Cooperative Binding of Sir Proteins to Nucleosomes and Its Implications for Silent Chromatin Assembly in Saccharomyces Cerevisiae

Cooperative binding of Sir proteins to nucleosomes and its implications for silent chromatin assembly in *Saccharomyces Cerevisiae*

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Chenning Lu

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Abstract

Silent chromatin, or heterochromatin, refers to regions of the genome in which genes are constitutively repressed. These regions are important for regulating developmental genes and for maintaining genome stability, and are epigenetically inherited. In *Saccharomyces cerevisiae,* subtelomeres and silent mating type loci are assembled into silent chromatin by the Silent Information Regulator (SIR) complex, composed of Sir2, Sir3 and Sir4, which deacetylates histones and spreads along chromatin. Many questions remain regarding the mechanism of Sir protein spreading along chromatin and the mechanism of epigenetic inheritance of silent chromatin domains. It has been hypothesized that the lateral Sir-Sir protein interactions together with Sir-nucleosome interactions cooperatively recruit Sir proteins to spread along chromatin.

In my thesis project, I set out to test this cooperativity hypothesis by examining the interaction of Sir proteins with well-defined *in vitro* reconstituted mono- and di-nucleosomes. Using electrophoretic mobility shift assay (EMSA), I find that Sir3, the main nucleosome-binding component of the SIR complex, associates with nucleosomes cooperatively, involving the dimerization of Sir3 bound to neighboring nucleosomes. I demonstrate that this inter-nucleosomal cooperativity is mediated by the Sir3 C-terminal winged helix (wH) dimerization domain and is further stabilized by the Sir4 coiled-coil (CC) domain, which mediates both Sir4 homodimerization and Sir3-Sir4 interactions.

There is functional redundancy from the two domains in mediating binding cooperativity, as suggested by the measurement of cooperativity free energy. Surprisingly, my binding measurements suggest that there are no Sir-Sir protein interactions on the same nucleosome. Moreover, by using an *in vitro* bridging assay, I show that Sir3 effectively bridges free nucleosomes in solution and that its wH domain is required for its bridging activity. My *in vitro* results are corroborated by *in vivo* ChIP-seq results showing that either the Sir3 wH domain or the Sir4 CC domain alone could mediate weak spreading of Sir3 protein, away from recruitment sites, under Sir3 overexpression conditions. However, mutations in both domains abolish the spreading completely.

Both histone H4 lysine 16 (H4K16) acetylation and histone H3 lysine 79 (H3K79) methylation are hallmarks of euchromatin in *S. cerevisiae*. I quantify the effect of either modification alone and both modifications in combination on Sir3-nucleosome binding affinity. This shows that either modification alone decreases Sir3 binding affinity towards nucleosomes by 3-4 fold, and that the two modifications work together to reduce the binding affinity even further. Statistical mechanical modeling of the nucleosome binding results indicate that the combined effect of H4K16 acetylation and H3K79 methylation can account for partitioning of Sir3 between silent and active chromatin regions *in vivo*.

Our findings and their quantitative analysis suggest that SIR complexes spread along chromatin discontinuously, arguing against the stepwise polymerization model for silent chromatin assembly.

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CHAPTER I

INTRODUCTION

Cooperative binding of Sir proteins to nucleosomes and its implications for silent chromatin assembly in *Saccharomyces Cerevisiae*

I. Nucleosomes, Chromatin, and the Genome

A. The Chromatin Structure

Eukaryotic genomes range in sizes from $12x10^6$ base pairs in *Saccharomyces cerevisiae* to $6x10^9$ base pairs in humans to up to 10^{11} base pairs in some flowering plants. These sizes correspond to DNA fibers with linear lengths of 4 mm in the former to more than 2 meters in humans, and this DNA must be compacted into nucleus with 2-10 μm in diameter in general (Redon et al., 2002). The way eukaryotes solve this problem is by packing their DNA into chromatin using basic proteins called histones and other proteins. The term chromatin was coined by Walther Flemming in the 1880s, due to its affinity to dyes (Flemming, 1882). Nucleic acids and histones were discovered around the same time (Kossel, 1911; Miescher, 1871). It was initially proposed that chromatin contains nucleic acid, or is equivalent to nucleic acid (Flemming, 1882). However, its exact chemical nature remained unknown. It was not until the 1970s that it was realized that chromatin is a complex of DNA and histones, and that chromatin is composed of repeating subunits as visualized under the Electron Microscope (Kornberg, 1974; Kornberg and Thomas, 1974; Olins and Olins, 1974). Nucleosome, the basic unit of chromatin folding, received its present name in 1975 (Oudet et al., 1975).

A nucleosome core particle is composed of a histone octamer core wrapped around by 147 base pairs of DNA in a left-handed supercoil in around 1.7 turns. The histone octamer consists of 2 copies each of histones H2A, H2B, H3, and H4 (Kornberg, 1977; Luger et al., 1997). *In vivo,* neighboring nucleosome core particles are connected via linker DNA of various lengths, ranging from 20 bp in *S. cerevisiae* to greater than 70 bp in human, to form nucleosomal arrays (Arya et al., 2010; Hayes and Hansen, 2001). Linker DNAs are associated with additional proteins, including linker histones and *trans*-

acting factors, and nucleosome arrays together with non-histone proteins form the chromatin fiber (Hansen, 2002; Hayes and Hansen, 2001). The precise structure that chromatin adopts *in vivo* remains uncharacterized. However, *in vitro* biophysical and imaging studies suggest that chromatin fiber can condense into a compact 30 nm fiber, which under higher divalent salt concentrations can self-associate into higher-order chromatin structures (Hansen, 2002; Hayes and Hansen, 2001; Schwarz et al., 1996). Chromosome compaction also involves proteins such as condensins and cohesins, which will not be discussed in this thesis.

B. Euchromatin and Heterochromatin

A general view of chromatin reveals that chromatin can be broadly divided into two regions, euchromatin and heterochromatin. The definition of heterochromatin and euchromatin was initially based on morphological criteria. In late 1920s, Emil Heitz discovered differentially stained chromatin regions through cytological studies – deeply staining and compact bodies throughout the cell cycle, including interphase, are termed heterochromatin; light-staining chromatin regions which appear diffuse at interphase, but condense at metaphase, are termed euchromatin (Heitz, 1928; Weiler and Wakimoto, 1995).

Around the time that Heitz was describing heterochromatin as a cytogenetic phenomenon, Hermann Muller discovered the first examples of position effect variegation (PEV) in *Drosophila*, where he isolated several X-ray induced mutations of the *white+* (*w+*) gene that resulted in variegated eye color phenotypes (Muller and Altenburg, 1930). It was later revealed that each of the mutations was associated with a chromosome rearrangement event that placed the normally euchromatic gene in close

proximity to heterochromatin, thus creating novel junctions of euchromatin and heterochromatin, which induced mosaic inactivation of euchromatin genes (Schultz, 1936). This PEV phenomenon provides the first palpable link between chromatin organizations and transcriptional status of genes, which has since been observed across different species, from yeast to mammals (Girton and Johansen, 2008; Huisinga et al., 2006).

Euchromatin regions are generally transcriptionally active, more accessible to DNA binding proteins, and typically replicate early in S phase. Histones within euchromatic regions tend to be hyperacetylated. Heterochromatin, in contrast, is believed to contain more regularly spaced nucleosomes, which contribute towards the formation of a more condensed structure that is less accessible to chromatin transaction machineries. In addition, heterochromatic regions contain hypoacetylated histones and patterns of histone methylation that differ from those found in heterochromatin, and typically replicate late in S phase (Elgin and Grewal, 2003; Grewal and Elgin, 2002; Richards and Elgin, 2002).

C. Post-translational Modifications of Nucleosomes

Histones are small basic proteins consisting of a highly conserved histone-fold globular domain, a more flexible, lysine-rich NH_3^+-terminus (histone N-terminal tail), and a shorter C-terminal tail. When wrapped in nucleosomes, the globular domain is wrapped inside the nucleosome core, while the tails protrude from the nucleosome core (Luger et al., 1997). Residues within both the histone globular domain and tails, particularly the N-terminal tail, are subject to a vast array of reversible posttranslational modifications, such as methylation, acetylation, phosphorylation, ubiquitination, sumoylation and ADP

ribosylation, making nucleosomes highly versatile in terms of their capacity for being regulated, and having an impact on various cellular processes, including transcription, replication, DNA repair and recombination (Groth et al., 2007; Jenuwein and Allis, 2001; Kouzarides, 2007; Li et al., 2007a). Some of these modifications may affect higher-order chromatin structure by affecting contacts between histones in adjacent nucleosomes or interactions of histones with DNA (Pepenella et al., 2014; Shogren-Knaak et al., 2006). A better characterized function of histone modifications is that they serve to facilitate or exclude the binding of numerous chromatin associated proteins, some of which contain enzymatic activities that further modify chromatin (Grewal and Moazed, 2003; Jenuwein and Allis, 2001; Kouzarides, 2007; Moazed, 2001a; Schreiber and Bernstein, 2002; Strahl and Allis, 2000; Turner, 2002).

In a few cases, combinations of different histone modifications are recognized by the same (Eustermann et al., 2011; Iwase et al., 2011; Moriniere et al., 2009; Ramon-Maiques et al., 2007), or different domains in a single protein or different subunits of a complex (Dhalluin et al., 1999; Li et al., 2007b; Li et al., 2006; Rothbart et al., 2013; Ruthenburg et al., 2011). The combined action of multiple histone modifications potentially provides better binding specificity and higher binding affinity (Du and Patel, 2014; Taverna et al., 2007).

Active and silent chromatin regions are associated with stereotypical patterns of histone modifications, with each type of region containing several different modifications (Ernst et al., 2011; Filion et al., 2010; Kharchenko et al., 2011). For example, in budding yeast *S. cerevisiae*, both H4K16 acetylation and H3K79 methylation are hallmarks of active euchromatin, and are depleted in heterochromatic regions (Braunstein et al., 1996; Imai et al., 2000; Landry et al., 2000; Ng et al., 2003; Ng et al., 2002; Smith et al.,

2000; Suka et al., 2001; van Leeuwen et al., 2002). In both fission yeast *S. pombe* and higher eukaryotes, H3K9 methylation and H3K4 methylation are primarily localized to heterochromatic and euchromatic regions, respectively (Elgin and Grewal, 2003; Noma et al., 2001; Richards and Elgin, 2002).

D. Epigenetic Inheritance

One of the key features of heterochromatin is that it can be faithfully maintained both structurally and functionally during mitosis and meiosis, exhibiting epigenetic inheritance (Grewal, 2000; Moazed, 2001a), which is defined as the process that involves heritable phenotypic change without alterations in the underlying DNA sequences, and in the absence of the initiating signals (Gottschling, 2004). Epigenetic inheritance is important for maintenance of expression patterns of genes and is essential for cell differentiation and cellular identity maintenance during eukaryotic development (Grewal and Moazed, 2003; Ringrose and Paro, 2004).

In the epigenetic inheritance of genomic CpG methylation pattern, the maintenance DNA methyltransferase, Dnmt1, is thought to re-establish DNA methylation by preferentially associating with and methylating hemimethylated DNA (Bestor and Ingram, 1983; Holliday and Pugh, 1975). In a similar fashion, the replication of heterochromatin has been proposed to involve a cycle of events, in which modifications are reestablished by complexes that recognize a specific modification on an inherited parental histone and catalyze the same type of modification on adjacent newly deposited histones (Dodd et al., 2007; Grewal and Moazed, 2003; Kaufman and Rando, 2010; Kouzarides, 2007; Moazed, 2011; Rusche et al., 2003; Strahl and Allis, 2000).

II. Silent Chromatin in *S. cerevisiae*

Silent chromatin domains in *S. cerevisiae* lack the morphological distinction of heterochromatin of more complex eukaryotes, i.e. the darkly stained and highly condensed appearance throughout the cell cycle (Moazed, 2011; Smith and Boeke, 1997). However, they are also referred to as heterochromatin or heterochromatin-like, as they are transcriptionally inactive (Schnell and Rine, 1986), recombinationally repressed (Gottlieb and Esposito, 1989), generally inaccessible to nucleases (Loo and Rine, 1994, 1995), enriched with hypoacetylated histones (Braunstein et al., 1993; Suka et al., 2001), replicated late during the S phase, have perinuclear localization, and are epigenetically inherited (Grewal, 2000; Moazed, 2001a; Pillus and Rine, 1989; Rusche et al., 2003).

Chromatin silencing is distinct from gene repression, in that the latter is promoter and gene specific. Silencing involves the general inactivation of a large region of chromatin and any genes within silent chromatin domains are inactivated (Rine, 1999; Rusche et al., 2003). Gene silencing has been identified as a major mechanism to maintain committed gene expression patterns during cell development, and to regulate cellular identity and differentiation during eukaryotic development (Grewal and Moazed, 2003; Moazed; Ringrose and Paro, 2004). In addition, silent chromatin or heterochromatin is essential for the maintenance of chromosome stability in eukaryotes. For example, regions of repetitive DNA in the genome that can be susceptible to detrimental recombination events, and parasitic DNA elements are typically located within heterochromatin (Grewal and Elgin, 2002; Henikoff, 2000).

There are three regions of silent chromatin in the *S. cerevisiea* genome, including the two silent mating type or homothallic (*HM*) loci (*HML* and *HMR*), subtelomeric

regions, and the rDNA tandem arrays (Rusche et al., 2003). Silencing at *HM* loci and subtelomeric regions share many mechanistic features, which involve the recruitment of silencer specificity factors to the silencers, and further recruitment and spreading of Sir (silent information regulator) proteins, including Sir2, Sir3, and Sir4, which together form the SIR complex (Aparicio et al., 1991; Rusche et al., 2002, 2003). Silencing at rDNA loci takes place through a distinct mechanism, requiring the recruitment of the silencing complex RENT (regulator of nucleolar silencing and telophase exit), which contains Sir2, Net1, and Cdc14 (Huang and Moazed, 2003; Huang, 2002; Smith and Boeke, 1997). Sir2 is the only Sir protein directly involved in rDNA silencing. There is no direct involvement of Sir1, Sir3 or Sir4. This thesis will focus on the silencing mediated by the SIR complex at *HM* loci and telomeres.

A. Silent Chromatin Domains

1) The *HM* loci

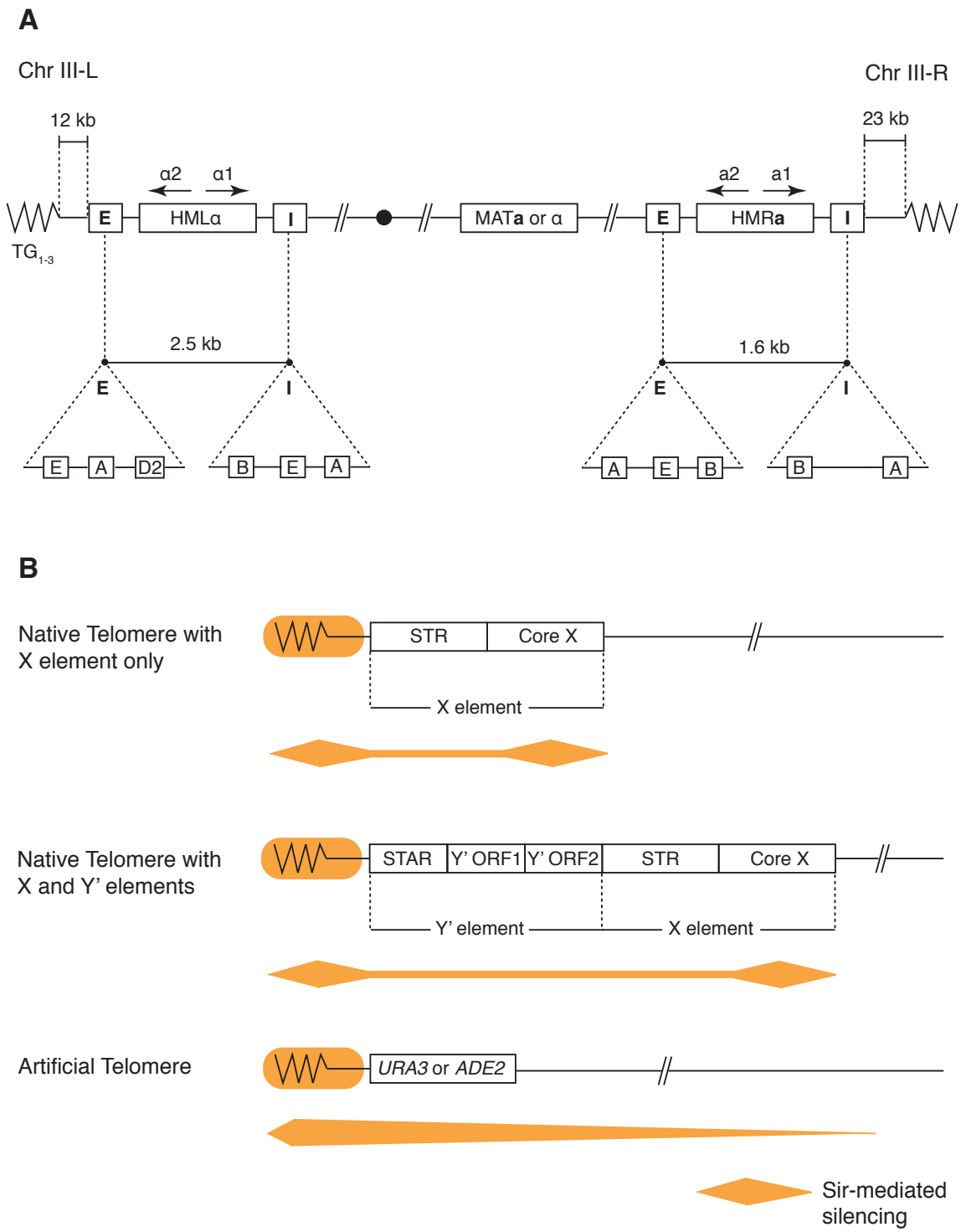
Haploid *S. cerevisiae* exhibits either of two mating types, **a** and α. The mating type is determined by the *MAT**a*** or *MATα* allele encoded at the mating type locus *MAT* on chromosome III. In addition to *MAT*, all *S. cerevisiae* strains have two silent mating type loci (*HM* loci), *HMR* and *HML*, each on either side of *MAT*. The *HMR* locus is around 23 kb from the right telomere of Chr III, and around 100 kb right to the *MAT* locus. The *HML* locus is around 12 kb from the left telomere of ChrIII, and around 220 kb left to the *MAT* locus (Figure 1-1A) (Grunstein and Gasser, 2013; Kueng et al., 2013; Rusche et al., 2003). In most strains, *HMR* contains a cryptic copy of the *MAT**a*** allele, and *HML* contains a cryptic copy of the *MATα* allele. *HMR* and *HML* confer the donor mating type information to *MAT*, and are responsible for mating type switch in some of the *S. cerevisiae* strains, known as homothallic strains. These strains carry the *HO* gene

Figure 1-1. Organization of Sir-mediated heterochromatin regions *in S. cerevisiae.*

(A) Structural organization of the homothallic mating-type (*HM*) loci and the *MAT* locus on Chr III. Telomeric tract of $C_{1-3}A/TG_{1-3}$ repeats is represented as wires. Genes that are normally located in the loci are indicated by arrow heads. The *E* and *I* silencers are indicated in bold. Silencer specificity factor recruiting elements found in *E* and *I* silencers are depicted below: E, Rap1 binding site; A, autonomously replicating sequences (ARS); B, Abf1 binding site; D2, Sum-1 binding element. CEN, centromere; TG_{1-3}, telomere repeat. The illustration is not drawn to scale. (Modified from Keung, *et al.* 2013.)

(B) Structural organization of native and artificial telomeres, and their silencing pattern. Telomeric tract of $C_{1-3}A/TG_{1-3}$ repeats is represented as wires. Subtelomeric elements, X and Y' are depicted with their major components. Native telomeres fall into two general classes: those containing the X element only, and those containing both X and Y' elements. For those containing the Y' element, individual telomere can have one and up to four tandem copies of Y' elements, located proximal to the telosome. Only one Y' element is depicted here for illustration purpose. The STAR (subtelomeric anti-silencing region) and STR (sub-telomeric repeats) elements block the propagation of silencing, and leave a region of reduced silencing within the Y' or X element, respectively. Artificially truncated telomeres, on the other hand, have a gradient of silencing that extends several kb from the TG repeat. Looping is proposed for native telomeres, so that repressed regions contact each other, leaving unrepressed chromatin in between. (Modified from Grunstein and Gasser, 2013.)

Figure 1-1 (Continued)



encoding the *HO* site-specific endonuclease, which cleaves the *MAT* locus, resulting in DNA repair and gene conversion between *MAT* and the cryptic copies of *MAT**a*** or *HMLα* sequences at *HMR* and *HML* loci, respectively. The mating type switch can happen as frequently as once per generation in these strains. When the *MAT**a*** allele at the *MAT* locus is replaced by *MATα*, a cell switches from mating type **a** to α; the opposite replacement results in the opposite switching. Silencing at *HM* loci is essential to maintain the ability of haploid cells to mate (Rine and Herskowitz, 1987; Rusche et al., 2003).

2) The Telomeres

Telomeres are specialized DNA-protein complex at the end of linear chromosomes. They facilitate the replication of chromosome ends, and are important for genomic stability and nuclear organization. Transcriptional gene silencing at telomeres was first observed in *D. melanogaster*, in a phenomenon called PEV (Muller and Altenburg, 1930). When genes are introduced near the ends of *S. cerevisiae* chromosomes, they exhibit a similarly reversible silencing phenomenon, termed as Telomeric Position Effect (TPE) (Gottschling et al., 1990).

In *S. cerevisiae*, the telomeric DNA consists of $C_{1-3}A/TG_{1-3}$ repeats around 300 ± 75 bps, with the exact number varying between individual telomeres and is under dynamic change. These repeats, together with its associated proteins, including Rap1, yKu70/80 complex, Rif, Sir3 and Sir4, constitute the non-nucleosomal cDNA-protein complex, the telosome, at the very end of linear chromosomes (Louis, 1995; Shampay et al., 1984; Wright et al., 1992; Wright and Zakian, 1995). Internal to telosomes, at the subtelomeric regions, there are two classes of middle repetitive elements, X and Y'

(Figure 1-1B). Y' is a highly conserved element, and can be found in 0 – 4 tandem copies at subtelomeric regions. There are two forms of Y' element, the long 6.7 kb and the short 5.2 kb form, which are different from each other with a series insertions/deletions. The X element is less conserved and heterogeneous, varying in sizes from 0.3 to 3 kb, and the element itself is composed of several sub repeats. However, almost all X elements share a ~500 bp core region (C-ACS), which contains an ARS consensus sequence (ACS), a binding site for origin replication complex (ORC), and in most cases, an Abf1 binding site (Louis, 1995). Y' is not present in every subtelomeric region, whereas almost all telomeres have at least a subset of the X element. When there is a Y' element, it is usually located more proximal to the telomere than the X element (Figure 1-1B). Both subtelomeric elements are organized into nucleosomes, which are hypoacetylated as compared to the rest of the genome, a feature shared with *HM* loci (Louis, 1995; Zakian, 1996).

Historically, most studies on telomeric silencing have been carried out by constructing an artificial truncated chromosome, where a reporter gene, usually *URA3* or *ADE2*, is integrated near the telosome, with the concomitant deletion of subtelomeric regions in between. These studies establish that at truncated telomeres, there is a continuous domain of TPE, and the strength of TPE diminishes with increasing distance from the telomere (Gottschling et al., 1990; Louis, 1995; Renauld et al., 1993). However, among native telomeres, the degree and robustness of silencing appears to exhibit high variability, which is attributed primarily to sequence divergence in subtelomeric elements (Pryde and Louis, 1999). Consistently, recent genome-wide analysis indicates a discontinuous binding mode for Sir proteins at natural telomeres, depending on the telomeric sequences (Ellahi et al., 2015; Radman-Livaja et al., 2011; Thurtle and Rine,

2014). The general model proposes that the telomere, with its associated Rap1 and Sir2, 3, 4 complex, folds back and interacts with proteins bound at X-ACS, leading to a confined region of silenced chromatin. The intervening Y' element is looped out and transcriptionally active (Pryde and Louis, 1999; Tham and Zakian, 2002).

B. The Main Components of Silent Chromatin

1) Silencers and Silencer Specificity Factors

Silencers refer to *cis*-acting DNA sequences which recruit sequence-specific silencer binding proteins (silencer specificity factors), and mediate the initiation of silenced chromatin formation. Silencers are able to function in a position-, orientation-, and promoter-independent manner, and can act over a long distance, up to 2.6 kb away (Brand et al., 1985). It has been shown that insertion of silencers to some ectopic loci can result in the silencing of adjacent genes (Lee and Gross, 1993; Maillet et al., 1996; Shei and Broach, 1995)

At the *HM* loci, each of *HMR* and *HML* is flanked by a pair of related but distinct silencers, designated *E* (essential) and *I* (important), with *E* on the left and *I* on the right side, respectively (Figure 1-1A) (Abraham et al., 1984; Feldman et al., 1984; Rusche et al., 2003). Genetic analyses show that *HMR-E* alone is sufficient for silencing of *HMR**a*** or other genes, whereas *HMR-I* cannot function alone without *HMR-E*. In contrast, either *HML-E* or *HML-I* alone is able to silence *HMLα* or other inserted genes (Brand et al., 1985; Mahoney and Broach, 1989).

Among the four silencers, *HMR-E* is the most thoroughly characterized, and offers a good understanding of what makes a functional silencer. *HMR-E* is ~140 bp in length, and has three functional sequence elements, known as A, E, and B elements,

which correspond to ACS which binds ORC, Rap1-binding site, and Abf1-binding site, respectively (Figure 1.1) (Brand et al., 1987; Buchman et al., 1988a; Diffley and Stillman, 1988; McNally and Rine, 1991; Shore et al., 1987). Mutations in any single element have little effect on *HMR* silencing, but mutations in any two lead to almost complete loss of silencing, indicating functional redundancy among these sequence elements (Brand et al., 1987).

The six-subunit ORC is well conserved throughout eukaryotes and is required for DNA replication initiation. Both Rap1 and Abf1 alone can act as transcription activators (Buchman et al., 1988b; Della Seta et al., 1990; Rhode et al., 1992; Shore, 1994). It is intriguing how the binding sites of these three factors together act as silencers, which is proposed to be an emergent characteristic by close juxtaposition (Haber, 1998; Rusche et al., 2003). Interestingly, synthetic silencers can be generated either by arrays of telomeric $C_{1\text{-}3}A$ tracts, which constitute multiple Rap1 binding sites (Conrad et al., 1990; Stavenhagen and Zakian, 1994), or by a combination of one Rap1-binding site, one ACS, and one Sum1-binding D2 element (Weber and Ehrenhofer-Murray, 2010).

Studies have shown that Abf1 directly interacts with Sir3. Rap1 interacts with both Sir3 and Sir4 through its C-terminal domain, and therefore is able to initiate silencing by recruiting Sir proteins to chromatin (Buck and Shore, 1995; Chen et al., 2011; Moretti et al., 1994; Moretti and Shore, 2001; Rusche et al., 2003). When the C-terminus of Rap1 is deleted, silencing defects are observed at both *HM* loci and telomeres (Cockell et al., 1995; Kyrion et al., 1993).

ORC is shown to be able to interact with Sir1 through its largest subunit, Orc1, through the N-terminus Orc1 BAH domain (Gardner et al., 1999; Hou et al., 2005; Triolo

and Sternglanz, 1996). This interaction enhances the probability of recruiting other Sir proteins, Sir2-4, to the silencer and silent chromatin regions (Zhang et al., 2002). Artificial tethering experiments showed that if targeted to the *HMR* locus as a GAL4 DNA-binding domain-Sir1 hybrid through the GAL4-binding site, Sir1 is sufficient to establish silencing without the silencer (Chien et al., 1993). Sir1 is not among the silent chromatin structural proteins, as it does not spread from the silencer, and is not detected within silent chromatin regions (Rusche et al., 2002). Studies show that Sir1 associates with the *HMR-E* silencer independently of other Sir proteins (Rusche et al., 2002). In the absence of Sir2 enzymatic activities, Sir1 takes part in the partial assembly of SIR complexes at the *HMR-E* silencer, indicating that initiation and spreading steps of silent chromatin can be separated. The Orc1 protein also helps recruit Sir4 through interacting with the Sir4 N-terminal regions (Triolo and Sternglanz, 1996).

At telomeres, Sir proteins are recruited not only by Rap1, which binds the terminal TG_{1-3} repeats at chromosomal ends (Buck and Shore, 1995; Conrad et al., 1990; Liu and Lustig, 1996; Lustig et al., 1996; Moretti et al., 1994), but also by the yKu complexes, composed of yKu70/80 heterodimers, which bind all chromosomal ends and interacts with Sir4 specifically (Gravel et al., 1998; Laroche et al., 1998; Mishra and Shore, 1999; Roy et al., 2004; Taddei and Gasser, 2004).

Conclusions that the major function of silencer specificity factors is to recruit Sir proteins have been demonstrated by tethering experiments. Deletion of a silencer or mutations in Rap1 could be fully functionally restored by targeting Sir1, Sir2, Sir3 or a Sir4 C-terminal fragment to an *HM* reporter through a DNA binding domain (Chien et al., 1993; Cockell et al., 1995; Marcand et al., 1996). In addition, tethering Sir3 to telomeres

via LexA binding sites in *rap1* mutant could restore telomeric silencing (Lustig et al., 1996).

2) Nucleosomes and Their Patterns of Post-translational Modifications in Silent Chromatin

Histones are hypoacetylated and hypomethylated in silent chromatin regions in *S. cerevisiae* (Braunstein et al., 1993; Braunstein et al., 1996; Jenuwein and Allis, 2001; Kouzarides, 2007; Kueng et al., 2013; Rusche et al., 2003; Strahl and Allis, 2000). H3 and H4 N-terminal tails, in particular, play important roles in chromatin silencing in *S. cerevisiae*, as indicated by genetic and biochemical studies (Chien et al., 1993; Hecht et al., 1995; Johnson et al., 1992; Johnson et al., 1990; Kayne et al., 1988; Megee et al., 1990; Thompson et al., 1994). Mutational studies suggest that H4K16 is of particular importance in silencing (Braunstein et al., 1996; Johnson et al., 1990; Park and Szostak, 1990). It has been estimated that more than 80% of H4K16 residues in yeast are acetylated (Clarke et al., 1993; Smith et al., 2003). H4K16 is deacetylated specifically by Sir2, an NAD-dependent deacetylase in the SIR complex (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000; Tanny et al., 1999), and its deacetylation is required for Sir3 binding to histone tails (Carmen et al., 2002; Liou et al., 2005) and nucleosomes (Armache et al., 2011; Johnson et al., 2009; Wang, 2013). Sir protein binding and nucleosome deacetylation appear to reinforce each other. A mutation of H4K16 to amino acids such as glutamine or glycine results in decreased binding of Sir3 to silent chromatin (Hecht et al., 1996); a deletion of Sir3 leads to an increase in histone acetylation levels at silent chromatin; whereas Sir3 overexpression results in Sir3 spreading into adjacent subtelomeric chromatin with concomitant hypoacetylation of H4 in that region (Suka et al., 2002). Sas2, a histone acetyltransferase (HAT), shows

specific activity towards H4K16 (Kimura et al., 2002; Sutton et al., 2003), and is postulated to act as a boundary factor that limits the spreading of the SIR silencing complex, due to its role in H4K16 acetylation in subtelomeric regions of the genome (Kimura et al., 2002; Shia et al., 2006; Suka et al., 2002). In addition, Sas2 is involved in silencing in mating type loci *HMR* and *HML* (Ehrenhofer-Murray et al., 1997). Its acetylation of a $tRNA^{Thr}$ gene close to the *HMR-I* silencer helps create a robust heterochromatin barrier (Donze and Kamakaka, 2001).

Contradictory roles of H4K16 acetylation on heterochromatic silencing have been proposed. On the one hand it was postulated that H4K16 acetylation serves to inhibit Sir protein binding and spreading. In particular, it has been shown that Sir3 binding to nucleosomes is inhibited by H4K16 acetylation (Armache et al., 2011; Johnson et al., 2009; Liou et al., 2005; Martino et al., 2009; Onishi et al., 2007; Wang, 2013). In addition, H4K16 acetylation stimulates the binding of Dot1 to nucleosomes, which in turn methylates the H3K79 residue, rendering the nucleosomes more unfavorable for Sir3 binding (Altaf et al., 2007), as discussed later in the section. However, recently it was proposed that H4K16 acetylation might play an additional positive role in establishing Sir-mediated silencing. Previous *in vitro* pull down experiments showed that Sir2/4 subcomplex binding to nucleosomes is insensitive to H4K16 acetylation (Johnson et al., 2009). However, the recent gel shift experiments from Oppikofer *et al* indicated that Sir2/4 binding to nucleosomes was actually enhanced by H4K16 acetylation, and that NAD-dependent deacetylation of H4K16 acetylation by Sir2 stimulated SIR complex loading onto nucleosomes (Oppikofer et al., 2011). Thus it is proposed that H4K16ac promotes the spreading of the SIR complex by recruiting the Sir2/4 subcomplex, yet prevents the ectopic spreading of Sir3 alone (Kueng et al., 2013; Oppikofer et al., 2011).

Recently, it has been shown that deacetylation of H3K56 in the H3 core globular domain is associated with silent chromatin in telomeres (Hyland et al., 2005; Xu et al., 2005; Xu et al., 2007; Yang et al., 2008). H3K56 deacetylation can be achieved *in vitro* by Sir2 (Xu et al., 2007), and *in vivo* by Hst3 and Hst4, two paralogues of Sir2 in *S. cerevisiae* (Maas et al., 2006; Yang et al., 2008). Mutations in H3K56 lead to silencing defects at telomeres. However, the effect is much less pronounced at *HM* loci, where there are redundant silencing mechanisms (Xu et al., 2007). Interestingly, H3K56 mutations that disrupt silencing do not decrease Sir protein binding at the telomere. Instead, they render telomeric chromatin more accessible to ectopic *dam* methylase. It is thus proposed that since H3K56 is located at the DNA entry and exit points of nucleosomes, its deacetylation leads to chromatin compaction, and therefore, heterochromatic gene silencing (Xu et al., 2007). Along this line, it is shown that acetylation at H3K56 increases the spontaneous and transient unwrapping of the nucleosomal DNA from the histone octamer core, referred to as nucleosomal “breathing” (Li et al., 2005; Neumann et al., 2009).

Another important histone residue in heterochromatic silencing is H3K79 in the H3 globular domain, on the nucleosome surface. H3K79 is methylated by the histone methyltransferase (HMT) Disruptor of Telomeric Silencing (Dot1). About 90% of H3K79 in the *S. cerevisiae* is methylated. In contrast, it is hypomethylated at all silenced loci. Methylated H3K79 is one of the most important anti-silencing markers, and the binding and spreading of Sir3 is inhibited by H3K79 methylation both *in vivo* (Altaf et al., 2007; Onishi et al., 2007; van Welsem et al., 2008) and *in vitro* (Altaf et al., 2007; Ehrentraut et al., 2011; Martino et al., 2009). H3K79A mutation disrupts silencing at both telomeres and *HM* loci (Ng et al., 2003; Ng et al., 2002; van Leeuwen et al., 2002). Genetic screens

for histone H3 have identified residues spanning amino acids 68-83 in the globular domain to be involved in silencing (Park et al., 2002; Thompson et al., 2003). Later crystal structural and biochemical studies confirmed that Sir3 interacts with the nucleosome core region, around H3K79, and that this interaction is inhibited by H3K79 methylation (Armache et al., 2011; Onishi et al., 2007; Wang, 2013).

Interestingly, there appears to be some crosstalk between the two anti-silencing markers, H4K16 acetylation and H3K79 methylation, as it has been shown that H4K16ac stimulates the methylation activity of Dot1 on H3K79 (Altaf et al., 2007; Fingerman et al., 2007). In addition, since Sir3 binds to the H4 N-terminal tail region around H4K16, the same binding site for Dot1, its binding hindered the H3K79 methylation by Dot1 (Altaf et al., 2007).

Similar to H3K79, methylation of H3K4 by another HMT, Set1, is frequently associated with euchromatin (Bernstein et al., 2002). H3K4 is hypomethylated at regions of silent chromatin, and Sir3 binding is sensitive to H3K4 methylation (Santos-Rosa et al., 2004). Mutation of Set 1, which leads to a global demethylation of H3K4, disrupts gene silencing at telomeres and *HM* loci (Fingerman et al., 2005; Nislow et al., 1997). Right upstream of H3K4, H3R2 can also be methylated. This unique asymmetric dimethylation of H3R2 in *S. cerevisiae* occurs specifically at heterochromatic loci and inactive euchromatic genes, and is mutually exclusive with trimethylation of H3K4. H3R2 methylation blocks Set1 binding to and methylating H3K4 (Kirmizis et al., 2007).

Molecular experiments in a population of cells indicate that heterochromatin assembly in *S. cerevisiae* appears to occur through two major phases: an early, rapid deacetylation phase mediated by active enzymatic removal of histone acetylation, which

results in incomplete transcriptional silencing, followed by a slower maturation phase, during which histone methylation are removed gradually by dilution with unmethylated histones, through 3-5 cell generations, stabilizing the silent state (Katan-Khaykovich and Struhl, 2005). However, phenotypic measurements at single-cell resolution show that in most cells silencing is completed within one to two cell divisions (Osborne et al., 2009).

3) Sir Proteins

The four *SIR* genes, *SIR1-4*, involved in heterochromatic silencing were identified by mutations that derepressed the *HM* loci (Ivy et al., 1986; Rine and Herskowitz, 1987). Among them, Sir2, Sir3 and Sir4 form the SIR complex, which constitutes the structural component of silent chromatin (Rusche et al., 2002), and are essential for silencing. *In vitro* experiments suggest that the SIR complex is composed of a stoichiometric assembly of Sir2, Sir3 and Sir4 proteins in a 1:1:1 complex (Cubizolles et al., 2006). However, the exact stoichiometry between the Sir proteins and nucleosomes is still debatable. It has been shown that Sir3 binds to nucleosomes in a 2:1 stoichiometry (Swygert et al., 2014), yet the SIR complex appears to bind to chromatin with one complex per linker, and thus in a (n-1):n stoichiometry with nucleosomes (Martino et al., 2009).

In contrast, Sir1 is not essential for silencing. It is not required for the maintenance (defined as the preservation of silencing within a cell cycle, through the G1, G2 and M phases), or the inheritance (defined as being inherited upon cell division, through S phase) of silent chromatin, but it does stabilize the interaction of other Sir proteins with silencers, promote the assembly of Sir proteins along the heterochromatin, and therefore is required for the establishment of silencing (Pillus and Rine, 1989;

Rusche et al., 2002; Triolo and Sternglanz, 1996). Mutants lacking Sir1 exhibit a bistable silencing phenotype (Dodson and Rine, 2015; Pillus and Rine, 1989; Xu et al., 2006).

Similar to other species, in which heterochromatin has a high propensity to cluster at the nuclear envelope, telomeres and *HM* loci in *S. cerevisiae* are enriched at the nuclear periphery, forming foci that sequester Sir proteins (Gotta et al., 1996; Maillet et al., 1996; Palladino et al., 1993; Taddei et al., 2009).

1. Sir2

Sir2 belongs to the highly conserved NAD (nicotinamide adenine dinucleotide)-dependent protein deacetylase family (Blander and Guarente, 2004; Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). In addition to being a structural component of silent chromatin, its enzymatic activity is required for silencing (Hoppe et al., 2002; Tanny et al., 1999). The targets of Sir2 have been shown to be H3K9, H3K14, and H4K16 *in vitro* (Imai et al., 2000). However, mutational studies suggest that H4K16 plays a much more substantial role than other lysine residues in silencing (Braunstein et al., 1996; Johnson et al., 1990). Unlike Sir3 or Sir4, Sir2 does not interact appreciably with either DNA or nucleosomes on its own (Martino et al., 2009). Sir2 interacts with Sir4 through a region upstream of its deacetylase domain (a.a. 99-237) (Figure 1-2A) (Cockell et al., 2000; Froyd and Rusche, 2011; Mead et al., 2007).

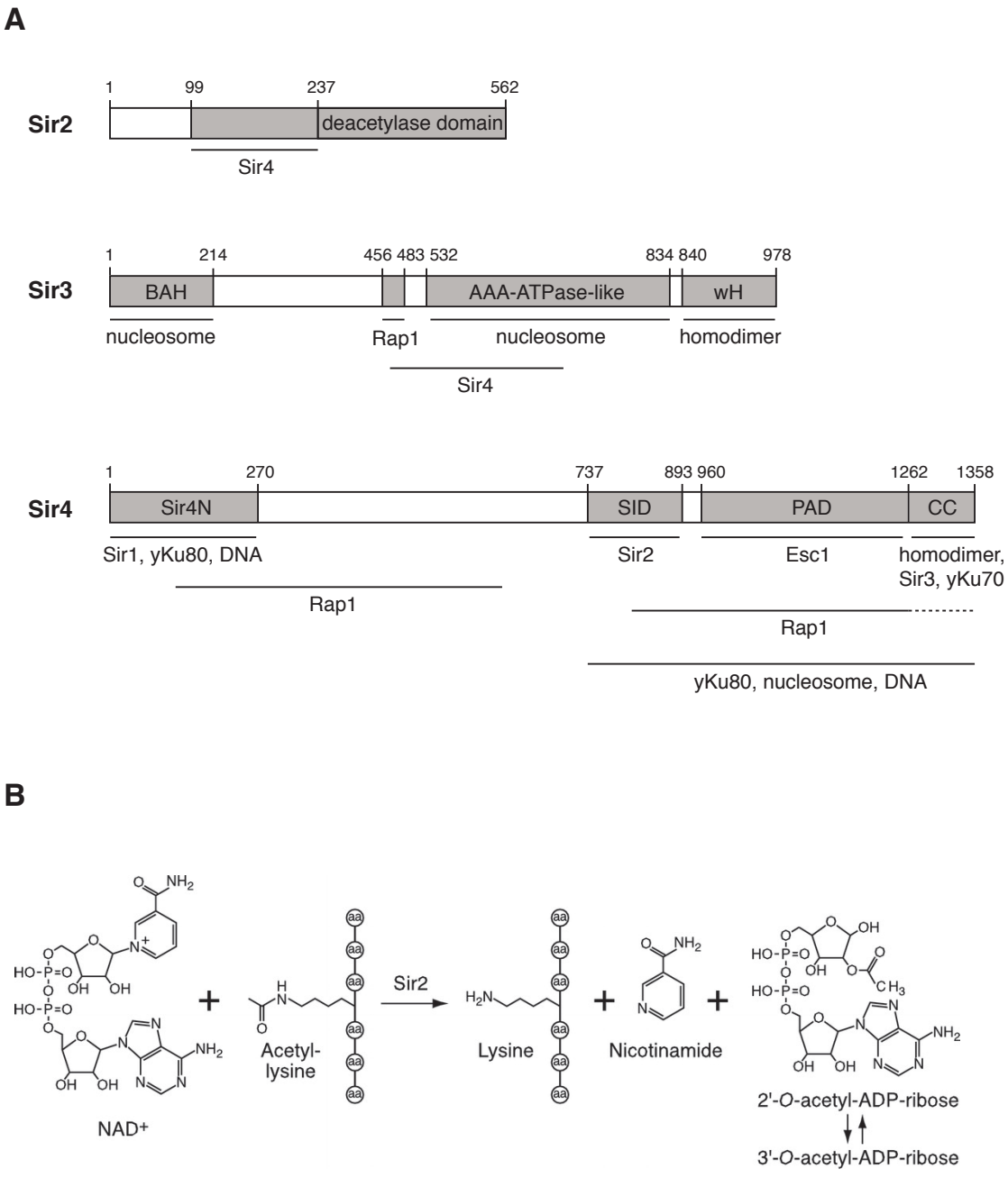
Sir2 couples the deacetylation reaction to the hydrolysis of NAD^+. In this reaction, for every acetyl group removed, one molecule of NAD^+ is cleaved at the N-glycosidic bond at the ribose C-1 into one ADP-ribose and one nicotinamide molecules. The acetyl group is transferred to ADP-ribose, forming a novel product, *O*-acetyl-ADP-ribose (AAR or OAADPR), which exists in equilibrium of two forms, 2'-*O*-acetyl-ADP-ribose and 3'-*O*-

Figure 1-2. The domain organization of Sir proteins, their relevant interactions and the deacetylation reaction of Sir2.

(A) Schematic representation of Sir2, Sir3 and Sir4 proteins, their domain organization, and their interaction partners. The numbering refers to the primary sequence of the proteins. Domains are in shaded boxes. Regions that mediate important interactions are highlighted. Only a weak interaction between Rap1 and Sir4 CC domain has been reported by yeast two-hybrid assays (Moretti et al., 1994), and the interaction is indicated by dotted line. BAH: bromo-adjacent homology domain; wH: winged helix domain; Sir4N: Sir4 N-terminal domain; SID: Sir2-interacting domain; PAD: partitioning-and-anchoring domain; CC: coiled-coil domain. (Modified from Oppikofer, *et al.* 2013).

(B) Schematic representation of Sir2-mediated NAD-dependent deacetylation reaction. Sir2 couples histone deacetylation to NAD^+ hydrolysis and acetyl group transfer from the lysine substrate to ADP-ribose. The overall reaction scheme and the structures of NAD^+, nicotinamide, and the novel reaction product, *O*-acetyl-ADP-ribose (AAR) are shown. AAR exists as an equilibrium between 2'- and 3'-*O*-acetyl-ADP-ribose in solution. (Adapted from Rusche, *et al.* 2003).

Figure 1-2 (Continued)



acetyl-ADP-ribose (Figure 1-2B) (Imai et al., 2000; Jackson and Denu, 2002; Tanny et al., 1999; Tanny and Moazed, 2001). Since other deacetylases exist in *S. cerevisiae* that are NAD-independent, and both deacetylation and N-glycosidic bond cleavage are energetically favorable, the requirement of Sir2 for silencing has generated much speculation about whether AAR has any physiological function (Moazed, 2001b).

2. Sir3

Sir3 does not have enzymatic activities, but rather plays a structural role in the silent chromatin assembly. It fulfills the special role as the primary anchoring factor for nucleosome binding within the SIR complex, and is key for SIR complex spreading. The domain organization of Sir3 is as shown in Figure 1-2A. The N-terminal 1-214 a.a. of Sir3 contains a Bromo-Adjacent Homology (BAH) domain, which is conserved throughout eukaryotes, and which is involved in protein-protein interactions, and has been identified in several chromatin-associated proteins, including Orc1 (Callebaut et al., 1999; Connelly et al., 2006). BAH is the primary nucleosome interaction domain of Sir3, as identified by co-immunoprecipitation, pull down, gel shift, and mutational studies (Buchberger et al., 2008; Onishi et al., 2007). *In vitro* peptide binding experiment shows that Sir3 BAH domain binds to two regions of nucleosomes, H4 N-terminal tail region around H4K16, and a region of H3 globular domain centered around H3K79 on the exposed surface of the nucleosome (Onishi et al., 2007). BAH domain binding, as well as the full length Sir3 binding to histones and nucleosomes, is sensitive to H4K16 acetylation and H3K79 methylation (Altaf et al., 2007; Carmen et al., 2002; Georgel et al., 2001; Johnson et al., 2009; Liou et al., 2005; Oppikofer et al., 2011). Since these two regions are adjacent on the nucleosome, it was proposed that they constitute a

composite binding site in the Sir3 BAH domain. This is confirmed by the recent crystal structures of Sir3-BAH-nucleosome complex (Armache et al., 2011; Wang, 2013).

In addition to the BAH domain, two regions within and near the Sir3 AAAL domain (a.a. 532-834), Sir3 (623-762 a.a.) and Sir3 (799-910 a.a.), are required for silencing, and have been shown to contribute to binding to H3 and H4 N-terminal tails (Hecht et al., 1995). Sir3 AAAL domain binds to nucleosomes with much lower affinity than the BAH domain, although the binding is similarly sensitive to H3K79 methylation (Ehrentraut et al., 2011). Sir3 interacts with the Sir4 coiled coil (CC) domain, through its AAAL domain (Chang et al., 2003; Ehrentraut et al., 2011; King et al., 2006). This Sir3-Sir4 interaction is required for the recruitment of Sir3 to silencers, and for the spreading of Sir proteins away from the silencer, and is essential for silencing (Chang et al., 2003; Ehrentraut et al., 2011; Rudner et al., 2005). However, Sir3 does not appear to form a stable complex with Sir2/4, as most Sir3 does not associate with Sir2/4 in yeast extracts (Moazed et al., 1997; Rudner et al., 2005). *In vitro* studies suggest Sir3-Sir4 interaction may be regulated, with the Sir4 N-terminal region appearing inhibitory (Moazed et al., 1997). Consistently, Sir4 C terminus overexpression disrupts silencing, and this dominant negative effect is suppressed by Sir3 overexpression (Ivy et al., 1986; Marshall et al., 1987). It is therefore proposed that Sir3 binds to the Sir2/4 complex upon the binding of the latter to chromatin during silent chromatin assembly (Ghidelli et al., 2001; Moazed et al., 1997).

The Sir3 AAAL domain shares some but poor similarity to the AAA (ATPase Associated with diverse cellular Activities) ATPase module (Neuwald et al., 1999). Key residues involved in ATP binding are not conserved in Sir3, suggesting Sir3 does not hydrolyze ATP (Stone and Pillus, 1998). However, because of its homology to the AAA

module, a nucleotide binding domain, it has been proposed that the AAAL domain binds AAR to assist the spreading of SIR complexes along the chromatin (Buchberger et al., 2008; Kueng et al., 2013). Interestingly, studies have generated results supporting either side. It has been shown that AAR increases the binding of Sir3 to immobilized Sir2/Sir4, and induces a conformational change in the SIR complex in the presence of H4 N-terminal peptide, as visualized by the electron microscope (Liou et al., 2005). In addition, AAR increases the binding affinity of both Sir3 and SIR complexes to oligonucleosomes *in vitro* (Martino et al., 2009). In contrast, a study from Gartenberg's group suggests that the requirement for Sir2 can be partially bypassed by a $Sir2^{78-252}$-$Hos3^{2-549}$-$Sir2^{522-562}$ fusion protein, in which the Sir2 catalytic core is replaced by the NAD-independent histone deacetylase Hos2 (Chou et al., 2008). Furthermore, the recent crystal structure of the Sir3 AAAL domain suggests that its putative nucleotide binding pocket is too narrow to be able to accommodate an AAR molecule (Ehrentraut et al., 2011). However, it remains possible that AAR binds to full-length Sir3, for example to the interface formed by different domains/regions of Sir3, or remains bound to Sir2 after deacetylation. Further experiments would be required to test this idea.

It has been shown that Sir3 forms dimer and oligomers *in vitro* (Liaw and Lustig, 2006; Liou et al., 2005; McBryant et al., 2006; Moretti et al., 1994), and it is proposed that the AAAL domain mediates this oligomerization, as the canonical AAA-ATPase domain forms hexamer. However, recent structural data suggest that the Sir3 AAAL domain does not form oligomers (Ehrentraut et al., 2011). The Sir3 C-terminal winged helix (wH) domain (a.a. 840-978) is both necessary and sufficient for Sir3 dimerization (Liaw and Lustig, 2006; Oppikofer et al., 2013). Deletion of the wH domain abolishes Sir3 association with silent chromatin regions, and disrupts silencing at both *HM* loci and

telomeres (Oppikofer et al., 2013). The wH domain can be functionally rescued by replacement with an orthologous dimerization domain (Oppikofer et al., 2013).

Sir3 itself is N-terminally acetylated on the A2 residue, by the NatA Nα-acetyltransferase (Geissenhoner et al., 2004; Wang et al., 2004). It has been shown that this N-terminal acetylation stabilizes Sir3 binding to nucleosomes (Arnaudo et al., 2013; Onishi et al., 2007; Yang et al., 2013), and is required for Sir3 spreading, and thus silencing at both telomeres and *HM* loci (Geissenhoner et al., 2004; Ruault et al., 2011; Sampath et al., 2009; Wang et al., 2004).

3. Sir4

Sir4 is an important structural component of silent chromatin regions, linking various proteins together (Figure 1-2A). Its N-terminal region (Sir4N, a.a. 1-270) binds directly to Sir1 (Triolo and Sternglanz, 1996) and yKu70/80 (Roy et al., 2004; Tsukamoto et al., 1997), a DNA-end binding heterodimer, which binds at DNA telomeric ends, plays an important role in the maintenance of telomeric structure, and is required for TPE (Gravel et al., 1998; Laroche et al., 1998; Mishra and Shore, 1999; Nugent et al., 1998; Polotnianka et al., 1998). Consistent with these interactions, Sir4N is dispensable for *HM* silencing, but is essential for TPE (Kueng et al., 2012). In addition, Sir4N has nonspecific binding activity towards free DNA (Martino et al., 2009), and contributes towards the protection of linker DNA when Sir2/4 or the SIR complex binds to chromatin (Kueng et al., 2012).

The C-terminal half of Sir4 (a.a. 747-1358) is sufficient for the formation of a functional SIR complex, and is sufficient to support *HM* but not telomeric silencing (Kueng et al., 2012; Moazed et al., 1997). This region contains interacting activity with

Rap1, a silencer specificity factor at both *HM* loci and telomeres (Cockell et al., 1995; Luo et al., 2002; Moretti et al., 1994; Moretti and Shore, 2001). The Sir2-interacting domain (SID) encompasses a.a. 737-893 (Hsu et al., 2013), leading to the formation of a stable 1:1 heterodimeric Sir2/4 subcomplex that enhances the deacetylation activity of Sir2 (Cubizolles et al., 2006; Ghidelli et al., 2001; Hoppe et al., 2002; Hsu et al., 2013; Moazed et al., 1997; Tanny et al., 2004). Sir2/4 subcomplex is able to bind nucleosomes *in vitro*, although Sir2 alone is unable to do so (Ghidelli et al., 2001). Yeast two-hybrid, co-immunoprecipitation, and *in vitro* pull-down experiments indicate that Sir4 interacts independently with both Sir3 and Sir2 (Cockell et al., 1995; Hecht et al., 1995; Moazed and Johnson, 1996; Moretti et al., 1994; Strahl-Bolsinger et al., 1997), while Sir3 and Sir2 do not interact with each other (Moazed et al., 1997). Probably as a result of all the above interactions, Sir4 is able to associate with the silencer at *HMR-E* in the absence of either Sir2 or Sir3, both of which, on the contrary, requires Sir4 for the association (Hoppe et al., 2002; Luo et al., 2002; Rusche et al., 2002). Sir4 also interacts directly with H3 and H4 N-terminal tails (Hecht et al., 1995). H4K16 acetylation appears to abolish Sir4 binding to H4 tails (Liou et al., 2005). However, the Sir2/4 subcomplex interacts with chromatin with little specificity (Johnson et al., 2009; Oppikofer et al., 2011). The subcomplex also interacts with free DNA nonspecifically, likely due to the N-terminal domain of Sir4, which may mask its specific interaction with nucleosomal histones (Kueng et al., 2012; Martino et al., 2009) (Figure 1-2A).

Downstream of the Sir4 SID is the partitioning-and-anchoring domain (PAD) (aa 960-1262), which is sufficient to tether a chromosomal locus to the nuclear periphery by binding to the nuclear envelope-associated protein Esc1 (Andrulis et al., 2002; Taddei et al., 2004). In addition to Esc1, Sir4 is able to interact with yKu80, and mediates the

anchoring of silent chromatin to the nuclear periphery in an Esc1-independent pathway (Gartenberg et al., 2004; Hediger et al., 2002; Taddei and Gasser, 2004; Taddei et al., 2004). This perinuclear anchoring contributes significantly to TPE, although *HM* silencing does not seem to be affected much by loss of anchoring (Hediger et al., 2002; Laroche et al., 1998). The importance of perinuclear anchoring for Sir-mediated silencing was demonstrated by tethering a reporter flanked by crippled silencers to the inner nuclear membrane (Andrulis et al., 2002). This anchoring supports Sir-mediated silencing of the reporter, albeit only when perinuclear Sir clusters are intact (Taddei et al., 2009). This supports the notion that it is the close proximity to high concentrations of Sir proteins, but not perinuclear anchoring per se, that enables weak silencers to support silencing (Marcand et al., 1996; Taddei et al., 2009).

The extreme C-terminal coiled-coil (CC) domain of Sir4 (a.a. 1262-1358) mediates both its homodimerization and its binding to Sir3 (Chang et al., 2003; Murphy et al., 2003; Rudner et al., 2005). The dimerization activity of Sir4 is required for silencing at both telomeres and the *HML* locus (Chang et al., 2003; Chien et al., 1991; Murphy et al., 2003). Interestingly, the Sir4 CC domain also interacts with Rap1 and yKu70, and therefore serves to recruit Sir4 to telomeres (Cockell et al., 1995; Moretti et al., 1994; Tsukamoto et al., 1997).

In addition, Sir4 interacts with Ubp10/Dot4, a ubiquitin protease targeting H2B for deubiquitylation (Kahana and Gottschling, 1999), and localizes it to telomeres. There, Ubp10 serves to maintain low level of ubiquitination at H2BK123, which in turn leads to low level of methylation at H3K4 and H3K79 (Emre et al., 2005; Gardner et al., 2005). Ubp10 also helps target Sir2 to telomeres (Emre et al., 2005).

C. Current Model of Silent Chromatin Assembly

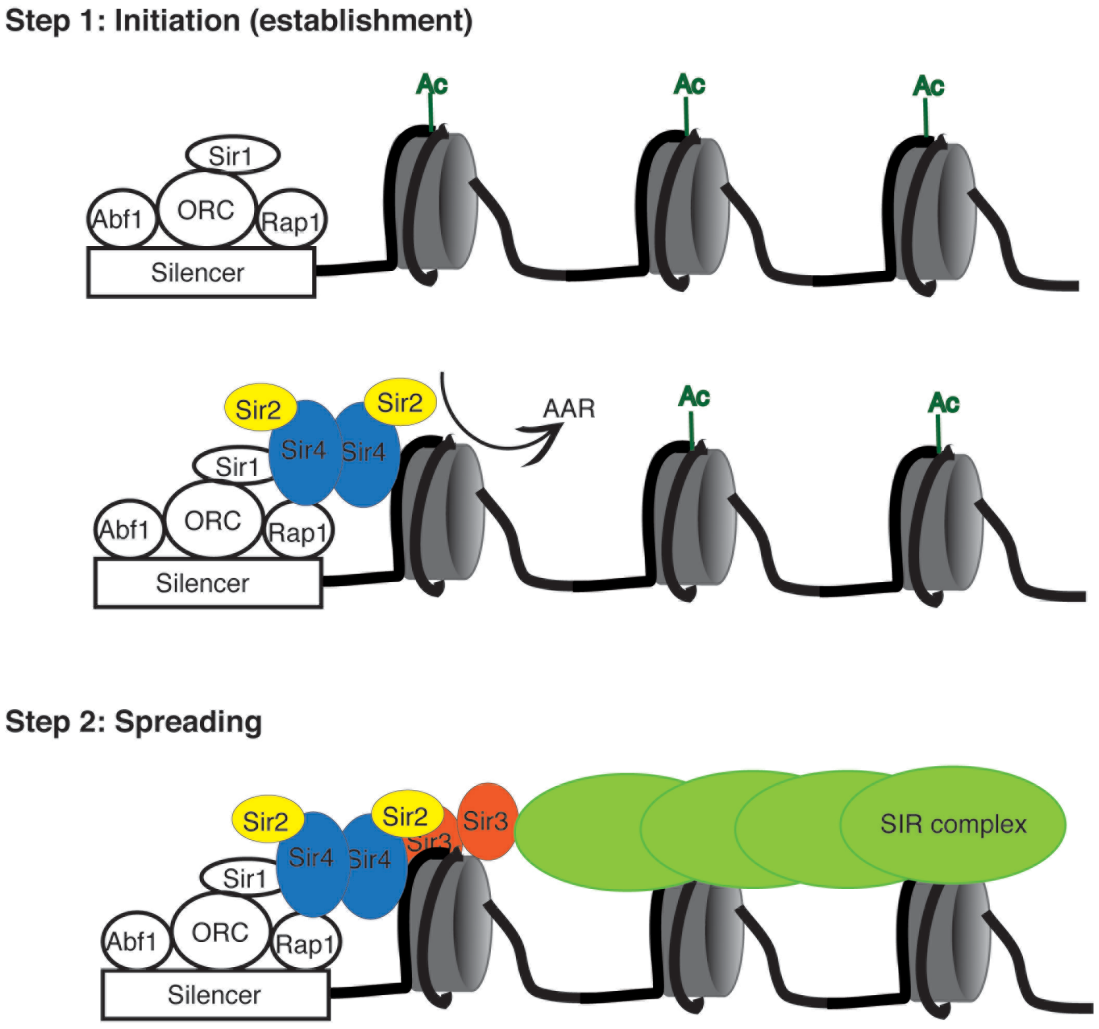
The current consensus model for assembly of silent chromatin at *HM* loci and telomeres involves two major steps, initiation or the establishment step, and the spreading step (Figure 1-3). At the initial establishment step, silencer specificity factors, including ORC, Rap1 and Abf1 at the *HM* loci, and yKu70/80 heterodimer and Rap1 at telomeres, bind to their cognate sequences; Sir1 is recruited to the *HM* loci silencer through its interaction with ORC. Sir4 is recruited via its binding to Sir1 and Rap1 at the *HM* loci, and Rap1 and yKu70/80 at telomeres. Sir4 brings along Sir2 as the Sir2/4 complex, which then deacetylates the nucleosome adjacent to the silencer. Deacetylation of histone tail lysine residues, especially H4K16, creates a binding site for Sir3 and Sir4, resulting in the joining of Sir3 into the Sir2/4 subcomplex. Newly joined Sir3 recruits additional Sir2/4 proteins, due to its affinity for Sir4. The subsequent iterative cycles of deacetylation and Sir protein binding leads to the spreading of SIR complexes along chromatin, which then forms the silent chromatin (Hoppe et al., 2002; Luo et al., 2002; Rusche et al., 2002, 2003; Tanny et al., 2004).

This model of silent chromatin assembly may suggest a linear stepwise spreading of Sir proteins and gene silencing. So far, the most convincing argument for linear spreading was presented by Renauld *et al* (Renauld et al., 1993), who showed that the efficiency of a reporter gene silencing decreased with increasing distance away from the Chr V-R telomere. In addition, Sir protein overexpression led to proportionately increased silencing further away from the telomere. However, as observed at native yeast telomeres, the spreading of Sir proteins can be discontinuous (Ellahi et al., 2015; Fourel et al., 1999; Pryde and Louis, 1999; Thurtle and Rine, 2014). At the *HM* loci, Sir

Figure 1-3. A Stepwise model for silent chromatin assembly in *S. cerevisiae.*

The model involves 2 major steps: Step 1 is the initiation/establishment step, where Sir2/Sir4 subcomplexes are recruited to the silencer through interactions with silencer-specificity factors. Here the silencer is depicted as the HMR-E silencer. Composition in other silencers may differ (refer to the main text for details). Step 2 is the spreading stage, in which nucleosomes deacetylated by Sir2 create a strong binding site for Sir3, which joins Sir2/Sir4, forming the SIR complex. Subsequent iterative cycles of histone deacetylation and Sir protein binding lead to spreading of SIR along the chromatin.

Figure 1-3 (Continued)



proteins show a heterogeneous distribution pattern (Thurtle and Rine, 2014). In addition, the catalytically inactive Sir2-N345A does not have a dominant negative effect on silencing at *HMR*, indicating that Sir protein spreading does not proceed in a strictly linear fashion at the *HMR* locus (Lynch and Rusche, 2009). Furthermore, computational simulation studies suggest that long-range Sir protein interactions and nucleosome deacetylation is required for the bistability and epigenetic maintenance of silent chromatin (Dodd et al., 2007).

Upon overexpression, Sir3 spreads further into chromatin, away from telomeres, extending the size of silent chromatin from 2-4 kb to around 20 kb (Hecht et al., 1996; Renauld et al., 1993). In the extended silent chromatin region, relatively higher amount of Sir3 than Sir2 or Sir4 are associated with chromatin. However, both Sir2 and Sir4 are required for the extended silencing regions (Renauld et al., 1993; Strahl-Bolsinger et al., 1997). Therefore, the structure of extended telomeric silencing formed by Sir3 overexpression may be different from that of the native telomeric silencing.

D. Boundaries of Silent Chromatin

As silent chromatin initiates at silencers and spread along the chromatin fiber, boundaries are required to limit the spreading of silencing proteins, and separate silent chromatin from euchromatin. This is especially important near the *HM* loci, which are flanked by essential genes. Multiple mechanisms for silent chromatin boundaries are employed in *S. cerevisiae*. For example, *cis* boundary element, such as the $tRNA^{Thr}$ gene to the right of the *HMR* locus, serves to prevent heterochromatin spreading to the nearby gene (Donze and Kamakaka, 2001). Anti-silencing histone post-translational modifications, including acetylation of H4K16 primarily by Sas2 (Kimura et al., 2002;

Suka et al., 2002) and secondarily by Esa1 (Suka et al., 2002; Suka et al., 2001), acetylation of H3K56 by Rtt109 (Yang et al., 2008), methylation of H3K4, H3K36 and H3K79 by Set1, Set2 and Dot1 respectively (Bernstein et al., 2002; Ng et al., 2003; Ng et al., 2002; Rao et al., 2005; van Leeuwen et al., 2002; Verzijlbergen et al., 2009), and H2B ubiquitylation by Rad6/Bre1 (Emre et al., 2005; Leung et al., 2011) are all anti-correlated with silent chromatin. In particular, deletion of Sas2 leads to hypoacetylation of H4K16 near subtelomeric regions and the spreading of Sir3 from 3kb to roughly 15kb away from telomeres, resulting in transcriptional repression of telomere-distal regions (Suka et al., 2002). Tethering of either Sas2 or Dot1 to a locus creates effective boundary activity blocking the spreading of heterochromatin (Donze and Kamakaka, 2001; Oki et al., 2004; Stulemeijer et al., 2011). Furthermore, replacement of canonical histones with histone variants, such as H2A.Z/Htz1 near telomeres and flanking the *HMR* locus, has been shown to block the spreading of Sir proteins into enchromatic regions (Meneghini et al., 2003). Finally, limited pool of Sir proteins inside the cell and their compartmentalization has been shown to help limit Sir protein to heterochromatic regions only (Buck and Shore, 1995; Hecht et al., 1996; Maillet et al., 1996; Marcand et al., 1996; Renauld et al., 1993). The requirement of boundary elements to limit the spreading of silent chromatin may argue for a processive spreading mechanism that cannot jump around the boundary element.

It is of interest to note that there is extensive crosstalk between these different boundary mechanisms, which may further reinforce boundary activities. For example, it has been shown that H4K16ac histone tails recruit and stimulate the methylation activity of Dot1 on H3K79 (Altaf et al., 2007). H2B ubiquitylation is required for efficient methylation of both H3K4 and H3K79 (Lee et al., 2007; Weake and Workman, 2008).

Deletion of Sas2 renders the $tRNA^{Thr}$ gene a less effective boundary element, suggesting that Sas2-mediated acetylation enhances the boundary activity of the tRNA gene (Donze and Kamakaka, 2001). Finally, H4K16ac recruits chromatin remodelers that deposit Htz1, and the presence of H4K16ac and Htz1 synergistically prevent the ectopic propagation of heterochromatin (Shia et al., 2006). Set1-mediated H3K4 methylation and Htz1 also cooperates to prevent the ectopic spreading of Sir proteins (Venkatasubrahmanyam et al., 2007).

E. Epigenetic Inheritance of Silent Chromatin

The phenomenon of epigenetic inheritance of silent chromatin in *S. cerevisiae* is revealed by PEV at telomeres and variegating states at the *HM* loci in cells lacking Sir1. When an *ADE2* reporter gene is inserted near a telomere, yeast cells produce sectored colonies with white and red sectors, in which cells have the *ADE2* gene in either ON or OFF state, respectively (Aparicio et al., 1991; Gottschling et al., 1990). This variegation results from stochastic loss and re-establishment of the silencing state, with daughter cells inheriting the expression state of the originally switched mother cells. Normally, silencing at the *HM* loci is stably inherited through many rounds of DNA replication. However, among a population of genetically identical *MAT**a** sir1* mutant cells, 20% of the cells have *HMLα* under the repressed state, whereas the other 80% have derepressed *HMLα*, as assessed by their insensitivity to the *α*-factor (Pillus and Rine, 1989). At the single cell level, both the repressed and derepressed states can be stably inherited for 10 generations or more (Pillus and Rine, 1989). These phenomena indicate that silent chromatin in genetically identical cells can exist in reversibly stable on and off expression states.

F. Maintenance of Silent Chromatin

As discussed in previous sections, silent chromatin is epigenetically inherited, that is they are structurally and functionally inherited from one generation to the next. Silent chromatin also has to be faithfully maintained throughout the duration of cell cycles. It has been shown that instead of being a static structure, heterochromatin is quite dynamic, with at least some of its structural components undergoing a constant flux of exchange with newly synthesized molecules of the same protein (Cheng and Gartenberg, 2000; Cheutin et al., 2003; Festenstein et al., 2003; Ficz et al., 2005). In particular, in *S. cerevisiae,* silencers and functional Sir proteins are continuously required for silencing and maintenance of a repressive chromatin structure, even in the absence of DNA replication (Bi and Broach, 1997; Cheng and Gartenberg, 2000; Cheng et al., 1998; Holmes and Broach, 1996; Miller and Nasmyth, 1984). In addition, newly synthesized Sir proteins are incorporated into silent chromatin even in the absence of S-phase passage (Cheng and Gartenberg, 2000). A recent experiment designed to capture transient losses of gene silencing in *S. cerevisiae* showed that in *HM* loci rare transcription events occurred in approximately 1/1000 cell divisions. These transcription events were transient, occurred in limited levels, and could occur at any stage of the cell cycle, indicating that the fluctuations in heterochromatin structure at the *HM* loci are rare and very likely of no functional consequence (Dodson and Rine, 2015). True epigenetic states in *S. cerevisiae* are only revealed by TPE at telomeres and the existence of stable on and off expression states at the *HM* loci when *SIR1* is deleted (Pillus and Rine, 1989).

III. Cooperative binding

Molecular binding is an interaction between molecules that results in physical association between them. Cooperative binding describes a type of allosteric interaction. It can occur if one of the molecules contains multiple binding sites, and it describes the changes that occur to the affinity of other binding sites, when one binding site is occupied. There can be positive or negative cooperativity, where in positive cooperativity, occupancy of one binding site increases the binding affinity of other binding sites. In addition, cooperativity can be either homotropic or heterotropic. In homotropic cooperative binding, only one type of ligand/substrate is involved, and the binding of the ligand/substrate is influenced by how much of that same kind of ligand/substrate is already bound. However, in heterotropic cooperative binding, the binding of one ligand/substrate affects the binding of a different kind of ligands/substrates. The phenomenon of cooperative binding is widespread in biological systems, including many enzymes and receptors. It can also be seen in large lattice-like molecules that are made of many identical, or near identical, subunits, such as DNA (Cantor and Schimmel, 1980), and as will be described later the association of Sir proteins with chromatin. Below, I will describe the major systems and equations that deal with cooperative binding.

One of the earliest and best-studied examples of cooperative binding is oxygen binding to hemoglobin, a protein made of four subunits with each subunit containing a binding site for oxygen. When plotting hemoglobin saturation with O_2 as a function of the partial pressure of O_2, the binding exhibits a sigmoidal curve (Bohr et al., 1904), instead of the hyperbolic curve as expected from the Michaelis and Menten behavior. This indicates that the more oxygen is bound to hemoglobin, the easier it becomes for more

oxygen to bind, until all binding sites are saturated. Thus, oxygen binding to hemoglobin exhibits homotropic positive cooperativity.

Throughout the twentieth century, cooperative binding of ligands with macromolecules has been treated by many authors, and various frameworks have been developed. The first description of cooperative binding was developed by AV Hill, working on oxygen binding to hemoglobin (Hill, 1910). The Hill Equation is shown in Figure 1-4A, and it remains the most well known framework to study cooperative binding among biologists. The Hill Equation is essentially a semi-empirical approach that interprets the physical significance of empirical parameters that are obtained. It is based on the assumption that the binding over part of the saturation range can be described by equations phenomenologically resembling those for an infinitely cooperative system. It assumes the cooperativity, n, to be constant, which does not change with saturation. It also assumes that binding sites always exhibit the same affinity regardless of the ligand concentration, described by the apparent dissociation constant, K_d (Cantor and Schimmel, 1980; Stefan and Le Novere, 2013).

Having demonstrated that hemoglobin contained four hemes, and therefore contains four binding sites for oxygen, and noting that the Hill plot for hemoglobin was not a straight line, GS Adair hypothesized that the cooperativity was not a fixed term, but instead was dependent on ligand saturation. He assumed that fully saturated hemoglobin was formed in stages, and introduced an apparent macroscopic association constant, K_i, at each intermediate stage (Adair, 1925). The resulting fractional occupancy can be expressed as shown in Figure 1-4B (Stefan and Le Novere, 2013). Working on calcium binding proteins, Irving Klotz deconvoluted Adair's association constants by considering the stepwise formation of intermediate stages, and expressed the cooperative binding in

Figure 1-4. Equations of different models for cooperative binding.

(A) The Hill Equation. Θ: fractional occupancy, defined as the number of ligand-bound binding sites divided by the total number of ligand binding site; K_d, apparent dissociation constant; n, Hill coefficient; [X], ligand concentration. If n=1, the system exhibits no cooperativity. If n<1, the system exhibits negative cooperativity. If n>1, the system exhibits positive cooperativity.

(B) The Adair equation. Equation 1 describes oxygen binding to hemoglobin. Equation 2 is for any protein with n ligand binding sites, where n denotes the number of binding sites, and K_i is the apparent macroscopic constant describing the binding of i ligand molecules.

(C) The Klotz equation (also called Adair-Klotz equation). K_i describes the macroscopic association constant when i binding sites are occupied. If all ligand binding sites are identical with a microscopic association constant K, one would expect $K_1 = nK$, $K_2 = (n-1)K/2$, … , $K_n = K/n$ (that is $K_i = (n-i+1)K/i$) in the absence of cooperativity. There is positive cooperativity if K_i lies above expected values for $i > 1$.

Figure 1-4 (Continued)

A

$$\Theta = \frac{[X]^n}{K_d + [X]^n}$$

B

$$\Theta = \frac{1}{4} \frac{K_I[X] + 2K_{II}[X]^2 + 3K_{III}[X]^3 + 4K_{IV}[X]^4}{1 + K_I[X] + K_{II}[X]^2 + K_{III}[X]^3 + K_{IV}[X]^4} \qquad \text{(Equation 1)}$$

$$\Theta = \frac{1}{n} \frac{K_I[X] + 2K_{II}[X]^2 + \ldots + nK_n[X]^n}{1 + K_I[X] + K_{II}[X]^2 + \ldots + K_n[X]^n} \qquad \text{(Equation 2)}$$

C

$$\Theta = \frac{1}{n} \frac{K_1[X] + 2K_1K_2[X]^2 + \ldots + n(K_1K_2 \ldots K_n)[X]^n}{1 + K_1[X] + K_1K_2[X]^2 + \ldots + (K_1K_2 \ldots K_n)[X]^n}$$

terms of elementary processes governed by the law of mass action (Klotz, 2004; Klotz, 1946). The equation describing fractional occupancy in his framework is shown in Figure 1-4C. It is often used in experimental literature to describe ligand binding in terms of sequential apparent binding constant (Stefan and Le Novere, 2013).

Adair and Klotz models are purely thermodynamic equations based on mass-law principles, which do not address the underlying molecular mechanisms of binding cooperativity. When it was realized that cooperative binding is accompanied by ligand-induced conformational changes of biological macromolecules, as exemplified by X-ray structural studies of hemoglobin in the 1960s (Perutz, 1965, 1970, 1976), additionalframeworks that attempted to incorporate the concept of allostery were proposed to explain cooperative binding, such as the concerted model (or MWC model) and sequential models, exemplified by the KNF model (or induced fit model). Due to space limitations, these schemes will not be described here. Interested readers can find more information in the review by Stefan and Novere (Stefan and Le Novere, 2013) and books dealing with the subject (Cantor and Schimmel, 1980; Wyman and Gill, 1990).

IV. Dissertation overview

The model for silent chromatin assembly in *S. cerevisiae* described in previous sections leaves many questions unanswered. For example, we do not know exactly how Sir proteins spread along the chromatin, what set of molecular interactions govern the spreading, and how their spreading is regulated by histone modifications and Sir-Sir protein interactions. My thesis research attempted to answer these questions by studying the association of Sir3 proteins with well-defined *in vitro*-reconstituted nucleosome templates, and determined how this association is affected by the Sir4

coiled-coil (Sir4 CC) domain and histone modifications. In Chapter II, I will describe my studies on Sir3 binding to mono-nucleosome versus binding to di-nucleosome templates. I find that Sir3 binds to nucleosomes with strong inter-nucleosomal cooperativity, and that this cooperativity requires the Sir3 wH dimerization domain. Sir4 CC also contributes towards the SIR inter-nucleosomal cooperative binding. In Chapter III, I will present quantitative analysis on how Sir3 binding to nucleosomes is affected by histone modifications, in particular by H4K16 acetylation, H3K79 methylation, and the two modifications in combination. In Chapter IV, I will discuss implications of this work for the mechanism of Sir3-nucleosome interaction and SIR complex-chromatin association. I will also discuss some open questions in the field, and propose future directions that could help shed light on the molecular mechanisms of silent chromatin assembly and its epigenetic inheritance in *S. cerevisiae.*

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CHAPTER II

Analysis of Cooperative Interactions Between Sir Proteins and Nucleosomes

AUTHOR CONTRIBUTION

This study was initially motivated by the cooperativity hypothesis proposed by Danesh Moazed to explain the epigenetic inheritance of silent chromatin in *S. cerevisiae* (Moazed, 2011). Results described in this chapter have been submitted for publication, whose authors include Chenning Lu, Ian Dodd and Danesh Moazed. Chenning Lu and Danesh Moazed designed the experiments. Chenning Lu prepared all samples for the chromatin immunoprecipitation (ChIP) experiment. Gloria Jih constructed the libraries for multiplex ChIP-seq experiments, and mapped the sequenced reads. Ruby Yu processed data for the ensemble plots. Chenning Lu performed all other experiments and all data analysis. Ian Dodd performed the statistical mechanical modeling.

ABSTRACT

Silent chromatin in *Saccharomyces cerevisiae* is analogous to heterochromatin in multicellular eukaryotes. Besides nucleosomes, its major structural component is the Silent Information Regulator (SIR) complex, composed of Sir2, an NAD-dependent histone deacetylase, Sir3, a nucleosome-binding protein, and Sir4. Sir2 and Sir4 form a stable heterodimer. Both Sir3 and Sir4 form homodimers, through their winged helix (wH) and coiled-coil (CC) domains, respectively. The Sir4 CC domain also interacts with the Sir3 AAA-ATPase-like (AAAL) domain. The SIR complex spreads along chromatin in a sequence-independent manner. However, the nature of the Sir-Sir and Sir-histone interactions that mediate SIR complex spreading is not fully understood. In this study, we investigate the interaction of Sir proteins with well-defined *in vitro* reconstituted mono- and di-nucleosomes. We find that Sir3 binds cooperativity to di-, but not mono-nucleosomes. The cooperative binding of Sir3 to di-nucleosomes is mediated by the wH dimerization domains of Sir3 proteins bound to adjacent nucleosomes. This inter-nucleosomal cooperative binding is further stabilized by Sir4 CC domain. Sir3 is furthermore able to bridge free mono-nucleosomes in solution in a wH domain-dependent manner. Surprisingly, the binding measurements suggest that there are no Sir-Sir interactions on the same nucleosome, arguing against polymerization-based models for SIR complex spreading. Finally, consistent with the *in vitro* binding studies, ChIP-seq experiments indicate that both Sir3 wH and Sir4 CC domains are required for efficient Sir3 spreading *in vivo* but could each mediate some Sir3 spreading in the absence of the other. Together these findings indicate that the SIR complex forms an inter-nucleosomal bridge and suggest a novel mechanism of its spreading on chromatin.

INTRODUCTION

Silent chromatin or heterochromatin is a conserved feature of eukaryotic chromosomes that plays important roles in regulation of gene expression and maintenance of chromosome stability (Grewal and Moazed, 2003; Moazed, 2001; Richards and Elgin, 2002; Rusche et al., 2003). Studies in yeast, flies, and mammals have revealed diverse mechanisms by which cells establish, maintain, and regulate heterochromatin. However, despite the divergence of molecular components, general mechanisms of heterochromatin assembly appear to remain strikingly similar during evolution (Beisel and Paro, 2011; Grewal and Moazed, 2003; Moazed, 2001, 2011; Richards and Elgin, 2002). Heterochromatin assembly involves an initial recruitment or nucleation event in which specificity factors bind to silencers, and recruit chromatin-modifying complexes that spread along chromatin in a sequence-independent manner. The spreading is regulated by histone post-translational modifications and interactions between the recruited proteins, but how specific and processive spreading occurs is poorly understood.

In eukaryotes, nuclear DNA is packaged with histones and other proteins into chromatin. The fundamental repeating unit of chromatin folding is the nucleosome, in which 147 base pairs of DNA wraps around an octamer of 2 copies of each histones H2A, H2B, H3, and H4 (Kornberg, 1977; Luger et al., 1997). The highly conserved histones contain N-terminal tails and core globular domains, both of which provide binding sites for numerous chromatin-associated proteins (Jenuwein and Allis, 2001; Schreiber and Bernstein, 2002; Strahl and Allis, 2000). The binding of these proteins to chromatin is regulated by reversible post-translational modifications of histones, such as acetylation, methylation, phosphorylation, and ubiquitylation of specific amino acids in

histones (Kouzarides, 2007; Li et al., 2007). In yeast and *Drosophila,* site-specific DNA-binding proteins, bound to specific DNA sequences called silencers, recruit histone-binding and –modifying proteins, and generate the specific histone modification patterns associated with silenced chromatin regions (Muller and Kassis, 2006; Rusche et al., 2003).

Due to its genetic and biochemical tractability, silent chromatin in budding yeast *S. cerevisiae* has served as a major model system for studies of heterochromatin establishment and epigenetic inheritance. There are three regions of silent chromatin in the *S. cerevisiae* genome, the silent mating type or homothallic loci (*HM* loci, *HMR* and *HML*), the subtelomeric regions, and the ribosomal DNA (rDNA) tandem arrays (Rusche et al., 2003). While silencing at rDNA loci takes place through a distinct mechanism, silencing at *HM* loci and telomeres share mechanistic features, and require silent information regulator (Sir) proteins, Sir2, Sir3, and Sir4, which together form the SIR complex, one of the structural components of silent chromatin (Aparicio et al., 1991; Klar et al., 1979; Moazed et al., 1997; Rine and Herskowitz, 1987; Rusche et al., 2003; Smith and Boeke, 1997).

Current models propose that the assembly of silent chromatin at *HM* loci and telomeres occurs in a stepwise manner. During the initiation or establishment step, the Sir2 and Sir4 proteins, which together form a stable Sir2/Sir4 heterodimer (Moazed et al., 1997), and Sir3, are recruited to the silencer, through interactions with silencer-specificity factors, ORC, Abf1, and Rap1 (Hoppe et al., 2002; Luo et al., 2002; Moretti et al., 1994; Moretti and Shore, 2001; Rusche et al., 2002; Triolo and Sternglanz, 1996). The Sir2 subunit, which is an NAD-dependent deacetylase (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000), then deacetylates silencer-proximal nucleosomes, particularly the H4

lysine 16 (H4K16) residue, creating a binding site for Sir3 (Armache et al., 2011; Carmen et al., 2002; Liou et al., 2005; Wang, 2013). Subsequent iterative cycles of deacetylation and Sir protein association lead to the spreading of SIR complexes along multiple kilobases of chromatin away from the silencer in a sequence-independent manner (Hoppe et al., 2002; Luo et al., 2002; Rusche et al., 2002). Consistent with this model, deletion of Sir2 or mutations that disrupt its catalytic activity results in inefficient recruitment and spreading of Sir3, and loss of silencing (Hoppe et al., 2002; Rusche et al., 2002; Tanny et al., 1999). However, the nature of the Sir-Sir interactions and Sir-histone interactions that promotes sequence-independent spreading is not fully understood.

Sir3, the major nucleosome-binding subunit in the SIR complex, interacts with nucleosomes in several regions, particularly the basic patch covering histone H4 residues 16-19 in the H4 N-terminal tail, and the region centered around histone H3 lysine 79 (H3K79) residue in the H3 globular domain, located on the nucleosome core surface (Armache et al., 2011; Wang, 2013). Sir3 binding to nucleosomes is sensitive to either H4K16 acetylation or H3K79 methylation, as indicated by a combination of genetic and biochemical experiments (Altaf et al., 2007; Carmen et al., 2002; Johnson et al., 2009; Liou et al., 2005; Martino et al., 2009; Ng et al., 2002; Onishi et al., 2007; van Leeuwen et al., 2002). Sir3-histone interactions are required for the efficient recruitment of SIR complex to the silent chromatin and silencing (Hoppe et al., 2002; Luo et al., 2002; Rudner et al., 2005; Rusche et al., 2002).

In addition to Sir-histone interactions, multiple homo- and hetero-typic interactions among Sir proteins are essential for silencing. Sir3 forms dimers and oligomers *in vitro* (King et al., 2006; Liaw and Lustig, 2006; Liou et al., 2005; McBryant et

al., 2006; Moretti et al., 1994). Sir3 dimerization is mediated by its C-terminal winged helix (wH) domain (Oppikofer et al., 2013). Deletion of the wH domain abolishes Sir3 association with silent chromatin regions, and disrupts silencing at both *HM* loci and telomeres (Oppikofer et al., 2013). Sir4 also forms homodimers, via its C-terminal coiled-coil (CC) domain, and this dimerization activity is required for silencing at both telomeres and the *HML* locus (Chang et al., 2003; Chien et al., 1991; Murphy et al., 2003). The Sir4 CC domain interacts with Sir3, through the Sir3 AAA ATPase-like (AAAL) domain (Chang et al., 2003; Ehrentraut et al., 2011; King et al., 2006). The interaction of Sir3 and Sir4 is required for the recruitment of Sir3 to silencers, and for the spreading of Sir proteins away from the silencer, and is essential for silencing (Ehrentraut et al., 2011; Rudner et al., 2005).

In order to determine how the Sir proteins associate with specific chromosome regions, and how their spreading is regulated by Sir-histone and Sir-Sir protein interactions, we compared the association of Sir3 proteins with *in vitro*-reconstituted mono- versus di-nucleosome templates. We also examined how this association is affected by the Sir4 CC domain. We find that Sir3 binds to di-nucleosomes with strong cooperativity. This cooperative binding requires the Sir3 wH dimerization domain, suggesting that this domain mediates lateral Sir3-Sir3 interactions across two nucleosomes. Consistent with this hypothesis, we demonstrate that Sir3 is able to bridge free mono-nucleosomes in solution, and that this bridging activity requires the wH domain. The loss of Sir3 binding cooperativity towards di-nucleosomes due to deletion of the wH domain can be restored by the addition of Sir4 CC, which also increases full-length Sir3 binding cooperativity, suggesting that Sir4 CC also contributes to the stable binding of the SIR complex to di-nucleosomes. Quantitative modeling of our binding data

supports the hypothesis that spreading of the SIR complex involves interactions between the wH domains of Sir3 proteins bound to adjacent nucleosomes and its stabilization by the Sir4 CC domain. Our findings furthermore suggest that there are no Sir-Sir interactions on the same nucleosome and argue against a polymerization-based spreading mechanism.

RESULTS

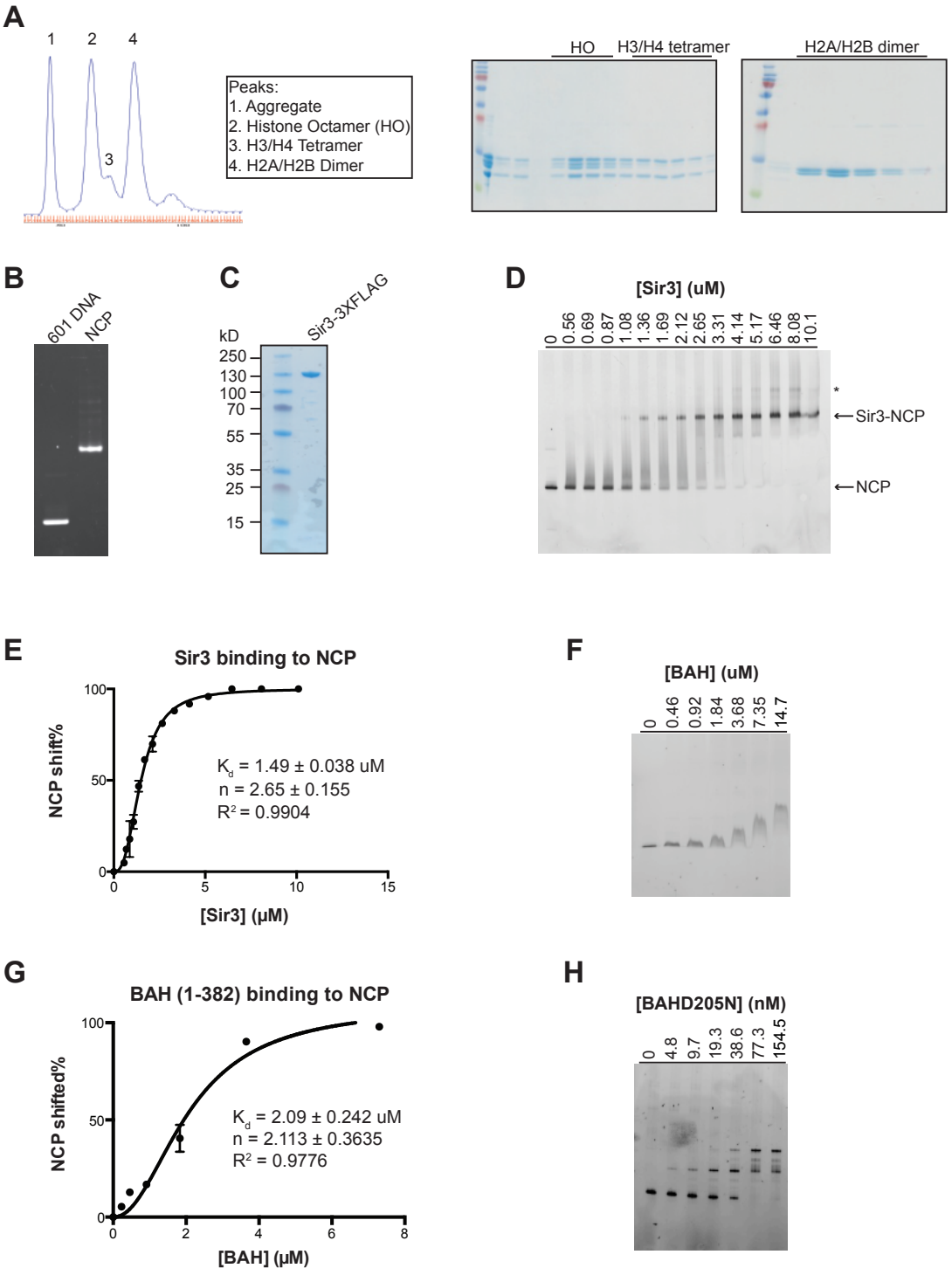
Sir3 and its BAH domain binds to mono-nucleosomes non-cooperatively

In order to investigate the mechanism of Sir3 association with chromatin, we started with studying Sir3 binding to the nucleosome core particle (NCP). Nucleosomes were reconstituted by the salt-gradient dialysis method, as described previously (Luger et al., 1999), using recombinant *S. cerevisiae* histones purified from *E. coli*, and the 147-bp 601 nucleosome positioning sequence (Lowary and Widom, 1998) (Figure 2-1A-B). Sir3 and its bromo-adjacent homology (BAH) domain were overexpressed and purified from *S. cerevisiae* (Figure 2-1C) (Buchberger et al., 2008; Johnson et al., 2009; Liou et al., 2005). Using electrophoretic mobility shift assays (EMSA) and fitting the binding curve with the Hill Equation, we found that Sir3 bound to unmodified NCP with a K_D around 1.5 μM (Figure 2-1D-E). The BAH domain bound to the NCP with slightly lower affinity (K_D around 2.0 μM) (Figure 2-1F-G), consistent with it being the major domain responsible for Sir3 binding to nucleosomes (Onishi et al., 2007). Sir3-NCP binding resulted in only one shifted band, and both Sir3 and BAH domain bound to the NCP with Hill coefficient around 2, suggesting that they might bind to the NCP cooperatively, with two sides of the

Figure 2-1. Sir3 and the bromo-adjacent homology (BAH) domain binding to unmodified nucleosome core particle (NCP).

(A) Purification and analysis of the yeast histone octamer (HO) using gel filtration. HO refolded from individual histones was purified on a gel filtration column. Fractions from the gel filtration column (left) were run on denaturing SDS polyacrylamide gel (right). Fractions containing HO were pooled and used in nucleosome reconstitution. **(B)** Native polyacrylamide gels showing reconstituted NCP. **(C)** Purified Sir3 protein used in EMSA experiments. **(D)** Sir3 binding to unmodified NCP. Purified Sir3 proteins were titrated onto a constant amount of NCP at 3 nM. Samples were separated on native gels, nucleosomes were stained with SYBR Gold, and the amount of unbound nucleosomes was quantified by the staining intensity of the unshifted nucleosome band. **(E)** Quantification and analysis of Sir3 binding to NCP. Binding curves from three experiments performed as in **D** were fitted with the Hill Equation ($\theta=B_{max}*[S]^n/(K_d^n + [S]^n)$. **(F)** BAH binding to unmodified NCP. Recombinant BAH proteins were overexpressed and purified from *S. cerevisiae*, and titrated onto a constant amount of NCP at 3 nM. **(G)** Quantification and analysis of BAH binding to NCP. Binding curves from three experiments performed as in **F** were fitted with the Hill Equation. *, higher mobility shifted band that may result from either bridging of mono-nucleosomes by Sir3 or other minor high molecular weight Sir3-NCP complexes. **(H)** BAH-D205N binding to unmodified NCP. Recombinant BAH-D205N proteins were overexpressed and purified from *S. cerevisiae*, and titrated onto a constant amount of NCP at 3 nM.

Figure 2-1 (Continued)



nucleosome simultaneously occupied. However, solution binding assays performed by Reza Behrouzi, a postdoc in the lab, suggested that Sir3 and the BAH domain bound to the NCP with weak cooperativity, with a Hill coefficient around 1.2 (personal communication). The apparent high Hill coefficient in EMSA might therefore be an artifact, caused by the dissociation of the singly bound Sir3/BAH species during gel electrophoresis, while the doubly bound species survived the gel shift assay. This is supported by the observation that instead of one shifted band, binding of the NCP by the hypermorphic BAH-D205N (Armache et al., 2011; Brogaard et al., 2012; Connelly et al., 2006; Johnson et al., 1990; Park et al., 1998) produced a strong and clear intermediate shifted band (Figure 2-1H). The smearing between the unshifted and shifted bands in the Sir3- and BAH-NCP EMSA may be due to dissociation of the unstable singly-bound species (Figure 2-1D and F). This lack of intra-nucleosomal cooperativity suggests that Sir3 proteins bound to the same nucleosome cannot interact with each other.

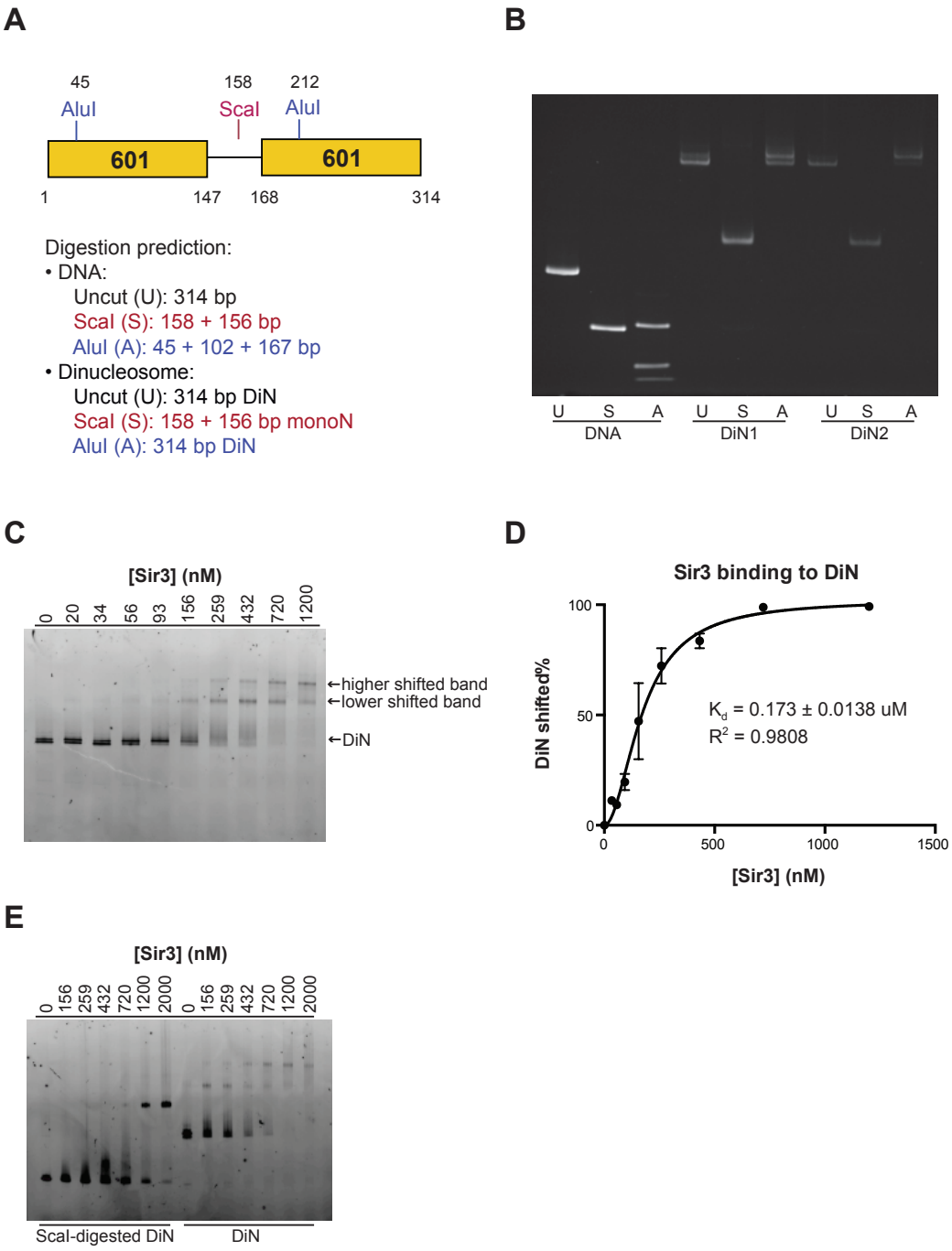
Sir3 binds to di-nucleosomes cooperatively

We also studied Sir3 binding to reconstituted di-nucleosomes (DiN). We reasoned that the potential ability of Sir3 proteins bound to adjacent nucleosomes to interact with each other should result in cooperative binding that would be reflected in measurements of binding affinities and calculations of a cooperativity coefficient for binding to di-nucleosomes. For the di-nucleosome reconstitution, the DNA template we used composed of direct repeats of the 601 sequence, separated by a 20-bp linker (Figure 2-2A). Inter-nucleosomal linker DNA in the *S. cerevisiae* silent chromatin regions has a heterogeneous length distribution (Brogaard et al., 2012; Ravindra et al., 1999; Weiss and Simpson, 1998). We chose the linker DNA to be 20 bp, which reflects the average

Figure 2-2. Sir3 binding to unmodified di-nucleosomes (DiN).

(A) Illustration of the DiN DNA construct, and the predicted restriction enzyme digestion pattern for naked DNA and fully reconstituted DiN. **(B)** Restriction enzyme protection assay of reconstituted DiN. **(C)** Sir3 binding to unmodified DiN. Purified Sir3 proteins were titrated onto a constant amount of DiN at 3 nM. **(D)** Binding curves from three experiments performed as in **C** were fitted with the Hill Equation. **(E)** Sir3 binding to DiN and to mono-nucleosome released from ScaI digestion of DiN.

Figure 2-2 (Continued)



linker DNA length in *S. cerevisiae* (Arya et al., 2010). The quality of the reconstituted DiN was assessed by restriction enzyme protection (Figure 2-2B).

Sir3 bound to DiN with an apparent K_D around 0.173 μM (Figure 2-2C-D), a more than 8 fold decrease relative to the K_D of binding to NCP, indicating that Sir3 bound to DiN cooperatively. Sir3 has been shown to exhibit nonspecific affinity towards free DNA. To eliminate the possibility that the increased Sir3 binding affinity towards DiN resulted from the presence of linker DNA, we assessed Sir3 binding to mono-nucleosomes released from ScaI digestion of the DiN. As shown in Figure 2-2E, Sir3 bound to mono-nucleosomes, released from the digestion of DiNs, with an affinity similar to that observed for the NCP (Figure 2-1D), and to the DiN prior to digestion with greater affinity.

To analyze the inter-nucleosomal binding cooperativity quantitatively, we used a cooperativity binding equation derived from the Klotz model, which breaks down molecular interactions into intermediate steps (Cantor and Schimmel, 1980; Klotz, 2004; Stefan and Le Novere, 2013) (Figure 2-3A, Equation 1). In this scheme, Sir3 binding to DiN was dissected into two steps: during the first step, one mono-nucleosome within the DiN was occupied by Sir3; during the second step, the second nucleosome became occupied (Figure 2-3A). Since we were interested in the inter-nucleosomal cooperativity, we simplified the Sir3-mononucleosome binding as a single-step event, with an apparent characteristic dissociation constant k (Figure 2-3A, Equation 1), such that Sir3 bound to the first nucleosome with affinity k, and bound to the second nucleosome with affinity k/c, with c being the parameter for inter-nucleosomal cooperativity (see Materials and Methods). Fitting the Sir3-DiN binding curve with Equation 1 gave a cooperativity value (c) of around 60 (Figure 2-3B). This c value translates into a cooperativity free energy, $\triangle G^0_{coop}$, of -2.4 kcal/mol (Figure 2-3C), which is in the same range as the net free

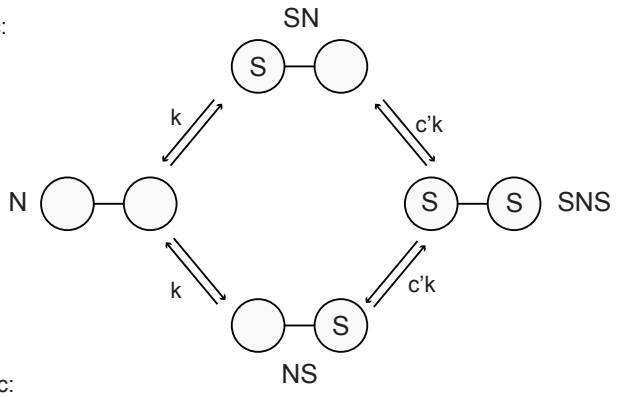
Figure 2-3. Thermodynamic model for analysis of Sir3 inter-nucleosomal cooperative binding to DiN.

(A) Sir3 binding to DiN can be dissected into two sequential binding steps, binding to one mono-nucleosome first, and followed by binding to the second mono-nucleosome. Our analysis model assumes that Sir3 binds to each mono-nucleosome within the DiN in exactly the same way as binding to NCP. This binding is characterized by the microscopic characteristic k, which equals to K_D measured from Sir3-NCP binding, with the exception that Sir3 binds to the second mono-nucleosome with a cooperativity coefficient, c. **(B)** Sir3 cooperative binding to unmodified DiN, as analyzed by Equation 1 (see Materials and Methods), indicating a c value of 60. **(C)** Calculation of free energy of cooperativity. ΔG^0_{coop} was calculated from the equation $\Delta G^0_{coop} = -RT\ln c$.

Figure 2-3 (Continued)

A

Microscopic:

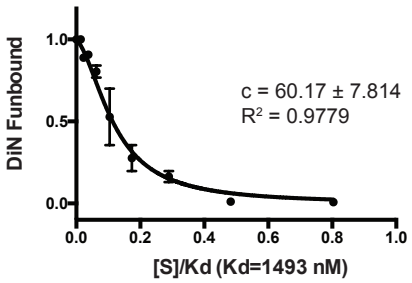


Macroscopic:

$$N \underset{}{\overset{K_1}{\rightleftharpoons}} NS + SN \overset{K_2}{\rightleftharpoons} SNS$$

- K_{D1}=k/2, K_{D2}= 2c'k, where k is the apparent characteristic dissociation constant of Sir3 binding to NCP
- **$F_{unbound}$=1/(1 + 2[S]/k + c*([S]/k)²)**, c = 1/c', c>1 indicates positive cooperativity **[Equation 1]**

B



C

$K_{Dc} = K_{D2} = 2k/c$, $K_{Dnc} = 2k/1 = 2k$

$\Delta\mathbf{G^0_{coop}} = \Delta\Delta G^0_{nc\text{-}c} = \Delta G^0_{nc} - \Delta G^0_{c}$

$= -RT\ln K_{Dnc} + RT\ln K_{Dc} = RT\ln(K_{Dc}/K_{Dnc})$

$\mathbf{= -RT\ln c}$

$\Delta\mathbf{G^0_{coop}}$ **=** -2.4 kcal/mol for unmodified DiN

energy change due to positive cooperativity between pairs of adjacently bound λ repressors, estimated to be 2-3 kcal/mol (Johnson et al., 1979).

Sir3 cooperative binding to di-nucleosome is mediated by its C-terminal wH dimerization domain

We next tried to elucidate the molecular mechanism of Sir3 cooperative binding to DiN. According to our hypothesis, the inter-nucleosomal cooperative binding is mediated by lateral interactions of Sir3 bound to adjacent nucleosomes. It has been shown that the Sir3 C-terminal wH domain is necessary and sufficient for Sir3 dimerization, and is required for Sir3 spreading and silencing (Figure 2-4) (Oppikofer et al., 2013). Sir3 may also form higher order oligomers *in vitro* that may result from interactions between its AAAL domain (Liaw and Lustig, 2006; Liou et al., 2005; McBryant et al., 2006; Moretti et al., 1994), although recent structural data suggest that the AAAL domain of Sir3 does not form oligomers (Ehrentraut et al., 2011). To test whether the wH domain is responsible for Sir3 cooperative binding to DiN, we overexpressed and affinity purified Sir3 lacking the wH domain (Sir3ΔwH) from *S. cerevisiae* (Figure 2-5A), and studied its binding to both NCP and DiN with EMSA.

We found that Sir3ΔwH bound to the NCP with a K_D around 1.3 μM (Figure 2-5B-C), similar to the K_D value (1.5 μM) we observed for the full-length Sir3 binding to the NCP. Deletion of the wH domain therefore did not affect Sir3 affinity for mono-nucleosomes. In addition, Sir3ΔwH bound to DiN with only about 2 fold increased affinity relative to the NCP (K_D around 0.69 μM for DiN, and 1.25 μM for NCP) (Figure 2-5B-C). This was in contrast with the greatly increased affinity of full-length Sir3 for DiN compared to NCP (Figure 2-2C-E). Cooperative binding analysis showed that the

Figure 2-4. Sir3 C-terminal winged helix (wH) dimerization domain is required for subtelomeric and *HMR* silencing.

(A) Silencing assay. The indicated plasmids, producing Sir3 wild type or Sir3ΔwH from a CEN vector under the Sir3 endogenous promoter, were introduced into strain W303a *sir3Δ::Kan*^{R} *hmrΔE::TRP1* TELVII-L::URA3, and assayed for silencing. Growth on medum with 5-fluoroorotic acid (+5FOA) and lack of growth on medium lacking tryptophan (-Trp) indicate silencing. Tenfold serial dilutions of wild type and mutant strains were plated on the indicated growth medium. **(B)** Western blot analysis showing that Sir3ΔwH is expressed in the same level as Sir3 wild type.

Figure 2-4 (Continued)

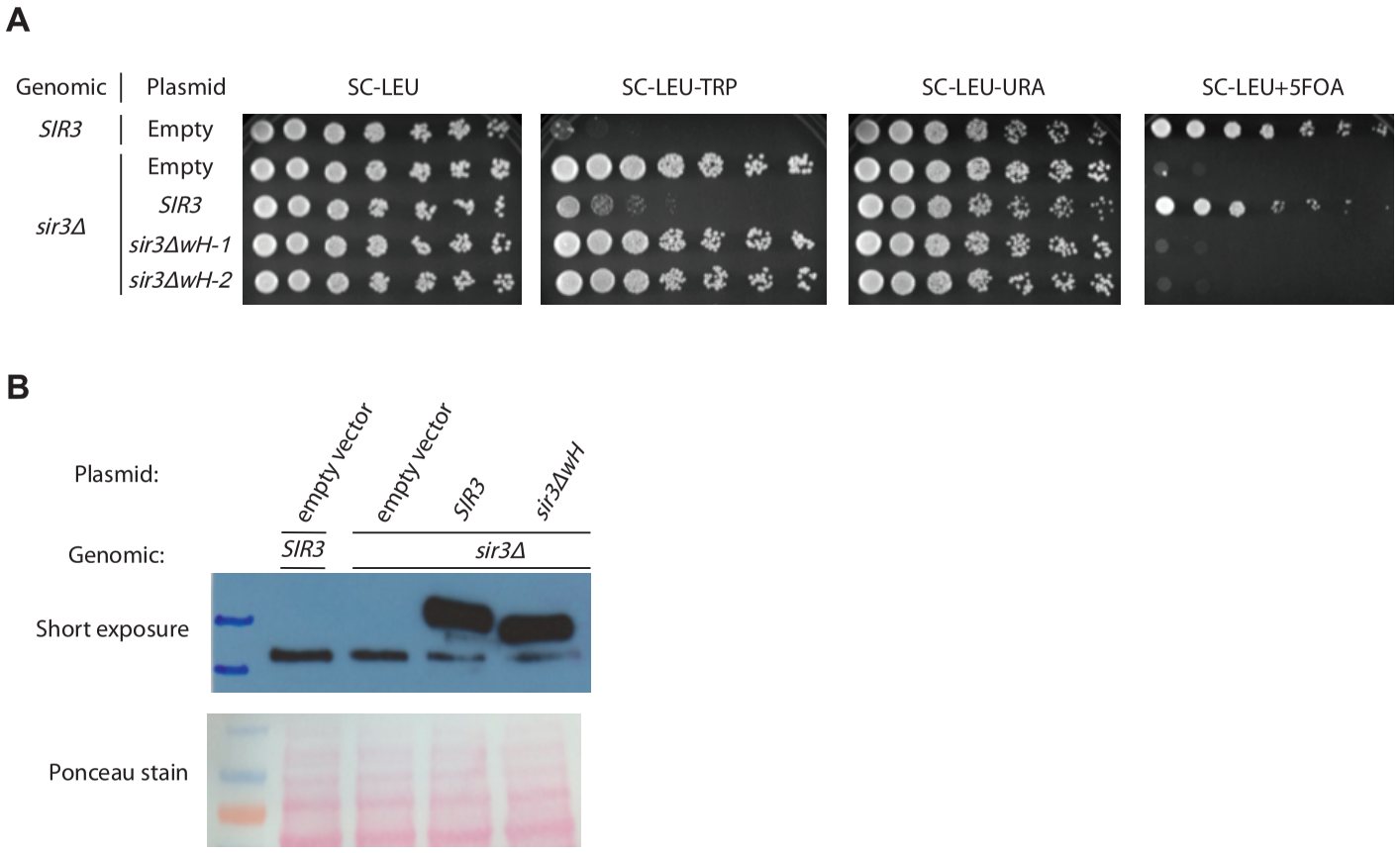
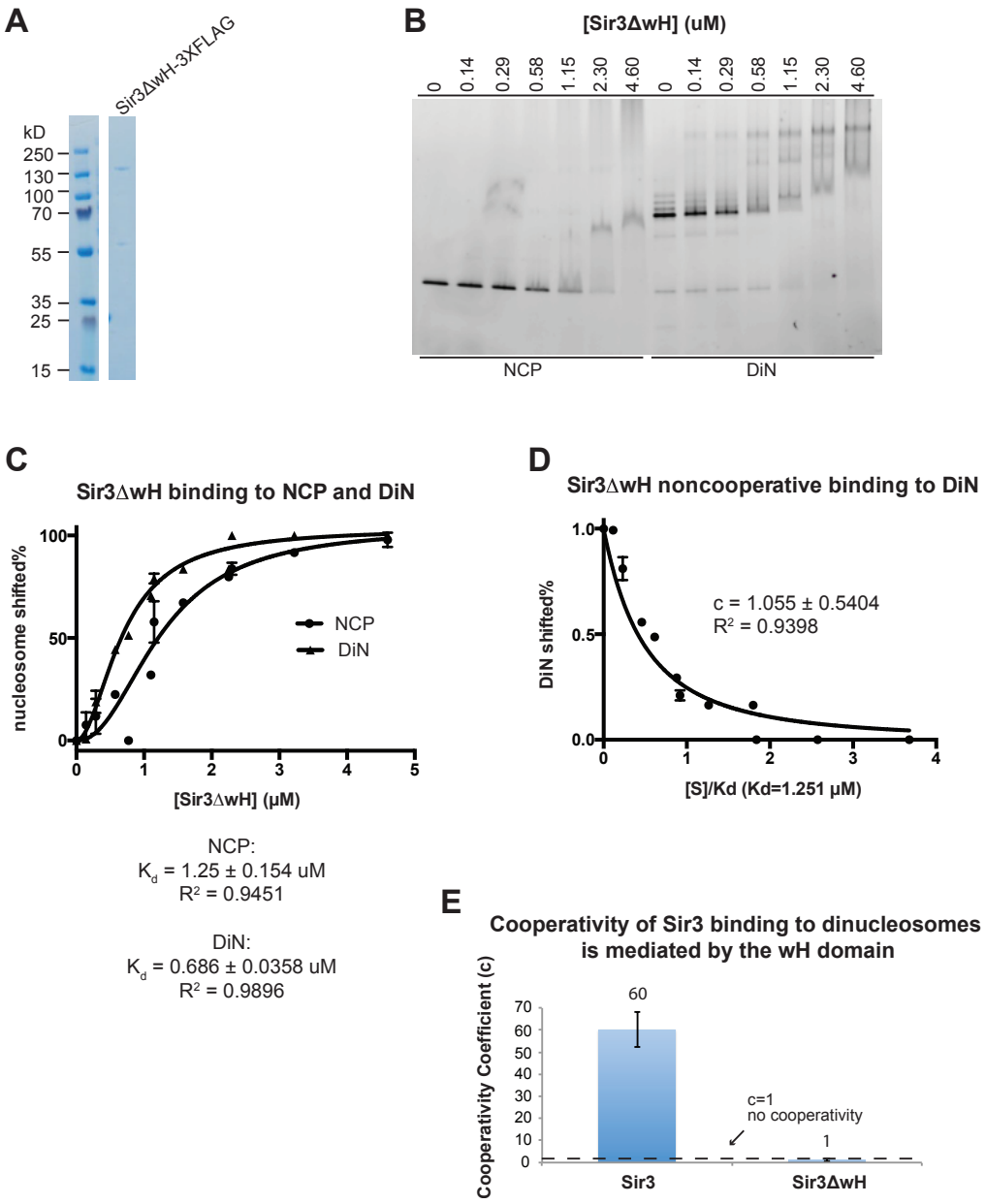


Figure 2-5. The winged helix (wH) domain of Sir3 mediates its cooperative binding to di-nucleosomes.

(A) Coomassie-stained gel showing purified Sir3ΔwH protein used in EMSA experiments. **(B)** EMSA experiments comparing Sir3ΔwH binding to unmodified NCP and DiN. Purified Sir3ΔwH proteins were titrated onto a constant amount of NCP or DiN at 3 nM. **(C)** Binding curves from three experiments performed as in **B** were fitted with the Hill Equation. Sir3ΔwH binds to NCP with a Hill coefficient of 1.76 ± 0.393, and to DiN with a Hill coefficient of 1.53 ± 0.220. **(D)** Sir3ΔwH binds non-cooperatively to DiN, with a cooperativity c value of 1, as analyzed by Equation 1. **(E)** Comparison of cooperativity of Sir3 (from Figure 2-3B) and Sir3ΔwH binding to unmodified DiN.

Figure 2-5 (Continued)



cooperativity coefficient of Sir3ΔwH binding to DiN was around 1, indicating that the ability of Sir3 to bind DiN cooperatively was lost when its wH domain was deleted (Figure 2-5D-E). We therefore conclude that the wH dimerization domain is required for Sir3 cooperative binding to DiN, without affecting Sir3 binding affinity to mono-nucleosomes. These results suggest that Sir3 wH domain bound to one nucleosome is able to interact with the wH domain of another Sir3 molecule bound on an adjacent nucleosome.

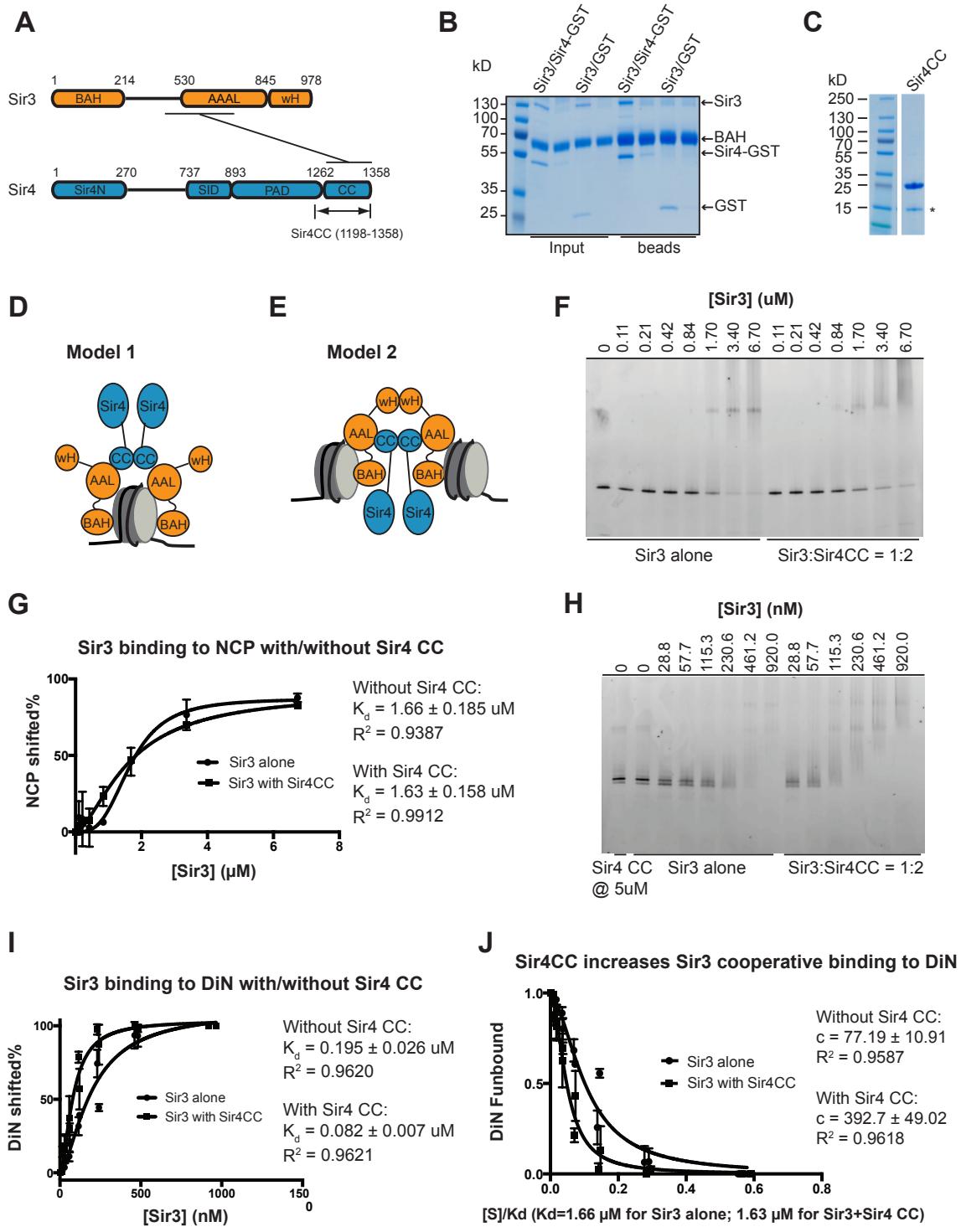
Sir4 coiled-coil dimerization domain enhances the Sir3 cooperative binding to di-nucleosomes

We next wanted to investigate how Sir4 interacts with Sir3 on the nucleosome. Sir4 forms dimers through its C-terminal CC domain (Chang et al., 2003; Murphy et al., 2003). The surface of the CC domain also provides binding sites for two Sir3 molecules via interactions with the Sir3 AAAL domains (Chang et al., 2003). Thus the Sir4 CC domain constitutes the smallest domain that is necessary and sufficient for both Sir4-Sir4 and Sir3-Sir4 interactions (Figures 2-6A-B). We therefore used Sir4 CC as a surrogate of the full length Sir4 to study its effect on Sir3 binding to nucleosomes. There are at least two possible models of Sir4 interacting with Sir3-nucleosome complexes. In the first model, the Sir4 CC mediates interactions between two Sir3 molecules bound to each side of the same nucleosome (Figure 2-6D). In the second model, the Sir4 CC interacts with two Sir3 molecules bound to two neighboring nucleosomes, adding a second layer of inter-nucleosomal interactions (Figure 2-6E). The two models predict different Sir4 CC effects on the binding affinities of Sir3 for the NCP, and different Sir4 CC effects on the binding cooperativity of Sir3 towards DiNs, and therefore can be distinguished by EMSA experiments. The first model predicts that Sir3/Sir4 CC has higher binding affinity towards NCP than Sir3 alone. Furthermore, because Sir3 already mediates

Figure 2-6. Sir4 coiled-coil (CC) domain does not change Sir3 binding affinity towards mono-nucleosomes, but increases its binding cooperativity towards di-nucleosomes.

(A) Schematic diagram showing the domain organization of Sir3 and Sir4. BAH: bromo-adjacent homology domain, AAAL: AAA-ATPase-like domain wH: winged helix domain, Sir4N: Sir4 N-terminal domain, SID: Sir2-interaction domain, PAD: partitioning and anchoring domain, CC: coiled-coil domain. Sir4 CC domain interacts with Sir3 AAAL domain and the region N-terminal to it, as illustrated by a line connecting the mapped interacting regions. The Sir4 CC domain (a.a. 1198-1358, indicated below Sir4) was purified and used in the biochemical assays in the current study. **(B)** Coomassie-stained gel of the pull down experiment showing that Sir3 interacts with Sir4 CC (1198-1358)-GST, but not with the GST negative control. **(C)** Coomassie-stained gel showing purified Sir4 CC protein (a.a. 1198-1358) used in EMSA experiments. *: Degradation product that corresponds to Sir4 a.a. 1242-1358, as confirmed by Mass Spectrometry. **(D, E)** Two models for the association of Sir3 and Sir4 proteins with nucleosomes. **(F)** EMSA experiments comparing Sir3 binding to NCP in the presence or absence of Sir4 CC. Proteins were titrated onto a constant amount of NCP at 3 nM. **(G)** Binding curves from three experiments performed as in **F** were fitted with the Hill Equation. **(H)** Comparison of Sir3 binding to DiN in the presence or absence of Sir4 CC. Proteins were titrated onto a constant amount of DiN at 3 nM. **(I)** Binding curves from three experiments performed as in **H** were fitted with the Hill Equation. **(J)** Cooperative binding analysis of Sir3/Sir4 CC binding to DiN showing that Sir4 CC increases the cooperativity of Sir3 binding to DiN.

Figure 2-6 (Continued)



inter-nucleosomal interactions, the Sir3 binding cooperativity towards di-nucleosomes will not change upon the addition of Sir4 CC (Figure 2-6D). In contrast, the second model predicts (1) higher binding cooperativity of Sir3/Sir4 CC towards di-nucleosomes compared with Sir3 alone, since in this case two layers of inter-nucleosomal cooperativity are provided, one by the dimerizing Sir3 wH domain, and the other by the interaction of Sir4 CC with Sir3 AAAL domains (Figure 2-6E), and (2) no change in the binding affinity of Sir3 towards mono-nucleosomes upon the addition of Sir4 CC.

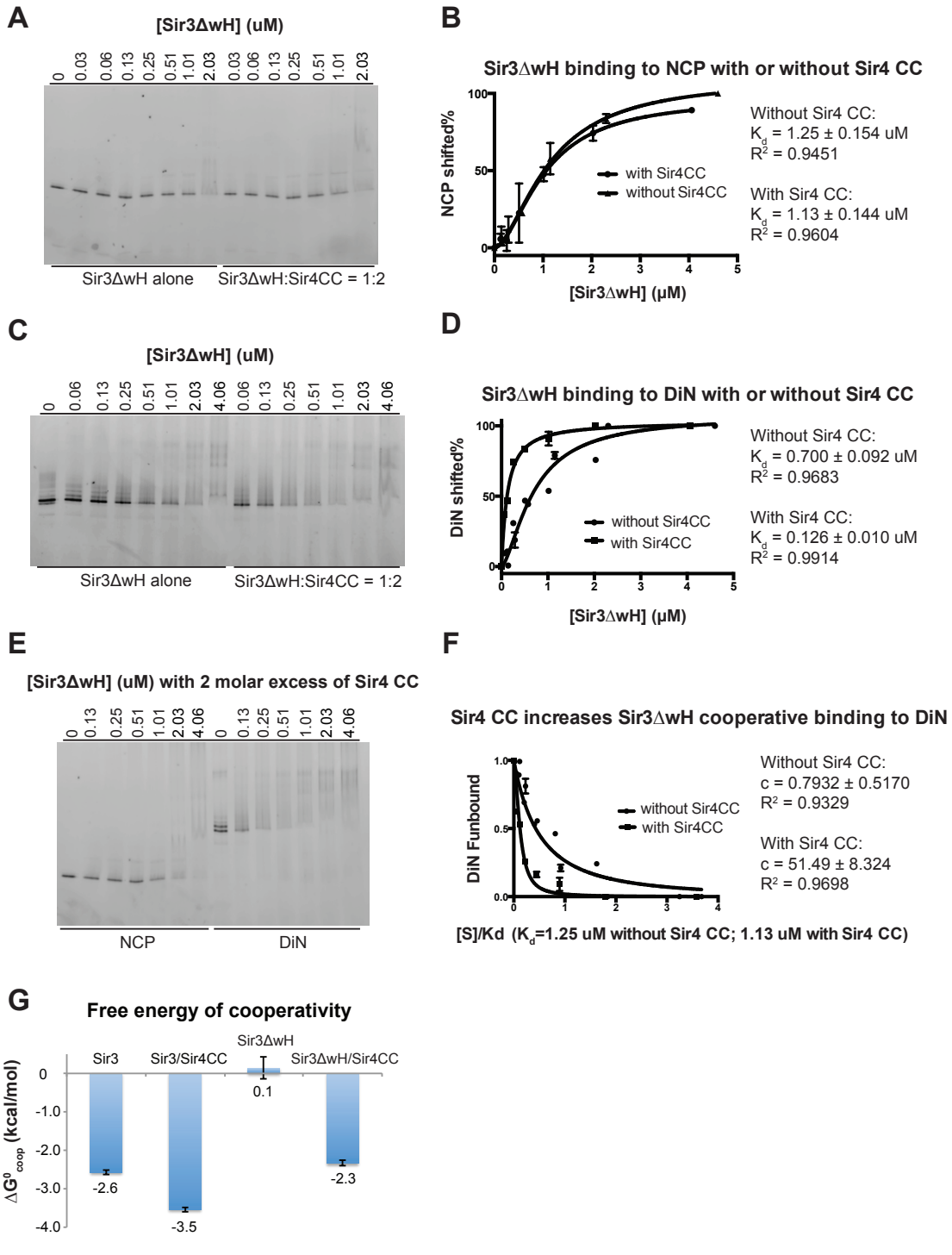
We overexpressed and affinity purified from *E. coli* the Sir4 fragment a.a. 1198-1358, containing the core Sir4 CC domain (a.a. 1262-1358) (Chang et al., 2003) (Figure 2-6C). EMSA experiments showed that although the addition of Sir4 CC caused a slight supershift of Sir3-NCP band, it did not affect Sir3-NCP binding affinity, as the K_D values were similar with or without Sir4 CC (Figure 2-6F-G). In contrast, Sir4 CC decreased the apparent K_D value of Sir3 binding to DiN about 2 fold, from 0.195 to 0.082 μM (Figure 2-6H-I). This translates to an increase in the Sir3 binding cooperativity, c, towards DiN from 77 to 393 (Figure 2-6J). These results agree with the predictions of the second model (Figure 2-6E) and suggest that Sir4 binds to Sir3 proteins that bridge neighboring nucleosomes.

Consistent with Sir3/Sir4 CC binding results (Figure 2-6), Sir4 CC did not affect the binding affinity of Sir3ΔwH towards NCP, but increased its binding cooperativity towards DiN (Figure 2-7A-F). In the absence of Sir4 CC, Sir3ΔwH did not show any binding cooperativity towards DiN, but the addition of Sir4 CC restored its binding cooperativity to about the same level as full-length Sir3 (Figure 2-7E-F). Either Sir3 wH or Sir4 CC alone conferred a cooperativity free energy of around -2.5 kcal/mol towards

Figure 2-7. Sir4 coiled-coil (CC) domain does not change Sir3ΔwH binding affinity towards mono-nucleosomes, but increases its binding cooperativity towards di-nucleosomes.

(A) EMSA experiments comparing Sir3ΔwH binding to NCP in the presence or absence of Sir4 CC. **(B)** Binding curves from three experiments performed as in **A** were fitted with the Hill Equation. **(C)** Comparison of Sir3ΔwH binding to DiN in the presence or absence of Sir4 CC. **(D)** Binding curves from three experiments performed as in **C** were fitted with the Hill Equation. **(E)** Comparison of Sir3ΔwH binding to NCP and DiN in the presence or absence of 2-fold molar excess of Sir4 CC. **(F)** Cooperative binding analysis of Sir3ΔwH/Sir4 CC binding to DiN. Sir4 CC increases the cooperativity of Sir3ΔwH binding to DiN. For all EMSA experiments, proteins were titrated onto a constant amount of nucleosomes at 3 nM. **(G)** Comparison of the contributions of Sir3 wH and Sir4 CC to the free energy of Sir3 inter-nucleosomal binding cooperativity.

Figure 2-7 (Continued)



Sir3 binding to DiN (Figure 2-7G). However, Sir3/Sir4 CC in combination contributed a cooperativity free energy around -3.5 kcal/mol (Figure 2-7G). This indicates that both Sir3 wH and Sir4 CC dimerization domains contribute to the cooperative binding of SIR complexes to chromatin, but they may be functionally partially redundant in this respect.

Sir3 bridges free mono-nucleosomes in solution and the bridging activity requires the wH dimerization domain

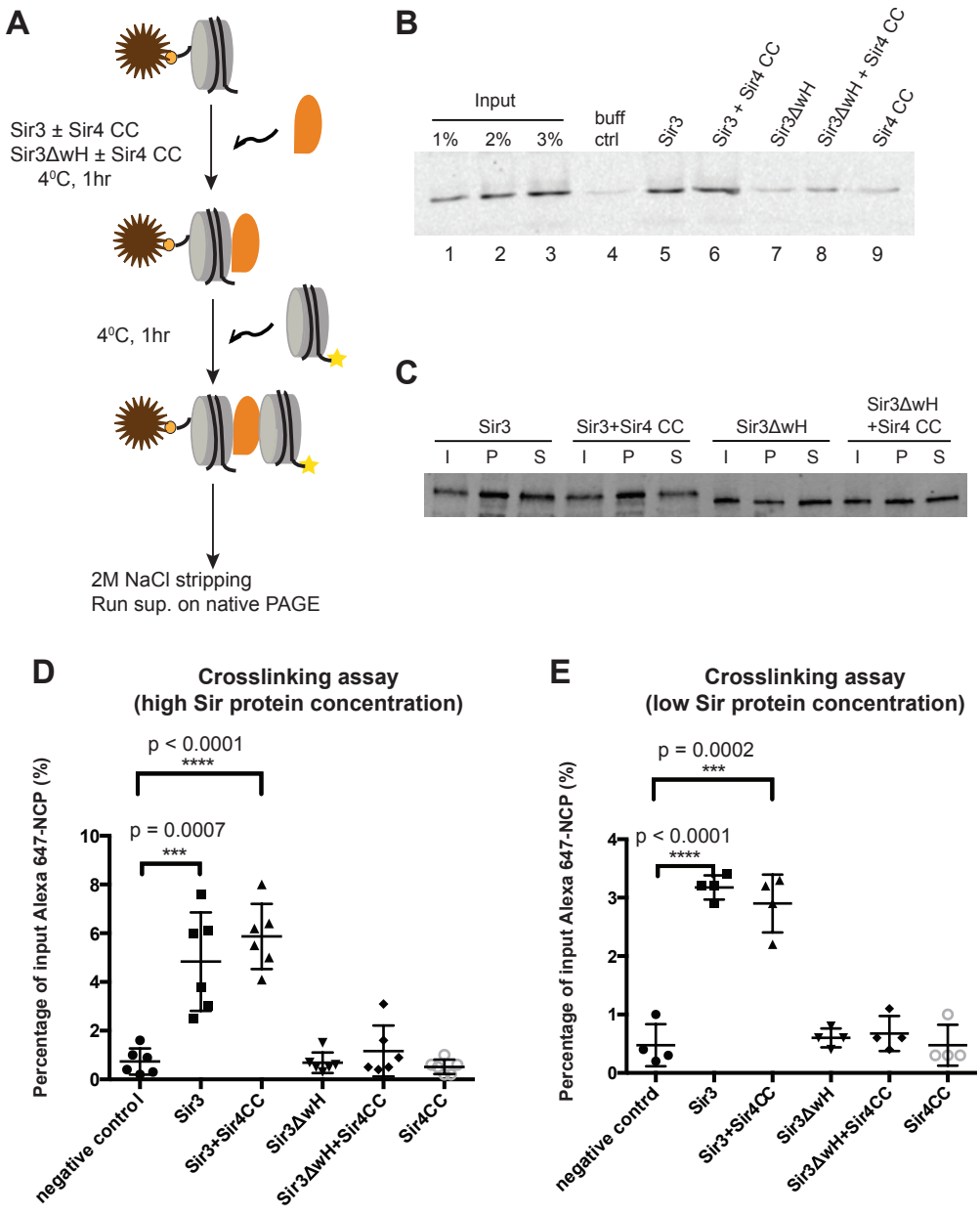
Since Sir3 binds to di-nucleosomes cooperatively, we tested whether it could act as a bridge linking free mono-nucleosomes in solution. To achieve this, we designed a bridging assay in which the ability of a nucleosome immobilized on a solid resin to bind to a free nucleosome could be tested (Figure 2-8A). We assembled mono-nucleosomes with 5' biotinylated 601 DNA containing a 20 bp linker 5' to the 601 sequence to allow sufficient space and flexibility of the nucleosome away from the solid support. The reconstituted biotinylated nucleosome was conjugated to the streptavidin magnetic beads and incubated with Sir3 or Sir3ΔwH, either alone or in combination with Sir4 CC. This was followed by incubation with Alexa-647-labeled NCP. The beads and their associated proteins and nucleosomes were then recovered by magnetic concentration and washed prior to elution of nucleosomal DNA from the beads with 2M NaCl. The resulting supernatant was analyzed by gel electrophoresis, and the amount of pulled down Alexa-647 nucleosomal DNA was quantified by the intensity of its fluorescent band (Figure 2-8A-B).

The addition of Sir3 to immobilized nucleosomes promoted the recovery of free labeled nucleosomes (Figure 2-8B, lane 5) and this recovery was not affected by the

Figure 2-8. Sir3 bridges free mono-nucleosomes in solution.

(A) Illustration of the bridging assay (see Materials and Methods for details). **(B)** A representative native polyacrylamide gel showing the fluorescent DNA pulled down from different reaction mixtures. **(C)** A representative western blot showing Sir3 and Sir3ΔwH in all reactions bound to nucleosomes efficiently. I: 0.2% of input; P: 4.5% of pellet; S: 0.2% of supernatant. **(D)** Percentage of input Alexa 647-nucleosomes that was pulled down in different reaction mixtures where high Sir3 or Sir3ΔwH protein concentration (2 uM) was used. Quantification for 6 independent experiments is presented. **(E)** Quantification of crosslinking assays where low Sir3 or Sir3ΔwH protein concentration (0.2 uM) was used. Quantification for 4 independent experiments is presented. In all experiments each species of nucleosome concentration was 0.1 uM.

Figure 2-8 (Continued)



addition of Sir4 CC (Figure 2-8B, lane 6). In contrast, Sir3ΔwH, alone or in the presence of Sir4 CC did not promote the recovery of free nucleosomes (Figure 2-8B, lanes 7-9). This indicated that Sir3 wH was required for the bridging activity of Sir3. We did not observe any bridging activity of Sir3ΔwH with the addition of Sir4 CC. This lack of bridging activity by Sir4 CC compared to the wH domain was unexpected, since the DiN EMSA assays (Figure 2-7G) indicated that Sir4 CC and Sir3 wH domain provided inter-nucleosomal cooperativity of similar strengths. The bridging assay is potentially a more stringent test of interaction than the DiN EMSA because the concentration of nucleosomes relative to each other is much lower (100 nM) than the effective local nucleosome concentration in the DiN EMSA, and we suspect that while Sir4 CC can readily link Sir3 bound to adjacent DNA-linked nucleosomes, the contact is not strong enough by itself to stably bring together separate Sir3-bound nucleosomes. A weak Sir4 CC-Sir3 interaction in solution is consistent with most Sir3 not being associated with Sir4 in yeast extracts (Moazed et al., 1997; Rudner et al., 2005). In contrast, the wH-wH interactions can mediate relatively stable dimers in solution (Oppikofer et al., 2013). A possible alternative explanation is that the relative orientation and position of the two nucleosomes in the di-nucleosome template is constrained by the 20 bp linker DNA. This constraint may be sub-optimal for wH-wH interactions between Sir3 proteins bound to adjacent DNA-linked nucleosomes. Thus the inter-nucleosomal cooperativity provided by Sir3 wH may be underestimated more than that provided by Sir4 CC in the DiN EMSA. These two explanations are not mutually exclusive.

The trivial explanation, that differences in bridging activity of different proteins were caused by unequal loading of proteins onto nucleosomes, was ruled out by the western blot showing that similar amounts of proteins were loaded onto the resin (Figure

2-8C). Furthermore, to rule out the possibility that a weak bridging activity in Sir4 CC was masked by high Sir3 protein concentration (2 μM) used in the bridging assay, we repeated the assay using lower Sir3 protein concentration (200 nM). However, we still did not observe a statistically significant increase in bridging activity with the addition of Sir4 CC (Figure 2-8D-E).

Statistical mechanical modeling of Sir3 inter-nucleosomal binding cooperativity

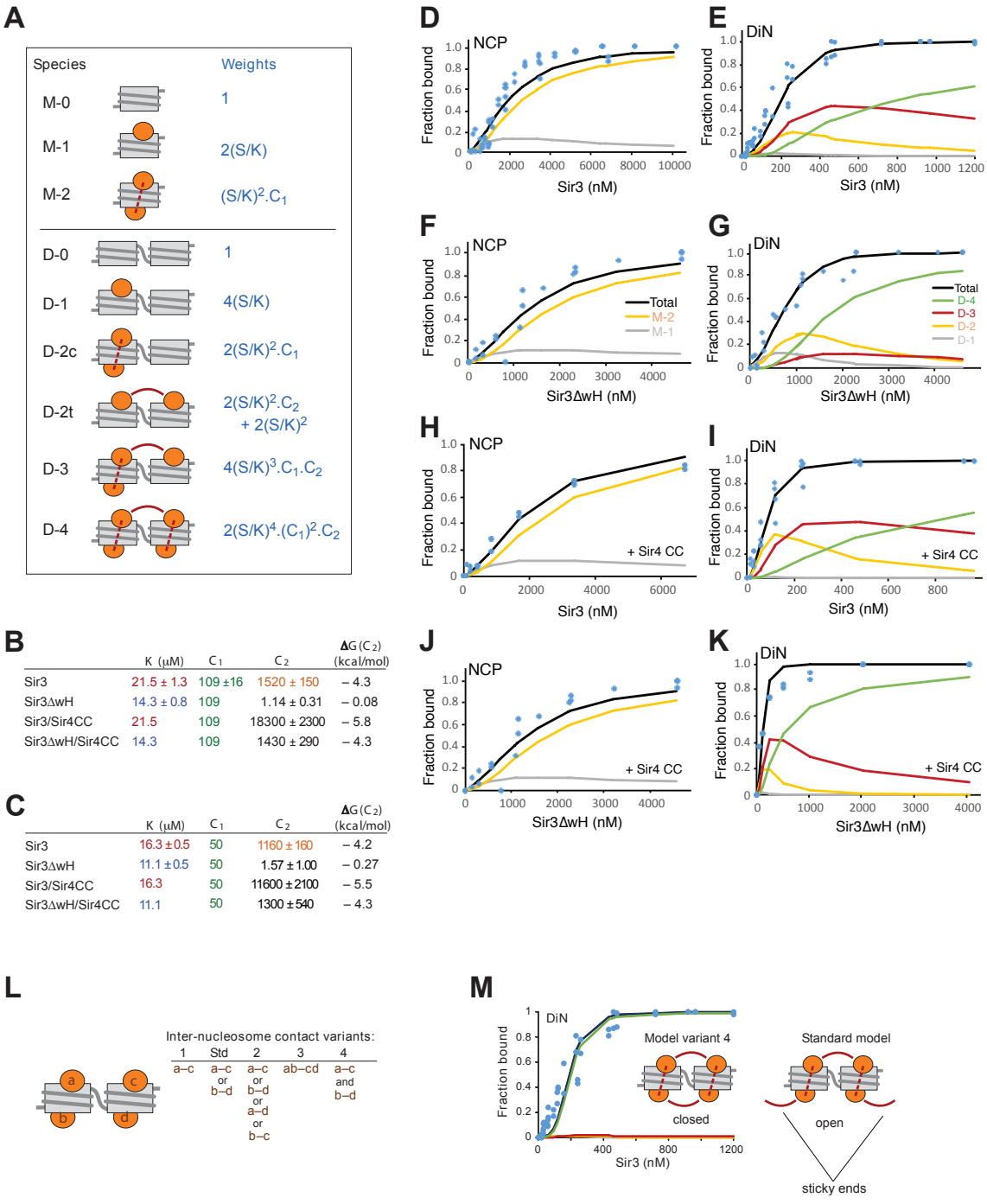
In the preceding analysis of Sir3 inter-nucleosomal cooperative binding using Equation 1 (Figure 2-3A), we made the simplifying assumption that Sir3 binds to mono-nucleosomes in a single step. To allay our concern that this simplification may affect our conclusions on Sir3 inter-nucleosomal cooperativity, we carried out a more detailed statistical mechanical modeling of the EMSA results, which considers elementary steps of Sir3 binding to nucleosomes, and which can predict the appearance of intermediate Sir3-bound nucleosome species in EMSA. This should allow a more rigorous test of our conclusions about the mechanism of Sir3 binding to chromatin.

In the modeling, each nucleosome can be bound by 0, 1 or 2 Sir3 monomers, and thus one di-nucleosome can be occupied by up to 4 Sir3 molecules (Figure 2-9A, also see Figure 2-6E). Each Sir3 molecule binds to nucleosome with a dissociation constant K (note that the dissociation constant K here is different from the apparent characteristic dissociation constant k used in Equation 1, Figure 2-3A, which denotes the Sir3 binding to both sides of the nucleosome). We also defined C_1 and C_2, which denote the intra-nucleosomal and inter-nucleosomal cooperativity factor of Sir3 binding, respectively. C_2 is akin to the c factor used in Equation 1 (Figure 2-3A). In the standard model, Sir3 monomers are able to form inter-nucleosomal interactions only when they

Figure 2-9. Statistical mechanical modeling of Sir3 cooperative binding to nucleosomes.

(A) Statistical-mechanical model for Sir3 monomer binding to mono- and di-nucleosomes. S is [Sir3], K is the dissociation constant for monomer binding. The strength of intra-nucleosomal interactions (dashed lines) is specified by C_1, and the strength of inter-nucleosomal interaction (solid lines) by C_2. Prefactors to the weights specify the number of ways each structure can be achieved. **(B)** Average optimal parameters for constrained global fits of the pooled bound-fraction data in the EMSAs for the 4 conditions. Values constrained to be the same are indicated by color. Errors are standard deviations of 200 optimal fits in which the data points were individually varied with a standard deviation of 20%. These errors underestimate the range of parameter values consistent with the data (see text). **(C)** Average optimal parameters for constrained global fits of the pooled bound-fraction data in the EMSAs for the 4 conditions, with C_1 constrained to be 50. **(D-K)** Data points (light blue filled circles) and model predictions (blue line) for NCP and DiN binding by Sir3 ± Sir4 CC and Sir3ΔwH± Sir4 CC, showing predicted fractions of different stoichiometries. **(L)** Variants of the standard model, with different possibilities for Sir3 inter-nucleosomal interactions. **(M)** Model variant 4, which allows two inter-nucleosomal interactions, predicts a lack of intermediates in the Sir3 DiN EMSA (The plot is for variant 4 combined with variant 2). This model favors a closed non-spreading structure as opposed to the open spreading-competent structure of the standard model and is not consistent with the EMSA results.

Figure 2-9 (Continued)



are bound at specific positions relative to each other on the di-nucleosome (Figure 2-9A, D-2t).

We used a parameter sampling approach (see Materials and Methods) to find values for K, C_1 and C_2 that optimized the global fit to pooled bound-fraction data from NCP and DiN EMSAs of the 4 different conditions used (Sir3 ± Sir4 CC binding to NCP or DiN; Sir3ΔwH ± Sir4 CC binding to NCP or DiN). Unconstraint fitting, allowing individual K, C_1 and C_2 parameters for each of the 4 conditions (a total of 12 parameters), produced a variety of acceptable fits to the data, as values of K, C_1 and C_2 could be changed to offset each other. However, fits of similar quality could be produced if certain parameter values were constrained to be the same across different conditions, generating a more parsimonious set of 7 free parameters (Figure 2-9B, two K parameters – 1 for Sir3 and 1 for Sir3ΔwH; one C_1 for all conditions; and 4 C_2 parameters – 1 for each of Sir3, Sir3ΔwH, Sir3+Sir4 CC, and Sir3ΔwH+Sir4 CC) that was able to explain the data quite well. Since the solution binding assay suggested weak intra-nucleosomal cooperativity, we explored lower C_1 values that could fit the data reasonably well. When we constrained the C_1 value to be 50, the fit was not much worse (Figure 2-9D-K). This gave rise to a set of optimal constrained parameter values for Sir3 binding to nucleosomes (Figure 2-9C), which are similar to the optimal values in the unconstrained fitting. The parameters specify very weak binding of individual Sir3 monomers (K = 16 μM), weak intra-nucleosomal cooperativity between Sir3 monomers (C_1 = 50; equivalent to ΔG^0 = -2.3 kcal/mol at room temperature (RT)), and moderate inter-nucleosomal cooperativity (C_2 = 1160; equivalent to ΔG^0 = -4.2 kcal/mol). We note that the model sometimes underestimates the steepness of the NCP binding curves (e.g.

Figure 2-9D), since it can only generate a Hill coefficient of 2, while the data gives a slightly higher Hill coefficient (nH = 2.7 for Sir3).

The effect of the deletion of Sir3 wH domain on Sir3 binding to nucleosomes is consistent with our previous analysis. There is little change on the affinity of Sir3 binding to NCP upon the deletion of the wH domain (K_d = 1.5 μM for Sir3 in Figure 2-1D-E, and 1.3 μM for Sir3ΔwH in Figure 2-5B-C; K = 16 μM for Sir3 monomer, and 11 μM for Sir3ΔwH monomer in Figure 2-9C). We note that optimal fits to the Sir3ΔwH binding data (Figure 2-9F-G) required a slight improvement in monomer dissociation constant compared to that of Sir3 (Figure 2-9C). It is possible that the wH domain might impose a slight inhibition of Sir3 monomer binding to nucleosomes. However, we think a more likely explanation is that there are different fractions of active proteins in Sir3 versus Sir3ΔwH protein preparations. The deletion of the wH domain reduces the Sir3 inter-nucleosomal cooperativity C_2 from 1160 to around 1, indicating the loss of cooperativity. Values of $C_2 > 20$ for Sir3ΔwH worsened the fit substantially, and could not be compensated for by changes in other parameters.

The effect of the addition of Sir4 CC on Sir3 binding to nucleosomes is also consistent with our previous analysis. While Sir4 CC has no effect on Sir3 binding affinity to NCP (K_d = 1.6 μM for Sir3 ± Sir4 CC in Figure 2-6F-G; K = 16 μM for Sir3 ± Sir4 CC in Figure 2-9C), it increases the Sir3 inter-nucleosomal cooperativity towards DiN. The addition of Sir4 CC increases Sir3 inter-nucleosomal cooperativity C_2 by 10 fold (Figure 2-9C, equivalent to $\Delta\Delta G^0$ = -1.3 kcal/mol), and increases the Sir3ΔwH C_2 by 830 fold (Figure 2-8C, equivalent to $\Delta\Delta G^0$ = -4.0 kcal/mol). The weaker enhancement of inter-nucleosomal cooperativity by Sir4 CC in the presence of the Sir3 wH domain is consistent with conclusions based on Equation 1 (Figure 2-7G). In fact, we were unable

to obtain good fits if we constrained the fold-enhancement of Sir4 CC on the C_2 for Sir3 to be the same as for Sir3ΔwH.

Our model predicts the population of nucleosome species with different Sir3 stoichiometries in a manner that is consistent with their appearance in EMSAs. For example, the single-monomer-bound NCP (species M-1) is expected to remain at low levels, (Figures 2-9D, F, H, J), consistent with the observations of only one major shifted band (species M-2) in NCP gel shifts (Figures 2-1D, 2-5B, 2-6F, 2-7A). In addition, only two Sir3 stoichiometries are predicted to accumulate to high levels in the Sir3-DiN binding (Figure 2-9E), the D-3 and D-4 species, consistent with there being only two major shifted bands observed in EMSA (Figure 2-2C). As seen in the EMSA, the intermediate D-3 band appears and peaks at lower Sir3 concentrations, when the upper D-4 band starts to appear and accumulates steadily with increasing Sir3 concentration. The predicted population of Sir3ΔwH stoichiometries in Sir3ΔwH-DiN binding (Figure 2-9G) is also consistent with the observed pattern in the Sir3ΔwH-DiN EMSA (Figure 2-5B), although in this case the intermediate shifted band corresponds to the D-2 species, instead of the D-3 species, in the absence of strong inter-nucleosomal cooperativity.

We also tested variants of models with more restricted or more relaxed rules for inter-nucleosomal cooperativity, allowing the single Sir3 inter-nucleosomal interactions to occur from different relative positions of Sir3 monomers on the di-nucleosome (Figure 2-9L, variants 1 and 2). These variant models did not alter the fits. The only difference was that a 2-fold increase or a 2-fold decrease in C_2 was required for variant 1 or 2, respectively. Therefore, the model cannot distinguish different ways in which a single Sir3 inter-nucleosomal contact can be made. We examined another variant in which inter-nucleosomal cooperative interactions occurs only when both nucleosomes within

the di-nucleosome are fully occupied by Sir3 (Figure 2-9L, variant 3). This model gave a somewhat worse fit to the data, and predicted an almost complete lack of intermediate species in the Sir3 DiN EMSA, inconsistent with the strong intermediate shifted band seen (Figure 2-2C).

In the models tested above, when one pair of Sir3 monomers makes inter-nucleosomal contact within a di-nucleosome unit, the other 2 bound Sir3 monomers are freely available to interact with Sir3 molecules bound on neighboring nucleosomes (Figure 2-9M). However, a maximum of 2 inter-nucleosomal contacts can be made within a fully Sir3-occupied di-nucleosome, forming a self-closed structure (Figure 2-9M). Thus we examined variant models in which two simultaneous inter-nucleosomal interactions are allowed (Figure 2-9L, variant 4). The fits produced by these models were slightly worse than the standard model, and also predicted an almost complete lack of intermediate species in DiN EMSAs (Figure 2-9M). Therefore, our EMSA experiments rule out a closed Sir3 di-nucleosome complex where all inter-nucleosomal interactions are satisfied, precluding cooperative spreading (Figure 2-9M). Instead, the gel shift data is consistent with an open complex (Figure 2-9M), in which Sir3 monomers bound to opposite sides of a nucleosome are available for inter-nucleosomal interactions with Sir3 bound to different neighboring nucleosomes, allowing the cooperative spreading of Sir3 along chromatin. Finally, we tested a variant of the model in which Sir3 is predominantly dimeric prior to its association with nucleosomes, and obtained similarly good fits to the EMSA data. This suggests that the mechanism of Sir3 inter-nucleosomal cooperative binding involves an increase in local concentration of either Sir3 proteins (promoting dimerization via the wH domain) or nucleosomes (one adjacent to a bound Sir3 protein).

Sir3 wH and Sir4 CC domains both contribute to Sir3 spreading *in vivo*

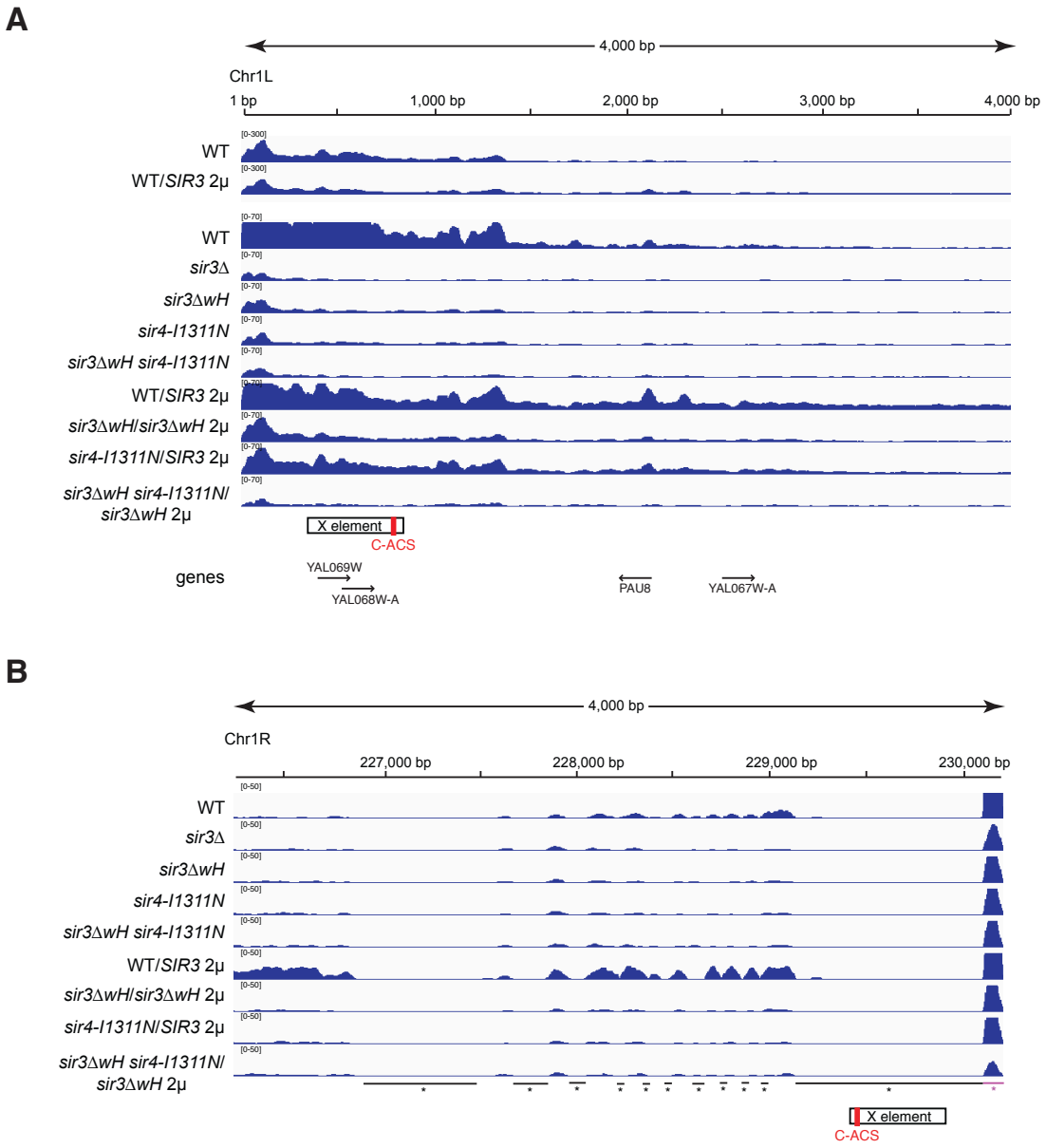
To explore the effect of Sir3 wH and Sir4 CC domain on Sir3 binding and spreading *in vivo*, we performed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) experiments. Using an antibody that recognizes Sir3, we determined the localization of Sir3 in the genome when its wH domain is deleted, in both wild-type (WT) and *sir4-I1311N* background, and under either endogenous or Sir3/Sir3ΔwH over expression (OE) conditions. Since Sir3 associates with subtelomeres, and shows the highest level of enrichment at the core X element (Ellahi et al., 2015; Radman-Livaja et al., 2011; Thurtle and Rine, 2014; Zill et al., 2010), we focused our analysis at these regions (Figure 2-10A-AF). We also generated ensemble plots of all 30 telomeres (excluding *TEL01R* and *TEL13R*, in which there are deletions in the X element sequence in the experimental W303-1a strain as compared to the reference S288C strain whose genome is annotated), by aligning them either at chromosome ends (Figure 2-10AI-AJ) or at the ACS sequence in the core X element (C-ACS) (Figure 2-10AK-AL).

Two features are apparent in Figure 2-10A-AF. First, there are gaps of no coverage in some telomeres (large gaps in *TEL01R, TEL07L* and *TEL13R,* and various small gaps in some other telomeres, indicated by black lines and asterisks in Figure 2-10A-AF). These gaps were observed in the input sample as well. This is because the laboratory strain (W303-1a), with which the ChIP-seq experiments were performed, had deletions in subtelomeric regions as compared to the S288C reference genome. Second, there are regions of enrichments in the *Δsir3* sample. This is due to nonspecific bindings of the polyclonal Sir3 antibody we used in the ChIP-seq experiments. Some of these

Figure 2-10. Sir3 winged helix (wH) domain and Sir4 coiled-coil (CC) domain mediate Sir3 inter-nucleosomal cooperative binding *in vivo.*

Normalized ChIP-seq read densities for Sir3 under different conditions at **(A-AF)** all telomeres, and **(AG)** *HML* and **(AH)** *HMR*. In **A-AF**, the core X element is indicated by a black box, and the ACS within (C-ACS) is indicated by a filled red box. The laboratory strain, W303-1a, which the ChIP-Seq experiements were performed on, has deletions in subtelomeric regions as compared to the S288C reference. This results in sequence alignment gaps, which are indicated in black lines and aristerisks. Repetitive sequences which lead to enrichment peaks in the input sample are indicated by magenta lines and aristerisks. **(AI-AJ)** Ensemble plots of ChIP-seq signals from 30 subtelomeres, excluding *TEL01R* and *TEL13R,* aligned at chromosome ends. ChIP signals at all 30 subtelomeric regions was summed up, normalized to the *Δsir3* sample, and plotted as a function of distance from chromosome end. **(AK-AL)** Ensemble plots of ChIP-seq signals from 30 subtelomeres, excluding *TEL01R* and *TEL13R,* aligned at ACS within the core X element (C-ACS). Negative values are towards chromosome end, and positive values are towards centromere.

Figure 2-10 (Continued)



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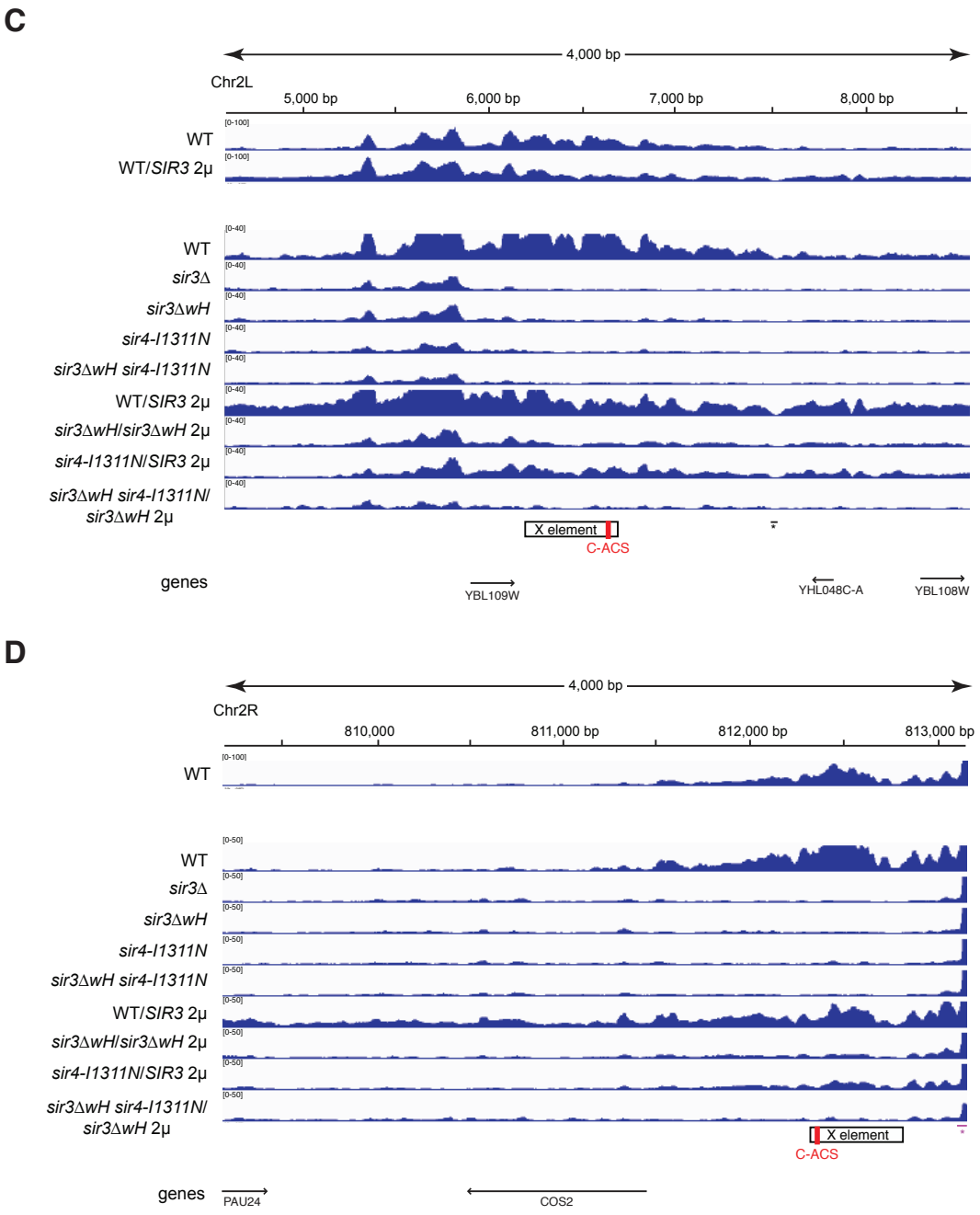

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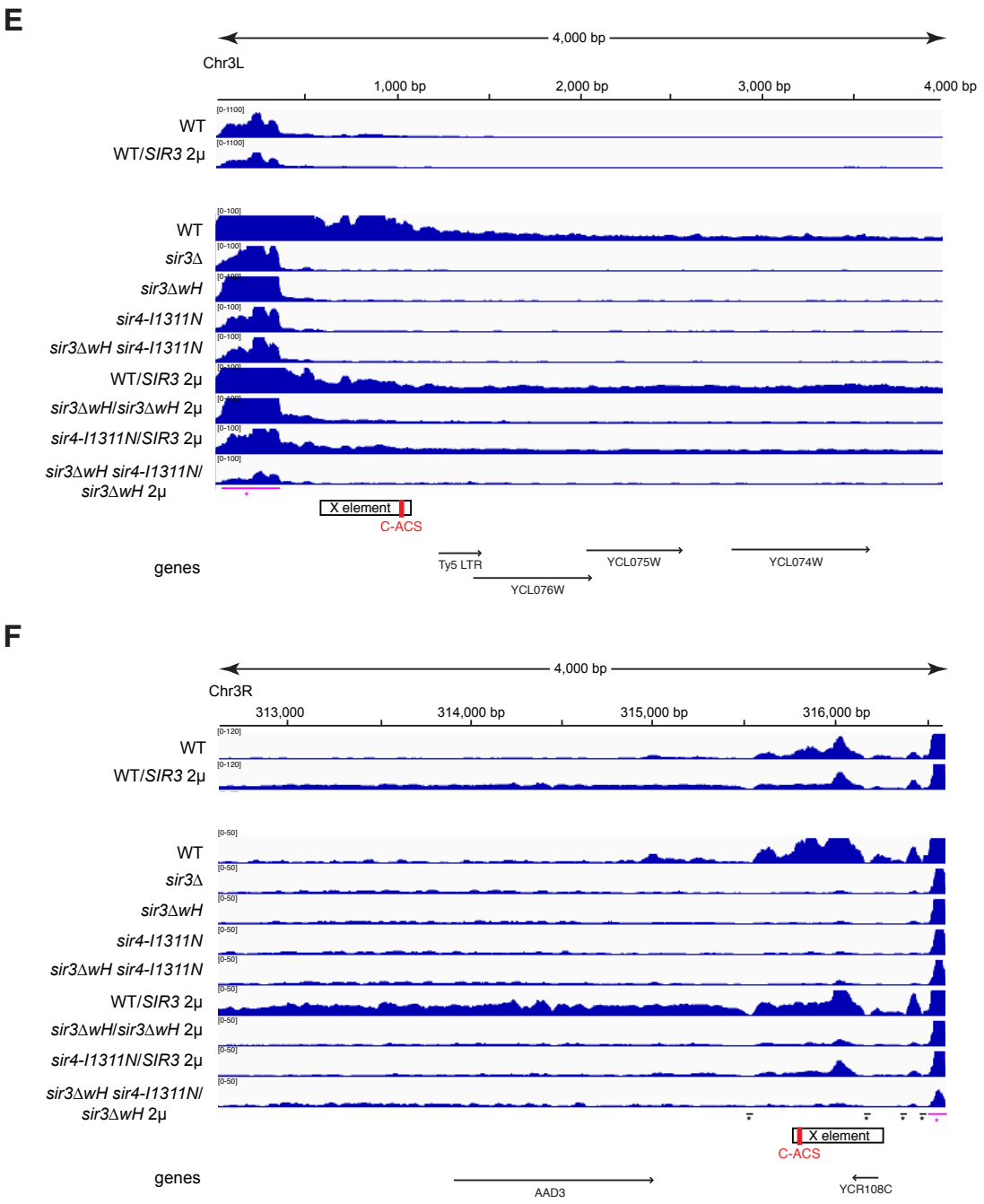

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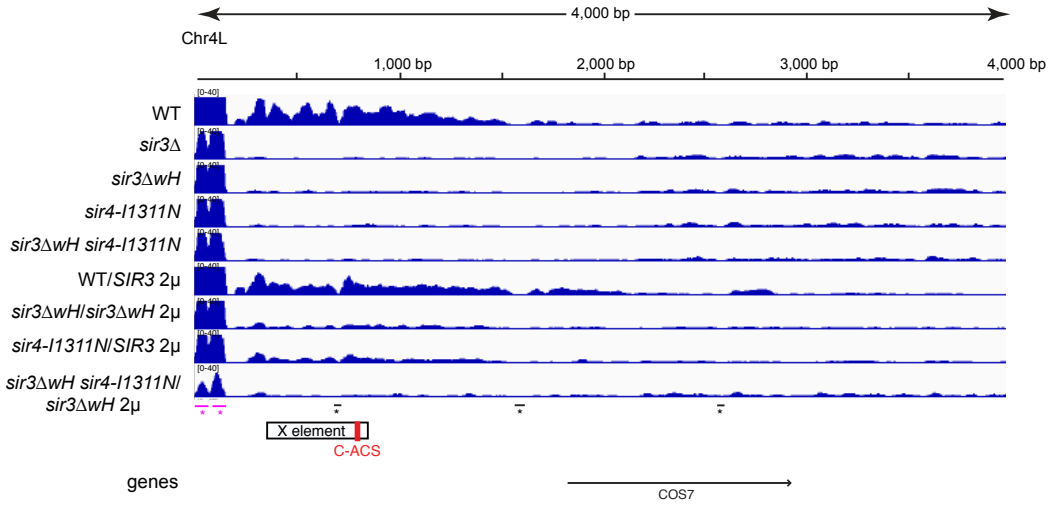

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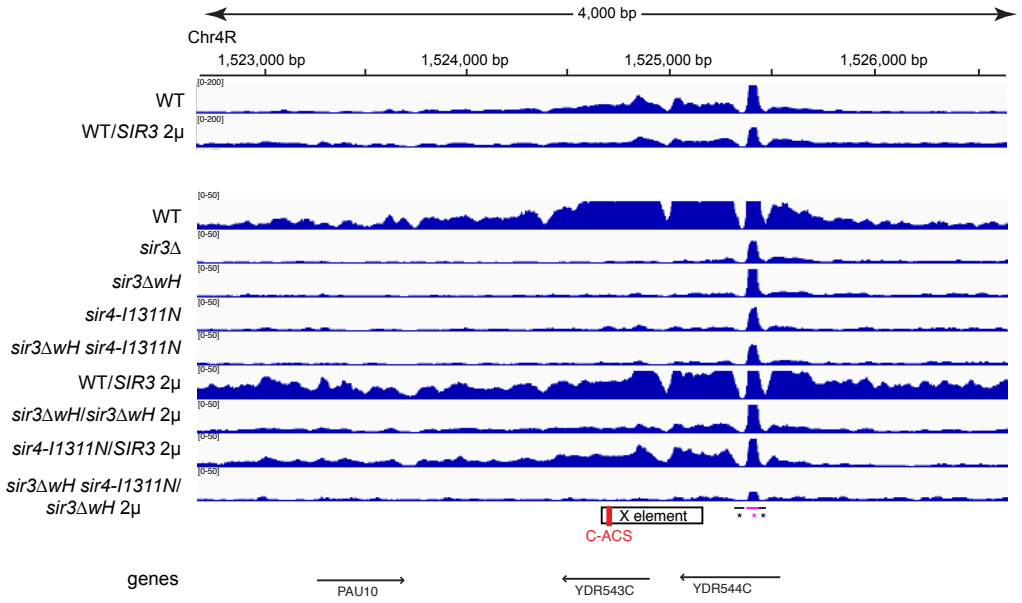

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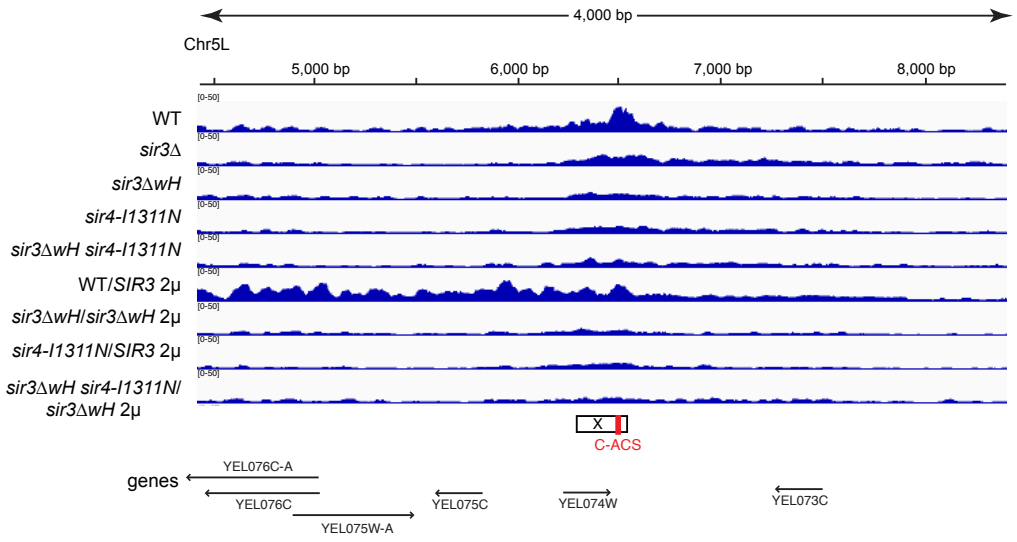

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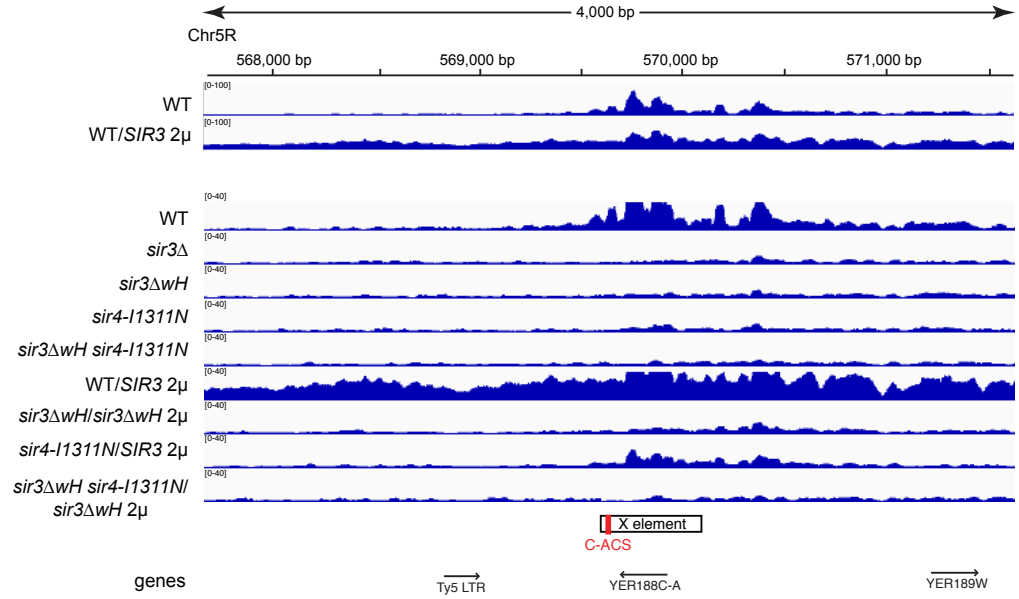

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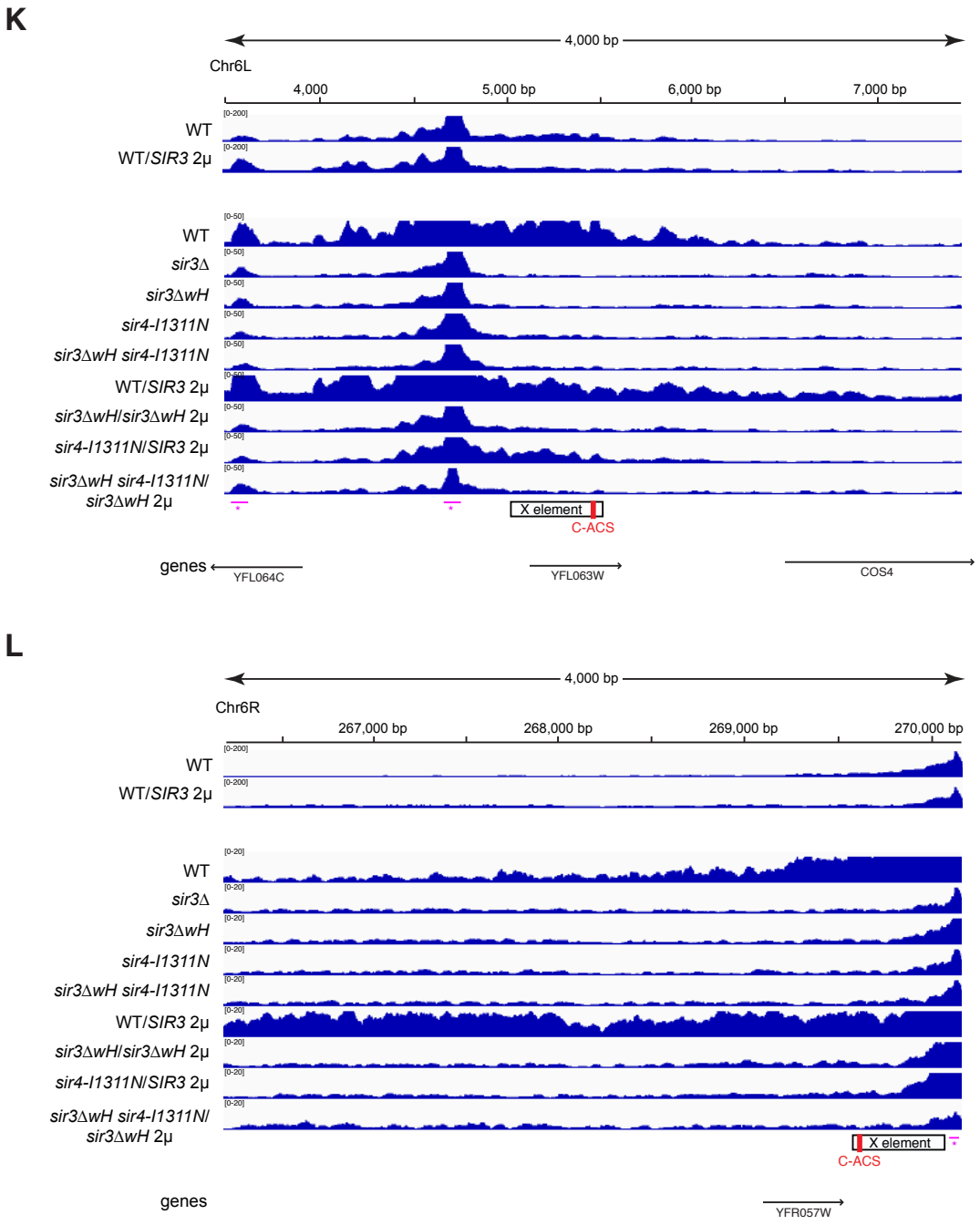

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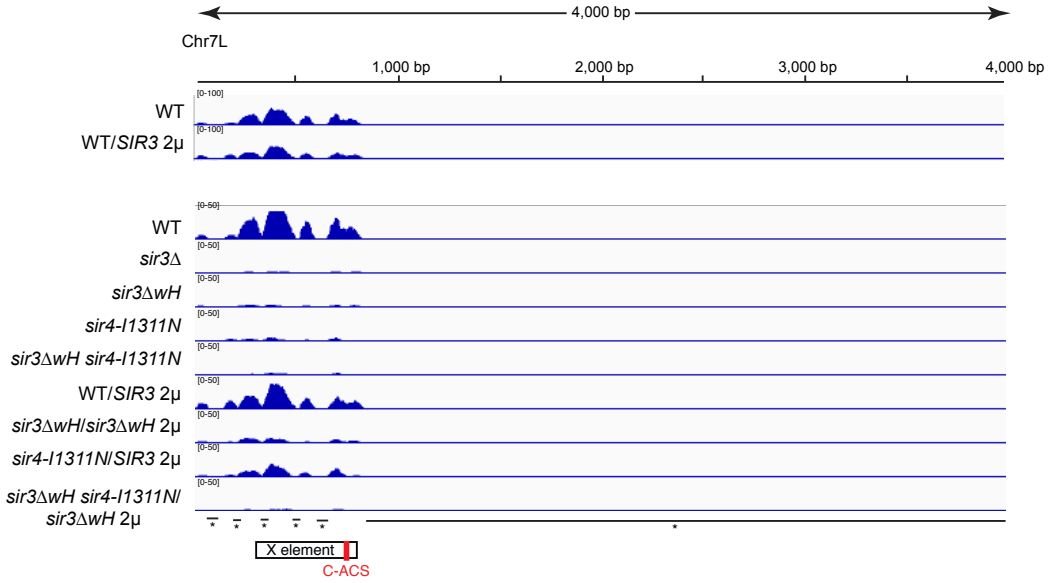

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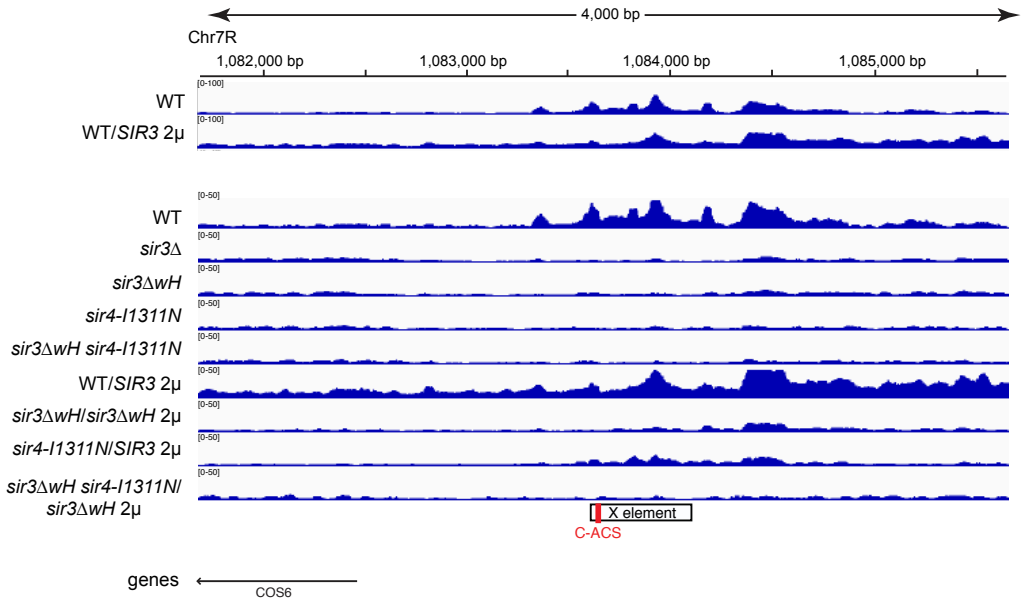

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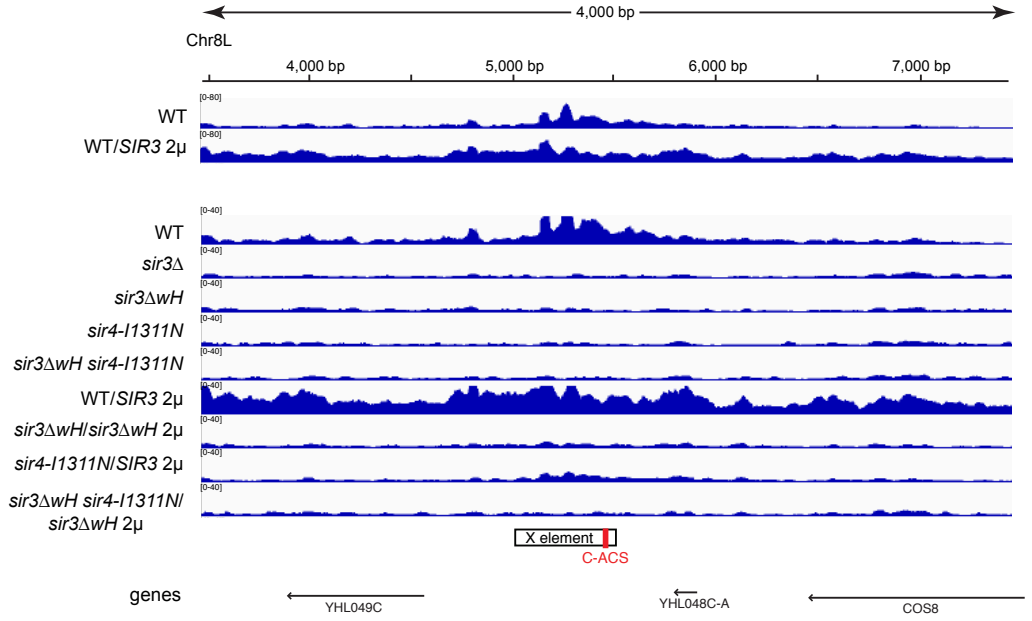

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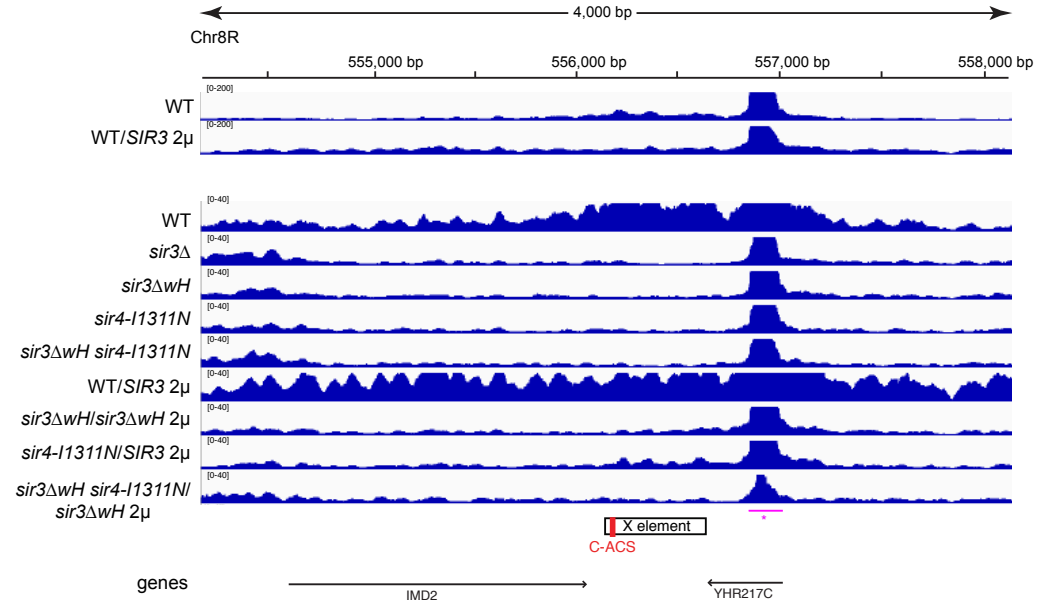

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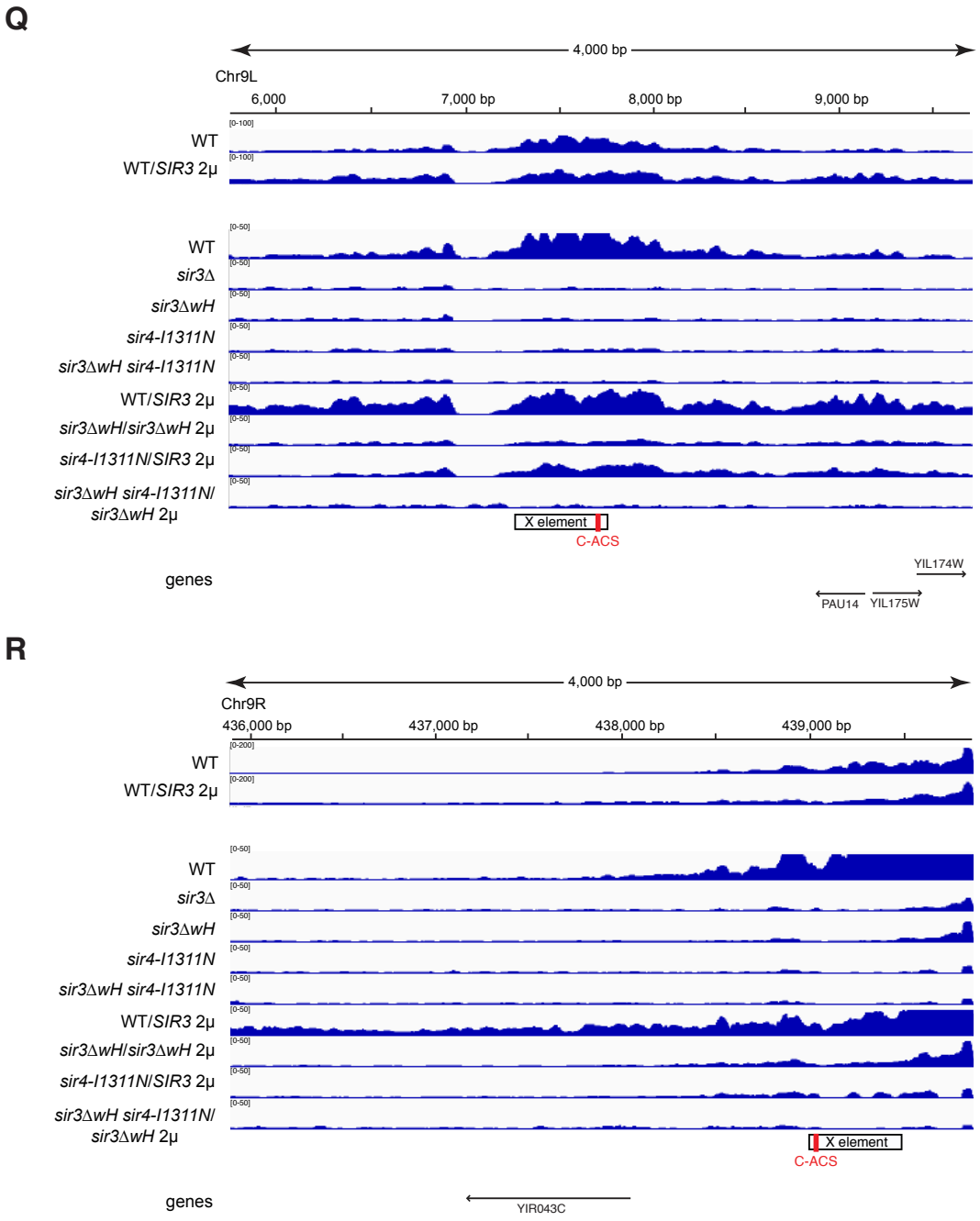

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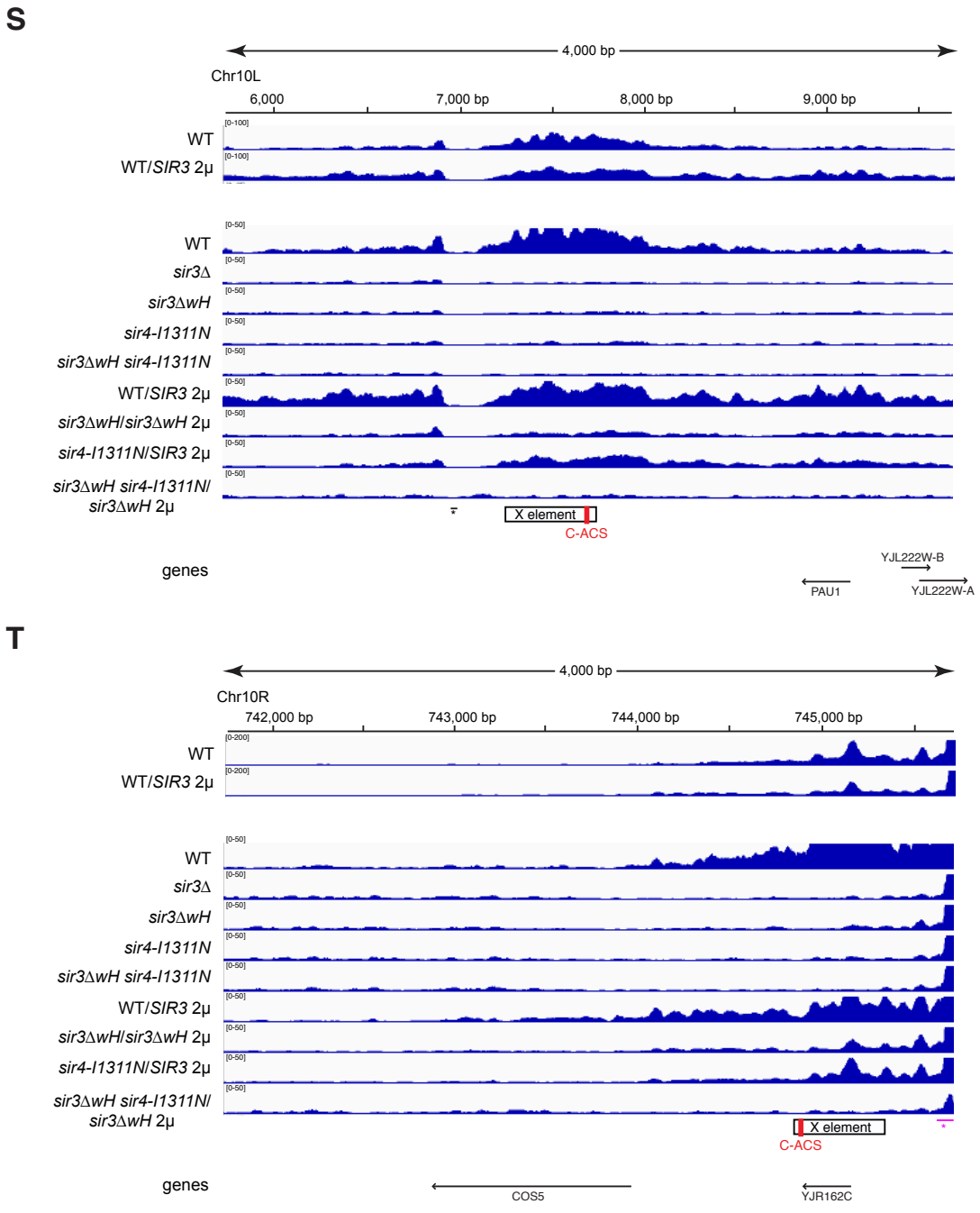

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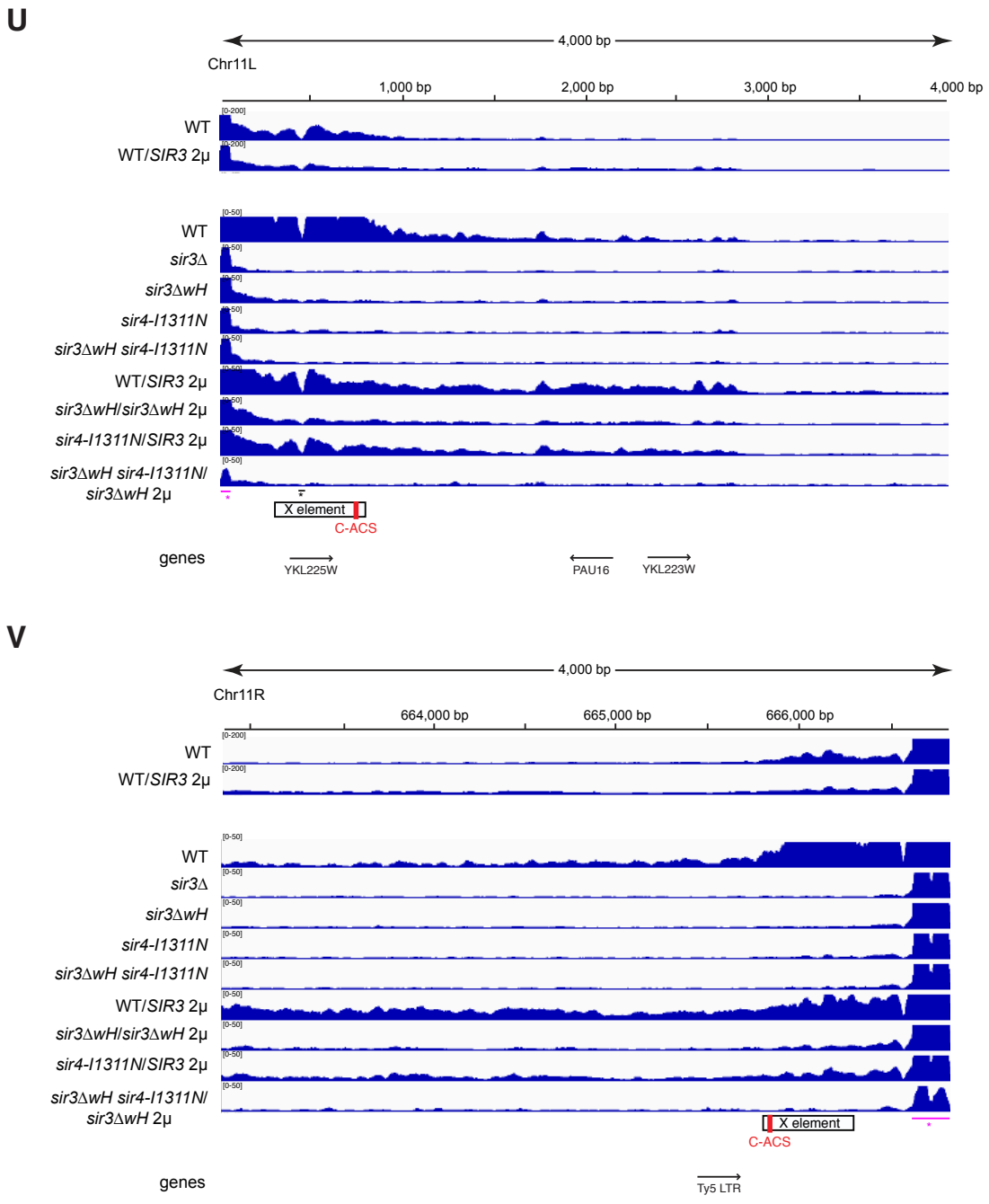

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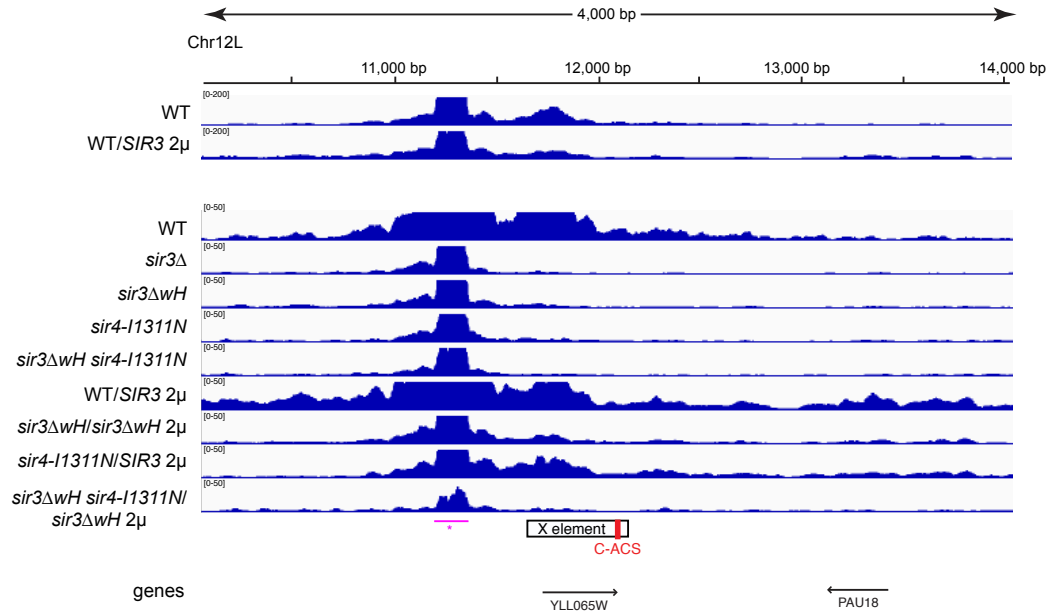

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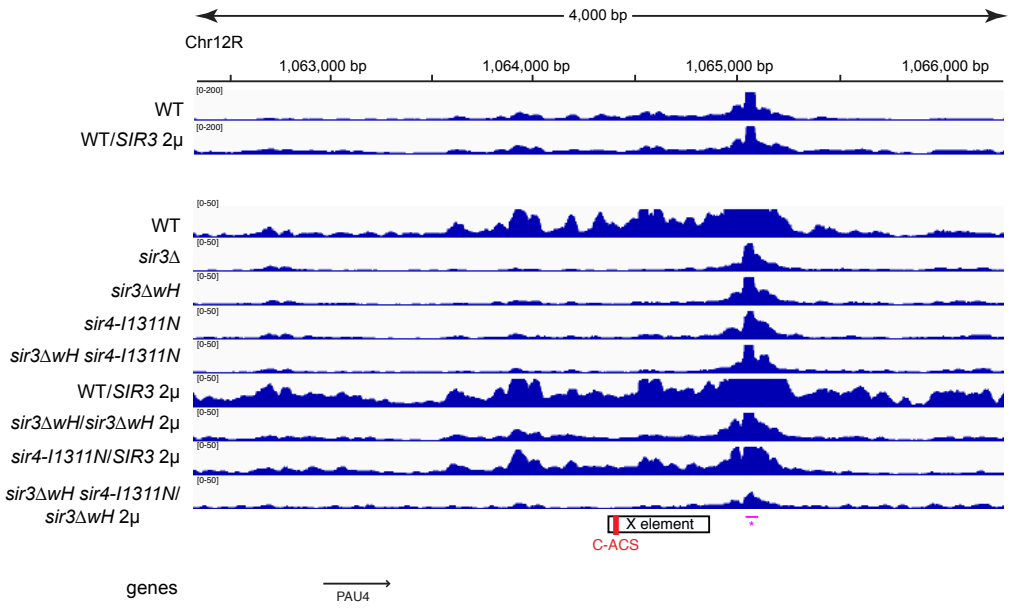

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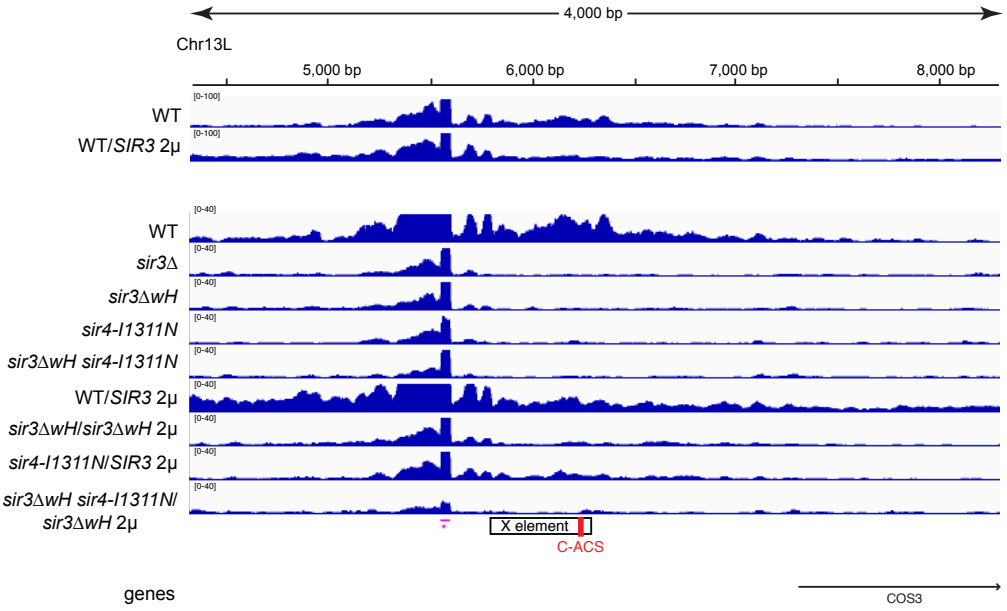

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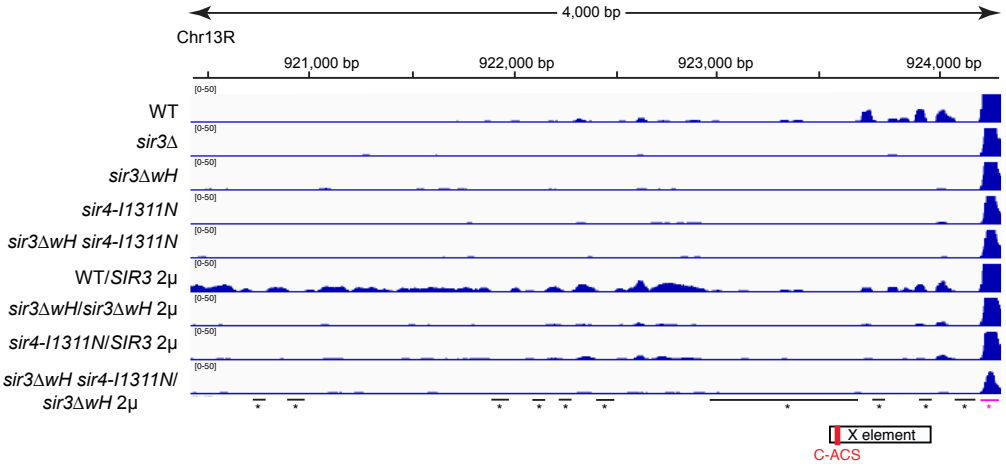

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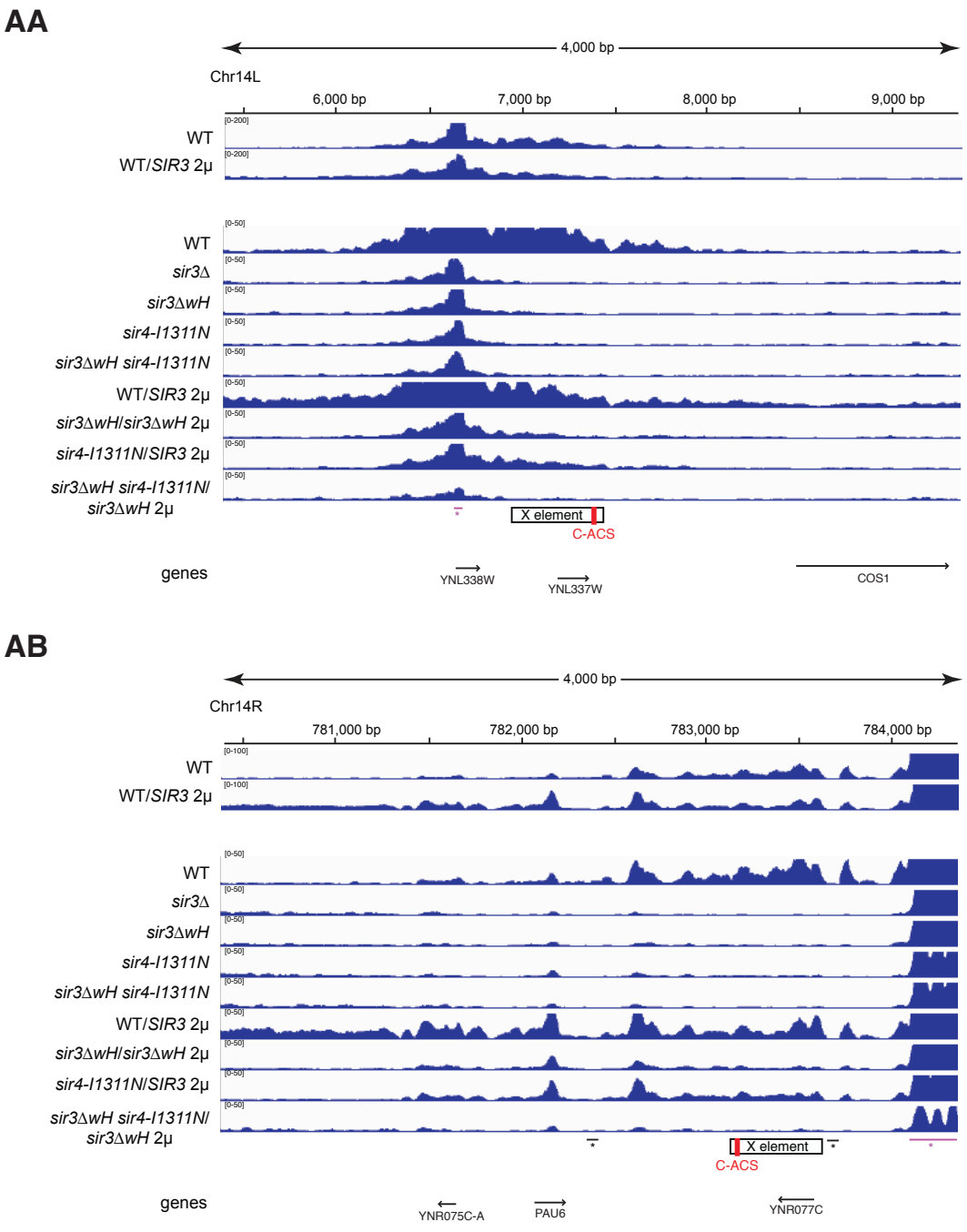

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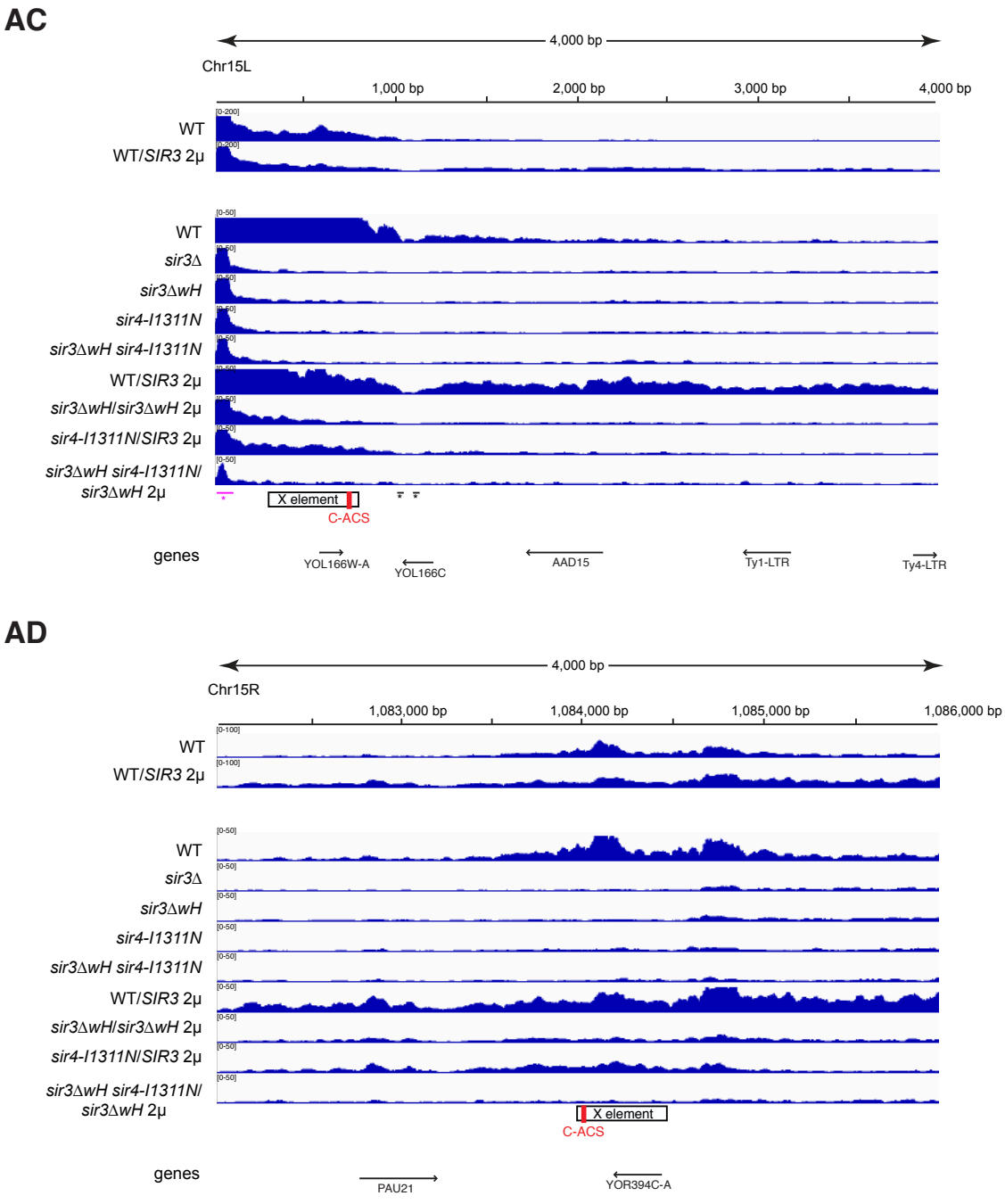

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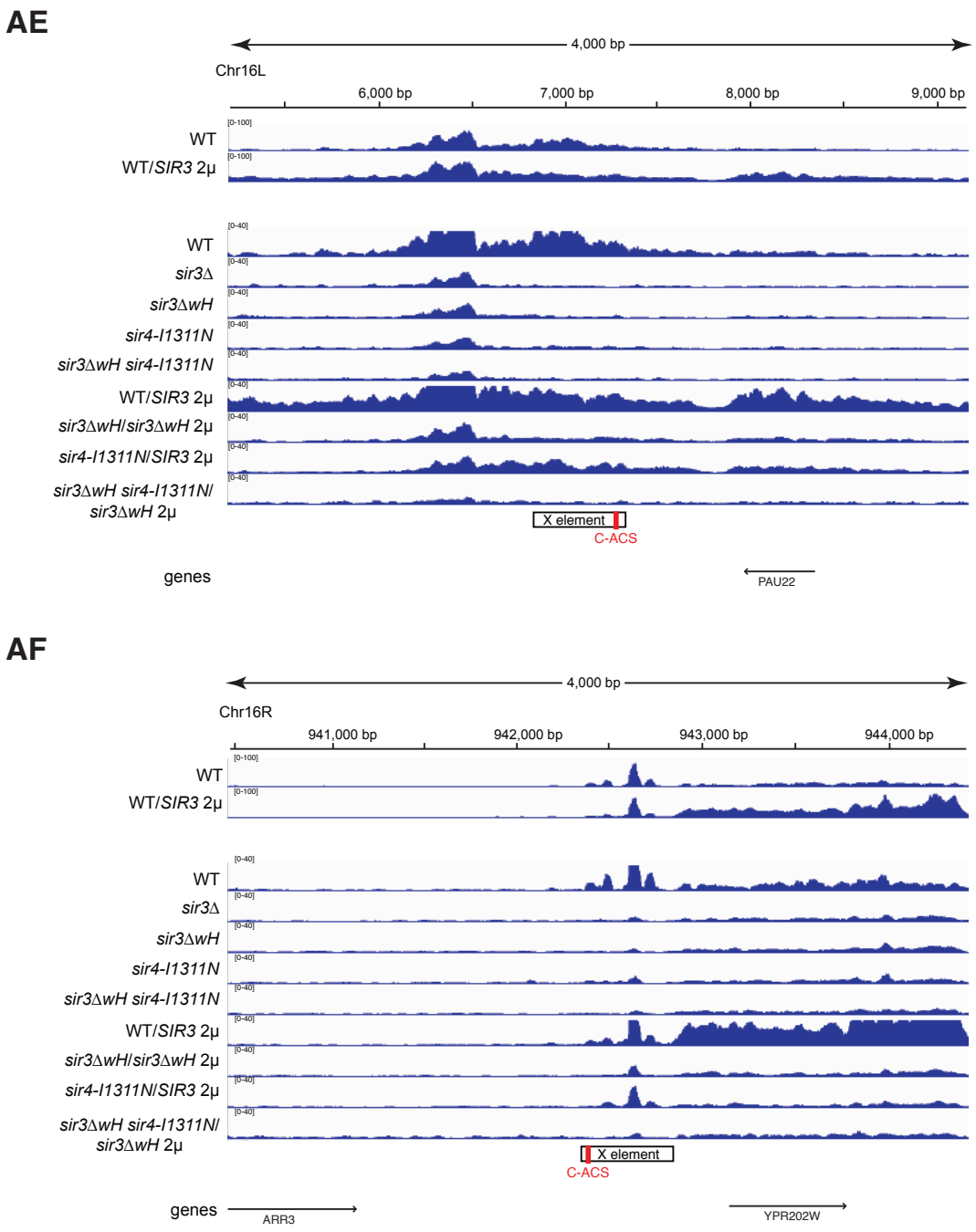

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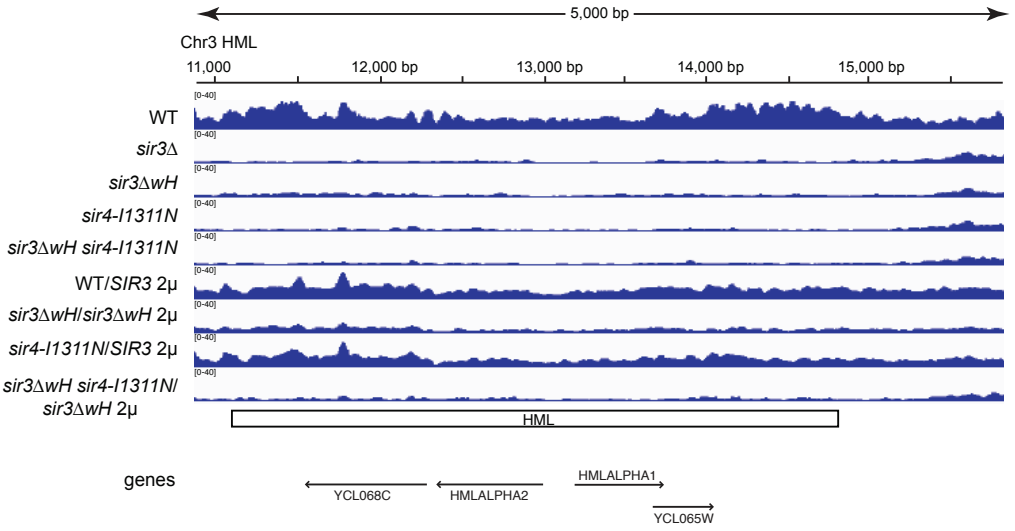

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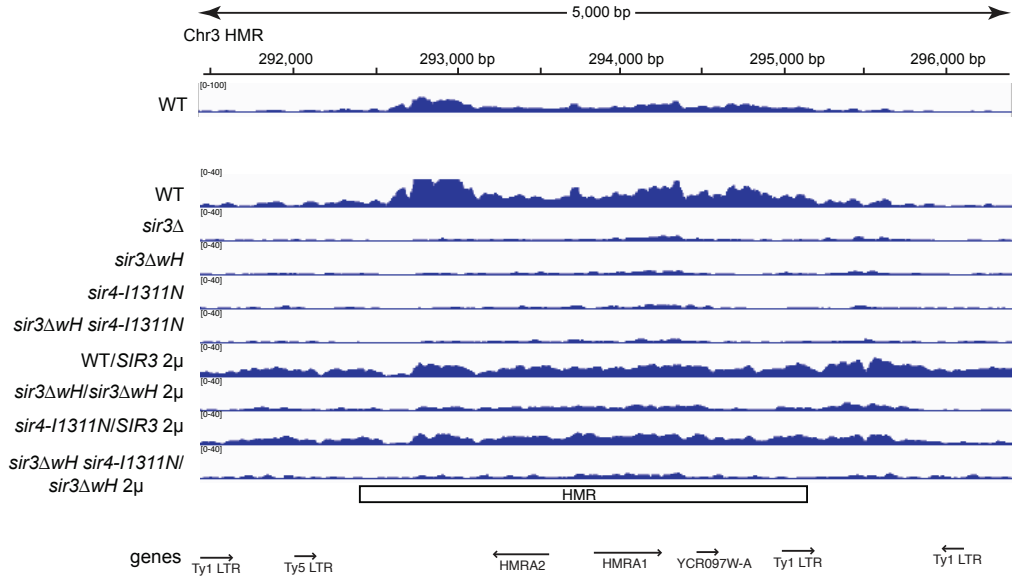

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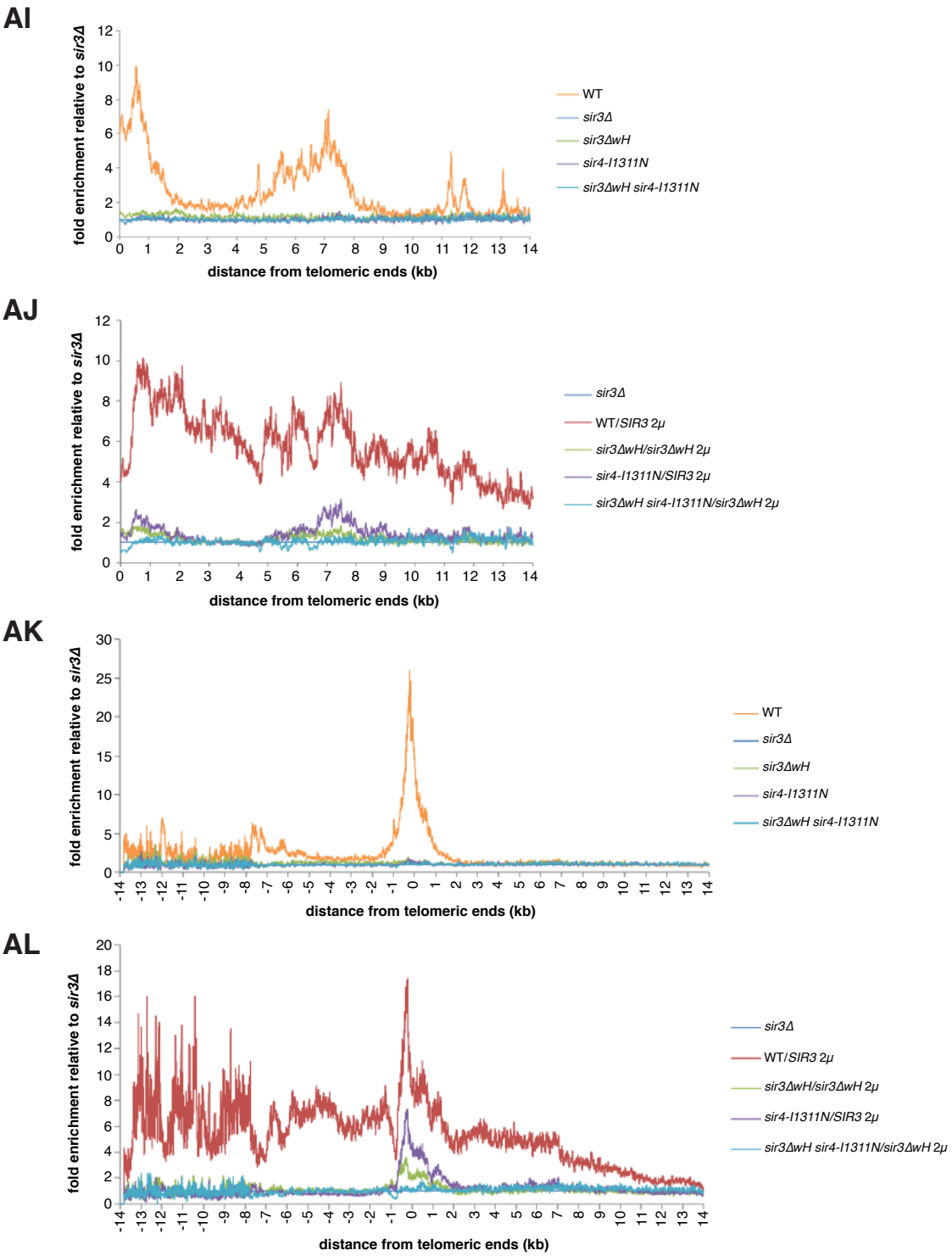

regions are repetitive sequences, which are not fully mapped, leading to “enrichment peaks” in the input sample. These regions are indicated by magenta lines and asterisks in Figure 2-10A-AF. Differences in the experimental strain and the reference genome is a common problem in ChIP-seq experiments with *S. cerevisiae* (Ellahi et al., 2015; Radman-Livaja et al., 2011), and do not affect the analysis of our result. In the ensemble plots, all samples were normalized to the *sir3Δ* sample, so that peaks due to nonspecific binding of antibodies were removed.

Examination of Sir3 localization at individual telomeres and *HM* loci indicated that Sir3ΔwH failed to localize to any of the 32 subtelomeres or *HM* loci, mirroring the phenotype of *sir3Δ* (Figure 2-10A-AH compare *sir3Δ* and *sir3ΔwH* tracks, Figure 2-10AI and AK). This indicates that the wH domain is required for the efficient recruitment and spreading of Sir3 on chromatin, and is consistent with previous findings showing that Sir3 wH-mediated dimerization is required for Sir complex spreading *in vivo* at telomeres 6R, 7L, 9R and both *HM* loci (Oppikofer et al., 2013). Previous studies show that the residue I1311 in the Sir4 CC domain is critical for the efficient interaction between Sir3 and Sir4 (Chang et al., 2003; Rudner et al., 2005), and that its mutation to glutamine disrupts the recruitment and spreading of Sir proteins (Rudner et al., 2005). Consistently, we found that Sir3 proteins failed to localize to subtelomeres or *HM* loci in the *sir4-I1311N* background (Figure 2-10A-AH *sir4-I1311N* tracks, Figure 2-10AI and AK). As expected, combined mutation of *sir3ΔwH* and *sir4-I1311N* also led to loss of Sir3 recruitment and spreading (Figure 2-10A-AH *sir3ΔwH sir4-I1311N* tracks, Figure 2-10AI and AK).

Consistent with previous findings showing that Sir3 OE leads to Sir3 spreading into chromatin regions further away from telomeres (Hecht et al., 1996; Renauld et al.,

1993), our studies showed that there was an extension of Sir3-bound chromatin domains in most subtelomeres under the Sir3 OE condition (Figure 2-10A-AF compare WT and WT/*SIR3* 2μ tracks, Figure 2-10AJ and AL). Interestingly, Sir3ΔwH OE in the *sir3ΔwH SIR4* background also led to weak spreading of Sir3ΔwH proteins near the core X element at all subtelomeres (Figure 2-10A-AF *sir3ΔwH/sir3ΔwH* 2μ tracks). In addition, there was some spreading of Sir3 at these regions with Sir3 OE in the *SIR3 sir4-I1311N* background (Figure 2-10A-AF *sir4-I1311N/SIR3* 2μ tracks). There appeared to be slightly more Sir3 spreading and higher level of Sir3 occupancy in *SIR3 sir4-I1311N/SIR3* 2μ than in *sir3ΔwH SIR4/sir3ΔwH* 2μ sample. However, the spreading was completely abolished when both Sir3 and Sir4 were mutated (Figure 2-10A-AF *sir3ΔwH sir4-I1311N/sir3ΔwH* 2μ tracks). These effects on spreading were even more obvious in the ensemble plots, where we observed enrichment and weak spreading of overexpressed Sir3ΔwH, spreading of overexpressed Sir3 in the *sir4-I1311N* background, and no spreading in the double mutant background, at both chromosome ends and near the core X element (we referred to these as “difference regions” in the following text) (Figure 2-10AJ and AL). In the ensemble plots aligning at chromosome ends, there were several peaks of “difference regions,” around 0-1 kb, 5-6 kb and 6-8 kb, respectively, away from chromosome ends. These peaks correspond to the distribution of subtelomeric C-ACS elements, which are most frequently located within 1 Kb (43%), between 5-6 kb (10%) or between 6-8 Kb (40%) from chromosome ends (Table 2-1). These findings indicate that the deletion of Sir3 wH domain has a more negative effect than the Sir4-I1311N mutation on Sir3 protein spreading, and that efficient Sir3 spreading requires both Sir3 wH and Sir4 CC domains.

Similar results were observed at both *HMR* and *HML* loci (Figure 2-10AG-AH). There was slight enrichment of Sir3ΔwH when it was overexpressed in the *sir3ΔwH SIR4* background (Figure 2-10AG-AH *sir3ΔwH/sir3ΔwH* 2μ tracks). There was even more enrichment when Sir3 was overexpressed in the *SIR3 sir4-I1311N* background (Figure 2-10AG-AH *sir4-I1311N/SIR3* 2μ tracks). However, Sir3 protein binding and spreading was completely lost in the double mutant background, even under Sir3ΔwH OE conditions (Figure 2-10AG-AH *sir3ΔwH sir4-I1311N/sir3ΔwH* 2μ tracks).

The *in vivo* Sir3 ChIP-seq results described above are consistent with our *in vitro* EMSA and bridging assay results, which suggest that Sir3 wH and Sir4 CC act redundantly in mediating Sir3 inter-nucleosomal cooperative binding, and that the level of cooperativity mediated by Sir3 wH is higher than that mediated by Sir4 CC.

DISCUSSION

Our results indicate that Sir3 binds to nucleosomes cooperatively, involving the dimerization of two Sir3 molecules bound to adjacent nucleosomes. This inter-nucleosomal cooperative binding is further stabilized by Sir3-Sir4 interactions. Both the Sir3 wH and Sir4 CC domains, which mediate the inter-nucleosomal cooperative binding, are required for the efficient spreading of Sir3 along chromatin. Our findings suggest that the inter-nucleosomal cooperative binding of Sir proteins mediate SIR complex spreading along heterochromatin. Sir3 molecules bound to adjacent nucleosomes interact with each other through their winged helix (wH) domain, and they interact with their bound nucleosomes through the bromo-adjacent homology (BAH) domain (Figure 2-11). This inter-nucleosomal cooperativity is further stabilized by the Sir4 coiled coil (CC) domain, which forms dimers, and interact with Sir3 via the AAA-ATPase-like

Table 2-1. Distribution of telomeres with different distances between chromosome end and the ACS sequence in the core X element.

Distance between chromosome end and C-ACS (bp)	# of telomeres (TEL01L and TEL13R ignored due to gaps in the X element)	%
0-1k	13	43.3
5k-6k	3	10.0
6k-7k	3	10.0
7k-8k	9	30.0
11k-12k	1	3.3
13k-14k	1	3.3
	30	100

(AAAL) domain of the latter. Sir2, which is an NAD-dependent histone deacetylase, and which stably associates with Sir4, deacetylates adjacent nucleosomes, providing binding sites for additional Sir3 (to the n+1 nucleosome, Figure 2-11). The ability of Sir3 to bridge free nucleosomes in solution, as indicated by our bridging assay, raises the possibility for Sir2 to deacetylate, and for Sir3 to bind to nucleosomes that are non-adjacent, but proximal (the n+X nucleosome, Figure 2-11), potentially facilitated by higher-order chromatin structure or DNA looping. Previous computational simulation work suggests that such long-range inter-nucleosomal contacts, even though occurring at a low rate is required for the bistability of epigenetic states (Dodd et al., 2007).

Our conclusions about the mechanism of SIR complex binding to chromatin are supported by extensive previous genetic and biochemical studies that revealed the importance of each of the Sir protein domains for silencing *in vivo* (Buchberger et al., 2008; Onishi et al., 2007; Oppikofer et al., 2013; Rudner et al., 2005), or nucleosome binding *in vitro* (Johnson et al., 2009; Martino et al., 2009; Onishi et al., 2007; Oppikofer et al., 2013; Sampath et al., 2009). Our analysis of Sir3 binding to defined mono- and di-nucleosome templates has allowed us to define primary roles for Sir3-Sir3 and Sir3-Sir4 interactions in bridging of adjacent nucleosomes, rather than stabilization of SIR complex association with single nucleosomes (Figure 2-11). In fact our results do not support the existence of any Sir-Sir interactions that form across a single nucleosome, suggesting that the architecture of the SIR complex may prohibit such interactions. The conclusions of binding results are further supported by statistical mechanical modeling of Sir3-nucleosome binding, which accurately predicts the main observed binding intermediates in our EMSA experiments. Importantly, this modeling indicates that Sir3 and nucleosomes interact in such a way that the formed “sticky ends” allow each

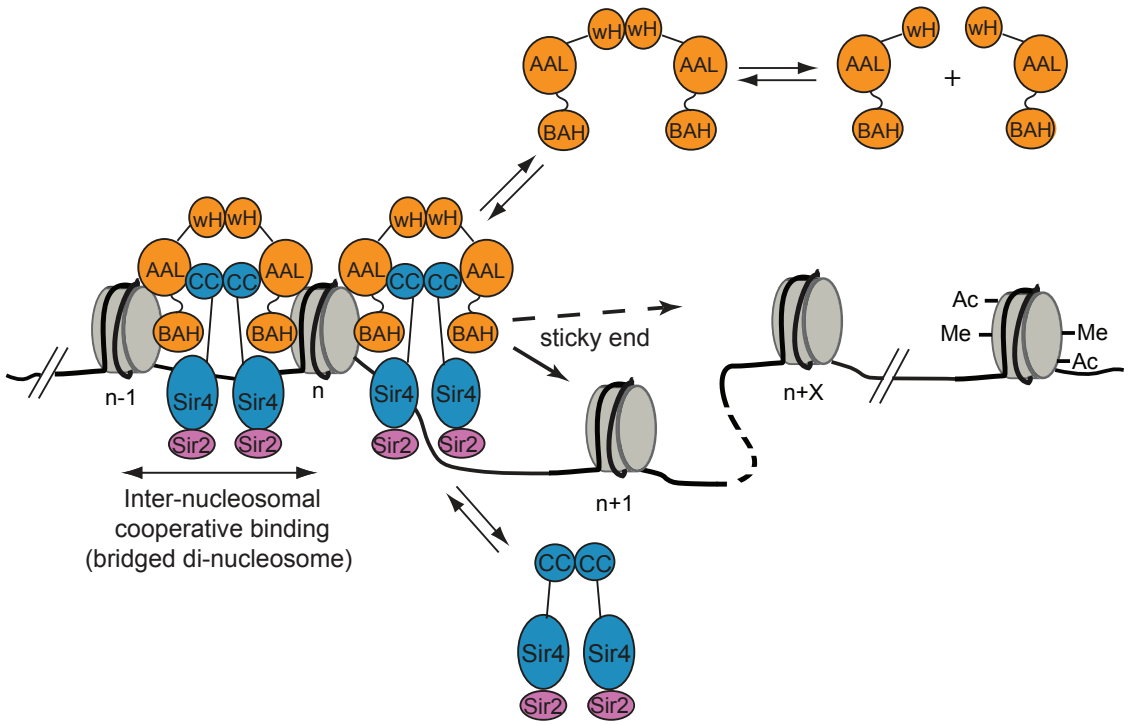

Figure 2-11. Model for the spreading of SIR complexes along heterochromatin.

Spreading is mediated by inter-nucleosomal cooperative binding. Inter-nucleosomal cooperativity depends on Sir3 dimerization through its wH domains and Sir3-nucleosome interactions mainly by its BAH domains. This inter-nucleosomal interaction is further stabilized by the Sir4 CC domain, which interacts with Sir3 AAAL domains. Sir4 also brings in the Sir2 NAD-dependent deacetylase, which deacetylates an adjacent nucleosome (n+1), or possibly a nearby non-adjacent nucleosome (n+x, dashed arrow) to create a binding site for the Sir3 sticky end. H4K16 acetylation and H3K79 methylation together promote the partitioning of Sir3 between active and silent chromosomal regions.

nucleosome to make further contacts along the nucleosome chain.

The mechanism of Sir3 binding to chromatin and its spreading along chromatin shares similarities as well as major differences with the spreading mechanism proposed for Swi6/HP1 in *S. pombe* (Canzio et al., 2011). The Swi6 protein, which like Sir3 forms dimers, associates with lysine 9 methylated histone H3 (H3K9me) tails on adjacent, rather than the same nucleosomes. Therefore in both *S. cerevisiae* and *S. pombe* dimerization of a nucleosome-bound protein mediates heterochromatin spreading. Unlike *S. pombe,* in which Swi6 has been proposed to self-associate on the same nucleosome via its chromodomain and spread on chromatin via an oligomerization mechanism (Canzio et al., 2011), our results reveal no Sir-Sir interactions on the same nucleosome. Therefore in contrast to polymerization models, which have long been thought of as a general feature of heterochromatin spreading (Canzio et al., 2011; Liaw and Lustig, 2006; Liou et al., 2005; McBryant et al., 2006; Moretti et al., 1994), our findings suggest that Sir3 spreads along chromatin discontinuously, with Sir-Sir protein interactions taking place only in-between nucleosomes (Figure 2-11). A non-polymerization model with the di-nucleosome as the fundamental binding unit (Figure 2-11) may be more consistent with *in vivo* Sir3 occupancy data which suggest that Sir3 binds discontinuously at native telomeres and the *HMR* locus (Ellahi et al., 2015; Fourel et al., 1999; Pryde and Louis, 1999; Radman-Livaja et al., 2011; Thurtle and Rine, 2014).

MATERIALS AND METHODS

Table 2-2. List of *S. cerevisiae* strains used.

Name	Genotype	Source
SF1	W303-1a	Jasper Rine
SF4	*sir3Δ::TRP1* in SF1	Jasper Rine
DMY4350	*sir3ΔwH::TRP1* in SF1	Chenning Lu
ADR2973	*sir4-I1311N* in SF1	Adam Rudner
DMY4351	*sir4-I1311N sir3ΔwH::TRP1* in SF1	Chenning Lu
DMY3315	W303-1a *sir3Δ::Kan^R hmrΔE::TRP1* TELVII-L::URA3	

Table 2-3. List of plasmids used.

Name	Genotype	Source
pJR104 (pDM602)	*SIR3* under endogenous promoter in YEp24	Jasper Rine
pDM1798	*sir3ΔwH* under endogenous promoter in YEp24	Chenning Lu
pDM832	Sir3-3XFLAG under endogenous promoter in pRS315	Johannes Buchberger
pDM1799	Sir3ΔwH-3XFLAG under endogenous promoter in pRS315	Chenning Lu

Table 2-1 lists the strains used in this study. Table 2-2 lists the plasmids used in this study. All deletions and mutations were confirmed by PCR and sequencing. Epitope-tagged strains were constructed by a PCR-based gene targeting method (Longtine et al., 1998; Rudner et al., 2005).

Protein Cloning and Purification

Sir3-3XFLAG and BAH-3XFLAG were purified from *S. cerevisiae* as described previously (Buchberger et al., 2008; Liou et al., 2005). Sir3ΔwH-3XFLAG was constructed by deleting the winged helix (wH) region on pDM1009 (GAL-Sir3-3XFLAG 2μ plasmid), and purified by the same FLAG purification protocol. Sir4 CC (1198-1358) was cloned into the pET47b(+) plasmid, and the protein was purified from *E.coli* by Ni^+-affinity purification, followed by PreScission protease cleavage and gel filtration, to

remove the His tag. A minor degradation product co-purified with Sir4 CC (1198-1358). Mass Spectrometry analysis identified this fragment as Sir4 (1242-1358), which covers the entire Sir4 CC core domain, and should therefore have the same Sir3 binding activity as the larger Sir4 CC (1198-1358). Sir4 CC (1198-1358) was also cloned into pGEX6P-1, and the resulting GST-Sir4 CC was affinity purified from *E.coli*. *S. cerevisiae* histones were overexpressed and purified from *E.coli* as previously described (Johnson et al., 2009).

Mono-nucleosome and Di-nucleosome Reconstitution

Mono-nucleosomes and di-nucleosomes were reconstituted using gradient salt dialysis as described previously (Luger et al., 1999). The NCP DNA template contains the 147 bp 601 positioning sequence (Lowary and Widom, 1998). The di-nucleosomal DNA template contains two direct repeats of the 601 sequence, separated by a 20 bp linker. The biotinylated nucleosomal DNA template contains the 601 sequences, with an extra 20 bp linker added to its 5' end, and was produced by PCR reactions using 5' biotinylated primer (Integrated DNA Technologies). The Alexa-647 labeled NCP DNA template was also made by PCR reactions using 5' Alexa-647 labeled primer (Integrated DNA Technologies).

Restriction Enzyme Protection Assay

Di-nucleosomes were incubated with 10U of either ScaI or AluI restriction enzyme (New England Biolabs) in 1XNEB CutSmart Buffer, at 37^0C for 1 hr. The resulting digestion products were separated on native polyacrylamide gels, and visualized by staining with ethidium bromide.

Electrophoretic Mobility Shift Assays (EMSAs)

Different amounts of Sir3 protein were incubated with 3 nM mono- or di-nucleosomes in binding buffer (25 mM Tris.HCl (pH7.5), 50 mM NaCl, and 5 mM DTT) at 4^0C for 1 hr. Samples were then run on native polyacrylamide gels, stained with SYBR Gold (Invitrogen), visualized on a Typhoon FLA7000 imager (GE Healthcare), and quantified using ImageQuant software. As observed previously by other groups, shifted nucleosome bands were stained less effectively than unshifted bands, probably due to protein loading onto nucleosomes (Oppikofer et al., 2013). Thus, as all previous literature, Sir3 binding to nucleosomes was quantified by the decrease in the intensity of the unbound nucleosome band. The K_D for each binding reaction was calculated with Prism Graphpad software by fitting the binding curve with the Hill Equation.

Cooperative Binding Analysis

Sir3 binding to di-nucleosomes was analyzed by the Klotz model, one of several models for analyzing cooperative binding. This model considers the stepwise formation of intermediate stages, and expresses the cooperative binding in terms of elementary processes governed by the mass action law (Cantor and Schimmel, 1980; Klotz, 2004; Stefan and Le Novere, 2013). According to the Klotz Equation, $\Theta = 1/n * (K_1[X] + 2K_1K_2[X]^2 + \ldots + n(K_1K_2\ldots K_n)[X]^n)/(1 + K_1[X] + K_1K_2[X]^2 + \ldots + (K_1K_2\ldots K_n)[X]^n)$, where Θ is the fractional occupancy; K_i describes the macroscopic association constant when i binding sites are occupied. If all ligand binding sites are identical with a microscopic association constant K, one would expect $K_1 = nK$, $K_2 = (n-1)K/2$, … , $K_n = K/n$ (that is $K_i = (n-i+1)K/i$) in the absence of cooperativity. There is positive cooperativity if K_i lies above these expected values for $i > 1$.

Direct fitting the Sir3 DiN binding curve with the Klotz Equation is complicated by two factors. First, in the Klotz Equation, the fractional occupancy is calculated from the

number of bound and total binding sites, but this information is elusive in the current EMSA data. As shown in Figure 2-2C, there are two shifted bands of Sir3 binding to DiN, but the molecular and binding stoichiometric nature of each band is uncertain. Second, a recent analytical ultracentrifugation (AUC) study suggests that Sir3 binds to nucleosome arrays with a stoichiometry of 2:1 (Swygert et al., 2014). However, even if we make the assumption that the lower shifted band in Sir3-DiN EMSA corresponds to the binding of 2 Sir3 molecules, and that the higher shifted band represents the binding of 4 Sir3 molecules, further complications arise from unequal band staining intensity caused by protein loading onto nucleosomes, which differentially affects the efficiency of DNA staining, a staining artifact also observed by others (Oppikofer et al., 2013). To overcome these limitations, we simplified the Sir3 DiN binding problem by making the following assumption: Sir3 binds to each mono-nucleosome within the DiN in exactly the same way, that is with the same stoichiometry and the same K_D, as its binding to NCP (an apparent characteristic "k" influenced only by histone modifications), except that the association of Sir3 with the second nucleosome may occur cooperatively, with a cooperativity coefficient, "c". With this assumption, Sir3 binding to DiN can be illustrated by a binding cycle as shown in Figure 2-3A, and analyzed accordingly. By combining the steady-state equilibrium equation of each binding step, a cooperative binding equation as shown in Figure 2-3A can be deduced. $F_{unbound} = 1/(1+2[S]/k+c*([S]/k)^2)$ (Equation 1), where $F_{unbound}$ is the fraction of unbound di-nucleosomes, [S] is Sir3 concentration used in the binding reaction, and k is the apparent characteristic dissociation constant of Sir3 binding to NCP, measured by Sir3-NCP EMSA. The c value of Sir3 binding to di-nucleosomes is deduced by fitting the Sir3-dinucleosome binding curve with Equation 1. c equals to 1 when there is no cooperativity, and c values larger than 1 indicate positive cooperativity.

In addition, it was deduced that on the macroscopic level, Sir3 binds to the first nucleosome with $K_{D1}=k/2$, and binds to the second nucleosome with $K_{D2}=2k/c$. The cooperativity c value was translated to the cooperativity free energy ($\triangle G^0_{coop}$) by comparing the difference between real Gibbs free energy of dissociation when Sir3 binds to the second mono-nucleosome within DiN and the free energy of this binding event when there is no cooperativity (null hypothesis, c=1). Thus $\triangle G^0_{coop} = \triangle\triangle G^0_{nc\text{-}c} = \triangle G^0_{nc} - \triangle G^0_{c} = - RT ln K_{Dnc} + RT ln K_{Dc} = RT ln(K_{Dc}/K_{Dnc}) = -RT ln c$.

Non-covalent nucleosome crosslinking assays

Nucleosomes assembled with biotinylated DNA were conjugated to Dynabeads M-280 streptavidin (Invitrogen) at RT for 1 hr with rotation, using 36 µl of beads slurry per µg of nucleosomes in the binding buffer (20 mM Tris.HCl (pH7.5), 0.3 mM EDTA, 50 mM NaCl, 10% glycerol, 5 mM DTT, 1 mg/ml BSA, and 0.02% NP-40). Bead-conjugated nucleosomes were washed, and resuspended in equal volume of binding buffer as the initial volume of beads taken. Equal amount of conjugated nucleosomes, in a final concentration of 100 nM, was added to tubes containing Sir3, Sir3/Sir4 CC, Sir3ΔwH, Sir3ΔwH/Sir4 CC, Sir4 CC, or buffer alone, and incubated with rotation at 4^0C for 1 hr. The concentration of Sir3 proteins was 2 µM in the case of high protein concentration binding assay, and 200 nM in the case of low protein concentration binding assay. Sir4 CC was in 2X molar excess of Sir3 proteins. Then Alexa-647 nucleosomes were added into each reaction at a final concentration of 100 nM, and reactions were incubated for another 1 hr at 4^0C. Finally, the beads were washed twice in the binding buffer before magnetic concentration. Alexa-647 nucleosomal DNA from the crosslinked nucleosomes was stripped from the beads with 2M NaCl, separated on native polyacrylamide gels, and quantified by the fluorescent intensity of the band.

Silencing Assays

Yeast strain DMY3315 (W303a *hmrΔE::TRP1 TELVII-L::URA3 sir3ΔKan*^{R}) was described previously (Rudner et al., 2005). DMY3315 was transformed with plasmids pRS315 (empty vector), pDM832 (Sir3-3XFLAG), or pCL21(Sir3ΔwH-3XFLAG). For silencing assays, cells were grown to OD_{600} of 1.0, plated as 10-fold serial dilutions on SC-LEU, SC-LEU-TRP, SC-LEU-URA, or SC-LEU+5-FOA plates, incubated at 30^0C for 3 days, and photographed.

Protein Interaction Assays

GST-Sir4 CC protein was expressed and purified from *E. coli*, and used in pull-down assays to confirm the interaction between Sir4 CC and Sir3. Either the fusion protein or GST was incubated with either Sir3 or BSA at 4^0C for 1 hr, in the binding buffer (25 mM Tris (pH7.5), 100 mM NaCl, 1 mM DTT, and 0.5 mM BSA). The mixtures were then incubated with pre-equilibrated glutathione Sepharose beads (GE Healthcare) at 4^0C for 1 hr, with end-to-end rotation. The beads were recovered by centrifugation at 500 g for 5 min, washed 3 times with binding buffer, and then resuspended in SDS-PAGE sample buffer to elute the bound proteins. The samples were denatured by heating, and the proteins in the complexes were visualized by SDS-PAGE and staining with Coomassie blue.

Statistical mechanical modeling of Sir3 binding to nucleosomes

The fraction of each species was calculated at a given [Sir3] and given values for K, C_1 and C_2, as its weight divided by the sum of all mono- or di-nucleosome species weights (Fig 2-9A). Parameter optimization involved random starting guesses for parameter values (K, C_1, and C_2) followed by random incremental parameter alterations to find values that minimized $\Sigma(observed–expected)^2/expected$ over all EMSA NCP and DiN

fraction-bound data points in the 4 conditions. The contribution of data error to error in the estimation of optimal parameters was assessed by randomly altering each data point by a 20% standard deviation according to the normal distribution for each fitting run (Fig 2-9B). The quality of the fit was generally not substantially diminished with moderate changes (~2-fold) in the values of the parameters. This is because the overall affinity of Sir3 for mono-nucleosomes is determined by C_1 and K, such that changes in one parameter can be compensated for by changes in the other. Similarly, the effects of changes in C_1 or K on di-nucleosome binding can be compensated to some degree by altering C_2.

ChIP-seq

Cells were cultured overnight in YEPD medium, or selective media for cells harboring overexpression plasmids (YEp24 2μ plasmid with Sir3 or Sir3ΔwH expressed from Sir3 endogenous promoter), diluted into fresh media to OD_{600} = 0.4, and harvested at late log phase (OD_{600} = 1.5). Cells were fixed with 1% formaldehyde for 15 min at room temperature (RT), then quenched with 130 mM glycine for 5 min at RT, harvested by centrifugation, washed twice with TBS (50 mM Tris, pH 7.6, 150 mM NaCl), and flash frozen. Cell pellets were resuspended in 600 μl lysis buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, 0.1% SDS, 1 mM PMSF, protease inhibitor tablet (Roche)), and disrupted by bead beating (MagNA Lyser, Roche) for 6x30 sec at 4500 rpm with 0.5 mm glass beads. Tubes were punctured and the flow-through was collected in a new tube by centrifugation. After sonication for 3x20 sec at 40% amplitude (Branson Digital Sonifier), the extract was centrifuged (Eppendorf 5415R) for 15 min at 13,000 rpm. The soluble chromatin was then transferred to a fresh tube. 10 μl lysate from the wild type sample was saved as input, to which 240 μl of

1XTE/1% SDS were added (TE: 50 mM Tris pH 8.0, 1 mM EDTA). Sir3 antibody (Rudner et al., 2005) was preincubated with washed Dynabeads Protein A, and for each immunoprecipuitation, 2 µg antibody coupled to 100 µl beads was added to soluble chromatin. Samples were incubated for 2 hours at 4^{o}C with rotation, after which the beads were collected on magnetic stands, and washed 3 times with 1 ml lysis buffer and once with 1 ml TE, and eluted with 100 µl preheated buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS) at 65 ^{o}C for 15 min. The eluate was collected, and 150 µl 1XTE/0.67% SDS was added. Input and immunoprecipitated samples were incubated overnight at 65^{o}C to reverse crosslink, diluted with 250 µl TE, and treated with 50 µg RNase A at 37^{o}C for 1h. 60 µg glycogen and 5 µl proteinase K (Roche) was added and incubation was continued at 55^{o}C for 1h. 22 µl of 10M LiCl was added and the samples were purified using PCR purification kit (Qiagen).

Libraries for Illumina sequencing were constructed following the manufacturer's protocols, starting with ~5 ng of immunoprecipitated DNA fragments. Each library was generated with custom-made adapters carrying unique barcode sequences at the ligating end (Wong and Struhl, 2011). Barcoded libraries were mixed and sequenced with Illumina HiSeq 2500. Raw reads were separated according to their barcodes and mapped to the *S. cerevisiae* S288C genome using Bowtie. Mapped reads were normalized to reads per million and visualized in IGV.

Ensemble plots aligned at chromosome ends were generated by aligning all 30 telomeres, excluding *TEL01R* and *TEL13R,* at chromosome ends, and calculating the total ChIP-seq signal across 14 kb regions towards the centromere. The cumulative ChIP-seq reads was then normalized, on a per-base basis, to that of the *Δsir3* sample. Ensemble plots aligned at the ACS within the core X element (C-ACS) were generated in

a similar manner, except that each telomere was aligned at C-ACS, and total ChIP-seq signal was computed for 14 kb in each direction.

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CHAPTER III

Analysis of Effects of Histone Modifications on Sir3-Nucleosome Interactions

AUTHOR CONTRIBUTION

Chenning Lu and Danesh Moazed designed the experiments. Chenning Lu performed all experiments and data analysis. Ian Dodd performed the statistical mechanical modeling. Results described in this chapter have been submitted for publication.

ABSTRACT

Histone H4 lysine 16 (H4K16) acetylation and histone H3 lysine 79 (H3K79) methylation are hallmarks of active chromatin in *Saccharomyces cerevisiae*, and are depleted in silent chromatin regions. Both modifications inhibit Sir3 binding to histones and nucleosomes. In the current study, we examine the effect of either modifications alone, or both modifications in combination, on Sir3 binding to nucleosomes, using electrophoretic mobility shift assay (EMSA) and quantitative analysis. We find that each modification reduces the affinity of Sir3 binding to nucleosomes by 3-4 fold, and that the presence of both modifications on the same nucleosome greatly reduces Sir3 binding. Our results suggest that the simultaneous presence of H4K16 acetylation and H3K79 methylation on euchromatic nucleosomes can account for the *in vivo* partitioning of Sir3 between silent and active chromosomal regions.

INTRODUCTION

Histone proteins that help organize eukaryotic nuclear DNA into chromatin are subject to a wide array of reversible post-translational modifications, such as methylation, acetylation, phosphorylation, ubiquitination, sumoylation and ADP ribosylation at specific amino acid residues. These post-translational modifications impact various cellular processes, including transcription, replication, DNA repair and recombination (Groth et al., 2007; Jenuwein and Allis, 2001; Kouzarides, 2007; Li et al., 2007).

A well-characterized function of histone modifications is that they regulate association of numerous chromatin associated proteins, serving to either promote or exclude the binding of these proteins (Grewal and Moazed, 2003; Jenuwein and Allis, 2001; Kouzarides, 2007; Moazed, 2001; Schreiber and Bernstein, 2002; Strahl and Allis, 2000; Turner, 2002). In some cases, combinations of different histone modifications are recognized by the same (Eustermann et al., 2011; Iwase et al., 2011; Moriniere et al., 2009; Ramon-Maiques et al., 2007), or different domains in a single protein, or different subunits of a complex (Dhalluin et al., 1999; Li et al., 2007; Li et al., 2006; Rothbart et al., 2013; Ruthenburg et al., 2011). The combined action of multiple histone modifications potentially provides better binding specificity and higher binding affinity (Du and Patel, 2014; Taverna et al., 2007).

Euchromatic and heterochromatic regions of the eukaryotic genome are associated with stereotypical histone modification patterns, with each type of region containing or excluding several different modifications (Ernst et al., 2011; Filion et al., 2010; Kharchenko et al., 2011). In budding yeast *S. cerevisiae*, both histone H4 lysine 16 (H4K16) acetylation and histone H3 lysine 79 (H3K79) methylation are hallmarks of

active euchromatin, and are depleted in the heterochromatic region (Braunstein et al., 1996; Imai et al., 2000; Landry et al., 2000; Ng et al., 2003; Ng et al., 2002; Smith et al., 2000; Suka et al., 2001; van Leeuwen et al., 2002).

In *S. cerevisiae*, 80% of H4K16 in the genome is acetylated (Clarke et al., 1993; Smith et al., 2003), but in silent chromatin regions, it is specifically deacetylated by Sir2, an NAD-dependent histone deacetylase (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000). Sir2 is a subunit in the SIR complex, composed of Sir2, Sir3 and Sir4. Mutagenesis studies suggest that H4K16 plays an essential role in silencing (Braunstein et al., 1996; Johnson et al., 1990). In addition, it has been shown that both Sir3 and Sir4 interact with deacetylated H3 and H4 N-terminal peptides (Hecht et al., 1995). In particular, Sir3 interacts directly with regions around H4K16, and its interaction with both histones and nucleosomes is sensitive to H4K16 acetylation (Armache et al., 2011; Carmen et al., 2002; Johnson et al., 2009; Liou et al., 2005; Swygert et al., 2014; Wang, 2013) . Deletion or the enzymatically inactive mutation of Sir2 results in inefficient recruitment of Sir3 to silent chromatin and defective silencing (Johnson et al., 2009; Rusche et al., 2002).

H3K79 resides in the H3 globular domain, and is located on the nucleosome core surface. It is another residue that is critically involved in silencing in *S. cerevisiae* (Ng et al., 2003; Ng et al., 2002; van Leeuwen et al., 2002). H3K79 is methylated by the histone methyltransferase, Disruptor of Telomeric Silencing 1 (Dot1), and its methylation is a hallmark of euchromatin in most eukaryotes. About 90% of H3K79 in the *S. cerevisiae* genome is methylated (van Leeuwen et al., 2002). In contrast, H3K79 is hypomethylated at all silenced loci (Ng et al., 2003; Ng et al., 2002; van Leeuwen et al., 2002). Sir3 interacts with the nucleosome core region, around H3K79, and this interaction is

inhibited by H3K79 methylation (Armache et al., 2011; Onishi et al., 2007; Wang, 2013). Although H4K16 acetylation and H3K79 methylation abolish the association of Sir3 with histone peptides, the effect of each modification on Sir3 binding to nucleosomes is modest (Johnson et al., 2009; Martino et al., 2009). It remains possible that specificity is achieved by the combined action of both modifications, but direct evidence in support of this idea is lacking.

In order to determine how Sir3 association with chromatin is affected by histone modifications, we studied Sir3 binding to *in vitro*-reconstituted nucleosomes that are unmodified, singly modified with H4K16 acetylation or H3K79 methylation, or doubly modified with both modifications. Consistent with previous results, we find that both H4K16 acetylation and H3K79 methylation decreases Sir3 binding affinity to nucleosomes. In combination, the two modifications work together to dramatically reduce the affinity of Sir3 for nucleosomes.

RESULTS

H4K16 acetylation and H3K79 methylation reduces Sir3 binding affinity to nucleosomes

For studies using acetylated nucleosomes, the reconstituted nucleosomes were acetylated by the catalytic Piccolo subcomplex of the NuA4 histone acetyltransferase (HAT) complex purified from *E. coli*, as described previously (Barrios et al., 2007; Johnson et al., 2009; Selleck et al., 2005) (Figure 3-1A). The Piccolo HAT complex acetylates both H2A and H4 (Selleck et al., 2005). However, among hypoacetylated histone residues within *S. cerevisiae* silent chromatin regions, H4K16 has been shown to be particularly critical for silencing, and for the interaction of Sir3 with H4 N-terminal

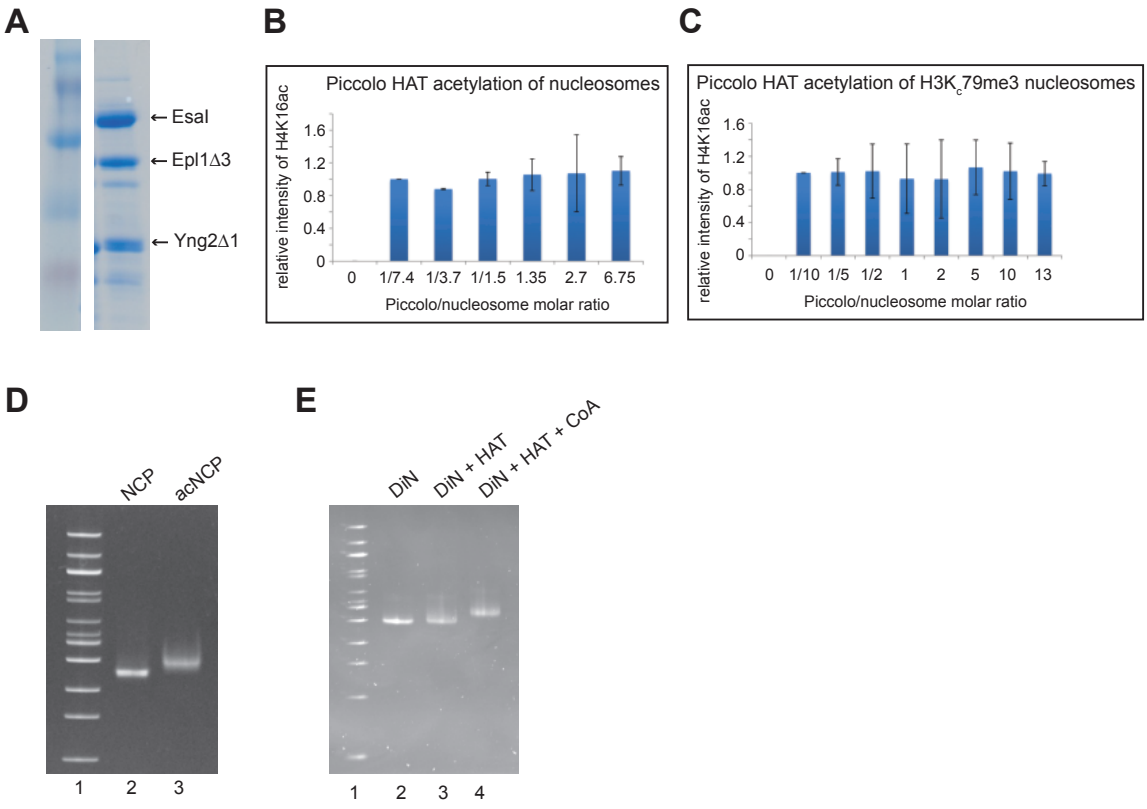


Figure 3-1. Nucleosome acetylation by purified Piccolo HAT complex.

(A) Coomassie stained gel showing purified Piccolo HAT complex. **(B)** Quantification, by quantitative western blot, of Piccolo HAT mediated acetylation on unmodified nucleosomes. Increased amounts of Piccolo HAT complex were titrated against a constant amount of unmodified nucleosomes. The resulting H4K16ac level was quantified using anti-H4K16ac antibody. There was no increase in acetylation signal with increasing amount of Piccolo HAT, indicating saturating levels of acetylation. **(C)** Quantification of acetylation reactions as in **B** but on H3K$_C$79me3 nucleosomes. **(D, E)** Nucleosome acetylation causes a slight upward shift in nucleosome mobility, consistent with previous findings. Acetylated nucleosomes are completely shifted, indicating complete acetylation.

peptide and nucleosomes (Braunstein et al., 1996; Johnson et al., 2009; Liou et al., 2005; Onishi et al., 2007; Rusche et al., 2003; Shahbazian and Grunstein, 2007). Thus we attribute the effect of acetylation on Sir3 binding to nucleosomes to H4K16 acetylation. The completion of H4K16 acetylation (H4K16ac) was assessed by western blot using antibody against H4K16ac (Figure 3-1B), and was confirmed by the complete shift of nucleosomes on Native PAGE, a behavior of acetylated nucleosomes described previously (Li et al., 2007) (Figure 3-1D-E).

Dot1, the H3K79 methyltransferase in *S. cereivise,* does not appear to be a robust enzyme *in vitro*, as assessed by western blot. The signal of H3K79 methylation (H3K79me) does not saturate with increasing amount of Dot1, so the methyl-lysine analog (MLA) method was used to generated K_C79me3 H3 histones (Figure 3-2A) (Simon et al., 2007), which was then reconstituted into nucleosome core particle (NCP) and di-nucleosome (DiN) (Figure 3-2B-C). We chose H3K79 trimethylation (H3K79me3) for our binding studies because it has been shown that the trimethylated state of H3K79 is the predominant *in vivo* state (Frederiks et al., 2008; Ng et al., 2002).

Consistent with previous studies showing that H4K16ac and H3K79me each inhibits Sir3 binding to histone peptides and nucleosomes (Altaf et al., 2007; Carmen et al., 2002; Hecht et al., 1995; Johnson et al., 2009; Liou et al., 2005; Swygert et al., 2014; Wang, 2013), our results showed that H4K16ac and H3K_C79me3 each decreased the affinity of Sir3 binding for NCP by 4-5 fold, with K_D values of about 4.7 μM and 6.5 μM, respectively (Figure 3-3A-D). Each modification also reduced the affinity of Sir3 for DiN with K_D values of 0.9 uM and 0.8 uM, respectively (Figure 3-3E-H), which represented about a 5-fold decrease in affinity relative to unmodified DiNs. We note that Sir3 bound H4K16ac and H3K_C79me3 NCPs shift to a lower position than Sir3 bound unmodified

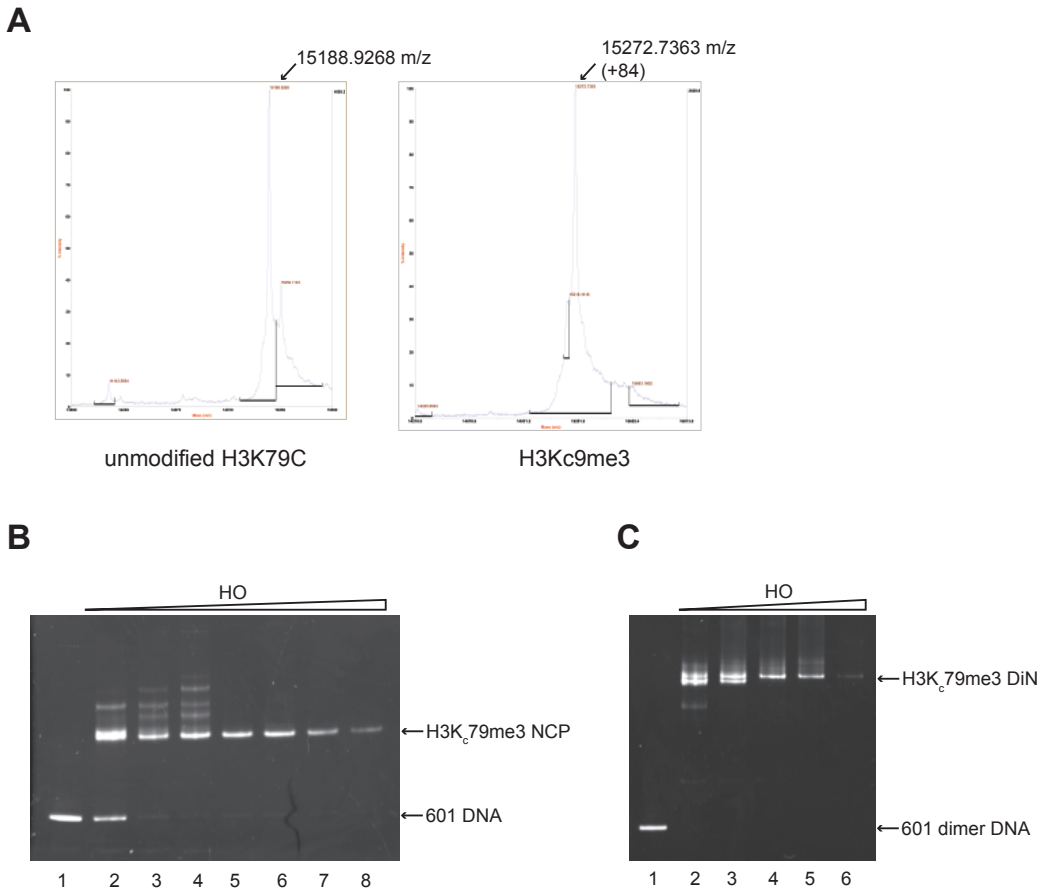


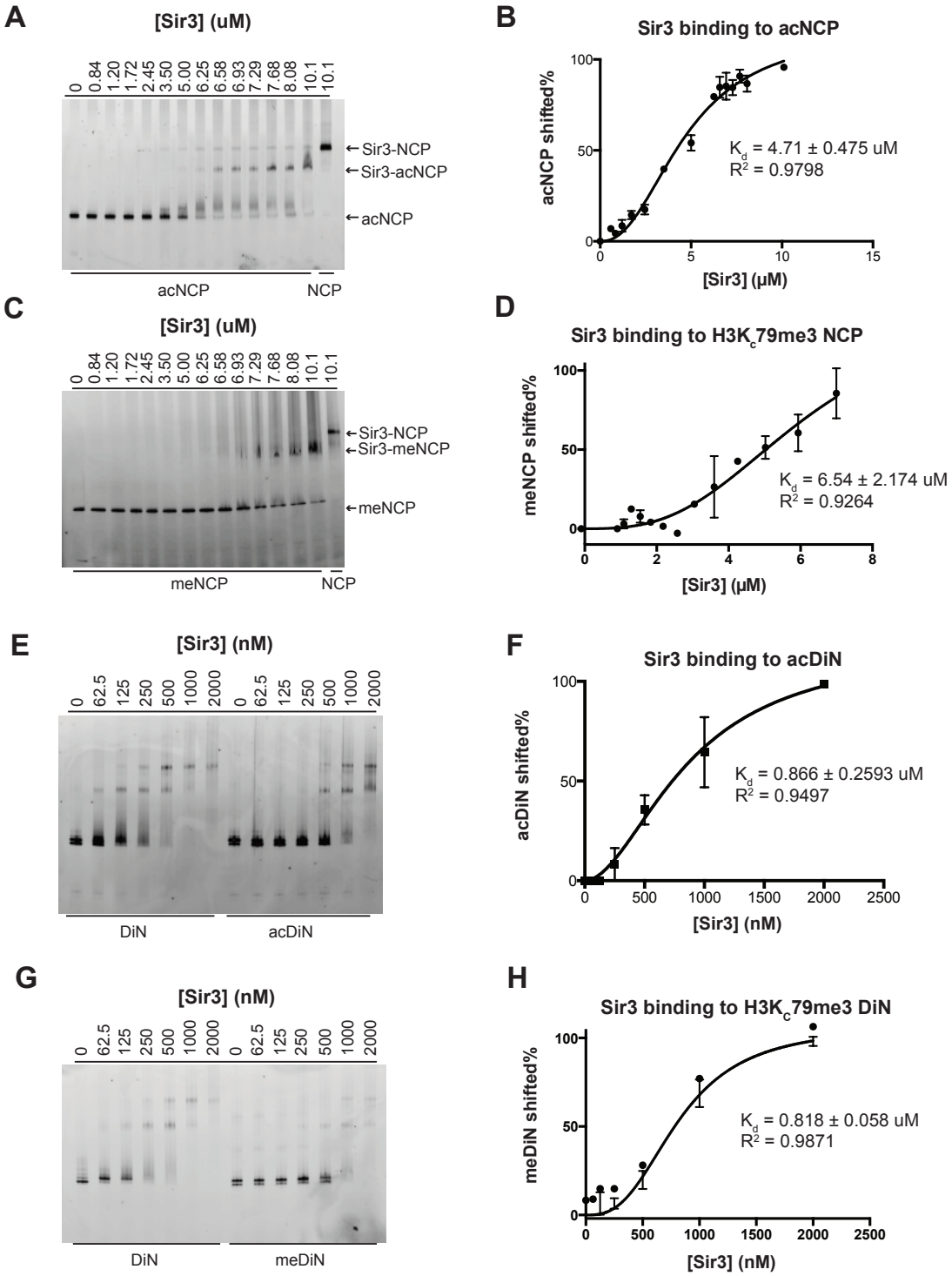
Figure 3-2. H3K79 trimethylation and reconstitution of H3K$_C$79me3 nucleosomes.

(A) Mass Spectrometry verified the complete alkylation of H3K$_C$79me3. **(B)** Reconstitution of H3K$_C$79me3 NCP. **(C)** Reconstitution of H3K$_C$79me3 DiN. Nucleosomes which produced a single band were used for biochemical assays.

Figure 3-3. H4K16 acetylation and H3K79 trimethylation reduces Sir3 binding affinity to nucleosomes.

(A) EMSA experiments of Sir3 binding to H4K16ac NCP. **(B)** Binding curves from three experiments performed as in **A** were fitted with the Hill Equation, which gives a Hill coefficient of 2.28 ± 0.280. **(C)** EMSA experiments of Sir3 binding to $H3K_C79me3$ NCP. **(D)** Binding curves from three experiments performed as in **C** were fitted with the Hill Equation, which gives a Hill coefficient of 3.02 ± 0.891. **(E)** EMSA experiments comparing Sir3 binding to unmodified and H4K16ac DiNs. **(F)** Binding curves from three experiments performed as in **E** were fitted with the Hill Equation, which gives a Hill coefficient of 1.86 ± 0.547 for Sir3 binding to H4K16ac DiN. **(G)** EMSA experiments comparing Sir3 binding to unmodified and $H3K_c79me3$ DiNs. **(H)** Binding curves from three experiments performed as in **G** were fitted with the Hill Equation, which gives a Hill coefficient of 2.77 ± 0.384 for Sir3 binding to $H3K_c79me3$ DiN. For all EMSA experiments, purified Sir3 proteins were titrated onto a constant amount of nucleosomes at 3 nM.

Figure 3-3 (Continued)



nucleosomes, suggesting that Sir3 binds to modified nucleosomes in a different conformation than to unmodified nucleosomes (Figure 3-3A and C).

H4K16 acetylation and H3K79 methylation does not change the cooperativity of Sir3 binding to nucleosomes

Sir3 binds to each of H4K16ac and H3K_C79me3 NCPs with a Hill coefficient around 2 (Figure 3-3B and D). This is the same as Sir3 binding to unmodified NCP, indicating that neither histone modifications affect Sir3 intra-nucleosomal binding cooperativity. We next examined whether either modifications affects Sir3 inter-nucleosomal binding cooperativity to DiN. As shown in Figure 3-4A and C, Sir3 bound to H4K16ac DiN and to H3K_C79me3 DiN with much higher affinity than binding to correspondingly modified mono-nucleosomes. Analysis of inter-nucleosomal binding cooperativity using Equation 1 in Figure 2-3A showed that the cooperativity coefficient for H4K16ac and H3K_C79me3 DiN was 33 and 59, respectively (Figure 3-4B and D). This indicates that neither modifications affect Sir3 cooperative binding to di-nucleosomes (compare with c value of 60 in unmodified DiN in Figure 2-3B).

H4K16 acetylation and H3K79 trimethylation act together to inhibit Sir3 binding to nucleosomes

As both H4K16ac and H3K79me are markers for euchromatin, and are deposited globally in *S. cerevisiae* (Kimura et al., 2002; Ng et al., 2002; Suka et al., 2002), it is highly likely that nucleosomes within the euchromatic regions harbor both histone modifications at the same time. Our *in vitro* system enables us to quantitatively analyze the effect of the co-existence of both modifications on Sir3 binding to nucleosomes.

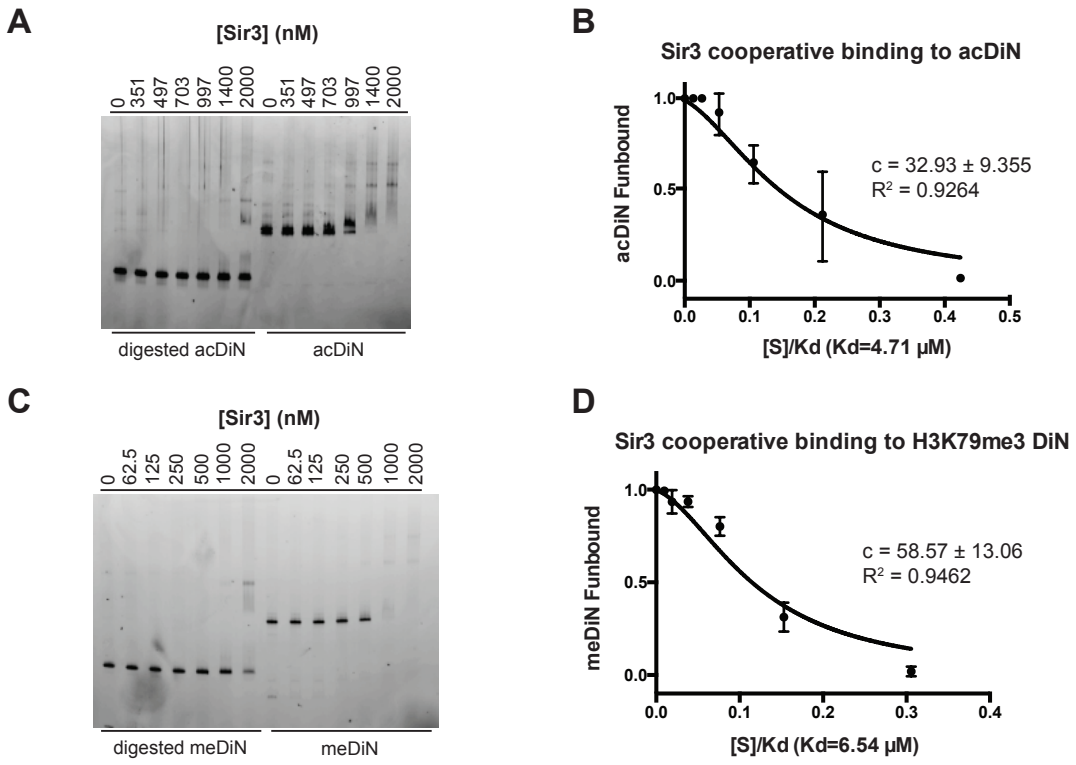


Figure 3-4. H4K16 acetylation and H3K79 trimethylation does not change Sir3 inter-nucleosomal binding cooperativity.

(A) EMSA experiments comparing Sir3 binding to H4K16ac DiN and to mono-nucleosomes released from digestion of H4K16ac DiN. **(B)** Sir3 cooperative binding to H4K16ac DiN, as analyzed by Equation 1 in Figure 2-3A. **(C)** EMSA experiments comparing Sir3 binding to H3K$_c$79me3 DiN and to mono-nucleosomes released from digestion of H3K$_C$79me3 DiN. **(D)** Sir3 cooperative binding to H3K$_c$79me3 DiN, as analyzed by Equation 1 in Figure 2-3A. For all EMSA experiments, Sir3 proteins were titrated onto a constant amount of nucleosomes at 3 nM.

H4K16ac/H3K_C79me3 doubly modified nucleosomes were generated by piccolo HAT complex acetylation of H3K_C79me3 nucleosomes (Figure 3-1C and 3-5A).

We found that the combination of the two modifications inhibited Sir3 binding to NCP and DiN, so that we could not obtain specifically shifted bands at the highest Sir3 concentration (11 μM) used in the assay (Figure 3-5B-C). This observation suggests that the two modifications act together to inhibit Sir3 binding, so that the K_D value for NCP is higher than 11uM. We also found that Sir3 bound to H4K16ac/H3K_C79me3 doubly modified DiN with a different shifting pattern than binding to either unmodified or singly modified DiN. Whereae the typical Sir3-DiN binding leads to two defined shifted bands (Figure 2-2C, 3-3E and G), the H4K16ac/H3K_C79me3 DiN band shifts upwards gradually with increasing Sir3 concentration (Figure 3-5C). This suggests that the binding of Sir3 to doubly modified DiNs we observed in EMSA may be nonspecific, and is caused by the artificially high Sir3 concentration used *in vitro*. In addition, Sir3 may bind to H4K16ac/H3K_C79me3 nucleosomes in a different conformation than binding to unmodified nucleosomes.

Statistical mechanical modeling of the effect of H4K16 acetylation and H3K79 trimethylation on Sir3 binding to nucleosomes

We used the same statistical mechanical modeling method described in Chapter 2 (Figure 2-9A) to fit the pooled bound-fraction data from H4K16ac and H3K_C79me3 nucleosome EMSAs. We could obtain reasonably good fits if we kept C_1 and C_2 values the same as values for Sir3 binding to unmodified nucleosomes, and only increased K values (Figure 2-9C and 3-6A). Thus, similar to the conclusions from Equation 1 in Figure 2-3A, the effect of H4K16 acetylation and H3K79 trimethylation on Sir3 binding

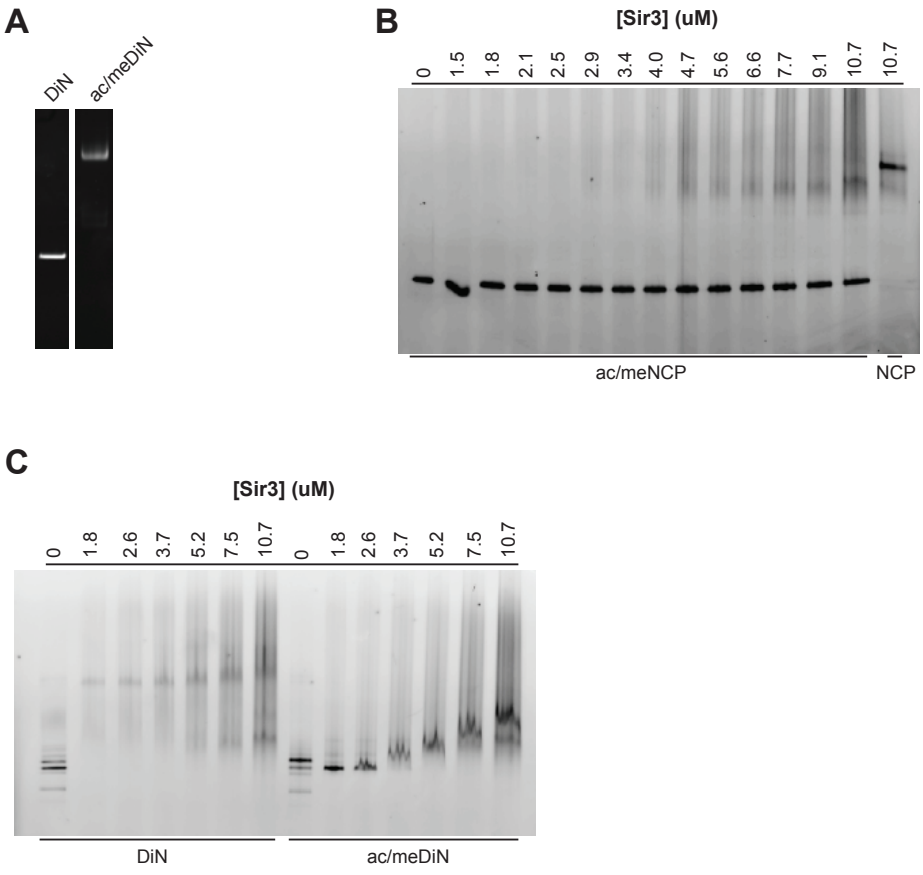


Figure 3-5. H4K16 acetylation and H3K79 methylation act together to strongly inhibit the binding of Sir3 to nucleosomes.

(A) Native PAGE showing the assembly of doubly modified H4K16ac/H3K_C79me3 DiN. **(B)** EMSA experiments of Sir3 binding to doubly modified H4K16ac/H3K_C79me3 NCPs. There is about 10% NCP shift at 10.7 μM Sir3. **(C)** EMSA experiments of Sir3 binding to unmodified and doubly modified H4K16ac/H3K_C79me3 DiNs. For all EMSA experiments, purified Sir3 proteins were titrated onto a constant amount of nucleosomes at 3 nM.

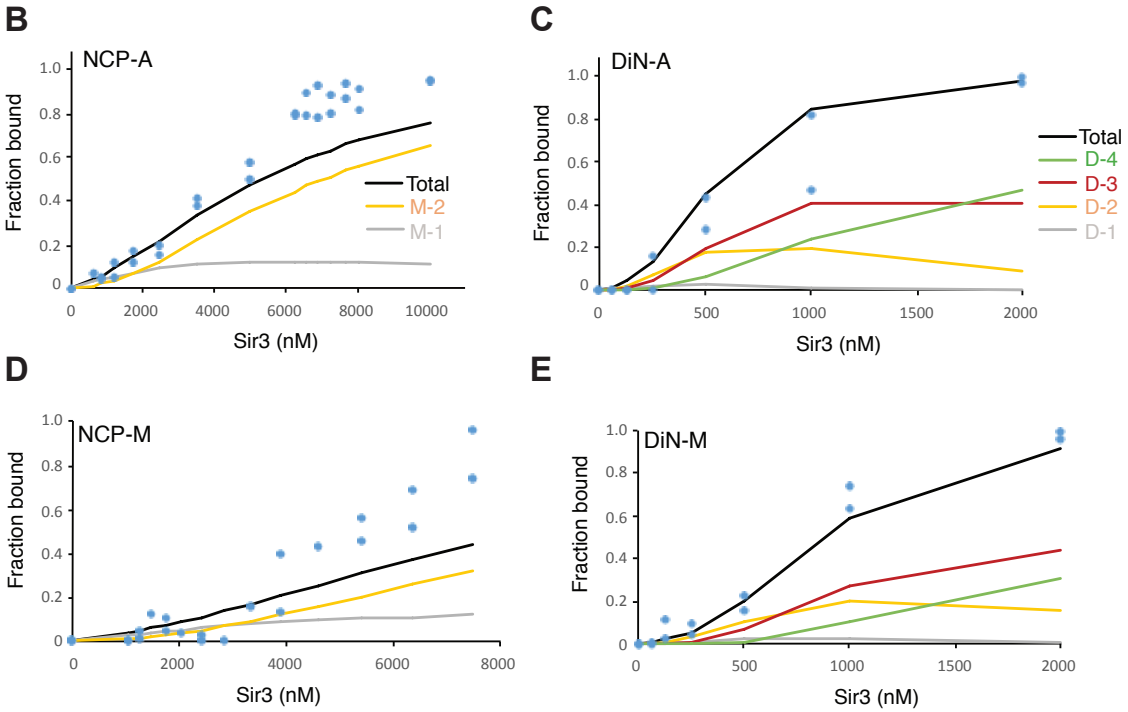
Figure 3-6. Statistical mechanical modeling of Sir3 binding to H4K16 acetylated and H3K$_C$79me3 nucleosomes.

(A) Average optimal parameters for constrained global fits of the pooled bound-fraction data in the EMSAs for the 6 conditions (4 conditions that used unmodified nucleosomes, Sir3 ± Sir4 CC; Sir3ΔwH ± Sir4CC, and the 2 conditions that used Sir3 with acetylated or methylated nucleosomes). Values constrained to be the same are indicated by color. Errors are standard deviations of 200 optimal fits in which the data points were individually varied with a standard deviation of 20%. **(B-E)** Data points (light blue filled circles) and model predictions (blue line) for modified NCP and DiN binding by Sir3, showing predicted fractions of different stoichiometries.

Figure 3-6 (Continued)

A

	Mod	K (μM)	C_1	C_2	$\Delta G(C_2)$ (kcal/mol)
Sir3	U	16.3 ± 0.5	50	1160 ± 160	– 4.2
Sir3ΔwH	U	11.1 ± 0.5	50	1.57 ± 1.00	– 0.27
Sir3/Sir4CC	U	16.3	50	11600 ± 2100	– 5.5
Sir3ΔwH/Sir4CC	U	11.1	50	1300 ± 540	– 4.3
Sir3	A	43.5 ± 2.1	50	1160	
Sir3	M	70.8 ± 2.9	50	1160	



could be reproduced solely by poorer monomer binding, with 2.7-fold and 4.3-fold increases in K, respectively (Figure 3-6A). However, the data do not exclude the possibility of reductions in C_1 or changes in C_2 in addition to increases in K. We note that similar to Sir3 binding to unmodified NCP (Figure 2-9D), the model underestimates the steepness of the modified NCP binding curves (Figure 3-6B and D), as it can only generate a Hill coefficient of 2, while the data gives a slightly higher Hill coefficient (nH = 2.28 for H4K16ac NCP and 3.02 for $H3K_C79me3$ NCP). However, the model predicts the presence of only one major shifted band in NCP EMSAs (Figure 3-6B and D), which is consistent with the pattern of gel shifts (Figure 3-3A and C). In addition, only two Sir3 stoichiometries, the D-3 species and the D-4 species, are predicted to accumulate to high levels in the DiN EMSAs; the D-3 species appears and peaks at lower Sir3 concentrations, while the D-4 species accumulates steadily with increasing Sir3 concentration (Figure 3-6C and E). These predictions are consistent with what we observed in the DiN gel shifts (Figure 3-3E and G). Modeling using the dimer only model produced similar results.

We tried to estimate the binding affinity of Sir3 to doubly modified nucleosomes. If H4K16 acetylation and H3K79 trimethylation have independent effects on Sir3 affinity, then the K value for binding of a Sir3 monomer to a doubly modified nucleosome is 200 µM (16.3 µM x 2.7 x 4.3). This value for K gives 50% NCP binding at ~22 µM Sir3 and 50% DiN binding at ~ 2.7 µM, which is consistent with the EMSA data (Figure 3-5B and C). However, the quality of the binding data at high Sir3 concentrations does not allow us to exclude some interdependence of the two modifications.

DISCUSSION

Active and silent chromatin regions are associated with stereotypical patterns of histone post-translational modifications, with each type of region containing or lacking a combination of different modifications (Ernst et al., 2011; Filion et al., 2010; Kharchenko et al., 2011; Taverna et al., 2007). It has been proposed that the binding of multivalent chromatin-associated proteins/complexes to a combination of different histone modifications increases binding affinity and specificity (Dhalluin et al., 1999; Du and Patel, 2014; Eustermann et al., 2011; Iwase et al., 2011; Li et al., 2007; Li et al., 2006; Moriniere et al., 2009; Ramon-Maiques et al., 2007; Rothbart et al., 2013; Ruthenburg et al., 2011; Taverna et al., 2007).

In budding yeast, both histone H4 lysine 16 (H4K16) acetylation and histone H3 lysine 79 (H3K79) methylation are hallmarks of euchromatin (Braunstein et al., 1996; Ng et al., 2003; Ng et al., 2002; Suka et al., 2001; van Leeuwen et al., 2002), and are likely to coexist in euchromatic regions. Previous studies indicate that H4K16 acetylation and H3K79 methylation each reduces Sir3 binding to nucleosomes, and therefore negatively regulates heterochromatin formation (Braunstein et al., 1996; Johnson et al., 2009; Liou et al., 2005; Martino et al., 2009; Onishi et al., 2007; van Leeuwen et al., 2002). Furthermore, recent structural studies of Sir3 BAH domain bound to the NCP indicate that both the H4K16 and H3K79 regions interact directly with the BAH domain (Armache et al., 2011; Arnaudo et al., 2013; Wang, 2013).

Studies in this chapter examine the effect of H4K16 acetylation and H3K79 methylation on Sir protein binding to nucleosomes *in vitro*. We show that each of the modifications reduces Sir3 binding affinity towards nucleosomes by 3-4 fold, and that the

two modifications in combination dramatically inhibit Sir3 binding. The reduction in binding affinity, combined with the strong cooperative binding, can provide a plausible explanation for the binding specificity of Sir3 and its partitioning between active and silenced chromosomal regions.

It is estimated that there are approximately 1400 Sir3 molecules per yeast haploid cell (Ghaemmaghami et al., 2003). Since Sir3 resides primarily inside the nucleus, and the mean nuclear volume is estimated to be around 2 μm^3 (Jorgensen et al., 2007), this copy number translates into an approximate intranuclear concentration of 1.2 μM. We estimate that there are ~72,000 nucleosomes per haploid cell (12.1 M genome size /167 bp per nucleosome), with ~400 of these in silenced regions (~2 kb silent chromatin at each sub-telomere and *HM* loci). Excluding ~5000 silent rDNA nucleosomes in the nucleolus, whose silencing does not require Sir3 (Smith and Boeke, 1997), leaves ~66,600 active region nucleosomes. We can use the Sir3 statistical mechanical monomer binding model to calculate the equilibrium distribution of free Sir3 and Sir3 bound to active and silenced chromatin, assuming that each nucleosome acts as a member of a di-nucleosome.

If all nucleosomes, active or silenced, were unmodified, then 1400 Sir3 monomers would exist as 45 free (39 nM), 1347 bound-active, and 8-bound-silenced. Obviously, the 8 Sir3 molecules shared among 400 silenced nucleosomes is insufficient for effective silencing, indicating that a pool of unmodified active nucleosomes titrate away too much Sir3. This is consistent with the requirement of anti-silencing modifications for efficient silencing (Verzijlbergen et al., 2009). On the other hand, if all nucleosomes were both H4K16 acetylated and H3K79 methylated (with a K = 200 μM), then 1400 Sir3 monomers would exist as 440 free (377 nM), 954 bound-active and 6

bound-silenced. Again, Sir3 binding to the silenced regions would be insufficient, in this case because of the low affinity due to modifications. However, if one assumes that active nucleosomes are modified (both H4K16 acetylated and H3K79 methylated), and silenced nucleosomes are unmodified, then a more reasonable partitioning occurs – 1400 Sir3 monomers would exist as 343 free (294 nM), 561 bound-active and 496 bound-silenced. This estimate of 1.2 Sir3 molecules per nucleosome in the silenced regions is close to the predicted value of 2 Sir3 per nucleosome (Swygert et al., 2014), and may be adequate for effective silencing, while the 148-fold lower Sir3 occupation of active region nucleosomes (0.0084 Sir3 per active nucleosome) would avoid unwanted silencing. On the other hand, if active nucleosomes were singly modified, either H4K16 acetylated or H3K79 methylated alone, Sir3 partitioning would not be sufficient for effective silencing. There would be 70 bound-silenced Sir3 in the case of H4K16 acetylation, representing 10-fold enrichment of Sir3 at silenced regions; and 176 bound-silenced Sir3 in the case of H3K79 methylation, representing 27-fold enrichment of Sir3 at silenced regions. This is in contrast with 148-fold enrichment of Sir3 at silenced regions when active nucleosomes are doubly modified.

The requirement of both modifications for effective silencing is consistent with previous findings indicating that loss of either modification results in promiscuous interactions of Sir proteins within euchromatin and disruption of silencing (Altaf et al., 2007; Frederiks et al., 2008; Johnson et al., 1990; Onishi et al., 2007; van Leeuwen et al., 2002; Verzijlbergen et al., 2009). Therefore the combined effect of H4K16 acetylation and H3K79 methylation, together with SIR-mediated deacetylation and inhibition of methylation of silenced region nucleosomes, as well as highly cooperative Sir3 binding, may be largely responsible for a self-sustaining confinement of Sir3 to silenced

chromatin regions. Other euchromatic histone modifications, such as H3 acetylation mediated by Gcn5 and Elp3, and H3K4 methylation mediated by Set1 (Verzijlbergen et al., 2009), as well as interactions that localize silent chromatin domains to foci at the nuclear periphery (Gasser et al., 2004) are likely to enhance this partitioning.

MATERIALS AND METHODS

Protein Cloning and Purification

Sir3-3XFLAG was purified from *S. cerevisiae* as described previously (Buchberger et al., 2008; Liou et al., 2005). *S. cerevisiae* histones were overexpressed and purified from *E.coli* as previously described (Johnson et al., 2009). $H3K_C79me3$ histone was prepared as previously described (Simon et al., 2007). The catalytic Piccolo subcomplex of the NuA4 histone acetyltransferase (HAT) complex was purified from *E. coli* as previously described (Barrios et al., 2007; Johnson et al., 2009; Selleck et al., 2005).

Mono-nucleosome and Di-nucleosome Reconstitution

Mono-nucleosomes and di-nucleosomes were reconstituted using gradient salt dialysis as described previously (Luger et al., 1999). The NCP DNA template contains the 147 bp 601 positioning sequence (Lowary and Widom, 1998). The di-nucleosomal DNA template contains two direct repeats of the 601 sequence, separated by a 20 bp linker. Trimethylated $H3K_C79$ ($H3K_C79me3$) histone was produced by the methyl-lysine analog (MLA) method as described previously (Simon et al., 2007). Nucleosome acetylation was carried out as described previously (Johnson et al., 2009). Briefly, nucleosomes were incubated with 1/10th molar ratio of the Piccolo HAT complex and 100X molar excess of acetyl-CoA in the HAT buffer (20 mM Tris.HCl, pH8.0, 50 mM KCl, 5% glycerol, 5 mM DTT, 1 mM PMSF, and 0.5 mg/ml BSA) at 30^0C for 1 hr. The completion of acetylation was assessed by the complete shift of the nucleosome band, and by quantitative western blot using antibody against acetylated H4K16, where saturated signal was achieved.

Electrophoretic Mobility Shift Assays (EMSAs)

Different amounts of Sir3 protein were incubated with 3 nM mono- or di-nucleosomes in binding buffer (25 mM Tris.HCl (pH7.5), 50 mM NaCl, and 5 mM DTT) at 4^0C for 1 hr. Samples were then run on native polyacrylamide gels, stained with SYBR Gold (Invitrogen), visualized on a Typhoon FLA7000 imager (GE Healthcare), and quantified using ImageQuant software. As observed previously by other groups, shifted nucleosome bands were stained less effectively than unshifted bands, probably due to protein loading onto nucleosomes (Oppikofer et al., 2013). Thus, as all previous literature, Sir3 binding to nucleosomes was quantified by the decrease in the intensity of the unbound nucleosome band. The K_D for each binding reaction was calculated with Prism Graphpad software by fitting the binding curve with the Hill Equation.

Cooperative Binding Analysis

Refer to Materials and Methods in Chapter 2 for details.

Statistical mechanical modeling of Sir3 binding to nucleosomes

Refer to Materials and Methods in Chapter 2 for details. Data fitting was performed for all EMSA NCP and DiN fraction-bound data points in the 6 conditions, including unmodified and modified nucleosomes. Since the model defines the fraction of each Sir3-bound species at any free [Sir3], it was used to examine the effect of histone modifications on genome partitioning of Sir3. For a given number of nucleosomes in a particular modification state and thus with a given Sir3 affinity (K=21.5 μM for unmodified, K=245 μM for A/M modified), there is a unique free [Sir3] at which the number of bound Sir3 monomers + the number of free Sir3 monomers = 1400. For this analysis, the effect of inter-nucleosomal cooperativity was represented by assuming that each genomic nucleosome is part of a di-nucleosome.

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CHAPTER IV

Discussion and Future Perspectives

Silent chromatin domains in the budding yeast *Saccharomyces cerevisiae* represent examples of epigenetically inherited chromatin domains and share properties with heterochromatin in multicellular eukaryotes (Grewal, 2000; Moazed, 2001; Rusche et al., 2003). The assembly of silent chromatin in *S. cerevisiae* requires Sir2, Sir3 and Sir4 proteins, which assemble into SIR complexes that spread along chromatin. Sir3 is the primary nucleosome-binding subunit of the SIR silencing complex. In addition, there are multiple homo- and heterotypic interactions between Sir2, Sir3 and Sir4, which are required for the spreading of Sir proteins along the chromatin fiber. However, exact molecular details that mediate Sir protein spreading are not fully understood. My thesis attempts to address these questions by quantitatively studying the interaction of Sir proteins with well-defined *in vitro* reconstituted mono- and di-nucleosome templates. My results indicate that Sir3 binds to nucleosomes cooperatively, mediated by the dimerization of Sir3 molecules bound to neighboring nucleosomes. This inter-nucleosomal cooperative binding is further stabilized by Sir3-Sir4 interactions, which occur via the interaction of the Sir4 homodimerization coiled coil (CC) domain with Sir3. I further demonstrate that the Sir3 winged helix (wH) domain and the Sir4 CC domain mediate the Sir inter-nucleosomal cooperative binding, and are both required for the efficient binding and spreading of Sir3 along chromatin *in vivo*. Surprisingly, my results suggest that there is no Sir-Sir interactions on the same nucleosome. Thus we propose that the inter-nucleosomal cooperative binding of Sir proteins mediate their spreading along chromatin, and that they spread along chromatin discontinuously.

Moreover, my results demonstrate that the simultaneous presence of H4K16 acetylation and H3K79 methylation determines the nucleosome binding specificity *in vitro*, and is likely the major determinant of the partitioning of Sir3 between silent and

active chromatic regions *in vivo.* However, these results do not entirely address the mechanism of epigenetic inheritance of silent chromatin in *S. cerevisiae*. In this chapter, I will discuss future work that is required for addressing this question.

FUTURE PERSPECTIVES

Cooperativity hypothesis for the mechanism of epigenetic inheritance

In budding yeast, heterochromatic regions are hypoacetylated and hypomethylated. This pattern of histone modifications is inherited mitotically and meiotically. In addition, it has been shown that silencer sequences are continuously required for the stable maintenance of the heterochromatin structure (Bi and Broach, 1997; Cheng and Gartenberg, 2000). Thus models of epigenetic inheritance must explain why the silencer is continuously required for maintenance of the silent state. A cooperativity hypothesis, which proposes that multiple protein-protein interactions together contribute to the cooperative recruitment of Sir proteins during the assembly of silent chromatin, has been put forward to explain the molecular requirement for epigenetic inheritance of silent chromatin in yeast (Moazed, 2011). In particular, it is proposed that during the silent chromatin re-establishment step, recruitment of the SIR complex involves cooperative interactions between the Sir proteins and silencer specificity factors as well as parentally inherited unacetylated nucleosomes. During the spreading stage, Sir-Sir and Sir-newly deacetylated nucleosome interactions cooperate to recruit more SIR complexes along the chromatin.

Results from my thesis project provide a strong support for the cooperativity hypothesis at the spreading stage of silent chromatin assembly. Additional experiments are required to test the role of silencer specificity factors in recruiting Sir proteins, and to

test the cooperativity hypothesis at the re-establishment stage. To do this, we will have to make a chromatin template composed of a silencer sequence ligated to a nucleosome positioning sequence, on which the nucleosome will be either unacetylated or acetylated, and to test the efficiency of the templates to recruitment Sir proteins, in the absence or presence of cognate silencer specificity factors. According to the cooperativity hypothesis, the efficient recruitment of Sir proteins requires the presence of both silencer specificity factors and unacetylated nucleosomes. If the hypothesis is correct, Sir proteins are expected to bind more strongly to the unacetylated template than to the acetylated template in the presence of silencer specificity factors. In addition, the binding to the unacetylated template is expected to be weaker in the absence of silencer specificity factors than in their presence.

Long-range deacetylation mediated by Sir2 and cooperativity between Sir3 bound to nonadjacent nucleosomes

Results from my nucleosome bridging experiments show that Sir3 efficiently bridges free nucleosomes in solution. This raises the possibility that there can be interactions between Sir proteins bound to nonadjacent nucleosomes. In fact, computational modeling suggests that such long-range interactions are required for the bistability of silent chromatin (Dodd et al., 2007). Moreover, if the distribution of parental unacetylated nucleosomes during DNA replication is truly random, as suggested by previous studies (Jackson and Chalkley, 1985; Sogo et al., 1986), then such long-range interactions are required if the cooperativity hypothesis for epigenetic inheritance is to be correct, as after DNA replication, the inherited parental nucleosome may be several nucleosomes away from the silencer and from each other. To test if such long-range interactions exist, we will have to make chromatin templates with unacetylated nucleosomes separated by at

least one acetylated nucleosome, and examine whether there is inter-nucleosomal cooperativity between Sir3 proteins bound to the unacetylated nucleosomes. Similarly, to test if long-range interactions exist between Sir3 bound to silencer specificity factor-loaded silencer and nonadjacent unacetylated nucleosomes, we will need to examine Sir3 binding to chromatin templates composed of a silencer and an unacetylated nucleosome with acetylated nucleosomes interspersed in between.

So far several pieces of *in vivo* data suggest that Sir proteins spread along chromatin non-linearly. First, it has been demonstrated that the association of Sir proteins with native telomeric regions is discontinuous (Ellahi et al., 2015; Fourel et al., 1999; Pryde and Louis, 1999; Radman-Livaja et al., 2011; Thurtle and Rine, 2014). Second, Sir proteins show a heterogeneous distribution pattern at the *HM* loci (Thurtle and Rine, 2014). In addition, the catalytically inactive Sir2-N345A mutant does not have a dominant negative effect on *HMR* silencing (Lynch and Rusche, 2009). On the other hand, the catalytically inactive Sir2-H364Y is strongly dominant negative for silencing of a *URA3* reporter gene at an artificial telomere (Tanny et al., 1999). Similarly, mutations in the Sir3 bromo-adjacent homology (BAH) domain are strongly dominant negative for both telomeric and *HMR* silencing (Buchberger et al., 2008). The working model of linear stepwise spreading by sequential deacetylation predicts that catalytically inactive Sir2 or mutations in the nucleosome binding domain of Sir3 should be dominant negative, as the incorporation of the inactive Sir proteins into silent chromatin during assembly would act as a polymerization terminator and prevent further spreading. Although there is some evidence in support for these models, the non-linear mode of Sir3 association with chromatin and our inability to observe Sir-Sir interactions during binding to mono-

nucleosomes suggest that either Sir spreading occurs without polymerization or factors that regulate Sir polymerization are absent in our *in vitro* system.

Biochemical experiments could be designed to directly test the ability of Sir2 to deacetylate nonadjacent nucleosomes. For example, nucleosomal arrays could be designed such that each nucleosome on the array has a unique nucleosomal positioning sequence, and is thus barcoded. Such arrays could be ligated to a silencer, and a time course Sir2-mediated deacetylation could reveal the extent of deacetylation of each individual nucleosome with time. If the deacetylation and Sir protein spreading is linear, we expect deacetylation to proceed progressively from the silencer-adjacent nucleosome to nucleosomes further away from the silencer. However, if longer-range deacetylation takes place, a more random deacetylation pattern will be observed. However, the set up of such experiments can be tricky, and interpretation of experimental results is limited by constraints of *in vitro* design. For example, results may be affected by differences in linker DNA lengths, and thus may not be physiologically relevant.

Ideally, models for the spatial order of Sir2 deacetylation and Sir3 binding should exploit the results of both *in vitro* and *in vivo* approaches (Lynch and Rusche, 2009; Osborne et al., 2009; Radman-Livaja et al., 2011). At the *HM* loci and most telomeres, during the initial phase of heterochromatin establishment, Sir protein spreading is extremely cooperative, occupying regions of 1-2 kb simultaneously (Lynch and Rusche, 2009; Radman-Livaja et al., 2011). However, the rate of spreading beyond this cooperative region and at some telomeres is much slower. A challenge for the future is to design *in vitro* and *in vivo* experiments that will lead to insight into the different modes of spreading within core and extended heterochromatin regions.

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APPENDIX

PUBLICATION

Heterochromatin protein Sir3 induces contacts between the amino terminus of histone H4 and nucleosomal DNA

Feng Wang[a,1], Geng Li[a,1], Mohammed Altaf[a], Chenning Lu[a], Mark A. Currie[a], Aaron Johnson[a,2], and Danesh Moazed[a,b,3]

[a]Department of Cell Biology and [b]Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115

Edited* by Michael Grunstein, David Geffen School of Medicine at UCLA, Los Angeles, CA, and approved April 5, 2013 (received for review January 7, 2013)

The regulated binding of effector proteins to the nucleosome plays a central role in the activation and silencing of eukaryotic genes. How this binding changes the properties of chromatin to mediate gene activation or silencing is not fully understood. Here we provide evidence that association of the budding yeast silent information regulator 3 (Sir3) silencing protein with the nucleosome induces a conformational change in the amino terminus of histone H4 that promotes interactions between the conserved H4 arginines 17 and 19 (R17 and R19) and nucleosomal DNA. Substitutions of H4R17 and R19 with alanine abolish silencing in vivo, but have little or no effect on binding of Sir3 to nucleosomes or histone H4 peptides in vitro. Furthermore, in both the previously reported crystal structure of the Sir3-bromo adjacent homology (BAH) domain bound to the *Xenopus laevis* nucleosome core particle and the crystal structure of the Sir3-BAH domain bound to the yeast nucleosome core particle described here, H4R17 and R19 make contacts with nucleosomal DNA rather than with Sir3. These results suggest that Sir3 binding generates a more stable nucleosome by clamping H4R17 and R19 to nucleosomal DNA, and raise the possibility that such induced changes in histone–DNA contacts play major roles in the regulation of chromatin structure.

gene silencing | histone H4 tail

Assembly of eukaryotic DNA into chromatin plays a central role in the regulation of gene expression and genome stability. The fundamental unit of chromatin folding, the nucleosome, is composed of 147 bp of DNA wrapped twice around an octamer of histones H2A, H2B, H3, and H4 (1). Posttranslational modifications of histones play important roles in the regulation of chromatin structure by affecting the interaction of histones with nucleosomal DNA and by recruiting effector molecules that perform downstream functions (2–6). Although many different types of chromatin domains have been defined based on histone modification patterns (7), the active gene-rich and inactive, repetitive, gene-poor chromosome regions are commonly referred to as euchromatin and heterochromatin, respectively.

In the budding yeast *Saccharomyces cerevisiae*, the silent mating type cassettes and telomeric DNA regions are assembled into heterochromatin-like structures that display epigenetic inheritance patterns and regional effects on gene expression (8–10). Studies by Grunstein and coworkers (11, 12) provided the first evidence of a specific role for histones in silencing. The conserved amino terminus of histone H4 is dispensable for growth but is required for repression of the silent mating type loci (11, 12). In the H4 amino terminus, substitutions within a basic patch region, composed of lysine 16 (K16), arginine 17 (R17), histidine 18 (H18), and arginine 19 (R19), abolish silencing, whereas substitution of lysine 20 (K20) has a partial silencing defect (12). Furthermore, H4K16 is hypoacetylated within silent domains, and although its substitution to arginine is tolerated, substitutions to alanine and glutamine abolish silencing, providing evidence that H4K16 acetylation regulates silencing in vivo (12, 13). In addition to histone H4, the globular domain of histone H3 surrounding lysine 79 (K79), termed the loss of rDNA silencing (LRS) surface, is required for silencing (14–17).

The establishment and maintenance of silent domains at telomeres and the mating type loci also requires the silent information regulator (Sir) 2, 3, and 4 proteins (18, 19). The Sir2 and Sir4 proteins form a subcomplex that associates with Sir3 into the silent infomation regulator (SIR) complex (20–23). Sir2 is a NAD-dependent deacetylase with preference for H4K16 (24-26), whereas Sir3 is a histone H4 and nucleosome binding protein that displays a strong preference for histone H4 peptides and nucleosomes that contain unacetylated H4K16 (22, 27, 28). The association of Sir3 with chromatin is also inhibited by dimethylation or trimethylation of histone H3K79 (29, 30) occurring in transcribed genomic regions (14, 16).

Sir3 associates with nucleosomes via a conserved N-terminal domain, the bromo adjacent homoloy (BAH) domain, as well as a less well-characterized C-terminal domain with similarities to AAA ATPases (AAL domain) but lacking ATP binding or hydrolysis activity (27, 29, 31–33). The recently solved 3-Å resolution crystal structure of the BAH–nucleosome complex reveals how the BAH domain binds to the nucleosome and how this binding is controlled by acetylation and methylation (34). H4K16 and H18 make multiple hydrogen-bonding interactions with a deep pocket in the BAH domain, which would be disrupted by acetylation of the H4K16 epsilon amino group. The importance of this BAH binding pocket is supported by studies indicating that mutations within the binding pocket and surrounding residues disrupt silencing in vivo (32, 33, 35). The H4 tail exits the nucleosome near the LRS regions, and within this region, H3K79 and its surrounding residues also have multiple bonding interactions with the BAH domain (34). Thus, although the BAH–nucleosome structure provides clear evidence of the participation of H3K79, H4K16, and H4H18 in Sir3–nucleosome binding, it does not address how this binding mediates the silencing function of Sir3. In particular, the roles of H4R17 and R19, two residues in the basic patch region that are as critical for silencing as H4K16 (12), remain unclear.

In this study, we examined the roles of amino acids in the histone H4 basic patch in histone peptide and nucleosome binding. As expected, we found that substitutions of H4K16 and H18 with alanine abolished histone H4 peptide and nucleosome binding.

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The authors declare no conflict of interest.

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Data deposition: Atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4JJN).

[1]F.W. and G.L. contributed equally to this work.

[2]Present address: Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, Denver, CO.

[3]To whom correspondence should be addressed. E-mail: danesh@hms.harvard.edu.

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However, substitutions of H4R17 and R19, which disrupted silencing to the same extent as K16 or H18 substitutions, had no effect on Sir3 binding in our in vitro assays. Consistent with the biochemical binding data, both H4R17 and R19 point away from the BAH domain in the crystal structure of the BAH domain in complex with the yeast nucleosome core particle (reported here), as well as the BAH domain in complex with the *Xenopus laevis* nucleosome core particle reported by Armache et al. (34). Rather than making contact with the BAH domain, these arginines make contacts with phosphates in the nucleosomal DNA backbone. Based on the foregoing findings, we propose that binding of Sir3 to the nucleosome induces a conformational change that clamps H4R17 and R19 onto nucleosomal DNA to create a silenced nucleosome.

Results and Discussion

We used a pull-down assay that examines the association of nucleosomes from a solubilized chromatin extract with tandem affinity purification (TAP)-tagged Sir3 to determine the contribution of H4R17, R19, and other amino acids in the H4 basic patch region to the binding of Sir3 to nucleosomes (Fig. 1*A*; see Tables S1 and S2 for yeast strains and plasmids). As a control and consistent with previous results (29), the substitution of H4K16 with either glutamine or alanine (H4K16Q and K16A, respectively) abolished the association of nucleosomes with Sir3 (Fig. 1*B*, compare lanes 10, 15, and 16; Fig. S1). Similarly, consistent with its interaction with the BAH domain (34), the substitution of H4H18 with alanine (H4H18A) abolished the binding of nucleosomes to Sir3 (Fig. 2*B*, compare lanes 10 and 13). In contrast, the substitution of either H4R17 or R19 with alanine (H4R17A and R19A, respectively) resulted in increased binding of nucleosomes to Sir3 (Fig. 1*B*, compare lanes 10, 12, and 14). As further controls, substitutions at H4K20 and K56 (H4K20A and K56A, respectively), which have weak silencing defects, had no effect on the Sir3–nucleosome interaction in this assay (Fig. 1*B*, lanes 11 and 17).

We next tested the importance of the H4 basic patch residues in Sir3 binding using a biotinylated peptide pull-down assay. Consistent with the nucleosome binding data, the acetylation of H4K16 (H4K16Ac) or the substitution of H4H18 with alanine diminished the ability of these residues to bind to either full-length Sir3 or the BAH domain (Fig. 1*C*, compare lanes 3, 4, and 6; Fig. 1*D*, lanes 6 and 7). On the other hand, the substitution of either H4R17 or R19 with alanine had little or no effect on the binding of full-length Sir3 or the BAH domain (Fig. 1*C*, compare lanes 3, 5, and 7). Thus, H4R17 and R19 are not required for the association of Sir3 with either nucleosomes or histone H4 N-terminal peptides.

Consistent with previous nucleosome-binding studies (29) and extensive contacts between the BAH domain and H3K79 (34), trimethylation of H3K79 abolished the binding of full-length Sir3 and the BAH domain to a peptide spanning amino acids 67–89 of histone H3 (Fig. 1*D*, lanes 3 and 4). Moreover, consistent with nucleosome-binding results (28), the substitution of H3K79 with alanine had no effect on binding (Fig. 1*D*, lane 5). This suggests that the methylation of H3K79 by Dot1 inhibits Sir3 binding via a steric hindrance mechanism that prevents interactions between Sir3 and the surrounding LRS region, rather than with H3K79 itself.

To verify that the point mutations used in our studies had the expected loss of silencing defects, we performed quantitative RT-PCR (qRT-PCR) to measure RNA levels for the subtelomeric *YFR057w* open reading frame on the right arm of chromosome VI, which is silenced in a Sir3-dependent manner. Substitutions of R17 and R19 with alanine displayed a similar increase in *YFR057w* RNA levels as seen with substitutions of H4K16 and H18, which are Sir3-contacting amino acids (Fig. 1*E*). However, ChIP experiments showed that H4R17A and R19A mutations disrupted the association of Sir3 with *YFR057w* in vivo (Fig. S2), likely owing to disruption of the Sir3–nucleosome interaction in cells carrying the arginine mutations during chromatin remodeling and transcription activation.

We note that the basic patch region of H4 also provides a binding site for the H3K79 methyltransferase disruptor of telomeric silencing 1 (Dot1), an event required for efficient silencing (30, 36). However, the complete loss of Dot1-mediated H3K79 methylation requires substitutions of both H4R17 and R19 with alanine (30, 36), and *dot1*Δ cells have near-WT silencing at the *homothalic left* (*HML*) mating type locus (37, 38), whereas the substitution of either H4R17 or R19 results in loss of silencing at *HML* and subtelomeric regions (12) (Fig. 1*E*). Thus, the silencing defects of H4R17 and R19 cannot be explained by a loss of Dot1 binding. In fact, the increase in Sir3 binding in the H4R17A and R19A mutations noted above (Fig. 1*B*) is similar to what we had previously observed comparing WT with Δ*dot1* cells using the same nucleosome pull-down assay (29), and suggests that increased Sir3 binding results from weaker Dot1 binding to the H4 basic patch region and reduced H3K79 methylation.

The crystal structure of the BAH domain (Sir3 amino acids 1–214) in complex with the heterologous *X. laevis* nucleosome core particle suggests that H4R17 and R19 do not contact the BAH domain (34). To determine whether these arginines occupy similar positions when the BAH domain is bound to the yeast nucleosome, we solved the crystal structure of a Sir3 N-terminal fragment from amino acids 2–382 (Sir3-382), which included the conserved BAH domain, in complex with the nucleosome core particle (NCP), reconstituted using the 147-bp Widom 601 positioning DNA and bacterially produced yeast histones. We used the Sir3-382 protein because it binds to the nucleosome with greater affinity than the smaller BAH domain (Sir3-214), and its binding is sensitive to substitutions at either H4K16 or H3K79 (Figs. S1 and S3). We further introduced a point mutation at position 205 (E205N), which was previously shown to increase the affinity of Sir3 for the nucleosome (34, 39). The 3.1-Å resolution structure of Sir3-382–*Sc*NCP shows two BAH domains bound symmetrically to each side of the nucleosome (Fig. 2*A* and Table S3). Most of the Sir3–nucleosome interaction interface in our structure was identical to the Sir3-214–*Xl*NCP complex (34) (Fig. S4), showing extensive contacts between the BAH domain and the globular domain of histone H3 surrounding H3K79 and the amino terminus of histone H4. Amino acids 216–382, beyond the BAH domain, displayed discontinuous electron density, which is consistent with predictions that this low-complexity region is unstructured, and are not presented in the structure.

We observed clear electron density for the H4R19 side chain and weaker electron density for the H4R17 side chain, but both arginines clearly pointed away from the BAH domain and were in a position to make contact with DNA (Fig. 2 *A* and *B*). H4R19 was located in a similar position as that previously described for the Sir3-214–*Xl*NCP structure (Fig. 2*C*). However, whereas in the Sir3-214–*Xl*NCP structure, both arginines were located close to the phosphate of nucleotide 100, in the Sir3-382–*Sc*NCP structure, H4R17 was located closer to the phosphate of nucleotide 52 (Fig. 2 *C* and *D*). Thus, H4R17 and R19 interact with phosphates 100 and 52, respectively, which are located across the minor groove on opposite strands of the DNA double helix in the BAH–nucleosome complex (Fig. 2 *C* and *D*). The different positions of H4R17 in the two structures may be related to alternative conformations or flexibility of R17, the location of which may be stabilized by additional Sir3 sequences or the other subunits of the SIR complex. Unlike H4K16 and H18, which penetrate binding pockets in the BAH domain, H4R17 and R19 make salt bridges with phosphates of nucleosomal DNA (Fig. 2*D*). Nucleotides 52 and 100, which contact H4R17 and R19, respectively, are located on complementary strands across the DNA minor groove (Fig. 2*D*). In addition, the contacts are symmetrical for the two histone H4 chains (Fig. S5 *A* and *B*). Thus, the locations of H4R17 and R19 in BAH complexes with yeast and

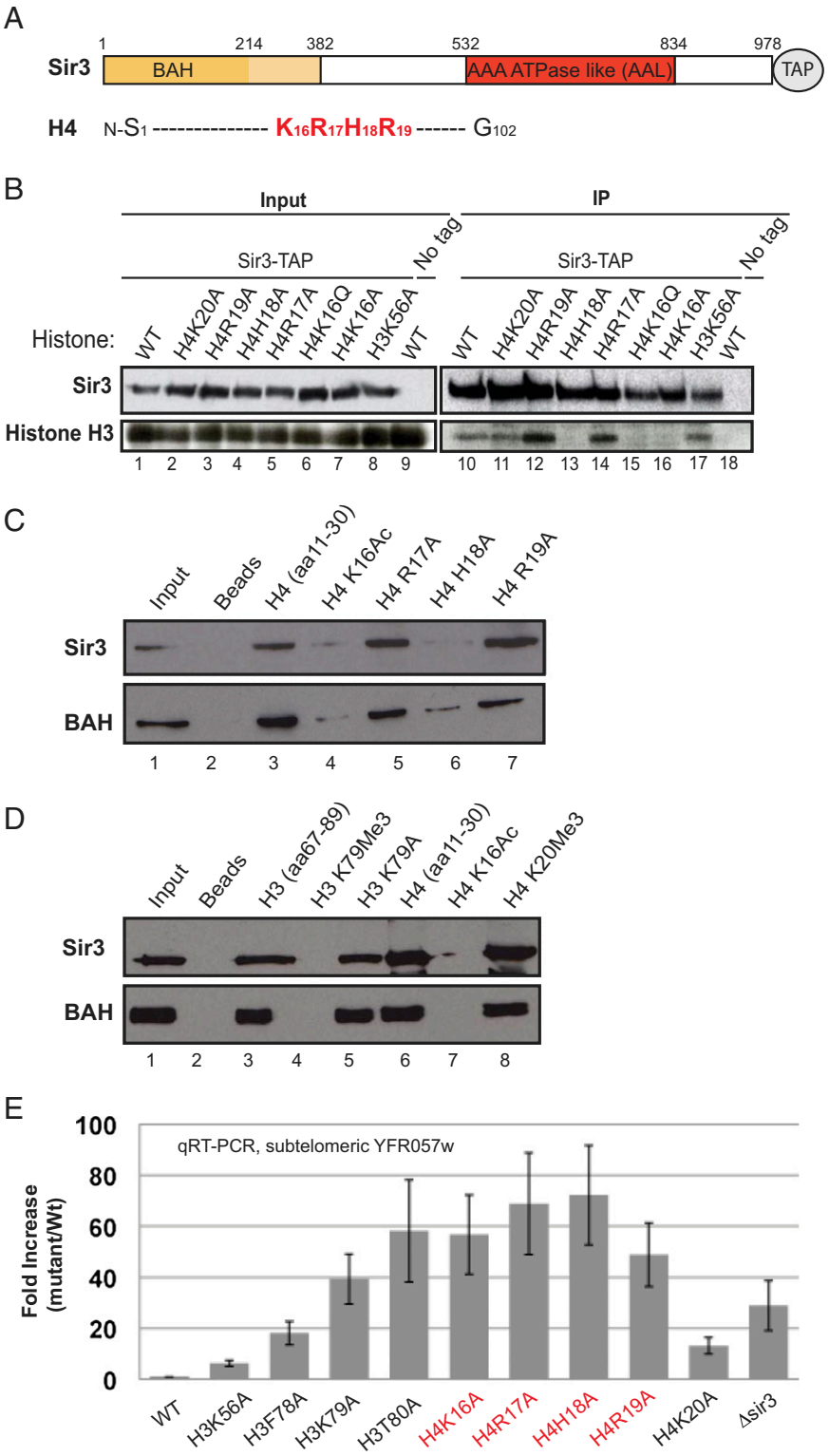

Fig. 1. Histone H4R17 and R19 are critical for silencing but do not affect the association of Sir3 with the nucleosome. (*A*) Schematic diagram highlighting the boundaries of the BAH and AAA ATPase-like (AAL) domains of Sir3, and the basic patch region of histone histone H4 (in red). (*B*) Sir3-TAP pull-downs showing the effect of histone H4 N-terminal basic patch and H3K56 mutations on the nucleosome–Sir3 association. Sir3-TAP was expressed in yeast under the control of its own promoter. (*C* and *D*) Peptide pull-down assays showing the association of Sir3 and the BAH domain (N-terminal subfragment, Sir3-BAH381) with WT histone peptides and histone peptides containing the indicated amino acid substitutions. In addition, the data show that trimethylation of the H3, amino acid 67–89 peptide at K79 abolishes its binding to Sir3 and its BAH-containing subfragment. H4K16Ac and K20me3 serve as positive and negative controls, respectively. Full-length Sir3 and Sir3-BAH381 (Sir3 amino acids 1–381) were purified from yeast. (*E*) qRT-PCR data comparing the silencing of the subtelomeric *YFR057w*, located on chromosome VI-R in WT and the indicated histone mutant cells. *sir3*Δ serves as a control.

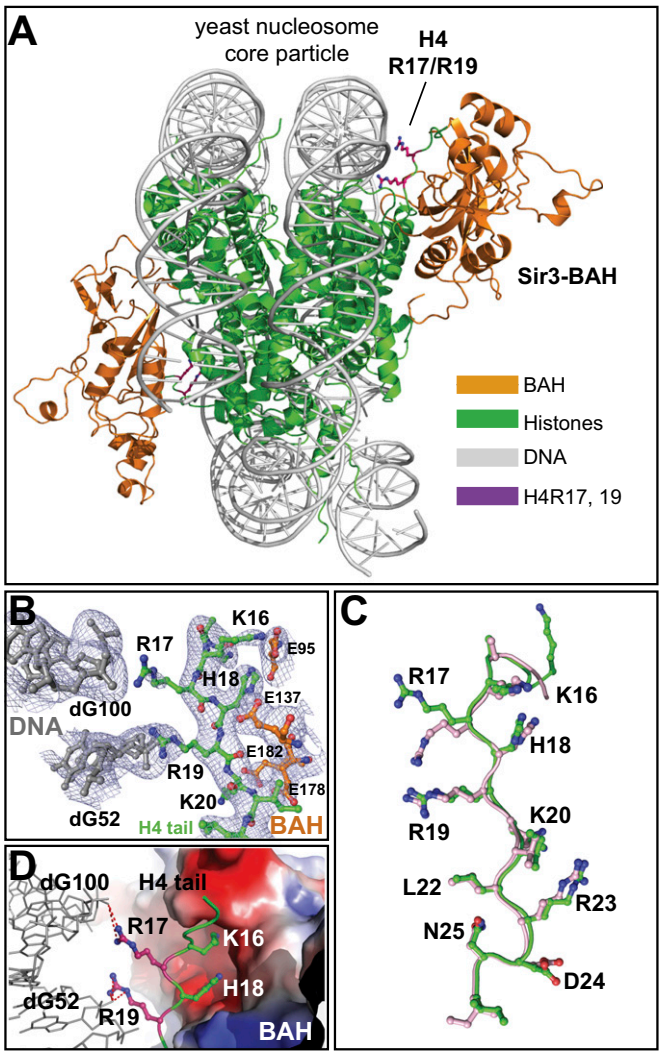

Fig. 2. Structure of the Sir3-BAH in complex with the yeast nucleosome. (*A*) Overall view of the structure highlighting interactions between histone H4R17 and R19 and nucleosomal DNA. The BAH domain is in orange; H4R17 and R19 are in magenta; histones H2A, H2B, H3, and H4 are in green; and the DNA is in gray. (*B*) Electron density map ($\sigma = 1.0$) showing the H4 basic patch region (green) and its interactions with DNA (gray) and the BAH domain (orange). (*C*) Overlay of the H4 tail in the BAH–*X*NCP (pink; ref. 34) and BAH–*Sc*NPC (green; this study) complexes. (*D*) High-magnification view of the interactions involving the H4 basic patch region. H4K16 and H18 interact with a deep pocket in the BAH domain, shown in electrostatic surface representation, whereas H4R17 and R19 point away from the BAH domain and make salt bridges with the phosphates of different strands of nucleosomal DNA at nucleotides 52 and 100.

frog nucleosomes are consistent, and support the idea that the silencing function of these amino acids involves their interaction with nucleosomal DNA rather than with Sir3.

To determine whether silencing requires H4R17 and R19 under conditions in which Sir3 is present on chromatin, we took advantage of the observation that overexpressed Sir3 associates with the *HML* locus and silences the expression of the *α1* and *α2* genes even in *sir4I1311N* mutant cells, in which it cannot interact with the Sir2/Sir4 deacetylase complex (Fig. S2). This silencing is not accompanied by the spread of the Sir2/Sir4 deacetylase and requires the substitution of H4K16 with arginine (H4K16R), which mimics deacetylated lysine. To determine whether the silencing mediated by this Sir3 binding requires H4R17 or R19, we constructed Sir3-overexpressing (*SIR3OE*) cells with H4R17A and R19A substitutions alone or in combination with H4K16R substitutions. Sir3 bound to the *HML* locus at near-WT levels in H4R17A and R19A cells, but this binding did not correlate with silencing of the *α1* and *α2* genes (Fig. S2 *B* and *C*). Furthermore, no silencing was observed even in cells in which the foregoing mutations were combined with H4K16R, indicating that the roles of H4R17 and R19 in silencing did not involve a contribution to the binding of overexpressed Sir3 or K16 deacetylation (Fig. S2 *B* and *C*). We emphasize that, as shown in Fig. S2*A* for the subtelomeric *YFR057W* gene, in the absence of overexpression, the H4R17A and R19A mutations disrupted Sir3 binding to chromatin, suggesting that even though they are not Sir3 contact residues, H4R17 and R19 affect the stability of Sir3 on chromatin in vivo.

Taken together, the biochemical and structural evidence presented here indicate that H4R17 and R19 play a role in silencing that is distinct from the role of H4K16 and H18. Histone tails generally display relatively weak electron density in the crystal structures of free nucleosomes reported to date (for examples, see refs. 1 and 40). Nonetheless, the conformation of the H4 tail and H4R17 and R19 observed in the Sir3–NCP complexes are unique. Comparison of the H4 tail conformation in the free yeast nucleosome (40) and the Sir3-382–*Sc*NCP complex indicates that the association of Sir3 with the nucleosome results in a conformational change in the H4 N-terminal tail involving a rotation around the main chain of asparagine 25 (Fig. 3 *A*–*C*). This rotation allows H4K16, H18, K20, L22, and R23 to interact with the BAH domain and brings the R17 and R19 side chains in close proximity to nucleosomal DNA. The locations of the R17 and R19 side chains may be further constrained by interactions of their backbone carbonyl groups with E137 in the BAH domain (Fig. 2*B* and Fig. S6). We propose that the salt bridges between these H4 arginines and the phosphates in the DNA backbone clamp the nucleosomal DNA to the histone octamer and help create a "silenced nucleosome" (Fig. 3*D*). This silenced nucleosome is likely to be more resistant to unwinding by chromatin remodeling complexes, a required step for DNA accessibility and transcription. The H4 amino terminus is likely to interact with nucleosomal DNA even in the absence of Sir3, such as in the high-resolution *Xenopus* nucleosome crystal structure (Fig. S4 *B*–*D*) (41). Previous studies have suggested that interactions between histone tails and DNA contribute to nucleosome stability and are regulated by acetylation (42). Thus, Sir3 may shift the equilibrium toward a conformation that has evolved to contribute to nucleosome stability.

Our findings suggest that silencing requires a specific mode of Sir3–nucleosome association and provide a possible explanation for previous reports suggesting that SIR complex binding does not correlate with silencing (28, 43, 44). The formation of a silenced nucleosome most likely requires multiple bonding interactions between Sir3 and the nucleosome that together stably clamp nucleosomal DNA to the histone octamer. The loss of a subset of the bonding interactions may allow for a more dynamic form of Sir3 binding that is insufficient to silence transcription. The functions of H4R17 and R19 proposed herein are reminiscent of the histone *sin* mutations that suppress defects in chromatin remodeling (45, 46). A large fraction of these mutations affect the interaction of histone H4R45 with a minor groove in nucleosomal DNA (46), providing another example of profound effects on chromatin structure resulting from histone point mutations that affect contacts with DNA.

Materials and Methods

Protein Expression and Purification. A DNA fragment encoding residues 2–382 of Sir3 was cloned into a modified pET28b vector with a His_6-Sumo tag fused at the N terminus. The D205N point mutation was introduced using the QuikChange II Site-Directed Mutagenesis Kit (Agilent). The Sir3 (2-382, D205N) plasmid was transformed and expressed in *Escherichia coli* Rosetta DE3 strain. After induction for 16 h with 0.1 mM isopropyl β-D-1-thiogalactopyranoside

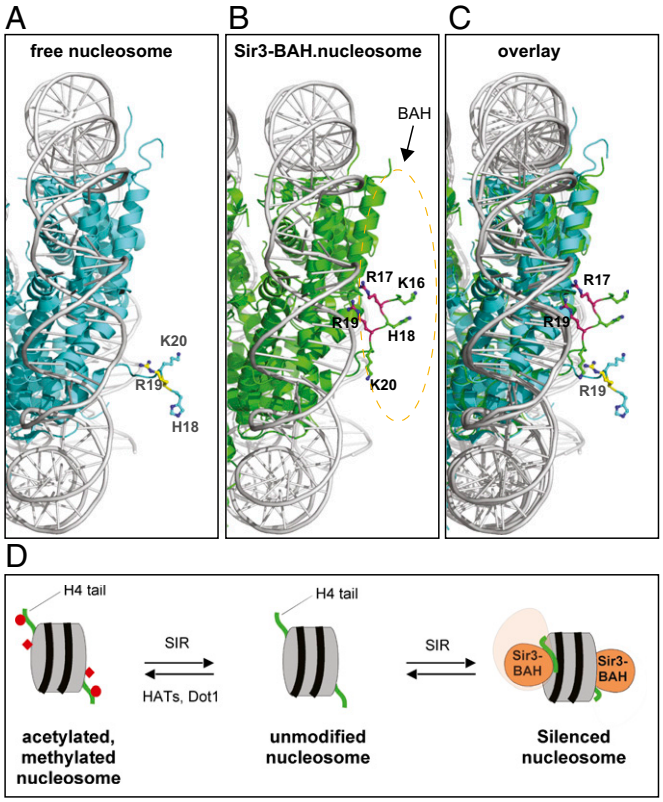

Fig. 3. Association of Sir3-BAH with the nucleosome induces a conformational change in histone H4. Comparison of the structure of the free yeast nucleosome (*A*; cyan, 1ID3) with the Sir3-BAH382–nucleosome complex (*B*; green) highlighting the basic patch region of histone H4. (*C*) Overlay of the two structures. The dotted oval indicates the location of the BAH domain, but the BAH atoms have been removed for clarity. The basic patch region in the free yeast nucleosome and the BAH–nucleosome complex are in yellow and magenta, respectively. H4K16 and R17 are absent in the free nucleosome structure. (*D*) Model for formation of the silenced nucleosome. Recruitment of the SIR complex results in the generation of nucleosomes containing deacetylated H4K16 and unmethylated H3K79. The SIR complex binds to such unmodified nucleosomes, and the association of the Sir3-BAH domain induces a conformational change in the H4 tail (in green; red circle, acetylated H4K16; red diamond, methylated H3K79) that clamps H4R17 and R19 to DNA to create a silenced nucleosome.

(IPTG) at 25 °C, the cells were harvested by centrifugation, and the resulting pellet was resuspended in lysis buffer [50 mM Tris·HCl (pH 8.0), 50 mM NaH_2PO_4, 400 mM NaCl, 3 mM imidazole, 10% glycerol, 1 mM PMSF, 0.1 mg/mL lysozyme, 2 mM 2-mercaptoethanol, and complete protease inhibitor mixture tablets (Roche)]. The cells were then lysed by sonication, and the cell debris was removed by ultracentrifugation. The supernatant was mixed with Ni-nitriloacetic acid (NTA) agarose beads (Qiagen) and rocked for 4 h at 4 °C, then washed twice with 20 column volumes of 10 mM imidazole in lysis buffer before elution with 250 mM imidazole. The ubiquitin-like specific protease 1 (ULP1) protease was added to remove the His_6-Sumo tag. Finally, the protein was further purified by passage through a Mono-Q ion-exchange column (GE Healthcare) and by gel-filtration chromatography on a Hiload Superdex 75 (GE Healthcare) equilibrated with 25 mM Tris·HCl (pH 8.0), 150 mM NaCl, and 5 mM DTT. The purified Sir3 (2-382, D205N) protein was concentrated to 25 mg/mL using centrifugal filters (Millipore) and stored at −80 °C. Budding yeast *S. cerevisiae* histones H2A, H2B, H3, and H4 were expressed in *E. coli*; purified; renatured in 2 M NaCl; and assembled into octamers through stepwise salt dialysis. The histone octamer core was assembled with the 601 Widom positioning sequence into the nucleosome core particle as described previously (1).

Sir3–NCP Complex Preparation and Crystallization. The complex of the Sir3-382 and the nucleosome core particle was assembled by mixing the Sir3 protein with nucleosome core particle in a 2:1 molar ratio before a final size-exclusion chromatography step (Superose 6; GE Healthcare). The assembled complex was concentrated to 8 mg/mL for crystallization screening trials. The crystals were grown by sitting drop vapor diffusion in 0.05 M sodium cacodylate (pH 7.5) and 32% (vol/vol) 2-methyl-2,4-pentanediol (MPD) at 4 °C. Crystals were transferred to 40% (vol/vol) MPD in 1% increments with 10 min between each step, and then flash-frozen in liquid nitrogen.

Data Collection and Crystallographic Analysis. Diffraction data were collected at the Advance Photon Source The Northeastern Collaborative Access Team (NE-CAT) beamline 24-ID-C, and processed using XDS and the CCP4 package (47, 48). The structure was solved by molecular replacement using Phaser (49), and a search model containing the budding yeast histone octamer core and the 147-bp human α-satellite DNA (PDB ID code 1ID3), with the DNA bases manually changed to the 601 Widom sequence. A clearly positive density for Sir3-382 was present on both sides of the nucleosome in the difference electron density map. Crystallographic refinement was carried out using PHENIX with manual model building in Coot (50, 51). All graphic presentations were prepared in PyMOL (www.pymol.org/). Secondary structural prediction was performed using the PredictProtein server (www.predictprotein.org).

Yeast Strains and Protein Purification. The strains and plasmids used in this study are listed in Tables S1 and S2. Yeast strains were made by a PCR-based gene targeting procedure (52–55). Histone mutant strains were constructed by introducing individual histone mutations by plasmid shuffle into the background strain of DMY3903 or DMY3985, as described previously (12, 56). Plasmids carrying histone mutations were kindly provided by Dr. Jef Boeke (57) and Dr. Sharon Dent (56). FLAG-tagged Sir3 proteins expressed in yeast were purified as described previously (22, 35).

TAP Protein Purification and Western Blot Analysis. TAP-pull downs were performed as described previously (29, 55). In brief, cells were resuspended in equal volumes of lysis buffer [50 mM Hepes KOH (pH 7.6), 10 mM magnesium acetate; 5 mM EGTA, 0.1 mM EDTA, 150 mM potassium chloride, 0.2% Nonidet P-40, 5% glycerol, 2 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, and 1 mg/mL each leupeptin, bestatin, and pepstatin] and lysed by bead beating. The resulting lysate was incubated with IgG-coupled M-270 Dynabeads (Invitrogen) for 90–120 min at 4 °C. Beads were washed four times with lysis buffer and resuspended in SDS sample buffer. Samples were loaded on SDS/PAGE, transferred to nitrocellulose membranes, and probed with peroxidase anti-peroxidase (Sigma-Aldrich) and anti-H3 (Abcam) antibodies.

Quantitative RT-PCR. Yeast cultures were grown in yeast extract peptone dextrose (YEPD) medium at 30 °C to an OD_{600} of 0.5. Total RNA was isolated by the hot phenol procedure and cleaned using the RNeasy Kit (Qiagen) to remove potential genomic DNA contamination. Gene-specific primers for *YFR057W* were used to prepare cDNA, followed by quantitative PCR using a LightCycler (Applied Biosystems). Relative RNA levels were calculated from *CT* values according to the *ΔCT* method and normalized to $act1^+$ RNA levels.

Native Gel Shift and Peptide Pull-Down Assays. Mononucleosomes were reconstituted as described previously (1, 28). Increasing molar ratios of full-length Sir3 protein or its subdomains were incubated with 8 nM mononucleosome, assembled on a 218-bp DNA fragment containing the 601 Widom positioning sequence (58) in binding buffer containing 20 mM Hepes (pH 7.5), 4 mM Tris (pH 7.5), 80 mM KCl, 0.1% Nonidet P-40, 0.2 mM EDTA, 2 mM DTT, 0.5 mg/mL BSA, and 5% glycerol. The binding reaction was carried out at 30 °C for 1 h, followed by chilling on ice for 15–20 min before loading onto the 3.5% native polyacrylamide gel. The native polyacrylamide gel was prerun at 50V for 1 h in 0.25× tris-borate EDTA (TBE), and then changed with fresh 0.25× TBE buffer. Samples were separated at 100V for 1.5–2 h at 4 °C or for 4–4.5 h in the presence of antibody. The gel was dried, analyzed by a storage phosphor screen, and quantified with Quantity One software (Bio-Rad). Saturation curves were analyzed using Kaleidograph software (Synergy Software). Peptide pull-down assays were carried out by incubating 1 μg of biotinylated histone peptides with Sir3 protein in 25 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 100 μg/mL BSA, 0.5 mM DTT, 0.1% Tween-20, and 1 μg/mL leupeptin, pepstatin, and aprotinin for 1 h at room temperature in a rotary shaker. Peptides were then isolated with magnetic streptavidin beads for 30 min at room temperature. Beads were resuspended in SDS sample buffer and loaded onto 12% SDS/PAGE, followed by Western blot analysis with anti-Flag antibody.

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