Asf2 mediates Sir3 availability during the assembly of heterochromatin

Sean E.K. Stephenson, B.Sc.

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Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
University of Ottawa

Abstract

Heterochromatin in *S. cerevisiae* is formed at telomeres, rDNA, and the mating type loci by the Silent Information Regulator (SIR) complex. Silencing requires the SIR complex that consists of Sir2, Sir3, and Sir4. The SIR proteins interact with each other, nucleosomes, and DNA binding proteins that are located at silencers. Although the interactions within the SIR complex are well defined, the requirements for each of these interactions during the nucleation and spreading of heterochromatin are not. This study uses genetic and biochemical techniques to assess silencing at various loci and to detect interactions between the SIR proteins. Asf2 (Anti-Silencing Factor 2) is a poorly characterized protein that interacts with Sir3 and is investigated in detail throughout this work. The overexpression of *ASF2* disrupts silencing and does so by outcompeting Sir4 for Sir3 binding. *ASF2* is a paralog of *SIR4,* and they share significant homology within their coiled-coil domains which is required for their interaction with Sir3. The Asf2 protein exists as a dimer that depends on Sir3 and may serve as a tool to alter Sir3 availability and impact heterochromatin stability. The evidence presented here categorizes the requirements for the Sir3-Sir4 interaction and the establishment of H4K16 acetylation in nucleation and spreading. Mutations in the AAA+ domain of Sir3 (*sir3-4A*) render it insufficient to nucleate heterochromatin but do not prevent Sir3-4A and Sir4 from spreading downstream of silencers. The Sir3-Sir4 interaction is therefore a nucleation-specific requirement. Cells lacking *SAS2* are defective for telomere silencing, but silencing is partially restored by overexpressing *SIR3* but not *sir3-4A*. Although the Sir3-Sir4 interaction is not required for Sir3 to spread on its own, Sir4/Sir2 are unable to spread without the establishment of H4K16 acetylation.

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List of Abbreviations

AAR – O-acetyl-ADP-ribose
ASF2 – anti-silencing factor 2
BAH – bromo-adjacent homology domain
CC – coiled-coil
CEN – centromeric plasmid
ChIP – chromatin immunoprecipitation
CRASH – cre-reported states of heterochromatin
DNA – deoxyribonucleic acid
DSB – double strand break
EDTA – ethylene diamine tetraacetic acid
EGTA -ethylene glycol tetraacetic acid
FOA – 5-fluoroorotic acid
G418 – Geneticin
GAL – galactose
GFP – green fluorescent protein
H2A – histone H2A
H2B – histone H2B
H3 – histone H3
H4 – histone H4
HAT – histone acetyltransferase
HDAC – histone deacetylase
HDM – histone demethylase
HDR – homology-directed repair
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HML – homothallic mating left / hidden mat left
HMR – homothallic mating right / hidden mat right
HMT – histone methyltransferase
IgA – immunoglobulin A
IgG – immunoglobulin G
IP – immunoprecipitation
LEU – leucine
MAT – mating type
MAPK – mitogen-activated protein kinase
MCM – mini-chromosome maintenance
NHEJ – non-homologous end joining
ORC – origin recognition complex
PCR – polymerase chain reaction
PMSF – phenylmethylsulfonyl fluoride
preRC – pre-replication complex
qPCR – quantitative polymerase chain reaction
rDNA – ribosomal deoxyribonucleic acid
RNA pol – RNA polymerase
SBF – SCB binding factor
SC – synthetic complete
SCB – Swi4, -6-dependent cell cycle box
SD – synthetic defined
SDS – sodium dodecyl sulfate
SIR – silent information regulator
TBST – tris buffered saline Tween-20
TRP – tryptophan
URA – uracil
RE – recombination enhancer
SUMO – small ubiquitin-like modifier
wH – winged helix domain
YEP – yeast extract peptone
YPD – yeast extract (peptone) dextrose

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Chapter 1: General Introduction

1.1 The historical significance of heterochromatin

The initial discovery of heterochromatin

The formation of DNA and proteins into chromatin is an everchanging landscape of genetic information. Chromatin has historically been categorized into two states based on the original observation that passive parts of the genome exist where active genes appear to be absent. These areas of highly condensed DNA with little to no gene expression were defined as heterochromatin, and are a direct contrast to euchromatin, which is much less compact and is associated with gene expression (Heitz, 1928). Heterochromatin was first visualized using a cytological staining method that involved boiling samples from plants and *Drosophila* to prepare the DNA (Passarge, 1979). After recognizing that heterochromatin was a common structure among several species, many studies emerged to help further categorize these condensed regions of the genome.

Heterochromatin can be further divided into constitutive and facultative heterochromatin. Constitutive heterochromatin generally persists throughout the entire cell cycle and is found at telomeres and the centromere. Facultative heterochromatin is a result of functional inactivation, and forms at a specific point during cell growth or development, such as the X chromosome in early embryos (Brown, 1996). Further work in *Arabadopsis* and maize revealed the complexity of centromere organization. Pericentric chromatin is defined as centromere-proximal chromatin that receives the mitotic spindle and localizes with sister chromatids to form a chromocenter in the nucleus during cell division. The packing, localization, protection, maintenance, and expression of DNA are all governed by the state of chromatin.

Position effect variegation

The state of chromatin is highly dynamic and offers a layer of complexity to the linear genetic code that allows for programmed cellular responses and processes. The ability to exclude genes from expression provides cells with a mechanism to differentiate physiological state, growth rate, and cellular identity depending on the environment or situation. The first evidence of gene silencing was observed in *Drosophila*, where genes were susceptible to position effect variegation (reviewed in Weiler and Wakimoto, 1995). Altering the location of the *white* gene to areas associated with heterochromatin resulted in the loss of wild-type eye color in the flies and was attributed to repression of the *white* gene. The limitation of the position effect variegation was determined to be up to 25 kb in *Drosophila*, indicating that a quite large region of the genome can be susceptible to variable expression states (Tartof et al., 1984). However, the suppression of genes was not completely linear, and a model was proposed to explain the skipping of genes that would be silenced by an uninterrupted stretch of heterochromatin.

Repetitive DNA elements cluster and form heterochromatin generating a loop that effectively passes over some active genes (Talbert and Henikoff, 2000). This was one of the first examples of *trans* activated silencing effects that hinted at the higher order structures and cooperativity of heterochromatin. Position effect variegation has been observed in many species including the telomeres of *S. cerevisiae*. Yeast telomeres containing *ADE2* and *URA3* reporters showed variable expression, and like *Drosophila*, the variable expression coincided with the presence of $TG_{1\text{-}3}$ repeats (Gottschling et al., 1990). The cross talk between non-coding DNA and proteins that make up chromatin give rise to the location of heterochromatin and its potential to spread.

1.2 Histones and Nucleosomes

The basic structure of nucleosomes

The assembly of DNA onto nucleosomes results in the formation of chromatin. A nucleosome is an octamer consisting of two copies of each histone H2A, H2B, H3, and H4. DNA wraps around the nucleosome 1.65 times occupying a length of 146 base pairs (Luger et al., 1997). Nucleosomes were first observed as 'beads on a string', or 'nu-bodies'; self-assembling structures at least 5 times more compact than free or extended DNA with a 125 Å diameter (Oudet et al., 1975). The histones that make up a nucleosome all contain a histone fold domain that is a dimer motif. Subtle differences in each histone fold dictate the core octamer arrangement of the nucleosome. This histone fold superfamily and octamer arrangement is conserved from archaea to high eukaryotes (Arents and Moudrianakis, 1995).

Experiments digesting chromatin with modified nucleosome cores highlight the main mechanisms of compaction by the histones. Digestion of reconstituted chromatin with DNAase and trypsin with only H3/H4 present produced similar digestion patterns to whole chromatin (Sollner-Webb et al., 1976). The arginine-rich profile of H3/H4 is sufficient to organize DNA to the same size as the full core nucleosome. Supporting this mechanism is the fact that H2A and H2B exchange out of the nucleosome much more often than H3 and H4. Core histones labeled in HeLa cells showed that H3/H4 were mainly retained in their original nucleosome while H2A/H2B were much more fluid (Kimura and Cook, 2001). This fluidity is crucial because compaction of DNA around nucleosomes and thus the stability of the core histone octamer must be relieved to allow for processes such as transcription and replication to occur.

The passage of transcriptional machinery through nucleosomes

Both replication and transcription require a polymerase to pass through the core nucleosome as the enzyme stays in association with template DNA. The TATA box is an example of a DNA element that is found in the promoter region of expressed genes. The TATA box is bound by the TATA-binding protein, TBP, which is an important step in initiating transcription. Inhibitory to this process is the incorporation of the TATA sequence into the nucleosome. The orientation of the TATA sequence dictates how shielded it is from TBP and highlights the need for active chromatin remodeling complexes to regulate expression (Imbalzano et al., 1994). During transcription RNA pol II causes a reversible structural change in nucleosomes. RNA pol II prefers to bind nucleosomes deficient in H2A/H2B but is unable to eject these histones through its own activity (Baer and Rhodes, 1983). Histone-interacting proteins such as Nap1 from *S. cerevisiae* and nucleoplasmin from *Xenopus* are required for successful transcription initiation. Both histone remodeling proteins are able to bind H2A/H2B to free up space around the nucleosome and allow transcription factors to bind DNA (Walter et al., 1995). Successful passage of RNA pol II through the nucleosome causes a slight change in location of the reassembled nucleosome, indicating the whole core is displaced during transcription (Clark and Felsenfeld, 1992).

In contrast to these *in vitro* experiments, genes that are only moderately expressed appear to not have their nucleosomes displaced. RNA polymerase II transcription is thought to form an intra-nucleosome DNA loop that results in exchange of H2A/H2B, but not H3/H4 (Kulaeva and Studitsky, 2010). As RNA polymerase II approaches the DNA-histone interface this small loop forms containing the transcriptional machinery. The loop

formation is important for both retaining H3/H4 and recovering the same DNA-histone interaction (Kulaeva et al., 2009). Similar to transcription, replication requires passage though the nucleosome. Distribution of parental H3/H4 dimers have a slight preference to assemble on lagging strands, but removal of DNA polymerase subunits Dpb3 and Dpb4 in yeast increase this preference for the lagging strand (Yu et al., 2018). The exchange of histones within existing nucleosomes during transcription and the formation of nucleosomes on newly synthesized DNA are regulated by many factors and have a direct impact on cell fate.

The distribution and de novo synthesis of histones

Existing histones are exchanged between nucleosomes and are distributed between new and existing DNA strands after replication. Histones are synthesized *de novo* during S-phase of the cell cycle and are distributed at the replication fork. New H3/H4 deposits as a tetramer, and new H2A/H2B deposits as a dimer (Jackson, 1988). New H2A/H2B dimers are deposited in association with old H2A/H2B, H3, and H4. In contrast, new H3/H4 are deposited as tetramers and only associate with old H2A/H2B dimers (Jackson, 1987). Newly replicated DNA requires histones that are sourced from parental nucleosomes and *de novo* synthesis. Most of the time it appears that parental H3/H4 tetramers are conserved and distriuted between existing and newly synthesized DNA strands (Annunziato, 2005). This observation makes a strong argument for the random segregation of histones, but exceptions to this rule continue to be found and indicate that parental histone distribution can be regulated by a variety of processes. Chaperones such as human CAF-1 help to deposit *de novo* synthesized histones to

recently replicated DNA (Kaufman, 1996). Mammalian H3/H4 tetramers can be distributed between strands of newly synthesized DNA but require the sub-complex formation with histone variants and chaperones (Tagami et al., 2004; Rowlands et al., 2019; reviewed in Stewart-Morgan et al., 2020). These intermediate complexes that form during replication can be regulated by a myriad of signals and factors that determine how histones are distributed.

1.3 Histone modifications

The histone code

The maintenance of histones and the role histones play in gene expression through position effect variegation requires thorough regulation. Histone-binding proteins, histone variants, and histone modifications all work in concert to dictate heterochromatin function. Not long after the discovery of heterochromatin it was found that histones are post-transcriptionally modified (Allfrey et al., 1964). With the help of high-resolution crystal structures, the N-terminus of histones was found to be unstructured in a way that presents itself to the solvent (Davey et al., 2002). Since their discovery, many examples of histone acetylation, phosphorylation, methylation, ubiquitylation, and SUMOylation have been noted (Bannister and Kouzarides, 2011). The seemingly countless number of histone modifications that work together to dictate transcriptionally active or inactive states is termed the histone code (Jenuwein and Allis, 2001). This code is highly conserved (Figure 1) and has become an important part of understanding nearly all other biological processes from DNA repair to cell differentiation. Just as important as the code itself are the epigenetic regulators that read and write the code, and mutations in these

regulators have been linked to human disease such as cancer and metabolic disorders (Portela and Esteller, 2010).

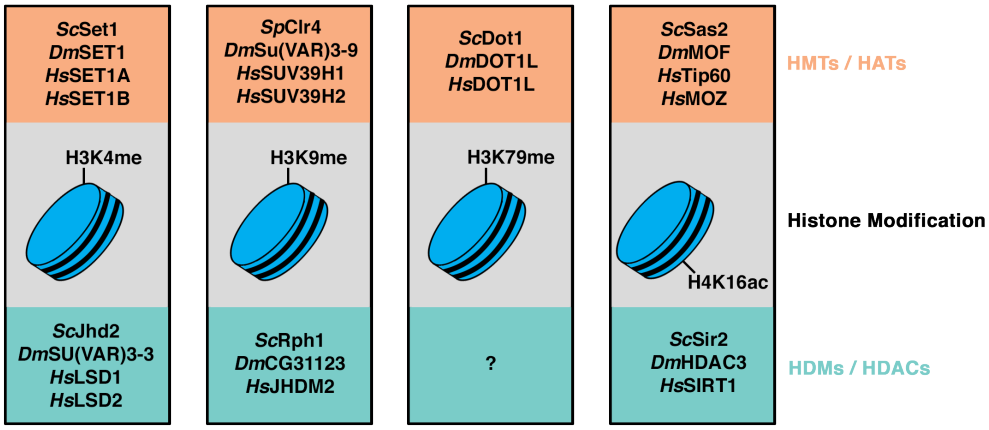

ScSet1
DmSET1
HsSET1A
HsSET1B
H3K4me
ScJhd2
DmSU(VAR)3-3
HsLSD1
HsLSD2
SpClr4
DmSu(VAR)3-9
HsSUV39H1
HsSUV39H2
H3K9me
ScRph1
DmCG31123
HsJHDM2
ScDot1
DmDOT1L
HsDOT1L
H3K79me
?
ScSas2
DmMOF
HsTip60
HsMOZ
H4K16ac
ScSir2
DmHDAC3
HsSIRT1
HMTs / HATs
Histone Modification
HDMs / HDACs

Figure 1. Histone modifications and conservation of the histone code. Residues H3K4, H3K9, H3K79, and H4K16 are examples of many conserved sites on histones found within nucleosomes (blue) that are post-transcriptionally modified in *Saccharomyces cerevisiae* (*Sc*), *Schizosaccharomyces pombe* (*Sp*), *Homo sapiens* (*Hs*), and *Drosophila melanogaster* (*Dm*). Post-transcriptional acetylation (ac) and methylation (me) of these residues are accomplished by histone acetyltransferases (HATs) and histone methyltransferases (HMTs) and are shown in orange. Removal of histone modifications are accomplished by histone deacetylases (HDACs) and histone demethylases (HDMs) and are shown in green.

Histone acetyltransferases

As with the conservation of histone proteins across species, the sites of modification on histone tails are strikingly similar amongst many organisms. The residues that act as acetylation sites on the N-terminal tails of H3 and H4 are good examples of how the spacing between modifications is conserved. Commonly conserved lysine residues that can be acetylated are H3K9, H3K14, H3K18, H3K23, H4K5, H4K8, H4K12, and H4K16 (Strahl and Allis, 2000). Acetylation of these residues are accomplished by a special set of enzymes called histone acetyltransferases, or HATs. HATs are divided into two types. B-type HATs are mainly cytoplasmic, act on free histones, and play a role in histone deposition. Hat1, the original HAT found in budding yeast, acetylates new H4 on residues K5 and K12 (Parthun, 2007). A-type HATs are usually nuclear and are commonly associated with multiprotein complexes. There are three families of A-type HATs: GNAT, or 'GCN5-Related N-Acetyltransferases', MYST, or 'MOZ-YBF2/SAS3-SAS2-TIP60', and CBP/p300, or 'CREB-/E1A-binding protein' (Hodawadekar and Marmorstein, 2007).

One of the first recorded histone modification by a HAT was in seen in yeast with Gcn5. The HAT activity of Gcn5 was linked to transcriptional activation, and the protein contained two domains common to other nuclear HATs, namely a bromodomain and the active site (Brownell et al., 1996). Shortly after it was discovered that Gcn5 was able to acetylate free histones on its own but required association with the multiprotein SAGA complex to act on nuclear histones. Transcriptional products of TBP-bound promoters are found to be expressed soon after SAGA complex formation, thus linking Gcn5 activity to transcriptional activation (Grant et al., 1997).

The MYST family of HATs was unveiled around the same time when the yeast genes *SAS2* and *SAS3* showed similarity to human MOZ and Tip60 (Reifsnyder et al., 1996). The human oncogene MOZ is a protein with HAT activity and is associated with myeloid leukemia. Sas3 is the yeast homolog of MOZ and is the catalytic subunit of the Gcn5-independent H3 HAT complex NuA3 that binds TBP-associated factors (Borrow et al., 1996; John et al., 2000). Another founding member of the MYST family is human Tip60, homolog to Esa1 in yeast, which acts on H2A, H3, and H4, but not H2B (Kimura and Horikoshi, 1998). In yeast, *ESA1* is an essential gene; mutations lead to failed cell cycle progression that occurs post-replication (Clarke et al., 1999). Esa1 is the catalytic subunit of the NuA4 complex, which is responsible for global acetylation. The NuA4 complex binds DNA for repair and transcriptional remodeling, and acetylates H4 and H2A (Doyon et al., 2004).

The third family of HATs contains the p300/CBP proteins, which were found to bind the E1a oncoprotein from the E1A adenovirus. The interaction between p300 and E1a promotes excess growth and leads to tumor formation (Shiama, 1997). The p300/CBP proteins are known to interact with many cofactors that dictate their specificity. The mechanism of specificity depends on limiting amounts of p300/CBP, allowing acute signals to conduct p300/CBP activity to a determined transcription pathway (Kamei et al., 1996). Some factors that interact with p300/CBP are c-Jun, an early response transcription factor, TFIID, part of the RNA Pol II preinitiation complex, MyoD, a skeletal muscle specific transcription factor, and E2F-1, and early response transcription factor (Roth et al., 2001). The ability of HATs to have a general function

that requires direction by co-factors to be specific is a common theme to histone modifying proteins.

Histone deacetyltransferases

The presence or absence of acetylation on histone tails can cause significant changes to chromatin structure and DNA accessibility, therefore regulating how and when acetylation is removed is equally important as when it is added. The enzymes responsible for removing histone acetylation are known as histone deacetylases, or HDACs. There are four main classes of HDACs. The class I and class II family of HDACs are most closely related to ScRpd3 and ScHda1 respectively. Class IV only contains one known member: mammalian HDAC11. Class III HDACs are classified as sirtuins, as they share homology with ScSir2 and all require nicotinamide adenine dinucleotide, or NAD+ (Yang and Seto, 2007; Cress and Seto, 2000).

Class II has two subclasses that categorize members based on structural differences. Class IIa have an adaptor domain at the N-terminus that binds the transcription factor myocyte enhancer factor 2, or MEF2. Class IIb have an extended tail domain at the C-terminus. Both classes contribute to multiple metabolic pathways and are differentially regulated by subtle differences in structure that promote specific subcomplex formation to dictate localization, activity, and overall specificity (Mihaylova and Shaw, 2013). Class IIa HDACs are regulated by the 14-3-3 family of proteins and that subsequently aid in subcellular localization and promote activity in the nucleus. While Class IIa HDACs are usually found in the nucleus, the location of Class IIa

HDACs requires checkpoint or nutrient sensing signals to relocate within the cell (Muslin, 2000).

Unlike the other three classes of HDACs that are more commonly found in higher eukaryotes, sirtuins are widely conserved from bacteria to humans. Originally, four homologs of *SIR2* in budding yeast, *HST1-4*, were discovered in a screen for effectors of telomere silencing. Overexpression of *HST1* was able to rescue silencing defects generated by *SIR2* mutants, and deletion of *HST3* and *HST4* together rendered silencing at the telomere defective (Brachmann et al., 1995). Humans contain seven sirtuin proteins, SIRT1-7. SIRT1 is the most like Sir2 based on significant homology between their catalytic domains (Frye, 1999). SIRT1 and SIRT2 are found in both the nucleus and cytoplasm, SIRT4 and SIRT5 are only found in the mitochondria, while SIRT6 and SIRT7 are only found in the nucleus (Seto and Yoshida, 2014). SIRTs help regulate the DNA damage response and lead to replicative senescence under prolonged stress, so mutations in mammalian SIRTs have been found to be linked to enhanced growth rates in some cancers (Saunders and Verdin, 2007). Although there are more members of class III HDACs in higher eukaryotes, ScSir2 is still used as a model for researching human disease tied to sirtuins.

Histone methyltransferases

Methylation is another histone modification that has a significant impact on heterochromatin formation and stability. As with acetylation, the residues on histones that accept methylation as a modification have many common examples across a variety of organisms. The pioneering type of histone methyltransferase, or HMT, are SET-domain

proteins that requires S-adenosyl-L-methionine (SAM) to function. Set-domain proteins methylate H3K4, H3K9, H3K27, and H3K36 (Herz et al., 2013). The SET domain is defined from Su(var)3-9, Enhancer-of-zeste, and Trithorax in *Drosophila*. All three HMTs contain similar homology within their chromodomain, and Su(var)3-9 mutants were shown to modify position effect variegation (Tschiersch et al., 1994). Human SUV39H1 and murine Suv39h1 are homologs to Su(var)3-9 in *Drosophila*, and they all have methylation activity for H3K9 (Rea et al., 2000). Clr4 is the sole HMT that acts on H3K9 in fission yeast, but H3K9 is not a target of methylation in budding yeast. However, *S. cerevisiae* does possess Rph1 which can remove H3K9 methylation and is indicative that this organism evolved away from H3K9 regulation (Nakayama and Tatchell, 2001; Klose et al., 2007). G9a-GLP is an example of a murine HMT that requires higher order complex formation to guide SET domain activity, which is a common theme for SET domain containing HMTs (Tachibana, 2005).

H3K4 is another methylation site on histones that regulates transcription and is common to algae, yeast, and humans (Strahl et al., 1999). H3K4 methylation is accomplished by Set1 alone in *S. cerevisiae*, and deletion of *SET1* abolishes H3K4 methylation at rDNA and de-represses it (Briggs, 2011). Yeast Set1 forms a large complex with many subunits known as COMPASS, or Complex Proteins Associated with Set1. Loss of certain COMPASS subunits alters complex stability and overall methylation patterns (Miller et al., 2001). Set1 homologs exist in *Drosophila*, namely SET1, TRX, and TRR. Human homologs of Set1 exist as well, such as SET1A, SET1B, and MLL1-4 (Shilatifard, 2012). Another screen for disruptors of telomere silencing led to the discovery of *DOT1*, as deletion of the gene appeared to effect telomere length

(Singer et al., 1998). Shortly after Dot1 was identified as a HMT that adds methylation modifications to H3K79 (van Leeuwen et al., 2002). DOT1L is conserved from *Drosophila* to humans and is responsible for all three methylation states of H3K79. This group of HMTs do not contain a SET domain but still contain a catalytic domain that requires SAM to function (Nguyen and Zhang, 2011).

H3K27 is another site that is susceptible to tri-methylation and was originally discovered to be acted on by E(z), or Enhancer of Zeste in *Drosophila*. The E(z) enzyme works with the Extra Sex Combs protein (ESC) to direct activity to polycomb binding sites (Czermin et al., 2002). The human counterpart to ESC-E(z) is EED-EZH2, where EZH2 is the homolog to E(z) and the catalytic subunit of the HMT complex. EED, or ectoderm embryonic development protein, acts similarly to ESC to help guide specific activity of EZH2 (Cao et al., 2002). The mammalian PRC2 complex contains many subunits such as EZH1, EZH2, SUZ12, EED, and RbAp46/48 that all act in concert to direct EZH2 activity (Margueron and Reinberg, 2011). H3K27 methylation is a modification found in flies, arabodopsis, worms, and mammals, but not yeast, as only one example of H3K27 methylation exists in the fungus *Neurospora crassa* (Jamieson et al., 2013). H3K36 is another site of methylation in *S. cerevisiae* and is generated by the HMT Set2, but its function is tied to transcription. Set2 can tri-methylate H3K36 and associate with C-terminus of RNA polymerase II to promote the transcription elongation process (Kizer et al., 2005). Other HMTs such as NSD1-3 and SETD2 that are present in mammals also play a role in transcription elongation, repression, alternative splicing, and DNA repair (Wagner and Carpenter, 2012). These examples highlight the broad impact that histone modifications can have on cellular function besides just promoting or

antagonizing transcription. As with HATs and HDACs, some enzymes also exist to remove the methylation marks left by HMTs. The yeast proteins Jhd1 and Jhd2 can remove H3K36 and H3K4 respectively. H3K9 and H3K36 can also be removed by Rph1 in yeast, but there are no known proteins that remove H3K27 methylation in yeast. In contrast, *Drosophila* and humans are able to remove H3K27 methylation, but no organisms are known to be able to remove methylation from a H3K79 site (Hyun et al., 2017).

Ubiquitylation and SUMOylaiton of histones

Histones are susceptible to modification by ubiquitylation and SUMOylation in the same way as other protein targets. The ubiquitin protein is well conserved from yeast to humans and is 76 amino acids in length. Only 3 of the 76 residues in human ubiquitin differ from that of *S. cerevisiae* (Ozkaynak et al., 1984). Yeast contains three ubiquitin genes that are fused to ribosomal proteins: UBI1, UBI2, and UBI3. UBI4 is a fourth ubiquitin gene that is linked by tandem repeats, and is induced by stress, high temperature, and starvation (Ozkaynak et al., 1987). The majority of ubiquitylation occurs on H2A/H2B but has been detected on H3/H4 and H1 as well. The addition of ubiquitin to histone has been shown to be involved in transcription and the DNA damage response (Cao and Yan, 2012; Meas and Mao, 2015). An example of this in yeast is the ubiquitin modification on H2B that is observed only when Rad6, a protein involved in DNA double strand break repair, is present (Robzyk, 2000).

The small ubiquitin-related modifier, or SUMO, does not have a role in protein degradation like its larger counterpart. The target residue for SUMOylation is lysine, a

common site shared with methylation, acetylation, and phosphorylation (Nathan et al., 2003). Humans have four SUMO genes, SUMO1-4, where *S. cerevisiae* has only one SUMO gene, *SMT3* (Huang et al., 2004). There are nine SUMO proteases in humans but only two in yeast, namely Ulp1 and Ulp2 (Hickey et al., 2012). SUMOylation can play a role in protein-protein interactions and alter subcellular localization of modified proteins. A compound effect between ubiquitinylated H2B and H3K4 methylation was shown to redirect the HDAC Set3 resulting in suppression of ncRNA transcription between regions coding genes (Ryu et al., 2020). Any given histone residue that is modified is susceptible to a combined effect from the type of modification and the crosstalk between modifications on the same and neighbouring nucleosomes (reviewed in Xu & Zhu, 2010).

1.4 Conserved functions of heterochromatin

DNA repair

Heterochromatin is involved in more processes than just transcriptional regulation. DNA repair is another cellular function that requires heterochromatic proteins and histone variants. One of the histone variants that play a role in DNA repair is H2A.Z and Htz1 in mammals and *S. cerevisiae* respectively. Chromatin remodeling complexes are recruited to double stranded breaks, or DSBs, to allow repair machinery to pass through nucleosomes by incorporating H2A.Z. Yeast use the chromatin remodeling complex RSC, whereas the mammalian machinery uses proteins such as INO80. This remodeling and incorporation of H2A.Z increases the accessibility repair machinery has for chromatin (Seeber et al., 2013). The SWR1 complex in *S. cerevisiae* and histone chaperones Nap1 and Chz1 are responsible for depositing H2A.Z at DNA damage

response sites (Dronamraju et al., 2017). DSB repair recruits H2A.Z to the break early and requires both Rad51 and SUMOylation of H2A.Z for proper localization to the nuclear periphery (Kalocsay et al., 2009).

In human cells H2A.Z is exchanged onto sites of DNA damage by p400 remodeling complexes and loading of H2A.Z onto chromatin also supports subsequent loading of the Ku70/80 DSB repair protein (Xu et al., 2012). The Ku complex binds DNA as a heterodimer of Ku70 and Ku80 and is one of the initial binding factors in DNA repair. Ku70/80 is the classical protein for non-homologous end joining, or NHEJ, as it forms a sequence-independent ring structure around the site of damage. Ku70/80 acts as a scaffold for the entire repair process and helps signal for the cell to arrest. Ku70/80 also functions in NHEJ of V(D)J recombination in immune processes (Fell and Schild-Poulter, 2014). Both homology directed repair, or HDR, and NHEJ can occur in S-phase, but the phosphorylation state of Ku70/80 helps to dictate that decision. Phosphorylation of Ku70/80 helps dissociate it from DSB ends and thus promotes HDR (Lee et al., 2016). In *S. cerevisiae*, Ku70/80 works similar to mammals in NHEJ, and this repair process is fully compromised if *YKU70* and *YKU80* are deleted. Mutations in these two genes also result in error prone DNA repair (Emerson et al., 2018). The yeast Ku70/80 complex plays the additional role of regulating telomere length, nuclear positioning, and transcription (Fisher and Zakian, 2005).

DSBs are also a site for H2A.X deposition, which becomes phosphorylated swiftly after incorporation into chromatin to make γH2A.X (Rogakou et al., 1998). The main role of γH2A.X is to initiate the signaling cascade for DNA repair. Incorporation of γH2A.X helps anchor chromatin ends together and initiates a checkpoint response giving

the cell time to properly repair the DSB (Mah et al., 2010; Kinner et al., 2008). Although *S. cerevisiae* does not possess H2A.X, an analogous phosphorylation occurs on H2A Ser-129 that helps to initiate repair (Redon et al., 2003). Both yeast and mammalian cells have inefficient γH2A.X signaling in regions of heterochromatin. This inefficient response is due to the association of H2A.X with heterochromatin related machinery such as NuA4 and Ino80. Interestingly, yeast have been shown to have transient γH2A.X signal at telomeres without any source of exogenous DNA damage (Kim et al., 2007). These transient signals demonstrate how dynamic heterochromatin formation is, and how similar some of the components are between constitutive heterochromatin like telomeres and DSB sites that become occupied by heterochromatic proteins.

Centromere formation and cohesion

Centromeres are another form of constitutive heterochromatin where the compaction of DNA has more to do with packaging and organization of chromatin regarding cell division. Centromeres require the centromere-specific histone H3 variant CENP-A. The centromere-associated network, or CCAN, is made up of 16 core proteins that help attach to the kinetochore (McKinley and Cheeseman, 2016). A homogeneous repetitive DNA alpha satellite is what governs centromere location and moving the satellite sequence results in CENP-A relocating to nearby euchromatin (Sullivan et al., 2016). The centromere serves as an attachment point for microtubules, a process in which CENP-A is required. The core of the centromere is flanked by pericentric heterochromatin that has H3K9 methylation marks and is associated with silencing proteins. In short, vertebrate CENP-A is like Cse4 in budding yeast and helps guide the

kinetochore network that is bound to microtubules to find the centromere (Schalch and Steiner, 2017).

The heterochromatin formed at the centromere also supports the kinetochore by allowing a high density of cohesin loading (Pidoux and Allshire, 2005). The cohesin-associated yeast protein Pds5 disrupts silencing if deleted and plays a role in cohesin acetylation. Pds5 helps regulate cohesin loading, and defects in Pds5 can be rescued by cohesin with mutations that mimic acetylation (Folco et al., 2019). The presence of the H3 variant CENP-A helps to distinguish other heterochromatin loci from centromeres. In fission yeast, the centromere also contains under-acetylated H3/H4 and methylated H3K9 that promotes Swi6 binding (Shareef et al., 2001). Swi6 is the yeast equivalent to HP1 in mammals and is required for proper kinetochore function (Nonaka et al., 2002). HP1 is also phosphorylated in response to DNA damage and is thought to help alter chromatin compaction to aid in the association of repair proteins (Dinant and Luijsterburg, 2009). HP1 has 3 paralogs in mammals, HP1α, HP1β and HP1γ. HP1α and HP1β are linked to DNA resection in HDR and single-stranded annealing, while HP1γ is inhibitory to these processes (Soria and Almouzni, 2013). Human Orc2 binds HP1α and HP1β in early G1/S-phase colocalizing at pericentric heterochromatin. Swi6/HP1 must read signals written by Clr4/SUV39H to associate with pericentric chromatin, as Swi6/HP1 has high affinity for methylated H3K9 (Prasanth et al., 2005). These centromere specific histone modifiers are a clear example of how the histone code can conduct the formation of heterochromatin with a specific function using much of the redundant machinery that exists in other condensed areas of the genome.

1.5 Heterochromatin in *S. cerevisiae*

Mating type

The life cycle of *S. cerevisiae* involves sporulation of diploids into haploid cells that take on the 'a' or 'α' mating type. Mutations linked to the mating type locus, or *MAT* locus, were identified by mating sterile spores and screening for the rare formation of diploids. These mutations were specific to either 'a' or 'α' cells and led to the suspicion that mating type specific alleles may exist within the *MAT* locus (Mackay, 1974). The α-specific allele is composed of α1 and α2 genes that produce the alpha1 and alpha2 proteins. Expression of α1, α2, and *MCM1* together leads to α-specific gene activation such as the α-factor pheromone pathway and production of the a-factor receptor (Hagen, 1993). *MCM1* is expressed constitutively and mutations in the gene lead to failed activation of α-specific genes (Bruhn, 1994). The alpha2-Mcm1 complex represses a-specific genes such as the production of a-factor and the α-factor receptor (Smith, 1992). Suppression of a-specific genes is necessary in *MAT*α cells because a-specific genes are constitutively expressed in the absence of alpha2. Concordantly, alpha-specific genes are unable to be expressed in the absence of alpha1 and cells that have the *MAT* locus removed default to a *MATa* mating phenotype (Strathem, 1981). After a successful mating both a1 and alpha2 come together to form a corepressor of haploid genes and an activator of diploid genes. The a1-alpha2 complex downregulates alpha1 to shut down α-specific genes, while the presence of alpha2 continues to repress a-specific genes and together render the diploid non-mating (Harashima, 1989).

Heterochromatin at the HM loci

The yeast *S. cerevisiae* are categorized as homothallic, meaning they possess a process for interconversion of their mating type. The mating type switch is driven by the *HO* gene that encodes an endonuclease with specificity for the *MAT* locus. This activity was recognized as a heritable event and gave rise to the 'cassette' hypothesis where extra copies of 'a' and 'α' information could be rearranged into the *MAT* locus (Hicks, 1977). This hypothesis was later confirmed using mutants that could change the mating type of heterozygous *MATa*/*MATa* diploids and rescue the ability to sporulate. These mutants disrupted a silent copy of the 'α' genes allowing the formation of the a1-alpha2 complex and restoration of the diploid phenotype (Haber, 1979). These extra copies of the homothallic mating type information were identified as *HMLα* and *HMRa* and contain the same information as *MAT*α and *MATa* but are continuously silenced by heterochromatin (Figure 2A). The mating type information is different between *HML*α and *HMRa* but the surrounding DNA elements are similar for both loci (Nasmyth, 1980).

Silencing of *HMLα* and *HMRa* and depends on two surrounding sites approximately 1kb to either side that have ARS activity. Deletion of one of these sites abolishes repression while the other only results in a partial loss of silencing (Abraham, 1984; Feldman, 1984). These silencer elements were termed 'E' for essential and 'I' for important, both of which are orientation-independent and can act on promoters up to 2.6kb away (Brand, 1985). Both *HML-E* and *HML-I* are each sufficient for the full repression of *HML*, and both must be removed to lose its suppression. In contrast, *HMR-E* is necessary, but *HMR-I* is dispensable for proper repression of *HMR*, so *HMR-E* must be removed for silencing to be lost at *HMR* (Mahoney, 1989). Placing an *ADE2* reporter

within *HMR* in conjunction with a compromised *HMR-E* element and mutations in *RAP1* revealed that both *cis* and *trans* regulation is required for proper silencing of the *HM* loci (Sussel, 1993).

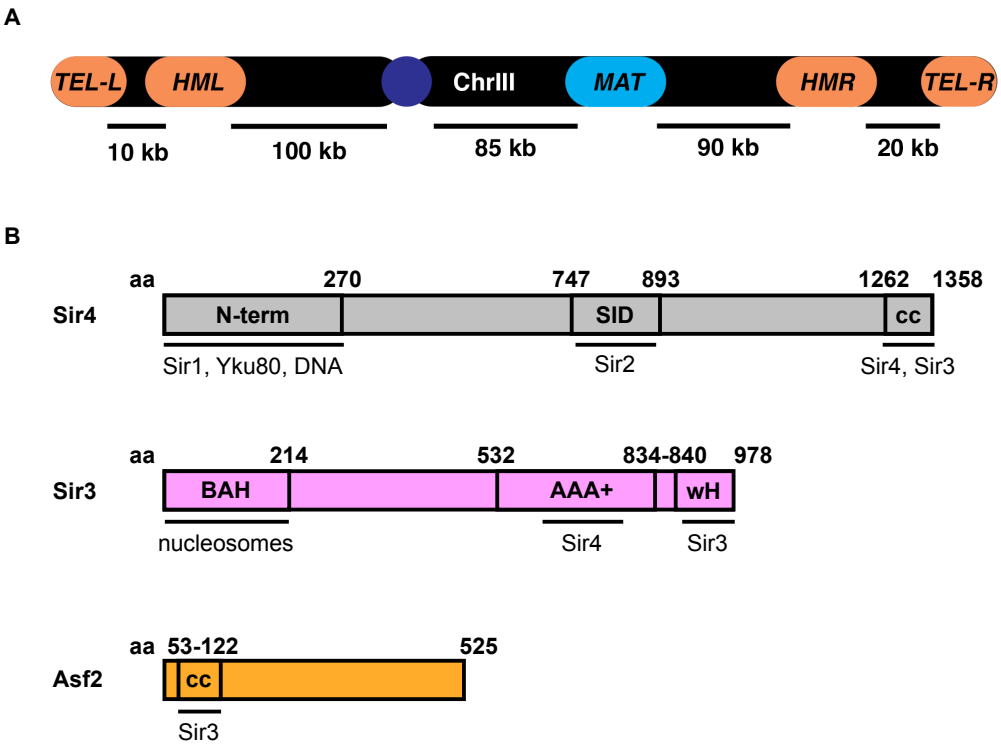

A
TEL-L
HML
ChrIII
MAT
HMR
TEL-R
10 kb
100 kb
85 kb
90 kb
20 kb
B
Sir4
aa
270
747
893
1262
1358
N-term
SID
cc
Sir1, Yku80, DNA
Sir2
Sir4, Sir3
Sir3
aa
214
532
834-840
978
BAH
AAA+
wH
nucleosomes
Sir4
Sir3
Asf2
aa
53-122
525
cc
Sir3
C
wH
CC
AAA+/CC
Sir4
Sir2
SID
Sir3

Figure 2. SIR-mediated heterochromatin in *S. cerevisiae*. (A) A schematic of chromosome III (ChrIII) indicating the regions of SIR-mediated silencing. Heterochromatin (orange) forms at the left and right end of the chromosome at telomeres (*TEL-L* & *TEL-R*) and at the two silent mating-type cassettes: homothallic mating left (*HML*) and homothallic mating right (*HMR*) relative to the centromere (dark blue). The mating type of the cell is determined by the genes expressed (light blue) from the mating type locus (*MAT*). **(B)** Linear maps of the amino acid sequences of Sir4, Sir3, and Asf2. Interaction surfaces are marked with a line and labeled with the respective interaction targets. The amino acid (aa) sequence number is indicated above the map. BAH = Bromo-adjacent homology domain. **(C)** The SIR complex is formed by two heterotrimers consisting of Sir2 (green), Sir3 (pink), and Sir4 (grey). Dimerization of Sir3 through a winged-helix domain (wH) and dimerization of Sir4 though a coiled-coil domain (CC) promote the formation of the core SIR complex. The complex is further stabilized by interactions between the AAA+ domain of Sir3 (AAA+) and the coiled-coil domain of Sir4 (CC). The histone deacetylase activity of Sir2 is guided by the SIR complex though its association with Sir4 (SID).

1.6 The SIR complex

Discovery of the SIR proteins

The Silent Information Regulator proteins, or SIR proteins, are the main components of heterochromatin at telomeres, rDNA, and the mating type loci in *S. cerevisiae*. The SIR proteins are unique in the fact that they are required for the silencing of the homothallic mating type loci *HML* and *HMR* (Ivy et al., 1986). Four genes were discovered that disrupted the silent state of the *HM* loci, namely *SIR1-4* (Rine and Herskowitz, 1987). Sir1 is required to keep the *HM* loci silent, and mutation of Sir1 renders haploid cells unable to mate (Rine et al., 1979). Mutation of *SIR2-4* leads to silencing loss at telomeres, but *SIR1* mutation has no effect on telomere silencing and argues for it having a specific role at the *HM* loci. These results highlight the fact that the heterochromatin structures at telomeres and *HM* loci are similar, yet distinct (Aparicio et al., 1991). Sir3 and Sir4 colocalize with Rap1 to Y' telomere sequences and appear as foci along the nuclear periphery. Mutating any of the involved proteins or overexpressing the C-terminal domain of Sir4 disrupts these foci indicating that a multicomponent complex is required for telomere silencing (Cockell et al., 1995; Gotta et al., 1996). Sir3 binds both Sir4/Sir2 and Rap1, all of which are present at both *HM* loci and telomeres (Hecht et al., 1996).

Sir2

The most evolutionarily conserved and main enzymatic component of the SIR complex is Sir2 (Figure 2C). Sir2 is an HDAC that is required for the silent state of heterochromatin. The enzymatically inactive mutant sir2-H364Y is unable to mate,

silence telomeres, or silence the rDNA loci (Tanny et al., 1999). A unique side product of Sir2 HDAC activity is O-acetyl-ADP-ribose, or AAR (Tanner et al., 2000). AAR, nicotinamide, and a deacetylated peptide are formed 1:1:1 during a single Sir2 reaction, an activity that requires NAD+ and is inhibited by nicotinamide (Landry et al., 2000). Sir2 can act on H3K9, H3K14, H4K5, H4K8, H4K12, and H4K16, however only H3K9, H3K14, and H4K16 are important for silencing (Imai et al., 2000). Sir2 is found at both *HM* loci and telomeres with Sir3 and Sir4 but acts independently of Sir3 and Sir4 at rDNA where it is recruited by Net1. Mutation of Sir2 can separate rDNA and *HM*/telomere function, although each category of mutations maps to the same core domain of Sir2 (Cuperus, 2000). Sir2 therefore exists in two distinct complexes, Sir4-Sir2 and Net1-Sir2. When in association with Sir4 and acting on *HM*/telomeres HDAC activity is NAD-dependent, whereas the Sir2-Net1is less dependent on NAD+ (Ghidelli, 2001). Sir2 is the only SIR protein required for rDNA repression, and Sir2 has been shown to form a homotrimer important for this function. Mutant sir2-P394L selectively disrupts homotrimer formation and rDNA silencing, while *HM* and telomere silencing remain intact (Cubizolles et al., 2006). These separate functions of Sir2 HDAC activity are governed by the complex it is associated with; the focus of which will be *HM* and telomere silencing when Sir2 is in association with Sir3 and Sir4.

Sir3

Sir3 is a paralog of Orc1, sharing homology between the N-terminal BAH domain and the C-terminal AAA+ domain (Figure 2B). One significant difference between Orc1 and Sir3 is that the AAA-like domain of Sir3 has lost the ability to bind ATP but gained

the ability to bind Sir4 (Ehrentraut et al., 2011). The central region of Sir3 between the BAH and AAA-like domains is still required for silencing as it is sufficient and necessary to bind the C-terminus of Rap1. Deletion of this central region of Sir3 however is less detrimental to silencing than removal of the C-terminus in Rap1 (Moretti and Shore, 2001). Expression of the N-terminal BAH domain of Sir3 alone can rescue some silencing defects brought on by the deletion of *SIR3*, although this rescue requires the overexpression of Sir1 (Connelly et al., 2006). Many mutations have been mapped to both the BAH and AAA-like domains of Sir3, and the majority that are found within the BAH domain interrupt the interaction between Sir3 and nucleosomes (Buchberger et al., 2008).

Basic residues on histone H4 are critical for *HM* silencing and mutating basic residues particularly near H4K16 hinders Sir3 binding (Johnson et al., 1990). The interaction between the C-terminus of Sir3 and histone H4 weakens in a cumulative manner as the tail of H4 becomes more and more acetylated. Sir3 binding to H4 is 50-fold stronger when residues H4K5, H4K8, H4K12, and H4K16 are not acetylated compared to when they all possess the acetylation modification (Carmen et al., 2002). The N-terminus of Sir3 also contains a winged helix domain that is required for Sir3 dimerization *in vitro* and *HM*/telomere silencing *in vivo*. The importance of this dimerization is highlighted by experiments replacing the winged helix domain with a bacterial dimerization domain where defects in the winged helix mutant are restored (Oppikofer et al., 2013). Sir3 must interact with itself, histones, and the other SIR proteins in a hierarchy of events that result in completely functional and mature heterochromatin.

Sir4

Sir4 is a large, 152 kDa, lysine and serine-rich protein that supports interactions between the other SIR proteins, DNA, and nucleosomes (Figure 2B). The expression of the C-terminus of Sir4 alone is enough to complement nonsense mutations in *SIR4* but not a full deletion of the gene. In addition, overexpression of the C-terminal domain disrupts silencing (Marshall et al., 1987). The C-terminal domain of Sir4 exists as a dimer *in vitro*, and this dimer formation is also thought to be required for silencing of *HM* loci and telomeres *in vivo* (Murphy et al., 2003). Sir4 has binding sites for Sir2 and Sir3, bridging the two proteins in a 1:1:1 ratio as a heterotrimer (Chang et al., 2003). Sir4 has subnuclear localization like Sir3 and telomeric Rap1, but Rap1 foci are lost when Sir3 or Sir4 are mutated. Sir3 and Sir4 are therefore required for proper subnuclear organization that plays a role in gene repression (Palladino et al., 1993). Sir4 can bind telomeres without Sir2 and Sir3, as it is recruited to telomeres by Rap1, however Sir4 and Rap1 do not reach distal regions without the other SIR proteins and Sir2 activity (Luo, 2002). Mutations in Sir4 have also been linked to aging capacity and lifespan, as these mutations prevent proper SIR protein binding to *HM* loci and telomeres causing an imbalance in the redistribution of SIR proteins amongst silent loci (Kennedy et al., 1995).

Acetylation of the SIR proteins

The SIR proteins are no exception to being targets of post-transcriptional modifications such as acetylation. Sir3 and its paralog Orc1 are both acetylated by NatA, an N-terminal acetyltransferase complex that is required for silencing at telomeres. Mutation of *ARD1* or *NAT1*, two subunits of NatA, leads to defective silencing

(Geissenhöner et al., 2004; Wang, Connelly et al., 2004). The acetylation of Sir3 has been shown to affect its silencing capacity but not its nuclear organization. Overexpression of Sir3 leads to telomere clustering as telomere foci get larger and localize to the nuclear interior. This reorganization of foci is still seen in *sir3-A2Q* mutants that block acetylation, but renders silencing defective (Ruault et al., 2011). The *sir3-A2Q* silencing defect is due to a compromised interaction with nucleosomes because incorporation of mutant histones H3-D77N and H4-H75Y can restore silencing to the *sir3-A2Q* mutant (Sampath et al., 2009). Crystal structures later confirmed that acetylation of the N-terminal BAH domain in Sir3 supports an interaction with the nucleosome (Arnaudo et al., 2013). The BAH domain interacts closely with histone H3 and binding of Sir3 to the nucleosome is coordinately enhanced when the BAH domain is acetylated and H3K79 is not methylated.

Mutations in *SIR1*, *POL32*, and *DOT1*, the HMT that acts on H3K79, can lead to non-specific binding of Sir3 to nucleosomes (van Welsem et al., 2008). Complementary to these findings, a set of *SIR3* mutants exists that enhance defects seen in *SIR1* mutants. This set of *SIR3* mutations disrupt telomere silencing on their own, but still have a high mating efficiency. When combined with mutations that disrupt the formation of the NatA complex, mating is lost (Stone et al., 2000). These findings suggest that the acetylation of Sir3 can change the affinity Sir3 has for nucleosomes, and that this effect can become more significant when combined with certain histone modifications or the absence of SIR protein binding partners.

Phosphorylation of the SIR proteins

As with acetylation, the SIR proteins are also susceptible to phosphorylation as a post-transcriptional modification. Sir3 is a target of hyperphosphorylation by MAPK in response to pheromone, heat shock, and starvation. When Sir3 is hyperphosphorylated silencing gets stronger at telomeres and mutations in the MAPK pathway block this enhancement (Stone and Pillus, 1996). Stress induced by rapamycin or chlorpromazine leads to hyperphosphorylation of Sir3 and results in a reduction of telomere silencing but no effect on silencing at *HML*. Mutations that mimic the hyperphosphorylated state of Sir3 placed near the BAH domain had no effect on silencing at *HM* loci or rDNA. Although previous studies observed changes in silencing at telomeres, the specific sites of phosphorylation and overall physiology induced by various stressors could account for the differences (Ai et al., 2002). When the cell commits to growth the MAPK pathway is activated and causes hyperphosphorylation of Sir3. However, this state of Sir3 has been demonstrated to decrease lifespan, and *sir3-S275A* mutants that block phosphorylation suffer a less detrimental effect. Overall, specific phosphorylation sites of Sir3 appear to have separate effects on silencing at the *HM* loci, telomeres, and rDNA (Ray et al., 2003).

Another gene that indirectly regulates Sir3 is *ZDS1*, a regulator of Swe1-dependent growth including mitotic entry and exit. Deletion of *ZDS1* results in an increased lifespan due to decreased Sir3 phosphorylation. Deletion of *ZDS2*, a paralog of *ZDS1*, causes decreased silencing at rDNA and increases Sir3 phosphorylation. The Zds2 protein interacts with Orc1 and the SIR proteins though a two-hybrid assay, but Zds1 does not. Zds1 may be involved in the recruitment of a kinase that acts on Sir3 (Roy et al., 2000). Sir3 phosphorylation has also been linked to cohesin-related silencing defects

at telomeres. The *mcd1-1* and *smc3-1* mutations are able to derepress some subtelomeric loci resulting from an increase of H4K16 acetylation, H3K79 methylation, and MAPK driven Sir3 hyperphosphorylation. These combined effects produce a similar effect as *mcd1-1 sir2*Δ cells where compaction of subtelomeric heterochromatin is compromised (Kothiwal and Laloraya, 2019).

Telomere silencing is also reduced by mutations in the RAN GTPase system, such as *rna1-1*, that leads to an accumulation of hyperphosphorylated Sir3. The Ran GTPase system regulates cellular timing during telophase to ensure proper centrosome function and nuclear envelope assembly. Interestingly, a high dosage of Sir3 can circumvent defects brought on by phosphorylation that is perhaps due to the incorporation of unmodified Sir3 into heterochromatin (Hayashi et al., 2007). Sir4 is also a target of phosphorylation as it has Cdk1 consensus phosphorylation sites such as S63 and S84. The N-terminal domain of Sir4 is the regulatory domain and can be hyperphosphorylated during mitosis, but the effect on silencing that these modifications have on their own are poorly categorized (Kueng et al., 2012).

Ubiquitylation and SUMOylation of the SIR proteins

The SIR proteins interact with ubiquitin-related modifiers to alter the state of heterochromatin by modifying histones. Ubp3 binds Sir4, and *ubp3*Δ cells have improved silencing at the *HM* loci and telomeres. It is likely that Ubp3 interacts with the SIR complex to render it inactive until it is degraded, or that it acts indirectly through another protein to accomplish the same task (Moazed and Johnson, 1996). Sir2 is responsible for keeping H4K16 acetylation low at sites of heterochromatin, but low levels of H2B

ubiquitylation are also important for silencing and is maintained by Ubp10. Removal of ubiquitin from H2B is thought to help Sir2 localize to telomeres (Emre et al., 2005). Disrupting Ubp10 leads to changes in subtelomeric genes; many of which are regulated in response to reactive oxygen species. Inactivating Sir4 in combination with Ubp10 mitigates changes in this set of genes. Ubp10 also interacts with Sir4, an interaction that is important for proper establishment of the histone code as demonstrated by *ubp10*Δ strains that have increased H3K4 and H3K79 methylation. Lower levels of these heterochromatin marks provide a more favorable environment for SIR protein binding (Orlandi et al., 2004; Gardner et al., 2005). Ubp10 co-assembles with the SIR proteins as silent chromatin transitions through a transcriptionally active state, supporting the proper reformation of heterochromatin (Zukowski et al., 2018).

Sir2 is the most recognized candidate for SUMOylation, a modification that plays a role in its subcellular localization. Sir2 is usually associated with telomere clusters at the nuclear periphery and with rDNA in the nucleolus. Sir2 has multiple lysine residues that are SUMOylated by Siz2, one of particular interest being K215. SUMOylation of Sir2 on K215 inhibits telomere silencing and blocking SUMOylation by mutating K215 can rescue this phenotype. Sir2 SUMOylation at K215 also compromises the Sir2-Sir4 interaction, but not the Sir2-Net1 interaction, and thus causes a change in the localization of Sir2 towards the nucleolus (Hannan et al., 2015).

The suppression of mutants in the pre-replication complex

The SIR proteins are involved in a variety of cellular processes by association. The entire genome must be replicated for successful cell division and some regions are

compacted into heterochromatin that hinder this process. The pre-replication complex, or pre-RC, is special machinery that exists to prime DNA for replication. The pre-RC is made up of the origin recognition complex (ORC), minichromosome maintenance proteins (MCM), and Cdc45. These complexes work together and are loaded onto replication origins during G1-phase of the cell cycle (Aparicio et al., 1997). The structure of the pre-RC is well conserved in eukaryotes and consists of Orc1-6, Cdc6, Cdt1, and Mcm2-7, however, there are differences in the DNA sequence elements that recruit these proteins between species (Speck et al., 2005). The DNA elements, such as the *ARS* sequence in yeast, is detected by ORC and allows the subsequent loading of Cdc6 and Cdt1 which are loading factors for the MCM proteins that possess helicase activity. This complex fully assembles before S-phase but is defective for assembly in *cdc6-4* mutants. Interestingly, disrupting Sir2 function can suppress the defective assembly of *cdc6-4* mutants. This genetic interaction indicates that Sir2 activity negatively regulates DNA replication initiation. It is important to mention that disruption of Sir3 and Sir4 also suppress the *cdc6-4* pre-RC assembly defect, but to a lesser extent than Sir2 (Pappas, 2004).

Human Mcm10 interacts with DNA, protein components of the replisome, and is an acetylated protein that is subject to SIRT1 activity. Deacetylation of Mcm10 by SIRT1 is an important step for proper fork initiation (Fatoba et al., 2013). Sirtuin-mediated deacetylation of pre-RC components seems to be a conserved process that is critical for proper origin firing. In yeast, five origins that are sensitive to Sir2 activity have been identified on chromosome II and VI. These origins were associated with an inhibitory sequence downstream of ORC binding sites where Sir2 activity leads to Abf1-mediated

nucleosome exclusion (Crampton et al., 2008). Deletion of Sir2 restored 80% of MCM loading in *cdc6-4* mutants with H4K16 acetylation present. Wild type cells have depleted H4K16 acetylation at origins suggesting that recruitment of Cdc6 and removal of H4K16 acetylation function cooperatively to promote timely origin firing. Sir3 has enhanced affinity for deacetylated H4K16 and is found on nucleosomes adjacent to origins. The nearby binding of Sir3 to origins can be promoted by the acetylation mimic H4K16Q and is enough to partially suppress *cdc6-4* mutants as well (Hoggard et al., 2018).

Early firing origins appear to be more dependent on Sir2-depleted H4K16 acetylation and Sir3 binding. Deletion of Sir2 leads to faster MCM loading at early firing telomere origins, while simultaneously causing late replicating origins to be delayed even further. It is thought that these early firing origins become hyperactive when Sir2 is deleted and ruins the distribution of MCM proteins between late and early firing origins (Hoggard et al., 2020). Sir2 activity on these origins renders chromatin more accessible for replication machinery and highlights how changes to H4K16 can provide a range of functions based on chromosomal location and cell cycle position.

1.7 Mechanisms of SIR-mediated heterochromatin

SIR recruitment to HM loci and telomeres

The SIR proteins are recruited to and spread along the *HM* loci and telomeres using similar yet distinct mechanisms. The silencers at the *HM* loci contain a combination of A, B, and E elements. These elements contain homology to activator sequences, such as the A element that resembles an autonomous replication sequence that is bound by Orc1 (Brand et al., 1987; Triolo and Sternglanz, 1996). Rap1 binds the E element, and the

related ARS binding factor Abf1 binds to the B element (Diffley and Stillman, 1989). Both *HML-I* and *HMR-E* contain A, B, and E elements, *HML-E* contains A, and E elements, and *HMR-I* contains A and B elements. The orientation of the silencers is asymmetrical to nucleosome positioning and leads to directional silencing specific to the *HM* loci, and SIR-silencing directionally coincides with the B element of the silencer (Zou et al., 2006). The A, B, and E elements can be circumvented if replaced with a *GAL4* DNA-binding motif and *GBD-SIR1* is expressed. Tethering Sir1 to these modified *HM* loci was sufficient to silence them and tethering Sir1 to telomeres also slightly increased silencing (Chien et al., 1993). However, Sir1 does not play a role in the nucleation of heterochromatin at telomeres as it does for the *HM* loci.

Rap1 binds the telomere at $TG_{1\text{-}3}$ repeat sites and is the main recruiter for Sir3 at telomeres. Mutations in Rap1 that disrupt the Sir3-Rap1 interaction are defective for telomere silencing, but tethering Sir3 to telomeres can restore it (Lustig et al., 1996). The yeast Ku complex, Ku70/Ku80, is another recruiter of SIR proteins that is essential for telomere silencing. The Ku complex is also involved in DNA repair, and becomes less efficient when *SIR2*, *SIR3*, or *SIR4* are deleted (Boulton, 1998). Ku70 and Ku80 are particularly important for binding the ends of DNA in DSB repair, and mutations in either protein that reduce their affinity for DNA compromise the repair process. These same mutants were still able to contact Sir4 as the N-terminal domain of Ku80 was left unaffected and binds Sir4 directly. Oppositely, if the residues important for interaction with Sir4 in Ku80 are mutated there is less Sir3 recruited to telomeres and silencing cannot be established (Lopez et al., 2011; Roy et al., 2004).

Mechanism of SIR spreading

Of all the SIR proteins, Sir1 is only found at silencers flanking the *HM* loci. The spread of heterochromatin from silencers is accomplished by the SIR complex and requires only Sir3, Sir4, and Sir2 HDAC activity (Figure 3). The N-terminal domain of Orc1 contains a BAH domain that is necessary for recruitment of Sir1 to the E element of silencers at the *HM* loci (Rusché et al., 2002; Zhang, 2002). Rap1 plays a role in recruitment of the SIR proteins to both *HM* loci and telomeres thus generating an inherent competition of heterochromatin establishment between these loci. This competition can be manipulated by tethering GBD-Rap1 to any given silent loci and results in a redistribution of SIR proteins amongst the other loci (Buck and Shore, 1995). The C-terminal domain of Rap1 is important for recruiting Sir3 and Sir4, and tethering of Rap1 to an internal location of the chromosome can cause weak silencing of genes not found within regular heterochromatin loci (Marcand et al., 1996).

The formation of stable heterochromatin with robust silencing requires several steps of maturation aside from just SIR protein recruitment. Although Sir4 and Sir2 can localize to chromatin and have HDAC activity without Sir3, they require Sir3 to spread. Inversely, Sir2 HDAC activity is not required for the initial recruitment of Sir3 and Sir4 but is required for the SIR complex to propagate along chromatin. The loss of H4K16 acetylation, and eventual disruption of H3K79 methylation are important steps in the maturation of heterochromatin and the spreading of SIR proteins (Hoppe et al., 2002; Yang et al., 2008). The different requirements for nucleation and spreading of heterochromatin are not fully understood and involve a combination of histone modifications and interactions between SIR proteins and other SIR-interacting factors.

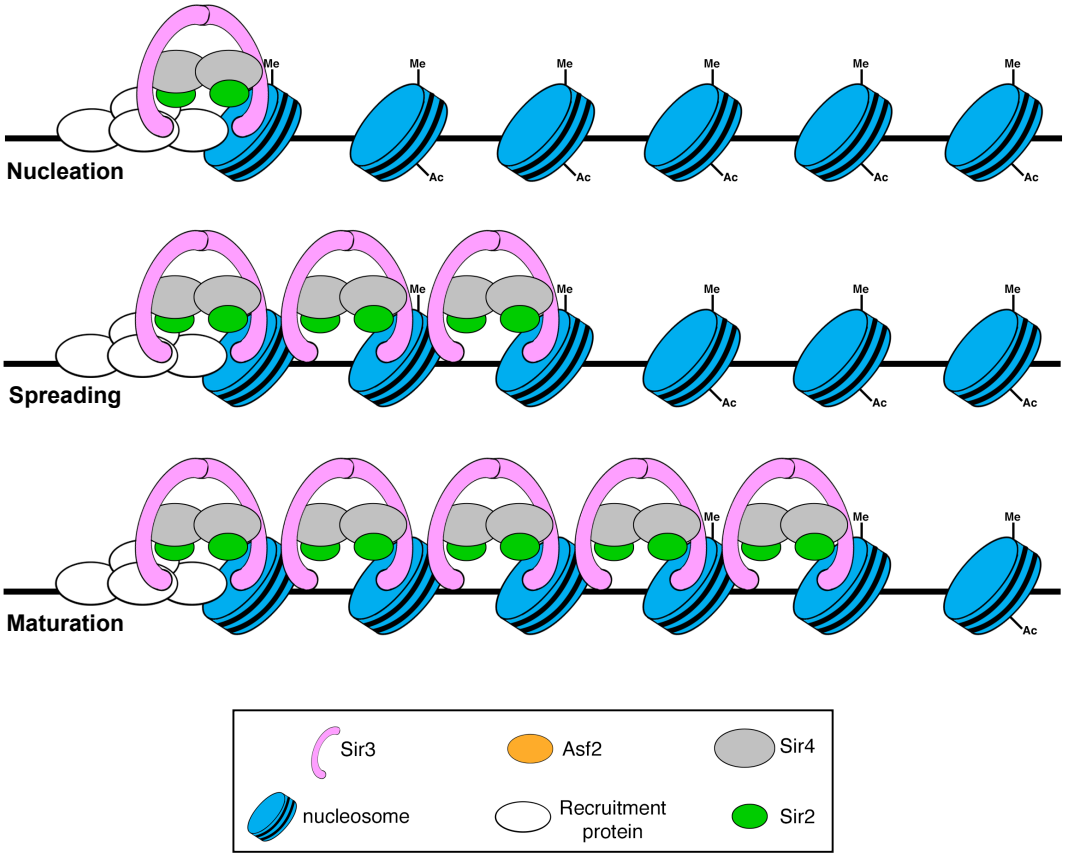

Me
Ac
Nucleation
Spreading
Maturation
Sir3
Asf2
Sir4
nucleosome
Recruitment protein
Sir2

Figure 3. The formation of SIR-mediated heterochromatin. The nucleation of heterochromatin begins with the SIR complex being recruited to chromatin through DNA binding proteins (white) such as Orc1, Sir1, Abf1, Rap1, and Ku70/80. The BAH domain of Sir3 is mainly responsible for interacting with nucleosomes that are originally acetylated (Ac) at H4K16 and methylated at H3K79 (Me). SIR-nucleosome and SIR-SIR interactions promote the spreading of the SIR complex and the formation of heterochromatin. As the SIR complex spreads Sir2 histone deacetylase activity removes acetylation from H4K16 and promotes subsequent interactions between the SIR proteins and neighboring nucleosomes. Limiting amounts of the SIR proteins and/or histone modifications acting as boundary signals modulate the length of heterochromatin and the eventual loss of methylation at H3K79 coincides with fully functional heterochromatin.

Chapter 2: Materials and Methods

2.1 Strain and Plasmid Construction

Standard methods were used for cloning and manipulation of yeast strains (Green et al., 2012; Sherman, 2002).

ASF2-TAP-TRP1 and *SIR-TAP-TRP1* strains were generated by transforming a PCR product amplified from *pBS1479* (Puig et al., 2001) with oligos ADR48/49 and DM445/446 respectively.

SIR3-13Myc-kanMX and *ASF2-13Myc-kanMX* strains were made by transformation using a PCR product amplified from the *pFA6a-13Myc-kanMX6* (P10, Longtine et al., 1998) plasmid with oligos ADR77/78 and ADR49/60 respectively. Cells with the *sir3*Δ*::kanMX* mutation were made by transformation using a PCR product amplified from the *pFA6a-kanMX6* (P1, Longtine et al., 1998) plasmid with oligos ADR77/78.

Cells with the *sir2*Δ*::LEU2* and *sir3*Δ*::LEU2* mutations were generated from pJH103.1 and pJH107.1 plasmids respectively (Ivy et al., 1986).

The *sir2*Δ*::HIS3* (JRY3433) and *sir3*Δ*::HIS3* (JRY3289) strains were kindly provided by Jasper Rine, University of California, Berkeley.

Strains with the *sir4*Δ*::natMX* mutation were constructed by transformation using a cassette amplified from *pAG25* (Goldstein et al., 1999) with oligos ADR102/103. Strains with the *sir4*Δ*::kanMX* mutation were constructed by transformation using a cassette amplified from *pFA6a-kanMX6* (P1, Longtine et al., 1998) with oligos ADR102/103.

Strains with the *asf2Δ::Sphis5* mutation were constructed by transformation using a cassette amplified from *pFA6a-HIS3MX6* (P3, Longtine et al., 1998) with oligos ADR49/50. Strains with the *asf2Δ::kanMX* mutation were constructed by transformation using a cassette amplified from *pFA6a-kanMX6* (P1, Longtine et al., 1998) with oligos ADR49/50.

The *ASF2-GFP-Sphis5* strain was generated by transforming a PCR product that was produced using the *pFA6a-GFP-HIS3MX6* plasmid (P6, Longtine et al., 1998) and oligos ADR49/60. The *SIR4-eGFP-HIS3* strain was generated by transforming a PCR product that was produced using the pAR482 plasmid and oligos ADR141/142. pAR482 was produced using the pKT128 plasmid (Sheff and Thorn, 2004).

The *asf2-NRN* mutation was generated by combining three PCR products; one from pAR25 (Goldstein et al., 1999) using ADR1451/1452, and two from wild-type genomic DNA using ADR1455/74 and ADR1449/312. The three PCR products were reamplified with ADR1451/312 to generate the final PCR product for transformation. Cells were selected based on their resistance to hygromycin and the presence of the *asf2-NRN* mutation was confirmed using ADR52/752 followed by digest with EcoRV.

Cells harboring *hphMX-ASF2* or the *hphMX-asf2-NRN* mutation were constructed by transforming a PCR product that was generated from pAR387 (pAG32, Goldstein et al., 1999) using oligos ADR49/50 or ADR309/312. Cells with the *Sphis5-asf2-NRN* mutation were constructed by transforming a PCR product into ADR8132 that was generated from *pFA6a-HIS3MX6* (P3, Longtine et al., 1998) using oligos ADR703/704.

Strains with the *hmrΔE-TRP1* reporter (Sussel et al., 1993) were constructed by crossing derivatives of CCFY100 (kindly provided by Kurt W. Runge, The Lerner

Research Institute, Cleveland, OH; Roy and Runge, 2000) to create ADR4062 which was subsequently used to create other *hmrΔE-TRP1* strains.

Strains harboring the *TEL-V-R-URA3* reporter were generated by cross using derivatives of CCFY100 (Ray et al., 2003, kindly provided by Kurt W. Runge, The Lerner Research Institute, Cleveland, OH,). The *TEL-VII-L::URA3* strains were made by using *pTEL::URA3* (kindly provided by Dan Gottschling, Fred Hutchison Cancer Research Center) and subsequent crosses to wild-type strains. Strains with the *TEL-IX-R-URA3* reporter were generated by transforming a PCR product amplified from wild-type genomic DNA with ADR2160/2161 that was inserted roughly 2kb from *TEL-IX-R*, a site of Sir protein enrichment (Ellahi et al., 2015). Insertion was confirmed by PCR with ADR2102/1227 and ADR2103/1235.

Cells with the *his3-11,15::pGAL-HIS3* integration were generated by transformation of the pAR380 plasmid cut with Nhe1. Cells with the *his3-11,15::pGAL-ASF2-HIS3* and *his3-11,15::pGAL-asf2-NRN-HIS3* integration were made by transforming pAR378 and pAR1326 plasmids cut with Nhe1 respectively. The pAR378 and pAR1326 plasmids were made by ligating a PCR fragment generated from either ADR4006 or ADR8082 with oligos ADR53/54 into the pAR380 plasmid cut with EcoRI and NotI.

Cells with the *sas2*Δ*::natNT2* mutation were made by transformation using a PCR product amplified from *pFA6-natNT2* (Euroscarf B1, Janke et al., 2004) using oligos ADR1112/1113. Cells with the *rpd3*Δ*::hphNT1* mutation were made by transformation using a PCR product amplified from *pFA6-hphNT1* (Euroscarf A1, Janke et al., 2004) using oligos ADR1378/1379.

Strains with the *LEU2::GAL-ppr1-1* and *LEU2::GALPPR1-1* promoter were a gift from Dan Gottschling that were originally used in Aparicio and Gottschling, 1994. Further mutations were introduced by cross.

The *sir3-2A*, *3A*, and *4A* mutations were generated by site-directed mutagenesis of a single-stranded plasmid template carrying wild type Sir3 (pAR466, Buchberger et al., 2008; Kunkel, 1985; Gerson, 2015) using oligos ADR977/978/979. Sir3 mutagenesis was accompanied by insertion of a cryptic PstI restriction site, allowing mutants to be screened by restriction digest. The pJB16-derived *sir3* mutant plasmids were screened for successful incorporation of *sir3-2A, sir3-3A,* and *sir3-4A* mutations by digestion with Pst1 (New England Biolabs). Plasmids containing the mutations were digested with NdeI and SalI or NotI and SalI (New England Biolabs) to generate *sir3* gene fragments containing the mutations and these were used to create pAR988, pAR986, and pAR1064 by gap repair of *CEN-LEU2-SIR3-FLAG3* (pAR979). Plasmids were screened for *sir3* mutation by restriction digest with PstI, and expression of *SIR3-FLAG* was confirmed by Western blot.

2.2 Serial growth assays

Cells were cultured in liquid media (YEP +2% glucose, SC-LEU +2% glucose, or SC-HIS +2%glucose) to saturation (OD_{600} = 1-2) at 30°C before being serially diluted 10-fold and spotted onto solid media. Plates were incubated at 30°C for 48-72 h then imaged using white light. Silencing of the *TRP1* reporter was assessed by the viability of cells on solid SC-TRP media and silencing of the *URA3* reporter was assessed by the

viability of cells on solid SC-URA media or by the lack of growth on solid SC+FOA media.

2.3 Quantitative mating assay

Cells were grown overnight on solid media (YEP +2% glucose) at 30°C before being diluted into liquid media (YEP +2% glucose) at an OD_{600} of 1-2 for tester strains and 0.4-0.8 for experimental strains. Diluted cells were grown at 25°C for 3-4 hours and the OD_{600} value of each culture was measured again. Approximately 10^6 cells of the tester strain were diluted into a small volume of liquid media (YEP +2% glucose) with 200 cells of the experimental strain and mixed well. The small volume of mixed cells was plated onto solid media (SD +2% glucose) and grown for 3 days at 25°C. Approximately 1000 cells of the tester strain alone were plated onto solid media (SD +2% glucose) as a negative control, and 200 cells of the experimental strain alone were plated onto solid media (SC +2% glucose) as a positive control and reference to the experimental count. Colonies were imaged using the Bio-Rad ChemiDoc and counted manually. Mating efficiency is reported as a percentage (mean +/- standard error, n=3) based on the number of colonies that formed on SD media from the mixture of tester and experimental cells compared to the number of colonies that formed on SC media from the experimental strain alone.

2.4 Antibodies Used

The following antibodies were used in this study: affinity-purified rabbit polyclonal anti-Sir2, anti-Sir3 (Moazed et al., 1997), and anti-Sir4 (Larin et al., 2015);

mouse monoclonal anti-c-Myc (9E10, Covance); rabbit polyclonal anti-FLAG (Sigma-Aldrich); IgG from rabbit serum (Sigma-Aldrich); anti-GFP serum (a gift of Aaron Straight, Stanford University, Standford, CA); anti-Asf2 antibody was generated as follows. GST-Asf2 (aa 1-90) was expressed and purified in bacteria from pAR741 in which Asf2 (aa 1-90) was amplified (oligos 55/649) and cloned as a BamHI/EcoRI fragment into *pGEX6P-1* (Promega). 1mg of the fusion protein was injected into rabbits every 4 weeks for 8 to 16 weeks (uOttawa animal facility). Rabbit serum was harvested, clarified by centrifugation and loaded on Affigel-10 (Bio-rad) columns coupled to purified malE-Asf2 (aa 1-90) which was expressed from pAR743 in which Asf2 (aa 1-90) was amplified (oligos 55/673) and cloned as a BamHI/SalI fragment into pMAL-c2 (NEB). Antibody was eluted from Affigel columns with either 100 mM triethylamine pH 11.5 or 100 mM glycine pH 2.3. The triethylamine and glycine elutions were neutralized, dialyzed in PBS + 50% glycerol and stored at -80°C. This study was performed in strict accordance with standards for animal care and use outlined in the Canadian Council on Animal Care Standards. The University of Ottawa is a registered research facility under the Province of Ontario's Animals for Research Act. The protocol was approved by the University of Ottawa Animal Care Committee (Permit Number: BMI-113). All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

2.5 Immunoprecipitation

Cells were cultured in liquid media (YEP +2% glucose) to an OD_{600} of 0.8 at 30°C before being harvested and frozen in liquid nitrogen. Frozen yeast pellets were

lysed using a Biospec Mini-Beadbeater-16 with 0.1 mm glass beads for two 30 s pulses in 2 pellet volumes of lysis buffer (50 mM HEPES-KOH [pH 7.6], 500 mM NaOAc, 5 mM $Mg(OAc)_2$, 0.1 mM EDTA, 0.25% NP-40, 5% glycerol, 0.1%, and added fresh before use, 1 mM PMSF, 100 mM $NaVO_4$, 1 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL bestatin, 1 μg/mL pepstatin). Lysate was incubated on ice for 5 min between pulses. Resulting lysate was separated from glass beads and centrifuged at 14,000 rpm for 5 min to remove insoluble material. The protein concentration of each lysate was determined using Bradford reagent (Bio-Rad) and used to normalize samples. Proteins were immunoprecipitated using 0.5-3 μg of anti-c-Myc, anti-GFP, or anti-FLAG antibodies bound to protein A Dynabeads (Invitrogen, 0.3 mg per reaction). Antibody was added to the lysate and incubated on ice for 30 min. Protein A Dynabeads were washed in lysis buffer, added to the lysate, and then rotated end over end at 4°C for 2 h. Following immunoprecipitation beads were washed three times with lysis buffer (transferring to a fresh tube after the second wash), and then eluted by resuspending in SDS sample buffer (4% SDS, 160 mM Tris-HCl [pH 6.8], 20% glycerol, 20 mM EDTA, 0.04% bromophenol blue, and 1 mM PMSF) and heating to 65°C for 10 min. Samples were stored at -20°C for Western blotting.

2.6 Western Blotting

Samples were run on 10% or 12.5% polyacrylamide gels (80:1 and 120:1 acrylamide:bis, respectively, with no added SDS). Proteins were transferred to nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM, 20% methanol) for 90 min at (60V / 2000 mA). Blots were stained with Ponceau S to confirm transfer and equal

loading of samples and then were blocked for 30 min in blocking buffer (4% nonfat dried milk [Carnation], in TBST [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20]). All antibodies were incubated overnight at 4°C. Blots were washed with TBST, incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:5000 in blocking buffer [Amersham]) for 30 min at 25°C, washed again, incubated with WesternBright Peroxide/ECL (advansta), and then imaged using Bio-Rad ChemiDoc. The following antibodies were used for Western blotting: Anti-c-Myc antibody (1:1000) in TBST +0.02% sodium azide. Anti-Sir2 (1:5000), anti-Sir3 (1:2500), anti-Sir4 (1:2500), anti-GFP (1:2500), and anti-Asf2 (1:1000) antibodies were diluted in antibody storage buffer (autoclaved 4% nonfat dried milk, TBST, 5% glycerol, 0.02% NaN3).

2.7 Chromatin Immunoprecipitation

Cells were cultured in liquid media (SC +0.01% FOA) to an OD_{600} of 0.8 at 30°C before being fixed with 1% formaldehyde for 15 min at 25°C. Cross-linking was quenched for 5 min at 25°C by the addition of glycine to 125 mM. Cells were harvested, washed with 20 mM Tris-HCl (pH 7.5)-150 mM NaCl, then pelleted and frozen in liquid nitrogen. Frozen yeast pellets were lysed using a Biospec Mini-Beadbeater-16 and 0.1 mm glass beads for two 30 s pulses in 2 pellet volumes of ChIP lysis buffer (50 mM HEPES-KOH [pH 7.6], 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, and added fresh before use, 1 mM PMSF, 1 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL bestatin, 1 μg/mL pepstatin). Lysate was incubated on ice for 5 min between pulses.

Resulting lysate was separated from glass beads and chromatin was sheared to an average size of 200-300 bp by sonication using the Covaris S220 (water level=10, peak power=140, duty factor=10, cycles/burst=200, duration=7 min). Sonicated lysate was centrifuged at 14,000 rpm for 5 min to remove insoluble material and stored in liquid nitrogen. Samples were normalized based on DNA concentration using purification methods described below. Lysate normalized to 12.5 µg in ChIP lysis buffer was subject to immunoprecipitation using rabbit IgG (Sigma-Aldrich) coupled to M-270 epoxy dynabeads (Invitrogen; Cristea and Chait, 2011), or 0.5-3 µg of anti-FLAG or anti-Sir4 antibodies bound to protein A Dynabeads (Invitrogen, 0.3 mg per reaction) for 2 h at 4°C. Beads were washed three times with ChIP lysis buffer, once with Li buffer (10 mM Tris-HCl [pH 8.0], 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and once with 50/10 TE (50 mM Tris-HCl [pH 8.0], 10 mM EDTA). Protein and cross-linked DNA were eluted from beads with 50/10 TE +1% SDS at 65°C for 15 min. Cross-links were reversed at 65°C overnight, incubated with 40 µg/ml Proteinase K for 1 h then 30 µg/ml RNAse A for 1h at 55°C, extracted with equal volume 1:1 unbuffered phenol:chloroform, and extracted with chloroform alone. DNA was precipitated by adding 400 mM LiCl, 10 µg glycogen, and 2 volumes of ethyl alcohol, washed with 70% ethyl alcohol, air dried, and resuspended in 10/1-TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Input DNA was purified using 10% of the normalized lysate added to 50/10 TE +1% SDS and was not subject to immunoprecipitation. Precipitated and input DNA were amplified using a qPCR reaction mixture (1X PCR Buffer [Qiagen], 0.15% Triton X-100, 500 nM primer mix, 0.2 mM dNTPs, 1X SYBR Green I [Sigma], 0.2 units HotStar Taq

per reaction). PCR reaction conditions were an initial 95°C for 15 min, then 35 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 15 s, followed by a final extension at 72°C for 30 s. Primers used for ChIP are listed in Table 3. PCR reactions were quantified using BioRad CFX96/Maestro software and reported as percent input or fold enrichment using percent input calculations normalized by dividing by *ACT1* signal [% input = $100*2^{(Ct_{ADJ-IN}-Ct_{IP})}$, $Ct_{ADJ-IN} = Ct_{IN} - \log2(DF)$]. Statistical significance was determined by one-way ANOVA excluding negative controls, and compared using Tukey's test between loci or by t-test within the same loci ($p < 0.05$).

2.8 Galactose induction time course

Cells were grown in liquid media (YEP +2% raffinose) at 25°C until early exponential phase and arrested with alpha-factor or nocodazole for 3 hours. Uninduced samples were harvested as the 0 minute time point before galactose was added to the media (YEP +2% raffinose +2%galactose) to induce *PPR1* expression. Additional harvests were done at 45, 90, and 135 minute time points post-induction then 200-5000 cells were plated onto solid media (SC or SC+FOA). Colonies were counted after two days of growth at 30°C. Viability (%) on FOA is the mean of three independent experiments +/- SEM after normalizing to total cell number.

2.9 Cre-Reported Altered States of Heterochromatin (CRASH) Assay

Methods were adapted from Dodson and Rine, 2015. Cells were grown on selective solid media (YPD+G418) to maintain selection of cells expressing *RFP* exclusively. To prepare for plating, cells were diluted in liquid media (YPD) and grown

overnight to low exponential phase (OD_{600} = 0.3-0.6). Approximately 200 cells were plated on solid media (SC-TRP) and grown for 3 days at 30°C. Cells were imaged with the Bio-Rad ChemiDoc using 546 nm and 488 nm light to visualize RFP and GFP signal respectively. Half-sector frequency = [# of half-sectors / (Total # of colonies - # of fully green colonies)]. Counts were performed in triplicate (n=3) and are reported as the mean +/- standard error.

2.10 Protein purification and identification by liquid-chromatography mass-spectrometry

Methods were adapted from Hoppe et al., 2002. Silver-stained gels were dried in a cellophane sandwich without heat prior to analysis. Bands were excised from the dried gel and rehydrated with purified water. The cellophane was removed, followed by in-gel digestion with trypsin. Digested samples were pressure loaded onto a fused silica microcapillary C18 column (Magic; Michrom BioResources, Auburn, CA) An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) was used to deliver a gradient across a flow splitter to the column over 40 min. The column eluant was directed into an LCQ-DecaXP electrospray ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA), and eluting peptides were dynamically selected for fragmentation by the operating software. The peptide fragmentation spectra were data searched against the nonredundant protein database using the Sequest computer algorithm.

Chapter 3: Asf2 is a novel component of heterochromatin in *S. cerevisiae*

3.1 Introduction

The role of the recombination enhancer in mating type switching

The SIR proteins are directly involved in silencing the *HM* loci, and by extension, are involved in mating type switching. Mating type switching is initiated by expression of *HO*, an endonuclease that specifically cuts the *MAT* locus. Expression of *HO* usually only occurs in early G1-phase of the cell cycle when Cdk1 is inactive. The *HO* gene is regulated by the SWI proteins, mainly Swi4-Swi6, that bind a 1.4 kb regulatory element containing 10 SCB sites (Haber, 2012). Cutting of the *MAT* locus initiates repair from either *HMR* or *HML* depending on the mating type genes that were previously expressed from *MAT*. In *MATα* cells, the default donor is *HMRa*, but *MATa* cells require the help of the recombination enhancer, or RE, to use *HMLα* as the donor for repair. The RE is a 700 bp regulatory element that is 17kb away from *HML*. Deletion of the RE reduces donor preference for *HML* in *MATa* cells, and the RE is also sufficient for donor activation if moved to *HMR* (Wu and Haber, 1996). Removal of the SCB sites that regulate the RE also leads to less donor preference for *HML* in *MATa* cells (Coïc et al., 2006).

The RE is split into left and right components. The left component is important for regulating donor preference through association with ncRNAs and Fkh1 proteins that aid in detection of the DSB induced at *MAT*. The right component recruits condensin and Sir2 to help organize a chromatin loop structure that brings *HML* and *MAT* closer to aid in non-default switching. Sir2 is recruited to the right component of the RE at a promoter of *RDT1* in *MATa* cells only (Li et al., 2019). RE activity is suppressed in *MATα* cells by alpha2-Mcm1 as recombination with *HML* is not desired in *MATα* cells. Binding of Fkh1

to the RE is blocked by alpha2-Mcm1 and deletion of Fkh1 largely reduced donor preference for *HML* in *MATa* cells. Mutations in Mcm1 can abolish RE function as Mcm1 represses the RE in *MATα* cells but aids in switching in *MATa* cells (Wu et al., 1998). Removal of the RE can be rescued by tethering lexA-Fkh1 to the RE location, and deletion of the left RE component and Fkh1 only affects *MATα* cells. In contrast, deletion of the right RE component alone causes defects in the spatial conformation of *MAT* with *HML* and compromises donor preference (Belton et al., 2015). Although Sir2 is the only SIR protein known to be directly involved at the RE, the distribution of Sir2 and the stability of heterochromatin at *HML* and *HMR* are impacted by the rest of the SIR complex and could influence RE activity or sensitivity to activation.

The overexpression phenotype of Asf2

The anti-silencing factor genes *ASF1* and *ASF2* were originally discovered in a screen for genes that disrupted the silent mating type loci when overexpressed. Overexpression of *ASF2* resulted in a more significant loss of silencing compared to *ASF1* but showed no sensitivity to DNA damage, where *ASF1* displayed a loss in viability. Both genes were shown have two MCB motifs upstream of their ORF that are bound by Mbp1 and Swi6 to activate transcription during the transition from G1 to S-phase (Le et al., 1997). Other studies complemented these findings by revealing binding sites for Yhp1 and Plm2, Plm2 being a genome duplicate of TOS4 which is a target of SBF (Swi4, -6-dependent cell cycle box binding factor). Yph1 is a homeobox transcriptional repressor and bocks a subset of genes throughout the M to G1-phase

transition. Therefore, *ASF2* is thought to be regulated by SBF before M-phase (Horak, 2002).

The localization of Asf2 demonstrated by Asf2-YFP appeared to be punctate along the nuclear periphery which is also characteristic of the SIR proteins (Sundin et al., 2004). To date there is no evidence that the overexpression of *ASF2* is sufficient to suppress DNA damage or replication blocks brought on by HU arrest or mutations in *RAD53* (Hu, 2001). In fact, evidence regarding *ASF2* is limited to silencing defects caused by its overexpression in *S. cerevisiae* and *K. lactis*. Although overexpression of *ASF2* in *S. cerevisiae* disrupts both *HML* and *HMR*, overexpression of *ASF2* in *K. lactis* appears to only disrupt silencing at *HML* (Hickman and Rusche, 2009).

Conservation of ASF2 and similarity to SIR4

The *ASF2* gene is an ancient paralog of *SIR4* arising from whole genome duplication. There are not many Sir4-like proteins outside of the *Saccharomycetaceae* family, and *SIR4* seems to have appeared in evolution along with the silent mating-type cassette systems found in these organisms (Hickman et al., 2011; Wolfe and Butler, 2017). Other yeast in this phylum that have only one or no silent cassettes and correlates with the absence of *SIR4* from their genome (Hanson and Wolfe, 2017). Asf2 has been shown to bind Sir3 and remains bound to Sir3 BAH mutants that have a dominant negative silencing defect. So far, no binding mechanism or biological function has been attributed to *ASF2* (Buchberger et al., 2008). The *ASF2* gene is also not required for telomere protection from NHEJ, a function that is governed by SIR-mediated heterochromatin in yeast.

Investigations into the sequence of *ASF2* unveiled a coiled-coil domain that shares homology with a coiled-coil domain found in *SIR4*. All species of the *Saccharomycetaceae* family that have *ASF2* contain the coiled-coil domain, including *K. lactis* and *E. gossypii*, which also have an additional Sir2-interacting domain (Faure et al., 2019). The anti-silencing phenotype of overexpressed *ASF2* and the similarity between the coiled-coil domains of Asf2 and Sir4 suggest that Asf2 may play a role in SIR-mediated heterochromatin.

3.2 General hypothesis & Specific Objectives

The SIR complex mediates heterochromatin formation in *S. cerevisiae*. The SIR proteins are regulated by dosage, post-transcriptional modification, the histone code, and other associated factors. The stability and distribution of heterochromatin can be influenced by any of these factors and is highly dynamic throughout the cell cycle. The core SIR complex is composed of Sir2, Sir3, and Sir4, but their modification and association with other factors dictates their specificity and the maturity of heterochromatin fibers. Asf2 is a poorly characterized protein that associates with the SIR complex, disrupt silencing when overexpressed, and shares homology with the coiled-coil domain of Sir4. This chapter investigates the hypothesis that Asf2 is a novel component of heterochromatin in *S. cerevisiae* and plays a role in modulating the stability and distribution of the SIR complex on chromatin.

1. Identify residues in Asf2 that interact with the SIR complex.
2. Propose a mechanism to explain the overexpression phenotype of *ASF2*.

3. Test the effects of overexpression, mutation, and deletion of *ASF2* on silencing at the *HM* loci and telomeres.

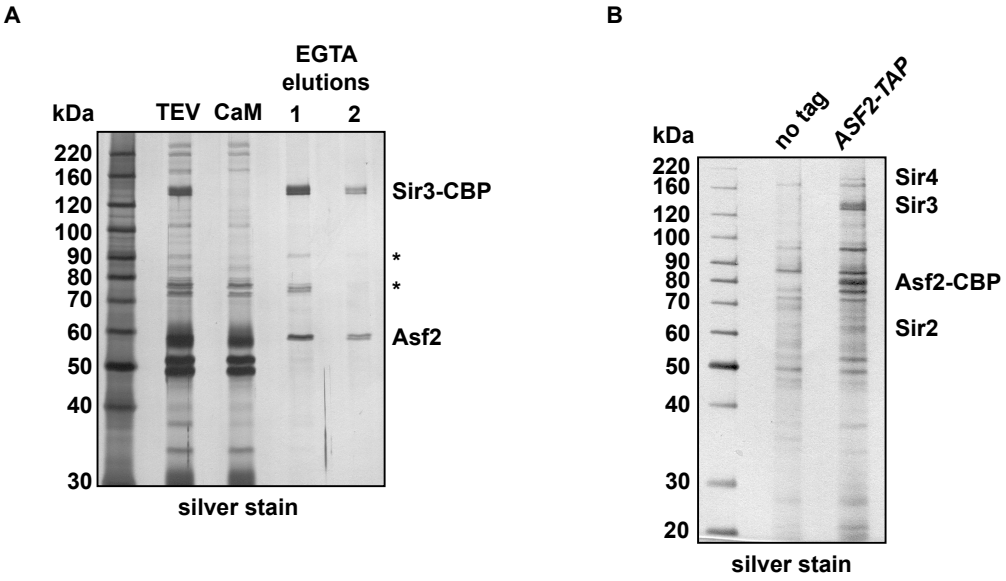
4. Determine if Asf2 co-localizes to regions of heterochromatin with the SIR complex.

5. Propose a biological function for *ASF2*.

3.3 Results

Asf2 interacts with the SIR complex

Previous work has shown that Sir3-CBP purifies independently of Sir4 and Sir2 from an endogenously TAP-tagged *SIR3* strain (Hoppe et al., 2002). Mass spectrometry was performed on samples from this strain and identified Asf2 as the 59kD protein present in the Sir3-CBP purifications (Figure 4A). This interaction was detected previously in purifications of Sir3-FLAG, but was not pursued (Buchberger et al., 2008). In contrast to this two-step Sir3-TAP purification, previous reports show that the full SIR complex can be isolated by immunoprecipitating a Myc-tagged Sir3, and this complex is stable in high salt and after treatments that remove chromatin (Rudner et al., 2005). A similar one-step pull down of TAP-tagged Asf2 pulls down Sir3, Sir4, and Sir2, thus confirming the interaction between Asf2 and the SIR complex (Figure 4B). Deletion of either *SIR2* or *SIR4* does not alter the interaction between Sir3 and a Myc-tagged Asf2. However, deletion of *SIR3* removes the association Asf2-13Myc has with both Sir4 and Sir2 (Figure 4C).


A
EGTA elutions
kDa
TEV
CaM
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160
120
100
90
80
70
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50
40
30
Sir3-CBP
*
*
Asf2
silver stain
B
kDa
no tag
ASF2-TAP
220
160
120
100
90
80
70
60
50
40
30
20
Sir4
Sir3
Asf2-CBP
Sir2
silver stain

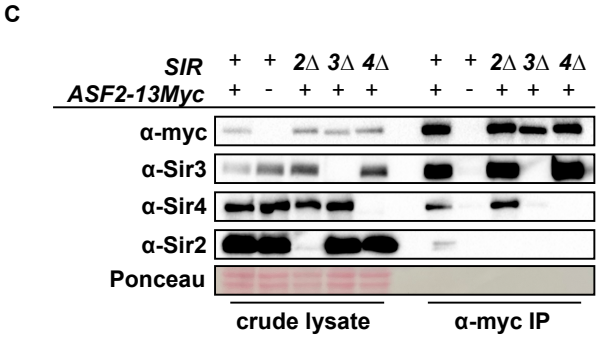

C
SIR + + 2Δ 3Δ 4Δ + + 2Δ 3Δ 4Δ
ASF2-13Myc + - + + + + - + + +
α-myc
α-Sir3
α-Sir4
α-Sir2
Ponceau
crude lysate
α-myc IP

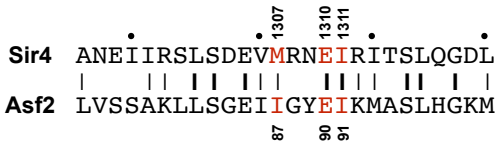
Figure 4. Asf2 interacts with Sir3 and the SIR complex. (A) Asf2 co-purifies with Sir3-CBP. Sir3-TAP was purified using a two-step affinity purification (Rigaut et al., 1999; Hoppe et al., 2002) and samples of the TEV elution (1st affinity purification), the CaM-bead supernatant (sup of 2nd affinity purification) and two EGTA elutions (2nd affinity purification) were silver stained. Sir3, Asf2 and two Sir3 degradation products (*) were excised from the dried gel and identified by LC-MS/MS. **(B)** The SIR complex co-purifies with Asf2-CBP. Asf2-TAP was purified using a two-step affinity purification from untagged control and *ASF2-TAP* cells. Samples of the EGTA elution (2nd affinity purification) were silver stained. Sir4, Sir3, Asf2-CBP and Sir2 were identified by LC-MS/MS on the mixture of the eluted proteins. **(C)** Lysates from *Asf2-13Myc*, *ASF2*, *Asf2-13Myc sir2Δ* (*2Δ*), *Asf2-13Myc sir3Δ* (*3Δ*), and *Asf2-13Myc sir4Δ* (*4Δ*) cells were subject to immunoprecipitation using anti-Myc magnetic beads. Immunoblots were generated with anti-Myc, anti-Sir3, anti-Sir4, and anti-Sir2 on crude lysate and immunoprecipitated (IP) samples.

Asf2 and Sir4 interact with Sir3 through a conserved coiled-coil domain

A few point mutants have already been identified in *SIR4* (M1307N, E1310R, I1311N) that highlight the importance of a coiled-coil domain and its role in Sir3 binding (Rudner et al., 2005). Another group recognized that a homologous coiled-coil domain exists in Asf2, which may also mediate interaction with Sir3 (Faure et al., 2019). Analogous mutations were made in *ASF2*, resulting in *asf2-NRN* (I87N, E90R, I91N), to test if the coiled-coil domain in Asf2 is important for Sir3 binding (Figure 5A). Immunoprecipitation of Sir3-13Myc from *ASF2* and *asf2-NRN* strains yielded very different results when blotting for Asf2 and the SIR complex proteins. An interaction was observed between Sir3-13Myc and wild type Asf2, but Asf2 signal is fully abolished in a strain possessing the *asf2-NRN* mutation.

No Asf2 signal is observed with *asf2Δ*, while Sir4 and Sir2 remain associated to Sir3-13Myc in all the strains where it is present. (Figure 5B). Complementary to these observations, it has been demonstrated that residues in a loop of the Sir3 AAA+ domain are required for silencing and interaction with Sir4 (Ehrentraut et al., 2011). These tagged mutants *sir3-2A-FLAG3*, *sir3-3A-FLAG3*, *sir3-4A-FLAG3* along with untagged and wild type *SIR3-FLAG3* were subject to immunoprecipitation and blotted for associated SIR proteins and Asf2. Strains with wild type *SIR3-FLAG3* yielded detectable levels of Sir4, Sir2, and Asf2, but all three mutants and untagged strains did not (Figure 5C).

A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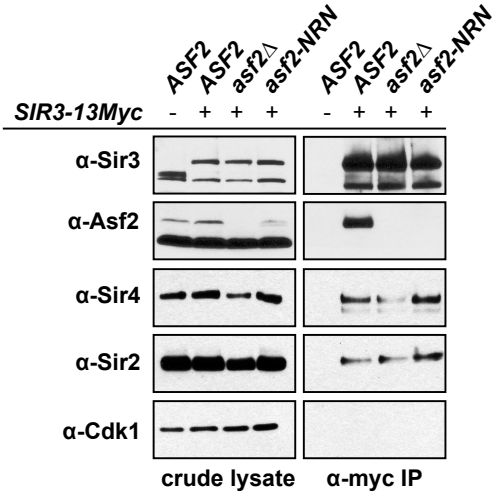
1310
1311
Sir4 ANEIIRSLSDEVMRNEIRITSLQGDL
Asf2 LVSSAKLLSGEIIGYEIKMASLHGKM
87
90
91

B


ASF2
ASF2
asf2Δ
asf2-NRN
ASF2
ASF2
asf2Δ
asf2-NRN
SIR3-13Myc - + + + - + + +
α-Sir3
α-Asf2
α-Sir4
α-Sir2
α-Cdk1
crude lysate
α-myc IP

C

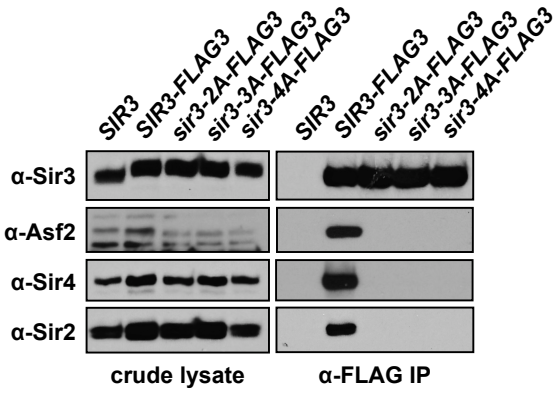
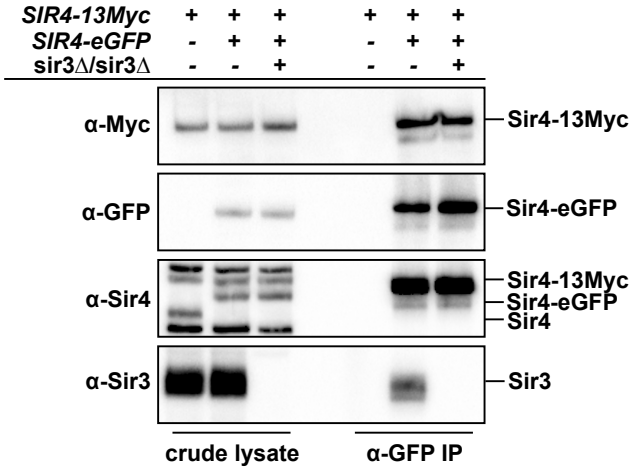

SIR3
SIR3-FLAG3
sir3-2A-FLAG3
sir3-3A-FLAG3
sir3-4A-FLAG3
SIR3
SIR3-FLAG3
sir3-2A-FLAG3
sir3-3A-FLAG3
sir3-4A-FLAG3
α-Sir3
α-Asf2
α-Sir4
α-Sir2
crude lysate
α-FLAG IP

Figure 5. Asf2 and Sir4 interact with Sir3 through a conserved coiled-coil domain. **(A)** The amino acid sequence alignment of the coiled-coil domain in Asf2 and Sir4. The coils are aligned by the heptad repeat (black dot). Identical (bold bar) and conserved (thin bar) residues are indicated. Amino acids that are required for interaction with Sir3 (M1307, E1310, I1311; I87, E90, I91) are shown in red. **(B)** Sir3 interacts with Asf2 via conserved amino acid residues in its coiled-coil. Lysates from *SIR3 ASF2*, *Sir3-13Myc ASF2*, *Sir3-13Myc asf2Δ*, and *Sir3-13Myc asf2-NRN* cells were subject to immunoprecipitation using anti-Myc magnetic beads. **(C)** A basic loop in Sir3 is required for interaction with both Sir4 and Asf2. Lysates from *SIR3*, *SIR3-FLAG3*, *sir3-2A-FLAG3*, *sir3-3A-FLAG3*, and *sir3-4A-FLAG3* cells were subject to immunoprecipitation using anti-FLAG magnetic beads. Immunoblots were generated with anti-Sir3, anti-Asf2, anti-Sir4, anti-Sir2, and anti-Cdk1 on crude lysate and immunoprecipitated (IP) samples.

Asf2 and Sir4 homodimerize in vivo

Valuable work has been done using peptide fragments of Sir4 to show that its coiled-coil domain is required for homodimer formation *in vitro*, but this interaction has not been demonstrated using the full-length protein *in vivo* (Chang et al., 2003). Diploid strains containing *SIR4-13Myc/SIR4-eGFP* were constructed and subject to immunoprecipitation with anti-GFP antibodies. Sir4-13Myc was detected in both *SIR4-13Myc/SIR4-eGFP* and *SIR4-13Myc/SIR4-eGFP sir3Δ/sir3Δ* strains, but not in a strain lacking the eGFP tag (Figure 6A). Analogous diploid strains containing *ASF2-13Myc/ASF2-GFP* were also generated and subject to immunoprecipitation with anti-GFP antibodies. Asf2-13Myc was detected in the *ASF2-13Myc/ASF2-GFP* strain but was barely detectable in the *ASF2-13Myc/ASF2-GFP sir3Δ/sir3Δ* strain (Figure 6B). No pulldown products were observed for the strain lacking the GFP tag. It is important to note that Asf2-GFP migrates directly on top of a non-specific band approximately 86 kDa in size.

A


SIR4-13Myc + + + + + +
SIR4-eGFP - + + - + +
sir3Δ/sir3Δ - - + - - +
α-Myc
Sir4-13Myc
α-GFP
Sir4-eGFP
α-Sir4
Sir4-13Myc
Sir4-eGFP
Sir4
α-Sir3
Sir3
crude lysate
α-GFP IP

B

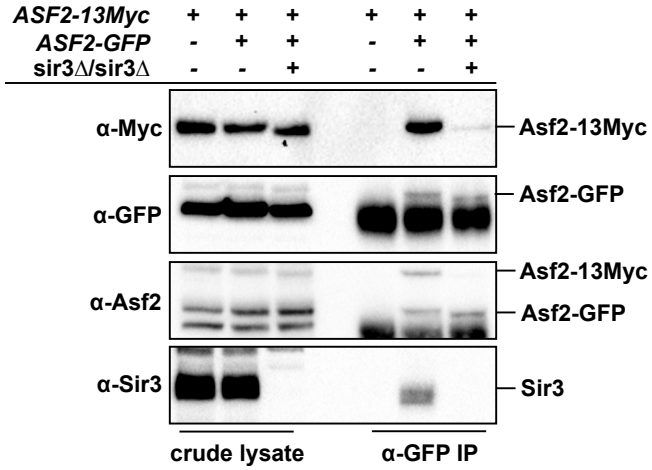

ASF2-13Myc + + + + + +
ASF2-GFP - + + - + +
sir3Δ/sir3Δ - - + - - +
α-Myc
Asf2-13Myc
α-GFP
Asf2-GFP
α-Asf2
Asf2-13Myc
Asf2-GFP
α-Sir3
Sir3
crude lysate
α-GFP IP

Figure 6. Asf2 and Sir4 homodimerize *in vivo*. (A) Sir4 dimerizes independently of Sir3. Lysates from *SIR4-13Myc/SIR4*, *SIR4-13Myc/SIR4-eGFP*, or *SIR4-13Myc/SIR4-eGFP sir3Δ/sir3Δ* diploid cells were subject to immunoprecipitation using anti-GFP magnetic beads. (B) Asf2 dimerization depends on Sir3. Lysates from *ASF2-13Myc/ASF2*, *ASF2-13Myc/ASF2-GFP*, or *ASF2-13Myc/SIR4-eGFP sir3Δ/sir3Δ* diploid cells were subject to immunoprecipitation using anti-GFP magnetic beads. Immunoblots were generated with anti-Myc, anti-GFP, anti-Sir4 or anti-Asf2, and anti-Sir3 on crude lysate and immunoprecipitated (IP) samples. Additional controls are shown in Appendix A3.

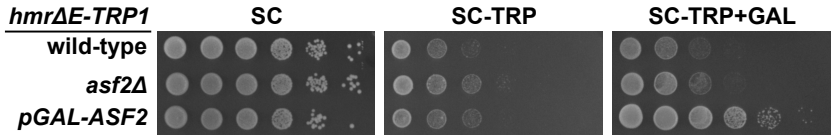
Overexpression of Asf2 disrupts heterochromatin and the SIR complex

ASF2 was originally identified as a gene that disrupts the SIR-dependent silent mating type loci when overexpressed (Le et al., 1997). This phenotype was confirmed by generating strains with an integrated copy of *ASF2* driven by a galactose-inducible promoter. The *pGAL-ASF2* strains also contain either a weakened *HMRa* (*hmrΔE-TRP1*) or a telomere-proximal (*TEL-VII-L-URA3*) reporter. Overexpression of *ASF2* induced by growth on galactose media resulted in a loss of suppression at the *hmrΔE-TRP1* reporter as demonstrated by improved growth on media lacking tryptophan (Figure 7A). Similarly, overexpression of *ASF2* derepressed the *TEL-VII-L-URA3* reporter and led to a severe growth defect for cells grown on media containing 5-Fluoroorotic acid (Figure 7B). It is important to note that overexpression of the mutant *pGAL-asf2-NRN* does not have the same growth defect when grown on 5-FOA indicating that *TEL-VII-L-URA3* remained silenced.

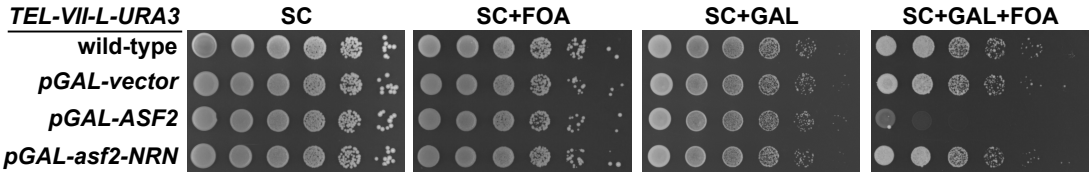
The same reporters were tested using *CEN* plasmids as a less severe overexpression system. Growth on SC-HIS+FOA plates was similar for both *empty-HIS-CEN* and *ASF2-HIS-CEN* plasmid containing strains, and *SIR4-HIS-CEN* containing cells displayed a very slight improvement in viability on SC-HIS+FOA. Larger differences were observed for the *hmrΔE-TRP1* reporter compared to *TEL-VII-URA3*. The *hmrΔE-TRP1* reporter lacks the E element that is found within the essential silencer region but still allows for repression of *HML*. Next to no growth was observed for either *empty-HIS-CEN* or *SIR4-HIS-CEN* on SC-HIS-TRP plates, but *ASF2-CEN-HIS* strains showed considerable viability (Figure 7C). The integrity of the SIR complex was questioned due to the fact these silent reporters were able to be disrupted by overexpressed *ASF2*. Strains

containing *pGAL-ASF2* and *SIR3-13Myc* were immunoprecipitated to observe how Sir3, Sir4, and Sir2 were interacting in the presence of excess Asf2. More Sir4 and Sir2 precipitated with Sir3-13Myc in samples that were grown in raffinose as opposed to those that had been induced with galactose (Figure 7D).

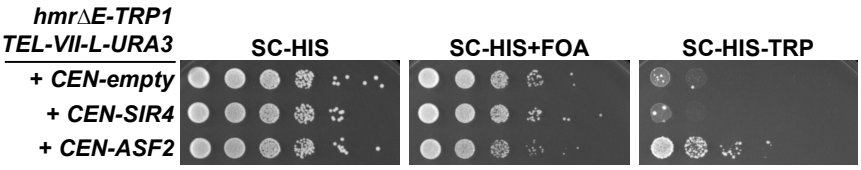
A


hmrΔE-TRP1
SC
SC-TRP
SC-TRP+GAL
wild-type
asf2Δ
pGAL-ASF2

B


TEL-VII-L-URA3
SC
SC+FOA
SC+GAL
SC+GAL+FOA
wild-type
pGAL-vector
pGAL-ASF2
pGAL-asf2-NRN

C


hmrΔE-TRP1
TEL-VII-L-URA3
SC-HIS
SC-HIS+FOA
SC-HIS-TRP
+ CEN-empty
+ CEN-SIR4
+ CEN-ASF2

D

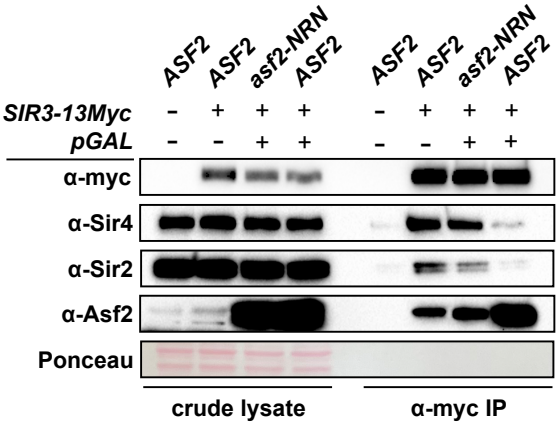
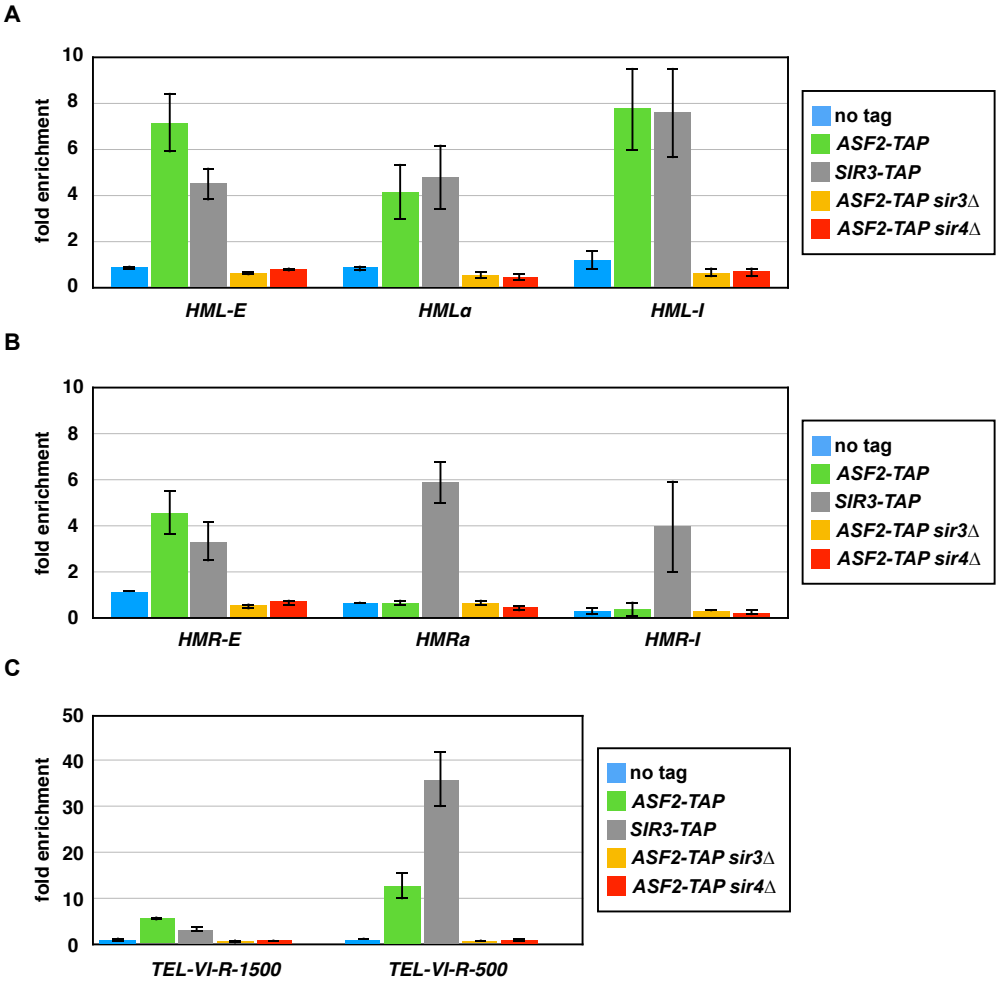

ASF2
ASF2
asf2-NRN
ASF2
ASF2
ASF2
asf2-NRN
ASF2
SIR3-13Myc
pGAL
α-myc
α-Sir4
α-Sir2
α-Asf2
Ponceau
crude lysate
α-myc IP

Figure 7. Overexpression of Asf2 disrupts heterochromatin and the SIR complex. **(A)** Overexpression of *ASF2* disrupts *HMR* silencing. Strains containing *hmr*Δ*E-TRP1, asf2*Δ *hmr*Δ*E-TRP1* and *pGAL-ASF2 hmr*Δ*E-TRP1* were grown in liquid YPD for 2 days at 30˚C and 10-fold serial dilutions were spotted onto the indicated plates (SC, SC-TRP, and SC-TRP+GAL) and incubated for 2 days at 30˚C. **(B)** Overexpression of *ASF2,* but not *asf2-NRN*, disrupts *TEL-VII-L* silencing. Strains containing *TEL-VII-L-URA3, TEL-VII-L-URA3 pGAL*, *TEL-VII-L-URA3 pGAL-ASF2*, and *TEL-VII-L-URA3 pGAL-asf2-NRN* were grown in liquid YPD for 2 days at 30˚C and 10-fold serial dilutions were spotted onto the indicated plates and incubated for 2 days (SC) or 3 days (SC+FOA, SC+GAL, SC+GAL+FOA) at 30˚C. **(C)** A small increase in Asf2 is sufficient to disrupt the *HMR*. Strains containing *TEL-VII-L-URA3 hmr*Δ*E-TRP1* and either empty-*HIS-CEN*, *SIR4-HIS-CEN*, or *ASF2-HIS-CEN* were grown in liquid SC-HIS media for 2 days at 30˚C and 10-fold serial dilutions were spotted onto the indicated plates and incubated for 2 days (SC-HIS and SC-HIS-TRP) or 3 days (SC-HIS+FOA) at 30˚C. **(D)** Overexpression of *ASF2* disrupts the interaction of Sir4/Sir2 with Sir3. Lysates from *SIR3*, *SIR3-13Myc*, *SIR3-13Myc pGAL-asf2-NRN*, and *SIR3-13Myc pGAL-ASF2* cells were subject to immunoprecipitation with anti-Myc magnet beads. Cells were shifted to galactose media for 3 hours prior to harvest. Immunoblots were generated with anti-Myc, anti-Sir4, anti-Sir2, and anti-Asf2, on crude lysate and immunoprecipitated (IP) samples.

Asf2 localizes to telomeres and the mating-type loci

Since Asf2 appears to associate with the SIR complex through Sir3, it is important to establish if Asf2 is also present at the same regions of heterochromatin as the SIR proteins. Chromatin immunoprecipitation was performed with strains containing *ASF2-TAP* and *SIR3-TAP* to test if Asf2 co-localizes with Sir3 at silent loci. The resulting DNA was analyzed by qPCR and the fold enrichment of *HML*, *HMR*, and *TEL-VI-R* was calculated relative to *ACT1*. Strains containing *ASF2-TAP* and *SIR3-TAP* produced a relative fold enrichment of 7.1 and 5.4 for *HML-E*, 4.2 and 4.8 for *HMLα*, and 7.8 and 7.6 for *HML-I* respectively (Figure 8A). Additionally, a relative fold enrichment of 4.6 and 3.3 for *HMR-E*, 0.7 and 5.9 for *HMRa*, and 0.4 and 3.9 for *HMR-I* were obtained from the same *ASF2-TAP* and *SIR3-TAP* strains respectively (Figure 8B). Two loci at different distances from the end of *TEL-VI-R* were also investigated, resulting in a relative fold enrichment of 12.9 at 500bp and 5.7 at 1500bp for *ASF2-TAP*. A fold enrichment of 36.0 at 500bp and 3.3 at 1500bp was detected for *SIR3-TAP* (Figure 8C). Control strains with no TAP tag and *ASF2-TAP* strains with *SIR3* and *SIR4* removed always produced less than a 2-fold enrichment for all loci tested.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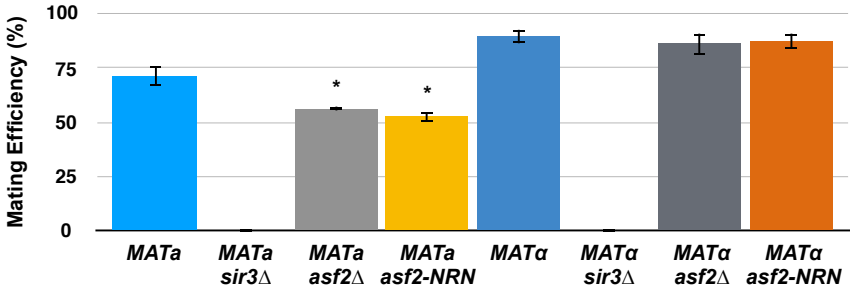
10
8
6
4
2
0
HML-E
HMLa
HML-I
no tag
ASF2-TAP
SIR3-TAP
ASF2-TAP sir3Δ
ASF2-TAP sir4Δ
B
fold enrichment
10
8
6
4
2
0
HMR-E
HMRa
HMR-I
no tag
ASF2-TAP
SIR3-TAP
ASF2-TAP sir3Δ
ASF2-TAP sir4Δ
C
fold enrichment
50
40
30
20
10
0
TEL-VI-R-1500
TEL-VI-R-500
no tag
ASF2-TAP
SIR3-TAP
ASF2-TAP sir3Δ
ASF2-TAP sir4Δ

Figure 8. **Asf2 localizes to telomeres and the mating type loci.** Wild-type (blue), *ASF2-TAP* (green), *SIR3-TAP* (grey), *ASF2-TAP sir3Δ* (orange) and *ASF2-TAP sir4Δ* (red) cells were fixed in 1% formaldehyde for fifteen minutes and processed for chromatin immunoprecipitation using IgG-coupled magnetic beads. The localization of the TAP-tagged protein to the (A) *HML*, (B) *HMR*, and (C) *TEL-VI-R* loci was determined by analyzing the immunoprecipitated DNA by qPCR with locus-specific primers (Table 3). Each sample was also tested at a non-silent locus, *ACT1*, as a control. Fold enrichment is the mean of the percent input +/- SEM after normalizing to *ACT1* signal (n=3). The means of each sample were grouped by loci and were determined to be statistically different based on one-way ANOVA ($p<0.05$), and means were compared using a paired t-test ($p<0.05$), see Appendix C, Table 4.

*asf2*Δ *has little impact on HM and telomeric heterochromatin*

A deficiency in mating has long been attributed to mutations in the *SIR1*, *SIR2*, *SIR3*, and *SIR4* genes (Rine and Herskowitz, 1987). A quantitative mating assay was conducted in strains containing *asf2Δ* and *asf2-NRN* mutations after recognizing the stability of *HML* & *HMR* is affected by how much Asf2 is bound to Sir3. The resulting average mating efficiency was 71% for *MATa*, 56% for *MATa asf2Δ*, and 52% for *MATa asf2-NRN*. Less variability was observed between *MATα* strains; 90%, 86%, and 88% for *MATα, MATα asf2Δ,* and *MATα asf2-NRN* respectively (Figure 9A). Control strains *MATa sir3Δ* and *MATα sir3Δ* both had mating efficiencies < 1%. To test if heterochromatin is affected in cells lacking *ASF2* telomeric silencing was assessed with regular *URA3*, or transcriptionally weakened *ppr1Δ URA3* reporters. Telomeric reporters *TEL-V-R-URA3*, *TEL-VII-L-URA3*, and *TEL-IX-R-URA3* were combined with *sir2Δ*, *sir3Δ*, *sir4Δ*, and *asf2Δ* mutations then grown on 5-FOA media selective for *URA3* silencing. Plating revealed that *sir2Δ*, *sir3Δ*, and *sir4Δ* all fully abolish silencing at every telomeric reporter, as these cells became inviable on 5-FOA. Oppositely, *asf2Δ* resulted in no growth difference compared to wild type for *TEL-VII-L-URA3* and *TEL-IX-R-URA3* but led to a slight increase in silencing stability and increased growth for cells containing *TEL-V-R-URA3* (Figure 9B).

A


Mating Efficiency (%)
100
75
50
25
0
*
*
MATa
MATa sir3Δ
MATa asf2Δ
MATa asf2-NRN
MATα
MATα sir3Δ
MATα asf2Δ
MATα asf2-NRN

B

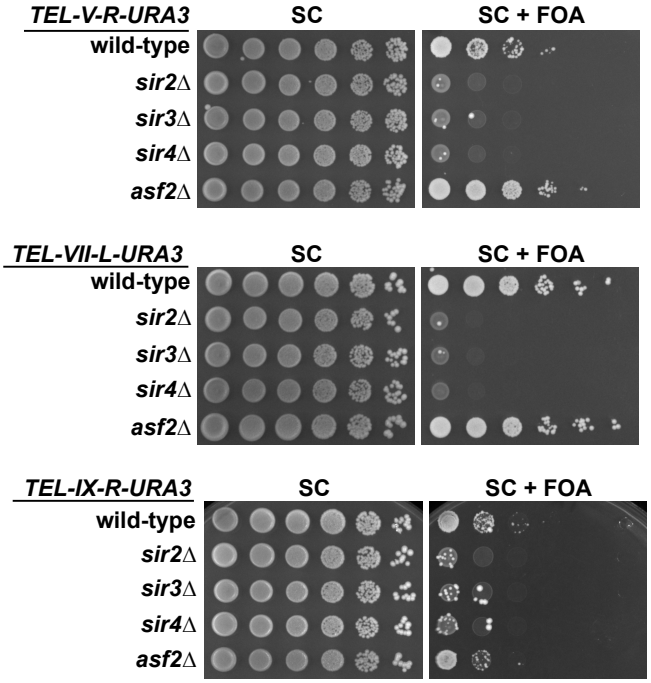

TEL-V-R-URA3
SC
SC + FOA
wild-type
sir2Δ
sir3Δ
sir4Δ
asf2Δ
TEL-VII-L-URA3
SC
SC + FOA
wild-type
sir2Δ
sir3Δ
sir4Δ
asf2Δ
TEL-IX-R-URA3
SC
SC + FOA
wild-type
sir2Δ
sir3Δ
sir4Δ
asf2Δ

Figure 9. *asf2*Δ has little impact on *HM* and telomeric heterochromatin. (A) Quantitative mating assays were performed by crossing the indicated strains to *MATa* and *MATα* tester strains. The absolute mating efficiency is the proportion of cells of each query strain that mated and formed colonies on synthetic media lacking amino acids. Statistical significance (*) was determined by paired one-way ANOVA and t-test comparing to wild-type samples, $p < 0.05$. **(B)** *asf2*Δ has no defects in telomeric silencing at the *TEL-V-R, TEL-VII-L* and *TEL-IX-R* loci. Wild-type, *sir2*Δ, *sir3*Δ, *sir4*Δ and *asf2*Δ strains containing either the *TEL-V-R-URA3, TEL-VII-L-URA3* or *TEL-IX-R-URA3* reporter were grown in liquid YPD for 2 days at 30˚C and 10-fold serial dilutions were spotted onto the indicated plates and incubated for 2 days (SC) or 3 days (SC+FOA) at 30˚C. Growth on FOA media is representative of repression at each *URA3* locus. Note that the *TEL-V-R-URA3* strains are also deleted for *PPR1* which increases the strength of silencing at this locus.

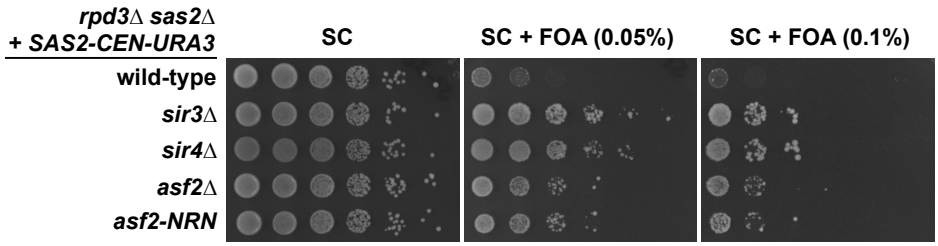
ASF2 acts as a positive regulator of silencing

The spreading of the SIR complex, and perhaps Asf2, from the nucleation site depends on histone modifications that act as boundary elements. These modifications states are altered by histone acetyltransferases and deacetylases, such as H4K16ac by *SAS2* and *RPD3*. Deletion of both genes (*rpd3Δ sas2Δ*) leads to lethality due to compromised heterochromatin boundary elements that allow subtelomeric essential genes to be silenced (Ehrentraut et al. 2010). Strains with the double mutation *rpd3Δ sas2Δ* were complemented with a *SAS2-CEN-URA3* plasmid to maintain viability before being assayed. These strains were combined with *sir3Δ*, *sir4Δ*, *asf2Δ*, and *asf2-NRN* mutations then plated onto 5-FOA media to select for plasmid loss and screen for lethality. All the combined mutants were able to grow on 5-FOA compared to *rpd3Δ sas2Δ* alone, but the viability of *rpd3Δ sas2Δ sir3Δ* & *rpd3Δ sas2Δ sir4Δ* was slightly better than *rpd3Δ sas2Δ asf2Δ* & *rpd3Δ sas2Δ asf2-NRN* (Figure 10A).

The importance of Asf2 in heterochromatin stability may not be highlighted in an endpoint experiment such as a serial growth assay, so *asf2Δ* was tested in conditions where chromatin is actively disassembled. A strain containing *TEL-V-URA3* and an inducible *URA3* transcription factor (*pGAL-PPR1*) was arrested in either G1 (α-factor) or M phase (nocodazole) prior to induction with galactose. Cells were plated at 0, 45, 90, and 135 minutes post-induction and grown on FOA media to select for cells with functional silencing. The percent of cells resistant to FOA decreased drastically after galactose induction for both α-factor and nocodazole arrests. Strains with *asf2Δ* led to a quicker loss of FOA resistance in nocodazole arrest noticeable as soon as 45min post-induction, but *asf2Δ* only had a minimal effect in the α-factor arrest (Figure 10B).

Importantly, inducing a nonsense version of the *URA3* transcription factor (*pGAL-ppr1*) has no significant effect on FOA resistance in these arrest conditions.

A


rpd3Δ sas2Δ
+ SAS2-CEN-URA3
SC
SC + FOA (0.05%)
SC + FOA (0.1%)
wild-type
sir3Δ
sir4Δ
asf2Δ
asf2-NRN

B

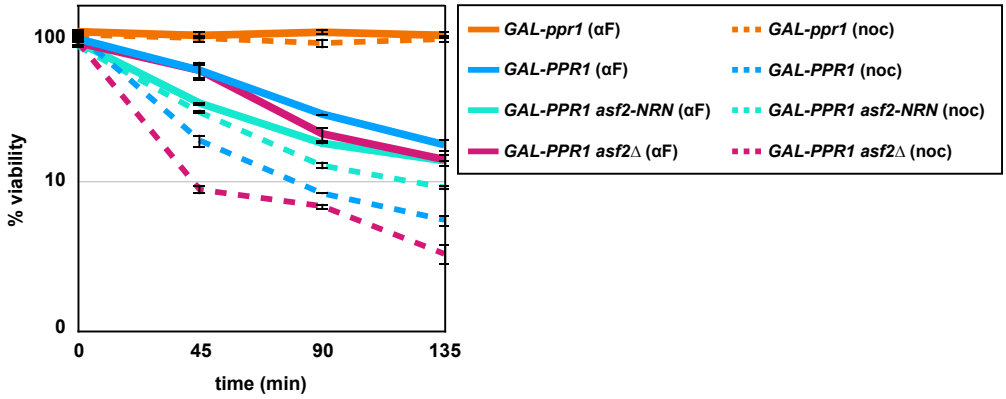

100
10
0
% viability
0
45
90
135
time (min)
GAL-ppr1 (αF)
GAL-ppr1 (noc)
GAL-PPR1 (αF)
GAL-PPR1 (noc)
GAL-PPR1 asf2-NRN (αF)
GAL-PPR1 asf2-NRN (noc)
GAL-PPR1 asf2Δ (αF)
GAL-PPR1 asf2Δ (noc)

Figure 10. *ASF2* acts a positive regulator of silencing. (A) *asf2Δ* and *asf2-NRN* suppress the lethality of *rpd3Δ sas2Δ*. Wild-type, *sir3Δ, sir4Δ, asf2Δ* and *asf2-NRN* strains containing *rpd3Δ sas2Δ SAS2-CEN-URA3* were grown in liquid SC-URA media for 2 days at 30˚C and 10-fold serial dilutions were spotted onto the indicated plates and incubated for 2 days (SC) or 3 days (SC+FOA) at 30˚C. Only cells that have lost the *SAS2-CEN-URA3* plasmid can grow on plates containing FOA. **(B)** *asf2Δ* and *asf2-NRN* have opposite effects on heterochromatin stability. Wild-type, *asf2Δ* and *asf2-NRN* cells containing a *TEL-V-R-URA3* reporter and an integrated *pGAL-PPR1* or a non-functional *pGAL-ppr1* were grown in YEP + raffinose media at 25°C until early exponential phase and arrested with alpha factor (αF) or nocodazole (noc) for 3h. Galactose was added to the media to induce *PPR1* expression and cells were harvested at the indicated time points and plated onto SC or SC+FOA plates. Colonies were counted after two days of growth at 30°C. Viability (%) on FOA is the mean of three independent experiments +/- SEM after normalizing to total cell number.

*asf2*Δ *does not cause increase transient expression of the HM loci*

Methods to detect transient losses of heterochromatin and expression of the *HM* loci has been developed using a fluorescent reporter. A cassette containing RFP and GFP is regulated by distance from the promoter, and is rearranged upon expression of *HMLα::cre* or *HMRα::cre* resulting in switch from *RFP* expression to *GFP* expression (Dodson and Rine, 2015). Combining this reporter system with mutations that disrupt heterochromatin, such as *sir1*Δ, cause a more frequent switching of the reporter and thus a higher half-sector frequency. This reporter system was combined with deletion of *ASF2* to probe for any instability in the *HM* loci brought on by a lack of *ASF2* (Appendix A1). Although the half-sector frequency of *sir1*Δ strains were similar to what was previously reported for both *HML* and *HMR*, the half-sector frequency of *asf2*Δ did not significantly deviate from that of wild type cells (0.0050 and 0.0056 for *HML*, 0.0138 and 0.0104 for *HMR* respectively).

*asf2*Δ *does not suppress preRC mutants like the other SIR proteins*

Investigation into a possible involvement of Asf2 in the formation of the preRC was conducted by combining mutations in the preRC and the SIR complex. Mutations in the preRC components such as *orc5-1*, *mcm2-1*, *cdc6-1*, and *cdc6-4* that lead to temperature sensitive growth restrictions can be suppressed by deletion of the SIR proteins with the strongest effect seen in a *sir2*Δ strain (Pappas, 2004). These preRC mutants were combined with either *sir3*Δ or *sir4*Δ and used in serial growth assays where strains were spotted and grown on YPD plates at 25°C, 30°C, 33°C, and 37°C (Appendix A2). Wild type cells were viable at all temperatures, but growth was restricted at 37°C for

orc5-1 and *mcm2-1*, and 33°C for *cdc6-1*, and *cdc6-4* mutants. Deletion of *SIR4* was able to slightly improve viability for all mutants except *orc5-1*, but deletion of *ASF2* did not alter viability in any of the mutants. Deletion of both *SIR4* and *ASF2* did not demonstrate a compound effect and resembled the viability of *sir4*Δ alone.

3.4 Discussion

Asf2 interacts with Sir3 directly

The SIR proteins interact with themselves, DNA-binding proteins, histones, and other regulating factors. This work aims to characterize Asf2 and its interaction with the SIR complex. Immunoprecipitation experiments indicate that Asf2 interacts with Sir2, Sir3, and Sir4 (Figure 4C). Asf2 still remains bound to Sir3 and Sir4 in *sir2*Δ cells, so it is likely that Sir2 is the most distal component of this complex relative to Asf2. Asf2 is not bound to Sir4 or Sir2 in *sir4*Δ cells which is not surprising since Sir4/Sir2 usually exists as a stable heterodimer. There appears to be no ability for Asf2 to bind Sir2 without Sir4 and this is consistent with the fact Asf2 does not possess a Sir2-interacting domain like Sir4 does (Faure et al., 2019). No interaction between Asf2 and the SIR proteins are observed in *sir3*Δ cells. Sir3 likely bridges the interaction between Asf2 and the other SIR proteins by binding Asf2 directly. It is also possible that the binding between Asf2 and Sir4/Sir2 is indirect and is a result of an Asf2-Sir3 complex co-immunoprecipitating with a neighboring SIR complex that contains Sir4/Sir2. It cannot be ruled out that the interaction observed between Asf2 and Sir4/Sir2 may be direct and dependent on Sir3.

Deletion of *ASF2* does not seem to interfere with the integrity of the SIR complex since immunoprecipitated Sir3 remains associated with Sir4 and Sir2 in *asf2*Δ cells (Figure 5B). Additionally, although mutations in the coiled-coil domain of Asf2 result in a complete loss of interaction between Sir3 and Asf2, cells expressing *asf2-NRN* do not display a loss in association between the other SIR proteins. These observations highlight how Asf2 is similar to Sir4, as mutation of the Sir4 coiled-coil domain has also been shown to disrupt the interaction between Sir3 and Sir4 (Rudner et al., 2005). It is important to note that mutations were directed to the surface of the coiled-coil domain in Sir4 in order to not interfere with its dimerization (Chang et al., 2003), and this is assumption is applied to Asf2 mutations as well. Complementary to these experiments, mutation of the AAA+ domain in Sir3 disrupts both the Sir3-Asf2 and Sir3-Sir4 interaction (Figure 5C). Based on this evidence it seems the association of Sir3 and Asf2 is due to direct binding between the coiled-coil domain of Asf2 and the AAA+ domain of Sir3.

Mechanism of the Asf2 overexpression phenotype

ASF2 was originally described as a gene that disrupts silencing when overexpressed (Le et al., 1997). Experiments performed using a galactose-inducible promoter to drive *ASF2* expression confirm its anti-silencing phenotype at both *HM* loci and telomeres (Figure 7A & 7B). Overexpression of *asf2-NRN* using the same system does not lead to a loss of silencing. Since the Asf2-NRN protein does not interact with Sir3, the anti-silencing phenotype of overexpressed *ASF2* must depend on the Asf2-Sir3 interaction. Immunoprecipitation of Sir3 when *ASF2* is overexpressed yields less

Sir4/Sir2 when compared to cells expressing wild-type levels of *ASF2* (Figure 7D). In contrast, overexpression of *asf2-NRN* does not reduce the amount of Sir4/Sir2 bound to immunoprecipitated Sir3. These results point to a mechanism in which Asf2 and Sir4/Sir2 compete for Sir3 binding. An excess level of Asf2 leads to an increase in the number of Sir3-Asf2 interactions and causes insufficient formation of the SIR complex (Sir3-Sir4-Sir2) that is required for silencing.

The overexpression of Sir4 or its C-terminal domain has also been shown to disrupt silencing (Ivy et al., 1986; Marshall et al., 1987). Although an excess amount of Sir4 disrupts silencing, a moderate increase in Sir4 dosage can stabilize some weaker silent loci. A decrease in Sir4 dosage also leads to a loss of silencing at a weakened *HMR* in heterozygous *SIR4*/*sir4*Δ diploid cells (Sussel et al., 1993). Taken together these observations show that silencing is susceptible to dosage of the SIR proteins and these dosage effects are loci specific. A moderate increase in Asf2 and Sir4 dosage was achieved using low copy replicating plasmids (Figure 7C). Cells containing *SIR4-CEN-HIS* had a very slight increase in silencing at *TEL-VII-URA3* and did not affect the silent state of *HMR*. However, cells containing *ASF2-CEN-HIS* did not affect *TEL-VII-URA3* but caused a loss in silencing at the *hmr*Δ*E-TRP1* reporter. Although the overexpression of *ASF2* titrates Sir3 away from Sir4/Sir2 and leads to widespread silencing loss, a more subtle increase in *ASF2* dosage is sufficient to disrupt weaker silent loci such as the *hmr*Δ*E-TRP1* reporter.

Asf2 incorporates into heterochromatin with the SIR complex

It seemed important to establish if Asf2 is present at silent chromatin since it affects heterochromatin stability and interacts with the SIR complex. Chromatin immunoprecipitation of Asf2 revealed that Asf2 is detectable at many of the same silent loci as Sir3 (Figure 8). Both Asf2 and Sir3 are present at *TEL-VI-R* and the *HM* loci, but the localization of Asf2 to heterochromatin depends on the presence of Sir3 and Sir4. Asf2 is not detectable at any of the known silent loci in *sir3*Δ cells and is more evidence that Asf2 primarily binds and is recruited to chromatin by Sir3. Asf2 is also not found at the silent loci in *sir4*Δ cells and suggests that Asf2 only associates with chromatin when SIR silencing is established. Therefore, Asf2 requires the core SIR complex to be recruited to heterochromatin during early steps of assembly prior to its recruitment.

Asf2 is present at both silencers and spans across the mating-type genes found at *HML*. In contrast, Asf2 is present at the E silencer of *HMR*, but not at *HMRa* genes or the *HMR-I* silencer. Sir3 is present at all these locations and indicates that there must be some additional factor, or lack thereof, that prevents or supports the recruitment of Asf2 to these loci. The *HMR-I* silencer is the only silencer amongst the *HM* loci that lacks the E element responsible for the recruitment of Rap1 (Zou et al., 2006). It is possible that the recruitment of Asf2 to heterochromatin is supported by a Sir3-dependent Rap1 interaction. This potential supporting interaction has not been explored but may help explain differences seen between *HML* and *HMR* regarding Asf2. Another speculation as to why Asf2 may be more abundant at *HML* than *HMR* is the involvement of the recombination enhancer. The recombination enhancer interacts with *HML* specifically and Asf2 may serve to support this interaction. Although they have not been investigated,

interactions between Asf2 and the RE machinery such as Fkh1 and Mcm1 proteins may exist directly or indirectly (Sun et al., 2002). Asf2 may also interact with Sir3 to change the chromatin structure of *HML* in a way that supports RE activity, or to alter the availability of Sir4/Sir2 in the SIR complex as Sir2 is relocated to the RE during its activation.

The absence of Asf2 causes a mating defect in MATa cells exclusively

A significant defect in mating efficiency was seen specifically for *MATa* cells with *asf2*Δ and *asf2-NRN* mutations (Figure 9A). This defect is similar in both *asf2*Δ and *asf2-NRN* and suggests that a lack of the Sir3-Asf2 interaction itself is sufficient to cause a loss in mating efficiency for *MATa* cells. Mating efficiency is determined by a combination of factors involving partner availability, mating-type switching capability, pheromone response, and fusion. In the conditions used partner availability and mating-type switching should not be limiting factors due to having an excess of mating partners available. Currently there is no known link between *ASF2* and other genes that play a role in pheromone response or cell fusion, but disruption of the silent mating-type loci can render cells non-mating. This could occur in *MATa* cells where *a1* is constitutively expressed if mutations in *ASF2* lead to weakened silencing and expression of *HMLα*. ChIP analysis showed that Asf2 binds the entirety of *HML* and only a portion of *HMR* and suggests that *HML* could be more susceptible to instability when *ASF2* is mutated or expressed abnormally. In agreement with this, anti-silencing effects using overexpressed *ASF2* in *K. lactis* only disrupts *HML* and not *HMR* (Hickman et al., 2011). Although there was no detected increase in silencing loss at either of the *HM* loci with *asf2*Δ cells

(Appendix A1), the assay used does not measure silencing in the steady state and does not account for the rate of reestablishment.

Another possible explanation for how *HML* may be exclusively affected in *asf2*Δ and *asf2-NR*N cells during the mating process is the phosphorylation state of Sir3. Sir3 becomes phosphorylated in alpha factor, and changes in the phosphorylation state of Sir3 has been linked to growth activation pathways that involve the redistribution of SIR proteins amongst silent loci (Stone and Pillus, 1996; Ray et al., 2003). Subtelomeric silencing is reduced when Sir3 becomes phosphorylated, but *HML* remains stable and unaffected (Ai et al., 2002). It is possible that a change in Sir3 phosphorylation state in combination with *ASF2* mutants have a compound effect that compromise *HML*, thus allowing its expression and loss of mating capability in *MATa* cells.

Asf2 is not required for silencing

Since an increase in *ASF2* expression causes a loss in silencing it is not unreasonable to predict that cells lacking *ASF2* may cause silent loci to become stronger or more stable. Experiments examining silencing at telomeres and the *HM* loci in *asf2*Δ cells demonstrates that the loss of *ASF2* has minor effects on silencing (Figure 9B & Figure 7A). Transcriptional silencing of the *URA3* reporter at telomeres *TEL-VII-L* and *TEL-IX-R*, and a *TRP1* reporter at *hmr*Δ*E*, is unchanged in *asf2*Δ cells. Although the difference is small, *asf2*Δ cells did provide a slight increase in viability on FOA with the *TEL-V-R-URA3* reporter relative to wild-type cells. The improved silencing in *asf2*Δ cells at *TEL-V-R* alone suggests that specific heterochromatin loci may be more susceptible to changes in *ASF2* expression than others. Deletion of *ASF2* does not affect the overall

integrity of the SIR complex (Figure 5B) but the presence or absence of Asf2 may alter Sir3 availability or the affinity Sir3 has for different heterochromatin loci. For example, the slight increase in silencing seen at *TEL-V-R* in *asf2*Δ cells may be caused by an increase in Sir3 availability that depends on the specific organization of *TEL-V-R*. The exact distance of the *URA3* reporter from the telomere end, the binding profile and abundance of Rap1 or Ku complexes, or the nuclear organization of *TEL-V-R* could be unique characteristics that make it particularly susceptible to increased Sir3 availability caused by the absence of *ASF2* expression.

Asf2 regulates heterochromatin in a silencer-dependent manner

Asf2 appears to have loci-specific dosage effects and situationally can have a positive or negative impact on silencing. A similar example can be seen for the Rap1-interacting factors Rif1 and Rif2. Silencing at the *HM* loci is limiting for Sir4 and is sensitive to Sir4 dosage, whereas silencing at telomeres is limited by both Sir3 and Sir4 dosage. Rif1 interferes with the Rap1-Sir4 interaction at telomeres and *rif1*Δ mutants display improved silencing at telomeres. Deletion of *RIF1* does not alter silencing at the *HM* loci and is attributed to the presence of Sir1-mediated nucleation sites that perhaps block the Rif1 protein from binding to the silencer (Buck and Shore, 1995). Low levels of Sir4 result in a slow establishment of silencing at the *HM* loci, and increased Sir4 levels lead to faster establishment. Similarly, *yku70*Δ and *ubp10*Δ mutants speed the assembly of heterochromatin at the *HM* loci but simultaneously induce defective telomere silencing (Larin et al., 2015). These loci-specific effects can be attributed to a redistribution of Sir4 protein from telomeres to the *HM* loci where Sir4 is a limiting factor. Complementary to

this fact, deletion of *RIF1* and *RIF2* can rescue telomere silencing in *yku70*Δ mutants presumably due to sufficient recruitment of Sir4 by Rap1 where the competition between SIR and RIF proteins for Rap1-binding is absent (Mishra and Shore, 1999).

The observation that a slight increase in *ASF2* expression disrupts *HM* loci but not telomeres support the idea that the Asf2 protein may have a variable influence on heterochromatin depending on the type of silencer (Figure 7C). Sir1-mediated silencers found at the *HM* loci appear to be more susceptible to increased Asf2 dosage, meanwhile telomeres require a more robust overexpression system to disrupt silencing. Sir1 is thought to interact with Sir4 in a nucleation complex that supports the recruitment of Sir3 and subsequent spreading of the SIR proteins (Bose et al., 2004). Excess amounts of Asf2 may out compete Sir4 for Sir3-binding at Sir1-mediated nucleation sites and interfere with the establishment of heterochromatin in a way that is not seen at Sir1-independent nucleation sites such as telomeres.

Asf2 can play a positive role in the formation of heterochromatin

Opposite to its name, the Asf2 protein can play a positive role in heterochromatin formation in certain situations. Although deletion of *ASF2* does not cause any major changes to the integrity of wild-type silencing, the presence of the Asf2 protein may support or stabilize intermediate heterochromatin fibers by interacting with Sir3. It has been proposed that intermediates in the formation of heterochromatin are present prior to the maturation of the chromatin fiber, such as prior to Sir2 HDAC activity or at the forefront of heterochromatin where Sir4/Sir2 has yet to be recruited to Sir3-bound histones (Shrahl-Bolsinger et al., 1997). Deletion of *SAS2*, a HAT that acts on H4K16,

and *RPD3*, a HDAC that acts redundantly with Sir2 on H4K16, produces extended heterochromatin at subtelomeric regions that is thought to cause lethality by blocking the expression of essential genes (Ehrentraut et al., 2010).

The lethality of *rpd3*Δ *sas2*Δ is suppressed by inclusion of the *SAS2* gene on a replicating plasmid by rescuing the heterochromatin boundary and allowing expression of essential genes near the telomere. The requirement for the *SAS2* plasmid can be circumvented by *sir4*Δ, *sir3*Δ, *asf2*Δ, or *asf2-NRN* mutations (Figure 10A). The lack of Sir3 or Sir4, which are critical for the establishment of heterochromatin, prevents silencing thus allowing the expression of essential genes and loss of the *SAS2* replicating plasmid when grown on FOA. Both the *asf2*Δ and *asf2-NRN* mutations also allow for growth on FOA and suggest that the Sir3-Asf2 interaction is required for heterochromatin to extend farther along telomeres to a region where essential genes are silenced. This phenotype demonstrates that the Asf2 protein can have a positive role in the formation of heterochromatin, but this function may depend on a certain chromatin landscape. The Asf2-Sir3 interaction could support intermediate steps in heterochromatin formation especially at the forefront of spreading where boundary elements are present.

Another example where Asf2 plays a positive role in heterochromatin stability can be seen during the active disassembly of silent loci (Figure 10B). Silencing of a *TEL-V-R-URA3* reporter is lost rapidly upon overexpression the *URA3* transcription factor gene *PPR1*. Overall, silencing is lost more quickly in cells arrested in mitosis with nocodazole compared to cells arrested in G1 with alpha-factor. Both *asf2*Δ and *asf2-NRN* mutations had little impact on the disassembly rate of heterochromatin with cells arrested in G1. Intriguingly, *asf2*Δ and *asf2-NRN* mutations had differential effects on the

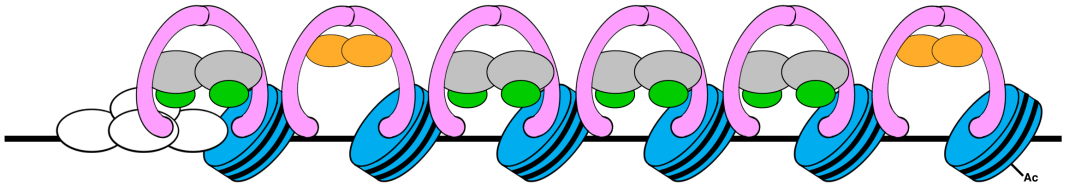
disassembly rate for cells arrested in nocodazole. Cells missing the entirety of the Asf2 protein have the fastest rate of disassembly and suggest that the presence of Asf2 stabilizes heterochromatin for longer as transcription competes with silencing. Cells with the *asf2-NRN* mutation lack the Sir3-Asf2 interaction but displayed a slower rate of disassembly compared to wild-type cells arrested in nocodazole. This observation may be explained by an undefined interaction between Asf2 and Rap1-bound promoter regions, or the SWI/SNF remodeler that is required for Sir3 displacement during replication stress (Lieb et al., 2001; Manning and Peterson, 2014) that can still occur in cells expressing *asf2-NRN*.

The Asf2 homodimer depends on Sir3

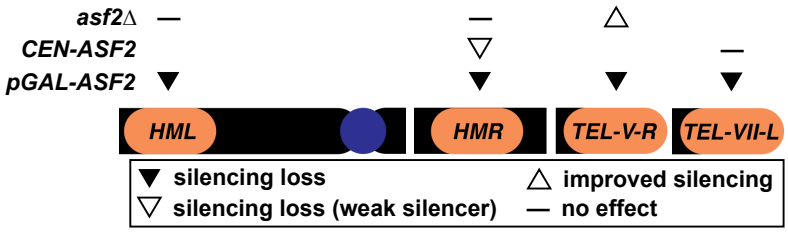
Sir4 homodimerization is a function of its coiled-coil domain, and is an interaction that is required for silencing and the proper recruitment of Sir2 to heterochromatin (Chang et al., 2003). Due to the homology observed between the coiled-coil domains of Asf2 and Sir4 the possible formation of an Asf2 homodimer was investigated. Although the self-interaction of the Sir4 coiled-coil has been show *in vitro*, this interaction has yet to be demonstrated *in vivo* for the full length Sir4 protein. Diploid cells containing differentially tagged copies of Sir4 were subject to immunoprecipitation, and an interaction between Sir4-eGFP and Sir4-13Myc was observed that does not depend on Sir3 (Figure 6A). Two different Sir4 molecules interacting is evidence that Sir4 homodimerizes *in vivo* and is an interaction that persists in the absence of Sir3. Complementary to these findings, diploid cells containing two distinctly tagged copies of Asf2 were also subject to immunoprecipitation (Figure 6B). An interaction between

Asf2-GFP and Asf2-13Myc was observed and is evidence that Asf2 can homodimerize *in vivo*, but in contrast to Sir4, the Asf2 homodimer is not detectable in the absence of Sir3. These results point to a mechanism where Sir3 first binds to chromatin and then incorporates the Asf2 homodimer, or Sir3 and Asf2 are recruited to chromatin simultaneously as a subcomplex. These observations support the idea that Asf2 requires Sir3 to be recruited to heterochromatin and impact its stability (Figure 11).

A


Ac

B


asf2Δ
CEN-ASF2
pGAL-ASF2
HML
HMR
TEL-V-R
TEL-VII-L
silencing loss
silencing loss (weak silencer)
improved silencing
no effect

C

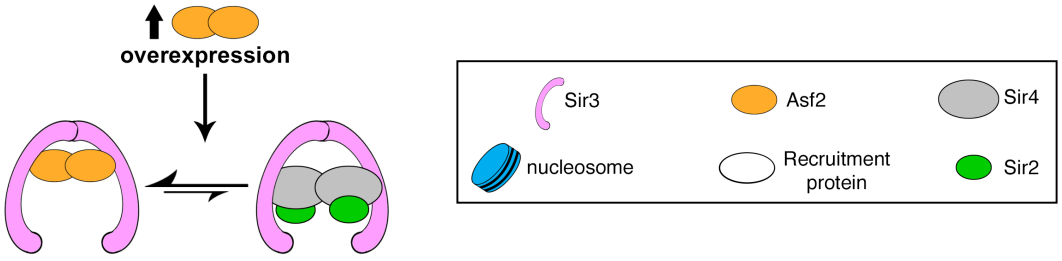

overexpression
Sir3
Asf2
Sir4
nucleosome
Recruitment protein
Sir2

Figure 11. *ASF2* expression as a mechanism to change Sir3 availability. (A) Depiction of Asf2 incorporated into the heterochromatin fiber. Asf2 is recruited to chromatin by Sir3 as a homodimer. The Asf2-Sir3 subcomplex is likely less abundant than the SIR complex in mature heterochromatin near the silencer. However, the Asf2-Sir3 subcomplex may be more prominent at the forefront of extended heterochromatin where it could stabilize Sir3 binding to nucleosomes as the fiber develops. The formation of a Sir4/Asf2 heterodimer bound to a Sir3 dimer (not shown) cannot be ruled out based on the data presented. **(B)** Summary of Asf2 dosage effects on silent loci. Cells with no *ASF2* (*asf2Δ*), an increased copy number of *ASF2* (*CEN-ASF2*), or overexpressed *ASF2* (*pGAL-ASF2*) have different effects on silencing at *HML*, *HMR*, *TEL-V-R*, and *TEL-VII-L*. A loss of silencing at loci with wild-type silencers is represented by a filled down arrow, and a loss of silencing at loci with a weakened silencer (*hmrΔE-TRP1*) is represented by a hollow down arrow. A slight improvement in silencing is represented by a hollow up arrow, and no effect is represented by a dash. **(C)** Overexpression of *ASF2* disrupts silencing by preventing Sir4 binding to Sir3. Excess amounts of Asf2 protein (black up arrow) result in an unbalanced competition (equilibrium arrows) between Asf2 and Sir4/Sir2 for Sir3-binding. When insufficient amounts of the complete SIR complex are formed silencing cannot be established or maintained.

Chapter 4: Roles of the Sir3-Sir4 interaction and H4K16 acetylation in heterochromatin formation

4.1 Introduction

The stabilization of heterochromatin through O-acetyl-ADP-ribose

Both SIR-SIR protein and SIR-histone interactions are required for fully functional heterochromatin to form. Small molecule factors and other proteins are also able to stabilize either of these interactions. Early experiments *in vitro* using the N-terminal half of Sir4 showed that it bound better to Sir3 than the full length Sir4 protein. Immunoprecipitation data revealed that Sir2/Sir4 barely associate with Sir3 *in vitro* and hinted that an early step in heterochromatin assembly alters the structure of Sir4 to allow for a stronger interaction with Sir3 (Moazed et al., 1997). The histone deacetylase activity of Sir2 produces O-acetyl-ADP-ribose, or AAR, a side product of the reaction that requires a deacetylated peptide and NAD+ hydrolysis. AAR binds Sir3 and pays a role in the formation of pre-heterochromatin filaments by stabilizing the interaction between the Sir3 BAH domain and C-terminal domains. This stabilization supports both inter Sir3 and Sir3-nucleosome interactions (Wang et al., 2019). A chimeric protein made of Sir3 and Hos3, another NAD+ dependent HDAC, still has silencing. In fact, removing the production of AAR still allows for silencing to occur but the removal of acetylation at H4K16 remains a requirement (Chou et al., 2008).

AAR has also been investigated *in vitro* using calorimetry to show the interaction between AAR and Sir3. The same group analyzed Sir3 localization by ChIP with AAR present and revealed that Sir3 was able to spread to extended regions of heterochromatin, but Sir2 was not present in these regions (Tung et al., 2019). Upstream of AAR

production is Rpd3, a HDAC that can act on the same residue as Sir2 thus removing the substrate for Sir2 that produces AAR. Deletion of Rpd3 leads to excess SIR spreading and silencing of subtelomeric genes. This corresponds with the fact that Rpd3 can create heterochromatin boundaries by being targeted to silent loci. Less AAR production leads to less extensive Sir3 binding and insufficient spreading, therefore highlighting the importance of Sir2 catalysis in heterochromatin formation (Ehrentraut, Weber, et al., 2010). Although the interaction is direct between Sir3 and AAR, the presence of AAR helps the entire SIR complex to have an increased affinity for reconstituted chromatin (Martino et al., 2009).

A model for SIR-mediated heterochromatin based on in vitro data

Although plenty of valuable work has been done *in vivo* and *in vitro* to generate hypothesized models of heterochromatin, it is difficult to fully explain the countless interactions that occur between SIR proteins and nucleosomes in a fully mature stretch of heterochromatin fiber. Sir3 has more flexible binding to chromatin than the binary nucleation and polymerization model suggests. Strong signals of Sir3 can be detected by ChIP at silencers, but the signal is discontinuous between silencers where heterochromatin is known to spread. Sir3 binding to telomeres is associated with highly condensed promoters and is stable, but widespread Sir3 binding to euchromatin regions suggests that subsequent stabilization is required for these interactions to persist (Radman-Livaja et al., 2011). Sir3 has two domains that support dimerization. One of these domains is shared with the Sir4 binding interface and this interaction is

strengthened when Sir3 exists as a stable dimer. Higher order assemblies have been observed *in vitro* with increasing concentrations of Sir3 (King et al., 2006).

Reconstituted chromatin has been used to try to understand how these higher order structures of Sir3 may bridge nucleosomes. The maximal binding of the SIR proteins occurred with di-nucleosomes and supports the idea of an interrupted SIR bridge model, where one part of the Sir3 dimer within the SIR complex interacts with one nucleosome and the counterpart to the Sir3 dimer interacts with a neighboring nucleosome (Behrouzi et al., 2016). This data somewhat contrasts the higher order structures seen with increasing concentrations of Sir3 but may be more reflective of what happens in a live cell. On the other hand, these higher order interactions seen *in vitro* using sedimentation and AFM reveal quaternary structure in Sir3 oligomers that may still be present *in vivo* when chromatin is further compacted or folds back onto itself in a three-dimensional structure (McBryant et al., 2006). Taken together, Sir3 is thought to interact with nucleosome arrays with a stoichiometry of 2-1, but Sir3 alone is not enough to compact chromatin fibers. The integrity of H4K16 and the Sir3-Sir4 interaction are both required to compact chromatin fibers and allow the Sir3 dimer to stabilize and bridge adjacent nucleosomes. Satisfying these requirements results in Sir3 being associated with a nucleosome trimer bridged by two Sir3 dimers that are supported by the Sir2-Sir4 heterotetramer (Swygert et al., 2014; Swygert et al., 2018).

Histone variants and modifications that are markers of euchromatin

Regulating how heterochromatin is limited in its spreading is just as important as its initial formation and subsequent stabilization. Histone modifications and variants are a

way to interrupt the regular interactions between the SIR proteins and nucleosomes. Both H3 methylation and the histone variant H2A.Z, or Htz1 in yeast, have been shown to antagonize spreading of the SIR complex. It is important to note that these modifications are also associated with a range of effects on transcriptional regions. H2A.Z acetylation is important for boundary function in *S. cerevisiae* and is established by NuA4 (Venkatasubrahmanvam et al., 2007). Some genes that are clustered near telomeres are regulated by the incorporation of Htz1 to protect them from the excess spreading of heterochromatin. Additionally, Htz1 deposits at active genes near *HMR* and acts as a boundary element to heterochromatin near the mating-type loci (Meneghini et al., 2003).

Other histone modifications on histone H3 and H4 that contact Sir3 also help to regulate the distance that the SIR complex can spread. Deletion of Set1 abolishes all H3K4 methylation, which is a modification important for rDNA silencing but also plays a role in telomere silencing. Methylation of H3K4 is generally associated with euchromatin and is removed by the JmjC domain-containing demethylase Jhd2 in budding yeast (Shia et al., 2006; Liang et al., 2007). The methylation of H3K4 is thought to antagonize silencing in two ways: first by inhibiting catalysis of H3K9 methylation that supports silencing, and by disrupting overall nucleosome stability. Tri-methylation of H3K4 is a modification commonly found at transcription start sites and di-methylation of H3K4 commonly corresponds with expressed genes (Greenstein et al., 2020). H3K4 can be methylated by Set1 or acetylated by Gcn5, and acetylation of H3K4 is also antagonistic to silencing. Removal of H3K4 acetylation can be accomplished by Hst1 in euchromatin but requires Sir2 to remove it from heterochromatin. H3K4 acetylation is usually found near promoters adjacent to H3K4 methylation and indicate a role for H3K4 acetylation in

preventing excess spreading of heterochromatin. (Thompson et al., 1994; Guillemette et al., 2011).

Active turnover of histone modifications & heterochromatin formation

The dosage of SIR proteins was previously thought to play a role in how far heterochromatin could spread, but more recent work has indicated that histone modifications such as H3K79 methylation and H4K16 acetylation may be more influential. Methylation of H3K79 does not reduce binding of the SIR complex to heterochromatin as shown by ChIP but does help with boundary formation. This is supported by the fact that removal of Dot1, the H3K79 HMT, still has silencing (Kitada et al., 2012; Sneppen and Dodd, 2015). Dot1 relies on an acidic patch in its C-terminal domain to bind a basic patch on histone H4 to conduct HMT activity and is an interesting example of trans-histone regulation. Strains lacking Dot1 have a complete loss of H3K79 methylation (Fingerman et al., 2007). In contrast to some *in vitro* data, mutating the H3K79 residue or using Dot1 catalytic mutants impaired telomere silencing and suggested that Dot1 activity and/or methylation of H3K79 promote SIR binding to telomeres (Ng, 2002). In addition to this, H3K79 methylation has been attributed to boundary function as silencing of extended silent domains by overexpressed *SIR3* seem to stop at areas enriched for H3K79 methylation. Overexpression of *SIR3* extends telomeres and reduces the wild-type profile of H3K79 methylation and is perhaps due to Dot1 being unable to establish methylation with extra SIR silencing present (Hocher et al., 2018; Ng et al., 2003).

H4K16 and its acetylation is another important residue that has long been known to influence SIR binding to chromatin (Johnson et al., 1992). Looking at the localization of SIR proteins by ChIP reveals that big peaks of SIR proteins coincide with hypoacetylated H4K16 at silencers and other non-expressed regions. Cross-linked samples have more peaks which indicate specialized chromatin organization imposed by the SIR proteins (Thurtle and Rine, 2014). Hypoacetylation decreases with distance from the telomere as Sir2 HDAC activity and Sas2 HAT activity work in concert to regulate the chromatin boundary. Rpd3 is antagonistic to Sir2 function as it steals the acetylated H4K16 substrate from Sir2 in early steps of assembly (Thurtle-Schmidt et al., 2016; Kimura et al., 2002). Sas2 acetylates H4K16 and deletion or mutation of this protein leads to excess Sir3 spreading. Deletion of both *SIR2* and *SAS2* still has some Sir3 binding at telomeres and is made possible by a lack of heterochromatin boundary (Suka et al., 2002). SIR-driven heterochromatin removes acetylation quickly, but it takes several generations for methylation to be removed. The gradual removal of histone methylation allows more Sir3 binding over time. Removal of *SAS2* and *DOT1* allow for Sir3 to accumulate faster at *HMR* indicating that Sir3 spreading depends highly on histone modifications (Katan-Khaykovich and Struhl, 2005). Removal of *SAS2* or *GCN5* also reduce tRNA barrier activity, a process where tRNA genes and partially assembled Pol III act as a critical barrier for heterochromatin near *HMR* (Donze, 2001; Simms et al., 2008). These examples highlight how dynamic heterochromatin is, and how much its establishment and ability to spread depends on not only the presence or absence of histone modifications, but the active process and timing of these changes as well.

Extended regions of heterochromatin driven by Sir3

Histone deacetylation by Sir2 is not sufficient for the full recruitment of SIR proteins. Mutations in Sir4 that disrupt the interaction between Sir3 and Sir4 are defective for silencing. Therefore, SIR-SIR interactions are required for functional silencing (Rudner et al., 2005). Silencing can be restored if the interaction between the Sir3 AAA+ domain and the coiled-coil domain of Sir4 are reconstituted (Samel et al., 2017). The acetylation mimic H4K16A is unable to bind Sir3 as efficiently as non-acetylated H4K16. In addition, the binding between the Sir3 AAA+ domain and non-methylated H3K79 is much stronger than that of methylated H3K79. Removal of the heterochromatin boundary by deletion of both *RPD3* and *SAS2* therefore promotes Sir3 binding to chromatin and has been shown to reduce viability in cells by silencing essential genes found in the subtelomere region (Johnson et al., 2009; Ehrentraut et al., 2010). These extended regions of heterochromatin that are also seen with overexpressed Sir3 are mainly composed of Sir3 and have less detectable levels of Sir4 and Sir2 compared to regions closer to the site of nucleation (Strahl-Bolsinger et al., 1997). The ability for Sir3 to spread and silence on its own is a process that is not well understood. The mechanism of SIR-nucleosome interactions in regions of extended heterochromatin, and the requirement for SIR-SIR protein interactions at the forefront of actively assembling heterochromatin has yet to be investigated fully.

4.2 General Hypothesis & Specific Objectives

SIR-mediated heterochromatin nucleates at silencers and spreads across nucleosomes to silence genetic information. The SIR proteins interact with several recruitment factors that bind DNA and guide the formation of heterochromatin to the appropriate loci. However, the SIR complex is the main component of heterochromatin that spreads along a given silent loci in *S. cerevisiae*. The interaction between SIR proteins and nucleation proteins, SIR proteins and nucleosomes, and SIR-SIR interactions are all involved in the formation of functional heterochromatin. This chapter addresses the hypothesis that the recruitment and spreading of SIR proteins require different combinations of these interactions. Mutations in *SIR3* that abolish the Sir3-Sir4 interaction may serve as a model to investigate separate requirements in nucleation and spreading.

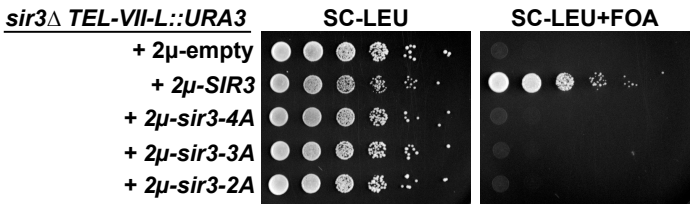
1. Characterize the silencing capability of *sir3-4A* strains.
2. Determine if the Sir3-4A protein incorporates into heterochromatin.
3. Identify if the Sir3-Sir4 interaction is required for nucleation and/or spreading of the SIR complex.
4. Propose a mechanism that allows for the recruitment of Sir4 to heterochromatin that contains Sir3-4A.

4.3 Results

Mutant sir3-4A is defective for silencing but is not dominant negative

It is thought that interactions between Sir3 and Sir4, and Sir3 and nucleosomes are important for the spreading of SIR-mediated heterochromatin. Mutations in *SIR3* were generated and expressed on 2μ plasmids based on previous reports of residues that are important for the Sir3-Sir4 interaction and complete SIR complex formation (Ehrentraut, 2011). These plasmids produce the mutant proteins Sir3-2A, Sir3-3A, and Sir3-4A that contain up to four amino acid substitutions within the AAA+ domain at K657A, K658A, R659A, and K660A. To test how each Sir3 mutant affects silencing the plasmids were incorporated into strains with a *TEL-VII-L-URA3* reporter. Strains lacking endogenous *SIR3* were transformed with the 2-micron plasmids containing either empty, wild-type *SIR3*, *sir3-2A*, *sir3-3A*, or *sir3-4A* and then grown to saturation before spotting 10-fold serial dilutions of cells onto solid SC-LEU and SC-LEU+FOA media (Figure 12A). Strains that maintained the plasmid were selected for by excluding leucine from growth media. All strains were viable on SC-LEU, but only strains expressing the 2μ-*SIR3* plasmid were viable on SC-LEU+FOA. The same plasmids were transformed into strains containing endogenous *SIR3* to test if the presence of the mutant Sir3-2A, Sir3-3A, or Sir3-4A proteins interfered with wild-type silencing. All the strains allowed for growth on both SC-LEU and SC-LEU+FOA media regardless of the plasmid they maintained (Figure 12B). Cells grown in the presence of FOA are not viable when *URA3* is expressed, and the growth defects brought on by these plasmids appear to depend on the absence of endogenous wild-type Sir3 protein.

A


sir3Δ TEL-VII-L::URA3
SC-LEU
SC-LEU+FOA
+ 2μ-empty
+ 2μ-SIR3
+ 2μ-sir3-4A
+ 2μ-sir3-3A
+ 2μ-sir3-2A

B

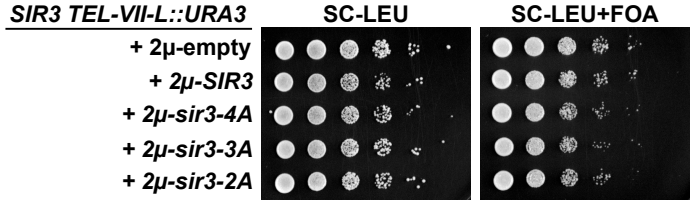
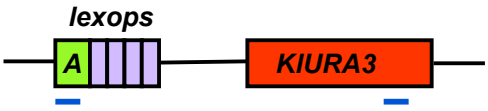

SIR3 TEL-VII-L::URA3
SC-LEU
SC-LEU+FOA
+ 2μ-empty
+ 2μ-SIR3
+ 2μ-sir3-4A
+ 2μ-sir3-3A
+ 2μ-sir3-2A

Figure 12. Sir3-4A cannot silence chromatin and is not dominant negative. (A) Sir3-2A, 3A, and 4A cannot nucleate silent chromatin. The Sir3 mutants were overexpressed from 2-micron plasmids in a *TEL-VII-L-URA3 sir3Δ* strain. Growth on FOA media is representative of repression at *TEL-VII-L-URA3*. **(B)** Expression of *sir3-2A*, *sir3-3A*, and *sir3-4A* do not interfere with silencing when co-expressed with wild-type *SIR3*. The mutants were overexpressed from 2-micron plasmids in a *TEL-VII-L-URA3* strain. All strains were grown in liquid SC-LEU media for 2 days at 30˚C and 10-fold serial dilutions were spotted onto the indicated plates and incubated for 2 days (SC-LEU) or 3 days (SC-LEU+FOA) at 30˚C. Data produced by Rosalind Gerson, Rudner Lab.

Tethered sir3-4A-LexA cannot nucleate silencing

Although Sir3-4A is detectable at heterochromatin loci it is unable to form functionally silent chromatin without the presence of wild-type Sir3 protein. To test if this defect occurs during the nucleation or spreading of SIR-mediated heterochromatin *SIR3-LexA* and *sir3-4A-LexA* fusions were generated and expressed from 2μ plasmids. These plasmids were transformed into cells containing four LexA-binding motifs inserted upstream of a weakened *HMR* reporter (*4lexops-AeB-hmr-klURA3*). Strains containing *2μ-LexA*, *2μ-SIR3-LexA*, and *2μ-sir3-4A-LexA* were grown to saturation before being serially diluted and spotted onto solid SC-HIS and SC-HIS+FOA media (Figure 13). Strains maintaining plasmids were selected for using media lacking histidine. Strains containing any of the three plasmids were viable on SC-HIS regardless of their *SIR*+, *sir3*Δ, *sir4*Δ, or *sir3*Δ *sir4*Δ genotype. Wild-type *SIR*+ strains were viable on SC-HIS+FOA with the *2μ-SIR3-LexA* and *2μ-sir3-4A-LexA* plasmids, but not the empty *2μ-LexA* plasmid. In contrast, strains with a *sir3*Δ background were only viable on SC-HIS+FOA with the *2μ-SIR3-LexA* plasmid and showed no growth for either the *2μ-LexA* or *2μ-sir3-4A-LexA* plasmid. Not surprisingly, strains lacking *SIR4* or both *SIR4* and *SIR3* had no growth on SC-HIS+FOA with any of the transformed plasmids. Similar to the previous results, strains with this *URA3* reporter were only able to grow on media containing FOA with wild-type *SIR3* present even when Sir3-4A is tethered to silencers.

A


lexops
A
KlURA3

B

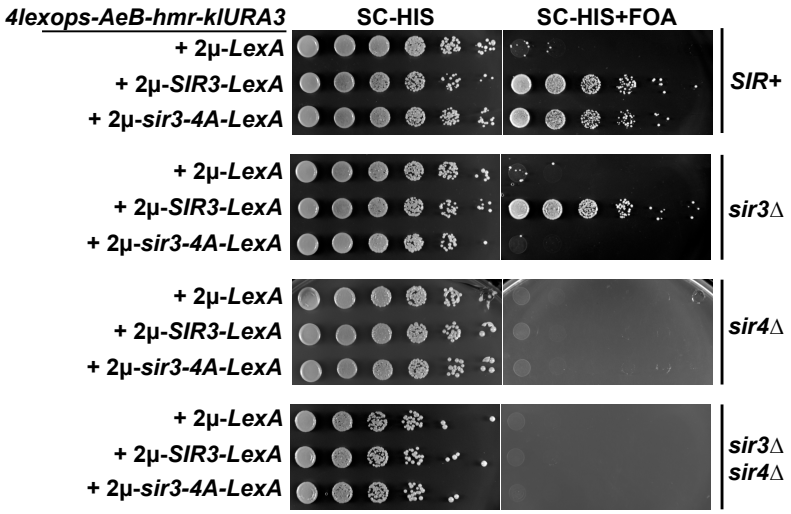

4lexops-AeB-hmr-klURA3
SC-HIS
SC-HIS+FOA
+ 2μ-LexA
+ 2μ-SIR3-LexA
+ 2μ-sir3-4A-LexA
SIR+
+ 2μ-LexA
+ 2μ-SIR3-LexA
+ 2μ-sir3-4A-LexA
sir3Δ
+ 2μ-LexA
+ 2μ-SIR3-LexA
+ 2μ-sir3-4A-LexA
sir4Δ
+ 2μ-LexA
+ 2μ-SIR3-LexA
+ 2μ-sir3-4A-LexA
sir3Δ
sir4Δ

Figure 13. Tethered Sir3-4A cannot nucleate silencing in the absence of wild-type Sir3. (A) Schematic diagram depicting construction of tethering strains. All strains contain six LexA binding motifs inserted upstream of a weakened *hmr-aEB* silencer. The *URA3* reporter was inserted at the *HMR* locus. Strains either expressed all SIR proteins or lacked one or both of Sir3 and Sir4. These strains were transformed with a 2-micron plasmid carrying either *LEXA* alone, *SIR3-LEXA*, or *sir3-4A-LEXA*. Growth on FOA media is representative of repression at *HMR*. **(B)** Strains were grown in liquid SC-HIS media for 2 days at 30˚C and 10-fold serial dilutions were spotted onto the indicated plates and incubated for 2 days (SC-HIS) or 3 days (SC-HIS+FOA) at 30˚C. Data produced by Rosalind Gerson, Rudner Lab.

Sir4 is recruited to chromatin independently of the Sir3-Sir4 interaction

The expression of *sir3-4A* was not dominant negative to *SIR3* and was insufficient to rescue growth defects seen in *sir3*Δ cells. It was unclear if this phenotype was due to the inability of the mutant protein to localize to heterochromatin. Chromatin immunoprecipitation was used to determine if Sir3-4A was being incorporated into heterochromatin at *TEL-V-R* in cells that contain wild-type Sir3. The enrichment of Sir3, Sir4, and Sir2 was analyzed for strains containing either 2µ-empty, *2µ-SIR3*, or *2µ-sir3-4A* and a *TEL-V-R-URA3* reporter at either 1.0 or 5.5 kb from the end of the telomere (Figure 14). Sir3 showed an enrichment of 0.19%, 0.39%, and 0.38% for *URA3* at 1.0kb compared to less than 0.1% for *ACT1* in strains containing 2µ-empty, *2µ-SIR3*, and *2µ-sir3-4A* respectively. Sir3 enrichment at *URA3* at 5.0kb was 0.33% and 0.34% for *2µ-SIR3* and *2µ-sir3-4A*, but only 0.03% for 2µ-empty. Sir4 enrichment was 0.49%, 0.25%, and 0.24% for *URA3* at 1.0kb compared to <0.1% for *ACT1* in strains containing 2µ-empty, *2µ-SIR3*, and *2µ-sir3-4A* respectively. Sir4 enrichment at *URA3* at 5.0 kb was 0.06%, 0.13%, and 0.11% for 2µ-empty, *2µ-SIR3*, and *2µ-sir3-4A* respectively. Sir2 enrichment at *URA3* at 1.0 kb was 0.18% for 2µ-empty, 0.10% for *2µ-SIR3*, and 0.12% for *2µ-sir3-4A*. Sir2 enrichment at *URA3* at 5.0 kb was 0.03% for 2µ-empty, 0.07% for *2µ-SIR3*, and 0.07% for *2µ-sir3-4A*. In all cases the relative enrichment of Sir3, Sir4, and Sir2 at *TEL-V-R* was similar for both *2µ-SIR3* and *2µ-sir3-4A*. Unexpectedly, the enrichment of Sir4 and Sir2 is higher in the 2µ-empty strain compared to *2µ-SIR3* and *2µ-sir3-4A* for *URA3* at 1.0 kb from the telomere.

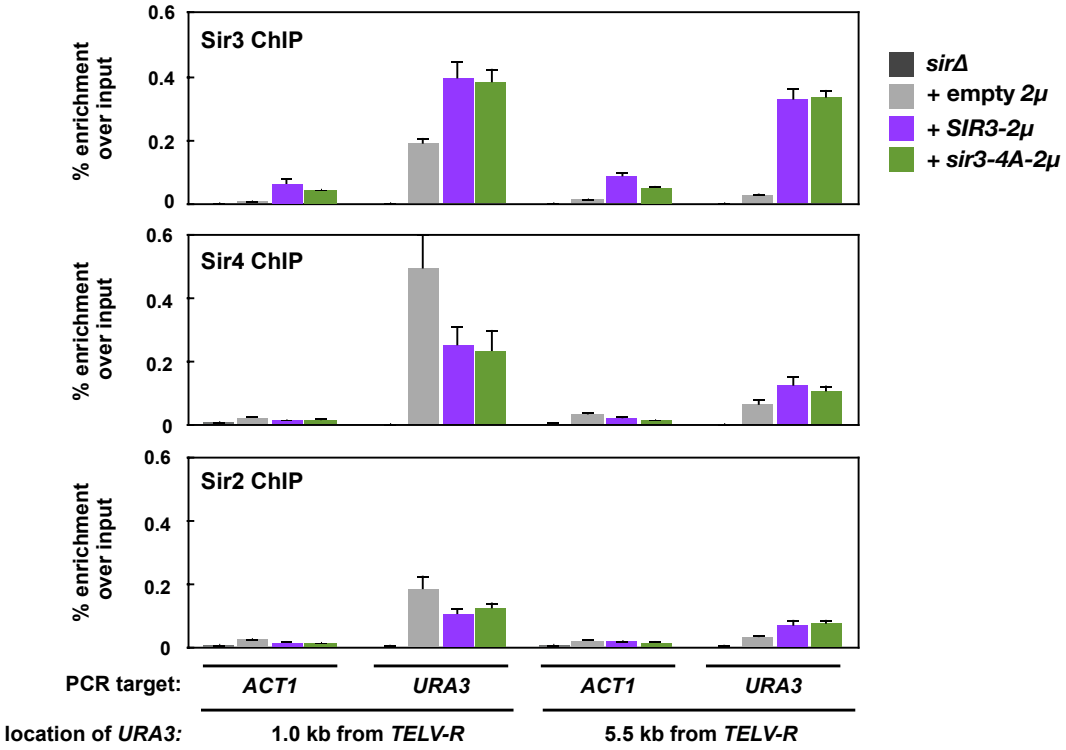

Sir3 ChIP
Sir4 ChIP
Sir2 ChIP
% enrichment over input
0.6
0.4
0.2
0
sirΔ
+ empty 2μ
+ SIR3-2μ
+ sir3-4A-2μ
PCR target:
ACT1
URA3
ACT1
URA3
location of URA3:
1.0 kb from TELV-R
5.5 kb from TELV-R

Figure 14. Sir4 and Sir2 are recruited to telomeric silent chromatin domains independently of the Sir3-Sir4 interaction. Strains with *URA3* located 1.0 or 5.5 kb from the end of *TEL-V-R* and overexpressing either *SIR3* or *sir3-4A* were grown in media containing FOA to select for cells that repress *URA3*. ChIP was performed to measure Sir3, Sir4 and Sir2 enrichment at the *URA3* locus in each strain. Cells were grown overnight in SC-LEU at 30°C before being processed for chromatin immunoprecipitation using anti-Sir3, anti-Sir4, or anti-Sir2 antibodies coupled to magnetic beads. PCR reactions were performed with locus-specific primers along *TEL-V-R*, and the *ACT1* locus was used as an internal control (Table 3). The mean +/- SEM of multiple replicates (n=3) are shown. Data produced by Rosalind Gerson, Rudner Lab.

Silencing is compromised in sas2Δ strains containing sir3-4A

Since Sir3-4A is recruited to chromatin but the interaction between Sir3 and Sir4 is compromised it was hypothesized that there must be a redundant mechanism for recruiting Sir4 to silencer-distant heterochromatin. Sir2 exists mainly as a heterodimer with Sir4 and has affinity for acetylated H4K16, which is a histone modification deposited by *SAS2*. Wild-type or *sas2*Δ strains containing *2μ-empty*, *2μ-SIR3*, or *2μ-sir3-4A* were spotted onto SC-LEU+FOA plates to test for silencing of the *TEL-V-R-URA3* reporter at 1.0 kb, 1.5 kb, 2.5 kb, and 3.5 kb (Figure 15). Wild-type strains (SAS2 2μ-empty) had moderate growth with *URA3* at 1.0 kb, the most growth with *URA3* at 1.5 kb, and minimal growth with *URA3* at both 2.5 and 3.5 kb. For every locus tested *2μ-sir3-4A* gave more growth on SC-LEU+FOA than *2μ-empty* but had slightly less growth than *2μ-SIR3* in strains containing wild-type *SAS2*. Oppositely, *sas2*Δ strains with *2μ-empty* showed no growth on SC-LEU+FOA at any of the loci. Expression of *2μ-SIR3* in *sas2*Δ strains was able to rescue some growth on SC-LEU+FOA relative to *2μ-empty* with the strongest effect being at 2.5kb. Expression of *2μ-sir3-4A* was not able to rescue growth on SC-LEU+FOA for any of the loci tested with a *sas2*Δ background.

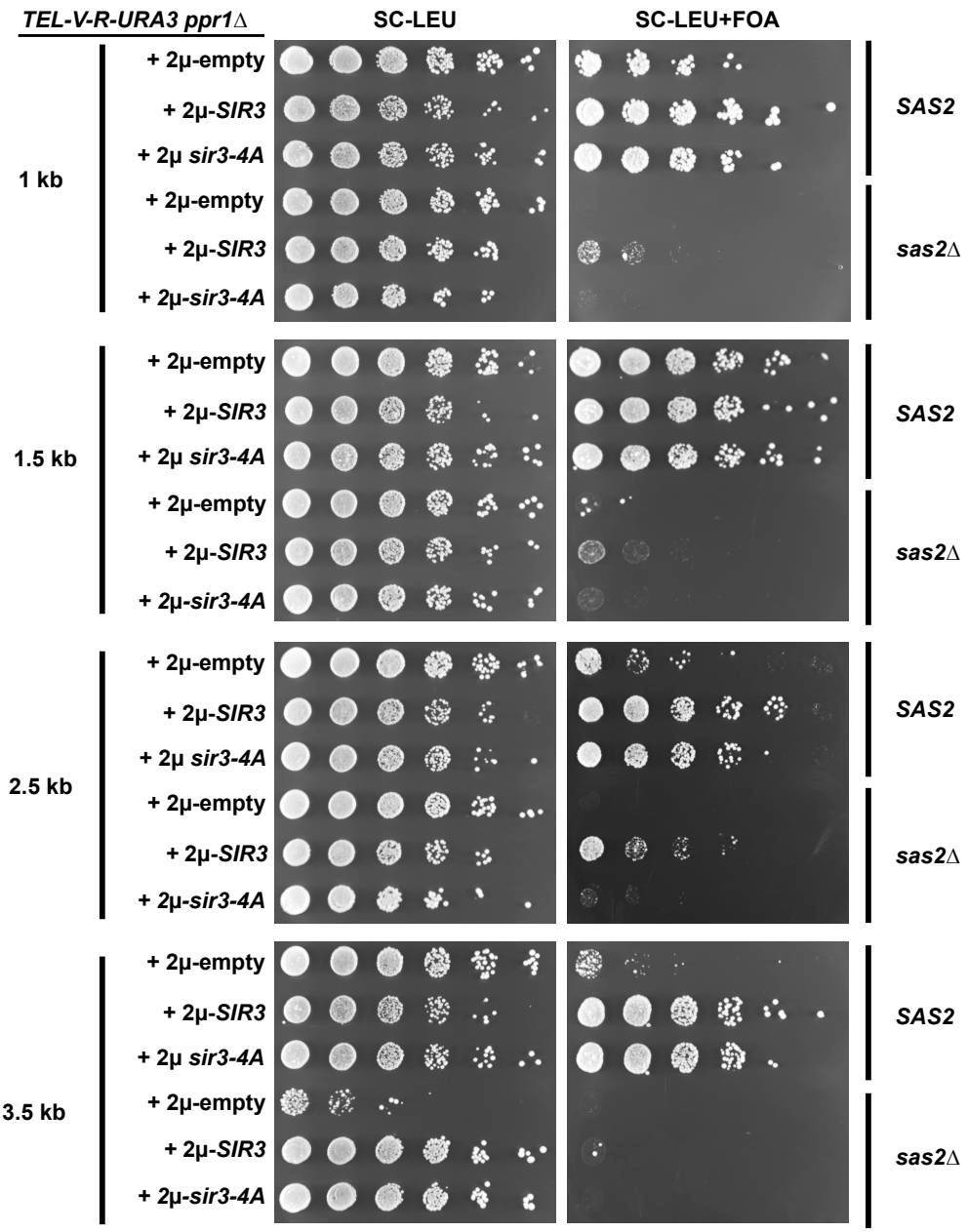

TEL-V-R-URA3 ppr1Δ
SC-LEU
SC-LEU+FOA
1 kb
1.5 kb
2.5 kb
3.5 kb
+ 2μ-empty
+ 2μ-SIR3
+ 2μ sir3-4A
+ 2μ-sir3-4A
SAS2
sas2Δ

Figure 15. Partial silencing is detected in *sas2Δ* strains expressing *sir3-4A*. Strains containing a *TEL-V-R-URA3* reporter in a wild-type or *sas2Δ* background with *2μ-empty*, *2μ-SIR3*, or *2μ-sir3-4A* were grown in liquid SC-LEU media for 2 days at 30˚C and 10-fold serial dilutions were spotted onto solid media and incubated for 2 days (SC-LEU) or 3 days (SC-LEU+FOA) at 30˚C. Growth on FOA media is representative of repression at *TEL-V-R-URA3*. Data produced by Rea Konci, Rudner Lab.

SIR4 spreading depends on H4K16 acetylation and interaction with Sir3

Chromatin immunoprecipitation was used to test if the presence of H4K16 acetylation supports the recruitment of Sir4 to heterochromatin in strains where Sir3-4A is present. The enrichment, represented by % input, was obtained for Sir3-FLAG and Sir4 at *HMR-E*, *URA3* (2.5 kb from the end of *TEL-V-R*), *TEL-VI-R* (0.5 kb and 1.5 kb from the telomere end), and *ACT1* (Figure 16). Strains containing wild-type *SAS2* and *2μ-SIR3-FLAG* produced a % input for Sir3 of 0.43% for *URA3*, 0.80% for *TEL-VI-R* 1.5 kb, 2.26% for *TEL-VI-R* 0.5 kb, 0.67% for *HMR-E*, and 0.05% for *ACT1*. Strains containing wild-type *SAS2* and *2μ-SIR3-FLAG* produced a % input of 0.15% for *URA3*, 1.14% for *TEL-VI-R* 1.5 kb, 0.96% for *TEL-VI-R* 0.5 kb, 1.21% for *HMR-E*, and 0.07% for *ACT1*. The enrichment of Sir3 for sas2Δ *2μ-SIR3-FLAG* and sas2Δ *2μ-sir3-4A-FLAG* strains were as follows: 0.16% and 0.12% for *URA3*, 0.35% and 0.35% for *TEL-VI-R* 1.5 kb, 1.15% and 0.81% for *TEL-VI-R* 0.5 kb, 0.27% and 0.28% for *HMR-E*, and 0.09% and 0.08% for *ACT1*. Sir4 enrichment was detectable in *2μ-SIR3-FLAG* and *2μ-sir3-4A-FLAG* strains at 0.23% and 0.18% for *TEL-VI-R* 1.5 kb, 1.58% and 1.63% for *TEL-VI-R* 0.5kb, and 0.30% and 0.50% for *HMR-E*. Sir4 enrichment was also detectable in *sas2*Δ *2μ-SIR3-FLAG* and *sas2*Δ *2μ-sir3-4A-FLAG* strains at 0.07% and 0.08% for *TEL-VI-R* 1.5 kb, 0.95% and 1.30% for *TEL-VI-R* 0.5kb, and 0.18% and 0.20% for *HMR-E*. The control strains *SAS2 2μ*-empty, *sas2*Δ *2μ*-empty, *SAS2 sir4*Δ *2μ*-empty, and sas2Δ sir4Δ *2μ*-empty all yielded a % input of <0.01% for both Sir3 and Sir4. Overall, the enrichment of Sir3 was similar for *sas2*Δ *2μ-SIR3-FLAG* and *sas2*Δ *2μ-sir3-4A-FLAG* at all loci except for TEL-VI-R 0.5 kb, which was still enriched for Sir3 in both strains. The enrichment of Sir3 was more variable in the *SAS2 2μ-Sir3-4A-FLAG* strain compared to

the *SAS2 2μ-SIR3-FLAG* strain for all loci except for *URA3*. Sir4 enrichment at *URA3* and *TEL-VI-R* 1.5 kb was nearly undetectable compared to Sir3 enrichment for both *sas2*Δ *2μ-SIR3-FLAG* and *sas2*Δ *2μ-sir3-4A-FLAG*. In general, removal of *SAS2* appears to lead to a lower yield of Sir3 and Sir4 for all loci regardless of the presence of either wild-type Sir3 or mutant Sir3-4A.

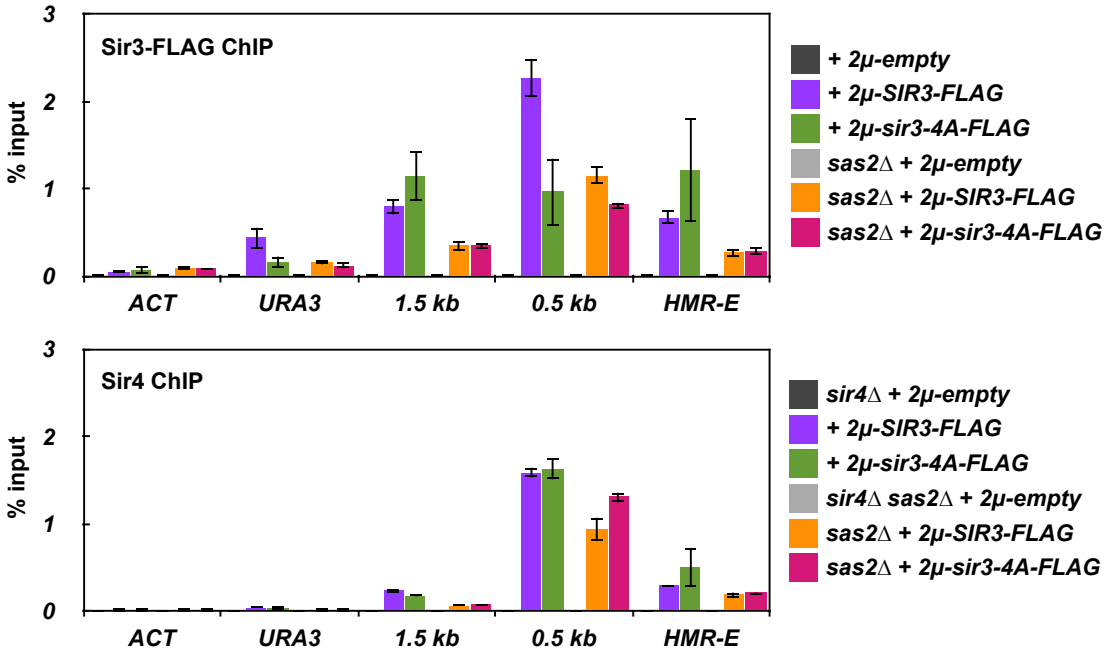

Sir3-FLAG ChIP
% input
3
2
1
0
ACT
URA3
1.5 kb
0.5 kb
HMR-E
+ 2μ-empty
+ 2μ-SIR3-FLAG
+ 2μ-sir3-4A-FLAG
sas2Δ + 2μ-empty
sas2Δ + 2μ-SIR3-FLAG
sas2Δ + 2μ-sir3-4A-FLAG
Sir4 ChIP
% input
3
2
1
0
ACT
URA3
1.5 kb
0.5 kb
HMR-E
sir4Δ + 2μ-empty
+ 2μ-SIR3-FLAG
+ 2μ-sir3-4A-FLAG
sir4Δ sas2Δ + 2μ-empty
sas2Δ + 2μ-SIR3-FLAG
sas2Δ + 2μ-sir3-4A-FLAG

Figure 16. Sir3 and Sir4 recruitment to heterochromatin is improved when H4K16 acetylation is established. Wild-type and cells lacking *SAS2* containing 2-micron plasmids expressing empty, *SIR3-FLAG*, or *sir3-4A-FLAG* were fixed in 1% formaldehyde for fifteen minutes and processed for chromatin immunoprecipitation using FLAG or Sir4-coupled magnetic beads. The localization of the FLAG-tagged protein to *TEL-V-R-URA3* (2.5kb from the telomere end), *TEL-VI-R* (1500 and 500bp from the telomere end), and the *HMR-E* loci was determined by analyzing the immunoprecipitated DNA by qPCR with locus-specific primers (Table 3). Each sample was also tested at a non-silent locus, *ACT1*, as a control. The mean of the percent input +/- SEM is shown (n=3). The means of each sample were grouped by loci and were determined to be statistically different based on one-way ANOVA ($p<0.05$), and means were compared using a paired t-test ($p<0.05$), see Appendix C, Table 5.

4.4 Discussion

The Sir3-Sir4 interaction is required to nucleate heterochromatin

Several interactions between Sir2, Sir3, and Sir4 are required for silencing. Nucleation and spreading of heterochromatin are separable processes that have independent requirements regarding SIR-SIR interactions. Mutations in the coiled-coil domain of Sir4 disrupt silencing and the interaction with Sir3 (Rudner et al., 2005). Additionally, mutation of the AAA+ domain of Sir3 (*sir3-4A*) disrupts both telomere and *HM* silencing, and removes the interaction seen between Sir3 and Sir4 in a two-hybrid assay (Ehrentraut et al., 2011). Interestingly, a *sir4-T1314S* mutation can restore *HM* silencing to a *sir3-4A* strain, but additional mutations (*sir4-E1310V,T1314S,K1325R*) are required restore telomere silencing (Samel et al., 2017). It is thought that an exposed loop of Sir3 within its AAA+ domain directly interacts with the coiled-coil domain of Sir4 and this Sir3-Sir4 interaction is necessary for the nucleation of heterochromatin. The requirement for additional mutations in Sir4 to restore telomere silencing suggests that the Sir3-Sir4 interaction may be more important to for the nucleation of telomeres than the *HM* loci. These differences may be attributed to the *HM* loci having less dependency on the Sir3-Sir4 interaction due to the presence of bi-directional silencers that contain Sir1.

The interaction between Sir3 and Sir4 is undetectable by immunoprecipitation and western blot with the *sir3-4A* mutant (Figure 5C). As expected, *sir3-4A* cells lacking the Sir3-Sir4 interaction are unable to silence telomeres when no wild-type Sir3 is present (Figure 12A). Expression of both *SIR3* and *sir3-4A* allows for telomere silencing when wild-type Sir3 is present and demonstrates that the *sir3-4A* mutation is not dominant

negative (Figure 12B). These results point to a mechanism where the *sir3-4A* mutant leads to a nucleation-specific defect. This mechanism is supported by evidence that the mutant Sir3-4A protein is still recruited to chromatin that is distant from the silencer when wild-type Sir3 is present (Figure 14). Tethering Sir3-4A-LexA proteins to *HM* silencers is also insufficient to establish silencing in the absence of wild-type Sir3 (Figure 13B). Based on these observations there are more requirements for silencing that involve Sir3 than just its recruitment to silencers. Although the Sir3-Sir4 interaction is thought to be the main mechanism for recruiting Sir3 to silencers, this interaction may also support the proper association of SIR proteins with DNA-binding proteins that are required for nucleation.

Acetylation at H4K16 is required for fully functional heterochromatin

The interaction between Sir3 and nucleosomes is highly dependent on the acetylation status of H4K16. Increasing the amount of acetylation on H4K16 and surrounding residues cumulatively decreased Sir3 binding to nucleosomes *in vitro* (Carmen et al., 2002). Sir3 binding to nucleosome arrays is also less efficient with the acetylation mimic H4K16A (Johnson et al., 1992). Although it may seem counterintuitive, cells lacking *SAS2*, the HAT that acts on H4K16, have worse silencing than cells that express *SAS2* (Figure 15). The establishment of H4K16 acetylation is therefore important for silencing and is crucial for the active assembly of heterochromatin brought on by Sir2 HDAC activity. The active removal of H4K16 acetylation produces AAR, which is a small molecule that supports both Sir3-Sir3 and Sir3-nucleosome interactions (Wang et al., 2019). The silencing defects in *sas2*Δ cells can be explained by

a lack of Sir2 substrate (Ehrentraut et al., 2010), but these defects can be partially circumvented by overexpressing *SIR3* (Figure 15). Overexpression of *SIR3* overcomes *sas2*Δ defects by providing an excess of Sir3 molecules that are re-incorporated into developing heterochromatin fibers faster than the rate of disassembly brought on by a lack of AAR production. Overexpression of *sir3-4A* however, does not rescue the silencing defects seen in *sas2*Δ cells (Figure 15). A lack of AAR production combined with a majority of Sir3 molecules that cannot bind Sir4 cumulatively reduce the incorporation of Sir4/Sir2 into heterochromatin. The Sir3-Sir4 interaction is therefore partially redundant to the function of AAR, as they both stabilize the SIR complex and improve its association with nucleosomes in a way that supports silencing.

Silencing is only partially functional up to 2.5kb from the telomere end with overexpressed *SIR3* and not *sir3-4A* in a *sas2*Δ background (Figure 15). Only Sir3 is detectable at the *URA3* reporter placed 2.5kb from the telomere end in the *2μ-SIR3-FLAG SAS2* strain, but neither Sir3 nor Sir4 are detected at the same reporter in *2μ-sir3-4A-FLAG SAS2* strains (Figure 16). The lack of Sir3 and Sir4 signal at the *URA3* reporter as seen by chromatin immunoprecipitation is possibly due to the presence of the FLAG tag, as FLAG tag-containing strains displayed less silencing when overexpressed compared to their wild-type counterparts (Appendix A4). Taken together, these results suggest that Sir3 can spread on its own and partial silencing is established without the Sir3-Sir4 interaction, but incorporation of Sir4/Sir2 into heterochromatin is less efficient when acetylation on H4K16 is absent.

Sir3 can spread independently of the Sir3-Sir4 interaction

The Sir3-Sir4 interaction is essential to nucleate heterochromatin, but it may not be necessary for Sir3 to bind nucleosomes at distances far from the silencer. Extended regions generated by overexpressing *SIR3* are mainly composed of Sir3 and have less detectable levels of Sir4 and Sir2 compared to the nucleation site (Strahl-Bolsinger et al., 1997). In agreement with this, more Sir3 signal is observed at 5.5 kb from the telomere end with overexpressed *SIR3* and *sir3-4A* than empty vector in a *SAS2* background (Figure 14). Although these strains contain wild-type *SIR3*, an increase in signal relative to the untagged constructs implies that the regions of heterochromatin assessed are mainly composed of proteins produced from the overexpression vectors. Silencing is also improved at sites up to 3.5kb away from the silencer with overexpressed *SIR3* and *sir3-4A* compared the empty plasmid (Figure 15). These observations support the idea that Sir3 can spread and promote heterochromatin formation without binding Sir4.

A lack of acetylation on H4K16 in *sas2*Δ strains has also been shown to influence how far Sir3 can spread from the telomere end. Sir3 has been detected up to 15kb away from the telomere end in *sas2*Δ strains, as acetylation of H4K16 is thought to act as a marker for heterochromatin boundary (Suka et al., 2002). The *sir3-4A* mutation was combined with *sas2*Δ to test if Sir3 and Sir4 rely on both H4K16 acetylation and the Sir3-Sir4 interaction to spread into these extended regions.

Both Sir3 and Sir3-4A were still detectable at chromatin up to 1.5kb from the telomere end in *sas2*Δ cells (Figure 16). These results demonstrate that Sir3 can localize to chromatin without binding Sir4 and in the absence of H4K16 acetylation. Despite the difference seen between Sir3 and Sir3-4A at 0.5kb, the amount of Sir3 and Sir3-4A

detected in *sas2*Δ cells at 1.5kb was similar. This difference points to a mechanism where the Sir3-Sir4 interaction plays a bigger role at nucleation sites compared to more distant regions where heterochromatin is spreading. A small amount of Sir4 was also detectable at 1.5kb from the telomere end in wild-type strains overexpressing either *SIR3-FLAG* or *sir3-4A-FLAG*, but no significant amount of Sir4 was detected in either *sas2*Δ strain at the 1.5kb loci. Therefore, Sir4 depends on H4K16 acetylation to efficiently spread from the silencer.

The requirements for nucleation are specific to each silencer

Silencers can contain different DNA elements and recruit various factors that make any given site of nucleation unique. The requirement for SIR-SIR interactions or the presence of acetylation at H4K16 may vary depending on the nucleation site. For example, less Sir3 is detected at *HMR-E* with *sas2*Δ strains compared to wild-type (Figure 16). This observation indicates that acetylation of H4K16 improves Sir3 recruitment during nucleation at *HMR*. Similar levels of Sir3 and Sir3-4A at the *HMR-E* silencer in *sas2*Δ cells suggest that the Sir3-Sir4 interaction does not support the recruitment of additional Sir3 molecules to *HMR-E*. Wild-type Sir3 protein is present in these samples and a small amount of wild-type Sir3 binding to the nucleation site is sufficient for the recruitment of subsequent SIR proteins that do contribute to the signal seen with Sir3-FLAG or Sir3-4A-FLAG. No significant difference was observed between Sir3 and Sir3-4A at *HMR-E* in wild-type *SAS2* cells.

Less binding of Sir3 is seen at 0.5kb from the telomere end in *sas2*Δ cells compared to wild-type, but in contrast to *HMR-E*, mutant Sir3-4A levels were also lower

with wild-type *SAS2*. Based on this evidence, nucleation at telomeres depends on both the Sir3-Sir4 interaction and the establishment of H4K16 acetylation to recruit SIR proteins. Consistent with this, slightly less Sir3-4A is seen at 0.5kb from the telomere end in *sas2*Δ compared to wild-type Sir3 in *sas2*Δ (Figure 16). The amount of Sir3 that is recruited to the telomere end is determined by a compound effect of H4K16 acetylation status and the Sir3-Sir4 interaction.

Difference seen between Sir3 and Sir3-4A in *sas2*Δ cells at *HMR-E*, but not at telomere ends, suggests that the requirements for nucleation at each of these sites are not the same. These differences can be influenced by the presence or absence of DNA-binding proteins that are unique to each silencer. Sir4 is also subject to differential regulation at silencers of the *HM* loci and telomeres mainly through interactions made by its N-terminal domain. The N-terminal portion of Sir4 binds both Sir1 and Yku80, which are found exclusively at the *HM* loci and telomeres respectively. Mutation of the N-terminal domain of Sir4 results in a loss of silencing at telomeres but not the *HM* loci. However, mutation of the Sir4 N-terminus and the removal of the Orc1 and Abf1 binding sites is sufficient to disrupt silencing at the *HM* loci (Kueng et al., 2012). In the same regard the C-terminal half of Sir4 is sufficient for silencing of the *HM* loci but not telomeres. The C-terminal half of Sir4 also has a higher affinity for Sir3 than the full length Sir4 protein, thus the Sir4 N-terminal domain may serve as a negative regulator of the Sir3-Sir4 interaction (Moazed et al., 1997). These observations led to the idea that Sir4 goes through a conformational change during early steps of heterochromatin assembly that improves its binding to Sir3.

The Sir3-Sir4 interaction may be more crucial for nucleation at telomeres than the *HM* loci. It is possible that the *sir3-4A* mutation blocks any benefit from the Sir4 conformational change during early steps in heterochromatin assembly and can account for the differences seen between Sir3 and Sir3-4A at 0.5kb from the telomere end. The Sir4 conformational change may be less important at *HMR-E* because its silencer contains elements that recruit Sir1 and Abf1. These DNA-binding proteins could occupy and orient the N-terminus of Sir4 in a way that renders the *HM* loci less dependent on the Sir3-Sir4 interaction for nucleation.

Chapter 5: General Discussion

Asf2 is a Sir3-interacting factor

This work has characterized the Asf2 protein as a Sir3-interacting factor that can alter the stability of heterochromatin. Asf2 binds Sir3 directly as the interaction between Asf2 and the SIR complex is completely abolished in the absence of Sir3, but not Sir4 or Sir2. Mutation of the coiled-coil domain in Asf2 disrupts the Sir3-Asf2 interaction, as does mutation of the AAA+ domain of Sir3. *ASF2* is a paralog of *SIR4* and they have significant homology within their coiled-coil domains. Both Asf2 and Sir4 contact Sir3 through their respective coiled-coil domain and do so in a competitive manner. As *ASF2* expression increases more Sir3 becomes bound to Asf2 and less of the Sir3-Sir4 interaction is observed. Incomplete formation of the SIR complex brought on by increased *ASF2* expression leads to silencing loss at the *HM* loci and telomeres in a dose-dependent manner. Overexpression of the coiled-coil mutant *asf2-NRN* however does not disrupt silencing because the Asf2-NRN protein cannot interact with Sir3. Asf2 localizes to known areas of heterochromatin such as the *HM* loci and telomeres, but it requires Sir3 and Sir4 to do so. The Asf2 protein is therefore an accessory to SIR mediated heterochromatin that can impact silencing by limiting Sir3 availability.

The Sir3-Sir4 interaction is a nucleation-specific requirement

Heterochromatin formation is highly dependent on a sufficient nucleation structure that originates at silencers. Interactions between the SIR proteins, nucleosomes, and DNA-binding proteins all work in concert to support the propagation of heterochromatin beyond the nucleation site. The Sir3-Sir4 interaction is necessary to

nucleate heterochromatin as expression of *sir3-4A* does not rescue silencing in *sir3*Δ cells. Importantly, expression of *sir3-4A* is not dominant negative and silencing is still functional when *SIR3* is co-expressed. Additionally, tethering the Sir3-4A protein to the nucleation site is also insufficient to rescue silencing in *sir3*Δ cells. This evidence argues that the Sir3-Sir4 interaction plays a role in nucleation that supports the hierarchy of binding requirements amongst all the proteins found at the silencer. ChIP experiments demonstrated that most of the SIR complex that formed on heterochromatin was composed of the tagged Sir3 or Sir3-4A constructs generated from plasmids as opposed to the endogenous Sir3. Based on these observations only a small amount of wild-type Sir3 is required to bind the silencer for nucleation.

Sir3 can spread independently of Sir4/Sir2

It was previously thought that the Sir3-Sir4 interaction is important to recruit additional SIR proteins during heterochromatin formation, but the evidence presented here suggest that Sir3 can spread on its own. Overexpression of *SIR3* or *sir3-4A* increases silencing up to 3.5kb away from the telomere end and demonstrates that both gene products are capable of supporting heterochromatin formation. Silent heterochromatin formed with Sir3-4A must also eventually incorporate Sir4 even though Sir3-4A does not interact with Sir4 directly. It is possible that Sir4/Sir2 is recruited to heterochromatin through the affinity Sir2 has for acetylated H4K16. The Sir3-Sir4 interaction is not necessary for Sir3 and Sir4 to spread, but the Sir3 dimer is required to compact chromatin by bridging adjacent nucleosomes (Behrouzi, et al., 2016) and this compaction likely supports Sir2 activity. The Sir3-Sir4 interaction and the affinity Sir2 has for its H4K16

acetylated substrate may function redundantly to recruit Sir4 to chromatin, but it is hypothesized that the establishment of heterochromatin is more efficient when both interactions are intact.

H4K16 acetylation is required for Sir4/Sir2 to spread efficiently

The excess spreading of Sir3 in *sas2*Δ cells is attributed to the widespread binding of Sir3 to nucleosomes that is enhanced when H4K16 is not acetylated (Suka et al., 2002). Both Sir3 and Sir3-4A are capable of localizing to chromatin at regions distant from the silencer, but *sas2*Δ strains producing excess Sir3-4A are unable to silence telomeric reporters like Sir3 can. The establishment of H4K16 acetylation and the Sir3-Sir4 interaction are therefore required for the SIR complex to spread. The silencing observed in *sas2*Δ cells with excess Sir3 is relatively weak and is attributed to the inefficient spreading of Sir4/Sir2. The incorporation of Sir4/Sir2 into heterochromatin in *sas2*Δ cells is compromised by a lack of affinity between Sir2 and acetylated H4K16, and the absence of AAR production. Although Sir3 can spread on its own, the recruitment of Sir4/Sir2 depends on the establishment and stepwise removal of acetylation at H4K16 (Figure 17).

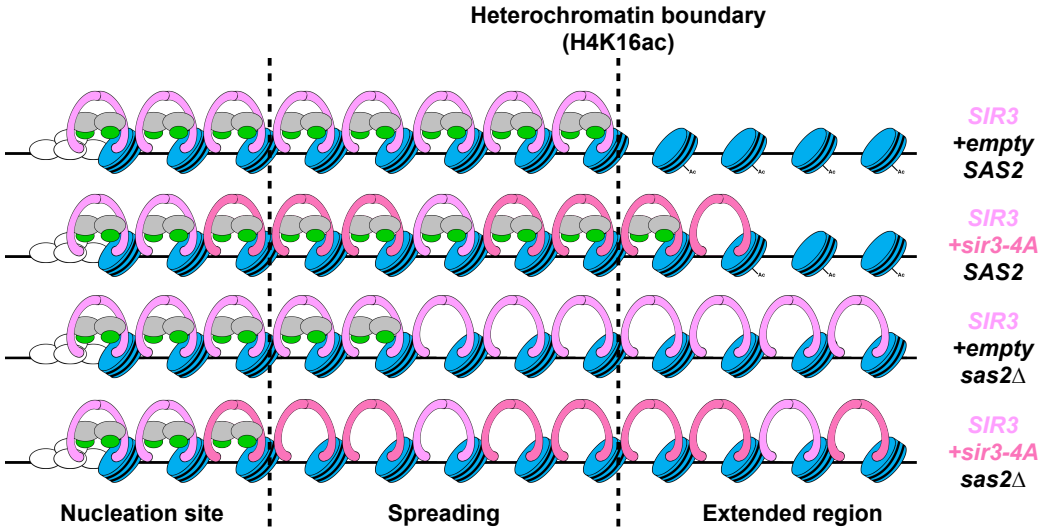

Heterochromatin boundary
(H4K16ac)
SIR3
+empty
SAS2
SIR3
+sir3-4A
SAS2
SIR3
+empty
sas2Δ
SIR3
+sir3-4A
sas2Δ
Nucleation site
Spreading
Extended region

Figure 17. H4K16 acetylation is required for Sir4/Sir2 to spread efficiently. In wild-type cells the spreading of the SIR complex (Sir2, green; Sir4, grey; Sir3, pink) is blocked by a heterochromatin boundary that is established by histone modifications such as H4K16 acetylation. Overexpression of *SIR3* or *sir3-4A* (Sir3-4A, dark pink) allows the SIR proteins to spread farther than wild-type cells. Cells lacking the establishment of H4K16 acetylation (*sas2Δ*) allow for Sir3 to extended farther but hinders the spreading of Sir4/Sir2.

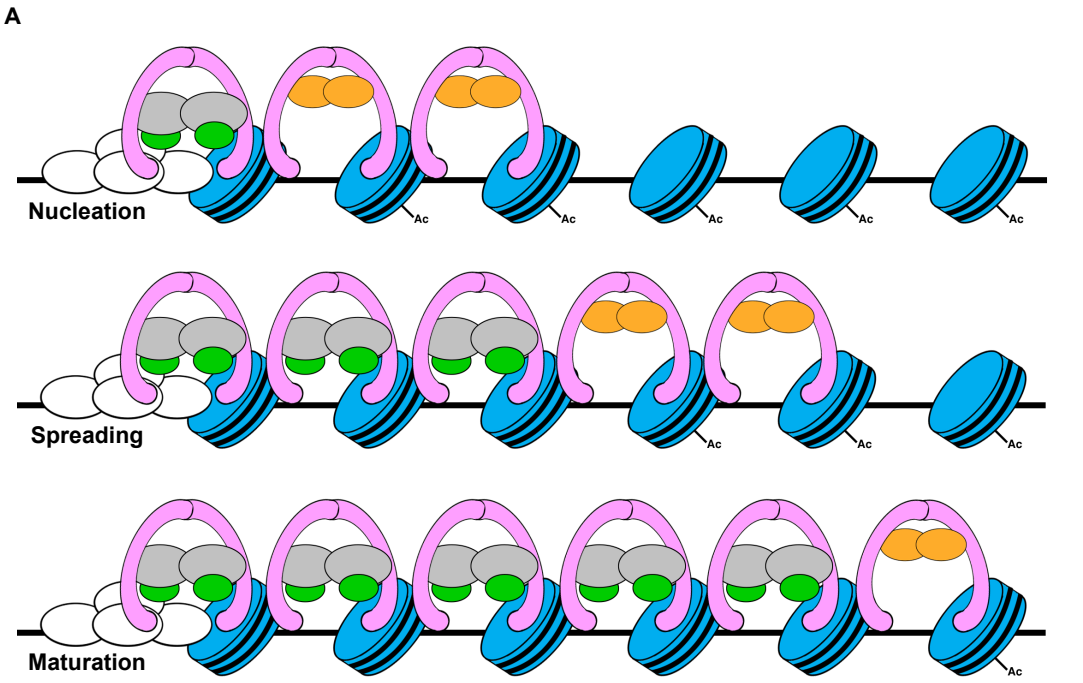
ASF2 can act as a negative regulator of heterochromatin

ASF2 plays both a positive and negative role in the formation of heterochromatin. Overexpression of *ASF2* is sufficient to disrupt silent loci at telomeres and the *HM* loci, but a more moderate increase in *ASF2* expression is only sufficient to disrupt heterochromatin with weakened silencers. Increases in *ASF2* expression may serve to weaken silencing or loosen chromatin structure at a given loci, whereas lowering *ASF2* expression would serve to stabilize heterochromatin or free up Sir3 molecules for heterochromatin formation. As an example, increasing *ASF2* expression may free up Sir4/Sir2 during activation of the recombination enhancer where Sir2 becomes relocated and translocation of information from *HML* to *MAT* occurs (Li et al., 2019). Alternately, lowering *ASF2* expression may improve silencing at specific loci that are particularly susceptible to Sir3 availability. As a speculative example, some telomeres can silence subtelomeric genes such as a subset of *pau* genes that are activated in response to variable stressors (Luo and van Vuuren, 2009). Asf2 may therefore function in a bet-hedging model where certain heterochromatin loci are more susceptible to repression or de-repression based on the current expression level of *ASF2* and may prime a population of cells for a stress response (Bagamery et al., 2020).

ASF2 can act as a positive regulator of heterochromatin

Due to the fact Asf2 appears to have a greater effect on disassembly in mitosis (Figure 10B) and is prominently transcribed in S-phase (Le et al., 1997) future experiments regarding Asf2 should be focused on processes during or shortly after replication. Although *ASF2* expression is not required to form heterochromatin, wild-type

levels of *ASF2* expression do support Sir3 spreading. Acetylation of H4K16 is not established in *sas2*Δ *rpd3*Δ cells and causes heterochromatin to extend further along the telomere into subtelomeric regions with essential genes (Figure 18). Viability is restored to *sas2*Δ *rpd3*Δ cells that lack *ASF2* indicating that heterochromatin does not extend as far and is stabilized by the presence of the Asf2 protein. The changes brought on by mutations in Rpd3 and Sas2 can have a global effect on both histone and non-histone targets and may explain the paradoxical effects seen with Asf2. In general, Asf2 may interact more efficiently with a modified version of Sir3, or if Asf2 is modified itself. Asf2 is likely modified in a manner that depends on the presence of Sir3 as Asf2 appears to be down-shifted in cells lacking *SIR3* (Figure 4C & Figure 6B). How Asf2 is modified and how global changes in post-translational modifications, such as those seen in *rpd3*Δ *sas2*Δ mutants, affect SIR spreading requires further investigation.


A
Nucleation
Spreading
Maturation
Ac

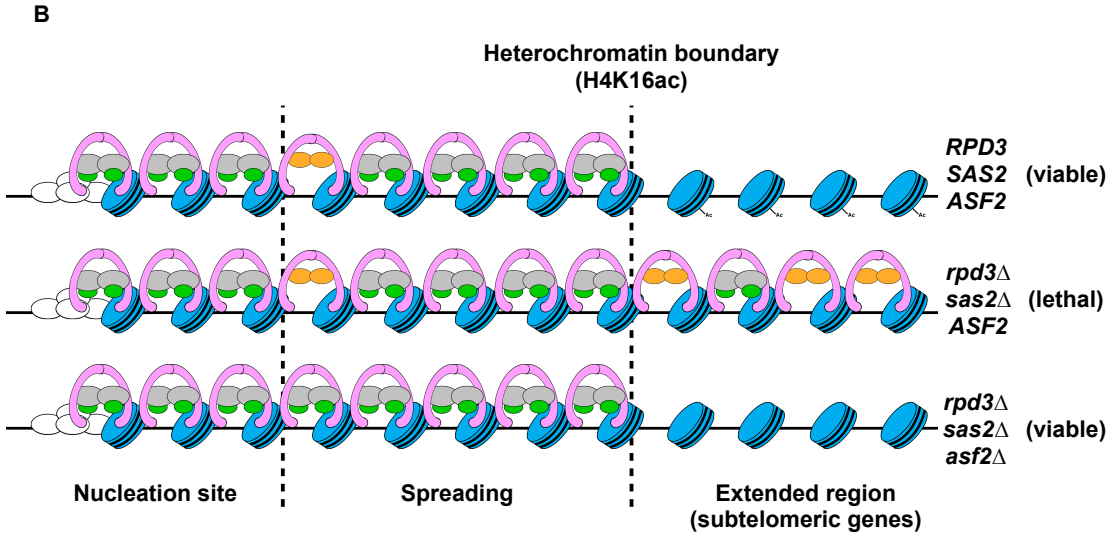
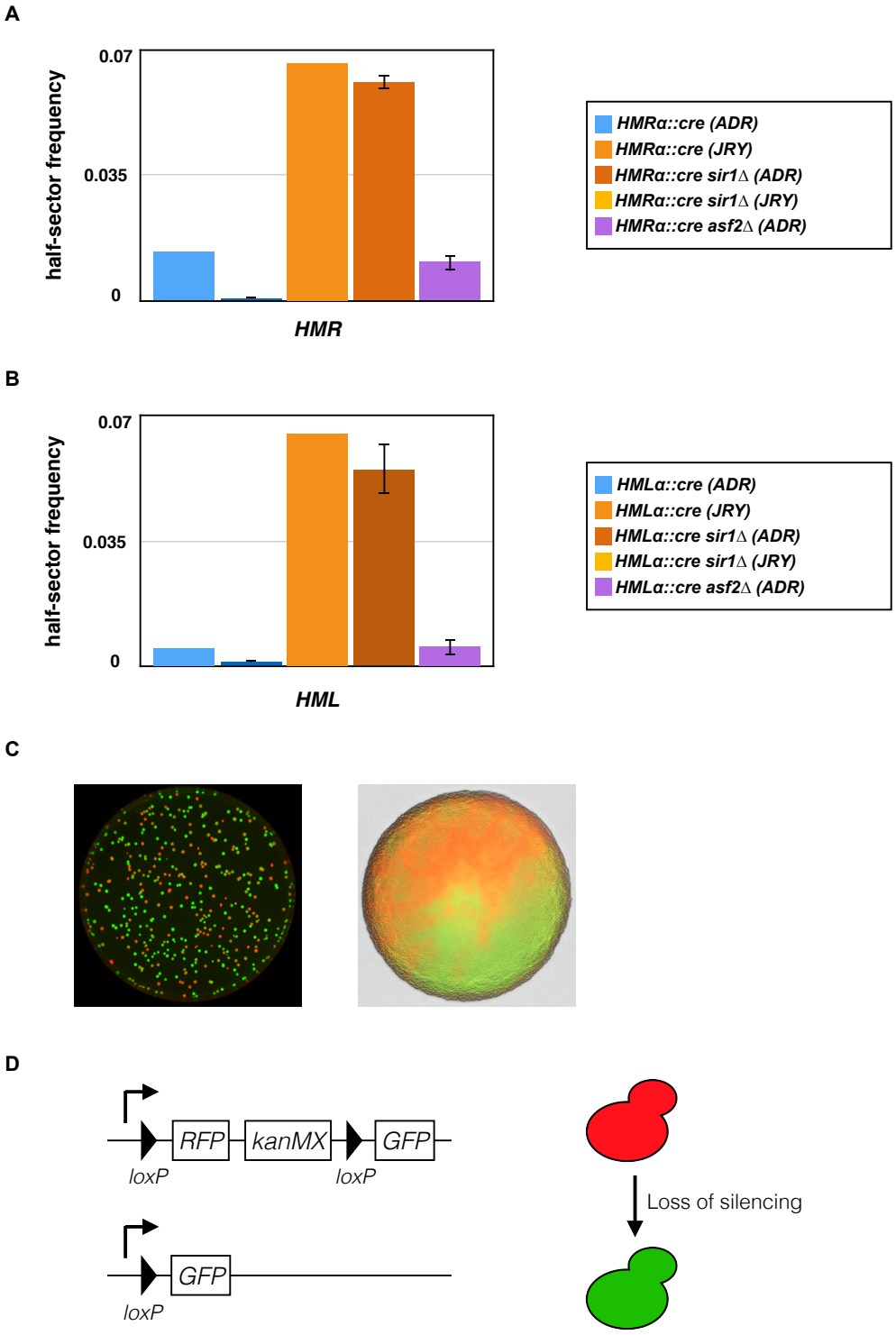

B
Heterochromatin boundary
(H4K16ac)
RPD3
SAS2
ASF2
(viable)
rpd3Δ
sas2Δ
ASF2
(lethal)
rpd3Δ
sas2Δ
asf2Δ
(viable)
Nucleation site
Spreading
Extended region
(subtelomeric genes)

Figure 18. Asf2 supports silencing in the absence of H4K16 acetylation. (A) Sir3 dimers bound to Asf2 may support the addition of Sir3 to the developing heterochromatin fiber. As Sir3 is loaded onto nucleosomes Asf2 is replaced by Sir2/Sir4 and removal of H4K16 acetylation occurs. The addition of Sir4/Sir2 and removal of H4K16 acetylation compacts the heterochromatin fiber and improves Sir3 binding to neighboring nucleosomes. **(B)** The Sir3-Asf2 interaction supports Sir3 loading to nucleosomes distant from the silencer that have limiting amounts of Sir2/Sir4. In wild-type cells the spreading of the SIR complex is blocked by a heterochromatin boundary that is established by histone modifications such as H4K16 acetylation. Cells lacking the establishment of H4K16 acetylation (*rpd3Δ sas2Δ*) allow for Sir3 to extended farther into regions of the subtelomere where essential genes are located. The presence of Sir3 at subtelomeric essential genes is inferred from a lack of viability in *rpd3Δ sas2Δ* cells. Cells lacking *ASF2* are able to restore viability to *rpd3Δ sas2Δ* mutants.

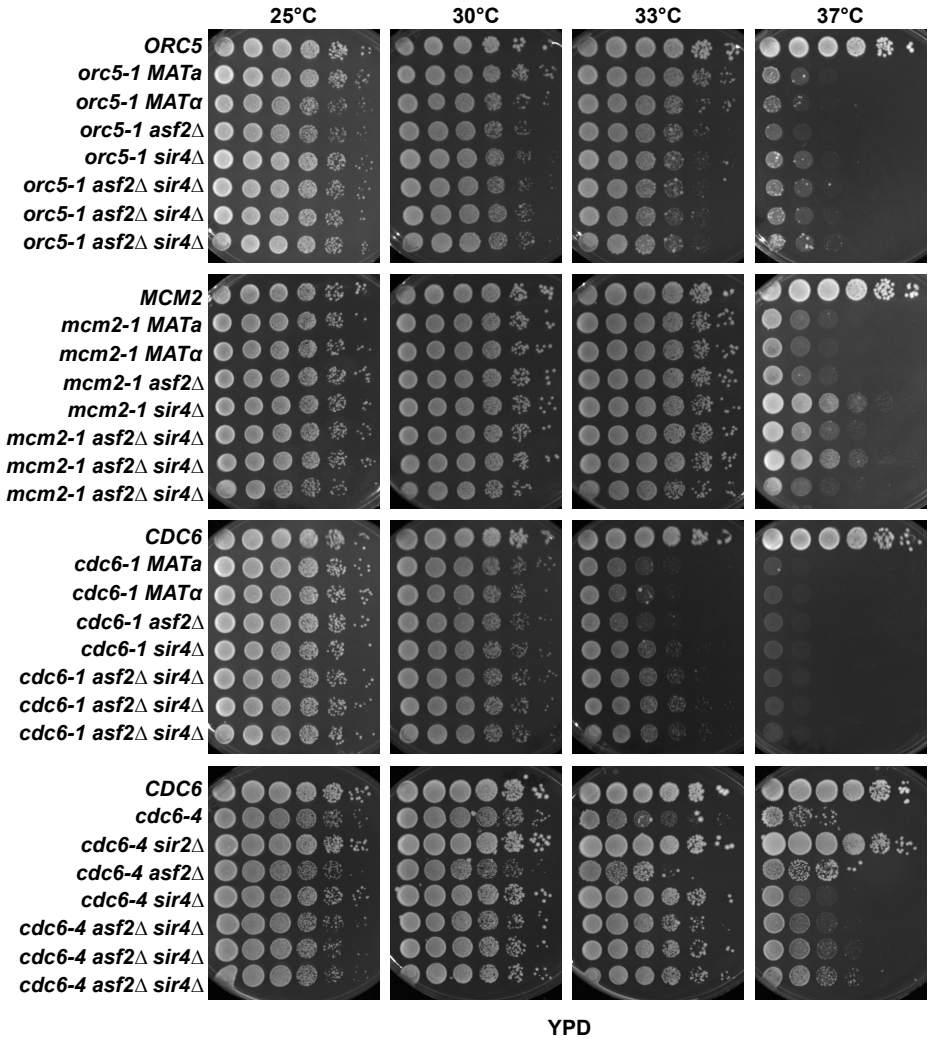
Appendix A

Appendix A1



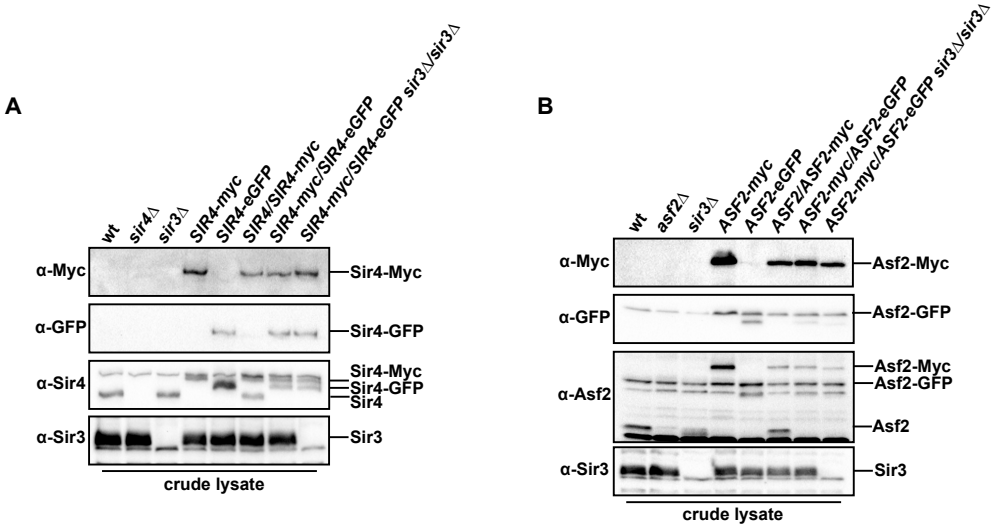
Appendix A1. Deletion of *ASF2* does not increase the transient expression of the *HM* loci. *SIR1* and *ASF2* were deleted in and compared to a wild-type strain that contains a reporter for half-sector analysis. **(A)** Half-sector frequencies for the *HMR* reporter. **(B)** Half-sector frequencies for the *HML* reporter. **(C)** Representative images of a plate for scoring (left) and an individual half-sectored colony (right). **(D)** A cassette containing RFP and GFP is regulated by distance from the promoter and is rearranged upon expression of *HMLα::cre* or *HMRα::cre* resulting in switch from *RFP* expression to *GFP* expression (Dodson and Rine, 2015). Cells that have lost silencing at either reporter before the second division after plating are represented by a half-sectored GFP colony. The frequency of half-sectors is proportional to the amount of silencing loss at the *HM* loci. The half-sector frequency = # of half-sectors/(Total # of colonies - # of fully green colonies). Counts were performed in triplicate for ADR strains (n=3) and are reported as the mean +/- SEM and are compared to previously reported values (JRY).

Appendix A2


25°C
30°C
33°C
37°C
ORC5
orc5-1 MATa
orc5-1 MATα
orc5-1 asf2Δ
orc5-1 sir4Δ
orc5-1 asf2Δ sir4Δ
orc5-1 asf2Δ sir4Δ
orc5-1 asf2Δ sir4Δ
MCM2
mcm2-1 MATa
mcm2-1 MATα
mcm2-1 asf2Δ
mcm2-1 sir4Δ
mcm2-1 asf2Δ sir4Δ
mcm2-1 asf2Δ sir4Δ
mcm2-1 asf2Δ sir4Δ
CDC6
cdc6-1 MATa
cdc6-1 MATα
cdc6-1 asf2Δ
cdc6-1 sir4Δ
cdc6-1 asf2Δ sir4Δ
cdc6-1 asf2Δ sir4Δ
cdc6-1 asf2Δ sir4Δ
CDC6
cdc6-4
cdc6-4 sir2Δ
cdc6-4 asf2Δ
cdc6-4 sir4Δ
cdc6-4 asf2Δ sir4Δ
cdc6-4 asf2Δ sir4Δ
cdc6-4 asf2Δ sir4Δ
YPD

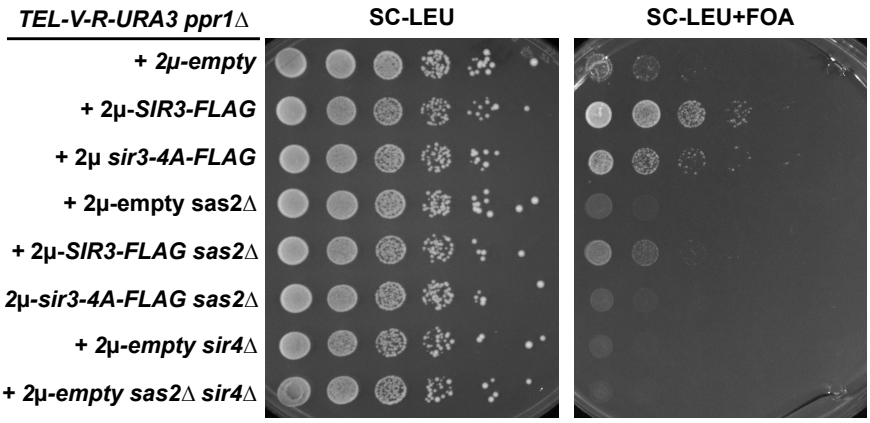
Appendix A2. Deletion of *ASF2* does not suppress defects in the preRC. (A) Wild-type, *orc5-1 MATa*, *orc5-1 MATα*, *orc5-1 asf2Δ*, *orc5-1 sir4Δ*, and *orc5-1 asf2Δ sir4Δ* strains were grown in liquid YPD for 2 days at 30˚C and 10-fold serial dilutions were spotted onto YPD plates and incubated for 2 days at 25˚C, 33˚C, 30˚C, and 37˚C. (B-D) The same protocol was followed as in (A), except with background mutants *mcm2-1*, *cdc6-1*, and *cdc6-4*. An example of lethality suppression can be seen in the *cdc6-4 sir2Δ* strain.

Appendix A3


A
wt
sir4Δ
sir3Δ
SIR4-myc
SIR4-eGFP
SIR4/SIR4-myc
SIR4-myc/SIR4-eGFP
SIR4-myc/SIR4-eGFP sir3Δ/sir3Δ
α-Myc
Sir4-Myc
α-GFP
Sir4-GFP
α-Sir4
Sir4-Myc
Sir4-GFP
Sir4
α-Sir3
Sir3
crude lysate
B
wt
asf2Δ
sir3Δ
ASF2-myc
ASF2-eGFP
ASF2/ASF2-myc
ASF2-myc/ASF2-eGFP
ASF2-myc/ASF2-eGFP sir3Δ/sir3Δ
α-Myc
Asf2-Myc
α-GFP
Asf2-GFP
α-Asf2
Asf2-Myc
Asf2-GFP
Asf2
α-Sir3
Sir3
crude lysate

Appendix A3. Asf2 and Sir4 homodimerize *in vivo*. (A) Lysates from wild type (wt), *sir4Δ*, *sir3Δ*, *SIR4-myc*, and *SIR4-eGFP* haploid cells, and *SIR4-13Myc*/*SIR4*, *SIR4-13Myc*/*SIR4-eGFP*, and *SIR4-13Myc*/*SIR4-eGFP sir3Δ*/*sir3Δ* diploid cells were subject to immunoprecipitation using anti-GFP magnetic beads. **(B)** Lysates from wild type (wt), *asf2Δ*, *sir3Δ*, *ASF2-myc*, and *ASF2-GFP* haploid cells, and *ASF2-13Myc*/*ASF2*, *ASF2-13Myc*/*ASF2-GFP*, and *ASF2-13Myc*/*SIR4-eGFP sir3Δ*/*sir3Δ* diploid cells were subject to immunoprecipitation using anti-GFP magnetic beads. Immunoblots were generated with anti-Myc, anti-GFP, anti-Sir4 or anti-Asf2, and anti-Sir3 on crude lysate and immunoprecipitated (IP) samples.

Appendix A4


TEL-V-R-URA3 ppr1Δ
SC-LEU
SC-LEU+FOA
+ 2μ-empty
+ 2μ-SIR3-FLAG
+ 2μ sir3-4A-FLAG
+ 2μ-empty sas2Δ
+ 2μ-SIR3-FLAG sas2Δ
+ 2μ-sir3-4A-FLAG sas2Δ
+ 2μ-empty sir4Δ
+ 2μ-empty sas2Δ sir4Δ

Appendix A4. Minimal silencing observed in FLAG-tagged strains with sas2Δ.
Strains containing a *TEL-V-R-URA3* reporter in a wild-type or *sas2Δ* background with *2μ-empty*, *2μ-SIR3-FLAG*, or *2μ-sir3-4A-FLAG* were grown in liquid SC-LEU media for 2 days at 30˚C and 10-fold serial dilutions were spotted onto solid media and incubated for 2 days (SC-LEU) or 3 days (SC-LEU+FOA) at 30˚C. Growth on FOA media is representative of repression at *TEL-V-R-URA3*.

Appendix B

Table 1. Strains used in this study

Strain (ADR#)	Genotype
21[a]	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*
2317	*MATα bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 SIR3-TAP-TRP1*
2366	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 pep4Δ::LEU2*
2381	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 pep4Δ::LEU2 ASF2-TAP-klTRP1*
2384	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ASF2-13Myc-kanMX*
2405	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 asf2Δ::Sphis5*
2432	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 pep4Δ::LEU2 SIR3-TAP-klTRP1*
2501	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ASF2-TAP-TRP1*
2586	*MATα bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir3Δ::LEU2*
2682	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmlΔ::TRP1 ASF2-13Myc-kanMX sir2Δ::HIS3*
2688	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmlΔ::TRP1 ASF2-13Myc-kanMX sir3Δ::HIS3*
2707	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmlΔ::TRP1 ASF2-13Myc-kanMX*
2708	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmlΔ::TRP1*
2752	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 SIR3-13Myc-kanMX*
2753	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 SIR3-13Myc-kanMX*
2754	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 SIR3-13Myc-kanMX asf2Δ::Sphis5*
2824	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ASF2-GFP-HIS3*
2828	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-VII-URA3*
2829	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-VII-L-URA3*

2836	*MAT(?) ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-VII-URA3 sir2Δ::HIS3*
2848	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-VII-URA3 asf2Δ::Sphis5*
3331	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1*
3353	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir3Δ::kanMX [CEN-SIR3-LEU2 (pAR395)]*
3353	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir3Δ::kanMX [CEN-SIR3-3XFLAG-LEU2 (pAR979)]*
3353	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir3Δ::kanMX [CEN-sir3-2A-3XFLAG-LEU2 (pAR988)]*
3353	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir3Δ::kanMX [CEN-sir3-3A-3XFLAG-LEU2 (pAR986)]*
3353	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir3Δ::kanMX [CEN-sir3-4A-3XFLAG-LEU2 (pAR1064)]*
3368	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir3Δ::kanMX ASF2-TAP-TRP1*
3391	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir4Δ::natMX ASF2-TAP-TRP1*
3688	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-LEU2 (pAR534)]*
3688	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-SIR3-LEU2 (pAR1020)]*
3688	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-Sir3-4A-LEU2 (pAR1017)]*
3689	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-LEU2 (pAR534)]*
3689	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-SIR3-LEU2 (pAR1020)]*
3689	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-Sir3-4A-LEU2 (pAR1017)]*
3689	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-LEU2 (pAR534)]*
3689	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-SIR3-LEU2 (pAR1020)]*
3689	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-Sir3-4A-LEU2 (pAR1017)]*
3690	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (2.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-LEU2 (pAR534)]*
3690	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (2.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-SIR3-LEU2 (pAR1020)]*
3690	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (2.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-Sir3-4A-LEU2 (pAR1017)]*

3691	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (3.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-LEU2 (pAR534)]*
3691	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (3.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-SIR3-LEU2 (pAR1020)]*
3691	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (3.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-Sir3-4A-LEU2 (pAR1017)]*
3813	*MATα bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 SIR4-eGFP-HIS3*
4006	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*
4062	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 sir3Δ::kanMX [2μ-LEU2 (pAR534)]*
4062	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 sir3Δ::kanMX [2μ-SIR3-LEU2 (pAR1020)]*
4062	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 sir3Δ::kanMX [2μ-sir3-4A-LEU2 (pAR1017)]*
4062	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 sir3Δ::kanMX [2μ-sir3-3A-LEU2 (pAR1023)]*
4062	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 sir3Δ::kanMX [2μ-sir3-2A-LEU2 (pAR1056)]*
5469	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 [2μ-LEU2 (pAR534)]*
5469	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 [2μ-SIR3-LEU2 (pAR1020)]*
5469	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 [2μ-sir3-4A-LEU2 (pAR1017)]*
5469	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 [2μ-sir3-3A-LEU2 (pAR1023)]*
5469	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 [2μ-sir3-2A-LEU2 (pAR1056)]*
5556	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 bar1Δ::TRP1 ppr1Δ::HIS3 TEL-VII-L-URA3 LEU2::GAL-ppr1-1*
5558	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 bar1Δ::TRP1 ppr1Δ::HIS3 TEL-VII-L-URA3 LEU2::GAL-PPR1-1*
5645	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 bar1Δ::TRP1 ppr1Δ::HIS3 TEL-VII-L-URA3 LEU2::GAL-PPR1-1 asf2Δ::kanMX*
5646	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 bar1Δ::TRP1 ppr1Δ::HIS3 TEL-VII-L-URA3 LEU2::GAL-PPR1-1 asf2Δ::kanMX*
6213	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (5.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-LEU2 (pAR534)]*

6213	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (5.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-SIR3-LEU2 (pAR1020)]*
6213	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (5.5kb) DIU5(2-13) ppr1Δ::HIS3 [2μ-Sir3-4A-LEU2 (pAR1017)]*
7092	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 sir3Δ::hphMX*
7097	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (5.5kb) DIU5(2-13) ppr1Δ::HIS3 sir3Δ::hphMX*
7098	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 sir4Δ::kanMX*
7101	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (5.5kb) DIU5(2-13) ppr1Δ::HIS3 sir4Δ::kanMX*
7322	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 sir2Δ::natMX*
7325	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (5.5kb) DIU5(2-13) ppr1Δ::HIS3 sir2Δ::natMX*
7598	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sas2Δ::natNT2 rpd3Δ::hphNT1 [CEN-SAS2-URA3 (pAR1176)]*
7599	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sas2Δ::natNT2 rpd3Δ::hphNT1 sir3Δ::kanMX [CEN-SAS2-URA3 (pAR1176)]*
7603	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sas2Δ::natNT2 rpd3Δ::hphNT1 asf2Δ::Sphis5 [CEN-SAS2-URA3 (pAR1176)]*
7607	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sas2Δ::natNT2 rpd3Δ::hphNT1 sir4Δ::natMX [CEN-SAS2-URA3 (pAR1176)]*
8129	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 SIR3-13Myc-kanMX hphMX-asf2-NRN*
8148	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I [2μ-LexA-HIS3 (pAR787)]*
8149	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I [2μ-SIR3-LexA-HIS3 (pAR788)]*
8150	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I [2μ-sir3-4A-LexA-HIS3 (pAR1189)]*
8152	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I sir3Δ::hphMX [2μ-LexA-HIS3 (pAR787)]*
8153	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I sir3Δ::hphMX [2μ-SIR3-LexA-HIS3 (pAR788)]*
8154	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I sir3Δ::hphMX [2μ-sir3-4A-LexA-HIS3 (pAR1189)]*
8156	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I sir4Δ::kanMX [2μ-LexA-HIS3 (pAR787)]*

8157	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I sir4Δ::kanMX [2μ-SIR3-LexA-HIS3 (pAR788)]*
8158	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I sir4Δ::kanMX [2μ-sir3-4A-LexA-HIS3 (pAR1189)]*
8160	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I sir3Δ::hphMX sir4Δ::kanMX [2μ-LexA-HIS3 (pAR787)]*
8161	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I sir3Δ::hphMX sir4Δ::kanMX [2μ-SIR3-LexA-HIS3 (pAR788)]*
8162	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmr::Aeb-4lexops-TRP1::klURA3-HMR-I sir3Δ::hphMX sir4Δ::kanMX [2μ-sir3-4A-LexA-HIS3 (pAR1189)]*
8658	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-LEU2 (pAR534)]*
8658	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-SIR3-LEU2 (pAR1020)]*
8658	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-Sir3-4A-LEU2 (pAR1017)]*
8659	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1.5kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-LEU2 (pAR534)]*
8659	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1.5kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-SIR3-LEU2 (pAR1020)]*
8659	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (1.5kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-Sir3-4A-LEU2 (pAR1017)]*
8660	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (2.5kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-LEU2 (pAR534)]*
8660	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (2.5kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-SIR3-LEU2 (pAR1020)]*
8660	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (2.5kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-Sir3-4A-LEU2 (pAR1017)]*
8661	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (3.5kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-LEU2 (pAR534)]*

8661	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (3.5kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-SIR3-LEU2 (pAR1020)]*
8661	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 TEL-V-R::URA3 (3.5kb) DIU5(2-13) ppr1Δ::HIS3 sas2Δ::hphMX [2μ-Sir3-4A-LEU2 (pAR1017)]*
9259[b]	*MATa his1-1*
9260[b]	*MATα his1-1 met1*
9612	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 ade2-1 can1-100 TEL-VII-L-URA3 his3-11,15::pGAL-HIS3*
9614	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 ade2-1 can1-100 TEL-VII-L-URA3 his3-11,15::pGAL-ASF2-HIS3*
9616	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 ade2-1 can1-100 TEL-VII-L-URA3 his3-11,15::pGAL-asf2-NRN-HIS3*
9845	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMLα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1*
9846	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMRα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1*
9847	*MATa lys2 his3-11,15 leu2-3,112 can1-100 sir1Δ::LEU2 HMLα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1*
9848	*MATa lys2 his3-11,15 leu2-3,112 can1-100 sir1Δ::LEU2 HMRα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1*
9850	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMLα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1 asf2Δ::hphMX*
9851	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMLα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1 asf2Δ::hphMX*
9852	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMLα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1 asf2Δ::hphMX*
9853	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMRα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1 asf2Δ::hphMX*
9854	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMRα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1 asf2Δ::hphMX*
9855	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMRα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1 asf2Δ::hphMX*
10301	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 [2μ-LEU2 (pAR534)]*
10302	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 [2μ-LEU2 (pAR534)]*

10303	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 [2μ-LEU2 (pAR534)]*
10304	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 [2μ-SIR3-3XFLAG-LEU2 (pAR1020)]*
10305	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 [2μ-SIR3-3XFLAG-LEU2 (pAR1020)]*
10306	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 [2μ-SIR3-3XFLAG-LEU2 (pAR1020)]*
10310	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 [2μ-sir3-4A-3XFLAG-LEU2 (pAR1017)]*
10311	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 [2μ-sir3-4A-3XFLAG-LEU2 (pAR1017)]*
10312	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 [2μ-sir3-4A-3XFLAG-LEU2 (pAR1017)]*
10316	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sas2Δ::hphMX [2μ-LEU2 (pAR534)]*
10317	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sas2Δ::hphMX [2μ-LEU2 (pAR534)]*
10318	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sas2Δ::hphMX [2μ-LEU2 (pAR534)]*
10319	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sas2Δ::hphMX [2μ-SIR3-3XFLAG-LEU2 (pAR1020)]*
10320	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sas2Δ::hphMX [2μ-SIR3-3XFLAG-LEU2 (pAR1020)]*
10321	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sas2Δ::hphMX [2μ-SIR3-3XFLAG-LEU2 (pAR1020)]*
10325	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sas2Δ::hphMX [2μ-sir3-4A-3XFLAG-LEU2 (pAR1017)]*
10326	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sas2Δ::hphMX [2μ-sir3-4A-3XFLAG-LEU2 (pAR1017)]*
10327	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sas2Δ::hphMX [2μ-sir3-4A-3XFLAG-LEU2 (pAR1017)]*
10335	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-4*
10336	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-4 sir2Δ::LEU2*
10431	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-1 asf2Δ::Sphis5 sir4Δ::natMX*
10432	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-1 asf2Δ::Sphis5 sir4Δ::natMX*
10433	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-1 asf2Δ::Sphis5 sir4Δ::natMX*
10441	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 orc5-1 asf2Δ::Sphis5 sir4Δ::natMX*
10442	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 orc5-1 asf2Δ::Sphis5 sir4Δ::natMX*

10443	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 orc5-1 asf2Δ::Sphis5 sir4Δ::natMX*
10444	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 mcm2-1 asf2Δ::Sphis5 sir4Δ::natMX*
10445	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 mcm2-1 asf2Δ::Sphis5 sir4Δ::natMX*
10446	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 mcm2-1 asf2Δ::Sphis5 sir4Δ::natMX*
10448	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 mcm2-1*
10449	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 mcm2-1*
10450	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 mcm2-1 asf2Δ::Sphis5*
10452	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 mcm2-1 sir4Δ::natMX*
10454	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 orc5-1*
10455	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 orc5-1*
10456	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 orc5-1 asf2Δ::Sphis5*
10458	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 orc5-1 sir4Δ::natMX*
10460	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-1*
10461	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-1*
10462	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-1 asf2Δ::Sphis5*
10464	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-1 sir4Δ::natMX*
10562	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-4 asf2Δ::Sphis5 sir4Δ::natMX*
10563	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-4 asf2Δ::Sphis5 sir4Δ::natMX*
10564	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-4 asf2Δ::Sphis5 sir4Δ::natMX*
10566	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-4 sir4Δ::natMX*
10568	*MAT? ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 cdc6-4 asf2Δ::Sphis5*
10579	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 SIR4-13Myc-kanMX*
10685	*MATa/MATα bar1Δ/bar1Δ ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 SIR4-13Myc-kanMX/SIR4-eGFP-HIS3*
10688	*MATa/MATα bar1Δ/bar1Δ ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/HIS ade2-1/ade2-1 can1-100/can1-100 SIR4-13Myc-kanMX/SIR4*

10715	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 bar1Δ::TRP1 ppr1Δ::HIS3 TEL-VII-L-URA3 LEU2::GAL-PPR1-1 asf2Δ::kanMX*
10717	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ppr1Δ::hphMX TEL-V-R-URA3*
10719	*MAT(?) bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ppr1Δ::hphMX TEL-V-R-URA3 sir2Δ::HIS3*
10722	*MAT(?) bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ppr1Δ::hphMX TEL-V-R-URA3 asf2Δ::Sphis5*
10725	*MAT(?) bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ppr1Δ::hphMX TEL-V-R-URA3 sir4Δ::natMX*
10731	*MATa/MATα bar1Δ/bar1Δ ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 ASF2-13Myc-kanMX/ASF2-GFP-HIS3*
10734	*MATa/MATα bar1Δ/bar1Δ ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/HIS ade2-1/ade2-1 can1-100/can1-100 ASF2-13Myc-kanMX/ASF2*
10737	*MAT(?) bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 ppr1Δ::hphMX TEL-V-R-URA3 sir3Δ::kanMX*
10774	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sir4Δ::kanMX [2μ-SIR3-3XFLAG-LEU2 (pAR1020]*
10775	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sir4Δ::kanMX [2μ-SIR3-3XFLAG-LEU2 (pAR1020]*
10776	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sir4Δ::kanMX [2μ-SIR3-3XFLAG-LEU2 (pAR1020]*
10777	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sir4Δ::kanMX sas2Δ::hphMX [2μ-SIR3-3XFLAG-LEU2 (pAR1020)]*
10778	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sir4Δ::kanMX sas2Δ::hphMX [2μ-SIR3-3XFLAG-LEU2 (pAR1020)]*
10779	*MATa ura3-52 leu2Δ1 trp1-Δ1 his3Δ200 lys2-801 ppr1Δ::HIS3 TEL-V-R-URA3 sir4Δ::kanMX sas2Δ::hphMX [2μ-SIR3-3XFLAG-LEU2 (pAR1020)]*
10788	*MAT(?) ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-VII-URA3 sir4Δ::natMX*
10792	*MAT(?) ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-VII-URA3 sir3Δ::kanMX*
10896	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-IX-URA3 ura3Δ::hphMX ppr1Δ::kanMX*
10899	*MATa/MATα bar1Δ/bar1Δ ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 ASF2-13Myc-kanMX/ASF2-GFP-HIS3 sir3Δ::natMX/sir3Δ::natMX*
10899	*MATa/MATα bar1Δ/bar1Δ ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 ASF2-13Myc-kanMX/ASF2-GFP-HIS3 sir3Δ::natMX/sir3Δ::natMX*

10902	*MATa/MATα bar1Δ/bar1Δ ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 SIR4-13Myc-kanMX/SIR4-eGFP-HIS3 sir3Δ::natMX/sir3Δ::natMX*
10902	*MATa/MATα bar1Δ/bar1Δ ura3-1/ura3-1 leu2-3,112/leu2-3,112 trp1-1/trp1-1 his3-11,15/his3-11,15 ade2-1/ade2-1 can1-100/can1-100 SIR4-13Myc-kanMX/SIR4-eGFP-HIS3 sir3Δ::natMX/sir3Δ::natMX*
10903	*MAT(?) ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-IX-URA3 ura3Δ::hphMX ppr1Δ::kanMX sir2Δ::HIS3*
10905	*MAT(?) ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-IX-URA3 ura3Δ::hphMX ppr1Δ::kanMX sir3Δ::natMX*
10907	*MAT(?) ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-IX-URA3 ura3Δ::hphMX ppr1Δ::kanMX asf2Δ::Sphis5*
10908	*MAT(?) ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 TEL-IX-URA3 ura3Δ::hphMX ppr1Δ::kanMX sir4Δ::natMX*
10926	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 asf2Δ::Sphis5*
10928	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 ade2-1 can1-100 hmrΔE-TRP1 his3-11,15::pGAL-ASF2-HIS3*
10956	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 bar1Δ::TRP1 ppr1Δ::HIS3 TEL-VII-L-URA3 LEU2::GAL-PPR1-1 hphMX-asf2-NRN*
10957	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 bar1Δ::TRP1 ppr1Δ::HIS3 TEL-VII-L-URA3 LEU2::GAL-PPR1-1 hphMX-asf2-NRN*
10958	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 bar1Δ::TRP1 ppr1Δ::HIS3 TEL-VII-L-URA3 LEU2::GAL-PPR1-1 hphMX-asf2-NRN*
10959	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sas2Δ::natNT2 rpd3Δ::hphNT1 Sphis5-asf2-NRN [CEN-SAS2-URA3 (pAR1176)]*
10974	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmlΔ::TRP1 ASF2-13Myc-kanMX sir4Δ::natMX*
10995	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*
10996	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*
10997	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*
10998	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*
10999	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*
11000	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100*
11001	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 asf2Δ::kanMX*
11002	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 asf2Δ::kanMX*
11003	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 asf2Δ::kanMX*

11004	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 asf2Δ::kanMX*
11005	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 asf2Δ::kanMX*
11006	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 asf2Δ::kanMX*
11007	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hphMX-asf2-NRN*
11008	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hphMX-asf2-NRN*
11009	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hphMX-asf2-NRN*
11010	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hphMX-asf2-NRN*
11011	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hphMX-asf2-NRN*
11012	*MATα ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hphMX-asf2-NRN*
11086	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 ade2-1 can1-100 SIR3-13Myc-kanMX his3-11,15::pGAL-ASF2-HIS3*
11089	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 ade2-1 can1-100 SIR3-13Myc-kanMX his3-11,15::pGAL-asf2-NRN-HIS3*
11163	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 [CEN-HIS3 (pAR440)]*
11165	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 [CEN-ASF2-HIS3 (pAR1449)]*
11168	*MATa ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 hmrΔE-TRP1 TEL-VII-L-URA3 [CEN-SIR4-HIS3 (pAR450)]*
JRY3433[c]	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir2Δ::HIS3*
JRY3289[c]	*MATa bar1Δ ura3-1 leu2-3,112 trp1-1 his3-11,15 ade2-1 can1-100 sir3Δ::HIS3*
JRY9628[c]	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMLα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1*
JRY9629[c]	*matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMRα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1*
JRY9739[c]	*MATa lys2 his3-11,15 leu2-3,112 can1-100 sir1Δ::LEU2 HMLα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1*
JRY9740[c]	*MATa lys2 his3-11,15 leu2-3,112 can1-100 sir1Δ::LEU2 HMRα-α2Δ::cre ura3Δ::pGPD-loxP-yEmRFP-tCYC1-kanMX-loxP-yEGFP-tADH1*

[a] strain is isogenic to the original W303-1a strain
[b] strains were kindly provided by Fred Winston, Harvard Medical School, Boston, MA
[c] strains were kindly provided by Jasper Rine, UC, Berkeley, CA

Table 2. Plasmids used in this study

#	**Plasmid**	**Origin**
P1	*pFA6a-kanMX6*	Longtine et al., 1998
P3	*pFA6a-HIS3MX6*	Longtine et al., 1998
P6	*pFA6a-GFP-HIS3MX6*	Longtine et al., 1998
P10	*pFA6a-13Myc-kanMX6*	Longtine et al., 1998
A1	*pFA6-hphNT1*	Janke et al., 2004 (Euroscarf)
B1	*pFA6-natNT2*	Janke et al., 2004 (Euroscarf)
pAR357	*CEN-LEU2*	Sikorski et al., 1989 (pRS315)
pAR378	*pGAL-ASF2-HIS3*	Rudner Lab
pAR380	*pGAL-HIS3*	Rudner Lab
pAR385	*pFA6-natMX* (pAG25)	Goldstein and McCusker, 1999
pAR387	*pFA6-hphMX* (pAG32)	Goldstein and McCusker, 1999
pAR393	*klTRP1-TAP*	Puig et al., 2001 (pBS1479)
pAR395	*CEN-SIR3-LEU2*	Rudner Lab
pAR440	*CEN-HIS3*	Sikorski et al., 1989 (pRS313)
pAR450	*CEN-SIR4-HIS3*	Rudner Lab
pAR482	*SIR4-eGFP-HIS3*	Rudner Lab
pAR534	*2μ-LEU2*	Rudner Lab
pAR787	*2μ-LEXA-HIS3*	Wu et al., 2011 (pBTM116His3)
pAR788	*2μ-SIR3-LEXA-HIS3*	Wu et al., 2011 (pCSW14)
pAR979	*CEN-SIR3-3XFLAG-LEU2*	Rudner Lab
pAR986	*CEN-sir3-3A-3XFLAG-LEU2*	Rudner Lab
pAR988	*CEN-sir3-2A-3XFLAG-LEU2*	Rudner Lab
pAR1017	*2μ-sir3-4A-LEU2*	Rudner Lab
pAR1020	*2μ-SIR3-LEU2*	Rudner Lab
pAR1023	*2μ-sir3-3A-LEU2*	Rudner Lab
pAR1056	*2μ-sir3-2A-LEU2*	Rudner Lab
pAR1064	*CEN-sir3-4A-3XFLAG-LEU2*	Rudner Lab
pAR1176	*CEN-SAS2-URA3*	Rudner Lab
pAR1189	*2μ-sir3-4A-LEXA-HIS3*	Rudner Lab
pAR1296	*2μ-SIR3-3XFLAG-LEU2*	Rudner Lab
pAR1298	*2μ-sir3-4A-3XFLAG-LEU2*	Rudner Lab
pAR1326	*pGAL-asf2-NRN-HIS3*	Rudner Lab
pAR1449	*CEN-ASF2-HIS3*	Rudner Lab

Table 3. Oligos used in this study

Name	ID#	Sequence
Pringle 5' check	35	GGACGAGGCAAGCTAAACAG
Pringle 3' check	36	TGGTCGCTATACTGCTGTCG
ASF2-TAP-5'	48	AAGCTTGGCGGATTCAAAGCATTGTTCAG GACTACATAAATCCATGGAAAAGAGAAG
ASF2-TAP-3'	49	GGCGGATGCCGTAGTGATCTCTACCGCTTC GTATTTATATTACGACTCACTATAGGG
ASF2-ko	50	CAGTGGAGAAAGGAGGGAAAAAGGCCAA GACTGAGTGCTTCAGCTGAAGCTTCGTAC GC
ASF2-check-ko	52	AAGCAGAGGACGGTGAAGAA
ASF2-orf-5'	53	GCGGAATTCACCATGCCAAAAAATCGTGG TG
ASF2-orf-3'	54	AGAATGCGGCCGCTTATTTATGTAGTCCTG AACAATG
ASF2-5'-MYC	60	AAGCTTGGCGGATTCAAAGCATTGTTCAG GACTACATAAAGGTCGACGGATCCCCGGG TT
Sir3 pringle 5'	77	CTTTTCGATGGATGAAGAATTCAAAAATA TGGACTGCATTCGGATCCCCGGGTTAATT
Sir3 pringle 3'	78	TAGGCATATCTATGGCGGAAGTGAAAATG AATGTTGGTGGGAATTCGAGCTCGTTTAA
SIR4-ko-3'	102	GGTACACTTCGTTACTGGTCTTTTGTAGAA TGATAAAAAGTACGACTCACTATAGGG
SIR4 check	103	TGAACAAAAGCGCATCACAT
ASF2 5'	309	TAGGGCCCCCAAGGCAGGTATGTTGCAT
ASF2-mut2	312	ATTTGCCCCTTTGTTTCTGA
ACT1 u (qPCR)	499	GCCTTCTACGTTTCCATCCA
ACT1 d (qPCR)	501	CGTAAATTGGAACGACGTGA
marker switch up check	703	CTGTTTAGCTTGCCTCGTCC
marker switch down check	704	CGACAGCAGTATAGCGACCA
ASF2-seq2	752	TCTTGATGGTTCGAATGCTG
sir3-4A mut	977	TGTCCCGAAAGCTGCAGCCGCCGCGACAC TAATATT
sir3-3A mut	978	TGTCCCGAAAGCTGCAGCCGCCAAGACAC TAATA
sir3-2A mut	979	TGTCCCGAAAGCTGCAGCAAGAAAGACAC TA

Sas2-up	1112	CCTATTTTCTAGTTGCTTTTTGTTTTCACTC GCAAAAAAAAGCTGAAGCTTCGTACGC
Sas2-down	1113	TGAAATACATATGCCATTAAGTTACATCCT GAATAGATTCTACGACTCACTATAGGG
URA3 ChIP	1227	GCAAGGGCTCCCTAGCTACT
qPCR URA up	1235	CAGGACTAGGATGAGTAGCAGC
URA3 u	1236	CCCAGTATTCTTAACCCAACTGC
URA3 d	1237	GCAGCACGTTCCTTATATGTAGC
Rpd3-ko-UP	1378	CATACAAAACATTCGTGGCTACAACTCGA TATCCGTGCAGAGCTGAAGCTTCGTACGC
Rpd3-ko-down	1379	TCACATTATTTATATTCGTATATACTTCCA ACTCTTTTTTTACGACTCACTATAGGG
asf2-NRN up	1449	CCGGCGAGATCAATGGATATCGAAATAAG ATGGCTAG
asf2-NRN down	1450	CTAGCCATCTTATTTCGATATCCATTGATC TCGCCGG
asf2-upmarker	1451	TCTGCGATGCTAGTATTAGCAAAGATGTA AAAGCGCTTTTCGTACGCTGCAGGTCGAC
asf2-downmarker	1452	AGATATGTTACTCACGCGTCGCGTCGCTCC TAAAGACATGATCGATGAATTCGAGCTCG
ASF2 up	1455	CATGTCTTTAGGAGCGACGC
KlURA3 u	1493	CATCA TTGTTGGCAGAGGAC
KlURA3 d	1494	CTGATTCTCTTTTGGTACGCTTC
HMR-E (A)	1501	ACGTATCTTGTACCCTTTTTATTGC
HMR-E (A)	1503	AAAGTTTTGTTTGCAAA TGTGG
HML-E d (qPCR)	1789	TTTCGGCACGGACTTATTTG
HML-E u (qPCR)	1790	CCCCCGAAATCGATAATAATG
HML-I d (qPCR)	1791	TGCTTTGTTGGGTGTTTGAC
HML-I u (qPCR)	1792	TCACCCAGAACCCCACTTAC
HMR-E d (qPCR)	1793	ATTGCAAAAACCCATCAACC
HMR-E u (qPCR)	1794	ATAACTTGGACGGGGATCG
HMRa d (qPCR)	1795	AGCCCAAAGGGAAAATCATC
HMRa u (qPCR)	1796	GCCACATTTCTTTGCAACTTC
T500 d (qPCR)	1797	AAATGAGGACTGGGTCATGG
T500 u (qPCR)	1798	ACCCACACCACCTCACTAGC
T1500 d (qPCR)	1799	TGCTAAAGGAATCCCCAGAG
T1500 u (qPCR)	1800	GAATGCGAAAGGATCTGTCC
HMR-I d (qPCR)	1825	AAAGCTGGATGCAAGGATTG
HMR-I u (qPCR)	1826	GGAATCCTCAAAAGGGAATCT
HMLalpha u (qPCR)	1828	ACTTCCAGACGCTATCCTGTGA

HMLalpha d (qPCR)	1827	CGTCTAGCTGAGCATGTGAGG
TEL-09R up	2100	TATTACAATATATTAAATAGAGCATAGTG GTACGACGATATACGACTCACTATAGGG
TEL-09R down	2101	TGAGCACTACCATGACGTCATTAATGTAA AAGTTCCTTAACAGCTGAAGCTTCGTACGC
TEL09-up check	2102	CTTCTCCCGCTTGTTTTGAG
TEL09-down check	2103	TCACATTCCACTCCACTCCA
9R-URA3-down	2160	TGAGCACTACCATGACGTCATTAATGTAA AAGTTCCTTAAAAGCTTTTCAATTCATCTT T
9R-URA3-up	2161	TATTACAATATATTAAATAGAGCATAGTG GTACGACGATAAAATTGGAAAGAAAAAGC TT
SIR3-TAP u	DM445	AATTACGCCTTTTCGATGGATGAAGAATTC AAAAATATGGACTGCATTTCCATGGAAAA GAGAAG
SIR3-TAP d	DM446	TGTGTACATAGGCATATCTATGGCGGAAG TGAAAATGAATGTTGGTGGTACGACTCAC TATAGGG

Appendix C

Table 4. Statistical Analysis (Figure 8)

Table 4.1
One-way ANOVA summary – 4 samples grouped for *0.5kb*

	SS	**DoF**	**MS**	**F**	**P**
Between	2766.097	4	691.5242	25.4921	3.16E-05
Within	271.2697	10	27.127		
Total	3037.367	14			

Table 4.2
One-way ANOVA summary – 4 samples grouped for *1.5kb*

	SS	**DoF**	**MS**	**F**	**P**
Between	53.7598	4	13.44	20.0615	9.10E-05
Within	6.6994	10	0.6699		
Total	60.4592	14			

Table 4.3
One-way ANOVA summary – 4 samples grouped for *HML-E*

	SS	**DoF**	**MS**	**F**	**P**
Between	103.7238	4	25.9309	19.6122	1.00E-04
Within	13.2218	10	1.3222		
Total	116.9456	14			

Table 4.4
One-way ANOVA summary – 4 samples grouped for *HMLalpha*

	SS	**DoF**	**MS**	**F**	**P**
Between	55.2628	4	13.8157	6.6218	0.00716
Within	20.864	10	2.0864		
Total	76.1269	14			

Table 4.5
One-way ANOVA summary – 4 samples grouped for *HML-I*

	SS	**DoF**	**MS**	**F**	**P**
Between	168.9216	4	42.2304	9.7234	0.00178
Within	43.4318	10	4.3432		
Total	212.3534	14			

Table 4.6
One-way ANOVA summary – 4 samples grouped for *HMR-E*

	SS	**DoF**	**MS**	**F**	**P**
Between	39.0086	4	9.7521	9.18	0.00221
Within	10.6233	10	1.0623		
Total	49.6319	14			

Table 4.7
One-way ANOVA summary – 4 samples grouped for *HMRa*

	SS	**DoF**	**MS**	**F**	**P**
Between	67.1231	4	16.7808	32.3627	1.07E-05
Within	5.1852	10	0.5185		
Total	72.3084	14			

Table 4.8
One-way ANOVA summary – 4 samples grouped for *HMR-I*

	SS	**DoF**	**MS**	**F**	**P**
Between	31.7065	4	7.9266	3.1146	0.066
Within	25.4503	10	2.545		
Total	57.1568	14			

Table 4.9
Two-tailed student's t-test

Loci	Comparison	p-value
0.5kb	Asf2 vs untagged	0.0156
0.5kb	Sir3 vs untagged	0.0046
1.5kb	Asf2 vs untagged	0.002
1.5kb	Sir3 vs untagged	0.0467
HML-E	Asf2 vs untagged	0.0083
HML-E	Sir3 vs untagged	0.007
HMLalpha	Asf2 vs untagged	0.0486
HMLalpha	Sir3 vs untagged	0.0497
HML-I	Asf2 vs untagged	0.0241
HML-I	Sir3 vs untagged	0.0324
HMR-E	Sir3 vs untagged	0.0253
HMR-E	Asf2 vs untagged	0.0727
HMRa	Sir3 vs untagged	0.949
HMRa	Asf2 vs untagged	0.0044
HMR-I	Sir3 vs untagged	0.907
HMR-I	Asf2 vs untagged	0.148

Table 5. Statistical analysis (Figure 16)

Table 5.1
One-way ANOVA summary – 4 samples grouped for *ACT1* (Sir3-FLAG)

	SS	**DoF**	**MS**	**F**	**P**
Between	0.0021	3	0.000701	0.3537	0.788
Within	0.0158	8	0.001981		
Total	0.0179	11			

Table 5.2
One-way ANOVA summary – 4 samples grouped for *URA3* (Sir3-FLAG)

	SS	**DoF**	**MS**	**F**	**P**
Between	0.193	3	0.06432	4.8309	0.0333
Within	0.1065	8	0.01331		
Total	0.2995	11			

Table 5.3
One-way ANOVA summary – 4 samples grouped for *HMR-E* (Sir3-FLAG)

	SS	**DoF**	**MS**	**F**	**P**
Between	1.7689	3	0.5896	2.1785	0.168
Within	2.1653	8	0.2707		
Total	3.9342	11			

Table 5.4
One-way ANOVA summary – 4 samples grouped for 0.5kb *TEL-VI-R* (Sir3-FLAG)

	SS	**DoF**	**MS**	**F**	**P**
Between	3.8908	3	1.2969	8.4315	0.00737
Within	1.2306	8	0.1538		
Total	5.1214	11			

Table 5.5
One-way ANOVA summary – 4 samples grouped for 1.5kb *TEL-VI-R* (Sir3-FLAG)

	SS	**DoF**	**MS**	**F**	**P**
Between	1.3498	3	0.44992	6.6134	0.0147
Within	0.5442	8	0.06803		
Total	1.894	11			

Table 5.6
Post-Hoc comparison using Tukey's HSD – *ACT1* vs. *URA3* (Sir3-FLAG)

ACT1 vs URA3	Means	Diff.	Lower	Upper	p-value
Sir3	[0.0526, 0.4343]	0.3817	0.0653	0.6981	0.02858
sir3-4A	[0.0691, 0.1533]	0.0842	-0.1267	0.2951	0.32991
*Sir3 sas2*Δ	[0.087, 0.15920]	0.0722	-0.0013	0.1457	0.05256
*sir3-4A sas2*Δ	[0.0816, 0.1185]	0.0369	-0.0479	0.1217	0.29372

Table 5.7
Post-Hoc comparison using Tukey's HSD – *ACT1* vs. *HMR-E* (Sir3-FLAG)

ACT1 vs HMR-E	Means	Diff.	Lower	Upper	p-value
Sir3	[0.0526, 0.6697]	0.6171	0.3904	0.8438	0.00164
sir3-4A	[0.0691, 1.2111]	1.1421	-0.5052	2.7894	0.12655
*Sir3 sas2*Δ	[0.087, 0.26532]	0.1783	0.0284	0.3282	0.02984
*sir3-4A sas2*Δ	[0.0816, 0.2846]	0.203	0.081	0.3251	0.00989

Table 5.8
Post-Hoc comparison using Tukey's HSD – *ACT1* vs. 0.5kb from *TEL-VI-R* (Sir3-FLAG)

ACT1 vs 0.5kb	Means	Diff.	Lower	Upper	p-value
Sir3	[2.2592, 0.0526]	-2.2066	-2.8225	-1.5907	0.00058
sir3-4A	[0.9615, 0.0691]	-0.8924	-1.9554	0.1706	0.08018
*Sir3 sas2*Δ	[0.087, 1.15210]	1.0651	0.7792	1.351	0.0005
*sir3-4A sas2*Δ	[0.0816, 0.8096]	0.728	0.6268	0.8292	0.00004

Table 5.9
Post-Hoc comparison using Tukey's HSD – *ACT1* vs. 1.5kb from *TEL-VI-R* (Sir3-FLAG)

ACT1 vs 1.5kb	Means	Diff.	Lower	Upper	p-value
Sir3	[0.7954, 0.0526]	-0.7428	-1.0056	-0.48	0.00143
sir3-4A	[1.1448, 0.0691]	-1.0757	-1.8585	-0.2929	0.01885
*Sir3 sas2*Δ	[0.3473, 0.0870]	-0.2603	-0.4287	-0.0919	0.01272
*sir3-4A sas2*Δ	[0.3457, 0.0816]	-0.2641	-0.3611	-0.1671	0.00164

Table 5.10
One-way ANOVA summary – 4 samples grouped for *ACT1* (Sir4)

	SS	**DoF**	**MS**	**F**	**P**
Between	8.30E-05	3	2.78E-05	8.778	0.00654
Within	2.50E-05	8	3.17E-06		
Total	1.10E-04	11			

Table 5.11
One-way ANOVA summary – 4 samples grouped for *URA3* (Sir4)

	SS	**DoF**	**MS**	**F**	**P**
Between	0.0022	3	0.000735	7.2582	0.0114
Within	0.00081	8	0.000101		
Total	0.00301	11			

Table 5.12
One-way ANOVA summary – 4 samples grouped for *HMR-E* (Sir4)

	SS	**DoF**	**MS**	**F**	**P**
Between	0.1841	3	0.06137	1.5912	0.266
Within	0.3085	8	0.03857		
Total	0.4926	11			

Table 5.13
One-way ANOVA summary – 4 samples grouped for 0.5kb from *TEL-VI-R* (Sir4)

	SS	**DoF**	**MS**	**F**	**P**
Between	0.8822	3	0.29407	9.9445	0.00448
Within	0.2366	8	0.02957		
Total	1.1188	11			

Table 5.14
One-way ANOVA summary – 4 samples grouped for 1.5kb from *TEL-VI-R* (Sir4)

	SS	**DoF**	**MS**	**F**	**P**
Between	0.0587	3	0.019582	38.1713	4.36E-05
Within	0.0041	8	0.000513		
Total	0.0629	11			

Table 5.15
Post-Hoc comparison using Tukey's HSD – *ACT1* vs. *URA3* (Sir4)

ACT1 vs URA3	Means	Diff.	Lower	Upper	p-value
Sir3	[0.00634, 0.04620]	0.0399	0.0201	0.0596	0.005
sir3-4A	[0.00908, 0.03546]	0.0264	0.005	0.0478	0.02678
Sir3 sas2Δ	[0.00619, 0.01285]	0.0067	0.0051	0.0082	0.0003
sir3-4A sas2Δ	[0.01269, 0.01711]	0.0044	-0.0105	0.0193	0.45618

Table 5.16
Post-Hoc comparison using Tukey's HSD – *ACT1* vs. *HMR-E* (Sir4)

ACT1 vs HMR	Means	Diff.	Lower	Upper	p-value
Sir3	[0.00634, 0.29605]	0.2897	0.2671	0.3123	2.7E-06
sir3-4A	[0.00908, 0.49551]	0.4864	-0.1346	1.1074	0.0953
Sir3 sas2Δ	[0.00619, 0.18150]	0.1753	0.0757	0.275	0.00814
sir3-4A sas2Δ	[0.01269, 0.20401]	0.1913	0.1729	0.2097	0.00001

Table 5.17
Post-Hoc comparison using Tukey's HSD – *ACT1* vs. 0.5kb from *TEL-VI-R* (Sir4)

ACT1 vs 0.5kb	Means	Diff.	Lower	Upper	p-value
Sir3	[1.57993, 0.00634]	-1.5736	-1.7329	-1.4143	0.00001
sir3-4A	[1.63016, 0.00908]	-1.6211	-1.9745	-1.2676	0.00022
Sir3 sas2Δ	[0.94828, 0.00619]	-0.9421	-1.3099	-0.5743	0.00207
sir3-4A sas2Δ	[1.30243, 0.01269]	-1.2897	-1.4253	-1.1542	0.00002

Table 5.18
Post-Hoc comparison using Tukey's HSD – *ACT1* vs. 1.5kb from *TEL-VI-R* (Sir4)

ACT1 vs 1.5kb	Means	Diff.	Lower	Upper	p-value
Sir3	[0.23360, 0.00634]	-0.2273	-0.2495	-0.205	0.00001
sir3-4A	[0.17544, 0.00908]	-0.1664	-0.2027	-0.13	0.00022
Sir3 sas2Δ	[0.06678, 0.00619]	-0.0606	-0.1087	-0.0125	0.02497
sir3-4A sas2Δ	[0.07500, 0.01269]	-0.0623	-0.0967	-0.028	0.00729

Table 5.19
Two-tailed student's t-test

Loci	Comparison	p-value
HMR-E	Sir3 vs Sir3-4A (Sir3-FLAG)	0.416
HMR-E	Sir3 vs Sir3 sas2Δ (Sir3-FLAG)	0.0132
HMR-E	Sir3 sas2Δ vs Sir3-4A sas2Δ (Sir3-FLAG)	0.782
0.5kb	Sir3 vs Sir3-4A (Sir3-FLAG)	0.042
0.5kb	Sir3 vs Sir3 sas2Δ (Sir3-FLAG)	0.0105
0.5kb	Sir3 sas2Δ vs Sir3-4A sas2Δ (Sir3-FLAG)	0.0324
1.5kb	Sir3 vs Sir3-4A (Sir3-FLAG)	0.0187
1.5kb	Sir3 vs Sir3 sas2Δ (Sir3-FLAG)	0.0009
1.5kb	Sir3 sas2Δ vs Sir3-4A sas2Δ (Sir3-FLAG)	0.719
0.5kb	Sir3 sas2Δ vs Sir3-4A sas2Δ (Sir4)	0.0662

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Sean Stephenson

EDUCATION

PhD in Biochemistry (2015-2021)
Department of Biochemistry, Molecular Biology, and Immunology, Faculty of Medicine, University of Ottawa, ON
Thesis: Asf2 mediates Sir3 availability during the formation of heterochromatin

Honours BSc in Biochemistry (2011-2015)
Faculty of Science, University of Ottawa, ON

High school diploma (2011)
Barrie North Collegiate, Barrie, ON

TEACHING EXPERIENCE

Teaching Assistant/Lab Demonstrator (2016-2019)
Department of Translational and Molecular Medicine, Faculty of Medicine, University of Ottawa, ON

- Supervised, assisted, and evaluated students during lab sessions
- Led introductory lectures explaining theory and experiments
- Prepared samples, lab manual protocols, and managed course data online

CONFERENCE PRESENTATIONS

Ottawa Institute of Systems Biology Biannual Retreat (2019)
Poster: Asf2: A novel component of heterochromatin
Authors: Sean Stephenson, Isabelle Callebaut, Scott Gerber, and Adam Rudner

Canadian Society for Molecular Biosciences 60th Annual Conference (2017)
Poster: Asf2: A novel component of heterochromatin
Authors: Sean Stephenson, Isabelle Callebaut, Scott Gerber, and Adam Rudner