Emerging Roles of the Sirtuin Family

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Submitted in partial fulfillment of the
requirements for the degree of
Master of Arts
in the Graduate School of Arts and Sciences

Program in Biotechnology
Department of Biological Sciences

COLUMBIA UNIVERSITY

2011

Abstract

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Studies using *Saccharomyces cerevisiae* revealed the existence of a unique family of proteins named Sirtuins. Sirtuins are highly conserved all the way from bacteria to humans. These proteins play important roles inside the cell, as nicotinamide adenine dinucleotide-dependent protein deacetylases. Sirtuins are major regulators of multiple cellular functions; they can modify the epigenetic landscape and are able to alter the proteome of the cell. Several studies have analyzed the role of sirtuins as a link between caloric restriction and aging. However, recent discoveries demonstrate new roles for these proteins. Sirtuins have also caught the attention of the biotechnology industry, and small molecule activators and inhibitors have been developed. The following review describes key aspects of sirtuin biology, presents the current situation of sirtuins in the biotechnology industry, and analyzes the emerging roles of sirtuins in cell metabolism, chromatin regulation, and cancer.

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Acknowledgements

Especial thanks to James J. Russo, Ph.D. for comments and critical reading of this work

Dedication

I dedicate this work to my family and friends, especially…

to Mom,

to Dad,

to David,

to my friends, who shared with me this incredible year in New York.

"In our midst are men and women… who are wholly absorbed in cracking the previously top secret codes governing the chemistry and physics of things alive. They are, moreover, enormously and almost continuously entertained by what they do. Great musicians experience a similar rapture, but only so long as they perform before an audience. Research scientists on quests to their liking are their own audiences. Their performances, which are in their heads, need never stop, not even when they are sleeping."

- Kurt Vonnegut

Introduction

Silencing Information Regulator-2 (SIR2) was identified by analyzing a spontaneous mutation that arose during routine transfers of *Saccharomyces cerevisiae*. The particular feature of this mutant strain was its inability to inhibit the expression of mating type loci HMa and HMα that resulted in sterility, characteristic of MATa/MATα diploid cells. However, the precise role of the protein encoded by the mutated gene remained elusive for several years. Sequence comparison to other known proteins shed some light on one of the roles of SIR2 as an ADP-ribosyltransferase. Further analysis elucidated the role of sirtuins as a new type of histone dependent deacetylases (HDACs class III) with the special characteristic that they use NAD^+ to exert their function. Because of this requirement for NAD, sirtuins act as sensors of the energy state of the cell and regulate several biological processes, such as response to DNA damage, cell survival, cell metabolism, cell-cycle regulation, and senescence.

Much of the work in the sirtuin field started in the lower organism *S. cerevisiae* and then extended to *C. elegans* and *D. melanogaster*. Highly conserved homologs of SIR2 are present in many organisms, ranging from bacteria to humans. In mammals seven sirtuins (SIRT1-SIRT7) have been identified, however only some of them have a well characterized function. All of the sirtuins contain a conserved core domain that comprises the (NAD^+)-binding domain and the catalytic site. However, the amino and carboxyl termini vary among all of the sirtuins, giving them the ability to recognize different substrates. Additionally the seven mammalian sirtuins are located in different cell compartments so each one has specific protein substrates depending on its localization (see Table 1).

Sirtuin	Localization
SIRT1	Nucleus, Cytoplasm
SIRT2	Cytoplasm, Nucleus
SIRT3	Mitochondria, Nucleus
SIRT4	Mitochondria
SIRT5	Mitochondria
SIRT6	Nucleus
SIRT7	Nucleolus

Table 1: Subcellular localization of mammalian sirtuins

Sirtuins became the subject of multiple publications when they were identified as the possible link between caloric restriction (CR) and aging. A lot of the work done in the sirtuin field is related to this association, and several reviews on these topics have been published [1, 2, 3]. However, during the last years sirtuin research has produced very interesting discoveries about the molecular mechanisms that link them to metabolic (*e.g.*, type 2 diabetes) and neurodegenerative disease, with recent publications implicating sirtuins as important players in cancer biology and tumorigenesis. Some sirtuins act as tumor suppressor genes, while others participate as oncogenes in tumor formation. The roles that sirtuins have in cancer include: promotion of the DNA damage repair response, maintenance of genomic stability, regulation of reactive oxygen species (ROS) levels and regulation of telomeric chromatin.

Because of the role of sirtuins in aging and different diseases, they have become promising drug targets. Small molecules that act as inhibitors or activators of sirtuins have been developed, and are currently in clinical trial.

This review presents the major breakthroughs, the ongoing work in the field of sirtuin research, and the emerging roles of these proteins in cell metabolism, chromatin regulation and cancer. In addition the current status of sirtuins as novel drug targets is addressed.

Discovery of sirtuins

SIRtuins (Silencing Information Regulators)

The mating-type locus (MAT) is responsible for determining the cell type (MATa or MATα alleles) in yeast. A complex and well studied process called “mating type switching” or interconversion enables some yeast strains to modify their cell type from MATa to MATα or vice versa. The mating type switching process depends on two additional genes HMLα and HMRa. It consists of copying and inserting the HMLα allele into the MAT locus to replace the MATa gene, thereby converting cells from MATa to MATα or inserting the HMRa allele into the MAT locus to convert cells from MATα to MATa. In their normal location, HMLα and HMRa should always be silent; in this way the cell type is exclusively defined by the expression of the MAT gene. Several mutants (SIR1-SIR4 [4]), with a sterile phenotype (like MATa/MATα diploid cells) were unable to silence the HM loci. One of the mutations was mapped to chromosome 4 and the gene locus was named MAR1 (then called SIR2), the founding member of the sirtuin family. Further studies revealed that SIR2 was responsible for silencing not just the mating-type loci, but also telomeric regions [5]. In addition, SIR2 silences ribosomal RNA genes (rDNA) in yeast [6].

ADP-ribosyl transferases and NAD+-dependent deacetylases

The exact molecular function by which SIR2 silenced different genomic regions (mating-type loci, rDNA, telomeres) remained unknown until the enzymatic activity of a related protein called CobB in *Salmonella typhimurium* was elucidated [7]. CobB catalyzes the reaction of 5'-phosphate transfer from nicotinic acid mononucleotide to a precursor of vitamin B12; this phosphoribosyl-transferase activity was confirmed in homologs of SIR2 [8].

Furthermore, additional work demonstrated that the overexpression of SIR2 in yeast resulted in general histone deacetylation [9]. Based on the previous observations, the breakthrough experiment that identified the precise enzymatic activity catalyzed by sirtuins consisted of incubating diacetylated histone peptides (that corresponded to the N-terminus) with purified SIR2 in the presence of NAD^+ [10]. Analysis of the incubated peptides by mass spectrometry (MS), demonstrated that SIR2 catalyzes an NAD dependent deacetylation reaction [11].

The enzymatic reaction (Figure 1) consists of the hydrolysis of NAD^+ that leads to the production of nicotinamide (NAM) and ADP-ribose; the latter is coupled to the enzyme, generating an enzyme ADP-ribose complex. The acetyl groups on the substrate (histone) react with ADP generating the deacetylated protein and 2'-O-acetyl-ADP-ribose (OAADRr). The use of NAD^+ as a cofactor classified sirtuins as a new type of histone deacetylase (HDACs class III).

To assess the intracellullar localization, a fusion of the human SIRT2 to GFP revealed that this protein resides in the cytoplasm [12]; this and other evidence [7] suggested that histones were not the only substrates of sirtuins. Now we know that sirtuins also

acetylate a wide variety of proteins including: TAFI68 [13], α-tubulin [14], acetylcoenzyme A synthase 2 (AceCS2) [15], glutamate dehydrogenase (GDH) [16], p53 [17], and FOXO transcription factors [18] among others.

From yeast to mammals: sirtuin homologs

Homologs of SIR2 (HSTs, HST1-HST4) were identified in yeast [19]. Mutations in these proteins have similar but not identical effects on silencing, for example hst3 and hst4 double mutants cannot silence telomeric regions. In contrast, overexpression of HST1 restores silencing at the HMR in sir2 cells [19].

Orthologs of SIR2 are present in several organisms (sir-2.1, *C. elegans* [20]; dSIR2, *D. melanogaster* [21]). Multiple sequence alignment revealed a highly conserved core domain, shared by all the SIR2-related proteins, that corresponds to the NAD binding pocket and the catalytic site. In addition, phylogenetic analysis of the sirtuin family identifies five different classes: Class I to IV, and U [22]. The core domain is comprised of 250 amino acids, while the amino and carboxyl termini have variable sequences and lengths. Furthermore, the variable regions can affect their localization [23], and might have an impact on substrate recognition and activity. For example, the mammalian SIRT3 amino-terminus needs to be proteolytically cleaved in order to activate its enzymatic activity [24].

Regulation of sirtuins

NADH/NAD^+ acts as a cofactor in many vital cell reactions, such as the ones that take place in the electron transport chain (ETC). NADH is involved in the generation of ATP,

acting as a carrier of electrons from the tricarboxylic acid (TCA) cycle to the ETC Complex I where it is oxidized to NAD^+, resulting in the translocation of protons to generate the gradient that later on will generate ATP. The ratio between NAD^+ and NADH defines the redox state of the cell, which has an impact on several enzymatic reactions including the activity of sirtuins [25].

Sirtuins are regulated by the [NADH]/[NAD^+] ratio inside the cell. Experiments done by measuring indirectly the [NADH]/[NAD^+] ratio (using lactate and pyruvate levels) demonstrate that transcription of SIRT1 is enhanced when the [NADH]/[NAD^+] ratio increases [25]. Additional experiments, in which nicotinamide phosphoribosyltransferase (Nampt) is overexpressed, resulting in the production of NAD^+, demonstrated that SIRT1 enzymatic activity is enhanced in the presence of high NAD^+ [26]. In contrast nicotinamide, a product of the reactions catalyzed by sirtuins (ADP-ribosylation reaction and deacetylation reaction), acts as an inhibitor [26]. A similar mechanism regulates the activity of poly(ADPribose) polymerase (PARP), described in the following sections.

NADH/NAD^+ levels can be altered by DNA damage and the nutritional status of a cell. Because of this and the fact that NAD^+ is required for their enzymatic activity, sirtuins are cellular sensors that link the energy status inside the cell to their different roles in cell metabolism, cancer, and aging.

Sir2, caloric restriction and aging

Yeast has been used as a model for studying lifespan extension by counting the number of mother cell divisions before senescence. Some studies showed that lifespan in yeast is related to the accumulation of extrachromosomal rDNA circles (ERCs), resulting in a reduction of lifespan. Recombination of the rDNA locus generates these

ERCs. The rDNA locus consists of a tandemly repeated unit of 9.1 kb which contains the 35S rRNA, the 5S rRNA and a nontranscribed spacer (NTS) in between.

A protein involved in the recombination process at the rDNA locus is FOB1. FOB1 mutants present a reduction in rDNA recombination and less ERCs are generated [27]. In contrast SIR2 yeast mutants accumulate ERCs and a reduction of lifespan is observed. Additional experiments confirm that overexpression of SIR2 homologs in other organisms has the same effect in lifespan extension [28]. As expected the interplay between *sir2* and *fob1* regulate ERC generation what results in lifespan alterations.

Separate observations revealed that mice fed with a calorie restricted (CR) diet have increased lifespan. However, the exact mechanism or at least some insights into the causes remained undiscovered. A landmark paper for the sirtuin area was published by Leonard Guarente's group [29]; this publication shed some light on the mechanism by which CR mediates lifespan extension. In this work it was demonstrated that the increase of lifespan induced by caloric restriction was dependent on SIR2. In this way sirtuins were linked to CR and aging.

These two publications [28, 29] opened a new line of research in the field of the biology of aging where sirtuins have a central role. From then on, many research groups began focusing on these molecules and an explosion of experimental data has been published. A large fraction of the current scientific literature comprises the role they might have in aging [1, 2, 3]. However growing evidence is now accumulating, which indicates that sirtuins have other important roles.

Sirtuins in cell metabolism

Cell metabolism involves all the reactions that keep a cell alive; among these reactions vital ones are those that provide energy to the cell. Mitochondria are the organelles where most of the energy producing pathways converge, so a great part of the cell's metabolism depends on them. SIRT3, SIRT4 and SIRT5 are mainly, if not exclusively [30], localized to the mitochondria [23]. In addition, sirtuins are able to sense energy levels ($NADH/NAD^+$), and depend on them to exert their function. Furthermore, a multiplicity of evidence demonstrates that these sirtuins are able to dramatically modify cellular metabolism by activating or inactivating important mitochondrial proteins.

SIRT3 energy metabolism

Just recently the role of acetylation, at the proteomic level, has been analyzed [31], revealing that a large fraction of the proteins that are acetylated are located in the mitochondria. However, the impact that this post-translational modification has on many of these proteins is still unknown. SIRT3 knockout mice exhibit hyperacetylation of mulitple mitochondrial proteins, whereas SIRT4 and SIRT5 knockouts do not [32], suggesting that SIRT3 seems to be the only protein responsible for the deacetylation of multiple mitochondrial targets. Because of its relevance to energy metabolism, the effects of deacetylation of two essential mitochondrial proteins mediated by SIRT3 will be reviewed in this section.

Acetyl-CoA synthetases (AceCSs) are essential enzymes that catalyze the conversion of acetate, CoA, and ATP into acetyl CoA (an important intermediate of fatty acid biosynthesis and the tricarboxylic acid cycle. Mammals have two of them: AceCS1 and AceCS2, and their activity is regulated by acetylation/deacetylation [33]. SIRT1 and SIRT3 are the proteins that control the deacetylation of AceCS1 and AceCS2 respectively [15]. Only the deacetylated form of AceCS2 is active; by this mechanism SIRT3 can regulate the levels of acetyl CoA in the mitochondria.

Another essential target of SIRT3 is a subunit that belongs to Complex I of the electron transport chain (ETC) called NDUFA9 [34]. Immunoprecipitation experiments demonstrate that SIRT3 directly interacts with NDUFA9 and is responsible for its acetylation. Furthermore, this interaction has an effect on the activity of Complex I; incubation of mitochondria with exogenous SIRT3 resulted in a higher activity of Complex I [34].

The regulation, mediated by SIRT3, of the two proteins described above has an impact on the energy levels of the cell and can explain the observed reduction of ATP in $SIRT3^{-/-}$ mouse embryonic fibroblasts [2]. Taking into consideration that Complex I is an NADH dehydrogenase and that SIRT3 depends on NAD^+ to exert its function, a feed-forward loop can be established whereby the oxidation of NADH mediated by Complex I produces NAD^+, which induces SIRT3 enzymatic activity that in turn activates Complex I.

A bigger picture emerges if we consider that SIRT3 promotes the activity of AceCS2 generating acetyl-CoA. Acetyl-CoA acts as a carrier molecule feeding the TCA cycle (the generator of NADH, which in turn is used by Complex I). All these data support the idea that SIRT3 is involved in essential mitochondrial metabolic reactions. However, a central

question of this field is: If SIRT3 has a vital role for the mitochondria, why do SIRT3$^{-/-}$ knock out mice have no altered phenotype under the analyzed conditions [32], despite the reduction of ATP that can be observed in SIRT3$^{-/-}$ tissues?

A possible hypothesis for these observations is that at there exists a mechanism able to compensate for the loss of SIRT3$^{-/-}$ in the knock out mice but acting only at the whole organim level, thereby producing no aparent phenotype. It is also important to consider that the search for a differential phenotype [32] was conducted under basal and mild nutritional stress scenarios, thus it cannot be ruled out that under different conditions an apparent phenotype would be observed.

SIRT4 in insulin secretion and energy metabolism

Incubation of SIRT4 with the core histone proteins (H2A, H2B, H3 and H4) in the presence of ^{32}P-labeled NAD showed that SIRT4 can transfer an ADP-ribose group to H2A [55], whereas no deacetylase activity for this sirtuin has been reported (SIRT4 is the only mammalian sirtuin lacking deacetylase activity). Moreover, it was demonstrated that an important mitochondrial protein, glutamate dehydrogenase (GDH), is a target of SIRT4.

GDH is an enzyme responsible for the conversion of glutamate into α-ketoglutarate (an intermediate of the TCA cycle). GDH can be ADP-ribosylated, and this modification inhibits its catalytic activity [35]. This inhibition has important effects in the physiology of the organism.

GDH favors the production of ATP by feeding the TCA cycle with α-ketoglutarate. The NADH and FADH generated in the TCA cycle are then used by the ETC to produce ATP. An increased amount of ATP induced by amino acids is known to stimulate the secretion of insulin. SIRT4 is expressed in pancreatic β-cells [56], and can regulate insulin secretion by altering the activity of GDH [35]. SIRT4$^{-/-}$ mice present high levels of insulin in response to feeding with glutamine and leucine, due to the unrestricted activity of GDH.

There is evidence that SIRT4 can also regulate insulin secretion in response to glucose by a different mechanism [36]. Mass spectrometric analysis of proteins that co-immunoprecipitate with SIRT4 identified three proteins involved in insulin secretion: insulin-degrading enzyme (IDE) [37], and the subunits ANT2 and ANT3 of the ATP/ADP translocase. However, no further experiments were done to demonstrate whether SIRT4 ADP-ribosylates these proteins, evidence only existing for their interaction. RNAi against SIRT4 in insulin secreting cells enhanced insulin secretion in the presence of glucose. More work is required to define the exact mechanism by which SIRT4 and its targets regulate insulin secretion.

Sirtuins function in chromatin regulation

Removing epigenetic marks

Transcriptional silencing of the mating-type loci and telomeric regions of the yeast genome correlates with hypoacetylation of core histones [9]. In contrast histone acetylation is enriched at promoters of actively transcribed genes. The rationale behind these observations is that acetylation neutralizes the positively charged lysines of histone N-terminal tails, which decreases its affinity for DNA [38], opens up chromatin

and allows the association of transcription factors. Furthermore, acetylation of specific residues is associated with nucleosome assembly resulting in different types of chromatin: euchromatin or heterochromatin (including constitutive heterochromatin (CH) and facultative heterochromatin (FH)).

It is not within the scope of this review to explain all the acetylation and methylation marks on histones, given their dynamism and complexity. However, to describe sirtuins' function in chromatin regulation, some of these marks will be presented. Acetylation of lysine residues (K5, K8, K12, and K16) in histone H4 and the K9 residue of histone H3 (H3K9) is typical of active chromatin; meanwhile hypoacetylation of these amino acids is associated with heterochromatin [39]. Methylation of H3K79 (H3K79me2) marks transcriptionally active regions, while methylation of H3K9 and H4K20 (H3K9me3, H4K20me1) marks repressed chromatin (see Table 2)

Histone marks	**Present in:**
H4K16ac	transcriptionally active chromatin
H3K9ac	transcriptionally active chromatin
H3K79me2	transcriptionally active chromatin
H3K9me3	repressed chromatin
H4K20me1	repressed chromatin

Table 2: Histone marks associated with SIRT1

As expected, nuclear sirtuins (SIRT1 and SIRT6) regulate chromatin structure by deacetylation of histone tails. SIRT1 is the best-studied member of the mammalian sirtuins and the mechanism by which it promotes heterochromatin formation has been

well characterized [40]. RNAi against SIRT1 correlates with: 1) an increase in H4K16 and H3K9 acetylation; 2) increase of H3K79me2, and 3) decrease of H3K9me3 and H4K20me1. Surprisingly SIRT1 also recruits the non-core histone H1, and deacetylates it at the K26 residue; this deacetylation might have the same effect as with the other histones (neutralization of the charges); however experiments to verify this have not been reported. What is known about H1 histone is the fact that it interacts with the linker DNA and favors DNA compaction. Interestingly, H1 has been related to aging suggesting a key relation with SIRT1 [41, 42]. However, the exact mechanism remains to be discovered.

SIRT1 lacks a DNA binding domain, so in order to deacetylate histones it should associate with a larger protein complex. Co-immunoprecipitation experiments for SIRT1 identified the histone methyl-transferase SUV29H1 as an interacting protein, which explains the increase of H3K9me3 associated with SIRT1 activity [43]. SIRT1 interacts with SUV39H1 and activates it by deacetylating lysine 266 (K266), a key residue that mediates the interaction of different protein domains. The interaction between SIRT1 and SUV39H1 is an example of a new mechanism, where a deacetylase is able to regulate the activity of a methyltransferase establishing an interplay between histone modifications, thereby allowing SIRT1 to act as a multifunctional regulator of heterochromatin formation. Like the other sirtuins, SIRT1 activity is dependent on NAD^+, suggesting that SIRT1 can mediate changes in the global transcription program based on the energy state of the cell by altering histone modifications, and activating other protein targets.

Sirtuins in cancer

Role in DNA repair

Alterations in the mechanisms responsible for DNA repair (base excision repair (BER), nucleotide excision repair (NER), nonhomologous end joining (NHEJ), homologous recombination (HR), and double-stranded base (DSB) repair) increase genomic instability. Genomic instability underlies the acquisition of most of the hallmarks of cancer, and refers to defects in genome repair and maintenance that promote tumor progression [44]. An example of a DNA repair mechanism mutated in cancer is the nucleotide-excision repair mechanism (NER). A well studied case is the xeroderma pigmentosum syndrome, where mutations in the proteins involved in NER fail to repair UV-induced mutations, resulting in skin cancer [45].

The sirtuin implicated in genomic stability is SIRT6. Experiments implicate SIRT6 in BER [46] and DSB (double-stranded breaks) repair [47]. $SIRT6^{-/-}$ mouse embryonic fibroblasts (MEFs) present genomic instability, such as chromosomal translocations and chromosome fragmentation. Genomic instability can be caused by alterations in one or several of the DNA repair mechanisms. Treatment of $SIRT6^{-/-}$ MEFs with several mutagens (ionizing radiations (IR), ultraviolet (UV) light, the alkylating agent methyl-methanesulphonate (MMS), and oxidative agents (H_2O_2)) demonstrated that these cells were sensitive to MMS and H_2O_2, suggesting a role for SIRT6 in BER. Separate studies demonstrate that SIRT6 is also involved in DSB repair [47], [48].

The BER pathway is responsible for removing small spontaneous mutations of the genome such as base oxidation, alkylation, depurination and deamination, caused by reactive species and free radical products of cell metabolism. The BER mechanism

consists of removal of the damaged DNA base, which is recognized by a lesion-specific DNA glycosylase and excised with an apurinic/apyrimidic endonuclease; the resulting gap is then filled in by a specialized polymerase called Polβ, and the remaining nicks are sealed with a DNA ligase (DNA ligase III). Expression levels of the proteins involved in the BER pathway were analyzed in $SIRT6^{-/-}$ and no differences were observed in comparison to controls. Additionally, no direct interaction between $SIRT6^{-/-}$ with a BER related protein has been described. Interestingly, Polβ can be inactivated by acetylation. It might be possible that the observed increase in sensitivity to MMS and H_2O_2 of $SIRT6^{-/-}$ is due to the inactivity of the acetylated form of Polβ; however no effect on the acetylation of Polβ was observed in the $SIRT6^{-/-}$ cells.

Another type of DNA lesion where sirtuins are involved is in DSB repair. Ionizing radiation creates DSBs, which consist on two free DNA ends that can recombine with other regions of the genome. DSBs generate genomic instability, with tumorigenic potential, by inducing mutations and chromosomal translocations. In eukaryotes there are conserved mechanisms to repair these lesions. SIRT6 has been implicated in DSB repair. Co-immunoprecipitation experiments identified a direct association of SIRT6 with a protein complex (DNA-PK holoenzyme) involved in the regulation of DNA DSB repair [49]. After exposure to DNA damage agents DNA-PK associates with chromatin at regions flanking the DSBs, the same is true of SIRT6 (it appears in the chromatin bound fraction). In SIRT6 knockdown cells, exposed to DNA damaging agents, 1) the levels of DNA-PK associated with chromatin remained unchanged, 2) H3K9 acetylation slightly increased, and 3) the number of DSBs increased. It is still not known how these three observed effects are related and if the small increase of H3K9 acetylation in SIRT knockdown cells is exclusive to regions flanking DSBs where DNA-PK and SIRT6 are present.

A separate study identified the poly-ADP-ribose polymerase (PARP) as a protein that is mono-ADP-ribosylated by SIRT6 [48]. PARP1 is a protein responsible for poly-ADP-ribosylating nuclear proteins, in response to DNA damage, and promoting DNA repair. The mono-ADP-ribosylation of PARP1 enhances its activity. This is consistent with the observations described above, regarding the increased genomic instability present in SIRT6 knockdown cells [46]. The role of SIRT6 in cancer needs to be assessed by analyzing the levels of SIRT6 in tumors; the results of such experiments will confirm if SIRT6 can be classified as a tumor suppressor gene.

Impact of the regulation of SIRT3 on ROS levels

Otto Warburg back in the 1920s made the following observation: All normal body cells meet their energy needs by oxygen respiration, whereas cancer cells meet their energy needs in great part by fermentation. The origin of this metabolic difference and its implications for cancer cell proliferation is a research area widely studied nowadays. This altered metabolism is known as the ‘Warburg effect’ or aerobic glycolysis. Cancer cells consume higher amounts of glucose than normal cells and have increased lactate production in the presence of oxygen. Proliferating cells incorporate most of the available nutrients into macromolecules. The metabolic features of cancer cells that distinguish them from normal cells can be summarized as follows: high glycolysis in the presence of oxygen and accumulation of building blocks necessary for continuous cell proliferation. This metabolic shift provides tumor cells a growth advantage. SIRT3 has been identified as a regulator of the cell metabolism shift to increased glycolysis in cancer cells, thus acting as a tumor suppressor gene [50].

Analysis of glucose uptake in SIRT3$^{-/-}$ cells revealed higher glucose consumption compared to WT cells. Separate studies identified that SIRT3$^{-/-}$ cells also presented with an increase level of reactive oxygen species (ROS) [51]. Now it is known how these two observations are connected; the role of SIRT3 as a tumor suppressor is accomplished in two different ways: 1) regulating the levels of ROS and 2) inhibiting the cell metabolic switch to glycolysis during low oxygen conditions mediated by HIF-α.

Increased ROS levels are associated with cancer. ROS (superoxide, hydrogen peroxide, hydroxyl radical) act as DNA-damaging agents causing mutations and genomic instability. SIRT3 regulation of ROS levels is mediated by the manganese superoxide dismutase (MnSOD) protein. Superoxide is generated as a byproduct of electron transfer reactions such as the ones that take place in complex I and III during respiration. MnSOD catalyzes the conversion of superoxide to hydrogen peroxide, which is then converted to water by other enzymes: catalase and peroxidases. MnSOD activity is regulated by the reversible acetylation of a lysine residue (lysine 122), which results in a decrease in its enzymatic activity [52]. SIRT3 deacetylates MnSOD, which explains the high ROS levels present in SIRT3$^{-/-}$ cells [53].

HIF-1α is a transcription factor responsible for the upregulation of several genes (such as the glucose transporters and glycolytic enzymes) that enable cell survival during low oxygen conditions. HIF-1α is stabilized under hypoxic conditions by a well-described mechanism that involves the inhibition of HIF prolyl-hydroxylases, however a new mechanism for HIF stabilization, independent of oxygen levels, has been described [50]. The new mechanism works as follows: the high levels of ROS observed in SIRT3$^{-/-}$ stabilize HIF-1α under normal oxygen conditions. As a result of HIF-1α stabilization

glucose uptake and glycolysis is enhanced resulting in a cellular metabolic shift that enables cell proliferation. In this way loss of SIRT3 reprograms cellular metabolism. Furthermore, overexpression of SIRT3 can repress the “Warburg effect” in breast cancer cell lines, confirming its role as a tumor suppressor.

Sirtuins as drug targets

Sirtuin activators

Caloric restriction in yeast mediates lifespan extension by increasing the activity of SIR2 [29]; in other organisms overexpression of SIR2 homologs has the same effect. In the case of mice, when they are fed a calorie restricted diet, they display an extension in lifespan, increase in SIRT1 expression in certain tissues, and a characteristic phenotype that consists of lower levels of cholesterol in the blood, high oxygen consumption and more glucose tolerance. Overexpression of SIRT1 in mice has no effect on lifespan but interestingly mimics some of the beneficial effects induced by caloric restriction [54] like improved glucose homeostasis.

If somehow the activity of sirtuins can be modulated then the effects of caloric restriction can be induced in the organism by these means. Based on this idea libraries of small molecules were used to screen for compounds that can activate SIRT1 (the most well studied mammalian sirtuin). The screening used a fluorescent deacetylation assay in which human SIRT1 was incubated with a synthetic acetylated peptide (which included the K382 residue present in p53, a well known target of SIRT1). The first screen identified several SIRT1 inhibitors and only two activators, both polyphenols

(quercetin and piceatannol). Identification of activators rather than inhibitors represents a bigger challenge. A secondary screen focusing more on polyphenol-like compounds discovered 15 SIRT1 activators, named sirtuin-activating compounds (STACs) [55]. The most potent activator identified was 3,5,4'-trihydroxystilbene (resveratrol) a compound present in red wine. Further experiments in yeast confirmed that resveratrol and two other compounds (butein and fistein) activate SIR2 and increase lifespan.

Experimental data showed that these compounds increased SIRT1 activity by lowering the Km for the acetylated peptide, and for the NAD^+ cofactor, explaining the observed activity enhancement. Assessing the levels of acetylated p53 in human cells treated with a low dose of resveratrol demonstrated that this compound was able to increase SIRT1 activity *in vivo* in one of its targets (p53) [55].

Additional screens help identified three small molecules (SRT1460, SRT1720 and SRT2183) with a structure distinct from resveratrol that activate sirtuins in a similar way by lowering the Km for acetylated peptide substrates only [56]. The next step was to analyze the effect of STACs in model organisms. Because of the effect that overexpression of SIRT1 has on glucose homeostasis the effects of SRT1460, SRT1720 and SRT2183 were tested in type 2 diabetes *in vivo* models [56]. The results indicated that these compounds are 100-fold more potent than resveratrol and have a beneficial effect on glucose homeostasis and insulin resistance. Based on the evidence that sirtuin activity can be modulated, different compounds (new chemical entities, NCEs) have been developed to improve SIRT1 targeting. The company called Sirtris is currently developing these NCEs and some of them are in clinical trials aimed at demonstrating if there are any side effects associated with sirtuin activation. Sirtuins have become

promising drug targets, but there is still a long way to go to understand better their roles and to modulate their activity for the treatment of human diseases.

Future Perspectives

Much of what we know today about sirtuins' effects in the cell have been obtained from experimental data where the particular acetylation state of a sirtuin target is assessed. For nuclear sirtuins a lot is known about their histone targets and some target trancription factors. However, increasing evidence demonstrates that several mitochondrial and cytoplamic proteins are acetylated [31]. Their acetylation state regulates their activity with an effect on multiple cellular processes. Because of this, a systematic analysis, using high-throughput technologies for assessing the acetylation state of the entire proteome in the presence and absence of each particular sirtuin, needs to be done. This type of analysis will help discover all the protein targets of sirtuins, all the pathways regulated by them, and how sirtuins regulate the mitochondrial and cytoplasmic acetylome.

Many studies have focused on SIRT1 and a lot is now known concerning regulation of its expression in different tissues and its multiple targets. But all the currently developed small molecule modulators have as drug target SIRT1. At the same time an analysis of the other mammalian sirtuins should be undertaken. Because all sirtuins are dependent on NAD, it is plausible to think that the entire cellular NAD pool is shared by all those sirtuins that localize to the same cell compartment. Overactivation of one sirtuin can lead to an imbalance in the NAD levels altering the activity of the other sirtuins.

High expectations were raised in the non-scientific community when work demonstrating that resveratrol (a component contained in red wine) was able to activate SIRT1 under the tested conditions, and led to increased lifespan in yeast, worms and flies. However, no beneficial effect on lifespan extension has been observed in SIRT1 transgenic mice treated with resveratrol. The better route is to analyze the role of sirtuins in specific diseases like type 2 diabetes and cancer, and determine if sirtuins can serve as drug targets for their treatment. The main goal of using sirtuins as drug targets should be to control and cure disease, not to extend lifespan.

Since the discovery that sirtuins are mediators of lifespan extension there has been an enormous amount of interest in them. In the past few years thousands of articles have been published on this topic, and growing evidence demonstrates that this family of proteins are key regulators of cellular metabolism, cancer and aging. However, only part of the picture has been discovered. Much more work needs to be done to fully understand their role inside the cell in a global context, and how the activity of these proteins can be modulated in order to use them as drug targets for the treatment of human diseases.

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Figures

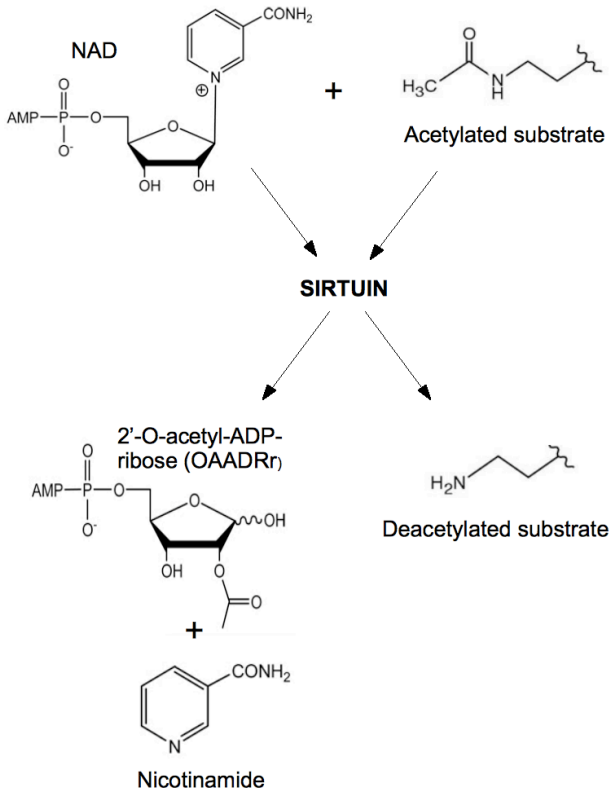

Figure 1. Deacetylation reaction catalyzed by sirtuins