Silencing proteins Sir3 and Sir4 have distinct roles in the assembly of silent chromatin in budding yeast

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Abstract

The Silent Information Regulator (SIR) complex is responsible for the formation of silent chromatin domains in *Saccharomyces cerevisiae*, and consists of the NAD-dependent histone deacetylase Sir2, and histone binding proteins Sir3 and Sir4. The current model of silent chromatin assembly proposes that histone deacetylation by Sir2 is required to promote recruitment of Sir3 and Sir4, and assembly of full SIR complexes on chromatin. However, recent work has suggested unique roles for the histone binding proteins Sir3 and Sir4 in this process. Here we present data suggesting that Sir3 is primarily responsible for mediating the spreading of silent chromatin from sites of nucleation, while regulation of Sir4 abundance controls the rate of silencing establishment. We have also investigated a potential novel dimerization domain in Sir3, which may represent a conserved function in vertebrates. Investigations into the regulation of silent chromatin assembly in budding yeast will facilitate our understanding of the mechanisms that control heterochromatin-mediated gene repression in higher organisms.

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List of abbreviations

5'-FOA – 5'-fluoroorotic acid
AAA$^+$ - ATPases associated with diverse cellular activities
AAR – 1-*O*-acetyl-ADP-ribose
ADP – adenosine diphosphate
BME – β-mercaptoethanol
BNGE – blue native gel electrophoresis
BSA – bovine serum antigen
BZ – benzamidine
ChIP – chromatin immunoprecipitation
DHO – dihyroorotic acid
DNA – deoxyribonucleic acid
DTT – dithiothreitol
EDTA – ethylenediaminetetraacetic acid
EGTA – ethylene glycol tetraacetic acid
GST – glutathione-S-transferase
HDAC – histone deacetylase
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HP1 – heterochromatin protein 1
ITC – isothermal titration calorimetry
LBPC – leupeptin-bestatin-pepstatin A-chymostatin cocktail
NAD – nicotinamide adenine dinucleotide
NAM – nicotinamide
OA – orotic acid
ORF – open reading frame
PCR – polymerase chain reaction
PEV – position-effect variegation
PMSF – phenylmethanesulfonyl fluoride
SIR – Silent Information Regulator
SPR – surface plasmon resonance
TPE – telomere position effect
UMP – uridine monophosphate
YEP – yeast extract with peptone

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Chapter 1: Introduction

General introduction

The assembly of heterochromatin is a highly regulated process that is conserved from budding yeast to humans. For all metazoans, heterochromatic regions of the genome are characteristically late replicating, transcriptionally silent and confined to the nuclear periphery. Heterochromatin is thus distinct from the euchromatic regions of the genome, which are highly expressed and replicated early during S phase of the cell cycle, and it plays a distinct role in cellular activities. For example, accurate chromosome segregation during mitosis is dependent on the formation of heterochromatin at centromeric loci. In multi-cellular organisms, temporal expression of developmental programs depends on changes in the location and boundaries of heterochromatic regions within the genome. Heterochromatin assembly is therefore a critical process whose timing and location must be tightly regulated.

Early studies in the field of genetics revealed that heterochromatin is a stable chromatin state that can be transmitted to daughter cells. Barbara McClintock first described the inheritability of gene silencing in the early 1950s, while studying patterns of mosaicism in maize (McClintock, 1953; 1961). Similar phenotypes of gene repression were soon observed in *Drosophila melanogaster* and *Saccharomyces cerevisiae*, and were named position-effect variegation (PEV) and telomere position effect (TPE), respectively (Gottschling et al., 1990; Tschiersch et al., 1994). A common mechanism for these effects was determined to be the juxtaposition of transcribed genes with heterochromatic regions of the genome, resulting in gene repression. This mechanism suggested the presence of dynamic silencing elements capable of forming extended regions of repressive chromatin.

The inheritability of these domains also suggested that the altered chromatin state is maintained through mitosis and passed on to daughter cells – it is epigenetically inherited.

Decades of research into these dynamic elements have revealed a well-conserved mechanism of gene regulation through transcriptional silencing. However, the details of the step-wise process that leads to the assembly of silent chromatin have not been well defined. This thesis examines the ability of silencing proteins to interact with and modify the surrounding chromatin environment in order to produce functionally silent regions of chromatin.

Silencing in *Saccharomyces cerevisiae*

The budding yeast *S. cerevisiae* is a powerful model organism to study silencing, due to its genetic tractability and rapid life span. Many of the processes involved in heterochromatin assembly in mammals are also present in the yeast system, which allows studies done in *S. cerevisiae* to be translated to higher organisms (see "Conservation of silencing" below). Indeed, much of the present knowledge on silencing comes from studies performed in *S. cerevisiae*. Silencing in budding yeast occurs at the silent mating loci, the telomeres and subtelomeric regions, and the ribosomal DNA (rDNA) repeats. The first two of these will be discussed in further detail in this thesis, but the latter will not be addressed as silencing of the rDNA repeats is dependent on Sir2 and the RENT complex, but does not require Sir3 or Sir4.

Silencing of the mating type loci

Silencing of specific gene loci was first described in budding yeast in the 1970s, with the discovery of the mating type loci and their regulation (Haber and George, 1979; Klar et al., 1979; Rine et al., 1979). *S. cerevisiae* exists in two mating types, **a** and α, which mate to

produce diploids that can undergo meiosis and sporulation to yield four haploid daughter cells. Mating type information is normally expressed from the active mating type (*MAT*) locus, which can contain the information required for the yeast to exist as an **a** or α cell (these are the **a**1/**a**2 or α1/α2 genes, respectively). In addition to the active mating loci, an additional copy of each mating type program is present in the genome: the silent mating type cassettes at the *HML* and *HMR* loci. Mating type interconversion can occur through translocation of the silent cassette to the active *MAT* mating locus through homologous recombination and the activity of an endonuclease encoded by the *HO* gene. Most laboratory strains of *S. cerevisiae* are ho^- to allow propagation of pure haploid cultures: mating type interconversion can occur in HO^+ strains after two cell divisions to produce clonal populations of haploids and diploids

Mating is dependent on the expression of only a single mating type program at a given time. The maintenance of silencing at *HML* and *HMR* is therefore critical for the propagation of genetic material through meiosis. The genomic structures of the silent mating loci are illustrated in Figure 1.1 (reviewed in Laurenson and Rine, 1992). The silent mating cassettes are identical in sequence and structure to those contained at the active *MAT* locus, indicating that the silencing observed at *HML/HMR* is dependent on genomic context. At the silent mating loci, the open reading frames (ORFs) encoding the **a**1/**a**2 or α1/α2 transcripts are flanked by the DNA elements E and I (termed "Essential" and "Important" for transcriptional repression, respectively), which have been named silencers. The E silencers contain distinct DNA motifs named the A, E and B elements (Figure 1.1) that are recognized by DNA-binding proteins including Orc1, Abf1, Sir1 and Rap1 (Cockell et al., 1995; Diffley and Stillman, 1988; Moretti et al., 1994; Shore and Nasmyth, 1987; Shore et al., 1987; Triolo and Sternglanz, 1996). There is a degree of functional redundancy within the silencer

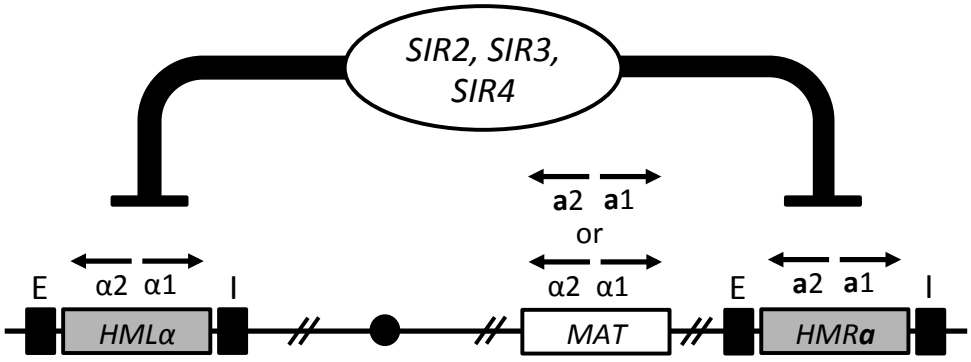

SIR2, SIR3,
SIR4
a2 a1
or
α2 α1
E
α2 α1
I
HMLα
MAT
E
a2 a1
I
HMRa

Figure 1.1 Organization of *Saccharomyces cerevisiae* mating type loci on chromosome III. The genomic organization of the active (*MAT*) and silent mating type loci (*HML* and *HMR*) is shown, along with the silencer elements E and I flanking the silent mating cassettes. The open reading frames coding for the mating type information and the direction of their transcription are also indicated. Transcriptional repression of *HML* and *HMR* is dependent on the *SIR2, SIR3* and *SIR4* genes. Figure adapted from Laurenson and Rine, 1992.

elements: none of the silencer elements are essential or sufficient for silencing on their own (Brand et al., 1987). *HML* and *HMR* differ slightly in the organization of their silencers: *HML* contains only the A and E elements, while *HMR* contains all three of A, E and B. *HML* and *HMR* are derepressed differently under various mutant conditions (Laurenson and Rine, 1992), and these differences are due at least in part to their distinct patterns of recruitment via their silencer elements.

Telomeric and subtelomeric silencing

Variegated expression of heterochromatin-proximal genes was first observed in *D. melanogaster*, where semistable transcriptional repression of genes involved in pigmentation resulted in sectoring within the developing compound eye (Tschiersch et al., 1994). Mutation of the protein *su(var)3-9* (suppressor of variegation) rescues the variegated expression by preventing the spread of heterochromatin into euchromatic regions (Bannister et al., 2001; Lachner et al., 2001; Tschiersch et al., 1994). A similar phenotype was observed in *S. cerevisiae* using a telomeric *ADE2* reporter gene: expression of the *ADE2* gene product results in white colonies, while repression of the gene produces red colonies. Clonal inheritance of the ON or OFF transcriptional state of *ADE2* results in red and white sectored colonies (Aparicio et al., 1991). The lack of an intermediate (pink) phenotype indicates that the ON-OFF transcriptional states are distinct and are bistably inherited through mitosis.

The formation of silent chromatin at telomere ends is critical to maintaining chromosome stability and genome integrity (Kyrion et al., 1992; Palladino et al., 1993). The telomeric repeats at the ends of chromosomes ($[TG_{1\text{-}3}]_n$) are bound by Rap1 (repressor-activator protein 1) and the yKu proteins (yKu70/80) to form a non-nucleosomal chromatin structure (Cockell et al., 1995; Gotta et al., 1996; Gravel, 1998; Wright et al., 1992). The

presence of these capping proteins at the chromosome ends protects the DNA strands from homologous recombination and non-homologous end joining (NHEJ). Binding of Rap1 and yKu70/80 to the tracts of telomeric repeats leads to recruitment of silencing proteins through direct protein-protein interactions (Luo et al., 2002; Roy et al., 2004). As structural proteins required for telomere integrity are also required for the recruitment of silencing proteins to the telomere ends, it is difficult to distinguish whether silencing is required for the formation of the telomere, or if it is an effect of the presence of telomere binding proteins such as the yKu70/80 dimer.

The Silent Information Regulator (SIR) proteins

Discovery of the SIR proteins

The Silent Information Regulator (SIR) genes were isolated in various screens for mutants that cause derepression of the silent mating loci and result in a nonmating phenotype (Haber and George, 1979; Klar et al., 1979; Rine et al., 1979; Rine and Herskowitz, 1987). *SIR1*, *SIR2*, *SIR3* and *SIR4* are non-essential genes that are all required for full transcriptional repression of the silent mating loci. *sir1* mutants display a weaker phenotype at *HML* and *HMR* than the other *sir* mutants, and do not have silencing defects at the telomeres (Aparicio et al., 1991). Early characterization of the *SIR* genes by linkage analysis confirmed that they represent four distinct gene products (Rine and Herskowitz, 1987). There has been no evidence found for transcriptional regulation of *SIR* genes by the Sir proteins themselves (Ivy et al., 1986; Shore et al., 1984).

The first function attributed to the *SIR* genes was transcriptional regulation of *HML* and *HMR*, as it was through screens for nonmating phenotypes that the genes were initially

discovered. Subsequent studies identified similar requirements for *SIR2*, *SIR3* and *SIR4* in producing telomeric position-effect variegation (also know as telomeric position effect [TPE]) in yeast (Aparicio et al., 1991; Gottschling et al., 1990). Studies using a telomeric *URA3* reporter gene confirmed that telomeric repression can be overcome using selective pressure for transcriptional activation (Aparicio and Gottschling, 1994; Gottschling et al., 1990; Kitada et al., 2012). This indicates that, similar to silencing at the mating loci, telomeric silencing is a reversible, dynamic process that is directly opposed by transcription (Pillus and Rine, 1989).

The SIR proteins

The dynamic properties of silencing suggest a mechanism through which silencing proteins are capable of directly sensing and responding to transcriptional cues. Investigations into the structure and function of the products of the *SIR* genes have identified key interactions with histones that are sensitive to regulatory histone modifications, as well as histone modifying activity within the SIR complex itself. Thus the Sir proteins have the capability to bind and modify chromatin to elicit transcriptional repression, while also maintaining responsiveness to opposing transcriptional machineries through the detection of histone modifications.

Many avenues of research suggested early on that transcriptional repression by the Sir proteins is due to an altered chromatin state. Sites within *HML* and *HMR* were found to be resistant to DNase I treatment, while the same sequence at the active *MAT* locus shows *SIR*-dependent DNase I-hypersensitivity (Nasmyth, 1982). A similar effect was shown for micrococcal nuclease digestion: SIR^{+} and sir^{-} strains exhibited shifts in nucleosome positioning, suggesting the Sir proteins alter chromatin structure (Nasmyth, 1982).

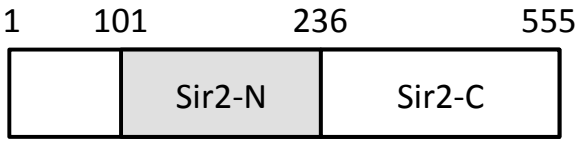
Purification of nucleosomes from *HML* and *HMR* indicate that the extent of their compaction *in vivo* is sufficient to prevent access to thiol-reactive residues within the nucleosome core (Chen et al., 1991). The telomeres are also resistant to methylation by the bacterial *Escherichia coli dam* methyltransferase (Gottschling, 1992). The results of these studies strongly indicate that Sir protein binding to chromatin alters nucleosomal conformations, which in turn leads to transcriptional repression.

Catalytic functions of Sir2

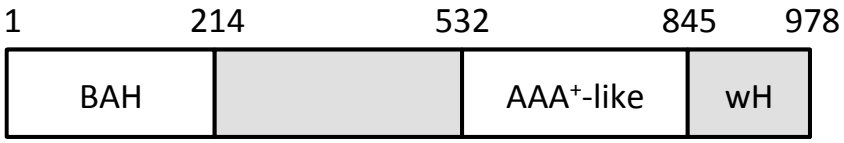
In particular, deacetylation of residues within the N-terminal tail of histone H4 was implicated in the adoption of a repressive state (Johnson et al., 1990; Kayne et al., 1988; Park and Szostak, 1990). Transcriptionally silent regions of the yeast genome are hypoacetylated, and overexpression of *SIR2* was shown to promote histone deacetylation *in vivo* (Braunstein et al., 1993). Two mechanisms could exist through which Sir2 could down-regulate histone acetylation: Sir2 could be directly responsible for histone deacetylation, or it could indirectly inhibit a histone acetyltransferase. The histone acetyltransferase responsible for acetylation of the histone H4 tail is *SAS2*. While Sir2 and Sas2 have opposing functions *in vivo* (Suka et al., 2002), there has been no evidence to show that Sir2 directly interacts with or inhibits the function of Sas2.

Further characterization of Sir2 confirmed that it possesses intrinsic histone deacetylation activity, and that this function is dependent on the co-factor nicotinamide adenine dinucleotide (NAD) (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000; Tanny et al., 1999). Sir2 is now recognized as the founding member of the Class III histone

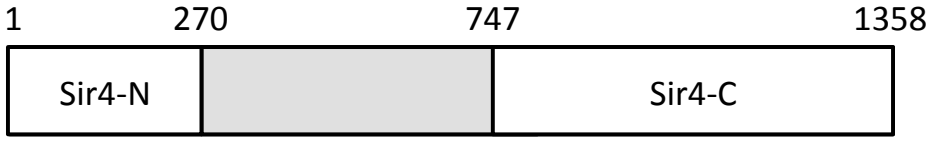
A Sir2



B Sir3



C Sir4



D

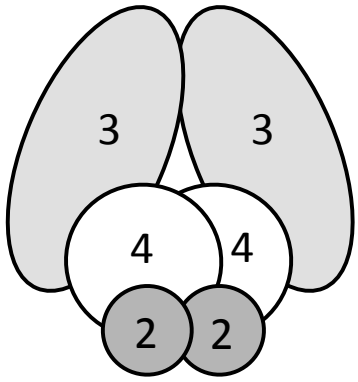


Figure 1.2 The Silent Information Regulator (SIR) Proteins. The domain architecture and organization of the individual SIR proteins is shown (**A**: Sir2, **B**: Sir3 and **C**: Sir4). Labeled areas of each protein indicate fragments that have been crystallized or to which a function has been assigned. Portions left unlabeled have no known function or are predicted to be highly disordered. **D**: A model of the SIR complex indicating inter-complex interactions between the SIR proteins. Sir4 bridges interactions with Sir2 and Sir3, while there is no detectable interaction between Sir2 and Sir3. The SIR proteins are present in stoichiometric amounts within the complex, and likely exist as dimers (Chang et al., 2003; Moazed et al., 1997).

deacetylase (HDAC) sirtuin family. Sir2 has two structural domains: a helical N-terminal domain (Sir2-N; amino acids 101-236) and a conserved C-terminal domain that contains the catalytic activity (Sir2-C; amino acids 237-555) (Hsu et al., 2013) (Figure 1.2A).

Multiple residues along the histone H4 N-terminal tail are modified by acetylation, most notably H4K5, K8, K12 and K16 (Braunstein et al., 1996). Genetic analyses suggest that deacetylation of H4K16 in particular is critical for silencing at the mating loci and telomeres (Hoppe et al., 2002; Johnson et al., 1990), and Sir2 preferentially deacetylates H4K16 *in vitro* (Imai et al., 2000; Tanny et al., 2004). Sir2 catalytic activity is required for the localization of both Sir3 and Sir4 to silent chromatin (Hoppe et al., 2002; Tanny et al., 1999). These results suggest that deacetylation of H4K16 is a major regulatory step in the establishment of silencing.

The biochemical mechanism through which Sir2 deacetylates lysine residues produces nicotinamide (NAM) and the unique product *O*-acetyl-ADP ribose (AAR) (Jackson and Denu, 2002; Tanner et al., 2000). Addition of AAR to the SIR complex *in vitro* causes structural rearrangements (Liou et al., 2005), leading some to predict that binding of this compound to the SIR complex may regulate silent chromatin assembly. However, a closer investigation into the direct role of AAR suggests that it is not necessary for silencing, and it is solely the histone deacetylation by Sir2 that is required for the assembly of silent chromatin (Chou et al., 2008). The requirement of NAD for Sir2's catalytic function means that Sir2 activity is inherently sensitive to the nutrient status of the organism. Sir2 activity is inhibited at high levels of NAM, and also by low intracellular NAD levels (Bitterman et al., 2002; Sauve et al., 2005; Smith et al., 2000). The coupling of Sir2's function to metabolite sensing has led to investigations into putative roles of related mammalian sirtuin proteins in a

wide range of biological processes, including cellular aging and metabolic disorders (Yamamoto et al., 2007).

Sir3 is a chromatin-binding protein

Similar to *SIR2*, *SIR3* is essential for silencing at both the mating loci and telomeres, although there has been no catalytic function attributed to the Sir3 protein. Overexpression of *SIR3* leads to extension of silent chromatin into subtelomeric regions (Hecht et al., 1996a; Renauld et al., 1993; Strahl-Bolsinger et al., 1997), suggesting that endogenous Sir3 is limiting for silencing, and that Sir3 has a direct positive effect on the establishment or spreading of silent chromatin. Sir3 binds directly to the N-terminal tails of both histones H3 and H4, and has also been shown to make contacts with the globular domain of histone H3 (Altaf et al., 2007; Armache et al., 2011; Connelly et al., 2006; Ehrentraut et al., 2011; Hecht et al., 1995; Johnson et al., 1990; Martino et al., 2009). Many of these nucleosomal contacts are sensitive to the modification status of the histone residues (discussed further below), suggesting that alteration of Sir3-nucleosome binding is the mechanism through which transcriptional repression and activation are effected.

Structurally, Sir3 is closely related to the origin recognition complex 1 (Orc1) protein. Sir3 was derived from Orc1 following a whole genome duplication event that occurred early in the phylogeny of *S. cerevisiae* (Kellis et al., 2004). The N-terminal bromo-adjacent homology (BAH) domain of Orc1 is similar enough to Sir3 that it can replace the N-terminal BAH domain of Sir3 (amino acids 1-214, Figure 1.2B) to produce functional silencing (Bell et al., 1995). Mutations in the BAH domain can suppress mutations in both histone H3 and H4, indicating a direct interaction between the BAH domain and the nucleosome (Buchberger et al., 2008; Johnson et al., 1990; Onishi et al., 2007; Thompson et

al., 2003). A hypermorphic allele of Sir3 (*SIR3-D205N*) suppresses histone H3 and H4 mutants, and rescues telomeric silencing in *rap1* mutants (Johnson et al., 1990; Liu and Lustig, 1996; Park and Szostak, 1990; Thompson et al., 1994). This phenotype was explained when Armache et al. (2011) solved the crystal structure of the Sir3-BAH^{D205N} in complex with the nucleosome, suggesting that the rescue is due to an increase in nucleosome binding affinity. Interestingly, overexpression of a fragment encompassing the BAH domain (amino acids 1-380, BAH$^{1-380}$) is capable of silencing in the absence of full length Sir3 (Connelly et al., 2006). This suggests that the minimum functions for silencing are contained in this fragment, which exhibits nucleosome binding capability and potentially self-association (discussed further below).

Acetylation of the N-terminus of Sir3 by the Ard1-Nat1 acetyltransferase complex is required for silencing (Wang et al., 2004). Acetylation occurs at the Ala2 residue, as the initiating methionine of Sir3 is proteolytically cleaved following translation (Polevoda et al., 1999). Crystallographic investigations into the role of N-terminal acetylation show that this modification alters the conformation of the BAH domain in complex with the nucleosome (Yang et al., 2013), indicating that stabilization of the BAH-nucleosome interaction is critical for silencing *in vivo*.

The homology between Sir3 and Orc1 is maintained in their overall domain architecture: in addition to the BAH domain, Sir3 shares an AAA$^+$-like C-terminal domain in common with Orc1 (amino acids 532–845, Figure 1.2B; AAA$^+$ is an abbreviation for "ATPases Associated with diverse cellular Activities"). This domain of Sir3 has retained structural similarity to the AAA$^+$-domain family but lacks the ATP-hydrolysis activity that is present in Orc1 (Bell et al., 1995; Ehrentraut et al., 2011; Gasser and Cockell, 2001; Martino et al., 2009). Deletion series analysis identified two C-terminal histone-binding domains

(CHB1 [amino acids 632-762] and CHB2 [amino acids 799-910]) within the AAA^+-like domain that are sufficient for interactions with the N-termini of histones H3 and H4 (Hecht et al., 1995). In addition, the AAA^+-like domain binds to the globular domain of histone H3, although with less affinity than the full length Sir3 protein (Altaf et al., 2007; Ehrentraut et al., 2011). The same study identified a cluster of basic residues (amino acids 657-660) within the AAA^+-like domain that mediates binding with Sir4 (Ehrentraut et al., 2011), confirming previous investigations that mapped Sir4 binding to this domain (King et al., 2006).

Finally, the C-terminal 138 amino acids of Sir3 (amino acids 845-978, Figure 1.2B) form a winged-helix-turn-helix variant domain (Sir3-wH) that is required for self-interaction *in vitro*, and for silencing *in vivo* (Oppikofer et al., 2013). While this study is the first to link Sir3 dimerization to a silencing function *in vivo*, there have been many investigations into Sir3 self-interactions. Native gel electrophoresis and analytical centrifugation of purified Sir3 have identified a range of multimeric forms of Sir3 (Liou et al., 2005; McBryant et al., 2006). These investigations indicate that Sir3 is capable of forming higher order quaternary structures *in vitro*, but it is not known which domains are responsible for these interactions. The Sir3-wH domain is sufficient to mediate dimerization, but the detection of multimeric complexes *in vitro* suggests the presence of at least one other self-interaction domain.

The presence of two distinct nucleosome-binding domains, a Sir4-interaction domain and at least one dimerization domain within Sir3 suggests that it mediates significant contacts between the SIR complex and the core nucleosome. The spatial arrangement of Sir3 and its multiple domains on chromatin remains unclear, as does the effect of Sir2/4-binding on this interaction. What is clear is that Sir3 possesses multiple redundant interaction domains that may serve to bridge both adjacent SIR complexes and nucleosomes, consistent

with its predicted role in modifying the local chromatin environment to produce a highly compact chromosomal structure.

Sir4 is a scaffolding protein

SIR4 is the final member of the SIR complex, and similar to Sir3, has no known catalytic function. Sir4 is a large protein (156kDa) that consists mainly of unstructured residues, which has hampered studies into its structure and function. Early investigations into the biological role of Sir4 determined that its function is separable into two distinct protein domains (Marshall et al., 1987). Co-expression of N-terminal and C-terminal fragments of Sir4 (N-terminus [Sir4-N]: amino acids 1-270; C-terminus [Sir4-C]: amino acids 747-1358; Figure 1.2C) *in trans* is sufficient to rescue silencing at the silent mating loci, although full-length Sir4 is required for TPE (Kueng et al., 2012b; Marshall et al., 1987). Overexpression of either full length Sir4 or Sir4-C results in derepression of both the silent mating loci and telomeres, possibly through titration of silencing proteins away from these sites (Ivy et al., 1986; Marshall et al., 1987). This explanation is supported by the fact that the C-terminus of Sir4 is sufficient to form a stable and functional SIR complex *in vitro* (Kueng et al., 2012b; Moazed and Johnson, 1996). Sir4 has also been shown to interact with the N-termini of histones H3 and H4 by GST-pull down assays (Hecht et al., 1995), but further investigations with reconstituted nucleosomal arrays suggest that Sir4 interacts preferentially with the nucleosomal core rather than the histone tails (Martino et al., 2009).

While it is clear that the Sir4 C-terminus has an important function in SIR protein interactions and silencing, the role of the N-terminal fragment is less well understood. Purification of Sir3-Sir4 subcomplexes suggests that the N-terminus of Sir4 has an inhibitory effect on the Sir3-Sir4 interaction, and that this may represent a regulatory mechanism of

SIR complex assembly (Moazed et al., 1997). Sir4-N has high affinity for naked DNA and mononucleosomes, although it shows no sequence specificity in binding (Martino et al., 2009). It is therefore possible that Sir4-N aids in SIR complex-nucleosome interactions through non-specific contacts with chromatin, while Sir4-C mediates interactions within the SIR complex itself. It has also been predicted that Sir4-N could regulate Sir2 catalytic activity through the stabilization of an active conformation (Hsu et al., 2013).

The extreme C-terminus of Sir4 (amino acids 1267-1358, Figure 1.2C) forms a parallel coiled-coil that mediates dimerization of Sir4 (Chang et al., 2003). Sir4 exists in solution in its dimeric form (Chang et al., 2003), suggesting that this interaction is highly stable and represents the physiological form of Sir4. Dimerization of Sir4 is thought to be required for interactions with Sir3 (Chang et al., 2003; Murphy et al., 2003), as it has proven difficult to isolate mutants that disrupt the coiled-coil structure but retain a Sir3 interaction (Rudner et al., 2005). A number of mutants within the coiled-coil domain disrupt Sir3-Sir4 interactions both *in vivo* and *in vitro,* and result in loss of silencing at both the silent mating loci and telomeres (Rudner et al., 2005), emphasizing the role of this structure in the integrity of the SIR complex.

SIR complex interactions with chromatin

Interactions within the SIR complex

Early SIR protein purifications from yeast indicated a hierarchy of protein interactions within the complex: Sir2 and Sir4 can be isolated as a soluble subcomplex, while Sir3 can be isolated independently of the other SIR proteins (Hoppe et al., 2002; Moazed et al., 1997; Strahl-Bolsinger et al., 1997). Sir3 and Sir4 have been shown to interact *in vitro* through GST-pull down assays and isothermal titration calorimetry (ITC), and *in vivo* by

two-hybrid assay and co-immunoprecipitation (Chang et al., 2003; Hecht et al., 1996a; Moazed and Johnson, 1996; Moretti et al., 1994; Rudner et al., 2005). Sir4 is required for the integrity of the complex and the recruitment of both Sir2 and Sir3 to chromatin, as there is no significant interaction between Sir2 and Sir3 (Hoppe et al., 2002; Rudner et al., 2005; Rusche et al., 2002; see Figure 1.2D). While results from initial SIR protein purifications suggested that stable subcomplexes may only interact as full SIR complexes on chromatin, it was later shown that full SIR complexes can be isolated by immunoprecipitating Sir3 (Rudner et al., 2005). In addition, it is likely that each of the proteins within the SIR complex exist as dimers *in vivo* (Chang et al., 2003; Moazed et al., 1997; Oppikofer et al., 2013), suggesting the presence of numerous interfaces through which both intra- and inter-complex contacts could be made.

The Sir4-binding domain of Sir3 was recently mapped by Ehrentraut et al. (2011) within the C-terminal AAA^{+}-like domain of Sir3. Mutation of multiple residues within a basic cluster (Sir3-K657 K658 R659 K660) to alanine (*sir3-2A, -3A* or *-4A*) abolishes the Sir3-Sir4 interaction both *in vitro* and *in vivo* (Ehrentraut et al., 2011 and Harding et al, in prep.). Work in the Rudner laboratory confirmed that these residues are specifically responsible for the interaction of Sir3 with the C-terminal coiled-coil of Sir4 (Chang et al., 2003; King et al., 2006 and Harding et al, in prep).

A panel of Sir4 mutants have also been isolated that provide additional confirmation that the Sir3-Sir4 interaction is mediated by the Sir4 coiled-coil. These mutants (*SIR4-M1307N, sir4-E1310R,* and *SIR4-I1311N)* prevent binding to Sir3 *in vitro* and *in vivo,* but retain their binding to Sir2 (Chang et al., 2003; Rudner et al., 2005). Interruption of the Sir3-Sir4 interaction by these mutants causes a loss of silencing at a weakened silent mating loci

(*hmrΔE::TRP1*) and telomere: the *SIR4-M1307N* and *SIR4-I1311N* mutants abolish all Sir3 binding and disrupt silencing, indicating that this interaction is required (Rudner et al., 2005).

The Sir2-Sir4 interaction involves a C-terminus of Sir4 (Sir2-interacting domain [SID]: amino acids 745-1172) and the majority of the Sir2 protein (Hsu et al., 2013; Moazed and Johnson, 1996; Strahl-Bolsinger et al., 1997). The Sir4-SID forms extensive contacts with both structural domains of Sir2, which stabilizes the substrate-binding channel of the Sir2 catalytic active site (Hsu et al., 2013). Sir2 has also been proposed to dimerize (Moazed et al., 1997) and is crystallized as a homodimer (Hsu et al., 2013), but it is unclear whether this interaction is physiologically relevant.

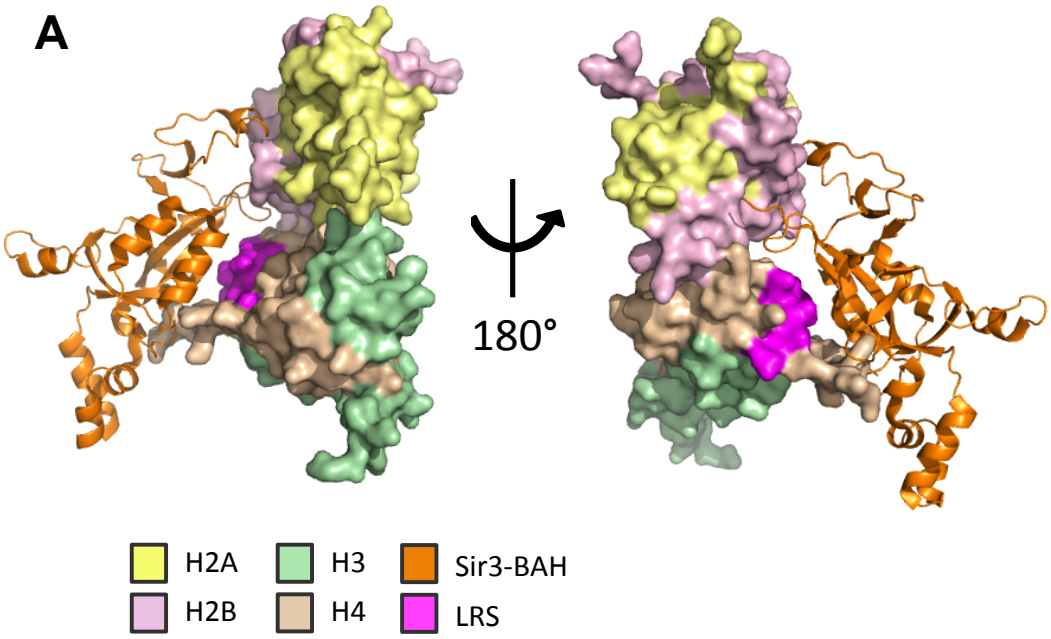
SIR protein-nucleosome interactions

As mentioned previously, extensive direct contacts are made between the SIR complex and chromatin, especially through Sir3. Structural investigations into these interactions have confirmed genetic analyses and provided insights into the function of the SIR complex. However, crystal structures represent static interactions between the nucleosome and isolated fragments of individual Sir proteins, and likely do not reflect the dynamic nature of silent chromatin. For example, the histone modifications deposited by chromatin modifying complexes likely affect silencing by altering binding of the SIR complex to chromatin. Obtaining a co-crystal of such large protein structures in various modification states may prove to be prohibitively complex, but studies thus far have provided a general overview of the SIR-nucleosome interactions that exist within silent chromatin.

Sir3 is able to interact with nucleosomes through both its BAH domain and AAA^{+}-like domain. Interestingly, the crystal structure of the BAH domain in complex with the

nucleosome shows interactions with the loss of rDNA silencing (LRS) region of the histones H3 and H4 (Armache et al., 2011; Fry et al., 2006; Park et al., 2002; Thompson et al., 2003). While Sir3 is not required for silencing at the rDNA sequences, this structure may indicate a conserved mechanism through which silencing proteins, including Net1 at the rDNA loci (Straight et al., 1999), interact with the nucleosome. Within this crystal structure, there are three potential hydrogen bonds between residues within the B6 and B8 β-sheets of Sir3-BAH (residues E84 and E140) and the histone H3K79 residue within the nucleosome core (Armache et al., 2011). The interaction between Sir3 and the nucleosome is altered *in vitro* by methylation of H3K79 (Buchberger et al., 2008; Onishi et al., 2007), which is catalyzed by the methyltransferase Dot1 (Lacoste et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002). Overexpression of Dot1 leads to a global increase in H3K79 methylation and disrupts silencing *in vivo* (Singer et al., 1998). Based on the crystal structure by Armache et al. (2011), the mechanism through which H3K79 methylation opposes silencing may be by altering Sir3 binding. However, a recent study found that Sir3 can occupy regions of the genome containing methylated H3K79, although this does not lead to functional gene silencing (Kitada et al., 2012).

Similarly, binding of the AAA^+-like domain to reconstituted nucleosomes *in vitro* is sensitive to methylation of H3K79, suggesting that residue is also involved in binding of the AAA^+-like domain to the nucleosome (Altaf et al., 2007; Ehrentraut et al., 2011). However, full length Sir3 binds to the nucleosome with much higher affinity than the AAA^+-like domain alone, and this binding is more sensitive to H3K79 methylation (Ehrentraut et al., 2011). It is possible that the binding observed by Ehrentraut et al. (2011) is mediated primarily through interactions of the AAA^+-like domain with the tails of histones H3 and H4 (Hecht et al., 1995) and that H3K79 methylation indirectly alters this interaction.


A
180°
H2A
H2B
H3
H4
Sir3-BAH
LRS

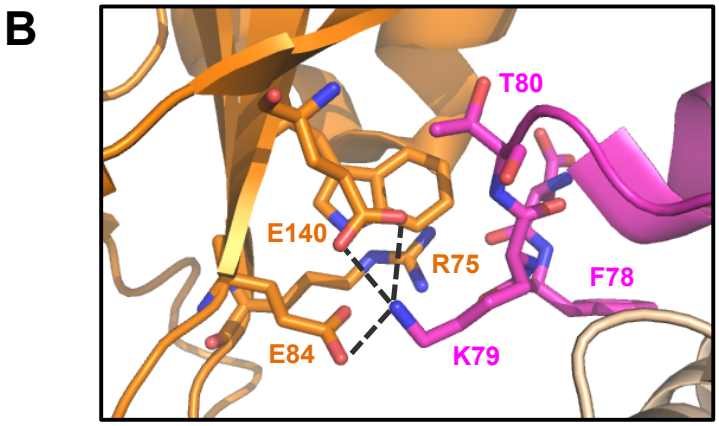

B
T80
E140
R75
F78
E84
K79

Figure 1.3 Structure of the Sir3-BAH domain in complex with the nucleosome. A. The crystal structure of recombinant Sir3-BAH in complex with a reconstituted *Xenopus* nucleosome core particle (NCP; Armache et al, 2011; PDB ID 3TU4) is shown. A single BAH domain and one half of the histone octamer are shown for clarity: Sir3-BAH in orange, H2A in light pink, H2B in yellow, H3 in green and H4 in tan. The LRS (loss of rDNA silencing) region is highlighted in magenta. The structure is rotated 180° around the *y* axis to produce two views. **B**. Detailed view of the region surrounding the H3K79 residue within the LRS (magenta), including residues from the Sir3-BAH domain (orange). Histone H3K79 may be involved in multiple interactions with the Sir3-BAH domain. Three putative hydrogen bonds (dashed lines) are shown between H3K79 and two residues within Sir3: E84 and E140 (Armache et al, 2011).

Therefore it is likely that the BAH domain is primarily responsible for binding to histone H3 and sensing the methylation status of H3K79 *in vivo* (Onishi et al., 2007).

These studies have provided strong evidence that Sir3 interacts directly with the globular domain of histone H3, but the potential interactions between Sir3 and the N-terminal H3 tail are less well understood. Hecht et al. (1995) observed an interaction between full length Sir3 and an N-terminal peptide of the histone H3 tail *in vitro* that required the CHB regions within the AAA^{+}-like domain. This interaction has a much lower affinity than that of the Sir3-H4 tail interaction, as determined by surface plasmon resonance (SPR) spectroscopy (Liou et al., 2005). In the crystal structure solved by Armache et al. (2011), the H3 tail does not come into contact with the BAH domain. However, deletion or mutation of the H3 tail increases Sir3 occupancy within subtelomeric regions, suggesting that it plays a role in Sir3 recruitment (Sperling and Grunstein, 2009).

The BAH domain also interacts directly with the N-terminal tail of histone H4 (Armache et al., 2011; Connelly et al., 2006; Hecht et al., 1995; Onishi et al., 2007). The variegated phenotype observed in TPE is susceptible to mutations in the N-terminal tail of histone H4 (Aparicio et al., 1991). Electrostatic interactions between the basic histone tail and an acidic patch within the BAH domain are predicted to stabilize the interaction between $Sir3^{BAH}$ and histone H4, and may be disrupted by acetylation of H4K16 (Armache et al., 2011). Mutation of H4K16 to glycine or glutamine (*H4K16G* and *H4K16Q*) reduces mating efficiency *in vivo,* while mutation to arginine (*H4K16R*) only shows a minor mating defect (Johnson et al., 1990). In addition, an *H4K16A* allele prevents Sir3 binding to chromatin arrays *in vitro* (Johnson et al., 2009), indicating that the positive charge of the lysine is critical for the Sir3-histone H4 interaction. Interestingly, binding of full-length Sir3 to nucleosome particles blocks Dot1-mediated methylation of H3K79 in an H4K16 dependent

manner (Altaf et al., 2007). Therefore, H4K16 deacetylation may promote Sir3 binding and inhibit methylation of H3K79 by Dot1, producing a feed-forward mechanism to allow silencing.

Sir4 interactions with the nucleosome are less well documented than those of Sir3. Sir4 can interact with the N-terminal tails of both histones H3 and H4 *in vitro* (Hecht et al., 1995), although the specificity of this interaction has been debated (Johnson et al., 2009; Kueng et al., 2012a; Martino et al., 2009). Interactions of the Sir2-Sir4 heterodimer with nucleosomal arrays are thought to be mediated more by the affinity of Sir4 for dsDNA, rather than direct recognition of the histones (Kueng et al., 2012b; Martino et al., 2009).

Assembly, maintenance and establishment of silencing

The assembly of a silent chromatin domain is a step-wise process, and distinctions between each step may involve different requirements for the silencing proteins and the presence of histone modifications. Initiation, or nucleation, of silencing refers to the recruitment of SIR proteins to chromatin, and is directed by interactions with DNA-binding proteins that assemble at the silencers. Following initiation, spreading of silent chromatin occurs through SIR protein interactions and contacts with the nucleosome, which are regulated through histone modification. It has proven difficult to isolate the requirements for the SIR proteins in each of nucleation and spreading of silencing, as spreading is itself dependent on nucleation.

Nucleation of silencing

Assembly of silent chromatin at both the silent mating loci and telomeres requires all of the SIR proteins and the presence of recruitment proteins. Silencers at *HML* and *HMR* are

bound by Orc1, Abf1, Sir1 and Rap1 (Cockell et al., 1995; Diffley and Stillman, 1988; Moretti et al., 1994; Shore and Nasmyth, 1987; Shore et al., 1987; Triolo and Sternglanz, 1996), which exhibit functional redundancy at the silencers. At telomeres, binding of Rap1 and yKu70/80 to the telomeric repeats results in recruitment of silencing proteins through direct interactions with Sir4 (Luo et al., 2002; Roy et al., 2004). Recruitment of Sir3 and the Sir4-Sir2 heterodimer to the silencers occurs through direct interactions with these recruitment proteins, although the exact sequence of binding is not well understood.

The establishment of functionally silent chromatin requires all three SIR proteins. Sir4 is required for recruitment of both Sir3 and Sir2 to the silent mating loci and the telomeres (Hoppe et al., 2002; Rusche et al., 2002). However, Sir4 can be recruited to silencers through interactions with non-SIR proteins, independently of Sir2 and Sir3 (Luo et al., 2002; Roy et al., 2004). Mutations that disrupt Sir3-Sir4 interactions prevent recruitment of Sir proteins to chromatin and cause a loss of silencing (Ehrentraut et al., 2011; Rudner et al., 2005 and Harding et al, in prep). In addition, the catalytic activity of Sir2 is required for silencing, although not for the recruitment of Sir proteins to chromatin (Hoppe et al., 2002). The Sir2-Sir4 heterodimer preferentially binds to nucleosomes containing acetylated H4K16 (Oppikofer et al., 2011), providing another possible recruitment mechanism through which SIR proteins recognize regions to be silenced.

Spreading of silent chromatin

Following the initial recruitment of SIR proteins to silencers, deacetylation of neighbouring histones by Sir2 (bound to Sir4) is thought to promote Sir3 binding to adjacent nucleosomes (Carmen et al., 2001). Successive rounds of deacetylation and nucleosome binding could then lead to polymerization of the SIR complex along chromatin. Telomeric

silent chromatin domains can spread up to around 4kb from the end of the telomere towards the centromere (Gottschling et al., 1990). Silencing is eventually countered by the activity of chromatin remodelers including Sas2 and Dot1, and the presence of boundary elements (Donze et al., 1999; Suka et al., 2002; van Leeuwen et al., 2002).

Overexpression of Sir3 extends regions of silent chromatin into subtelomeric regions (Renauld et al., 1993; Strahl-Bolsinger et al., 1997 and Harding et al, in prep). These regions show enrichment of Sir3, and low levels of Sir2 or Sir4 (Strahl-Bolsinger et al., 1997 and Harding et al, in prep), suggesting that Sir3 has a unique role in the formation of extended regions of silent chromatin, independently of the other Sir proteins. It has been shown many times that Sir3 is able to bind nucleosomal arrays *in vitro* in the absence of both Sir2 and Sir4 (Ehrentraut et al., 2011; Johnson et al., 2009; Martino et al., 2009; Oppikofer et al., 2011; 2013). The ability of Sir3 to oligomerize *in vitro* is also consistent with this model (Liou et al., 2005). In addition, Sir3 dimerization was found to improve loading of the SIR complex onto chromatin arrays *in vitro* (Oppikofer et al., 2013), suggesting a role for Sir3 self-interactions in directing the assembly of silent chromatin domains. However, the requirement for the Sir3-Sir4 interaction for nucleation may suggest a unique role for an intact SIR complex to template Sir3 oligomerization, beginning at the site of recruitment. While the current model suggests that SIR protein spreading causes transcriptional repression, Kitada et al. (2012) observed Sir3 occupancy and histone deacetylation at a transcriptionally ON telomeric locus, suggesting that the spreading of SIR proteins is not in itself sufficient to repress transcription.

Conservation of silencing

The assembly of heterochromatin in yeast shares many characteristics with the process in mammalian systems and there is a growing body of evidence that supports the conservation of some of the proteins involved. Sir2 is the founding member of the sirtuin family of HDACs that are conserved in organisms up to humans. Silencing in *S. pombe* is dependent on both Sir2 and RNAi, a mechanism that is not present in *S. cerevisiae* (Bühler et al., 2006). The human homologues to Sir2, the *SIRT* family, have been associated with transcriptional regulation, and the substrates of the hSIRT proteins include transcription factors and transcriptional regulators, in addition to acetylated histones H1, H3 and H4 (Yamamoto et al., 2007). However, the mechanisms that regulate the targeting of SIRT proteins to chromatin remain unclear. Sir3 is a homologue of Orc1, which is highly conserved in vertebrates as a member of the Origin Recognition Complex (ORC) (Bell et al., 1995; Callebaut et al., 1999; Connelly et al., 2006). Orc1 is required for localization of HP1 to chromatin in vertebrates (Prasanth et al., 2010), suggesting a mechanism of silencing protein recruitment similar to that found in *S. cerevisiae*. While there are no obvious homologues of Sir4 in higher organisms, collaboration between the Rudner laboratory and Dr. Isabelle Callebaut (Université Pierre et Marie Curie, Paris, France) identified conserved subdomains within Sir4 and Asf2 (anti-silencing factor 2). Data from the Rudner laboratory has shown that Asf2 is capable of binding Sir3 *in vivo*, indicating functional conservation between Sir4 and Asf2.

Research objectives and hypotheses

General hypothesis

Transcriptional repression through gene silencing is responsible for diverse cellular processes including the temporal expression of developmental programs in mammalian systems, and the regulation of cellular identity in organisms from yeast to humans. The Sir proteins found in *S. cerevisiae* perform an analogous function to heterochromatin proteins in vertebrates, and provide a genetically tractable system in which to study the processes involved in heterochromatin assembly.

The SIR proteins have been well characterized individually, but the process through which they assemble on chromatin and repress transcription is not clear. The establishment of a region of silent chromatin must be regulated in order to prevent spurious repression of essential genes. This thesis will examine the roles of Sir3 and Sir4, and their interaction, in the assembly of silent chromatin, in order to provide a better understanding of the events that lead to the creation of repressive chromatin.

Specific hypothesis

I hypothesize that Sir3 and Sir4 have distinct roles within the SIR complex: Sir3 is responsible for mediating spreading of silent chromatin, while Sir4 determines the conditions of establishment. We believe that Sir3 spreads along chromatin independently of Sir4, but its spreading creates a chromatin template that allows recruitment of Sir2 and Sir4, independently of Sir4 binding to Sir3. In addition, we hypothesize that regulation of Sir4 abundance at the site of nucleation controls the rate of silencing assembly.

Statement of objectives

1. To examine the requirement of the Sir3-Sir4 interaction in the spreading of silent chromatin
2. To determine if the Sir3-ARK loop mediates Sir3-Sir3 interactions
3. To assess the role of Sir4 dosage in silencing establishment

Chapter 2: General Methodology

Physiology and strain construction

Yeast strains were grown in either rich media containing yeast extract (Fisher) and peptone (Fisher) (YEP) supplemented with 2% sugar (Wiesent) and 0.005% adenine and tryptophan (Sigma), or minimal media containing 0.5% ammonium sulfate (Fisher) and 0.15% yeast nitrogen base lacking amino acids (Fisher), supplemented with 2% sugar (Wiesent) and synthetic amino acids (Sigma). α-factor was used at a final concentration of 1μg/mL, and nocodazole was used at a final concentration of 10μg/mL. 5'-FOA was used at a concentration of 1g/L in solid media.

GAL-SIR4-C2 and GAL-PPR1 overexpression strains: Overexpression strains were created in derivatives of the reporter strain ADR4062. For GAL-SIR4-C2, the C-terminal fragment of Sir4 (amino acids 1267-1358) was amplified from genomic DNA using primers (1057/1059) that introduced ClaI and EcoRI restriction sites at the 5' and 3' ends of the PCR product. The resulting PCR product and the GAL-promoter plasmid (pAR380) were doubly digested with the restriction enzymes ClaI/EcoRI (New England Biolabs) to remove the blunt ends of the PCR product and linearize the plasmid. The resulting fragments were then purified by gel extraction and ligated together using T4 DNA ligase (New England Biolabs). The cloned *GAL-SIR4-C1* plasmid (pAR1063) was then linearized with NheI enzyme and transformed into the reporter strain ADR4062. Similarly, the *GAL-PPR1* overexpression strains were created by linearizing the Bodo103 plasmid with Nhe1 (New England Biolabs), and transforming the linearized plasmid into the reporter strain ADR7503.

ARK mutants: PCR-mediated mutagenesis was performed in an ADR4062 background to create the Sir3-ARK mutants (ADR7415, 7418, 7420, 8060). Specific oligonucleotides were designed for each mutation, containing nucleotide substitutions that resulted in codon changes and introduced cryptic endonuclease restriction sites to facilitate screening (primers listed in Table A1.3). For each mutation, PCR products were created upstream and downstream of the mutagenesis site, using a mutagenesis primer and an upstream or downstream Sir3 genomic primer. These PCR products were then stitched together *in vitro* using the flanking Sir3 genomic primers, to create a full-length PCR product containing the mutation internal to two flanking regions of *SIR3* genomic homology. The individual full-length PCR products were transformed into ADR4062 simultaneously with an antibiotic marker containing homology immediately upstream to the *SIR3* gene. Transformants were screened by both growth on media containing antibiotic and by genomic PCR followed by restriction enzyme digestion.

Serial dilution assays

Yeast cultures were grown to saturation in 10mL of the appropriate media for 2 days at 30°C. Ten-fold dilutions of starting culture were made in a 96-well plate (Fisher). Wherever cultures were grown in rich media, the starting cultures were diluted to 1/5 prior to performing ten-fold dilutions, in order to detect loss of viability in the lower density growth spots. Dilutions were spotted on the appropriate control and treatment plates, and grown at 30°C for 2-3 days. Plates were photographed using epi-white imaging on an ImageQuant LAS4000 system (GE Healthcare Bio-sciences).

Quantitative silencing assays

Yeast cultures were grown to saturation for 2 days at 30°C in 10mL of synthetic minimal media lacking leucine to maintain plasmid selection. Cultures were normalized by optical density (OD_{600}; Biochrom Ultrospec 2100 *pro*, Fisher) These were plated at appropriate dilutions on synthetic minimal media plates lacking leucine, in the presence or absence of 5'-fluoroorotic acid (5'-FOA; Toronto Research Chemicals) and grown at 30°C for three days. Viability on 5'-FOA is expressed as the percent ratio of absolute survival on 5'-FOA to absolute survival on control plates, and error bars represent standard error. Values of percent survival below 0.001% were considered below the detection limit for this assay (Renauld et al., 1993).

Silencing disassembly assays

GAL-PPR1 and GAL-SIR4-C2: Yeast cultures were grown overnight in 50mL of rich media containing raffinose (YEP+2% RAFF) to an OD_{600} of 0.6-0.8. Expression of *PPR1* or *SIR4-C2* was induced through the addition of 2% galactose to the cultures. 1 x 10^7 cells were harvested at each time point and plated at appropriate dilutions on rich media plates in the presence or absence of 5'-FOA.

Silencing during α-factor arrest: Yeast cultures were grown overnight in rich media containing dextrose (YEP+2% DEX) to an OD_{600} of 0.3-0.5. Cultures were split into two, and α-factor was added to one culture to a final concentration of 1μg/mL while the other culture was grown asynchronously. G1 arrests were confirmed visually after approximately 3 hours by the presence of >90% cells with mating projections. 1 x 10^7 cells were harvested at each time point and plated at appropriate dilutions on rich media plates in the presence or absence of 5'-FOA.

Western blotting

Western blotting was performed as previously described (Rudner et al., 2005). In general, 3 x 10^7 cells were harvested from cultures for each sample, and flash frozen in liquid nitrogen. Whole cell extracts were made by bead beating frozen pellets for 1 minute in 250µL 1X sample buffer (80mM Tris-HCl [pH 6.8], 2% SDS, 0.1% bromophenol blue, 10% glycerol, 10mM EDTA, 5% BME; with 1mM PMSF added immediately prior to use) with an equal volume 0.5mm glass beads (BioSpec). Samples were spun at 14,000rpm for 5 minutes at 4°C to pellet insoluble material and the glass beads. 15µL of each sample was loaded on a polyacrylamide gel for detection by Western blotting.

Standard methods were used for polyacrylamide gel electrophoresis and protein transfer to nitrocellulose. Polyacrylamide gels were run for 2h at a constant amperage (25mA) and ramping voltage (up to 200V) in SDS-Tris running buffer. Nitrocellulose blots were stained with Ponceau S to confirm transfer and equal loading of samples, and then blocked in blocking buffer (4% non-fat dried milk [Carnation] in TBS-T [20mM Tris-HCl pH 7.5], 150mM NaCl, 0.1% Tween-20) for 30 minutes prior to incubation with primary antibodies. All primary antibodies were incubated overnight at 4°C.

The following antibodies were used for Western blots: rabbit polyclonal α-Sir2 (1:5000), α-Sir3 (1:1000), α-Sir4 (1:2500) and α-pan-methyl H3K79 (1:1000; Millipore) were diluted in TBS-T with 4% non-fat dried milk (Carnation), 5% glycerol and 0.02% NaN_3. Rabbit polyclonal α-Cdk1 (1:1000) and α-Clb2 (1:2500) were diluted in 5% BSA solution. Membranes blotted for Cdk1 and Clb2 were washed twice with TBS-T following blocking in order to remove milk proteins prior to incubation with the primary antibody. After incubation with primary antibody, membranes were washed twice with TBS-T and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Amersham) at

a 1:5000 dilution in blocking buffer, for 30 minutes to an hour at 25°C. Blots were washed again in TBS-T, incubated with Western Lightning reagents (Perkin-Elmer) according to manufacturers instructions, and exposed to HyBlot-CL autoradiography film (Denville Scientific).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitations were performed as described previously (Rudner et al., 2005). Briefly, cultures were grown at 30°C to OD_{600} of 1.5, and fixed with 1% formaldehyde for 15 minutes. Cultures treated with α-factor or nocodazole were arrested for 5 hours prior to fixing. Cross-linking was quenched with 125mM glycine for 5 minutes, and cells were washed once with 20mM Tris-HCl (pH7.5)-150mM NaCl. Pellets were immediately flash frozen in liquid nitrogen and stored at -80°C. Cells were lysed from frozen pellets in ChIP lysis buffer (50mM HEPES-KOH [pH 7.6], 500mM NaCl, 1mM EDTA, 1% Triton X-100). The following were added to the lysis buffer just before use: 0.1% sodium deoxycholate, 1mM PMSF, 1mM benzamidine, and 1mM leupeptin-bestatin-pepstatin A-chymostatin cocktail (LBPC). Chromatin in the lysates was sheared to an average size of 500bp, using a Covaris S220 Ultra-Sonicator (Covaris). Lysates were clarified with a 5-minute centrifugation step (15,000rpm at 4°C). Supernatants were collected and normalized using Bradford reagent (Biorad). Input DNA was prepared from 50μL normalized lysate in 200μL 50/10-TE + 1% SDS (50/10-TE is 50mM Tris-HCl [pH 8.0], 10mM EDTA); samples were incubated at 65°C overnight and then processed in the same manner as the immunoprecipitates.

Sir2, Sir3, and Sir4 were immunoprecipitated using 1μL affinity-purified rabbit polyclonal antibody (Rudner lab). Histone H3 and histone H3 methylated at K79 was

immunoprecipitated using 1μL commercial α-H3 or α-pan-methyl H3K79 rabbit polyclonal antibody (Millipore). Lysates were incubated with antibodies for 30 minutes, after which 10μL Protein A Dynabeads (Invitrogen, 10001D) were added to each sample and incubated at 4°C for 1-2h. Beads were washed three times with ChIP lysis buffer, once with Li buffer (10mM Tris-HCl [pH 8.0], 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA), and one time with 50/10-TE. Protein and cross-linked DNA were eluted in 100μL 50/10-TE + 1% SDS at 65°C for 10 minutes. This eluate was removed, and the beads were washed with 150μL 50/10-TE + 1% SDS and this wash was combined with the eluate. The samples were incubated at 65°C overnight to reverse the crosslinks, then sequentially incubated with RNase A (100 μg per input sample and 10 μg per IP sample; 1h at 55°C) and with 50μg/mL proteinase K (2h at 55°C). The samples were extracted twice with an equal volume of 1:1 phenol:chloroform and extracted once with chloroform alone. The remaining DNA was precipitated with 1/10 volume 3M NaOAc (pH 5.2), 2.5 volumes ethyl alcohol and 10μg glycogen. DNA pellets were washed once with 70% ethyl alcohol, air-dried and resuspended in 50μL 10/1 TE (10mM Tris-HCl [pH 8.0], 1mM EDTA).

PCR reactions were performed with either 5μL of a 1:10 dilution of immunoprecipitated DNA, or 5μL of a 1:1000 or 1:2500 dilution of input material (DNA-Engine Thermocycler; BioRad). Reaction mixtures were a total volume of 12.5μL, containing PCR buffer (Invitrogen), 0.1mM deoxynucleoside triphosphates (Fisher), 0.1mCi of [α-^{32}P]dCTP (Perkin Elmer), and 1μM of each primer (primers listed in Table A1.3). PCR reaction conditions were 1 cycle of 95°C for 2 min, 55°C for 30 s and 72°C for 1 min; 26-28 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; and a final step of 72°C for 4 min. PCR reactions were run on 6% acrylamide (30:1 acrylamide-bis)-Tris-borate-EDTA gels for 45-60 minutes at 100V. Samples were loaded with 1.5μL loading dye (50mM Tris-HCl [pH

8.0], 50% sucrose, 4M urea, 0.05% bromophenol blue). Gels were dried and subjected to autoradiography (Storage Phosphor Screen; GE Healthcare) and quantification by densitometry (ImageQuant). Relative fold enrichment values for Sir4 and H3 were calculated as follows: [silent locus (IP)/*ACT1* (IP)]/[silent locus (input)/*ACT1* (input)]. Values are expressed as mean and standard deviation of three replicates, and scaled with average values for control strains set to 1.

Statistical analyses

ChIP quantification data were analyzed using a one-way ANOVA and Bonferroni post-hoc test for statistical significance. P-values less than 0.05 were considered indicative of a statistically significant difference. Quantitative silencing assays and disruption of silencing assays were analyzed using a two-way ANOVA and Bonferroni post-hoc test, or non-linear regression analysis and a Wald-Wolfowitz runs test for goodness of fit.

Protein visualization

Protein images were obtained from the National Center for Biotechnology Information (NCBI) Protein Databank (PDB; Madej et al., 2014) and visualized using PyMOL (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.).

Chapter 3: Defining the role of the Sir3-Sir4 interaction

Introduction

The assembly of silent chromatin domains is a multi-step process that involves the recruitment of SIR proteins to chromatin, followed by the spreading of the silent domain along the chromosome. Sir2, Sir3 and Sir4 are all essential for silencing at the mating type loci and at the telomeres, and the Sir3-Sir4 interaction is absolutely required for nucleation (Rine and Herskowitz, 1987 and Harding et al, in prep). In addition, tethering of either Sir3 or Sir4 directly to the chromatin does not result in silencing in the absence of Sir2 (Marcand et al., 1996). The Sir2-H364Y catalytic mutant blocks the nucleation of silencing, indicating that active histone deacetylation is required for the assembly of silent chromatin (Hoppe et al., 2002). These findings all indicate that the nucleation of silencing is dependent on the contribution of all SIR proteins to the formation of SIR complexes, and their association with nucleosomes containing deacetylated H4K16. However, overexpression of Sir3 leads to the creation of extended silent chromatin domains that are enriched in Sir3 but not Sir2 or Sir4 (Hecht et al., 1996a; Renauld et al., 1993; Strahl-Bolsinger et al., 1997). The ability of Sir3 to interact directly with itself and the nucleosome independently of the other SIR proteins (Armache et al., 2011; Buchberger et al., 2008; Liou et al., 2005; Onishi et al., 2007) suggests a major role for Sir3 in mediating the spreading of silent chromatin following initiation. In light of this evidence, we wanted to test if the role of the Sir3-Sir4 interaction is restricted to the nucleation of silent chromatin.

In order to investigate the function of Sir3 independent from the full SIR complex, we employed a mutant allele of *SIR3*, *sir3-4A*, that has been shown to disrupt Sir3-Sir4

interactions (Ehrentraut et al., 2011 and Harding et al, in prep). This allele mutates four basic residues in a loop of the AAA^{+}-like domain to alanine (K657A K658A R659A K660A, Figure 3.1A). Previous work in the Rudner laboratory has confirmed that the *sir3-4A* mutant is unable to initiate silencing, probably due to its inability to bind to Sir4 at the silencer, and that it does not have a dominant negative phenotype that would indicate poisoning of the SIR complex. We used the *sir3-4A* allele in the context of strains also expressing wild type *SIR3* in order to examine the role of the Sir3-Sir4 interaction following initiation.

Results

Overexpression of Sir3-4A extends silent chromatin domains

In light of prior evidence that Sir3 is enriched in abundance over other SIR proteins in extended silent chromatin domains (Strahl-Bolsinger et al., 1997), we wanted to test whether overexpression of Sir3-4A was sufficient to form similarly extended domains. Using a series of reporter strains containing *URA3* directly integrated into the subtelomeric region of Tel-VR at various loci (the strains are a kind gift from Dr. Gottschling's lab) we quantified gene repression along the telomere. We transformed high copy 2μ-plasmids containing either the *SIR3* or the mutant *sir3-2A,-3A* or *-4A* gene, or the empty 2μ-vector alone, into these strains, resulting in an approximately 10-fold increase in Sir3 protein compared to endogenous levels (Figure 3.1B).

Silencing of the *URA3* marker along Tel-VR was assessed by counter-selection using the drug 5'-fluoroorotic acid (5'-FOA). In the presence of the *URA3* gene product (orotidine 5'-phosphate decarboxylase), 5'-FOA is converted into the toxic metabolite 5'-fluorouracil, which irreversibly inhibits the production of thymidine by thymidylate synthase. Overexpression of *SIR3* (*SIR3-2μ*) represses transcription of *URA3* and improves growth on 5'-FOA compared to strains expressing only endogenous levels of Sir3 (Empty *2μ*) (Figures 3.2A & B). Strains overexpressing *sir3-3A* or *sir3-4A* showed growth patterns similar to each other when the *URA3* marker was placed at 1kb or 2kb, while the *sir3-2A* showed a slightly stronger growth defect. All of these Sir3 mutants also improved growth on 5'-FOA compared to the empty *2μ* vector alone, although the growth of the mutants was less than that of wild type.

Expression of the *URA3* reporter gene is regulated by the transcriptional activator Ppr1 (pyrimidine pathway regulator 1). Ppr1 is a member of the Zn_2Cys_6 binuclear cluster family of transcriptional regulators, which also includes Gal4, an activator of galactose-inducible genes (Liang et al., 1996). The DNA-binding activity of Ppr1 is found in its N-terminal domain (amino acids 29-123) and it recognizes a $CGGN_6CCG$ motif found upstream of the *URA3* transcriptional start site (Liang et al., 1996; Marmorstein and Harrison, 1994). Interestingly, Ppr1 is sensitive to the levels of metabolic intermediates within the pyrimidine biosynthetic pathway, and its activity is increased in the presence of two uridine monophosphate (UMP) precursors: dihydroorotic acid (DHO) or orotic acid (OA) (Flynn and Reece, 1999).

Deletion of the *URA3*-transactivator, Ppr1, improved silencing at all loci tested, as has been shown previously (compare Figures 3.2A & B; Renauld et al., 1993). Comparison of silencing between $PPR1^+$ and *ppr1Δ* strains allows us to directly correlate the effect of the *sir3-4A* mutants to the function of the silent chromatin: the ability to compete with transcriptional activators acts as a readout of silent chromatin stability and efficiency of transcriptional repression.

We next focused on the ability of *sir3-4A* to cause TPE by monitoring viability of strains containing the *URA3* reporter gene, as a function of distance from the telomere end. Silencing of *URA3*, as measured by growth on 5'-FOA, decreased as the reporter gene was moved internally along the chromosome arm (Figures 3.3A & B). Overexpression of *sir3-4A* improved viability on 5'-FOA compared to endogenous levels of Sir3 alone, although not to the same extent as overexpression of wild type *SIR3*. As expected, deletion of *PPR1* improved silencing in both *SIR3* and *sir3-4A*-overexpressing strains (Figures 3.3A & B).

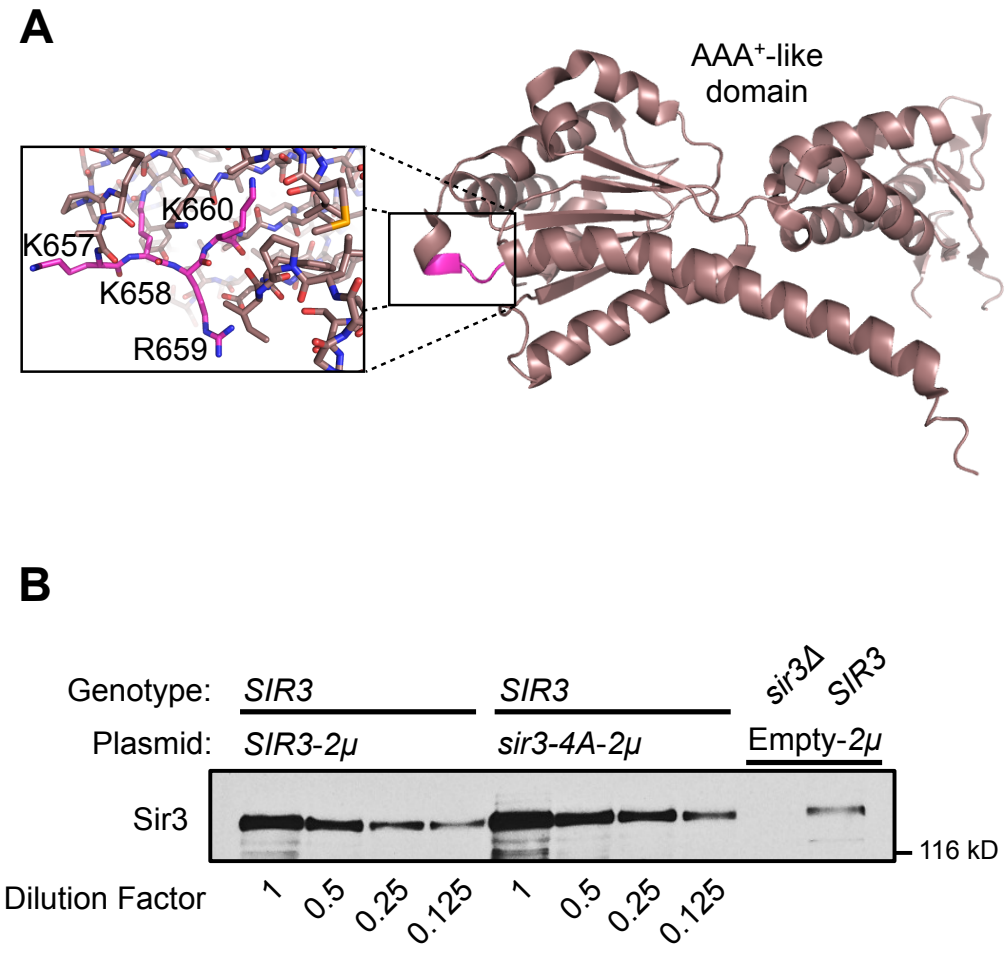
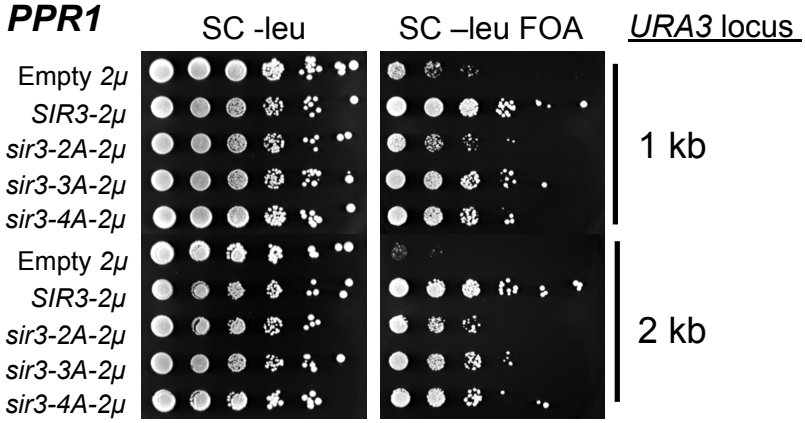

A
AAA+-like
domain
K660
K657
K658
R659
B
Genotype:
SIR3
SIR3
sir3Δ
SIR3
Plasmid:
SIR3-2μ
sir3-4A-2μ
Empty-2μ
Sir3
116 kD
Dilution Factor
1
0.5
0.25
0.125
1
0.5
0.25
0.125

Figure 3.1 Overexpression of *SIR3* and *sir3-4A* alleles. A: Structure of the Sir3-AAA^{+}-like domain highlighting residues 657-660 (shown in inset, in pink), which are mutated to alanine in the *sir3-4A* mutant. Image obtained from the Protein Data Bank (PDB, PDB ID: 3TE6). **B**: Western blot against Sir3 in two-fold dilutions of whole cell extract from strains overexpressing *SIR3* or *sir3-4A* from high-copy 2μ plasmids. Overexpression results in protein levels approximately 10-fold higher than endogenous levels, and both *SIR3* and *sir3-4A* are overexpressed to a similar extent.

A


PPR1
SC -leu
SC –leu FOA
URA3 locus
Empty 2μ
SIR3-2μ
sir3-2A-2μ
sir3-3A-2μ
sir3-4A-2μ
Empty 2μ
SIR3-2μ
sir3-2A-2μ
sir3-3A-2μ
sir3-4A-2μ
1 kb
2 kb

B

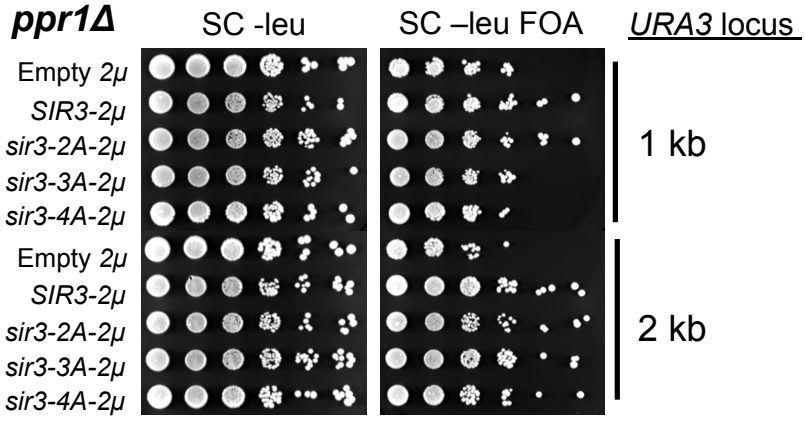

ppr1Δ
SC -leu
SC –leu FOA
URA3 locus
Empty 2μ
SIR3-2μ
sir3-2A-2μ
sir3-3A-2μ
sir3-4A-2μ
Empty 2μ
SIR3-2μ
sir3-2A-2μ
sir3-3A-2μ
sir3-4A-2μ
1 kb
2 kb

Figure 3.2 Sir3-2A, -3A and -4A mutant alleles display silencing defects. Serial dilutions of strains containing a telomeric *URA3* reporter and overexpressing wild type *SIR3* or mutant *sir3-2A, -3A* or *-4A* alleles from high-copy 2μ plasmids were spotted on media in the presence and absence of the counter-selection drug 5'-FOA. Growth on 5'-FOA is indicative of silencing of the telomeric *URA3* reporter, which was placed at two locations along *TEL-VR* (1kb and 2kb). Viability was assessed in the presence (**A**) and absence (**B**) of *PPR1*, a *URA3*-transactivator. Deletion of *PPR1* decreases the sensitivity of these strains to 5'-FOA.

In order to more directly compare the phenotype of strains overexpressing *SIR3* or *sir3-4A*, we developed a quantitative silencing assay to measure the repression of *URA3* within a population. The population is monitored as a whole in order to determine the proportion of cells that are able to silence the reporter gene, giving an accurate measure of silencing efficiency within the population (expressed as percent viability on 5'-FOA), rather than qualitative viabilities as assayed after growth on plates for several days.

Strains expressing endogenous levels of Sir3 show a strong decrease in silencing efficiency as the *URA3* reporter is placed at increasing distances from the telomere end, similar to previous reports (Renauld et al., 1993; Figure 3.4A, +Empty *2μ*). Overexpression of wild type *SIR3* resulted in a significant increase in viability when *URA3* was located closer than 2.5kb from the telomere end (Figure 3.4A, +*SIR3-2μ*). Overexpression of mutant *sir3-4A* also improved silencing efficiency at all loci tested, compared to expression of endogenous levels of *SIR3*, although this did not reach statistical significance (Figure 3.4A, +*sir3-4A-2μ*). In *ppr1*Δ strains, overexpression of both *SIR3* and *sir3-4A* significantly improved silencing compared to the empty vector alone, when the *URA3* marker was placed within 3.5kb of the telomere end (Figure 3.4B). Deletion of Ppr1 significantly improved silencing at almost all loci in both the wild type *SIR3* and the *sir3-4A* mutant, whereas there was not a significant change in silencing between $PPR1^{+}$ and *ppr1*Δ strains at most loci when only endogenous levels of *SIR3* were expressed (Figure 3.4C, summarized in Appendix A1.4). The difference in efficiency between wild type and mutant Sir3 is approximately 10-fold at all loci tested, and does not seem to be dependent on the distance from the telomere.

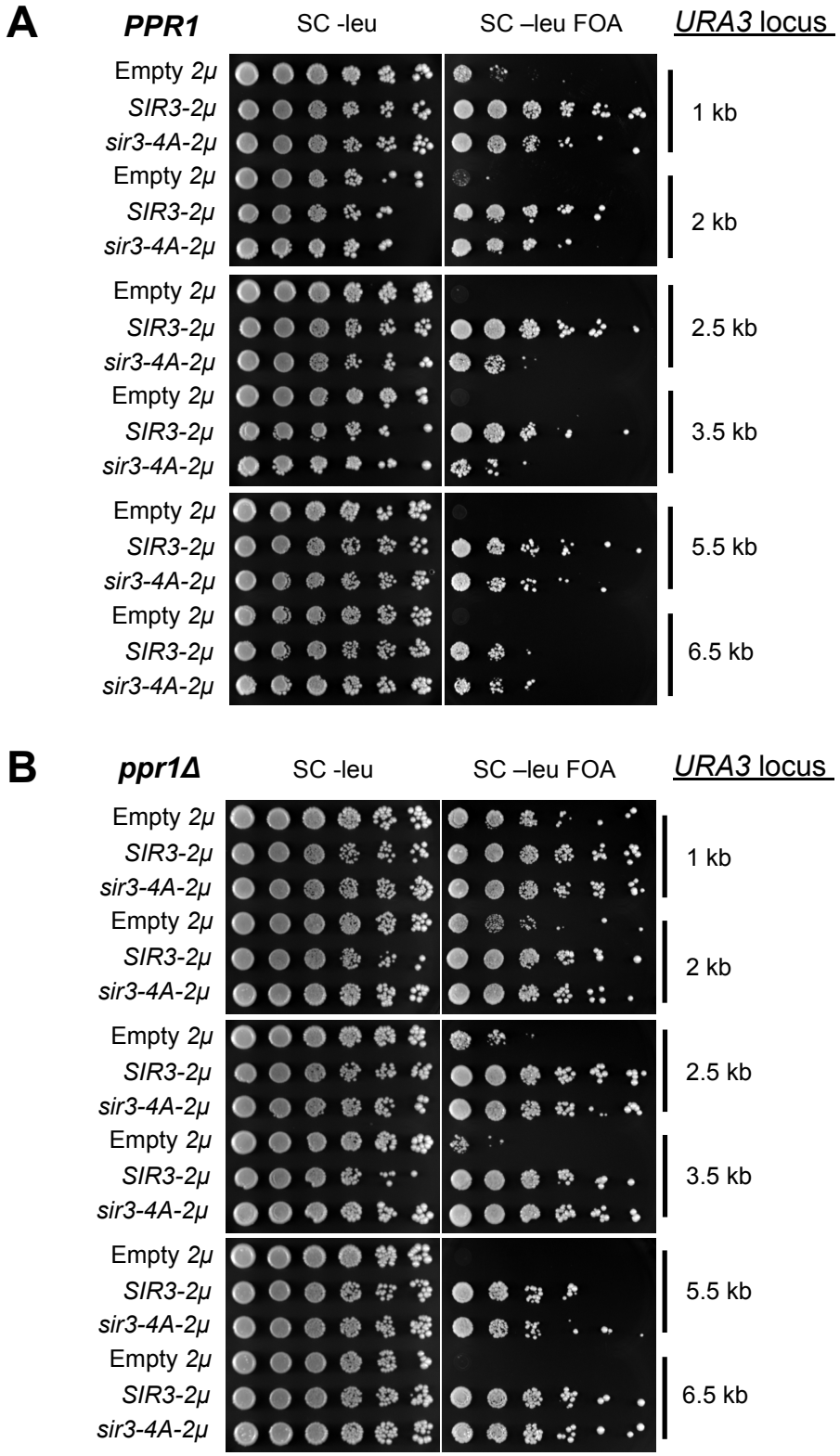

A
PPR1
SC -leu
SC –leu FOA
URA3 locus
Empty 2μ
SIR3-2μ
sir3-4A-2μ
1 kb
2 kb
2.5 kb
3.5 kb
5.5 kb
6.5 kb
B
ppr1Δ
SC -leu
SC –leu FOA
URA3 locus
Empty 2μ
SIR3-2μ
sir3-4A-2μ
1 kb
2 kb
2.5 kb
3.5 kb
5.5 kb
6.5 kb

Figure 3.3 Silencing of a *URA3* reporter is subject to telomeric position effect. Serial dilutions of strains were plated in the presence and absence of 5'-FOA, as discussed in Figure 3.2. Growth on 5'-FOA was assayed in strains containing a telomeric *URA3* marker placed at increasing distances along *TEL-VR*, into which were transformed high-copy 2μ plasmids containing *SIR3* or *sir3-4A* alleles, or the vector alone. Viability was assessed in the presence (**A**) or absence (**B**) of *PPR1*, as in Figure 3.2

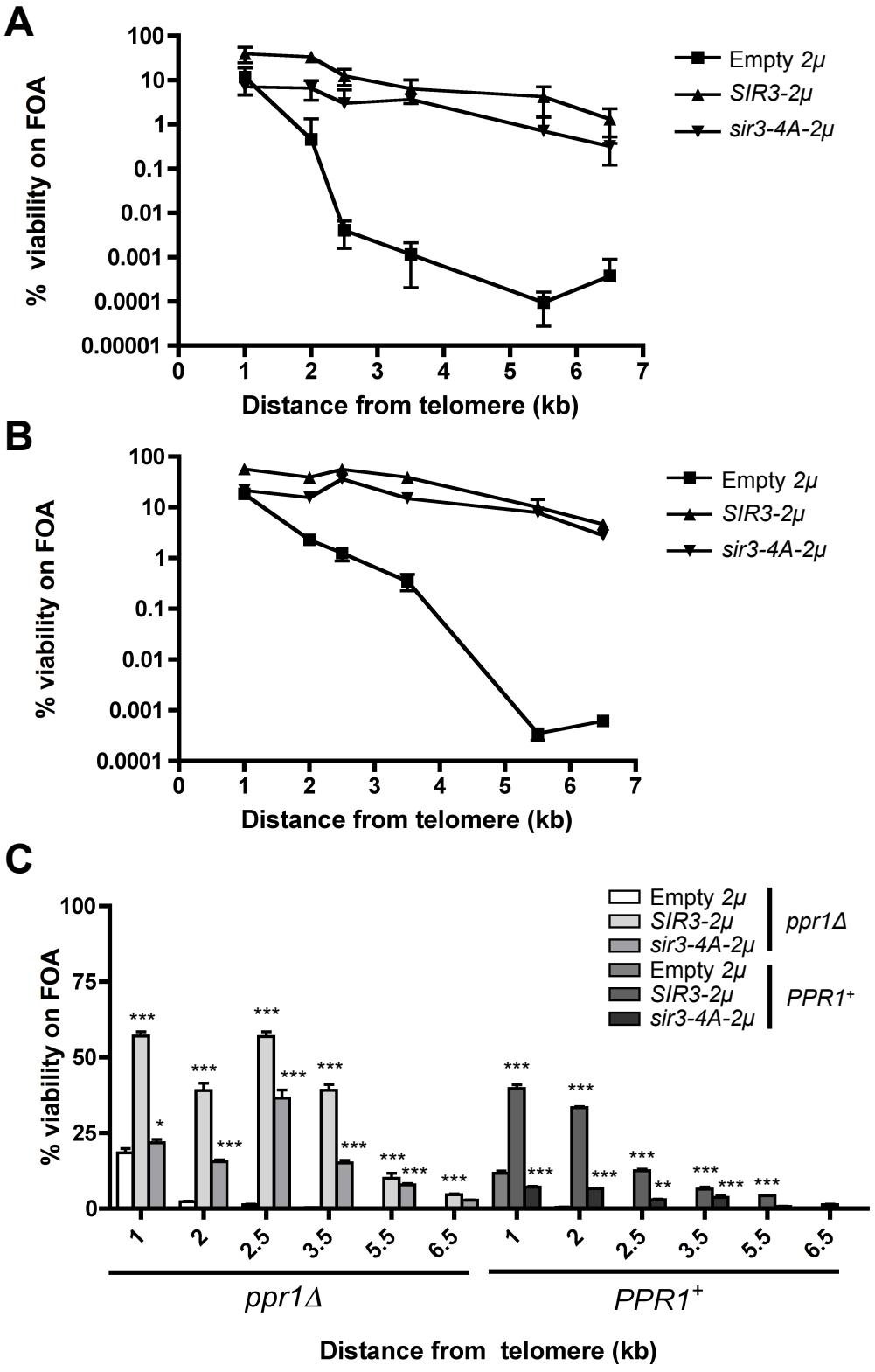

A
% viability on FOA
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0 1 2 3 4 5 6 7
Distance from telomere (kb)
Empty 2μ
SIR3-2μ
sir3-4A-2μ
B
% viability on FOA
100
10
1
0.1
0.01
0.001
0.0001
0 1 2 3 4 5 6 7
Distance from telomere (kb)
Empty 2μ
SIR3-2μ
sir3-4A-2μ
C
% viability on FOA
100
75
50
25
0
Empty 2μ
SIR3-2μ
sir3-4A-2μ
ppr1Δ
Empty 2μ
SIR3-2μ
sir3-4A-2μ
PPR1+
1 2 2.5 3.5 5.5 6.5 1 2 2.5 3.5 5.5 6.5
ppr1Δ
PPR1+
Distance from telomere (kb)

Figure 3.4 Deletion of *PPR1* improves silencing of a telomeric *URA3* reporter gene. A quantitative silencing assay was developed to measure silencing of a telomeric *URA3* reporter gene with overexpression of *SIR3* or *sir3-4A* from high-copy 2μ plasmids. Viability on 5'-FOA is expressed as ([absolute survival on 5'-FOA]/[absolute survival on control] x100%) and error bars represent standard error of at least three replicates. Values of percent survival below 0.001% were considered below the detection limit for this assay (Renauld et al., 1993). Viability was assessed in the presence (**A**) and absence (**B**) of *PPR1*. **C**: Bar graph quantification of values shown in **A** and **B**. Two-way ANOVA with Bonferroni post-hoc tests were performed to determine statistical significance: *PPR1* Empty 2μ vs *PPR1 SIR3-2μ*, $p<0.001$ at 1, 2 & 2.5kb; *PPR1* Empty 2μ vs *PPR1 sir3-4A-2μ*, $p<0.01$ at 2.5kb, $p<0.001$ at 1, 2 & 3.5kb; *ppr1Δ* Empty 2μ vs *ppr1Δ SIR3-2μ*, $p<0.001$ at all loci; *ppr1Δ* Empty 2μ vs *ppr1Δ sir3-4A-2μ*, $p<0.05$ at 1kb, $p<0.001$ at 2, 2.5, 3.5 & 5.5kb. All comparisons shown are to the Empty 2μ control strain for the given genotype at each loci (*$p<0.05$, **$p<0.01$, ***$p<0.001$; all comparisons to Empty 2μ).

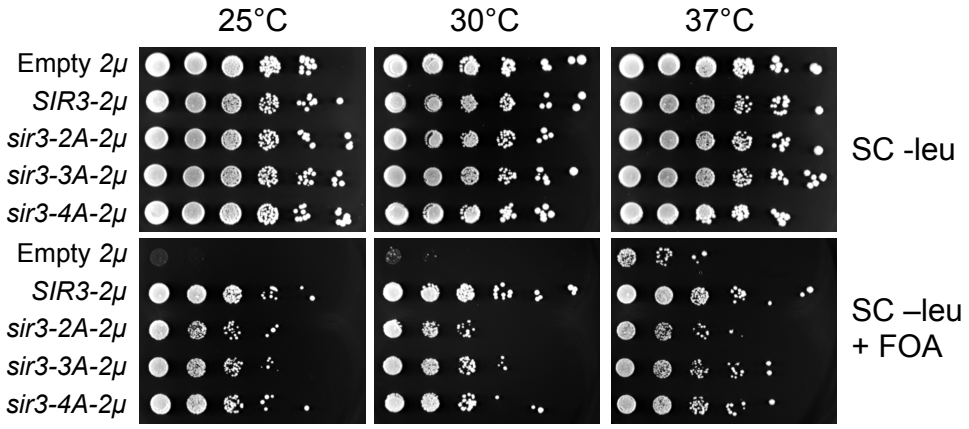
Sir3-4A silent domains do not exhibit inherent instability

The Sir3-Sir4 interaction is required for the efficient recruitment of SIR proteins to chromatin, but it is unknown whether this interaction must be maintained during the subsequent spreading of silent chromatin. Our previous results have shown that overexpression of Sir3-4A is sufficient to cause the extension of silent chromatin, in the presence of wild type Sir3 required to nucleate silencing. This suggests that the Sir3-Sir4 interaction may not be required for the recruitment of Sir4 during the formation of functionally silent domains. We therefore wanted to assess the stability of silent chromatin formed by Sir3-4A, to determine if the loss of the Sir3-Sir4 interaction reduces the affinity of the repressive SIR complex with chromatin and sensitizes the silent chromatin to disassembly.

Silencing exhibits temperature sensitivity, and is impaired at both 25°C and 37°C, compared to 30°C. However, Figure 3.5 shows that silent chromatin formed by any of the *sir3-2A, -3A* or *-4A* mutants does not increase temperature sensitivity, relative to wild type *SIR3*. This suggests that the stability of these domains is similar to that of silent chromatin formed by wild type Sir3, with respect to their temperature sensitivity.

Disruption of silent chromatin can also be achieved by driving transcriptional read-through of promoters found within the silent domain. Overexpression of the *URA3* trans-activator Ppr1 placed under the control of a galactose-inducible promoter (*GAL-PPR1*) results in the induction of *URA3* transcription, as demonstrated by loss of viability on media containing 5'-FOA in the presence of galactose (Figure 3.6A). We wanted to test the ability of silent chromatin domains formed by either wild type Sir3 or mutant Sir3-4A to resist disassembly in the presence of high levels of Ppr1. Ppr1 expression was induced through the

A ***PPR1***



B ***ppr1Δ***

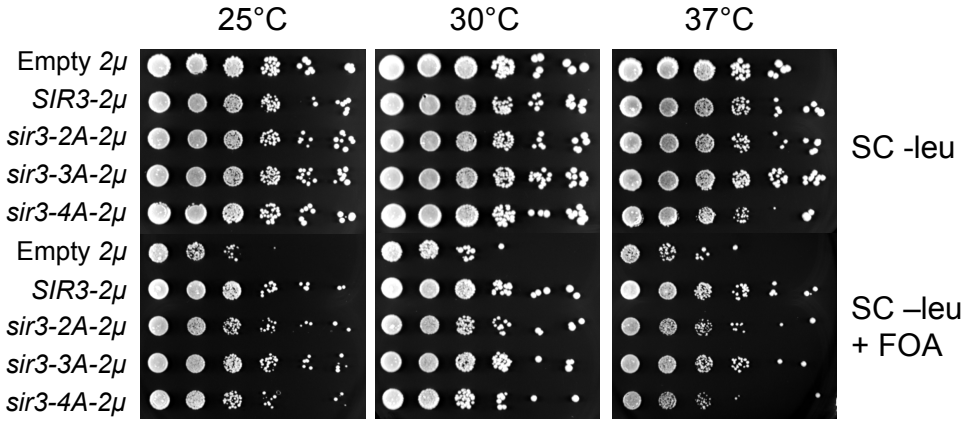
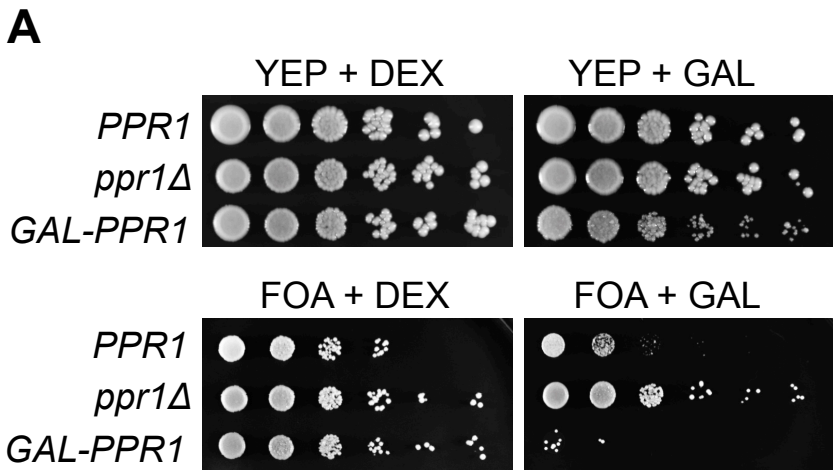


Figure 3.5 Sir3-4A-mediated silent domains do not display increased temperature sensitivity. Serial dilutions of strains were spotted on media in the presence and absence of 5'-FOA to assay silencing of a telomeric *URA3* reporter gene when wild type *SIR3* or the mutant *sir3-2A*, *-3A* or *-4A* alleles are overexpressed from high copy 2μ plasmids (as in Figure 3.2). Strains were grown for three days at 25°C, 30°C or 37°C to test for temperature sensitivity. Viability was assessed in the presence (**A**) or absence (**B**) of *PPR1*.

addition of galactose to the culture media, and silencing of the *URA3* reporter over time was measured through viability on solid media containing 5'-FOA. As shown in Figure 3.6B, induction of Ppr1 causes a loss of silencing at *URA3* over time. Viability is improved when either Sir3 or Sir3-4A is overexpressed, compared to endogenous levels of Sir3 alone. However there is no detectable difference between the disassembly of silent chromatin formed by Sir3 or Sir3-4A, as measured by this assay.

Finally, overexpression of the C-terminus of Sir4 (Sir4-C2, amino acids 1267-1358) has been shown to disrupt silencing (Ivy et al., 1986; Marshall et al., 1987), possibly through the titration of Sir3 away from chromatin. We created a construct in which expression of the Sir4-C2 fragment is under the control of a galactose-inducible promoter (*GAL-SIR4-C2*). Growth in the presence of galactose induces overexpression of this Sir4-C2 fragment. We used a reporter strain containing a telomeric *URA3* reporter and a *TRP1* reporter inserted at a weakened mating locus (*hmrΔE*) in order to monitor silencing following overexpression of Sir4-C2. Consistent with previous reports (Marshall et al, 1987), overexpression of Sir4-C2 alone causes complete derepression of silencing at both the telomere and the *hmrΔE* locus. Overexpression of either Sir3 or Sir3-4A improves silencing when Sir4-C2 is induced compared to endogenous levels of Sir3 only, with the difference reaching statistical significance at the t=1 time-point (Figure 3.7B). However, there are no significant differences between the viability of strains overexpressing Sir3 or Sir3-4A at any time-point. It is possible that the lack of difference between these strains is due to the titration of wild type Sir3 away from the site of nucleation in both cases. This would suggest that the disassembly of silent chromatin from the site of nucleation is dominant over any effect within the extended domain.


A
YEP + DEX
YEP + GAL
PPR1
ppr1Δ
GAL-PPR1
FOA + DEX
FOA + GAL
PPR1
ppr1Δ
GAL-PPR1

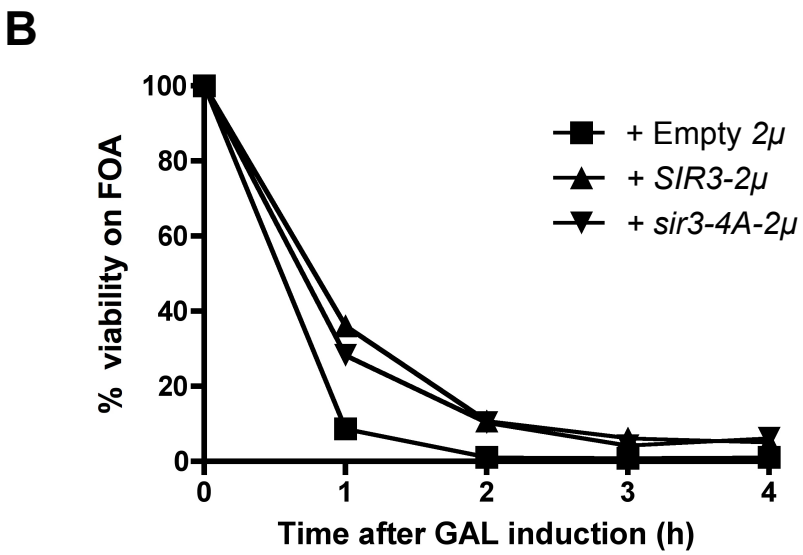
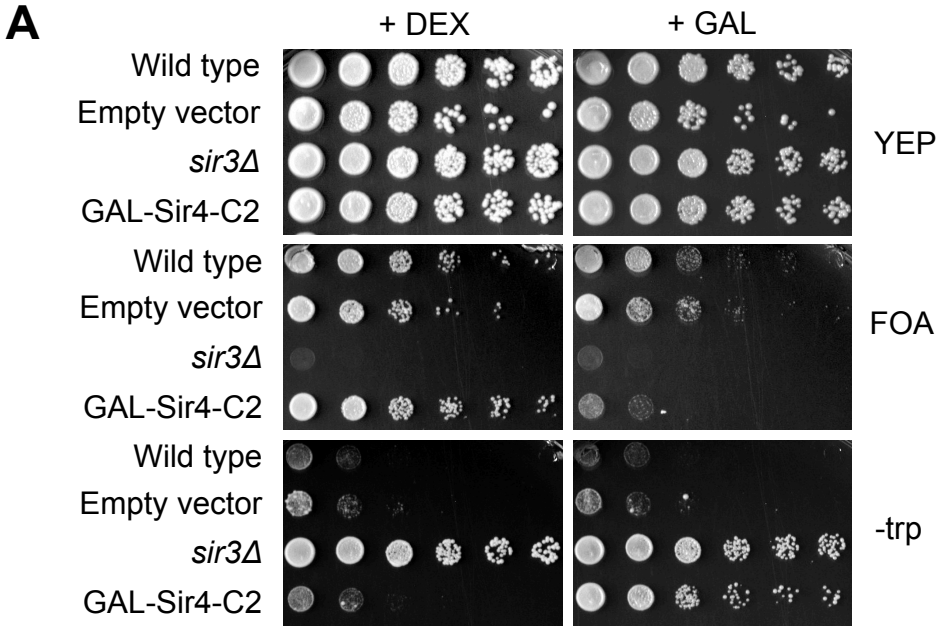

B
% viability on FOA
100
80
60
40
20
0
0
1
2
3
4
Time after GAL induction (h)
+ Empty 2μ
+ SIR3-2μ
+ sir3-4A-2μ

Figure 3.6 Sir3-4A-mediated silent chromatin domains do not exhibit higher sensitivity to disruption by *GAL-PPR1*. **A**: Phenotypic confirmation of *GAL-PPR1* integration in a telomeric *URA3* reporter strain. Serial dilutions of strains were spotted on media containing either 2% dextrose or 2% galactose as a carbon source, in the presence or absence of 5'-FOA. Induction of *GAL-PPR1* in the presence of 2% galactose is confirmed by the loss of viability in the presence of 5'-FOA, while no loss of viability is observed on 5'-FOA plates containing 2% dextrose. A non-inducible *PPR1* strain and a *ppr1Δ* strain are included as growth controls. **B**: Time course of viability on 5'-FOA following *GAL-PPR1* induction. *GAL-PPR1* strains overexpressing *SIR3* or *sir3-4A* (or the empty vector control) were grown overnight in synthetic media lacking leucine and containing 2% raffinose, and *GAL-PPR1* expression was induced with the addition of 2% galactose. Cells were harvested every hour and plated on media with or without 5'-FOA. Percent viability is expressed as [absolute viability on 5'FOA]/[absolute viability on control] x 100%, and normalized to t=0.


A
+ DEX
+ GAL
Wild type
Empty vector
sir3Δ
GAL-Sir4-C2
YEP
FOA
-trp

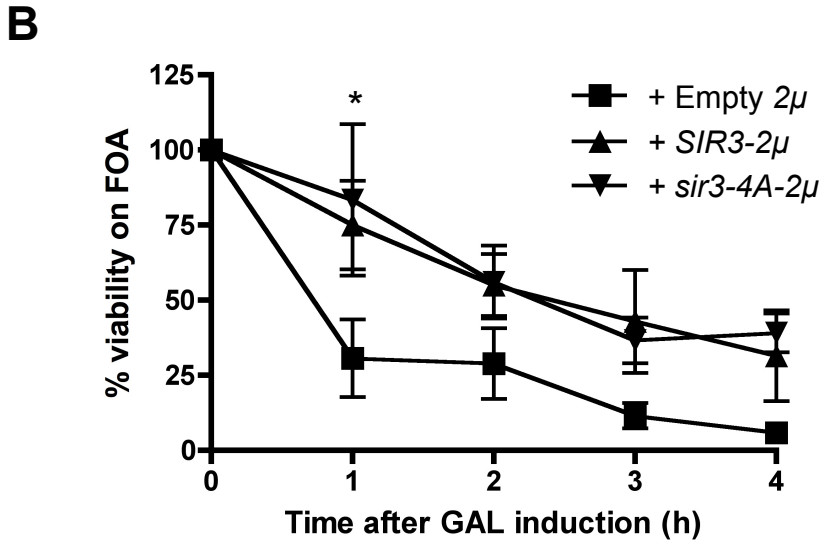

B
% viability on FOA
125
100
75
50
25
0
*
+ Empty 2μ
+ SIR3-2μ
+ sir3-4A-2μ
0
1
2
3
4
Time after GAL induction (h)

Figure 3.7 Sir3-4A-mediated silent chromatin domains do not exhibit higher sensitivity to disruption by *GAL-SIR4-C2*. **A**: Phenotypic confirmation of *GAL-SIR4-C2* integration in a telomeric *URA3* reporter strain. Serial dilutions of strains were spotted on media containing either 2% dextrose or 2% galactose as a carbon source, in the presence or absence of 5'-FOA and the amino acid tryptophan. Induction of *GAL-SIR4-C2* in the presence of 2% galactose is confirmed by the loss of viability in the presence of 5'-FOA and growth in the absence of tryptophan. The parental strain (ADR4062) and a *sir3Δ* derivative (ADR5469) are included with an empty vector control. **B**: Time course of viability on 5'-FOA following *GAL-SIR4-C2* induction. *GAL-SIR4-C2* strains overexpressing either *SIR3* or *sir3-4A* (or the empty vector control) were grown overnight in synthetic media lacking leucine and containing 2% raffinose, and *GAL-SIR4-C2* expression was induced with the addition of 2% galactose. Cells were harvested every hour and plated on media with or without 5'-FOA. Percent viability is expressed as [absolute viability on 5'FOA]/[absolute viability on control] x 100%, and normalized to t=0. Statistical significance was determined by two-way ANOVA with a Bonferroni post-hoc test: Empty 2μ vs. *SIR3-2μ*, $p<0.05$ at t=1; Empty 2μ vs. *sir3-4A-2μ*, $p<0.05$ at t=1 (* $p<0.05$, all comparisons to Empty 2μ).

Sir3-4A, but not Sir2 or Sir4, is enriched in extended silent chromatin domains

To support our phenotypic data suggesting that Sir3-4A is capable of forming functionally silent domains, we wanted to investigate more directly the binding of SIR proteins to chromatin. Strahl-Bolsinger et al. (1997) have previously shown that extended telomeric silent chromatin is enriched for Sir3, whereas little Sir2 or Sir4 are found outside of the site of silencing nucleation. This data supports our model that Sir3 is the primary driver of silent chromatin spreading, and that the Sir3-Sir4 interaction is not required for this process.

We assessed SIR protein binding to the right telomeric arm of chromosome VI (*Tel-VIR*) using chromatin immunoprecipitation (ChIP). We immunoprecipitated each of Sir2, Sir3 and Sir4 from cross-linked yeast extracts, purified the DNA present in the immunoprecipitations and used this as a template for specific PCR reactions using radiolabelled nucleotide (αP^{32}-dCTP). As shown in Figure 3.8A, Sir2 and Sir4 protein levels were similar between all strains used for ChIP, and Sir3 overexpression was similar to that seen in previous strains (compare to Figure 3.2B). Similar to results shown previously by Strahl-Bolsinger et al. (1997), we have confirmed that overexpression of Sir3 results in its enrichment along the telomere. Overexpression of Sir3 or Sir3-4A significantly increased the fold enrichment of Sir3 at numerous loci along the chromosome, including up to 15kb away from the telomere end, when compared to the *sir3Δ* control (Figure 3.9B). In contrast, overexpression of Sir3 or Sir3-4A did not result in a significant increase in Sir2 enrichment at any loci, compared to the *sir2Δ* control strain (Figure 3.9A). Sir4 was only significantly enriched at the two closest loci tested on the telomere arm, towards the end of the telomere and the site of silencing nucleation (Strahl-Bolsinger et al., 1997; Figure 3.9C). These

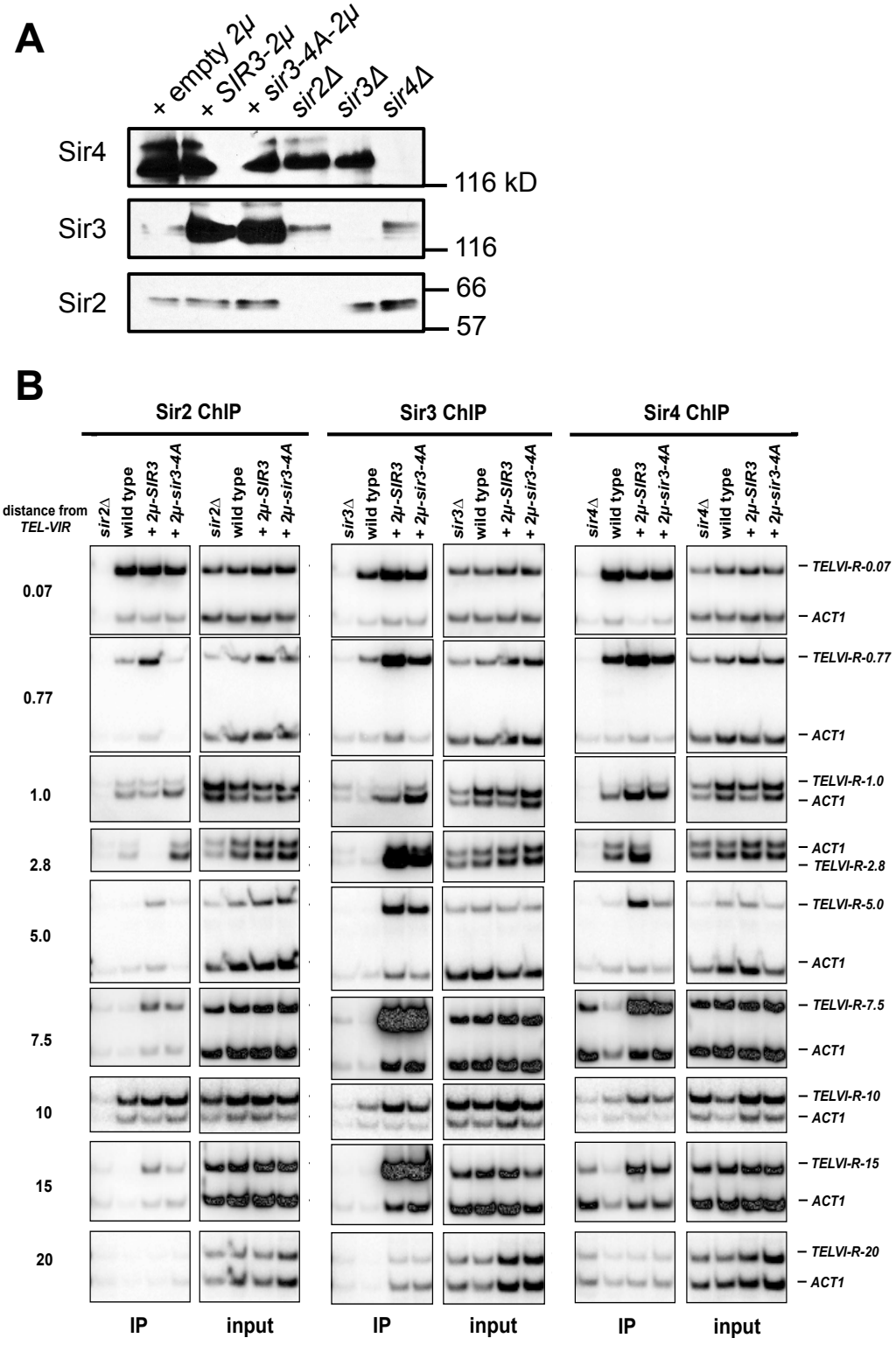

A
+ empty 2μ
+ SIR3-2μ
+ sir3-4A-2μ
sir2Δ
sir3Δ
sir4Δ
Sir4
116 kD
Sir3
116
Sir2
66
57
B
Sir2 ChIP
Sir3 ChIP
Sir4 ChIP
distance from TEL-VIR
sir2Δ
wild type
+ 2μ-SIR3
+ 2μ-sir3-4A
sir3Δ
sir4Δ
0.07
0.77
1.0
2.8
5.0
7.5
10
15
20
TELVI-R-0.07
ACT1
TELVI-R-0.77
TELVI-R-1.0
TELVI-R-2.8
TELVI-R-5.0
TELVI-R-7.5
TELVI-R-10
TELVI-R-15
TELVI-R-20
IP
input

Figure 3.8 Chromatin immunoprecipitation (ChIP) of SIR proteins along *TEL-VIR*. A: Western blots for Sir2, Sir3 and Sir4 from non-cross-linked whole cell extracts of strains used for chromatin immunoprecipitation (ADR21, 3101, 3353 & 3632, transformed with pAR534 [empty 2μ], 469 [*SIR3-2μ*] or 1017 [*sir3-4A-2μ*]). **B**: Autoradiography images of P^{32}-dCTP-labelled ChIP-PCR products. PCR products were separated on 6% polyacrylamide-TBE gels, vacuum-dried and exposed to Storage Phosphor screens to obtain images for densitometry analysis. Representative images are shown for immunoprecipitations of Sir2, Sir3 and Sir4, and specific PCR reactions at each locus tested along *TEL-VIR*.

findings conclude that overexpression of Sir3 or Sir3-4A is sufficient to extend silent chromatin along the telomere arm, and that Sir3 is the only SIR protein significantly enriched in those extended domains.

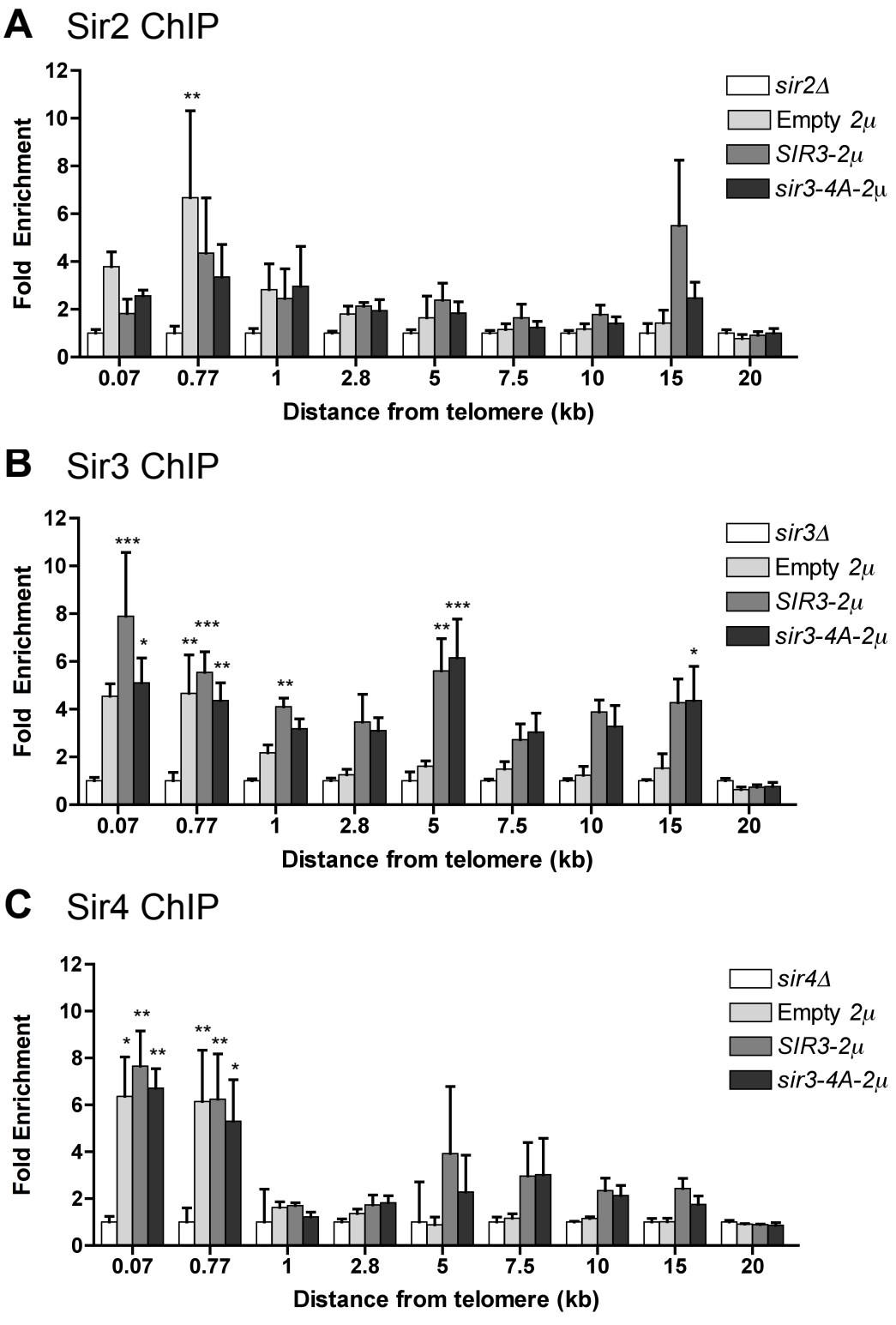

A Sir2 ChIP
Fold Enrichment
Distance from telomere (kb)
sir2Δ
Empty 2μ
SIR3-2μ
sir3-4A-2μ
B Sir3 ChIP
Fold Enrichment
Distance from telomere (kb)
sir3Δ
Empty 2μ
SIR3-2μ
sir3-4A-2μ
C Sir4 ChIP
Fold Enrichment
Distance from telomere (kb)
sir4Δ
Empty 2μ
SIR3-2μ
sir3-4A-2μ

Figure 3.9 Sir3-4A is enriched within extended silent chromatin domains. Quantification of densitometry analysis from autoradiography images, as seen in Figure 3.8 (**A**: Sir2-IP, **B**: Sir3-IP, **C**: Sir4-IP). PCR products obtained from immunoprecipitations were first normalized to an internal *ACT1* PCR control, and then to the input reaction [specific locus/*ACT1*] ratio. Enrichment values for each locus are normalized to the respective *sir2Δ*, *sir3Δ* or *sir4Δ* strain, whose mean enrichment value is set to 1 at each locus. Error bars represent standard error of at least two replicates. Two-way ANOVA with a Bonferroni post-hoc test were performed to determine statistical significance: *sir2Δ* vs Empty 2μ, $p<0.01$ at 0.77kb; *sir3Δ* vs Empty 2μ, $p<0.01$ at 0.77kb; *sir3Δ* vs *SIR3-2μ*, $p<0.001$ at 0.07kb & 0.77kb, $p<0.01$ at 1kb & 5kb; *sir3Δ* vs *sir3-4A-2μ*, $p<0.001$ at 5kb, $p<0.01$ at 0.77kb, $p<0.05$ at 0.07kb & 15kb; *sir4Δ* vs Empty 2μ, $p<0.01$ at 0.77kb, $p<0.05$ at 0.07kb; *sir4Δ* vs *SIR3-2μ*, $p<0.01$ at 0.07kb & 0.77kb; *sir4Δ* vs *sir3-4A-2μ*, $p<0.01$ at 0.07kb, $p<0.05$ at 0.77kb (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; all comparisons to *sir2Δ, sir3Δ* or *sir4Δ*, as appropriate).

Discussion

The current model of silent chromatin assembly proposes that recruitment of the SIR proteins to silencers leads to spreading of silent chromatin through iterative rounds of histone deacetylation and Sir3-nucleosome binding. As all SIR proteins are required for the nucleation of silencing, it has proven difficult to test their functions in the distinct process of silent chromatin spreading. Using an allele of Sir3 that is deficient in Sir4-binding, this chapter has sought to explain the role of the Sir3-Sir4 interaction in the spreading of silent chromatin.

We have confirmed previous reports that overexpression of Sir3 extends silent chromatin into subtelomeric regions (Hecht et al., 1996a; Renauld et al., 1993; Strahl-Bolsinger et al., 1997). I was able to quantify the effect of Sir3 overexpression by monitoring transcriptional repression along the telomeric arm of chromosome V-R, under different conditions of transcriptional activation. Overexpression of Sir3 or Sir3-4A increases the proportion of the population that is able to silence a *URA3* reporter gene, suggesting that endogenous levels of Sir3 are limiting for the spread of silencing. As additional support for this theory, we show that deletion of Ppr1 does not have a significant effect on silencing when only endogenous levels of Sir3 are present. It is possible that, when Sir3 is expressed at endogenous levels, the threshold required to compete efficiently with transcriptional activation has not been reached, and so deletion of Ppr1 does not result in an improvement in silencing. Finally, the detection of transcriptional repression by Sir3-4A confirms that this mutant protein is able to interact with chromatin sufficiently to prevent access by the transcriptional machinery, albeit less well than wild type.

While Sir3 and Sir3-4A proteins are overexpressed at similar levels (Figure 3.1B), the efficiency of *URA3* repression by wild type Sir3 is higher than that of the mutant Sir3-4A. Initiation requires the Sir3-Sir4 interaction, and so this first assembly step depends on the incorporation of endogenous wild type Sir3 into silent chromatin. When Sir3-4A is overexpressed, the effective concentration of Sir3 available for initiation is decreased compared to the case of overexpression of wild type Sir3. The difference in silencing efficiencies may therefore reflect the inability of the strains overexpressing Sir3-4A to initiate silencing as efficiently as those overexpressing wild type Sir3. This hypothesis is consistent with the observation that the rate of decrease in silencing efficiency is the same when either Sir3 or Sir3-4A is overexpressed and suggests that the difference in efficiency between Sir3 and Sir3-4A is not related to distance from the telomere, and only reflects the efficiency of establishment. This is in contrast to the silent chromatin formed by limiting amounts of Sir3, where silencing efficiency is directly proportional to the distance from the telomere end.

Following our examination of silencing in the Sir3-4A mutant, we wanted to directly relate this phenotype to Sir3-chromatin binding. Our hypothesis predicted that silent chromatin domains created by Sir3-4A would be deficient for Sir4, if the recruitment of Sir4 to chromatin is dependent on the Sir3-Sir4 interaction. We have shown that Sir3 is significantly enriched in these domains, while Sir4 and Sir2 show no significant increase in enrichment within extended silent chromatin. Importantly, overexpression of a Sir4-binding deficient allele of Sir3 (*sir3-4A*) shows a similar enrichment pattern as overexpression of wild type Sir3 (Figure 3.10). With respect to our hypothesis, the levels of Sir2 and Sir4 recruitment when either Sir3 or Sir3-4A was overexpressed were not significantly different from the background detected in the *sir2Δ* and *sir4Δ* control strains, respectively (Figure

3.10A & C). The low enrichment of Sir2 and Sir4 prevents us from commenting on differences between the effect of Sir3 or Sir3-4A overexpression.

The results of our ChIP analysis strongly suggest that Sir3 is able to extend silent chromatin independently of the Sir3-Sir4 interaction. However, these experiments were performed in strains expressing endogenous wild type Sir3, and the possibility of some contribution of the wild type protein to silencing cannot be ruled out. Future ChIP experiments will address this issue by comparing the enrichment of a tagged endogenous Sir3 on chromatin at sites of nucleation and spreading, in cases where either wild type Sir3 or mutant Sir3-4A are overexpressed.

While the levels of Sir3 present in extended domains are higher than that of Sir2 and Sir4, these results do not address whether Sir2/Sir4 are dispensable for the assembly of extended domains. By extension, it is still unknown whether the histone deacetylation by Sir2 that is required at nucleation is also necessary during spreading of silent chromatin. A C-terminal fragment of Sir3 (amino acids 510-970) binds with higher affinity to a deacetylated histone H4 tail than to the acetylated form (Carmen et al., 2001), and full length Sir3 shows some preference for unmodified nucleosomal arrays at low concentrations *in vitro* (Oppikofer et al., 2011). However, the overall affinity of Sir3 for the full nucleosome is likely due to the incremental contributions of multiple weak interactions. These are mediated by numerous interfaces including the BAH domain, which was not included in the fragment used by Carmen et al. (2001).

It is also possible that binding of Sir4 to Sir3, or Sir3 to itself, results in a conformational change in Sir3 that alters its nucleosome binding activity. The full SIR complex shows preference for deacetylated nucleosomes only in the presence of NAD (Oppikofer et al., 2011). NAD is predicted to alter the conformation of the SIR complex on

chromatin (Liou et al., 2005). Together these data suggest that conformational changes within the complex may contribute to determining sensitivity to histone modifications, in addition to the inherent binding properties of Sir3.

Chapter 4: Mechanisms of Sir3 oligomerization

Introduction

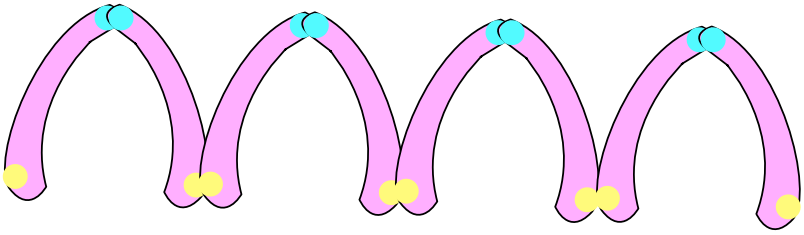
The hypothesis of Sir3-mediated silencing predicts the presence of multiple interaction domains within Sir3. Previous studies have confirmed that Sir3 forms multimers *in vitro* (Connelly et al., 2006; Liou et al., 2005; McBryant et al., 2006). Liou et al. (2005) observed multimeric complexes whose molecular weights suggest that Sir3 first self-associates in dimer form, and that larger complexes are the result of dimer-dimer interactions. This result is consistent with the presence of two interaction domains in Sir3: one to mediate dimerization, and the other to mediate higher order oligomerization between dimers (Figure 4.1).

The C-terminal Sir3-wH dimerization domain was recently identified and is required for silencing (Oppikofer et al., 2013). It has often been proposed that the BAH domain is capable of dimerization, but as of yet no self-interactions within this domain have been shown *in vivo* (Buchberger et al., 2008). Interestingly, Armache et al. (2011) detected self-association of the BAH domain by sedimentation velocity analytical ultracentrifugation. It is unclear if silencing mediated by the BAH^{1-380} domain reflects Sir3-BAH multimerization: the fragment crystallized by Connelly et al. (2006) formed a regular superhelical structure that could possibly be stabilized by an existing dimerization domain.

A clue to the second dimerization domain in Sir3 that is predicted by our model comes from the related yeast, *Schizosaccharomyces pombe*. Silent chromatin in *S. pombe* is more similar to that of vertebrates than of *S. cerevisiae*, but certain processes are predicted to be common to both: multimerization of a protein complex along chromatin that alters the

A **Oligomerization with one asymmetric interaction domain**

B **Oligomerization with two symmetric interaction domains**



C **Oligomerization with one symmetric and one asymmetric interaction domains**

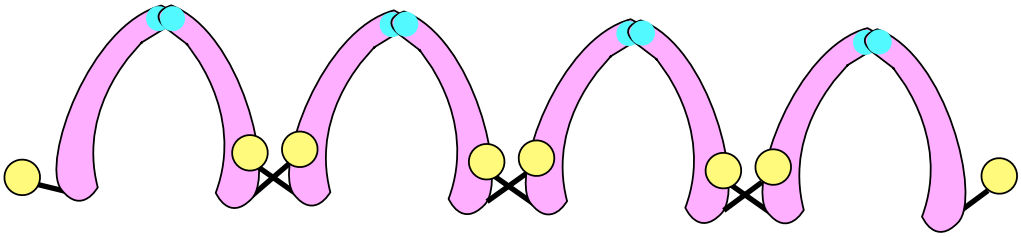


Figure 4.1 Potential mechanisms of oligomerization involving symmetrical or asymmetrical interactions. **A**: Oligomerization may occur through a single asymmetrical domain, involving two distinct interfaces that interact to form oligomers. Both interfaces must be intact for the interaction to take place. **B**: Oligomerization could also occur through interactions of multiple symmetric domains. Mutation of either domain individually could result in the formation of dimers, but not higher order oligomers. **C**: Finally, oligomerization may require both a symmetrical and an asymmetrical domain. This requires three distinct interfaces to be present and intact in order for higher order oligomerization to occur. We favor this model for the self-interaction of Sir3: the wH domain mediates symmetrical interactions, while the ARK loop is involved in asymmetrical interactions with an unidentified binding pocket within Sir3.

underlying histone modifications and prevents transcription. A major silencing protein in *S. pombe* is the chromodomain protein Swi6, which is thought to play a role analogous to that of Sir3 in budding yeast. A recent study confirmed the presence of a second dimerization domain in the N-terminal chromodomain of Swi6, named the ARK loop (amino acids 92-97) (Canzio et al., 2013). This is in addition to a previously mapped dimerization domain at the C-terminus (Cowieson et al., 2000), suggesting that Swi6 is capable of forming multimeric complexes. We therefore sought to identify a similar domain in Sir3 to determine if the multimerization mechanism seen in Swi6, and conserved in other mammalian heterochromatin proteins, is present in *S. cerevisiae*.

Results

Identification of the Sir3-ARK loop

We first examined the primary sequence of the Sir3 N-terminus in order to identify any similarity with the sequence of the Swi6 dimerization domain, in particular the ARK loop. A multiple alignment of Sir3 (sequences from *S. cerevisiae* and *Candida galibrata*), Swi6 (sequence from *S. pombe*) and related proteins is shown in Figure 4.2A. Orc1 (sequence from *S. cerevisiae*) is a homologue of Sir3, and HP1 (sequences from *D. melanogaster*, *Xenopus laevis*, and *Homo sapiens*) is a vertebral homologue of Swi6. Although there is some divergence between sequences, we identified a region of strikingly similarity across the primary sequences of Sir3, Swi6 and HP1 that retains a lysine at position 0, and basic residues (Arg or Lys) at positions -1, -3 and -4. Through collaboration with Dr. Isabelle Callebault (Université Pierre et Marie Curie, Paris, France), we attempted to quantify the homology of the ARK loop within Sir3-related proteins, however the region of interest is too small to determine the statistical significance of the similarity that we observed.

However, the secondary structures of these regions of Sir3, Swi6 and HP1 suggest there may be structural similarity in this region as well (Figure 4.2B). All three structures have an anti-parallel consecutive three-stranded β-sheet, and in all cases the residues of the ARK loop are on exposed loops between β-strands 1 and 2. Conservation of secondary structure suggests that these residues have an important functional role, however it is possible that the same structure was achieved in these proteins through convergent evolution rather than homology.

A

```
scOrc1   21 NIIDGGQ-KRLRRRGAKTEHYLK 42
cgSir3   21 NVMDERD-RKVRRKRNVSTDLFI 42
scSir3   21 RVIDDNN-RRRSRKRGG-ENVFL 41
spSwi6   81 YVVEKVLKHRMARKGGGYEYLLK 103
dmHP1a   24 YAVEKII-DRRVRKGKV-EYYLK 44
xlHP1a   36 YVVEKVL-DRRVVKGQV-EYLLK 56
hsHP1a   20 YVVEKVL-DRRVVKGQV-EYLLK 40
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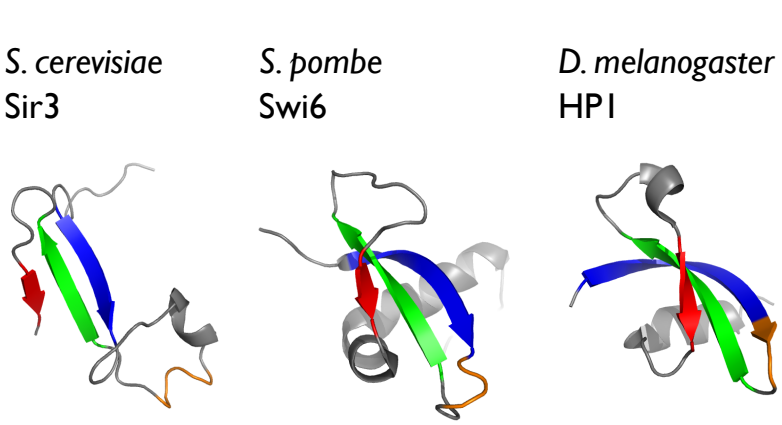
B



C

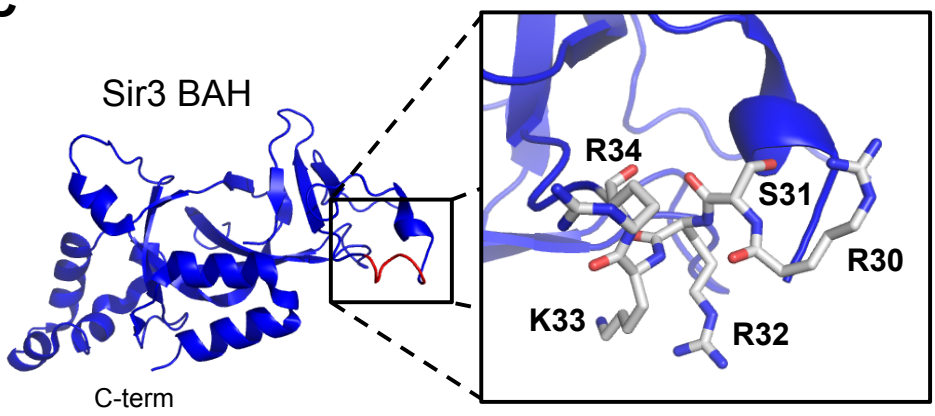


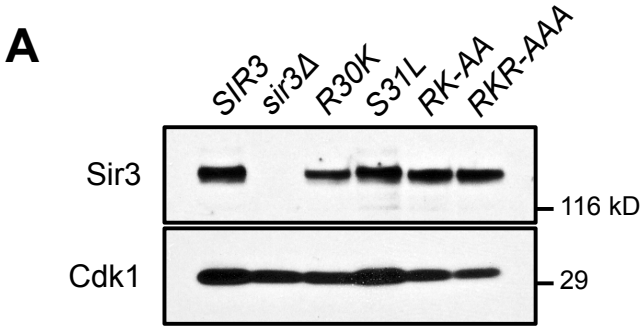
Figure 4.2 A Sir3 dimerization domain may be conserved in heterochromatin proteins in higher organisms. A: A manual alignment of primary amino acid sequences of Sir3 and Sir3-related proteins from various organisms. Sequences were obtained from the National Center for Biotechnology Information (NCBI) Protein Database: scOrc1 (AAB38248.1), cgSir3 (AGK26813.1), scSir3 (NP_013547.3), spSwi6 (CAA50668.1), dmHP1a (ACI96787.1), xlHP1a (NP001080863.1), hsHP1a (NP_001120794.1). Red text indicates the ARK loop region of interest, and green or blue text indicates conserved residues of interest. **B**: Secondary structures of Sir3 and Sir3-related proteins obtained from PDB (PDB IDs: 3TU4 [Sir3], 2RSO [Swi6] and 1KNE [HP1]). Consecutive anti-parallel β-sheets are highlighted in each structure (red, green and blue), and ARK residues are highlighted in orange. Amino acids 1-54 of scSir3, 80-142 of spSwi6 and 23-74 of dmHP1 are shown. **C**: The N-terminal BAH domain of Sir3 is shown (PDB ID: 2FVU), with the ARK residues (amino acids 30-34) highlighted in red (also shown in inset).

Mutations in the Sir3-ARK loop abolish silencing and are dominant negative

In order to determine the role of the Sir3-ARK loop in silencing, we created a panel of mutants within this region of the BAH domain (Figure 4.2C). Two mutants in the ARK loop had been previously identified in two separate screens for enhancers of sir1 mating defects (Stone et al., 2000) and suppressors of Rap1 mutant alleles (Liu and Lustig, 1996): the *sir3-R30K* and *sir3-S31L* mutants, respectively. While these Sir3 mutants were previously documented, they were not examined in the context of a possible role in Sir3 self-interactions. In addition to those mutants, we also created *sir3-RK-AA* (R32A K33A) and *sir3-RKR-AAA* (R32A K33A R34A) in order to examine the role of these basic residues within the ARK loop. All of the Sir3-ARK mutant proteins were expressed at levels similar to wild type Sir3 (Figure 4.3A).

Oppikofer et al. (2013) showed that dimerization of Sir3 via the winged helix-turn-helix domain is required for silencing. We predicted that if the Sir3-ARK loop were involved in mediating Sir3-Sir3 interactions, we would see a similar loss of silencing. *sir3-R30K*, *sir3-RK-AA* and *sir3-RKR-AAA* show significant silencing defects at both the telomere and a weakened mating locus, whereas *SIR3-S31L* shows only a mild defect at the weakened mating locus and none at the telomere (Figure 4.3B). This result implicates the residues within the ARK loop in the silencing function of Sir3, but does not indicate whether this effect is through Sir3-Sir3 interactions.

To address whether the Sir3-ARK mutants interfered with Sir3-mediated spreading of silent chromatin, we tested if the ARK loop mutants cause a dominant negative silencing defect. Dominance of the mutant alleles would suggest that the mutant is able to interact with the wild type protein, but prevents it from functioning normally. We transformed centromeric plasmids containing wild type *SIR3* into the strains containing mutations in their


A
SIR3
sir3Δ
R30K
S31L
RK-AA
RKR-AAA
Sir3
116 kD
Cdk1
29

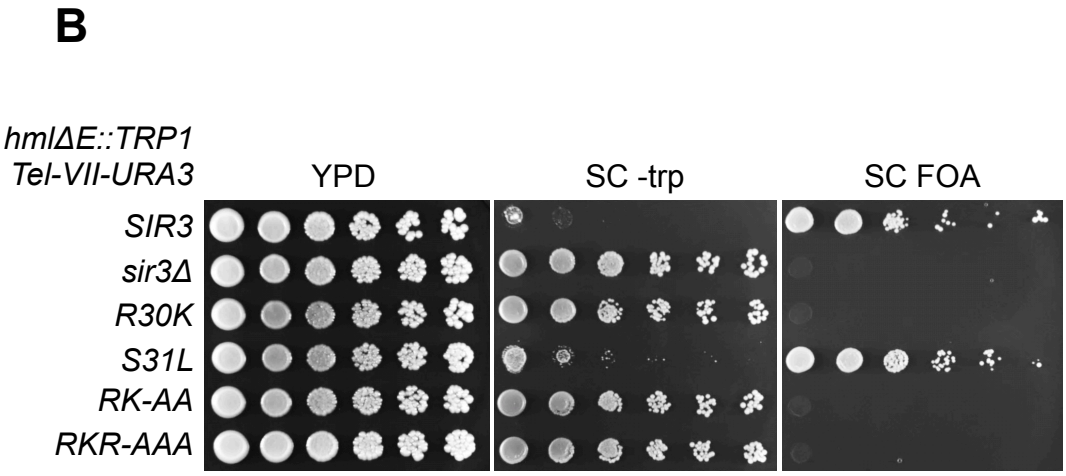

B
hmlΔE::TRP1
Tel-VII-URA3
YPD
SC -trp
SC FOA
SIR3
sir3Δ
R30K
S31L
RK-AA
RKR-AAA

Figure 4.3 Mutations in the Sir3-ARK loop abolish silencing. A: Western blot for Sir3 in whole cells extracts from Sir3-ARK mutants to confirm protein expression levels. Cdk1 is shown as a loading control. **B**: Serial dilutions of ADR4062-derivative strains expressing Sir3-ARK mutant alleles (*sir3-R30K, sir3-S31L, sir3-RK-AA* or *sir3-RKR-AAA*) were plated on media lacking tryptophan or media containing 5'-FOA for positive and negative selection for silencing, respectively. Each of these strains contains a *TRP1* reporter gene inserted at a weakened *HML* locus, and a *URA3* reporter gene inserted at *TEL-VII*. All of the ARK mutants show silencing defects at both the silent mating type loci and the telomere, with the exception of *sir3-S31L*.

genomic copy of Sir3. Expression of *SIR3* from the centromeric plasmid rescued silencing function in a *sir3Δ* strain, confirming that the plasmid produces functional Sir3. Expression of *SIR3* rescues silencing at both the mating loci and the telomere in the *sir3-R30K* mutant, and improves the silencing defect seen at the mating loci in the *sir3-S31L* mutant (Figure 4.4). Both *SIR3-RK-AA* and *SIR3-RKR-AAA* display strong dominant negative phenotypes: their silencing defects persist in the presence of wild type Sir3, at both the mating loci and the telomere. This result is consistent with our prediction that the Sir3-ARK mutants interfere with silent chromatin assembly by poisoning Sir3-Sir3 interactions.

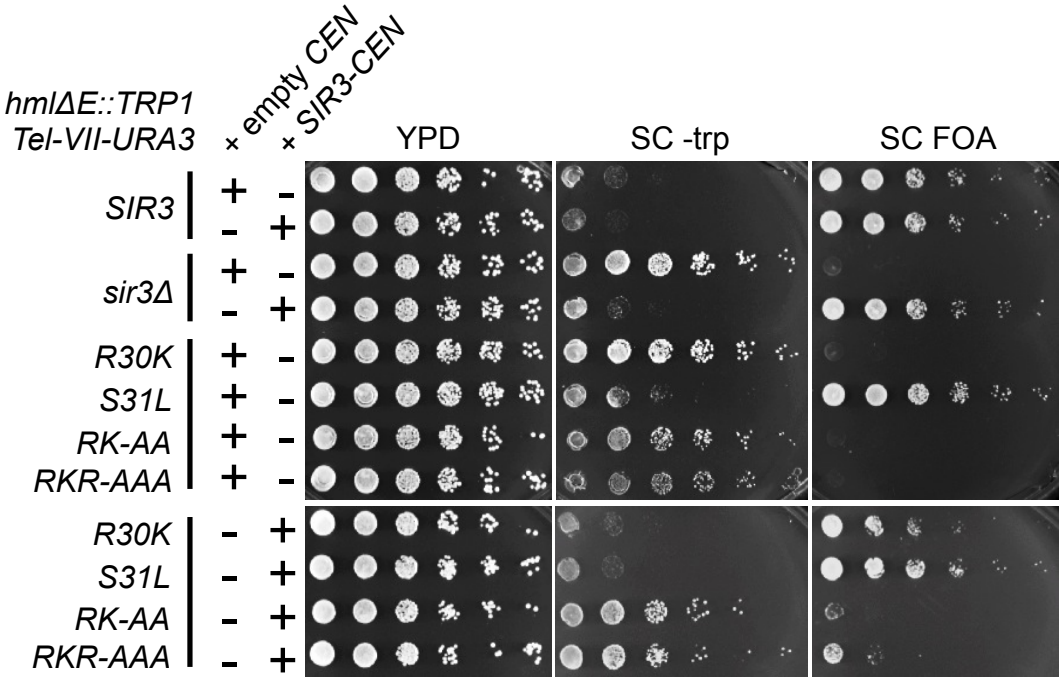

hmlΔE::TRP1
Tel-VII-URA3
× empty CEN
× SIR3-CEN
YPD
SC -trp
SC FOA
SIR3
sir3Δ
R30K
S31L
RK-AA
RKR-AAA
R30K
S31L
RK-AA
RKR-AAA

Figure 4.4 Sir3-ARK mutants interfere with the assembly of silent chromatin. The Sir3-ARK mutant strains were transformed with *SIR3-CEN* or an empty *CEN* plasmid to assess dominant negative phenotypes. Serial dilutions of the strains were plated on media lacking tryptophan or media containing 5'-FOA (as seen in Figure 4.3). Silencing defects persisted in the presence of wild type *SIR3* in the *sir3-RK-AA* and *sir3-RKR-AAA* mutants, and to a lesser extent in the *sir3-R30K* mutant.

Discussion

The assembly of silent chromatin domains is dependent on protein-protein interactions both within the SIR complex and between adjacent complexes. Sir3 has been shown to mediate interactions with the nucleosome and Sir4, and therefore has a critical role in maintaining the integrity of silent chromatin. In addition, there is strong evidence that Sir3 interacts directly with itself to form higher order oligomers (Armache et al., 2011; Liou et al., 2005; McBryant et al., 2006). This chapter discusses the identification of a putative novel self-interaction domain within the N-terminus of Sir3 and its potential homology with mammalian heterochromatin proteins, and begins to characterize its role in the assembly of silent chromatin.

We have identified a motif of residues in *S. cerevisiae* Sir3 that appear to be conserved in various yeast and vertebrate homologues of heterochromatin protein 1 (HP1). These residues are part of the ARK loop, first described in the *S. pombe* HP1 homologue Swi6 (Canzio et al., 2013). We show that these residues are required for silencing in *S. cerevisiae*, and that ARK loop mutants display dominance over wild type Sir3. Further experiments will be required to confirm that the ARK loop directly mediates Sir3-Sir3 interactions *in vitro* and *in vivo*. Immunoprecipitation of ARK mutant proteins with a tagged wild type Sir3 would indicate that Sir3 is capable of self-interaction *in vivo*, although these experiments should be performed in strains lacking Sir4 in order to avoid the formation of complete SIR complexes, which could appear to bridge adjacent molecules of Sir3.

The Sir3 C-terminal wH domain interacts through a symmetrical interface (Oppikofer et al., 2013), however dimerization can also occur asymmetrically (Figure 4.1). The ARK loop of Swi6 is predicted to act as a histone H3 tail mimic that binds to a hydrophobic cage

within the chromodomain (amino acids 74-81, 104-105; Canzio et al., 2013). The ARK loop shows primary sequence similarity to residues 7-12 of the histone H3 tail (7-Ala-Arg-Lys-Ser-Thr-Gly-12) and the H3 tail is able to compete with the ARK loop for binding to the Swi6 chromodomain (Canzio et al., 2013). The Swi6 binding pocket specifically recognizes a modified H3K9me3 tail peptide. Interestingly, the lysine within the ARK loop of HP1 is post-translationally modified by methylation (LeRoy et al., 2009), suggesting that competition of the H3 tail and the ARK loop could be regulated by their modification status. While H3K9 is not methylated in *S. cerevisiae*, it is possible that a similar competition mechanism regulates the assembly of SIR complexes on chromatin via Sir3-histone H3 interactions. Hecht et al. (1995) observed specific binding of Sir3 to the N-terminal tail of histone H3, and mapped this activity to two C-terminal histone binding domains: CHB1 and CHB2 (amino acids 623-762 and 808-910). These CHB domains are therefore potential locations within Sir3 for ARK loop and histone H3 tail binding, but they encompass the majority of the AAA^{+}-like domain. The search for a hydrophobic cage in Sir3 similar to that found in Swi6 may be confounded by the divergence of the two proteins, and the possibility that the tertiary structure of the cage may be formed by non-consecutive residues.

We would also like to examine the role of the winged-helix domain in the multimerization of Sir3. Oppikofer et al. (2013) confirmed that the Sir3-wH domain is required for silencing, and that this domain mediates Sir3-Sir3 interactions *in vitro*. While dimerization via the wH domain is necessary for silent chromatin assembly (Oppikofer et al., 2013), it may not be sufficient. As Buchberger et al. (2008) did not observe self-interaction of the Sir3-BAH domain, it is possible that multimerization of Sir3 by the ARK loop is dependent on prior dimerization. However, contradictory to Oppikofer et al. (2013), Buchberger et al. (2008) did not observe self-interaction of full length Sir3, so it remains

possible that the assay used to test dimerization via the BAH domain was not sensitive enough to detect Sir3 self-interactions. We will examine this hypothesis in future experiments by performing co-immunoprecipitations between various forms of Sir3 in which the ARK loop is mutated and/or the wH domain is absent. These mutants will allow us to examine the dependency of interactions via the ARK loop on dimerization mediated by the wH domain.

Our model suggests that Sir3 binding to the nucleosome is dependent on the formation of higher order complexes, but does not indicate whether Sir3-Sir3 contacts are retained once bound to the chromatin. Canzio et al. (2013) determined that binding of the H3 tail to the Swi6-CD destabilized oligomerization of Swi6. We therefore consider that the ARK loop may stabilize the formation of Sir3 oligomers prior to their binding to the nucleosome and, when freed up by the binding of the histone H3 tail, the ARK loop acts to then bind to a Sir3 dimer on an adjacent nucleosome. This mechanism has been predicted for Swi6 (Canzio et al., 2011), and may be conserved for Sir3 in *S. cerevisiae*.

Chapter 5: Sir4 abundance regulates establishment of silencing

Introduction

Silencing of transcription at *HML* and *HMR* was originally shown to require passage through S phase (Miller et al., 1984), suggesting that some aspect of the establishment of silent chromatin is regulated by the cell cycle. Two major possible targets of this cell cycle regulation could be the association of SIR proteins with chromatin, or regulation of the SIR proteins themselves. Katan-Khaykovich and Struhl (2005) determined that the establishment of silencing coincides with the dilution of H3K4 and H3K79 methylation marks during DNA replication and histone replacement. These findings suggest that recruitment of SIR proteins to chromatin is dependent on the loss of H3K4 and H3K79 methylation. Consistent with this hypothesis, loss of the histone methyltransferases Set1 (H3K4) and Dot1 (H3K79) significantly speeds the rate of silencing establishment at *HMR* relative to the time required for cell division (Osborne et al., 2009). However, deletion of *DOT1* results in loss of silencing at the telomeres (Singer et al., 1998), which is unexpected given its role in catalyzing H3K79 methylation and its positive effect on silencing at the mating loci when deleted. This result suggests that histone methylation is not the sole cell cycle-dependent regulator of silencing establishment.

Loss of Dot1-mediated H3K79 methylation is thought to result in promiscuous binding of SIR proteins throughout the genome (Ng et al., 2002; van Leeuwen et al., 2002). As the mating loci contain stronger silencers than the telomeres, free SIR proteins may be recruited in a biased manner to *HML* and *HMR*. Therefore the effect of *dot1Δ* on telomeric silencing may not be directly due to its methylation activity, but rather to its ability to

indirectly regulate local abundance of SIR proteins. Intriguingly, silencing is incredibly sensitive to Sir4 dosage: high overexpression of Sir4 causes derepression at both the telomeres and the silent mating loci (Marshall et al., 1987), while modest increases in Sir4 speed the rate of *de novo* assembly (Parsons et al, in prep; Sussel and Shore, 1991; Sussel et al., 1993). In addition, *SIR4* is haploinsufficient for silencing at the telomeres and silent mating loci when expressed in diploid cells (Parsons et al, in prep; Sussel et al., 1993). This sensitivity to Sir4 dosage raises the possibility that *de novo* assembly may be regulated through Sir4 abundance.

We have recently identified a potential link between the cell cycle and regulation of the *de novo* assembly of silent chromatin. We have shown that Sir4 protein is lost during α-factor arrest, where cells accumulate in G1 phase (Parsons et al, in prep). While the mechanisms regulating Sir4 translation or degradation are unknown, we propose that regulation of Sir4 abundance is a critical step in controlling the assembly of silent chromatin. In this chapter, we therefore sought to examine the effects of Sir4 abundance and genomic localization on the establishment of silencing throughout the cell cycle.

Results

Increasing Sir4 dosage improves maintenance of silencing

Previous work in the Rudner laboratory has shown that increased dosage of Sir4 speeds the rate of silencing establishment at *HMR*, relative to the time required for cell division, and that halving the Sir4 concentration slows assembly but does not affect maintenance (Parsons et al, in prep). Heterochromatin proteins are dynamically associated with chromatin (Cheutin, 2003), which suggests that maintenance of transcriptional silencing is a function of the rate of spontaneous assembly and disassembly of silent chromatin (Ahmad and Henikoff, 2001). We hypothesized that by increasing the pool of Sir4 available for silencing, the rate of assembly should surpass the rate of disassembly, resulting in the maintenance of transcriptional silencing. Conversely, loss of Sir4 protein may indirectly promote the disassembly of silent chromatin by preventing re-assembly in the case of spontaneous disassembly. We therefore wanted to assess the maintenance of silencing during an α-factor pheromone arrest, where Sir4 levels are depleted.

We developed an assay to monitor the kinetics of silencing of a telomeric *URA3* reporter gene over the course of an α-factor pheromone arrest. As shown in Figure 5.1, Sir4 protein levels are depleted following a 6 hour arrest in α-factor, but are stable in nocodazole-arrested cells. I created strains expressing *SIR4* from either one or two uniquely marked *CEN* plasmids (1X *SIR4-CEN* and 2X *SIR4-CEN*, respectively), in order to monitor the effect of increasing Sir4 dosage on the rate of silencing disassembly. Cells were grown asynchronously or arrested in α-factor, and were then plated on media containing FOA as negative selection for silencing.

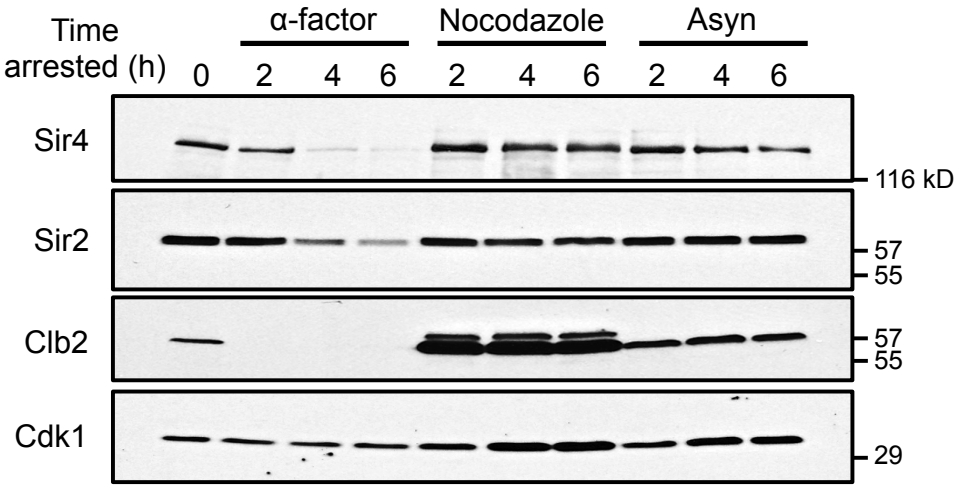

Time
arrested (h)
α-factor
Nocodazole
Asyn
0
2
4
6
2
4
6
2
4
6
Sir4
Sir2
Clb2
Cdk1
116 kD
57
55
57
55
29

Figure 5.1 Sir4 protein levels are responsive to pheromone. Western blots for Sir4, Sir2, Clb2 and Cdk1 in whole cell extracts from cells arrested in either α-factor or nocodazole, or grown asynchronously, for 6 hours. Clb2 indicates cell cycle phase: it is low in G1-arrested cells but is stabilized during nocodazole arrest. Cells treated with α-factor show a loss of Sir4 protein, while Sir4 is stable in nocodazole-treated and asynchronous cells. Cdk1 is included as a loading control.

Increased expression of Sir4 led to an increase in the absolute viability of the reporter strain at all time points (Figure 5.2A). Viability of the 2X *SIR4-CEN* strain was increased relative to both the strain expressing endogenous *SIR4* only, and the strain expressing a single additional copy of *SIR4-CEN*. This indicates that higher expression levels of Sir4 results in a higher proportion of cells that have established silent chromatin at *URA3*. Treatment with α-factor consistently decreased viability of all strains, in a timespan that correlated with loss of Sir4 protein (compare Figures 5.1 and 5.2). Our results are in contrast to the findings of Aparicio and Gottschling (1994), who observed that α-factor increased the stability of silent chromatin against disruption by the transcriptional activator *PPR1*. This discrepancy may be due to the different approaches taken to disrupt silencing: Aparicio and Gottschling (1994) were testing the resistance to an exogenous activator, while we are assessing the inherent stability of chromatin as Sir4 becomes limiting.

In order to compare rates of silencing disassembly, we determined the relative viability of these strains at each time point, compared to t=0 (Figure 5.2B). As expected, asynchronous cultures did not show any decrease in viability over time. Cells arrested with α-factor showed a similar decrease in viability over time, although high expression of Sir4 was correlated with increased viability at each time point in arrested cells. We next performed non-linear regression to quantify the rates of disassembly in each of these strains (Figure 5.2C). Curve fitting of an exponential decay function produced R^2 values > 0.95 for each strain, indicating strong goodness of fit for this function and allowing us to compare rates of decay between strains. However, we found no significant difference between the optimal parameters of each exponential decay function, suggesting that the rate of silencing disassembly in α-factor arrest is not changed by increasing expression of Sir4.

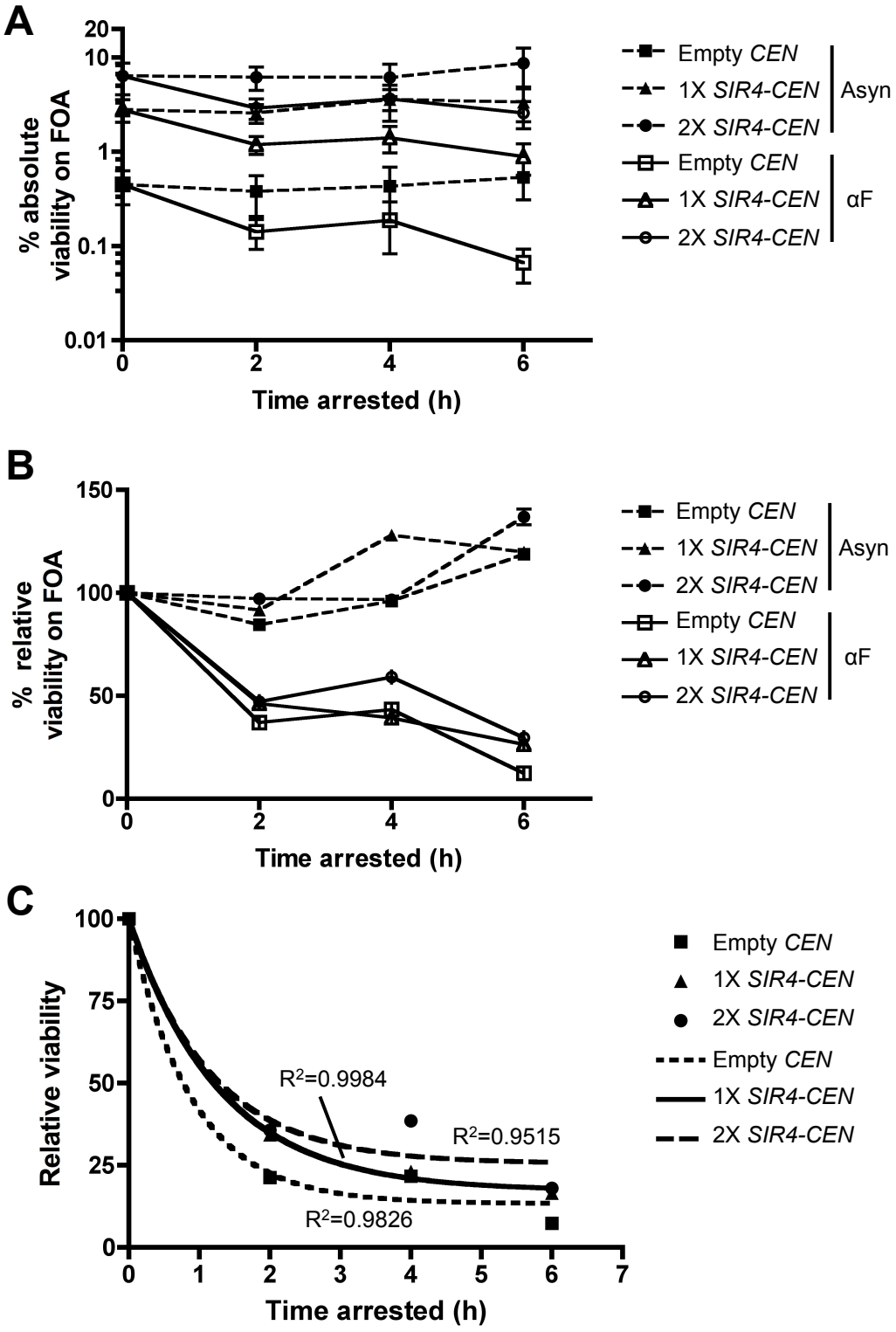

A
20
10
1
0.1
0.01
% absolute viability on FOA
0
2
4
6
Time arrested (h)
Empty CEN
1X SIR4-CEN
2X SIR4-CEN
Asyn
Empty CEN
1X SIR4-CEN
2X SIR4-CEN
αF
B
150
100
50
0
% relative viability on FOA
0
2
4
6
Time arrested (h)
Empty CEN
1X SIR4-CEN
2X SIR4-CEN
Asyn
Empty CEN
1X SIR4-CEN
2X SIR4-CEN
αF
C
100
75
50
25
0
Relative viability
R2=0.9984
R2=0.9515
R2=0.9826
0
1
2
3
4
5
6
7
Time arrested (h)
Empty CEN
1X SIR4-CEN
2X SIR4-CEN
Empty CEN
1X SIR4-CEN
2X SIR4-CEN

Figure 5.2 Increasing Sir4 dosage does not change the rate of silencing disassembly. A quantitative silencing assay was developed to monitor silencing of a telomeric *URA3* reporter during α-factor arrest, in cells expressing additional *SIR4* from one or two uniquely marked *CEN* plasmids. Cultures were arrested with α-factor and cells were harvested every 2 hours, and plated on media in the presence and absence of 5'-FOA. **A**: Absolute viability of strains is expressed as the ratio [absolute viability on 5'-FOA]/[absolute viability on control] x 100%. Error bars represent standard error of at least three replicates. Increasing *SIR4* expression is correlated with increased viability on 5'-FOA during pheromone arrest and in asynchronous cultures. **B**: Relative viability on 5'-FOA is calculated by normalizing growth curves to the t=0 time point. Statistical significance was determined by two-way ANOVA with a Bonferroni post-hoc test: Empty vs. 1X *SIR4-CEN*, $p<0.001$ at t=2 & 6; Empty vs. 2X *SIR4-CEN*, $p<0.001$ at t=2, 4 & 6. (*** $p<0.001$; all comparisons against α-factor-treated strains with Empty *CEN*). **C**: Non-linear regression analysis of curves from **B**, normalized to asynchronous cultures. Data points were fitted with a one phase exponential decay curve: Empty *CEN*, $R^2=0.9826$; *1X SIR4-CEN*, $R^2=0.9984$; *2X SIR4-CEN*, $R^2=0.9515$. A goodness of fit comparison was performed using the Wald-Wolfowitz runs test: $y = span^{(-K \cdot x)} + plateau$; parameters *span*, *K* and *plateau* are shared, p-value=1. Optimal curve equations and standard error are included in Appendix A1.5.

Sir4 association with chromatin is cell cycle-regulated

Previous work in the Rudner laboratory has shown that Sir4 protein levels decrease in the presence of the mating pheromone α-factor (Parsons et al, in prep; and Figure 5.1A). At first glance, this is in contrast to reports that silent chromatin is more stable in α-factor, when compared to a nocodazole arrest (Aparicio and Gottschling, 1994). This is also in contrast to the absolute necessity that the *HM* loci remain silenced in pheromone in order for mating to occur. We therefore wished to test if the direct association of Sir4 with chromatin is cell cycle-regulated, in order to resolve these findings.

We used chromatin immunoprecipitation (ChIP) to directly assess the binding of Sir4 to both the silent mating loci and telomeres. Cultures were treated with either α-factor or nocodazole, or grown asynchronously prior to fixing. We assessed Sir4 binding to multiple locations within the silent mating loci and the right telomeric arm of chromosome VI (*TEL-VIR*). Arrest in α-factor consistently increased the association of Sir4 with chromatin compared to nocodazole arrest at all loci tested (Figure 5.3B), although this difference is not statistically significant. Sir4 enrichment was also higher at *HML-E* and at 0.07kb from the end of *TEL-VIR* in α-factor arrested cells, compared to asynchronously grown cells. This increase was not observed at *HML-α* or at 0.77kb from *TEL-VIR*. The specificity of the Sir4 enrichment that we see may reflect the difference in strength between the silencers at these loci. These results suggest that Sir4 localization on chromatin is altered between G1 and M phases, although the mechanism through which this occurs is not yet clear.

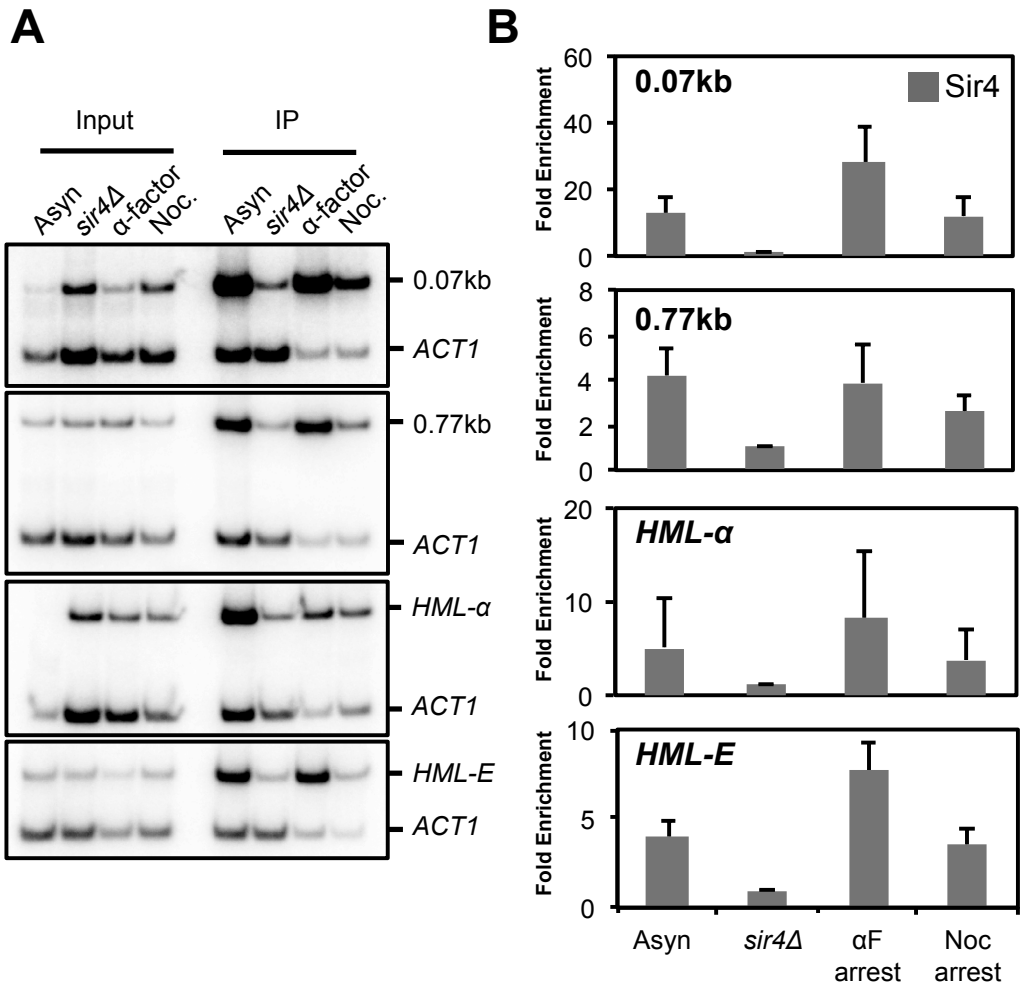

A
Input
IP
Asyn
sir4Δ
α-factor
Noc.
Asyn
sir4Δ
α-factor
Noc.
0.07kb
ACT1
0.77kb
ACT1
HML-α
ACT1
HML-E
ACT1
B
0.07kb
Sir4
Fold Enrichment
60
40
20
0
0.77kb
Fold Enrichment
8
6
4
2
0
HML-α
Fold Enrichment
20
10
0
HML-E
Fold Enrichment
10
5
0
Asyn
sir4Δ
αF arrest
Noc arrest

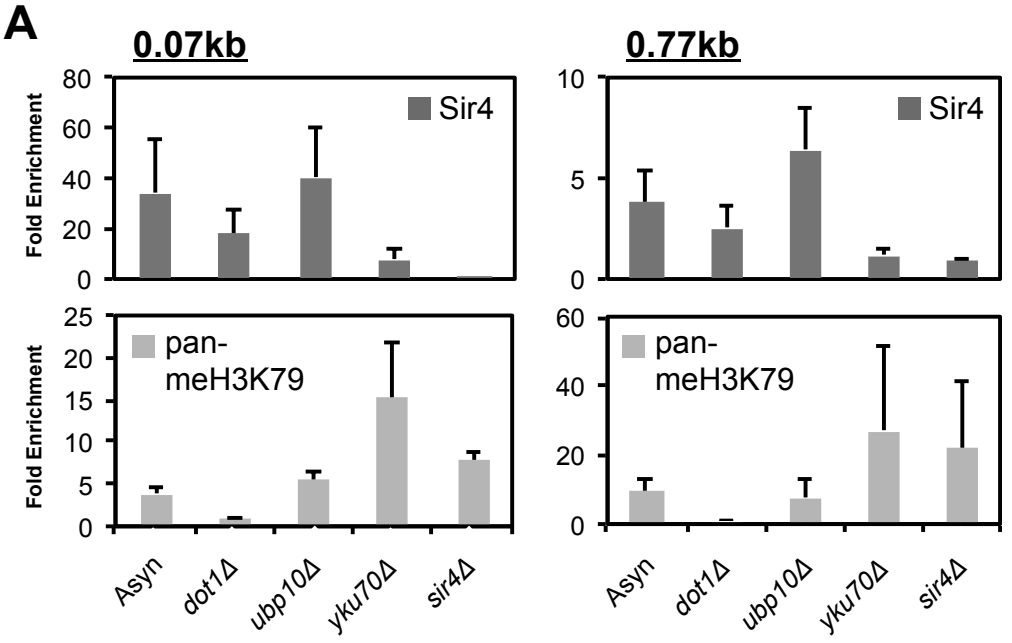
Figure 5.3 Cell cycle regulation of Sir4 does not reduce chromatin bound Sir4. A: Representative autoradiography images of P^{32}-dCTP labeled ChIP-PCR products. Cultures were treated with α-factor or nocodazole, or grown asynchronously prior to fixing with formaldehyde, and Sir4 was then immunoprecipitated from cell lysates. PCR products were separated on 6% polyacrylamide-TBE gels, vacuum dried and exposed to Storage Phosphor screens to generate images for densitometry analysis. **B**: Quantification of autoradiography images, including those shown in **A**. Enrichment values were calculated as in Figure 3.9, and normalized to the values from the *sir4Δ* strain. Mean and standard error of three replicates is shown. Treatment with α-factor results in a subtle but consistent increase in chromatin-bound Sir4, in contrast to the loss of soluble Sir4 protein seen during pheromone arrest.

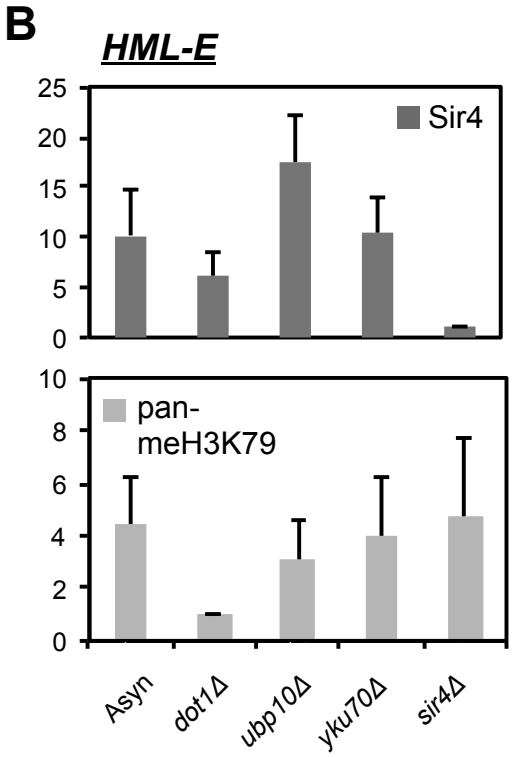
Loss of H3K79 methylation is independent of Sir4 binding

It is not yet clear what role H3K79 methylation plays in the establishment of silencing: is loss of methylation a cause or effect of silencing? In order to address his question, we wanted to investigate the effect of *dot1Δ* on Sir4 localization more closely, in addition to studying mutants that alter Sir4 localization without a direct impact on H3K79 methylation. Deletion of proteins responsible for Sir4 recruitment to silencers can disrupt silencing establishment, independently from changes in Sir4 protein levels (Luo et al., 2002; Roy et al., 2004; Singer et al., 1998 and Parsons et al, in prep). We therefore wanted to monitor H3K79 methylation in strains where Sir4 recruitment to silencers is lost, which would allow us to assess whether H3K79 methylation is an upstream or downstream regulator of silent chromatin assembly.

We performed chromatin immunoprecipitation (ChIP) in strains lacking Dot1 (H3K79 methyltransferase), Ubp10 (H2BK123 ubiquitin protease) or yKu70 (a telomere-binding protein). All of these mutants have silencing defects that are the result of altered SIR protein recruitment (Kahana and Gottschling, 1999; Mishra and Shore, 1999; Ng et al., 2002; van Leeuwen et al., 2002). Dot1 directly regulates SIR protein binding to the nucleosome through H3K79 methylation (Altaf et al., 2007; Ng et al., 2002; van Leeuwen et al., 2002), while Ubp10 indirectly promotes SIR binding by removing H2BK123 ubiquitylation, which is required for H3K4 and H3K79 methylation mediated by Set1 and Dot1, respectively (Gardner et al., 2005). yKu70 is required for Sir4 recruitment to the telomere, but *yku70Δ* mutants do not have a silencing defect at the silent mating loci (Laroche et al., 1998; Mishra and Shore, 1999 and Parsons et al, in prep).

As expected, deletion of Dot1 resulted in a global loss of H3K79 methylation (Figure 5.4C), while the other mutants did not have an effect on this histone mark. Consistent with its


A
0.07kb
0.77kb
Fold Enrichment
Sir4
pan-meH3K79
Asyn
dot1Δ
ubp10Δ
yku70Δ
sir4Δ


B
HML-E
Sir4
pan-meH3K79
Asyn
dot1Δ
ubp10Δ
yku70Δ
sir4Δ

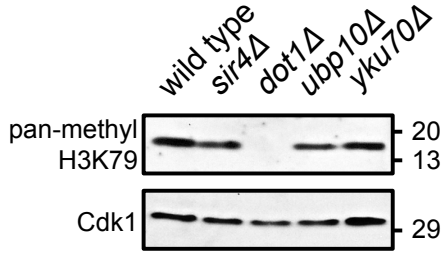

C
wild type
sir4Δ
dot1Δ
ubp10Δ
yku70Δ
pan-methyl H3K79
Cdk1
20
13
29

Figure 5.4 Loss of H3K79 methylation alters Sir4 recruitment to silent chromatin. **A**: Quantification of ChIP-PCR (as discussed in Figure 3.9): PCR reactions using primers specific to telomeric loci were performed using template obtained from Sir4 and pan-meH3K79 immunoprecipitates. Enrichment values for pan-meH3K79 samples were normalized to total histone H3, and values obtained from *dot1Δ* strains were set to 1. Mean and standard error of three replicates are shown. **B**: Same as **A**, using primers specific to the *HML-E* silencer region. **C**: Western blot for pan-meH3K79 in non-cross-linked whole cell extracts from strains used for ChIP. Cdk1 is included as a loading control.

telomeric silencing defect (Singer et al., 1998), the *dot1Δ* strain had lower enrichment of Sir4 at both 0.07kb and 0.77kb from *TEL-VIR* (Figure 5.4A, top panels). However, we also observed a slight decrease in Sir4 enrichment at *HMR-E* when Dot1 is deleted (Figure 5.4B, top panel), indicating that loss of Sir4 at the telomere does not directly result in accumulation of Sir4 at this silencer. Deletion of Ubp10 did not change the enrichment of H3K79 methylation at telomeric loci compared to wild type, however there was a subtle increase in Sir4 binding at the most distal telomeric locus tested (Figure 5.4A; 0.77kb, bottom panel). Sir4 binding at *HML-E* was higher in the *ubp10Δ* strain compared to wild type, and this was accompanied by a slight decrease in H3K79 methylation (Figure 5.4B). Very little Sir4 was detected at telomeric loci in the *yku70Δ* mutant (Figure 5.4A, top panels), as expected from previous reports (Mishra and Shore, 1999). Interestingly, the loss of Sir4 at the telomere in the *yku70Δ* mutant was associated with an increase in H3K79 methylation at both loci (Figure 5.4A, bottom panels). There was no change in enrichment of either Sir4 or H3K79 methylation at *HML-E* between the *yku70Δ* mutant and wild type (Figure 5.4B), consistent with a telomere-specific role for yKu70. Deletion of Sir4 did not result in significant increases in H3K79 methylation at any loci tested (Figures 5.4A & B, bottom panels). These results suggest that loss of H3K79 methylation is not an effect of Sir4 binding, and lead us to predict that Dot1-mediated H3K79 methylation is regulated independently of silencing establishment.

Discussion

The establishment of silencing is sensitive to both cell cycle progression and Sir4 dosage as well as the presence of histone modifications, specifically histone H3K79 methylation (Katan-Khaykovich and Struhl, 2005; Miller et al., 1984; Osborne et al., 2009; Sussel and Shore, 1991; Sussel et al., 1993; Parsons et al, in prep). In order to clarify the role of the H3K79 methyltransferase Dot1 in regulating silencing establishment, we directly investigated the effect of *dot1Δ* on Sir4 localization. Deletion of Dot1 resulted in a loss of Sir4 occupancy at telomeric loci, which is consistent with the findings of Singer et al. (1998). However, we did not observe a concomitant increase in Sir4 localization to *HML-E*. This finding is inconsistent with the results of Osborne et al. (2009), who determined that *dot1Δ* strains establish silencing at *HML* earlier than wild type, relative to the time required for cell division. The discrepancy between our results may reflect the difference between single cell- and population-based assays: the overall Sir4 enrichment as detected by our ChIP experiments may not be representative of the small proportion of cells that have rapidly established silencing (Osborne et al., 2009). This hypothesis presupposes that there is a threshold of Sir4 required to establish silencing, and may suggest that the total amount of Sir4 detected at fully silenced loci is the same irrespective of the presence of additional soluble Sir4.

We also examined other strains with documented silencing defects, including *ubp10Δ* and *yku70Δ* mutants, in order to assess the effect of Sir4 mislocalization on H3K79 methylation. Surprisingly, deletion of *UBP10* did not result in a strong increase in H3K79 methylation, even though its ubiquitin protease activity directly inhibits Dot1-mediated H3K79 methylation (Gardner et al., 2005). While we did not observe a change in H3K79

methylation, Sir4 enrichment was higher in the *ubp10Δ* mutant than wild type at all loci tested. As expected, deletion of yKu70 resulted in a loss of Sir4 binding to the telomere but did not change Sir4 occupancy at *HML-E* (Mishra and Shore, 1999). We did observe increased H3K79 methylation upon release of Sir4 from telomeric loci in the *yku70Δ* mutant. These results suggest that, although an anti-correlation exists between Sir4 binding and H3K79 methylation, Sir4 recruitment to chromatin does not lead directly to H3K79 demethylation, nor does the loss of H3K79 methyl mark lead directly to an increase in Sir4 occupancy.

As we predict that Dot1 may exert its effect on silencing by indirectly regulating local Sir4 abundance, we also examined the effect of Sir4 dosage on silencing establishment. Recent work in our lab has shown that Sir4 protein levels are down-regulated in response to α-factor, suggesting to us that regulated Sir4 protein production or turnover may control silencing establishment. As expected, treatment of cells with α-factor results in a loss of silencing that correlates with the loss of Sir4 protein over time, while cells grown asynchronously do not show a decrease in silencing efficiency. The expression of higher levels of Sir4 partially improves the loss of silencing during pheromone arrest, although these results are not statistically significant. These findings indicate a dose dependent response of silencing to Sir4 abundance, where higher expression of Sir4 leads to an increase in silencing establishment within a population.

Our results support a model in which upstream regulation of Sir4 protein levels through a pheromone-responsive pathway controls the nucleation of silent chromatin. We have also examined the association of Sir4 with chromatin during pheromone arrest, and have shown that treatment with α-factor results in a subtle but consistent increase in Sir4 enrichment at telomeric loci and at *HML*. It is possible that loss of the soluble protein in

whole cell extracts (seen in Figure 5.1A) is due to active transport of Sir4 to chromatin in the presence of α-factor, which is consistent with the increase in chromatin-bound Sir4 seen in our ChIP experiments. For example, the activity of the SCF^{Dia2} (Skp1/Cullin/F-box protein) ubiquitin E3 ligase complex is required for proper localization of Sir4 throughout the cell cycle (Burgess et al., 2012). While Sir4 ubiquitylation by SCF^{Dia2} does not change its expression levels (Burgess et al., 2012), this modification may be sufficient to alter Sir4 interactions with the nucleosome or the SIR complex, and promote its binding to chromatin. It is also possible that chromatin-bound Sir4 is more resistant to degradation than soluble Sir4 protein, and that recruitment of Sir4 to chromatin following exposure to α-factor protects this pool of Sir4 from being turned over rapidly, although the mechanism through which this would occur is unknown.

Future experiments will address the potential role of the pheromone response pathway in regulation of Sir4 abundance, as preliminary data from the Rudner laboratory suggests that the down-regulation of Sir4 levels is α-factor-dependent, rather than an effect specific to G1 phase. The components of this pathway have been well characterized (Kurjan, 1993), and they present many potential targets for regulation of Sir4, including the downstream effectors Far1 (a Cdk1 inhibitor), Ste12 (a transcription factor) or Bni1 (a formin required for actin polarization and mating projection morphogenesis). Recent work has shown that pheromone induces a Bni1 and polarization-dependent repression of cellular translation (Goranov et al., 2009), suggesting the intriguing possibility that Sir4 may be regulated at the level of translation. IF Sir4 has an intrinsically short half-life, changes in the rate of translation could cause the dramatic drop in abundance that we observe. We will examine the requirement of components of the pheromone pathway in mediating sensitivity

of Sir4 expression levels to α-factor, in order to more fully understand the regulation of Sir4 abundance in the context of silencing establishment.

Chapter 6: General Discussion

Summary

This study was undertaken to provide a better understanding of the regulatory processes and protein interactions that mediate recruitment and spreading of the SIR complex along chromatin. Many years of genetic, biochemical and structural studies have led to a strong characterization the individual SIR proteins, but mechanisms underlying their assembly on chromatin remain unclear. Regulated assembly and maintenance of silent chromatin is of critical importance in budding yeast, as the ability of *S. cerevisiae* to mate is predicated on the presence of functional silencing at the silent mating cassettes, *HML* and *HMR* (Haber and George, 1979; Klar et al., 1979; Rine et al., 1979). The regulation of mating-type information in budding yeast is similar to the concept of developmental program regulation in higher organisms, which depend on the heterochromatic gene repression to regulate developmental choices and cell identity (Andrés et al., 1999; Cirillo and Zaret, 1999; O'Carroll et al., 2001).

Our investigations into the potential homology between *S. cerevisiae* Sir3 and mammalian HP1 draw upon a functional analogy of heterochromatin assembly in these two systems, and suggest that this process may be more conserved than has previously been appreciated. While much emphasis has been placed on the role of histone modifications in transcriptional regulation and the definition of heterochromatic regions (Kitada et al., 2012; Oppikofer et al., 2011), we still do not fully understand the physical and regulatory interactions between these modifications and the silencing proteins responsible for transcriptional repression.

The first objective of this thesis was to examine the role of the Sir3-Sir4 interaction following the nucleation of silencing. We determined that overexpression of a *sir3-4A* mutant allele that is defective in Sir4 binding is sufficient to spread silent chromatin into subtelomeric regions. This mutant was not competent for silencing in the absence of wild type Sir3, highlighting the requirement for the Sir3-Sir4 interaction at the site of nucleation. The ability of Sir3-4A-mediated silent chromatin to compete with transcription was similar to domains formed by wild type Sir3, and the overall stability of the silent chromatin domains were also similar, indicating that there is no inherent instability in extended silent chromatin when the Sir3-Sir4 interaction is lost. We also directly examined binding of the SIR proteins to chromatin within extended domains and observed that only Sir3 was significantly enriched away from sites of nucleation. These findings support a model in which Sir3 is primarily responsible for mediating the spreading of silent chromatin following the initial recruitment of SIR complexes to the silencer, and that this spreading occurs independently of the Sir3-Sir4 interaction.

The second objective of this thesis was to examine the potential oligomerization activity of a novel N-terminal motif in Sir3, the ARK loop. Mutation of residues within this motif abolishes silencing, and some of these mutants display a dominant negative phenotype. The presence of similar residues within the primary sequence and a potentially conserved secondary structure in vertebrate HP1 proteins suggests a conserved mechanism of heterochromatin assembly. Our data support a model in which oligomerization of Sir3 dimers is dependent on asymmetrical interactions between the ARK loop and an unidentified binding pocket within a second domain of Sir3.

The final objective of this thesis was to assess the effect of Sir4 dosage on the establishment of silencing, and to investigate endogenous mechanisms that may regulate Sir4

abundance at the chromatin level. Treatment of cells with α-factor results in loss of Sir4 protein over time, whereas cells arrested with nocodazole show constant levels of Sir4 protein. However, this result does not directly address whether Sir4 is regulated in G1, or in response to the pheromone. We observe that higher expression of Sir4 increases the rate of silencing establishment at *HML*, but does not noticeably change the rate of silencing disassembly during α-factor arrest at *TEL-VR*. In addition, changes in local Sir4 abundance may mediate the silencing defects documented in *dot1Δ* mutants, although we have observed that loss of H3K79 methylation does not directly promote Sir4 recruitment. Based on our findings, regulation of Sir4 dosage may present a novel mechanism controlling silent chromatin assembly.

Defining the role of the Sir3-Sir4 interaction

All SIR proteins are required for silencing, and recruitment to the site of nucleation is dependent on Sir3-Sir4 interactions. Nucleation is also dependent on Sir2 catalytic activity, indicating that histone deacetylation at the site of nucleation alters the interaction of the SIR complexes with chromatin, and allows silent chromatin to nucleate and spread. Overexpression of Sir3 can extend domains of silent chromatin, which contain more Sir3 than either Sir2 or Sir4, suggesting a structural difference between core and extended silent chromatin. This difference may be due to a conformational change in the SIR complex caused by a lack of histone deacetylation outside the site of nucleation, consistent with a lack of Sir2 in these domains.

We developed the hypothesis that the Sir3-Sir4 interaction is not required for Sir3 to spread along chromatin, although it is absolutely required for nucleation. We confirmed that the Sir3-4A mutant protein is able to spread along chromatin, but our initial ChIP

experiments made it hard to determine if this spreading and silencing occurred independently of Sir4. Parallel work in the Rudner lab using a sensitized assay to enrich for silencing has shown that Sir4 is in fact present to the same extent in extended domains mediated by either Sir3 or mutant Sir3-4A. The presence of Sir4 within the extended domains created by Sir3-4A suggests that Sir4 can be recruited to the nucleosome independently of its interaction with Sir3. We have no evidence that Sir3-4A has any affinity for Sir4 in solution either *in vitro* or *in vivo*, but additional testing could be performed using isothermal titration calorimetry (ITC). This technique has been used previously to identify interactions between Sir3 and Sir4 (Chang et al., 2003). However, this technique would not involve the full-length proteins and would not tell us if there is affinity between the proteins that does not involve the fragments being tested. In addition, Sir4 could interact with Sir3-4A when they are on a chromatin template if there is a second interface through which the Sir3-Sir4 interaction occurs. We could test this interaction in the future by adding purified Sir3 or Sir3-4A with Sir4 to nucleosomal arrays *in vitro* to determine if Sir4 becomes incorporated into the arrays, and if this depends on the Sir3-Sir4 interaction. This assay would also be able to provide us with information about the affinity of Sir4 for nucleosomes, if performed in the absence of Sir3.

The levels of Sir4 enrichment in the presence of mutant Sir3-4A were similar to those of the wild type, so it is possible that the binding of Sir4 may have the same properties in both cases: even in the presence of wild type Sir3, Sir4 recruitment could be independent of the Sir3-Sir4 interaction. However, as ChIP assesses protein binding at a static point, there could be a difference in the kinetics of association of Sir4 between Sir3- and Sir3-4A-mediated silent chromatin. Once Sir4 is recruited by either mechanism, Sir2-dependent

deacetylation of the chromatin may stabilize the Sir4-chromatin interaction to a similar extent.

Very little is known of Sir4-nucleosome binding, although Sir4 can interact with a histone H4 tail peptide *in vitro* (Hecht et al., 1995) and may bind to dsDNA with little sequence affinity (Kueng et al., 2012b). The Sir3-4A mutant could be used in a screen to identify residues in Sir4 required for Sir4-histone interactions. We predict that these interactions are secondary to the recruitment of Sir4 by DNA-binding proteins at the silencers, and will therefore screen for Sir4 mutants that are still able to nucleate silencing at *HML* or *HMR* but are unable to contribute to the spreading of silencing to a distal locus along the telomere.

Additional data from the Rudner lab has shown that tethering of Sir3-4A to the mating loci is not sufficient for silencing. Sir4 is therefore required at the silencer for more than simply the recruitment of Sir3, because tethering Sir3-4A directly to the silencer does not bypass the requirement for the Sir3-Sir4 interaction. Tethering of either Sir3-4A or the wild type Sir3 to the silencer in the absence of Sir4 also does not allow silencing, suggesting that Sir4 is also required for spreading of functional silent chromatin.

It is possible that Sir4 plays some role in silencing following nucleation, although it is not likely directly responsible for mediating the spreading of silent chromatin. There is very strong evidence that Sir3 is capable of interacting directly with itself and with chromatin (Armache et al., 2011; Ehrentraut et al., 2011; Liou et al., 2005; Martino et al., 2009; Oppikofer et al., 2011), so it is unlikely that Sir4 is required to bridge these interactions. We predict that Sir4 binding to chromatin, independently of the Sir3-Sir4 interaction, results in recruitment of Sir2 and deacetylation of chromatin. This change in the chromatin template allows for tighter binding of Sir3, and perhaps Sir4, stabilizing the SIR

complex and silent chromatin. Deacetylation may also be a requirement for robust demethylation of histone H3K79, which has been shown to correlate with transcriptional repression within silent chromatin.

While nucleation involves all SIR proteins and requires the histone deacetylase activity of Sir2, we predict that spreading of silent chromatin is mediated primarily by Sir3, and the role of the Sir3-Sir4 interaction is restricted to nucleation. Our results support the following model for spreading of silent chromatin: Sir3 and Sir4 association with chromatin is not dependent on the Sir3-Sir4 interaction, but incorporation of Sir4/Sir2 into Sir3-mediated domains allows histone deacetylation and alters the interactions between Sir3 and chromatin to allow efficient silencing (Figure 6.1). We propose that after Sir3 spreading, Sir4 can be recruited to chromatin independently of the Sir3-Sir4 interaction, although the domains on histone responsible for this are as of yet unknown.

Mechanisms of Sir3 oligomerization

Sir3 is capable of forming higher order oligomers *in vitro* (Armache et al., 2011; Liou et al., 2005), but currently only one self-interaction domain has been characterized (Oppikofer et al., 2013). We believe that we have identified residues that mediate oligomerization of Sir3, based on a similar motif in Swi6, an HP1 homologue found in fission yeast (Canzio et al., 2013). Although Swi6 and Sir3 are not apparently homologous, they perform analogous function in budding and fission yeasts. The similarity between the CD-ARK loop of Swi6 and the histone H3 tail suggests that the ARK loops acts as a histone tail mimic, and that release of the H3 tail mimic from Swi6 promotes heterochromatin assembly. The ubiquity of heterochromatin suggests a conserved function of assembly, and this process in both yeasts and in vertebrate systems involves the polymerization of

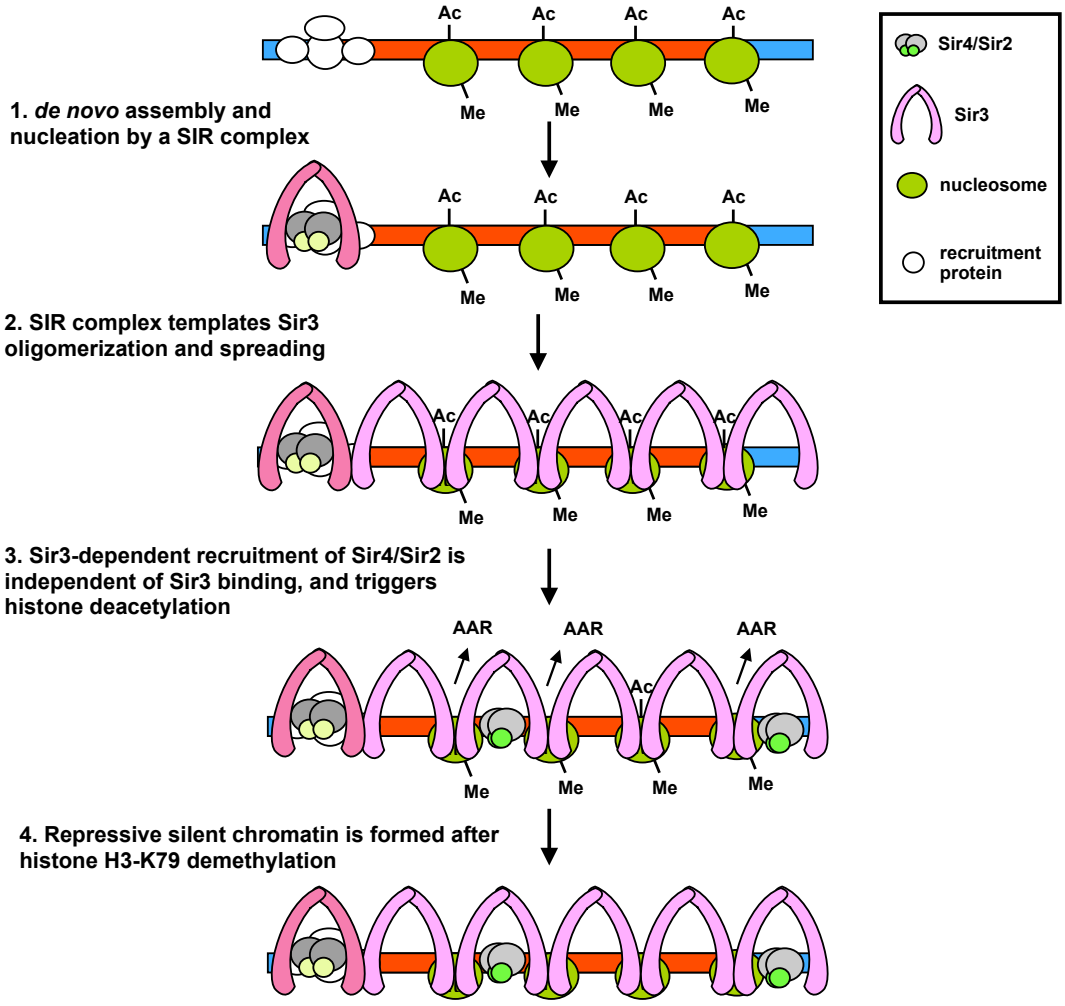

1. *de novo* assembly and nucleation by a SIR complex
2. SIR complex templates Sir3 oligomerization and spreading
3. Sir3-dependent recruitment of Sir4/Sir2 is independent of Sir3 binding, and triggers histone deacetylation
4. Repressive silent chromatin is formed after histone H3-K79 demethylation
Ac
Me
AAR
Sir4/Sir2
Sir3
nucleosome
recruitment protein

Figure 6.1 Model of Sir3-dependent silent chromatin spreading. We propose a novel model of silent chromatin assembly, in which spreading of silencing is mediated primarily by Sir3. Nucleation of silencing (Step 1) requires all SIR proteins and the histone deacetylase activity of Sir2. Spreading of Sir3 (Step 2) can occur independently of Sir3-Sir4 binding, and likely requires the ability of Sir3 to oligomerize. Incorporation of Sir2 and Sir4 into silenced regions (Step 3) is independent of Sir3-Sir4 binding, and histone deacetylation by Sir2 tightens the interaction of the SIR complex with chromatin. Demethylation of histone H3K79 (Step 4) then produces fully transcriptionally silent chromatin.

heterochromatin proteins along chromatin, along with the modification of the underlying histones to repress transcription.

If the Sir3-ARK loop acts in a manner similar to the CD-ARK loop in Swi6, there will be a second domain involved in the asymmetrical self-interaction. We predict that this domain is within the AAA^{+}-like domain, which has been shown to possess histone H3 tail binding activity (Hecht et al., 1995). Ehrentraut et al. (2011) found a severe silencing defect in a *sir3-D640A S642L* mutant, and these residues are within the CHB1 domain identified by Hecht et al. (1995). These residues may represent part of an acidic patch or hydrophobic cage, similar to that found in Swi6 (Canzio et al., 2013). Interestingly, deletion or mutation of the histone H3 tail increases Sir3 occupancy on chromatin: this data is consistent with a model in which the H3 tail inhibits Sir3-Sir3 interactions, and release of the H3 tail mimic produces "sticky ends" that promote interactions with an adjacent Sir3 dimer (Canzio et al., 2013).

Dimerization of Sir3 mediated by the winged-helix (wH) domain is necessary for silencing, and this domain is required for interactions of full-length Sir3 *in vitro* (Oppikofer et al., 2013). This may suggest a hierarchy of self-association: dimerization via the wH domain is necessary prior to oligomerization via the ARK loop. Overexpression of the wH domain disrupts silencing (Oppikofer et al., 2013), probably by titrating free Sir3 away from chromatin, indicating a strong interaction between full-length Sir3 and the wH domain that is not dependent on the ARK loop. In contrast, overexpression of the BAH domain, that contains the ARK loop, does not disrupt silencing (Connelly et al., 2006). The potential Sir3-Sir3 interactions mediated by the ARK loop would therefore be dependent on prior dimerization by the wH domain.

We propose that Sir3 self-interactions are required for assembly of silent chromatin, although their role following the association of Sir3 with chromatin is not yet clear (Figure 6.2). Mutation or deletion of the Sir3-wH domain reduces binding of the SIR complex to chromatin *in vitro* (Oppikofer et al., 2013), suggesting that dimerization of Sir3 promotes interaction of the SIR complex with the nucleosome. Oligomerization mediated by the ARK loop may be more apparent *in vitro* if it is dependent on prior dimerization by the wH domain. We propose that this oligomerization promotes a "chromatin-binding competent" conformation in soluble Sir3, and that release of the ARK loop upon binding to the histone H3 tail promotes interactions with Sir3 of the next adjacent SIR complex.

Sir4 abundance regulates the establishment of silencing

Sir4 abundance is a limiting factor during the establishment of silencing (Sussel et al., 1993 and Parson et al, in prep), and Sir4 protein levels are altered in response to pheromone. Therefore we predicted that regulation of Sir4 dosage might present a novel mechanism controlling silent chromatin assembly. Increasing Sir4 dosage increases the rate of establishment, while halving the expression levels slows establishment, relative to the time required for cell division. However, there is no change in maintenance of silencing: once silencing is established and stable, the abundance of Sir4 does not matter. Our finding that treatment with α-factor causes a loss of Sir4 protein is very surprising. Mating is dependent on the maintenance of silencing at *HML* and *HMR*, so it is interesting that pheromone changes the levels of a silencing protein. Many of the experiments studying cell-cycle dependence of silencing have used α-factor to arrest cells (Kirchmaier, 2001; Kirchmaier and Rine, 2006; Lau et al., 2002), so this finding may confound the interpretation

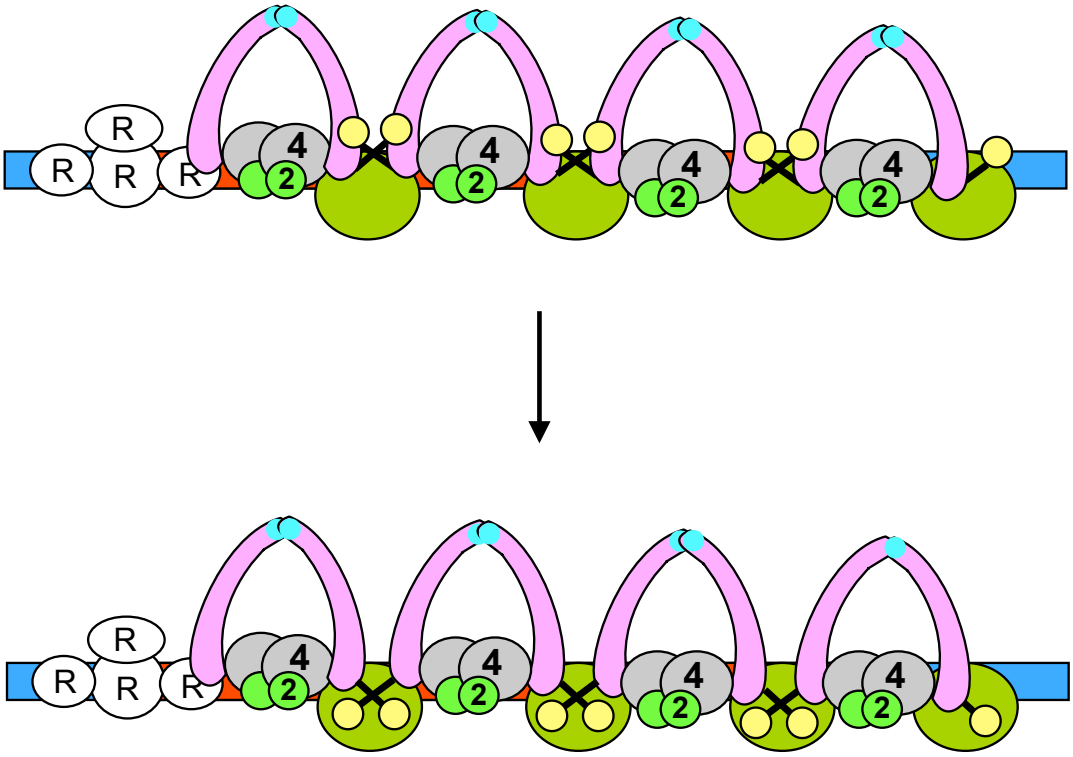

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Figure 6.2 Model of ARK-loop mediated spreading of Sir3 on chromatin. We propose that oligomerization of Sir3, mediated by the C-terminal wH domain and the N-terminal ARK loop, is integral to the ability of Sir3 to spread on chromatin. We predict that the ARK loop binds to a yet unidentified binding pocket within the AAA^{+}-like domain of Sir3, and this region may also be responsible for binding to the histone H3 tail. Oligomerization of soluble Sir3 may promote the chromatin-binding activity of Sir3, and release of the ARK loop upon Sir3 binding to the histone H3 tail may promote the incorporation of an adjacent SIR complex.

of their results, as it is not clear if the effect they observe is due to the cell cycle phase or is in some way caused by the loss of Sir4 protein.

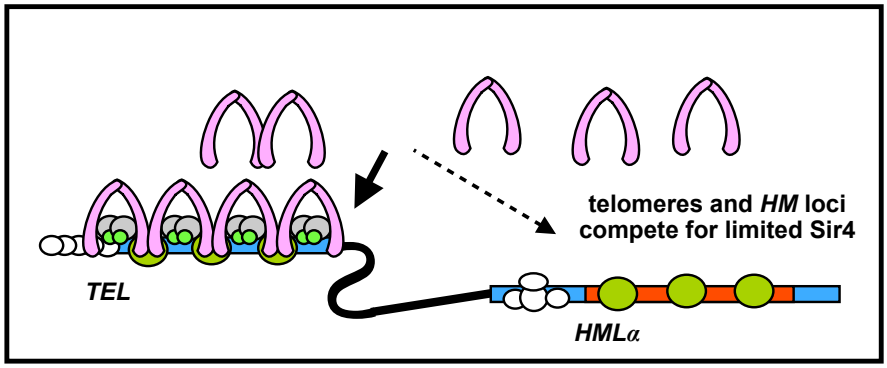
Sir4 levels alter the rate of silencing establishment: increasing Sir4 expression with ectopic *CEN* plasmids increases the proportion of cells within a population that have established silencing. It is possible that a threshold of Sir4 is required to establish silencing, and that once that threshold is reached the silencing is maintained at a constant rate. Single-cell analysis of silencing establishment in *SIR4-CEN* strains indicates that increased Sir4 expression directly increases the rate of establishment, relative to the time required for cell division (Parsons et al, in prep). However, the outputs of the single-cell analysis are categorical rather than continuous, and the resolution of the assay to detect establishment is limited to a full cell division.

The rate of silencing disassembly is similar between all Sir4 expression levels, as measured using our *URA3* reporter assay. We had predicted that higher expression of Sir4 would result in a decreased rate of disassembly, because there would be a higher effective concentration of Sir4 to initiate re-assembly. Aparicio and Gottschling (1994) showed that silent chromatin is more stable in α-factor arrest than in nocodazole arrest, as measured by the ability to antagonize transcriptional activation. While this thesis did not directly compare the rates of disassembly between α-factor and nocodazole arrests, we were measuring the effect of an inherent property of silent chromatin (Sir4 protein levels) rather than looking at the effect of an exogenous activator. It is possible that α-factor treatment stabilizes the association of Sir4 with chromatin in a manner that represses transcription more efficiently than in the absence of pheromone, while also inhibiting the association of additional soluble Sir4 with chromatin by promoting its degradation. This hypothesis is consistent with the results of Aparicio and Gottschling (1994) and with our ChIP data (Parsons et al, in prep).

We did not observe a causal relationship between Sir4 binding and H3K79 methylation, although there was a general anti-correlation between their enrichment on chromatin. We predict that the silencing defects of mutants such as *dot1Δ*, *ubp10Δ* and *yku70Δ* are due to indirect effects on Sir4 localization. This is in contrast to the current model of silencing establishment, which suggests a strong dependency on loss of the H3K79 methylation mark prior to de novo assembly of silent chromatin (Osborne et al., 2009). Our results suggest a more subtle interaction between histone modifications and SIR protein binding. The presence or absence of a particular mark at a particular locus may affect silencing on both local and global scales by directly regulating the binding of SIR proteins to nucleosomes within that locus and thus altering their relative concentrations within the genome (Figure 6.3). This model is consistent with many reports that SIR proteins are limiting in concentration for silencing (Hecht et al., 1996b; Marcand et al., 1996; Renauld et al., 1993; Strahl-Bolsinger et al., 1997; Sussel and Shore, 1991; Sussel et al., 1993), allowing their relative abundances to be used as a mechanism of regulation.

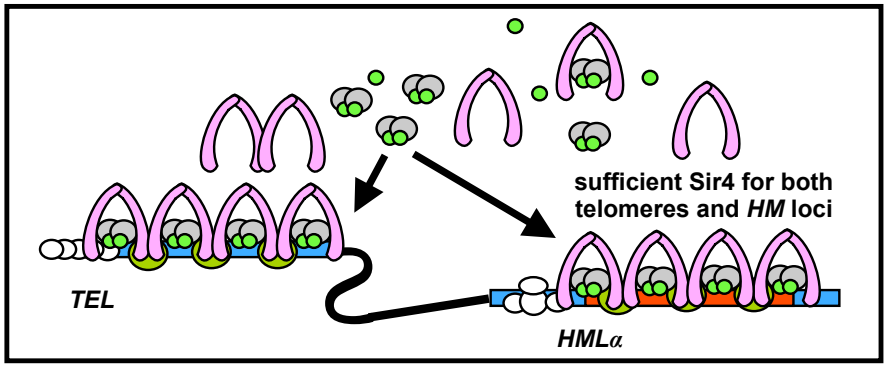
A

de novo assembly of heterochromatin at *HMLα*



B

de novo assembly of heterochromatin in cells with increased Sir4



C

de novo assembly of heterochromatin in *dot1*Δ or *yku70*Δ cells

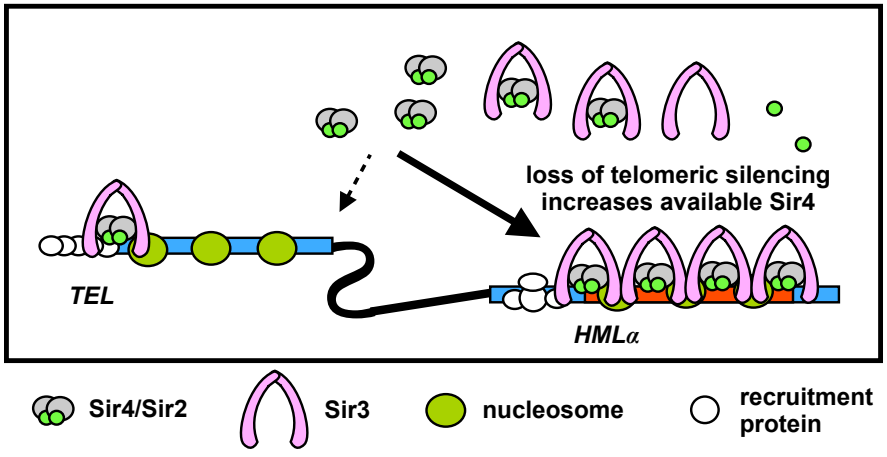


Figure 6.3 Sir4 abundance regulates *de novo* establishment of heterochromatin. We propose that Sir4 protein abundance present a novel mechanism of regulation of silencing establishment. **A**: Silent chromatin loci compete for limiting pools of soluble Sir4. The presence of strong silencer elements and a high concentration of recruitment proteins at the *HM* loci promote biased association of Sir4 with the silent mating cassettes over the telomeres. **B**: Increasing the pool of available Sir4 through ectopic expression provides sufficient Sir4 for efficient silencing at both the *HM* loci and at telomeres, increasing the rate of silencing establishment. **C**: Liberation of Sir4 from telomeres through the disruption of telomeric silencing, such as *yku70Δ* or *dot1Δ* mutants, also increases the rate of silencing establishment at the *HM* loci.

Appendix

A1.1 Strains used in this study

ADR Number	Genotype	Source	Mating type
21	wild type W303	Rudner lab	*MAT**a***
22	wild type W303	Rudner lab	*MATα*
617	*bar1Δ::LEU2*	Rudner lab	*MAT**a***
2829	*TelVII-URA3*	Rudner lab	*MAT**a***
3101	*sir4Δ::NAT*	Rudner lab	*MAT**a***
3252	*TelVR-URA3*	Rudner lab	*MATα*
3353	*sir3Δ::KAN*	Rudner lab	*MAT**a***
3387	*sir4Δ::NAT*	Rudner lab	*MATα*
3632	*sir2Δ::HIS3*	Rudner lab	*MAT**a***
3684	*DIU5-2kb PPR1+*	Renauld et al, 2003	*MAT**a***
3685	*DIU5-1kb PPR1+*	Renauld et al, 2003	*MAT**a***
3686	*DIU5-3.5kb PPR1+*	Renauld et al, 2003	*MAT**a***
3687	*DIU5-2.5kb PPR1+*	Renauld et al, 2003	*MAT**a***
3688	*DIU5-2kb ppr1Δ::HIS*	Renauld et al, 2003	*MAT**a***
3689	*DIU5-1kb ppr1Δ::HIS*	Renauld et al, 2003	*MAT**a***
3690	*DIU5-3.5kb ppr1Δ::HIS*	Renauld et al, 2003	*MAT**a***
3691	*DIU5-2.5kb ppr1Δ::HIS*	Renauld et al, 2003	*MAT**a***
4006	*bar1Δ*	Rudner lab	*MAT**a***
4062	*TelVII-URA3 hmrΔE::TRP1*	Rudner lab	*MAT**a***
5469	*TelVII-URA3 hmrΔE::TRP1 sir3Δ::KAN*	Rudner lab	*MAT**a***
6204	*DIU5-6.5kb PPR1+*	Renauld et al, 2003	*MAT**a***
6205	*DIU5-5.5kb PPR1+*	Renauld et al, 2003	*MAT**a***
6212	*DIU5-6.5kb ppr1Δ::HIS*	Renauld et al, 2003	*MAT**a***
6213	*DIU5-5.5kb ppr1Δ::HIS*	Renauld et al, 2003	*MAT**a***
6260	*dot1Δ::KAN*	Rudner lab	*MATα*
6262	*ubp10Δ::KAN*	Rudner lab	*MATα*
6264	*ku70Δ::KAN*	Rudner lab	*MATα*
6383	*TelVII-URA3 hmrΔE::TRP1 GAL-SIR4-C2-HIS*	This study	*MAT**a***
6384	*TelVII-URA3 hmrΔE::TRP1 GAL-SIR4-C2-HIS*	This study	*MAT**a***
7254	*DIU5-1kb ppr1Δ::HIS bar1Δ::LEU*	This study	*MAT**a***
7257	*DIU5-5.5kb ppr1Δ::HIS bar1Δ::LEU*	This study	*MAT**a***
7415	*HYG-sir3-R30K*	This study	*MAT**a***
7418	*HYG-sir3-RK-AA*	This study	*MAT**a***
7420	*HYG-sir3-RKR-AAA*	This study	*MAT**a***

7503	*TelVII::URA3 hmrΔE::TRP1 ppr1Δ::NAT*	This study	*MAT**a***
7524	*TelVII::URA3 hmrΔE::TRP1 GAL-PPR1-HIS ppr1Δ::NAT*	This study	*MAT**a***
8048	*bar1Δ ppr1Δ::NAT*	This study	*MAT**a***
8049	*bar1Δ ppr1Δ::NAT*	This study	*MAT**a***
8060	*HYG-sir3-S31L*	This study	*MAT**a***
8122	*ppr1Δ::NAT TelVR-URA3*	This study	*MAT**α***
8123	*ppr1Δ::NAT TelVR-URA3*	This study	*MAT**α***

A1.2 Plasmids used in this study

pAR number	Genotype	Source
Bodo103	*GAL-PPR1-HIS3*	Rudner lab
P1	*KanMX*	Rudner lab
121	*GAL1-HIS3*	Rudner lab
359	GST tag	Rudner lab
380	*GAL1-HIS*	Rudner lab
411	*GST-SIR4-C2(1267-1358)*	Rudner lab
419	GST-Precision Protease	Rudner lab
441	Vector *CEN-TRP1*	Rudner lab
447	*SIR4-CEN-TRP1*	Rudner lab
450	*SIR4-CEN-TRP1*	Rudner lab
453	*sir4-I1311N-CEN-TRP1*	Rudner lab
469	*SIR3-LEU2-2μ*	Rudner lab
472	pGEX-G1-1	Rudner lab
504	MBP tag	Rudner lab
512	*SIR3-CEN-LEU2*	Rudner lab
534	Vector *LEU2-2μ*	Rudner lab
1009	*sir3-2A(464-978)-His_6*	Rudner lab
1011	*sir3-4A(464-978)-His_6*	Rudner lab
1012	*SIR3(464-978)-His_6*	Rudner lab
1017	*sir3-4A-LEU2-2μ*	Rudner lab
1023	*sir3-3A-LEU2-2μ*	Rudner lab
1042	*sir3-3A(464-978)-His_6*	Rudner lab
1056	*sir3-2A-LEU2-2μ*	Rudner lab
1057	*SIR4(1165-1358)-MBP*	This study
1061	*GST-SIR4-C1(1198-1358)*	This study
1062	*GST-sir4-I1311N-C1(1198-1358)*	This study
1063	*GAL-SIR4-C2(1267-1358)-HIS3*	This study

A1.3 Primers used in this study

Primer number	Primer name	Sequence	Source
101	sir4Δ UP	GCTTCAACCCACAATACCAAAAAAGCGAAGAAA ACAGCCACAGCTGAAGCTTCGTACGC	Rudner lab
102	sir4Δ DOWN	GGTACACTTCGTTACTGGTCTTTTGTAGAATGAT AAAAAGTACGACTCACTATAGGG	Rudner lab
133	SIR3-seq3 (+1285 top)	AACGAAATGAACGGAAATGG	Rudner lab
134	SIR3-seq4 (+1953 top)	AAATGTCCCGAAAGCAAAAA	Rudner lab
135	SIR3-seq5 (+2645 top)	CTCTGGAGACCGTCCTGATT	Rudner lab
137	SIR3-seq7 (+2385 bottom)	ACGTTGGCTACGTTCTTTGC	Rudner lab
138	SIR3-seq8 (+1712 bottom)	GGGAAGACATCAAACTGTCG	Rudner lab
140	SIR3-seq10 (+325 bottom)	ATCTGGCCTGAATTGTTCGT	Rudner lab
149	ChIP Tel6R 0.07 UP	CATGACCAGTCCTCATTTCCATC	Grunstein lab (OAR149)
150	ChIP Tel6R 0.07 DS	ACGTTTAGCTGAGTTTAACGGTG	Grunstein lab (OAR 150)
289	bar1Δ::LEU2 UP	CTCCGACATCATGCTGAAAC	Rudner lab
290	bar1Δ::LEU2 DS	CAAAATTGTGATGGCTGCAT	Rudner lab
295	sir2Δ UP	CGTGTATGTCGTTACATCAGATGAACATCCCAA AACCCTCGGTGACGGTGCTGGTTTA	Rudner lab
296	sir2Δ DS	TATTAATTTGGCACTTTTAAATTATTAAATTGCC TTCTACTCGATGAATTCGAGCTCG	Rudner lab
499	ChIP ACT1 UP	GCCTTCTACGTTTCCATCCA	Rudner lab
500	ChIP ACT1 DS	GGCCAAATCGATTCTCAAAA	Rudner lab
679	ChIP HML-α UP	AGACGGCCAGAAACCTC	Rudner lab (formerly GH81)
680	ChIP HML-α DOWN	TCGCCTACCTTCTTGAAC	Rudner lab (formerly GH82)
1038	MBP-Sir4(1165-1358) UP	CGCGGATCCAACAAAGCAAAAATAATCCCTGG	This study
1039	MBP-Sir4(1165-1358) DS	ACGCGTCGACTCAATACGGTTTTATCTCCTT	This study
1057	GST-Sir4-C1(1198-1358) UP	CGCGGATCCAAAAGGGTTCCAATCGTTG	This study
1058	GST-SIR4-C1(1198-1358) DS	CCGGAATTCTCAATACGGTTTTATCTC	This study

1059	GAL-Sir4-C2(1267-1358) UP	TGCCATCGATACCATGAAAGAAGATGGTCTTTCC	This study
1085	ChIP Tel6R 0.77 UP	TACAAGGGAACAATGAGCAGAGGG	This study
1087	ChIP Tel6R 0.77 DS	GCGCCTAGTGCAACTAGTGCATAT	This study
1088	ChIP Tel6R 1.0 UP	GGACAGATCCTTTCGCATTCCTAC	This study
1089	ChIP Tel6R 1.0 DS	GGCCGAGCACTTACTATAGACACT	This study
1096	ChIP Tel6R 2.8 UP	GCTAGCATCAAAGACGGGTGTTGG	This study
1097	ChIP Tel6R 2.8 DS	ATTCTTGGGGCGTCTTGAGGTGTA	This study
1098	ChIP Tel6R 5.0 UP	GCGAGGAATGCGTACATTGCACTT	This study
1100	ChIP Tel6R 5.0 DS	AACCCAGTATTCATGTCCGGGACA	This study
1103	ChIP Tel6R 7.5 UP	GATACCGGCAACCCATAGTGACTT	This study
1104	ChIP Tel6R 7.5 DS	GCAGATTAGCCTAGGCCTCTATTG	This study
1105	ChIP Tel6R 15 UP	TCCCAAGGAAGTGAAACCGATTGC	This study
1106	ChIP Tel6R 15 DS	GGAGCAACCAGGAAAAATACTCTGG	This study
1107	ChIP Tel6R 20 UP	GGATTGACGGGTAACCCTAAAAGG	This study
1108	ChIP Tel6R 20 DS	GTTGTCATGGCCAATGACCACGAT	This study
1223	ChIP Tel6R 10 UP	AACTGAGGCCATCATTCGTC	This study
1224	ChIP Tel6R 10 DS	TTTCAGTGTCCACCACCGTA	This study
1225	ChIP Tel6R 5.0 UPc	CCCTTTTTGGCGTACCTACA	This study
1226	ChIP Tel6R 5.0 DSb	GGACATTGCACCAAGGAAGT	This study
1281	Sir3 UP	CGCGAAAACGCTATTCTTTC	This study
1282	Sir3 checking UP	CAAGGGCTGTAAGGTTCTGG	This study
1283	Sir3 checking DS	ACTTTTCCGCAGTAGGTTCG	This study
1284	Sir3 R30K mutation UP	ACAACAATAGAAGAAAATCTAGAAAAAGAGGAGGAG	This study
1285	Sir3 R30K mutation DS	CTCCTCCTCTTTTTCTAGATTTTCTTCTATTGTTGT	This study
1286	Sir3 S31L mutation UP	AACAATAGAAGAAGACTTCGAAAAAGAGGAGGAG	This study
1287	Sir3 S31L mutation DS	CTCCTCCTCTTTTTCGAAGTCTTCTTCTATTGTT	This study
1288	Sir3 RK-AA mutation UP	AACAATAGAAGAAGAAGCGCTGCTAGAGGAGGAGAAAAC	This study
1289	Sir3 RK-AA mutation DS	GTTTTCTCCTCCTCTAGCAGCGCTTCTTCTTCTATTGTT	This study

1290	Sir3 RKR-AAA mutation UP	CAATAGAAGAAGATCAGCGGCCGCAGGAGGAG AAAACGT	This study
1291	Sir3 RKR-AAA mutation DS	ACGTTTTCTCCTCCTGCGGCCGCTGATCTTCTTCT ATTG	This study
1292	Sir3 HYG marker UP	TGTTCATTTTAACTTTTTTGGAAAGAATAGCGTT TTCGCGAGCTGAAGCTTCGTACGC	This study
1293	Sir3 HYG marker DS	AAGAAATAATTGTAAATTTTCATCGCATCAGTA ATATGACTACGACTCACTATAGGG	This study
1319	GST-SIR4-C1(1198-1358) XhoI DS	CCGCTCGAGTCAATACGGTTTTATCTC	This study
1375	ppr1Δ::HIS UP	CTTTAGAGTACATAAAACATACGAAGATGATGA TTAAATCAGCTGAAGCTTCGTACGC	This study
1376	ppr1Δ::HIS DS	CCTACTTTCAATCCATTGATCATGATTCTTCGAG CTATCGTACGACTCACTATAGGG	This study
1377	ppr1Δ::HIS check	CCCTGAACCGATGACTTGAT	This study
1490	Sir3-seq11 (+14 top)	TGAAAGATTTGGACGGTTGG	This study

A1.4 Statistical analysis

Table A1.4. **p-values obtained from comparisons of silencing in *PPR1/ppr1Δ* strains at *TEL-VR*** (with Figure 3.4)**.** Two-way ANOVA with a Bonferroni post-hoc test were performed on silencing data obtained from strains containing a telomeric *URA3* reporter gene and overexpressing *SIR3* or *sir3-4A* from high copy 2µ plasmids. Comparisons are between *PPR1* and *ppr1Δ* strains within each locus and expressing each 2µ plasmid.

***URA3* locus (kb)**	Empty *2µ*	*SIR3-2µ*	*sir3-4A-2µ*
1	<0.001	<0.001	<0.001
2	>0.05	<0.001	<0.001
2.5	>0.05	<0.001	<0.001
3.5	>0.05	<0.001	<0.001
5.5	>0.05	<0.001	<0.001
6.6	>0.05	<0.05	>0.05

A1.5 Non-linear regression analysis summary

Table A1.5. **Non-linear regression analysis summary of the effect of Sir4 dosage on silencing disassembly during pheromone arrest** (with Figure 5.2)**.** Data was obtained from α-factor-arrested cells plated on 5'-FOA to assess silencing through the arrest. Relative viability is expressed as [absolute viability on 5'-FOA]/[absolute viability on control] x100%, normalized to t=0 and asynchronous cultures at each time point. Data points were fitted with a one-phase exponential decay curve, using Prism Graphpad Software. Best fit and standard error values are shown.

	Empty *CEN*	*1X SIR4-CEN*	*2X SIR4-CEN*
Best-fit values			
SPAN	86.60	82.70	74.26
K	1.116	0.7696	0.8603
PLATEAU	13.33	17.24	25.48
Half life	0.6211	0.9007	0.8057
Standard Error			
SPAN	12.34	3.659	18.27
K	0.6839	0.1079	0.7159
PLATEAU	7.787	2.617	12.49

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