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The conserved role of sirtuins in chromatin regulation

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ABSTRACT The members of the Sir2 family, or Sirtuins, have garnered considerable attention because of their key roles as metabolic sensors and mediators of cell survival under stress. Sirtuins may play roles in myriad human pathologies such as cancer, neurological diseases, malaria, leishmaniasis and hormone-related disorders. They are present from prokaryotes to humans and show a high degree of functional diversification that has led to two different enzymatic activities, a wide range of substrates and a highly diversified pattern of cellular localization. Throughout chromatin evolution, Sirtuins have maintained an intimate functional relationship in regulating its structure and function via their targeting of histones, particularly H4K16Ac, as well as other non-histone chromatin proteins. This link permitted fast communication from metabolic fluctuations to chromatin allowing efficient adaptation to environmental stimuli. Therefore, understanding the common path of Sirtuins and chromatin development over the course of evolution might be important for understanding not only the remarkable diversity of functions of these proteins in mammals, but also the path followed by chromatin evolution. Herein is provided an overview of current knowledge of Sirtuin function, from bacteria to humans, including a discussion on its implications for chromatin dynamics, organization and integrity.

KEY WORDS: *sirtuins, chromatin, SirT1-7, deacetylase, heterochromatin*

The remarkable size of eukaryotic genomes dictates that nuclear DNA be assembled into a compact - yet dynamic - structure that can efficiently store and correctly manage genetic information. Hence, DNA associates with histones into the nucleoproteic structure known as chromatin (Kornberg and Lorch, 1999). However, chromatin structure is not homogeneous; it is organized into a hierarchy of successive levels of compaction that range from the basic unit of chromatin, the nucleosome, to the most compacted form, the metaphase chromosome. Chromatin structure not only determines the physical distribution of the genome, but plays a key role in controlling access to genetic information and, therefore, to gene expression (Kornberg and Lorch, 1999; Wu and Grunstein, 2000). Thus, the first level of chromatin organization, the 11 nm fiber, corresponds to a nucleosome array or *beads on a string*, in which the DNA is compacted seven-fold. This fiber is accessible to the transcriptional machinery and is associated with transcriptionally active regions, which are also known as *active chromatin* or *euchromatin* (Trojer and Reinberg, 2007; Vaquero *et al.*, 2003). In contrast to euchromatin, heterochromatin refers to the regions of the genome that associate to inactive regions, stain differentially with certain dyes, and correspond to a more compacted chromatin structure. The best candidate for heterochromatin structure, supported by numerous *in vitro* studies, is the 30-nm fiber, which corresponds to a considerably more compacted (42-fold) DNA structure that is refractory to transcription. However, the 30nm fiber, has still not been clearly detected *in vivo* (Tremethick, 2007; Trojer and Reinberg, 2007).

The transition between these two levels of chromatin organization is vital to the control of transcription, replication and many other cellular functions associated with DNA.

One of the major findings of the last decade is the fact that histones participate in virtually every aspect of chromatin regulation. A major mechanism by which histones mediate this regulation is the modification of their N-terminal domains, or *histone tails*, which are accessible, unstructured domains that protrude from nucleosomes. Certain residues in histone tails undergo specific post-translational modifications such as acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination,

Abbreviations used in this paper: HDAC, histone deacetylase; HMT, histone methyltransferase; MEF, mouse embryonic fibroblast; Sir, silencing information regulator.

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sumoylation, and biotinylation (Vaquero *et al.*, 2003). Among these modifications, the acetylation and methylation of lysine residues in histones H3 and H4, are critical to the regulation of chromatin structure and gene expression (Shahbazian and Grunstein, 2007). The acetylation of lysine 16 in histone H4 (H4K16Ac) stands out for its involvement in epigenetic phenomena seen throughout chromatin evolution as well as for its unique role in chromatin structure, gene expression, DNA repair and recombination (Vaquero *et al.*, 2007b). These post-translational modifications are partly epigenetic, meaning that organisms can pass them on to their progeny. Alteration of epigenetic processes has remarkable consequences for numerous human pathologies, including cancer (Esteller, 2007; Ting *et al.*, 2006).

Histone marks have provided important information on the defining features of euchromatin and heterochromatin as well as on the factors involved in the transition between these two forms of chromatin (Vaquero *et al.*, 2003). Overall, euchromatic active regions correlate with lysine acetylation and certain combinations of methylation in both lysine and arginine. These modifications provide regulatory factors with access to promoter regions and allow efficient transcription by RNA polymerase II (Eissenberg and Shilatifard, 2006). These marks are H4K16Ac, H3K4me3, H3K36me3, H3K79me2 and 3, and H4R3me2. In contrast, compacted heterochromatin regions are generally hypoacetylated and methylated in a discrete combination of lysine methylated marks such as H3K9me2 and 3, H4K20me1 and 3, and H3K27me3 (Trojer and Reinberg, 2007). In particular, methylation of lysine 9 in H3 (H3K9me2 and 3) is a hallmark of global chromatin organization, and its recognition by specific structural proteins such as HP1 is required for heterochromatin assembly and spreading (Lachner and Jenuwein, 2002).

Histone deacetylases (HDACs) are part of a group of proteins that regulate acetylation marks. These enzymes are responsible for removing the acetyl group from ε-lysine residues and, consequently, are involved in gene repression and heterochromatin formation. This role also makes HDACs key players in epigenetic regulation and management of specific spatial-temporal programs of expression, such as development and cellular differentiation (Ahringer, 2000; Margueron *et al.*, 2005; Vaquero *et al.*, 2003; Verdin *et al.*, 2003). Moreover, HDACs have important roles in DNA repair, DNA replication, cell cycle control, apoptosis and other functions (de Ruijter *et al.*, 2003; Kurdistani and Grunstein, 2003). Interestingly, HDACs are not restricted to histone deacetylation, but rather have a broad range of non-histone targets. Acetylation/deacetylation of non-histone proteins has emerged as a more general mechanism for modulating cellular protein functions than previously anticipated. Currently available data suggest three main levels of regulation: DNA-binding, protein stability and enzymatic activity (Glozak *et al.*, 2005).

HDACs have been divided into four phylogenetic groups (Class I to IV) (Yang and Seto, 2008). Classes I, II and IV are highly related from a structural and mechanistic point of view, whereas Class III comprises the members of the Sir2 family, a group of proteins with unique features crucial for cell fitness, adaptation to environmental stimuli and genomic integrity.

The Sir2 family

The Sir2 family is defined by its homology to the budding yeast silencing factor Sir2p (Silencing Information Regulator 2), an NAD^+-dependent histone deacetylase (Imai *et al.*, 2000; Landry *et al.*, 2000) involved in processes that include epigenetic silencing, DNA repair, replication, recombination and cell cycle control (Saunders and Verdin, 2007; Vaquero *et al.*, 2007b). The degree of conservation among Sir2 family members is restricted to the catalytic domain, a region of approximately 250 residues (Frye, 1999) that contains two well-defined structural motifs: an NAD^+-binding, reverse Rossman fold domain and a small zinc ribbon that provides structural stability (Finnin *et al.*, 2001; Min *et al.*, 2001).

Sir2 family members are characterized by their dependence on NAD^+ for conducting enzymatic reactions (Imai *et al.*, 2000). The dinucleotide NAD^+ is a metabolic cofactor required for electron transfer in intermediary metabolism together with its reduced form, NADH. In the catalytic mechanism mediated by these enzymes, NAD^+ is catabolized into nicotinamide and ADP-ribose in the presence of an acetylated target (Sauve and Schramm, 2003). In contrast to the other classes of HDACs, Class III proteins do not release acetyl groups into solution as acetate. Instead, the acetyl groups are transferred to ADP-ribose, releasing O-acetyl-ADP-ribose as final product (Sauve *et al.*, 2001).

The fact that sirtuins require NAD^+ is fundamental to understanding most of their functions, since it suggests that they can act as sensors of a cell's metabolic state (Fulco *et al.*, 2003; Haigis and Guarente, 2006). Hence, sirtuins are able to relay changes in metabolism to chromatin through deacetylation of histones and other proteins. As we will see, throughout evolution, there have been two primary functions associated with sirtuins: metabolism and chromatin regulation. A third role, stress response, is widely found in eukaryotes, but not to the extent of the former two, suggesting that it was acquired later in evolution.

The Sir2 family catalytic mechanism not only conducts deacetylation, but can also have mono ADP-ribosyltransferase activity (Tanny *et al.*, 1999), which is present in almost all sirtuins tested to date—although in many, this is only residual (Saunders and Verdin, 2007; Yamamoto *et al.*, 2007). Although Sirtuins were originally ribosyltransferases, the fact that they perform protein deacetylation in archaea as well as in bacteria suggests that they probably acquired this function relatively early in evolution (Starai *et al.*, 2002; Tsang and Escalante-Semerena, 1998). The discovery of HDAC activity in Sir2 family proteins led to their immediate classification as HDAC Class III. However, not all sirtuins show detectable deacetylase activity (North *et al.*, 2005). In some cases, they only exhibit mono-ADP ribosyltransferase activity. Therefore, the scientific community might want to reconsider the definition of Class III HDACs. Nonetheless, sirtuins lacking known deacetylase activity might actually be true deacetylases of unidentified substrates.

As mentioned before, the Sir2 family is much older than histones and chromatin. In fact, its members are found in all three life domains (eubacteria, archaea and eukaryotes) (Frye, 2000) and findings suggest that the original role of sirtuins was to regulate metabolism via key enzymes such as acetyl-CoA synthetase (Starai *et al.*, 2002). However, the functional link between sirtuins and chromatin regulation is particularly striking for various reasons. First, sirtuins have been involved in chromatin regulation throughout evolution from the first examples of chromatin-like organization of DNA in archaea (Bell *et al.*, 2002) to the more complex and developed mammalian genome (Kuzmichev *et al.*,

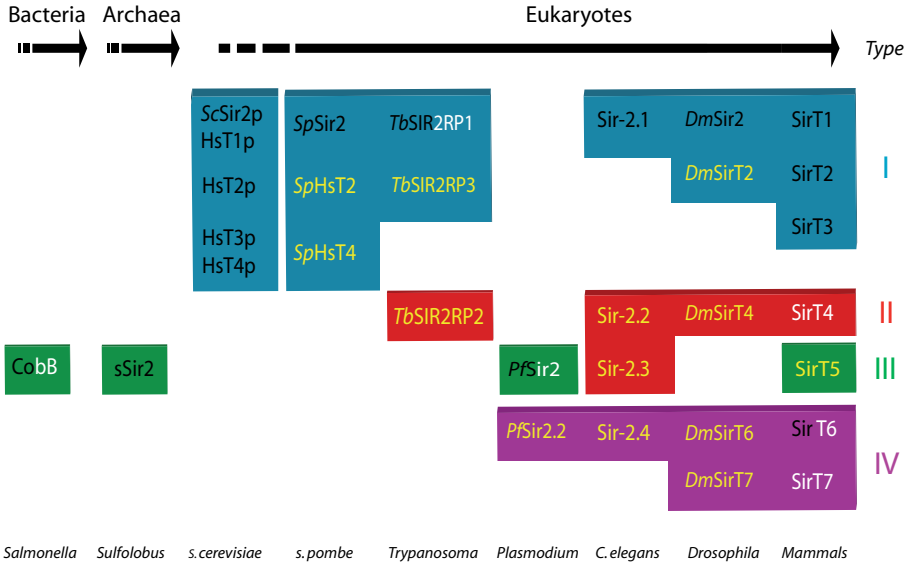

Fig. 1. Classification of sirtuins. *List of sirtuins from selected organisms from bacteria to humans classified phylogenetically (Type) according to Frye (2000). The denomination "Type" here and elsewhere in the text has been used instead of "Class", to avoid confusion with HDAC classification (HDAC Class I to III). The font color used for the sirtuins indicates current knowledge on their enzymatic activity: black for those with known deacetylase activity; white for those whose main activity is ADP-ribosylation; and black and white for both. The sirtuins shown in yellow have not yet been characterized; hence, no information on them is available.*

2004; Vaquero *et al.*, 2007a, Vaquero *et al.*, 2004; Vaquero *et al.*, 2006). Secondly, eukaryotic sirtuin activity is closely related to the regulation of a particular mark, H4K16Ac and, to a lesser extent, H3K9Ac (Vaquero *et al.*, 2007b). Interestingly, these are the only two residues with a well-defined role in chromatin structure regulation (Shahbazian and Grunstein, 2007), which suggests that sirtuin specificity has closely evolved with the control of chromatin organization. Thirdly, sirtuin activity not only targets histones, but also various other chromatin-related factors such as chromatin modifying enzymes, structural proteins, and transcription factors (Saunders and Verdin, 2007; Shahbazian and Grunstein, 2007). Lastly, although the sirtuins of higher organisms are involved in diverse functions implying myriad targets, many of these roles are executed through gene expression, and therefore, through chromatin regulation.

Interestingly, sirtuin catalytic activity—in contrast to that of other HDACs—is tightly regulated by different mechanisms. One mechanism, found from yeast to man, is the non-competitive effect of excess free nicotinamide, which is released upon NAD^+ catabolism (Bitterman *et al.*, 2002). This inhibition could represent a way of limiting sirtuin activity and thereby avoiding NAD^+ depletion, which can prove lethal in certain conditions, as was shown for Poly-ribosyltransferase 1 (PARP1) (Pieper *et al.*, 2000). A second mechanism involves the metabolic intermediary nicotinamide riboside, a NAD^+ precursor in the yeast salvage pathway that enhances Sir2 activity *in vivo* (Belenky *et al.,* 2007). A third mechanism through which sirtuins are regulated involves O-acetyl-ADP ribose, the main product of Sir2 activity. In the case of Sir3p, O-acetyl-ADP ribose binding has been shown to cause a conformational change that increases its binding to *Sc*Sir2p and promotes loading onto chromatin (Liou *et al.*, 2005). However, the way in which O-acetyl-ADP ribose regulates the other mammalian sirtuins is unclear. The only available clue is that this molecule can bind to the histone macroH2A (mH2A), an H2A variant involved in certain forms of heterochromatin (Kustatscher *et al.*, 2005). Although this might suggest downstream signaling of sirtuin action, the effect of O-acetyl-ADP ribose on mH2A is unknown.

Metabolites are not the only type of molecule that can modulate sirtuin activity. Recent studies have identified several proteins that can enhance or inhibit the activity of certain sirtuins such as mammalian SirT1 (Kim *et al.*, 2007; Kim *et al.*, 2008; Zhao *et al.*, 2008). However, the conditions in which these modulators act remain unknown.

An interesting consequence of sirtuin involvement in metabolic signaling is a possible link to lifespan control. This has been demonstrated in organisms including yeast, *C. elegans* and *Drosophila* (Longo and Kennedy, 2006; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). Given that caloric restriction, which is associated with lifespan increase, induces sirtuin activity and expression, sirtuins have been speculated to be mediators of caloric restriction effects on longevity, perhaps through enhancing cell viability. This has only been demonstrated in yeast and *Drosophila* (Longo and Kennedy, 2006; Rogina *et al.*, 2002), although data suggest that it might also the case in mammals (Boily *et al.*, 2008).

The number of sirtuins varies among different organisms and generally correlates with greater complexity. For instance, prokaryotes have one to two sirtuins, yeast has three to five, *Drosophila* has five and mammals have seven (Fig. 1). However, plants are an exception to this rule: *Arabidopsis thaliana* and rice only contain two sirtuins (Frye, 2000).

Sirtuins have been phylogenetically divided into five types: I to IV, and U (Frye, 2000) (Fig. 1). Type I members are clearly linked to chromatin regulation—although not exclusively—and show true histone deacetylase activity. Type I sirtuins include all yeast sirtuins, human SirT1-3, and others. Type II comprises sirtuins of prokaryote origin that seem to be involved in metabolic control. These sirtuins seem to have a primary ADP-ribosyltransferase activity and are mainly located in the mitochondria. Type III is another prokaryote-related group of sirtuins; in fact, it includes the majority of eubacterial and archeal Sirtuins. The eukaryotic members are, like Type II sirtuins, located in the mitochondria. Type IV contains eukaryotic sirtuins that also appear to be functionally related to chromatin, such as mammalian SirT6 and SirT7. Although previous findings suggested that Type IV sirtuins were only ADP-ribosyltransferases, the very recent discovery of H3K9Ac-specific deacetylase activity for SirT6 in telomeres has changed the functional view of Class IV, and suggests that both activities coexist in this family (Michishita *et al.*, 2008). Finally, Type U contains other uncharacterized prokaryotic sirtuins. Strikingly, the most common combination of sirtuins present through evolution is of Types II and IV. Further studies should help

elucidate the features of each type and determine how the different evolutionary lines developed.

Prokaryotes

Although sirtuins are widely distributed, they are not present in all prokaryotes. Current knowledge of bacterial Sir2-family members and their functions is quite limited. Sirtuins from Gram-negative bacteria are phylogenetically related to archaea members and have been shown to possess both ADP-ribosyltransferase and deacetylase activity (Starai *et al.*, 2002; Tsang and Escalante-Semerena, 1998). In contrast, Gram-positive sirtuins are completely uncharacterized. The *Salmonella enterica* sirtuin encoded by *CobB* is involved in the regulation of acetyl-CoA synthetase (Acs), the enzyme that generates acetyl-CoenzymeA (Ac-CoA) from acetate, ATP and CoenzymeA (CoA) (Starai *et al.*, 2002). Deacetylation of Acs lysine 609 by CobB results in a two-fold increase in Acs activity and allows Ac-CoA generation under growth conditions in which the carbon and energy sources are short-chain fatty acids (*e.g.* propionate and acetate). Additionally, CobB can compensate for the loss of CobT, a phosphoribosyltransferase involved in cobalamin (vitamin B12) synthesis (Tsang and Escalante-Semerena, 1998). These findings suggest that both activities have coexisted since the early development of the sirtuin family.

Archaea provide a complex and unique view of the early stages of chromatin evolution. Firstly, archaeal chromosomes contain two widely distributed groups of chromatin proteins: histones and Alba (Sandman and Reeve, 2005). Some members of the Euryarchaeota and Nanoarchaeota phyla contain histones that are packed into nucleosomes comprised of 80 bp of DNA wrapped around a histone octamer (Sandman and Reeve, 2005). These histones contain the characteristic *histone fold* of eukaryotic histones and can form chromatin fibers *in vitro* similarly to eukaryotic histones, but they do not have exposed N-terminal tails nor do they undergo post-translational modifications (Luijsterburg *et al.,* 2008). Alba, on the other hand, is more ubiquitous than histones: it is found in all archaea, with the exception of some Euryarchaeota (Sandman and Reeve, 2005). Some archaea contain both types of chromatin proteins, whereas others contain only one type, reflecting the evolution of different strategies to efficiently organize DNA. Interestingly, eukaryotes contain both histone and Alba proteins. However, eukaryotic histones evolved to acquire histone N-terminals that undergo a wide range of post-translational modifications, whereas eukaryotic Alba relatives acquired a more specialized RNA-related role (Bernander, 2003)

Because Alba has been studied almost exclusively in *Sulfolobus*, which is a member of the Crenarchaea phylum, and therefore lacks histones, the degree of interplay between Alba and histones, and the possible implications of this interaction on DNA organization in other archaea, are poorly understood. Alba is a protein dimer formed by 10 kDa subunits (Zhao *et al.*, 2003). It nonspecifically binds to double-stranded DNA and is uniformly distributed across the genome. Consistent with a role as general chromatin structure component, Alba binds to DNA with a stoichiometry of 5-10 bp per protein dimer, creating a chromatin-like structure (Zhao *et al.*, 2003). Like eukaryotic histones, Alba has an N-terminal domain that is acetylated at lysine 16, which negatively affects Alba's binding affinity for DNA (Wardleworth *et al.*, 2002). Interestingly, *Sulfolobus* Sir2 has been shown to increase Alba binding to DNA through deacetylation of K16, which results in the formation of a compact, chromatin-like structure that is refractory to transcription (Bell *et al.*, 2002; Wardleworth *et al.*, 2002). This is a remarkable finding, since it involves one of the earliest known stages of chromatin evolution. The functional involvement of Sir2 suggests not only that sirtuins have been involved in chromatin structure regulation from the very beginning, but also that Sir2 might be among the factors that have shaped chromatin evolution.

Lower eukaryotes

The transition from prokaryotes to eukaryotes was brought about by a revolution in many aspects, including the creation of the nuclear membrane, the development of new organelles and the organization of DNA into true chromatin. It is in lower eukaryotes that specialized chromatin machinery capable of efficient packing is first observed. This allowed the increase in genome size and complexity necessary for evolution of multicellular organisms, cell type differentiation and development.

There is actually a considerable amount of data on the role of sirtuins in lower eukaryotes. This has mainly come from genetic studies on the budding yeast *Saccharomyces cerevisiae*, in which the first sirtuin (*Sc*Sir2p) was identified (Rine and Herskowitz, 1987; Rine *et al.*, 1979). Studies in lower eukaryotes generally implicate sirtuins in chromatin structure regulation. In fact, nuclear sirtuins have two conserved cellular localizations: telomeres and the nucleolus (Guarente, 2000). In both cases, sirtuins participate in heterochromatin formation. In the first, they play an important role in genome structure and gene silencing, whereas in the second, they mediate silencing of rDNA expression, cell cycle control, recombination and other processes (Gartenberg, 2000).

Yeast

Studies in the budding yeast, *Saccharomyces cerevisiae*, have been key to understanding chromatin regulation in higher organisms. This is partly due to the fact that *S. cerevisiae* is an atypical eukaryote in terms of chromatin structure. Chromatin in *S. cerevisiae* does not compact in the same way as it does in other eukaryotes; instead, it is generally found in a euchromatic, or lightly packed, conformational state that is more amenable to transcription. In fact, only three genomic locations in *S. cerevisiae* undergo chromatin compaction: telomeres, mating-type loci and nucleolar rDNA genes (Gartenberg, 2000). But even these heterochromatic structures are significantly different from those of higher organisms because they lack important hallmarks of chromatin compaction such as H3K9 methylation and heterochromatin-specific structural proteins (Buck *et al.*, 2004). In fact, heterochromatin formation in *S. cerevisiae* is based mainly on histone hypoacetylation, which, although capable of producing a certain degree of compaction in the chromatin fiber, cannot do so to the extent seen in the heterochromatin of higher eukaryotes (Kurdistani *et al.*, 2004; Robyr *et al.*, 2002). Consequently, *S. cerevisiae's* particular type of chromatin makes histone deacetylases major players of chromatin organization in yeast. This is a rather surprising finding considering that, after histone deacetylation, H3K9 methylation is the most conserved feature of silenced chromatin in eukaryotes (Klose *et al.*, 2007; Krauss, 2008; Schotta

et al., 2002). Given that this modification is already present in members of the kingdom Amoebozoa, H3K9 methylation probably developed in the early stages of eukaryotic evolution. Comparisons with *Neurospora* and *Schizosaccharomyces*, which are close relatives of *Saccharomyces* that conduct H3K9 methylation, suggest that, whereas their shared ancestor probably had this activity, *Saccharomyces* lost it as it evolved a more compact genome (Krauss, 2008).

The establishment of heterochromatic loci in *Saccharomyces* depends on the activity of the first described sirtuin, *Sc*Sir2p. The four *SIR* genes, *SIR1* to *4*, were identified as mutations that allowed the abnormal expression of the normally silent mating-type genes (Rine *et al.*, 1979). Their gene products are involved in the epigenetic silencing of the mating-type loci through binding to the silencer regions of the *HMR* and *HML* loci, located in the different arms of chromosome three. Sir1p is responsible for establishing gene silencing via its interaction with ORC1, a component of the origin of replication complex (ORC) and transcription factors such as Rap1p and Abf-1 in the *HMR/HML* silencer region (Fig. 2A). The process of mating-type loci silencing involves the arrival of Sir1p, followed by the recruitment of Sir3p and the complex Sir4p-*Sc*Sir2p (Moazed *et al.*, 1997). Both Sir3p and Sir4p bind chromatin through direct interaction with histones H3 and H4. The recruitment of *Sc*Sir2p (as a Sir4p-*Sc*Sir2p complex), results in deacetylation of H3 and H4 tails, and particularly H4K16Ac. Once loaded, the heterochromatin structure spreads via binding of Sir3p and Sir4p-*Sc*Sir2p to the adjacent hypoacetylated H3 and H4, which in turn recruits and causes further deacetylation (Fig. 2A) (Gartenberg, 2000). Moreover, H4K16 hypoacetylation is recognized as a signature of *Sc*Sir2p-silenced regions (Robyr *et al.*, 2002). Conversely, it is known that the Sir complex spreading is inhibited by H4K16 acetylation and that boundary regions in silenced heterochromatin have hyperacetylated H4K16 (Ekwall, 2005; Kimura *et al.*, 2002; Suka *et al.*, 2002). It has actually been shown that H4K16Ac HAT (histone acetyltransferase) Sas2p creates gradients of H4K16Ac that control *Sc*Sir2p-silencing extension (Kimura *et al.*, 2002; Suka *et al.*, 2002).

Although *Sc*Sir2p is involved in the silencing of telomeres, mating-type loci and nucleolar rDNA genes, the Sir proteins that act on these regions of heterochromatin are not always the same (Fig. 2 A-C). For example, whereas telomere silencing involves Sir3p and Sir4p but not Sir1p, rDNA silencing does not require any of these (Grunstein, 1997) (Fig. 2C). *Sc*Sir2p-induced telomere silencing (Fig. 2B) can extend into a chromosome anywhere from 2 kb to 19 kb and participate in position effect variegation (PEV) (Aparicio *et al.*, 1991; Lieb *et al.*, 2001). Sir4p is responsible for establishing the extent of *Sc*Sir2p-induced telomere silencing by binding to Rap1p and the DNA repair protein Ku70p. Upon arrival of theSir4p-*Sc*Sir2p complex, Sir3p is also recruited, initiating

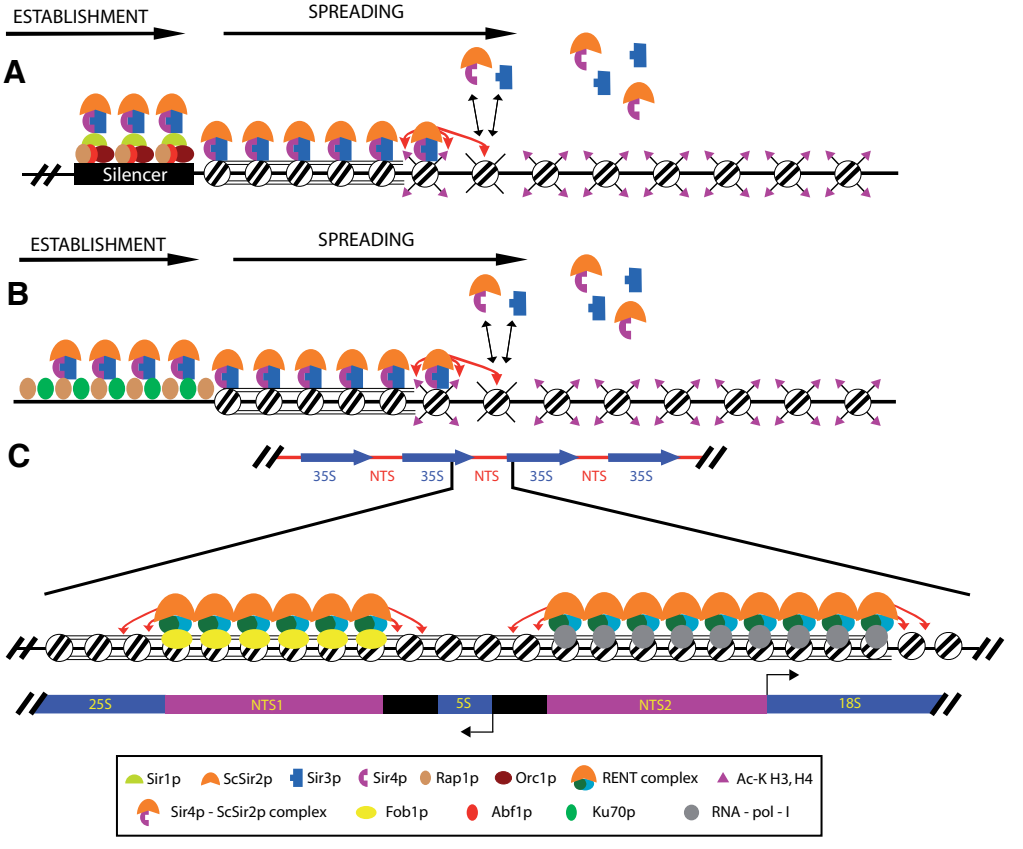

Fig. 2. Model of Sir2-dependent Heterochromatin formation in S. cerevisiae. (A) *Mating-type loci: Sir1 participates in the establishment of the heterochomatin structure during S-phase through binding to silencer-bound proteins such as ORC1p, Rap1 and Abf1p and recruitment of the rest of the SIR machinery. Different models have been proposed to describe this process but it is commonly accepted that Sir4p (present in vivo as Sir4-ScSir2p complex) is probably responsible for binding to Sir1p, which in turn is believed to bring Sir3p to chromatin, resulting in hypoacetylation of H3 and H4 as well as silencing. Once the structure is established, it can spread without Sir1p.* **(B)** *Telomeres: The SIR complex is formed in the telomeric regions, through binding to Rap1. Sir3p and Sir4p bind to Rap1p independently and recruit ScSir2p. The complex spreads similarly as in the mating-type loci.* **(C)** *Nucleolar silencing of rDNA. Each of the 100-200 repeats distributed in tandem is around 9.1kb and composed of two regions: 35S (blue arrows), formed by 18S and 25S, and NTS (Non transcribed spacer). The latter consists of a 5S gene and two flanking regions named NTS1 and NTS2. Transcription of each repeat renders a RNA pol-I dependent 35S precursor rRNA and a RNA-pol III –dependent 5S rRNA precursor. The RENT complex binds to two regions, NTS1 and NTS2. In both cases, RENT recruitment to chromatin requires binding to specific factors, fob1p in the case of NTS1, and RNA-polymerase Ip (RNA-pol-I) in the case of NTS2. Binding of RENT to NTS2 spreads into the coding region of 18S (Huang and Moazed, 2003). Based on Huang and Moazed, 2003.*

heterochromatic spreading through a mechanism very similar to the mating-type loci (Fig. 2B). Interestingly, *Sc*Sir2p and Sir3p have been found to relocalize from telomeres to non-homologous end-joining (NHEJ) DNA repair sites in double-strand break (DSB) events, suggesting that they have a role in these processes (Mills *et al.*, 1999). However, it is unknown whether these sirtuins directly participate in DNA repair.

The third example of heterochromatin in *S. cerevisiae*, the rDNA loci, is located on chromosome 12 and comprises 100 to 200 tandem copies of rDNA genes. In this locus, *Sc*Sir2p induces epigenetic silencing in the context of a complex called RENT (regulator of nucleolar silencing and telophase exit), where it associates with Net1p and the phosphatase Cdc14p. Net1p interacts directly with *Sc*Sir2p and is responsible for keeping *Sc*Sir2p in the nucleolus (Fig. 2C) (Straight *et al.*, 1999). It binds to DNA polymerase I (Pol I) and seems to be directly involved in regulating the nucleolus structure (Shou *et al.*, 2001). In contrast, Cdc14p is involved in mitosis control, particularly during anaphase exit (Hogan and Koshland, 1992). In non-mitotic stages, Cdc14p is sequestered in the nucleolus by the RENT complex and released in early anaphase by phosphorylation (Shou *et al.*, 1999). Interestingly, *Sc*Sir2p is also linked to meiosis in two ways: firstly, through Cdc14p, which is also involved in meiosis-I exit and control of the sequential meiotic program (Marston *et al.*, 2003); and secondly, as revealed by genetic studies, through the meiosis checkpoint protein Pch2p, with which it is involved in meiotic regulation—specifically, in the pachytene phase of meiosis I (San-Segundo and Roeder, 1999). Pch2p is tethered to the nucleolus through a *Sc*Sir2p-dependent mechanism, but whether this effect is due to direct interaction between the two factors is unknown.

An outcome of *Sc*Sir2p nucleolar silencing is its inhibitory effect on recombination events that occur within rDNA genes. This has linked *Sc*Sir2p to the replicative lifespan increase observed in yeast, since rDNA recombination produces extrachromosomal rDNA circles (ERC) whose accumulation is toxic and seems to be associated with ageing in this organism (Gottlieb and Esposito, 1989; Sinclair *et al.*, 1998).

In addition to *SIR2*, *S. cerevisiae* contains four other sirtuins, named *HST* (homologs of Sir-two) 1 through 4. Interestingly, all of these are Class I sirtuins, like *Sc*Sir2p, suggesting that their functions might be related to chromatin and may include histone deacetylase activity (Fig. 1).

Hst1p is the closest homolog of *Sc*Sir2p and is product of a relatively recent gene duplication event, since close relatives of *S. cerevisiae* such as *Kluyveromyces lactis* or *S. pombe* only contain one gene (Hickman and Rusche, 2007). Hst1p is located in the nucleus like *Sc*Sir2p and is involved in silencing middle sporulation genes. Hst1p gets recruited to its target genes by the transcription factor Sum1p and induces repression by deacetylating H3 and H4 tails (Xie *et al.*, 1999). Hst1p also participates in the regulation of some genes of the kynureine pathway, which is involved in the *de novo* formation of NAD^+(Bedalov *et al.*, 2003; Robert *et al.*, 2004). Interestingly, Sum1p is actually responsible for recruiting Hst1p to virtually all its target genes; indeed, deletion of *SUM1* impairs Hst1p localization in ChIP-on-chip experiments. Additionally, Hst1p has been found in SET3C, a complex which participates in meiotic repression and sporulation together with other factors, among which is the HDAC Hos2p (Pijnappel *et al.*, 2001). However, given the ChIP-on-chip results described above, and the fact that Hst1p is not responsible for the activity of the complex, the implications of this finding are unknown.

Studies have shown that Hst1p function can rescue some of the silencing defects in *SIR2Δ* strains, whereas *Sc*Sir2p still retains the capacity of interacting with Sum1p and rescues most of *HST1Δ* defects in sporulation (Hickman and Rusche, 2007). This finding suggests that the capacity to bind to Sum1p existed in the original *SIR2/HST1* encoded protein, whereas the SIR-mediated heterochromatin developed after the duplication event. This conclusion is supported by the lack of the rest of SIR proteins outside of *S. cerevisiae*.

Hst2p is located in the cytoplasm, although it seems to be present in the nucleus in small levels. This is caused by an active nuclear export mechanism that has been recently described (Wilson *et al.*, 2006). Hst2p overexpression is known to be able to affect *Sc*Sir2p silencing: it increases rDNA repression while derepressing *Sc*Sir2p-dependent telomeric silencing (Perrod *et al.*, 2001). These genetic observations might be explained by recent evidence suggesting that Hst2p is involved in nuclear epigenetic silencing in certain cases. Hst2p and Hst1p participate in silencing of the subtelomeric flocculation gene *FLO10*, which encodes a cell-wall glycoprotein, important for cell-surface and cell-cell adhesion (Halme *et al.*, 2004). Moreover, ChIP-on-chip studies in *S. pombe* have shown that *Sp*Hst2, the Hst2p homolog, is involved in rDNA and centromeric silencing, as well as in gene repression of a specific subset of genes (Durand-Dubief *et al.*, 2007). However, the exact role for Hst2p in both cases is currently unknown.

Like its mammalian homolog SirT2, Hst2p has histone deacetylase activity specific for H4K16Ac and, to a lesser extent, H3K9Ac (Vaquero *et al.*, 2006). In contrast to ScSir2p or Hst1p, loss of Hst2p in yeast produces global hyperacetylation of H4K16Ac. These observations might suggest a role for Hst2p in control of global cell cycle-dependent H4K16Ac levels. This hypothesis is reinforced by the fact that SirT2 has been shown to localize to chromatin during the G_2/M transition and deacetylate H4K16Ac prior to cell cycle entry into mitosis (Vaquero *et al.*, 2006). However, it is not known whether Hst2p in yeast is responsible for the drop in H4K16Ac prior to mitosis analogously to SirT2 in mammals. Another facet of Hst2p function that remains unknown is its reported involvement in lifespan increase through an uncharacterized *Sc*Sir2p-independent pathway (Lamming *et al.*, 2005).

Hst3p and Hst4p are involved in the deacetylation of H3K56Ac, a residue present in the globular domain of histone H3, which occurs outside of S-phase (Celic *et al.*, 2006; Maas *et al.*, 2006). Global levels of H3K56Ac depend on S-phase progression and seem to be associated with a transient stage of histone H3 deposition. This modification is part of the replication-dependent DNA damage response mediated by the Mec1p-dependent pathway. In fact, Hst3p and Hst4p are cell-cycle regulated and are responsible for keeping H3K56Ac levels low through cell cycle except during replication (Celic *et al.*, 2006; Maas *et al.*, 2006). However, upon DNA damage, their expression is downregulated, rendering H356Ac hyperacetylation, a mechanism that seems to be key for DNA damage response and survival under these conditions (Masumoto *et al.*, 2005). Additionally, recent studies suggest that *Sc*Sir2p itself can deacetylate H3K56Ac, and in

doing so, somehow enforces heterochromatin formation (Xu *et al*., 2007).

Chromatin organization and regulation in the fission yeast *Schizosaccharomyces pombe* is very different from that of *S. cerevisiae*. Firstly, the chromatin in *S. pombe* is organized much more similarly to that of higher eukaryotes. Specifically, it conducts H3K9 methylation and has a heterochromatin specific protein, Swi6p, which is a homolog of metazoan HP1 (Grewal and Jia, 2007). Unlike *S. cerevisiae*, which encodes five sirtuins (*Sc*Sir2p, Hst1p to Hst4p), *S. pombe* only has three (*Sp*Sir2, *Sp*Hst2 and *Sp*Hst4) (Fig. 1) (Freeman-Cook *et al*., 2005). *Sp*Sir2, like *Sc*Sir2p, is not essential for viability and localizes to the nucleus. ChIP-on-chip assays show that *Sp*Sir2 is involved in silencing heterochromatin in telomeres, mating-type loci, and centromeres, but apparently not in nucleolar rDNA (Freeman-Cook *et al*., 2005). The model of *Sp*Sir2-mediated heterochromatin implies deacetylation of H3 and H4, particularly H3K9Ac and H4K16Ac, methylation of H3K9 and Swi6p recruitment to these regions (Freeman-Cook *et al*., 2005; Shankaranarayana *et al*., 2003). However, at least in centromeric heterochromatin, the main target of *Sp*Sir2p is H3K9Ac and not H4K16Ac (Mellone *et al*., 2003). Thus, deacetylation of H3K9Ac by *Sp*Sir2p is required for trimethylation of H3K9 by Clr4, and the subsequent arrival of Swi6 (Shankaranarayana *et al*., 2003).

*Sp*Hst2 protein is cytoplasmic, but resides in low amounts in certain heterochromatic regions of the nucleus with the remaining sirtuins (Durand-Dubief *et al*., 2007). Meanwhile, *Sp*Hst4 is cell cycle regulated like Hst4p and is probably involved in H3K56Ac control (Haldar and Kamakaka, 2008), although in some conditions it has been reported to localize with *Sp*Sir2 and *Sp*Hst2. Notably, all three *S. pombe* sirtuins are involved in retrotransposon silencing, but their exact role in this context is unknown (Durand-Dubief *et al*., 2007).

Protozoa

Knowledge of sirtuin function in unicellular eukaryotes has also benefited from studies of the parasitic protozoa that cause human diseases such as malaria, Chagas' disease, sleeping sickness and leishmaniasis. The studies have focused on three genera; *Plasmodium*, *Trypanosoma* and its close relative *Leishmania*.

Among the different species of the *Plasmodium* genus, *Plasmodium falciparum* is responsible for the most severe form of malaria. A major factor that determines the virulence of the infection is a group of *Plasmodium falciparum* genes that encode cell surface proteins. These proteins are expressed by infected erythrocytes during the blood stage of the parasite's infective life cycle and are recognized by the host's immune system (Merrick and Duraisingh, 2006). Much of the parasite's success is due to mechanisms it has developed to avoid immune system recognition. One such mechanism known as antigenic variation, which is used by *Plasmodium* and other parasites, is based on switching the expression of these surface proteins so that only one of their genes is expressed at a time (Scherf *et al*., 1998). Most important among the antigenic proteins are the adhesins encoded by the *var* gene family. *Plasmodium* contains around 60 *var* genes, which are mainly distributed in clusters along subtelomeric regions, although some copies exist around the center of chromosomes. The *var* genes are controlled by three types of promoters: upsA, B and C. UpsA and B are located mainly in telomeric regions, whereas UpC are located in more central position of the chromosomes (Merrick and Duraisingh, 2006).

Contrary to *Trypanosoma*, *var* gene antigenic variation in *Plasmodium* is not based on recombination events, but rather on epigenetic silencing of inactive genes, which greatly depends on the sirtuin *Pf*Sir2(Duraisingh *et al*., 2005; Freitas-Junior *et al*., 2005). Although *P. falciparum* contains two sirtuins, only *Pf*Sir2 has been characterized. *Pf*Sir2 shows both histone deacetylase and ADP-ribosyltransferase activities (Merrick and Duraisingh, 2007). It is predominantly located in subtelomeric regions and in the nucleolus. Interestingly, yeast *Sc*Sir2p also localize to these two loci, suggesting a conserved sirtuin role in both locations.

Disruption of *Pf*Sir2 function leads to deregulation of many inactive subtelomeric *var* genes, especially those regulated by upsA and, to a lesser extent, by upsB (Gardner *et al*., 2002). Chromatin immunoprecipitation (ChIP) experiments have shown that *var* gene activation is associated with histone H4 hyperacetylation in its promoter and 5´ coding regions as well as loss of *Pf*Sir2 from these regions (Duraisingh *et al*., 2005). These finding suggest that, as with sirtuins in budding yeast, those in *P. falciparum* are involved in generating heterochromatin-like structures in subtelomeric regions via deacetylation of histone H4 and possibly, H3.

Although not completely understood, the role of Sir2 in telomeric *var* gene silencing might resemble, in some aspects, to *S. cerevisiae* mating-type loci. However in this case, contrary to *Sc*Sir2p, the *Pf*Sir2-dependent silencing also involves methylation of H3K9. This is supported by studies demonstrating that *var* gene silencing correlates with a loss of acetylation in histone H3 lysine 9 and with trimethylation of lysine 9 throughout the gene (Lopez-Rubio *et al*., 2007). However, whether *Pf*Sir2 is directly responsible for histone H3 lysine 9 acetylation and H3K9me3 methyltransferase recruitment, and the reason why loss of *Pf*Sir2 affects certain promoter-controlled *var* genes, remain unknown. Additionally, the roles of *Pf*Sir2 in the nucleolus, and whether the other uncharacterized *P.falciparum* sirtuin plays a role in the organism's pathogenicity, have yet to be determined. Such studies could reveal a way to inhibit antigenic selection of *P. falciparum* by directly targeting its Sir2 family members. The fact that *Pf*Sir2 is sensitive to nicotinamide *in vitro* but is not affected by other drugs that modulate eukaryotic sirtuins, suggests that drugs capable of exclusively inhibiting this parasite's sirtuin activity without affecting that of the host could be developed (Merrick and Duraisingh, 2007).

Other well characterized protozoan sirtuins belong to the flagellate *Tripanosoma brucei*, which is responsible for African sleeping sickness. *T. brucei* contains three sirtuins: two Type I members, *Tb*SIR2RP1 and *Tb*SIR2RP3, and a Type II member, *Tb*SIR2RP2, related to bacterial sirtuins (Garcia-Salcedo *et al*., 2003). Like *Plasmodium Pf*Sir2, *Tb*SIR2RP1 is nuclear and shows *in vitro* both deacetylase and ADP-ribosyltransferase activity of histones, and in particular H2A and H2B, and is located in subtelomeric regions where it participates in heterochromatin formation (Garcia-Salcedo *et al*., 2003). However, *Tb*SIR2RP1 is not involved in antigenic variation. Additionally, although loss of *Tb*SIR2RP1 does not result in lethality under normal conditions, it renders *T. brucei* hypersensitive to DNA-damaging drugs such as methanesulfonic acid methyl ester (MMS), suggesting that *Tb*SIR2RP1 may also be implicated in DNA repair (Alsford *et al*.,

2007).

In contrast to *Trypanosoma*, its close relative *Leishmania* does not appear to have nuclear sirtuins. Its only sirtuin studied to date, *Lm*SIR2, is localized to the cytosol (Vergnes *et al.*, 2002). The other sirtuin appears to be related to bacterial sirtuins and therefore is likely located in the mitochondria (Frye, 2000). *Lm*SIR2 has not yet been characterized enzymatically, but it is known to be indispensable to the parasite's survival, making it one of the few described essential sirtuins. The only role attributed to *Lm*SIR2 is the promotion of survival under stressful conditions during certain stages of the parasite's life cycle (Vergnes *et al.*, 2002; Vergnes *et al.*, 2005). However, the mechanism through which *Lm*SIR2 accomplishes this is completely unknown, although chromatin regulation is a possibility. In fact, *Lm*SIR2 belongs to the Type I sirtuins, whose members are generally chromatin related. There is already a precedent for this in humans, in which the cytoplasmic sirtuin SirT2 modulate H4K16Ac levels throughout the cell cycle (Vaquero *et al.*, 2006).

C. elegans and *Drosophila*

As in previous stages of chromatin evolution, sirtuins adapted to new functions that arose with multicellular eukaryotes, cellular differentiation and development. One obvious adaptation is an increase in the number of sirtuins compared to that of lower eukaryotes, with the exception of *S. cerevisiae*.

Evidence suggests that sirtuins are involved in development and cell identity in both *Drosophila* and *C. elegans*. The *C. elegans* genome contains four sirtuins, denoted as Sir-2.1 to 2.4. (Fig. 1). Current knowledge on these is limited to a few studies and functional comparisons with better-known mammalian counterparts. Presently, there is no biochemical evidence to support any enzymatic activity for the nematode sirtuins.

Sir-2.1 is Type I sirtuin and the sirtuin most closely related to *Sc*Sir2p. The fact that Sir-2.1 has a nuclear localization and is phylogenetically close to mammalian SirT1 (Frye, 2000) suggests that it is probably a histone deacetylase and might have a role in chromatin regulation. This idea is reinforced by the fact that Sir-2.1 participates in the silencing of repetitive transgenes in *C.elegans* (Jedrusik and Schulze, 2003) and is involved in germline silencing (Jedrusik and Schulze, 2007). Nevertheless, most studies of Sir-2.1 have focused on whether extra copies of this gene promote lifespan increase (Tissenbaum and Guarente, 2001). Said effect has been observed under conditions of stress when Sir-2.1 interacts with the insulin-like signaling pathway via the forkhead transcription factor DAF-16 and the chaperone protein 14-3-3 (Berdichevsky *et al.*, 2006). Although loss-of-function mutations in the insulin-like receptor DAF-2 are known to negate the lifespan-increasing ability of Sir-2.1 (Tissenbaum and Guarente, 2001), the mechanism by which Sir-2.1 extends lifespan is unidentified. Since mammalian sirtuins can deacetylate FOXO forkhead factors and modulate their transcriptional activities (Giannakou and Partridge, 2004), the same could occur in *C. elegans*. Interestingly, Sir-2.1 has been linked to survival phenomena in other studies. For instance, it has been found to protect against polyglutamine-induced neuronal dysfunction (Bates *et al.*, 2006) and to regulate levels of key mediators of the endoplasmic reticulum (ER) unfolded-protein response (Viswanathan *et al.*, 2005).

Sir-2.2 and Sir-2.3 are Type II sirtuins, and like mammalian SirT4, share homology with some mitochondrial proteins. However, considering that Sir-2.2 has been found in a genome-wide RNAi screen for promoters of genome stability together with other chromatin-related factors (*e.g.* HDACs 1 to 4, lysine methyltransferases, the HDAC-related factor Sin3, and many DNA repair proteins) (Pothof *et al.*, 2003), it may be localized to the nucleus, where it could directly regulate chromatin functions. In fact, although not involved in lifespan increase, Sir-2.2 seems to share Sir-2.1's ability to protect *C. elegans* from neurodegeneration whereas no effect has been observed with Sir-2.3 (Bates *et al.*, 2006).

Sir-2.4, like mammalian SirT6 and SirT7, is a Type IV sirtuin, and like Sir-2.2, is not involved in lifespan increase. However, beyond this, their function is completely unknown.

Drosophila melanogaster contains five sirtuins, which, due to their homology with mammalian sirtuins, are named *Dm*Sir2 (or *Dm*SirT1), *Dm*SirT2, *Dm*SirT6 and *Dm*SirT7.

The only one of them studied so far is *Dm*Sir2, the ortholog of *C. elegans* Sir-2.1.

Like *Sc*Sir2p and Sir-2.1, *Dm*Sir2 promotes lifespan increase through a mechanism that involves caloric restriction (Rogina *et al.*, 2002). *Dm*Sir2 is clearly involved in chromatin functions, particularly those related to development. Although *Dm*Sir2 is involved in PEV (Position effect variegation) (Newman *et al.*, 2002; Rosenberg and Parkhurst, 2002)—suggesting that it is required for heterochromatin-mediated silencing—and despite its shared conservation with sirtuins in lower eukaryotes, it does not seem to regulate telomere heterochromatin (Rosenberg and Parkhurst, 2002). Rosenberg and Parkhurst reported some controversial genetic and biochemical data suggesting that *Dm*Sir2 interacts with the transcriptional repressors Hairy and Deadpan, which are members of the HES (Hairy Deadpan Enhancer of Split) family (Rosenberg and Parkhurst, 2002). The HES family of transcription factors contains a bHLH (basic helix-loop-helix) domain, and plays a key role in developmental processes through transcriptional repression of certain key genes (Younger-Shepherd *et al.*, 1992). Hairy and Deadpan recruit repressors, such as the histone deacetylases *Dm*Rpd3 and maybe *Dm*Sir2, to their target genes. (Bianchi-Frias *et al.*, 2004). However, this data is currently being challenged (Astrom *et al.*, 2003).

Another finding that suggests a role for *Dm*Sir2 in developmental processes is the identification of *Dm*Sir2 as part of a Polycomb complex, which contains factor E(Z) (Enhancer of zeste), a histone methyltransferase involved in long-range epigenetic silencing of the spatially-restricted expression pattern exhibited by homeotic genes during *Drosophila* development (Furuyama *et al.*, 2004). Epigenetic silencing by Polycomb factors is based on the trimethylation of H3K27 and resembles in some aspects *Sc*Sir2p-dependent silencing of heterochromatin. *Dm*Sir2's involvement in development seems to be very dynamic (Furuyama *et al.*, 2004; Newman *et al.*, 2002; Rosenberg and Parkhurst, 2002). For example, *Dm*Sir2 mRNA levels peak at early stages of embryogenesis, then decrease progressively until stabilizing in adults. Furthermore, localization of *Dm*Sir2 can change from the nucleus to the cytoplasm or encompass both locations simultaneously during different stages of the differentiation program (Rosenberg and Parkhurst, 2002). However, *Dm*Sir2 loss-of-function mutants are viable, suggesting a strong redundancy

among the *Drosophila* sirtuins (Astrom *et al.*, 2003; Newman *et al.*, 2002). Further studies are needed to clarify the role of other *Drosophila* sirtuins and determine the existence of a common function in development.

Mammalian sirtuins

Knowledge of sirtuin function has recently surged. This has partly been the result of characterization of mammalian sirtuins and their functional implications in lifespan regulation, cancer, neurological diseases (*e.g.* Alzheimer's and Parkinson's), and hormone-related pathologies.

Due to the lack of information on *C. elegans* and *Drosophila* sirtuins, current views on the adaptative course of sirtuins in throughout the evolution of higher eukaryotes are based almost exclusively on mammalian sirtuins. All evidence suggests that sirtuins adapted to increasing complexity by acquiring new functions through the targeting of a wide range of substrates (Fig. 3).

Thus, the seven mammalian sirtuins (SirT1 through 7) are a clear example of evolutionary diversification. In terms of function, SirT1 through 3 show deacetylase activity *in vivo*, whereas SirT4 and SirT6 exhibit specific and strong mono ADP ribosyl-transferase activity, and the main catalytic activities of SirT5 and SirT7 are currently unknown (Saunders and Verdin, 2007; Vaquero *et al.*, 2007b). SirT1, 2, 3 and 6 preferentially target histones, specifically H4K16Ac and/or H3K9A (Vaquero *et al.*, 2004). However, SirT1 through 3 also target non-histone proteins, including various nuclear factors (SirT1), tubulin (SirT2) and mitochondrial targets (SirT3) (Saunders and Verdin, 2007; Vaquero *et al.*, 2007b).

Mammalian sirtuins offer a prime example of the diversity and complexity of sirtuin cellular localization patterns. SirT1, SirT6 and a small fraction of SirT3 and SirT2 are present in the nucleus, SirT2 is cytoplasmic, SirT3 through 5 are mitochondrial, and SirT7 is nucleolar (Michishita *et al.*, 2005). Furthermore, these localizations can be dictated by cellular identity, developmental stage, environmental stimuli or cell cycle progression (Saunders and Verdin, 2007; Vaquero *et al.*, 2007b, Yamamoto *et al.*, 2007). For example, the localization of SirT1 varies with cell type, developmental stage and stress conditions.

SirT1

SirT1 and yeast *Sc*Sir2p are the best known of all sirtuins. In fact, SirT1 is the phylogenetic and functional ortholog of both yeast *Sc*Sir2p and Hst1p and shows the broadest range of functions as well as the widest array of substrates among sirtuins (Yamamoto *et al.*, 2007). Said functions can be classified into four groups: chromatin organization; metabolic regulation; cell survival in stress conditions; and cell differentiation and development (see below and Fig. 3).

Although a nuclear protein, the localization of SirT1 varies with cell type and stage of differentiation; it can be exclusively cytoplasmic or present throughout the cell (Chen *et al.*, 2006; Moynihan *et al.*, 2005).

Loss of SirT1 results in prenatal or perinatal death in half of individuals; the remaining half suffer defects in gametogenesis and sterility, eyelid opening problems, chronic lung infection and pancreatic atrophy (McBurney *et al.*, 2003a). SirT1 is expressed in all organs, but is most prevalent in the most energy dependent tissues (Michishita *et al.*, 2005).

SirT1 and chromatin regulation

SirT1 has strong histone deacetylase activity *in vitro*, especially toward H4K16Ac and H3K9Ac. RNAi studies have revealed that loss of SirT1 correlates with a global increase in H4K16Ac and H3K9Ac together with a loss of heterochromatin marks such as H3K9me3 and H4K20me1, suggesting that SirT1 is involved in the formation of heterochromatin (Vaquero *et al.*, 2004). Heterochromatin is divided into two main forms according to their distinct structural functional dynamics: constitutive heterochromatin (CH) and facultative heterochromatin (FH) (Trojer and Reinberg, 2007). CH refers to the regions that are always maintained as heterochromatin, span large portions of the chromosome, and have a rather structural role. These regions contain few genes and are located primarily in pericentromeric regions and telomeres. In contrast, FH refers to those regions that can be formed as heterochromatin in certain situations (*e.g.* certain stages of the developmental program, and the cell cycle) but can revert to euchromatin once required. Facultative heterochromatin can span from a few kilobases to a whole chromosome and generally includes regions with a high density of genes (Craig, 2005; Trojer

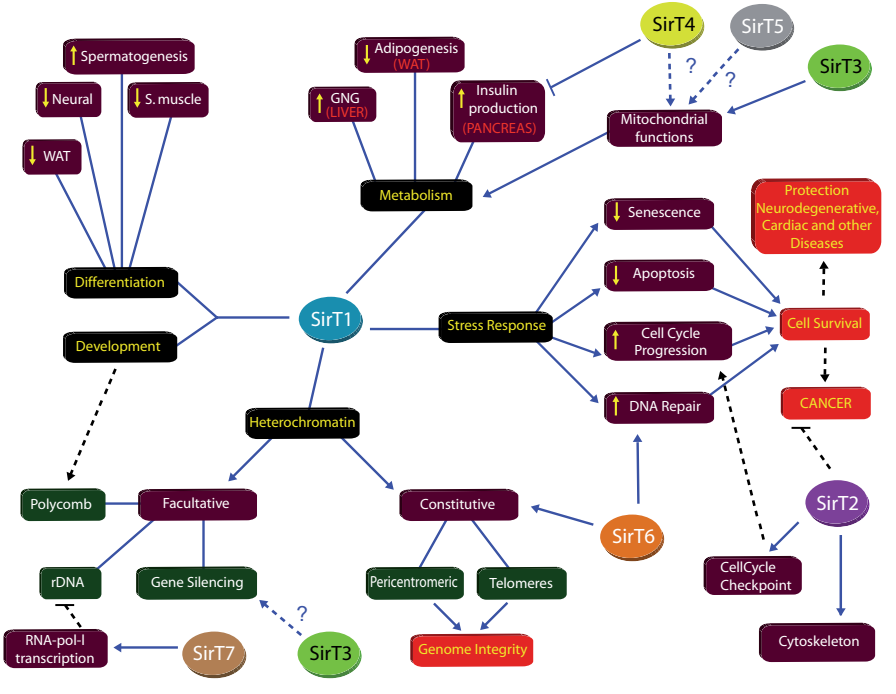

Fig. 3. Function of mammalian sirtuins. *The main functions of SirT1 are indicated in black boxes. They are mediated through promotion of the indicated actions (maroon and green boxes) and consequences (red boxes). The functions of the remaining sirtuins are also listed. A question mark is used to indicate functions which have not been demonstrated. Polycomb, Ezh2 (Polycomb)-dependent silencing; WAT, white adipose tissue.*

and Reinberg, 2007).

Current evidence suggests that SirT1 is actually involved in both forms of heterochromatin. Among the most important and distinctive features of SirT1 is that, in contrast to other Class I and II HDACs, it is more than just a histone deacetylase: due to its remarkable capacity to bind to many factors and target many substrates, SirT1 is actually a coordinator of heterochromatin formation (Vaquero *et al.*, 2004; Vaquero *et al.*, 2007b) (Fig. 4).

The main biological form of SirT1 is an homotrimer complex of *ca.* 350 kDa (Vaquero *et al.*, 2004). One other biochemically stable SirT1-containing complex, PRC4, has been described (Kuzmichev *et al.*, 2005). Like other sirtuins, SirT1 lacks the ability to directly bind DNA and must be recruited to the target DNA regions. Although numerous SirT1-targeted genes are unidentified, all data suggest that SirT1 participates in the regulation of many genes via formation of FH by coordinating several events (Fig. 4) (Vaquero *et al.*, 2004).

The first event, arrival of SirT1 to chromatin, results in deacetylation of H4K16Ac and H3K9Ac, and direct recruitment of the linker histone H1, a key factor in the formation of the 30 nm fiber (Hansen, 2002). In fact, SirT1 seems to interact specifically with one of the five somatic variants, H1b (also known as H1.4), which is particularly enriched in heterochromatin and assembled into chromatin during late S-phase (D'Incalci *et al.*, 1986; Parseghian *et al.*, 2001; Parseghian *et al.*, 2000). SirT1 not only interacts and recruits histone H1, it can also deacetylate histone H1 at lysine K26 *in vitro* and *in vivo* (Vaquero *et al.*, 2004). There are findings that suggest a role for H1K26 deacetylation in heterochromatin formation, although this has yet to be demonstrated. Firstly, acetylation of H1 N-terminal domain could act similarly as in the core histones, weakening intra- and inter-nucleosomal interactions and thus favoring a less compacted chromatin structure (Vaquero *et al.*, 2004). Secondly, H1K26 is methylated by the Polycomb protein Ezh2, homolog of *Drosophila* E(Z) (Kuzmichev *et al.*, 2004). Since in mammals, as in *Drosophila*, SirT1 coexists with Ezh2 in the PRC4 complex, deacetylation of H1K26Ac by SirT1 could allow coordinated methylation by Ezh2. Thirdly, HP1 proteins bind to dimethylated H1bK26 through its chromodomain, an interaction inhibited by phosphorylation of the adjacent serine 27 (Daujat *et al.*, 2005; Nielsen *et al.*, 2001a). This mechanism might explain the targeting of HP1 to certain regions in the absence of H3K9 methylation in *Xenopus* (Meehan *et al.*, 2003). Finally, H1b is also present in a complex formed by L3MBTL1, core histones, HP1γ and retinoblastoma (Rb). The MBT (malignant brain tumor) factor L3MBTL1 promotes heterochromatin formation through binding to mono- and di- methylated H4K20 and H1K26me3 (Trojer *et al.*, 2007).

Upon arrival of SirT1, loss of the active chromatin mark H3K79me2 is observed (Fig. 4). Loss of this mark—which is involved in transcriptional active chromatin, DNA damage response and meiotic checkpoint control and is located in the globular domain of histone H3 (Feng *et al.*, 2002; Kouskouti and Talianidis, 2005)—spreads a few kilobases away from the promoter regions. Interestingly, this modification is very important for establishing the boundaries of ScSir2p silencing in *S. cerevisiae*, since it inhibits the spread of the ScSir2p/3p/4p complex and is absent in Sir2-silenced regions (Ng *et al.*, 2002). Nevertheless, the mechanism behind that loss is not understood. Whether SirT1 is directly involved, or if the mechanism requires histone exchange activity or a demethylase activity, should be addressed in future studies.

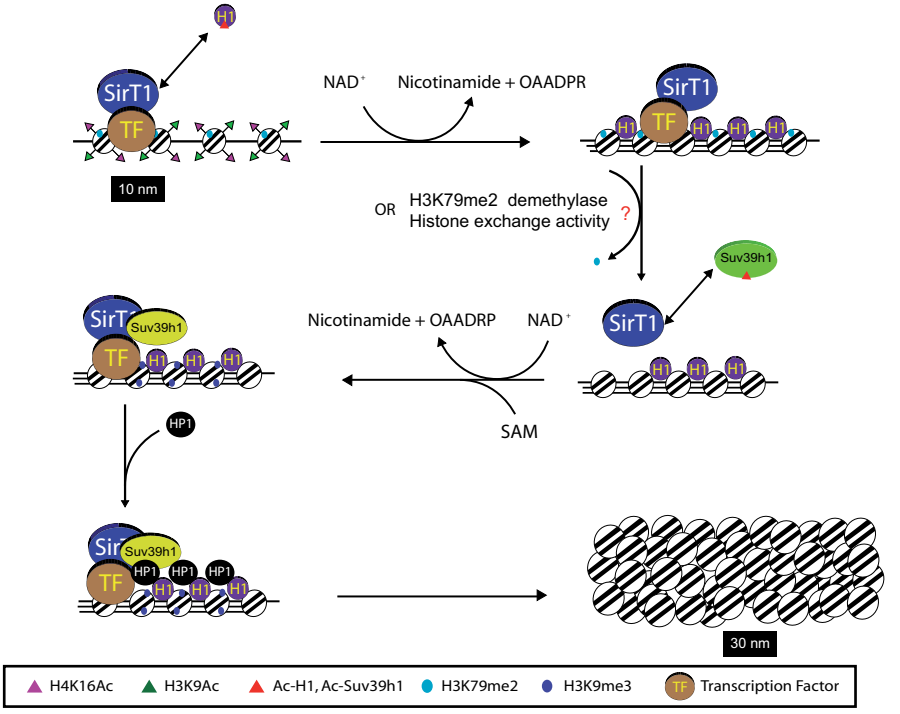

Fig. 4. Model of SirT1-mediated facultative heterochromatin. *Upon recruitment by a DNA-binding factor (TF), SirT1 promotes histone deacetylation, particularly of H4K16Ac and H3K9Ac, as well as recruitment and deacetylation of H1 (upper panel). This arrival correlates with loss of the active mark H3K79me2. Next, SirT1 recruits and deacetylates Suv39h1, which becomes "superactive" (middle panel) and methylates the neighboring nucleosomes. This in turn attracts HP1 and triggers the spread of heterochromatin, resulting in the formation of the 30 nm fiber (lower panel). OAADPR, O-Acetyl-ADPribose; SAM, S-adenosyl-methionine.*

Lastly, SirT1 arrival promotes the establishment of heterochromatin marks, particularly H3K9me3 and H4K20me1, which spread throughout the coding region of the gene (Vaquero *et al.*, 2004). The mechanism involved in the establishment of these marks is known for H3K9me3, but not for H4K20me1. SirT1 promotes the establishment of H3K9me3 through an intimate functional relationship with Suv39h1, the main enzyme responsible for the modification (Vaquero *et al.*, 2007a). Suv39h1 was the first lysine methyltransferase ever described (Rea *et al.*, 2000)and is a key player in chromatin organization, through maintenance of H3K9me3 levels in both pericentromeric and telomeric CH (Peters *et al.*, 2001). It is also involved in formation of FH in certain regions (Nielsen *et al.*, 2001b). The importance of this activity is reflected by several pieces of evidence. Firstly, loss of both Suv39h1 and its close relative Suv39h2 (Suv39DN) during murine development leads to a complete loss of H3K9me3 in the pericentromeric heterochromatin as well as to a substantial re-

duction in H3K9me3 levels in telomeres (Peters *et al.*, 2001). This produces a loss of heterochromatin, demonstrated by loss of HP1 proteins and relocalization of H4K16Ac to the heterochromatic foci (Vaquero *et al.*, 2007a), chromatin segregation problems, delay in the G_2 to M transition, and DNA damage. Interestingly, loss of either of these does not affect H3K9me3 levels, suggesting the importance of maintaining the levels of the modification through the developmental program (Peters *et al.*, 2001). Moreover, in adult tissues Suv39h1 is ubiquitously expressed, whereas Suv39h2 is restricted to the testes, where it seems to be involved in heterochromatic regulation during meiosis (O'Carroll *et al.*, 2000). The functional relationship between SirT1 and Suv39h1 challenges current views on the interplay between histone deacetylases and methyltransferases and suggests a more intimate association between these groups of enzymes than previously understood. In fact, evidence in *S. pombe* suggest that this relationship is conserved from yeast to humans (Shankaranarayana *et al.*, 2003).

SirT1 promotes Suv39h1-dependent H3K9me3 methylation through four different mechanisms (Vaquero *et al.*, 2007a). Firstly, SirT1 directly recruits Suv39h1 to regulatory regions through its N-terminal region (SirT1-Nt), the same one involved in H1 recruitment (Vaquero *et al.*, 2004). In the case of Suv39h1, the domain involved in the interaction involves the first 88 residues, which include the chromodomain (residues 44 to 88), involved in binding to HP1, and an N-terminal region (residues 1 to 43) with an unknown function. Interestingly, the chromodomain alone retains the capacity of SirT1 binding, which suggests that SirT1 might compete with HP1 for the binding to Suv39h1. Secondly, SirT1 deacetylates H3K9Ac to allow methylation of the same residue by Suv39h1 in the regulatory regions. Thirdly, SirT1-Nt specific binding to Suv39h1 increases its methyltransferase activity *in vitro* and *in vivo*, probably through a conformational change. As with H1, this binding capacity is specific for SirT1, given that overexpression of the SirT1 N-t domain, a domain specific to SirT1, results in a global increase in H3K9me3 (Vaquero *et al.*, 2007a). Finally, SirT1 deacetylates K266 of Suv39h1, a residue located in the catalytic SET domain, rendering the enzyme more active. K266 is conserved through evolution in all high eukaryotic Suv39h1 orthologs and a remarkable number of SET-containing methyltransferases. Although the role of K266 in heterochromatin formation, and its implications for Suv39h1 functions, remain unknown, comparative studies with the crystal structure of *S. pombe* Clr4 strongly suggest that this residue is located in an exposed loop important for proper folding of the SET domain (Min *et al.*, 2002). This would explain how acetylation and deacetylation can modulate the enzymatic activity of Suv39h1.

Although this is the only known case of acetylation/deacetylation of a methyltransferase to date, the degree of conservation in the SET-containing family of HMTs—which includes almost all lysine methyltransferases—suggests that this mechanism may be more general. One obvious case is Ezh2, the H3K27me3 and H1K26me2/3 methyltransferase that has conserved K266 from *Drosophila* to humans (Vaquero *et al.*, 2007a). It is possible that in the context of PRC4, SirT1 might modulate Ezh2 activity, not only via histone deacetylation, but also through a direct effect on the SET domain of Ezh2. Further studies are required to determine the extent of this modification.

An intriguing and unexplained aspect of the relationship of SirT1 to Suv39h1 and to Ezh2 in heterochromatin formation is the role of these in DNA methylation. Both Suv39h1 and Ezh2 have been found to bind directly to Dnmt1—and also to Dnmt3a in the case of the former—and direct DNA methylation to specific genes (Fuks *et al.*, 2003; Vire *et al.*, 2006). SirT1 has also been found to interact with Dnmt1 in nucleolar rDNA. However, the SirT1 knockdown does not affect DNA methylation levels, whereas loss of Dnmt1 leads to hyperacetylation of H4K16Ac and H3K9Ac (Espada *et al.*, 2007).

All current evidence suggests that in the genes or affected regions, proper DNA methylation requires the presence of certain methyltransferases (*e.g.* Suv39h1, Ezh2 and G9a). In contrast, SirT1 heterochromatin-induced silencing is a phenomena that occurs downstream of the DNA methylation. In agreement with this, loss of SirT1 leads to a loss of silencing in certain tumor suppressor genes, although it does not alter DNA methylation levels (Pruitt *et al.*, 2006). A possible explanation for this is that different Suv39h1 complexes are involved in both stages: DNA methylation establishment and heterochromatin formation. This premise is supported by several lines of evidence: first, Suv39h1 interacts with many chromatin and transcription factors, including HDAC1, 2 and 3, and is part of the Rb-repressing complex (Vaute *et al.*, 2002); second, SirT1 was recently found to interact with Suv39h1 and the H3K9me binding protein Nuclomethylin in rDNA loci and induce silencing of these genes in conditions of energetic stress (Murayama *et al.,* 2008).

The relevance of the relationship between SirT1 and Suv39h1 actually goes beyond FH; as in the case of *S. pombe*, it also affects CH, which supports a general role for SirT1 in the global organization of mammalian chromatin (Vaquero *et al.*, 2007a). Mouse embryonic fibroblasts (MEFs) derived from $SirT1^{-/-}$ knockout mice show a complete loss of H3K9me3 levels in the CH foci in approximately 50% of their cells, which correlates with mislocalization of HP1α in the same foci. Transfection of SirT1 in these cells produces a full recovery of H3K9me3 levels, demonstrating direct involvement of SirT1 in this phenotype. Interestingly, and in agreement with biochemical data, this recovery requires both the N-terminal domain and the catalytic activity of SirT1, since transfection of either SirT1 lacking the N-terminal domain or a catalytically-inactive full length SirT1 point mutant leads to only partial recovery (Vaquero *et al.*, 2007a).

Many questions arise from these observations. Since SirT1 has never been found in the HC foci in immunofluorescence studies, it is unclear how SirT1 promotes H3K9 methylation in these regions. Possible explanations are that the levels of SirT1 could be so low that they fall below the threshold of immunofluorescence detection, or that the protein is only present during a very restricted time window in the cell cycle (*e.g.* certain stages of S-phase)—an idea supported by the massive invasion of H4K16Ac to CH foci upon loss of Suv39h1 and 2(Vaquero *et al.*, 2007a). Given the obvious limitations of immunofluorescence techniques, ChIP techniques would appear to be the most appealing alternative for tracking SirT1.

Another interesting question is why loss of SirT1 only affects 50% of the MEFs. Loss of SirT1 during development may be partially covered by other HDACs. Indeed, TSA treatment of mouse L929 cells induces loss of H3K9me3 in CH foci, suggesting that, as in *S. pombe* Clr3p and Clr6p, other Class I and II HDACs might be involved and may have partial functional redun-

dancy (Taddei *et al.*, 2001). Another possibility is that SirT1 involvement in maintenance of H3K9me3 levels is restricted to a certain cell cycle stage or to certain conditions. Due to the closeness of Suv39h1 to its relative Suv39h2, the effect of SirT1 may be mediated not only through the former, but also through the latter. Further studies are required to elucidate these points.

Finally, SirT1 is also involved in the degradation of the H2A variant H2A.Z, which is associated with active chromatin and is essential in development. Deacetylation of H2A.Z by SirT1 under cardiac hypertrophy conditions induces cell growth and inhibits apoptosis (Chen *et al.*, 2006).

SirT1 and metabolic regulation

In addition to chromatin regulation, SirT1 is crucial to metabolism during fasting conditions, during which it is upregulated. This role actually relates to two main aspects of metabolism: firstly, through the direct control of certain enzymes of the intermediary metabolism (*e.g.* AceCS1, the cytosolic isoform of Acetyl-CoA synthetase involved in fatty-acid formation from acetate) (Hallows *et al.*, 2006); and secondly, through regulation of endocrine signaling, particularly of carbohydrate and lipid metabolism. In fact, SirT1 has also been linked to the signaling of several hormone receptors such as that of the insulin-like growth factor (IGF) pathway (Lemieux *et al.*, 2005)and those of the hepatic LXR α and β, androgen and glucocorticoid receptors (Amat *et al.*, 2007; Fu *et al.*, 2006; Li *et al.*, 2007b). Particularly important is the role of SirT1 in glucose homeostasis, through its combined effect on the pancreas, liver, and white adipose tissue (WAT) (Amat *et al.*, 2007; Feige and Auwerx, 2008). It promotes insulin production in pancreatic β-cells (Bordone *et al.*, 2006; Moynihan *et al.*, 2005), hepatic gluconeogenesis through interaction and deacetylation of the transcription regulator PGC1α (Rodgers *et al.*, 2005), and inhibition of adipogenesis and differentiation via binding to the transcriptional activator peroxisome proliferator-activated receptor γ (PPARγ) (Picard *et al.*, 2004b).

SirT1 and cell survival

Among the functions of SirT1 that has garnered the most interest is its role in the responses to oxidative and genotoxic stress conditions (Giannakou and Partridge, 2004; Haigis and Guarente, 2006). As in the case of heterochromatin regulation, SirT1 seems to coordinate many processes such as DNA damage sensing, DNA repair and detoxifying machinery induction, inhibition of apoptosis and of senescence, cell proliferation, and autophagy stimulation. It interacts with many different factors which are often related to its transcriptional silencing capacity. This coordinated response appears to protect nerve, cardiac, liver and others types of tissues, and links SirT1 activity to cancer processes (Fig. 3).

SirT1 interacts and deacetylates key factors involved in response to stress, including the forkhead FOXO family of transcription factors (Brunet *et al.*, 2004; Motta *et al.*, 2004; van der Horst *et al.*, 2004) and NF-κB (Yeung *et al.*, 2004), by modulating their transcriptional activity. SirT1 activity induces transcriptional activation of DNA repair and DNA detoxifying machinery together with repression of cell-cycle control genes or induce apoptosis. Another interesting and not completely understood aspect of SirT1 function is its antagonism with the tumor suppressor p53, a major regulator in cell cycle control. Deacetylation of p53 by SirT1 inhibits p53-dependent apoptosis and senescence (Luo *et al.*, 2001; Vaziri *et al.*, 2001). Interestingly, SirT1$^{-/-}$ MEFs show p53 hyperacetylation (Cheng *et al.*, 2003) and an inhibitory effect on p53 translocation to mitochondria upon stress (Han *et al.*, 2008).

The second level of SirT1 cell survival induction is through direct interaction and targeting of DNA repair machinery, for which two different targets have been reported to date. The first, Nijmegen breakage syndrome 1 (NBS1), is a checkpoint protein involved in DNA damage sensing and induction of DNA repair (Yuan *et al.*, 2007). SirT1 binding and deacetylation of NBS1 induces DNA repair and cell survival. The second is the DNA repair factor Ku70, which is involved in double strand breaks (DSB) repair through non-homologous end joining (NHEJ). SirT1 binds to and deacetylates Ku70, which promotes its binding to the pro-apoptotic factor Bax. Binding to Ku70 sequesters Bax away from the mitochondria, thereby inhibiting Bax-dependent apoptosis (Cohen *et al.*, 2004). This SirT1-Ku70 interaction might have implications in DSB repair. In fact, recent studies have shown that upon DSB breaks, SirT1 is recruited to the damaged site, an event that correlates with chromatin compaction and silencing in the same region (O´Hagan *et al.,* 2008). This recruitment seems to be associated with SirT1 relocalization from its native loci, which induces a change in the pattern of gene expression that resembles mammalian aging (Oberdoerffer *et al.,* 2008). However, the role of SirT1 in the process of DNA repair is now known.

The third level of regulation is through cell cycle control. SirT1 is not only involved in efficient response to environmental stress, but also in promoting cell progression. In fact, there is evidence of SirT1 downregulation upon cell-cycle exit (Sasaki *et al.*, 2006). Although not totally understood, current data suggest that SirT1 regulates certain major players in cell cycle control, such as retinoblastoma (Rb) and E2F1. Rb is a tumor suppressor that controls G_1 to S transition by binding to E2F transcription factors. Cell-cycle dependent phosphorylation of Rb induces its binding to E2F and subsequent repression of E2F-responsive genes, thereby inducing cell cycle progression (Wong and Weber, 2007). Acetylation of Rb inhibits this binding; therefore suggesting that deacetylation of Rb by SirT1 promotes its phosphorylation and cell proliferation (Wong and Weber, 2007). In fact, Rb forms a complex with Suv39h1 and HDAC1, 2 and 3 (Vaute *et al.*, 2002), suggesting that the SirT1 functional link between Suv39h1 may also be involved. Additionally, SirT1 binds and deacetylates E2F1, inhibiting its pro-apoptotic activity and inducing cell proliferation (Wang *et al.*, 2006). Recent findings suggest that SirT1 can also interfere in the transcriptional repressive activity of HDAC1-containing Rbp1 complex, inhibiting its growth arrest activity (Binda *et al.*, 2008). However, data suggest that this SirT1 induction of proliferation turns to growth arrest in chronic stress conditions, suggesting a more complex and fine-tuned mechanism of SirT1 control on cell survival (Chua *et al.*, 2005).

Finally, very recent observations suggest that SirT1 promotes autophagy, a mechanism involved in degradation of damaged proteins and organelles resulting from stress. SirT1$^{-/-}$ MEFs cannot sustain autophagy activation upon stress conditions (Lee *et al.*, 2008). However, whether this effect is direct or indirect is currently unknown.

SirT1, development and cell differentiation

In mouse ES (Embryonic stem) cells, SirT1 levels are high in

non-differentiated cells and decrease upon differentiation (Kuzmichev *et al.*, 2005), which suggests that SirT1 has an antagonistic relationship with differentiation. Evidence shows that SirT1 function is key in two of the most metabolically-dependent tissue types: skeletal muscle and WAT. In regular conditions, both tissues are heavy consumers of systemic glucose. Upon fasting, SirT1 upregulation induces silencing of certain key genes in both types to inhibit differentiation. In skeletal muscle, *myogenin* and *MHC* genes are silenced after SirT1 forms a complex with the transcription factor MyoD and the HAT PCAF (p300/CBP associating factor). Silencing is achieved through deacetylation of MyoD and PCAF and likely through formation of FH (Fulco *et al.*, 2003). In WAT, SirT1 inhibits activation of genes such as fatty-acid-binding protein (aP2) through recruitment of the corepressors NCoR and SMRT to the PPARγ-response genes, resulting in mobilization of fat as well as inhibition of WAT differentiation (Picard *et al.*, 2004a). Recent data suggest that differentiation in nerve tissue is likewise dependent on metabolic changes and regulated by SirT1. Under oxidative stress, mouse neural progenitor cells (NPCs) stop proliferating and differentiate into astroglial cells (instead of neurons) through a SirT1-dependent mechanism (Prozorovski *et al.*, 2008). This mechanism relies on modulation of the transcription factor Hes1 by SirT1, which induces silencing of the pro-neuronal gene Mash1.

SirT1 is also involved in activation of differentiation in gametogenesis. Both male and female SirT1$^{-/-}$ knockout mice are sterile and show depletion of differentiating germ cells (McBurney *et al.*, 2003b). However, the mechanisms involved are currently unknown.

SirT2

SirT2 is a type I sirtuin like its yeast ortholog Hst2p, and is located in the cytoplasm except during the G_2 to M transition, when it is transported to the nucleus and localizes to chromatin (North and Verdin, 2007a, Vaquero *et al.*, 2006). Although many aspects of SirT2 function remain unknown, all evidence suggests that it participates in cell cycle control, particularly at the G_2 to M checkpoint. Interestingly, SirT2 overexpression delays mitosis exit and shortens G_1 (Bae *et al.*, 2004; Dryden *et al.*, 2003), whereas SirT2$^{-/-}$ MEFs are associated with longer G_1 and shorter S-phase (Vaquero *et al.*, 2006). Data suggest that the levels, activity and localization of SirT2 are tightly regulated by phosphorylation (Dryden *et al.*, 2003; North and Verdin, 2007b). In contrast to SirT1, which is associated with cell survival and cancer processes, SirT2 acts as a tumor suppressor (Inoue *et al.*, 2007). Overexpression of mammalian SirT2 under conditions of uncontrolled proliferation or mitotic stress promotes cell cycle arrest before mitotic entry (Inoue *et al.*, 2007), and overexpression of either SirT2 or Hst2p in starfish oocytes delays cell division (Borra *et al.*, 2002). Consistent with this, SirT2 is downregulated in certain cancers (*e.g.* glial and gastric carcinomas) (Hiratsuka *et al.*, 2003; Inoue *et al.*, 2007) and it has been found mutated in melanomas. (Lennerz *et al.*, 2005)

Like SirT1, SirT2 has also been linked to inhibition of differentiation of adipocyte and neural oligodendroglial cells (Jing *et al.*, 2007; Li *et al.*, 2007a). Furthermore, SirT2 has also been imputed in neurodegenerative diseases like SirT1, but apparently with an opposite role which is not understood (Outeiro *et al.*, 2007).

So far, two major targets have been described for SirT2: chromatin and the cytoskeleton.

Despite its cytosolic localization, SirT2 has histone deacetylase activity—highly specific for H4K16Ac, and to a lesser extent, H3K9Ac—, and in RNAi experiments, loss of SirT2 leads to high levels of H4K16Ac (Vaquero *et al.*, 2006). SirT2 is responsible for the global drop of H4K16Ac levels just before mitosis, which may promote proper compaction of chromosomes during mitosis. Given that H4K16Ac shows a unique capacity to inhibit the formation of high orders of chromatin organization (Shogren-Knaak *et al.*, 2006), its removal should be necessary for the cell cycle to proceed. However, SirT2$^{-/-}$ MEFs, which show hyperacetylation of H4K16 during mitosis, do not exhibit any clear delay in mitosis progression, but do show a delay in S-phase entry (Vaquero *et al.*, 2006). This might suggest that any possible defect in chromatin produced by mitotic progression in the presence of H4K16 hyperacetylation might pay a toll in S-phase entry, when the G_1/S checkpoint needs to decide whether to proceed with DNA replication. The source of this defect is unknown, but it could involve DNA repair processes, given that the main H4K16 acetyltransferase in mammalian cells, MOF (Gupta *et al.*, 2008; Taipale *et al.*, 2005), is associated with DNA damage-sensing machinery and key elements of the G_1 to S checkpoint (*e.g.* p53) (Gupta *et al.*, 2005). Considering that global levels of H4K16Ac peak during S-phase and that this modification has been involved in histone deposition in plants (Belyaev *et al.*, 1997), there is clearly a close but unexplained relationship between H4K16Ac and the S-phase. Further studies should clarify whether the cell cycle defects described upon SirT2 loss are completely related to aberrant H4K16Ac levels or to other possible targets.

The other known SirT2 target described so far is α-tubulin, whose deacetylation has been hypothesized to be important for regulation of microtubule dynamics (North *et al.*, 2003). Since tubulin acetylation seems to stabilize microtubule structures, deacetylation might disrupt it and consequently inhibit cell progression (Piperno *et al.*, 1987). SirT2 has actually been found to interact with another tubulin deacetylase, HDAC6, but the implications of this finding are not completely understood (North *et al.*, 2003).

SirT3

SirT3 is a close relative of SirT2, is phylogenetically related to Hst2p and is the only sirtuin directly involved in human longevity (Rose *et al.*, 2003). It is present mainly in the mitochondria, to which it is translocated upon cleavage of 142 residues from its N-terminus (Onyango *et al.*, 2002; Schwer *et al.*, 2002). The cellular role of SirT3 is related to metabolism and mitochondrial function, although it is not clearly understood. Despite the fact that SirT3 loss has been shown to produce general hyperacetylation of mitochondrial proteins (Lombard *et al.*, 2007) and to interact with the Foxo factor Foxo3a (Jacobs *et al.,* 2008), only one target has been described to date: acetyl-CoA synthetase 2 (AceCS2) (Hallows *et al.*, 2006; Schwer *et al.*, 2006). Mitochondrial AceCS2 is activated upon deacetylation by SirT3, inducing the production of Acetyl-CoA (Ac-CoA), which in turn causes the mitochondrial metabolic rate to increase. Although widely expressed, SirT3 is particularly important in brown adipocyte tissue (BAT), brain and kidney, but is very poorly expressed in WAT (Shi *et al.*, 2005).

Surprisingly, SirT3 might have a function in chromatin regulation. Like SirT2, SirT3 shows strong histone deacetylation activity

specific for H4K16Ac and H3K9Ac (Scher *et al.*, 2007). Moreover, some full-length SirT3 resides in the nucleus in certain uncharacterized foci. SirT3 transfection in human embryonic 293 cells can induce silencing of a reporter gene integrated into euchromatic regions through deacetylation of H4K16Ac and H3K9Ac in promoter regions. However, in contrast to loss of SirT2, loss of SirT3 does not correlate with a global increase of H4K16Ac or H3K9Ac, suggesting that, if indeed involved in transcriptional regulation, it might only target a small subset of genes. However, no candidate genes have yet been identified.

Stress conditions induced by DNA damaging agents such as Etoposide and UV radiation, as well as SirT3 overexpression, induce extensive relocalization of SirT3 from the nucleus to mitochondria for an unknown function (Scher *et al.*, 2007). Nuclear SirT3 might represent a different functional population than the majority present in the mitochondria; hence, translocation to mitochondria might imply new roles, such as the described involvement of SirT3 in apoptosis (Allison and Milner, 2007). Another possibility is that, as with SirT2, SirT3 levels require very tight regulation, and translocation to mitochondria might overcome a certain threshold beyond which specific responses might occur. A nuclear shuttling mechanism, like the recently described for SirT2, has been hypothesized for SirT3 to support these observations. It is based on the fact that leptomycin A can block the translocation (Scher *et al.*, 2007). Other data suggest that overexpression of another mitochondrial sirtuin, SirT5, induces nuclear accumulation of SirT3 through a completely unknown mechanism (Nakamura *et al.*, 2008).

SirT4 and SirT5

SirT4 is a mitochondrial Type II sirtuin involved in ADP-ribosylation of mitochondrial proteins and does not have any deacetylase activity or any described role in chromatin. In contrast, it seems to have a role in metabolic control and insulin production in pancreatic β-cells via modulation of the activity of glutamate dehydrogenase (GDH), which is involved in the catabolism of glutamic acid and glutamine (Haigis *et al.*, 2006).

SirT5 is a Type III sirtuin related to the prokaryotic sirtuins. Its function remains unknown.

SirT6

SirT6 is a Type IV nuclear chromatin-bound sirtuin essential for viability (Mostoslavsky *et al.*, 2006). Its loss is associated with genomic instability, increased sensitivity to ionizing radiation (IR), and both oxidative and genotoxic stress, which lead to lifespan shortening and an aging-like phenotype. SirT6 is functionally associated with base excision repair (BER), a DNA repair mechanism responsible for single-stranded break repairs (Mostoslavsky *et al.*, 2006). However, it is unclear whether this involvement is direct, since SirT6 does not seem to localize to BER foci, and its loss does not impair BER mechanism. SirT6 actually binds to GCIP, a putative tumor suppressor and a cell proliferation inhibitor, which suggests that SirT6 might have more of a role in sensing and signaling DNA repair and in certain conditions of growth arrest (Ma *et al.*, 2007). In agreement with this role, a recent report described a positive role for p53 in the control of SirT6 protein levels in normal growth conditions (Kanfi *et al.*, 2008). Interestingly, SirT6 levels are also upregulated in caloric restriction or nutrient deprivation conditions (Kanfi *et al.*, 2008), which together with the evidence described above support a role for SirT6 in oxidative stress response.

SirT6 was originally found to show strong ADP-ribosyltransferase activity, but no other target was described except itself. However, SirT6 has also recently been observed in telomeric regions, where it seems to exhibit very specific H3K9Ac deacetylase activity (Michishita *et al.*, 2008). SirT6 loss induces hyperacetylation of H3K9Ac as well as telomeric defects such as end-to-end chromosomal fusions, leading to senescence. Additionally, SirT6 is involved in the telomeric localization of Werner syndrome gene protein (WRN), a DNA helicase involved in telomeric replication during S-phase that also interacts with SirT1 (Narala *et al.*, 2008). This body of evidence suggests that SirT6 is involved in ensuring proper telomeric replication. However, how these data correlate with the previously described functional link to DNA repair, and how the H3K9Ac deacetylation activity of SirT6 participates in these functions, are unknown.

SirT7

SirT7, the other Type IV mammalian sirtuin, is located in the nucleolus and seems to have major ADP-ribosylation activity (Michishita *et al.*, 2005). Surprisingly, it does not seem to be implicated in rDNA silencing; instead, it appears to directly activate RNA polymerase-I. Loss of SirT7 produces a loss of RNA-polymerase I bound to the rDNA regions, inducing inhibition of cell proliferation and apoptosis (Ford *et al.*, 2006). This role is dependent on SirT7 catalytic activity, but no targets have been described to date. In contrast, $SirT7^{-/-}$ mice show heart hypertrophy and inflammatory cardiopathy as well as a significant loss of resistance capacity to stress (Vakhrusheva *et al.*, 2008). How these observations are related to the SirT7 role in nucleolar rDNA regulation is now known and should be addressed in the future.

Interestingly, the other sirtuin found in the nucleolus, SirT1, has an antagonistic role to SirT7: it binds to rDNA copies through interaction with DNA-methyltransferase Dnmt1 and participates in silencing of these regions (Espada *et al.*, 2007). In addition to histones, SirT1 also inhibits RNA polymerase-I by deacetylating the basal factor TAF_I68 (Muth *et al.*, 2001). To date, the functional relationship between these two sirtuins is unknown.

Conclusions

The relevance of sirtuins to chromatin in archaea to humans makes them exceptional witnesses of the path followed by chromatin regulation and reflects the importance of chromatin functions in metabolism and stress adaptation. Despite this, the conserved role of sirtuins in chromatin dynamics has not garnered the same attention as most of the newly acquired sirtuin functions (*e.g.* cell survival under stress), and in particular in the context of human pathologies such as cancer and neurodegenerative diseases. Interestingly, a growing body of evidence suggests that in a significant number of these new functions, the main effect of sirtuins is exerted via a direct effect on chromatin. Elucidating sirtuins' roles in chromatin is not only relevant to understanding said pathologies, but also to explaining the evolution and specialization of these proteins. Next years should be crucial to build a more complete and integrated

perspective of all described sirtuin roles into a more accurate description of sirtuin global contribution to cell life basic functions.

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