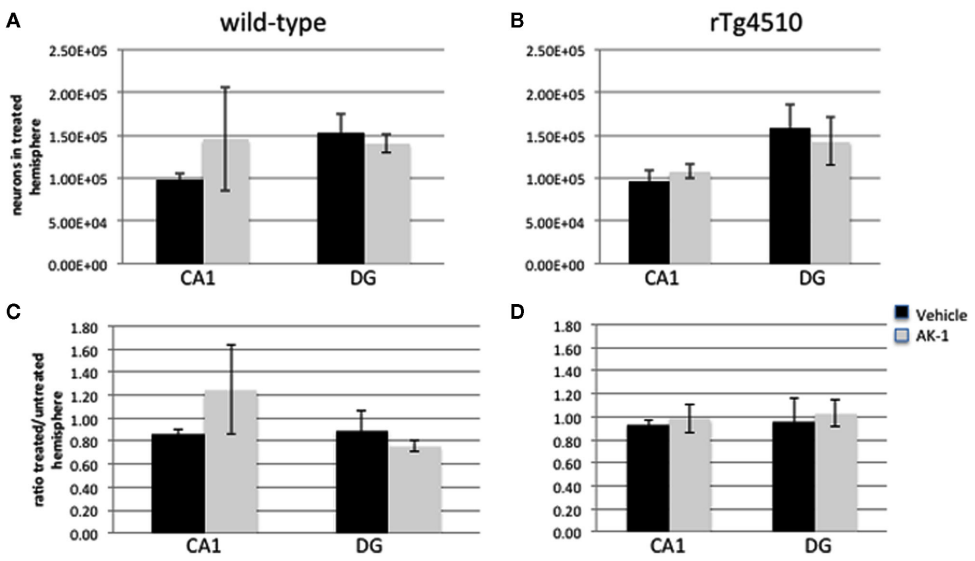

FIGURE 2 | AK1 delivery to the hippocampus is non-toxic. AK1 administration from 4 to 5.5 months of age does not cause neurodegeneration in wild-type **(A)** or rTg4510 hippocampus **(B)**. Placement of the cannula in the treated hemisphere does not cause unilateral neuron loss in wild-type **(C)** or rTg4510 **(D)** hippocampus.

$0.9 \times 10^5 \pm 0.1 \times 10^5$ neurons/hemisphere ($p = 0.009$, Wilcoxon test). This loss was partially prevented by AK1 treatment (**Figure 4**). rTg4510 mice treated with AK1 had significantly higher numbers of DG neurons than vehicle treated rTg4510 mice at 7 months ($p = 0.03$, Wilcoxon test), and they are not significantly different from control animals (control $p > 0.05$). These results indicate that AK1 treatment may be neuroprotective in tauopathy.

DISCUSSION

There is a pressing need to develop neuroprotective therapies for tauopathies including tau-associated FTD and Alzheimer's disease, for which there are currently no effective treatments (Robertson, 2011). One promising therapeutic avenue is manipulation of sirtuins. In particular, SIRT2 inhibition has been shown to be neuroprotective in neuronal cell culture and invertebrate animal models of Parkinson's and Huntington's diseases (Outeiro

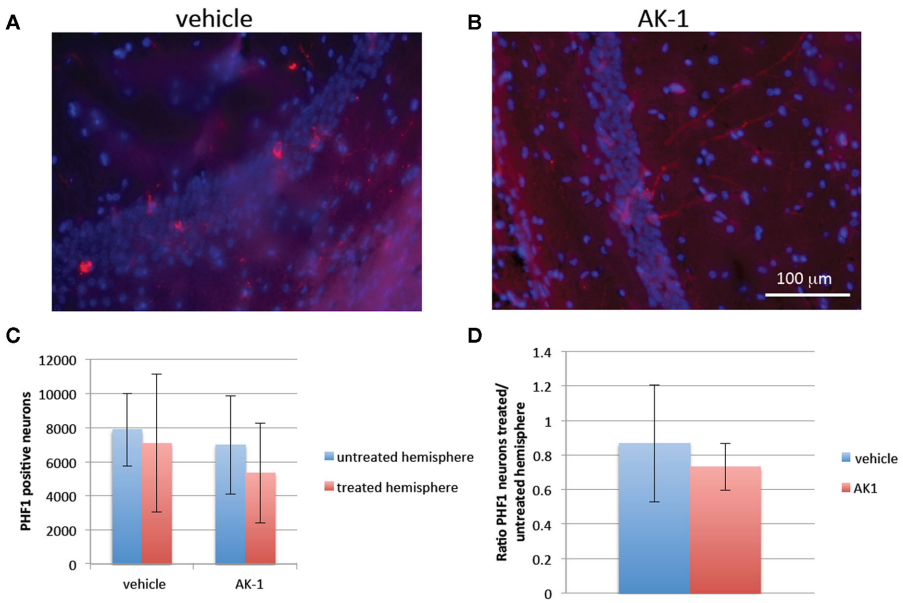

FIGURE 3 | AK1 treatment does not affect numbers of PHF1-positive neurofibrillary tangles. Micrographs from rTg4510 mice treated from 4 to 5.5 months with vehicle **(A)** or AK1 **(B)** show that both treatment groups have PHF1 positive neurofibrillary tangles (red) in the treated CA1. Quantification reveals no difference in the numbers of tangles in the treated and untreated hemispheres either when all animals are averaged **(C)** or when a ratio of treated/untreated hemisphere is calculated for each animal before averaging **(D)**.

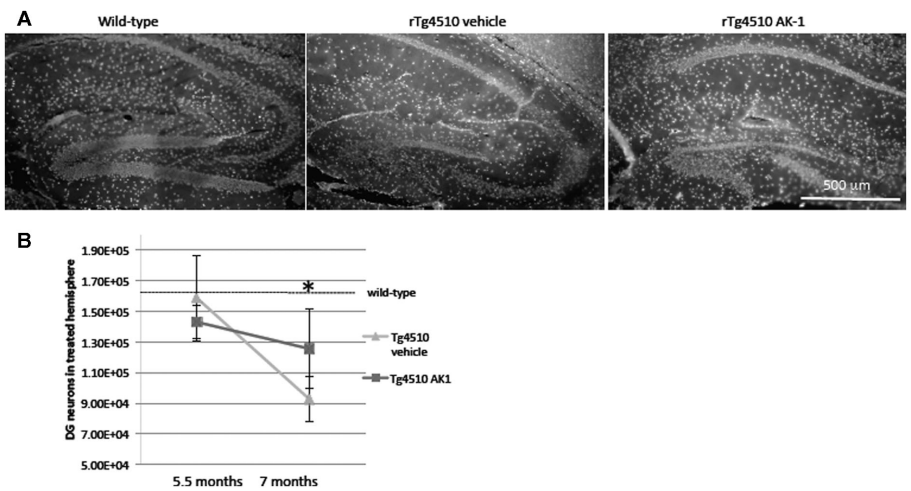

FIGURE 4 | AK1 treatment partially protects against tau-induced neurodegeneration. Micrographs of DAPI staining of the dentate gyrus **(A)** demonstrate the loss of neurons in rTg4510 mice compared to control mice at 7 months of age. rTg4510 mice treated with vehicle from 5.5 to 7 months of age underwent neuronal loss, compared both to wild-type and 5.5-month-old rTg4510 animals (**B**, $*p < 0.05$). AK1 treatment partially prevented this loss. AK1 treated rTg4510 DG does not have significant loss compared to wild-type or 5.5-month-old rTg4510 brain.

et al., 2007; Luthi-Carter et al., 2010). Due to the ongoing discussion and controversy on the therapeutic role of sirtuins in neurodegeneration, it was imperative to assess the properties of SIRT2 inhibition on the brain directly. While we cannot rule out off-target effects of the AK1 compound, it is the case that most marketed drugs interact with multiple targets yet mediate therapeutic effects. The purpose of this study was to assess preliminary toxicity and efficacy of a SIRT2 inhibitor belonging to a therapeutically promising structural scaffold of sulfobenzoic acid derivatives, prior to medicinal chemical optimization for drug-like properties, brain permeability, bioavailability, and bioactivity.

Tauopathies are characterized by the accumulation of aggregated tau pathology and neuronal loss. In this study, we tested whether inhibition of SIRT2 with AK1 delivery directly to the hippocampus could prevent neuronal loss in rTg4510 mice. We find that in both wild-type and rTg4510 brain, AK1 treatment was non-toxic to neurons (no neurons were lost with treatment from 4 to 5.5 months). Furthermore, between 5.5 and 7 months of age, neurons are lost in the DG of rTg4510 mice, and this is partially prevented by AK1 treatment. It is worth noting that the neuronal counts in this study show a phenotypic drift toward delayed neuronal loss in our rTg4510 colony with no neuronal loss in CA1 or DG between 4 and 5.5 months and no loss in CA1 between 5.5 and 7 months, whereas previous characterization of this model by our group found a steep decline in hippocampal neuron numbers in rTg4510 mice between 4 and 5.5 months of age (Spires et al., 2006). In Alzheimer's disease, neurofibrillary tangles were classically viewed as toxic since they correlate with cognitive decline, but new evidence has emerged suggesting that soluble forms of tau may be more toxic than aggregates (Spires-Jones et al., 2009). As such, it is important when developing new pharmacological targets to assess their effects both on neuronal loss and on tau aggregates. Here, we observe neuroprotection in the hippocampus without affecting neurofibrillary tangle pathology, lending support to the hypothesis that soluble tau is neurotoxic.

The mechanisms of tau toxicity are unclear but there is some evidence implicating altered cholesterol processing in the neurodegenerative process (Klunemann et al., 2002; Yu et al., 2005; Belarbi et al., 2011), and SIRT2 inhibition is known to modulate cholesterol biosynthesis in cultured neurons (Taylor et al., 2011). Here we confirm *in vivo* that the expression of ABCA1, which is a major regulator of cholesterol homeostasis, is affected in the brain with SIRT2 inhibition by AK1.

While the complexity of osmotic minipump studies resulted in the methodological limitation of a small number of animals that completed the study successfully in each group, we were still able with these small numbers to observe a modest beneficial effect of AK1 treatment and confirm that there is no overt neuronal loss induced by this compound. This study provides three important new pieces of information to the field of treatment for neurodegenerative tauopathies: (1) SIRT2 inhibition in the brain is non-toxic in wild-type and FTD model mice, (2) SIRT2 inhibition with AK1 does not affect neurofibrillary pathology in this model, and (3) AK1 treatment protects hippocampal neurons to some extent from degeneration. Osmotic minipump administration of drugs directly to the brain circumvents the blood–brain-barrier and allowed us to test the safety and efficacy of SIRT2 inhibition *in vivo*. However, this technique is technically difficult and not ideal for human patients. Our results suggest that the development of a potent, brain permeable compound to inhibit SIRT2 would be a good therapeutic avenue for tauopathies including FTD and Alzheimer's disease.

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Interactomic and pharmacological insights on human Sirt-1

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Sirt-1 is defined as a nuclear protein involved in the molecular mechanisms of inflammation and neurodegeneration through the de-acetylation of many different substrates even if experimental data in mouse suggest both its cytoplasmatic presence and nucleo-cytoplasmic shuttling upon oxidative stress. Since the experimental structure of human Sirt-1 has not yet been reported, we have modeled its 3D structure, highlighted that it is composed by four different structural regions: N-terminal region, allosteric site, catalytic core and C-terminal region, and underlined that the two terminal regions have high intrinsic disorder propensity and numerous putative phosphorylation sites. Many different papers report experimental studies related to its functional activators because Sirt-1 is implicated in various diseases and cancers. The aim of this article is (i) to present interactomic studies based human Sirt-1 to understand its most important functional relationships in the light of the gene–protein interactions that control major metabolic pathways and (ii) to show by docking studies how this protein binds some activator molecules in order to evidence structural determinants, physico-chemical features and those residues involved in the formation of complexes.

Keywords: Sirt-1, molecular docking, interactome, activators, interaction map

INTRODUCTION

In complex biological systems the protein–gene interactions operate under protein–protein or gene–gene interaction maps where they have specific functional roles (Barabási and Oltvai, 2004). In this context well-connected hubs are of high functional importance (Jeong et al., 2001; He and Zhang, 2006). Consequently, studies based on protein–protein interaction (PPI) networks can be inferred from centrality statistics of proteins associated with disease and biological processes associated with genes and proteins. Genes associated with a particular phenotype or function are not randomly positioned in the PPI network, but tend to exhibit high connectivity; they may cluster together and can occur in central network locations (Goh et al., 2006; Oti and Brunner, 2006). Seven different homologous proteins compose Sirtuin family, and in particular Sirt-1 exhibits a high degree of structural disorder as demonstrated in a recent work of our group (Autiero et al., 2009). In general it has been already that the protein disorder plays a crucial role in PPIs and in regulatory processes for understanding the phenomenon of interactome (Tompa and Fuxreiter, 2008). Therefore, it is important to focus the attention on Sirtuins because they are involved in numerous processes and implicated in different diseases. Importantly the second-degree interaction maps related to these family present 5786 neighbors with average number of neighbors equal to 84.22. However some sirtuins have not yet been well studied and not much information are known in regard to their interaction with other proteins (*data not shown*) in second order interactome. In particular, Sirt-1 is defined as a nuclear protein even if experimental data suggest also its cytoplasmatic presence and indicate that it is involved into nucleo-cytoplasmic shuttling upon oxidative stress (Autiero et al., 2009). Sirt-1 is a NAD+ dependent histone deacetylates that play important functional roles in many biological processes causing various modifications of histone/protein acetylation status by several class I and II histone deacetylase (HDAC) inhibitors (Kyrylenko et al., 2003). In literature it is reported that Sirt-1 regulates gene silencing, cell cycle, DNA-damage repair and life span. In specific diseased conditions, Sirt-1 regulates or interacts with many proteins: TP53, NEDD8, SMAD4, DYNC1H1, TUBULIN, NUDC, DYNACTIN, HDAC4, POLR2H, and BRCA1. For example, Sirt-1

Abbreviations: ADP, adenine diphosphate; AR, androgen receptor; ARNTL, Aryl hydrocarbon receptor nuclear translocator-like; BRCA1, Breast cancer type 1 susceptibility protein; DLD, dihydrolipoamide dehydrogenase; DYNC1H1, dynein, cytoplasmic 1, heavy chain 1; EP300, E1A binding protein p300; FOXOs, forkhead box protein O; HIC1, hypermethylated in cancer 1; HDAC, histone deacetylase; KAT2, K (lysine) acetyltransferase 2; KRT1, keratin 1; MCF2L2, MCF.2 cell line derived transforming sequence-like 2; MYOD1, myogenic differentiation 1; NAD, nicotinamide adenine dinucleotide; NCOR1, nuclear receptor co-repressor 1; NEDD8, neural precursor cell expressed, developmentally down-regulated 8; NFkB, nuclear factor of kappa light polypeptide gene enhancer in B-cells; NUDC, nuclear distribution gene C homolog; PARP1, poly (ADP-ribose) polymerase 1; PPARGC1A, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; RELA, V-rel reticuloendotheliosis viral oncogene homolog A; RPS27L, ribosomal protein S27-like; RRP8, ribosomal RNA processing 8, methyltransferase, homolog; RTN4, reticulon 4; SLC25A3, solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; SMAD4, SMAD family member 4; SYNCRIP, synaptotagmin binding, cytoplasmic RNA interacting protein; TP53, tumor protein 53; WRN, Werner syndrome, RecQ helicase-like.

interacts with TP53 which is a very short lived protein involved in the acetylation processes and gene activation as consequent target (Appella and Anderson, 2001). In fact, the inactivation of HIC1 leads to an up-regulation of Sirt-1 which deacetylates and deactivates TP53. This allows the cells to bypass apoptosis and survive DNA damage (Chen et al., 2005). It is also known that Sirt-1 is involved in inflammatory processes and in neurodegenerative diseases like Huntington (Pallkes et al., 2008). Moreover, in literature it is reported that Sirt-1 interacts also with HDAC2, HDAC4, MEF2, SUMO, and UBIQUITIN and that HDAC4 might function to integrate sumoylation and deacetylation signals via its interaction with UBC9 and Sirt-1 and that acetylation and sumoylation occur on the same lysine residue (Zhao et al., 2005). This evidences the reason for which the analysis of the Sirt-1 interactome is of great interest in order to find the relationships between nodes (i.e., genes, proteins) and their positions as well as the overall relationships in the entire system along with structural inferences of activators associated with it.

Since the 3D structure of Sirt-1 has not yet been obtained experimentally, we have recently modeled this protein by computational methods and highlighted that it is composed by four different regions: N-terminal region, allosteric site, catalytic core and C-terminal region and underlined that the two extended terminal regions of about 250 residues each are highly disordered (Autiero et al., 2009). Sirt-1 is implicated in numerous diseases and cancers and many different papers report experimental studies related to the effects of its activation. In fact, Sirt-1 activation by natural activators seems to show a wide spectrum of beneficial effects in cardiovascular, metabolic, and neurodegenerative diseases and, hence, interest is increasing in testing more potent Sirt-1 activators for the treatment of these aging associated diseases. The natural activator resveratrol has been largely studied because of its low toxicity in humans and its anti-aging properties (Orallo, 2006; Harikumar and Agarwal, 2008). In particular, it is an important constituent of red wine (Zhuang et al., 2003) that increases the cell survival in several animals by stimulating the Sirt-1 dependent deacetylation of TP53 (Howitz et al., 2003). Since natural compounds failed to induce an increased activity of Sirt-1 (Yang et al., 2007), new activators (SRT1460, SRT1720, SRT2183) with a good affinity for Sirt-1 have been synthesized. Recently, a pharmaceutical biotechnology company, starting from these activators, discovered novel selective Sirt-1 activators using a high-throughput screening methodology (Smith et al., 2009; Vu et al., 2009; Yamazaki et al., 2009). In this article we will report studies on the Sirt-1 interactome and on molecular complexes between Sirt-1 and four different activators, i.e., SRT1460, SRT1720, SRT2183, and resveratrol, by molecular docking (Camins et al., 2010). Since the human sirtuin is proving to be a multifunctional protein with a large spectrum of biological activities and partners, the analysis of its interactome is an important step to define which biological process is directly or indirectly controlled by this molecule. This information is preliminary to understand the structural characteristics of complexes between sirtuin and those ligands that have been shown to regulate its biological activity. Starting from this knowledge we can design new molecules in a targeted way to control specific biological functions dependent on sirtuin.

MATERIALS AND METHODS

INTERACTOMIC STUDIES

Cytoscape software (Kohl et al., 2011) is used to visualize the network of Sirt-1 family. The experimentally evidenced interactions of Sirt family proteins were filtered from Bio grid, HPRD, MINT, and Pathway Interaction Database which are curated from both high-throughput data sets and individual focused studies along with interaction published in peer reviewed journals (Watts and Strogatz, 1998; Stark et al., 2006; Chatr-Aryamontri et al., 2007; Keshava Prasad et al., 2009; Schaefer et al., 2009). Further more the manually curated PPI network is obtained from Center for BioMedical Computing (CBMC) at University of Verona. Centrality statistics of the protein network are vitals for attaining properties of the network (Assenov et al., 2008; Scardoni et al., 2009). In particular, we focused most of our attention on central vertices in complex networks since they might play the role of organizational hubs. Betweenness centrality (BC; Freeman, 1977; Joy et al., 2005) and closeness centrality (CC; Wuchty and Stadler, 2003) are based on the calculation of shortest paths. Przulj et al. showed bottleneck's importance in protein interaction networks and their correlation with gene essentiality (Przulj et al., 2004; Yu et al., 2004). Lin et al. (2008) proposed two characteristic analysis algorithms: maximum neighborhood component (MNC) and density of maximum neighborhood component (DMNC) for exploring essential proteins (Hub proteins) from protein interaction networks (Lin et al., 2008). Most of these different methods for identifying essential nodes from the network have been stated in literature (Mason and Verwoerd, 2007). We utilized Maximal Clique Centrality (MCC), MNC, and DMNC, EPC, and other centrality based measure are taken into account for exploring the potential hubs in interaction maps of Sirt-1. Gene ontological data were mapped to nodes (Proteins) in the network. Gene Ontological study of a network infers about biological process, molecular function, and cellular location of the interactants present in the interactome. Significant clustering of genes, mapped with proteins, are layered into Graphs of the Gene Ontology and they are identified using the GO enrichment analysis plugin BiNGO (Maere et al., 2005).

MOLECULAR DOCKING STUDIES

Most cellular processes are carried out by PPIs. Predicting the 3D structures of protein–protein complexes by docking, it can shed light on their functional mechanisms and roles in cell. Docking can assist in predicting PPIs, in understanding signaling pathways and in evaluating the affinity of complexes (Andrusier et al., 2008). In this work, docking studies were done both to get structural models of those Sirt-1 complexes suggested by the interactome analysis and to understand the structural determinants underlying the interaction of Sirt-1 with small molecules that have the function of effectors. Automated docking is widely used for modeling biomolecular complexes in structure/function analysis and in molecular design. There are several effective methods available, incorporating different parameters such as algorithm and scoring function to provide reasonably good predictions. AutoDock4 is resulted a very useful tool for predicting the complexes conformation and the related binding energies of ligands with proteins. The basic algorithm used for conformational searching in AutoDock4

is Lamarckian genetic algorithm (LGA; Morris et al., 1998). This algorithm works on the basis of the stepwise generation selection. In fact, during the docking simulation a test population of docking conformation is created and in subsequent stepwise generations these individual conformations are selected for the next generation and in this way the best conformation is obtained. LGA has an additional feature called "Lamarckian" that allows to the individual conformation of searching the local conformation space, of finding the local minima and, then, of passing this to next generation. In particular, AutoDock4 uses a semi empirical free energy force field to predict binding free energy of small molecules and macromolecules, presents other traditional features such as Simulated Annealing and genetic algorithm and uses a force field that refers to the form and parameters of mathematical function used to describe the potential energy of a system of particles and leads to calculate the intermolecular energies for predicting free energy of binding. AutoDock 4 is composed by two software packages, i.e., AutoDock and AutoGrid, and consists of Rigid Docking and Flexible Docking modules. Rigid Docking (called also Grid-based approach) allows the ligand to have a large and a fixed conformational space around protein. In this approach the target macromolecule is embedded in the grid, the interaction energy between the probe and the target is computed and stored in this grid and is used as input for docking simulation. In this case, the relative orientation of molecules interacting with each other are allowed to change whereas the internal geometry of the target molecule is kept fixed. On the other hand, Flexible Docking module includes the side chain flexibility. In fact, in this approach a specific part of the target molecule is made flexible and, during the docking time, these flexible parts are treated explicitly allowing rotations of bond angles around torsion degrees of freedom.

The most important part in docking is the selection of the correct active binding site. In certain cases the binding site area on the surface of the protein is found with the help of specific software but the selection is also addressed on the basis of prior knowledge of the protein. Before setting up the docking run, ligands and receptor or target molecule were prepared by adding charges, torsions, and hydrogen atoms by specific tools. This preparation is important to mimic the "*in vivo*" conditions of molecular interaction (see **Figure A1** in Appendix). After the preparation of parameter and map files, AutoDock suite was launched for the process of docking that generates as output a log file (DLG) containing all the information of docked complexes (see **Figure A2** in Appendix). The description of AutoDock procedure used to simulate the complexes between Sirt-1 and the four activators is shown in **Figure 1**. The first docking methods or rigid docking

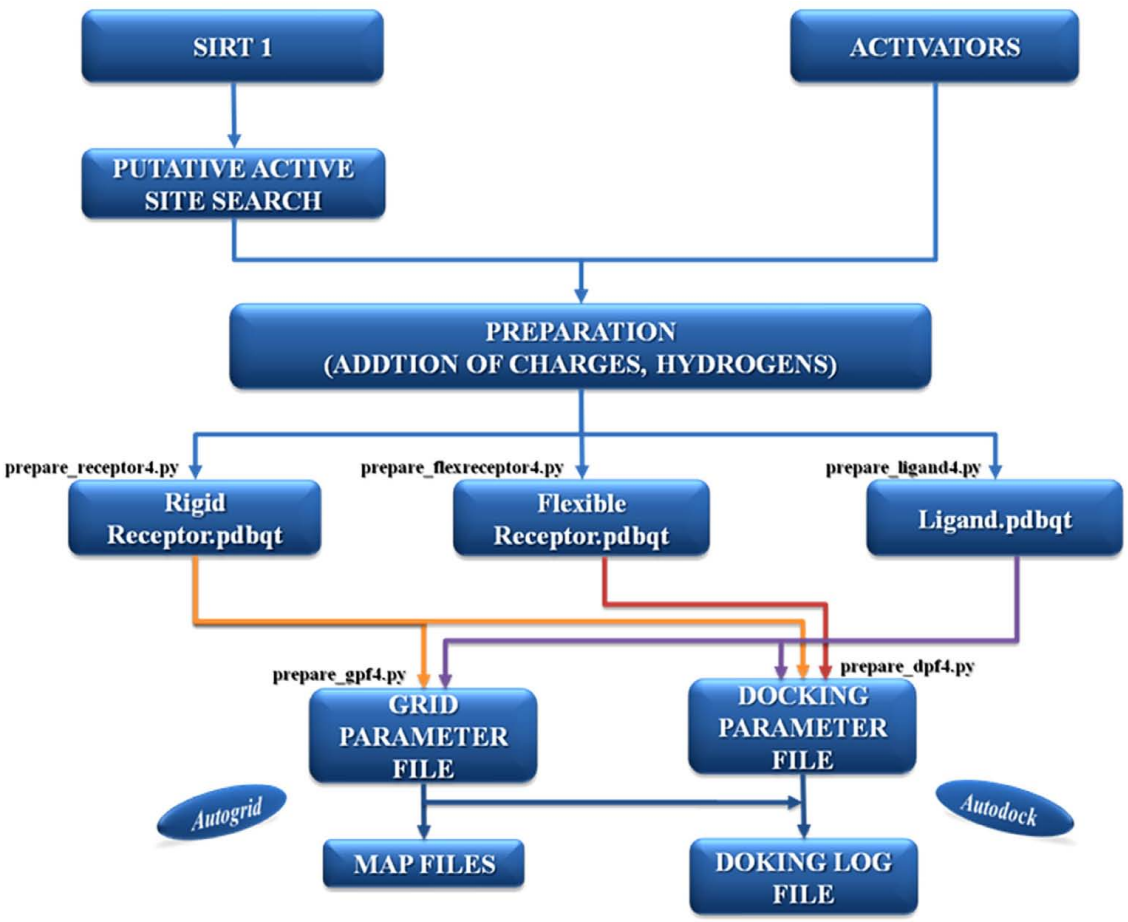

FIGURE 1 | The description of the AutoDock protocol used to simulate the complexes between Sirt-1 and its four activators.

treated proteins as rigid bodies (means the internal geometry of the molecule are kept fixed) in order to reduce the search space for optimal structure of complexes (Wodak and Janin, 1978; Halperin et al., 2002). However ignoring flexibility could prevent docking algorithms from recovering native associations (Andrusier et al., 2008) and specially in the case of unordered proteins or highly flexible proteins one cannot ignore the importance of flexible docking. Moreover, flexibility in docking should be taken into account if docked structures were determined by homology modeling (Marti-Renom et al., 2000) or if loop conformations were modeled (Soto et al., 2008) and this scenario implies in our case the presence of two unordered loops/regions, i.e., N-terminus and C-terminus (Autiero et al., 2009). The benefit of rigid docking procedure is relatively low in computational time and is less complex (Andrusier et al., 2008) but we cannot ignore the structural characteristic of Sirt-1. Therefore we have used this peculiar protocol that use steps of rigid docking followed by steps of flexible docking to generate near native models of complexes made with flexible Sirt-1 protein.

RESULTS

CENTRALITY STATISTICS OF FIRST ORDER INTERACTION OF Sirt-1

Sirt family first order interaction maps, obtained concerning experimental data reported in protein databases (see Methods section), have 228 nodes and 3769 edges (interactions). The extraction of first order interaction map of Sirt-1 has 136 nodes and 1503 edges with Sirt-1 as a central node of the network (**Figure 2**). A statistic analysis of first order interaction map of Sirt-1 was performed. In particular, given undirected networks, the clustering coefficient C_n of a node n is defined as $C_n = 2e_n/[k_n(k_n - 1)]$, where k_n is the number of neighbors of n and e_n is the number of connected pairs between all neighbors of n (Barabási and Oltvai, 2004). In directed networks, the definition is slightly different: $C_n = e_n/[k_n(k_n - 1)]$. The evaluation of the average clustering coefficient distribution gives the average of the clustering coefficients for all nodes n with k neighbors and identifies a modular organization of networks. The clustering coefficient C_n for undirected network of the Sirt-1 interaction map is 0.717. The mean shortest path length between any two proteins is 1.836 (**Figure 3A**).

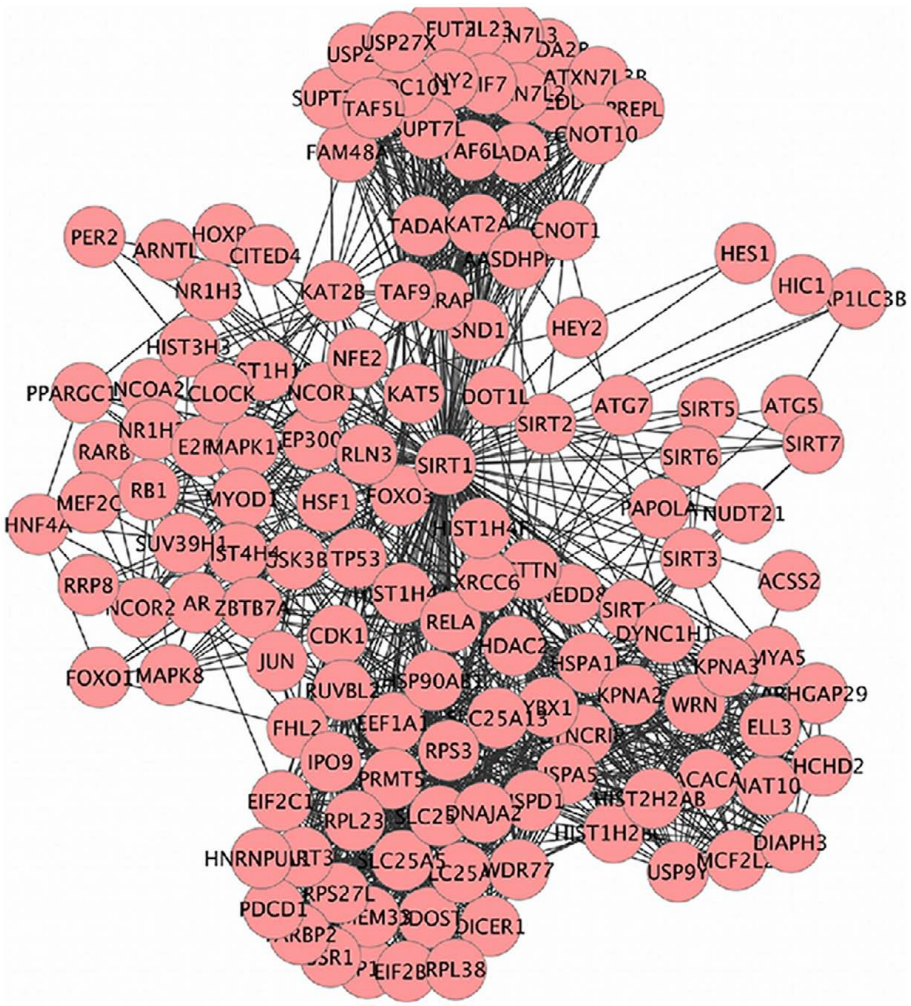

FIGURE 2 | SIRT family interaction maps containing 136 nodes and 1503 edges. Black lines are interactions and nodes (proteins) are represented by Circles.

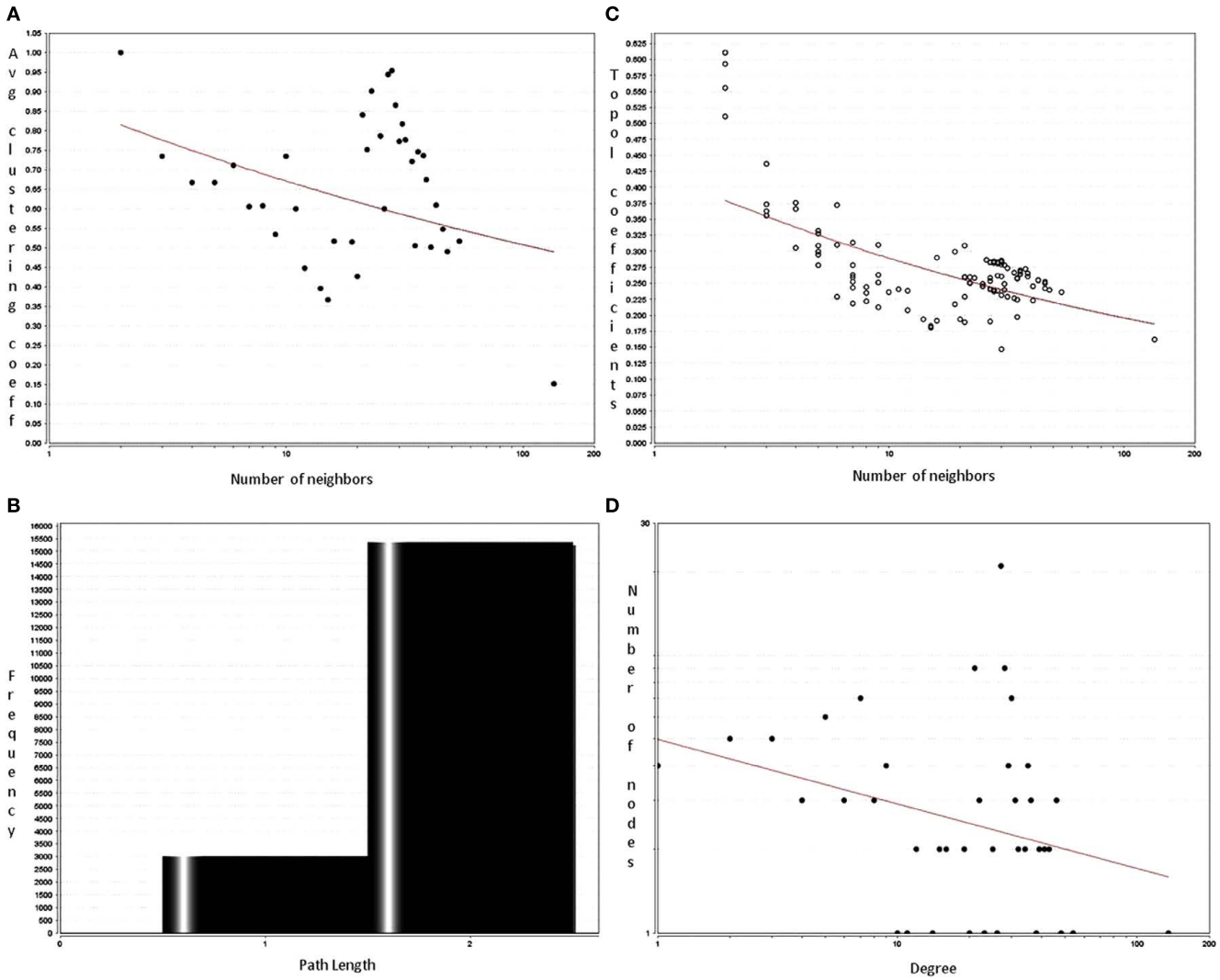

FIGURE 3 | Analysis of first order interactome for Sirt-1 composed by 136 nodes and 1504 edges. The shortest path length distribution **(A)** indicates that the network possesses small-world property. The node degree distribution **(B)** shows a scale free network property. **(C)** Average clustering coefficient of Sirt-1 network showing constant decrease **(D)** topological coefficient giving insights on the measure of extent to which a protein in the network shares interaction partners with other proteins.

The top 30 best-connected nodes obtained by average path length, as calculated by Centiscape (see **Table 1**), have relatively lesser average path length in respect to TP53 Interactome (Dartnell et al., 2005). The node distribution degree of Sirt-1 interactome gives information of the protein interactions with the k other proteins (**Figure 3B**). In details, it tends to decrease slowly complying with the power law $y = ax^b$ where "a" is 4.971 and "b" is -0.232 with a correlation coefficient of 0.113. This value indicates a scale free network (Barabási and Oltvai, 2004) and in general these are very robust against failure, such as removal of arbitrary network elements. This evaluation suggests that the Sirt-1 interaction map is assortative and has a low value of vertices. In this network the average number of interacting partners was evaluated and it resulted equal to 22.10. Moreover, since Jeong et al. (2001) showed that a protein acting as hub is more important than those sparsely connected with a small number of interactions (Jeong et al., 2001), we calculated the putative hub proteins present in our network by using different algorithms like MCC, DMNC, MNC and Edge Percolated component and different centrality based measures. In **Table 2** are reported the top 10 hub proteins obtained by these analysis but only five of them (SLC25A3, Sirt-1, JUN, MCF2L2, and EP300) were selected as hub by all used different algorithms.

Parameters related to topological aspects of Sirt-1 neighbors are acquired by calculating the average clustering coefficient of proteins that shows tendency to form clusters or groups (Barabási and Oltvai, 2004). Sirt-1 network has a constant decrease in clustering coefficients due to the higher number of interaction of each protein (**Figure 3C**). This suggests that it is a small-world network having hierarchical modularity. Ravasz et al. (2002) showed that

Table 1 | Top 30 neighbors based on smallest average path length.

Average path length	Proteins
1	SIRT-1
1.64	YBX1
1.66	HSP90AB1, HSPA5, EEF1A1
1.68	RPS3
1.69	HDAC2, HSPA1L
1.71	RUVBL2, RPL23, WDR77
1.73	SLC25A6, SLC25A3, DNAJA2
1.74	TP53, XRCC6, TAF9, SYNCRIP, TRRAP, SLC25A5
1.76	KAT2A, SLC25A5
1.77	EP300, DYNC1H1, SND1, RPS27L, TADA 3, SART3, AASDHPPT, EIF2C1, RPL38, DDOST

In particular, Sirt-1 is having the least Avg path length of 1 as it is central node of the network. Average path length denotes average number of steps along the shortest paths for all possible pairs of network nodes.

AASDHPT, aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase; DDOST, dolichyl-diphosphooligosaccharide – protein glycosyltransferase; DNAJA2, DnaJ (Hsp40) homolog, subfamily A, member 2, DYNC1H1, dynein, cytoplasmic 1, heavy chain 1; EEF1a1, eukaryotic translation elongation factor 1 alpha 1; EIF2C1; eukaryotic translation initiation factor 2C, 1; EP300; E1A binding protein p300; HDAC2, histone deacetylase 2; HSPA1L, heat shock 70 kDa protein 1-like; HSPA5, heat shock 70 kDa protein 5; HSP90AB1, heat shock protein 90 kDa alpha (cytosolic) class B member 1; KAT2A, K(lysine) acetyltransferase 2A; RPL38, ribosomal protein L38; RPS27L, Ribosomal protein S27-like; RPS3, ribosomal protein S3; RUVBL2, RuvB-like 2; RPL23, ribosomal protein L23; SART1, squamous cell carcinoma antigen recognized by T cells 3; SLC25A3, solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3; SLC25A5, solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5; SLC25A6, solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator) member 6; SND, staphylococcal nuclease and tudor domain containing 1; SYNCRIP, synaptotagmin binding, cytoplasmic RNA interacting protein; TADA3, transcriptional adaptor 3; TAF9, RNA polymerase II; TATA, box binding protein (TBP)-associated factor; TP53, tumor protein 53; TRRAP, transformation/transcription domain-associated protein; WDR77, WD repeat domain 77; XRCC6, X-ray repair complementing defective repair in Chinese hamster cells 6; YBX1, Y box binding protein 1.

highly connected regions connect sparsely connected nodes. In fact they classified networks into two modular organizations: local clustering and global networks. Local networks are considered to have functionality similar to biological processes whereas global connectivity is related to hub proteins present in the network connecting high-end nodes (higher order communication points between protein complexes; Han et al., 2004). Sirt-1 network is showing a tendency to connected global networks.

The decrease of the topological coefficient with the number of interacting partners gives information regarding interaction of proteins with common neighbor (**Figure 3D**). This shows that hub proteins (except SLC25 protein family) share fewer common neighbors then sparsely connected nodes and it also proves that the early inference of modular organization of Sirt-1 network is correct. A stressed node in the network is Sirt-1 having the highest number of distribution degrees (see supplementary material for details in **Table A1** in Appendix). BC has been evaluated as the amount of traffic that a vertex or edge has to handle in a network. In Sirt-1 interactome, the number of nodes has a high degree of BC and this is reported in (see supplementary material for details in **Table A1** in Appendix). It has been shown that high degree of connectivity correlates well with pleiotropic effects (Tyler et al., 2009). This indicates also that the most part of Proteins in Sirt 1 interactome map are involved in many different biological processes with different cellular localizations, more precisely AR, RELA, and SYNCRIP are present in nucleus as well as cytoplasm whereas SLC25A5 is in inner mitochondrial membrane as well as cytoplasm. Sirt 1 is found to interact with proteins involved in numerous pathways like Foxo Signaling, Regulation of Androgen receptor activity (**Table A3** in Appendix).

GENE ONTOLOGICAL STUDIES OF Sirt-1

GO studies on the hub proteins inferred from our analysis suggest that they are involved in important biological processes related to gene regulation, Metabolism and proton co-transport (**Table A2** in Appendix). In details, SLC25A3 is responsible for the inorganic phosphate transport into the mitochondrial matrix, either by proton co-transport or in exchange for hydroxyl ions (k, Entrez Gene description), while JUN interacts directly with specific target DNA sequences to regulate gene expression. The centrality analysis based on hub proteins showed SLC25A3, JUN, Sirt-1, RUVBL2, and MCF2L2 as important proteins of the network. Other Methods based on MCC, DMNC, MNC, and EPC evidenced the same proteins as hub nodes along with EP300, YBX1, RPL38, AR, and Sirt-2.

Genes associated with proteins and found significant in the interactome were analyzed by the BiNGO package in Cytoscape. Sirt-1 first order interacting partners are involved into numerous biological processes. Sirt-1 interactome is significantly involved Metabolism modulation related processes (**Figure 4**). Sirt-2 in chromatin silencing at rDNA, RPS27L, and RTN4 in regulating anti-apoptotic phenomena.

Certain processes, like chromatin remodeling and modification, involve many important proteins of the network like KAT2B, NCOR1, HDAC6, RRP8, HDAC2, and KAT2A. Moreover, TP53, Sirt-2, PPARGC1, CPS1, and JUN are responsible for the processes related to the response to starvation whereas the response to stress is regulated by NCOR1, MYOD1, KRT1, SIRT2, HDAC2, RPS3, RELA, FOXO1, HDAC6, and other proteins involved in ncRNA metabolic processes and in negative regulation of signaling pathways. In particular, the important processes like DNA binding activity transcription factor regulation and DNA repair are shown to have an involvement with proteins like Sirt-1, Sirt-2, TP53, PPARGC1A, JUN, EP300, HDAC2, HDAC6, KAT2A, Kat 2B, RELA, RB1, WRN, XRCC5, and XRCC6 (**Figure A3** in Appendix).

In particular, the sirtuin network shows that Sirt-2, HDAC6, HDAC2, Sirt-1, PPARGc1A, TRRAP are implicated in histone modification and histone deacetylation whereas SUV39H1 and DICER1 are involved in gene silencing phenomenon.

The proteins in Sirt 1 interaction maps showed also different cellular localization and molecular function (**Figure 4** and **Table A4** in Appendix). In details, Sirt family, ARNTL, WRN, EP300, SYNCRIP, JUN, RPS3 are proteins showing pleiotropicity in biological as well as in the cellular localization in the GO analysis

Table 2 | The 10 hub proteins present in Sirt-1 first order interaction maps obtained by different algorithms and centrality measures.

MCC	DMNC	MNC	EPC	Degree	Bottleneck	Betweenness	Stress	Closeness
SIRT1	RPL38	RPL38	JUN	JUN	JUN	JUN	JUN	SLC25A5
YBX1	RELA	SIRT-1	SIRT-1	SIRT-1	SIRT-1	SIRT-1	SIRT-1	TADA2B
SIRT2	SLC25A3	SLC25A3	RELA	RUVBL2	RUVBL2	RUVBL2	RUVBL2	HSF1
JUN	MCF2L2	SIRT2	SLC25A3	SLC25A3	KPNA3	KPNA3	KPNA3	HNF4A
SLC25A13	AR	MCF2L2	SIRT2	SIRT2	MCF2L2	MCF2L2	MCF2L2	TADA3
RUVbl2	SIRT6	AR	RPS3	MCF2L2	CNOT10	CNOT10	CNOT10	HIST1H2BC
EP300	SYNCRIP	SIRT6	SIRT6	DYNC1H1	WRN	WRN	WRN	EIF2C1
HDAC2	EP300	SYNCRIP	EP300	SLC25A13	RPS27L	RPS27L	RPS27L	CMYA5
SIRT6	YBX1	EP300	YBX1	YBX1	SIRT4	SIRT4	SIRT4	NAT10
SLC25A3	HDAC2	YBX1	SIRT3	HDAC2	HDAC2	HDAC2	HDAC2	ARNTL

AR, androgen receptor; CMYA, cardiomyopathy associated 5; CNOT10, CCR4-not transcription complex subunit 10; DMNC, density of maximum neighborhood component; EIF2C1, eukaryotic translation initiation factor 2C, 1; EPC, edge percolated component; HIST1H2BC, histone cluster 1, H2bc 2; HNF4A, hepatocyte nuclear factor 4 alpha; HSF1, heat shock transcription factor; KPNA3, karyopherin alpha 3 (importin alpha 4); MCC, Maximal Clique Centrality; MCF2L2, MCF.2 cell line derived transforming sequence-like; MNC, Maximum Neighborhood Component; NAT10, N-acetyltransferase 10; RELA, V-rel reticuloendotheliosis viral oncogene homolog A; RPS27L, ribosomal protein S27-like, RecQ helicase-like; TADA2b, transcriptional adaptor 2B, ribosomal protein L38; TADA3, transcriptional adaptor 3; WRN, Werner syndrome.

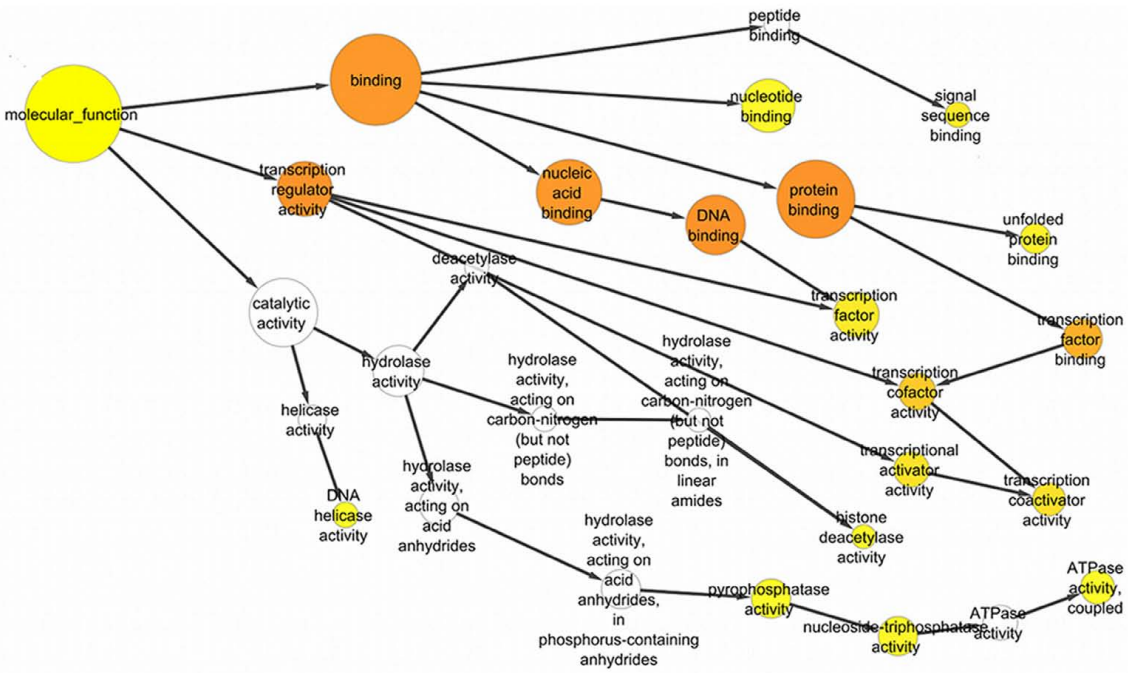

FIGURE 4 | The significant GO ontological data related to molecular function with the GO nodes are listed in circles connected by black arrows to the GO nodes. These yellow and orange color nodes correspond to the statistically significant nodes.

maps. In fact, Sirt-1 interacts with cytoplasmic, nuclear, extracellular and mitochondrial proteins as found with a significant p value, i.e., $p < 0.05$ to $p > 0.0000005$ that measures the statistical significance of the different essentialities of proteins implicated in the biological processes. RELA and JUN show interactions with mitochondrial proteins, Sirt-1 interacts with other cellular proteins in activating DNA repair and stress protection mechanisms.

SECOND ORDER INTERACTION OF Sirt-1

The Sirt-1 second-degree interaction map is composed of 4691 nodes. These nodes correspond to different partners interacting by 221595 edges (Interactions). The second order network of Sirt-1 is scale free and small-world network interacting with numerous proteins implicated in transcription and metabolism related processes. Sirt 1 has a high degree of interactions in second order

interaction maps as it is having interactions with high number of proteins like PARP1 (inhibits Sirt activity) and NAMPT (regulates NAD+ levels; Yang et al., 2006). Analyzing centrality statistics and pattern of rearrangement of interacting nodes in Second order interactome of Sirt-1 will provide further insights on the variability in functionality, cellular localization, and pleiotropicity nature of the SIRT interaction map.

MOLECULAR DOCKING STUDIES

Dai et al. (2010) suggested that Sirtuin activating compounds (STACS) interact directly with Sirt-1 activating the deacetylation through an allosteric mechanism. This mechanism requires the presence of an allosteric site on the protein; therefore, we have used for modeling the same structural site on which we have recently found that binds AROS, the allosteric effector of Sirt-1 known as endogenous activator (Autiero et al., 2009). So we acquired from the structural model of the complex AROS–SIRT1, obtained after docking and molecular dynamics, the putative residues of interaction (see **Table 3**). Hence, our docking studies have focused on the interactions between the allosteric site found on the native modeled structure of Sirt-1 and STACs like SRT1720, SRT2183, SRT1460, and resveratrol (**Figure A4** in Appendix). The best docking results were obtained by implementing flexible docking in AutoDock4. In particular, the reason for the selection of this site depends from the fact that many experimental data have suggested that the modulation of the catalytic activity of Sirt-1 is exerted through the adjustment implemented by the allosteric site. Recent works also show that the interaction of Sirt-1 with small effectors has a functional relevance for its activation (Zhao et al., 2004; Milne et al., 2007; Bemis et al., 2009). However, the modeled structure of Sirt-1 shows that the allosteric site selected as binding area for activators is near to N-terminal region predicted as unordered (Autiero et al., 2009).

We have also focused our attention on the disordered residues flanking the allosteric site (see **Table 3**) considering them as flexible during the process of docking. This structural region is close to the highly disordered N-terminal segment and involved into the regulation of the enzyme activity (Tanno et al., 2007; Ford et al., 2008; Sasaki et al., 2008).

The grid-based approach was implemented defining a rigid box (of dimension $4.14 \times 19.56 \times -24.21$ Angstroms) on the surface of the protein and around the residues of the allosteric site (see **Table 3** and **Figure A6** in Appendix) to specify the docking area for the activators. Parametric details of the grid parameters such as "number of spacing," "number of grid points," and "center grid box" in all three directions are given in **Figure A5** in Appendix.

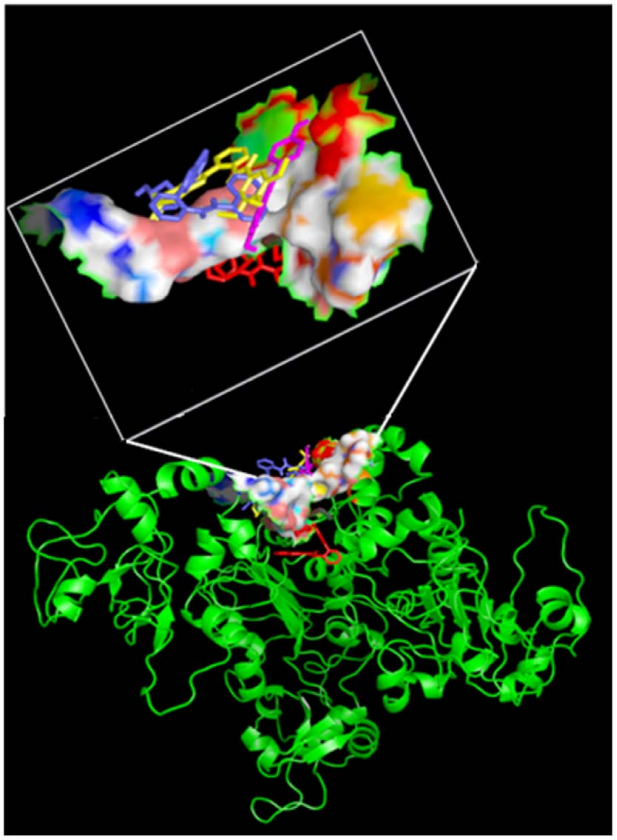

FIGURE 5 | Side view of four activators docked on the active site. All the four activators are shown in "stick" confirmation with different colors: SRT1460 in yellow, SRT2183 in blue, resveratrol magenta and SRT1720 in red). The active site residues are shown in "surface" confirmation whereas Sirt-1 by cartoon. The active site region with the docked activators is highlighted in a white box.

Figures 5 and **6** and **Figure A7** in Appendix show the best docking models computed for all the four activators against Sirt-1. The complexes present negative values of the binding free energy, which indicates that the models between Sirt-1 and its activators are reliable. Their analysis in terms of interaction residues confirms that the binding region is conserved with the involvement of charged and aromatic residues which suggests complexes stabilized by electrostatic and stacking interactions. Moreover, the complex between Sirt-1 and resveratrol resulted not so stable in respect to the complexes of the other three ligands. This can be inferred from

Table 3 | Sirt-1 residues resulted at the interaction interface with AROS (Autiero et al., 2009) and used during the docking studies.

Sirt-1 residues interacting with AROS	MET1; ALA2; ASP3; LEU7; GLU161; ASP166; SER169; **HIS170; ALA171; SER172; SER173; SER174; ASP175; TRP176; PRO184; TYR185; PHE187; VAL188; HIS191; LEU192**; ILE194; GLY195; THR196; ASP197; THR219; TRP221; GLN222; ILE223; TRP624; ARG627VAL628
Sirt-1 residues of the allosteric site considered flexible during the docking studies	**HIS170; ALA171; SER172; SER173; SER174; ASP175; TRP176; PRO184; TYR185; PHE187; VAL188; HIS191; LEU192**

Residues common in the interaction surface are indicated in bold. The smaller number of residues involved in the interaction is due to the different molecular sizes of AROS and small activators.

the absence of H-bonds and relatively lesser number of charged interaction residues (**Table 4**). However, the $EC_{1.5}$ values related to the Sirt-1 activity, and reported in the literature, supports the observation that the resveratrol is a less potent activator (Milne et al., 2007; Bemis et al., 2009). A remarkable observation that supports our models is that the experimental $EC_{1.5}$ values are linearly correlated with the binding energy values found by AutoDock for the Sirt-1 complexes with the four activators. In fact, a correlation coefficient of 0.73 demonstrates the good agreement between functional data and computational results.

DISCUSSION

Human Sirt-1 is an unordered protein (IDP) and may therefore adopt types of order (and conformations) that are not easily recognized by current secondary or tertiary structure prediction algorithm, which primarily recognize higher order assemblies stable in the time. Even the classic experimental structural techniques often fail in studying structural aspects of these proteins. Sirt-1 is a Hub protein because of its numerous partners and for its structural characteristic. Structural features that affect the ability of hubs in PPI networks to recognize and bind multiple partners are numerous. In this article we primarily focused on the role of intrinsic disorder in the Sirt-1 structure. However, a study in progress in our laboratory focuses on the charged residues on the surfaces of this protein and on the role of phosphorylations. Preliminary data support the idea that it has highly charged surfaces as compared to large, disorder containing hubs indicating its possible involvement in promiscuous binding (Patil and Nakamura, 2006).

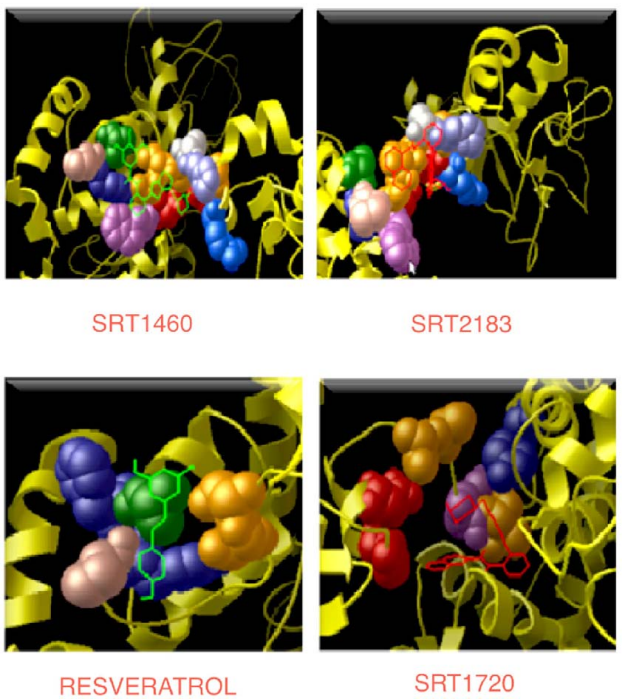

FIGURE 6 | Interaction regions in four complexes. All the four activators are shown in the line/bond conformation with different colors and the residues (reported in **Table 4**), which are interacting with their respective activators, are shown in the CPK conformation.

Our interactomic analysis showed for the first time how much is vast the number of physiological partners of this hub protein. Sirt-1 is an interesting case because we are just beginning to understand some of the mechanisms that lead to multi-specificity in the binding of hub proteins. In particular, a huge number of articles have been published on the clinical, biological and, functional aspects of human sirtuins but we know only general details about their structures and molecular mechanisms which govern the functional behavior of these proteins.

The aim of this study was to evaluate and integrate functional and structural features by computational methods to predict the involvement of the human Sirt-1, the most studied of sirtuins, into the basic molecular mechanism describing the complex regulation of this protein. Since *in vitro* or *in vivo* experiments is time consuming and expensive; *in silico* prediction can provide functional candidates and help narrow down the experimental efforts. Moreover, we have also analyzed multiple large-scale experimental data sets describing the metabolic involvement of the Sirt-1 to understand the basic mechanism underlying the function of this hub protein. We have examined objective criteria that could infer organizations of the Sirt-1 network and the structural determinants featuring the interaction between Sirt-1 and some biological activators which are reported in the literature as potent modulators of the metabolic activities of sirtuin 1 (Milne et al., 2007; Dai et al., 2010). At the same time, we can make suggestions about the structural mechanisms underlying the interaction of small molecule activators on which there is currently much disagreement (Pacholec et al., 2010). This knowledge may also be used to direct the design of new and more specific sirtuin activators.

Table 4 | Interaction details of four complexes compared to experimental data reported in literature.

Name of activators	Number of interacting residues	Name of interacting residues	Number and residue name of H-bond	Energy score (Kcal/mol)	$EC_{1.5}$ value
SRT1460	13	D166, R167, S169, H170, A171, S172, S173, S174, D175, **W176**, P184, Y185, V188	1 H-BOND (S173)	−5.62	0.36
SRT1720	9	D163, D166, S172, S173, S174, D175, **W176**, P177, Y185	1 H-BOND (D175)	−4.98	0.16
SRT2183	10	D166, R167, H170, A171, S172, S173, S174, D175, Y185, V188	1 H-BOND (S173)	−2.18	2.9
RESVERATROL	8	S173, S174, **W176**, P180, R181, P184, Y185, V188	NO H-BOND	−1.48	36.2

The charged residues are underlined but those aromatic are shown in bold.

Sirt-1 interactomic study holds the key for understanding associations and interactions between various proteins to develop knowledgeable insights of highly diverse and complex biological systems, which are interwoven into each other. On the basis of the experimental data (see Materials and Methods) at disposal on various public databases, we have performed an interactomic analysis and found 136 direct partners interacting with Sirt-1 that are involved in the important pathways discussed above. Several proteins are biologically active in metabolic processes whereas several others proteins perform gene regulatory functions. Scale freeness of the Sirt-1 interaction map is exhibited by a trend shown by many proteins with logarithmically decreased connectivity and Sirt-1 interactome shows small-world property with smaller diameter and high connectivity (**Figure 3**). These properties make a network more robust to perturbations like mutations and viral infections. However, these parameters imply that the pleiotropicity nature or the complex associations of the proteins governing different biological processes are found implicated in many pathways. In particular, some proteins that connect more nodes in different pathways are, for example, the hub nodes like JUN, HDAC2, RELA, and SLC25A3. Promislow (2004) showed that the pleiotropicity is linked to higher connectivity of nodes, especially, in senescence. However, there is a significant amount of inferences on possible associations between Sirt-1 and caloric restriction and senescence. Probably, we could suggest that the pleiotropic nature of the proteins interacting with Sirt-1 may address the senescence through the involvement of multiple factors possibly related to stress and mitochondrial proteins or the processes associated with mitochondria. This derives from the fact that with aging there is the progression of many diseases like Parkinson, Huntington and Alzheimer that depend on mitochondrial dysfunction. In Sirt-1 interactome the mitochondrial sirtuins, i.e., Sirt-3, Sirt-4, and Sirt-5, interact with proteins implicated in different metabolic processes (**Figure A8** in Appendix) and the deregulation of these proteins by any factor can lead to chronic metabolic disorders. Moreover, the direct interaction provides some insights about the involvement of Sirt-1 in cancer as this protein is also found to be acetylating TP53. In our studies of the Sirt-1 interactome, this protein and some hub proteins like JUN, RELA, and EP300 show to have interactions with many different proteins involved in some processes and in different cellular localizations. Further, Sirt-1 interaction map or other protein interaction networks often demonstrate static picture of bulk amount of complex dynamic interactions. To get perspective on modulation of Sirt-1, there will be necessary studies on dynamics interactions considering the interaction levels in strength, chronology in PPI maps and rate order reaction in case of metabolic processes. It would be very interesting to know the affinity values for the NAD moiety in Sirt family and PARP's (second order interacting partner) of human Sirt-1 (Kolthur-Seetharam et al., 2006; Bai et al., 2011). Sirt-1 network analysis confined with GO studies showed agreement to the observations of Bai et al. (2011). Moreover, Sirtuin genes are found to be controlling the organism's health in the times of adversity like in diseased conditions. CR is one of the phenomenon's that switch on the Sirt-1 genes for regulatory functionality and controlling the metabolic pathways. Therefore, hyper activation of the sirtuin genes might be one of the possible contributory causes for healthier life.

Since Sirt-1 became an interesting and promising target for its importance in life span and for its role in various diseases (Camins et al., 2010), the exploration of its pharmacological aspects has been the topic of key research in last decade. In particular, the attention has been focused on the role of certain small activator molecules that affect the activity of Sirt-1. In literature there are some articles on the interaction between Sirt-1 and activators (Milne et al., 2007; Dai et al., 2010; Huber et al., 2010; Pacholec et al., 2010). In particular, Milne et al. (2007) showed that three synthetic activators, namely SRT1460, SRT1720, and SRT2183, are Sirt-1 activators better than the natural resveratrol because EC values of these three synthetic activators are lower than the natural ones. Moreover, these compounds were reported to bind the Sirt-1 enzyme – peptide substrate complex at an allosteric site. Therefore, these Authors suggested the possibility of developing a new therapeutic approach using both caloric restriction and the direct activation of Sirt-1 using these activators. In 2010, in contrast to Milne et al. (2007), other Authors (Huber et al., 2010; Pacholec et al., 2010) have evaluated the same Sirt-1 activators (SRT1460, SRT1720, SRT2183, and resveratrol) by employing biochemical assays containing native substrates such as the p53-derived peptide lacking the fluorophore as well as purified full-length protein p53 or acetyl-CoA synthetase 1. In these experiments the four activators did not lead to apparent activation of Sirt-1 with native peptide or full-length protein substrates, whereas they activated Sirt-1 with peptide substrate containing a covalently attached fluorophore. In particular, Huber et al. (2010) showed that SRT1720 and SRT2183 effectively decreased acetylated p53 in cells treated with DNA damaging agents but did so in cells that lack Sirt-1. Also Pacholec et al. (2010) evidenced that SRT1720, SRT2183, SRT1460, and resveratrol exhibited multiple off-target activities against receptors, enzymes, transporters, and ion channels. Therefore, they concluded that these four molecules were not direct activators of Sirt-1 and required a fluorophore (named TAMRA) for activating Sirt-1 (Pacholec et al., 2010). Recently, in contrast to Pacholec et al. (2010) and Huber et al. (2010) but in agreement with Milne et al. (2007), Dai et al. (2010) have demonstrated that there are many Sirtuin activating compounds (STACs) that produce biological effects consistent with direct Sirt-1 activation. In this study they evaluated again the three STACs (SRT1720, SRT2183, and SRT1460) and showed that they can accelerate the Sirt-1 catalyzed deacetylation of specific unlabeled peptides composed only of natural amino acids in contrast with those Authors which stated that fluorophores were required for Sirt-1 deacetylation. Therefore, they suggested that these three molecules interact directly with Sirt-1 and activate Sirt-1-catalyzed deacetylation through an allosteric mechanism demonstrating that the complex between STACs and specific fluorophores was not necessary for SIRT1 activation (Dai et al., 2010). As one can see the controversy essentially arises because of the lack of details on both structural and functional activity of Sirt-1. Moreover, in our opinion, authors do not take into account that disordered regions allow binding to multiple partners modulating their function. To achieve this capacity, these regions are able to interact with numerous and various enzymes that operate post-translational modifications

of which the kinases are certainly the most studied. They phosphorylate sites that are found almost always in disordered zones modifying in this way both the ability to interact that the function. Therefore, the presence of specific kinases in the various cellular districts, where intrinsically disordered proteins have to be post-translationally modified, is fundamental for the activity of these proteins. In other words, if Sirt-1 with its long disordered terminal arms is controlled by its phosphorylation state (Autiero et al., 2009), its activity for the recognition of protein partners at any one time will be directly dependent on the activity of the kinases and phosphatases that act on it in a specific cellular district. In this regard, it is worth of note that we have found more than 90 putative sites on the human Sirt-1 arms specific for about 40 different human kinases (manuscript in preparation). All the above suggests that *in vitro* testing of one of these proteins should have in the assay also the kinase necessary for the specific recognition of partners or, at least, a sirtuin already post-translationally modified for the specific substrate. Only reasoning on this basis it will be possible to properly test and compare the functional activity of these proteins. However, often the experimentalists act with the traditional structure centric view characteristic of globular enzymes that cannot be applied to IDPs because their activity in respect of a substrate is strongly dependent on those post-translational modifications required to correctly recognize that substrate. It seems evident that a computational approach in these cases is useful for understanding and directing studies in solution. This has led to the lack of conclusive data particularly on small molecule activators due to the not easy comparability of the results of *in vitro* and *in vivo* experiments. We think that the field has been over-focused mainly with functional studies performed without taking account at structural level of the different structural behavior of the intrinsically disordered proteins and of the necessary recognition specificity determined by the presence of the numerous kinases. Moreover, conflicts at physiological level are probably due to animal models that are not genetically appropriate. The issue of longevity is extremely complicated because the aging involves many genes and the small molecules like polyphenols have gained attention because they can enter cellular machinery and exert epigenetic changes in hundreds of genes; therefore, higher standards for genetic analysis are required and it is important to assess if the longevity is due to a direct binding to Sirt-1 or to other physiological effects sirtuin independent. Therefore, in this work we have modeled by flexible docking studies the complexes between Sirt-1 and the four activators (SRT1460, SRT1720, SRT2183, and resveratrol) reported by Milne et al. (2007). Given that we recently modeled the interaction between AROS and the allosteric site of Sirt-1 (Autiero et al., 2009), Milne et al. (2007) and Dai et al. (2010) showed that these molecules can interact directly with Sirt-1 and activate it through an allosteric mechanism, therefore, we have decided to simulate these interactions. In particular, flexible docking study was chosen because of the highly flexible and unordered nature of Sirt-1 protein, that is composed of four different regions (Autiero et al., 2009), of which the two terminal domains are resulted highly unordered. In particular, the area selected for binding of these activators is a flexible loop joining N-terminal and allosteric site. In this particular scenario it is important to concern flexible binding area, as it will add more authenticity to the docking results. In fact, flexible docking environment can mimic the "*in vivo*" conditions of molecular interaction such as change in certain bond angles or bond lengths take place when two molecules tend to interact. In this work 13 residues present in the allosteric site were chosen to be flexible. In details, these 13 residues selected from the selective binding site area comprise four hydrophilic (SER172, SER173, SER174, TYR185), three hydrophobic (ALA171, VAL188, LEU192), one negatively charged (ASP175), two positively charged (HIS170, HIS191), and three aromatic residues. The significance of aromatic residues and charged residues in the area of active site is very important because they are involved in putative stacking and electrostatic interactions, respectively. Moreover our results evidence that aromatic residues form H-bonds that is important for the structural compactness and stability of the docked complexes. The comparison between flexible docking results and the experimental data indicates that the well known natural activator, resveratrol, does not show good binding affinity for Sirt-1 respect to other synthetic activators (SRT1460, SRT1720, SRT2183). In fact, resveratrol has lower affinity than its synthetic counterparts as shown from binding free energy values (expressed in Kcal/mol) and the lack of H-Bond formation with Sirt-1. **Figure A9** in Appendix shows the correlation between the energy values found for the four tested small molecules and the values of EC, experimentally determined (see **Table 4**). As one can see, while for synthetic molecules there is a correlation coefficient of 0.97 which indicates a good agreement between our structural data of direct binding and physiological data, the resveratrol is the only molecule that does not correlate with the others due to its poor correlation coefficient. This suggests that the biological activity does not depend on a direct binding. Thus, our docking model resveratrol–sirtuin-1 clearly shows that resveratrol is a poor allosteric modulator. Its binding energy is lower than that of the other modulators (see **Table 4**).

On the basis of these results we can highlight that the use of a flexible docking in the case of intrinsically unordered and highly flexible proteins such as Sirt-1 is able to successfully simulate protein complexes since our docking data are in agreement with the functional data. This is the first example, to our knowledge, that a docking between a flexible and disordered protein and ligands is not only able to simulate the experimental data but also to clearly discriminate between different hypothesis. However recently it has also been reported that ligand-receptor docking studies of CXCR4 (Kufareva et al., 2011) failed to correctly predict the ligand binding sites despite the availability of template GPCR crystal structures. We observe that in the X-ray structure of CXCR4 (PDB: 3ODU) is missing the N-terminus of about 50 residues. This point as we will discuss later is important. Each chemokine receptor has an extracellular N-terminal region, seven helical transmembrane domains with three intracellular and three extracellular hydrophilic loops, and an intracellular C-terminal region. The first and second extracellular loops are linked together by disulfide bonding between two conserved cysteine residues. The N-terminal region of a chemokine receptor is structurally important because it is crucial for ligand specificity whereas the intracellular C-terminal region couples G-proteins and this mechanism is implicated for receptor signaling transduction. In a study in progress in our lab (manuscript in preparation) we have found diffuse presence of

disorder in the family of the human chemokine membrane receptors. N and C terminal arms possess structural characteristics such that they can be considered intrinsically disordered with a high structural flexibility and the presence of numerous charged patches and phosphorylation sites. Without any consideration of these important structural aspects of CXCR4 (not resolved by X-ray), we think that dockings failed because evidently the structural features of N-terminus play a crucial role in the binding of those ligands and most of all the flexibility also plays a structural role which must carefully taken into account in docking as we have done with Sirt-1.

To better validate our docking results, we have compared the complexes between Sirt-1 and the four activators (SRT1460, SRT1720, SRT2183, and resveratrol) obtained by AutoDock4, a very useful tool for predicting the complexes conformation (Morris et al., 1998, 2009), with those performed by Glide, a program that uses a different protocol indicated as "flexible" (Halgren et al., 2004). The best complexes generated by this last program in terms of energetic values showed that (i) the four molecules bind the same allosteric site predicted for AROS with good affinity and use about 90% of interactions evidenced by AutoDock4 but with the same number and type of H-bonds and (ii) the correlation coefficient between energy score by AutoDock4 and Glide programs is 0.91 (**Figure A9** in Appendix).

These results have evidenced the good accuracy of our complexes between Sirt-1 and four molecules even if the certainty of the result can be obtained only by experimental studies. Hence, further studies will be performed to validate experimentally our computational results by biochemistry assays.

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APPENDIX

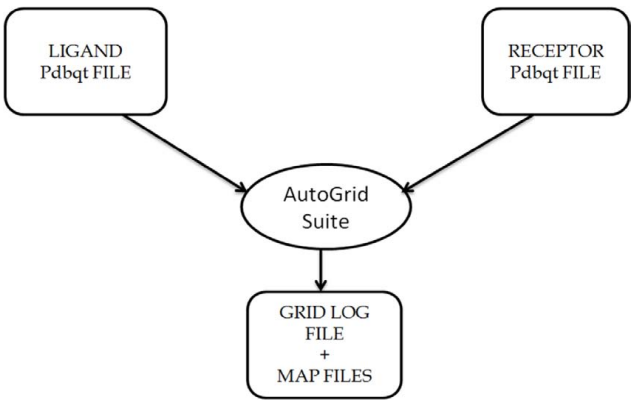

FIGURE A1 | Description of the usage of AutoGrid Suite in the AutoDock4 software package, that generates map files specifying the area over the surface of protein for ligand binding on the basis of information provided in GPF files, i.e., Grid Parameter file.

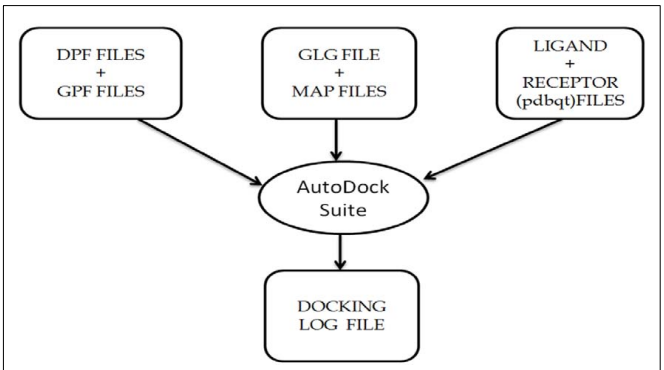

FIGURE A2 | Description of the usage of AutoDock Suite in the AutoDock4 software package, that generates the final docking log file containing information about the final docked complex.

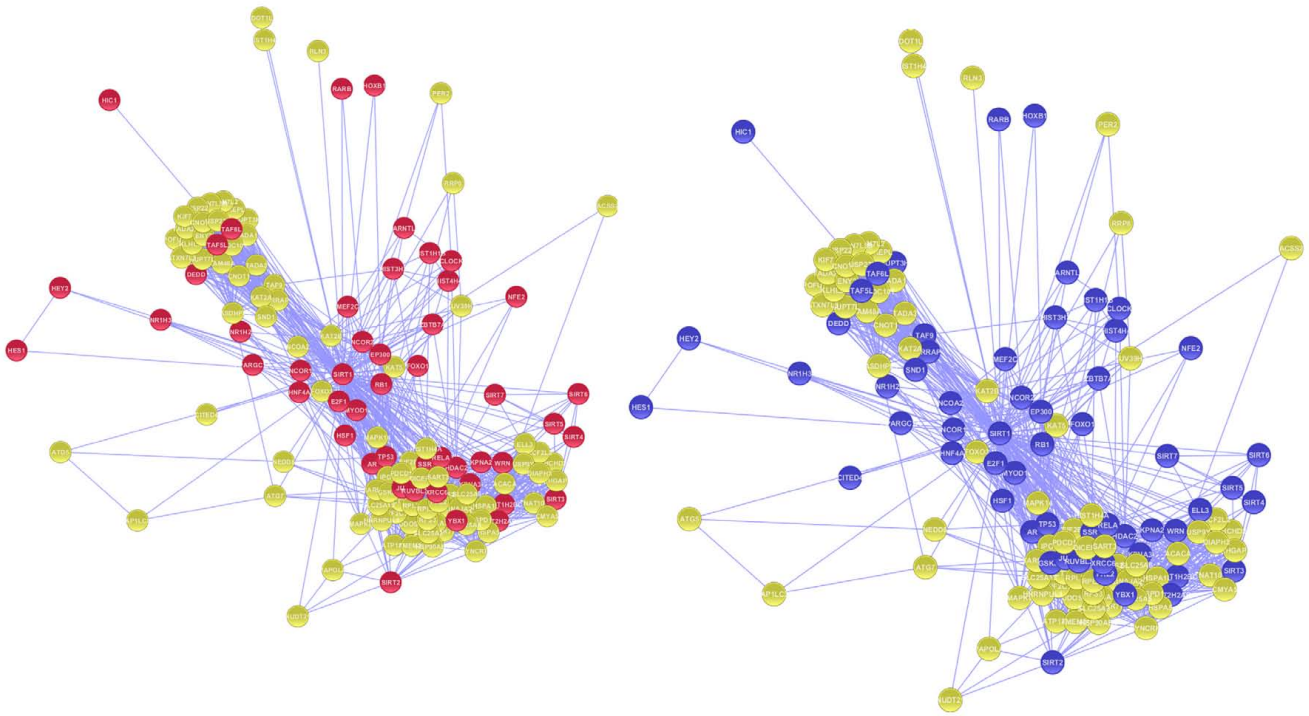

FIGURE A3 | Nodes in magenta represents the proteins involved in DNA binding Activity whereas proteins involved in Transcription regulating activity in GO studies are represented in purple.

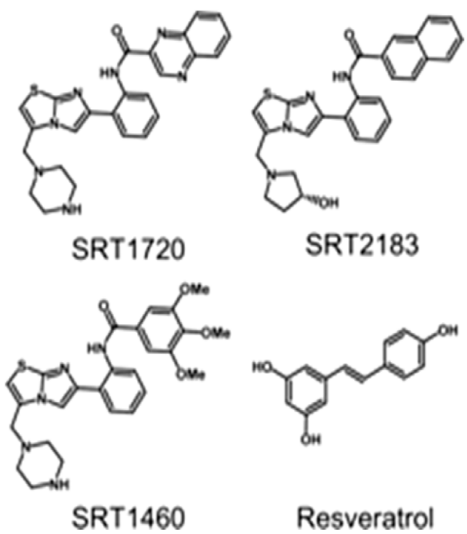

FIGURE A4 | Chemical structures of four activators which are used in molecular docking studies against Sirt-1 (Pacholec et al., 2010).

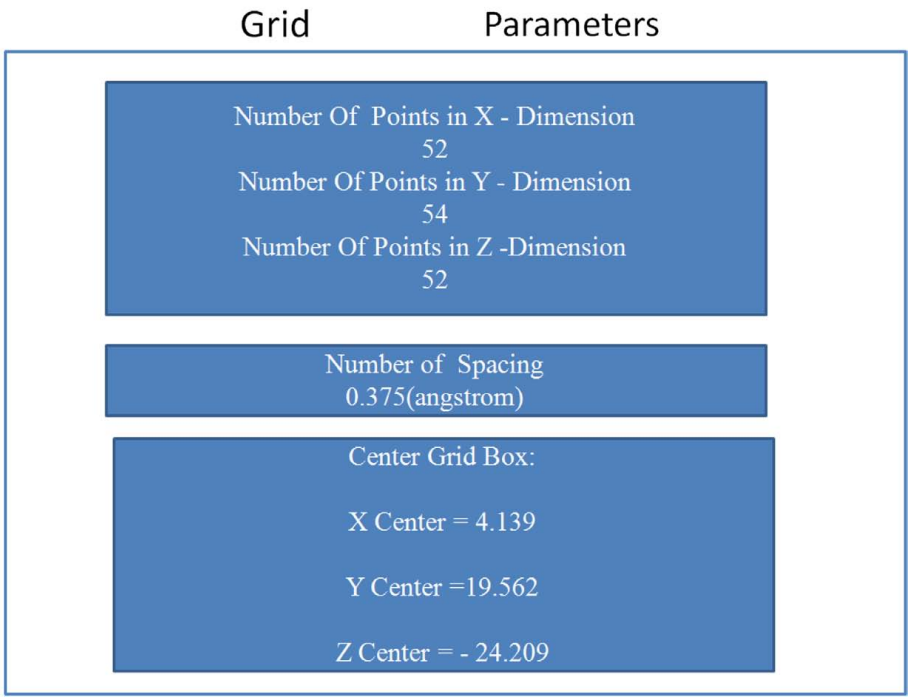

FIGURE A5 | Details of the grid parameters such as "number of spacing," "number of grid points," and "center grid box," in all three directions.

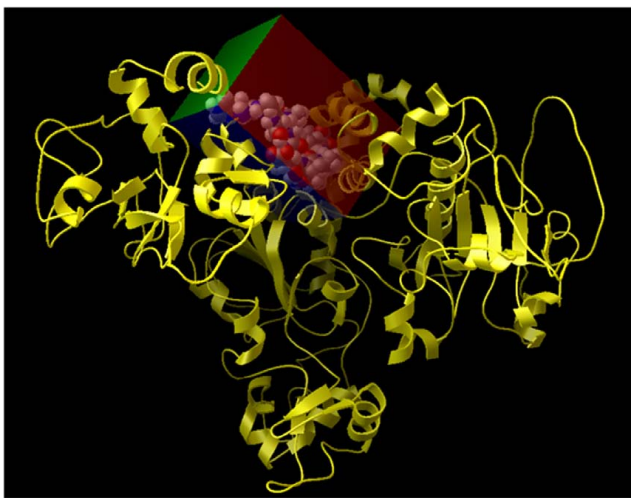

FIGURE A6 | Representation of the grid box created on the surface of Sirt-1 around the allosteric site. In details, Sirt-1 is reported in yellow cartoon conformation and the area of active site in the cpk conformation.

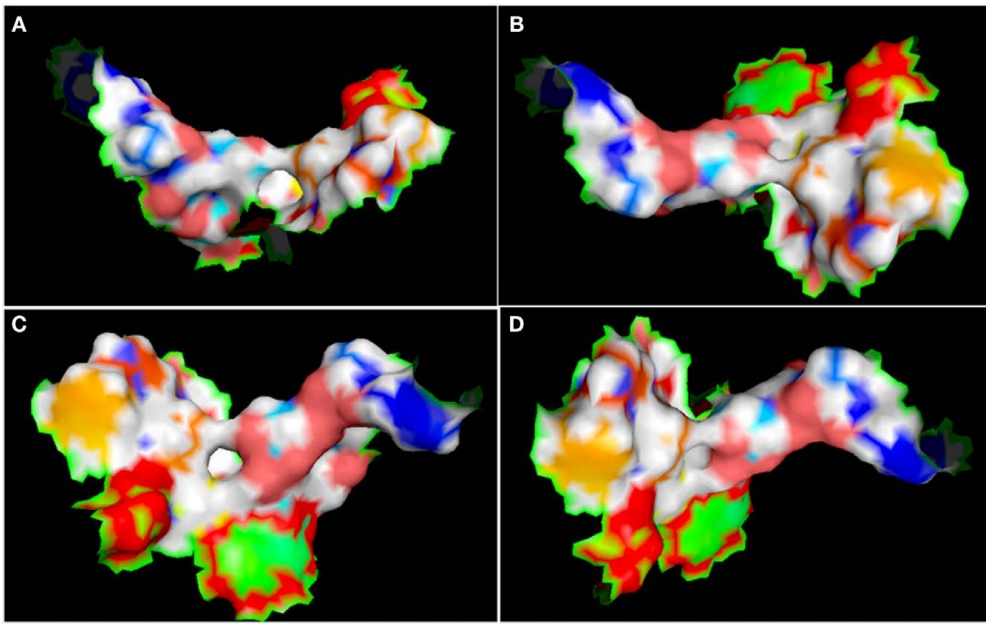

FIGURE A7 | Different view of active site (represented in the surface conformation by Pymol) of four directions. Concerning a clockwise direction, the first view shows the front view **(A)**, the second shows the top view **(B)**, the third shows the side view **(C)**, and the fourth shows the rear side view of active site residues **(D)**.

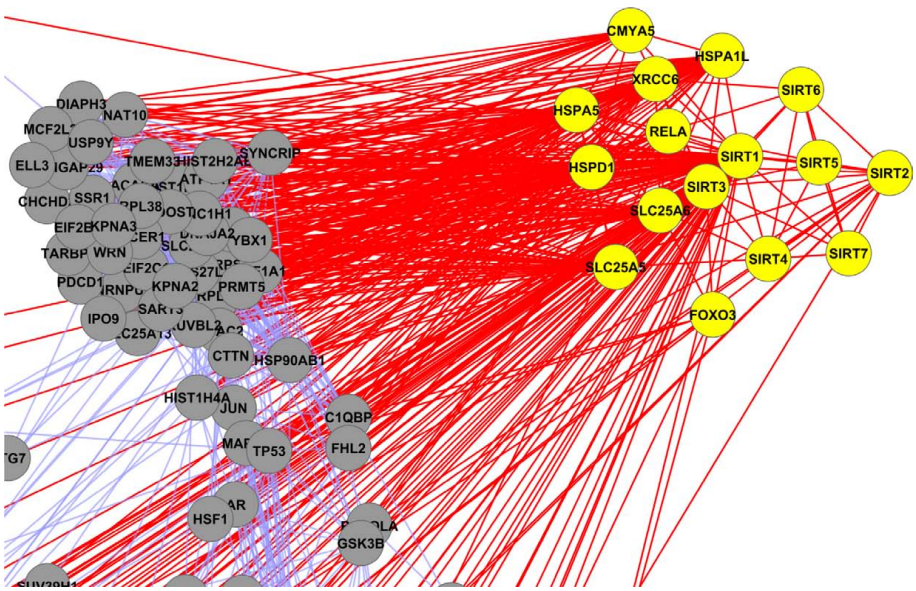

FIGURE A8 | Yellow colored nodes are showing the interaction of mitochondrial sirtuins (Sirt-2, Sirt-3 and Sirt-4).

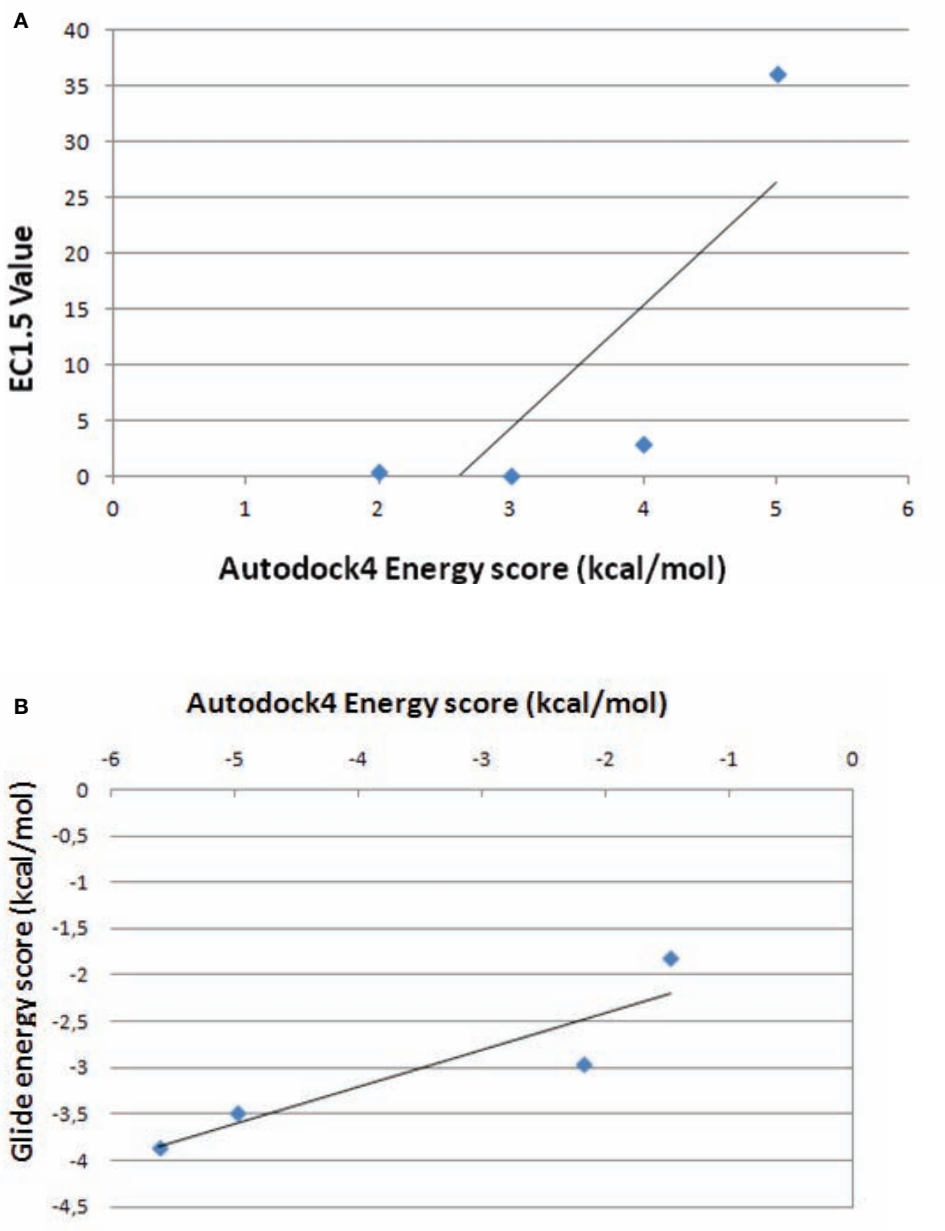

FIGURE A9 | Correlation between energy scores by AutoDock4 and the values of $EC_{1.5}$, experimentally determined (A) between energy scores by AutoDock4 and Glide programs (B).

Table A1 | The interacting partners and the centrality values associated with Sirt1 direct interacting partners.

ID	Eccentricity	Radiality	Node degree	Stress	Closeness	betweenness	Centroid
HIST1H4F	0.5	1.007407407	1	0	0.003717472	0	−134
DIAPH3	0.5	1.155555556	21	0	0.004016064	0	−114
CTTN	0.5	1.192592593	26	260	0.004098361	61.65401265	−109
USP22	0.5	1.2	27	0	0.004115226	0	−108
YBX1	0.5	1.355555556	48	1150	0.004504505	236.8117886	−87
HSF1	0.5	1.051851852	7	20	0.003802281	3.261976912	−128
PER2	0.5	1.022222222	3	0	0.003745318	0	−132
ATG7	0.5	1.044444444	6	14	0.003787879	4.981818182	−129
USP9Y	0.5	1.162962963	22	30	0.004032258	3.382936508	−113
RLN3	0.5	1.007407407	1	0	0.003717472	0	−134
CITED4	0.5	1.014814815	2	0	0.003731343	0	−133
HNRNPUL1	0.5	1.207407407	28	44	0.004132231	5.115295815	−107
HOXB1	0.5	1.014814815	2	0	0.003731343	0	−133
NUDT21	0.5	1.02962963	4	2	0.003759398	0.4	−131
C1QBP	0.5	1.155555556	21	90	0.004016064	15.56143024	−114
ATP1A1	0.5	1.2	27	0	0.004115226	0	−108
HDAC2	0.5	1.303703704	41	880	0.004366812	241.4581972	−94
JUN	0.5	1.155555556	21	244	0.004016064	60.87072927	−114
ZBTB7A	0.5	1.051851852	7	14	0.003802281	2.370707071	−128
E2F1	0.5	1.081481481	11	44	0.003861004	7.796897547	−124
TADA3	0.5	1.22962963	31	186	0.0041841	46.44718615	−104
POFUT2	0.5	1.2	27	0	0.004115226	0	−108
NR1H2	0.5	1.037037037	5	6	0.003773585	1.233333333	−130
SIRT5	0.5	1.051851852	7	8	0.003802281	1.752380952	−128
GSK3B	0.5	1.140740741	19	212	0.003984064	51.19381729	−116
SIRT2	0.5	1.111111111	15	146	0.003921569	58.07142857	−120
CCDC101	0.5	1.207407407	28	34	0.004132231	3.075757576	−107
HES1	0.5	1.014814815	2	0	0.003731343	0	−133
SIRT3	0.5	1.088888889	12	70	0.003875969	23.90622711	−123
SYNCRIP	0.5	1.259259259	35	444	0.004255319	70.4048396	−100
PAPOLA	0.5	1.037037037	5	8	0.003773585	1.819047619	−130
DNAJA2	0.5	1.266666667	36	324	0.004273504	34.0688319	−99
HSPA1L	0.5	1.303703704	41	756	0.004366812	129.3411642	−94
CLOCK	0.5	1.051851852	7	18	0.003802281	6.071428571	−128
HSP90AB1	0.5	1.340740741	46	910	0.004464286	139.8057991	−89
ARHGAP29	0.5	1.155555556	21	0	0.004016064	0	−114
NAT10	0.5	1.155555556	21	0	0.004016064	0	−114
TMEM33	0.5	1.207407407	28	20	0.004132231	1.061319967	−107
CHCHD2	0.5	1.155555556	21	0	0.004016064	0	−114
TRRAP	0.5	1.251851852	34	364	0.004237288	122.354329	−101
DOT1L	0.5	1.007407407	1	0	0.003717472	0	−134
ELL3	0.5	1.155555556	21	0	0.004016064	0	−114
FHL2	0.5	1.118518519	16	110	0.003937008	27.81993562	−119
KLHL23	0.5	1.2	27	0	0.004115226	0	−108
HEY2	0.5	1.022222222	3	2	0.003745318	1	−132
SIRT7	0.5	1.044444444	6	0	0.003787879	0	−129
AASDHPPT	0.5	1.222222222	30	156	0.004166667	65.43333333	−105
AR	0.5	1.148148148	20	218	0.004	49.62665945	−115
FAM48A	0.5	1.207407407	28	50	0.004132231	14.68571429	−107
MYOD1	0.5	1.088888889	12	76	0.003875969	17.44062049	−123
NEDD8	0.5	1.044444444	6	12	0.003787879	4.244444444	−129

(Continued)

Table A1 | Continued

ID	Eccentricity	Radiality	Node degree	Stress	Closeness	betweenness	Centroid
HIC1	0.5	1.007407407	1	0	0.003717472	0	−134
WDR77	0.5	1.281481481	38	372	0.004310345	34.81392157	−97
ATXN7L3B	0.5	1.2	27	0	0.004115226	0	−108
RPL23	0.5	1.288888889	39	468	0.004329004	53.71204567	−96
USP27X	0.5	1.2	27	0	0.004115226	0	−108
NR1H3	0.5	1.02962963	4	6	0.003759398	1.4	−131
CNOT10	0.5	1.2	27	0	0.004115226	0	−108
HIST1H2BC	0.5	1.2	27	174	0.004115226	22.27959818	−108
EP300	0.5	1.222222222	30	666	0.004166667	228.5605339	−105
RPS27L	0.5	1.22962963	31	116	0.0041841	11.38406389	−104
DEDD	0.5	1.2	27	0	0.004115226	0	−108
EIF2C1	0.5	1.222222222	30	90	0.004166667	7.877714563	−105
RPS3	0.5	1.318518519	43	708	0.004405286	93.02002658	−92
PPARGC1A	0.5	1.051851852	7	24	0.003802281	5.652380952	−128
SND1	0.5	1.22962963	31	208	0.0041841	93.26666667	−104
CMYA5	0.5	1.162962963	22	34	0.004032258	6.219047619	−113
SART3	0.5	1.222222222	30	98	0.004166667	15.94178383	−105
SIRT1	1	2	135	15352	0.007407407	10940.83505	81
HSPD1	0.5	1.4	54	1384	0.00462963	242.3024192	−81
TAF5L	0.5	1.207407407	28	34	0.004132231	3.075757576	−107
NCOR2	0.5	1.111111111	15	120	0.003921569	32.02236652	−120
SSR1	0.5	1.2	27	0	0.004115226	0	−108
EEF1A1	0.5	1.340740741	46	916	0.004464286	144.4425679	−89
HIST3H3	0.5	1.059259259	8	28	0.003816794	9.085714286	−127
MCF2L2	0.5	1.155555556	21	0	0.004016064	0	−114
SIRT4	0.5	1.059259259	8	20	0.003816794	6.285714286	−127
MAPK14	0.5	1.155555556	21	270	0.004016064	75.87614053	−114
CN0T1	0.5	1.214814815	29	102	0.004149378	38.86666667	−106
PREPL	0.5	1.2	27	0	0.004115226	0	−108
PDCD1	0.5	1.2	27	0	0.004115226	0	−108
FOXOI	0.5	1.037037037	5	6	0.003773585	1	−130
HIST1H1B	0.5	1.037037037	5	8	0.003773585	2.566666667	−130
TAF6L	0.5	1.207407407	28	34	0.004132231	3.075757576	−107
ATG5	0.5	1.022222222	3	2	0.003745318	1	−132
KAT5	0.5	1.066666667	9	32	0.003831418	5.803174603	−126
ATXN7L2	0.5	1.2	27	0	0.004115226	0	−108
PRMT5	0.5	1.318518519	43	708	0.004405286	131.1924652	−92
DICER1	0.5	1.214814815	29	78	0.004149378	9.36469364	−106
HNF4A	0.5	1.066666667	9	28	0.003831418	6.469047619	−126
ACSS2	0.5	1.014814815	2	0	0.003731343	0	−133
DDOST	0.5	1.222222222	30	66	0.004166667	3.923840779	−105
FOX03	0.5	1.066666667	9	46	0.003831418	14.66753247	−126
TP53	0.5	1.259259259	35	852	0.004255319	247.7405667	−100
SLC25A13	0.5	1.237037037	32	200	0.004201681	58.17817533	−103
NCOA2	0.5	1.066666667	9	28	0.003831418	5.857142857	−126
MAPK8	0.5	1.074074074	10	24	0.003846154	4.271428571	−125
RBI	0.5	1.118518519	16	122	0.003937008	27.02950938	−119
SLC25A5	0.5	1.251851852	34	262	0.004237288	39.46214475	−101
IP09	0.5	1.214814815	29	64	0.004149378	10.93376623	−106
MEF2C	0.5	1.051851852	7	14	0.003802281	1.934199134	−128
TARBP2	0.5	1.2	27	0	0.004115226	0	−108
NCOR1	0.5	1.103703704	14	110	0.00390625	33.77150072	−121

(Continued)

Table A1 | Continued

ID	Eccentricity	Radiality	Node degree	Stress	Closeness	betweenness	Centroid
WRN	0.5	1.17037037	23	50	0.004048583	5.246184371	−112
SIRT6	0.5	1.059259259	8	18	0.003816794	4.004761905	−127
SUPT3H	0.5	1.207407407	28	34	0.004132231	3.075757576	−107
KIF7	0.5	1.2	27	0	0.004115226	0	−108
SLC25A6	0.5	1.266666667	36	340	0.004273504	62.29482874	−99
TADA1	0.5	1.207407407	28	34	0.004132231	3.075757576	−107
RUVBL2	0.5	1.288888889	39	500	0.004329004	92.11672012	−96
SUV39H1	0.5	1.051851852	7	18	0.003802281	5.785714286	−128
KAT2B	0.5	1.2	27	472	0.004115226	157.352381	−108
ATXN7L3	0.5	1.2	27	0	0.004115226	0	−108
NFE2	0.5	1.02962963	4	4	0.003759398	0.7	−131
RPL38	0.5	1.222222222	30	64	0.004166667	3.657072047	−105
XRCC6	0.5	1.259259259	35	626	0.004255319	162.0582313	−100
KPNA2	0.5	1.2	27	196	0.004115226	30.50607726	−108
ACACA	0.5	1.185185185	25	120	0.004081633	29.98435813	−110
HIST4H4	0.5	1.037037037	5	8	0.003773585	2.333333333	−130
EIF2B4	0.5	1.2	27	0	0.004115226	0	−108
KPNA3	0.5	1.185185185	25	136	0.004081633	30.1805203	−110
ENY2	0.5	1.2	27	0	0.004115226	0	−108
DYNC1H1	0.5	1.222222222	30	248	0.004166667	39.60332249	−105
RARB	0.5	1.014814815	2	0	0.003731343	0	−133
HIST2H2AB	0.5	1.214814815	29	194	0.004149378	18.36998916	−106
SLC25A3	0.5	1.266666667	36	300	0.004273504	25.33091014	−99
KAT2A	0.5	1.237037037	32	246	0.004201681	72.24401154	−103
HIST1H4A	0.5	1.140740741	19	120	0.003984064	39.88333333	−116
SUPT7L	0.5	1.207407407	28	34	0.004132231	3.075757576	−107
MAP1LC3B	0.5	1.022222222	3	2	0.003745318	1	−132
RELA	0.5	1.162962963	22	282	0.004032258	72.94258519	−113
RRP8	0.5	1.022222222	3	2	0.003745318	0.4	−132
TADA2B	0.5	1.2	27	0	0.004115226	0	−108
ARNTL	0.5	1.037037037	5	4	0.003773585	1.333333333	−130
TAF9	0.5	1.259259259	35	434	0.004255319	159.8090909	−100
HSPA5	0.5	1.340740741	46	986	0.004464286	135.950784	−89

Table A2 | Biological processes associated with interacting proteins in the Sirt 1 interaction maps with significant *p*-value.

GO-ID	*p*-Value	Description	Genes
6333	2.81 E – 07	Chromatin assembly or disassembly	HIST1H2BC|SIRT4|SIRT6|SIRT1|SIRT2|S
16763	1.39E – 06	Transferase activity, transferring pentosyl	SIRT4|SIRT6|SIRT1|SIRT3
16575	1.54E – 06	Histone deacetylation	HDAC2|SIRT1|SIRT2
6355	2.05E – 06	Regulation of transcription, DNA-dependent	AR|RELA|SIRT4|SIRT6|ARNTL|SIRT1|YB)
31323	2.28E – 06	Regulation of cellular metabolism	AR|EIF2C1|RELA|SIRT4|SIRT6|ARNTL|SI
6476	2.45E – 06	Protein amino acid deacetylation	HDAC2|SIRT1|SIRT2
32774	2.83E – 06	RNA biosynthesis	AR|RELA|SIRT4|SIRT6|ARNTL|SIRT1|YB)
17136	3.21 E – 06	NAD-dependent histone deacetylase activity	SIRT1|SIRT2
6259	3.67E – 06	DNA metabolism	HIST1H2BC|HDAC2|SIRT4|RUVBL2|SIRT
45449	4.65E – 06	Regulation of transcription	AR|RELA|SIRT4|SIRT6|ARNTL|SIRT1|YB)
16070	5.39E – 06	RNA metabolism	AR|RELA|SIRT4|SYNCRIP|SIRT6|ARNTL|
6996	6.66E – 06	Organelle organization and biogenesis	HIST1H2BC|HDAC2|SIRT4|RUVBL2|SIRT
6139	8.88E – 06	Nucleobase, nucleoside, nucleotide and	rHIST1H2BC|AR|RELA|SIRT4|SYNCRIP|SI
3950	9.44E – 06	NAD+ ADP-ribosyltransferase activity	SIRT4|SIRT1|SIRT3
5667	1.70E – 05	Transcription factor complex	EP300|HDAC2|JUN|RELA|RUVBL2
45892	1.84E – 05	Negative regulation of transcription, DNA	SIRT4|SIRT6|SIRT1|SIRT2|SIRT3
16570	2.87E – 05	Histone modification	HDAC2|SIRT1|SIRT2
43170	4.14E – 05	Macromolecule metabolism	HIST1H2BC|AR|EIF2C1|RELA|SIRT4|SYN
3700	5.96E – 05	Transcription factor activity	AR|EP300|HDAC2|HNF4A|HSF1|JUN|REL
123	1.15E – 04	Histone acetyltransferase complex	EP300|RUVBL2
30528	1.86E – 04	Transcription regulator activity	AR|EP300|HDAC2|HNF4A|HSF1|JUN|REL
19538	3.16E – 04	Protein metabolism	HIST1H2BC|EIF2C1|RELA|SIRT4|RPS27L
5488	3.41 E – 04	Binding	EIF2C1|SYNCRIP|RPS27L|RPL38|YBX1|F
43283	3.81 E – 04	Biopolymer metabolism	HIST1H2BC|AR|RELA|SIRT4|SYNCRIP|SI
16932	9.70E – 04	Transferase activity, transferring glycosyl	SIRT4|SIRT6|SIRT1|SIRT3
6950	1.12E – 03	Response to stress	AR|EP300|HNF4A|HSF1|RELA|RUVBL2|S
3678	1.44E – 03	DNA helicase activity	RUVBL2|WRN
15320	1.82E – 03	Phosphate carrier activity	SLC25A3
31509	1.82E – 03	Telomeric heterochromatin formation	SIRT2
31509	1.82E – 03	Telomeric heterochromatin formation	SIRT2
183	1.82E – 03	Chromatin silencing at rDNA	SIRT2
35026	1.82E – 03	Leading edge cell differentiation	JUN
6348	1.82E – 03	Chromatin silencing at telomere	SIRT2
7517	2.76E – 03	Muscle development	EP300|SIRT1|SIRT2
8080	2.77E – 03	*N*-acetyltransferase activity	EP300|NAT10
16282	3.43E – 03	Eukaryotic 43S preinitiation complex	EIF2C1|RPS3
16410	3.58E – 03	*N*-acyltransferase activity	EP300|NAT10
3707	3.58E – 03	Steroid hormone receptor activity	AR|HNF4A
15207	3.64E – 03	Adenine transporter activity	SLC25A5
42903	3.64E – 03	Tubulin deacetylase activity	SIRT2
10224	3.64E – 03	Response to UV-B	RELA
4882	3.64E – 03	Androgen receptor activity	AR
15810	3.64E – 03	Aspartate transport	SLC25A13
5496	4.02E – 03	Steroid binding	AR|HNF4A
4879	4.02E – 03	Ligand-dependent nuclear receptor activity	AR|HNF4A
45137	4.02E – 03	Development of primary sexual characteristics	AR|SIRT1
8406	4.02E – 03	Gonad development	AR|SIRT1
8134	5.18E – 03	Transcription factor binding	EP300|HDAC2|RELA|SIRT2
40009	5.45E – 03	Regulation of growth rate	WRN
5345	5.45E – 03	Purine transporter activity	SLC25A5
4032	5.45E – 03	Aldehyde reductase activity	AR
48511	6.78E – 03	Rhythmic process	ARNTL|SIRT1

(Continued)

Table A2 | Biological processes associated with interacting proteins in the Sirt 1 interaction maps with significant *p*-value.

GO-ID	*p*-Value	Description	Genes
5497	7.26E – 03	Androgen binding	AR
6980	7.26E – 03	Redox signal response	SIRT2
40007	8.18E – 03	Growth	AR|RUVBL2|WRN
15205	9.07E – 03	Nucleobase transporter activity	SLC25A5
42301	9.07E – 03	Phosphate binding	RELA
45120	9.07E – 03	Pronucleus	HSF1
5850	9.07E – 03	Eukaryotic translation initiation factor 2 complex	EIF2C1
30850	9.07E – 03	Prostate gland development	AR
19899	9.08E – 03	Enzyme binding	HDAC2|RELA|SIRT2
35267	1.09E – 02	NuA4 histone acetyltransferase complex	RUVBL2
35035	1.09E – 02	Histone acetyltransferase binding	SIRT2
45084	1.09E – 02	Positive regulation of interleukin-12 biosynthesis	RELA
1889	1.09E – 02	Liver development	RELA
42177	1.09E – 02	Negative regulation of protein catabolism	RELA
9887	1.10E – 02	Organ morphogenesis	AR|EP300|RELA|SIRT1
6310	1.11E – 02	DNA recombination	RUVBL2|WRN
45935	1.12E – 02	Positive regulation of nucleobase, nucleoside, nucleotide al	EP300|JUN|RELA
3702	1.17E – 02	RNA polymerase II transcription factor activity	HNF4A|JUN|RELA
5313	1.27E – 02	l-Glutamate transporter activity	SLC25A13
8143	1.27E – 02	Poly(A) binding	SYNCRIP
43189	1.27E – 02	H4/H2A histone acetyltransferase complex	RUVBL2
43565	1.29E – 02	Sequence-specific DNA binding	AR|HNF4A|HSF1|JUN
8270	1.36E – 02	Zinc ion binding	AR|EP300|HNF4A|SIRT4|SIRT6|RPS
32615	1.45E – 02	Interleukin-12 production	RELA
15172	1.45E – 02	Acidic amino acid transporter activity	SLC25A13
8139	1.45E – 02	Nuclear localization sequence binding	KPNA3
45075	1.45E – 02	Regulation of interleukin-12 biosynthesis	RELA
51059	1.45E – 02	NF-kappaB binding	RELA
5868	1.45E – 02	Cytoplasmic dynein complex	DYNC1H1
8026	1.45E – 02	ATP-dependent helicase activity	RUVBL2|WRN

Table A3 | Interacting protein partner of Sirt-1 involved in various pathways.

Pathways (in human)	Interacting proteins in Sirt-1 interactome
E2F transcription factor network	E2F1
FoxO family signaling	FOX03A, FOXOl
HIF-2 alpha transcription factor Network	HIF2A, ARNT
Regulation of Androgen receptor activity	AR, NCOA1
Regulation of retinoblastoma protein	RB1
P73 transcription factor network	P300, P73
Signaling events mediated by HDAC class III	P300, HISTH1B, FOX04, PGC1A, MEF2D, HDAC4, TP53, MYOD, PCAF, FHL2, BAX, XRCC6

Table A4 | Cellular localization of the Sirt-1 interacting proteins by GO studies.

GO-ID	*p*-Value	Cellular component	Genes
5677	1.81E – 12	Chromatin silencing complex	SIRT4|SIRT6|SIRT1|SIRT2|SIRT3
44451	1.10E – 09	Nucleoplasm part	HDAC2|EP300|JUN|RELA|SIRT4|RUVBL2|SIRT6|SIRT
16585	5.30E – 09	Chromatin remodeling complex	SIRT4|SIRT6|SIRT1|SIRT2|SIRT3
5654	6.05E – 09	Nucleoplasm	HDAC2|EP300|JUN|RELA|SIRT4|RUVBL2|SIRT6|SIRT
44428	8.71E – 08	Nuclear part	HDAC2|EP300|JUN|RELA|SIRT4|SYNCRIP|RUVBL2|SI
31981	9.86E – 08	Nuclear lumen	HDAC2|EP300|JUN|RELA|SIRT4|RUVBL2|SIRT6|SIRT
43234	1.54E – 07	Protein complex	EIF2C1|RELA|SIRT4|SYNCRIP|RPS27L|SIRT6|RPL38|S
44422	1.66E – 07	Organelle part	SLC25A5|RELA|SIRT4|SYNCRIP|SIRT6|RPL38|SIRT1|S
44446	1.66E – 07	Intracellular organelle part	SLC25A5|RELA|SIRT4|SYNCRIP|SIRT6|RPL38|SIRT1|S
43233	6.77E – 07	Organelle lumen	HDAC2|EP300|JUN|RELA|SIRT4|RUVBL2|SIRT6|SIRT
31974	6.77E – 07	Membrane-enclosed lumen	HDAC2|EP300|JUN|RELA|SIRT4|RUVBL2|SIRT6|SIRT
43229	6.11E – 06	Intracellular organelle	SYNCRIP|RPS27L|RPL38|YBX1|RPS3|HSF1|SLC25A3|
43226	6.13E – 06	Organelle	SYNCRIP|RPS27L|RPL38|YBX1|RPS3|HSF1|SLC25A3|
5622	1.55E – 05	Intracellular	EIF2C1|SYNCRIP|RPS27L|RPL38|YBX1|RPS3|MCF2L2
44424	1.95E – 05	Intracellular part	EIF2C1|SYNCRIP|RPS27L|RPL38|YBX1|RPS3|HSF1|SL
5634	2.98E – 05	Nucleus	HISTlH2BC|AR|RELA|SIRT4|SYNCRIP|SIRT6|WRN|Af
5667	3.71E – 05	Transcription factor complex	HDAC2|EP300|JUN|RELA|RUVBL2
123	1.59E – 04	Histone acetyltransferase complex	EP300|RUVBL2
43231	2.31E – 04	Intracellular membrane-bound organelle	HIST1H2BC|AR|SLC25A5|RELA|SIRT4|SYNCRIP|SIRTE
43227	2.39E – 04	Membrane-bound organelle	HIST1H2BC|AR|SLC25A5|RELA|SIRT4|SYNCRIP|SIRTE
16282	4.74E – 03	Eukaryotic 43S preinitiation complex	EIF2C1|RPS3
31967	8.64E – 03	Organelle envelope	SLC25A13|SLC25A5|SLC25A3|KPN A3
31975	8.96E – 03	Envelope	SLC25A13|SLC25A5|SLC25A3|KPN A3
5743	9.48E – 03	Mitochondrial inner membrane	SLC25A13|SLC25A5|SLC25A3
5850	1.07E – 02	Eukaryotic translation initiation factor 2 complex	EIF2C1
45120	1.07E – 02	Pronucleus	HSF1
19866	1.13E – 02	Organelle inner membrane	SLC25A13|SLC25A5|SLC25A3
5830	1.15E – 02	Cytosolic ribosome (sensu Eukaryota)	RPL38|RPS3
35267	1.28E – 02	NuA4 histone acetyltransferase complex	RUVBL2
5737	1.45E – 02	Cytoplasm	AR|EIF2C1|SLC25A5|RELA|SYNCRIP|RPS27L|RPL38|S
43189	1.50E – 02	H4/H2A histone acetyltransferase complex	RUVBL2
31966	1.50E – 02	Mitochondrial membrane	SLC25A13|SLC25A5|SLC25A3
5868	1.71E – 02	Cytoplasmic dynein complex	DYNC1H1
5740	1.87E – 02	Mitochondrial envelope	SLC25A13|SLC25A5|SLC25A3

Excel sheets with more the details can be found on these links.

- Complete details about the Biological processes of Sirt1 and its interacting partners as analyzed by GO studies: http://bit.ly/s0XBTz
- Details regarding HUB proteins, Average path length and biological processes and cellular localization associated with Sirtlhub nodes at bit.ly/hubproteinsofSIRT1Network

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SIRT2 as a therapeutic target for age-related disorders

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Sirtuin proteins are conserved regulators of aging that have recently emerged as important modifiers of several diseases which commonly occur later in life such as cancer, diabetes, cardiovascular, and neurodegenerative diseases. In mammals, there are seven sirtuins (SIRT1-7), which display diversity in subcellular localization and function. SIRT1 has received much of attention due to its possible impact on longevity, while important biological and therapeutic roles of other sirtuins have been underestimated and just recently recognized. Here we focus on SIRT2, a member of the sirtuin family, and discuss its role in cellular and tissue-specific functions. This review summarizes the main scientific advances on SIRT2 protein biology and explores its potential as a therapeutic target for treatment of age-related disorders.

Keywords: SIRT2, aging, metabolic syndrome, cancer, neurodegenerative disorder

INTRODUCTION

Silent information regulator 2 (SIR2) is the eponymous gene of a whole family of conserved genes named sirtuins, which are present in organisms ranging from bacteria to plants, and animals (Haigis and Sinclair, 2010). Original studies of aging in *S. cerevisiae* led to the discovery that extra copies of the *SIR2* gene extended lifespan by 50%, whereas *SIR2* deletion reduced longevity (Kaeberlein et al., 1999). In yeast, *SIR2* promotes longevity by suppressing the formation of toxic extrachromosomal rDNA circles (Sinclair and Guarente, 1997). Remarkably, the fly and worm *SIR2* orthologs also play a role in the regulation of lifespan (Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). However, the increase of longevity due to Sir2 overexpression in fly and nematode models has recently been challenged, and is now considered to be about 15% in nematodes (Burnett et al., 2011; Viswanathan and Guarente, 2011). Nevertheless, several studies in yeast and fly models suggested that SIR2 could be a critical mediator of the beneficial effects of caloric restriction (CR) (Chen and Guarente, 2007). CR is a dietary regimen that reduces caloric intake by a total of 30% without malnutrition. CR has been shown to slow down the aging process and increase lifespan in all laboratory models tested (Koubova and Guarente, 2003). In mammals, several studies found that CR leads to an increase of sirtuins protein expression in a variety of tissues (Cohen et al., 2004; Shi et al., 2005). Importantly, SIRT1 was found to be required for the increase of physical activity induced by CR (Chen et al., 2005). These studies suggest that mammalian sirtuins are involved in the activation of biological responses during CR required for the increased lifespan.

The discovery that SIR2 extended lifespan in yeast, worms, and flies incited scientists to further study mammalian sirtuins, since questions such as whether sirtuins promote health and protect against aging-associated disorders are of great scientific and social/economic interest. In mammals there are seven sirtuins, SIRT1-7, all possessing a highly conserved central NAD^+-binding site and common catalytic domain (Frye, 2000; Landry et al., 2000; Smith et al., 2000; Tanner et al., 2000). Sirtuins are NAD^+-dependent deacetylases, linking their enzymatic activity to the energy state of the cell (Nakagawa et al., 2009). Therefore it is crucial to study the effect of nicotinamide phosphoribosyltransferase (NAMPT) on SIRT2 activity, a key enzyme that regulates NAD biosynthesis. The sirtuin-mediated deacetylation reaction couples lysine deacetylation to NAD^+-hydrolysis. This hydrolysis yields *O*-acetyl-ADP-ribose, the deacetylated substrate and nicotinamide, which is an inhibitor of sirtuins activity (Tanner et al., 2000). During glycolysis and the citric acid cycle NAD^+ is reduced to NADH, hence the ratio of NAD^+ to NADH is inversely proportional to energy availability. Interestingly, in yeast cells NADH can competitively inhibit the deacetylase reaction catalyzed by sirtuins and NADH levels are decreased by CR (Lin et al., 2004). Thus it is tempting to speculate that mammalian sirtuins should have augmented enzymatic activity under conditions of low energy, high NAD^+ to NADH ratio, and therefore they function as sensors for the cellular energy status. Although the catalytic domain of mammalian sirtuins is conserved, they are structurally different with respect to their N- and C-termini, to their localization within the cell, and in

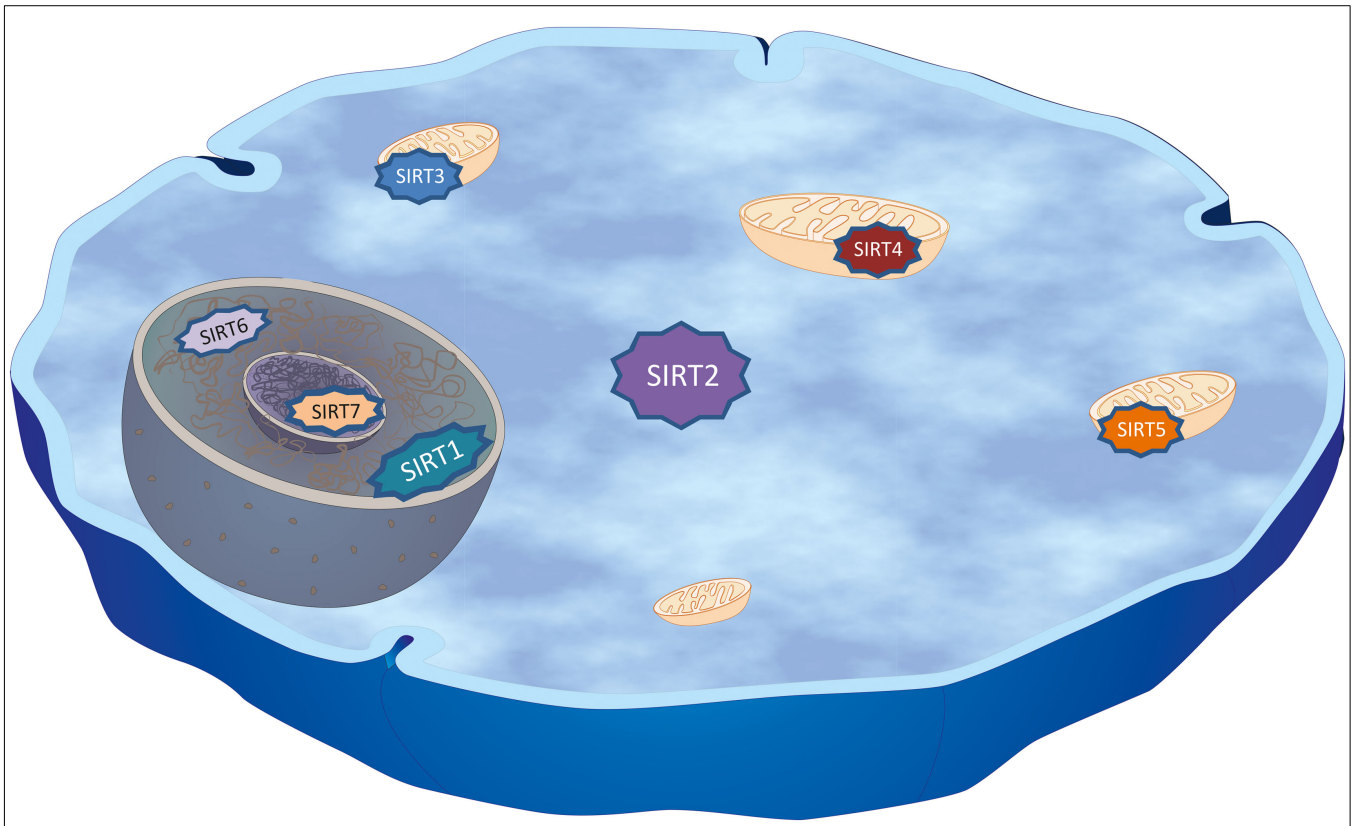

FIGURE 1 | Cellular distribution of mammalian sirtuins. Mammals possess seven sirtuins, SIRT1–7, that have a NAD^+-dependent catalytic core domain in common. Additional variable N-terminal and C-terminal sequences flank this core domain leading to diverse subcellular localizations that may account for differences in their biological functions as well as different substrates and binding partners within the cell. SIRT1 is mainly nuclear, modulating, amongst others, chromatin structure by deacetylating specific lysine residues in histones H1, H3, and H4, although it can transiently be found in the cytoplasm. SIRT2, the closest homolog of SIRT1, is primarily a cytoplasmic protein, but can transiently shuttle into the nucleus during mitosis, where it deacetylates Lys-56 of histone H3 and Lys-16 of histone H4. In the cytoplasm, SIRT2 deacetylates microtubules, in particular α-tubulin at Lys-40 and a variety of other substrates (**Table 1**). SIRT3, SIRT4, and SIRT5 were found to co-localize with mitochondria indicating key regulative roles in metabolism and energy usage of the cell. SIRT6 is a nuclear protein found to regulate DNA repair, while SIRT7 co-localizes with the nucleolus and was shown to be involved in rRNA transcription.

that they utilize different substrates and protein binding partners (Blander and Guarente, 2004). SIRT1 is mainly nuclear, although it can transiently be found in the cytoplasm (Haigis and Guarente, 2006). While SIRT2 is primarily a cytoplasmic protein, it can transiently shuttle into the nucleus in a cell cycle-dependent manner (North et al., 2003; North and Verdin, 2007a). SIRT3, SIRT4, and SIRT5 are mitochondrial proteins. SIRT6 is a nuclear protein and SIRT7 is nucleolar (Liszt et al., 2005; Ford et al., 2006; **Figure 1**). Despite the fact that some sirtuins can have redundant functions, different biological roles may be determined by intracellular compartmentalization, and by different tissue expression pattern.

Among all mammalian sirtuins, SIRT1 has been the most extensively studied. Nevertheless, studies of the other mammalian sirtuins have uncovered a variety of substrates, interacting partners and biological relevance in diverse cellular processes. Here we focus on the biological role of SIRT2, the only cytoplasmic member of the family, explore its various roles in age-associated disorders and discuss possible therapeutic applications.

THE BIOLOGICAL PROPERTIES OF THE SIRT2

SIRT2 is the mammalian ortholog of yeast HST2 (Perrod et al., 2001). Similar to SIR2, HST2 is upregulated by CR as well as oxidative stress and extends lifespan by a SIR2-independent mechanism (Lamming et al., 2005; Zhu et al., 2012). In future experiments, it will be important to determine if mice overexpressing SIRT2 mice show an altered lifespan. It would be equally important to determine whether SIRT2 is required for any of the protective phenotypes mediated by CR.

SIRT2 is found primarily in the cytoplasm, co-localizes with microtubules and deacetylates the major component of microtubules, α-tubulin at lysine 40 (North et al., 2003). In the same study the authors identified the microtubule deacetylase HDAC6 as a binding partner of SIRT2. It is unclear whether SIRT2 deacetylates soluble or polymerized α-tubulin, and thus could be formally considered as a microtubule deacetylase.

SIRT2 transiently migrates to the nuclei during G2/M transition and deacetylates histone H4 at lysine 16, thereby modulating chromatin condensation during metaphase (Vaquero et al., 2006).

Interestingly, SIRT1 also deacetylates lysine 16 of histone H4, indicating a possible synergistic relationship or functional redundancy between these sirtuins (Imai et al., 2000; Vaquero et al., 2004). When SIRT2 is overexpressed it delays cell cycle progression (Dryden et al., 2003). Moreover, SIRT2 protein levels vary accordingly to the cell cycle phase, increasing during mitosis. Altogether, these observations implicate SIRT2 in the regulation of the cell cycle (Inoue et al., 2007).

In addition to α-tubulin and histone H4 substrates, SIRT2 deacetylates forkhead transcription factors of class O, FOXO1, and FOXO3 (Li et al., 2007; Wang and Tong, 2009; Zhu et al., 2012). Since FOXO transcription factors are involved in multiple cellular processes, such as DNA repair, cell cycle, apoptosis, metabolism, and aging, SIRT2 is therefore also connected with these diverse pathways (Calnan and Brunet, 2008).

SIRT2 deacetylates lysine residues in the catalytic domain of p300, a histone acetyltransferase, which maintains its active form by autoacetylation (Black et al., 2008). The known consequence of SIRT2-dependent deacetylation of p300 is the de-repression of p53 transcriptional activity (Han et al., 2008). Notably, p300 is capable of inhibiting SIRT2 activity by direct acetylation, thus demonstrating a complex relationship and regulation of acetylase and deacetylase activities in cells. Interestingly, posttranslational phosphorylation also negatively modulates SIRT2 activity. Phosphorylation at serine 331 inhibits SIRT2 catalytic activity (Pandithage et al., 2008). SIRT2 is phosphorylated by various cycline-CDK complexes at serine 331 and is dephosphorylated by CDC14B phosphatase (Dryden et al., 2003; Southwood et al., 2007; Pandithage et al., 2008).

In addition, several proteins such as 14-3-3 β/γ and homeobox transcription factor 10 are binding partners but not deacetylation substrates of SIRT2 (Bae et al., 2004; Jin et al., 2008). The currently known SIRT2 substrates/binding partners suggest a complex and apparently diverse function for this sirtuin in the cell (**Table 1**).

SIRT2 IN METABOLIC SYNDROMES

In western societies high fat and low fibber diets, together with a sedentary life style, are associated with a high prevalence of metabolic syndrome, that increases with age (Ford et al., 2002). Metabolic syndrome is the condition brought about by: obesity; insulin resistance; hypertension; and elevated lipid content in the blood. Metabolic syndrome increases the risk of serious health problems (Moller and Kaufman, 2005). In the obese it is the proportion of body fat which is significant, not purely weight *per se.* Dramatically, obesity is associated with a decrease in life expectancy (Haslam and James, 2005).

Among all the mammalian sirtuins, the SIRT2 transcript is the most abundant in adipocytes (Li et al., 2007); this alone may indicate a role in the regulation of adipose tissue functionality. Together with the observation that SIRT2 decreases during preadipocyte differentiation and regulates adipocyte differentiation in a negative manner by deacetylating FOXO1, it raises the exciting hypothesis that this protein has a pivotal role in the regulation of fat abundance (Jing et al., 2007; Wang and Tong, 2009). Thus, it can be speculated that modulating SIRT2 activity may ameliorate, at least in part, the metabolic disturbances in the obese resulting from increased fat mass. In support of this hypothesis, retroviral expression of SIRT2 in adipocytes was found to promote

Table 1 | SIRT2 substrates and interactors and their biological relevance.

Interactor/substrate	Kind of interaction	Biological relevance	Reference
α-Tubulin	Substrate (deacetylation of Lys-40)	Cytoskeleton modulation Oligodendroglial differentiation	North et al. (2003), Tang and Chua (2008), Zhu et al. (2012)
Histone H3	Substrate (deacetylation of Lys-56)	Cell cycle regulation	Das et al. (2009)
Histone H4	Substrate (deacetylation of Lys-16)	Cell cycle regulation	Vaquero et al. (2006)
FOXO1	Substrate (deacetylation of Lys-residues surrounding Ser-253)	Adipocyte differentiation	Li et al. (2007), Wang and Tong (2009)
FOXO3	Substrate (residues not yet identified)	Regulation of oxidative stress Cell growth arrest apoptosis	Zhu et al. (2012)
Par-3	Substrate (deacetylation of Lys-831, 848, 881, 1327)	Modulation of peripheral myelination	Beirowski et al. (2011)
P300	Substrate (deacetylation of several Lys-residues)	Regulation of p300 autoacetylation	Black et al. (2008)
PEPCK1	Substrate (residues not yet identified)	Blood glucose homeostasis	Jiang et al. (2011)
p65	Substrate (deacetylation of Lys-310)	Regulation of NF-κB dependent gene expression	Rothgiesser et al. (2010)
HOXA10	Binding partner	Not known	Bae et al. (2004)
HDAC6	Binding partner	Cytoskeleton dynamics	North et al. (2003)
14-3-3β/γ	Binding partners	Downregulation of p53 activity	Jin et al. (2008)
CDK1, cyclinE/CDK2, cyclinA/CDK2, p35/CDK5	Phosphorylation of SIRT2 at Ser-331,-368	Inhibition of SIRT2 catalytic activity	Southwood et al. (2007), Pandithage et al. (2008)

CDC14A/B, Dual-specificity protein phosphatase 14A/B; CDK1, cyclin-dependent kinase 1; cyclinA/CDK2, cyclin A/cyclin-dependent kinase 2 complex; CyclinE/CDK2, cyclin E/cyclin-dependent kinase 2 complex; p35/CDK5, p35/cyclin-dependent kinase 5 complex; FOXO, Forkhead box protein of class O; HDAC6, Histone deacetylase 6; HOXA10, Homeobox protein A10; Lys, Lysine; NF-κB, Nuclear factor-kappa B; Par-3, Polarity protein par-3; PEPCK1, phosphoenolpyruvate carboxykinase 1; P300, Histone acetyltransferase ac-P300; p53, tumor suppressor protein 53; p65, transcription factor p65; Ser, Serine; 14-3-3β/γ, 14-3-3 Protein β/γ isoforms.

lipolysis (Wang and Tong, 2009). Thus, SIRT2 activators could be used as a novel therapeutic approach to obesity and in a more general way to metabolic syndrome.

SIRT2 expression is elevated in white adipose tissue and kidney of caloric-restricted mice (Zhu et al., 2012). It is interesting to note that CR reverses the modifications to some physiological parameters caused by metabolic syndrome. Moreover CR protects against the diseases to which metabolic syndrome is a risk factor (Guarente, 2006). Another exciting study raised the possibility that SIRT2 plays a role in blood glucose homeostasis by deacetylating and stabilizing phosphoenolpyruvate carboxykinase 1 (PEPCK1), an important enzyme in gluconeogenesis (Jiang et al., 2011). When SIRT2 is activated by low glucose conditions, PEPCK1 is stabilized and it shifts the equilibrium toward the generation of glucose from non-carbohydrate carbon sources, mimicking a fast or exercise state in the organisms. Conversely, in the presence of high glucose SIRT2 expression is suppressed, leading to PEPCK1 degradation by the ubiquitin proteasome system (Jiang et al., 2011). This study raises the question of how glucose availability regulates SIRT2 transcription. Since other sirtuins are also linked to metabolic homeostasis, another question arises: how does SIRT2 communicate with other family members to promote metabolic homeostasis? Further insights into the intricate regulation of the metabolic network will likely emerge when we identify novel SIRT2 targets in organs such as liver, fat, pancreas and muscle. The study of tissue-specific knockouts will also contribute to a better understanding of the regulatory pathways that control metabolism in health and disease and assess a possible therapeutic role of SIRT2 modulation in metabolic syndromes. Genetic studies should also address the safety of targeting SIRT2 activity pharmacologically and the extent of the possible benefits.

SIRT2 IN CANCER

Cancer cells have the ability to divide and grow in an uncontrolled manner. A common event in various cancers is the presence of mutant proteins, namely the machinery responsible for cell cycle control and differentiation (Vogelstein and Kinzler, 2004). Several studies are in favor of SIRT2 working as a mitotic check-point, thus suggesting a role in tumorigenesis (Inoue et al., 2007). SIRT2 protein and RNA levels are decreased in gliomas, melanomas, and gastric carcinomas (Hiratsuka et al., 2003; Peters et al., 2010). We can speculate that in human gliomas this decrease may be associated with the deletion of the SIRT2 gene, since SIRT2 is located at 19q13.2, a region frequently deleted in this kind of carcinoma (von Deimling et al., 1994; Rasheed et al., 1999; Hiratsuka et al., 2003). Moreover, in a glioma cell line SIRT2 inhibits colony formation (Hiratsuka et al., 2003). In melanomas SIRT2 is mutated in the catalytic domain, eliminating its enzymatic activity (Lennerz et al., 2005). Another important observation is that when the serine 368 SIRT2 mutant (phosphorylation site) is overexpressed in a glioblastoma cell line, it leads to a reduction of hyperploid cells under mitotic stress exposure (North and Verdin, 2007b). These two studies indicate that the enzymatic function of SIRT2 may play a role in cancer, at least in the case of melanoma and glioblastoma. It remains to be determined whether SIRT2 enzymatic activity is also important in other kinds of cancers. In a recent report, SIRT2 deficiency in mice led to augmented levels of Aurora-A and -B, known to direct centrosome amplification, aneuploidy, and mitotic cell death. Surprisingly, gender-specific tumorigenesis was observed in the SIRT2-deficient mice, where females primarily developed mammary tumors, while males developed more hepatocellular carcinoma (Kim et al., 2011). The same study showed SIRT2-dependent regulation of the anaphase-promoting complex/cyclosome activity through deacetylation of its coactivators, APC (CDH1) and CDC20. The previous data combined with other study showing that lysine 53 of histone H3, which is deacetylated by SIRT2 and SIRT1, was hyperacetylated in cancer cells, implies that SIRT2 may act as a tumor suppressor gene (Das et al., 2009). Downregulation of SIRT2 renders cancer cells to apoptosis. For example, SIRT2 downregulation caused apoptosis in a cancer cell line such as HeLa but not in normal cells, suggesting a possible therapeutic avenue for intervention (Li et al., 2011). In addition, SIRT2 regulates the expression of the pro-apoptotic protein BIM due to its ability to deacetylate FOXO3 (Wang et al., 2012). Overall, SIRT2 overexpression is found to reduce cell proliferation and to regulate cell death in response to DNA damage stress. These and previously published results with cancer cell lines suggest SIRT2 as a tumor suppressor gene (Das et al., 2009). The dual role of sirtuins in cancer has recently been reviewed and although most evidences point to SIRT2 as a tumor suppressor gene. Further studies will be important to elucidate the role of SIRT2 activation in specific human cancers (Bosch-Presegue and Vaquero, 2011). These critical studies will illuminate potential and feasible therapeutic interventions for cancer treatment.

SIRT2 IN THE CENTRAL NERVOUS SYSTEM

All seven members of the mammalian SIR2 family are expressed in the brain. SIRT2 is the most abundant, although not much is known about its role in this organ (Zhu et al., 2012). SIRT2 is expressed in nearly all brain cells including olfactory and hippocampal neurons, while primarily found in the myelin producing cells of the central nervous system (CNS): oligodendrocytes (OL; Pandithage et al., 2008; Zhu et al., 2012). There are three known alternatively spliced SIRT2 isoforms in mammals. The full-length SIRT2 protein (isoform SIRT2.1) showed only moderate expression in the CNS, comparable to that seen in peripheral tissues. In sharp contrast, a significant increase of the second isoform, SIRT2.2, was observed during postnatal mouse development. SIRT2.2 expression levels remained remarkably high in the adult CNS, with maximal accumulation in aged brains (Maxwell et al., 2011).

SIRT2 expression in OL plays a role in myelinogenesis, while in neurons its function is essentially unknown. In OLs, SIRT2 is rather localized to the myelin sheath than to the cell body (Werner et al., 2007). SIRT2 appears to decelerate cell differentiation of OL precursor cells through deacetylating α-tubulin (Li et al., 2007). In accordance with the suggested role for SIRT2 as an OL differentiation inhibitor, gene knockdown causes upregulation of myelin basic protein and promotes OL differentiation. Moreover, it appears that SIRT2 functions in myelin sheaths at sites of microtubule remodeling (Southwood et al., 2007; Harting and Knoll, 2010). Based on a recent report, SIRT2 is highly abundant during active myelination, and protein levels are regulated by the QKI-dependent pathway and mediated through selective regulation

of proteolipid protein PLP (Zhu et al., 2012). Consistent with this mechanism, the presence of the SIRT2.2 isoform is severely reduced in brain of Plp1 knockout mice. Recently, mice with tissue-specific knockout of SIRT2 in Schwann cells were found to display a transient delay in myelination (Beirowski et al., 2011). It appears that in Schwann cells SIRT2 deacetylates an important regulator of cell polarity Par-3. SIRT2 deacetylates Par-3 and leads to a decrease in the activity of the polarity complex signaling component aPKC, thus regulating myelin formation. Notably, SIRT2 knockout did not lead to an increase of α-tubulin acetylation in Schwann cells. It remains to be tested whether pharmacological stimulation of SIRT2 can facilitate re-myelination in neuronal injuries or to be therapeutically beneficial in human conditions such as multiple sclerosis, characterized by progressive loss of myelin.

In neurons, SIRT2 is evenly distributed within the cytoplasm, neurites, and their growth cones (Pandithage et al., 2008). One important feature regulated by SIRT2 is neuronal motility, which is strongly dependent on the dynamic properties of the cytoskeleton, particularly those of actin filaments and microtubules. The obvious connection is the ability of SIRT2 to deacetylate α-tubulin, which is the main component of microtubules, together with β-tubulin. SIRT2 inhibits neurite outgrowth and growth cone collapse in postmitotic hippocampal neurons (Pandithage et al., 2008). Interestingly, this effect was antagonized by CDK-dependent phosphorylation of SIRT2, raising the important question of how posttranslational modifications, namely SIRT2 phosphorylation regulates its activity (Pandithage et al., 2008). Recent studies reveal that focal areas of endogenous SIRT2 expression correlate with reduced α-tubulin acetylation in primary mouse cortical neurons, further suggesting that SIRT2 may function as a microtubule deacetylase in mature neurons (Maxwell et al., 2011). Noteworthy, there are no direct evidences convincingly demonstrating that SIRT2 deacetylates microtubules in neurons. Overexpression of SIRT2 leads to inhibition of neurite elongation and impairment of migration in primary hippocampal neurons (Pandithage et al., 2008). However, no studies have been published so far indicating a direct role for SIRT2 in neuronal differentiation. The fact that SIRT2 deacetylates FOXO3 and thereby regulates its transcriptional activity, suggests a role for SIRT2 in neural stem cells homeostasis; because FOXO3 regulates the neuronal stem cell pool by maintaining quiescence (Renault et al., 2009). Besides, SIRT1 modulates neuroblastoma cell differentiation by deacetylating FOXO3 (Kim et al., 2009). Thus, it is likely to assume that SIRT2 might also play a role in neuronal differentiation. The tumor suppressor p53, has recently been ascribed a role in the regulation of neuronal cell migration, neurite outgrowth, growth cone motility, and axonal regeneration (Di Giovanni et al., 2006; Tedeschi and Di Giovanni, 2009). Since SIRT2 was shown to deacetylate p53 *in vitro*, we cannot rule out the possibility of p53 being an *in vivo* substrate for SIRT2 (Nahhas et al., 2007). Whether SIRT2 mediates these p53 dependent processes, is an intriguing question. Clearly more studies are needed to assess the precise role of SIRT2 in neuronal networks.

SIRT2 in neurodegenerative disorders

Neurodegenerative disorders affect mostly the elderly population and strongly contribute to a tremendous increase in health expenditures due to the augmentation in life expectancy. A common hallmark to several neurodegenerative disorders is the presence of abnormal protein inclusions in the brain containing specific misfolded proteins (Mattson and Magnus, 2006). However, the precise functions of those proteins under physiological and pathological conditions remain unclear. Nevertheless, SIRT2 was associated with the aggregation of proteins such as α-synuclein and huntingtin, involved in Parkinson's and Huntington's disease (HD), respectively (Outeiro et al., 2007; Pallos et al., 2008; Luthi-Carter et al., 2010). In addition, SIRT2 was indirectly associated with cellular processes implicated in the pathophysiology of neurodegenerative disorders, namely autophagy, oxidative stress, and inflammation.

Modifications in autophagy pathways were described in neurodegenerative diseases and in the normal aging brain (Wong and Cuervo, 2010). Importantly, activation of autophagy may mitigate or even prevent these disorders. Autophagy is a degradation mechanism by which cells clear out organelles, proteins, and protein aggregates that may be too large to be degraded by the ubiquitin proteasome system (Kraft et al., 2010). Several stimuli can activate autophagy and, notably, one of the main triggers is nutrient deprivation. In response to oxidative stress or serum deprivation SIRT2 releases FOXO1, which is then acetylated and binds to ATG7 and thus induce autophagy in the context of cancer (Zhao et al., 2010a,b). Accordingly, it would be interesting to test whether SIRT2 also mediates autophagy through deacetylation of FOXO1 in the context of neurodegeneration. This hypothesis seems plausible concerning the stimulus for SIRT2 to release FOXO1 and making it available to activate autophagy. Interestingly, SIRT1 also plays a role in the regulation of autophagy through deacetylation of ATG5, 7, and 8 (Lee et al., 2008). It is widely accepted that oxidative stress is implicated in the pathogenesis of neurodegenerative diseases. SIRT2 elevates the expression of the antioxidant mitochondrial superoxide dismutase (MnSOD) due to its ability to deacetylate FOXO3 and consequent increase of FOXO3 DNA-binding activity (Wang et al., 2007). Interestingly, MnSOD enzymatic activity is regulated by SIRT3 deacetylation in response to stress (Tao et al., 2010).

Another interesting link between SIRT2 and neurodegeneration is the nuclear factor-kappa B (NF-κB). NF-κB plays a pivotal role in regulating gene expression programs related to aging and inflammation, namely by inducing the expression of pro-inflammatory cytokines. New emerging data point to an association between chronic neuroinflammation and the exacerbation of several neurodegenerative diseases (Salminen and Kaarniranta, 2009). For instance, as the organism ages NF-κB transcription is activated and its incorrect regulation may elicit neurodegeneration. SIRT2 has been reported to interact with p65, an NF-κB family member, in the cytoplasm and to deacetylate it at lysine 310 after stimulation with TNF-α (Rothgiesser et al., 2010). Interestingly, deacetylation of p65 by SIRT1 antagonizes NF-κB activity (Yeung et al., 2004).

SIRT2 in Parkinson's disease. Parkinson's disease (PD) is the second most common neurodegenerative disorder, after Alzheimer's disease affecting around 4 million people worldwide. The disease is characterized by the dysfunction and degeneration of

dopaminergic neurons in the substantia nigra, which alters neurotransmitter balance in the striatum resulting in the progressive loss of movement control (Gelb et al., 1999; Forman et al., 2005). The vast majority of PD cases are sporadic and aging is the main risk factor, although inherited forms account for 5–10% of the cases. The pathological hallmark of PD and other synucleinopathies is the accumulation of protein aggregates called Lewy bodies (LB), which consist mainly of α-synuclein (Spillantini et al., 1997). LBs occur in the central, peripheral, and autonomic nervous system. The biological significance of LBs, their impact on neurodegeneration, and whether they are detrimental, and interfere with normal cell function, or are a structural manifestation of a cytoprotective response to confine and eliminate cytotoxic proteins is currently unclear.

SIRT2 regulates α-synuclein inclusion number, size, and cytotoxicity. Inhibition of SIRT2 function, either pharmacologically or genetically, led to a rescue of α-synuclein toxicity in models of PD, namely dopaminergic neurons and in an *in vivo* fly model (Outeiro et al., 2007). Inhibition of SIRT2 promoted the development of fewer and bigger aggregates. Interestingly, a smaller quantity of LB-like inclusions which are bigger in size was ascribed a protective role in neuronal cell death *in vitro* (Outeiro et al., 2007). However, the exact molecularSIRT2 inhibition-dependent neuroprotective mechanism is still elusive. It is tempting to speculate that SIRT2 inhibition increases microtubule-dependent transport of putative neurotoxic α-synuclein oligomers to the nucleation aggregation site, which facilitates formation of large benign inclusions. Consistent with this idea, an interaction between microtubules and α-synuclein was observed by several groups (Iseki et al., 2000; Payton et al., 2001; Alim et al., 2004). Speculatively, the affinity of interaction between oligomeric α-synuclein and acetylated microtubules may be enhanced by inhibition of microtubule deacetylase SIRT2 or/and HDAC6. We envision that genetic crosses between PD and SIRT2 brain-specific knockout mice will provide valuable clues on neuroprotective mechanism(s) and further report on therapeutic potentials of SIRT2.

SIRT2 in Huntington's disease. Huntington's disease is an autosomal dominant neurodegenerative disorder caused by mutant huntingtin protein containing pathologically extended polyglutamine repeats (Bates, 2003). The disease is characterized by a gradual and progressive loss of neurons, predominantly in the cortex and striatum leading to impairment in muscle coordination, cognitive decline, and dementia. Currently, there is no cure for HD and treatments can only mildly relieve some of its symptoms. Pharmacological inhibition of SIRT2 achieved neuroprotection in cellular and invertebrate models of HD through a negative regulation of sterol biosynthesis (Luthi-Carter et al., 2010). In primary striatal neurons expressing a mutant huntingtin fragment, genetic or pharmacological inhibition of SIRT2 was associated with significant reduction of polyglutamine inclusions. The reduction of inclusion formation, which is believed to be dependent on microtubule transport, may represent a benign biomarker. Another major finding from this study is that this protective effect is intimately related to transcriptional regulation of genes controlling metabolism, including sterol and fatty acid biosynthesis, carbohydrate metabolism, and purine metabolism (Luthi-Carter et al., 2010). More specifically, SIRT2 inhibition reduced sterol levels via decreased nuclear trafficking of sterol regulatory element-binding protein 2 (SREBP-2) and resulted in lower cholesterol levels. Notably, in the same model, manipulation of sterol biosynthesis at the transcriptional level mimicked SIRT2 inhibition, demonstrating that the metabolic effects are sufficient to diminish mutant huntingtin toxicity. Genetic manipulation of SREBP-2 expression levels and/or subcellular localization had no effect on the aggregation state of mutant huntingtin fragments. In follow-up studies, pharmacological inhibition of SIRT2 in wild type primary neurons resulted in SREBP-2 cytoplasmic retention, transcriptional downregulation of cholesterol biosynthetic genes, and reduction of neuronal cholesterol (Taylor et al., 2011). The experiments were extended to Neuro-2a (N2a) neuroblastoma cells and to hippocampal slice cultures from wild type mice, where SIRT2 inhibition-dependent reduction of cholesterol levels was observed as well (Taylor et al., 2011). These results illuminate an emerging novel role of SIRT2 in regulation of neuronal metabolism, and specifically of cholesterol biosynthesis. Accordingly, in a HD fly model, decreased levels of SIRT2 promoted viability of photoreceptor neurons (Pallos et al., 2008).

The fact that the same small molecule is protective in PD and HD, two devastating disorders, is highly promising, suggesting it might also have a beneficial impact on other neurodegenerative diseases.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Despite one decade of research, the precise function of SIRT2 in age-related disorders remains unclear. Therefore, elucidating the cellular function of SIRT2 and the mechanisms underlying its protective and/or pathogenic effects in those disorders is essential for the development of efficient therapies for preventing and treating age-related maladies. The identification of novel SIRT2 substrates will be crucial in pursuing such endeavor. Although a lack of direct evidence supporting the hypothesis that SIRT2 can regulate lifespan in mice exists, it deacetylates a variety of proteins, which play diverse roles in fundamental cellular processes related to the healthy state of the organisms. In this list we have FOXO transcription factors, α-tubulin, PEPCK1, and NF-κB. The effect of SIRT2 seems to vary in a tissue-specific and disease-specific manner, indicating we are still lacking a complete understanding of its mode of action.

Although persuasive, when considering the usage of pharmacological inhibitors/activators of sirtuins as candidates for protection against aging-related maladies, one has to be aware of the apparent opposite effects of SIRT1 and SIRT2 as well as the possible cross talk between other family members. We might not be able to use general inhibitors/activators since they might evoke opposite response from different family members. The development of specific inhibitors/activators for the mammalian sirtuins will enclose the potential to be beneficial to neurodegenerative diseases, cancer, diabetes, and cardiovascular diseases.

Currently unanswered questions in this fast growing field are: (i) How is SIRT2 transcription, stability, and enzymatic activity regulated by pathological stress conditions? (ii) How does SIRT2 communicate with other sirtuins to modulate the pathophysiology of diseases such as cancer, metabolic syndrome, PD, and HD?

(iii) Can SIRT2 regulate lifespan in mice? (iiii) Is SIRT2 together with SIRT1 a mediator of CR?

Regardless of the current lack of answers to these questions, SIRT2 biology grew substantially in the last decade. It will be exciting to see which new pieces to the puzzle will undoubtedly be added in the years to come. This will yield tremendous profits for both basic and clinically applied age-related research. In addition, understanding the molecular mechanisms underlying the protective role of all sirtuins in different organs could bring us closer to the development of novel drug targets, which could be used to design new and more successful therapies for these diseases and even postpone the normal aging process.

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