Toward a molecular understanding of yeast silent chromatin: roles for H4K16 acetylation and the Sir3 C-terminus

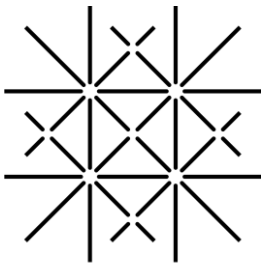
Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Mariano Oppikofer

aus
Ascona, Ticino



Basel, 2012

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Dr. Susan M. Gasser
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Basel, den 18.09.2012

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To my parents, Alessandra and Danilo Oppikofer,
for encouraging my curiosity.

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ABSTRACT: KEY FINDINGS AND IMPLICATIONS

Discrete regions of the eukaryotic genome assume a heritable chromatin structure that is refractory to gene expression. In budding yeast, silent chromatin is characterized by the loading of the Silent Information Regulatory (Sir) proteins – Sir2, Sir3 and Sir4 – onto unmodified nucleosomes. This requires the deacetylase activity of Sir2, extensive contacts between Sir3 and the nucleosome, as well as interactions between Sir proteins forming the Sir2-3-4 complex. During my PhD thesis I sought to advance our understanding of these phenomena from a molecular perspective.

Previous studies of Sir-chromatin interactions made use of histone peptides and recombinant Sir protein fragments. This gave us an idea of possible interactions, but could not elucidate the role of histone modifications in the assembly of silent chromatin. This required that we examine nucleosomal arrays exposed to full length Sir proteins or the holo Sir complex. In Chapter 2, I made use of an *in vitro* reconstitution system, that allows the loading of Sir proteins – Sir3, Sir2-4 or Sir2-3-4 – onto arrays of regularly spaced nucleosomes (Cubizolles *et al*, 2006; Martino *et al*, 2009), to examine the impact of specific histone modifications on Sir protein binding and linker DNA accessibility. The "active" H4K16ac mark is thought to limit the loading of the Sir proteins to silent domain thus favoring the formation of silent regions indirectly by increasing Sir concentration locally. Strikingly, I found that the Sir2-4 subcomplex, unlike Sir3, has a slight higher affinity for H4K16ac-containing chromatin *in vitro*, consistent with H4K16ac being a substrate for Sir2. In addition the NAD-dependent deacetylation of H4K16ac promotes the binding of the holo Sir complex to chromatin beyond generating hypoacetylated histone tails. We conclude that the Sir2-dependent turnover of the "active" H4K16ac mark directly helps to seed repression.

The tight association of the holo Sir complex within silent domains relies on the ability of Sir3 to bind unmodified nucleosomes. In addition, Sir3 dimerization is thought to reinforce and propagate silent domains. However, no Sir3 mutants that fail to dimerize were characterized to date. It was unclear which domain of Sir3 mediates dimerization *in vivo*. In Chapter 3, we present the X-ray crystal structure of the Sir3 extreme C-terminus (aa 840-978), which folds into a variant winged helix-turn-helix (Sir3 wH) and forms a stable homodimer through a large hydrophobic interface. Loss of wH homodimerization impairs holo Sir3 dimerization *in vitro* showing that the Sir3 wH module is key to Sir3-Sir3 interaction. Homodimerization mediated by the wH domain can be fully recapitulated by an unrelated bacterial homodimerization domain and is essential for stable association of the Sir2-3-4 complex with chromatin and the formation of silent chromatin *in vivo*.

1 INTRODUCTION

The heritable information underlying the generation of all organisms is contained in polymers of deoxyribonucleic acid (DNA). Formed by a non-repetitive series of only 4 chemical units paired in a double helical chain, the DNA molecule holds the information that is necessary, albeit insufficient, to build an entire organism, whether unicellular like yeast or multicellular like man. Within this heritable information are shorter DNA sequences called genes, which can be transcribed into a ribonucleic acid (RNA) polymer and translated into a chain of amino acids, or protein. Together with RNA, proteins are the basic structural and catalytic constituent of cells. DNA, RNA and proteins build a tightly interconnected frame that constitutes life and supports its propagation.

1.1 THE NUCLEOSOME

In order to accommodate the long linear chains of DNA in the nucleus of eukaryotic cells, DNA is generally found associated with histone proteins to form nucleosomes. In the late nineties, the first high resolution crystal structure of the nucleosome core particle (NCP) was solved (Figure 1; (Luger *et al*, 1997)). Technically, a NCP corresponds to 147 base pairs (bp) of DNA wrapped around a histone octamer in 1.65 superhelical turns without any linker DNA. The four core histone proteins (H2A, H2B, H3 and H4; two copies of each) are highly basic proteins that contain a characteristic structural motif called "histone fold" which consists of three α-helices separated by two loops (Luger *et al*, 1997; Davey *et al*, 2002). This structured histone fold is flanked by disordered N- and C-terminal extensions called histone "tails", which protrude from the nucleosomal core. Although histone tails are not required for the formation of a nucleosome, they have a great effect on its thermal stability (Ausio *et al*, 1989). The histone tails make up roughly 30% by mass of the histone octamer and have important regulatory functions described below (Luger and Richmond, 1998b; Zheng and Hayes, 2003). In the crystal lattice of the NCP, the N-terminal tail of the histone H4 (aa 16-25) makes contacts with an acidic patch on the surface of the H2A-H2B dimer of an adjacent NCP particle with important implications for the formation of high-order structures (Luger *et al*, 1997).

In the absence of DNA, histones form heterodimers (H2A-H2B) and heterotetramers (H3-H4). Histones dimerize through their central and longest α-helices in an anti-parallel orientation and in the case of the heterotetramers (H3-H4), two H3-H4 dimers make extensive H3-H3 contacts forming a 4-helix bundle (Luger *et al*, 1997). Given the highly basic charge of all four histones, a stable octamer of $(H2A\text{-}H2B)_2(H3\text{-}H4)_2$ only forms at very high salt concentrations (2M NaCl) or in the presence of DNA, which wraps around the histone octamer to form a nucleosome. The histone octamer is held together through a hydrophobic cluster formed by extensive interactions between H4 and H2B (Luger *et al*,

1997). Both the side chains and main chain amides of histone proteins make over 120 contacts with the DNA backbone phosphates (Luger *et al*, 1997; Luger and Richmond, 1998a). Therefore, the histone octamer binds DNA in a non-specific manner (i.e. absence of a binding motif) consistently with its ubiquitous distribution along the genome.

While histones are remarkably conserved throughout evolution, several variant forms have been identified. Examples are the centromere-specific CENP-A (also referred to as CenH3 or Cse4 in *S. cerevisiae*) which is essential for the formation of the kinetochore (Santaguida and Musacchio, 2009) and H2A.Z which is found at transcriptional start sites (Zlatanova and Thakar, 2008) where it promotes RNA Polymerase II recruitment (Adam *et al*, 2001). Interestingly, these alternative histone proteins are restricted to H2A and H3, while no variants of H2B and H4 have been found to date (reviewed in (Malik and Henikoff, 2003; Talbert and Henikoff, 2010)).

Along with histones, the structure of the NCP is also remarkably conserved, with an electron density maps displaying an overall root mean square deviation (r.m.s.d) of only 1.57 Å between *X. laevis* and *S. cerevisiae* (White *et al*, 2001). To date, the structure of more than 25 different NCP has been solved to high resolution (Chakravarthy *et al*, 2005b; Andrews and Luger, 2011), including some containing histone variants (Suto *et al*, 2000; Chakravarthy *et al*, 2005a; Tachiwana *et al*, 2011a; Tachiwana *et al*, 2011b) and post-translationally modified histones (Lu *et al*, 2008). Moreover, the structures of few protein-bound NCPs have also become available, such as the fly protein RCC1 (Makde *et al*, 2010) and the budding yeast Sir3 BAH domain (Armache *et al*, 2011).

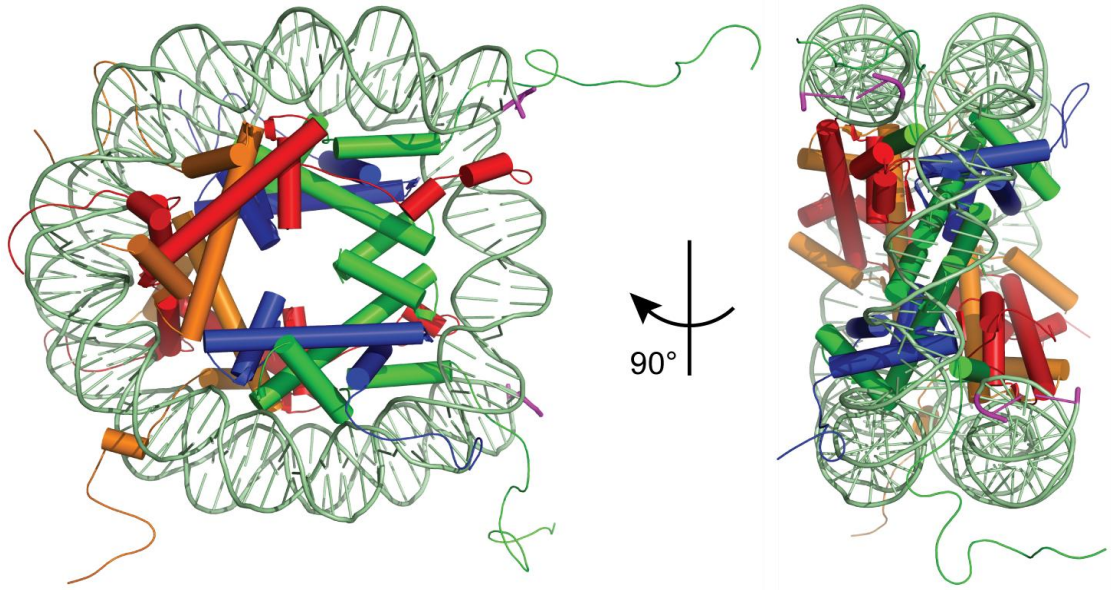

Figure 1 - The nucleosome Representation of the nucleosome core particle (NCP) at 1.9Å resolution solved by X-ray crystallography (PDB 1KX5, (Davey *et al*, 2002)). The DNA double helix is colored in light green, the terminal bases are highlighted in magenta. The histones are labeled as follow: H2A red, H2B orange, H3 green and H4 blue.

1.2 STRUCTURAL ASPECTS OF THE CHROMATIN FIBER

Organization of DNA into arrays of nucleosomes – referred to as chromatin – not only allows for compaction of the genetic information but plays important regulatory roles in DNA replication (Hayashi and Masukata, 2011), genomic stability (Bao, 2011; Greenberg, 2011; Luijsterburg and van Attikum, 2011; Lukas *et al*, 2011) and regulating gene expression (Rando and Winston, 2012), as discussed below. Hereafter, the linear "beads-on-a-string" organization of nucleosomes and linker DNA is referred to as chromatin primary structure. The secondary structure of chromatin corresponds to the non-linear arrangement of nucleosomes resulting from interactions between nucleosomes. Interactions between distant regions of secondary structure are referred to as the tertiary structure of chromatin.

The primary structure of the chromatin fiber corresponds to the unfolded nucleosomal array. Under conditions of low ionic strength, chromatin extracted from cells (i.e. "native") as well as nucleosomal arrays reconstituted with purified components *in vitro* (see below) exist in an extended "beads on a string" configuration, also referred to as the 11 nm fibre (Olins and Olins, 1974; Thoma and Koller, 1977). However, in presence of physiological salt concentration, both native and reconstituted chromatin fibers adopt a secondary structure which further compacts the DNA polymer and can be stabilized by the presence of linker histones H1 and H5 (Thoma *et al*, 1979; Carruthers *et al*, 1998; Hizume *et al*, 2005; Huynh *et al*, 2005; Scheffer *et al*, 2011; Scheffer *et al*, 2012). This second level of compaction involves the non-linear arrangement of nucleosomes (see next section; (Hansen, 2002; Staynov, 2008)) and is thought to recapitulate the compaction of chromatin into the 30 nm fibre observed by electron microscopy (EM) on native starfish sperm and chicken erythrocytes chromatin preparations (Bazett-Jones, 1992; Horowitz *et al*, 1994).

The existence of a defined secondary structure of chromatin *in vivo* is still highly debated and is likely to vary in different cell types, cell-cycle stages and specific genomic locations. Nonetheless, filtered transmission EM studies on mammalian cells *in situ* suggest that the majority of chromatin during interphase is in the form of 11 nm or 30 nm fibres, indicative of an important role for these structures in DNA processes (Dehghani *et al*, 2005). These studies are supported by vitreous sectioning and cryo-EM studies of chicken erythrocyte nuclei, in conditions that avoid most of the artefacts due to sample preparation in standard filtered transmission EM (Scheffer *et al*, 2011; Scheffer *et al*, 2012). It is reasonable to argue that the 30 nm fibre provides the structural basis for further chromatin compaction. However, cryo-EM of vitreous sections and X-ray scattering studies failed to detect 30 nm chromatin fibres in human mitotic chromosomes *in situ* suggesting instead the existence of irregularly arranged and interdigitated nucleosomal fibres that may allow a more flexible organization of the genome (Eltsov *et al*, 2008; Fussner *et al*, 2011; Hansen, 2012; Nishino *et al*, 2012).

Finally, interactions between distant regions of secondary structure define the tertiary structure of chromatin, which involves the loading of non-histone proteins (Moser and Swedlow, 2011) and the extreme DNA density characteristic of the mitotic chromosome (Belmont and Bruce, 1994; Moser and Swedlow, 2011). Folding of the chromatin fiber into secondary and high-order tertiary structures is cooperative and reversible and requires the histone tails (Allan *et al*, 1982; Schwarz *et al*, 1996; Hansen, 2002; Dorigo *et al*, 2003). Indeed, cross-linking studies have shown that the tails of the histone H3 and H4 make both intra- and inter-nucleosomal interactions upon salt-dependent array folding (Zheng *et al*, 2005; Kan *et al*, 2007; Kan *et al*, 2009). While chromatin tertiary structures are still largely unexplained, the effort of many has led to the postulation of two main models for the secondary structure of chromatin discussed in the next section (Figure 2).

1.2.1 PURSUING THE SECONDARY STRUCTURE OF CHROMATIN

Given its potential of regulating DNA accessibility, the packing of nucleosomes into secondary chromatin structure has attracted a lot of attention and, over the years, several models have been proposed for the hypothetical 30 nm fibre. Two models, based on native chromatin preparations, have endured over time. Electron microscopy and X-ray diffraction from oriented samples suggested the existence of an one-start solenoid structure where adjacent nucleosomes are connected by linker DNA bent between them to follow a superhelical path, with about 6 to 8 nucleosomes per turn (Finch and Klug, 1976; Widom and Klug, 1985). However, using a similar approach, other researchers put forward a two-start helix model, in which adjacent nucleosomes are connected by straight linker DNA and follow a "zig-zag" arrangement (Williams *et al*, 1986).

Although the nucleosome is a conserved unit of chromatin, native chromatin is highly heterogeneous as it contains variable DNA sequences, histone composition and modifications, and linker DNA length (van Holde and Zlatanova, 2007). Therefore, researchers have developed *in vitro* reconstitution systems that make use of strong position sequences such as the 5S rDNA sequence (Gottesfeld and Bloomer, 1980) or the synthetic (i.e. not found in nature) Widom-601 sequence (Lowary and Widom, 1998). The consecutive alignment of several Widom-601 sequences strongly positions recombinant histone octamers, generating well-defined, regularly spaced nucleosomal arrays (Dorigo *et al*, 2003; Huynh *et al*, 2005). The use of such a homogeneous array made possible the crystallization of a tetra-nucleosome (Schalch *et al*, 2005). This X-ray structure was combined to the salt-dependent compaction (0.5 mM $MgCl_2$) of a cross-linked 48-mer nucleosomal array (48 x 167 bp Widom-601) studied by EM (Dorigo *et al*, 2004) to model a twisted ribbon structure where "zig-zag" oriented nucleosomes form a two-start crossed-linker helix with a diameter of 25 nm and a nucleosome packing density of 5-6 nucleosome per 11 nm. However, four nucleosomes are not enough to generate a solenoid structure and pair-wise disulfide cross-linking of the 48-mer nucleosomal arrays required two amino acid substitutions in H4 and H2A which may affect nucleosomal packing. Therefore, it is not clear to which extent the model proposed by Richmond and colleagues can be generalized to be *the* secondary structure of chromatin.

On the other hand, Rhodes and colleagues used the positioning Widom-601 sequence to generated long arrays (up to 80 nucleosomes) with linker DNA length from 30 to 90 bp which were compacted in presence of 1-1.6 mM of $MgCl_2$ and the linker histone H5 (Robinson *et al*, 2006; Routh *et al*, 2008). An unequivocal structure could not be determined by EM and cryo-EM measurements. Instead, three classes of fibers were created based on length diameter and nucleosome packing density. Importantly, the biophysical properties of these nucleosomal arrays were directly correlated to the length of the linker DNA used. Arrays characterized by 30-60 bp of linker DNA formed fibers with roughly 33 nm of diameter and a packing density of 11 nucleosomes per 11 nm, while linker DNA length of 70-90 bp led to structures with a diameter of approximately 43 nm and 15 nucleosomes per 11 nm of packing density. The non-linear relationship between the linker DNA length and the dimensions of these fibers are consistent with a one-start solenoid model. However, arrays containing only 20 bp of linker DNA were thinner, with a diameter of roughly 21 nm and a nucleosome packing density of only 6.1 nucleosomes per 11 nm. Importantly, these arrays appeared in a "zig-zag" arrangement consistent with the two-start helix, and their compaction was poorly dependent on the presence of a linker histone (Routh *et al*, 2008). This set of experiments nicely pointed out that the length of the linker DNA has a strong influence on secondary structure formation and dependency on the linker histone (Routh *et al*, 2008).

To summarize, linker DNA length greater than 30 bp favours the formation of two classes of one-start solenoid fibres *in vitro* (Robinson *et al*, 2006). The stabilization into one of these two conformations may be modulated by the abundance of linker histone. On the other hand, linker DNA length shorter than 30 bp conveys a zigzag arrangement of the nucleosomes in a two-start helix which relies more on nucleosome stacking and less on linker histones (Dorigo *et al*, 2004; Schalch *et al*, 2005; Routh *et al*, 2008). While this set of rules helps explaining different secondary structures observed *in vitro*, nucleosomal remodeling and the association of non-histone proteins with the chromatin fiber is likely to impact linker DNA length and chromatin secondary structure *in vivo*.

Genome-wide techniques such as tiling array, ChIP-chip and ChIP-Seq have been used to measure linker DNA lengths genome-wide in *S. pombe* (~12 bp) (Lantermann *et al*, 2010), *S. cerevisiae* (~21 bp) (Yuan *et al*, 2005), *C. elegans* (~29 bp) (Valouev *et al*, 2008) and humans (~54 bp) (Schones *et al*, 2008). However, linker DNA length and nucleosomal positioning and spacing *in vivo* is likely to be tightly regulated by *cis*- as well as *trans*-acting factors ((Bell *et al*, 2011; Rando and Winston, 2012), see below) and may vary at discrete genomic locations. Structural variation within a single chromatin fiber is defined as chromatin "heteromorphism" and is supported experimentally by EM-assisted nucleosome interaction capture (EMANIC) coupled with Monte Carlo simulation (Grigoryev *et al*, 2009) and mesoscale simulation modeling (Schlick and Perisic, 2009). Finally, it is important to keep in mind that loading of non-histone proteins onto the chromatin fiber can play major roles in determining chromatin secondary structure (Li and Reinberg, 2011). *In vivo*, it is very likely that, at a given time within a given chromatin fiber, multiple secondary organizations exist simultaneously (at different loci) conveying variable DNA accessibilities.

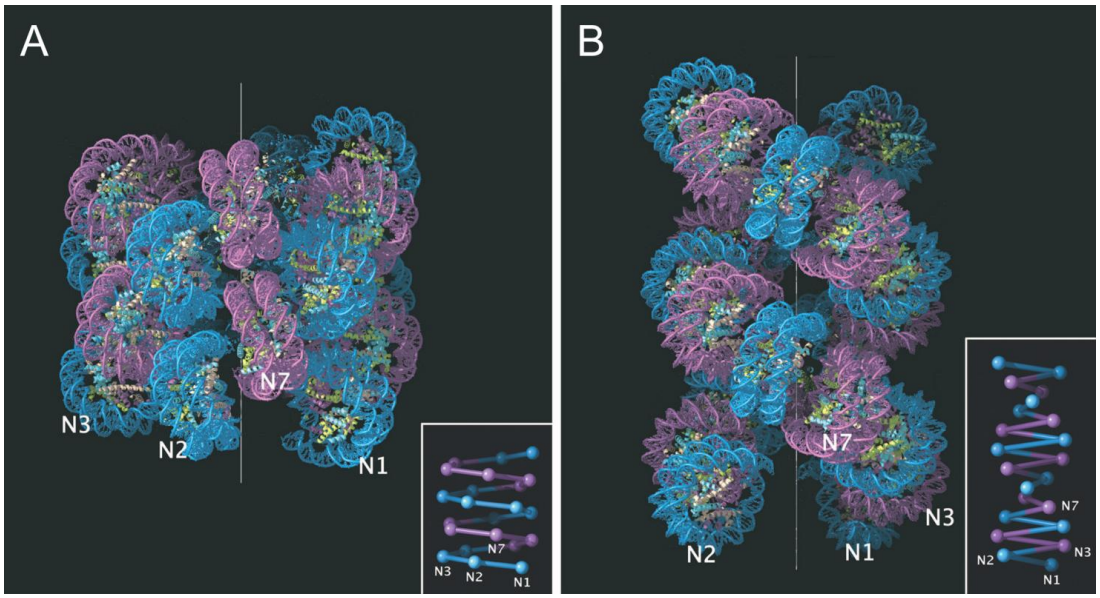

Figure 2 - Models for the 30 nm chromatin fiber (A) Interdigitated one-start helix model based on measurements obtained in (Robinson *et al*, 2006). Alternate helical gyres are colored in marine and magenta. (B) Two-start helical crossed linker model adapted from the model reported in (Schalch *et al*, 2005). Alternate nucleosome pairs are colored in marine and magenta. The positions of the first, second, third, and seventh nucleosome in the linear DNA sequence are marked on both models with N1, N2, N3, and N7. (Insets) Schematic representations of both atomic models showing the proposed DNA connectivity. Adapted from (Robinson *et al*, 2006).

1.3 CHROMATIN REGULATES DNA-TEMPLATED PROCESSES

Packaging of DNA into chromatin hinders DNA accessibility to proteins and thus regulate cellular processes that require DNA as a template. However, nucleosomes and chromatin are not static entities, and modulation of chromatin organization – at the primary, secondary and tertiary levels – is likely to have important regulatory functions.

One of the most studied regulatory roles for chromatin concerns DNA transcription. The primary structure of chromatin – the nucleosomal organization of DNA – plays a fundamental role in regulating gene expression. The next sections will cover some of the crucial experiments that linked histone proteins and nucleosomes to gene expression *in vitro* and *in vivo*, with a particular focus on *S. cerevisiae*. The implication of secondary and tertiary high-order structures as well as the nuclear organization of the genome in regulating gene expression is less well understood and will be discussed later in the thesis (see (Akhtar and Gasser, 2007; Sexton *et al*, 2007; Arib and Akhtar, 2011; Geyer *et al*, 2011; Szerlong and Hansen, 2011; Zimmer and Fabre, 2011; Albert *et al*, 2012; de Wit and de Laat, 2012) and references cited therein).

1.3.1 TRANSCRIBING THROUGH CHROMATIN: EARLY STUDIES *IN VITRO* AND CURRENT VIEW

Early studies *in vitro*, based on bacterial and eukaryotic systems, demonstrated that the organization of DNA into a chromatin template impairs transcription.

In a pioneering study, *in vitro* assembled chromatin prepared from SV40 DNA and calf thymus histones inhibited for transcription by the *E. coli* RNA polymerase. Inhibition seemed to occur at both the initiation and elongation levels and depended on the number of nucleosomes formed in the reconstituted complexes (Wasylyk *et al*, 1979). However, it could not be excluded that the mRNA production observed using under-reconstituted templates was actually a result of transcription of contaminating free DNA. In a subsequent study, transcription by human RNA polymerase II from the adenovirus-2 major late promoter was tested *in vitro* using HeLa nuclear extracts and circular DNA templates onto which varying numbers of nucleosomes had been reconstituted with Xenopus oocyte extracts (Knezetic and Luse, 1986). This system was better controlled (Glikin *et al*, 1984), as manipulation of ATP and Mg^{2+} levels in the reconstitution reaction allowed the authors to fine-tune the amount of assembled nucleosomes (Knezetic and Luse, 1986). Importantly, no initiation occurred on reconstituted templates with more than two-thirds of the "physiological" nucleosome density (considered to be ~ 40 bp of linker DNA (Kornberg, 1977)), whereas templates with less than one-third of the physiological nucleosome density were transcribed as efficiently as naked DNA (Knezetic and Luse, 1986). These studies showed that the organization of DNA in chromatin can block transcription depending on the amount of nucleosomes found in the template, probably reflecting the likelihood to find a nucleosome on the promoter (see below).

Importantly, further experiments demonstrated the existence of one or more components in cell extracts that were able to activate transcription through chromatin – on normally refractory conditions – if incubated with DNA prior to reconstitution of the chromatin template (Matsui, 1987; Workman and Roeder, 1987). One of these components is the TATA box-binding factor TFIID which was sequestered into the nucleosome-assembled templates and allowed for transcriptional initiation by RNA polymerase II (Workman and Roeder, 1987).

At this point it was not clear whether the RNA polymerase could transit through nucleosomes or had the ability to evict or displace histones from the chromatin fiber. In conditions that allow for transcriptional initiation, purified RNA polymerase II elongation partially removed histones from a DNA template (adenovirus-2 promoter) as determined by restriction enzyme digestion and a gel shift observed in the electrophoretic mobility of the transcribed template (Lorch *et al*, 1987). However, combining sedimentation velocity, electrophoretic mobility and digestion with restriction nucleases it was shown that the bacteriophage T7 polymerase was able to transcribe through nucleosomes without removing the histones from the DNA template (based on the murine α-globin gene cloned with a T7 promoter) (Kirov *et al*, 1992).

Building on these early observations *in vitro* a great body of work has refined our understanding of how the primary structure of chromatin relates to transcription. The emerging picture is one in which histone release/exchange is strongly influenced by the rate of RNA polymerase II activity. The H3 and H4 histones seem to be more stably associated with DNA while the H2A-H2B heterodimer has a higher likelihood to be released (Kulaeva *et al*, 2007; Hodges *et al*, 2009; Kulaeva *et al*, 2009; Kulaeva *et al*, 2010). Similar results were observed *in vivo* (Lee *et al*, 2004; Schwabish and Struhl, 2004; Thiriet and Hayes, 2005; Dion *et al*, 2007). Interestingly the passage of the RNA polymerase seems to displace nucleosomes "backwards" in the direction of the promoter both *in vitro* (Hodges *et al*, 2009) and *in vivo* (Weiner *et al*, 2010). It is important to keep in mind that, while nucleosomes *per se* pose a barrier to DNA transcription (Bondarenko *et al*, 2006; Hodges *et al*, 2009), the post-translational modification of histones can facilitate the recruitment of additional factors such as ATP-dependent chromatin remodelers and histone chaperones which highly facilitate the passage of RNA polymerase II *in vivo* (see below and (Bell *et al*, 2011; Luse *et al*, 2011; Luse and Studitsky, 2011; Kremer *et al*, 2012; Rando and Winston, 2012)). For instance, the conserved histone chaperone FACT (FAcilitates Chromatin Transcription) is critical for nucleosome reorganization during transcription – as well as DNA replication and repair ((Orphanides *et al*, 1998; Orphanides *et al*, 1999; Brewster *et al*, 2001; Formosa *et al*, 2001; Belotserkovskaya *et al*, 2003; Saunders *et al*, 2003) and reviewed in (Belotserkovskaya and Reinberg, 2004; Winkler and Luger, 2011; Rando and Winston, 2012)). FACT is thought to reversibly destabilize nucleosomes to facilitate both transcription initiation and the passage of RNA polymerase II through the disruption of core histone-histone and histone-DNA interactions (reviewed in (Belotserkovskaya and Reinberg, 2004; Winkler and Luger, 2011; Rando and Winston, 2012)). Finally, it is clear now that nucleosomal positioning at the gene promoter, as opposed to the coding region, is the key determinant for gene expression by modulating DNA accessibility to upstream regulatory factors (see below and (Bell *et al*, 2011; Rando and Winston, 2012)).

1.3.2 PIONEERING STUDIES IN *S. CEREVISIAE*: THE IMPORTANCE OF HISTONES IN GENE REGULATION *IN VIVO*

Much of the knowledge on how chromatin structure controls gene expression comes from pioneering studies in *S. cerevisiae*. While higher eukaryotes, such as flies, mice and humans, have very high copy numbers of histone genes

(50-100 copies) (Lifton *et al*, 1978; Marzluff *et al*, 2002), *S. cerevisiae* has only 2 genes encoding for each of the four histone proteins: H2A, H2B, H3 and H4. The low histone genes copy number, coupled with the ease of its genetic manipulation, promoted budding yeast as a formidable tool to study histone function *in vivo* by mutational analysis.

Experiments in yeast led to the discovery that histone genes are essential for viability (Rykowski *et al*, 1981). Two strains were constructed individually lacking either of the genes coding for the histone H2B (*HTB1* and *HTB2*). While mutant strains survived as haploids and heterozygous or homozygous diploids, no spores could be derived, from a heterozygous diploid, that were mutated for both H2B genes, thus unable to produce the histone protein H2B (Rykowski *et al*, 1981). Early studies in yeast also led to the discovery that histone levels play important regulatory role for gene expression *in vivo*. Indeed, when histone H4 levels were decreased, by means of a glucose-repressible *GAL1-10* promoter system, the *PHO5* gene was activated under normally repressive conditions and the nucleosomal distribution at the *PHO5* promoter was greatly altered as probed by micrococcal nuclease digestion (Han *et al*, 1988). Similar results were also obtained for engineered *CYC1* and *GAL1* promoters if the upstream activator sequences (UAS) – which normally mediates glucose-dependent repression – were deleted (Han and Grunstein, 1988). Moreover, nuclease accessibility studies of the *PHO5* and the *GAL1-10* genes established the principle that nucleosomes occupy promoters in repressive conditions but are removed following induction (reviewed in (Lohr, 1997)). These studies showed that the nucleosome is a fundamental determinant of DNA accessibility and transcription. Consistently, the abundance of histone proteins in yeast is highly regulated on many levels (see (Rando and Winston, 2012) and references cited therein). This includes, but is note restricted to, the level of saturation of chaperones with histones (Osley and Lycan, 1987; Dollard *et al*, 1994; Eriksson *et al*, 2012) and regulation of histone protein stability (Gunjan and Verreault, 2003; Singh *et al*, 2009).

In addition to the core histones H2A, H2B, H3 and H4, *S. cerevisiae* has three single copy genes coding for an equal number of additional histones. The histone H1 (*HHO1*), is not required for cell viability and plays a limited role in regulating chromatin structure and gene expression (Patterton *et al*, 1998; Levy *et al*, 2008; Schafer *et al*, 2008; Yu *et al*, 2009). The histone Cse4 (*CSE4*) is a centromere-specific histone H3 variant essential for cell viability and crucial for centromere function (Meluh *et al*, 1998; Furuyama and Biggins, 2007). Finally, the histone H2A.Z (*HTZ1*) is a histone H2A variant enriched at genes promoters, but whose specific role is still under examination (Rando and Winston, 2012).

1.3.3 NUCLEOSOME OCCUPANCY AND TRANSCRIPTION IN YEAST

Genome-wide analysis of nucleosome occupancy in budding yeast has revealed important features of gene regulation (Kaplan *et al*, 2009; Zhang *et al*, 2009; Brogaard *et al*, 2012). From a global perspective, genes in yeast can be divided in two classes: “housekeeping” and “stress” genes. “Housekeeping” genes are associated with biomass production and are strongly expressed during rapid cellular growth. These genes, often regulated by TFIID binding, are characterized by a nucleosome-depleted region (NDR) located upstream of the coding region and bordered by two highly positioned nucleosomes often containing the histone variant H2A.Z (Rando and Winston, 2012). These features are well conserved from yeast to man (Bell *et al*, 2011). On the other hand, “stress” genes are poorly expressed in normal conditions but become rapidly induced in stress conditions in a manner often dependent on the SAGA complex. The promoter architecture at these genes is rather variable and, while associated with delocalized nucleosomes, a clear NDR region is less obvious (Rando and Winston, 2012). This may result from the specific mechanism of regulation of different stress genes that evolved to respond to very characteristic conditions. Parallel to this global classification several *cis*- and *trans*-factors (see below) are responsible for conveying specific chromatin structures at discrete genomic location by modulating nucleosomal positioning.

1.3.3.1 NUCLEOSOME POSITIONING: *CIS*-REGULATORS ARE NOT ENOUGH

The nucleosome does not require a binding motif on the DNA, consistent with its general packing function. However, the DNA polymer has to wrap tightly around the histone octamer and the propensity of a given DNA sequence to bend affects the tendency of a nucleosome to occupy that position ((Drew and Travers, 1985; Travers and Drew, 1997; Lowary and Widom, 1998) and reviewed in (Travers, 2004; Segal and Widom, 2009; Travers *et al*, 2012)). Since the biophysical properties of the DNA molecule rely on its sequence, DNA sequence indirectly affects nucleosome positioning. Of particular relevance, is the dA/dT content. Poly(dA/dT) stretches have a non-canonical conformation of the double-helix that renders them intrinsically stiff and less prone to be bent (Nelson *et al*, 1987). On the other hand, dA/dT dinucleotides spaced in 10 bp intervals create an intrinsic curvature which facilitates nucleosome assembly (Anselmi *et al*, 1999; Thastrom *et al*, 1999).

In yeast, poly(dA/dT) stretches – which disfavor nucleosome formation – are enriched in NDR *in vivo* (Kaplan *et al*, 2009). However, intrinsically bendable DNA sequences poorly explain the nucleosome positioning observed genome-wide (Kaplan *et al*, 2009; Zhang *et al*, 2009). The analysis of specific genes also revealed a variable dependency on the DNA sequence. For example, the chromatin structure observed at the *HIS3* promoter *in vivo* could be recapitulated *in vitro* using purified DNA and recombinant histones (Sekinger *et al*, 2005). On the other hand, this was not the case for the *PHO5* promoter region (Korber *et al*, 2004). Strikingly, the *in vivo* nucleosomal organization at the *PHO5* promoter

could be reproduced *in vitro* upon addition of whole-cell extract, indicating that *trans*-acting factors were responsible for promoter nucleosomal organization at this specific locus (Korber *et al*, 2004). A recent study showed that, outside of promoter regions, nucleosomal occupancy relies mostly on ATP-dependent processes (Zhang *et al*, 2011).

Indeed, a large family of ATP-dependent chromatin remodelers works in concert with histone chaperones and histone modifying enzymes to regulate chromatin structure and DNA-templated processes, including gene expression (see below and (Clapier and Cairns, 2009; Das *et al*, 2010; Hondele and Ladurner, 2011; Rando and Winston, 2012)). Eukaryotic cells contain four different families of chromatin remodeling complexes: Swi/Snf, Iswi, Chd, and Ino80 (Clapier and Cairns 2009). Although there is some variation in the precise protein composition of these multi-protein complexes in different organisms, individual families are well conserved from yeast to man. All four families share a similar ATPase domain and use ATP hydrolysis to alter histone-DNA contacts. However, each family member bears unique flanking domains and associated subunits specialized for particular purposes and biological contexts (see (Clapier and Cairns, 2009; Conaway and Conaway, 2009; Morrison and Shen, 2009; Rando and Winston, 2012) and references cited therein).

1.3.3.2 TRANSCRIPTION FACTOR BINDING AND NUCLEOSOME REMODELING

Binding of transcription factors is fundamental to gene expression. Whilst in *S. cerevisiae* transcription factor binding sites often lie within nucleosome-depleted regions (Segal *et al*, 2006), binding of transcription factors to nucleosomal DNA can directly displace histones *in vitro* (Workman and Kingston, 1992). In addition, transient unwrapping of the DNA from the histone octamer – referred to as "nucleosomal breathing" – can expose transcription factor binding sites (and other DNA sequences) covered by a nucleosome *in vitro* (Li *et al*, 2005; Poirier *et al*, 2009) and *in vivo* (Bucceri *et al*, 2006).

Interestingly, binding of a transcription factor to a binding site within a NDR can lead to the recruitment of histone modifying and nucleosome remodeling machineries that expose secondary binding sites. In the case of the *PHO5* promoter, which is induced by low levels of phosphate, the Pho4 transcription factor binds first to an accessible site and recruits three histone acetyltransferases (NuA4, Gcn5 and Rtt109; see below), two nucleosome-remodeling complexes (Swi/Snf and Ino80) and a histone chaperone (Asf1) to free a secondary binding site and convey full activation of the *PHO5* promoter ((Almer and Horz, 1986; Almer *et al*, 1986) and reviewed in (Rando and Winston, 2012)). This and other studies (e.g. on the *GAL1-10* promoter) highlight the key role of nucleosomal positioning and chromatin structure dynamics in regulating gene expression (reviewed in (Rando and Winston, 2012)). Building on the Pho4 example, the role of "pioneer" transcription factors that bind DNA first and "open up" the local chromatin structure to allow binding of further activators is now considered a broadly conserved phenomenon in eukaryotes (Magnani *et al*, 2011; Zaret and Carroll, 2011).

1.3.3.3 ATP-DEPENDENT NUCLEOSOME REMODELING AT PROMOTERS

A recent *in vitro* reconstitution study showed that ATP-dependent remodeling explains most of the nucleosome positioning observed outside of gene promoters (Zhang *et al*, 2011). However, as introduced above with the example of the *PHO5* gene, ATP-dependent nucleosome remodeling plays also an important role in determining chromatin structure and DNA accessibility at promoters. A well-characterized factor is the essential SWI/SNF-family member RSC (Remodels the Structure of Chromatin), an ATP-dependent nucleosome remodeling complex (Cairns *et al*, 1996). RSC is required for activation of many genes in *S. cerevisiae* (Parnell *et al*, 2008). RSC seems to synergize with intrinsic "anti-nucleosomal" DNA sequences (high A/T content) to generate NDR at the 5' of genes (Hartley and Madhani, 2009). In the other hand, another ATP-dependent nucleosome remodeling complex, Isw2, has been shown to play the opposite role (Whitehouse *et al*, 2007). Isw2 seems to slide nucleosomes into unfavorable A/T rich sequences to inhibit aberrant transcription from canonical and cryptic promoters (Whitehouse *et al*, 2007).

The emerging picture is one where DNA sequence can favor the formation of NDR, however the actual position of nucleosomes is highly dependent on *trans*-acting factors such as transcription factors and ATP-dependent remodeling complexes, which ultimately define transcriptional states (Zaugg and Luscombe, 2012).

1.4 THE YEAST NUCLEOSOME

The X-ray crystal structure of a NCP reconstituted with recombinant *S. cerevisiae* histones has been solved at 3.1 Å resolution (White *et al*, 2001). Importantly, the overall architecture of the histone octamer and the residues that directly contact the DNA in *S. cerevisiae* are unchanged compared to the available structural information for the NCP of higher eukaryotes (Luger *et al*, 1997; Harp *et al*, 2000). However, the yeast nucleosome exhibits some structural particularities that may affect chromatin organization in this organism. Two amino acid changes within the H2A-H2B dimeric interface (H2A-Q42 and H2B-A85, in yeast) reduce the surface between two H2A-H2B dimers from 150 to 90 $Å^2$ (White *et al*, 2001). Weaker interaction in this interface can affect the stability of the yeast octamer and indeed, salt-dependent and thermal unfolding show a less constrained structure of the yeast nucleosome as compared to higher eukaryotes (Lee *et al*, 1982; Pineiro *et al*, 1991).

Other structural differences between the yeast and the *X. laevis* octamer reside in the C-terminal regions of H2A and H2B. The C-terminus of H2A contains a well ordered helix which is exposed on the surface of the nucleosome. While playing a minor role in the context of the *X. laevis* octamer, this C-terminal H2A helix forms essential inter-nucleosomal contacts in the crystal packing of the yeast nucleosome (White *et al*, 2001). Moreover, in yeast, H2B T128 and Q129 form hydrogen bonds with H3 K121 and K125 of a neighboring nucleosome but none of these residues are conserved in *X. laevis*. This results in an altered nucleosomal packing (in the crystal lattice) which prevents the basic H4 N-terminal tail of a yeast nucleosome to interact with the acidic patch on the H2A-H2B surface of a neighboring nucleosome (Luger *et al*, 1997; White *et al*, 2001). Instead, the H4 N-terminal tail in yeast makes crystal contacts with the DNA molecule of a neighboring nucleosome (White *et al*, 2001). Nonetheless, it is very likely that contacts within the crystal lattice are not descriptive of the situation *in vivo* (Finch *et al*, 1981). Consistent with is crucial role in regulating chromatin processes (see below), the H4 N-terminal tail is highly conserved and given that the acidic patch on the H2A-H2B surface is largely maintained as well, this raises the intriguing possibility that the interaction partners of the H4 N-terminal tail may be modulated *in vivo*, for example by histone post-translational modifications or loading of non-histone proteins (e.g. Sir3 BAH; see section - Is the BAH domain involved in nucleosomal stacking?).

1.5 REGULATION OF GENE EXPRESSION BY HISTONE MODIFICATIONS

It is now clear that post-translational modification of histones is a key factor modulating the dynamic organization of the chromatin fiber. This section outlines the modification of histones and its broad role in regulating chromatin processes. The specific role of histone modifications in regulating silent chromatin in *S. cerevisiae* will be presented in great detail below (see section - Histone modifications regulates yeast silent chromatin).

In the early 1960s, pioneering studies from Vincent Allfrey showed that histones can be acetylated and methylated "very probably after completion of the polypeptide chain" and that "acetylation in particular, may affect the capacity of the histones to inhibit ribonucleic acid synthesis *in vivo*" (Allfrey *et al*, 1964). We now know that Vincent Allfrey was largely right and that histone proteins are subjected to a wide range of post-translational modification bearing regulatory functions (Bannister and Kouzarides, 2011; Tan *et al*, 2011).

An intensively studied histone modification – or "mark" – is the acetylation of lysines which is carried out by histone acetyltransferases (HAT) transferring an acetyl group from acetyl-CoA to the ε-amino group of lysines lying both on the nucleosomal surface and the tails of already assembled nucleosomes (type-A; e.g. MYST, *Sc*Sas2) (Ehrenhofer-Murray *et al*, 1997; Pillus, 2008) or newly synthesized histones (type-B; e.g. p300/CBP, *Sc*Rtt109) (Driscoll *et al*, 2007; Wang *et al*, 2008b; D'Arcy and Luger, 2011). Acetylation of lysines is a very dynamic process, and this histone modification can be removed by the action of histone deacetylases (HDAC). Based on homology, HDACs can be grouped in four classes: class I is related to *Sc*Rpd3 (Taunton *et al*, 1996), class II is related to *Sc*Hda1(Yang and Seto, 2008), class III is also referred to as "Sirtuins" (founded by the NAD-dependent deacetylase *Sc*Sir2; see below) (Blander and Guarente, 2004; Toiber *et al*, 2011) and class IV whose only member is HDAC11 (Yang and Seto, 2007). Similarly to histone acetylation, the phosphorylation of histones is an important and highly dynamic process. It occurs on serines, threonines and tyrosines, predominantly but not exclusively on the N-terminal histone tails (Bannister and Kouzarides, 2011). Histone phosphorylation is carried out by kinases which transfer a phosphate group from ATP to the hydroxyl group of the target amino acid side chain and is removed by phosphatases (Oki *et al*, 2007; Banerjee and Chakravarti, 2011).

While acetylation neutralizes the positive charge of the lysine's side chain, histone methylation, which mainly occurs on lysines and arginines, does not alter the charge of histone proteins but adds an adduct. Since the discovery of SUV39, a histone lysine methyltransferase (HMT; which targets H3K9) (Rea *et al*, 2000), many HMTs have been identified. All known HMTs use S-adenosylmethionine (SAM) as donor to transfer a methyl group to the ε-amino group of lysine residues. Whereas the large majority of HMTs have a SET domain – named for its concurrent occurrence in the *Drosophila* *S*u(var), *E*(Z) and *T*rithorax genes – and target histone tails, an important exception is the Dot1 (disruptor of telomeric silencing 1) enzyme. Dot1 is conserved from yeast to man, lacks a SET domain and methylates H3K79 on the core nucleosomal surface (Feng *et al*, 2002; Ng *et al*, 2002; van Leeuwen *et al*, 2002). It is unclear why Dot1 is structurally different from all others HMTs, but perhaps it reflects the relative inaccessibility of its substrate H3K79. Importantly, unlike acetylation, HMT enzymes can methylate a target lysine to a specific degree: mono-, di- or tri-methylation. This specificity is an intrinsic property of the enzyme and is originated by the architecture of the enzyme's catalytic pocked as elegantly shown by X-ray crystallography (Cheng *et al*, 2005; Collins *et al*, 2005). In addition to lysine residues, arginine can be mono-, symmetrically or asymmetrically di-methylated (reviewed in (Bedford and Clarke, 2009; Lan and Shi, 2009; Ng *et al*, 2009). All arginine methyltransferases use SAM to transfer a methyl group to the ω-guanidino group of arginine residues and major histone arginine methyltransferases are PRMT1, 4, 5 and 6 (Bedford and Clarke, 2009; Wolf, 2009). Both lysine and arginine methyltransferases have the SAM-binding pocket on one face of the enzyme and the peptidyl acceptor channel on the opposite face. This suggest that the SAM molecule and the histone substrate come together from opposing sides of the enzyme (Copeland *et al*, 2009). For long time, histone methylation was thought to differ from acetylation and phosphorylation, given its inability to be reverted by an enzymatic activity. We now know that several histone demethylases exist (Tsukada *et al*, 2006; Hou and Yu, 2010). All

histone demethylases contain a catalytic "jumonji" domain with the exception of LSD1 which utilizes Flavin Adenine Dinucleotide (FAD) as a co-factor for demethylation (Shi *et al*, 2004; Tsukada *et al*, 2006; Mosammaparast and Shi, 2010).

Histone proteins can also be modified by the covalent attachment of an entire protein: the 76 amino acids long ubiquitin or the SUMO (small ubiquitin-like modifier) protein. Histone ubiquitination and sumoylation follow the typical E1-activacting, E2-conjugating and E3-ligating enzymatic pathway and in the case of histones mono-ubiquitination and mono-sumoylation seem most relevant (Wright *et al*, 2012). Another very dramatic way of modifying histone tails is to cleave them off – referred to as histone tail clipping – a process described so far only for the N-terminus of H3. First identified in *Tetrahymena* (Allis *et al*, 1980), this presumably irreversible way of altering the nucleosome has also been found in yeast (Santos-Rosa *et al*, 2009) and mouse (Duncan *et al*, 2008).

Other histone post-translational modification include: deimination, propionylation, ADP ribsoylation, butyrylation, formylation, citrullination, crotonylation and histone proline isomerization ((Tan *et al*, 2011) and reviewed in (Bannister and Kouzarides, 2011)). Notably through the advance of mass spectrometry-based proteomics a multitude of histone modifications have been discovered (Tan *et al*, 2011), yet the functional importance of most of these remains to be addressed. Modification of histones can alter the structural organization of chromatin *per se* or function as docking sites for *trans*-acting factors.

1.5.1 DIRECT REGULATION OF CHROMATIN STRUCTURE BY HISTONE MODIFICATIONS

Acetylation of lysines reduces the positive charge of histone proteins, decreasing the possibility of compacting the chromatin fiber through electrostatic interactions. The N-terminal tails of the histones H3 and H4 can be heavily acetylated at positions including H3K9, H3K14, H3K18, H4K5, H4K8, H4K16 and H4K12 (Kouzarides, 2007). Consistent with a role in "relaxing" the chromatin fiber, multiple acetyl marks are enriched at promoters of active genes, presumably facilitating the access to transcription factors (Wang *et al*, 2008c). Importantly, the acetylation of the single lysine 16 within the tail of the histone H4 (H4K16) is sufficient to inhibit compaction of the chromatin fiber *in vitro* (Shogren-Knaak *et al*, 2006), presumably by disrupting the electrostatic interaction between the positive H4 tail and a negative patch of the H2A-H2B dimer on the adjacent nucleosome (Luger *et al*, 1997).

While acetylation of H4K16 seems to affect inter-nucleosomal interaction, single particle analysis of nucleososmes acetylated on H3K56 have shown that the H3K56ac mark increases the spontaneous (but transient) unwrapping of the DNA from the histone octamer, referred to as nucleosomal "breathing" (Neumann *et al*, 2009).

A more dramatic effect was observed for chemically engineered ubiquitination of H2BK120 which inhibits salt-dependent compaction of a nucleosomal array *in vitro*, as tested by sedimentation velocity (Fierz *et al*, 2011). Importantly, ubiquitination of H2BK120 and acetylation of H4K16 were synergistic and surprisingly, this effect could not be reproduced by substituting ubiquitin with the ubiquitin-related protein Hub1 indicating that decreased compaction by ubiquitin may not be merely a steric effect (Fierz *et al*, 2011).

The H3 histone tail promotes both intra- and inter-nucleosomal interactions upon salt-dependent array folding (Zheng *et al*, 2005; Kan *et al*, 2007). Therefore, removal of the first 21 amino acid by histone tail clipping likely favours a less compacted chromatin structure.

While understanding the impact of histone modifications on the dynamic properties of nucleosomal arrays *in vitro* helps defining their role in the genome, it is important to consider that chromatin is associated with a multitude of proteins *in vivo* and a growing amount of studies directly links histone modifications to the regulation of protein-chromatin interactions.

1.5.2 HISTONE MODIFICATIONS REGULATE THE BINDING OF CHROMATIN FACTORS

As described above, some of the direct effects of histone modification on chromatin structure can be elegantly recapitulated *in vitro*, in particular the direct effect of histone modifications on chromatin structure. However, *in vivo* histone modifications play an additional and crucial role in regulating protein-chromatin interaction.

Specialized modules such as chromodomains, PHD domains, Tudor domains and WD40 domains have been shown to selectively bind methylated histones while bromodomains bind to acetylated histones (Taverna *et al*, 2007). These domains are very often included into multivalent proteins and complexes which can directly alter the structure of chromatin or further modify histones in the genomic location they bind. Indeed, there is an important level of cross-talk between different histone modifications (Kouzarides, 2007). For example, in *S. cerevisiae*, methylation of H3K4 by *Sc*COMPASS and methylation of H3K79 by *Sc*Dot1 both depend upon ubiquitination of H2BK123 by *Sc*Rad6/Bre1 (Lee *et al*, 2007). Additionally, the H3K4me3 mark recruits *Sc*Yng1, a PHD finger-containing subunit of the NuA3 complex which in turn acetylates H3K14 (Taverna *et al*, 2006).

Tri-methylated H3K4 also appears to directly recruit chromatin remodelling activities. For instance, the H3K4me3 mark can be bound by the tandem chromodomains within CHD1, an ATP-dependent chromatin remodelling complex capable of displacing nucleosomes (Sims *et al*, 2005). Similarly, histone acetylation at promoter regions recruits the SWI/SNF

chromatin remodeler complex through the bromodomain-containing subunit Swi2/Snf2, facilitating gene activation (Hassan *et al*, 2002). However, histone modifications do not always function as docking site for other factors, but can also have a repulsive role. For instance, the H3K4me3 mark, which is associated with gene expression, can inhibit the binding (to the H3 N-terminal tail) of the NuRD complex, a transcriptional repressor with both chromatin remodelling and HDAC functions (Zegerman *et al*, 2002) and the Polycomb repressive complex 2, PRC2 (Schmitges *et al*, 2011). Thus, in addition to altering the chromatin structure *per se*, histone modifications can stimulate or disfavour the deposition of additional marks and recruit chromatin remodelling complexes to modulate gene expression.

1.6 UNEQUAL DISTRIBUTION OF HISTONE MODIFICATIONS LABELS DIFFERENT GENOMIC REGIONS

It has been appreciated for some time that the conformation of chromatin within the nucleus is not uniform and can be differentiated using dyes such as DAPI (4',6-diamidino-2-phenylindole). DAPI stains the DNA (preferring AT-rich regions) and yielded fluorescence images showing characteristic regions of high and low density, which occupy distinct regions of the nucleus. This was confirmed by uranyl acetate staining and EM techniques (Monneron and Bernhard, 1969; Olins and Olins, 1974; Belmont and Bruce, 1994; Dehghani *et al*, 2005). This observations have inspired a classic model in which dense staining compartments – referred to as "heterochromatin" – reflect higher levels of chromatin compaction and reduced DNA accessibility, whereas lighter staining regions – referred to as "euchromatin" – reflect lower levels of chromatin compaction which facilitates DNA accessibility.

However, the bimodal classification into euchromatin and heterochromatin is likely to be too simplistic (van Steensel, 2011). Indeed, the use of integrative computational analysis to map the binding of non-histone proteins to chromatin and the location of histone modifications genome-wide has revealed that both euchromatin and heterochromatin can be divided in 2-3 subgroups each, at least in fly and man ((Filion *et al*, 2010; Kharchenko *et al*, 2011; Ram *et al*, 2011) and reviewed in (van Steensel, 2011)). Moreover, the actual contribution of chromatin compaction in the repression of gene expression is still debated (Bell *et al*, 2011; van Steensel, 2011). When DNA accessibility, in *C. elegans* or yeast, was probed *in vivo* by means of the DNA adenine methyltransferase (DAM) method, euchromatin-located DNA was barely 2-fold more accessible compared to heterochromatic regions when averaged over a population of cells (Chen *et al*, 2005; Sha *et al*, 2010). Whether this relatively small difference is due to dynamic changes within a population and whether it would be sufficient to prevent efficient transcription is still unclear. Whilst our understanding of chromatin domains *in vivo* has been refined and will continue to evolve, the "euchromatin" and "heterochromatin" nomenclature has survived to date.

1.6.1 HISTONE MODIFICATIONS WITHIN EUCHROMATIN

Euchromatin is not all the same but, based on known regulatory protein binding profiles genome-wide, can be divided into at least three groups (van Steensel, 2011). While the first two groups are very similar and approach the classical definition of euchromatin (see below), a third group is specifically marked with the heterochromatic proteins HP1 and SUV39. There is still much to learn about this type of chromatin (Kwon and Workman, 2011; van Steensel, 2011).

Euchromatin is the first to replicate during S phase and this correlates with high levels of H4K16 acetylation (Bell *et al*, 2010). The histone H4 is highly acetylated at active genes especially in proximity of the transcriptional start site (TSS) which also bears the H3K4me3 mark (Bell *et al*, 2011). In contrast, transcriptional enhancers are enriched with mono-methylated H3K4 (Barski *et al*, 2007) and the H3K36me3 mark is highly enriched within the body of active genes (Bannister *et al*, 2005). Interestingly, it has been recently shown that H3K4me3 and H3K36me2/3 significantly inhibit the methylation of H3K27 by PRC2 (see below) in actively transcribed regions (Schmitges *et al*, 2011).

Finally, within euchromatin, active marks (H3K4me3 and H3K36me3) are not homogenously distributed but often seem to be enriched at discrete regions (Barski *et al*, 2007). Spatial clustering of these regions in so-called transcription "factories" is an attractive hypothesis still under debate (Sutherland and Bickmore, 2009; Cook, 2010).

1.6.2 TRANSCRIPTIONAL REPRESSION WITHIN HETEROCHROMATIN IN HIGHER EUKARYOTES

In the light of what discussed above, it is important to note that active genes can also be found in chromatin regions traditionally considered as heterochromatic ((Wakimoto and Hearn, 1990; Clegg *et al*, 1998) and reviewed in (van Steensel, 2011)). The next paragraph outlines the molecular machineries responsible for transcriptional repression in heterochromatic regions of higher eukaryotes, whereas silent chromatin in *S. cerevisiae* will be described in detail in the next section.

It is currently understood that different machineries are involved in various types of gene repression – or silencing – and act on a diverse set of genes (Beisel and Paro, 2011). Genes that are differentially expressed through development and differentiation are often regulated by the Polycomb family (reviewed in (Muller and Verrijzer, 2009; Morey and Helin, 2010; Sawarkar and Paro, 2010; Margueron and Reinberg, 2011)). Given that the subset of genes silenced by Polycomb factors varies among different cells and throughout development, Polycomb associated silencing is often

referred to as "facultative" heterochromatin. The molecular mechanism for Polycomb-mediated silencing is still a very active field of research. The Polycomb repressive complex 2 (PRC2) catalyses the deposition of the H3K27me3 mark while PRC1 ubiquitinates H2AK119 which may impede transcriptional elongation (Stock *et al*, 2007). Intriguingly, PRC1 has been shown to compact a nucleosomal array *in vitro* with potential repercussion on DNA accessibility (Francis *et al*, 2004). The exact mechanism by which Polycomb-mediated silencing can be maintained through cell division is still unclear, but it may involve the recruitment of PRC2 by H3K27me3 itself (Hansen *et al*, 2008) and the somehow persistent interaction of PRC1 with chromatin during DNA replication (Lo *et al*, 2012).

In contrast to Polycomb-associated domains, genomic locations commonly silenced among different cell types, such as centromeres and telomeres, are organized into so-called "constitutive" heterochromatin. This class of heterochromatin is characterized by histone hypoacetylation, methylation of H3K9 and H4K20 and recruitment of the heterochromatin protein 1 (HP1). Most eukaryotes have several HP1 isoforms that carry out different functions, also associated with euchromatic gene expression (Kwon and Workman, 2011). HP1 dimers bind to H3K9me2/3 through their chromodomains but also interact with the SUppressor of Variegation 3-9 (SUV39), a H3K9 methyltransferase (Bannister *et al*, 2001; Lachner *et al*, 2001). Following association of HP1 dimers with H3K9me3-containing chromatin, HP1 is thought to oligomerize through multiple self-interaction interfaces, involving both its chromo- and chromoshadow- domains, and bring together nearby nucleosomes (Canzio *et al*, 2011). Therefore, HP1 is thought to repress gene expression by limiting the DNA accessibility. The observation that HP1 interacts H3K9me3-containing nucleosomes and also the enzyme depositing this mark (SUV39) has led to an intriguing hypothesis for the epigenetic heritability of "constitutive" heterochromatin. Whilst H3K9me3-containing nucleosomes are diluted during DNA replication, the remaining H3K9me3 mark would recruit HP1 which will bring along SUV39 to methylate newly deposited nucleosomes (Bannister *et al*, 2001; Lachner *et al*, 2001). In *S. pombe*, the *clr4* and *swi6* genes – which encode the fission yeast homologs of SUV39 and HP1 – are required for heterochromatic gene silencing (Thon *et al*, 1994; Allshire *et al*, 1995; Ivanova *et al*, 1998; Nakayama *et al*, 2000; Nakayama *et al*, 2001). Deletion of either *clr4* or *swi6*, while viable, leads to high rates of chromosomal missegregation in *S. pombe* (Ekwall *et al*, 1996; Nonaka *et al*, 2002). Deletion of both SUV39 isozymes does not disrupt pericentric heterochromatin formation in mice and, while H3K9 di- and tri-methylation levels are strongly reduced, H3K9 mono-methylation is unaffected. However, double mutant mice display genome instability and several developmental abnormalities (Rea *et al*, 2000; Peters *et al*, 2001). Interestingly, Cbx5 (HP1α, heterochromatic isoform) null mice are viable and do not exhibit obvious abnormalities. Therefore, it appears that while the SUV39 pathway is not absolutely required for the formation of pericentric heterochromatin in mammalian systems, it plays an important and conserved role in protecting genome stability and normal development.

Despite an increasing knowledge of molecular details, the mechanism how chromatin modifications and chromatin-protein interactions repress gene expression remains unclear. Chromatin-mediated silencing has been extensively investigated in budding yeast. While the molecular machinery involved differs significantly between yeast and higher eukaryotes, these studies have given important conceptual insights on how chromatin associated factors can control gene expression.

1.7 SILENT CHROMATIN IN *S. CEREVISIAE*

As discussed above, heterochromatic regions are characterized by a subset of histone modifications and specific protein composition. Heterochromatic regions in *S. cerevisiae* – hereafter referred to as "silent chromatin" because transcriptionally repressed – are characterized by the absence of histone modifications and are brought about by loading of the Silent Information Regulator (Sir) proteins Sir2, Sir3 and Sir4 onto the chromatin fiber (Rusche *et al*, 2003). The Sir proteins come together in a Sir2-3-4 heterotrimeric complex – referred to as the Sir complex – which is considered the basic silencing machinery of budding yeast (Gasser and Cockell, 2001; Liou *et al*, 2005; Cubizolles *et al*, 2006; Johnson *et al*, 2009; Martino *et al*, 2009).

Silent chromatin in yeast occurs at the subtelomeric regions (Gottschling *et al*, 1990) and the cryptic homothallic mating-type loci (*HM*), *HML* and *HMR* (Rine and Herskowitz, 1987). Deletion of either *SIR2*, *SIR3* or *SIR4* disrupts silencing at telomeres and the *HM* loci (Haber and George, 1979; Klar *et al*, 1979; Rine and Herskowitz, 1987; Aparicio *et al*, 1991). In addition Sir2, but not Sir3 and Sir4, plays an important role in suppressing rDNA recombination (Gottlieb and Esposito, 1989; Fritze *et al*, 1997; Straight *et al*, 1999) and silencing of RNA Polymerase II-driven reporter genes inserted in the rDNA locus (Bryk *et al*, 1997; Fritze *et al*, 1997; Smith and Boeke, 1997). A fourth Sir protein, Sir1, is important for efficient silencing at the *HML* locus but not at telomeres (see below) (Rine *et al*, 1979; Ivy *et al*, 1986; Pillus and Rine, 1989).

The Sir proteins themselves do not recognize specific DNA sequences, but are recruited to discrete loci through protein-protein interactions with multifunctional factors such as ORC, Abf1 and Rap1 that recognize specific DNA motifs – referred to as silencers – at the *HM* loci called the E and I elements (Brand *et al*, 1985; Brand *et al*, 1987; Shore *et al*, 1987; Buchman *et al*, 1988a; Bell *et al*, 1993; Foss *et al*, 1993). These sequences were shown to repress ectopic promoters if placed into their vicinity (Brand *et al*, 1985). Hence, analogous to enhancers that activate transcription,

these elements were termed "silencers" (Brand *et al*, 1985). Generally, silencers flank repressed promoters. In addition, "protosilencers" are sequence elements that display no silencing activity on their own but can cooperate with silencers over a distance to establish or maintain repression at silent domains (Fourel *et al*, 1999; Fourel *et al*, 2002). Similarly, at the telomeres, TG_{1-3} repeats tracts can be considered as minimal silencers consisting solely of a long array of binding sites for Rap1 ((Conrad *et al*, 1990) reviewed in (Rusche *et al*, 2003)).

From these initial nucleation sites the Sir complex spreads for 3-20 kb along the chromatin fiber (Hecht *et al*, 1996; Strahl-Bolsinger *et al*, 1997). Spreading of silent chromatin requires the NAD-dependent deacetylase activity of Sir2 (Tanny *et al*, 1999; Imai *et al*, 2000; Smith *et al*, 2000; Yang and Kirchmaier, 2006), which generates high affinity binding sites for Sir3 and Sir4 and are thought to play structural roles in the establishment of silent chromatin. Loading of Sir4 in turns brings in additional Sir2 to deacetylate neighbor nucleosomes and promoting the farther binding of Sir3 and Sir4 (see below and reviewed in (Gasser and Cockell, 2001; Rusche *et al*, 2003; Moazed *et al*, 2004)). The spread of Sir-mediated repression is limited both by histone modifications (and the machineries involved in their deposition) that lower affinity of Sir3 for chromatin and by the limited concentration of Sir proteins in the nucleus (Buck and Shore, 1995; Maillet *et al*, 1996; Marcand *et al*, 1996; van Leeuwen *et al*, 2002; Martino *et al*, 2009; Oppikofer *et al*, 2011).

1.7.1 SILENCERS ENSURE SIR-DEPENDENT REPRESSION AT THE *HM* LOCI ALLOWING FOR MATING IN *S. CEREVISIAE*

S. cerevisiae can exist in two different mating-types of haploid cells, expressing **a** or α mating factors from the mating-type (*MAT*) locus, which can then mate to form an **a**/α diploid. The *MAT* locus is found near the centromere of chromosome III. Complete copies of the either **a** or α genes are located at the left (*HML*α) and right (*HMR***a**) arm of the chromosome III, which under normal conditions are kept in a transcriptionally repressed state. These sequences are called cryptic homothallic mating-type loci. A wild-type haploid yeast cell is called "homothallic" as it can switch mating-type by replacing the genetic information present in the *MAT* locus with the information stored at either *HML* or *HMR* through a gene conversion event, dependent on the site-specific endonuclease HO (reviewed in (Haber, 2012)). Importantly, if silencing mechanisms are compromised (e.g. deletion of either *SIR2*, *SIR3* or *SIR4*), both *HML*α and *HMR***a** become expressed simultaneously and the haploid cell becomes sterile. Thus, Sir-dependent silencing of the *HM* loci is essential for mating in yeast. As a result, *S. cerevisiae* evolved a rather sophisticated mechanisms that ensures recruitment of the Sir complex through partially redundant pathways to silence the cryptic *HM* loci (reviewed in (Haber, 2012)).

As introduced above, the *HM* loci are both flanked by two silencers called, E and I – referred to as *HML*-E, *HMR*-E and *HML*-I, *HMR*-I. The E and I silencers are composed of a series of DNA motifs named A, B, E and D2. The A motif is an autonomous replication sequence (ARS) bound by the origin recognition complex protein Orc1 (Brand *et al*, 1987). The B motifs are bound by the transcription factor Abf1 (Diffley and Stillman, 1989), while E sequences are recognized by Rap1 (Shore and Nasmyth, 1987). The E and B motifs directly recruit Sir4 through its interaction with Rap1 (Moretti and Shore, 2001) or Sir3 by its interaction with either Rap1 or Abf1 (Gasser and Cockell, 2001; Moretti and Shore, 2001). In the case of the A motif, which binds the hexameric ORC complex, Orc1 further binds Sir1 which in turn recruits Sir4 (Triolo and Sternglanz, 1996). Importantly, Sir-recruitment appears to be the main function of silencers as efficient gene repression could be obtained by targeting either Sir1 (which binds Sir4) or Sir3 to an artificial Gal4 binding sequence by means of Gal4 domain fusions in a strain lacking the *HMR*-E element (Chien *et al*, 1993; Lustig *et al*, 1996). It may sound counterintuitive that Rap1 and Abf1, known to be transcription activators, play a crucial role in establishing silent chromatin (Brand *et al*, 1987; Shore and Nasmyth, 1987; Shore *et al*, 1987; Buchman *et al*, 1988a; Buchman *et al*, 1988b). However, this reveals the importance of the chromosomal context in conveying specific functions to these factors. Interestingly, a recent study showed that replication stress arising from tight DNA-protein interactions – such as transcription factors binding – can favor gene silencing through the recruitment of Sir proteins (Dubarry *et al*, 2011). While Rap1 and Abf1 (and Orc1-Sir1) directly recruits the Sir complex through protein-protein interaction, additional mechanisms ensuring stable repression of the *HM* loci – thus the ability to mate – may involve the local increase of Sir protein concentration due to the genomic context and high order chromatin structure (see section - how is gene repression achieved in yeast silent chromatin?)

1.7.2 SUBTELOMERIC SILENCING: TELOMERE POSITION EFFECT

Yeast telomeres exert Sir-dependent transcriptional repression on genes located in their vicinity in a process called Telomere Position Effect (TPE) (Gottschling *et al*, 1990; Aparicio *et al*, 1991). TPE is similar to the Position Effect Variegation (PEV) observed at centromeric regions in flies (Raffel and Muller, 1940). Indeed, TPE has no DNA sequence specificity and reporter genes (such as *URA3*) can be transcriptionally silenced when inserted in a subtelomeric region (Gottschling *et al*, 1990). Importantly, gene silencing is inherited through cell division yet it appears to be bi-stable. A silent gene that stochastically escapes telomeric repression and becomes active, also sees is active state stably transmitted between cell generations (Gottschling *et al*, 1990). This epigenetic heritability of active and inactive states characterizes both TPE in yeast and PEV in flies (Raffel and Muller, 1940; Gottschling *et al*, 1990).

Analogous to the situation at the *HM* loci, the Sir complex is recruited to subtelomeric regions by the Rap1 protein which binds to the TG_{1-3} repeats as well as to Sir4 and Sir3 (Conrad *et al*, 1990; Buck and Shore, 1995; Liu and Lustig, 1996; Gasser and Cockell, 2001; Moretti and Shore, 2001). One molecule of Rap1 binds approximately ~20 bp of TG_{1-3} repeats tract (Gilson *et al*, 1993), which varies in length from 250 to 630 bp at different telomeres (Shore and Nasmyth, 1987; Buchman *et al*, 1988b; Longtine *et al*, 1989; Conrad *et al*, 1990; Klein *et al*, 1992). Temperature-sensitive mutants of Rap1 resulted in a telomere shortening phenotype (Conrad *et al*, 1990). Consistently, the Rap1 C-terminus (RCT domain, aa 695-827) binds to Rif1 and Rif2 which are negative regulators of telomeric length (Hardy *et al*, 1992a; Hardy *et al*, 1992b; Wotton and Shore, 1997). Importantly, the same domain of Rap1 also interacts with Sir3 and Sir4 which are thought to compete with Rif1 and Rif2 for binding to Rap1 (Liu *et al*, 1994; Moretti *et al*, 1994; Buck and Shore, 1995; Feeser and Wolberger, 2008; Chen *et al*, 2011). Deletion of *RIF1* and *RIF2* has been proposed to favor subtelomeric silencing directly by increasing the Rap1-dependent recruitment of Sir proteins and concurrently by increasing TG_{1-3} repeats length which in turn would recruit more Rap1 and thus Sir proteins (Hardy *et al*, 1992b; Buck and Shore, 1995; Wotton and Shore, 1997). Importantly, increased recruitment of Sir4 at telomeres weakens silencing of the *HM* loci, supporting the model that the amount of Sir proteins in the nucleus is limiting for silencing (Buck and Shore, 1995; Maillet *et al*, 1996; Marcand *et al*, 1996; Cockell *et al*, 1998a).

In addition to Rap1, silencing at telomeres, but not at the *HM* loci, also relies on the Yku70/Yku80 heterodimer. Indeed, mutations in the *HDF1* and *HDF2* genes (coding for Yku70 and Yku80 respectively) derepress telomeric silencing (Boulton and Jackson, 1998; Laroche *et al*, 1998; Mishra and Shore, 1999). Consistently, Yku70/Yku80 was shown to interact directly with Sir4 and to play an important role (together with Esc1; (Andrulis *et al*, 2002)) in positioning telomeres at the nuclear periphery (Hediger *et al*, 2002; Roy *et al*, 2004; Taddei *et al*, 2004). Additional evidence of the important role of Yku70/Yku80 to promote subtelomeric silencing reside in the observation that a 350 bp long TG_{1-3} repeats tract is unable to nucleate silencing when inserted away from the end of the chromosome (Stavenhagen and Zakian, 1994) while as little as 80 bp of TG_{1-3} repeats – roughly recruiting only four Rap1 molecules – are enough to induce subtelomeric silencing (Gottschling *et al*, 1990). In addition to their role in subtelomeric silencing, it has been shown that Yku70/Yku80 also participate in the formation of silencing at the *HM* loci where their recruitment is dependent on Sir4 (Patterson and Fox, 2008; Vandre *et al*, 2008).

The clustering of silent domains at the nuclear periphery increases the local concentration of Sir proteins thus facilitating the formation a repressed state (Maillet *et al*, 1996; Marcand *et al*, 1996; Andrulis *et al*, 1998; Taddei *et al*, 2004; Taddei *et al*, 2009). Interestingly, a recent study indicates that, in conditions of Sir3 overexpression, clustering of telomeres is mostly mediated by Sir3 which, presumably by recruiting high concentrations of Sir proteins locally, supports silencing independently of the actual localization of the telomeric clusters at the nuclear periphery (Ruault *et al*, 2011). Sir3 overexpression may imitate the situation at the *HMR* locus where Sir recruitment is more efficient and transcriptional repression can be sustained without perinuclear anchoring (Gartenberg *et al*, 2004). Therefore, it appears that the determinant for gene silencing is the local concentration of Sir proteins which may be increased by strong silencers or clustering of weaker recruitment sites. In addition to the silencer-dependent recruitment of Sir proteins to specific genomic location, histone modifications are thought to play an important role in inhibiting the ectopic binding – and the consequent dilution – of the Sir proteins into euchromatic regions.

1.8 HISTONE MODIFICATIONS REGULATE YEAST SILENT CHROMATIN

The establishment of silent domains in budding yeast relies on the loading of the Sir complex onto unmodified nucleosomes. Based on its primary structure, the size of the holo Sir complex (Sir2-3-4; 326 kDa) exceeds the size of a nucleosome (205 kDa) by about 60%. This suggests that the Sir complex can have multiple interaction sites with the nucleosome spanning the DNA, the histone octamer surface as well as the histone tails.

We still lack a complete picture of how the Sir complex binds to the nucleosome. However, a combination of genetic, biochemical and recent structural approaches have mapped some of the key Sir-nucleosome interaction sites, particularly in relation to Sir3 (and (Norris and Boeke, 2010; Armache *et al*, 2011; Ehrentraut *et al*, 2011) and references cited therein). Importantly, the interaction of Sir3 with the nucleosome needs to be fine-tuned as both too weak and too strong interactions can impair silent chromatin formation (see below). The Sir4 N-terminus (aa 1-344) has high affinity for naked DNA, plausibly favoring the interaction of the Sir complex with the nucleosomal linker DNA (Martino *et al*, 2009; Kueng *et al*, 2012). On the other hand, the Sir2 protein fails to interact appreciably with either DNA or nucleosomes (Martino *et al*, 2009).

As described above, histone modifications play a major role in modulating gene expression. While high eukaryotes possess specific heterochromatic histone modifications (e.g. H3K9me3 and H4K27me3), yeast silent chromatin appears to be depleted of histone marks. Consistently, several histone marks have been found to exhibit "anti-silencing effects", including the direct repulsion of the Sir proteins – and particularly Sir3 – from the chromatin fiber thus limiting spread of Sir-mediated silencing. The following sections will review the mechanisms by which the modification of some key residues (Figure 3) is thought to regulate the formation of yeast silent domains.

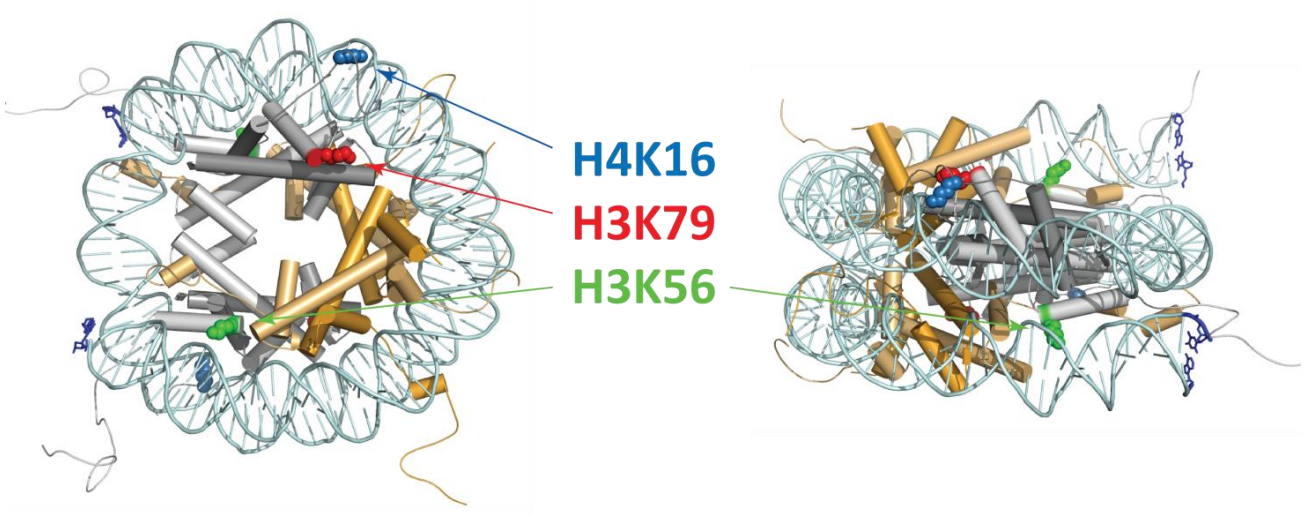

Figure 3 - The position of H3K56, H3K79 and H4K16 is indicated on a representation of the nucleosome core particle (Davey *et al*, 2002). H3K56 (green) is located at the entry/exit point of the DNA wrapped around the histone octamer. H3K79 (red) is exposed on the lateral surface of the nucleosome, while H4K16 (blue) is located on the H4 N-terminal tail.

1.8.1 THE H4 N-TERMINAL TAIL: A DOCKING SITE REGULATED BY ACETYLATION

Early evidence for the important role played by histone proteins in yeast silent chromatin formation, came from the observation that removal of the H4 N-terminal tail derepressed silencing at the *HM* loci, thus disrupting mating (Kayne *et al*, 1988). Importantly, this activation was specific for heterochromatic-like regions since regulation of other genes (e.g. *PHO5* or *GAL10*) was unaltered (Kayne *et al*, 1988). In particular, a basic domain spanning from glycine 14 to arginine 19 was essential for mating. Mutagenesis studies revealed that substitution of lysine 16, arginine 17, histidine 18 or arginine 19, to a neutral amino acid (glycine or alanine) derepressed *HM* (Johnson *et al*, 1990; Park and Szostak, 1990) and telomeric silencing (Aparicio *et al*, 1991). Importantly, a search for suppressors of histone H4 tail mutants, revealed that mutations in the N-terminal BAH domain of Sir3 (W86R, *sir3R1* and D205N, *sir3R3*; see below) could restore *HM* silencing (Johnson *et al*, 1990). This strongly suggested that Sir3 could contact the H4 N-terminal tail, and in particular this acidic patch (aa 16-19), during the assembly of silent chromatin. Indeed, pull-down experiments showed that *in vitro* translated Sir3 (and Sir4) binds to bacterially expressed histone H4 tails *in vitro* in a manner sensitive to mutations near lysine 16 (Hecht *et al*, 1995). While, the lysine 16 to glutamine substitution – mimicking the acetylated state – didn't appreciably affect the interaction between Sir3 and the H4 tail peptides (Hecht *et al*, 1995) by pull-down, Surface Plasmon Resonance (SPR) experiments using a immobilized Sir3 C-terminal fragment (aa 503-970; expressed in bacteria) and synthetic histone H4 tails peptides revealed that acetylation of lysine 5, 8, 12, or 16 can decrease the interaction between Sir3 and the H4 tail in a cumulative manner (Carmen *et al*, 2002). A recent study using reconstituted chromatin *in vitro*, instead of isolated histone tail peptides, showed that Sir3 purified recombinantly from insect cells has less affinity for chromatin lacking the histone H4 N-terminal tails (Martino *et al*, 2009; Sinha *et al*, 2009). Supporting a regulatory role for acetylation, it was recently found that the H4K16A substitution, or the NuA4-dependent unspecific acetylation of the nucleosome (including at H4K16), reduced binding of Sir3 to a chromatin template *in vitro* (Johnson *et al*, 2009). Together, the H4 N-terminal tail is an important docking site for Sir3 and the acetylation of the H4 tail, particularly at the H4K16 residue, is likely to regulate Sir3 binding.

1.8.2 ACETYLATION OF H4K16: IS ALL ABOUT REMOVING A POSITIVE CHARGE?

Acetylation of H4K16 (H4K16ac) marks transcriptionally active chromatin in most species, peaking at the transcriptional start site and decreasing through the body of active genes (similar to H3K4me3), and is enriched at early firing origins in yeast and flies (Kimura *et al*, 2002; Suka *et al*, 2002; Bell *et al*, 2010). Consistent with the majority of the yeast genome being in an active state, H4K16ac is very abundant, with ~ 80% of histone H4 molecules bearing this mark (Clarke *et al*, 1993; Smith *et al*, 2003b; Millar *et al*, 2004). Interestingly, an intact H4 N-terminal tail, and in particular unmodified H4K16, promotes nucleosomal array folding *in vitro* (Dorigo *et al*, 2003; Shogren-Knaak *et al*, 2006; Robinson *et al*, 2008) arguing that H4K16ac mark helps maintain chromatin in an "open" state accessible to regulatory and transcriptional machineries.

In yeast, H4K16 is acetylated primarily by the nonessential histone acetyltransferase (HAT) Sas2 (Kimura *et al*, 2002; Suka *et al*, 2002) and secondarily by the essential HAT Esa1, which also targets H4K5, H4K8 and H4K12 (Suka *et al*, 2001; Suka *et al*, 2002; Chang and Pillus, 2009). A recent, genome-wide study suggests that the H4K16ac mark is deposited by Sas2 independently of transcription and histone exchange (Heise *et al*, 2012). The *SAS2* gene was identified independently in two different screens: one for enhancers of *sir1* silencing defects (Something About

Silencing 2; (Reifsnyder *et al*, 1996)) and the other for suppressors of a silencing-deficient *HMR*-E, crippled by mutations in the Rap1 and Abf1 binding sites (Ehrenhofer-Murray *et al*, 1997). Thus, ever since its initial characterization, loss of Sas2 seemed both to disfavor (Reifsnyder *et al*, 1996) and to promote silencing (Ehrenhofer-Murray *et al*, 1997), depending on the assay used. Several laboratories have explored the effect of *sas2* deletion and H4K16 mutation in order to define the precise roles of *SAS2* and the H4K16ac mark in gene silencing. However, the situation has not been straightforward. Deletion of *SAS2* completely derepressed a reporter gene inserted at telomere 7L and was partially defective in mating (Reifsnyder *et al*, 1996). This agrees with the previous observation that even a conservative substitution of lysine 16 to arginine (H4K16R) impaired *HML* silencing (Johnson *et al*, 1990) and repression at the telomere 7L (Meijsing and Ehrenhofer-Murray, 2001). Importantly, this suggests that the conservation of a positive charge at this residue is not sufficient for efficient silencing. Consistently, an elegant study monitoring the onset of silencing with single-cell resolution showed that the establishment of silencing at *HML* was slower in the absence of Sas2 (Osborne *et al*, 2009). However, the same H4K16R mutation, which again phenocopied a *SAS2* deletion, promoted silencing of a reporter inserted at a crippled *HMR*, where Sir recruitment is favored (Ehrenhofer-Murray *et al*, 1997). Nonetheless, the rate of Sir3 recruitment to *HMR* was slower in cells that lack Sas2 and, therefore, H4K16 acetylation (Katan-Khaykovich and Struhl, 2005).

In summary, maintaining a positive charge at H4K16 by either a conservative substitution (H4K16R) or deletion of the *SAS2* gene, exhibits phenotypes which are in apparent disagreement. Intriguingly, more often than not, blocking the deposition of the H4K16ac "active" mark seemed to disfavor the formation of silencing. At first glance, this is counter-intuitive and seems to contradict *in vitro* data showing that Sir3 binds specifically to unacetylated H4 tails (Carmen *et al*, 2002; Johnson *et al*, 2009), which suggests that a lack of H4K16ac should promote silencing. However, two mutually non-exclusive models can explain this conundrum.

1.8.2.1 H4K16 ACETYLATION PREVENTS THE DILUTION OF SILENCING FACTORS

It was shown that deletion of *SAS2*, as well as the conservative H4K16R substitution, results in the spread of Sir proteins (Sir2, Sir3 and Sir4) away from subtelomeric regions leading to the inactivation of genes found in euchromatic domains (Kimura *et al*, 2002; Suka *et al*, 2002). Given that the steady state level of Sir proteins is limiting for the establishment of silencing (Buck and Shore, 1995; Maillet *et al*, 1996; Marcand *et al*, 1996; Cockell *et al*, 1998a), loss of H4K16ac would generate binding sites for Sir3 genome-wide diluting Sir proteins – in particular Sir3. Thus efficient silencing would be possible only in presence of strong recruitment sites, such as the *HMR* locus (Ehrenhofer-Murray *et al*, 1997). While this model is consistent with our current understanding of yeast silent chromatin, recent findings suggest that genetic loss of H4K16ac has an additional negative impact on silencing.

1.8.2.2 THE TURNOVER OF THE H4K16AC MARK MAY FAVOR SILENCING DIRECTLY

Using *in vitro* reconstituted chromatin immobilized on beads, it was shown that, unlike Sir3 alone, the loading of the holo Sir complex onto chromatin was not impaired by NuA4-dependent nucleosomal acetylation, including of H4K16 (Johnson *et al*, 2009). This suggests that the role of H4K16ac may not be merely to prevent "dilution" of the Sir proteins into euchromatic regions. Strikingly, by means of chromatin immunoprecipitation (ChIP), it was shown that the Sir complex can spread efficiently in cells expressing the histone mutant H4K16R – mimicking the H4K16 hypoacetylated state – yet silencing was impaired (Yang and Kirchmaier, 2006). In other words, Sir proteins were not completely dispersed in a H4K16R background, yet even when loaded onto chromatin depleted from the H4K16ac mark, silencing was compromised. Given that H4K16ac is a key substrate of Sir2 (Imai *et al*, 2000; Kimura *et al*, 2002; Suka *et al*, 2002; Borra *et al*, 2004), and that the catalytic activity of Sir2 is absolutely required for Sir-silencing in cells expressing wild-type histones (Tanny *et al*, 1999; Imai *et al*, 2000; Smith *et al*, 2000), it seems that the active Sir2-mediated deacetylation of H4K16ac facilitates the establishment of silencing directly. As detailed in the next section (see - Sir2: from histone deacetylation to *O*-AADPR production), Sir2-dependent deacetylation uses a NAD moiety and is coupled to the production of *O*-acetyl-ADP-ribose (*O*-AADPR). Importantly, purified *O*-AADPR has been proposed to alter the stoichiometry and the conformation of reconstituted Sir2-3-4 particles bound to a synthetic histone H4 tail (Liou *et al*, 2005). Furthermore, it was shown that purified *O*-AADPR increases the binding affinity of the Sir complex (and Sir3 alone) for a nucleosomal array *in vitro* (Martino *et al*, 2009). Therefore, there is good evidence for an additional role of the H4K16ac other than merely repelling Sir proteins from most of the genome. The Sir2-dependent turnover of this "active" mark could convey an important silencing role to H4K16ac (see Chapter 2 - An active mark promotes silencing).

1.8.3 METHYLATION OF H3K79 BY DOT1: AN INVARIANT BARRIER?

The H4K16ac mark has been found to stimulate the methylation of lysine 79 on the histone H3 (H3K79me) by the methyltransferase Dot1, possibly by decreasing the affinity of Sir3 for the nucleosome (Altaf *et al*, 2007). Indeed, the H4 N-terminal tail is required for Dot1-dependent methylation of H3K79 *in vitro* and *in vivo* and acetylation of H4K16 displaces Sir3 allowing Dot1 binding *in vitro* (Altaf *et al*, 2007; Fingerman *et al*, 2007).

DOT1 was isolated in a screen for factors that impair telomeric silencing when overexpressed (Singer *et al*, 1998). It was shown that Dot1 specifically methylates H3K79 which lies on the surface of the nucleosome and does not contact

DNA or other histones (Luger *et al*, 1997; Feng *et al*, 2002; Ng *et al*, 2002; van Leeuwen *et al*, 2002). This shows that modulation of Sir-silencing is not restricted to modification of the histone tails. Dot1 and methylation of H3K79 is conserved from yeast to man (Feng *et al*, 2002; Ng *et al*, 2003) and is essential for mammalian development and cell proliferation (Jones *et al*, 2008; Kim *et al*, 2012). The X-ray crystal structure of human and yeast Dot1's catalytic core revealed a very conserved α/β structure composed of a large seven-stranded β-sheet characteristic of the class I SAM-dependent methyltransferases which lack a SET domain (Min *et al*, 2003; Sawada *et al*, 2004).

In yeast, loss of Dot1 completely abolishes H3K79 methylation *in vivo*, showing that Dot1 is the only HMT for this specific residue (Ng *et al*, 2002; van Leeuwen *et al*, 2002). Importantly, as shown *in vitro*, Dot1-dependent methylation of H3K79 occurs only when the histone H3 is assembled into chromatin (Ng *et al*, 2002; van Leeuwen *et al*, 2002). The observation that Dot1 only methylates the nucleosomal histone H3 likely has to do with the fact that Dot1 docks on the H4 N-terminal tail (Altaf *et al*, 2007). Combining ChIP and mass spectrometry it was shown that Dot1 has the ability of carry out mono-, di- and tri-methylation of H3K79 *in vivo*. Moreover, the transition to di- and tri-methylation requires H2BK123 ubiquitination, which is dispensable for mono-methylation (Sun and Allis, 2002; Shahbazian *et al*, 2005). It is not yet clear how H2BK123 ubiquitination (H2BK123Ub) increases the processivity of Dot1 (Frederiks *et al*, 2008) and three non-mutually exclusive models have been proposed. 1) Dot1 could interact directly with H2BK123Ub through a lysine-rich region within the Dot1 N-terminus (McGinty *et al*, 2008; Chatterjee *et al*, 2010; Oh *et al*, 2010). 2) A bridging protein could bind both Dot1 and H2BK123Ub, candidates are the proteasomal ATPases Rpt4 and Rpt6 (Ezhkova and Tansey, 2004) as well as Cps35, a component of the COMPASS complex (Lee *et al*, 2007). 3) Alternatively, ubiquitination of H2B has been shown to disrupt chromatin compaction *in vitro* (Fierz *et al*, 2011) and this may facilitate the accessibility of H3K79 to Dot1.

Importantly, not only overexpression but also deletion of *DOT1* compromised telomeric silencing and the non-conservative substitution of H3K79 with alanine (H3K79A) completely derepressed silencing of reporter genes inserted at telomeres and the *HM* loci (Ng *et al*, 2002; van Leeuwen *et al*, 2002). Consistently, and to a greater extent than H4K16R, disruption of silencing in the H3K79A mutant correlated with a redistribution of Sir2, Sir3 and Sir4 away from the subtelomeric region (Ng *et al*, 2002; van Leeuwen *et al*, 2002). This strongly suggested a model in which the unmodified H3K79 residue makes important contacts with the Sir complex and that methylation of H3K79 in euchromatic regions disfavor Sir-chromatin interaction. This concentrates the Sir proteins into silent regions (Ng *et al*, 2002; van Leeuwen *et al*, 2002; Verzijlbergen *et al*, 2009). Indeed, Sir3 binding and spreading of Sir-mediated silencing was shown to be hindered by methylation of H3K79 *in vivo* (Altaf *et al*, 2007; Onishi *et al*, 2007; van Welsem *et al*, 2008) and *in vitro* (Altaf *et al*, 2007; Martino *et al*, 2009; Ehrentraut *et al*, 2011). Consistently, the recent crystal structure of the N-terminal BAH domain of Sir3 bound to the nucleosome shows that the H3K79 residue makes hydrogen bonding with the loop 3 of the BAH domain which would be displaced by progressive methylation (Armache *et al*, 2011) (see section - Sir3: selective nucleosome binding and spreading of silencing).

As discussed earlier, there is some similarity in the mode of action of H3K79me and H4K16ac: both limit the spread of silent domain by disfavoring the binding of Sir3 to the nucleosome. Yet, while the H4K16ac mark can be removed by Sir2, no enzyme has been found so far that removes the H3K79me mark, and its removal may depend on sequential dilution through rounds of DNA replication (Katan-Khaykovich and Struhl, 2005; Osborne *et al*, 2009). However, to date, all other histone lysine residues subjected to methylation were shown to undergo enzymatic removal of the methyl mark (Klose *et al*, 2006; Klose and Zhang, 2007; Cloos *et al*, 2008; Liu *et al*, 2010; Qi *et al*, 2010). Is H3K79me the only exception? There is evidence suggesting that H3K79me may be reversible and regulated. For instance, H3K79me2 has been shown to fluctuate during the cell cycle in both yeast and human cells (Feng *et al*, 2002; Schulze *et al*, 2009). With the exception of Lsd1, all histone demethylases discovered to date contain a "jumonji" domain and can be competitively inhibited by 2-hydroxyglutarate (2-HG) (Tsukada *et al*, 2006; Mosammaparast and Shi, 2010; Xu *et al*, 2011). Intriguingly, when the level of 2-HG was artificially increased in cultured human cancer cells, global histone methylation was increased, including the H3K79me2 mark (~ 10-fold). This suggests that a not yet characterized jumonji-containing enzyme responsible for H3K79 demethylation was inhibited by 2-HG (Xu *et al*, 2011). While indirect effects cannot be excluded, these lines of evidence suggest that a demethylase for H3K79 may exist, at least in higher eukaryotes. Clearly, more work is required to understand the turnover of the H3K79me mark.

1.8.4 ACETYLATION OF H3K56: SILENCING IS NOT JUST ABOUT LOADING OF SIRS

A further histone modification that interferes with Sir-mediated repression is the acetylation of K56 on the histone H3 (Xu *et al*, 2005; Xu *et al*, 2007; Yang *et al*, 2008b). In contrast to Dot1-dependent methylation of H3K79, acetylation of H3K56 is deposited by Rtt109 during S phase prior to the loading of the histones onto DNA, and therefore serves as a marker for newly assembled nucleosomes (Hyland *et al*, 2005; Masumoto *et al*, 2005; Han *et al*, 2007a; Li *et al*, 2008). H3K56 is acetylated while H3-H4 dimers are bound to the chaperone Asf1 (Han *et al*, 2007b; Tsubota *et al*, 2007) and after peaking in S phase, H3K56ac is removed by Hst3 and Hst4 during G2/M (Masumoto *et al*, 2005; Celic *et al*, 2006; Maas *et al*, 2006). Consistently, expression of Hst3 and Hst4 peaks precisely during the G2/M phase (Maas *et al*, 2006).

In chromatin the H3K56 residue is located near the entry/exit point of DNA around the nucleosome, suggesting that it may play an important role in regulating nucleosomal architecture (Luger *et al*, 1997). Supporting this model,

nucleosomal arrays isolated from yeast cells expressing the acetylmimic H3K56Q mutation are more sensitive to micrococcal nuclease digestion, than are wild-type histones or the H3K56R mutant, which mimicks the deacetylated state (Masumoto *et al*, 2005). Accordingly, plasmids isolated from *rtt109* mutants are more supercoiled compared to wild-type, suggesting that acetylation of H3K56 induces a less compacted chromatin organization (Driscoll *et al*, 2007). Using Fluorescence Resonance Energy Transfer (FRET) it was shown that the DNA can transiently and rapidly unwrap from the histone octamer surface – referred to as nucleosomal "breathing" (Li *et al*, 2005). Supporting a regulatory role for H3K56 in nucleosomal architecture, homogeneous acetylation of H3K56 increased nucleosomal "breathing" roughly 7-fold (Neumann *et al*, 2009).

Genetic analysis of H3K56 mutants strongly implicated this residue in modulating yeast silent chromatin (Xu *et al*, 2007; Yang *et al*, 2008b). While H3K56 residue seems to play a minor role at the *HM* loci (Xu *et al*, 2007; Yang *et al*, 2008b), non-conservative substitutions of H3K56 with either glycine or glutamine (acetylmimic) dramatically impaired telomeric silencing of both reporter genes and native telomeres (Xu *et al*, 2007). In addition, the conservative substitution of H3K56 with arginine (deacetylmimic) also derepressed silencing yet to a lesser extent (Xu *et al*, 2007). The observation that a conservative substitution at H3K56 still impaired silencing supports the notion that this residue plays an important role in modulating nucleosomal architecture, likely due to its characteristic localization (Luger *et al*, 1997). Importantly, mutation of H3K56 neither altered the acetylation state of other histone residues, including H4K16, nor did it induce histone loss (Xu *et al*, 2007). This indicates that the modification of H3K56 may regulate silencing directly.

Intriguingly, while mutation of the H3K56 residue derepresses telomeric silencing, the binding of Sir2, Sir3 and Sir4 was unchanged (Xu *et al*, 2007), suggesting that H3K56, unlike H4K16 and H3K79, is not a direct binding site for the Sir complex. Using an *in vivo* bacterial DAM methylase assay (Gottschling, 1992) it was shown that substitution of H3K56 with either glycine, glutamine or arginine increases telomeric DNA accessibility by ~ 6-fold, despite the continuous presence of the Sir complex. However, deletion of *SIR2* had a more dramatic effect as DNA accessibility was increased roughly 12-fold (Xu *et al*, 2007), consistent with the notion that Sir2 is essential to reduce DNA accessibility by deacetylating other residues, notably H4K16. Together, *in vitro* analysis of nucleosomes bearing the H3K56ac mark (Neumann *et al*, 2009) or the acetylmimic H3K56Q mutation (Masumoto *et al*, 2005; Driscoll *et al*, 2007) combined with *in vivo* accessibility assay (Xu *et al*, 2007) suggest that H3K56 plays an important role in regulating DNA accessibly beyond the loading of the Sir complex onto the chromatin fibre, implying that the removal of the H3K56ac mark is required for efficient silencing *in vivo*.

1.8.4.1 IS THERE A SPECIFIC HDAC FOR H3K56AC WITHIN SILENT CHROMATIN?

Using a tiling array, it was shown that acetylation of H3K56 is indeed strongly reduced at subtelomeric regions, while it is enriched in the coding region of actively transcribed genes (Xu *et al*, 2007). While it is accepted that Hst3 and Hst4 deacetylate H3K56ac during cell cycle progression (Masumoto *et al*, 2005; Celic *et al*, 2006; Maas *et al*, 2006), the enzymatic machinery responsible for removing this mark specifically within silent chromatin may be either the Hst3/Hst4 pair (Yang *et al*, 2008b), the silencing protein Sir2 (Xu *et al*, 2007) or both.

In favour of a role for Sir2 in the removal of the H3K56ac mark, Xu and colleagues showed that deletion of *SIR2*, but not of *HST3/HST4*, increases acetylation of H3K56 at telomere 6R and the *HM* loci 4-6 fold, as assessed by ChIP with an anti-H3K56ac antibody (Xu *et al*, 2007). However, the authors normalized the data to an internal locus: *SPS2*, located in the middle of chromosome 4. The amount of *SPS2* DNA pulled-down using the anti-H3K56ac antibody is markedly increased in the *hst3/hst4* mutant, since the *hst3/hst4* mutant increases global H3K56 acetylation, including at the *SPS2* locus (Masumoto *et al*, 2005; Celic *et al*, 2006; Maas *et al*, 2006). As a result the H3K56 acetylation levels at silent loci appeared artificially low in the *hst3/hst4* mutant as the amount of *SPS2* DNA used for normalization was specifically increased in the *hst3/hst4*, but not in the *sir2* mutant background (Xu *et al*, 2007).

Supporting a role for Hst3/Hst4 in the removal of the H3K56ac mark at silent regions, an early study showed that deletion of *HST3/HST4* impairs silencing of a reporter gene inserted at the telomere 7L (Brachmann *et al*, 1995). In addition, again using ChIP, another study showed that H3K56ac levels at the natural *YFR057W* gene (telomere 6R) were strongly increased in the *hst3/hst4* mutant and silencing at the *YFR057W* gene was lost (Yang *et al*, 2008b). Importantly, in agreement with the previous study (Xu *et al*, 2007), Sir2 was still present on chromatin and H4K16 was hypoacetylated (Yang *et al*, 2008b), suggesting that the H3K56ac mark impairs silencing without decreasing the affinity of Sir proteins for chromatin. Importantly, H3K56ac levels were not further increased by genetic removal of the Sir2 catalytic activity (*sri2-345*) in a *hst3/hst4* background. Moreover, overexpression of Sir2 did not reduce H3K56ac levels nor restore telomeric silencing (Yang *et al*, 2008b). On the other hand, expression of Hst3 from a low-copy plasmid – or deletion of *rtt109*, the HAT responsible for depositing the H3K56ac mark – reduced H3K56ac levels and restored silencing of the *YFR057W* gene (Yang *et al*, 2008b). This suggests that Sir2 is unable to deacetylate H3K56ac *in vivo* and that the role of Hst3/Hst4 is not limited to cell cycle regulation of H3K56ac level, but also extends to silent regions.

In vivo, overexpression of Hst3/Hst4 but not Sir2, reduces H3K56ac levels at telomeres, *HM* loci and genome-wide. On the other hand, using recombinant Sir2 expressed in bacteria and synthetic histone peptides acetylated on H3K56 or native histones extracted from yeast, Sir2 efficiently deacetylated the H3K56ac residue *in vitro* (Xu *et al*, 2007). While

the experiments where well-controlled, the material used presents several caveats. First, Sir2 expressed in bacteria may lack post-translational modifications key for specificity of the deacetylation reaction. Second, and more importantly, the specific location of H3K56 at the entry/exit point of the DNA around the histone octamer may greatly affect its accessibility. It is unlikely that histone peptides or extracted histones accurately reflect the situation where H3K56ac is embedded into chromatin. Whether Sir2 is able to remove the H3K56ac mark from a nucleosomal template *in vitro* was not tested heretofore. The data presented in Chapter 2 reveal that Sir2, in complex with Sir3 and Sir4, is unable to remove the H3K56ac mark from a nucleosomal array although it efficiently deacetylates H4K16ac *in vitro* (see Chapter 2 - An active mark promotes silencing).

1.9 SIR-MEDIATED SILENCING: A *COMPLEX* STORY WITH THREE PROTAGONISTS

The establishment and maintenance of yeast silent chromatin requires Sir2, Sir3 and Sir4 at telomeres, while Sir1 is crucial for recruitment at the *HM* loci (Rine and Herskowitz, 1987; Gottschling *et al*, 1990). Genetic experiments showed that the correct dosage of Sir2, Sir3 and Sir4 is crucial for silent chromatin formation. For instance, overexpression of a Sir4 C-terminal fragment derepressed *HML* silencing unless Sir3 was overexpressed simultaneously (Marshall *et al*, 1987; Maillet *et al*, 1996). In addition, overexpression of Sir4 C-terminus delocalized Sir3 (and Rap1) and derepressed telomeric silencing (Cockell *et al*, 1995). Similarly, overexpression of either Sir4 full-length or a Sir4 C-terminal fragment strongly derepressed telomeric silencing and clustering, yet both phenotypes could be suppressed by co-overexpression of Sir3 (Cockell *et al*, 1998a). Moreover, overexpression of a C-terminal fragment of Sir3 (aa 568-978) alone – but not a N-terminal portion (aa 1-503) – impaired silencing at telomeres (Gotta *et al*, 1998). In addition, overexpression of Sir4 reduced Sir2 levels in the nucleolus thus impairing silencing of a reporter gene inserted at the rDNA locus (Smith *et al*, 1998). Together, the genetic interdependency between Sir proteins for proper functioning strongly suggested both that Sir4 interacts with Sir2 and Sir3 and that stoichiometric assembly of a Sir2-3-4 complex is required for proper silencing.

Consistently, yeast-two-hybrid showed that Sir3 and Sir4 interact (Moretti *et al*, 1994) and co-immunoprecipitation and co-purification confirmed that Sir4 interacts with both Sir3 and Sir2 (Cockell *et al*, 1995; Hecht *et al*, 1996; Moazed and Johnson, 1996; Strahl-Bolsinger *et al*, 1997). Importantly, pull-down experiments using purified substrates revealed that Sir4-Sir3 interaction occur in the absence of Sir2 and that Sir4-Sir2 interaction occurs in the absence of Sir3 (Strahl-Bolsinger *et al*, 1997). Co-immunoprecipitation experiments showed that Sir3 and Sir2 do not interact (Moazed *et al*, 1997). Together, this argues that Sir4 functions as a scaffold for the assembly of the Sir2-3-4, or Sir complex.

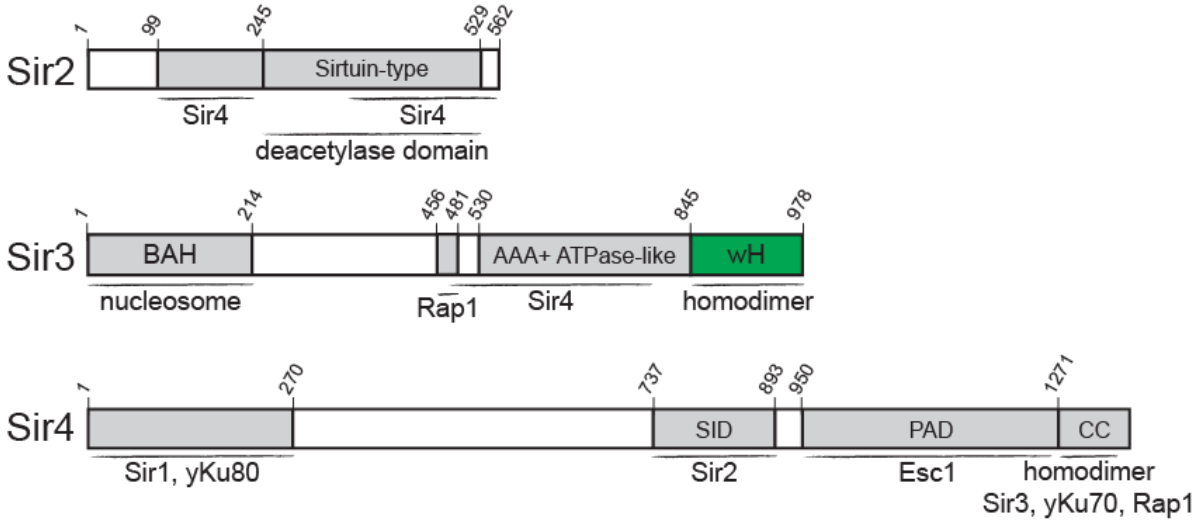

Figure 4 - The Sir proteins Schematic representation of the Sir proteins and their modular organization. Important interaction sites and other features are highlighted. The numbering refers to the primary sequence of the proteins. Details on Sir interactions and appropriate references can be found in the manuscript. The structure and function of Sir3 wH, highlighted in green has been revealed in the Chapter 3 of this PhD thesis.

Although we lack a comprehensive structural characterization of the holo Sir complex, several contacts between Sir4 and Sir3 or Sir2 can be recapitulated *in vitro* using purified proteins or domains (see Figure 4). An N-terminal fragment of Sir4 (aa 142-590) interacts with a large Sir3 C-terminus (aa 623-978) and with full length Sir2 (Strahl-Bolsinger *et al*, 1997). On the other hand, the Sir4 C-terminus (aa 1144-1358) interacts with a large Sir3 N-terminal fragment (aa 1-762) (Strahl-Bolsinger *et al*, 1997). The region of Sir3 that interacts with Sir4 was further narrowed down to aa 464-728 (King *et al*, 2006) and successive truncation showed that aa 495-521 in Sir3 are essential to interact with the Sir4 C-terminus (Chang *et al*, 2003). Based on a recent – yet unpublished – crystal structure, the tight interaction between Sir2 and Sir4 relies on the so-called Sir2 Interaction Domain (SID, aa 737-893) of Sir4 which interacts primarily with a Sir2

N-terminal domain (aa 101-236) and secondarily with a Sir2 C-terminal fragment (aa 237-555) involving mostly hydrophobic interactions (Rolf Sternglanz and Rui-Ming Xu, unpublished).

Initial attempts to purify the Sir complex from yeast yielded only a Sir2-4 heterodimer with estimated molecular weights ranging from 0.7 to 1 MDa leaving open the issue of stoichiometry (Ghidelli *et al*, 2001; Hoppe *et al*, 2002; Tanny *et al*, 2004). Moreover, some of these preparations contained contaminant activities as histone deacetylation could be performed in the absence of NAD, an obligate Sir2 co-factor (Ghidelli *et al*, 2001). A stoichiometric assembly of the Sir2, Sir3 and Sir4 proteins was observed by co-purification from insect cells where the Sir proteins came together in a 1:1:1 Sir2-3-4 complex (Cubizolles *et al*, 2006). Such a Sir complex maintained a stoichiometric assembly when loaded onto chromatin arrays *in vitro* (Martino *et al*, 2009). Alternatively, a 1:1:1 Sir2-3-4 complex could also be reconstituted by mixing Sir2-4 and Sir3 purified independently from overexpression in yeast (Liou *et al*, 2005). However, when this Sir complex was incubated with synthetic tetra-acetylated histone H4 tails and NAD, the Sir3 to Sir2 ration increased of approximately 2.5 fold and that of Sir3 to Sir4 of roughly 4.5 fold (Liou *et al*, 2005). This suggests that the architecture of the Sir complex may vary following the Sir2-dependent deacetylation of nucleosomes during the formation of silent chromatin. Further experiments are needed to confirm or disprove this hypothesis.

While the assembly of the Sir2, Sir3 and Sir4 proteins into the Sir complex is important to repress gene expression, Sir2, Sir3 and Sir4 each plays a very different but complementary role as described below. The NAD-dependent deacetylase activity of Sir2, is required for silencing and it may do more than generate hypoacetylated nucleosomes. Sir3 is the key player in binding the nucleosome and, by "sensing" the modification state of histones, restricts the loading of the Sir complex to regions to be silenced. In addition, Sir3 may play a major role in promoting the spread of silent domains. Finally, Sir4 plays a crucial scaffolding role, bridging between Sir2 and Sir3 as well as localizing Sir-repression on the chromatin fiber and at the nuclear periphery.

1.9.1 SIR2: FROM HISTONE DEACETYLATION TO *O*-AADPR PRODUCTION

The only Sir protein with a catalytic activity is Sir2, a class III NAD-dependent histone deacetylase. Sir2 is the founding member of the Sirtuin family conserved from yeast to man whose members are known to deacetylate a wide variety of histone and non-histone substrates ((Imai *et al*, 2000; Smith *et al*, 2000) and reviewed in (Greiss and Gartner, 2009)). Although Sirtuins are involved in various cellular processes, there is at least one Sirtuin involved in gene silencing in most organisms. Interestingly, the role of Sirtuins in controlling gene expression is very old, since even in Archaea a Sir2 homolog is reported to regulate silencing through deacetylation of the major archaeal chromatin protein Alba (Bell *et al*, 2002).

In yeast, there are five Sirtuins: Sir2, Hst1, Hst2, Hst3 and Hst4. Hst1 is the paralog of Sir2 and these two proteins arose from the genome duplication event that generated *S. cerevisiae* (Hickman and Rusche, 2007). Sir2 and Hst1 repress a different subset of genes and their common ancestor *K. lactis* Sir2 fulfils both functions indicating that they specialized following duplication (Hickman and Rusche, 2009). Hst1 has a promoter-specific repression function and works in complex with Sum1 and Rfm1 (Xie *et al*, 1999; Sutton *et al*, 2001; McCord *et al*, 2003). On the other hand, Sir2 specialized in non-specific promoter silencing and repression of rDNA recombination, as part of the SIR or the RENT complex, respectively. The catalytic core of Sir2 and Hst1 are highly conserved and can be interchanged, yet the specific silencing functions of Sir2 and Hst1 require protein interfaces that mediate binding to Sir4 (aa 101-236 of Sir2) or Rfm1 (aa 266-325 of Hst1), respectively (Mead *et al*, 2007; Froyd and Rusche, 2011; Hickman *et al*, 2011). Specific interactions for Sir2 and Hst1 were likely achieved through the acquisition of divergent N-terminal domains and mutation of two residues in the catalytic core (yet not directly involved in catalysis; N378 and L379 in Sir2 (Mead *et al*, 2007). Consistently, when the N-terminal region of Hst1 was swapped with the N-terminus of Sir2, the Hst1::$Sir2_{12-155}$ chimera was able to interact with Sir4 and silence the *HM* loci and telomeres in a *sir2*Δ strain (Mead *et al*, 2007). In addition, swapping only two Sir2 residues with the Hst1 counterparts (N378Q and L379I) generated a Sir2 mutant which efficiently associates with Rfm1 and can suppress Hst1 promoter-specific target genes in a *hst1*Δ strain (Mead *et al*, 2007). Less clear is the evolutionary origin of the interactions found in the rDNA-localized RENT complex (containing Sir2, Cdc14, Net1 (Shou *et al*, 1999; Straight *et al*, 1999; Ghidelli *et al*, 2001; Tanny *et al*, 2004)). In addition, Sir2 has been suggested to self-associate in a homotrimeric complex specifically at the rDNA (Cubizolles *et al*, 2006), a feature not yet reported for Hst1.

1.9.1.1 GENETIC AND STRUCTURAL DISSECTION OF SIR2 FUNCTIONS

Sir2 has been subjected to extensive mutagenesis which revealed functionally important domains and residues, notably mapping to the catalytic core region (Cockell *et al*, 2000; Cuperus *et al*, 2000; Armstrong *et al*, 2002; Garcia and Pillus, 2002; Cubizolles *et al*, 2006; Matecic *et al*, 2006; Hickman and Rusche, 2007; Wang *et al*, 2008a). Indeed, the single amino acid substitution N345A, maps to the nucleotide binding motif (Rossman fold) and disrupts Sir2 enzymatic activity *in vitro* and *in vivo* (Imai *et al*, 2000; Armstrong *et al*, 2002). Similarly, the substitution of a conserved histidine within the catalytic core with tyrosine abrogates the enzymatic activity of yeast Sir2 (H364Y in *Sc*Sir2) (Tanny *et al*, 1999) and its human counterpart (Frye, 1999). In agreement with the dual role of Sir2 acting either within the SIR or the RENT complex, several laboratories have identified Sir2 domains or point mutations that specifically affect silencing at

telomeres and *HM* loci or the rDNA locus (Cockell *et al*, 2000; Cuperus *et al*, 2000; Cubizolles *et al*, 2006). For instance, the P394L mutation within the Sir2 core, selectively disrupts rDNA repression and impairs Sir2 homotrimerization (Cubizolles *et al*, 2006). Depending on its binding partners, Sir2 is either localized in telomeric foci at the nuclear periphery, co-localizing with Sir3, Sir4 and Rap1, or it is found in the nucleolus with Cdc14 and Net1, associated with tandem rDNA repeats from which Sir3 and Sir4 are normally excluded (Gotta *et al*, 1997; Kennedy *et al*, 1997). The balance between the two Sir2 populations has to be tightly regulated, since Sir2 levels in the nucleolus can be reduced by high Sir4 concentrations, impairing rDNA silencing dramatically (Smith *et al*, 1998).

Many mutations identified within the catalytic core (aa 237-562) can nicely be mapped to the active site in the crystal structure ((Imai *et al*, 2000; Armstrong *et al*, 2002) and Rolf Sternglanz and Rui-Ming Xu, unpublished). Structurally, Sir2 is highly similar to its yeast paralog Hst2 or other homologs found in various species, including man (i.e. SIRT1) (Finnin *et al*, 2001; Min *et al*, 2001; Zhao *et al*, 2003). Sir2 is composed of a catalytic domain (aa 237-555) highly conserved from Archaea to man (Sauve *et al*, 2006; Sanders *et al*, 2010), and a N-terminal domain (aa 99-236) arranged in a horseshoe shape (Rolf Sternglanz and Rui-Ming Xu, unpublished). The interface between this two domains is limited (622 Å) suggesting a relative dynamic positioning. However, interaction with the Sir4 SID (aa 737-893), which becomes buried between the two Sir2 domains, is proposed to stabilize the orientation of the catalytic domain and the N-terminal module of Sir2, enhancing Sir2 catalytic activity (Rolf Sternglanz and Rui-Ming Xu, unpublished). This is in agreement with earlier reports showing that that Sir4 binding increases the activity of Sir2 *in vitro* (Tanny *et al*, 1999; Cubizolles *et al*, 2006).

1.9.1.2 BEYOND HYPOACETYLATED HISTONES: A ROLE FOR *O*-AADPR?

The catalytic activity of Sir2 is essential for Sir proteins spreading and the establishment of silencing (Tanny *et al*, 1999; Yang *et al*, 2008a). While an early study reported that Sir2 overexpression correlates with global histone hypoacetylation (Braunstein *et al*, 1993), the crucial substrate of Sir2 is acetylated lysine 16 on the tail of the histone H4 (H4K16ac) (Imai *et al*, 2000; Borra *et al*, 2004; Millar *et al*, 2004). As introduced above, H4K16ac is mainly deposited by the nonessential histone acetyltransferase Sas2 (Kimura *et al*, 2002; Suka *et al*, 2002). Sir2 has been reported to deacetylate other substrates, however their relevance is either unclear or debated ($H3K79^{ac}$ (Bheda *et al*, 2012), $Pck1\text{-}K514^{ac}$ (Lin *et al*, 2009), $H3K56^{ac}$ (Xu *et al*, 2007)). Like other Sirtuins, the NAD-dependent Sir2 deacetylation reaction is coupled to the production of nicotinamide (NAM), which inhibits Sir2 activity *in vivo* and *in vitro* (Bitterman *et al*, 2002; Sauve *et al*, 2005), and *O*-acetyl-ADP-ribose (*O*-AADPR; (Tanner *et al*, 2000; Tanny and Moazed, 2001) and reviewed in (Tong and Denu, 2010)). The generation of NAM and *O*-AADPR makes this class of enzymes interesting for several reasons: the NAD dependency makes the catalytic activity reliant on cellular NAD concentration, a measure of nutrient availability. Even though Hst1 seems to be more sensitive to NAM/NAD concentrations (Li *et al*, 2010), *NDT1* and *PCN1*, two enzymes regulating cellular NAM or NAD concentrations, impact silencing especially at the rDNA (Bitterman *et al*, 2002; Gallo *et al*, 2004; McClure *et al*, 2008). On the other hand, *O*-AADPR has been hypothesized to function as a second messenger molecule since several proteins contain potential binding sites for this metabolite (Gasser and Cockell, 2001; Tong and Denu, 2010). The observation that microinjecting purified *O*-AADPR into starfish oocytes leads to a delay in maturation, led to the speculation that *O*-AADPR may have a physiological activity and thus needs to be regulated (Borra *et al*, 2002). Consistently, several enzymes have been shown to further metabolize *O*-AADPR at least *in vitro,* like the budding yeast NUDIX family member Ysa1, which hydrolyses *O*-AADPR into *O*-acetylribose-5-phosphate and AMP ((Rafty *et al*, 2002) and reviewed in (Tong and Denu, 2010)).

In yeast, *O*-AADPR has been suggested to alter the architecture of the SIR complex and promote loading of the Sir complex and Sir3 to chromatin (Gasser and Cockell, 2001; Liou *et al*, 2005; Martino *et al*, 2009). However, direct binding of *O*-AADPR was so far only detected for Sir2 (Tung *et al*, 2012) while the AAA domain of Sir3 has lost its ability to bind nucleotides (see below and (Ehrentraut *et al*, 2011)). Alternatively, the acetyl group of *O*-AADPR could be transferred to an unknown substrate (Rafty *et al*, 2002) and promote silencing indirectly. While Sir2 activity is required for silencing in normal conditions (Tanny *et al*, 1999; Imai *et al*, 2000; Yang and Kirchmaier, 2006; Yu *et al*, 2006), the presence of *O*-AADPR is not absolutely essential. Silencing can be accomplished, yet not at wild-type levels, in a strain depleted of all five Sirtuins – thus presumably *O*-AADPR – if Sir3 is fused to the NAD-independent histone deacetylase Hos3 (Chou *et al*, 2008). Silencing can also be restored to a partial level if H4K16 is substituted with arginine (H4K16R, mimicking the deacetylated state). However, silencing in these conditions requires the overexpression of Sir3 (Yang and Kirchmaier, 2006), which *per se* has the ability to extend silent domains (Renauld *et al*, 1993). Importantly, the Sir complex can associate with chromatin in a H4K16R background, yet silencing is only partial (Yang and Kirchmaier, 2006). This again suggests that the simple association of the Sir complex with chromatin does not convey maximal repression which may require H4K16ac turnover and production of *O*-AADPR. Consistently, *O*-AADPR is not required for a basal level of chromatin compaction, linker DNA protection from enzymatic attack and decreased transcription *in vitro* (Johnson *et al*, 2009; Martino *et al*, 2009). Nevertheless, Sir-chromatin interaction and protection of the linker DNA can be reinforced by *O*-AADPR directly *in vitro* (Johnson *et al*, 2009; Martino *et al*, 2009). The same can be achieved by the enzymatic activity of Sir2 (Oppikofer *et al*, 2011), suggesting that production of *O*-AADPR enhances silencing *in vivo* (see Chapter 2 - An active mark promotes silencing).

1.9.2 SIR4: SCAFFOLDING, NUCLEATION AND ANCHORING

Sir4 is one of the fastest evolving proteins in the budding yeast genome and is not conserved except in closely related *Saccharomycetales* (Zill *et al*, 2010). The C-terminal half (aa 747-1358) is essential for silencing and mediates most interactions, especially the coiled-coil domain at its extreme C-terminus (aa 1262-1358). The extreme Sir4 N-terminus (aa 1-270) binds DNA and is thought to help recruiting Sir4 to silent regions (Martino *et al*, 2009; Kueng *et al*, 2012). The central domain of Sir4 (aa 300-730) appears to be unique to *Sc*Sir4. This domain does not contain mapped mutations or known functions, yet it is necessary for silencing at telomeres, suggesting a structural role (Kueng *et al*, 2012).

1.9.2.1 THE SIR4 N-TERMINUS: RECRUITING AND REGULATING SILENCING

The Sir4 N-terminus (Sir4N; aa 1-270) is dispensable for Sir-mediated repression of the *HM* loci in normal conditions. However, Sir4N is required for efficient repression of a crippled *HMR* locus where either Orc1 or Abf1 binding sites have been deleted (Kueng *et al*, 2012). While Sir4N is not required for the assembly or the enzymatic activity of the holo Sir complex (Kueng *et al*, 2012), Sir complexes lacking the Sir4N domain bind nucleosomal arrays and protect linker DNA from nucleolytic digestion less effectively than wild-type Sir complexes *in vitro*. Consistently, Sir4N was shown to readily bind to DNA *in vitro* (Martino *et al*, 2009), plausibly through the many basic residues found in this region (Martino *et al*, 2009; Kueng *et al*, 2012). Together with the observation that the Sir4N is required for efficient repression if Sir-recruitment is impaired (Kueng *et al*, 2012), this suggests that the Sir4N enhances or stabilizes the loading of Sir complexes onto chromatin *in vivo* through its interaction with DNA. Additionally, Sir4N interacts with Yku80 and Sir1 (Triolo and Sternglanz, 1996; Roy *et al*, 2004) suggesting a Sir-recruiting role for this domain in silent regions. In addition Sir4N also interacts with Sif2, a component of the transcriptional activator Set3C complex (Wang *et al*, 2002), but the functional relevance of this interaction is unclear (Cockell *et al*, 1998b).

Together with the uncharacterized central part (aa 271-746), the N-terminal half of Sir4 has been suggested to function as a regulatory domain (Zill *et al*, 2010). Indeed, this region of Sir4 becomes heavily phosphorylated during mitosis, when silencing is released (Laroche *et al*, 2000; Smith *et al*, 2003a; Kueng *et al*, 2012). Phosphorylation of Sir4N may decrease its affinity for DNA, as suggested by acetylmimic substitutions at serine 63 and 84 (Kueng *et al*, 2012), thus decreasing the affinity of Sir4 and the Sir complex to chromatin.

1.9.2.2 MANY INTERACTIONS WITHIN THE SIR4 C-TERMINUS: ANCHORING AND BEYOND

The C-terminal half of Sir4 (aa 747-1358) is sufficient for silencing at *HM* loci but not at telomeres, consistent with the recruiting function of Sir4N which becomes more important at telomeres where recruitment is less redundant (Kueng *et al*, 2012). At the beginning of this half resides the Sir2 Interaction Domain (SID; aa 737-893), which binds the catalytic domain and the N-terminus of Sir2, enhancing its catalytic activity ((Tanny *et al*, 1999; Cubizolles *et al*, 2006) and (Rolf Sternglanz and Rui-Ming Xu, unpublished). Between the SID and the C-terminal coiled-coil domain is the Partitioning and Anchoring Domain (PAD; aa 950-1262), which is sufficient to tether a chromatin locus to the nuclear periphery through its interaction with Enhancer of Silent Chromatin 1 (Esc1; the interacting region in Esc1 is aa 1395-1551; (Andrulis *et al*, 2002; Taddei *et al*, 2004)). However, the peripheral localization of Sir proteins – and therewith telomeres and the *HM* loci – is mediated by both Esc1 and Yku interactions with Sir4 (Hediger *et al*, 2002; Taddei *et al*, 2004).

The C-terminal half of Sir4 also interacts with Yku80 and Ubp10/Dot4 (Kahana and Gottschling, 1999; Taddei *et al*, 2004). Ubp10 de-ubiquitinates H2BK123 leading to a more compacted chromatin structure (Fierz *et al*, 2011) and reduced levels of H3K4 and H3K79 methylation (Foster and Downs, 2009). Thus, Ubp10 counteracts the deposition of active chromatin marks, favoring the establishment of silencing (Emre *et al*, 2005; Gardner *et al*, 2005).

The Sir4 C-terminal coiled-coil domain (aa 1262-1358) mediates Sir4 homodimerization by forming a parallel coiled-coil structure as revealed by X-ray crystallography (Chang *et al*, 2003; Murphy *et al*, 2003). Disruption of this dimerization interface leads to a loss of silencing (Murphy *et al*, 2003). However, this domain also interacts with Sir3, which binds to the surface of the Sir4 coiled-coil region (Chang *et al*, 2003), and the mutations introduced by Murphy and colleagues also disrupt the Sir4-Sir3 interaction (Stephanie Kueng and Susan Gasser, unpublished). Therefore, it is still unclear whether Sir4 coiled-coil homodimerization is required for silencing *per se*. The Sir4 coiled-coil module also interacts with Rap1 and Yku70, playing an important role in recruitment of Sir4 to telomeres and silencers (Moretti *et al*, 1994; Tsukamoto *et al*, 1997; Mishra and Shore, 1999). While Sir3 can bind a mutant of Sir4 with impaired dimerization function (Stephanie Kueng and Susan Gasser, unpublished), it is unclear whether mutations that disrupt Sir4 coiled-coil dimerization also disrupt its interaction with Yku70 and Rap1.

In summary, the C-terminal half of Sir4 forms a scaffold for silent chromatin by mediating 1) interactions with both Sir2 and Sir3 leading to the formation of the holo Sir complex, 2) Sir recruitment to silent sites through Rap1 and Yku, and 3) nuclear anchoring through Esc1 and Yku70/80, all of which are crucial for silencing.

1.9.2.3 SIR4 INTERACTION WITH CHROMATIN

Sir4 binds DNA with no sequence specificity (Martino *et al*, 2009). Pull-down experiments showed that *in vitro* translated Sir4 also binds to histone H4 tails (expressed in bacteria) in a manner sensitive to mutations within the basic patch (aa 16-22) (Hecht *et al*, 1995). However, *in vivo*, Sir4 is most likely to be found in complex with Sir2 as a stoichiometric Sir2-4 complex could be purified by overexpressing a TAP-tagged Sir4 in yeast (Hoppe *et al*, 2002; Liou *et al*, 2005). Consistently, Sir4 alone seems to be unstable *in vitro*, but could be readily co-purified with stoichiometric amounts of Sir2 after co-infection of insect cells (Martino *et al*, 2009). The binding affinity of Sir2-4 to an array of reconstituted nucleosomes was not appreciably affected by deletion of the H4 tails which may be due to the high affinity of Sir4 for DNA (Martino *et al*, 2009). In contrast, loading of the holo Sir complex to reconstituted nucleosomal arrays lacking the H4 N-terminal tail was mildly but reproducibly decreased (Martino *et al*, 2009). Specific recognition of the H4 tail is likely to be mediated by Sir3 as deletion of the H4 tail decreased the binding affinity of Sir3 to nucleosomal arrays and mononucleosomes *in vitro* (Martino *et al*, 2009; Sinha *et al*, 2009). Consistently, acetylation of a nucleosomal array *in vitro* including the key residue H4K16 (Millar *et al*, 2004), by means of a NuA4 subcomplex, impaired Sir3-chromatin interactions yet it did not decrease Sir2-4 or Sir2-3-4 loading onto chromatin (Johnson *et al*, 2009). This result was surprising as acetylation of H4K16 was thought to inhibit the spread of Sir-mediated silencing (Kimura *et al*, 2002; Suka *et al*, 2002; Millar *et al*, 2004) and the functional implication of this interaction needed to be tested further (see above and Chapter 2 - An active mark promotes silencing).

1.9.2.4 NUCLEATION OF SILENCING: SIR4 IS KEY BUT "UNITED WE STAND, DIVIDED WE FALL"

Sir4 is the only protein within the Sir complex detected at silencers and telomeric TG_{1-3} repeats in the absence of the other Sir proteins (Hoppe *et al*, 2002; Luo *et al*, 2002; Rusche *et al*, 2002). Immunoblotting analysis of a whole cell lysate revealed that the level of the Sir4 protein is mildly reduced if *SIR3* or *SIR2* are deleted (Rusche *et al*, 2002). This may result from the instability of the Sir4 protein when a Sir2-4 heterodimer or the holo Sir complex cannot be formed (Martino *et al*, 2009). Consistently, in a *sir3*Δ or *sir2*Δ background, the recruitment of Sir4 at silencers drops by about 50% (Hoppe *et al*, 2002; Luo *et al*, 2002). Importantly, the recruitment of Sir4 at silencers is also reduced to roughly 50% in a strain carrying a catalytic dead Sir2 (H346Y) which still supports the formation of the holo Sir2-3-4 complex (Luo *et al*, 2002). In addition, Sir3 is also thought to participate to nucleation of silencing through its interaction with Rap1 and Abf1 (Gasser and Cockell, 2001; Moretti and Shore, 2001). This suggests that Sir4 plays an important scaffolding role in assembling the Sir complex at silencers, yet a functional and holo Sir complex is required for efficient nucleation.

1.9.3 SIR3: SELECTIVE NUCLEOSOME BINDING AND SPREADING OF SILENCING

Sir3 is a paralog of Orc1 that arose through the whole genome duplication that created *S. cerevisiae* approximately 100 million years ago (Kellis *et al*, 2004; Hickman *et al*, 2011). It shares the same architecture as Orc1 having an N-terminal Bromo-Adjacent Homology domain (BAH; aa 1-214 (Connelly *et al*, 2006)), a central AAA+ ATPase-like domain (AAA; aa 532-834 (Ehrentraut *et al*, 2011)) and a predicted C-terminal winged helix-turn-helix domain (wH; aa 840-978; see Figure 4). Interestingly, a chimeric protein containing the Orc1 BAH domain and the C-terminus of Sir3 can restore *HM* silencing in a *sir3*Δ background, indicating a high degree of functional conservation between these two paralogs (Bell *et al*, 1995). Inversely, swapping the N-terminal part of Orc1 with the Sir3 counterpart generates a protein functional in replication, but not silencing (Bell *et al*, 1995; Stone *et al*, 2000). Consistently, the C-terminus of Sir3 interacts with proteins important for silencing such as Sir4 and Rap1 (see below).

1.9.3.1 THE BAH DOMAIN FAVORS BINDING TO UNMODIFIED NUCLEOSOMES

The main function of the BAH domain is to interact specifically with non-modified nucleosomes. Johnson and colleagues hypothesized that the Sir3 BAH contacts chromatin when they identified suppressor mutations within the Sir3 BAH that could restore silencing in strains carrying point mutations in the basic domain of the H4 N-terminal tail (Johnson *et al*, 1990). One of the mutations they identified, D205N (*sir3R3*), was later found to strongly increase the affinity of the BAH domain for nucleosomes *in vitro* and to rescue telomeric silencing in a *rap1-22* (H810Y) silencing deficient strain (Liu and Lustig, 1996). The D205N mutation also made possible the co-crystallization of the BAH domain with a mononucleosome (Armache *et al*, 2011). This recent structure nicely confirmed several features of the interaction surface between the BAH domain and the nucleosome previously mapped using mutagenesis (Buchberger *et al*, 2008; Sampath *et al*, 2009), especially the mutations in the so called LRS region (Loss of rDNA Silencing) around H3K79 on the nucleosomal surface (Park *et al*, 2002; Thompson *et al*, 2003). Importantly, the interaction of the BAH domain with the nucleosome is specific for non-modified histones, and two modifications that are inhibitory to silent chromatin formation *in vivo* – H4K16ac and H3K79me – have been shown to strongly decrease the binding affinity of both, full length Sir3 and the BAH domain for chromatin (Altaf *et al*, 2007; Onishi *et al*, 2007; Johnson *et al*, 2009; Martino *et al*, 2009; Armache *et al*, 2011; Oppikofer *et al*, 2011). The crystal structure provided molecular details on how methylation of H3K79 decreases the BAH-nucleosomes association: progressive methylation reduces the hydrogen bonding between K79 and the loop 3 of the BAH domain as well as increasing the hydrophobicity of this region,

thereby reducing affinity of the BAH domain for H3K79^{me} nucleosomes (Armache *et al*, 2011). Similarly, the H4K16 ε-amino group interacts with polar or negatively charged side chains of the BAH domain (D60, Y69, E95, and S67) and acetylation of H4K16 could disrupt most of the electrostatic contacts in this negatively charged binding pocket of the Sir3 BAH (Armache *et al*, 2011).

The N-terminal acetylation of Sir3-BAH by NatA (a dimer of *ARD1* and *NAT1*) is essential for silencing (Aparicio *et al*, 1991; Park and Szostak, 1992), which explains why adding an N-terminal tag to Sir3 disrupts its function (Wang *et al*, 2004; Onishi *et al*, 2007). Sir3 N-terminal acetylation is likely to enhance chromatin affinity, as defects in *HMR* silencing in a non-acetylatable Sir3 mutant (A2G) can be suppressed by mutations in the histone H4 and H3 tails (Sampath *et al*, 2009).

1.9.3.2 IS THE BAH DOMAIN INVOLVED IN NUCLEOSOMAL STACKING?

By disclosing the binding mode of the BAH domain to the H4 N-terminal tail, the crystal structure of the Sir3 BAH bound to the nucleosome (Armache *et al*, 2011) suggests the hypothesis that budding yeast may have evolved a unique chromatin compaction mechanism. Based on the *X. laevis* NCP structure, the H4 tail has been suggested to function in chromatin compaction by contacting the acidic path of the H2A/H2B dimer of the neighboring nucleosome (Luger *et al*, 1997; Luger and Richmond, 1998b). Indeed the H4 tail is essential for chromatin compaction *in vitro* (Dorigo *et al*, 2003) and acetylation of H4K16 releases compaction of reconstituted nucleosomal arrays (Shogren-Knaak *et al*, 2006; Robinson *et al*, 2008). However, in the structure of the yeast nucleosome, the H4 tail is more distorted, contacting the DNA rather than the H2A/H2B dimer of the neighboring nucleosome (see section - The yeast nucleosome and (White *et al*, 2001)). In the BAH-nucleosome structure, the H4 tail becomes ordered upon interaction with the BAH domain making contacts prevalently with lysine 16 and histidine 18. This suggests that upon binding to the H4 N-terminal tail, the Sir3 BAH may reinforce and/or alter the architecture of the nucleosomal stacking. Intriguingly, based on contacts present in the crystal packing, the BAH domain seems to self associate and form a dimeric bridge between two nucleosomes (Armache *et al*, 2011). However, the relevance of this finding outside of the crystal context is unclear, as dimerization of the Sir3 BAH could not be detected by co-immunoprecipitation (Buchberger *et al*, 2008) and only a very weak interaction (dimerization constant ~ 2 mM) was observed by analytical ultracentrifugation (Armache *et al*, 2011).

1.9.3.3 THE C-TERMINAL SIR3 AAA DOMAIN ALSO BINDS THE NUCLEOSOME

The BAH domain is not the only part of Sir3 interacting with chromatin. *In vitro* studies identified two domains in the Sir3 C-terminus (aa 623-762 and aa 808-910) that bind the N-terminal tail of the histone H4 (Hecht *et al*, 1995). In addition to the H4 tail, *in vitro* studies showed that a Sir3 C-terminal fragment (aa 620-978) interacts with a H3 histone peptide (aa 67-89) in a manner sensitive to the methylation of H3K79, indicating that this domain, together with the BAH, can also contact the LRS surface of the nucleosome (Altaf *et al*, 2007). Whether the Sir3 AAA alone was sufficient for this interaction and whether Sir3 AAA could interact with the LRS surface in the context of chromatin was not tested prior to my work (see Chapter 3 - The AAA+ ATPase-like domain of Sir3 binds nucleosomes in an H3K79me sensitive manner). A screen for dominant negative *sir3* alleles, discovered a point mutation within the first chromatin interaction domain of the Sir3 C-terminus (L738P) indicating that the interaction of the Sir3 AAA domain with chromatin may also be important *in vivo* (Buchberger *et al*, 2008). Indeed, this mutation enhanced the interaction of Sir3 with chromatin and impaired spreading of the Sir complex (Buchberger *et al*, 2008).

1.9.3.4 SIR3 MAY BIND THE NUCLEOSOME IN MORE THAN ONE CONFORMATION

The BAH D205N and the AAA L738P mutants, both impair silencing yet bind the nucleosome with higher affinity compared to wild-type (Connelly *et al*, 2006; Buchberger *et al*, 2008). Similarly, ectopically expressed Orc1 BAH cannot replace Sir3 BAH in silencing, yet it co-immunoprecipitates more histone H3 than Sir3 BAH (Stone *et al*, 2000; Onishi *et al*, 2007). This suggests that strong binding does not correlate with efficient silencing. A possible explanation is that the Sir3 protein may need to continuously bind and release the nucleosome for efficient silencing, however the benefit of such a binding mode is unclear. A better explanation for this apparent conundrum comes from the observation that both the BAH and the AAA domain (or a larger Sir3 C-terminal fragment, aa 623-910) bind to the nucleosomal surface around H3K79 as well as the H4 N-terminal tail (Hecht *et al*, 1995; Carmen *et al*, 2002; Altaf *et al*, 2007; Onishi *et al*, 2007; Armache *et al*, 2011; Ehrentraut *et al*, 2011). This strongly suggests that Sir3 can bind the nucleosome in at least two different conformations where either the BAH or the AAA interact with the H3K79/H4 N-terminal region of a given nucleosome. Within a single Sir3 molecule, the domain not involved in this interaction, may contact the same region of a neighbor nucleosome, the linker DNA or being involved in additional protein-protein interactions. Such a mechanism may facilitate the spreading of the Sir3 protein (and the Sir complex) along an appropriately unmodified chromatin fiber.

1.9.3.5 A CENTRAL ROLE IN SPREADING FOR THE SIR3 PROTEIN?

The most striking features of Sir3 are its multiple and modifications specific interactions with chromatin, and its multimerization properties. Multimerization of full length Sir3 has been observed *in vitro* at high concentrations and

low ionic strength (Georgel *et al*, 2001). Moreover, Sir3 has a strong tendency to form higher order structures if bound to DNA and nucleosomal arrays (Georgel *et al*, 2001). This property may reflect the role of Sir3 in spreading silencing *in vivo*. Indeed, when overexpressed, Sir3 extends telomeric silencing (Renauld *et al*, 1993) and accumulates on chromatin at distances further away from the nucleation site with only substoichiometric amounts of Sir2-4 (Strahl-Bolsinger *et al*, 1997). This property of Sir3 seems to be mediated, at least in part, by the Sir3 BAH, which increases both the frequency and extent of subtelomeric silencing when expressed ectopically in SIR+ strain (Gotta *et al*, 1998). Intriguingly, overexpression of an N-terminal construct encompassing the BAH domain (aa 1-380; which also interacts with the DNA) or a BAH-lexA fusion can partially restore silencing in a *sir3*Δ background (Connelly *et al*, 2006). This function of the Sir3 BAH was suggested to result from spreading of the BAH domain on chromatin given that BAH-BAH interactions could be observed in crystallographic conditions (Connelly *et al*, 2006). However, the lexA tag can dimerize *per se* and Sir3 BAH oligomerization could not be observed *in vitro* (Connelly *et al*, 2006; Armache *et al*, 2011), making it unclear how relevant this is *in vivo*. Similarly, an atypical N-terminal α-helical extension within the Sir3 AAA domain (see above) mediates oligomerization in the crystal lattice (Ehrentraut *et al*, 2011), yet these interactions may have no relevance *in vivo*. It is not clear which domain contributes most to the extended spread of Sir3 observed *in vivo* upon overexpression (Renauld *et al*, 1993; Strahl-Bolsinger *et al*, 1997) or to the oligomeric Sir3-chromatin structures found *in vitro* (Georgel *et al*, 2001). Moreover, it is unclear whether these interactions are also responsible for spreading of the holo Sir-complex under normal conditions, which likely involves additional interactions such as Sir3-Sir4 and Sir4 homodimerization.

1.9.3.6 THE SIR3 AAA DOMAIN LOST ATPASE ACTIVITY AND EVOLVED TO BIND TO SIR4

The homologous AAA+ ATPase-like domain of yeast Orc1 hydrolyses ATP and this function is essential for cell viability (Klemm *et al*, 1997). However, the residues required for binding and catalysis of ATP are not conserved in Sir3 (Bell *et al*, 1995). Moreover, the recent crystal structure of the Sir3 AAA domain (aa 530-845) revealed not only that the key catalytic residues are missing but that the overall nucleotide binding pocket is altered in the Sir3 AAA domain (Ehrentraut *et al*, 2011). While Sir3 AAA posses typical AAA+ ATPase-like features, such as the "base" sub domain (or Rossman fold) and the "lid" module, the orientation of the "lid" compared to the "base" is unusual and the flexible N-terminal region that in Orc1 participates in the nucleotide binding pocket, in Sir3 AAA forms a protruded α-helical extension directed away from the core structure. This results in a rather shallow groove incompatible with nucleotide binding (Ehrentraut *et al*, 2011). Whether Sir3 acquires the ability to bind a nucleotide once assembled in the holo Sir complex and/or loaded onto chromatin remains to be tested. Ehrentraut and colleagues also conducted a systematic alanine scan on charged residues within Sir3 AAA highlighting several potential protein-protein interfaces required for silencing (Ehrentraut *et al*, 2011). Notably, alanine substitutions at residues 657-659 disrupt the Sir3-Sir4 interaction and assembly of the Sir complex *in vitro* (Ehrentraut *et al*, 2011). However, it remains to be determined whether these residues directly bind to the Sir4 coiled-coil domain – the region of Sir4 interacting with Sir3 – and if they could complement the mutations in the Sir4 coiled-coil disrupting its interaction with Sir3 (single mutation either M1307N, E1310R or I1311N; (Chang *et al*, 2003; Rudner *et al*, 2005)). Thus, it seems that after the whole genome duplication that generated *S. cerevisiae*, the Sir3 AAA domain has lost ATPase activity and acquired a protein-protein interaction function crucial for gene silencing.

1.9.3.7 SIR3-RAP1 AND SIR3 INTERACTING FACTORS

Apart from itself and chromatin, other important Sir3 interaction partners are Sir4, Abf1, and Rap1 (Moretti *et al*, 1994; Moazed *et al*, 1997; Gasser and Cockell, 2001; Moretti and Shore, 2001). Whereas the Abf1 interaction has never been fully investigated (Gasser and Cockell, 2001), the last 27 amino acids of Rap1 have been shown to bind Sir3 and are essential for silencing of reporter genes inserted at telomeres or compromised *HM* loci (Liu *et al*, 1994; Moretti *et al*, 1994). The Rap1 binding domain within Sir3 is upstream of the AAA domain (aa 456-481 (Moretti *et al*, 1994)) and the structure of the Rap1-Sir3 interaction surface has recently been solved by X-ray crystallography (Chen *et al*, 2011). The short Sir3 fragment forms an α-helix that binds to a hydrophobic groove within the C-terminal domain of Rap1 (RCT; aa 695-827). Interestingly, *S. pombe* Rap1 and human Rap1 bind with the same mode to their telomeric binding partners Taz1 and TRF2, respectively, indicating that this interaction module has been conserved across evolution (Chen *et al*, 2011). The same fragment of Rap1 also interacts with Sir4, Rif1 and Rif2, even though the four proteins do not all share the exact same binding mode (Feeser and Wolberger, 2008), indicating a competition between silencing and telomeric proteins (Buck and Shore, 1995; Marcand *et al*, 1996; Wotton and Shore, 1997). This competition is important for telomere length regulation as removing either of the Rif proteins increases the length of the TG_{1-3} repeats tract (Buck and Shore, 1995; Wotton and Shore, 1997) while deletion of Sir3 or Sir4 has the opposite effect (Palladino *et al*, 1993) (see section - Subtelomeric silencing: Telomere Position Effect).

1.9.3.8 PURSUING THE FUNCTION OF THE EXTREME SIR3 C-TERMINUS

The extreme C-terminus of Sir3 (aa 840-978) is predicted to adopt a winged-helix-turn-helix (wH) fold based on its homology with the crystallized archaeal Orc1 C-terminus (Dueber *et al*, 2007; Gaudier *et al*, 2007). Structural as well as biochemical studies show that the wH of homologs archaeal Orc1 bind DNA and participate in the assembly of the replication origin (De Felice *et al*, 2004; Dueber *et al*, 2007; Gaudier *et al*, 2007). However, in budding yeast, Sir3-

DNA interactions had only been mapped to a N-terminal fragment containing the BAH domain (aa 1-380; (Connelly *et al*, 2006)). In contrast to the archaeal homolog, the Sir3 wH domain has been reported to self-associate in a yeast-two-hybrid assay (Liaw and Lustig, 2006) and to interact *in vitro* with a central domain of Sir3 (aa 464-728), which contains a N-terminal portion of the AAA domain (King *et al*, 2006). *In vivo*, the Sir3 wH can mediate repression of a subtelomeric reporter gene when bound to the promoter-proximal region as a lexA fusion protein (Park *et al*, 1998; Liaw and Lustig, 2006). Ectopic establishment of silencing is thought to involve the Sir3 wH dimerization activity which would recruit full-length, endogenous Sir3 which in turn brings in Sir4 and Sir2 (Liaw and Lustig, 2006). However, the function of this predicted Sir3 wH module and Sir3 dimerization had never been tested and remained elusive (see Chapter 3 - Homodimerization of Sir3 wH is essential for silent chromatin formation).

1.10 HOW IS GENE REPRESSION ACHIEVED IN YEAST SILENT CHROMATIN?

Despite the extensive genetic and interaction information cited above, the precise mechanism through which Sir proteins mediate gene repression is not fully understood. Several mutually non-exclusive models have been proposed, going from simple steric hindrance involving higher order chromatin structures, to an active inhibition of recruitment and activation of the transcriptional machinery.

1.10.1 THE STERIC HINDRANCE MODEL: HIGHER-ORDER FOLDING

The steric hindrance model proposes that loading of Sir proteins onto chromatin physically blocks the access of site-specific activator proteins to their DNA target sites. This model is supported by the observation that silent chromatin *in vivo* is less accessible to the HO endonuclease (Nasmyth, 1982; Weiss and Simpson, 1997), the DAM methyltransferase (Gottschling, 1992; de Bruin *et al*, 2000) and protein required for DNA repair (Terleth *et al*, 1989; Sinha *et al*, 2009). In addition, loading of Sir proteins *in vitro* limits the accessibility of the linker DNA to micrococcal nuclease digestion (Martino *et al*, 2009).

Steric hindrance could be generated at different levels. At the primary level, the binding of the Sir complex to the nucleosome could inhibit the transient unwrapping of the DNA from the histone octamer (Li *et al*, 2005; Bucceri *et al*, 2006; Poirier *et al*, 2009) – referred to as "nucleosomal breathing" – and limit the exposition of transcription factors binding sites. Second, Sir-bound chromatin could form higher-order structures further limiting the accessibility of regulatory factors. The existence of higher-order folding at Sir-silenced regions is supported by the discontinuous mode of silencing observed at native telomeres illustrated by the "looping out" of the active Y' element (Fourel *et al*, 1999; Pryde and Louis, 1999). In addition, back folding or looping of truncated telomeres, was suggested by the association of Rap1 to subtelomeric regions (beyond the TG_{1-3} nucleating repeats) (de Bruin *et al*, 2000) and, more convincingly, by the Gal4-dependent activation of reporter genes located up to 2 kb away from a Gal4 recruiting sequence (de Bruin *et al*, 2001). Additionally, as monitored by EM, DNA fragments containing the *HM* loci appeared to form loops between E and I silencers and between the silencers and the promoter region *in vitro*, in a manner dependent on Rap1 (Hofmann *et al*, 1989). Finally, a recent study combined boundary trap assays, Gal4 enhancer activity assay, ChIP and chromosome conformation capture (3C) to show that *HMR*-E and *HMR*-I are found in close proximity *in vivo* in a manner dependent on Sir-mediated silencing (Valenzuela *et al*, 2008). Higher order folding (i.e. back folding or looping) does not directly imply decreased DNA accessibility and the presumed strengthening of silencing by folding or clustering of silent domains could also be due to a local increase in Sir proteins concentration favoring silencing by other means than simple steric hindrance (see below).

1.10.2 SIR-MEDIATED SILENCING: A FINE-TUNED PROCESS

While the steric hindrance model could be a component of Sir-mediated silencing, there is evidence against such a simple mode of gene repression. The promoter region of the silent *HML* locus was shown to be nucleosome-free and the DNA between two TATA boxes is more sensitive to micrococcal nuclease digestion compared to the active *MAT*α (Weiss and Simpson, 1998). In addition, silencing of reporter genes inserted in the rDNA occurs in regions actively transcribed by RNA polymerase I (Buck *et al*, 2002). Together, these results suggest that Sir-mediated silencing involves a more regulated mechanism that simple unspecific steric hindrance.

In an attempt to understand the molecular basis of Sir-repression, Chen and colleagues monitored LexA binding, as well as restriction enzyme and DAM methylase activity to quantify DNA accessibility at promoters of reporter genes and endogenous loci in active (*sir2*Δ) and repressed ($SIR2^{+}$) conditions (Chen and Widom, 2005). In conflict with previous interpretations (i.e. not necessarily the data in (Gottschling, 1992; Loo and Rine, 1994)) – Chen and colleagues found at best a 2 fold reduction in DNA accessibility in silenced compared to actively transcribed chromatin loci (Chen and Widom, 2005). As investigated by ChIP, Sir-silencing only slightly decreased (< 2 fold) occupancy of the endogenous activator protein of the *URA3* gene, Prp1, yet occupancy of RNA polymerase II at the same location was drastically decreased (> 20 fold) (Chen and Widom, 2005). Similarly, Sir-silencing had a striking effect on the occupancy of the general transcription factors TFIIB and TFIIE which were virtually absent from silent loci (Chen and Widom, 2005). Given that neutral reporters (LexA, EcoRV and DAM methylase), as well as the endogenous Prp1 protein, are still able to access DNA within Sir-silenced regions, it appears that loading of the Sir complex onto chromatin does not markedly

hinder DNA accessibility. Yet, RNA polymerase II occupancy and assembly of the preinitiation complex seems to be efficiently inhibited at promoters of silent genes (Chen and Widom, 2005). Additionally, based on ChIP analysis, Gao and colleagues observed that even when an activated form of RNA polymerase II (Serine 5 phosphorylation) can be detected at the promoter of silenced genes, Sir-mediated silencing dramatically reduces the occupancy of mRNA capping enzymes (Cet1 and Abd1), elongation factors (Spt5, Par1C and TFIIS) and the Mediator (Sekinger and Gross, 2001; Gao and Gross, 2008).

The emerging picture is one where loading of the Sir complex onto chromatin may not efficiently inhibit the binding of all upstream gene regulatory factors (e.g. Prp1) yet silencing impairs the formation of a stable RNA polymerase II preinitiation complex and inhibits the recruitment of elongation factors. Mechanisms for such a specific Sir-dependent regulation of chromatin accessibility are both fascinating and speculative. For instance, silent regions are specifically depleted from histone modifications, unmodified nucleosomes could be a poor binding partner for components of the transcriptional machinery. Alternatively, Sir2 could directly deacetylate transcription factors thereby reducing their affinity to chromatin. Yet, it is also possible that small effects on affinity and occupancy of singular proteins could accumulate cooperatively to strongly reduce the occupancy of a multiprotein complex, such as the RNA polymerase holoenzyme.

2 AN ACTIVE MARK PROMOTES SILENCING

The work presented in this experimental section was published in *A dual role of H4K16 acetylation in the establishment of yeast silent chromatin*. **Oppikofer M**, Kueng S, Martino F, Soeroes S, Hancock SM, Chin JW, Fischle W, Gasser SM (2011) *EMBO J* 30: 2610-2621

Author contributions: MO, SK, and SMG designed the experiments and interpreted results. MO performed the experiments. SK and FM contributed reagents. SS and WF contributed the H4K16ac histone octamers. SMH and JC contributed the H3K56ac histone octamers. MO, SK and SMG wrote the manuscript. SMG supervised the work.

In this experimental section, I made use of an *in vitro* reconstitution system, which allows the loading of Sir proteins onto arrays of regularly spaced nucleosomes, to examine the impact of specific histone modifications on SIR protein binding and linker DNA accessibility. I studied the role of H3K79 methylation, H3K56 acetylation and H4K16 acetylation (see section - Histone modifications regulate yeast silent chromatin, Figure 3) and addressed their molecular role in regulating yeast silent chromatin formation from a biochemical perspective.

The most important finding of this work is that acetylation of H4K16, while it repels Sir3, increases the affinity of the Sir2-4 heterodimer to chromatin. Moreover, the deacetylation of H4K16ac by Sir2 actively promotes the high-affinity binding of the holo Sir complex and reduces the accessibility of the linker DNA. We propose that H4K16ac is actively implicated in the establishment of yeast silent chromatin, being the first histone mark shown to directly increase the binding affinity of a Sir subcomplex to chromatin.

The EMBO Journal (2011) 30, 2610–2621 | © 2011 European Molecular Biology Organization | All Rights Reserved 0261-4189/11
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THE EMBO JOURNAL

A dual role of H4K16 acetylation in the establishment of yeast silent chromatin

Mariano Oppikofer[1], Stephanie Kueng[1], Fabrizio Martino[2], Szabolcs Soeroes[3], Susan M Hancock[2], Jason W Chin[2], Wolfgang Fischle[3] and Susan M Gasser[1,*]

[1]Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland; [2]Medical Research Council Laboratory of Molecular Biology, Cambridge, UK and [3]Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

Discrete regions of the eukaryotic genome assume heritable chromatin structure that is refractory to transcription. In budding yeast, silent chromatin is characterized by the binding of the Silent Information Regulatory (Sir) proteins to unmodified nucleosomes. Using an *in vitro* reconstitution assay, which allows us to load Sir proteins onto arrays of regularly spaced nucleosomes, we have examined the impact of specific histone modifications on Sir protein binding and linker DNA accessibility. Two typical marks for active chromatin, H3K79^{me} and H4K16^{ac} decrease the affinity of Sir3 for chromatin, yet only H4K16^{ac} affects chromatin structure, as measured by nuclease accessibility. Surprisingly, we found that the Sir2-4 subcomplex, unlike Sir3, has higher affinity for chromatin carrying H4K16^{ac}. NAD-dependent deacetylation of H4K16^{ac} promotes binding of the SIR holocomplex but not of the Sir2-4 heterodimer. This function of H4K16^{ac} cannot be substituted by H3K56^{ac}. We conclude that acetylated H4K16 has a dual role in silencing: it recruits Sir2-4 and repels Sir3. Moreover, the deacetylation of H4K16^{ac} by Sir2 actively promotes the high-affinity binding of the SIR holocomplex.

The EMBO Journal (2011) **30,** 2610–2621. doi:10.1038/emboj.2011.170; Published online 10 June 2011
Subject Categories: chromatin & transcription
Keywords: H4K16; histone deacetylation; methylation; silent chromatin; SIR complex

Introduction

Heterochromatin is a heritable, specialized chromatin structure that silences discrete regions in eukaryotic genomes. Among other features, gene silencing within heterochromatic regions is thought to involve compaction of the chromatin fibre in order to structurally limit DNA accessibility. In budding yeast, silent chromatin requires the binding of Silent Information Regulatory (Sir) proteins to unmodified nucleosomes. Work of many laboratories has identified Sir2, Sir3 and Sir4 proteins as the core components of silent chromatin at telomeres and silent mating type loci (Rine and Herskowitz, 1987; reviewed in Rusche *et al* (2003)). Two-hybrid and protein binding studies suggested that they form a complex, with Sir4 being a scaffold protein that bridges between Sir2 and Sir3 (Moazed *et al*, 1997; Strahl-Bolsinger *et al*, 1997; Rudner *et al*, 2005; Cubizolles *et al*, 2006). Although initial attempts to purify the Sir proteins from yeast yielded only an Sir2-4 heterodimer (Ghidelli *et al*, 2001; Hoppe *et al*, 2002), a stable Sir2-Sir3-Sir4 heterotrimer with 1:1:1 stoichiometry (hereafter SIR complex) was purified from insect cells (Cubizolles *et al*, 2006). A fourth Sir protein, Sir1, is important for the establishment of silencing at the silent mating type loci, but is not required for repression at telomeres (Pillus and Rine, 1989; Aparicio *et al*, 1991).

Sir proteins do not bind DNA in a sequence-specific manner, yet zones of silencing are restricted to specific domains in the yeast genome. To achieve targeted silencing, Sir proteins are recruited by bifunctional DNA binding factors, such as Rap1, Abf1 and Orc1, which bind yeast silencer elements. The SIR complex then spreads from this nucleation site for 3–20 kb, depending on the abundance and balance of available Sir proteins (reviewed in Gasser and Cockell (2001) and Rusche *et al* (2003)). SIR complex association decreases the ability of enzymes, like DNA methylases or endonucleases, to access the DNA (Gottschling, 1992; Loo and Rine, 1994). Transcription in these regions is repressed, most likely by reducing RNA polymerase II occupancy at promoters (Chen and Widom, 2005; Lynch and Rusche, 2009), although other studies suggest that Sir protein binding interferes with RNA polymerase II elongation (Sekinger and Gross, 2001; Gao and Gross, 2008).

All three Sir proteins, Sir2, Sir3 and Sir4, are essential for transcriptional repression. Sir3 and Sir4 are primarily thought to be structural proteins of silent chromatin (Gasser and Cockell, 2001). *SIR3* arose from a duplication of the *ORC1* gene, with which it shares an N-terminal BAH domain and a related AAA+ ATPase domain (Hickman and Rusche, 2010). Sir4 is found only in related ascomycetes species, while Sir2 is a NAD-dependent histone deacetylase conserved from bacteria to man. Its enzymatic activity is required for gene silencing (Tanny *et al*, 1999; Imai *et al*, 2000; Smith *et al*, 2000).

The key substrate of Sir2 is histone H4 acetylated on lysine 16 (H4K16^{ac}; Imai *et al*, 2000; Smith *et al*, 2000; Borra *et al*, 2004). This mark is found on transcriptionally active chromatin in most species and marks early firing origins in yeast and flies (Kimura *et al*, 2002; Suka *et al*, 2002; Schwaiger *et al*, 2010). It has been shown that unmodified H4K16 promotes compaction of the chromatin fibre *in vitro* and *in vivo* (Smith *et al*, 2003; Shogren-Knaak *et al*, 2006; Robinson *et al*, 2008). Consistently, in budding yeast H4K16^{ac} is found throughout the genome except at silent loci (Suka *et al*, 2001; Smith *et al*, 2003).

Recombinant fragments of Sir3 and Sir4 were shown to bind to the histone H4 tail *in vitro*, in a manner sensitive to

*Corresponding author. Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland.
Tel.: +41 61 697 5025; Fax: +41 61 697 3976;
E-mail: susan.gasser@fmi.ch

Received: 17 December 2010; accepted: 28 April 2011; published online: 10 June 2011

mutations near K16 and to lysine acetylation (Hecht *et al*, 1995; Carmen *et al*, 2002). Using recombinant proteins and nucleosomal substrates, it was found that the H4K16A mutation can decrease binding of Sir3 *in vitro* (Johnson *et al*, 2009), while mutations of H4K16 to glycine or glutamate, and to a lesser extent arginine, diminished mating efficiency *in vivo* (Johnson *et al*, 1990; Megee *et al*, 1990; Park and Szostak, 1990). Finally it was shown that the H4K16G phenotype could be suppressed by a compensatory mutation in Sir3, suggesting that Sir3 contacts the H4 tail in an acetylation-sensitive manner (Johnson *et al*, 1990).

In yeast, H4K16 is acetylated primarily by the histone acetyltransferase (HAT) Sas2 (Kimura *et al*, 2002; Suka *et al*, 2002) and secondarily by the essential HAT Esa1, which also targets H4K5 and H4K12 (Suka *et al*, 2001, 2002; Chang and Pillus, 2009). Similar to conservative mutations in H4K16, the deletion of *SAS2* impairs repression of a reporter gene at telomeres or the *HML* locus, although the same mutation favours silencing of a reporter at *HMR*, which has much stronger silencer elements (Reifsnyder *et al*, 1996; Ehrenhofer-Murray *et al*, 1997; Meijsing and Ehrenhofer-Murray, 2001). However, the rate of Sir3 recruitment to *HMR* was slower in cells that lack Sas2 (Katan-Khaykovich and Struhl, 2005), as was the establishment of silencing at *HML* (Osborne *et al*, 2009). This, together with the observation that the catalytic activity of Sir2 is required for silencing (Tanny *et al*, 1999; Imai *et al*, 2000; Smith *et al*, 2000; Yang and Kirchmaier, 2006; Yang *et al*, 2008a), suggests that Sir-mediated deacetylation of H4K16^{ac} might have an active role in the formation of silent chromatin.

The H4K16^{ac} mark is also required for efficient methylation of lysine 79 on the histone H3 (H3K79^{me}) by the methyltransferase Dot1 (Altaf *et al*, 2007). *In vivo* H3K79^{me} appears to impair the spreading of the Sir proteins, and is thought to act by reducing association of Sir3 to chromatin (Ng *et al*, 2002, 2003; van Leeuwen *et al*, 2002; Altaf *et al*, 2007; Onishi *et al*, 2007). Consistently, a recent study with recombinant proteins has suggested that both Sir3 and the SIR holocomplex have lower affinities for reconstituted chromatin bearing H3K79^{me} (Martino *et al*, 2009). This shows that, in addition to the histone tails, the Sir3 protein also interacts with the nucleosomal core, a property that has been assigned both to the N-terminal BAH domain (Onishi *et al*, 2007; Buchberger *et al*, 2008; Norris *et al*, 2008; Sampath *et al*, 2009) as well as the Sir3 C-terminal region (Altaf *et al*, 2007).

A further histone modification that interferes with SIR-mediated repression is the acetylation of K56 on histone H3. In budding yeast, H3K56^{ac} is deposited by Rtt109 during S phase before the loading of the histones onto DNA, and therefore serves as a marker for newly assembled nucleosomes (Hyland *et al*, 2005; Masumoto *et al*, 2005; Han *et al*, 2007; Li *et al*, 2008). A large number of studies have also linked H3K56^{ac} to gene transcription from yeast to man (Xu *et al*, 2005, 2007; Schneider *et al*, 2006; Williams *et al*, 2008; Yang *et al*, 2008b; Michishita *et al*, 2009; Xie *et al*, 2009). In yeast, amino-acid substitutions at H3K56 severely disrupt silencing without completely displacing the SIR complex (Xu *et al*, 2007; Yang *et al*, 2008b). Moreover, elimination of the histone deacetylases responsible for removal of H3K56^{ac}, Hst3 and Hst4, disrupts SIR-mediated repression as well (Yang *et al*, 2008b). A recent report has shown that acetylation of H3K56 favours transcriptional elongation through yeast heterochromatin, generating speculation that H3K56^{ac} promotes the displacement of the Sir proteins (Varv *et al*, 2010). However, there is as yet no direct evidence that the affinity of Sir proteins for nucleosomes is lowered by H3K56^{ac}.

To gain insight into the role played by these histone modifications in the assembly of silent chromatin, we reconstituted SIR-bound chromatin *in vitro* using nucleosomes homogeneously modified on only one residue. Our system recapitulates many of the characteristics of silent chromatin (Martino *et al*, 2009) and allows us to probe both Sir protein binding and accessibility of the linker DNA to micrococcal nuclease (MNase). We find that both H3K79^{me} and H4K16^{ac} decrease the affinity of Sir3 for chromatin, while only H4K16^{ac} has an effect on MNase accessibility. Surprisingly, we found that Sir2-4 prefers to bind to chromatin acetylated on H4K16. The binding of Sir2-4, in presence of NAD and Sir3, leads to the removal of the H4K16^{ac} mark and couples stable binding of the Sir2-3-4 complex with a significant decrease in linker DNA accessibility. On the other hand, H3K56^{ac} slightly increases MNase accessibility and reduces the interaction of chromatin with the SIR complex. Importantly, we find that H3K56^{ac} is not a substrate for Sir2-mediated deacetylation. We, thus, show how the anti-silencing properties of different histone modifications differentially affect silent chromatin. Of particular interest are the two contradictory roles played by H4K16^{ac}, which reduces the binding of Sir3 and favours the recruitment of Sir2-4. The acetylation and deacetylation of H4K16 thus appear to orchestrate the sequential binding of Sir proteins in order to establish a stable silent chromatin.

Results

The H4K16^{ac} mark differentially affects the binding of Sir2-4 and Sir3 to chromatin

It is generally accepted that the H4K16^{ac} mark plays an important role in silent chromatin by preventing the ectopic spread of the Sir proteins from the non-acetylated silent domains (Kimura *et al*, 2002; Suka *et al*, 2002; Millar *et al*, 2004; Yang *et al*, 2008a). However, accumulating evidence suggests that not only the absence of the acetyl mark but its Sir2-dependent removal may be required for efficient establishment of silencing (Liou *et al*, 2005; Yang and Kirchmaier, 2006; Yang *et al*, 2008a; Martino *et al*, 2009; Osborne *et al*, 2009). To shed light on this matter, we analysed in detail the binding of SIR subcomplexes to nucleosomal arrays bearing a fully acetylated H4K16.

Nucleosomes were reconstituted with recombinant histones that were either unmodified or fully acetylated on H4K16. These were generated by expressing a truncated version of H4 and adding the N-terminal tail by native chemical ligation (NCL) using a synthetic peptide (Shogren-Knaak *et al*, 2006; Supplementary Figure S1). Nucleosomal arrays were then reconstituted by salt dialysis using a DNA template containing repeated arrays of a 167-bp histone octamer positioning sequence (Widom 601) as described previously (Huynh *et al*, 2005; Martino *et al*, 2009). Recombinant Sir proteins were purified from insect cells (Figure 2F; Cubizolles *et al*, 2006; Martino *et al*, 2009).

We first compared acetylated and non-acetylated arrays of a 6mer of nucleosomes by monitoring the accessibility of linker DNA to MNase in the absence of Sir proteins. Previous studies have shown that acetylation of H4K16 inhibits chromatin compaction both *in vitro* and *in vivo* (Shogren-Knaak *et al*, 2006; Robinson *et al*, 2008). By challenging this chromatin with increasing amounts of MNase, we found that H4K16^{ac} enhances linker DNA accessibility of a chromatin template as short as six nucleosomes (Figure 1A).

Sir3 has been reported to be more susceptible than Sir4 to modifications on histone tails (Carmen *et al*, 2002; Johnson *et al*, 2009). Therefore, we examined first the effect of H4K16^{ac} on the binding of Sir3 to nucleosomal arrays. Increasing amounts of Sir3 were titrated onto unmodified or H4K16^{ac} arrays of nucleosomes. The binding was analysed by gel shift and quantified by scoring the loss of the unbound 6mer. In agreement with previous studies (Carmen *et al*, 2002; Johnson *et al*, 2009), we found that H4K16^{ac} reduces the binding affinity of Sir3 to an *in vitro* reconstituted nucleosomal array by roughly two-fold (Figure 1B). In contrast, the binding affinity of the Sir2-4 heterodimer to chromatin was increased nearly two-fold by the presence of the H4K16^{ac} mark (Figure 1C). Superficially, this appears to contradict the fact that silent chromatin is depleted for this mark, although it is consistent with the notion that H4K16^{ac} is a key substrate of Sir2-4 (see also Johnson *et al* (2009)). Therefore, we decided to perform competition experiments in order to reinforce this observation. The binding of increasing amounts of Sir2-4 to an unmodified Cy5-labelled array was competed with a four-fold excess of either unlabelled unmodified or unlabelled H4K16^{ac} chromatin. Confirming our previous results, H4K16^{ac} chromatin competed roughly two-fold more efficiently for the binding of Sir2-4 compared with unmodified chromatin (Figure 1D).

We previously showed that our recombinant Sir2-4 has efficient histone deacetylase activity in the presence of its co-factor NAD (Cubizolles *et al*, 2006). We reasoned that if Sir2-4 bound H4K16^{ac} with higher affinity because it is a preferred substrate of Sir2, then the complex should have less affinity once H4K16^{ac} had been deacetylated. To test this, we quantified the binding of the Sir2-4 heterodimer to H4K16^{ac} chromatin in the absence or presence of NAD. Confirming our hypothesis, Sir2-4 bound more readily to acetylated chromatin and less readily following deacetylation (Figure 1E). Surprisingly, this shows an enhanced affinity of Sir2-4 for acetylated H4K16, while the opposite is true for Sir3.

Sir2-dependent deacetylation of H4K16^{ac} stabilizes the association of Sir2-3-4 to chromatin

Removal of H4K16^{ac} through the catalytic activity of Sir2 has been reported to be important for silencing (Johnson *et al*, 1990; Suka *et al*, 2001, 2002; Carmen *et al*, 2002; Kimura *et al*, 2002). However, it is not clear whether this is due exclusively

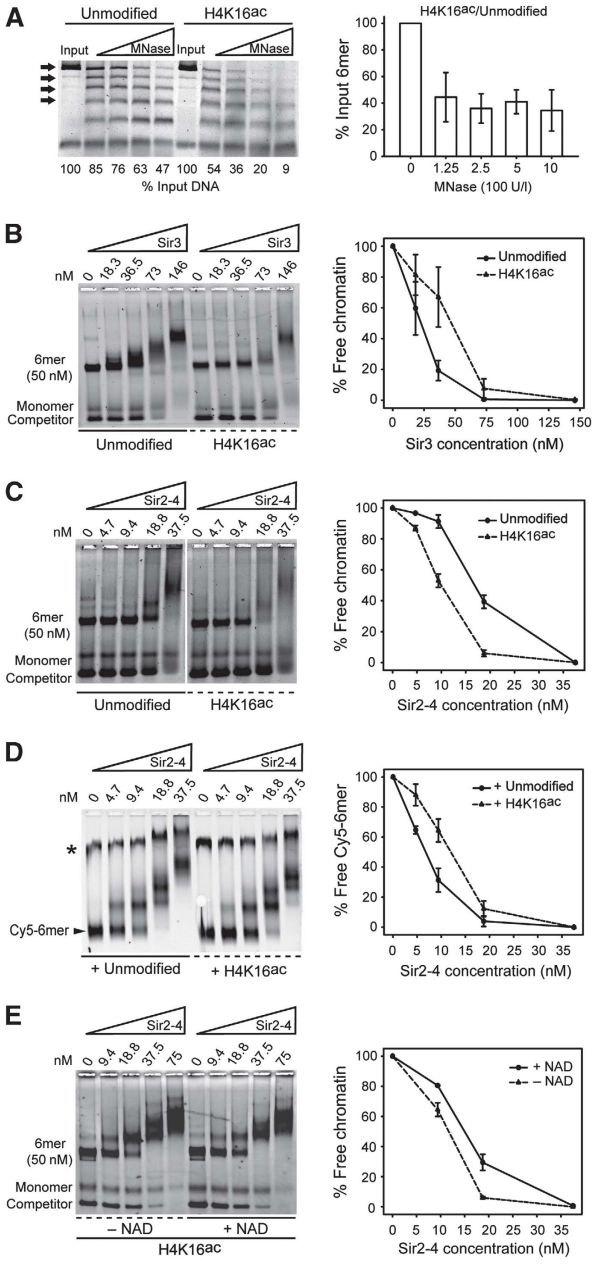

Figure 1 Acetylation of H4K16 decreases the binding affinity of Sir3 but favours the loading of Sir2-4 onto chromatin. (**A**) Equally saturated 6mer of 601 nucleosomes with either unmodified or acetylated H4K16 was digested with increasing amount of MNase, as indicated. After protein digestion, the denatured DNA was separated by electrophoresis and visualized by SYBR® Safe staining. The bands showed by an arrow (6-, 5-, 4- and 3mers) were quantified and normalized to input. The histograms show the ratio between the amount of intact 6-3mers of H4K16^{ac} over unmodified chromatin for the indicated MNase titration point. The Sir3 protein (**B**) or the Sir2-4 heterodimer (**C**) were titrated into a constant amount of unmodified or H4K16^{ac} 6mer of 601 nucleosomes. Samples were separated by native agarose gel electrophoresis and visualized by SYBR® Safe staining. (**D**) The binding of increasing amounts of Sir2-4 to 8 nM of unmodified Cy5-labelled 6mer of nucleosomes (indicated by the arrowhead) was competed with 32 nM of either unlabelled unmodified or unlabelled H4K16^{ac} 6mer of nucleosomes. Cy5 fluorescence was used to monitor the binding of Sir2-4 to the unmodified labelled chromatin. The asterisk indicates a Cy5-labelled contaminant DNA. (**E**) The Sir2-4 heterodimer was titrated into a constant amount of H4K16^{ac} 6mer of 601 nucleosomes. Deacetylation is allowed by the addition of 150 μM NAD where indicated. Samples were separated and visualized as in (**B**, **C**). The images are representative of at least three independent experiments, quantifications show the mean value ± s.e.m. of the % of unbound chromatin compared with the input.

to the generation of unmodified H4K16 or whether it additionally involves a conformational change coupled to *O*-acetyl-ADP-ribose (*O*-AADPR) production (Liou *et al*, 2005; Martino *et al*, 2009). To gain insight into the molecular consequences of H4K16^{ac} deacetylation on the establishment of silencing, we compared the binding of the SIR complex with unmodified or H4K16^{ac} chromatin in the presence or absence of NAD. We first examined the effect of H4K16^{ac} on the binding of the Sir2-3-4 heterotrimer in absence of NAD (Figure 2A) and found that, similar to Sir2-4 (Figure 1C) but in a less pronounced manner, the SIR holocomplex bound slightly better to acetylated chromatin. We then confirmed that our purified Sir2-3-4 complex was able to efficiently deacetylate H4K16^{ac} within chromatin in the presence of NAD (Figure 2E), as shown previously for chemically acetylated histone octamers (Cubizolles *et al*, 2006). In the following experiments, the term 'deacetylated chromatin' will be used whenever H4K16^{ac} marks were actively removed by Sir2 in the presence of NAD, to distinguish it from chromatin assembled from unmodified histones.

We next compared the binding affinity of Sir2-3-4 with unmodified or H4K16^{ac} chromatin in the presence of NAD. We found that the active removal of the H4K16^{ac} mark increased the binding affinity of the SIR complex to chromatin by roughly two-fold (Figure 2B). This effect is not caused by NAD alone as enhanced binding was not observed when H4K16^{ac} chromatin was replaced with unmodified chromatin (Supplementary Figure S2A). Indeed, in absence of Sir2,

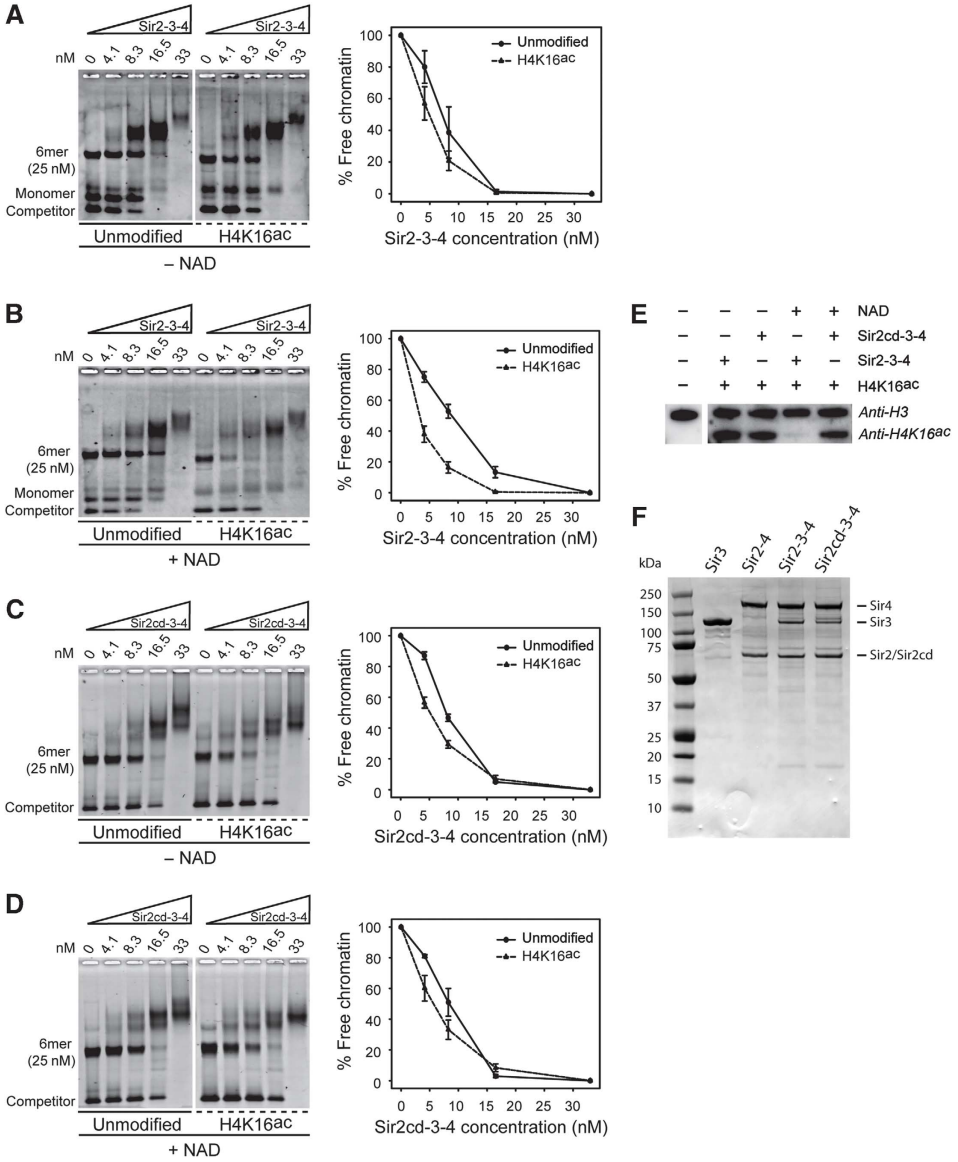

Figure 2 Sir2-dependent deacetylation of H4K16^{ac} stabilizes the association of Sir2-3-4 to chromatin. The Sir2-3-4 complex (**A**, **B**) or the catalytically dead Sir2cd-3-4 mutant (**C**, **D**) was titrated into a constant amount of unmodified or H4K16^{ac} 6mer of 601 nucleosomes. Where indicated, 150 μM NAD was added to the samples. Scatter plot quantifications show the mean value ± s.e.m. of the % of unbound chromatin compared with the input for at least three experiments. (**E**) Reconstituted chromatin fully acetylated on H4K16 was subjected to NAD-dependent deacetylation in presence of a 2.5-fold molar excess of the Sir2-3-4 complex or the Sir2cd-3-4 mutant. The acetylation state was then determined by immunoblotting using acetylation mark-specific antibodies and H3 for loading. (**F**) Two micrograms of the indicated Sir protein were denatured in sample buffer and run on a 4–12% NuPAGE® Novex® Bis-Tris Gel.

NAD does not affect the acetylation state of chromatin (Supplementary Figure S2B). This shows that the binding affinity of the SIR complex for deacetylated chromatin is higher compared with chromatin assembled from unmodified histones.

In order to reinforce this finding, we tested whether the deacetylase activity of Sir2 itself was required for the enhanced binding of acetylated template in the presence of NAD. We generated a catalytic inactive Sir2 (Sir2cd) by introducing the point mutation N345A, which maps to the nucleotide binding motif (Rossman fold). This mutation disrupts Sir2 enzymatic activity *in vitro* and *in vivo* (Imai *et al*, 2000; Armstrong *et al*, 2002). The N345A substitution, however, did not affect the stability of Sir2 or its interaction with Sir3 and Sir4 and we were able to purify the mutated Sir2cd-3-4 from insect cells with the same efficiency as for the Sir2-3-4 complex (Figure 2F). We could furthermore confirm that this mutant did not retain significant deacetylase activity (Figure 2E).

In order to confirm that the Sir2cd-3-4 mutant was still able to recognize its substrate, we monitored the binding of Sir2cd-3-4 to unmodified or H4K16^{ac} chromatin in the absence of NAD. We found that, like Sir2-3-4, Sir2cd-3-4 has a slight preference for H4K16^{ac} chromatin (compare Figure 2A and C). We then monitored the loading of the Sir2cd-3-4 mutant onto unmodified or H4K16^{ac} chromatin in the presence of NAD. Unlike the Sir2-3-4 complex, the catalytic dead Sir2cd-3-4 showed the same slight preference for the H4K16^{ac} chromatin, as it did in the absence of NAD (compare Figure 2C and D). This data reinforce our observation that the deacetylation reaction has a positive role on the loading of the SIR complex onto chromatin (Figure 2B). Given that the deacetylation of H4K16^{ac} chromatin reduced the binding affinity of the Sir2-4 heterodimer (Figure 1E), these results suggest that Sir3 and the deacetylation of H4K16^{ac} by Sir2 jointly promote the binding of the SIR holocomplex to chromatin.

The Sir3 protein and Sir2-dependent deacetylation of H4K16^{ac} are both required to decrease nuclease accessibility of the linker DNA

SIR complex bound chromatin is thought to have a more compact structure *in vivo* as it is less accessible to enzymatic attack (Gottschling, 1992; Loo and Rine, 1994). The SIR complex could compact chromatin in two ways: first by deacetylating H4K16, and second by binding to chromatin. We observed that loading of the SIR complex onto unmodified chromatin greatly reduces the accessibility of the linker DNA to MNase and the restriction enzyme *Ava*I *in vitro*, consistent with a direct role for binding (Supplementary Figure S3A and B; Martino *et al*, 2009). In order to test the impact of H4K16^{ac} deacetylation on compaction *in vitro*, we first incubated H4K16^{ac} chromatin with the Sir2-4 subcomplex in the presence or absence of NAD and then challenged it with increasing amounts of MNase. We found that the presence of NAD did not significantly change the accessibility of the linker DNA (Figure 3A). Since acetylation of chromatin usually results in greater accessibility (Figure 1A), this result is likely a combination of changed accessibility due to removal of H4K16^{ac} and reduced binding affinity of Sir2-4 for deacetylated chromatin (Figure 1E).

We then performed the same experiment but replaced Sir2-4 with the SIR holocomplex. The concentration of Sir2-3-4

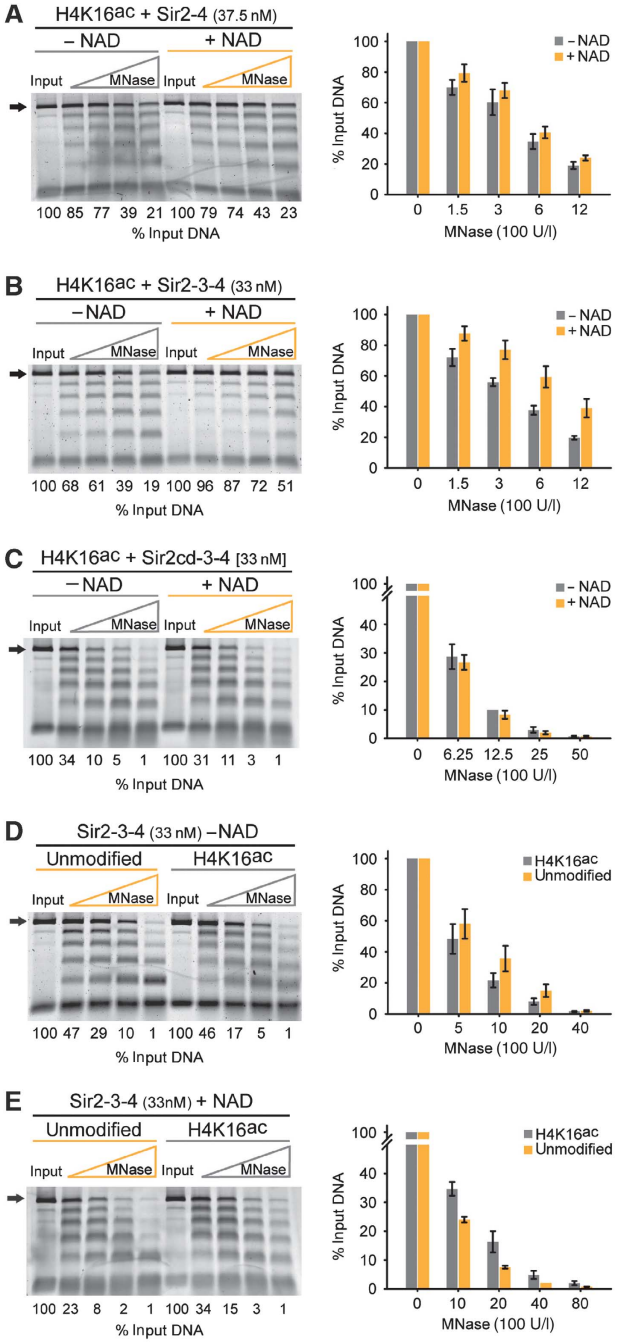

Figure 3 Sir3 is required to translate the Sir2-dependent deacetylation of H4K16^{ac} into a decrease of nuclease accessibility of the linker DNA. Unmodified or H4K16^{ac} 6mer of 601 nucleosomes (50 nM) was incubated with the indicated amount of Sir2-4 (**A**), Sir2-3-4 (**B**, **D** and **E**) or Sir2cd-3-4 (**C**) and was challenged with increasing amounts of MNase. Where indicated, SIR-bound chromatin was supplemented with 150 μM NAD and incubated for 15 min at 30°C before MNase digestion. Deproteinated samples were separated by electrophoresis and the amount of intact 6mer DNA (black arrow) was quantified and normalized to input. Quantification of at least three experiments was used to generate the vertical bar charts, data represent mean value ± s.e.m.

used resulted in a complete upshift of both acetylated and unmodified chromatin in a binding assay, ruling out differential accessibility due to incomplete ligand occupancy. Interestingly, we observed that in the presence of NAD the linker DNA was more protected from MNase than SIR-bound

chromatin in the absence of NAD (Figure 3B). Importantly, the addition of NAD did not change the protection of linker DNA of an array bound by the catalytic inactive Sir2cd-3-4 (Figure 3C). The same analysis in the absence of Sir3 did not increase protection against MNase attack (Figure 3A), arguing that the protective effect of NAD-dependent deacetylation of H4K16^{ac} by Sir2 requires Sir3 (compare Figures 3A and B). Finally, the increased linker DNA protection observed in Figure 3B was not caused by the NAD molecule *per se*, as no NAD-dependent differences were scored for linker DNA accessibility when Sir proteins were bound to unmodified chromatin (Supplementary Figure S3C). These results suggested that the deacetylation of H4K16^{ac} by the SIR complex promotes linker DNA protection.

When comparing unmodified with acetylated chromatin bound by Sir2-3-4 in the absence of NAD, we found that the linker DNA is slightly more protected (Figure 3D), indicating that at least some of the protection observed in Figure 3B is due to loss of H4K16^{ac} *per se*. However, given our previous observation that deacetylated chromatin is bound with higher affinity than unmodified chromatin (Figure 2B), we decided to explore the possibility that the deacetylation reaction itself may also contribute to the increased linker DNA protection observed in Figure 3B. Therefore, we compared the linker DNA accessibility of unmodified and H4K16^{ac} chromatin in the presence of Sir2-3-4 and NAD. We found that deacetylated chromatin is reproducibly more protected from MNase attack compared with chromatin assembled from unmodified histones (Figure 3E). Together, these results show that both Sir2-dependent deacetylation of H4K16^{ac} and Sir3 are required to decrease the nuclease accessibility of linker DNA, which presumably reflects the tighter binding of the SIR holocomplex to chromatin. In addition, there may be a conformational change that enhances linker DNA protection.

H3K56^{ac} loosens Sir protein binding to chromatin, slightly increasing linker DNA accessibility

To ask if our observation for H4K16^{ac} can be generalized to other acetylation marks we tested the effects of H3K56 acetylation, which is found on newly assembled nucleosomes in S phase. Since there are contradictory reports about which enzyme deacetylates H3K56^{ac} (Xu *et al*, 2007; Yang *et al*, 2008b) we first tested if Sir2 can deacetylate H3K56^{ac} as suggested earlier. We incubated H3K56^{ac} chromatin with SIR complex in the presence or absence of NAD. Chromatin homogenously acetylated at H3K56 was obtained by purifying acetylated H3 from *E. coli* using an expanded genetic code strategy (Neumann *et al*, 2008). Probing the histones with H3K56^{ac} antibodies after incubation showed that, unlike for H4K16^{ac}, the level of H3K56 acetylation remained unchanged (Figure 4B). We conclude that H3K56^{ac} is not a substrate of the NAD-dependent deacetylase activity of Sir2. This supports previous work reporting that two Sir2-related enzymes, Hst3 and Hst4, are required for H3K56^{ac} deacetylation *in vivo* (Celic *et al*, 2006; Maas *et al*, 2006; Yang and Kirchmaier, 2006) and suggests that Hst3 and Hst4 are the exclusive deacetylases for this residue.

To address whether acetylation of H3K56 has an effect on Sir protein loading, we compared the binding of the SIR holocomplex with unmodified and H3K56^{ac} chromatin. We found that H3K56^{ac} reduces the affinity of the SIR holocomplex for chromatin by roughly two-fold (Figure 4C). The binding affinity of the Sir2-4 heterodimer was also reduced in presence of the H3K56^{ac} mark (Figure 4D), while the binding affinity of the Sir3 protein alone was mostly unchanged (Figure 4E). To explore whether the slight affinity decrease observed here for the SIR complex could be responsible for the silencing defects seen *in vivo*, we investigated whether the SIR complex efficiently protects linker DNA in chromatin bearing the H3K56^{ac} mark. The acetylation on H3K56 *per se* has been shown to increase transient unwrapping of the DNA from the histone octamer but not to change the higher order structure of a 61mer nucleosomal array (Neumann *et al*, 2009). Consistently we show, by means of an MNase digestion assay, a slight increase in linker DNA accessibility for the chromatin bearing H3K56^{ac} over the unmodified control (Figure 4F). Subsequently, after adding the SIR complex in saturating concentrations (Supplementary Figure S4A), the H3K56^{ac} chromatin continued to show slightly higher linker DNA accessibility as compared with unmodified chromatin (Figure 4G). This is consistent with a previous *in vivo* study indicating that H3K56^{ac} chromatin is more sensitive to DNA methylation by an ectopically expressed bacterial *dam* methylase (Xu *et al*, 2007). Moreover, H3K56 point mutations disrupted silencing at telomeres without affecting Sir protein spreading (Xu *et al*, 2007). We conclude that H3K56^{ac} does not have a role similar to that of H4K16^{ac}, neither in the recruitment of Sir2-4, nor by being a substrate for Sir2.

Methylation of H3K79 by Dot1 neither increases linker DNA accessibility nor reduces Sir2-4 loading

Another mark associated with active chromatin in yeast is methylation of lysine 79 of histone H3 (H3K79). This methylation is exclusively catalysed by Dot1 and is thought to act as a boundary for the inappropriate spreading of the Sir proteins on chromatin (van Leeuwen *et al*, 2002; Frederiks *et al*, 2008; Martino *et al*, 2009; Verzijlbergen *et al*, 2009). Moreover, *in vitro* studies showed that interaction of the Sir3 protein with histone peptides was sensitive to the methylation of H3K79 (Altaf *et al*, 2007; Onishi *et al*, 2007). Previous work from our laboratory showed that we can make use of recombinant Dot1 in order to methylate reconstituted nucleosomal arrays *in vitro* (Martino *et al*, 2009). We have previously shown that even partial methylation of H3K79 decreases the binding affinity of both the SIR complex and the Sir3 protein alone to chromatin (Martino *et al*, 2009). We now provide further evidence that the lowered affinity indeed affects Sir3 binding, since the Sir2-4 heterodimer associates with unmodified and H3K79^{me} chromatin with nearly equal affinity (Figure 5A), while Sir3 clearly prefers unmodified chromatin (Figure 5B).

We then decided to test whether H3K79^{me} also impacts the structure of SIR-bound or SIR-depleted chromatin. To examine the potential impact of H3K79^{me} on linker DNA protection, we challenged *in vitro* methylated chromatin lacking Sir proteins with increasing amounts of MNase. Unlike the case for H3K56^{ac}, the accessibility of the linker DNA in the absence of SIR complex was unaffected by H3K79^{me} (Figure 5C). However, in the presence of substoichiometric amounts of Sir2-3-4 (Supplementary Figure S4B), the accessibility of the linker DNA was higher for H3K79^{me} chromatin than for unmodified chromatin (Figure 5D), consistent with notion that better SIR complex binding enhances linker DNA protection. When we added additional Sir2-3-4 such that

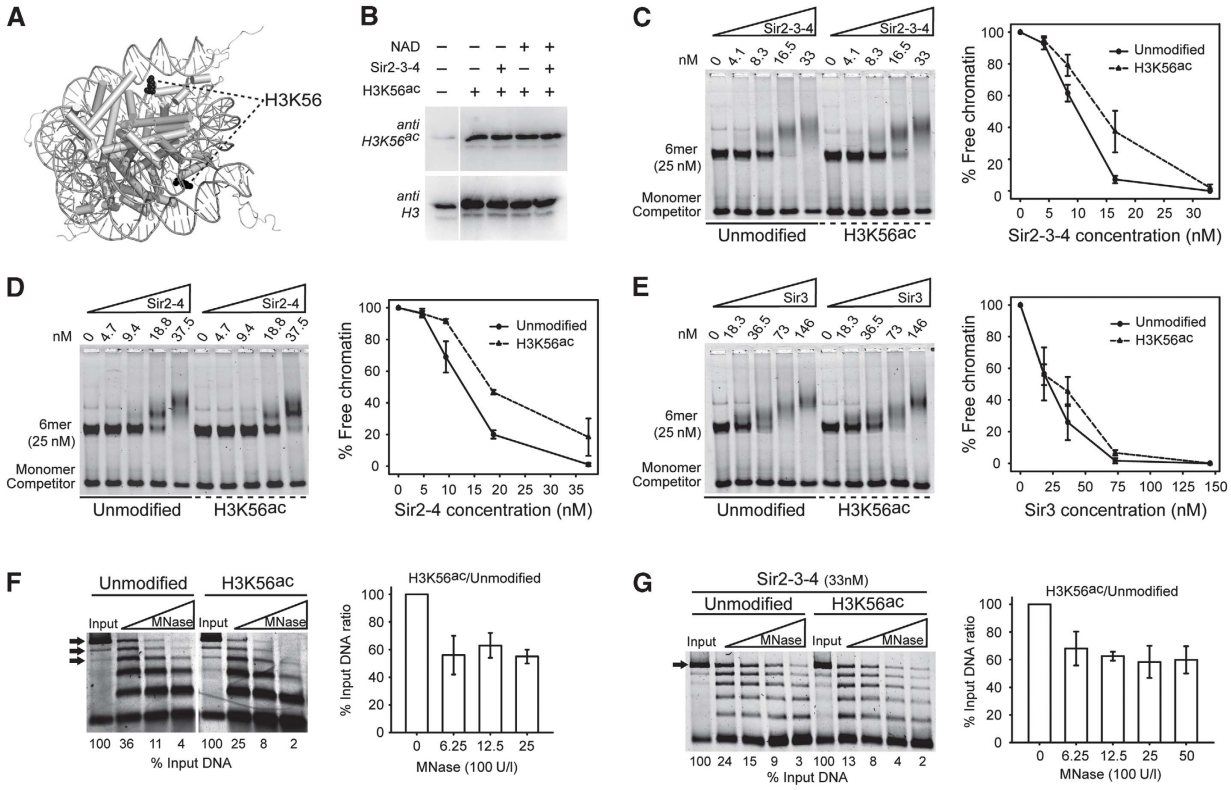

Figure 4 H3K56^{ac} decreases Sir protein binding affinity and slightly increases linker DNA accessibility. (**A**) Cartoon representation of the nucleosome core particle (NCP147; Davey *et al*, 2002) highlighting the position of H3K56 (black) at the entry/exit point of the DNA around the histone octamer. (**B**) Reconstituted chromatin fully acetylated on H3K56 was subjected to NAD-dependent deacetylation in presence of a 2.5-fold molar excess of the SIR complex. The acetylation state was then determined by immunoblotting using acetylation mark-specific antibodies and H3 for loading. The SIR complex (**C**), Sir2-4 heterodimer (**D**) or Sir3 (**E**) were titrated into a constant amount of unmodified or H3K56^{ac} 6mer of 601 nucleosomes. Samples were analysed as in Figure 2. Unmodified or H3K56^{ac} 6mer of nucleosomes were challenged with an increasing amount of MNase in absence (**F**) or presence (**G**) of the SIR complex. The 6mer, 5mer and 4mer bands (**F**) or the band corresponding to the intact 6mer alone (**G**), shown by black arrows, were quantified and normalized to the input. The histograms show the ratio between the amounts of quantified DNA from H4K16^{ac} chromatin over unmodified for the indicated MNase titration point ± s.e.m.

we score an equal degree of binding on both substrates (Supplementary Figure S4C), we observed no difference in accessibility of linker DNA (Figure 5E). This suggests that H3K79^{me} neither changes chromatin structure nor prevents the SIR complex from compacting it, but decreases the affinity of the SIR complex for chromatin. Thus, it antagonizes silencing through a mechanism distinct from H3K56^{ac}.

Discussion

Silent chromatin in *Saccharomyces cerevisiae* is the best studied system of heterochromatic gene silencing, yet we still do not fully understand the molecular mechanisms of its assembly and the role of histone modifications in this process. *In vitro* binding analysis between Sir protein domains and histone peptides have been informative, yet they only reflect a small part of the chromatin template. To examine the molecular basis of SIR-dependent silencing, we have established a fully recombinant system that recapitulates key features of silent chromatin in budding yeast (Martino *et al*, 2009). Here, we extend this system to examine how histone modifications participate in the formation of stable silent and active domains.

We show here that the H4K16^{ac} mark has both a positive and a negative role in SIR binding in a sequential manner (see Figure 6). Importantly, we show that H4K16^{ac} decreases the binding affinity of Sir3, but, in contrast, promotes the association of the Sir2-4 heterodimer to chromatin. Even the binding affinity of the SIR holocomplex is slightly increased by the presence of H4K16^{ac} in the absence of NAD (see also Johnson *et al* (2009)). This result, while initially counter-intuitive, helps elucidate the dual role of H4K16^{ac} in heterochromatin formation. On one hand, H4K16^{ac} prevents the dispersion of its key ligand, Sir3, into euchromatic chromatin. On the other hand, the high affinity of Sir2-4 for H4K16^{ac} may help nucleate silent chromatin, since it is likely in yeast that the targeted nucleosomes are acetylated before SIR complex loading.

In support of this dual role, it was shown that the substitution of H4K16 by not only an acetyl-mimicking residue, but also unacetylatable amino acids, disrupts silencing at telomeres and mating type loci (Johnson *et al*, 1990, 1992; Megee *et al*, 1990; Park and Szostak, 1990; Aparicio *et al*, 1991; Millar *et al*, 2004). Moreover, the deletion of *SAS2*, which encodes the HAT responsible for most H4K16^{ac} in yeast, impaired the repression of reporter genes at certain loci, such as TelVIIL or within the *HML* locus (Reifsnyder *et al*, 1996; Meijsing and Ehrenhofer-Murray, 2001) and led to the spreading of Sir proteins into subtelomeric regions that usually lack SIR-mediated repression (Kimura *et al*, 2002;

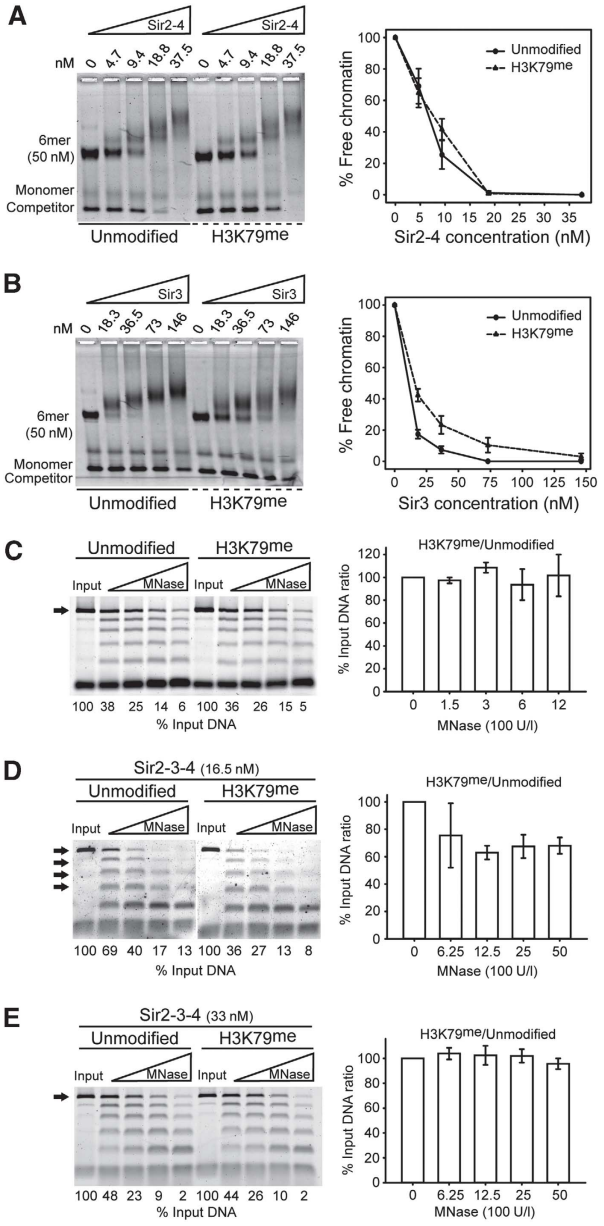

Figure 5 Methylation of H3K79 by Dot1 affects neither linker DNA accessibility nor Sir2-4 loading onto chromatin. The Sir2-4 heterodimer (**A**) or Sir3 (**B**) was titrated into a constant amount of unmodified or H3K79^{me} 6mer of nucleosomes. Samples were analysed as in Figure 2. Unmodified or H3K79^{me} chromatin were challenged with an increasing amount of MNase in absence (**C**) or presence (**D**, **E**) of the indicated amount of the SIR complex. The 6mer DNA band alone (**C**, **E**) or the 3mer to 6mer bands (**D**), shown by black arrows, were quantified and normalized to the input. The histograms show the ratio between the amount of quantified DNA from H3K79^{me} over unmodified chromatin for the indicated MNase titration point.

Suka *et al*, 2002). In contrast, the repression of a reporter at *HMR* was enhanced, probably because this locus has much stronger silencers, which dominate over an indiscriminate spreading of Sirs (Ehrenhofer-Murray *et al*, 1997). Importantly, both the kinetics of Sir3 recruitment to *HMR* and the establishment of silencing at *HML* were slower in cells that lack the H4K16-specific HAT, Sas2 (Katan-Khaykovich and Struhl, 2005; Osborne *et al*, 2009), suggesting a positive role for H4K16 acetylation. Collectively, these results support the model that Sas2-mediated acetylation of H4K16 has more than one role in silent chromatin formation (see also Zou and Bi (2008)). Reporter context appears to determine which role is rate limiting: the recruitment of Sir2-4, or the assembly and propagation of the Sir3-containing holocomplex along nucleosomes.

Sequential assembly of nuclease-resistant SIR-bound chromatin requires H4K16^{ac} deacetylation

In vivo the absence of H4K16^{ac} from silent chromatin suggests that it is removed by Sir2 as soon as the SIR complex is loaded. Moreover, it was shown that in the absence of Sir2 catalytic activity, H4K16^{ac} prevents the formation of silent domains (Yang and Kirchmaier, 2006). On the other hand, as mentioned above, even conservative substitutions at H4K16 decrease silencing efficiency at *HML* and at telomeres (Johnson *et al*, 1990; Meijsing and Ehrenhofer-Murray, 2001; Yang and Kirchmaier, 2006). This supports the notion that not only the recruitment of Sir2-4 by H4K16^{ac}, but the deacetylation reaction itself helps to seed repression (Johnson *et al*, 1992; Imai *et al*, 2000; Millar *et al*, 2004; Liou *et al*, 2005; Yang *et al*, 2008a; Martino *et al*, 2009).

Why is the deacetylation reaction important for silencing? The recapitulation of these steps *in vitro* helped us to address this question. Indeed, by adding NAD to SIR-bound H4K16^{ac} chromatin, we catalysed deacetylation of H4K16 and increased the binding affinity of the SIR holocomplex to chromatin. This shows in a well-defined recombinant system that removal of the single H4K16^{ac} mark by Sir2 increases SIR complex binding. Even more importantly, we found that the linker DNA was better protected from MNase digestion when the SIR complex was assembled on chromatin in the presence of H4K16^{ac} and NAD, as compared with its being loaded onto unmodified chromatin. This protection nicely mimics the DNA shielding observed in SIR-silenced chromatin regions *in vivo* (Gottschling, 1992; Loo and Rine, 1994; Xu *et al*, 2007) and argues that the SIR complex may associate with chromatin in more than one conformation. It was previously proposed that a by-product of Sir2 NAD-dependent deacetylation, *O*-AADPR, might trigger a conformational change of the SIR–chromatin complex to favour repression (Liou *et al*, 2005; Onishi *et al*, 2007; Martino *et al*, 2009).

Importantly, the deacetylation-dependent increase in affinity of the SIR holocomplex for chromatin, and the increase in linker DNA protection, depends crucially on the presence of Sir3. This is consistent with previous results which showed that exogenously added *O*-AADPR enhances the binding of both Sir3 and the SIR holocomplex to chromatin (Martino *et al*, 2009). A further study showed that addition of an excess of acetylated peptides and NAD to SIR–chromatin assemblies generated a structure that appeared more compact by electron microscopy (Johnson *et al*, 2009), perhaps reflecting a conformational change in the SIR complex (Liou *et al*, 2005). Nevertheless, *O*-AADPR is probably neither absolutely required for SIR complex loading nor for silencing, since repression can be achieved in a strain devoid of NAD-dependent deacetylases if an ectopic HDAC is fused to Sir3 (Chou *et al*, 2008) or if Sir3 is overexpressed in a H4K16R background (Yang and Kirchmaier, 2006). Taken together, our data support a scenario in which the sequential loading of Sir2-4

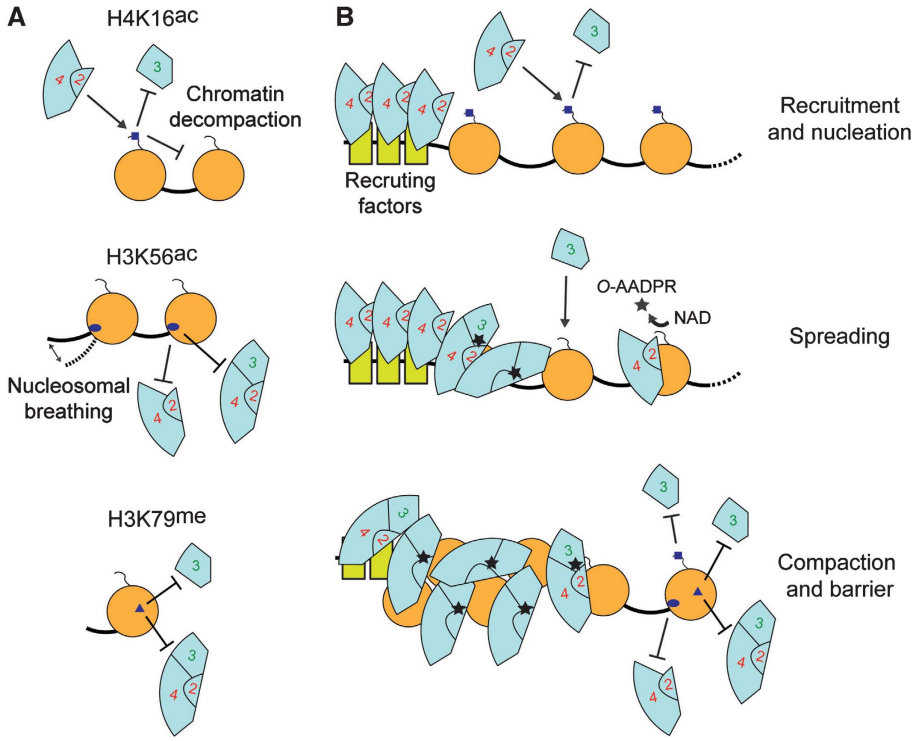

Figure 6 Combinatorial histone modifications distinguish silent and active chromatin regions. (**A**) Outline of the role played by different histone modifications on Sir protein loading and chromatin structure. (**B**) The Sir proteins are recruited onto chromatin by protein–protein interactions and bind tightly unmodified nucleosomes driving gene silencing. Spreading of the SIR complex is promoted by H4K16^{ac} that recruits the Sir2-4 heterodimer yet prevents the ectopic spreading of Sir3 alone. The NAD-dependent deacetylation reaction of H4K16^{ac} by Sir2 generates a high-affinity binding substrate for Sir3 and the synthesis of *O*-AADPR favours the tight association of the SIR complex to unmodified nucleosomes. H3K56^{ac} and H3K79^{me} generate a boundary to the spreading of the SIR complex mainly by reducing the binding affinity of the Sir2-4 heterodimer and the Sir3 protein, respectively. The acetylation of H3K56 and H4K16 also enhance the accessibility of the chromatin fibre, unlike methylation of H3K79.

onto nucleosomes containing H4K16^{ac}, its NAD-dependent deacetylation and the loading of Sir3, sequentially promote a stable assembly that protects linker DNA from exogenous factors (Figure 6).

Boundary formation and reduction of SIR holocomplex affinity by histone modifications

The observation that H4K16^{ac} might provide a boundary for heterochromatin spreading (Kimura *et al*, 2002; Suka *et al*, 2002) seems counterintuitive given the results described above. However, H4K16^{ac} does affect other processes beyond SIR complex association to chromatin, most notably, the recruitment of the histone methyltransferase Dot1 to chromatin (Altaf *et al*, 2007). Given that Sir2-4 preferentially binds chromatin carrying H4K16^{ac}, we propose that Dot1 competes with the recruitment of Sir2-4, and not as proposed earlier, with Sir3 (Altaf *et al*, 2007). On the other hand, the anti-silencing role of the methylation mark itself, H3K79^{me}, is most likely a reflection of reduced interaction between Sir3 and methylated chromatin (Ng *et al*, 2002, 2003; van Leeuwen *et al*, 2002; Altaf *et al*, 2007; Onishi *et al*, 2007; Martino *et al*, 2009). Consistently, we found that nucleosomes bearing H3K79^{me} neither affect the binding of the Sir2-4 heterodimer, nor was there an inherent change in structural properties of H3K79^{me}-containing chromatin. This is consistent with crystallographic analyses which argue that H3K79^{me} does not alter the structure of the nucleosome (Lu *et al*, 2008). Given that no enzyme has been found so far that removes the H3K79^{me} mark, depletion of this mark may depend on histone eviction or on sequential dilution through rounds of DNA replication. Its slow removal renders H3K79^{me} a more stable barrier to the spreading of the SIR complex than H4K16^{ac}, which instead recruits Sir2-4 and promotes the spread of repression (Figure 6).

The third histone mark correlated with active chromatin in yeast is the acetylation of H3 on K56. In contrast with H3K79^{me}, H3K56^{ac} is clearly subject to active deacetylation. Here, we show that Sir2 is unable to remove the H3K56^{ac} mark *in vitro*, which indirectly supports previous work showing that H3K56^{ac} is primarily deacetylated by two Sir2-related enzymes: Hst3 and Hst4 (Celic *et al*, 2006; Maas *et al*, 2006). Indeed, Sir-mediated repression cannot be established in the absence of these two enzymes, although Sir proteins still bind telomeres in an *hst3*Δ*hst4*Δ mutant (Yang *et al*, 2008b). Consistent with our work, this suggests that the H3K56^{ac} mark does not completely block SIR–chromatin interaction.

How then does H3K56^{ac} impair the formation of silent chromatin? Using our *in vitro* system, we found that H3K56^{ac} affects both the affinity with which SIR complexes bind chromatin and the formation of a chromatin structure that is less accessible to MNase attack. The observed drop in affinity of SIR holocomplex for chromatin agrees with an *in vivo* study, which suggested that H3K56^{ac} facilitates Sir protein displacement and RNA polymerase II elongation within heterochromatin regions (Varv *et al*, 2010). Our

observation that H3K56^{ac} increases accessibility of the linker DNA is consistent with an increase in spontaneous (but transient) unwrapping of the DNA from the histone octamer, which may reflect the position of H3K56 at the entry/exit point of the nucleosomal DNA (Figure 4A; Neumann *et al*, 2009). It is striking that even SIR-saturated arrays showed increased linker DNA accessibility in the presence of H3K56^{ac}, indicating that SIR binding cannot overcome the effect of H3K56^{ac} on nucleosomal structure. Although it is unclear why this modification reduces SIR complex binding, this and the increased linker DNA exposure are likely to account for the anti-silencing effect of the H3K56^{ac} mark.

To conclude, we propose that the euchromatic mark H4K16^{ac} is required for the formation of both active and silent chromatin. The process of creating stable silent and active states is not an one-step event, but requires positive feedback loops. H4K16^{ac} may be the starting point for silent domains, which are reinforced by the Sir2 deacetylation reaction and possibly the generation of *O*-AADPR, and active domains, where it promotes H3K79 methylation. These interdependent pathways are conserved throughout evolution and mathematical modelling clearly shows that such networks are required to establish a stable binary switch (Dodd *et al*, 2007; Mukhopadhyay *et al*, 2010). Here, we have demonstrated that H4K16^{ac} is actively implicated in the establishment of yeast silent chromatin, being the first histone mark shown to recruit Sir proteins to chromatin.

Materials and methods

SIR purification and chromatin reconstitution

In vitro reconstitution of SIR-bound chromatin was carried out essentially as described (Cubizolles *et al*, 2006; Martino *et al*, 2009). Briefly, the Sir proteins were expressed in sf21 insect cells with baculoviruses generated using BD BaculoGold^{TM}, BD-Biosciences. Co-infection was used to produce the Sir2-3-4 complex, the catalytic dead Sir2cd-3-4 and the Sir2-4 heterodimer, a single infection was used to produce the Sir3 protein alone (Cubizolles *et al*, 2006). Recombinant *X. laevis* histones were use to reconstitute histone octamers as described previously (Luger *et al*, 1997). Chromatin was assembled *in vitro* by adding increasing amounts of purified histone octamer to a constant amount of DNA arrays containing six 601-Widom positioning elements separated by 20 bp of linker DNA, referred as 601-167-6mer (Lowary and Widom, 1998). An unspecific DNA sequence of 147 bp (referred as 'competitor' on the figures) was added to the mix in order to bind the excess of histone octamers subsequent to the saturation of the 601-167-6mer (Huynh *et al*, 2005). Cy5-labelled 601-167-6mer was generated by filling the 5′ overhang-ends of an *Eco*RI site with Klenow enzyme (NEB, accordingly to manufacturer's instruction), using d-CTP-Cy5 (GE Healthcare). The free nucleotides were then separated from the Cy5-labelled array using small Bio-spin columns (Bio-Rad). DNA and histones were mixed in 40 μl of buffer A (10 mM TEA pH 7.4 and 1 mM EDTA) and 2 M NaCl on ice, and chromatin was reconstituted by step dialysis in buffer A containing 1.2, 1, 0.8 or 0.6 M NaCl for 2 h at 4°C and in buffer A overnight (Lee and Narlikar, 2001). The 601-167-6mer was routinely prepared at a final nucleosomal concentration of 10^{-6} M. Increasing amounts of Sir proteins were added to the 601-167-6mer diluted to 5×10^{-8} M or 2.5×10^{-8} M in 10 mM TEA pH 8, 25 mM NaCl, 0.05% Tween-20 on ice and after 10 min incubation the samples were fixed with 0.0025% glutaraldehyde for 10 min on ice. The fixation yields slightly sharper bands but the results are very similar without fixation. When chromatin deacetylation was coupled to Sir protein loading, SIR-bound chromatin was incubated with or without 150 μM NAD for 15 min at 30°C before incubation on ice for a 10 min fixation as above. The samples were routinely run at 80 V for 90 min at 4°C in a 0.7% agarose gel 0.2 × TB: 18 mM Tris, 18 mM Boric acid. The gel was soaked for 20 min in 1 × SYBR® Safe and the DNA was visualized in a Typhoon 9400 scanner.

Preparation of the histone modifications

Methylation of H3K79 was carried out on reconstituted chromatin as described before (Martino *et al*, 2009). Briefly, 0.8 pmol of recombinant Dot1 was incubated with 8 pmol of reconstituted 601-167-6mer in 25 mM Tris pH 7.9, 20 mM NaCl, 0.4 mM EDTA, with or without 160 pmol of S-adenosylmethionine (SAM) at 30°C for 30 min, then 160 pmol of SAM was added and the reaction was continued for 30 min. Mass spectrometry analysis showed that H3K79 is mono-, di- and, to a lesser extent, tri-methylated on at least 50% of the available K79 residues (Frederiks *et al*, 2008; Martino *et al*, 2009). The chromatin was then stored at 4°C.

Homogeneous acetylated histone H3 at the lysine 56 was obtained using an aminoacyl-tRNA synthetase and $tRNA_{CUA}$ pair created by directed evolution in *E. coli* (Neumann *et al*, 2008). An unmodified control was prepared in parallel. Histone octamers were assembled as described previously (Luger *et al*, 1997) and kept at 4°C before chromatin reconstitution.

Full acetylation of H4K16 was obtained by NCL as described previously (Shogren-Knaak *et al*, 2006). Briefly, the H4 N-terminal peptide containing residues 1–22 and acetylated lysine at position 16 was synthesized using Fmoc (N-(9-fluorenyl)methoxycarbonyl)-based solid-phase synthesis and activated at the C-terminus by thioesterification. Subsequently, the globular *X. laevis* H4Δ1-22,R23C was ligated to the activated H4 peptide and the ligation product was purified as described previously (Shogren-Knaak *et al*, 2006). Identity and purity of the histones were verified by SDS–PAGE as well as ESI-MS (Supplementary Figure S1). Histone octamers were assembled as described previously (Luger *et al*, 1997) and kept at 4°C before chromatin reconstitution.

MNase digestion assay

MNase digestion was carried out in 20 μl of 10 mM TEA pH 8, 1.5 mM $CaCl_2$, 25 mM NaCl, 0.05% Tween-20. In all, 1 pmol of 601-167-6mer was digested with increasing amounts of MNase, as detailed in the figures, for 12 min on ice. The digestion was stopped by adding 10 mM EGTA, proteins were removed by proteinase K digestion for 15 min at 30°C and the samples were run at 65 V for 60 min in a 1.2% agarose gel 1 × TBE: 90 mM Tris, 90 mM Boric acid, 2 mM EDTA. Digestion of SIR-bound chromatin was performed on 601-167-6mer pre-incubated with the indicated amount of Sir proteins for 10 min on ice. MNase digestion of deacetylated chromatin was performed on 601-167-6mer incubated with 0.66 pmol of Sir2-3-4, Sir2cd-3-4 or Sir2-4 for 15 min at 30°C with or without 150 μM NAD and recovered on ice. Concerning the Sir2-3-4 complex, similar results were obtained by incubating the nucleosomal array with 0.66 pmol of Sir2-4 at first and adding 0.66 pmol of Sir3 before the recovery on ice. In order to strengthen our observations, different batches of modified and unmodified chromatins were compared.

Deacetylation reaction

Deacetylation of 2 pmol of reconstituted chromatin was performed in 30 μl of 25 mM Tris pH 8, 50 mM NaCl in presence of 5 pmol of the Sir2-3-4 complex, the Sir2cd-3-4 mutant or Sir2-4 and 150 μM NAD for 30 min at 30°C and stopped by addition of 4 × Laemmli buffer. Similar results were obtained in 25 mM Tris pH 8, 137 mM NaCl, 2.7 mM KCl and 1 mM $MgCl_2$. The acetylation state was determined by immunoblotting using acetylation mark-specific antibodies (anti-H3K56^{ac} Upstate #07-677, anti-H4K16^{ac} Serotec AHP417) and H3 for loading (anti-H3 Abcam ab1791-100).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We would like to thank the Gasser laboratory and in particular Helder Ferreira for discussion and support as well as Simon Lattman for assistance in analysing the data. We thank Heinz Neumann for providing a preliminary batch of H3K56^{ac} histone octamers. The Gasser laboratory is supported by the Novartis Research Foundation and the EU network Nucleosome 4D. SK was supported

by an EMBO long-term fellowship and an FWF Schroedinger fellowship.

Author contributions: MO, SK and SMG designed the experiments and interpreted results. MO performed the experiments. SK and FM contributed reagents. SS and WF contributed the H4K16^{ac} histone octamers. SMH and JC contributed the H3K56^{ac} histone octamers. MO, SK and SMG wrote the manuscript. SMG supervised the work.

Conflict of interest

The authors declare that they have no conflict of interest.

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3 THE SIR3 C-TERMINUS BINDS THE NUCLEOSOME AND MEDIATES SIR3 HOMODIMERIZATION

3.1 THE AAA+ ATPASE-LIKE DOMAIN OF SIR3 BINDS THE NUCLEOSOME IN A H3K79ME SENSITIVE MANNER

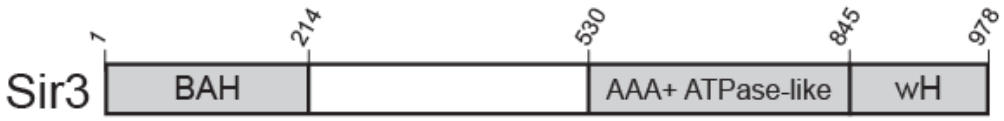
The work presented in this experimental section was published, together with work done by others, in *Structural basis for the role of the Sir3 AAA+ domain in silencing: interaction with Sir4 and unmethylated histone H3K79*. Ehrentraut S, Hassler M, **Oppikofer M**, Kueng S, Weber JM, Mueller JW, Gasser SM, Ladurner AG, Ehrenhofer-Murray AE (2011) *Genes Dev* 25: 1835-1846

The text and the figure have been edited to fit in the present PhD thesis. I performed and interpreted all experiments shown here and wrote the text which was edited by SK, SMG and AEE prior to publication. MH purified the Sir3 AAA domain. SMG supervised the work presented here. Recombinant yeast Dot1 was a kind gift of Fred van Leeuwen. Detailed material and methods can be found in the original publication.

The most important finding of this work is that the Sir3 AAA domain is able to bind the nucleosome *in vitro*, and this interaction is influenced by the methylation state of H3K79.

Brief introduction

Full-length Sir3 has the ability to bind to chromatin (Georgel *et al*, 2001), and this binding is sensitive to methylation of H3K79 in the nucleosome core region (Martino *et al*, 2009; Oppikofer *et al*, 2011). Recognition of the nucleosomal face has been attributed to both the Sir3 N-terminal BAH domain as well as a large C-terminal fragment (aa 620-978) that encompasses the AAA domain (Hecht *et al*, 1995; Connelly *et al*, 2006; Altaf *et al*, 2007; Onishi *et al*, 2007). Genetic and biochemical evidence indicates that the BAH domain is sensitive to H3K79 methylation (Onishi *et al*, 2007; Buchberger *et al*, 2008; Sampath *et al*, 2009), yet the binding of the large C-terminal region of Sir3 to an H3 peptide (aa 67-89) is also sensitive to methylation of H3K79 (Altaf *et al*, 2007).

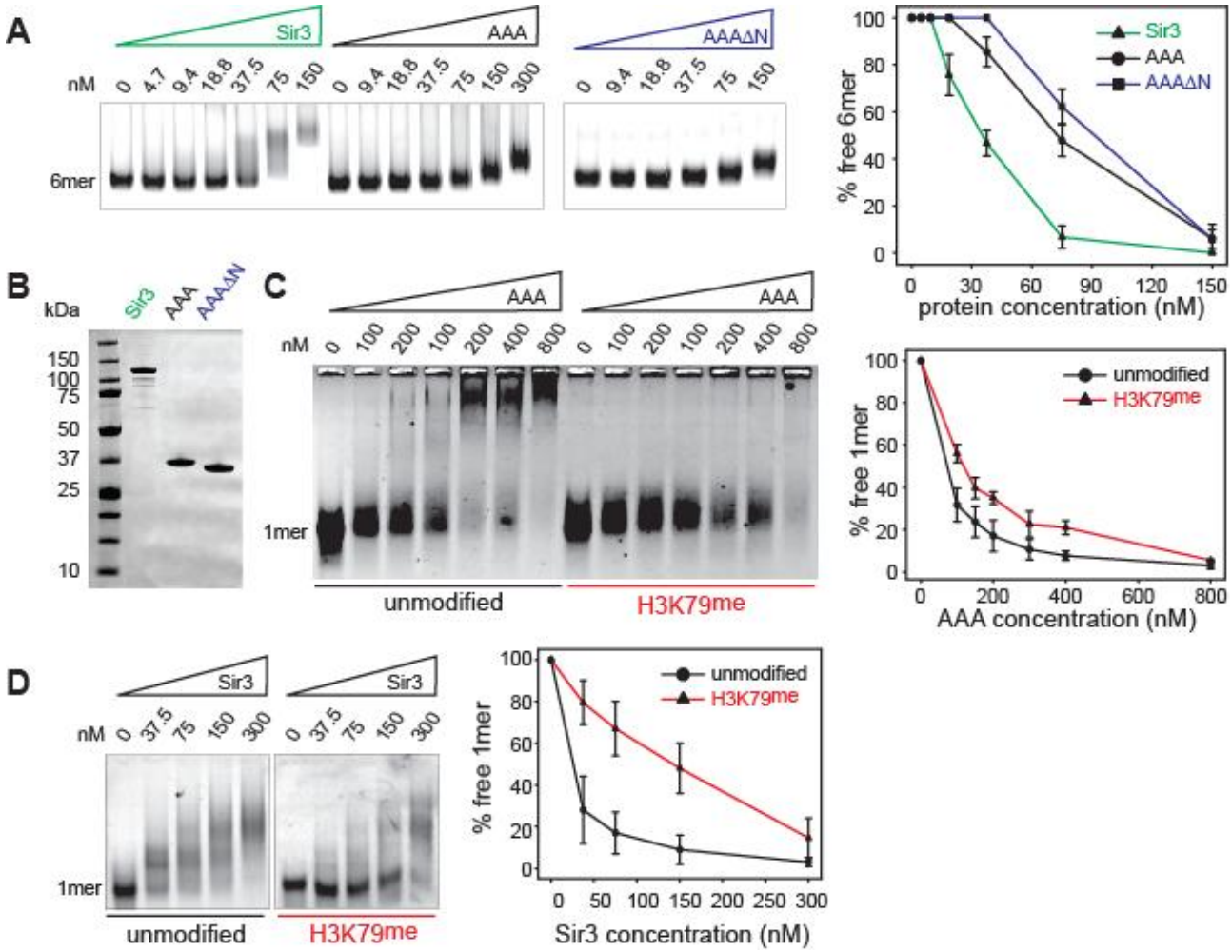


The Sir3 protein - Schematic representation of the modular organization of the Sir3 protein encompassing an N-terminal bromo-adjacent homology (BAH) domain and C-terminal AAA+ ATPase-like (AAA) and winged helix-turn-helix (wH) modules. The numbering refers to the primary sequence of the protein.

Results

To address the contribution of the AAA domain to chromatin binding, we compared its association with recombinant nucleosomal arrays with that of full-length Sir3. Increasing amounts of recombinant full-length Sir3 and AAA domain (aa 530-845) were incubated with a constant amount of a 6-mer of regularly spaced nucleosomes reconstituted on the 601-Widom sequence, as described earlier (Martino *et al*, 2009). Binding was analyzed by native agarose gel electrophoresis. We also included a shortened AAA domain lacking the N-terminal α-helical extension involved in oligomerization of this domain in the crystal (AAAΔN, aa 545-845), since the absence of these residues in full-length Sir3 caused a significant loss of telomeric silencing *in vivo* (Ehrentraut *et al*, 2011). The titrations show that the AAA domain has chromatin-binding capacity, although it is 2-3 fold reduced compared with full-length Sir3 (Figure 3A). The AAAΔN protein bound chromatin like the full-length AAA domain, indicating that this N-terminal arm is not a major chromatin interaction site (Figure 3A).

To test whether the binding of the AAA domain was sensitive to H3K79 methylation in the context of nucleosomes, we incubated the AAA domain with mononucleosomes that had been methylated by recombinant yeast Dot1 (Figure 3C). As monitored by mass spectrometry, these nucleosomes are methylated between 50% and 70%, containing mono-, di-, and tri-methylated H3K79 residues (Martino *et al*, 2009). Previous studies showed that all methylated forms are functional in disrupting silencing (Frederiks *et al*, 2008). Indeed, the binding affinity of the AAA domain to nucleosomes was reduced by roughly 2 fold upon H3K79 methylation (Figure 3C). In the same conditions, the binding affinity of full-length Sir3 was reduced by approximately 6 fold by H3K79 methylation (Figure 3D). We conclude that the Sir3 AAA domain binds both a hexamer and a mononucleosome, with a preference for unmethylated H3K79, yet it is likely that the N-terminal BAH domain also contributes to the pronounced sensitivity of full-length Sir3 to H3K79 methylation.



Binding of the Sir3 AAA to chromatin is sensitive to methylation of H3K79 (***A***) The Sir3 protein, the Sir3 AAA+ ATPase-like domain (AAA; aa 530-845) or an N-terminal truncation (AAAΔN; aa 545-845) were titrated over a constant amount (25 nM) of unmodified 6-mer nucleosomes. (***B***) SDS-PAGE gel of 1 µg of the Sir3 protein, the Sir3 AAA domain and the N-terminal truncation used in the experiments above stained with Coomassie brilliant blue. The Sir3 AAA domain (***C***) or Sir3 full-length (***D***) were titrated over a constant amount (25 nM) of unmodified or H3K79me mononucleosomes. Samples were separated by native agarose gel electrophoresis, and Cy3- or Cy5-labeled DNA was visualized. The images are representative of at least three independent experiments, quantifications show the mean value ± s.e.m. of the % of unbound template compared to the input.

3.2 THE HOMODIMERIZATION OF THE SIR3 C-TERMINAL WINGED-HELIX DOMAIN IS ESSENTIAL FOR SILENT CHROMATIN FORMATION

The work presented in this experimental section corresponds to a manuscript currently under review at *The EMBO Journal*.

Dimerization through the yeast Sir3 C-terminal winged helix domain is essential for heterochromatin formation. **Oppikofer M**, Kueng S, Keusch JJ, Hassler M, Ladurner AG, Gut H, Gasser SM

Author contributions: MO, SK and SMG designed most experiments and interpreted results. MO performed most experiments with the following exceptions. HG collected X-ray diffraction data, solved the Sir3 wH structure, produced the wH alignment and supervised the structural work. SK performed pull-down experiments from insect cells and helped with *in vivo* assays and ChIP. JJK performed SEC-MALS analysis and helped with cloning and purification of proteins from *E. coli*. MH purified the Sir3 AAA domain and performed ITC analysis under the supervision of AGL. MO, SK, HG, AGL and SMG wrote the manuscript. SMG supervised the work.

This experimental section combines biochemistry, X-ray crystallography and molecular genetics, to elucidate the role of the last 138 amino acids of Sir3. The most important finding of this work is that the extreme Sir3 C-terminus folds into a winged helix-turn-helix (wH) domain that drives Sir3 homodimerization. Strikingly, Sir3 homodimerization is essential to form silent chromatin at telomeres and *HM* loci.

Manuscript under review at The EMBO Journal

Dimerization through the yeast Sir3 C-terminal winged helix domain is essential for heterochromatin formation

Mariano Oppikofer[1,2], Stephanie Kueng[1], Jeremy J. Keusch[1], Markus Hassler[3], Andreas G. Ladurner[3], Heinz Gut[1] and Susan M. Gasser[1,2,*]

[1]Friedrich Miescher Institute for Biomedical Research, 4058 Basel, Switzerland, [2] University of Basel, Faculty of Natural Sciences, 4003 Basel, Switzerland, [3] Butenandt Institute of Physiological Chemistry, Faculty of Medicine, Ludwig Maximilian University of Munich, 81377 Munich, Germany

*Corresponding author. Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland. Tel.: +41 61 697 5025; Fax: +41 61 697 3976;
E-mail: susan.gasser@fmi.ch

Abstract

Gene silencing in budding yeast relies on the binding of the Silent Information Regulator (Sir) complex to chromatin, which is mediated by extensive interactions between the Sir proteins and nucleosomes. Sir3, a divergent member of the AAA+ ATPase-like family, contacts both the histone H4 tail and the nucleosome core. Here we present the structure and function of the conserved C-terminal tail domain of Sir3, comprising 138 amino acids. This module adopts a variant winged helix-turn-helix (wH) architecture that exists as a stable homodimer in solution. Mutagenesis shows that the self-association mediated by this domain is essential for holo-Sir3 dimerization. Its loss impairs Sir3 loading onto nucleosomes *in vitro* and eliminates silencing at telomeres and *HM* loci *in vivo*. Replacing the Sir3 wH domain with an unrelated bacterial dimerization motif restores both *HM* and telomeric repression in s*ir3*Δ cells. In contrast, related but monomeric wH domains of archaeal and human members of the Orc1/Sir3 family have DNA binding activity. We speculate that a dimerization function for the wH evolved with Sir3's ability to facilitate heterochromatin formation.

Introduction

Large regions of eukaryotic chromatin assume an epigenetically heritable structure that is refractory to gene expression, called heterochromatin or, in budding yeast, silent chromatin. From yeast to man, gene silencing is thought to stem from a more compact folding of the chromatin fiber that sterically restricts DNA accessibility (Gottschling, 1992; Singh and Klar, 1992; Loo and Rine, 1994). Covalent modifications of histone proteins play a major role in determining chromatin compaction, and hypoacetylated histones are characteristic of silent chromatin in budding yeast (Braunstein *et al*, 1993; Suka *et al*, 2001). The binding of non-histone complexes along the nucleosomal fiber appears to repress productive transcription. In budding yeast this is achieved by the binding of a heterotrimeric complex of Silent Information Regulator (Sir) proteins Sir2, Sir3 and Sir4, each of which is essential for gene silencing (Rine and Herskowitz, 1987).

The Sir proteins themselves do not recognize specific DNA sequences, but are recruited to discrete loci through protein-protein interactions with mutifunctional factors like Rap1, ORC and Abf1, which recognize specific DNA motifs at telomeres and the silent homothallic mating type (*HM*) loci. At *HM* loci an intermediary protein called Sir1 bridges between these factors and the Sir complex (reviewed in (Rusche *et al*, 2003)). From these initial nucleation sites, or

silencers, the Sir complex spreads for 3-20 kb along the chromatin fiber (Hecht *et al*, 1996; Strahl-Bolsinger *et al*, 1997).

The establishment of silent chromatin requires the NAD-dependent deacetylase activity of Sir2 (Tanny *et al*, 1999; Imai *et al*, 2000; Smith *et al*, 2000; Yang and Kirchmaier, 2006), while Sir3 and Sir4 are thought to play structural roles in the silent chromatin formation (reviewed in (Gasser and Cockell, 2001; Rusche *et al*, 2003; Moazed *et al*, 2004)). Sir4 forms a tight complex with Sir2, and stimulates Sir2 activity *in vitro* (Ghidelli *et al*, 2001; Hoppe *et al*, 2002; Tanny *et al*, 2004; Cubizolles *et al*, 2006). The C-terminal coiled-coil domain of Sir4 dimerizes and interacts with Sir3; this interaction is essential for silencing as point mutations in this Sir3-Sir4 interface abolish assembly of the trimeric Sir complex (Chang *et al*, 2003; Rudner *et al*, 2005; Ehrentraut *et al*, 2011).

The spread of Sir-mediated repression is limited both by histone modifications that lower affinity of Sir3 for chromatin and by the limited concentration of Sir proteins in the nucleus (Maillet *et al*, 1996; Marcand *et al*, 1996; van Leeuwen *et al*, 2002; Martino *et al*, 2009; Oppikofer *et al*, 2011). The overexpression of Sir3, or a balanced overexpression of Sir4 and Sir3, leads to the extension of the silent chromatin domains at telomeres or enhanced repression at silencer-flanked reporter genes (Renauld *et al*, 1993; Gotta and Gasser, 1996; Hecht *et al*, 1996; Maillet *et al*, 1996; Strahl-Bolsinger *et al*, 1997), suggesting that Sir3, and its dosage, play major roles in Sir complex spreading and transcriptional repression.

SIR3 arose from the duplication of the *ORC1* gene (Kellis *et al*, 2004), and both encode a highly conserved N-terminal BAH domain, a C-terminal AAA+ ATPase-like domain, plus an extreme C-terminal domain, which assumes a winged helix structure in archaeal Orc1 (Gaudier *et al*, 2007). A chimeric protein formed by exchanging the BAH domain of Sir3 with that of Orc1 can restore mating in a *sir3*Δ strain, but no other Orc1 subdomain is able to support silencing when integrated into Sir3 (Bell *et al*, 1995). Overexpression of a N-terminal domain of Sir3 reinforces telomere-proximal silencing in a SIR^+ strain and partially restores mating at *HM* loci in a *sir3*Δ background if Sir1 is overexpressed (Gotta *et al*, 1998; Connelly *et al*, 2006). Consistently, biochemical and structural analyses show that the Sir3 BAH domain interacts with the nucleosome (Onishi *et al*, 2007; Buchberger *et al*, 2008; Sampath *et al*, 2009; Armache *et al*, 2011). Both H3K79 methylation by Dot1 and H4K16 acetylation by Sas2 reduce the association of Sir3 BAH with chromatin (Onishi *et al*, 2007), or of holo-Sir3 with nucleosomes *in vitro* (Martino *et al*, 2009; Oppikofer *et al*, 2011), indicating that the BAH domain helps to restrict Sir3-mediated silencing to unmodified nucleosomes.

The AAA+ ATPase-like (AAA) domain of Sir3 (aa 530-845) has lost its ATPase activity, but has gained specific contacts with both Sir4 and unmodified nucleosomes (Ehrentraut *et al*, 2011). Mutagenesis confirmed that both interactions are essential for silencing. The third structural subdomain of Sir3 occupies its extreme C-terminus (aa 843-978). When fused to lexA and targeted to a subtelomeric reporter, this fragment can recruit Sir proteins and nucleate transcriptional repression (Liaw and Lustig, 2006). It was also reported to self-associate in a yeast-two-hybrid assay (Liaw and Lustig, 2006). Pull-down experiments argued that a slightly longer Sir3 C-terminal fragment (aa 832-978) might bind a central domain of Sir3 (aa 464-728), which contains the N-terminal portion of the AAA+ ATPase module (King *et al*, 2006). While these results suggested that the Sir3 C-terminus might harbor homo- or hetero-dimerization activities, the importance of such interactions for silencing or their redundancy with other potential dimerization domains in Sir3 was never rigorously tested. It is important to note that this C-terminal domain is conserved among Orc1 homologues and corresponds to the domain III of ORC/CDC6, which in *A. pernix* and other archaea binds DNA (Bell *et al*, 1995; Liu *et al*, 2000; De Felice *et al*, 2004; Singleton *et al*, 2004). X-ray structure analysis showed that the archaeal domain adopts a winged helix-turn-helix fold (Gaudier *et al*, 2007). Given that the sequence identity between the C-termini of archaeal Orc1 and yeast Sir3 is very low (13%), structure-function predictions remain highly speculative.

To sort out the role of this extreme C-terminal domain of Sir3, we first solved its X-ray crystal structure to 2.7Å resolution. These 138 amino acids form a winged helix-turn-helix variant (Sir3 wH) that is reminiscent of domain III of Orc1/Cdc6, yet the Sir3 wH has acquired a strong homodimerization function and fails to bind DNA. We show that deletion of the domain (Sir3ΔwH), or loss of its dimerization potential, ablates silencing, which can be restored by attaching a bacterial dimerization motif to Sir3ΔwH. With a combination of structural, biochemical and genetic analyses we demonstrate that the last 138 aa of Sir3 evolved as the crucial homodimerization motif within Sir3 that is essential for the assembly of Sir-dependent silent chromatin in yeast. Distinct features of the wH structure distinguish human and archaeal Orc1 from the silencing-competent *S. cerevisiae* Sir3 paralogue.

Results

The C-terminal 138 aa of Sir3 form a winged helix-turn-helix variant that homodimerizes

SIR3 arose from the duplication of the *ORC1* gene with which it shares a multidomain organization (Figure 1A) (Bell *et al*, 1995; Kellis *et al*, 2004). While the extreme C-terminal region of archaeal *A. pernix* Orc1 folds as a winged helix-turn-helix domain that binds DNA (De Felice *et al*, 2004; Dueber *et al*, 2007; Gaudier *et al*, 2007), the function of the homologous domain in *Sc*Sir3 was unclear. The multiple sequence alignment of selected archaea, vertebrate, and yeast Orc1/Sir3 wH domains revealed important evolutionary changes (Figure 1B). Notably, yeast Orc1/Sir3 wH domains possess a ~30 residues insertion (Figure 1B, orange residues) which is missing in archaea and higher eukaryotic Orc1

wH domains. In addition, the specific *Ap*Orc1 residues implicated in DNA binding (Gaudier *et al*, 2007) are poorly conserved in yeast and higher eukaryotes (Figure 1B, blue residues).

To determine the biochemical and structural features of the predicted Sir3 wH we expressed the last 138 aa of Sir3 (aa 840-978) in *E. coli* and purified the protein to ≥ 95 % purity (Figure 1C). Surprisingly, molecular mass determination of the purified Sir3 840-978 aa (15.9 kDa) using size exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) yielded a mass of 33.1 kDa, consistent with recombinant Sir3 840-978 aa being a homodimer in solution (Supplemental Figure S1). The folding of this C-terminal module was determined by solving its X-ray crystal structure.

The extreme C-terminal domain of Sir3 crystallized in space group $P3_212$ with one molecule per asymmetric unit and its structure was solved to 2.7 Å resolution by the multi-wavelength anomalous diffraction (MAD) method using a seleno-methionine derivative (Table I). The final crystal structure displays clear electron density for residues 849-975, that fold into a wH variant featuring a total of 7 α-helices and a 3 stranded β-sheet wing (Figure 1D). A search of the Protein Data Bank (PDB) using DALI (Holm and Rosenstrom, 2010) indicated that the wH domains of archaeal Orc1 (*A. pernix*, 2V1U) (Gaudier *et al*, 2007), Orc2 (*A. pernix*, 1W5T) (Singleton *et al*, 2004), CDC6P (*P. Aerophilum*, 1FNN) (Liu *et al*, 2000), and CDCP6 (*S. solfataricus*, 2QBY) (Dueber *et al*, 2007) are most similar.

Superposition of Sir3 wH with an archaeal homolog (*Ap*Orc1 wH; (Gaudier *et al*, 2007); Figure 1E) showed that the core helix-turn-helix structures and the β-sheet wing are highly similar, but also revealed important structural differences. The most significant differences are due to an additional stretch of 30 aa we identified by sequence alignment (Figure 1B, orange residues), which is absent in the archaeal *Ap*Orc1. This insert is found between the wH core α-helices three (α3, aa 883-896) and four (α4, aa 928-938) and forms two additional α-helices (a, b) that are connected by an elongated turn in coiled conformation (Figure 1B, 1E; highlighted in orange).

Whereas the asymmetric unit of Sir3 in the crystal contains one Sir3 wH module, the crystal packing reveals two symmetry-related Sir3 wH proteins in the crystal lattice (Figure 1F). The 30 aa α-helical insertion does not alone mediate Sir3 wH-wH dimerization, but contributes most to the buried solvent-accessible surface area of the protein-protein interface covering 1500 $Å^2$, with leucine 861 and valine 909 being located in the hydrophobic core of this contact (Figure 1F). This large contact surface is likely to account for the strong homodimerization observed in solution (Supplemental Figure S1), as predicted by PISA (Protein Interfaces, Surfaces and Assemblies) calculations based on the crystal packing (Krissinel and Henrick, 2007).

In order to test the importance of the wH module for Sir3 dimerization in a competitive assay, we co-expressed in insect cells a His-tagged full length Sir3 (Sir3-His) with a Myc-tagged full length Sir3 (Sir3-Myc) or Sir3 lacking the wH module (Sir3ΔwH-Myc; aa 1-850). Affinity purified Sir3-His was tested for interaction with Sir3-Myc and Sir3ΔwH-Myc using an anti-Myc antibody on proteins recovered on Ni-beads, which bind the His-tag. Strikingly, we observed that the deletion of the wH domain reduced Sir3 self-association to background levels, whereas full length Sir3-Myc was efficiently recovered with full length Sir3-His (Figure 1G). This suggests that Sir3 wH-mediated homodimerization accounts for most, if not all, Sir3 self-interaction, at least in its soluble form.

The wH fails to interact with DNA or chromatin but promotes Sir3 loading onto nucleosomes

Past work has implicated both the BAH and the AAA domains in dimerization, and in the spreading of Sir3 along the nucleosomal fiber (Connelly *et al*, 2006; Armache *et al*, 2011; Ehrentraut *et al*, 2011). Moreover, the Sir3 wH has been proposed to heterodimerize by binding a central Sir3 domain (King *et al*, 2006; Liaw and Lustig, 2006). In contrast, the archaeal ORC/CDC6 wH modules were shown to bind to DNA in concert with the AAA+ ATPase-like domain (De Felice *et al*, 2004; Singleton *et al*, 2004; Dueber *et al*, 2007; Gaudier *et al*, 2007). The residues implicated in DNA binding within *Ap*Orc1, however, are not conserved in Sir3 wH Figure (Figure 1B, 2A; blue residues; (Gaudier *et al*, 2007)).

To test various models of DNA binding, domain interaction and cooperation in chromatin association, we purified two additional Sir3 C-terminal fragments: the AAA+ ATPase-like (AAA) domain alone (aa 530-845; (Ehrentraut *et al*, 2011)) and the entire Sir3 C-terminal half, which includes both AAA+ and wH domains (Sir3C; aa 527-978; Figure 2B; Supplemental Figure S2A). We first incubated increasing amounts of the Sir3 wH and the AAA module with a constant amount of a Cy3-labelled 147 bp DNA (Huynh *et al*, 2005; Martino *et al*, 2009), and monitored interaction by gel shift assay. Scoring the loss of unbound DNA template, we found that Sir3 AAA readily binds DNA, while Sir3 wH does not, even at 2500-fold molar excess (Figure 2C). To see if the Sir3 wH instead binds a chromatin template, we repeated the titration into a constant amount of hexameric (6-mer) nucleosomal arrays, reconstituted from bacterially expressed histones and the Widom repeat (Martino *et al*, 2009). The AAA domain efficiently interacted with this array (see also (Ehrentraut *et al*, 2011)), while Sir3 wH was unable to bind chromatin, even at 2500-fold molar excess (Figure 2D).

In order to test whether the dimerization capacity of the wH domain contributes to the DNA and chromatin binding properties of Sir3 AAA, we compared the binding of the AAA domain with the larger Sir3C fragment, which includes the wH domain (Figure 2B). Strikingly, the presence of the wH, which does not interact with DNA *per se*, increased Sir3C-DNA binding affinity by roughly 4-fold over that of the AAA module alone (Figure 2E). Similarly, when we compared the loading of AAA and the larger Sir3C onto the 6-mer array, the chromatin binding affinity of the larger

Sir3C fragment was again roughly 4-fold higher (Figure 2F). Nonetheless, the pull-down experiments shown in Figure 1G indicated that the wH does not interact with the AAA module. Indeed, we failed to co-precipitate purified wH and AAA domains under a variety of conditions, nor could we detect interaction by isothermal titration calorimetry (Supplemental Figure S2B, S2C). Thus, the simplest explanation is that homodimerization of the wH module within the full Sir3C domain promotes the observed cooperative binding of neighboring Sir3C molecules onto chromatin, indirectly increasing affinity for the 6-mer array. Direct contacts with nucleosomes and DNA appear to be mediated exclusively by the AAA domain (Figure 2, see also (Ehrentraut *et al*, 2011)).

Sir3 wH is essential for silencing *in vivo*, and can be substituted by a bacterial dimerization motif

To test whether Sir3 dimerization through its wH module is important to form silent chromatin *in vivo*, we examined the restoration of silencing in a *sir3*Δ strain complemented with various Sir3 constructs expressed from a plasmid (Figure 3A). The constructs were cloned together with 1 kb of Sir3 5'- and 3'-UTR to ensure endogenous expression levels, which were checked routinely by Western blot (Figure 3B). As expected, expression of full length *SIR3* fully restored silencing of reporter genes inserted at telomere 7L and to a large extent at the *HMR* locus (Figure 3E). We then tested the complementation efficiency of a construct lacking the wH module (Sir3ΔwH; aa 1-850). Consistent with pull-down data (Figure 1G), deletion of the wH module disrupted the dimerization capacity of Sir3 as tested by glycerol gradient sedimentation (Supplemental Figure S3A, S3B). Importantly, we found that Sir3ΔwH, which was expressed at wild-type Sir3 levels, failed to restore silencing at telomere 7L and *HMR* (Figure 3B, 3E).

We next examined whether Sir3 wH homodimerization was necessary for silencing. Because PISA analysis suggested that leucine 861 (L861) and valine 909 (V909) contribute strongly to the wH-wH hydrophobic interface, we mutated these two key residues to alanines (Sir3-AA; L861A-V909A). V909 is located at the C-terminal end of the α-helix "a", which is part of the yeast specific insert described above. It contacts L861 of the other Sir3 wH unit within the crystallized homodimer (Figure 1B, 1F, Figure 3C). Like Sir3ΔwH, the Sir3-AA mutant had impaired dimerization capacity and sedimented as a monomer in a glycerol gradient (Supplemental Figure S3A , S3B). Importantly, the Sir3-AA double mutant, which was again expressed at levels comparable to wild-type Sir3 (Figure 3B), led to a complete loss of silencing of the telomere 7L reporter gene, mimicking a full deletion of *SIR3* (Figure 3E; Supplemental Figure S4A, S4B). Sir3-AA also weakly decreased silencing at *HMR* compared to wild-type Sir3 (Figure 3E). Several other mutations, such as the triple alanine substitution mutant, L861A-V909A-F926A, or mutation of L861 and V909, singly or in combination, into tryptophan (W), arginine (R) or aspartic acid (D) residues, as well as swapping the residues 900-914 with a short glycine-serine-glycine (GSG) linker, strongly reduced Sir3 protein levels, although they were expressed from the same promoters. These mutations, which rendered Sir3 unstable, of course, also compromised repression at telomere 7L (hereafter, TPE for Telomere position effect (Gottschling *et al*, 1990)); Supplemental Figure S4A, S4B).

We reasoned that if Sir3 wH homodimerization were the only essential function of the wH in gene repression, a chimeric protein consisting of the Sir3ΔwH construct fused to an ectopic dimerization domain might be able to restore silencing. To test this, we made use of the well-characterized transcription factor HlyU from *Vibrio vulnificus*, which dimerizes readily and is similar in overall size to Sir3 wH, although it shares no sequence identity (Nishi *et al*, 2010; Liu *et al*, 2011) (Figure 3D). This chimeric construct (*sir3-HlyU*), like the AA mutant, was expressed at wild-type Sir3 levels (Figure 3B) in a *sir3*Δ strain. Using reporters at both telomere 7L and at the *HMR* locus, we found that the hybrid protein Sir3-HlyU was able to restore silencing completely, with an efficiency indistinguishable from that of full-length Sir3 protein (Figure 3E). The fact that an ectopic bacterial dimerization module can functionally replace the wH domain of Sir3, conferring silencing function and protein stability, strongly suggests that dimerization is the only essential function of the Sir3 wH domain for Sir-mediated repression *in vivo*.

Overexpression of Sir3 wH competes with endogenous Sir3 dimerization to relieve TPE

Given the above findings, and past results showing that SIR3 gene dosage affects repression (Renauld *et al*, 1993; Hecht *et al*, 1996; Maillet *et al*, 1996; Strahl-Bolsinger *et al*, 1997), we reasoned that an excess of Sir3 wH might compete for endogenous full length Sir3 dimerization and impair silencing. To test this hypothesis, we cloned the wH module under the control of the *GAL1* promoter, which allows overexpression in galactose-containing media (Mumberg *et al*, 1994). We introduced this construct in a *SIR3*$^{+}$ strain and monitored TPE at telomere 7L and 5R, as well as silencing at the *HMR* locus on plates, containing galactose to induce Sir3 wH expression. Indeed, overexpression of Sir3 wH abrogated TPE at the two telomeres scored (Figure 4A; Supplemental Figure S5). On the other hand, Sir3 wH overexpression did not affect repression at *HMR*, where redundant silencers ensure more stable repression (Figure 4B; (Maillet *et al*, 1996; Marcand *et al*, 1996)).

To confirm that the Sir3 wH-provoked derepression stems from its ability to dimerize and thereby disrupt essential Sir3-Sir3 interactions, we introduced structure-based point mutations in the dimerization interface of Sir3 wH. As detailed in Figure 3, by introducing the L861A and V909A mutations in the overexpressed Sir3 wH module (LV-AA) we reduced the extent of the wH-wH hydrophobic dimerization interface (Figure 3C). We also replaced V909 by an aspartic acid (V-D), destabilizing Sir3 wH dimerization by inserting a negative charge at the hydrophobic interface. Both mutated Sir3wH domains, LV-AA and V-D, were expressed at similar levels as the wild-type Sir3 wH domain (Figure 4C).

Although overexpression of the V-D mutant failed to impair TPE at telomeres 7L and 5R (Figure 4A; Supplemental Figure S5), the LV-AA mutant derepressed both sites, albeit less efficiently than the wild-type domain (Figure 4A; Supplemental Figure S5). This is consistent with the notion that overexpression of the Sir3 wH domain has a dominant negative effect on TPE by interfering with endogenous Sir3 dimerization, and again confirms the importance of the large dimerization interface within Sir3 wH domains for Sir-mediated repression.

Sir3 dimerization is dispensible for Sir complex formation, but is essential for Sir complex assembly on chromatin *in vivo*

Our *in vitro* assays revealed that the Sir3 wH domain promotes the loading of Sir3 C-terminus onto a reconstituted chromatin template (Figure 2). Given the multiplicity of contacts between the Sir complex and chromatin, we next examined whether Sir3 wH homodimerization also promotes the binding of the holo-Sir complex to chromatin *in vitro*. We first expressed Sir3-AA, Sir3ΔwH, or wild-type Sir3 in insect cells, together with Sir2 and Sir4, and found that all generate stable trimeric complexes with Sir2-4 that can be purified from cell extracts (Figure 5A). The purified holo-Sir complexes were then incubated with the 6-mer of reconstituted nucleosomes. Strikingly, disruption of Sir3 dimerization by deletion (Sir3ΔwH) or mutation (Sir3-AA) of the Sir3 wH domain reduced the binding affinity of the holo-Sir complex to chromatin *in vitro* by roughly 2-fold (Figure 5B). This is consistent with the loss of TPE in cells expressing only the Sir3ΔwH or Sir3-AA forms of Sir3.

Given the fact that loss of Sir3 wH homodimerization weakened Sir complex loading *in vitro* (Figure 5B) and compromised TPE *in vivo* (Figure 3), we asked whether Sir3 wH-mediated dimerization was also necessary for the stable binding and spread of Sir complexes along subtelomeric chromatin *in vivo*. Alternatively, the mutant Sir complexes might bind chromatin, but simply fail to repress. To address this, we performed chromatin immunoprecipitation (ChIP) analysis using an antibody against Sir4 in strains that express the Sir3 constructs described above (Figure 3), driven by the *SIR3* promoter and flanked by *SIR3* terminator sequences. All constructs are expressed at levels comparable to wild-type Sir3 (Figure 3B). Using qPCR on chromatin sheared to obtain a resolution of roughly 500 bp, we monitored Sir4 binding to the subtelomeric *URA3* reporter gene that monitors silencing at telomere 7L, along with other genomic locations that support Sir-mediated repression.

We found that Sir3ΔwH failed to support Sir4 loading at native telomeres 6R and 9R, and at the *HML-α1* gene, while there was reduced Sir4 binding at telomere 7L and at the *HML-E* silencer (Figure 5C). In addition, the dimerization mutant *sir3-AA* reduced Sir4 loading at all telomeres tested (Tel6R, 7L, 9R) as well as at *HML-α1*, while it did not affect binding of Sir4 at the *HML-E* silencer. Consistent with its ability to restore repression, expression of Sir3-HlyU restored binding of the Sir4 protein at all loci tested (Figure 5C). This confirms that Sir3 dimerization through the wH domain is essential for Sir complex spreading *in vivo*, although Sir4-silencer binding is independent of Sir3 dimerization activity. Our ChIP results correlate perfectly with the silencing phenotypes described in Figures 3, and reveal that Sir3 wH-mediated dimerization is critical for holo-Sir complex loading or stable association on chromatin *in vivo*. Since the bacterial dimerization domain (HlyU) restores holo-Sir binding, we propose that the Sir3 wH performs no other silencing-specific function.

The *S. cerevisiae* Orc1/Sir3 wH domain evolved to self-associate prior to gene duplication

As discussed above, the Orc1/Sir3 family is conserved throughout evolution. The archaeal A. PERNIX Orc1 wH was shown to bind DNA (Gaudier *et al*, 2007), and based on this observation, a similar function was predicted for other Orc1 homologues, including the Sir3 wH domain (Norris and Boeke, 2010; Hickman *et al*, 2011). Having shown here that Sir3 wH does not bind DNA, and evolved instead into a dimerization module that is crucial for Sir-mediated silencing, we were curious whether the *S. cerevisiae* and *H. sapiens* Orc1 wH domains would be more similar to Sir3 or to the archaeal protein.

To shed light on the functional conservation of these wH modules, we purified the equivalent domains of *Sc*Orc1 and *Hs*Orc1 after expression in *E. coli* (Figure 6A). To test whether the *Sc*Orc1 or *Hs*Orc1 wH domains interact with DNA, we titrated an increasing amount of each purified protein into a constant amount of Cy3-labelled 147-bp DNA, and monitored interaction by gel shift assay. We found that the human Orc1 wH domain bound DNA readily, while the yeast Orc1 wH did not (Figure 6B). Next, we investigated the oligomeric states of *Sc*Orc1 and *Hs*Orc1 by SEC-MALS. We found that *Sc*Orc1 wH oligomerizes in solution and, in the conditions used here, forms a range of oligomers, primarily consistent with 5-7 molecules, but ranging in one experiment up to 27 (Figure 6C). This may reflect a need for other subunits of ORC for proper oligomerization. In contrast, the *Hs*Orc1 wH showed no self-association and retained a stable monomeric state in solution (Figure 6D). We conclude that there is a fundamental difference between *Sc*Orc1/Sir3 and both the archaeal and human Orc1 proteins, in that the *Sc*Orc1/Sir3 wH evolved to promote protein-protein interactions, losing DNA binding ability. This most likely occurred before an ancient yeast underwent the genome duplication that generated the two related *SIR3* and *ORC1* genes.

Discussion

The formation of yeast silent chromatin depends on the recruitment of the Sir proteins by factors that bind specific DNA elements, followed by the establishment of Sir-chromatin and Sir-Sir contacts. These layers of interaction contribute to

a stable chromatin structure that impairs transcription and protects the linker DNA from nuclease attack. Whereas Sir protein recruitment by Rap1, ORC, Abf1 and yYku has been studied extensively, as have interactions between Sir domains and chromatin, only a few studies have examined the importance of Sir protein dimerization for repression.

The Sir3 C-terminal wH domain is necessary and sufficient for Sir3 dimerization

Sir4 homodimerizes through a C-terminal coiled-coil domain, which also interacts with Sir3, Yku70 and Rap1 (Moretti *et al*, 1994; Tsukamoto *et al*, 1997; Chang *et al*, 2003; Murphy *et al*, 2003; Rudner *et al*, 2005). Sir4 dimerization has been proposed to be necessary for silencing by providing a binding site for Sir3 (Chang *et al*, 2003; Murphy *et al*, 2003). The importance of Sir3 dimerization has never been tested directly, even though Sir3 has the ability to spread along chromatin with substoichiometric amounts of Sir2 and Sir4 (Hecht *et al*, 1996; Strahl-Bolsinger *et al*, 1997). This implied that Sir3-Sir3 interaction might be important for its function *in vivo*.

Over the years, various Sir3 domains have been proposed to mediate homo- or hetero-dimerization. Both the AAA domain and the BAH domain made suggestive contacts in the crystal packing when crystallized alone (Connelly *et al*, 2006; Ehrentraut *et al*, 2011) or together with a nucleosome (Armache *et al*, 2011). However, the relevance of this finding outside of the crystal context is unclear. Dimerization of the Sir3 BAH could not be detected by co-IP (Buchberger *et al*, 2008) and only a very weak interaction was observed by analytical ultracentrifugation (Armache *et al*, 2011). A Sir3 C-terminal fragment that contained both the AAA and the wH domains appeared to dimerize in solution (Chang *et al*, 2003), while the Sir3 wH and a fragment containing a large N-terminal portion of the AAA domain showed some interaction upon bacterial co-overexpression (King *et al*, 2006). However, no study to date had carefully mapped the dimerization interface between Sir3 molecules, nor mutated important residues to monitor both a loss of dimerization and of silencing function *in vivo*. Here we demonstrate unequivocally that the Sir3 C-terminal 138 residues form a wH module that is necessary and sufficient for dimerization, and that Sir3 homodimerization is crucial for Sir complex loading along nucleosomes *in vitro* and *in vivo*. Genetic, biochemical and structural data support this conclusion rigorously.

The crystal structure of the wH domain presented here reveals a large hydrophobic dimerization interface (1500 $Å^2$), which we show is biologically relevant in contrast to the contact surfaces observed for the BAH and AAA domains (Connelly *et al*, 2006; Ehrentraut *et al*, 2011). We confirmed Sir3 wH dimerization in solution by SEC-MALS. Importantly, Sir3 dimerization in insect cell extracts was reduced to background levels when full length Sir3 was co-expressed with Sir3 lacking the wH domain (Figure 1G). Finally, while holo-Sir3 was shown to migrate as a dimer in a glycerol gradient, Sir3ΔwH or Sir3-AA shifted the Sir3 protein toward a monomeric state. Together, these studies indicate that dimerization of Sir3 is mostly, if not entirely, mediated by the wH domain. In contrast, we found that the AAA domain does not stably dimerizes (Connelly *et al*, 2006; Ehrentraut *et al*, 2011), nor does it interact with the wH domain by either pull-down or isothermal titration calorimetry (this study). While we cannot rule out that novel dimerization interfaces are revealed in Sir3 upon assembly into the holo-Sir complex or upon binding to chromatin, it is made less likely by the fact that either deletion or mutation of the Sir3 wH domain reduces the binding affinity of the holo-Sir complex to chromatin *in vitro*.

The Sir3 wH does not bind DNA or chromatin, but stabilizes the Sir complex on chromatin

Due to its homology with the crystallized archaeal *Ap*Orc1 C-terminus (Gaudier *et al*, 2007), the Sir3 wH domain was predicted to bind DNA. However, Sir3 wH neither binds DNA nor chromatin *in vitro.* Instead it forms a homodimer that greatly reinforces the loading of the Sir3 C-terminus onto both templates in binding assays (Figure 2). Previous work using full-length Sir3 showed that holo-Sir3 can bind DNA and nucleosomal templates, forming oligomeric structures *in vitro* (Georgel *et al*, 2001; McBryant *et al*, 2006; McBryant *et al*, 2008). We attribute these chromatin interactions to contacts demonstrated for the BAH and AAA domains (Connelly *et al*, 2006; Armache *et al*, 2011; Ehrentraut *et al*, 2011), while Sir3 wH specifically mediates dimerization.

Our biochemical data suggest that the dimerization of Sir3 wH promotes Sir3 and holo-Sir complex loading onto chromatin *in vitro*, while ChIP data show that it also promotes the propagation and/or stable binding of the holo-Sir complex along the chromatin fiber *in vivo*. Reducing the Sir3 wH dimerization interface by targeted mutagenesis (Sir3-AA) or deletion of the wH domain, significantly decreased the loading of Sir4 along subtelomeric nucleosomes at telomeres 6R, 7L, 9R and across the *HML* locus *in vivo*. Consistently, the Sir3-AA or Sir3ΔwH mutants failed to silence subtelomeric reporter genes. Remarkably, repression could be completely restored to the wH-deficient Sir3 mutant by attaching a bacterial dimerization motif that has no homology with the native Sir3 domain. Reinforcing our conclusion that dimerization of Sir3 is crucial for repression, it was reported that expression of the BAH domain could support measurable *HM* silencing only when fused to the dimerization-competent lexA module (Connelly *et al*, 2006). This suggests that dimerization capacity is a general requirement for gene repression, even when it is mediated by an alternative mechanism that involves only a subdomain of Sir3. In summary, our data show unequivocably that Sir3 wH-mediated homodimerization is essential for loading of Sir proteins onto chromatin and for the silencing of underlying genes.

Consistent with the loss- and gain-of function constructs described above we also demonstrate that overexpression of the Sir3 wH domain, but not of a dimerization deficient mutant, ablates TPE. This refines previous data showing that overexpression of a large Sir3 C-terminal construct (aa 437-978) derepresses TPE, but only if it includes the Sir3 wH domain (Enomoto *et al*, 2000). Although TPE has been shown to be more easily disrupted than *HMR* silencing in overexpression studies, we note that the deletion of the wH domain fully derepressed both *HMR* and telomeric reporters, demonstrating the general need of Sir3 wH dimerization for silencing.

A previous study showed that a few amino acid substitutions that do not affect Sir3 wH dimerization in yeast-2-hybrid assays, interfered with a tethered repression assay (Y900A, Y964A and K973A; (Liaw and Lustig, 2006)). Our structural studies reveal that these residues are not involved in dimerization, and thus their effects may stem instead from altered intramolecular secondary structure within Sir3, or Sir3 interfaces with other proteins. However, the fact that the *HlyU* dimerization motif can replace wH to restore TPE, argues against other silencing-specific functions in this C-terminal domain.

Evolutionarily distinct wH domains among Orc1/Sir3 family members

The basic wH fold that we present here for the Sir3 C-terminal domain is conserved from archaea to man, and appears generally to mediate macromolecular interactions, either between proteins or between proteins and nucleic acids (Aravind *et al*, 2005). The yeast Sir3 wH domain, however, binds neither chromatin nor DNA, in striking contrast to the wH domain of archaeal (Dueber *et al*, 2007; Gaudier *et al*, 2007) or human Orc1 (this study). Indeed, we show here that a recombinant wH domain of *Hs*Orc1, like the archaeal Orc1 wH, forms a stable monomer in solution and readily binds DNA.

Sequence analysis predicts that only budding yeast lineage (*Saccharomycetales*) homologues, i.e. those bearing an insertion in the wH domain, will have a sufficiently large hydrophobic surface to mediate dimerization (Figure 1B). Indeed, the *Sc*Orc1 wH, unlike *Hs*Orc1 wH, self-associates in solution, and fails to bind DNA. These functional similarities between *Sc*Orc1 and *Sc*Sir3, place the yeast Orc1 wH closer to Sir3 than to the human Orc1 wH, which more closely resembles the archaeal domain, at least with respect to macro-molecular interactions. However, given that the critical residues for *Ap*Orc1-DNA interaction are not conserved in man (Figure 1B), the two may bind DNA in different ways.

The fact that both yeast Sir3 and yeast Orc1 wH domains have a ~30-aa insert, which in Sir3 contributes to a large protein-protein interface, and the fact that both multimerize and fail to bind DNA, argues that these changes in the wH domain arose prior to the genome duplication event that generated *S. cerevisiae* and the *SIR3*/*ORC1* gene pair (Kellis *et al*, 2004). Consistently, the characteristic insertion in the wH domain is also found in the budding yeast *kluyveromyces lactis*, whose genome was not duplicated (Hickman and Rusche, 2010). Moreover, Orc1 from *K. lactis* which diverged from *S. cerevisiae* before genome duplication, acts in conjunction with the deacetylase Sir2 and the histone-binding protein Sir4 to generate telomeric and mating type heterochromatin (Hickman and Rusche, 2010). Although it is not known how important *Sc*Orc1 self-association is for either its replication or silencer functions, it is clear that changes in the wH domain that promote dimerization, correlate with a divergent branch of the Orc1/Sir3 family that is involved in silencing. We note that mechanisms of chromatin-mediated repression, such as those involving Heterochromatin Protein 1 or Polycomb repressive complex 1, also have components with homodimerization potential (Fujisaki *et al*, 2003; Czypionka *et al*, 2007; Canzio *et al*, 2011). The work presented here, however, is the first to our knowledge to show genetically and biochemically, that restoration of homodimerization through a bacterial motif can restore heterochromatin to a silencing-deficient repressor mutant. Indeed, Sir3's dimerization capacity is essential for efficient Sir complex loading onto chromatin *in vitro* and *in vivo*, and for mating type maintenance, which is essential for the survival of the species.

Finally, we speculate that some of the differences between mammalian and yeast Orc1 reflect the divergence in domain structure reported here. Whereas Orc1 supports replication origin licensing in both species, *Hs*Orc1 has other functions, such as centrosome duplication (Hemerly *et al*, 2009), cell cycle control and a role in centromeric heterochromatin (Prasanth *et al*, 2010), distinct from the activities attributed to *Sc*Orc1. The fact that *Sc*Orc1 cannot substitute for Sir3 in budding yeast silencing, despite having a similar wH domain, most likely reflects the specialization of the Sir3 AAA+ domain for Sir4 interaction (Ehrentraut *et al*, 2011). At the very least, our findings call into question models that require a *Sc*Orc1 wH DNA binding function (Chen *et al*, 2008; Sun *et al*, 2012).

Materials and Methods

Multiple sequence alignment

Orc1 wH sequences from higher eukaryotes were aligned with ClustalW (www.ebi.ac.uk) and submitted to HHPRED (Soding *et al*, 2006) to yield an alignment with the *A. pernix* Orc1 wH sequence of which the structure is known (Gaudier *et al*, 2007). A ClustalW alignment of yeast Sir3 and Orc1 wH sequences was then added according to a structural alignment computed with DALI (Holm and Rosenstrom, 2010) using crystal structures of the *A. pernix* Orc1 and Sir3 wH domains.

Purification of protein domains

The Sir3 wH (aa 840-978), Sir3C (aa 527-978), *Sc*Orc1 wH (aa 783-914) and *Hs*Orc1 wH (aa 760-861) constructs were cloned into a pOPINF vector using the In-Fusion system (Clontech)(Berrow *et al*, 2007), expressed in the *E. coli* strain *BL21 Rosetta pLysS* and affinity purified *via* a His-tag using ProBond Ni-NTA resin (Invitrogen) according to manufacturer's instructions. Tags were removed by HRV 3C protease digestion (Novagen) and Sir3 domains were further purified by gel filtration on a HiLoad 16/60 Superdex 75 column in 50 mM Tris pH 7.5, 200 mM NaCl, 0.02% NaN_3 and 1 mM TCEP. The Sir3 AAA (aa 530-845) was purified as described previously (Ehrentraut *et al*, 2011). Point mutations were introduced by site directed mutagenesis using *Pfu* DNA polymerase (Promega). For the chimera Sir3-HlyU, the coding region for aa 851-978 was deleted and replaced with *Asc*I and *Xba*I sites using site directed mutagenesis. The cDNA of HlyU_Vv was amplified by PCR (without the ATG start codon) inserting *Asc*I and *Xba*I sites and a SGSG linker. The cDNA of *Hs*Orc1 (IRAK013J04) was obtained through RIKEN BRC, Japan (Ota *et al*, 2004; Otsuki *et al*, 2005; Itoh *et al*, 2006; Kimura *et al*, 2006). Purity was confirmed by SDS PAGE and Coomassie blue staining, protein concentration was measured by UV spectroscopy.

Structure determination

Native and selenomethionine substituted Sir3 wH crystal were grown at 20°C by sitting-drop vapor diffusion in 96-wells crystallization plates from mixtures containing equal volumes of protein (22 mg/ml in 20 mM Tris pH 8.5, 200 mM NaCl, 0.02% NaN_3 and 1 mM TCEP) and reservoir solutions (0.1 M sodium citrate pH 5.6, 0.2 M ammonium acetate and 30% (w/v) PEG-4000) and were flash-frozen in mother liquor made up to 25% (v/v) ethylene glycol. Diffraction data were collected at beam lines X06DA and X10SA at the Swiss Light Source synchrotron in Villigen, Switzerland. Detailed Materials and Methods for structure determination and refinement can be found in supplemental material. Coordinates have been deposited in the Protein Data Bank database under accession code XXXX.

SEC-MALS experiments

Purified domains were concentrated to 2 -8 mg/ml and filtered through a 0.1 µM Amicon filter before injection. 100 µl of each protein was separated on a Wyatt SEC 300A 7.8 x 300 mm column equilibrated in 20 mM Tris pH 7.4, 200 mM NaCl, 0.02% NaN_3 at a flow rate of 0.5 ml/ min at 4°C. Light scattering was recorded on an in-line miniDAWN TREOS 3-angle light scattering detector (Wyatt Technology) and protein concentration detected with an in-line Optilab Trex refractive index detector. Data analysis was done using the Zimm fitting method in Astra V software (Wyatt Technology).

Co-immunoprecipitation from insect cells

Full length or truncated Sir3-ΔwH were co-infected in Hi5 cells as indicated in Figure 1G. Cells were lysed by gentle sonication in 50 mM HEPES pH 7.5, 150 mM NaCl, 0.1% NP-40, and Sir3-HIS-HA purified in batch using ProBond Ni-NTA resin (Invitrogen). Beads were washed with 50 mM Na_2HPO_4 pH 8.0, 500 mM NaCl, 50 mM imidazole, 0.05% NP-40 and eluted in 2x the bead volume with 50 mM Na_2HPO_4 buffer pH 8.0, 300 mM NaCl, 250 mM imidazole, 0.05% NP-40. Co-purification of Myc-tagged Sir3 constructs was analyzed by immunoblotting using HIS-hrp (Abcam, ab1187) or Myc (9E10) antibodies.

Chromatin and DNA interaction assays

In vitro reconstitution of nucleosomal arrays and binding assays were performed as described previously (Oppikofer *et al*, 2011). See also descriptions in supplemental material.

Overexpression and complementation assays in yeast

For overexpression assays the genomic sequence coding for Sir3 wH (aa 840-978) was HA-tagged, amplified by PCR and cloned into the pRS425-GAL1 vector (Mumberg *et al*, 1994) using *Pst*1 and *Sal*1 sites. For complementation assays the *SIR3* gene together with 1 kb 5'- and 3'-UTR was amplified by PCR and cloned into pRS415 using *Bam*HI and *Not*I sites. Deletion and point mutations were introduced by site directed mutagenesis using *Pfu* DNA polymerase (Promega). Telomeric silencing used GA503 (*SIR3*$^+$) and GA7055 (*sir3*Δ) strains, while *HMR* silencing used GA484 (*SIR3*$^+$) and GA7292 (*sir3*Δ) strains. *SIR3* deletion was achieved by replacing the endogenous gene together with 1 kb 5'- and 3'-UTR with a kanamycin resistance cassette (Supplemental Table S1). Silencing of the indicated reporter genes (*URA3* or *TRP1*) was monitored as described (Gotta *et al*, 1998), by growth on synthetic media lacking either uracil or tryptophan.

ChIP and qPCR experiments

ChIP experiments were carried out as previously described (Braunstein *et al*, 1993), with an antibody raised against GST-Sir4 C-terminus used at 750 ng/ 20 µL MagSi-proteinA 1.0 beads (MagnaMedics). Precipitated DNA was purified using the AccuPrep PCR purification kit (Bioneer) after overnight incubation at 65 °C to reverse the formaldehyde cross-linking. The GA5822 *sir4*Δ strain (Kueng *et al*, 2012) was used as control. Quantification of the precipitated DNA was performed by qPCR on a StepOnePlus instrument (Applied Biosystems), enrichments were normalized to

input and the *ACT1* locus. Primers have been used previously (see Supplemental Table S2). Experiments were performed in biological and technical duplicates.

Acknowledgments

We would like to thank the Gasser laboratory for discussion and support. We thank L. Pillus (University of California, San Diego, California) for Sir3 antibody and Jeong-Sun Kim (Chonnam National University, Gwangju, Republic of Korea) for the HlyU_Vv cDNA. We thank the Research Association for Biotechnology, Dr. Yoshihide Hayashizaki (RIKEN OSC) and Dr. Sumio Sugano (Tokyo University Graduate School) for the *Hs*Orc1 cDNA (IRAK013J04). The Gasser laboratory is supported by the Novartis Research Foundation and the EU network Nucleosome 4D. SK was supported by an EMBO long-term fellowship, an FWF Schroedinger fellowship and the Swiss SystemsX initiative C-CINA.

Author contributions: MO, SK and SMG designed most experiments and interpreted results. MO performed most experiments with the following exceptions. HG collected X-ray diffraction data, solved the Sir3 wH structure, produced the wH alignment and supervised the structural work. SK performed pull-down experiments from insect cells, and helped with *in vivo* assays and ChIP. JJK performed SEC-MALS analysis and helped with cloning and purification of proteins from *E. coli*. MH purified the Sir3 AAA domain and performed ITC analysis under the supervision of AGL. MO, SK, HG, AGL and SMG wrote the manuscript. SMG supervised the work.

Conflict of interest

The authors declare that they have no conflict of interest.

Figure legends

Figure 1 The C-terminal 138 residues of Sir3 fold into a wH domain and homodimerize. (**A**) Schematic representation of the Sir3 protein. The C-terminal wH domain in green has been crystallized. Purple bars show the position of the 3 stranded β-sheet wing. (**B**) Multiple sequence alignment of Orc1/Sir3 wH modules from archaea, budding yeasts and higher eukaryotes. Conserved residues are highlighted in black (100%), dark grey (50%), and light grey (20%). *Sc*Sir3 wH residues that contribute to the dimerization interface are in red, the 30 aa insertion is highlighted in orange, and *Ap*Orc1 wH residues that contact DNA are in blue (Gaudier *et al*, 2007). Above the alignment is the secondary structure of Sir3 wH color coded as in panel *D*. The L861 and V909 residues mutated in the Sir3-AA construct are highlighted. (**C**) 2 µg of purified Sir3 wH (aa 840-978) were loaded on a SDS-PAGE and stained with Coomassie blue. (**D**) Representation of the Sir3 wH structure; α-helices are represented in green, β-sheets in purple and coils in yellow. (**E**) Superposition of Sir3 and *Ap*Orc1 (PDB 2v1u; (Gaudier *et al*, 2007). The insertion, absent in *Ap*Orc1, forming most of the Sir3 wH homodimerization interface is in orange (see sequence alignment) and displayed as dumbbell shaped helices (a, b). (**F**) Representation of a Sir3 wH dimer, residues involved in the hydrophobic interface in red and cyan for the green and grey Sir3 wH monomers, respectively. The insertion crucial for Sir3 wH dimerization is displayed as dumbbell shaped helices. (**G**) His-tagged full length Sir3 (Sir3-His) was co-expressed in insect cells with Myc-tagged full length Sir3 (Sir3-Myc) or Sir3 lacking the wH module (Sir3ΔwH-Myc; aa 1-850). Sir3-His was affinity purified, input and His elution were analyzed by Western blotting using the indicated antibody.

Figure 2 The association of the Sir3 C-terminus to DNA and chromatin is promoted by wH homodimerization. (**A**) Details on the residues of *Ap*Orc1 wH (blue) interacting with DNA and superposition with the corresponding region of Sir3 (green). (**B**) Scheme of the modular organization of the Sir3 protein. Constructs used are highlighted.

The indicated Sir3 domains were titrated onto a constant amount of Cy3-labeled 147 bp DNA (**C**, **E**) or a 6-mer of nucleosomes (**D**, **F**). Samples were separated on a native agarose gel, the nucleosomal template was stained with SYBR safe and the amount of unbound probe was quantified by the intensity of the fluorescent signal. The images are representatives for three or more experiments, quantification represent mean values and s.e.m of the % of free probe normalized to the input.

Figure 3 Sir3 wH homodimerization is essential for telomeric and HMR silencing. (**A**) Schematic representation of the Sir3 constructs used. Highlighted are Sir3 wH wild-type (green), the L861A-V909A mutant (blue) and the HlyU module from *Vibrio vulnificus* (orange). (**B**) Western blot analysis of alkaline yeast extracts (Ruault *et al*, 2011) of the strains used in panel *E* show wild type levels of Sir3 expression, using a polyclonal antibody raised against full length Sir3 (a kind gift from L. Pillus). (**C**) Modeled representation of the impact L861A and V909A mutations on the extent of the hydrophobic Sir3 wH dimerization interface. (**D**) Representation of the Sir3 wH or the HlyU dimer. The most C-terminal residues are highlighted by a black dot. (**E**) The indicated strain was grown overnight in selective media and 10-fold dilutions were dropped on the indicated media. Ability to repress a *URA3* gene inserted at the telomere 7L was tested by growth on synthetic media lacking uracil while ability to repress a *TRP1* gene inserted at the *HMR* locus was tested by growth on synthetic media lacking tryptophan. Synthetic complete media (only selecting for plasmids) was used to control for equal deposition.

Figure 4 Overexpression of Sir3 wH, but not of dimerization mutants, derepresses silencing. (**A**) The indicated strain was grown overnight in selective media and 10-fold dilutions were dropped on the indicated media containing galactose

as carbon source, to induce p*GAL1*^{promoter}-*SIR3*wH construct overexpression. Ability to repress a *URA3* gene at telomere 7L was tested as in Figure 3E. (**B**) The ability to repress *TRP1* inserted at the *HMR* locus was tested as in Figure 3E. (**C**) Western blot analysis from alkaline yeast extracts (Ruault *et al*, 2011) of strains showed in Figure 4A, grown on galactose, to confirm domain expression.

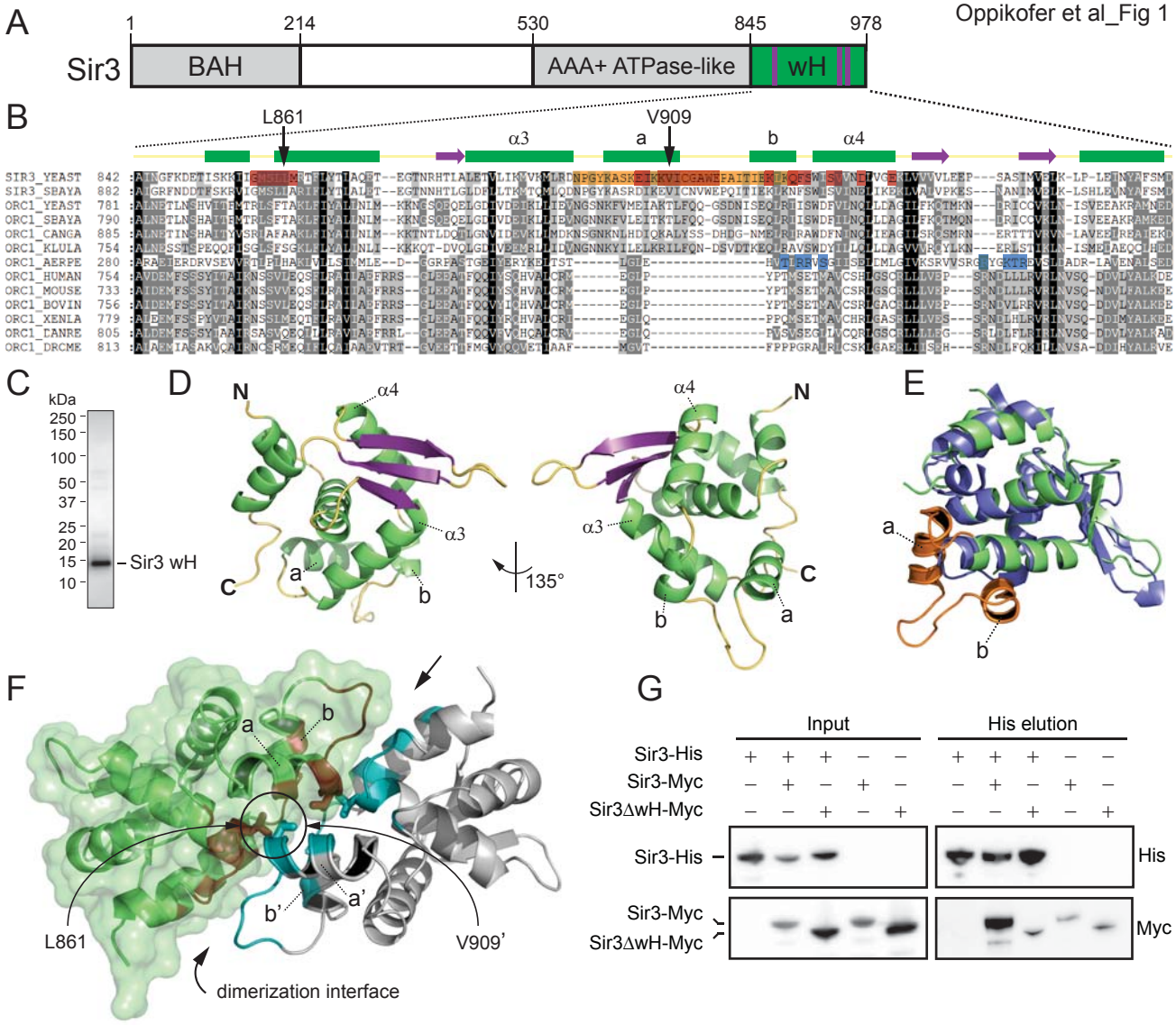
Figure 5 Sir3 wH homodimerization is critical for loading or stable binding of Sir complex onto chromatin *in vivo*. (**A**) Hi5 insect cells were co-infected with Sir2, Sir4 and the indicated Sir3 expressing virus. Sir4 was affinity purified as described previously (Martino *et al*, 2009) and a sample elution was loaded on a SDS-PAGE and stained with Coomassie blue. The asterisk indicates a common Sir4 degradation product. (**B**) The holo-Sir complex or mutant complexes containing either Sir3ΔwH (aa 1-850) or Sir3-AA (L861A and V909A) were titrated into a constant amount of 6-mer of nucleosomes. The images are representative of at least three independent experiments; quantifications show the mean value ± s.e.m of the % of unbound chromatin compared to the input. The asterisk indicates a contaminant DNA, and the bracket on the right indicates Sir/chromatin complexes. (**C**) Sir4 ChIP analysis of the same strains used for silencing assay in Figure 3E, the amount of precipitate DNA was measured by qPCR. Enrichment over the *ACT1* locus was normalized to the input.

Figure 6 Evidence for a functional conservation of Sir3 wH in *Saccharomycetales species*. (**A**) 2µg of the indicated domains were run on a SDS-PAGE and stained with Coomassie blue. (**B**) The indicated domain was titrated onto a constant amount of a 147 bp Cy3-DNA probe, binding was scored as in Figure 2. Purified *Sc*Orc1 wH, 3 mg/ml (**C**) or *Hs*Orc1 wH, 7.8 mg/ml (**D**) were analyzed by SEC-MALS as described in the Materials and Methods. The measured molar mass is represented by black dots (grey arrow) and superposed to the UV (280 nm) trace. The *Sc*Orc1 wH domain was injected five times yielding the following results: 78, 81, 108, 123 and 417 g/mol.

Table I. Data collection and refinement statistics

	Sir3 wH native	**Sir3 wH Se-Met peak**[b]	**Sir3 wH Se-Met inflection**[b]	**Sir3 wH Se-Met remote**[b]
Data collection				
Space group	$P3_212$	$P3_212$	$P3_212$	$P3_212$
Cell const. *a, b, c* [Å]	75.3, 75.3, 55.6	75.1, 75.1, 55.3	75.2, 75.2, 55.3	75.2, 75.2, 55.4
Wavelength [Å]	1.000	0.9793	0.9798	0.9716
Resolution range [Å][a]	30.0-2.7 (2.77-2.70)	30.0-3.1 (3.21-3.10)	30.0-3.1 (3.21-3.10)	30.0-3.1 (3.21-3.10)
Unique reflections	5076	6078	5990	5854
Completeness [%][a]	99.9 (100.0)	96.3 (73.1)	94.4 (64.8)	91.9 (50.6)
Multiplicity	8.2	5.8	5.8	5.7
R_{sym} [%][a]	4.9 (52.5)	6.9 (60.3)	6.8 (63.5)	7.0 (78.8)
$I/\sigma(I)$[a]	25.7 (4.7)	34.8 (1.4)	33.6 (1.2)	31.6 (0.84)
Phasing power ano	-	1.46	0.82	1.19
Refinement				
Resolution range [Å]	30.0-2.7			
Reflections (all)	5076			
Reflections (test set)	728 (14.3 %)			
R_{crys} [%]	20.0			
R_{free} [%]	25.2			
RMSDs				
Bond lengths [Å]	0.01			
Bond angles [°]	1.38			
Ramachandran plot [%]				
allowed	97.6			
outliers	2.4			

[a] Values in parentheses refer to the highest resolution shell
[b] Data collection statistics is reported for unmerged Friedel pairs


Oppikofer et al_Fig 1
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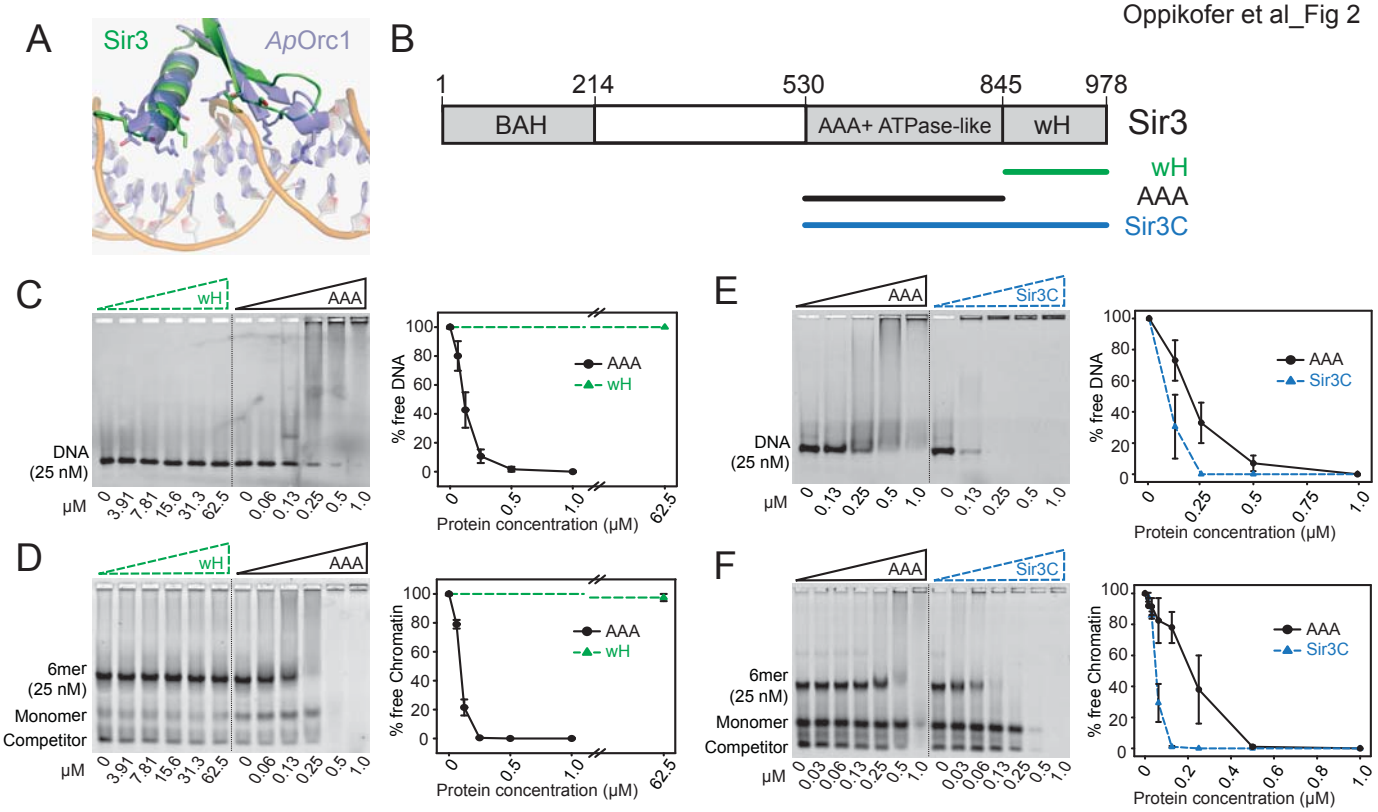
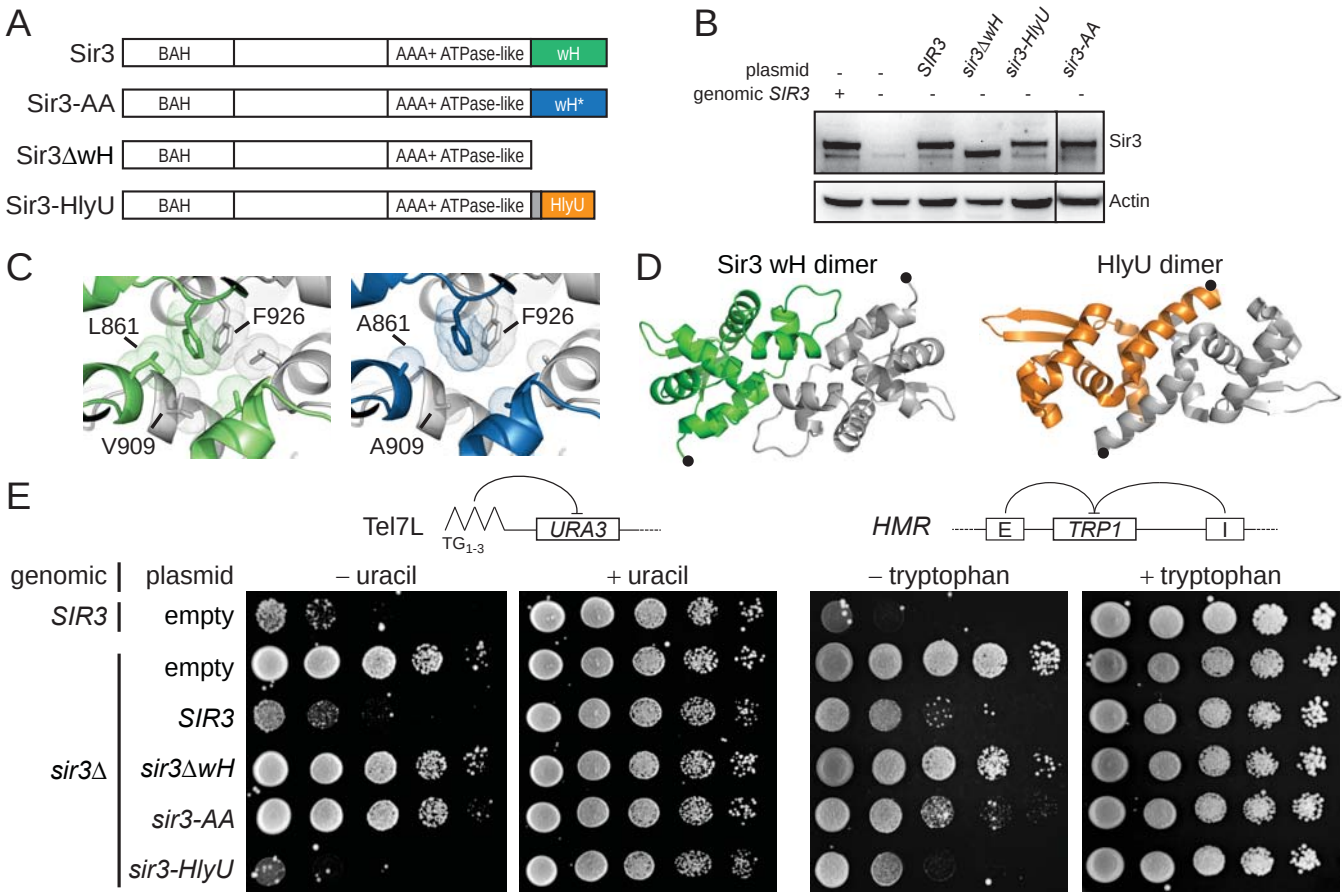
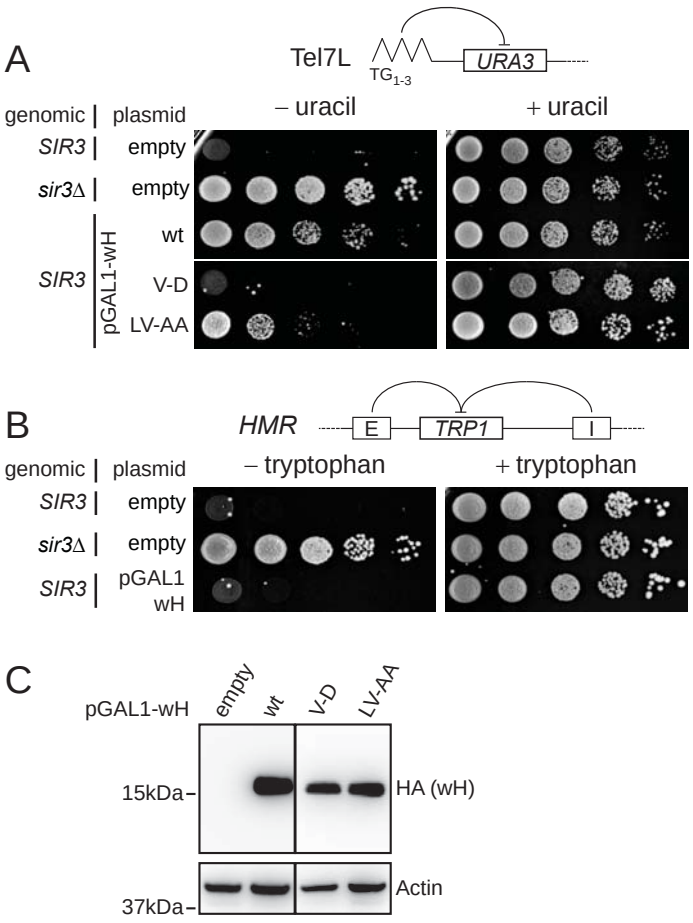
Sir3
1
214
530
845
978
BAH
AAA+ ATPase-like
wH
B
L861
V909
α3
a
b
α4
C
kDa
250
150
100
50
37
25
20
15
10
Sir3 wH
D
N
α4
α3
a
b
C
135°
E
a
b
F
a
b
b'
a'
L861
V909'
dimerization interface
G
Input
His elution
Sir3-His
Sir3-Myc
Sir3ΔwH-Myc
His
Myc

Oppikofer et al_Fig 2



Oppikofer et al_Fig 3

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Tel7L
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URA3
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+ uracil
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empty
sir3Δ
empty
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pGAL1-wH
wt
V-D
LV-AA
B
HMR
E
TRP1
I
genomic
plasmid
– tryptophan
+ tryptophan
SIR3
empty
sir3Δ
empty
SIR3
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empty
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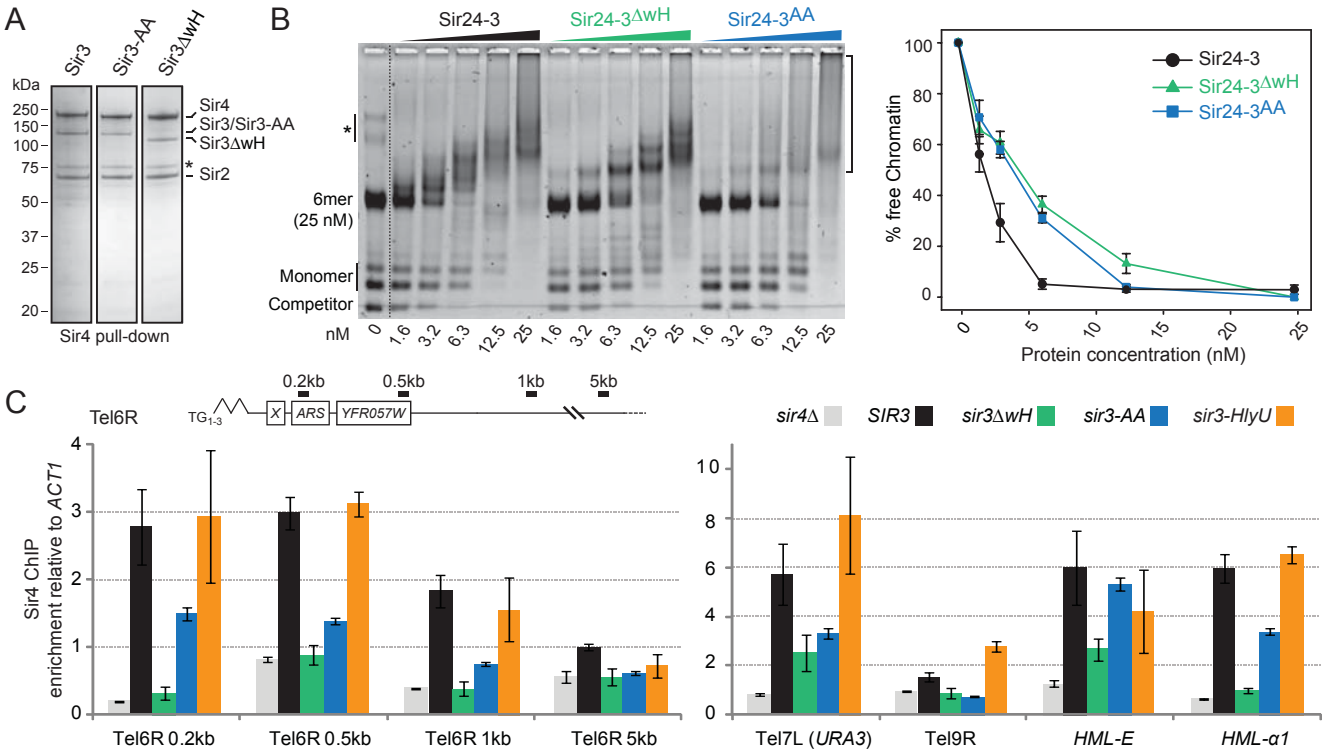
HA (wH)
37kDa
Actin


A
Sir3
Sir3-AA
Sir3ΔwH
kDa
250
150
100
75
50
37
25
20
Sir4
Sir3/Sir3-AA
Sir3ΔwH
Sir2
Sir4 pull-down
B
Sir24-3
Sir24-3ΔwH
Sir24-3AA
6mer
(25 nM)
Monomer
Competitor
nM
0
1.6
3.2
6.3
12.5
25
% free Chromatin
Protein concentration (nM)
C
Tel6R
TG1-3
X
ARS
YFR057W
0.2kb
0.5kb
1kb
5kb
sir4Δ
SIR3
sir3ΔwH
sir3-AA
sir3-HlyU
Sir4 ChIP
enrichment relative to ACT1
Tel6R 0.2kb
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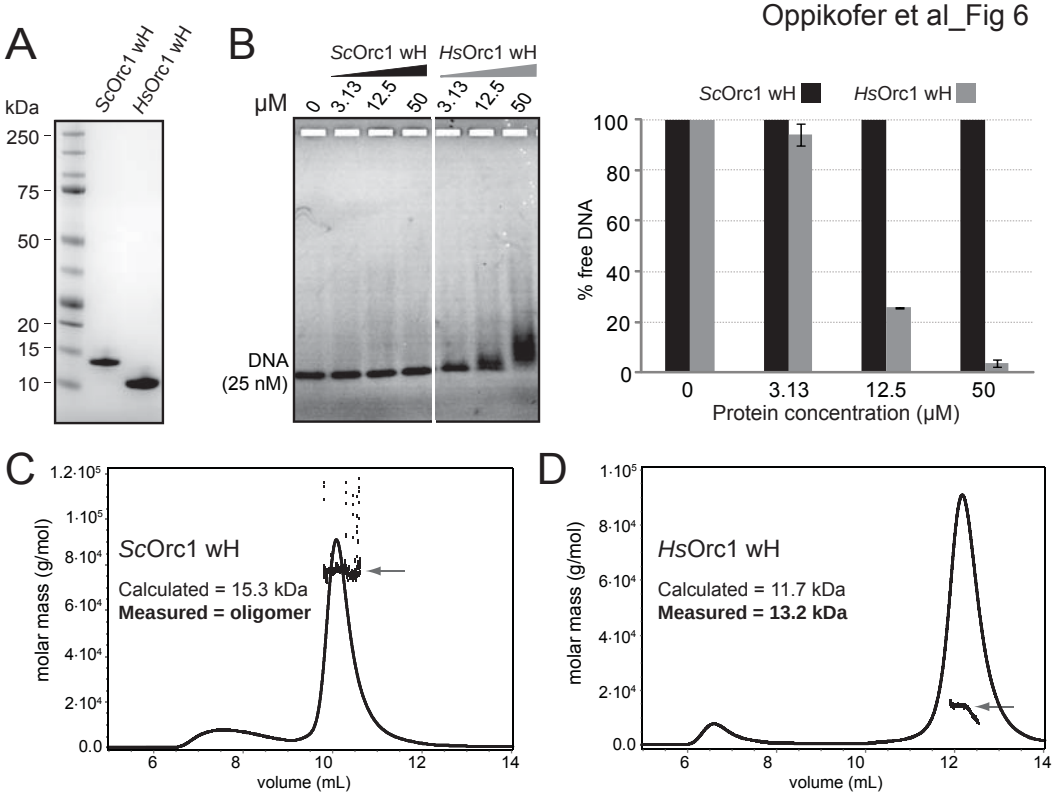
Tel9R
HML-E
HML-α1

Oppikofer et al_Fig 6



SUPPLEMENTAL MATERIAL for Oppikofer et al. "Dimerization of the yeast Sir3 C-terminal winged helix domain is essential for heterochromatin formation"

Supplemental Figures and legends

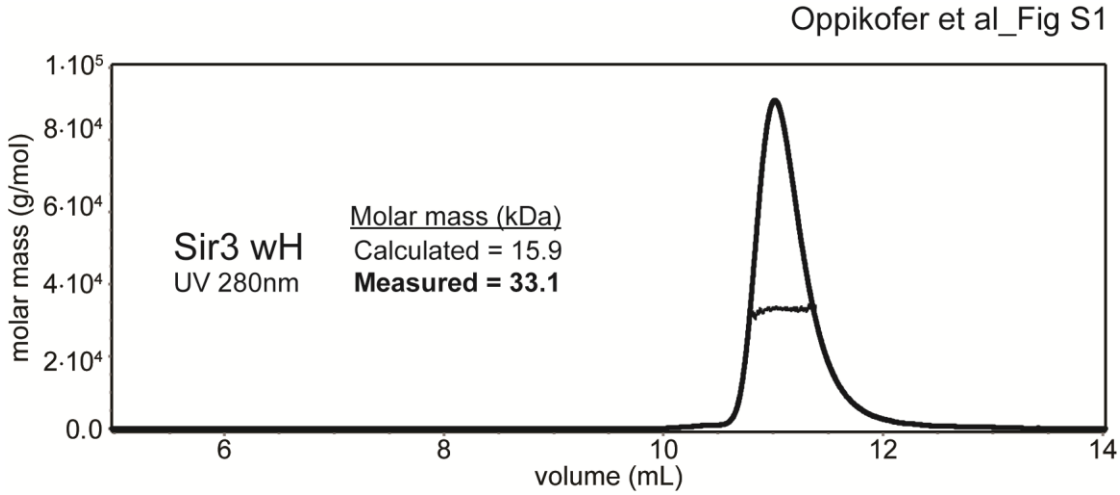


Figure S1 SEC-MALS analysis of the wild-type Sir3 wH domain. Purified Sir3 wH, 2 mg/ml was analyzed by SEC-MALS as described in the Materials and Methods. The measured molar mass is represented by black dots and superposed to the UV (280 nm) trace.

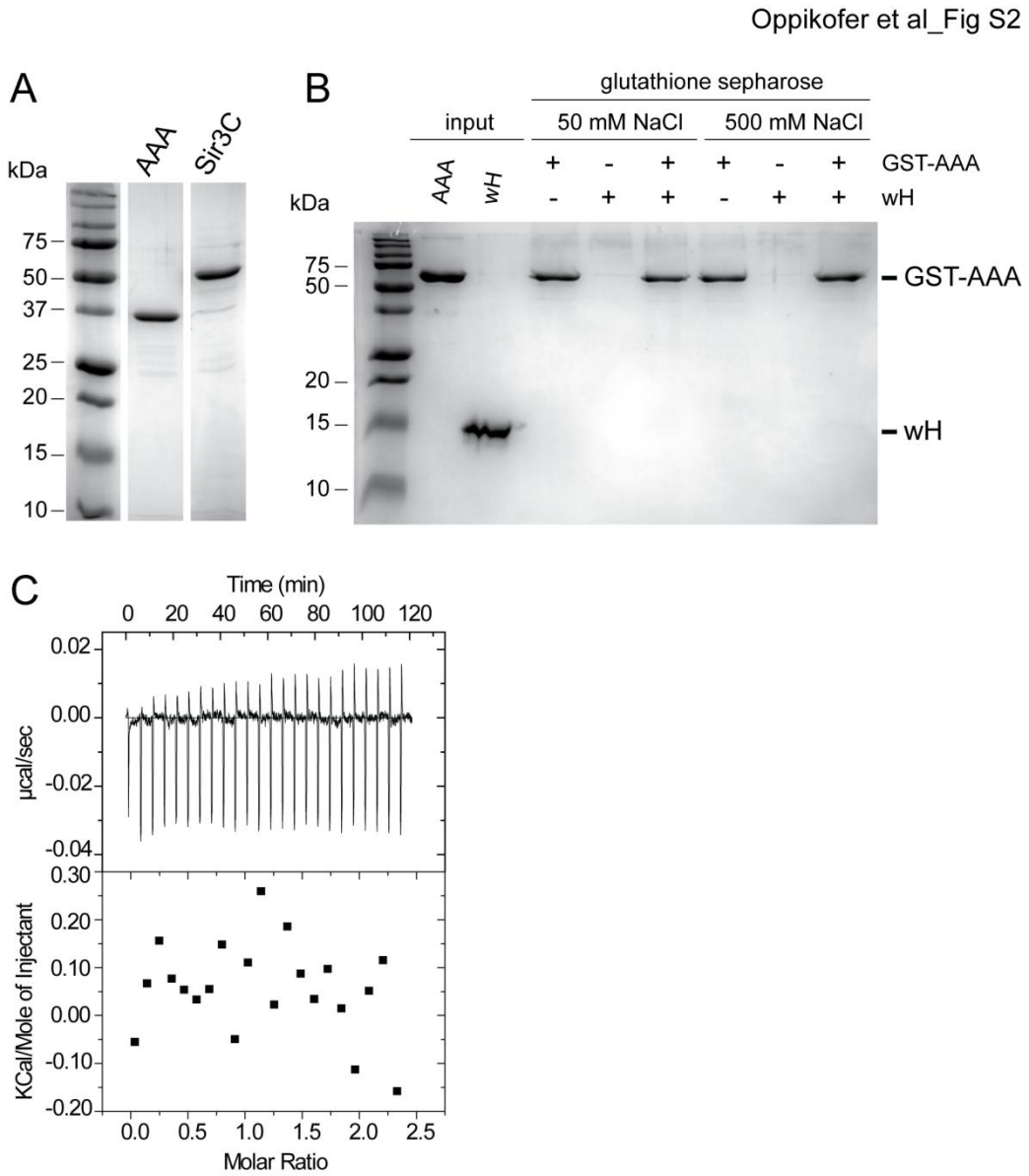


Figure S2 The Sir3 wH fails to interact with the AAA module *in vitro*. (**A**) 2µg of the indicated domain were run on a SDS-PAGE and stained with Coomassie blue. (**B**) Pull-down experiment using a GST-tagged AAA module in 50 mM Tris pH 7.5, 1 mM TCEP, 0.01 % Triton-X containing the indicate amount of NaCl. Input are 100% and 40 % for the AAA and the wH module respectively. (**C**) Isothermal titration calorimetry (ITC) binding was measured using a MicroCal Omega VP-ITC machine (MicroCal, GE) at 25°C with 20 µM Sir3 AAA domain in the sample cell and 254 µM Sir3 wH domain in the syringe. Proteins had been dialyzed against 25 mM Tris pH 7.5, 100 mM NaCl and 0.5 mM DTT. Calorimetric data were analyzed with the evaluation software MicroCal ORIGIN V5.0 (MicroCal Software, GE).

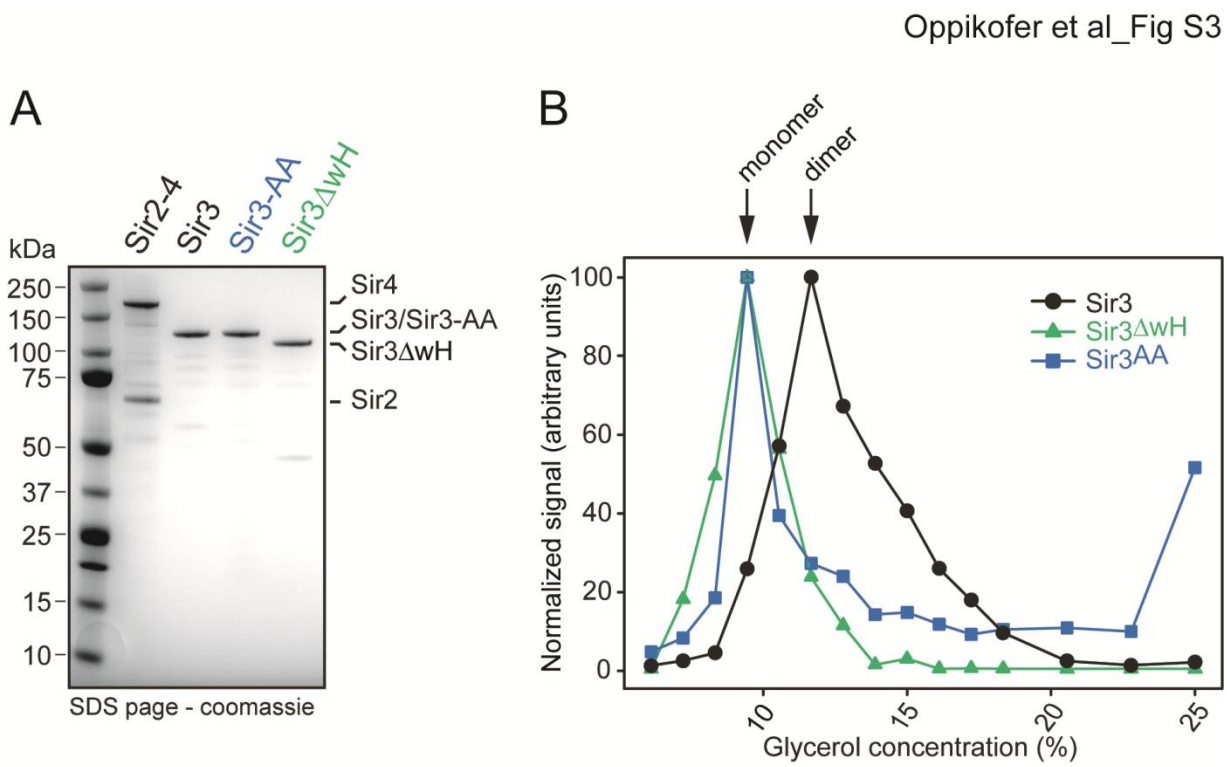


Figure S3 Deletion of the Sir3 wH domain or mutation of the crystallographic dimerization interface disrupt dimerization of purified Sir3 in solution. (**A**) 7 pmol of Sir2-4, full-length Sir3, Sir3ΔwH (aa 1-850) and Sir3-AA (L861A and V909A) were loaded on a SDS-PAGE and stained with Coomassie blue. (**B**) The sedimentation profiles of full-length Sir3, Sir3ΔwH and Sir3-AA were monitored in a 5-25% glycerol gradient by ultra centrifugation (16 h, 120'000 g, 4 °C) without fixation. After fractionation, 15 consecutive fractions were loaded on a SDS-PAGE and stained with SYPRO Ruby (data not shown). The graph represents the quantification of protein distribution in the gradient normalized to the fraction containing the highest amount of protein. Monomeric and dimeric states were determined based on molecular mass by running the same gradients in presence of 0.025 % Glutaraldehyde to fix the samples (GraFix method (Kastner *et al*, 2008)) prior to SDS-PAGE analysis (data not shown). The data is representative for two or more independent experiments. While wild-type Sir3 is mainly found in a dimeric form, Sir3ΔwH and Sir3-AA co-sediment as monomers in the conditions tested here.

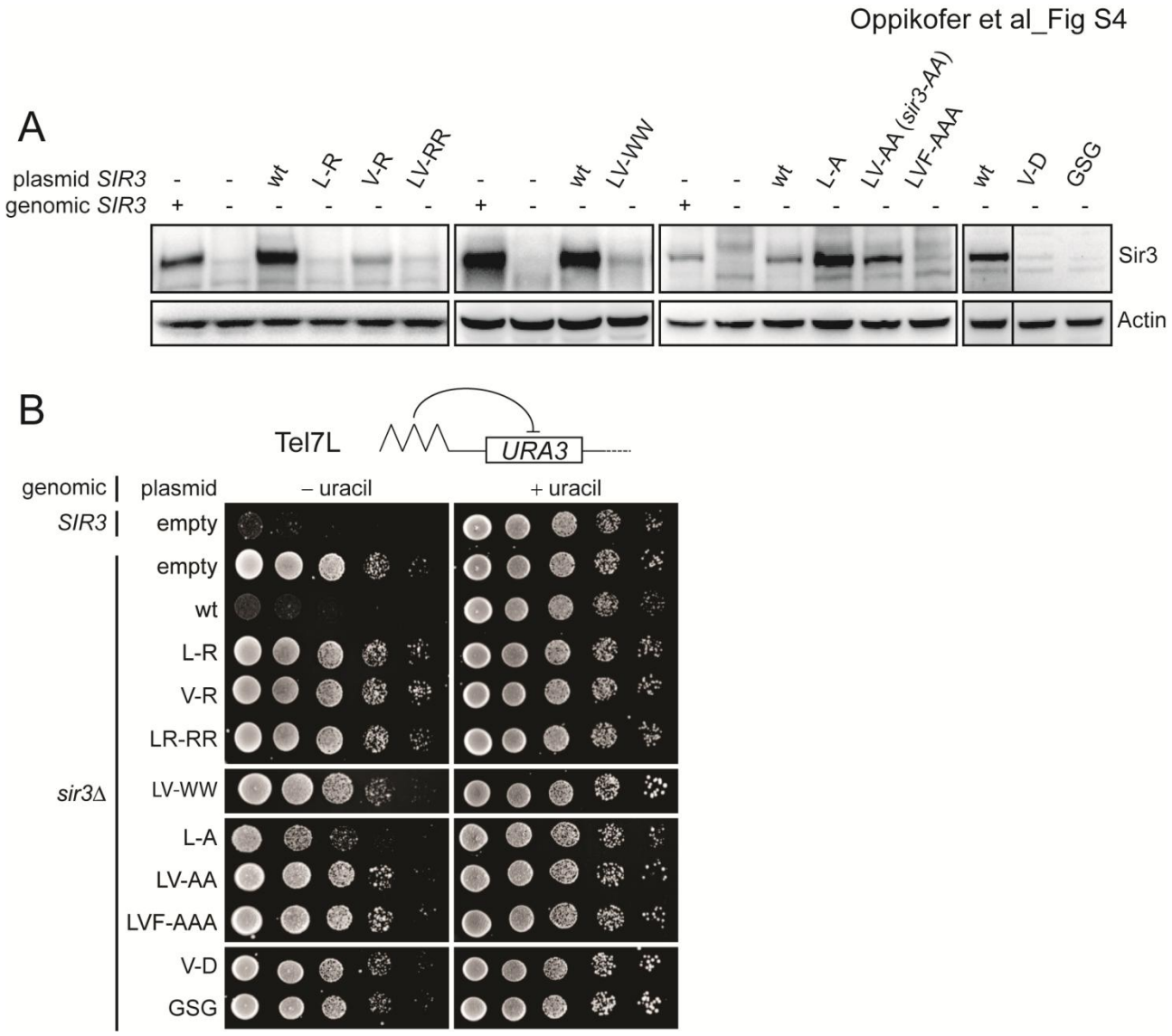


Figure S4 Several substitutions in the wH affect Sir3 stability *in vivo*. (**A**) Western blot analysis of alkaline yeast extracts (Ruault *et al*, 2011) of *sir3* mutants designed to disrupt wH homodimerization. In contrast to those characterized in Figures 1G, 3 and 4, namely *sir3-ΔwH* or *sir3-AA,* several point mutations in the Sir3 wH domain destabilize the mutant sir3 protein. (**B**) Indicated strains were grown overnight in selective media and 10-fold dilutions were dropped on the indicated media. Ability to repress *URA3* inserted at the telomere 7L was tested by growth on synthetic media lacking uracil, synthetic complete media was as plating control.

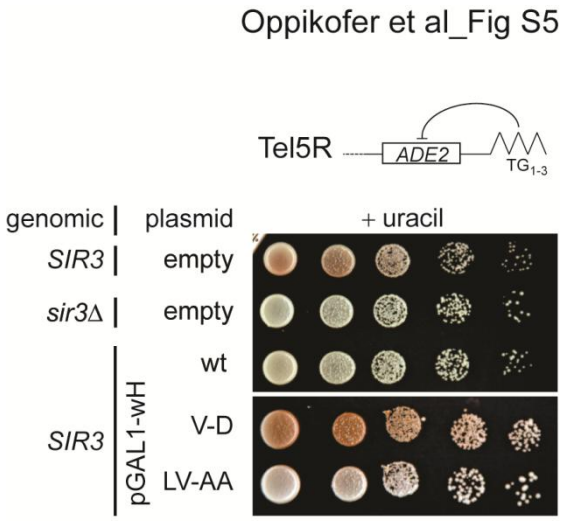


Figure S5 Overexpression of Sir3 wH, but not of dimerization mutants, derepresses telomeric silencing. The indicated strain was grown overnight in selective media and 10-fold dilutions were dropped on media containing galactose as carbon source. Ability to repress *ADE2* at the telomere 5R was monitored by accumulation of a red pigment; red color illustrates efficient repression.

Table S1. Yeast strains used in this study

strain	genotype	source
GA484	W303*; MAT*alpha*; hmr::TRP1*	(Singh and Klar, 1992)
GA503	PH499*; MATa ppr1::HIS3 adh4::URA3-TEL 7L 5R::ADE2-TEL*	(Gottschling, 1992)
GA5822	GA503*; sir4::kanMX6*	(Kueng *et al*, 2012)
GA7055	GA503; *sir3*::*kanMX6*	this study
GA7292	GA484; *sir3*::*kanMX6*	this study

Table S2. Primers used for qPCR

name	sequence	gene	source
SG4791	TTGACCCATACCGACCATGATA	*ACT1*	(Schawalder *et al*, 2004)
SG5788	AGGTTGCTGCTTTGGTTATTGA	*ACT1*	(Kueng *et al*, 2012)
SG6041	AAATGGCAAGGGTAAAAACCAG	Tel6R 0.2kb	(Darst *et al*, 2008)
SG6042	TCGGATCACTACACACGGAAAT	Tel6R 0.2kb	(Darst *et al*, 2008)
SG5783	GGAATGATCTTGGAAATCGATCA	Tel6R 0.5kb	(Yang and Kirchmaier, 2006)
SG5784	CTAGTGTCTATAGTAAGTGCTCGG	Tel6R 0.5kb	(Darst *et al*, 2008)
SG6043	GGACCTACTAGTGTCTATAGTAAGTG	Tel6R 1kb	(Darst *et al*, 2008)
SG5785	CTCTAACATAACTTTGATCCTTACTCG	Tel6R 1kb	(Darst *et al*, 2008)
SG6044	GGCTAGAAAAGCTTCAACATGGCCTTAC	Tel6R 5kb	(Darst *et al*, 2008)
SG6045	CTCCAGCCTGCCTAAGACAAGCTATAG	Tel6R 5kb	(Darst *et al*, 2008)
SG5861	CCGCCAAGTACAATTTTTTAC	Tel7L (*URA3*)	(Martins-Taylor *et al*, 2004)
SG5862	CAACCAATCGTAACCTTCATC	Tel7L (*URA3*)	(Martins-Taylor *et al*, 2004)
SG5789	AGCTGCGGTGTTTACAAGT	Tel9R	(Kueng *et al*, 2012)
SG5790	ACTACCGGAAACAAGAAACGTG	Tel9R	(Kueng *et al*, 2012)
SG418‘	CTTGTATTAGACGAGGGACGGAGTG	*HML-E*	(Martin *et al*, 1999)
SG419	ACAGAGGGTCACAGCACTACTACAG	*HML-E*	(Martin *et al*, 1999)
SG5780	CACAGTTTGGCTCCGGTGTA	*HML-alpha1*	(Yang and Kirchmaier, 2006)
SG5781	CCGCGTGCCATTCTTCAG	*HML-alpha1*	(Yang and Kirchmaier, 2006)

Supplemental Materials and Methods

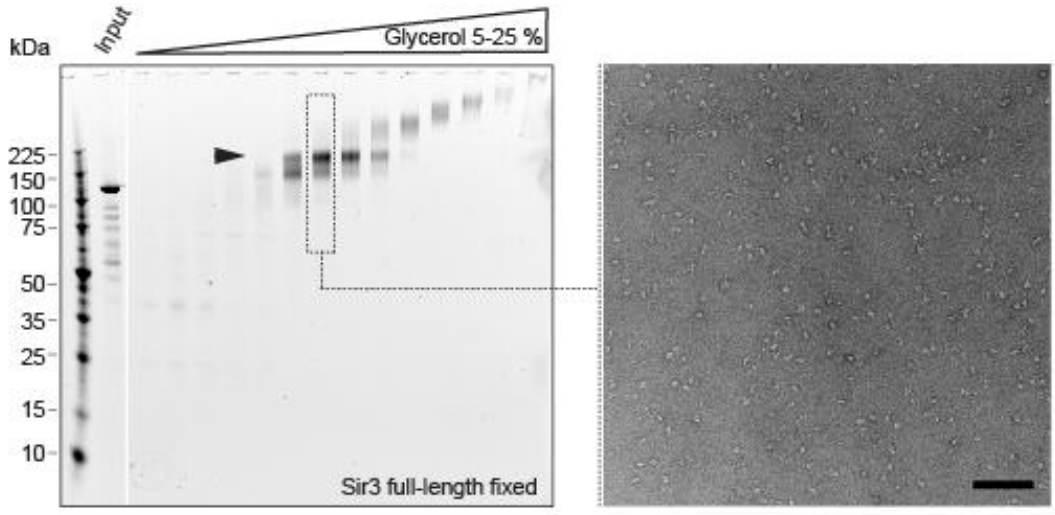
Structure determination and refinement

Diffraction images were processed and scaled with HKL-2000 (Otwinowski *et al*, 1997) and XDS (Kabsch, 2010). The structure of Sir3 wH was solved by MAD using five Seleno-Methionine sites per molecule identified in SHELXD (Sheldrick, 2008). Selenium sites were used for phase calculation and refinement of sites in Sharp (Bricogne *et al*, 2003) followed by density modification using Solomon (Abrahams and Leslie, 1996). Phases from density modification were then used for automatic model building in PHENIX (Adams *et al*, 2011) and BUCCANEER (Cowtan, 2006) followed by manual completion of the model using COOT (Emsley *et al*, 2010). The Sir3 wH structure was refined by the crystallographic simulated annealing routine followed by individual *B*-factor refinement in PHENIX. The final structure was validated using Molprobity (Chen *et al*, 2010) and COOT. Structural images for figures were prepared with PyMOL (http://pymol.sourceforge.net/).

Chromatin and DNA interaction assays

In vitro assembled recombinant *X. laevis* histone octamers (Luger *et al*, 1997) were used to reconstitute nucleosomes onto 6mer arrays of 601-Widom positioning sequence (Lowary and Widom, 1998; Huynh *et al*, 2005) by sequential salt dialysis (Lee and Narlikar, 2001). Increasing amount of the indicated protein was added to 25 nM of 601-167-6mer nucleosomal probe in 10 mM TEA pH 7.4, 50 mM NaCl and 0.05 % Tween-20. For bidning using the holo-Sir complex, the purified Sir2-4 heterodimer and the Sir3 constructs (Figure S3A) were added sequentially. After 10 min incubation on ice, the samples were separated by electrophoresis on a native 0.7 % agarose gel in 18 mM Tris, 18 mM boric acid at 80 V for 90 min at 4 °C. The probe was stained using SYBR® Safe. A Cy3-labeled 147 bp DNA probe, routinely used as competitor DNA for nucleosomal reconstitutions, was PCR amplified from a region outside the multiple cloning site of pUC18 and used to investigate DNA interaction in 10 mM TEA pH 7.4, 100 mM NaCl and 0.05 % Tween-20. The fluorescent signal was detected using a Typhoon 9400 scanner.

3.3 ADDENDUM - STRUCTURAL ANALYSIS OF SIR3 DIMERS BY ELECTRON MICROSCOPY



Dimers of full-length Sir3 can be enriched by sedimentation in a glycerol gradient and visualized by electron microscopy. The sedimentation properties of purified full-length Sir3 (110 kDa) were analyzed by ultracentrifugation using a 5-25% glycerol gradient. Glutaraldehyde (0.025 %) was added to the gradient in order to fix the sample during sedimentation (Kastner *et al*, 2008). The Sir3 protein mostly forms dimers (~ 225 kDa) indicates by the arrow head. The indicated fraction was used to prepare floated carbon film grids stained with standard uranyl acetate negative stain and visualized with a CM10-TEM microscope, scale bar 100 nm.

Grids preparation and imagining was performed by Dr. Mohamed Chami, laboratory of Prof. Dr. Henning Stahlberg, University of Basel at the Department for Biosystems Science and Engineering (D-BSSE).

4 CONCLUDING REMARKS AND OUTLOOK

Despite an increasing understanding of the molecular details, the mechanistic interplay between chromatin modifications and chromatin-associated factors in establishing heterochromatin still presents a formidable challenge. Chromatin mediated silencing has been extensively investigated in the budding yeast *S. cerevisiae*. While the molecular machinery involved differs significantly between budding yeast and higher eukaryotes, these studies have given important conceptual insights into how chromatin associated factors can repress gene expression. In the case of *S. cerevisiae*, there is good evidence that this is achieved primarily through occlusion of the promoter sequence by Sir proteins and by spatial segregation in the nucleus.

The first aim of this thesis was to understand the precise role of individual histone modifications in the establishment of yeast silent chromatin. In order avoid the caveats of histone modifications cross-talk *in vivo*, we turned to a highly controlled *in vitro* reconstitution system. As detailed in Chapter 2, we found that each histone modification tested – H3K79me, H3K56ac and H4K16ac – affects Sir-chromatin interactions in a different way. Strikingly, we highlighted a new role for the H4K16ac mark, which does not act as barrier to the spread of silent chromatin, but rather as a prerequisite to the formation of both active and silent domains.

The second aim of this thesis was to characterize the function of the extreme Sir3 C-terminus. We found that the last 138 amino acids of Sir3 fold into a wH domain which mediates homodimerization. Sir3 wH homodimerization accounts for most of Sir3-Sir3 interaction and is required for silencing at telomeres and *HM* loci. Intriguingly, we found that Sir3 wH homodimerization is required for efficient loading of the Sir2-3-4 complex onto chromatin *in vitro* and *in vivo*, possible reasons for this are discussed below.

4.1 A EUCHROMATIC HISTONE MARK IS ACTIVELY INVOLVED IN THE ESTABLISHMENT OF SILENCING IN YEAST

The role of histone modifications in the establishment of Sir-mediated silent chromatin has been extensively studied genetically (Ehrenhofer-Murray *et al*, 1997; Kimura *et al*, 2002; Ng *et al*, 2002; Suka *et al*, 2002; van Leeuwen *et al*, 2002; Xu *et al*, 2005; Yang *et al*, 2008b). These pivotal studies showed that the presence of Sir proteins and histone modifications are mutually exclusive within yeast chromatin *in vivo*. Consistently, by monitoring the interaction between modified histone peptides and full-length Sir proteins or domains, it was shown that histone modifications generally inhibit the loading of Sir proteins *in vitro* (Carmen *et al*, 2002; Liou *et al*, 2005; Altaf *et al*, 2007; Onishi *et al*, 2007), while the unmodified H4 histone tail promotes the loading of Sir3 onto chromatin *in vitro* (Martino *et al*, 2009; Sinha *et al*, 2009). Together, these studies generated the dogma that histone marks as a whole prevent the association of Sir proteins to euchromatic regions and form a barrier to the spread of heterochromatin.

These studies have been instrumental in understanding the basic mechanisms for separating active and silent domains in yeast, yet *in vivo* studies and peptide-based approaches present some caveats. *In vivo*, the cross-talk between histone modifications and the recruitment of additional non-histone factors may mask the specific contribution of a single histone mark in modulating Sir-chromatin interactions. On the other hand, when embedded in a nucleosomal template, the accessibility of histone marks may change compared to when in the context of free histone peptides. For instance, Sir4 was shown to interact with an isolated H4 N-terminal tail (Hecht *et al*, 1995) and binding of Sir2-4 – through Sir4 – to an isolated N-terminal H4 tail was sensitive to acetylation of H4K16 (Liou *et al*, 2005). However, the Sir2-4 heterodimer has poor specificity for the H4 tail when in the context of chromatin as it binds chromatin reconstituted with full-length or tailless histone with similar affinity (Martino *et al*, 2009). Consistently, the acetylation of the H4 tail by NuA4 did not decrease the affinity of Sir-2-4 to chromatin (Johnson *et al*, 2009). The different results obtained with isolated peptides and nucleosomes are best explained by the high affinity of the Sir4 N-terminus for DNA which likely overcomes the interaction between Sir4 and the H4 tail (Martino *et al*, 2009). Consistently, a Sir complex lacking the Sir4 N-terminus binds nucleosomal arrays less effectively than a wild-type Sir complex *in vitro* (Kueng *et al*, 2012). Finally, while the use of Sir domains allows us to assign a specific function of a given protein region, studying the interaction of chromatin with full-length Sir proteins and Sir complexes may better represent the situation *in vivo*.

To better understand the role of histone marks in the establishment of yeast silent chromatin, we therefore expanded the previously established *in vitro* reconstitution system (Cubizolles *et al*, 2006; Martino *et al*, 2009) using homogenously modified nucleosomal arrays and recombinant full-length Sir3, Sir2-4 and holo Sir complexes. This well-defined system allowed us to assess not only Sir-chromatin affinities, but also the sensitivity of the linker DNA to micrococcal nuclease, a neutral reporter for DNA accessibility. We made use of the Widom 601 positioning sequence (Lowary and Widom, 1998) to generate regularly spaced arrays which mimic the linker DNA length found in living yeast cells (~21 bp) (Yuan *et al*, 2005). As detailed in Chapter 2, we attributed specific functions to three established histone marks – H4K16ac, H3K56ac and H3K79me – and revealed that, in contrast to what was generally assumed, acetylation of H4K16 is likely to precede the formation of both active and silent domains.

Previous peptide-based studies suggested that H4K16ac resulted in a similar decrease in the binding of both Sir3 and the Sir2-4 heterodimer to isolated histone tail domains (Liou *et al*, 2005). Yet, it was shown that NuA4-dependent

acetylation – notably of H4K16 – only reduced the binding affinity of Sir3, but not Sir2-4, for a reconstituted chromatin template (Johnson *et al*, 2009). However, Johnson and colleagues used reconstituted chromatin arrays covalently attached to beads and only analysed the amount of bound protein at a single concentration point by Western blotting (Johnson *et al*, 2009). Taking advantage of more comprehensive titration experiments, we found that H4K16ac-containing chromatin in fact has an increased affinity for the Sir2-4 heterodimer, whilst decreasing Sir3 binding affinity (Oppikofer *et al*, 2011). Strikingly, the Sir2-dependent turnover of H4K16ac does not only recruit Sir3, but directly promotes the loading of the holo Sir complex. Therefore, the active Sir2-dependent removal of the H4K16ac mark promotes the formation of silent chromatin beyond the generation of a hypocetylated substrate and can be considered as a prerequisite for silencing (see Chapter 2).

The notion that H4K16ac plays a positive role in the establishment of silent chromatin was previously proposed, yet the role of H4K16ac was thought to be indirect by preventing the binding of Sir proteins within euchromatic loci thus concentrating them at regions to be silenced (Kimura *et al*, 2002; Suka *et al*, 2002; Millar *et al*, 2004). However, in light of the discoveries presented in this thesis, I propose a revised model that directly implicates the H4K16ac mark in the establishment of yeast silent chromatin. Based on the results detailed in Chapter 2, I propose that the boundary between silent and active chromatin is established by a competition between Sir2-4 and Dot1 for binding to H4K16ac-containing nucleosomes. This disputes the previous notion that there is a competition between Dot1 and Sir3 for binding the nucleosome (Altaf *et al*, 2007). Indeed, binding of Dot1 the H4 tail is unaffected by the acetylation state of H4K16 (Altaf *et al*, 2007), yet H4K16ac selectively repels Sir3 (Altaf *et al*, 2007; Oppikofer *et al*, 2011). Given that more than 80% of H4K16 residues are acetylated *in vivo* (Clarke *et al*, 1993; Smith *et al*, 2003b), Sir3 and Dot1 do not strictly compete for the same substrate but rather the euchromatic acetylation of H4K16 favors Dot1 over Sir3 binding to the nucleosome. However, at the boundary between silent and active domains it is likely that a given nucleosome can undergo one of the following two scenarios: 1) H4K16ac stimulates the binding of Dot1 which further inhibits the loading of Sir3 and the Sir complex by depositing the H3K79me mark. 2) H4K16ac recruits the Sir2-4 heterodimer which deacetylates H4K16ac, favoring the loading of Sir3, whose recruitment is additionally facilitated by its interaction with Sir4. Progressive Sir2-4 recruitment by H4K16ac, followed by cycles of histone deacetylation, helps the spread of silent regions. Importantly, the reduced affinity of Sir3 for H4K16ac chromatin assures that Sir3 binds preferentially in presence of Sir2-4, where deacetylation of H4K16ac – coupled to *O*-AADPR production – reinforces the association of the holo Sir complex to the chromatin fiber ensuring efficient gene repression. The question remains what is the actual role of *O*-AADPR.

The prevalence of either one of these scenarios is most likely dependent on the relative abundance of Sir2-4 and Dot1 at a given genomic location. Away from silencers, the concentration of Sir proteins drops dramatically, therefore allowing Dot1 a higher chance to bind to H4K16ac-containing chromatin first. However, in the proximity of silencers, the abundance of Sir proteins favors the establishment of a silent state. Therefore, H4K16ac *per se* is not a barrier to the spreading of silencing as suggested before (Kimura *et al*, 2002; Suka *et al*, 2002) but rather precedes the formation of both active and silent domains. This model is supported by the observation that H4K16ac-containing chromatin can be readily bound by the Sir complex (Johnson *et al*, 2009; Oppikofer *et al*, 2011) and its turnover is likely to be required for efficient silencing as loading of the Sir complex on H4K16R chromatin does not support full repression (Yang and Kirchmaier, 2006; Yang *et al*, 2008a). Coupling the turnover of the H4K16ac mark to *O*-AADPR production links the formation of silent chromatin to NAD levels. This is interesting in that it renders the spread of silent chromatin sensitive to the metabolic state of the cell. Consistently, Sir-mediated silencing decreases under conditions that lower intracellular NAD levels (Smith *et al*, 2000).

Intriguingly, Sir2, Dot1 and the H4K16ac mark are well conserved through evolution suggesting that mechanism described here may be at work in higher eukaryotes as well.

4.2 SIR3 EVOLVED SPECIFIC SILENCING FUNCTIONS

The *SIR3* gene arose from *ORC1* through a whole genome duplication event that took place ~100 million years ago (Wolfe and Shields, 1997; Kellis *et al*, 2004). *ORC1* codes for the largest subunit of the Origin Recognition Complex (ORC) which defines DNA replication origins in a variety of species.

Following gene duplication the fate of the new gene pair can follow at least three different scenarios (Ohno *et al*, 1968; Conant and Wolfe, 2008; Hahn, 2009; Hickman *et al*, 2011). 1) The function of the ancestor gene may be duplicated and conserved in both new genes which thus become redundant. 2) Evolution may lead to neofunctionalization where one of the duplicated genes acquires new functions, while the other copy retains the original function. 3) In a process called subfunctionalization, the functions of the ancestor gene are shared out among the new pair of genes which together retain the entire set of ancestral functions. Subfunctionalization also gives the opportunity to evolve new specialized functions. In this case the divergence of functions among paralogs also involves the accumulation of mutations in at least one of the duplicated genes, enabling it to acquire new functions.

Several line of evidence indicates that the *ORC1/SIR3* pair underwent subfunctionalization and that *SIR3* and the opportunity to evolve specialized silencing functions. Indeed, ORC interaction with several heterochromatin proteins is

evolutionarily conserved. For instance, both human and Drosophila ORC bind to HP1 (Pak *et al*, 1997; Lidonnici *et al*, 2004; Prasanth *et al*, 2004; Auth *et al*, 2006) and human ORC associates with pericentromeric and telomeric heterochromatin (Deng *et al*, 2007; Deng *et al*, 2009; Prasanth *et al*, 2010). Moreover, Orc1 has been shown to function together with Sir2 to establish telomeric silencing in the evolutionarily distant organism *P. falciparum* (Mancio-Silva *et al*, 2008; Deshmukh *et al*, 2012). This suggests that the *ORC1* gene had an ancestral and conserved role in silencing, yet the gene duplication event in *S. cerevisae* gave the chance to Sir3 to specialize further and outperform Orc1 in silencing functions (Hickman and Rusche, 2010).

The best evidence supporting a subfunctionalization and specialization of the Sir3 protein comes from studies in the budding yeast *K. lactis* which diverged from *S.cerevisiae* before the whole genome duplication event that produced Sir3 and thus possesses only *Kl*Orc1 (Hickman and Rusche, 2010). *Kl*Orc1 supports both DNA replication and silent chromatin formation in association with *Kl*Sir2 and *Kl*Sir4 in a manner analogous to Sir-dependent silencing in *S. cerevisiae* (Hickman and Rusche, 2010). This strongly suggests that the Orc1/Sir3 pair in *S. cerevisiae* subfunctionalized and while Orc1 maintained a role in DNA replication, Sir3 evolved as key player – together with Sir2 and Sir4 – in yeast silent chromatin formation (Hickman and Rusche, 2010).

Within multidomain proteins it is very likely that subfunctionalization and specialization occurs within discrete domains. For instance, as discussed in Chapter 1, the BAH domain of Sir3 (aa 1-214) is absolutely essential for silencing as it plays a major role in interacting with the nucleosome (Bell *et al*, 1995; Rusche *et al*, 2002; Connelly *et al*, 2006; Onishi *et al*, 2007; Armache *et al*, 2011). Interestingly, a chimera protein containing the Orc1 BAH domain and the C-terminus of Sir3 can restore *HM* silencing in a *sir3*Δ background, indicating that both Orc1 and Sir3 BAH domains retained an ancestral silencing function which existed prior to the genome duplication event (Bell *et al*, 1995). Consistently, despite its high conservation, the N-terminal BAH domain of Orc1 is dispensable for DNA replication in *S. cerevisiae* (Bell *et al*, 1995). However, this chimeric protein does not support DNA replication suggesting that the C-terminus of Sir3 has lost important functions for DNA replication after it arose from Orc1, namely the ability to hydrolyse ATP (Bell *et al*, 1995; Ehrentraut *et al*, 2011). The "inverse" chimera protein containing the Sir3 BAH domain and the C-terminus of Orc1 is not able to support silencing. This shows that while the Sir3 C-terminus has lost the ability to function in DNA replication, the gene duplication event has given the Sir3 C-terminus the opportunity to evolve new functions crucial for silencing (Bell *et al*, 1995), such as its interaction with Rap1 and Sir4 (see Chapter 1 and (Moretti *et al*, 1994; Cockell *et al*, 1995; Strahl-Bolsinger *et al*, 1997; Moazed, 2001; Moretti and Shore, 2001; Rudner *et al*, 2005; Chen *et al*, 2011; Ehrentraut *et al*, 2011)).

The work presented in Chapter 3, reveals new important functions for both the Sir3 AAA domain (aa 532-834) and the heretofore mysterious extreme Sir3 C-terminal wH motif (aa 840-978). As mentioned before, the nucleosome binding function of Sir3 AAA, as well as its interaction with Sir4, are likely to have evolved after the gene duplication event. On the other hand, the self-association function contained in the Sir3 wH module is also present in the extreme Orc1 C-terminus, suggesting that it evolved prior to the formation of the Orc1/Sir3 pair by gene duplication. While the Sir3 wH homodimerization is crucial for silencing, the role of the self-association of the predicted Orc1 C-terminal wH motif remains to be explored.

4.2.1 THE SIR3 AAA DOMAIN BINDS THE NUCLEOSOME: MULTIPLE BINDING MODES?

A screen for dominant negative *sir3* alleles revealed that a point mutation within the Sir3 AAA (L738P) enhances the interaction of Sir3 with chromatin and impaired spreading of the Sir complex (Buchberger *et al*, 2008). This suggests that the AAA module can bind to the nucleosome. Consistent with a previous study using a longer Sir3 C-terminal fragment (aa 620-978) (Altaf *et al*, 2007), the work presented in Chapter 3 shows that the Sir3 C-terminal AAA domain binds the nucleosome in a manner sensitive to the methylation of H3K79. This suggests that the Sir3 AAA module indeed contacts the surface of the nucleosome encompassing the H3K79 residue and that the regulation of this interaction is important for silent chromatin formation. Importantly, the Sir3 N-terminal BAH domain also binds the region of the nucleosome encompassing the H3K79 residue (Onishi *et al*, 2007; Sampath *et al*, 2009; Armache *et al*, 2011). This suggests that Sir3 can bind the nucleosome in at least two different conformations where either the BAH or the AAA interact with the H3K79 region of a given nucleosome. Within a single Sir3 molecule, the domain not involved in this interaction may contact the same region of a neighboring nucleosome, the linker DNA or other factors. Importantly, we show that the AAA binds free DNA and mononucleosomes with similar affinity, suggesting that a Sir3 binding conformation where the AAA domain interacts with DNA is possible. Whether such a combinatorial binding mode exists and may be at work to facilitate the spread of the Sir3 protein (and the Sir complex) along the chromatin fiber remains to be investigated.

4.2.2 HOMODIMERIZATION OF SIR3 WH IS REQUIRED FOR SILENCING, BUT WHY?

Essential to Sir-mediated silencing are the interactions within and between Sir-complexes that presumably contribute to the stable loading and spreading of Sir proteins along the chromatin fiber. Interaction between Sir proteins has been extensively monitored by genetics, yeast-2-hybrid assays and co-immunoprecipitation (see section - Sir-mediated silencing: a *complex* story with three protagonists). Yet, atomic level structural knowledge of how the Sir proteins

interact is very limited. Previously, only the dimerization of the Sir4 coiled-coil domain and the interaction between a central region of Sir4 and Sir2 have been elucidated by X-ray crystallography ((Chang *et al*, 2003; Murphy *et al*, 2003); Rolf Sternglanz and Rui-Ming Xu, unpublished).

Here we revealed a new interface essential for silent chromatin formation. The last 138 amino acids of Sir3 fold into a wH domain, whose homodimerization accounts for most of Sir3 dimerization and we show through mutagenesis that it is essential for silencing at telomeres and *HM* loci. The question remains: why is Sir3 dimerization indispensable for silencing?

At least part of the answer comes from monitoring the recruitment of Sir proteins to silencers. Given its interactions with recruiting factors such as Rap1 and Yku70/Yku80 and its bridging function between Sir2 and Sir3, Sir4 is believed to play a major role in the recruitment of Sir proteins to nucleation sites (Moretti *et al*, 1994; Strahl-Bolsinger *et al*, 1997; Roy *et al*, 2004; Taddei *et al*, 2004). In addition, Sir4 is the only component of the Sir complex detected at silencers in the absence of other Sir proteins (Hoppe *et al*, 2002; Luo *et al*, 2002; Rusche *et al*, 2002). Yet, in a *sir3*Δ or *sir2*Δ background, the overall level of Sir4 protein is mildly reduced and Sir4 recruitment at silencers is decreased by more than 50% (Hoppe *et al*, 2002; Luo *et al*, 2002; Rusche *et al*, 2002). Consistently, Sir3 also participates in the nucleation of silencing through its interaction with Rap1 and Abf1 (Gasser and Cockell, 2001; Moretti and Shore, 2001). Alternatively, the stoichiometric assembly of the holo Sir complex may stabilize Sir4 and facilitate the recruitment of silencing by reinforcing Sir-chromatin interactions.

Deletion of the Sir3 wH domain does not affect the assembly of the holo Sir complex, yet in a *sir3*ΔwH background the amount of Sir4 recruited to nucleation sites is strongly reduced (virtually abolished at telomere 6R and reduced by more than 50% at telomere 7L and *HML*-E). It is unlikely that the wH domain serves as an interaction platform for other Sir recruiting factors as an unrelated bacterial dimerization motif (HlyU) fully substitutes for the Sir3 wH domain. Therefore, I propose that the homodimerization of Sir3 through the C-terminal wH domain is required for the stable association of the holo Sir complex with chromatin at nucleation sites. It is likely that Sir3 wH and Sir4 coiled-coil homodimerization, in concert with Sir2-Sir4 and Sir3-Sir4 heterodimerization, are responsible for increasing the local concentration of Sir proteins at silencers, from whence gene repression can then spread. Consistently, the Sir3 wH domain alone can mediate repression of a subtelomeric reporter gene (in a TPE-deficient *rap1-17* strain) when bound to the promoter-proximal region as a lexA fusion protein plausibly by recruiting full-length, endogenous Sir3 which in turn brings in Sir4 and Sir2 (Liaw and Lustig, 2006). It remains unclear whether Sir3 wH homodimerization, in concert with Sir3-Sir4 and Sir4 coiled-coil homodimerization, also orchestrates the spread of Sir-mediated silencing along the chromatin fiber.

4.3 FUTURE DIRECTIONS

Owing to the work of many, we have a thorough understanding of the genetic requirements for yeast silencing. The precise molecular steps that assemble repressive chromatin are less well understood and the work presented here moves us a step closer to understanding how histone modifications, Sir-chromatin and Sir-Sir interactions regulate gene silencing. Many important questions remain unanswered.

It is unclear how and to which extent Sir3 wH and Sir4 coiled-coil homodimerization – together with the interactions that make up the holo Sir complex – cooperate to promote nucleation and/or the spread of silencing. While Sir4 dimerization has been reported to be necessary for silencing (Murphy *et al*, 2003), the mutations introduced by Murphy and colleagues also disrupt the Sir4-Sir3 interaction (Stephanie Kueng and Susan Gasser, unpublished). Therefore, it is still unknown whether Sir4 coiled-coil homodimerization *per se* is required for silencing. Recent work in our laboratory identified a Sir4 coiled-coil mutant with impaired dimerization function but consistent binding to Sir3 (Stephanie Kueng and Susan Gasser, unpublished). This will allow us to monitor the impact of Sir4 coiled-coil homodimerization on silencing as well as nucleation and spreading of Sir proteins. The combinatorial analysis of mutants defective for Sir3 or Sir4 homodimerization (see Chapter 3 of this thesis and (Stephanie Kueng and Susan Gasser, unpublished)) and the Sir3-Sir4 interaction (Murphy *et al*, 2003; Rudner *et al*, 2005; Ehrentraut *et al*, 2011) will shed light on the overlap in Sir-Sir interactions between nucleation and spreading of Sir-mediated repression *in vivo*. In addition, it is not clear how deacetylation of H4K16 – and *O*-AADPR production – alters the Sir-chromatin assembly and whether Sir3 wH or Sir4 coiled-coil homodimerization (or other Sir-Sir interactions dispensable for the formation of the holo Sir complex) play a role in this process. Building on the work presented here, it will be possible to shed light on this matter by monitoring the change in binding affinity of Sir complexes containing Sir3 and Sir4 dimerization mutants for chromatin deacetylated *in vitro*.

Finally, our understanding of the structural organization of the holo Sir complex *per se* and the Sir-bound chromatin template is still very limited. Yet, our ability to prepare well-defined Sir complexes and Sir-nucleosome assemblies is constantly improving. The use of the Sir3 dimerization mutants presented here combined to Sir4 dimerization mutants (Stephanie Kueng and Susan Gasser, unpublished) will allow us to prepare more homogeneous samples, unable to undergo oligomerization. Combining X-ray crystallography and electron microscopy we will soon be able to gain exciting insights into the structure of yeast silent chromatin.

ACKNOWLEDGEMENTS

I would like to thank Susan Gasser for giving me the chance to carry out my PhD thesis in her laboratory, the continuous support and for maintaining a highly communicative and stimulating environment in the lab. I would like to express my gratitude to David Shore, Nicolas Thomä and Andreas Ladurner for the precious guidance over the years beyond the participation to my thesis committee meetings. I am grateful to Robbie Loewith who first taught me that no luck comes without hard work.

I am deeply thankful to present and past members of the Gasser's Lab, for the precious help and the great working atmosphere, in particular Monika Tsai and Razel Arpagaus for the outstanding organization of the lab. I am especially grateful to Stephanie Kueng for the enormous and daily help at the bench and for the many stimulating conversations. I am thankful to Fabrizio Martino for teaching me the *in vitro* reconstitution system of yeast silent chromatin and helping me settle down at the FMI. I am very grateful to Helder Ferreira for proof-reading numerous abstracts and applications and the many inspiring discussions. Also, I would like to express my gratitude to the protein analysis and protein structure facilities at the FMI for the constant help during the years. In particular, Heinz Gut and Jeremy Keusch who played a crucial role in the Sir3 wH homodimerization project. I thank all the collaborators whose important contributions are detailed in the manuscripts above.

I would like to thank the many people that make FMI such a pleasant and special place to work in. I hope that many of the great relationships we built during the years will last long after the completion of my PhD degree. Finally, I would like to thank my parents for supporting in many ways my enthusiasm for science.

LIST OF ABBREVIATIONS

2-HG	2-hydroxyglutarate
3C	chromosome conformation capture
AAA	AAA+ ATPase-like
aa	amino acid
BAH	bromo-adjacent homology
bp	base pairs
ChIP	chromatin immunoprecipitation
DAM	DNA adenine methyl-transferase
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EM	electron microscopy
EMANIC	EM-assisted nucleosome interaction capture
FAD	Flavin Adenine Dinucleotide
FRET	fluorescence resonance energy transfer
HAT	histone acetyltransferases
HDAC	histone deacetylases
HMT	histone lysine methyltranferase
HP1	heterochromatin protein 1
LRS	loss of rDNA silencing
NAD	nicotinamide adenine dinucleotide
NCP	nucleosome core particle
NDR	nucleosome-depleted region
O-AADPR	*O*-acetyl-ADP-ribose
ORC	origin recognition complex
PAD	partitioning and anchoring domain
PDB	protein data bank
PEV	position effect variegation
PRC1	polycomb repressive complex 1
PRC2	polycomb repressive complex 2
r.m.s.d	root mean square deviation
RNA	ribonucleic acid
SAM	S-adenosylmethionine
SID	Sir2 interaction domain
Sir	silent information regulatory
SPR	surface plasmon resonance
SUV39	suppressor of variegation 3-9
TPE	telomere position effect
TSS	transcriptional start site
UAS	upstream activator sequences
wH	winged helix-turn-helix

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CURRICULUM VITAE

MARIANO OPPIKOFER
Swiss, 28.11.1983

Home Address Clarahofweg 25
4058 Basel
Switzerland

E-mail mariano.oppikofer@fmi.ch
Telephone 0041 61 69 755 83

Institute Address Friederich Miescher Institute for Biomedical Research
Maulbeerstrasse 66
4058 Basel
Switzerland

RESEARCH INTERESTS AND VISION

To understand a little more how life works, to disentangle a few more pieces of its complexity; this is what fascinates me. For this reason I have engaged myself in a scientific vocation at university and have been captivated by **biochemistry** as well as **structural and molecular biology**. I believe that the huge complexity of life is explainable on the basis of interactions that are enormous in number but relatively simple in nature. So far, I mainly invested my energies in fundamental, curiosity-driven research. Next, I would like to tackle the problem of deleterious **macromolecular interactions** which lead to the development of **diseases**. I am particularly fascinated by dissecting pathological phenomena at the **molecular and structural level**. I strongly believe that my research can make an essential contribution to the progress of medicine and human health.

EDUCATION AND RESEARCH EXPERIENCES

Present **PhD student in Biochemistry and Molecular Biology**
Since August 2008, under the supervision of Prof. Susan Gasser, Friedrich Miescher Institute for Biomedical Research and University of Basel.

In vitro reconstitution of yeast silent chromatin: towards a molecular understanding of gene silencing. Discovered a new function for the acetylation of lysine 16 on the histone H4 ($H4K16^{ac}$), as the first histone mark shown to recruit a silent protein (Sir2) to chromatin. This work shed light on years of contradictory reports, as we now know that $H4K16^{ac}$ is a prerequisite for the formation of both silent and active chromatin.

Applied X-ray crystallography to solve the structure of the last 138 amino acids of Sir3 C-terminal, which fold into a winged helix-turn-helix module which mediates Sir3 homodimerization and is essential for silencing in yeast.

2008 **Master in Biochemistry**

6 months research project under the supervision of Dr. John Rouse, MRC Protein Phosphorylation Unit, University of Dundee. *Functional analysis and characterisation of the HIRAN domain of yeast Rad5 and related proteins*. Single point mutations within the HIRAN domain of Rad5 drastically reduce viability of yeast cells under a variety DNA damage conditions. Used yeast genetics and biochemistry to dissect the function of the HIRAN domain in the error-free DNA damage bypass process.

8 weeks research project under the direction of Prof. Robbie Loewith, laboratory of molecular biology, University of Geneva. *TORC1 controls the temporal aspects of cell growth.* Performed a library based screen to identify new components of the TORC1 pathway.

4 weeks research project under the supervision of Prof. Claude Penel, laboratory of biochemistry and vegetal physiology, University of Geneva. *The impact of peroxidase on root morphology in Arabidopsis thaliana*. Combined measurements of peroxidase activity and roots morphological analysis.

2006 **Voluntary clinical research experience**

4 weeks research project under the guidance of Dr. Ariane de Agostini, Foundation for Medical Research "La Tulipe", Andrology Unit. *Dissecting the role of heparan sulfate proteoglycans (HSPG) in murine ovulation.* Analyzed the HSPG content of a variety of tissues from healthy and transgenic murine models.

2006 **Bachelor in Biochemistry**

Bibliography research under the guidance of Prof. Marc Ballivet, *The Huntington Disease*. Deep understanding and summary of the current literature (**maximum grade**).

KEY SKILLS

- Extensive knowledge of molecular biology and biochemistry techniques
- Experienced in recombinant proteins purification from *E. Coli* and Sf21 insect cells
- Advanced biochemical studies of proteins and DNA: interactions and properties
- Complex genetic manipulation of *Saccharomyces cerevisiae*
- Basic knowledge of X-ray crystallography and structure solving
- Efficient analysis of the literature and scientific writing
- Enjoy team work and collaborative personality

PEER-REVIEWED PUBLICATIONS

Oppikofer M, Kueng S, Martino F, Soeroes S, Hancock SM, Chin JW, Fischle W, Gasser SM. (2011) *A dual role of H4K16 acetylation in the establishment of yeast silent chromatin.* EMBO J **30:** 2610-2621

Ehrentraut S, Hassler M, **Oppikofer M**, Kueng S, Weber JM, Mueller JW, Gasser SM, Ladurner AG, Ehrenhofer-Murray AE. (2011) *Structural basis for the role of the Sir3 AAA+ domain in silencing: interaction with Sir4 and unmethylated histone H3K79.* Genes Dev **25:** 1835-1846

Kueng S, Tsai M, **Oppikofer M**, Ferreira H, Sack R, Gasser SM *Regulating repression: roles for the Sir4 N-terminus in linker DNA protection and the stabilization of epigenetic states.* PLoS Genet **8:** e1002727

Oppikofer M, Kueng S, Keusch JJ, Hassler M, Ladurner AG, Gut H, Gasser SM *An evolutionarily distinct dimerization function in the yeast Sir3 C-terminus is required for silent chromatin formation.* EMBO J (*under revision*)

GRANTS AWARDING

2011 Swiss Society for Biochemistry travel Grant (**maximum allowance**)

FASEB Summer Research Conference: Epigenetics, Chromatin & Transcription, Snowmass Village, USA.

CONFERENCES ATTENDANCE

2011 FASEB Summer Research Conference: Epigenetics, Chromatin & Transcription, Snowmass Village, USA. **Poster:** *In vitro reconstitution of yeast silent chromatin: towards a molecular understanding of silencing.*

2011 Joint PhD meeting between FMI and the Center for Genomic Regulation (CRG), Barcelona, Spain. **Talk:** *Toward the characterization of yeast silent chromatin: H4K16^{ac} recruits Sir2-4 and Sir3 C-terminal folds in a winged-helix domain.*

2010 Wellcome Trust Conference: Signalling to Chromatin, Cambridge, UK. **Poster:** *A dual role of H4K16 acetylation in the establishment of yeast silent chromatin.*

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2009 DNA and Chromosomes: Physical and Biological Approaches 5th international summer school; Cargèse, France. **Talk:** *Influence of histone modifications on yeast silent chromatin and characterization of Sir3 domains.*

2009 Joint PhD meeting between FMI and MRC-LMCB (University College London), Emmetten, Switzerland. **Poster:** *Characterizing the role played by active and inactive histone modification on silent chromatin assembly and accessibility.*

2009 EMBO Conference Series on Chromatin and Epigenetics; EMBL Heidelberg, Germany. **Poster:** *In vitro reconstitution of yeast silent chromatin bearing specific histone marks.*

REFERENCES

PhD Thesis Supervisor

Prof. Susan Gasser
Director of the Friedrich Miescher Institute
for Biomedical Research
Maulbeerstrasse 66
CH-4058 Basel, Switzerland

tel +41 61 697 7255 (assistant)
tel +41 61 697 5025 (direct)
fax +41 61 697 3976
susan.gasser@fmi.ch

External PhD Thesis Committee Member

Prof. Andreas Ladurner
Professor of Physiological Chemistry
Butenandt Institute of Physiological Chemistry
Faculty of Medicine
Ludwig Maximilians University of Munich
Butenandtstrasse 5
81377 München, Germany

tel +49 89 2180 77095
fax +49 89 2180 77093
andreas.ladurner@med.uni-muenchen.de

Master Thesis Supervisor (long project)

Prof. John Rouse
The MRC Protein Phosphorylation Unit
The Sir James Black Centre
College of Life Sciences
University of Dundee
Dow Street, Dundee
Scotland DD1 5EH

tel +44 1382 385490
fax +44 1382 223778
j.rouse@dundee.ac.uk

Master Thesis Supervisor (short project)

Prof. Robbie Loewith
Swiss National Science Foundation Professor
Department of Molecular Biology
University of Geneva, Sciences III
30, quai Ernest-Ansermet
CH-1211, Geneva 4, Switzerland

tel + 41 22 379 61 16
fax +41 22 379 68 68
Robbie.Loewith@unige.ch