The Contributions of Histones H3 and H4 to Gene Regulation in Saccharomyces cerevisiae:

A Closer Look at Sum1 Repression and Sum1-1 Silencing

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in the Graduate School of Duke University

2011
ABSTRACT

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Abstract

Chromatin is composed of DNA, histones, and other proteins and contributes to DNA packaging, controlling gene expression and DNA replication. This work focuses on the contributions of histones H3 and H4 to gene regulation in the yeast *Saccharomyces cerevisiae*. I identified a region of the nucleosome that is critical for three types of long-range transcriptional silencing but not for local repression mediated by some of the same proteins.

In *S. cerevisiae*, the Sir complex performs long-range silencing of the mating type loci, while the promoter-specific Sum1 complex represses mid-sporulation genes. Interestingly, the *SUM1-1* mutation changes the Sum1 repression complex into a silencing complex capable of long-range spreading. Sum1-1 provides a good model to distinguish between properties of nucleosomes important for long-range silencing (common to Sum1-1 and Sir silencing), and specific interactions nucleosomes might make with the Sum1 complex (common to Sum1 and Sum1-1 complexes). Interactions between nucleosomes and silencing proteins are critical to Sir silencing, and the spreading ability of Sum1-1p suggests that a component of the Sum1-1 complex may also interact with nucleosomes. Since the Sum1-1 and Sum1 complex components are shared, histone contacts may also contribute to wild type Sum1 repression.
I investigated the contributions of histones H3 and H4 to Sum1-1 silencing and Sum1 repression using a genetic screen. Interestingly, I found histone mutations that disrupt Sum1-1 silencing and cluster in the H3 core/H4 region of the nucleosome, which was previously identified to disrupt silencing at the mating type loci, telomeres, and rDNA. Therefore, this region of the nucleosome is important to silencing mediated by three distinct complexes- Sir, RENT, and Sum1-1. The Sir3p bromo-adjacent homology (BAH) domain binds this region of the nucleosome to facilitate Sir spreading and silencing, and I tested Orc1p, a paralog of Sir3p, to determine if it makes similar contributions to Sum1-1 silencing. Using reporter mating assays and chromatin immunoprecipitation, I found that mutations and deletion of the BAH domain of Orc1p disrupt Sum1-1 silencing. These results suggest that Orc1p may interact with this region of the nucleosome and contribute to Sum1-1 silencing outside of recruitment.

Surprisingly, Sum1 repression was not disrupted by histone mutations. I conducted in vitro binding assays to identify a region in Sum1p that may interact with histones and account for the spreading ability of Sum1-1p. Consistent with results that histones do not contribute to Sum1 repression, I did not find evidence of Sum1p binding to histone peptides. Therefore, interactions with histones H3 and H4 are important to Sir and Sum1-1 silencing and not Sum1 repression. These interactions with histones may facilitate the formation of higher-order chromatin structures necessary for long-range silencing complexes.
I also identified mutations in the H3 tail that disrupt Sum1-1 silencing. Surprisingly, these mutations did not disrupt the enrichment of Sum1-1p. Similar observations have been made for Sir proteins in the absence of the H3 tail, and the H3 tail may contribute to chromatin compaction and silencing after the assembly of silencing proteins. Therefore, the Sir and Sum1-1 complexes may share several features that facilitate silencing. The use of the LRS/H4 region of the nucleosome may be a common interaction surface with silencing proteins, and the H3 tail may assist in the formation of a specialized chromatin structure. These interactions may also be utilized in the formation of heterochromatin in higher eukaryotes.
Dedication

Ben Baird was a great friend during my time as an undergrad at the University of Northern Iowa. He was always able to make studying and research fun and was a great guy to be around. We both went on to pursue doctorate degrees and were in a race to see who would be the first to publish. Ben was never able to see his dream realized, and this work is dedicated to his memory.
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1. Introduction

Chromatin is the combination of DNA, histones, and other proteins that make up chromosomes. Chromatin packages DNA into a smaller volume to fit in the cell nucleus, strengthens DNA to allow mitosis and meiosis, and controls gene expression and DNA replication. Modification and mutation of the histones can result in changes to chromatin structure that affect DNA compaction, nucleosome interactions, and gene regulation. This work focuses on the contributions of histones H3 and H4 to gene regulation in the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* is a well-studied unicellular eukaryotic model organism that is easily manipulated and has a compact genome containing a small number of histone genes. It does not possess all of the features of heterochromatin found in higher eukaryotes, such as H3 K9Me and recognition by heterochromatin protein 1 (HP1). However, *S. cerevisiae* heterochromatic regions are hypoacetylated, and the origin recognition complex (ORC) contributes to heterochromatin formation. Thus, *S. cerevisiae* serves as a representative model organism for the study of heterochromatin. Our findings in *S. cerevisiae* can be applied to understand heterochromatin dynamics and gene regulation in higher eukaryotes based on these shared properties and evolutionarily conservation.

1.1 Heterochromatin and silencing

The genomes of eukaryotic organisms are packaged with histones and other proteins into organized regions of euchromatin and heterochromatin. Euchromatic
regions are transcriptionally active, replicate early, and de-condense as the cell cycle progresses. Heterochromatic regions are poorly transcribed, replicate late, and remain condensed throughout the cell cycle. Heterochromatin was originally identified cytologically in *Drosophila melanogaster* (Schultz 1936) and has been studied in many organisms from yeast to humans. It plays an important role in transcriptional gene silencing and structural stability of the chromosome and is commonly found at repetitive DNA sequences including the pericentromeric and sub-telomeric regions in most organisms. Heterochromatin is also heritable over multiple cell divisions, resulting in inheritance of the silenced state.

One of the first examples demonstrating a correlation between chromosomal organization and gene expression came in 1930 from Hermann Muller. In *D. melanogaster*, the white gene, which controls eye color in fruit flies, was translocated via X-irradiation induced chromosomal rearrangement from a euchromatic location to a position adjacent to pericentromeric heterochromatin (Muller 1930). The white gene is normally expressed at its euchromatic location and produces a red eye. However, when adjacent to heterochromatin, the white gene exhibited variegated expression, resulting in a mosaic red and white eye color. This effect is known as position effect variegation (PEV) and results in variegated expression when genes are located in close proximity to a heterochromatic locus.
PEV has also been observed in other organisms, including yeast and mice. In S. cerevisiae, ADE2 is involved in the biosynthesis of purine nucleotides, and repression of ADE2 leads to the accumulation of purine precursors that cause the colony to turn red. When ADE2 is used as a reporter gene and placed near the telomeres, red pigment accumulates, resulting in sectored red and white yeast colonies (GOTTSCHLING et al. 1990). These changes in gene expression are heritable and can last for multiple generations, propagating a heterochromatic state.

While the exact mechanisms and proteins involved in heterochromatin formation vary in different organisms, the use of histone modifying proteins and proteins that recognize these modifications is common throughout eukaryotes to achieve a silenced state (reviewed in (MOAZED 2001)). In most eukaryotes, including the fission yeast Schizosaccharomyces pombe, the fruit fly D. melanogaster, and many mammals, heterochromatin is defined by the absence of histone acetylation (DILLON 2004) and the presence of histone H3 K9 methylation and binding by HP1 (ELGIN and GREWAL 2003). HP1 contains a chromodomain that recognizes and binds H3 K9Me, which is enriched in heterochromatic regions.

S. cerevisiae does not contain these “classical” heterochromatin features (BRIGGS et al. 2001). Instead, it utilizes the Sir complex containing a histone modifying deacetylase and silencing proteins that recognize deacetylated nucleosomes to achieve a silenced state (RUSCHE et al. 2003). Hypoacetylated histones tails in S. cerevisiae (BRAUNSTEIN et al.
1993; SUKA et al. 2001) are a defining mark of heterochromatin that is shared with higher eukaryotes. Additionally, the involvement of the origin recognition complex (ORC) in heterochromatin is common. In S. cerevisiae, ORC is associated with silencer sequences and recruits Sir proteins to silenced domains. Similarly, in Drosophila and humans, ORC interacts with HP1 (AUTH et al. 2006; LIDONNICI et al. 2004; PAK et al. 1997; PRASANTH et al. 2004) and is enriched in telomeric and pericentromeric heterochromatin (DENG et al. 2007; DENG et al. 2009; PRASANTH et al. 2010; SHEN et al. 2010). From these observations, it has been concluded that ORC serves as a platform for recruiting heterochromatin proteins. Therefore, S. cerevisiae serves as a representative model organism for the study of heterochromatin.

1.1.1 The nucleosome and its role in chromatin compaction

Heterochromatin is less accessible to DNA modifying enzymes, nucleases, and transcriptional machinery than euchromatin and serves to regulate gene transcription. The principal packaging unit of heterochromatin is the nucleosome (Figure 1). It consists of approximately 147 base pairs of DNA wrapped in 1.67 left-handed superhelical turns around a histone octamer, containing two molecules each of histones H2A, H2B, H3 and H4 (LUGER et al. 1997; RICHMOND and DAVEY 2003). Nucleosomes form the fundamental repeating units of eukaryotic chromatin, which assists in packing the large eukaryotic genomes into the nucleus while still ensuring accessibility of the DNA when needed. The structure of the nucleosome core at the atomic level has been well established
(Luger et al. 1997), where two histone H2A-H2B dimers associate with a histone H3-H4 tetramer and DNA to properly form the nucleosome core particle. However, the mobile histone tails have not been crystallized. A fifth histone protein, linker histone H1, is not considered part of the nucleosome core, but does play an important role in associating with linker DNA between nucleosomes and promoting higher-order chromatin compaction.

Figure 1: The structure of the nucleosome core particle.

The nucleosome core particle was created in Pymol using PDB 1ID3 (White et al. 2001) and shows H2A (yellow), H2B (orange), H3 (blue), and H4 (green) wrapping the DNA (grey).

Nucleosomes are folded through successive higher-order structures to eventually form a metaphase chromosome, compacting DNA into the chromosome while allowing for regulatory gene control. Arrays of nucleosomes separated by linker DNA form the “beads on a string” repeating structure of chromatin, which can fold into higher-order
structures such as the 30-nm chromatin fiber (Schalch et al. 2005; Woodcock and Dimitrov 2001; Woodcock and Ghosh 2010). Two main models have been proposed for the structure of the 30-nm fiber: the solenoid model (Finch and Klug 1976), in which the linker DNAs are coiled between adjacent nucleosomes, and the zigzag model (Woodcock et al. 1984), in which zigzag arrays of nucleosomes form a condensed ribbon. One model has not been definitively accepted, and higher-order chromatin compaction is not very well understood.

Histone H4 is one of the most well conserved proteins among eukaryotes (McGhee and Felsenfeld 1980; Wells 1986) and plays an important role in compaction and higher-order chromatin structure. Nucleosome arrays lacking histone N-terminal domains are unable to form 30-nm fibers and fiber-fiber associations in high-salt solutions (Fletcher and Hansen 1995; Schwarz et al. 1996; Tse and Hansen 1997). The N-terminal domain of histone H4 appears to have the largest contribution to stability of higher-order chromatin structures (Dorigo et al. 2004; Gordon et al. 2005). Histone H4 basic residues 16-25 and acidic residues in histone H2A are thought to participate in nucleosome-nucleosome interactions and assist in nucleosome compaction (Dorigo et al. 2004); acetylation of H4 at K16 likely disrupts this interaction and inhibits 30-nm fiber formation (Shogren-Knaak et al. 2006). Single amino acid mutations in histone-fold domains of H4 (Horn et al. 2002) also disrupt 30-nm fiber formation. Additionally, histone H3 has been shown to play a role in chromatin interactions and compaction
(KAN et al. 2009; SPERLING and GRUNSTEIN 2009). Therefore, changes to the histone proteins within the nucleosome can affect the ability of the nucleosomes to properly package the DNA, resulting in changes to gene regulation.

1.1.2 Histone modifications

Histone proteins can be extensively modified post-translationally, and these modifications regulate gene expression and define heterochromatic and euchromatic regions of the genome. Common modifications include lysine and arginine methylation, lysine acetylation, lysine ubiquitylation, and serine phosphorylation (KOUZARIDES 2007). Modifications occur predominantly on the amino terminal tails, but can also be found on residues within the histone core (Figure 2). Histone modifications are dynamic and can be added by enzymes such as histone acetyltransferases (HATs) and methyltransferases (HMTs) or removed by enzymes such as histone deacetylases (HDACs) and demethylases (HDMs). Acetylation of histones H3 and H4, along with di- and tri-methylation of H3 K4 are modifications associated with active transcription. In contrast, heterochromatic marks include H3 K9 and H3 K27 methylation. While H3 K9Me and H3 K27Me are important heterochromatic marks in most eukaryotes, they are not found in \textit{S. cerevisiae} (MARTIN and ZHANG 2005). Instead, heterochromatin in \textit{S. cerevisiae} is defined by a lack of acetylation and methylation.
Histone tails can be modified in many ways, including methylation, acetylation, phosphorylation, and ubiquitylation. H3 K9Me and H3 K27Me modifications are found in *S. pombe* and higher eukaryotes, but are not found in *S. cerevisiae*. Figure from (ALLIS 2007).

Histone modifications are recognized and bound by proteins that regulate chromatin. Methylated lysine residues are recognized by chromodomains, WD40 domains, Tudor domains, or MBT domains (KIM et al. 2006; LACHNER et al. 2001), while acetylated lysines are recognized and bound by bromodomains (DHALLUIN et al. 1999; ZENG and ZHOU 2002). The interplay between these proteins allows for a changing chromatin environment that regulates gene transcription. For example, acetylated and methylated residues marking active regions of the genome can be recognized by the Spt-Ada-Gcn5-Acetyl transferase (SAGA) complex, a transcriptional co-activator complex (BAKER and GRANT 2007). In contrast, the Tup1-Ssn6 co-repression complex binds unacetylated histone tails to facilitate gene repression (EDMONDSON et al. 1996).
In addition to recruiting regulatory proteins, histone modifications can directly impact the structure of chromatin. In particular, acetylation of histone tails can have a profound effect on chromatin compaction and gene regulation, making them a focus of this work. Acetylation neutralizes the charge of lysine and can alter histone-DNA and nucleosome-nucleosome interactions, destabilizing the chromatin fiber and inhibiting folding of nucleosome arrays (SHOGREN-KNAAK et al. 2006; WANG and HAYES 2008). In contrast, hypoacetylated loci are generally silenced and able to form higher-order chromatin structures.

1.1.3 Other contributions to heterochromatin and gene regulation

1.1.3.1 Histone variants

Distinct from post-translational histone modifications, histone variants contain amino acid changes to the canonical histones and assist in marking regions of chromatin for specialized functions. Many sites of modification are maintained in histone variants although they are not highly conserved (MCKITTRICK et al. 2004). The canonical histones are expressed only in S phase, whereas histone variants are expressed outside of S phase and incorporated into chromatin in a DNA-replication independent fashion. Some common histone variants that are found in many eukaryotes include CenH3, H3.3, and H2A.Z, which are used in place of histones H3 and H2A.

CenH3 (chromosome segregation protein 4 (Cse4p) in S. cerevisiae) is a centromere-specific histone variant that marks the location of centromeres and is
essential for kinetochore formation and chromosome segregation (AMOR et al. 2004). In contrast, H3.3 serves to mark active regions of chromatin (HENIKOFF and AHMAD 2005) or those that are poised for activation. H2A.Z (Htz1p in S. cerevisiae) is found at transcription start sites (TSS) where nucleosomes turn over readily. It plays a role in many diverse processes, including gene activation, heterochromatin silencing, chromosome segregation, and nucleosome turnover (reviewed in (ALTAF et al. 2009; ZLATANOVA and THAKAR 2008).

1.1.3.2 Nucleosome positioning and chromatin remodeling

Selective positioning of nucleosomes through the use of chromatin remodeling complexes and DNA sequences assists in regulating gene function. Studies of nucleosome position in S. cerevisiae reveal that a nucleosome free region (NFR) over promoters is flanked by two well-positioned nucleosomes (BERNSTEIN et al. 2004; LEE et al. 2004; LEE et al. 1993; YUAN et al. 2005). These nucleosomes flanking the NFR often contain histone acetylation and methylation (KOUZARIDES 2007; LI et al. 2007) and histone variants, including H3.3 and H2A.Z (MALIK and HENIKOFF 2003; RAISNER et al. 2005), marking the region for activation and nucleosome eviction.

Chromatin remodeling complexes can unwrap the ends of DNA from histone octamers, form a DNA loop, or slide nucleosomes along the DNA. The first identified chromatin remodeling complex was the ATP-dependent SWItch/Sucrose Non Fermenting (SWI/SNF) complex, which was shown to serve as a positive regulator of
transcription (PETERSON et al. 1994; PETERSON and HERSKOWITZ 1992). SWI/SNF helps to move nucleosomes by exposing DNA regulatory sequences through the formation of DNA loops on the nucleosome surface (GUTIERREZ et al. 2007; SMITH and PETERSON 2005). Another chromatin remodeling complex, the Imitation SWItch (ISWI) chromatin remodeling complex, is involved in transcriptional repression, histone deposition, and creation of regions of heterochromatin at specific promoters (SMITH and PETERSON 2005). This complex uses the energy of ATP hydrolysis to bind nucleosomes and adjacent linker DNA and move the nucleosome in the direction of the linker DNA (FERREIRA and OWEN-HUGHES 2006; RIPPE et al. 2007), thus shortening the linker region and repositioning the nucleosome on the DNA. Nucleosomes can also be evicted from DNA through the cooperation of transcription factor binding, chromatin remodeling complexes, and actively transcribing Polymerase II (LI et al. 2007), allowing for better access to the DNA. Regulating DNA accessibility with nucleosome dynamics is crucial to proper gene regulation, but is not a focus of this work.

1.2 Sir silencing

*S. cerevisiae* utilizes the Silent Information Regulator (Sir) complex to form heterochromatic regions similar to those in higher eukaryotes. The Sir complex contains a histone deacetylase and silencing proteins that recognize and bind deacetylated nucleosomes (RUSCHE et al. 2003). It also utilizes ORC to recruit and assemble the Sir proteins, which participates in heterochromatin in higher eukaryotes. A role for specific
histone residues in Sir silencing has been well documented, and Sir silencing serves as a
good model for my interest in the contributions of histones H3 and H4 to gene
regulation.

The Sir proteins regulate silenced chromatin in *S. cerevisiae*. They were originally
identified genetically (IVY *et al.* 1986; RINE and HERSKOWITZ 1987) as essential for
repression of the silent mating type genes. The Sir silencing complex consists of Sir2p,
Sir3p, and Sir4p, which interact and form a complex in the cell (RUDNER *et al.* 2005).
Sir2p is a NAD\(^+\)-dependent histone deacetylase (IMAI *et al.* 2000b; LANDRY *et al.* 2000b;
SMITH *et al.* 2000), and Sir3p and Sir4p preferentially bind to deacetylated tails of
protein, Sir1p, acts independently of the core Sir silencing complex.

Chromatin immunoprecipitation studies in *S. cerevisiae* show enrichment of the
Sir proteins throughout silenced chromatin at the telomeres and silent mating type loci
Sir4p interacts with both Sir2p and Sir3p (HOPPE *et al.* 2002; MOAZED *et al.* 1997; RUDNER
*et al.* 2005) and plays an important structural role in the formation of the complex. In
contrast, Sir1p is not enriched throughout domains of silenced chromatin (RUSCHE *et al.*
2002) and is not essential to maintain silenced chromatin (PILLUS and RINE 1989).
1.2.1 Genomic regions subject to silencing

Silencing at the mating type loci

Silenced chromatin at the mating-type loci has been studied extensively. Haploid yeast can exist as one of two mating types, a or α, determined by the information expressed at the active MAT locus. Two cryptic HM loci, HMLa and HMRa, contain additional copies of the mating type genes utilized in mating type switching. For proper haploid cell identity and mating ability, it is essential to form a specialized chromatin structure at the HM loci and keep the extra mating information silent. A sequence-independent mechanism involving the Sir complex is utilized to silence the HM loci, while allowing for the identical information at the active MAT locus to be expressed.

Silencing at telomeres

The biological function of Sir silencing at telomeres is not fully understood, but is thought to primarily serve a structural role (GOTTSCHLING et al. 1990). Silencing at the telomeres was originally discovered by insertion of reporter genes near the telomeres, which resulted in variegated expression of the reporter genes (GOTTSCHLING et al. 1990). Similar to PEV in flies (MULLER 1930), the model of gene silencing as determined by proximity to the telomere was termed telomere position effect (TPE).

Silencing at the rDNA

An alternative form of silenced chromatin forms at the ribosomal RNA encoding repeats (rDNA) using the RENT complex. Sir2p is also involved in the RENT complex
(STRAIGHT et al. 1999), but the other Sir proteins do not participate in rDNA silencing. Silencing by the RENT complex is mechanistically distinct from Sir silencing and is not a focus of this work.

### 1.2.2 Establishment and mechanism of Sir silencing

**Establishment at the mating type loci**

The establishment of Sir silencing requires cis-acting elements, despite the ability of Sir proteins to bind nucleosomes. This ensures that Sir proteins are restricted to the appropriate regions of the genome. At the *HM* loci, these elements are termed silencers and include DNA binding sites for ORC, Rap1p, and Abf1p. Two silencers known as E and I flank the mating type genes located at *HMLα* and *HMRa*, and the composition of each silencer is unique (Figure 3). *HMR-E* is the only silencer that contains binding sites for all three silencer binding proteins and is thought to be the strongest silencer.

![Figure 3: The silent mating type loci HMLα and HMRa.](image)

The E and I silencers at *HMLα* and *HMRa* contain binding sites for ORC, Rap1, and Abf1p. The Sir silencing complex (yellow ovals) silences the silent mating type loci.
ORC, specifically Orc1p as part of ORC, recruits Sir1p, which interacts with Sir4p to recruit Sir2p and Sir3p to the HM loci (GARDNER et al. 1999; TRIOLO and STERNGLANZ 1996). Rap1p is able to bind to Sir3p and Sir4p (MORETTI et al. 1994; MORETTI and SHORE 2001), and Abf1p is thought to interact with Sir3p (GASSER and COCKELL 2001). Sir4p is required for Sir2p (HOPPE et al. 2002) and Sir3p recruitment to the silencer and is the only Sir protein that is recruited in the absence of the other Sir proteins (LUO et al. 2002; MOAZED et al. 2004; RUSCHE et al. 2002).

Establishment at the telomeres

Sir proteins are recruited to the telomeres through an array of Rap1 binding sites generated in the telomeric TG1-3 repeats (HECHT et al. 1996; LONGTINE et al. 1989). These binding sites likely recruit Sir4p and subsequently the Sir complex. In close proximity to the Rap1p binding sites is the X-core sequence, which contains binding sites for ORC and sometimes Abf1p (LOUIS 1995). Despite the close proximity of ORC binding sites, Sir1p is not recruited to the telomeres and does not appear to participate in telomeric silencing (APARICIO et al. 1991).

Mechanism of silencing

Once the Sir complex is recruited to the silencer, the complex is thought to spread via sequential deacetylation (RUSCHE et al. 2003). Sir2p, a NAD⁺-dependent deacetylase, removes acetyl marks on nearby nucleosomes, allowing for Sir3p and Sir4p to bind unacetylated nucleosomes. Sir3p and Sir4p can then interact with additional
Sir2p, which deacetylates the next nucleosome and allows for propagation of the Sir silencing complex along the chromatin fiber (Figure 4). This generates a chromatin structure that is restrictive to transcription and independent of DNA sequence.

**Figure 4: The sequential deacetylation mechanism of Sir silencing.**

Sir proteins are recruited to the silencers by DNA binding proteins including ORC, Rap1p (R), and Abf1p (A). ORC recruits Sir1p (1), which interacts with Sir4p (4) to recruit Sir2p (2) and Sir3p (3) to the HM loci. Sir2p, a NAD⁺-dependent deacetylase, removes acetyl marks on nearby nucleosomes, allowing for Sir3p and Sir4p binding and propagation of the Sir silencing complex along the chromatin.

A recent study showed that the rates of Sir assembly onto nucleosomes was profoundly different between the silent mating type loci and telomeres (LYNCH and RUSCHE 2009). At HMRα, Sir proteins assembled across more than 3 kilobases in a matter of minutes, while spreading over a similar distance at the telomeres took several hours.
Contributions from \textit{HMR}-E and a higher-order chromatin structure likely account for the improved silencing of \textit{HMRa}, and evidence of interactions within \textit{HMRa} from Chromatin Conformation Capture (3C) experiments (Miele et al. 2009; Valenzuela et al. 2008) support the formation of a specialized chromatin structure.

\textbf{1.2.3 Importance of histones to Sir silencing}

The importance of histone H3 and H4 tails to gene silencing has been well studied in \textit{S. cerevisiae}, and a summary of some characterized histone mutations is listed in Table 1. Critical lysine residues within the amino termini of H3 and H4 are subject to reversible acetylation, and hypoacetylation is correlated with telomeric and silent mating type regions (Braunstein et al. 1993; Suk et al. 2001). Mutations and deletion of the amino terminal residues of histones H3 and H4 disrupt telomeric and mating-type silencing (Aparicio et al. 1991; Johnson et al. 1992; Kayne et al. 1988; Mann and Grunstein 1992; Martin et al. 2004; Megee et al. 1990; Morgan et al. 1991; Park and Szostak 1990; Thompson et al. 1994). In contrast, deletion of the amino termini of histones H2A and H2B have little effect on silencing at the mating type loci (Kayne et al. 1988). Histones H3 and H4 may serve as better carriers of epigenetic marks than H2A and H2B because of their central location within the nucleosome.
Table 1: Characterized histone mutations that disrupt silencing

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sir HMRa</th>
<th>HMLα</th>
<th>Tel</th>
<th>rDNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2AΔ4-20</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Kayne et. al. 1988</td>
</tr>
<tr>
<td>H2BΔ3-32</td>
<td>+</td>
<td>~</td>
<td></td>
<td></td>
<td>Kayne et. al. 1988, Thompson et. al. 1994</td>
</tr>
<tr>
<td>H2B K49A</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Kyriss et. al. 2010</td>
</tr>
<tr>
<td>H2B R102A</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>Kyriss et. al. 2010</td>
</tr>
<tr>
<td>H2B K111A</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Kyriss et. al. 2010</td>
</tr>
<tr>
<td>H3Δ1-28</td>
<td>~</td>
<td></td>
<td></td>
<td></td>
<td>Morgan et. al. 1991</td>
</tr>
<tr>
<td>H3Δ4-30</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Marin et. al. 1992, Thompson et. al. 1994</td>
</tr>
<tr>
<td>H3 K9,14,18,23,27G</td>
<td>~</td>
<td></td>
<td></td>
<td></td>
<td>Jin et. al. 2009</td>
</tr>
<tr>
<td>H3 K4,K36,K79G</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Jin et. al. 2009</td>
</tr>
<tr>
<td>H3 R2G</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 T6K</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 L10P</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Xu et. al. 2005</td>
</tr>
<tr>
<td>H3 K12E</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Xu et. al. 2005</td>
</tr>
<tr>
<td>H3 A27V</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 K56A</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Hyland et. al. 2005</td>
</tr>
<tr>
<td>H3 K56Q</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Hyland et. al. 2005</td>
</tr>
<tr>
<td>H3 K56R</td>
<td>~</td>
<td>~</td>
<td></td>
<td></td>
<td>Hyland et. al. 2005</td>
</tr>
<tr>
<td>H3 Q68R</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 L70S</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 V71A</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 R72G</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003, Park et. al. 2002</td>
</tr>
<tr>
<td>H3 E73D</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 A75V</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Park et. al. 2002</td>
</tr>
<tr>
<td>H3 Q76R</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 D77G</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Smith et. al. 2002, Norris et. al. 2008</td>
</tr>
<tr>
<td>H3 D77N</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Norris et. al. 2008</td>
</tr>
<tr>
<td>H3 F78L</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Park et. al. 2002</td>
</tr>
<tr>
<td>H3 K79A</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Hyland et. al. 2005</td>
</tr>
<tr>
<td>H3 K79E</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003, Park et. al. 2002</td>
</tr>
<tr>
<td>H3 K79Q</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Hyland et. al. 2005</td>
</tr>
<tr>
<td>H3 K79R</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Park et. al. 2002</td>
</tr>
<tr>
<td>H3 T80A</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 T80P</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 D81G</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Smith et. al. 2002</td>
</tr>
<tr>
<td>H3 L82S</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Park et. al. 2002</td>
</tr>
<tr>
<td>H3 R83A</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Park et. al. 2002</td>
</tr>
<tr>
<td>H3 F84L</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 A114T</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Thompson et. al. 2003</td>
</tr>
<tr>
<td>H3 K122Q</td>
<td>~</td>
<td>-</td>
<td></td>
<td></td>
<td>Hyland et. al. 2005</td>
</tr>
<tr>
<td>H3 K122R</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>Hyland et. al. 2005</td>
</tr>
</tbody>
</table>
Characterized histone mutations that disrupt Sir silencing of \textit{HMRa}, \textit{HMLa}, and telomeres, or rDNA silencing mediated by RENT. Histone mutations that disrupt silencing (-), enhance silencing (+), or do not appear to have a significant effect on silencing (~) are indicated. Histones are colored to correspond with the structure of the nucleosome in Figure 1.

Several regions of the histone H4 tail contribute to silencing. Mutation or deletion of the modifiable lysine residues in the H4 tail results in silencing defects.
(KAYNE et al. 1988; MEGEE et al. 1990). Mutation of H4 K16 is very disruptive to silencing of the mating type loci, while other lysine mutations have a small defect in mating (PARK and SZOSTAK 1990), and H4 K16 is the residue primarily acetylated in histone H4 (CLARKE et al. 1993). The basic charge of H4 residues 16-19 is also important to silencing (JOHNSON et al. 1990; MEGEE et al. 1990; PARK and SZOSTAK 1990), and SIR3 suppressors have been identified that overcome the silencing defects in H4 16-19 mutations (JOHNSON et al. 1990). Finally, a non-basic patch of residues 21-29 in H4 are also required for silencing at mating type loci (JOHNSON et al. 1992).

In contrast, H3 tail deletions or mutations do not cause a dramatic defect in silencing of the mating type loci (MANN and GRUNSTEIN 1992; MORGAN et al. 1991), unless mating is weakened (THOMPSON et al. 1994), which suggests that the histone H3 tail plays a supporting role in silencing at the mating type loci. The histone H3 tail plays a more prominent role in telomeric silencing, and H3 residues 4-20 are needed for efficient telomeric silencing of a URA3 reporter (THOMPSON et al. 1994). Mutating lysines 4, 9, 14, 18, 23, and 27 to glycine in histone H3 significantly reduces subtelomeric gene silencing (MARTIN et al. 2004), and silencing at the telomere is dependent on the histone H3 N-terminal domain. Therefore, there are locus-specific requirements for histones that enable efficient silencing.

In addition to the histone tails, the histone H3 core is also important for silencing. Mutations to the LRS region disrupt silencing of the mating type loci, telomeres, and
rDNA (Park et al. 2002). While these residues were identified based on a disruption to rDNA silencing, only a few mutations are disruptive to mating. A separate screen for histone H3 mutations that disrupt telomeric silencing identified many of the residues in the LRS region (Thompson et al. 2003), and mutation of modifiable core residues also identified residues that disrupt rDNA silencing, including H3 K79Q, H4 K59A, and H4 K59Q (Hyland et al. 2005). H3 K79 is centrally located in the LRS domain and is methylated by Dot1p in euchromatin (Ng et al. 2003; Van Leeuwen et al. 2002). Methylation of H3 K79 prevents Sir3p binding to this region of the nucleosome (Altaf et al. 2007; Onishi et al. 2007). Sir3p and Dot1p compete for binding to the same region of the H4 tail (Altaf et al. 2007), which is located in close proximity to the LRS region of H3 in the nucleosome structure. Mutations in the LRS region disrupt methylation of H3 K79 (Fry et al. 2006) and Sir3p binding to the LRS region (Onishi et al. 2007; Sampath et al. 2009), resulting in silencing defects at the telomeres (Buchberger et al. 2008) and the mating type locus HMLα (Thompson et al. 2003).

Histone mutations have also been identified that enhance silencing. A screen for histone mutations that increase telomeric silencing identified H3 Q5, A15, S22, and A24 tail residues, along with H4 G7, L10, G11, G13, and A15. Residues in the core of H3 and H4 were identified but not studied further (Smith et al. 2002). These tail mutations are located in close proximity to the acetylated lysine residues in the tails or methylated H3 K4 lysine and likely disrupt the recognition of these lysines by histone-modifying
enzymes. This would allow for maintenance of the deacetylated state, inhibition of H3 K4 methylation, and an increase in silencing. Most of the core residues identified are located in the LRS region and likely increase Sir3p binding to this region of the nucleosome and/or disrupt H3 K79 methylation, explaining the enhancement of silencing. Other studies have also identified H3 and H4 core residues that increase telomeric silencing (Hylland et al. 2005; Xu et al. 2005).

Several large-scale histone mutation collections have also been recently studied (Dai et al. 2008; Matsubara et al. 2007), where surface residues or modifiable residues in all of the histone genes have been created. These studies have expanded our knowledge of the effects of histone mutations on silencing and on other biological functions. With the expansion of histone studies of H2A and H2B, recent work has identified contributions of H2B residues to silencing. H2B K111 disrupts telomeric silencing and Sir4 binding, while H2B R102 enhances silencing at yeast telomeres and the HMLα silent mating locus and increases Sir4 binding to these regions (KyriSS et al. 2010). However, with the large collection of data showing a role for histones H3 and H4 in silencing, my focus is on the contributions from H3 and H4.

1.3 Local repression

While silencing complexes spread over several kilobases and silence the underlying genes in a gene-independent manner, local repression is gene-specific and does not extend to neighboring genes. Interestingly, the SUM1-1 mutation in S. cerevisiae
changes a local repression complex into a silencing complex capable of long-range spreading and silencing at a new genomic location. Interactions between histones and silencing proteins are critical to Sir silencing and may also be important for the spreading ability of the Sum1-1 complex. Additionally, it is unlikely that the $SUM1-1$ mutation would create a novel function from a single amino acid change, so histone interactions may also be important for wild type Sum1 repression. In this dissertation, I have used the mutant Sum1-1 complex to study common requirements for histones in long-range silencing complexes (Sum1-1 and Sir complexes) and local repression complexes (Tup1-Ssn6 and wild-type Sum1 complexes).

1.3.1 The Tup1-Ssn6 repression complex

1.3.1.1 Tup1p-Ssn6p-mediated co-repression

The Tup1-Ssn6 complex in *S. cerevisiae* is one of the best studied co-repressor complexes. It consists of 3-4 molecules of Tup1p and 1 molecule of Ssn6p (REDD *et al.* 1997; VARANASI *et al.* 1996), which associate with sequence specific DNA binding proteins to turn off several subsets of genes. Over 180 diverse genes are repressed by the Tup1-Ssn6 complex, including genes regulated by glucose, DNA damage, mating type, and oxygen availability (GREEN and JOHNSON 2004; SMITH and JOHNSON 2000). Multiple DNA binding proteins (Mig1p, Crt1p, Rox1p) allow for customized recruitment of Tup1-Ssn6 to the particular gene families and subsequent repression through interactions with particular histone deacetylases (HDACs Rpd3p, Hos1p, Hos2p) (DAVIE *et al.* 2003).
The N-terminus of Tup1p facilitates tetramerization and interactions with Ssn6p, while the C-terminus folds into a propeller structure that facilitates interactions with other protein partners. Ssn6p contains 10 tetratricopeptide repeats (TPR) (Schultz et al. 1990), and different TPRs are required for interactions with different HDACs (Davie et al. 2003; Davie et al. 2002) and repression of specific genes (Tzamarias and Struhl 1995). Thus, the conformation of Ssn6p appears to be somewhat flexible and allows for versatility of the complex in its repression of several families of genes. The localization of the complex is also versatile. Tup1-Ssn6 remains localized at the promoter of some genes (Davie et al. 2002; Li and Reese 2001), but also spreads from the recruitment point into the coding region at other genes (Davie et al. 2002; Ducker and Simpson 2000).

1.3.1.2 The role of histones in Tup1-Ssn6 repression

The N-terminus of Tup1p, in addition to its interactions with Ssn6p and itself, also interacts with histones H3 and H4 (Edmondson et al. 1996). The histone tails are necessary and sufficient for this association, and preferential association is seen with hypoacetylated histones. Interactions with histones are thought to stabilize the association of the Tup1-Ssn6 complex with the target promoter, reinforcing repression (Malave and Dent 2006). Histone mutations that disrupt the association of Tup1p with histone tails (Edmondson et al. 1996) or increase histone acetylation (Watson et al. 2000) disrupt repression mediated by the Tup1-Ssn6 complex.
In addition, simultaneous deletion of RPD3, HOS1, and HOS2 HDACs abolishes repression at many genes and shows increased acetylation and decreased Tup1p binding (Davie et al. 2003; Davie et al. 2002; Watson et al. 2000). This suggests that HDACs are important to the mechanism of Tup1-Ssn6 repression. It is not clear if multiple HDACs interact with Ssn6p at once or if different HDACs are utilized at different sets of genes. Whatever the mechanism, HDACs and histone tail deacetylation are important to repression mediated by the Tup1-Ssn6 complex.

1.3.1.3 Conservation of Tup1-Ssn6 repression

Tup1 and Ssn6 have homologs in other eukaryotes including S. pombe and Caenorhabditis elegans, but no homologs have been identified in higher eukaryotes (Malave and Dent 2006). However, flies and vertebrates possess proteins with similar domain structures and functions in repression. For instance, the Groucho protein in D. melanogaster has an amino terminal domain important for tetramerization (Li 2000). It is recruited to many gene families through interactions with DNA-binding repressors (Courey and Jia 2001) and interacts with histones and HDACs (Malave and Dent 2006), behaving very similar to Tup1p. Mammalian transducin beta-like (TBL) proteins also interact with several repressors and bind to histones (Yoon et al. 2003). These mechanistic features are used in multiple repression complexes, so aspects of Tu1p-Ssn6 repression may be conserved in higher eukaryotes.
1.3.2 The Sum1 repression complex

Histones H3 and H4 are known to be important for Sir silencing and Tup1-Ssn6 co-repression. The Sum1 complex, like the Tup1-Ssn6 complex, is a promoter-specific repression complex (PIERCE et al. 2003; XIE et al. 1999). A single amino acid mutation to Sum1p forms the Sum1-1 complex, which is capable of regional silencing (RUSCHE and RINE 2001; SUTTON et al. 2001). The spreading ability of the Sum1-1 complex and its use of Hst1p, a presumed histone deacetylase, suggests that the complex may utilize interactions with histones to facilitate spreading. Since the complex components are the same between Sum1 repression and Sum1-1 silencing, histones may also be involved in Sum1 repression and Sum1-1 silencing. The Sum1-1 complex provides a good model system to learn about the requirements for histones H3 and H4 in silencing and repression.

1.3.1.1 Sum1p-mediated repression

The Sum1 complex (Figure 5) represses over fifty genes involved in sporulation, NAD⁺ biosynthesis, and α-cell identity (BEDALOV et al. 2003; XIE et al. 1999). Sum1p is a DNA binding protein that associates with a conserved middle sporulation element (MSE) sequence found in the promoters of its target genes (PIERCE et al. 2003; XIE et al. 1999). Sum1p recruits Rfm1p, which is thought to play a structural role in the complex and is required for Sum1p to associate with the NAD⁺-dependent deacetylase Hst1p (MCCORD et al. 2003).
Figure 5: The Sum1 repression complex.

Sum1p (S) recognizes and binds the middle sporulation element (MSE) consensus sequence, and Rfm1p (R) allows for Hst1p (H) association. Hst1p deacetylates histones and contributes to repression.

Sum1p contains two consensus AT-hook motifs in the N-terminus of the protein. AT-hook motifs are found in many eukaryotic chromatin and DNA-binding proteins (ARAVIND and LANDSMAN 1998) and bind the minor groove of DNA. The C-terminus of Sum1p was shown to bind to MSE sequences in vitro (PIERCE et al. 2003) and is thought to contain a DNA binding domain, although a specific domain has not been identified. Hst1p is a paralog of Sir2p and is thought to deacetylate histone tails in a similar fashion, contributing to its repressive function. However, the exact target of Hst1p deacetylation has not been identified.

1.3.2.2 The role of histones in Sum1 repression

In the absence of HST1, an increase in histone H3 and H4 acetylation levels occurs at Sum1p-repressed promoters (HICKMAN and RUSCHE 2007; ROBERT et al. 2004), suggesting that histone deacetylation is important for Sum1 repression. However, little is known about the requirements for histones H3 and H4 in Sum1 repression. Hst1p
contributes to the repressive function of the Sum1 complex (MCCORD et al. 2003), but is not required for regulation of all genes. This may suggest that histone H3 and H4 residues may not be required for repression of all Sum1p-regulated genes.

1.3.1.3 Conservation of Sum1 repression

Homologs of Sum1p and Rfm1p can be found in most closely related yeast species, but they are not conserved in more distantly related yeasts such as Candida and higher eukaryotes. However, the repression mechanism and utilization of histone residues and a deacetylase may be conserved. A whole genome duplication occurred in the lineage of the hemiascomycetes approximately 100 million years ago after the divergence of the Kluyveromyces and Saccharomyces species (WOLFE and SHIELDS 1997). The Sum1 complex in K. lactis, which did not undergo the whole genome duplication, consists of KlSum1p, KlSir2p, and KlRfm1p. These proteins work in complex to repress mid-sporulation genes in a local, promoter-specific manner (HICKMAN and RUSCHE 2009), similar to Sum1 repression in S. cerevisiae. However, KlSum1p also acts with KlSir2p to silence the cryptic mating-type loci (HICKMAN and RUSCHE 2009), where KlSum1p has the added capability to spread across the locus. These data suggest that the common ancestor of KlSum1p and ScSum1p most likely had a role in silencing. While this function was lost in S. cerevisiae, a mutation identified in Sum1p appears to uncover this ancestral ability.
1.4 Sum1-1 silencing

Aspects of Sum1-1 silencing appear to be evolutionarily conserved, and this complex bridges local repression and long-range silencing, making it an interesting model to study and learn about histone requirements to gene regulation.

1.4.1 Silencing proteins and their genomic localization

Sum1p was originally identified in a genetic screen for mutations that restore silencing in a \( \text{MAT}^a \ sir2\Delta \) strain (Klar et al. 1985). The dominant \( \text{SUM}1-1 \) mutant allele was found to contain a single amino acid substitution, threonine 988 to isoleucine, which restores silencing at the cryptic mating type loci in \( \text{sir}2\Delta \), \( \text{sir}3\Delta \), or \( \text{sir}4\Delta \) (Chi and Shore 1996; Laurensen and Rine 1991; Livi et al. 1990; Rusche and Rine 2001). Mutant Sum1-1p (Figure 6) continues to require Rfm1p and Hst1p (Lynch et al. 2005; Rusche and Rine 2001), but is not recruited to mid-sporulation genes as effectively (Lynch et al. 2005; Sutton et al. 2001). This is most likely due to its additional recruitment to the \( \text{HM} \) loci, where it forms a repressive chromatin structure (Rusche and Rine 2001; Sutton et al. 2001). Thus, wild type Sum1p participates in localized, gene-specific repression that does not extend to neighboring genes, while Sum1-1p has the added capability of long-range silencing across the \( \text{HM} \) loci (Lynch et al. 2005), similar to the Sir silencing complex.
Figure 6: The Sum1-1 complex.

A. ORC recruits Sum1-1p (S) to the mating type loci. Rfm1p (R) and Hst1p (H) associate and propagate along the chromatin to silence the mating type loci.

B. Sum1-1 silences HMRa more efficiently than HMLa.

While Sum1-1p can restore silencing of HMRa and HMLa independently of the Sir proteins, it works most effectively to silence HMRa. Full silencing of the a1-a2 genes at HMRa and robust mating is seen in MATα sirΔ SUM1-1 strains, while MATα sirΔ SUM1-1 strains maintain transcription of a1-a2 genes at HMLa and recover modest mating (Chi and Shore 1996; Klar et al. 1985; Laurenson and Rine 1991; Livi et al. 1990). As a result, much of the experimental work investigating Sum1-1 silencing has been done in MATα sir2Δ SUM1-1 strain backgrounds to assess silencing of HMRa, and the focus of this work is on understanding the role of histones in Sum1-1 silencing at HMRa.
1.3.2 Establishment and mechanism of the Sum1-1 complex

Similar to Sir silencing, Sum1-1 silencing is established through the use of DNA binding proteins. This allows for recruitment of silencing proteins to the proper genomic locations, where nucleosome modifying proteins can be recruited to create a silenced chromatin state. However, recruitment of Sum1-1p to the cryptic mating type loci occurs through interactions with ORC (LYNCH et al. 2005; RUSCHE and RINE 2001; SUTTON et al. 2001), rather than through use of the DNA binding domain of Sum1-1p. Sum1-1 silencing also requires the deacetylation activity of Hst1p (LYNCH et al. 2005; RUSCHE and RINE 2001; SUTTON et al. 2001), and SUM1-1 strains containing a deletion of HST1 or mutations to the catalytic domain experience silencing defects.

Sum1-1p has been shown to interact with Orc5p via yeast two-hybrid, and Orc5p and Sum1-1p CoIP if HMRa DNA is present (SUTTON et al. 2001). This suggests that the ORC binding sites present at the E and I silencers facilitate an interaction between ORC and Sum1-1p. Additionally, silencing was completely abolished in temperature sensitive mutations of orc2-1 and orc5-1, which is consistent with reduced association of Sum1-1p at all HM silencers in an orc5-1 strain (RUSCHE and RINE 2001). Finally, the N-terminus of Orc1p is required for Sum1-1 silencing (RUSCHE and RINE 2001). These data suggest an important role for ORC in Sum1-1p recruitment and silencing of HM loci.

In the absence of RFM1 or HST1, Sum1-1p enrichment is seen only at the E silencer (LYNCH et al. 2005), despite an ORC binding site at the I silencer. These data are
not consistent with the current model of Sum1-1 silencing, which suggests that the E and I silencers both serve as recruitment sites for Sum1-1p (SUTTON et al. 2001). Therefore, the E silencer appears to be the major recruitment site for Sum1-1p, and spreading of the complex across the loci through sequential deacetylation of histone tails may propagate Sum1-1p silenced chromatin. Although a nucleosome binding activity is predicted to enable spreading, it is not known whether the Sum1-1 complex has this property, something I will address in this dissertation. The ability of Sum1-1p to self-associate (SAFI et al. 2008) may also assist in the silencing ability of Sum1-1p, serving to stabilize the complex as it spreads.

1.3.3 The role of histones in Sum1-1 silencing

Little is known about the role of histones in Sum1-1 silencing, but the requirement of Hst1p for Sum1-1 silencing suggests an important role for histones H3 and H4. The removal of acetyl marks on lysine residues in the tails of histones H3 and H4 is important for creating a silenced state, and hypoacetylation of histones H3 and H4 has been confirmed in Sum1-1 silencing (RUSCHE and RINE 2001). Additionally, deletion of the H4 tail results in an enrichment of Sum1-1p at mid-sporulation genes and abolished Sum1-1 silencing (LYNCH et al. 2005), supporting the involvement of histone tails in silencing.

The spreading ability of the Sir complex is due to nucleosome binding proteins Sir3p and Sir4p, which bind to deacetylated nucleosomes and propagate the silenced
chromatin, and a nucleosome modifying protein, the Sir2p histone deacetylase. While Sum1-1 silencing utilizes a similar nucleosome modifying protein, the deacetylase Hst1p, a nucleosome binding protein has not been identified in the Sum1-1 complex. It seems unlikely that a novel histone binding property would result from a single amino acid change to Sum1p, so the ability to bind histones may be a feature of the wild type Sum1 complex and may also be important in repression.

Therefore, this work is focused on understanding the role of histone modifications and nucleosome binding ability to the propagation and stability of the Sum1-1 silencing complex and Sum1 repression complex. I performed a genetic screen for histone H3 and H4 mutations that disrupt Sum1-1 silencing or Sum1 repression (Chapter 2), examined the ability of Sum1p to bind to histone tails and serve as a nucleosome binding protein in these complexes (Chapter 3), and investigated the contribution of Sir silencing proteins to Sum1-1 silencing (Chapter 4).
2. A role for Orc1p and the LRS/H4 region of the nucleosome in Sum1-1 silencing

2.1 Introduction

The formation of silenced chromatin, or heterochromatin, in eukaryotes is important for proper gene regulation and chromosome stability and helps define centromeres and telomeres. One interesting property of heterochromatin is its capacity to spread along a chromosome to form an extended, repressive domain. This spreading is enabled by specific interactions between silencing proteins and nucleosomes. Consequently, particular surfaces on the nucleosome can be critical to the assembly of heterochromatin. This study reveals that in Saccharomyces cerevisiae, the same nucleosomal surface is critical for the formation of three types of silenced chromatin, mediated by the Sir, RENT, or Sum1-1 complexes, suggesting that this region of the nucleosome may be generally important to long-range silencing.

In the budding yeast S. cerevisiae, silenced chromatin domains are found at the cryptic mating-type loci \textit{HML}\alpha and \textit{HMR}\alpha and most sub-telomeric regions (RUSCHE \textit{et al}. 2003). The Sir proteins are structural components of this chromatin, and strains lacking Sir2p, Sir3p or Sir4p lose silencing. Consequently, \textit{sir} strains express both \textit{a} and \textit{\alpha} mating-type information, resulting in the loss of cell-type identity and an inability to mate. Interestingly, silencing and mating can be restored by the \textit{SUM1-1} mutation, which was originally identified as a suppressor of a \textit{sir2}\Delta mutation (KLAR \textit{et al}. 1985). The \textit{SUM1-1} mutation results from a single amino acid change (CHI and SHORE 1996),
which enables the Sum1 repressor, which normally does not spread, to form an extended silenced domain along the chromatin fiber (Rusche and Rine 2001; Sutton et al. 2001). The ability of mutant Sum1-1p to spread suggests that Sum1-1p or an associated protein interacts with nucleosomes and raises the question of whether the wild-type, non-spreading Sum1 complex also interacts with nucleosomes. To address these issues, I identified histone residues important for silencing mediated by the mutant Sum1-1 complex and the wild-type Sum1 complex.

Sir silencing at the cryptic mating-type loci is initiated at the E and I silencers, which flank HMLα and HMRα. These silencers contain binding sites for the origin recognition complex (ORC) and the transcription factors Rap1p and Abf1p, which recruit the Sir proteins. Notably, Orc1p, the largest subunit of ORC, interacts directly with Sir1p, stabilizing the Sir proteins at the silencer (TrioLO and Sternglanz 1996). Sir2p is a NAD⁺-dependent deacetylase (Imai et al. 2000a; Landry et al. 2000a; Smith et al. 2000), and deacetylation of nucleosomes in the vicinity of the silencers facilitates the binding of Sir3p and Sir4p, allowing the Sir complex to propagate along the chromosome through a sequential deacetylation mechanism (Hoppe et al. 2002; Rusche et al. 2002).

Sir3p is proposed to have two distinct histone binding domains that contribute to silenced chromatin assembly. A C-terminal histone-binding domain interacts with histone tails (Hecht et al. 1995; Liou et al. 2005), and the N-terminal BAH domain binds
the base of the histone H4 tail and an adjacent surface on the nucleosome core termed
the LRS region (BUCHBERGER et al. 2008; NORRIS et al. 2008; ONISHI et al. 2007; SAMPATH et al. 2009). Many mutations in histones that disrupt silencing are thought to act by
decreasing the affinity of Sir3p for nucleosomes. These include mutations in the histone
H4 tail (ALTAF et al. 2007; JOHNSON et al. 1992; JOHNSON et al. 1990; PARK and SZOSTAK
1990) and the LRS region (BUCHBERGER et al. 2008; NORRIS et al. 2008; PARK et al. 2002;
SAMPATH et al. 2009; THOMPSON et al. 2003). In the cell, interactions between Sir proteins
and nucleosomes are regulated by post-translational modifications. For example,
decacetylation of histones by Sir2p is thought to increase their affinity for Sir3p.
Moreover, mutation of lysines in the histone tails to glutamine, mimicking an acetylated
state, reduces Sir3p binding in vitro (ALTAF et al. 2007; CARMEN et al. 2002) and silencing
in vivo (JOHNSON et al. 1992; MEGEE et al. 1990). In addition, the affinity of Sir3p for
nucleosomes is modulated by methylation of H3 K4 (SANTOS-ROSA et al. 2004) and H3
K79, located within the LRS region that interacts with the Sir3p BAH domain (VAN
LEEUWEN et al. 2002).

Unlike the Sir proteins, wild-type Sum1p does not form an extended domain.
Sum1p is a DNA binding protein that recognizes a consensus sequence found in the
promoters of some mid-sporulation, NAD+ biosynthesis, and alpha-specific genes
(BEDALOV et al. 2003; PIERCE et al. 2003; XIE et al. 1999; ZILL and RINE 2008). Sum1p works
with Hst1p, an NAD+-dependent deacetylase related to Sir2p (MCCORD et al. 2003; XIE et
The mutant form of Sum1p, Sum1-1p, contains a single amino acid mutation (T988I) that redirects Sum1-1p to the cryptic mating-type loci through interactions with ORC. It has been proposed that the SUM1-1 mutation acts by changing the binding partners of the Sum1 protein. In particular, loss of T988 reduces the affinity of Sum1p for its DNA consensus sequence, and the presence of isoleucine at position 988 increases the tendency of the protein to self associate (SAFI et al. 2008), which may increase the ability of Sum1-1p to spread along chromatin. Additionally, the T988I mutation increases the affinity of Sum1-1p for ORC, leading to the recruitment of Sum1-1p to HMRa and other ORC binding sites (LYNCH et al. 2005; RUSCHE and RINE 2001; SUTTON et al. 2001). Thus, the SUM1-1 mutation changes the location and function of the complex, from a locus-specific repressor to a regional silencer. Sum1-1p or another component of the Sum1-1 complex may interact with the histones to enable the complex to spread. However, such an interaction has not been documented.

To investigate how the mutant Sum1-1 complex contacts nucleosomes and to determine whether such an interaction is also important for repression mediated by wild-type Sum1p, I conducted genetic screens for mutations in histones H3 and H4 that disrupt Sum1-1 silencing or SUM1 repression. I discovered that the same region of the nucleosome is important for regional silencing mediated by the mutant Sum1-1 or Sir complexes but this region is not critical for Sum1 repression. In addition, I present
evidence that this region interacts with the Orc1p BAH domain to facilitate Sum1-1 silencing.

### 2.2 Methods

#### Yeast strains

Strains used in this study were derived from W303-1b. The $SUM1-1$, $myc-SUM1$, $7myc-SUM1-1$, $sir2Δ::HIS3$, $sir3Δ::LEU2$, $hst1Δ::KanMX$ (Rusche and Rine 2001), $pPES4$-$HIS3$ (Hickman and Rusche 2007), $URA3$::$(lexAop)_8$-$lacZ$, $LYS2$::$(lexAop)_4$-$HIS3$ (Hollenberg et al. 1995) and VR-ADE-TEL (Singer and Gottschling 1994) alleles were previously described. The $hht1-hhf1Δ::NatMX$, $hht2-hhf2Δ::HygMX$, $sum1Δ::URA3$, $sir2Δ::LEU2$, $hmrΔ::URA3$, $set1Δ::URA3$, and $dot1Δ::URA3$ alleles are complete deletions of the open reading frames generated by one step gene replacement. To generate the p$YGL138C$-$LEU2$ reporter allele, the open reading frame of $YGL138C$ was replaced precisely with the $LEU2$ open reading frame by one-step gene replacement. The correct integration was confirmed by PCR using primers flanking the sites of insertion. These alleles were moved into the desired genetic backgrounds through standard genetic crosses to generate the strains listed in Table 4.

To generate $ADE2$::Gal4DBD-$7myc-SUM1-1$, an integrating plasmid was first constructed. The Gal4 DNA binding domain (DBD; amino acids 1-94) was amplified from genomic DNA using oligos that contain NcoI restriction sites at the ends. This DNA fragment was digested with NcoI and ligated into pLR20 ($7myc-SUM1-1$ $ADE2$) to
generate pLR40 (Gal4DBD-7myc-SUM1-1 ADE2). A NotI/BamHI fragment containing Gal4DBD-7myc-SUM1-1 was isolated from plasmid pLR40 and ligated into the integrating plasmid pRS402 to generate pLR836. A unique AgeI site was created within the ADE2 ORF by site-directed mutagenesis to generate plasmid pLR855. pLR855 was linearized with AgeI and integrated at the ADE2 locus.

To generate ORC1::3HA-HIS3, a 3HA-HIS3 tagging cassette was amplified from plasmid pLR541 (derived from pMPY-3xHA (Schneider et al. 1995)) with primers having homology to the 3’ end of ORC1. This PCR product was integrated at the ORC1 locus to create LRY1991 (W303-1b MATα ORC1::3HA-HIS3). To generate ORC1E84K::3HA-HIS3 and ORC1P179L::3HA-HIS3, the ORC1::3HA-HIS3 allele was amplified from LRY1991 and incorporated into pLR560 (ORC1 URA3) by homologous recombination to obtain pLR776 (ORC1::3HA-HIS3 URA3). The E84K and P179L mutations were created by site-directed mutagenesis of pLR776, and these mutated orc1 genes were then integrated at the ORC1 locus. The orc1Δbah::3HA-HIS3 allele, which lacks amino acid residues 2-235, was generated in two steps. First, orc1Δbah from plasmid pSPB1.48 (Bell et al. 1995) was integrated at the ORC1 locus by one-step gene replacement in an orc1Δ::URA3 strain. Then, a 3HA-HIS3 tagging cassette was integrated at the 3’ end of the orc1Δbah gene.
Plasmids

Plasmids used in this study are described in Table 5. pDM9 (HHT1 HHF1), pDM18 (HHT2 HHF2) (Duina and Winston 2004), pJR2292 (3myc-SUM1), pJR2291 (3myc-SUM1-1) (Rusche and Rine 2001), pLR052 (3HA-SUM1), pLR047 (3HA-SUM1-1) (Safi et al. 2008), pTT93 (LexA-ORC5), pGAD424 (GAD), pRH01 (GAD-SUM1), pRH02 (GAD-SUM1-1) (Sutton et al. 2001), pRM430 (HHT2Δ4-30) and pGF29 (HHF2Δ4-28) (Ling et al. 1996) were previously described.

Single point mutations to histones H3 and H4 were obtained from Jef Boeke (Fry et al. 2006) or made in one of two ways. Some histone mutations were created by site directed mutagenesis of pDM18. Other mutations were generated by homologous recombination to integrate desired mutations from other plasmids into pDM18. A BstNI-SpeI fragment containing the HHF2 gene with the desired mutation was isolated from plasmids generated in the Grunstein lab. HHF2 R17G, H18G, and K20G mutations were isolated from yeast strains LJY921, LJY933, LJY952 (Johnson et al. 1990), and HHF2 D24P and G28P were isolated from yeast strains containing modified versions of pUK499 HHF2 URA3 (Kayne et al. 1988). A wild-type yeast strain was transformed with the mutated HHF2 fragment of interest and linearized pDM18 in which the HHF2 gene had been removed using BglII and HpaI. Intact plasmids regenerated by homologous recombination were isolated from yeast, amplified in E. coli, and sequenced to verify the presence of the mutation of interest. Histone H3 and H4 tail deletion plasmids pLR497
(HHT2Δ5-20), pLR501 (HHT2Δ4-28), and pLR547 (HHT2Δ30-40) were created by site-directed deletion of pDM18.

Multiple point mutations to histones H3 and H4 were made using several strategies. Plasmids containing H4 K16Q mutations in combination with H3 mutations were constructed using site directed mutagenesis of single histone H3 mutant plasmids to create pLR819 (HHT2 K4R HHT2 K16Q), pLR820 (HHT2 K4I HHT2 K16Q), pLR821 (HHT2 E73G HHT2 K16Q), and pLR822 (HHT2 A75V HHT2 K16Q). Plasmids containing multiple lysine mutations were created by ligating synthetic histone genes obtained from Junbiao Dai and Jef Boeke (Dai et al. 2008) into pDM18. Plasmids pLR673 (HHTS K9,14,18,23R), pLR674 (HHTS K9,14,18,23Q), and pLR675 (HHTS K9,14,18,23A) were created by ligation of a NotI-KpnI fragment containing mutated HHTS (Dai et al. 2008) into the NotI-KpnI sites of pDM18, replacing the H3 gene. Plasmids pLR672 (HHFS K5,8,12,16Q), pLR676 (HHFS K5,8,12,16A), pLR677 (HHFS K5,8,12,16R), and pLR747 (HHTS K4A) were created by ligation of a SalI-NotI fragment containing mutated HHFS (Dai et al. 2008) into the SalI-NotI sites of pDM18, removing the H4 gene. Plasmids pLR700 (HHTS K9,14,18,23Q HHFS K5,8,12,16Q) and pLR702 (HHTS K9,14,18,23A HHFS K5,8,12,16A) were created by ligation of a MluI-BamHI fragment containing HHTS from pLR674 or pLR675 and a MluI-BamHI fragment containing pDM18 vector and HHFS. Plasmid pLR763 (HHTS K9,14,18,23Q HHT2 K5,8,12Q) was created using site directed mutagenesis of pLR700. Plasmid pLR766 (HHTS K9,14,18,23R HHT2 K5,8,12R) was
created by ligation of a PstI-BamHI fragment containing HHF2 K5,8,12R (Grunstein lab) and a PstI-BamHI fragment containing mutant HHTS and pLR673 vector. Plasmid pLR701 (HHTS K9,14,18,23R HHF2 K8,12,16R) was created by ligation of a PstI-BamHI fragment containing HHF2 K8,12,16R (Grunstein lab) and a PstI-BamHI fragment containing mutant HHTS and pLR673 vector.

A modified HMR synthetic silencer (HMRα-Gal4) was created in several steps. First, pLR495 containing a HindIII fragment of HMR was altered by site-directed mutagenesis to replace the ACS binding sites at the E and I silencers with Gal4 binding sites. HMR-E ARS317 aatattaatacCTAAATATAAAAAATgttattatat was mutated to aatattaatacGGAATTCTGAATTCCgttattatat, and HMR-I ARS318 aatgacacctATTAATTAA TATTTATtaataaccttt was mutated to aatgacacctGGAAATCTGATTTCCGtaataaccttt, creating pLR559. This modified HMR still bound ORC, so additional mutagenesis was performed to remove a 9/11 bp match to the ACS at ARS318 that was essential for ARS function (CHANG et al. 2008) and a cryptic ACS near ARS317. Specifically, at HMR-E atatatttatTTGTTTActttttctat was changed to atatatttatTCGTAGAc tttttctat containing a 7 bp mismatch and XhoI restriction site, and HMR-I cgatataattTATCATGttttggtatga was changed to cgatataattGGTCGACttttggtatg containing a 5 bp mismatch and SalI restriction site to generate plasmid pLR805.
**Genetic screen for histone mutations**

To generate mutations in histones H3 or H4, the gene of interest was amplified using error-prone PCR (0.2 mM dNTPs, 0.5 μM primers, 1.2 mM MnCl2, 5 ng plasmid pDM18, and 0.75 units Taq (Roche) [5U /μl] denatured for 3 minutes at 94°C, followed by 32 cycles of 15 seconds at 94°C, 15 seconds of 55°C, and 2 minutes at 68°C). The *HHT2* gene (H3) was amplified from pDM18 using primers oLR1025 (GGCTATGGCTCGGTGTCAAA) and oLR1026 (GCCCGCAATTATGTCTGTAAA), and the *HHF2* gene (H4) was amplified using primers oLR785 (TACATACGTGTGTTGTGCGTAT) and oLR788 (CCAGGGTTTTCCCCAGTCAC). The resultant collection of PCR products was integrated into plasmid pDM18 by homologous recombination in yeast cells (MUHLRAD et al. 1992). Specifically, pDM18 was digested with BglII and EcoRI to remove *HHF2* or AflII and RsrII to remove *HHT2*, and yeast (LRY1450 or LRY1849) were simultaneously transformed with the gapped plasmid (100 ng), PCR product (200 ng) and 30 μg salmon sperm DNA (Strategene). Colonies containing repaired plasmids, many of which contained mutations in histones H3 or H4, were selected on medium lacking tryptophan and then replica plated to medium lacking tryptophan with 0.1% 5-fluoro-orotic acid (5-FOA) to select for cells that lost pDM9 bearing wild type H3 and H4 and URA3.

To identify histone mutations that disrupted Sum1-1 silencing, LRY1450 colonies were replica plated to a lawn of cells of the opposite mating type (LRY1021 *MATa his4*).
on minimal plates to select for prototrophic diploids. Over 13,000 colonies were screened, and 163 colonies failed to mate. Plasmids were isolated from these yeast using a modified Qiagen miniprep procedure in which cells were vortexed with 0.5 mm Zirconia/Silica beads (BioSpec Products) for 5 minutes in buffer P1. Plasmids were amplified in DH5α *E. coli* cells. To determine whether the isolated plasmids were responsible for the mating defects, they were incorporated back into the reporter strain and mating was assessed. Plasmids contained in colonies that failed to mate a second time are listed in Table 7.

To identify histone mutations that disrupted Sum1 repression, colonies in LRY1849 were replica plated to medium lacking histidine and leucine to identify colonies that failed to repress Sum1p regulated reporters *pPES4-HIS3* and *pYGL138C-LEU2*. Over 17,000 colonies were screened, and 80 colonies grew on media lacking histidine and leucine. Plasmids were isolated and retested as described above. No plasmids were identified that enabled growth on medium lacking histidine and leucine. Twelve representative plasmids were sequenced and confirmed to have nucleotide and amino acid substitutions in the histone genes.

**Reporter assays**

For the semi-quantitative mating assay, one optical density (OD) equivalent of cells was collected from logarithmically growing cultures by centrifugation and resuspended in 100 μl yeast minimal medium (YM). Tenfold serial dilutions were
prepared, and 3 μl of each dilution was spotted onto rich medium (YPD) to monitor growth. For mating, an equal volume of a tester strain of the opposite mating type (LRY1021 MATa his4 or LRY1022 MATa his4) resuspended at 10 OD equivalents per ml in YPD was mixed with each sample in the dilution series. 3 μl of this mixture was spotted onto minimal medium to select for the growth of prototrophic diploids. Yeast were grown at 30°C for 3 days before imaging. For yeast containing mutated histone genes on plasmids, 3 μl of each sample in the dilution series was spotted onto medium containing 5-FOA and lacking tryptophan to monitor growth in the presence of mutant histone plasmids.

To test the dominance of histone mutations that disrupt Sum1-1 silencing, yeast were grown on medium lacking tryptophan and uracil to maintain both wild-type and mutant histone plasmids. Alternatively, yeast with chromosomal histone genes (LRY1363) were transformed with mutant histone plasmids and mating was assessed in medium lacking tryptophan to select for the mutant plasmids. For these experiments, LRY2386 MATa his4-519 trp1 ura3-52 was the mating partner, and prototrophic diploids were selected on YM plates supplemented with uracil.

To assess telomeric silencing, mutant histone plasmids were transformed into reporter yeast strain LRY2423 containing an ADE2 reporter gene integrated at telomere VR (SINGER and GOTTSCHLING 1994) and lacking chromosomal histone genes. One OD equivalent of cells was collected from logarithmically growing cultures by centrifugation
and resuspended in 100 μl YM medium. Ten-fold serial dilutions were prepared for each strain, and 10 μl of the 10,000 fold dilution for each strain was spread onto YPD medium. Yeast were grown at 30°C for 3 days and placed at 4°C for 7 days to allow color development before imaging. Mutations were assigned to groups based on the colorimetric phenotype observed. All white colonies were considered to lack telomeric silencing and scored as (-). Light pink, red, and sectored colonies were scored as (+) for maintaining telomeric silencing.

To assess Sum1p repression of PES4, mutant histone plasmids were transformed into yeast strain LRY1793 containing reporter pPES4-HIS. One OD equivalent of cells was collected from logarithmically growing cultures by centrifugation and resuspended in 100 μl yeast minimal medium (YM). Tenfold serial dilutions were prepared, and 3 μl of each dilution was spotted onto rich medium (YPD) to monitor growth or media lacking histidine to monitor Sum1p repression.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as previously described (Rusche and Rine 2001). Two independent logarithmically growing cultures of each strain were grown and 50 OD equivalents of cells were cross-linked with 1% formaldehyde for 30 min. Lysis and subsequent steps were as described. 3 μl of antibody was used; anti-myc (Millipore 06-549), anti-HA (Millipore H6908), anti-H3 (Upstate 06-755), anti-H3 K4 Me2 (Upstate 07-030), and anti-H3 K4 Me3 (Millipore 04-745). Samples
were quantified using real-time PCR with SYBR Green on a Bio-Rad iCycler. A standard curve was prepared from input DNA, and input DNA and samples were amplified using primers for a control locus and the locus of interest in separate reactions using real time PCR cycling parameters as described (SAFI et al. 2008). Oligonucleotides used are listed in Table 6. Data represent the average of two independent IPs for each strain, each analyzed in two independent PCR reactions. Error bars represent the standard error.

For immunoprecipitation of Gal4DBD-7myc-SUM1-1 and ORC1-3HA to the HMRa-Gal4 plasmid, immunoprecipitation was performed as previously described (HICKMAN and RUSCHE 2007) using a second cross-linking agent (KURDISTANI et al. 2002). Two independent logarithmically growing cultures of each strain were grown, and 50 OD equivalents of cells were cross-linked for 1 hour in 10 mL cold DMA (PBS with 10 mM dimethyl adipimidate, 0.25% DMSO), followed by 1 hour cross-linking in 1% formaldehyde. Lysis and subsequent steps were as described above. A standard curve was prepared from input DNA from each sample, to control for plasmid copy number differences in the various strains.

Co-Immunoprecipitation (Co-IP)

Co-immunoprecipitations were performed as previously described (RUSCHE and RINE 2001; SAFI et al. 2008) using 30 ODs of cells grown in medium lacking leucine and adenine to maintain plasmids. Cells were lysed in a buffer containing 50 mM tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% NP40, 1.5 mM DTT, and protease inhibitors.
Cleared lysates were incubated with 5 μl rabbit polyclonal IgG antibodies (Upstate; 06-549 and 05-902) at 4°C overnight and then 60 μl protein A agarose beads (Upstate) for 1 hr. Samples were electrophoretically fractionated on 7.5% polyacrylamide-SDS gels, transferred to membranes, and probed using mouse monoclonal antibodies (Upstate; 05-724 and 05-904).

**RNA Isolation and quantitative reverse transcriptase PCR (RT-PCR)**

RNA was isolated from two independent logarithmically growing cultures as described previously (SCHMITT *et al.* 1990), and cDNA was synthesized and quantified as previously described (HICKMAN and RUSCHE 2007). The standard curve was generated with genomic DNA isolated from wild type W303-1b. Oligonucleotides used are listed in Table 6. *SUM1, HST1, RFM1*, and *ORC1* transcript levels were calculated relative to *NTG1* and then normalized to the wild type strain. Results represent the average of two independently grown cultures for each strain, each analyzed in two independent PCR reactions. Error bars represent the standard error.

**Two-hybrid assay**

Two-hybrid interactions were detected through activation of a LacZ reporter construct as previously described (SAFI *et al.* 2008). To assay for β-galactosidase activity, yeast were grown for 18 hours directly on a Magna Graph Nylon membrane (GE, NJOHY08250) placed on a YPD plate. The filter was flash frozen in liquid nitrogen, placed onto filter paper soaked in developing solution (65 mM sodium phosphate, pH
7.0, 10 mM KCl, 1 mM MgSO4, 0.6% β-mercaptoethanol, 1% x-gal stock (100 mg/ml in N,N-dimethyl formamide), and developed for 2 hours at 37° in a closed container.

2.3 Results

*Genetic screen for histone mutations that disrupt Sum1-1 silencing*

To identify mutations in histones H3 and H4 that disrupt Sum1-1 silencing, I developed a genetic screen employing a plasmid shuffling strategy (Figure 7A, left column). A reporter yeast strain contained the *SUM1-1* mutation and a *sir2* deletion so Sum1-1 silencing could be assessed independently of Sir silencing. In addition, the essential histone H3 and H4 genes were deleted from their genomic locations and provided on a plasmid bearing a *URA3* marker. Mutations were generated in the histone H3 or H4 genes using error-prone PCR, and these mutated histone genes were incorporated into the reporter strain on plasmids bearing the *TRP1* marker. Finally, the wild-type histone genes were shuffled out by selecting against the *URA3* marker. The efficacy of Sum1-1 silencing in the presence of these histone mutations was assessed using a mating assay (Figure 7B), as loss of silencing at *HMR*α in these *MATα* cells leads to simultaneous expression of a and α mating-type information and sterility. Plasmids from colonies that failed to mate were isolated, amplified in *E. coli*, and re-examined in the reporter strain to confirm that the mating defect was linked to the plasmid. Over 13,000 colonies were screened; 7,500 for H3 and 5,700 for H4. 48 plasmids were recovered that conferred a mating defect, each containing 1 to 6 mutations (Figure 7C).
Figure 7: Genetic screens for mutations in histones H3 and H4 that disrupt Sum1-1 silencing or Sum1 repression.
A. Reporter yeast strains were transformed with a library of mutated histone genes generated by homologous recombination between a PCR product, generated under error-prone conditions, and a linearized vector (pDM18), the ends of which were homologous to the PCR product. Grey bars indicate the digestion sites for removal of wild type copies of H3 or H4 from the plasmid. The reporter strains had their only copies of the genes for histones H3 and H4 on a URA3-containing plasmid (pDM9), which was subsequently shuffled out by selecting for 5-FOA resistant yeast. To identify mutations that disrupted Sum1-1 silencing, a MATα sir2Δ SUM1-1 strain (LRY1450) was used, and colonies that failed to mate were identified. To identify mutations that disrupted Sum1 repression, the starting strain had two reporter constructs in which the promoters of the Sum1p-regulated genes PES4 and YGL138C drive the expression of HIS3 and LEU2 (LRY1849). Colonies that grew in the absence of histidine and leucine were selected.

B. Mutations were assessed in semi-quantitative assays. Sum1-1 silencing of HMRa was assessed using a mating assay, shown here for representative histone mutations. Sum1 repression of the pPES4-HIS3 and pYGL138C-LEU2 reporters was assessed by growth on medium lacking histidine and leucine, shown here for yeast strains SUM1 HST1 (LRY1849), sum1Δ HST1 (LRY1854), SUM1 hst1Δ (LRY1853), and sum1Δ hst1Δ (LRY1855). For both assays, 10-fold serial dilutions were plated on selective medium.

C. The numbers of colonies screened, plasmids identified, and disruptive mutations identified in each screen are indicated.

To identify those mutations that disrupted Sum1-1 silencing, plasmids containing single amino acid mutations were generated and tested in a semi-quantitative assay for their ability to disrupt mating. These single mutations were generated by site-directed mutagenesis or were obtained from existing sources (DAI et al. 2008; FRY et al. 2006; JOHNSON et al. 1990; KAYNE et al. 1988). For each mutation, the fraction of cells that mated in the presence of the mutation was estimated relative to a strain expressing wild-type histones (Figure 7B). Once a disruptive residue was identified on a particular plasmid, the remaining mutations were not investigated. 31 of the 71 histone H3 mutations and 11 of the 18 histone H4 mutations were tested (Table 7). To confirm that
the loss of mating resulted from the loss of silencing, as opposed to a defect in the mating pathway, the level of *HMRA1* mRNA was assessed in the presence of H3-K4I, H3-E73G, and H4-G28P mutations. In each mutant strain, *HMRA1* mRNA was induced compared to a strain with wild-type histones (data not shown), confirming the loss of silencing.

This screen was sensitive enough to identify histone mutations that caused a 10-fold loss of mating, but I focused on mutations conferring 100-fold or greater defects in mating. Ten mutations in histone H3 and four mutations in histone H4 fell into this category (Figure 8A, red bars; Figure 8B; Table 8). These 14 amino acid residues mapped to three distinct regions of the nucleosome; residues 2-4 of the H3 tail, the LRS region in the H3 core, and residues 18-26 of the H4 tail. Interestingly, mutations in two of these regions, the LRS domain and H4 tail, disrupt Sir silencing (JOHNSON et al. 1990; PARK et al. 2002). Since the Sir and Sum1-1 complexes both generate extended silenced domains, these regions of the nucleosome may contribute similarly to the formation of both types of silenced chromatin. Therefore, 14 additional mutations in the LRS domain and H4 tail that were previously demonstrated to disrupt Sir silencing were tested for their effect on Sum1-1 silencing. Seven additional residues that disrupted Sum1-1 silencing were identified in this manner (Figure 8B, light grey boxes; Table 8). The remaining seven residues did not have an effect on Sum1-1 silencing (Tables 9-10).
Figure 8: Histone mutations that disrupt Sum1-1 silencing cluster in discrete regions of the nucleosome.

A. Positions of histone mutations tested (x axis) and the number of mutations tested at each position (y axis). Mutations that did not have a silencing defect are in blue for H3 and green for H4. Disruptive mutations (>10X decrease in mating) are in red. 57 H3 and 31 H4 mutations were tested, and 22 mutations caused a silencing defect. 
B. The 22 histone mutations that caused silencing defects map to three distinct regions: the H3 tail, the H3 LRS region, and the H4 tail. Mutations in white were identified in the genetic screen, mutations in light grey were identified by testing previously described mutations, and the mutation in dark grey was identified through alanine scanning of modifiable residues.  
C. The nucleosome structure, displaying H2A (yellow), H2B (orange), H3 (blue), and H4 (green). Disruptive mutations are shown in red. A dashed line represents the point at which the H3 tail exits the nucleosome.
Post-translational modifications of histones modulate the assembly of chromatin structures. Therefore, I specifically examined the importance of 35 modifiable residues by replacing each one with alanine. Only H3-K4A was found to disrupt Sum1-1 silencing (Figure 8B, dark grey box), consistent with our recovery in the original screen of 10 independent plasmids bearing a mutation in H3-K4. After examining 96 individual mutations in H3 and H4, I identified 22 that caused a defect in Sum1-1 silencing; 14 in H3 and 8 in H4 (Figure 8A; Table 8). These mutations cluster in three discrete regions. Interestingly, the H3 LRS region and H4 tail are adjacent in the nucleosome structure (Figure 8C), and could form a surface that interacts with the Sum1-1 complex or another protein important for silencing.

To determine if histone mutations that disrupt Sum1-1 silencing are dominant, mating was assessed under conditions that maintain plasmids expressing wild-type and mutant histones. H3-K4A and K4I resulted in a 10-fold mating defect (data not shown). These mutations also disrupted mating when expressed in a yeast strain having wild-type chromosomal histone genes (data not shown). No other mutations were dominant. Thus, H3-K4 is particularly critical for Sum1-1 silencing.

Histone mutations do not disrupt Sum1 repression

I reasoned that residues in histone H3 and H4 that are important for Sum1-1 silencing might be important contact points for the Sum1 complex. Therefore, these residues might also play a role in repression mediated by wild-type Sum1p, which
differs from Sum1-1p by a single amino acid. A role for nucleosomes in Sum1 repression is also suggested by the requirement for the deacetylase Hst1p (Lynch et al. 2005; Rusche and Rine 2001; Sutton et al. 2001), which could act on histones. To investigate whether histones contribute to Sum1 repression, a second genetic screen was conducted (Figure 7A, right). The starting strain contained two reporters in which the Sum1p-repressed PES4 and YGL138C promoters drive the expression of HIS3 and LEU2, allowing Sum1 repression to be assessed by growth on medium lacking histidine and leucine. Yeast with wild-type SUM1 and HST1 genes were unable to grow in the absence of histidine and leucine, whereas control strains with sum1Δ or hst1Δ mutations grew well (Figure 7B). The starting strain also had its only copy of the histone H3 and H4 genes on a plasmid bearing the URA3 gene, and a plasmid shuffle strategy was used to screen for mutated histone genes that disrupt Sum1 repression. Over 17,000 colonies were screened, 11,200 for H3 and 6,100 for H4, but no plasmids were identified that enabled cells to grow on medium lacking histidine and leucine (Figure 1C). I also specifically examined each histone mutation that was found to disrupt Sum1-1 silencing, and none of these mutations disrupted Sum1 repression. Therefore, Sum1 repression could not be disrupted by single mutations in the H3 or H4 genes.

It is possible that multiple lysine residues must be deacetylated by Hst1p to achieve repression. Therefore, targeted mutations and deletions were tested for their ability to disrupt Sum1 repression (Table 2).
Table 2: Histone mutations and deletions tested for disruption of Sum1 repression

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Histone H3</th>
<th>Histone H4</th>
</tr>
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<tbody>
<tr>
<td>pLR501</td>
<td>Δ4-28</td>
<td>WT</td>
</tr>
<tr>
<td>pRM430</td>
<td>Δ4-30</td>
<td>WT</td>
</tr>
<tr>
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<td>Δ30-40</td>
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<td>Δ4-28</td>
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<tr>
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<td>K9,14,18,23 A</td>
<td>WT</td>
</tr>
<tr>
<td>pLR673</td>
<td>K9,14,18,23 R</td>
<td>WT</td>
</tr>
<tr>
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</tr>
<tr>
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<td>K5,8,12,16 A</td>
</tr>
<tr>
<td>pLR672</td>
<td>WT</td>
<td>K5,8,12,16 Q</td>
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<tr>
<td>pLR676a</td>
<td>WT</td>
<td>K5,8,12,16 R</td>
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<tr>
<td>pLR702</td>
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<td>K5,8,12,16 A</td>
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<tr>
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</table>

LRY1793 was used as the reporter strain, monitoring growth on medium lacking histidine. None of these mutations affected Sum1 repression. pLR676 H4 K5,8,12,16 R was lethal.

In particular, lysines known to be acetylated were replaced by glutamine to mimic the acetylated state, arginine to mimic the deacetylated state, or alanine. The tails of histone H3 or H4 were also deleted to remove the lysine residues entirely. In addition, mutations in H3 that disrupt Sum1-1 silencing were combined with H4 K16Q, as K16 is
a critical substrate of Sir2p (IMAI et al. 2000a; JOHNSON et al. 1990), the paralog of Hst1p. However, none of these alterations to histones H3 or H4 disrupted Sum1 repression of PES4 or YGL138C. Therefore, although particular regions of the nucleosome are critical for silencing mediated by the mutant Sum1-1 complex, no such requirements exist for Sum1 repression.

Histone mutations in the H3 LRS region and H4 tail disrupt Sir silencing

Our ability to identify histone mutations that disrupt silencing mediated by Sum1-1p, which acts over several kilobase pairs, but not Sum1p, which acts over a more restricted region, suggests that nucleosomes are more critical in generating long-range chromatin structures involved in Sum1-1 silencing. Moreover, it is striking that mutations disruptive to Sum1-1 silencing fall in the same regions of the nucleosome important for Sir silencing at the cryptic mating-type loci, telomeres, and rDNA (JOHNSON et al. 1992; PARK et al. 2002; THOMPSON et al. 2003). This suggests that related higher-order structures may be generated by the Sir and Sum1-1 complexes.

To determine whether histone mutations that affect Sum1-1 silencing also affect Sir silencing, a semi-quantitative mating assay was used to assess silencing at the HMLα locus and silencing of an ADE2 gene integrated at telomere VR (SINGER and GOTTSCHLING 1994). Examining both loci enabled us to distinguish mild from more severe mutations, as Sir silencing is more easily disrupted at the telomeres than at
Histone mutations that disrupted Sum1-1 silencing were described in Figure 7. Sir silencing of \textit{HML}_{\alpha} was tested in LRY1793 in the presence of histone mutations using a reporter mating assay. Mutations causing more than a 10-fold loss in mating were scored as defective (-). Silencing of telomere VR in the presence of histone mutations was tested in LRY2423 containing an \textit{ADE2} reporter at telomere VR. White colonies expressing the \textit{ADE2} gene were scored defective in silencing (-). Sectored, pink and red colonies maintained Sir silencing (+). Sum1 repression in the presence of histone mutations was tested in LRY1793 containing the Sum1p-regulated \textit{pPES4-HIS3} reporter. No growth was seen on media lacking histidine, and repression was maintained (+).
Sir silencing of HMLα was less disrupted by histone mutations, and only H3 E73 and residues 17-21 in the H4 tail cause a more severe silencing defect at HMLα. These results are consistent with H3 E73D having the strongest effect on HM silencing of any known H3 mutation (THOMPSON et al. 2003), and supports a role for the LRS/H4 region in silencing. Interestingly, mutations in the H3 tail were not disruptive to Sir silencing, with the exception of H3-K4I and K4R that disrupted telomeric silencing. Therefore, the H3 tail may serve a specific role in Sum1-1 silencing, whereas the LRS/H4 tail region may have a common function in Sum1-1 and Sir silencing.

**Mutations in the H4 tail did not act by interfering with the ISW complex**

One possible explanation for silencing defects in H4 tail mutants could be due to a defect in chromatin remodeling. H4 tail residues R17, H18, and R19 form a binding site for the ATP-dependent chromatin remodeler ISW (CLAPIER et al. 2002), and mutation of these residues could disrupt Sir and Sum1-1 silencing by inhibiting chromatin remodeling. However, epistasis experiments revealed that Sum1-1 silencing was more disrupted in strains bearing histone H4 mutations and isw1Δ isw2Δ mutations, compared to isw1Δ isw2Δ alone (data not shown). Therefore, histone H4 mutations do not act by disrupting the ISW complex, but may disrupt interactions with another chromatin binding protein.
The BAH domain of Orc1p contributes to Sum1-1 silencing by binding nucleosomes

The LRS/H4 tail domain of the nucleosome, in which many of the mutations fall, interacts with the BAH domain of Sir3p, and this interaction is required for the spreading of the Sir complex (Buchberger et al. 2008; Connelly et al. 2006; Norris et al. 2008; Onishi et al. 2007; Sampath et al. 2009). Therefore, it is possible that the LRS/H4 tail domain plays a similar role in Sum1-1 silencing and that a BAH domain-containing protein is also critical for Sum1-1 silencing. In particular, Orc1p, which is a paralog of Sir3p, might function in Sum1-1 silencing. Consistent with this idea, the BAH domain of Orc1p is required for Sum1-1 silencing (Rusche and Rine 2001), and ORC has been implicated in the recruitment of Sum1-1p to chromatin (Irlbacher et al. 2005; Lynch et al. 2005; Rusche and Rine 2001; Sutton et al. 2001). I investigated whether an interaction between the BAH domain of Orc1p and the LRS/H4 tail domain of nucleosomes is important for Sum1-1 silencing. Such an interaction is not anticipated by the previous model that ORC recruits Sum1-1p to silenced loci. Therefore, to determine whether the BAH domain of Orc1p contributes to Sum1-1 silencing by recruiting the Sum1-1 complex or by acting at subsequent steps in silencing, several experiments were conducted.

First, the BAH domain of Orc1p was tested for its role in a previously described two-hybrid interaction between Sum1-1p and Orc5p. If so, the primary role of the BAH domain is most likely in recruitment. As in previous assays (Safi et al. 2008; Sutton et al.
2001), the C-terminal portion of Sum1p (786-1062) was fused to the activation domain of Gal4p, and Orc5p was fused to the LexA DNA binding domain. An interaction between Sum1p and Orc5p resulted in the activation of a LacZ gene driven by a minimal promoter containing LexA binding sites. As previously described, Orc5p interacted with the mutant Sum1-1p but not the wild-type Sum1p (Figure 9A). Importantly, this interaction still occurred in an orc1Δbah strain (right panel), indicating that the BAH domain of Orc1p was not required. Therefore, the BAH domain of Orc1p may act at a step other than recruitment of the Sum1-1 complex.

Figure 9: The BAH domain of Orc1p was required for Sum1-1 silencing.

A. The BAH domain of Orc1p was not required for the interaction of Sum1-1p with ORC. MATa sum1Δ yeast strains with wild-type ORC1 (LRY1729) or orc1Δbah (LRY1835) were transformed with plasmids expressing LexA-Orc5 (pTT93) and either the Gal4 activation domain (pGAD424), the Gal4 activation fused to Sum1p775-1062 (pRH01) or the Gal4 activation fused to Sum1-1p775-1062 (pRH02). Two-hybrid interactions between LexA-Orc5p and GAD-Sum1-1p775-1062 resulted in activation of a LacZ reporter gene. Yeast were transferred to filters, which were developed with x-gal to detect the expression of the LacZ reporter gene. This experiment was performed by Alexias Safi.

B. The BAH domain of Orc1p was required for mating. Semi-quantitative mating assays of MATa SUM1-1 sir2Δ sir3Δ yeast with ORC1 (LRY2762), ORC1-HA (LRY2763), orc1E84K-HA (LRY2764), orc1P179L-HA (LRY2765) and orc1Δbah-HA (LRY2766).
To investigate whether the function of the Orc1p BAH domain in Sum1-1 silencing requires its nucleosome-binding capacity, mutations in Orc1p were created analogous to mutations in Sir3p that disrupt its ability to bind nucleosomes (BUCHBERGER et al. 2008). Such mutations should disrupt Sum1-1 silencing if the BAH domain acts by binding nucleosomes. Indeed, orc1-E84K and orc1-P179L mutations disrupted Sum1-1 silencing, as assessed by mating (Figure 9B). These data suggest that the BAH domain acts by binding nucleosomes rather than the Sum1-1 complex.

*The BAH domain of Orc1p contributes to Sum1-1 silencing at a step in addition to recruitment*

To investigate whether the ability of the Orc1p BAH domain to bind nucleosomes is important for a step in Sum1-1 silencing subsequent to recruitment, a situation was created in which Sum1-1p could be recruited to the HMRa locus independently of ORC. A plasmid containing the entire HMRa locus was modified to replace the ORC binding sites in the E and I silencers with Gal4 binding sites (Figure 10A). Yeast cells which lacked SIR2 and HMRa and expressed both a Gal4 DNA binding domain (Gal4DBD)-7myc-Sum1-1 fusion protein and untagged Sum1-1p were transformed with this HMRa-Gal4 plasmid, and silencing was assessed using a mating assay. These strains also lacked Sir3p, to prevent it from substituting for its paralog Orc1p. If the BAH domain of Orc1p acts at a step subsequent to recruitment, it should still be required to silence this modified HMR locus. Indeed, silencing was dependent on the presence of the Gal4DBD-Sum1-1 protein (Figure 10B, rows 2-3), indicating that the
recruitment of Sum1-1 was occurring through the Gal4DBD rather than ORC. Importantly, mutation or deletion of the BAH domain disrupted mating 100-fold (Figure 10B, rows 4-6), consistent with the BAH domain providing a function other than recruitment of Sum1-1p.

The requirement for the Orc1p BAH domain in silencing the HMRa-Gal4 locus suggests that Orc1p acts directly at the locus despite the absence of ORC binding sites in the silencers. To examine this possibility, I performed a chromatin IP assay and found Orc1p enrichment at the E silencer even in the absence of Gal4DBD-Sum1-1p (Figure 10C). Thus, a cryptic ORC binding site must still be present near the E silencer. Nevertheless, in the presence of Gal4DBD-Sum1-1p, the enrichment of Orc1p was significantly increased at the E silencer and was also observed at other sites within HMRa-Gal4. Therefore, Orc1p is stabilized at the HMRa-Gal4 locus by Sum1-1p. As anticipated, Gal4DBD-Sum1-1p was enriched across the modified HMRa-Gal4 locus (Figure 10D).
Figure 10: The BAH domain of Orc1p is required for Sum1-1 silencing.

A. HMRa schematic showing the E and I silencer binding sites. ORC binding sites were replaced with Gal4 binding sites to create HMRa-Gal4. A yeast strain lacking SIR2, SIR3, HMR and expressing a Gal4DBD-7myc-SUM1-1 fusion protein was transformed with plasmid pLR805 containing the modified HMRa-Gal4. This strain background was used to recruit Sum1-1p to HMRa-Gal4 independent of ORC.
B. Reporter mating assay of MATα sir2Δ sir3Δ hmrΔ SUM1-1 yeast containing ORC1-HA (LRY2804) and MATα sir2Δ sir3Δ hmrΔ Gal4DBD-7myc-SUM1-1 yeast with ORC1 (LRY2752, ORC1-HA (LRY2753), orc1-E84K-HA (LRY2754), orc1-P179L-HA (LRY2755), and orc1ΔBAH-HA (LRY2756) were transformed with HMRa-Gal4 and tested for silencing ability of HMRa-Gal4.

C-D. ChIP of Orc1p-HA and Gal4DBD-7myc-Sum1-1p at HMRa-Gal4. MATα sir2Δ sir3Δ hmrΔ Gal4DBD-7myc-SUM1-1 yeast containing ORC1 (LRY2752), ORC1-HA (LRY2753) and MATα sir2Δ sir3Δ hmrΔ SUM1-1 yeast containing ORC1-HA (LRY2804) were transformed with HMRa-Gal4 to assess the ability of Sum1-1p and Orc1p to be recruited to HMRa-Gal4 independent of ORC. Plus sign indicates addition of antibody (+anti-myc or +anti-HA). Genotype of ORC1 and SUM1-1 is shown for each strain. Primers are spaced ~1kb across HMRa-Gal4, and enrichment values are relative to PHO5, which is not associated with Sum1-1p. Significance values (p<0.0001) were calculated relative to SUM1-1 strains not recruited to HMRa-Gal4.

E-F. ChIP of Orc1p-HA and Gal4DBD-7myc-Sum1-1p at HMRa-Gal4. MATα sir2Δ sir3Δ hmrΔ Gal4DBD-7myc-SUM1-1 yeast with ORC1 (LRY2752), ORC1-HA (LRY2753), orc1-E84K-HA (LRY2754), orc1-P179L-HA (LRY2755), and orc1ΔBAH-HA (LRY2756) were transformed with HMRa-Gal4 to assess the ability of Sum1-1p and Orc1p to be recruited to HMRa-Gal4 in the presence of Orc1p BAH domain mutation or deletion. Minus sign indicates no antibody (-myc), and plus sign indicates addition of antibody (+anti-myc or +anti-HA). Genotype of ORC1 and Gal4DBD-7myc-SUM1-1 is shown for each strain. Primers are spaced ~1kb across HMRa-Gal4, and enrichment values are relative to PHO5, which is not associated with Sum1-1p. Significance values (p<0.0001) were calculated relative to ORC1-HA strains that do not contain BAH mutations.

Our initial hypothesis was that the interaction of the Orc1p BAH domain with nucleosomes promotes the distribution of Orc1p and Sum1-1p across the silenced locus, as is the case for Sir3p. However, the distribution of Orc1p was more restricted than that of Gal4DBD-Sum1-1p (Figures 10C-D), suggesting that Orc1p does not spread with Sum1-1p and makes a different contribution to silencing. To determine how the BAH domain of Orc1p promotes silencing at HMRa-Gal4, I performed a chromatin IP in strains bearing ORC1 mutations that disrupt nucleosome binding. If the ability of the
BAH domain to bind nucleosomes is important for stabilizing the Sum1-1 complex across *HMRA-Gal4*, these mutations should decrease the associations of Sum1-1p and Orc1p. However, although a significant decrease in enrichment of Orc1p was observed (Figure 10E), these mutations only had a slight effect on the enrichment of Sum1-1p (Figure 10F). Therefore, the BAH domain of Orc1p stabilizes its association with *HMRA-Gal4* and correlates with the maximally silenced state, suggesting that the presence of Orc1p is critical for complete silencing. It is likely that mutations in the LRS/H4 tail region of the nucleosome have similar consequences for Orc1p association with *HMRA*, but I was unable to test this idea directly due to technical complications of combining the *HMRA-Gal4* plasmid with histone mutations.

*The BAH domain of Orc1p was not required for the self-association of Sum1-1p*

In addition to binding nucleosomes and thereby stabilizing the association of Orc1p with chromatin, the BAH domain of Orc1p could contribute to Sum1-1 silencing in other ways. For example, the *SUM1-1* mutation increases the ability of Sum1-1p to self-associate (*SAFI* et al. 2008), and the BAH domain could stabilize this interaction. Two differently tagged alleles of Sum1-1p were previously demonstrated to co-precipitate (*SAFI* et al. 2008), whereas similarly tagged wild-type proteins do not co-precipitate. This co-precipitation was not disrupted by DNaseI and therefore may be independent of association with silenced loci. To determine whether the BAH domain of Orc1p was required for the self-association of Sum1-1p, the co-precipitation experiment was
repeated in an \textit{orc1\textDelta BAH} strain. The co-precipitation still occurred in this strain (Figure 11A and 11B), indicating that the BAH domain was not required for self-association.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure11.png}
\caption{The BAH domain of Orc1p is not required for Sum1-1p self-association.}
\end{figure}

A. myc-Sum1-1p or myc-Sum1p was immunoprecipitated from yeast of the genotype \textit{sum1\Delta} (LRY144) or \textit{sum1\Delta orc1\textDelta BAH} (LRY1879) transformed with plasmids expressing HA- and myc-tagged Sum1p (pLR052 and pLR021) or Sum1-1p (pLR047 and pJR2291). The samples were analyzed by immunoblotting with antibodies against the HA or myc tags.

B. HA-Sum1-1p or HA-Sum1p was immunoprecipitated from yeast as described in A. These experiments were performed by Alexias Safi.

\textit{Mutations in the N-terminus of H3 disrupt Sum1-1 silencing at a step subsequent to recruitment of Sum1-1p}

In addition to mutations in the LRS/H4 tail domain, I obtained seven mutations at the N-terminus of H3 that disrupted Sum1-1 silencing. These mutations may act through a different mechanism than mutations in the LRS/H4 tail domain as they have distinct properties. In particular, H3-K4A and K4I were the only mutations with a dominant phenotype, and most H3 tail mutations were not disruptive to Sir silencing. The H3 tail residues important for Sum1-1 silencing cluster around H3-K4, suggesting
that the methylation status of H3-K4 could affect silencing. In particular, mutation of H3-K4 would block methylation, and mutations of neighboring residues could interfere with the recognition of H3-K4 as a substrate. Consistent with this notion, Sum1-1 silencing was severely disrupted in a strain lacking Set1p, the methyltransferase specific for H3-K4 (Figure 12A). In contrast, deletion of Dot1p, which methylates H3-K79, located in the LRS domain, resulted in a very modest disruption of Sum1-1 silencing.

Methylation of H3-K4 in silenced domains could be critical for the assembly of Sum1-1p chromatin. Alternatively, methylation of euchromatin may help restrict Sum1-1 silenced chromatin to appropriate domains, as has been proposed for Sir silenced chromatin (SANTOS-ROSA et al. 2004). To determine whether H3-K4 is methylated or unmethylated at HMRa in the presence of Sum1-1p, chromatin IP was performed using antibodies specific for di- or tri-methylated H3-K4 (Figure 12B). In a background where Sir proteins silence HMRa, there was no detectable enrichment of di- or tri-methylated H3-K4, as previously reported (BERNSTEIN et al. 2002; SANTOS-ROSA et al. 2002). In a sir3Δ strain, in which HMRa1 is expressed, di- and trimethylation of H3-K4 were enriched at HMRa, as expected. In a sir3Δ SUM1-1 strain, in which Sum1-1p restores silencing of HMRa, methylation of H3-K4 was not observed, indicating that methylation of H3-K4 within the silenced domain is not necessary for Sum1-1 silencing.
Figure 12: Mutations in the N-terminus of H3 disrupt Sum1-1 silencing at a step subsequent to recruitment of Sum1-1p.

A. Deletion of Set1p disrupts Sum1-1 silencing. Mating assay of MATα 7myc-SUM1-1 strains with SIR2 (LRY529), sir2Δ (LRY459), sir2Δ dot1Δ (LRY1364), and sir2Δ set1Δ (LRY1384).

B. Sum1-1 silencing does not change the methylation status of HMRa. ChIP of di and tri-methylated H3 K4 at HMRa in MATα strains with SUM1 SIR3 (LRY1007), SUM1 sir3Δ (LRY341) and SUM1-1 sir3Δ (LRY344). Primers are spaced ~1kb across HMRa, and enrichment values are relative to downstream ATG1, shown to be devoid of methylation (Pokholok et al. 2005). Plus sign indicates addition of antibody (+anti-H3 K4Me2 or +anti-H3 K4Me3). Significance values (p<0.001) were calculated relative to sir3Δ SUM1-1 strains which are not silenced.

C. Histone H3 tail mutations do not disrupt expression of the Sum1 complex. RT-PCR analysis of SUM1, HST1, RFM1, and ORC1 in MATα sir2Δ 7myc-SUM1-1 strains with SET1 (LRY459) or set1Δ (LRY1384) and a MATα sir2Δ 7myc-SUM1-1 h3Δ h4Δ strain (LRY1450) transformed with a plasmid expressing wild-type histones (pDM18) or H3 K4I and wild-type H4 (pLR619). mRNA amounts were quantified relative to the control mRNA NTG1 and normalized to LRY459, containing wild type genomic histones.

D. Histone H3 tail mutations do not disrupt Sum1-1p enrichment at HMRa. Chromatin IP of 7myc-Sum1-1p at HMRa in MATα sir2Δ 7myc-SUM1-1 h3Δ h4Δ
strain (LRY1450) transformed with plasmids expressing wild-type histones (pDM18), H3 K4I (pLR619), or H3 E73G (pLR615). Minus sign indicates no antibody, and plus sign indicates addition of antibody (+anti-myc). Primers are spaced ~1kb across HMRa, and enrichment values are relative to PHO5, which is not associated with Sum1-1p. Significance values (p<0.001) were calculated relative to strains containing wild-type histones with antibody.

A trivial explanation for the disruption of Sum1-1 silencing by mutations in the H3 tail is that improper methylation of H3-K4 alters the expression of a component of the Sum1-1 complex. To examine this possibility, the steady-state mRNA levels of SUM1-1, HST1, RFM1, and ORC1 were compared by quantitative RT-PCR in strains expressing wild-type or mutant histones, and in a strain lacking Set1p. The levels of these mRNAs did not change significantly (Figure 12C). In addition, immunoblot analysis of Sum1-1p in these strains revealed no significant differences (data not shown). Therefore, a change in the expression of the Sum1-1 complex does not account for the silencing defect in strains expressing H3 tail mutations.

It is possible that mutations in the H3 tail reduce an interaction between this portion of H3 and the Sum1-1 complex, thereby disrupting silencing. To examine this, the association of Sum1-1p with HMRa was assessed in the presence of H3-K4I, the most disruptive mutation. In a strain expressing wild type histones, Sum1-1p was enriched across HMRa, as expected (Figure 12D). Interestingly, in the presence of H3-K4I, the enrichment of Sum1-1p was equivalent to the strain with wild-type histones, suggesting that the Sum1-1 complex assembles properly at HMRa and that a subsequent step in the
silencing process must be disrupted. In contrast, a mutation in the LRS region, H3-E73G, caused a modest reduction in the association of Sum1-1p with HMRa (Figure 12D), consistent with the modest decrease in Sum1-1p enrichment in the presence of Orc1p BAH mutations (Figure 10E).

2.4 Discussion

In this study, I identified two regions of the nucleosome that are important for long-range silencing mediated by the mutant Sum1-1 complex but not for wild-type Sum1 repression. The LRS/H4 tail region is also important for Sir silencing and is thought to interact with the Sir3p BAH domain. By analogy, I suggest that the BAH domain of Orc1p facilitates Sum1-1 silencing. The second region, the extreme N-terminus of H3 is more important for Sum1-1 than Sir silencing and disrupts silencing without reducing the association of Sum1-1 with the silenced domain.

The LRS/H4 tail region of the nucleosome is important for multiple types of silencing

It is striking that the same region of the nucleosome, the LRS region in the core and the adjacent base of the H4 tail, plays an important role in silencing mediated by three distinct protein complexes—Sir, RENT, and Sum1-1 (ALTAF et al. 2007; BUCHBERGER et al. 2008; JOHNSON et al. 1992; JOHNSON et al. 1990; NORRIS et al. 2008; PARK and SZOSTAK 1990; PARK et al. 2002; SAMPATH et al. 2009; THOMPSON et al. 2003), suggesting a common mechanism of action. This region could contribute to silencing by
promoting proper chromatin compaction, providing a binding site for a common factor, or serving as a multi-purpose recruitment site for silencing proteins.

One possibility is that the LRS/H4 tail region is important for multiple types of silencing because it facilitates intra- and inter-nucleosome interactions necessary for chromatin compaction. For example, histone H4 basic residues 14-19 and acidic residues in histones H2A and H2B have been proposed to interact (Dorigo et al. 2004; Kan et al. 2009). In addition, acetylation of H4 at K16 inhibits 30-nm fiber formation (Shogren-Knaak et al. 2006). However, mutations of H2A and H2B acidic residues did not disrupt Sir silencing (data not shown) and the LRS region is not reported to interact with other regions of the nucleosome. Therefore, assisting in chromatin compaction may not be the primary contribution of the LRS/H4 tail region.

A second possibility is that the LRS/H4 tail region associates with a common factor required for multiple types of silencing. One candidate is the ISW chromatin remodeling complex, which is associated with transcriptional repression and requires contacts with H4 tail residues 17-19 for remodeling (Clapier et al. 2002). However, Sum1-1 silencing was more severely affected by mutations in H4 tail residues than by an isw1Δ isw2Δ double deletion (data not shown), indicating that the histone tail mutations do not act solely by blocking the activity of ISW.

A third possibility is that the LRS/H4 tail region is relatively accessible and consequently is bound by different silencing proteins, including the BAH domains of
Sir3p and its paralog Orc1p. Whether a nucleosome binding protein is also important for silencing in the rDNA is unclear, as Sir3p and Orc1p are not thought to contribute to rDNA silencing.

Orc1p contributes to Sum1-1p silencing in a capacity in addition to recruitment

Given the ability of mutations in the LRS/H4 tail region to disrupt Sum1-1 silencing, I suspected that the Orc1p BAH domain, previously shown to be required for Sum1-1 silencing, makes important contacts with this domain. This model is interesting in light of the association of ORC with heterochromatin in a variety of species. For example, in Drosophila and humans, ORC interacts with HP1 (AUTH et al. 2006; LIDONNICI et al. 2004; PAK et al. 1997; PRASANTH et al. 2004) and is enriched in telomeric and pericentromeric heterochromatin (DENG et al. 2007; DENG et al. 2009; PRASANTH et al. 2010; SHEN et al. 2010). Based on the paradigm from S. cerevisiae, ORC is thought to serve as a platform for recruiting heterochromatin proteins. However, another potential function of ORC is to bind nucleosomes via the BAH domain of Orc1p, such that in some situations Orc1p may act like its paralog Sir3p, which enables spreading of the Sir complex by binding nucleosomes (BUCHBERGER et al. 2008; CARMEN et al. 2002; HECHT et al. 1995; ONISHI et al. 2007). In fact, in the yeast K. lactis, which lacks a distinct Sir3p, Orc1p acts in a Sir3p-like manner (HICKMAN and RUSCHE 2010).

Consistent with the “platform” model, ORC is proposed to recruit Sum1-1p to HMRa (LYNCH et al. 2005; RUSCHE and RINE 2001; SUTTON et al. 2001). However, the role
of the BAH domain in Sum1-1 silencing remained a mystery. Our identification of histone mutations that fall in the LRS/H4 tail region of the nucleosome and disrupt Sum1-1 silencing suggests that the ability of the Orc1p BAH domain to bind nucleosomes may be a second important function for Sum1-1 silencing. Indeed, when Sum1-1p was recruited to HMRα independently of ORC, the BAH domain of Orc1p was still required for silencing (Figure 10). Moreover, mutations in ORC1 predicted to decrease the affinity of the BAH domain for nucleosomes also disrupted silencing. Therefore, Orc1p contributes to Sum1-1 silencing through recruitment of Sum1-1p and at another step.

In theory, Orc1p could act in a Sir3p-like manner to promote the spreading of Sum1-1p across HMRα. However, the enrichment of Sum1-1p was only moderately decreased in strains containing mutations in the Orc1p BAH domain (Figure 10) or a LRS histone mutant (Figure 12). Moreover, Orc1p was not uniformly distributed across HMRα, as would be expected if it acted like Sir3p. Instead, Orc1p was localized to the E silencer. Thus, the presence of Orc1p at the E silencer may promote transcriptional silencing by altering the conformation of the chromatin fiber or recruiting factors that make the region less permissive for transcription.

It is interesting to note that the ancestral Sum1p likely acted with Orc1p to achieve silencing, and the SUM1-1 mutation may recapitulate this cooperation. In the yeast K. lactis, the Sum1 complex silences HMLα in conjunction with KlSir4p and
KIOrc1p (HICKMAN and RUSCHE 2009; HICKMAN and RUSCHE 2010). Therefore, the common ancestor of KlSum1p and ScSum1p most likely had a similar function, which was lost in the S. cerevisiae lineage.

The H3 tail is important for silencing

I also identified mutations in the H3 tail that disrupted Sum1-1 silencing but had less impact on Sir silencing. The greater susceptibility of Sum1-1 silencing to these mutations could indicate that a specific interaction occurs between the Sum1-1 complex and the H3 tail. However, this interpretation is inconsistent with the robust enrichment of Sum1-1 at HMRa in H3-K4I strains (Figure 10D). Interestingly, similar observations were made for Sir proteins, which remain associated with silenced loci when the H3 tail is deleted, although this deletion disrupts transcriptional silencing (SPERLING and GRUNSTEIN 2009). These authors concluded that the H3 tail contributes to chromatin compaction and hence silencing at a step after Sir protein assembly, and it is possible that the H3 tail contributes similarly to Sum1-1 silencing.

Histones in heterochromatin formation in other species

Little is known about regions of the nucleosome important for heterochromatin structure in species other than S. cerevisiae, in which heterochromatin has a distinct molecular composition. In other eukaryotic species, H3-K9Me and heterochromatin protein 1 (HP1) are hallmarks of heterochromatin, and different portions of the nucleosome might be critical in heterochromatin formation. In fact, in
*Schizosaccharomyces pombe*, mutation of histone H3-K9, S10, or K14 disrupted pericentromeric heterochromatin formation, but mutation of H4-K8 or K16 did not (Mellone *et al.* 2003), consistent with the importance of H3-K9Me in heterochromatin. A recent study showed that the BAH-domain containing protein BAHD1 contributes to heterochromatin in humans (Bierne *et al.* 2009), raising the possibility that BAHD1 utilizes the LRS/H4 tail region of the nucleosome to facilitate heterochromatin assembly. **Histones do not disrupt promoter-specific repression mediated by Sum1p**

A goal of this study was to identify mutations in histones H3 and H4 that disrupt a potential interaction between the Sum1 complex and nucleosomes, which could contribute to repression. However, no such mutations were isolated in the genetic screen, and candidate approaches did not identify more complex mutations that disrupt repression. Nevertheless, the deacetylase activity of Hst1p is required for Sum1 repression (Hickman and Rutsche 2007), and it has been assumed that histones are the relevant substrate of Hst1p. In fact, acetylation of histones H3 and H4 at Sum1-repressed genes increases in the absence of *HST1* (Hickman and Rutsche 2007; Robert *et al.* 2004; Weber *et al.* 2008). However, given that mutations that mimicked the acetylated state did not disrupt repression, the key substrate of Hst1p may not be histone H3 or H4. Instead, Hst1p may deacetylate other proteins such as histones H2A and H2B or the Sum1 complex.
The lack of histone mutations that disrupt Sum1 repression contrasts with other repressors in *S. cerevisiae* that can be disrupted by histone mutations (LENFANT *et al.* 1996; PARRA *et al.* 2006; RECHT *et al.* 1996; SABET *et al.* 2003). For example, histone mutations that disrupt the association of the co-repressor Tup1p with histone tails (EDMONDSON *et al.* 1996) or mimic acetylation (WATSON *et al.* 2000) disrupt repression mediated by the Tup1-Ssn6 complex. Unlike Tup1, the Sum1 complex may not require a stabilizing interaction with histones to achieve repression.

These studies have identified contributions of histones H3 and H4 to Sum1-1 silencing, highlighted the LRS/H4 region of the nucleosome that is used by several complexes to achieve full silencing. The LRS/H4 tail region may serve as a common binding site for silencing proteins, which assists in the formation and stabilization of silenced chromatin. The H3 tail serves a more specific role in Sum1-1 silencing as compared to Sir silencing, but may contribute to the formation of specialized chromatin structures that are required for full silencing. Higher eukaryotes may also utilize these regions of the nucleosome in order to achieve silencing and proper heterochromatin formation.
3. The search for a histone binding domain in Sum1p

3.1 Introduction

The SUM1-1 mutation results from a single amino acid change (threonine 988 to isoleucine) to Sum1p that increases the affinity of Sum1p-1p for ORC, leading to the recruitment of Sum1-1p to HMR and other ORC binding sites (LYNCH et al. 2005; RUSCHE and RINE 2001; SUTTON et al. 2001). In addition, loss of T988 reduces the affinity of Sum1p for its DNA consensus sequence, and the presence of isoleucine at position 988 increases the tendency of the protein to self associate (SAFI et al. 2008), which may increase the ability of Sum1-1p to spread along chromatin. Thus, the SUM1-1 mutation changes the location and function of the complex from a locus-specific repressor to a regional silencer at HMR. It is predicted that Sum1-1p or another component of the Sum1-1 complex interacts with histones to enable the complex to spread along the chromosome. However, such an interaction has not been documented.

The single amino acid change that creates SUM1-1 is unlikely to create a novel histone binding domain on its own. Therefore, an interaction with histone tails might be functionally important to wild-type Sum1 repression as well. I searched for a histone binding domain in Sum1p to uncover the role of histones in regulating promoter-specific gene repression. Using predicted secondary structures and multiple sequence alignments to identify conserved regions of the protein, deletions and mutations were tested for disruptions to Sum1 repression. I identified a conserved region in the N-
terminus of Sum1p that is important for proper Sum1p recruitment and complex formation, but do not have data to support a role for histone binding in Sum1 repression.

3.2 Materials and methods

Yeast strains

Strains used in this study were derived from W303-1b. The pPES4-HIS3 (HICKMAN and RUSCHE 2007), sum1Δ::URA3, sum1Δ::LEU2 and HST1-5HA (RUSCHE and RINE 2001) alleles were previously described. These alleles were moved into the desired genetic backgrounds through standard genetic crosses to generate the strains listed in Table 4.

Plasmids

Plasmids used in this study are described in Table 5. DMC326 (SUM1) (CHI and SHORE 1996), pJR2292 (3myc-SUM1), pJR2294 (SUM1), and pJR2296 (3myc-SUM1) (RUSCHE and RINE 2001) were previously described. To construct pLR387 (HIS-SUM1 1-530 AmpR), the desired region of SUM1 was amplified by PCR with primers containing Ndel (5') and BamHI (3') restriction sites. This PCR product was ligated into the Ndel and BamHI sites of the pET15b expression vector. Additional truncations of Sum1p, including HIS-SUM1 1-275 (pLR491), HIS-SUM1 1-376 (pLR492), HIS-SUM1 1-452 (pLR498), HIS-SUM1 85-179 (pLR530), and K. lactis HIS-SUM1 1-336 (pLR574) were created similarly. pJR2292 (3myc-SUM1 ADE2) was modified using PCR with mutagenic
primers to construct pLR521 (3myc-SUM1Δ85-179 ADE2) and Sum1p single point mutations pLR550 (3myc-Sum1p D147A, D148A ADE2), pLR555 (3myc-Sum1p D157A ADE2), and pLR569 (3myc-Sum1p D147A, D148A, D157A ADE2).

**Immunoblotting**

Whole cell protein samples were collected from 3 optical density (OD) equivalents of cells grown in media lacking adenine for plasmid retention. Trichloroacetic acid (TCA) was added to the cells to a final concentration of 10% and the cells were incubated on ice for 20 minutes. Cells were pelleted, washed with 500 μl 1M Tris, pH 8, and resuspended in 75 μl 2X protein sample buffer. Cells were lysed using glass beads and incubated at 95°C. The soluble fraction was collected and stored at -80°C. Samples were electrophoretically fractionated on 7.5% polyacrylamide-SDS gels, transferred to nitrocellulose membranes (Amersham), and probed using mouse anti-myc monoclonal antibody (Upstate 05-724) or 3-phosphoglycerate kinase (Molecular Probes/Invitrogen A-6457) as a loading control.

**Co-immunoprecipitation**

Co-immunoprecipitations were performed as previously described (RUSCHE and RINE 2001; SAFI et al. 2008) using 30 OD equivalents of cells grown in medium lacking adenine to maintain plasmids. Cells were lysed in a buffer containing 50 mM tris, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% NP40, 1.5 mM DTT, and protease inhibitors. Cleared lysates were incubated with 4 μl rabbit polyclonal IgG anti-myc or anti-HA
antibodies (Upstate 06-549 and 05-902) at 4°C overnight and then 60 μl protein A agarose beads (Upstate) for 1 hr. Samples were electrophoretically fractionated on 7.5% polyacrylamide-SDS gels, transferred to membranes, and probed using mouse anti-myc and anti-HA monoclonal antibodies (Upstate 05-724 and 05-904).

**Chromatin immunoprecipitation (ChIP)**

Chromatin immunoprecipitation was performed as previously described (RUSCHE and RINE 2001). Two independent logarithmically growing cultures of each strain were grown and 50 OD equivalents of cells were cross-linked with 1% formaldehyde for 30 min. Lysis and subsequent steps were as described using 4 μl of anti-myc antibody (Millipore 06-549). Samples were quantified using real-time PCR with SYBR Green on a Bio-Rad iCycler. A standard curve was prepared from input DNA, and input DNA and samples were amplified using primers for a control locus and the locus of interest in separate reactions using real time PCR cycling parameters as described (SAFI et al. 2008). Data represent two IPs for each strain, each analyzed in two independent PCR reactions. Error bars represent the standard error.

**Reporter spot assay**

One OD equivalent of cells was collected from logarithmically growing cultures by centrifugation and resuspended in 100 μl yeast minimal medium (YM). Tenfold serial dilutions were prepared, and 3 μl of each dilution was spotted onto medium
lacking adenine to monitor growth in the presence of the plasmid or medium lacking adenine and histidine to monitor derepression of \textit{pPES4-HIS3}.

\textit{Induction of recombinant \textit{Sum1p} in \textit{E. coli}}

HIS-tagged proteins were expressed in Rosetta2 \textit{Escherichia coli} cells (Novagen) grown in 200 ml Luria-Bertani (LB) broth + 0.05 mg/mL ampicillin + 0.035 mg/mL chloramphenicol at 37°C overnight. In the morning, 25 ml of the overnight culture was used to inoculate 1L of LB + 0.05 mg/mL ampicillin + 0.035 mg/mL chloramphenicol, which was grown at 25°C until the culture reached an optical density (OD600) of 0.8 OD. For an uninduced sample, 1 ml of the culture was pelleted and resuspended in 100 μl 3X protein sample buffer/OD. To the remaining culture, 200 μl of 1M IPTG was added for induction of \textit{Sum1p}. \textit{Sum1p} 85-179, \textit{Sum1p} 1-530, \textit{Sum1p} 1-375, \textit{Sum1p} 1-275, and \textit{K. lactis} \textit{Sum1p} 1-336 were induced for 3 hours at 37°C. \textit{Sum1p} 1-452 was induced at room temperature overnight. For an induced fraction, 1 ml of the culture was pelleted and resuspended in 100 μL 3X protein sample buffer/OD. The remaining culture was pelleted at 3500 rpm for 15 minutes, resuspended in 25 ml LB, transferred to pre-weighed 50 mL conicals, and pelleted at 3,000 rpm for 20 minutes. Pellets were weighed and frozen at -80°C.

\textit{Lysis and purification of \textit{Sum1p}}

Keeping everything on ice, \textit{E. coli} pellets were lysed in 3 ml/g lysis buffer containing 300 mM NaCl, 10 mM imidazole, 0.1% TritonX-100, 10 mM β-
mercaptoethanol, 1X Bug Buster (Novagen 70921), 50 mM sodium phosphate buffer, pH 8 (93.2 ml 1M Na₂HPO₄, 6.8 ml 1M NaH₂PO₄), benzonase (0.5 µl/mL lysis buffer), and 1X protease inhibitors (1000X stocks: 35.2 mg/mL TPCK, 174 mg/mL PMSF, 5 mg/mL chymostatin, 2 mg/mL pepstatin A, 156 mg/mL benzamidine and 100X stock of one complete tablet from Boehringer-Mannheim in 500 µL H₂O). Cells were incubated at room temperature for 15 minutes with rocking. A 1 ml aliquot was removed and pelleted at 4°C for 15 minutes to collect soluble (20 µl of supernatant + 10 µl 3X protein sample buffer) and insoluble samples (resuspended pellet in 1 ml lysis buffer, 20 µl of the resuspension + 10 µl 3X protein sample buffer). The remaining cell lysate was spun at 10,000 rpm for 30 minutes at 4°C and the supernatant was saved.

To prepare the Ni resin, 0.25 ml nickel-nitrilotriacetic acid slurry (Qiagen)/ml lysis buffer was spun 10 minutes at 1,000 rpm at 4°C to pellet the beads. The beads were washed 3 times with 50 ml of cold binding buffer (300 mM NaCl, 10 mM imidazole, 50 mM sodium phosphate buffer, pH 8). Cleared lysate was added to the nickel-nitrilotriacetic acid matrix with 1X protease inhibitors and rocked for 1 hour at 4°C. Proteins were purified from the cleared lysate over the Ni column, which was washed with 300 ml cold wash buffer (300 mM NaCl, 20 mM imidazole, 50 mM sodium phosphate buffer, pH 8), and protein was eluted with 20 ml cold elution buffer (300 mM NaCl, 150 mM imidazole, 50 mM sodium phosphate buffer, pH 8, 10% glycerol) at a rate
of ~1 drop/3 seconds. 1.5 ml fractions were collected and quantified using a Bradford Assay.

20 μl of each Ni column fraction was combined with 10 μl 3X protein sample buffer and run on SDS-PAGE gels. Samples containing the highest amounts of Sum1p were combined and applied to a S200 size-exclusion column in buffer containing 10 mM Tris, 1 mM EDTA, 100 mM NaCl, and 10% glycerol using an AKTA fast protein liquid chromatography system. 5 ml fractions containing the expected size of Sum1p were combined and concentrated using Amicon Ultra (Millipore) centrifuge tubes. Glycerol was added to a final concentration of 20%. The protein concentration was quantified using a Bradford Assay, and the protein sample was flash frozen on dry ice and stored at -80°C.

**Binding assays**

Protein samples were diluted to a concentration of 0.3 μg/μl with dilution buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol) or used at their starting concentrations if lower than 0.3 μg/μl. Input samples contained 6.25 μl protein (0.3 μg/μl) and 3 μl 3X protein sample buffer. Binding reactions contained 25 μl protein (0.3 μg/μl), 2 μl biotinylated histone peptide (2 μg/μl), (H2A: Upstate 12-406, H2B: Upstate 12-407, H3: Upstate 12-357, H4: Upstate 12-347, AcH4: Upstate 12-379) and 123 μl binding buffer (10 mM Tris, pH 7.5, 40 mM NaCl, 4 mM MgCl2, 6% glycerol). Control reactions contained 2 μl water rather than histone
peptide. Samples were incubated for 1 hour at 25°C. Streptavidin beads (Upstate 16-126) were washed twice with 5 volumes of binding buffer and resuspended as a 50% slurry in binding buffer. 75 μl of beads were added to each sample and incubated at 4°C for 1 hour with rotation. Beads were washed 4 times with 750 μl binding buffer, and proteins were eluted from beads with 50 μl 2X protein sample buffer. 12.5 μl of each sample was electrophoretically fractionated on 10% polyacrylamide-SDS gels with the total input sample to determine percent binding.

In a modified binding assay, 2 μl of the biotinylated H4 peptide (2 μg/μL) was pre-incubated with 75 μl washed beads and 123 μl binding buffer for 1 hour at 4°C with rocking. Beads were washed 2 times with 750 μl binding buffer and incubated with 25 μl protein (0.3 μg/μl) for 1 hour at 4°C with rocking. Beads were washed 4 times with 750 μl binding buffer and proteins were eluted from beads with 50 μl 2X protein sample buffer and electrophoretically fractionated on 10% polyacrylamide-SDS gels.

3.3 Results

The N-terminus of Sum1p binds to histone tails in vitro

To investigate whether interactions between Sum1p and histones are important for repression, the N and C-terminal portions of Sum1p and Sum1-1p were purified and tested for binding to histone tails in vitro. Sum1p is a large 118 KDa protein, making purification of the entire protein challenging, but the C-terminal half of Sum1p (residues 523-1062) has been successfully purified (PIERCE et al. 2003; SAFI et al. 2008). Using this
puriﬁcation scheme, the C-terminus of Sum1p and Sum1-1p were puriﬁed along with an 
N-terminal truncation containing residues 1-530. Commer- 
cially available biotinylated 
peptides containing up to the ﬁrst 22 amino acids of human histones were incubated 
with puriﬁed Sum1p truncations and streptavidin beads. After incubation, the beads 
were washed and the bound fraction was analyzed (Figure 13, unpublished work by 
Alexias Safi).

Figure 13: The N-terminus of Sum1p binds to histone tails in vitro.

Histone binding assays of N-terminal or C-terminal Sum1p and Sum1-1p truncations 
and histone peptides. Input contains 100% of the protein used in the binding assay. 
Other samples contain protein incubated with H2O or H2A, H2B, H3, H4, or AcH4 
peptides. AcH4 peptide contains acetyl groups at lysines 5, 8, 12, and 16. Samples 
were run on SDS-PAGE gels to detect Sum1p binding to histone tails using 
Coomassie staining.

The N-terminal half of Sum1p bound histone tails H3, H4, and H2A very well, 
while the C-terminal half of Sum1p and Sum1-1p did not bind above background. As 
expected, no binding is seen in reactions containing water in place of peptide. Although 
histone peptides are very well conserved, the commercially available human histone
peptides used in these experiments were not representative of *S. cerevisiae* histones H2A and H2B (Figure 14, differences shown in red). Therefore, the interactions of Sum1p with H2A and H2B peptides are not representative of what may be going on in yeast cells *in vivo*. However, the N-terminal half of Sum1p, which is common between Sum1p and Sum1-1p, demonstrates binding to histone H3 and H4 tails. Since the N-terminus of Sum1p is identical between Sum1p and Sum1-1p, the histone interaction does not appear to be a result of the *SUM1-1* mutation. Therefore, if the ability of the Sum1-1p complex to spread is due to the ability of the N-terminus of Sum1-1p to bind histone tails, this interaction with histone tails may be conserved in Sum1 repression.

![Figure 14: Comparison of human and yeast N-terminal histone sequences.](image)

**Histone sequences for commercially available human histone peptides (h) and the corresponding yeast sequence (y). Residues in red show the differences between human and yeast sequences.**

The histone tails can undergo many modifications, including acetylation, phosphorylation, and methylation, which could modify Sum1p binding. Hst1p, a NAD+-dependent deacetylase, is required for repression and is thought to deacetylase histone
tails like its paralog Sir2p, which preferentially deacetylates histone H4 K16 in vitro (IMA\textit{i} \textit{et al.} 2000a; TANN\textit{y} and MOA\textit{zed} 2001). Strains with \textit{hst1}\textit{\textDelta} have increased acetylation of H3 and H4 tails at Sum1p-regulated genes (ROBERT \textit{et al.} 2004), consistent with the role of Hst1p as a histone deacetylase in the Sum1 complex. Enzymatically inactive Hst1pN291A, described in (LYNCH \textit{et al.} 2005), still associates with Sum1p-regulated genes, but its catalytic activity is required for repression (HICKMAN and RUSCHE 2007). Thus, deacetylation by Hst1p could regulate Sum1p binding to histone tails.

To determine if Sum1p binding to histone H4 was affected in the presence of acetylation, histone H4 peptide containing acetylated lysine residues was used. In the presence of acetylation on histone H4, Sum1p is no longer able to bind (Figure 13, AcH4 column), consistent with the role of Hst1p as a histone deacetylase. These data suggest that the deacetylation of histone H4 by Hst1p allows for the interaction of Sum1p and histone H4, which may be an important aspect of Sum1 repression.

\textit{Conservation and predicted secondary structure of Sum1p}

To further characterize the role of histone binding in Sum1 repression, I sought to identify a minimal region within the N-terminus of Sum1p that was important for histone binding. I used several programs to predict secondary structure and globular or disordered regions in the protein before designing truncations of the Sum1p protein to test for binding ability to histone peptides. This information assisted in the design of Sum1p N-terminal truncations, since the structure of Sum1p has not been solved. Eight
secondary structure prediction programs were used to predict the secondary structure for Sum1p (Appendix A, Figure 25). The program Globplot was also employed to determine regions that were likely to be globular (solid lines) versus disordered (dashed lines) (Joshua Warren, personal communication), and overlayed with the secondary structure information. As expected, the C-terminal region of the protein shows a large stretch of predicted globular domain, consistent with the observed DNA binding ability of the C-terminal half (Pierce et al. 2003) and presumed DNA binding domain. The central region of Sum1p contains two smaller stretches of predicted globular domain, one of which is disrupted by the truncation site chosen for the Sum1p N and C-terminal halves. The N-terminus of Sum1p is predicted to be more disordered, but does contain one region of predicted globular domain.

To complement the protein predictions, the conservation of SUM1 was investigated using a multiple sequence alignment with 11 yeast species. A phylogenetic tree of these related yeast species shows their evolutionary relationship to one another (Figure 15, adapted from Meleah Hickman). The regions of SUM1 that are evolutionarily conserved correspond nicely with the predicted globular domains (Appendix A, Figure 26), with the highest amount of conservation in the C-terminus and smaller regions of conservation in the central and N-terminal regions of Sum1p.
Figure 15: A phylogenetic tree of *Saccharomyces cerevisiae* and related yeast species. These species were used for a multiple sequence alignment to investigate the conservation of Sum1p. Figure by Meleah Hickman.

Figure 16 combines secondary structure predictions, globular and disordered predictions, and conservation among closely related yeast species to highlight regions of potential interest for a histone binding domain. The C-terminus of the protein shows a high degree of conservation (height of black vertical bars corresponds with degree of conservation) along with predicted globular (solid black horizontal line) and secondary structure (ribbons for alpha helix, arrows for beta sheet), as would be expected for a region containing DNA binding ability. I expected a potential histone binding domain to possess similar characteristics, and searched for regions of Sum1p that were conserved and structured. Since Sum1p 1-530 bound histones *in vitro*, it was likely that this region was located in the N-terminus. A region within the first 200 amino acids stood out as a potential histone binding domain and was studied further.
Figure 16: Conservation and predicted structure of Sum1p.

Vertical black bars represent the degree of conservation of Sum1p residues among 11 yeast species. Solid horizontal black bars represent predicted globular domains. Alpha helix and beta sheet predictions are represented by red spirals and green arrows, respectively. The conserved AT hook motifs are highlighted in yellow.

A conserved, globular region in the N-terminus of Sum1p is important for proper functioning of the Sum1p complex

To test the importance of this conserved N-terminal region of Sum1p to histone binding and Sum1 repression, residues 85-179 were deleted to create Sum1pΔ85-179. These residues were chosen based on the overlap of globular protein prediction and conserved sequence. Sum1pΔ85-179 is stably expressed but is not enriched at Sum1p-repressed genes (Figure 17 A-B), suggesting that this region is important for proper recruitment of Sum1p to the promoters of Sum1p-regulated genes. Sum1pΔ85-179 is slightly less abundant than wild-type Sum1p, which could be due to some protein misfolding in the absence of this region, which would result in degradation. Additionally, Sum1pΔ85-179 does not maintain interactions that allow for co-immunoprecipitation with Hst1p (Figure 17 C), providing evidence that this complex is not properly functioning in the absence of residues 85-179. Sum1 repression is most
likely disrupted in this deletion since the associations of Sum1p with target promoters and Hst1p have been disrupted, although this was not directly tested.

Figure 17: Sum1p residues 85-179 are important for functioning of the Sum1 complex.

A. Western Blot of LRY0144 MATα sum1Δ::URA3 yeast containing SUM1 (DMC326), 3myc-SUM1 (pJR2296), or 3myc-sum1Δ85-179 (pLR521). Anti-myc antibody was used to detect SUM1.

B. ChIP of SUM1 from the above yeast at Sum1p-regulated genes SMK1 and DTR1. Enrichment values are relative to enrichment at the control locus ATS1. An enrichment of 1 represents background.

C. Co-IP of SUM1 and HST1 in LRY1866 MATα sum1Δ::URA3 HST1-5HA yeast with SUM1 (DMC326), 3myc-SUM1 (pJR2296), or 3myc-sum1Δ85-179 (pLR521). Samples were immunoprecipitated with anti-myc antibody and interaction with HST1 was detected with anti-HA antibody.

D. HIS-Sum1p 85-179 (pLR530) was expressed and purified from Rosetta2 E. coli cells. Uninduced (U) and induced (I) fractions show the successful induction of Sum1p 85-179 at 12 KDa on Coomassie stained SDS-PAGE gels.
E. Histone binding assay with purified Sum1p 85-179 and histone peptides. Input contains 100% of the protein used in the binding assay. Other reactions contain Sum1p 85-179 incubated with H2O, H3, or H4. Samples were run on SDS-PAGE gels to detect Sum1p binding to histone tails using Coomassie staining.

These *in vivo* studies of the N-terminus of Sum1p uncovered the importance of residues 85-179 in the proper functioning of the Sum1 complex but did not help to determine why this region is important. To test this region as a histone binding domain, I purified Sum1p 85-179 from Rosetta2 *E. coli* cells and tested it for *in vitro* binding to histone tails. Sum1p 85-179 was successfully induced (Figure 17 D) but was the wrong size to assess binding in this assay because a product of the streptavidin beads runs at the same size as Sum1p 85-179 (Figure 17 E, 12 KDa band), making binding to histone peptides indistinguishable from control reactions containing water. In retrospect, immunoblotting could have been used to identify Sum1p. However, a conclusive explanation for the importance of this region to the functioning of the Sum1 complex was not reached.

If residues 85-179 were primarily responsible for binding to histone tails, I would expect that deletion of this region would not disrupt interactions between Sum1p and Hst1p or recruitment to promoters of Sum1p-regulated genes. However, Sum1 repression would be disrupted if interactions with histones are important for achieving repression. Sum1pΔ85-179 causes disruption to all functions of the complex that were tested, which is most likely due to a disruption in Sum1p folding as a result of the
deletion. Alternatively, this region may be important for more than just binding to histones.

*Mutation of conserved residues within Sum1p 85-179 does not disrupt the Sum1 complex*

To study this region of Sum1p in a more conservative manner and determine if it interacts with histones, amino acid mutations were made in this region. If this region of Sum1p is important for binding to histone tails, then negatively charged residues in Sum1p are likely to strengthen interactions with positively charged, unacetylated lysine residues in the histone tails. Therefore, several highly conserved, negatively charged aspartate (D) and glutamate (E) residues were mutated to alanine (A) to study their importance in Sum1 repression (Figure 18 A, red asterisks denote mutated residues).

Sum1p D147A, D148A (DD) and Sum1p D157A (D), along with triple point mutant Sum1p D147A, D148A, D157A (DDD) were created. These mutant proteins were expressed and did not disrupt Sum1p binding to Sum1p-regulated promoters (Figure 18 B-C), consistent with proper functioning of the Sum1 complex. The presence of these mutations did not have a dramatic effect on Sum1 repression (Figure 18 D), suggesting that mutation of these residues is not enough to disrupt Sum1 repression. However, in the presence of *HST1-5HA*, repression in the presence of Sum1p mutations is only ~10-fold better than the fully derepressed state (Figure 18 E, compare mutations to vector alone).
Figure 18: Mutating conserved residues within Sum1p 85-179 does not disrupt the Sum1 complex.

A. Multiple sequence alignment of Sum1p in 11 related yeast species, which focuses on the conserved, globular region in the N-terminus of Sum1p containing residues 85-179 in *S. cerevisiae*. Horizontal black bars underneath the alignment represent the degree of conservation of Sum1p residues. Red asterisks show the conserved, negatively charged residues that were mutated to alanine.

B. Western Blot of LRY1623 MATa sum1Δ::LEU2 pPES4-HIS3 yeast containing SUM1, 3myc-SUM1 (pJR2294), 3myc-SUM1 D147A D148A (DD) (pLR550), 3myc-
SUM1 D157A (D) (pLR555), and 3myc-SUM1 D147A D148A D157A (DDD) (pLR569). Anti-myc antibody was used to detect the expression of SUM1 and anti-PGK was used as a loading control.

C. ChIP of SUM1 from the above yeast at Sum1p-regulated genes SMK1 and DTR1. Enrichment values are relative to enrichment at the control locus ATS1. An enrichment of 1 represents background.

D. Reporter spot assay of the above yeast with the above plasmids or vector pRS412. 10-fold serial dilutions of cells were plated on CSM-ADE plates to monitor growth and CSM-ADE-HIS to monitor Sum1 repression of reporter pPES4-HIS3.

E. Reporter spot assay of LRY2105 MATa sum1Δ::LEU2 HST1-5HA pPES4-HIS3 yeast with the above plasmids or vector pRS412. Cells were treated as in D.

F. Histone binding assay with purified HIS-Sum1p 1-530 (pLR387) or HIS-Sum1p 1-530 D147A D148A (pLR544) and histone peptides. Input contains 100% of the protein used in the binding assay. Other reactions contain protein incubated with H2O, H3, or H4. Samples were run on SDS-PAGE gels to detect Sum1p binding to histone tails using Coomassie staining.

Individually, Sum1p mutations or HST1-5HA do not disrupt Sum1 repression, but the combination likely destabilizes interactions within the Sum1 complex, causing the subsequent loss of repression. In a strain background containing HST1 and Sum1p mutations, Sum1p is successfully recruited to promoters of Sum1p-regulated genes and repression is not disrupted. To determine if these mutations disrupt histone binding, Sum1p 1-530 D147A D148A was purified in Rosetta2 E. coli cells and tested for in vitro binding to histone peptides. The presence of these mutations did not have a significant effect on Sum1p binding to histone H4 (Figure 18 F), as binding was comparable to binding with wild type Sum1p 1-530. Therefore, these mutations on their own are not disruptive to histone binding, consistent with their minimal effect on repression.
**Additional truncations of N-terminal Sum1p do not bind histones in vitro**

These data do not rule out the possibility that residues 85-179 contain a histone binding domain. Other conserved residues which were not tested in this region may be important to histone binding and/or repression. Alternatively, a region larger than residues 85-179 may be required for histone binding. To find a minimal histone binding domain in the N-terminus of Sum1p, smaller regions of the Sum1p N-terminus were designed with reference to secondary structure, globular predictions, and homology considerations to maximize the chances for proper folding and function of the truncated protein. Starting with Sum1p 1-530, which was previously shown to bind to histones H3 and H4, four additional truncations of the N-terminus were created (Figure 19).

All truncations were successfully induced in Rosetta2 *E. coli* cells (Figure 19 B, compare uninduced U to induced I lanes), although much of the induced protein was present in the insoluble pellet (compare pellet P to supernatant S lanes), dramatically decreasing the potential yield of protein. This lack of solubility is likely due in part to the unstructured nature of the N-terminus. The N-terminus of Sum1p from *K. lactis* was also purified to see if solubility and purification yield might improve, but the *K. lactis* Sum1p did not have dramatically different results as compared to *S. cerevisiae* Sum1p.
Figure 19: Design and purification of Sum1p N-terminal truncations.

A. Schematic of full length Sum1p and designed N-terminal truncations HIS-Sum1p 1-530 (pLR387), HIS-Sum1p 1-452 (pLR498), HIS-Sum1p 1-376 (pLR492), and HIS-Sum1p 1-275 (pLR491). Light gray HIS-Sum1p 1-336 (pLR574) is from *K. lactis*.

B. Uninduced (U), induced (I), pellet (P) and supernatant (S) fractions were run on SDS-PAGE gels and Coomassie stained to confirm the successful induction of N-terminal Sum1p truncations. Asterisks denote the Sum1p truncations at their expected sizes.

To purify Sum1p, lysed *E. coli* cells were run over a Ni column to select for HIS-Sum1p and eluted with imidazole. Concentrated Ni fractions were further purified on a S200 size exclusion column, and fractions containing protein at the expected size of Sum1p were concentrated and saved. Size exclusion chromatography revealed a large amount of aggregate Sum1p in the early fractions, providing further evidence that the
expression and purification of Sum1p in *E. coli* cells may not be ideal conditions for proper folding.

Several parameters were varied to optimize the purification conditions and improve the solubility of Sum1p, including induction temperature, induction time, imidiazole concentration, salt concentration, and volume of Ni beads. The only significant improvement in yield was a result of decreased Ni beads, which decreased the amount of nonspecific proteins that bound the Ni beads and increased the final yield of Sum1p. Therefore, the previously described purification protocol was used (SAFI *et al.* 2008) with fewer Ni beads.

Purified Sum1p truncations were tested for binding to histone tails (Figure 20 A). A small amount of full-length Sum1p 1-1062 (purified by Alexias Safi) was obtained and did not show binding to histone H3 and H4 tails at the expected size of 160 KDa. However, three smaller proteins present in the input bind to H2A and H4, and are close in size to N-terminal Sum1p 1-530 (67 KDa) and a presumed degradation product of Sum1p 1-530 (27 KDa). These proteins may represent more stable truncations of full length Sum1p that maintain binding to histones, or may be native *E. coli* proteins.
Figure 20: Binding of Sum1p to histones only occurs with Sum1p 1-530.

Histone binding assays of purified Sum1p and histone peptides. Input contains 100% of the protein used in the binding assay. Other samples contain protein incubated with H2O or H2A, H2B, H3, H4, or AcH4 peptides. Samples were run on SDS-PAGE gels to detect Sum1p binding to histone tails using Coomassie staining. Sum1p 1-376 and *K. lactis* Sum1p 1-336 were not tested for histone binding.

As previously shown by Alexias Safi in Figure 13, Sum1p 1-530 binds well to histone H3 and H4 peptides, while acetylated H4 prevents Sum1p binding. However, smaller truncations to Sum1p 1-530 did not bind to histone tails. Sum1p 1-452 shows binding to histones H3 and H4 just above background levels seen with H2A and H2B, and Sum1p 1-275 shows no binding to histone tails. Sum1p 1-376 did not purify well enough to be tested for binding to histone tails, as all of the protein loaded onto the size exclusion column ran in the early aggregated fractions rather than at the expected size of 50 KDa, likely a result of instability or improper folding of this truncation. Therefore,
these data suggest that some property within Sum1p 1-530 allows for binding to histone peptides. This may be due to sequence, stability, or folding that may affect the interaction with histone peptide and make it most favorable.

To determine if the order of binding affects Sum1p binding to histone tails, the binding assay was modified to allow histone peptide and streptavidin beads to incubate before the addition of Sum1p. Two independent protein preps of Sum1p 1-530 (purified by myself or Alexias Safi) previously shown to bind histone H4 were tested for binding to H4 in the modified binding assay. Sum1p binding to histone H4 was abolished when the order of binding was changed, and binding was not dependent on the concentration of histone H4 used (data not shown). Restricted accessibility of the histone peptide due to pre-binding with streptavidin beads could explain this result, but to ensure that the previously observed binding of Sum1p with histone peptides was real, several control experiments were performed.

**Sum1p is not detectable in binding samples**

An anti-HIS antibody was used to detect HIS-Sum1p 1-530 through the purification and binding to histone peptides. As expected, Sum1p 1-530 is present in the induced (I), supernatant (S), and pellet (P) fractions through the purification (Figure 21 A) and absent from the uninduced (U) sample. Fractions from the Ni column (Figure 21 B) and concentrated Sum1p 1-530 (Figure 21 A, sample C) reveal several degradation products around 45 KDa and 27 KDa. This result confirms instability of Sum1p through
the protein prep. However, despite this instability and degradation, Sum1p 1-530 remains in the concentrated sample used for binding (asterisk at 67 KDa).

Figure 21: Sum1p 1-530 is not detected in binding assays using anti-HIS antibody.

A. Western of uninduced (U), induced (I), supernatant (S), pellet (P), and concentrated (C) fractions from HIS-Sum1p 1-530 purification. Samples were run on SDS-PAGE gels and tested for the presence of HIS-Sum1p 1-530 using an anti-HIS antibody. An asterisk denotes Sum1p 1-530 at 67 KDa.

B. Western Blot of eluted Ni column fractions from HIS-Sum1p 1-530 purification. Ni column fractions (1-11) were tested for the presence of HIS-Sum1p 1-530 using an anti-HIS antibody.

C. Histone binding assays of aggregate (left) and monomer (right) protein samples from size exclusion purification with histone peptides. Input contains 100% of the protein used in the binding assay. Other samples contain protein incubated with H2O, H2B, or H4 peptides. Samples were run on SDS-PAGE gels to detect Sum1p binding to histone tails using Coomassie staining.
D. Western Blot of histone binding assays shown in C. HIS-Sum1p 1-530 was detected using an anti-HIS antibody.

E. Expression and purification of pET15 vector. Uninduced (U), induced (I), and Ni column fractions (1-10) show the presence of contaminant *E. coli* proteins around 70 KDa and 23 KDa that bind to the Ni column under these purification conditions. These *E. coli* proteins run near the expected size of Sum1p1-530 (67 KDa) and a major degradation product of Sum1p1-530 (27 KDa) (compare Ni fraction bands in B and D).

To incorporate additional controls into the histone binding assay, early fractions from the S200 containing aggregated proteins were saved along with the usual fractions running as monomer at ~67 KDa, the expected size for Sum1p 1-530. Aggregate protein contains improperly folded and degraded Sum1p, and binding of these proteins to histone peptides could indicate that the interaction is nonspecific. Monomeric protein should be specific to binding with histone H4, since Sum1p should be properly folded. Aggregate and monomeric protein fractions were incubated with H2O or H2B as negative controls or histone H4 peptide.

Identical binding reactions were run on SDS-PAGE gels and stained with Coomassie Blue to assess binding (Figure 21 C), or immunoblotted with anti-HIS antibody to detect HIS-Sum1p 1-530 (Figure 21 D). Aggregate protein samples show faint binding to H2B and H4 on the Coomassie stained gels (Figure 21 C, left panel), and an interaction with histone H4 and a truncation protein at ~27 KDa. Monomeric protein samples show specific binding of Sum1p 1-530 to histone H4 on the Coomassie stained gels (Figure 21 C, right panel), along with a truncation protein at ~27 KDa. There is a
background level of binding to histone H2B, which should not bind with sequence specificity.

Immunoblotting confirms that some HIS-Sum1p 1-530 is present in the aggregate fraction (Figure 21 D, left panel), along with several HIS-Sum1p degradation products, and some faint background binding to histone tails is detected at 67 KDa. Since Sum1p is not properly folded in these fractions, binding above background would indicate nonspecific interactions with histone peptides. For the monomeric protein samples, a very faint amount of HIS-Sum1p 1-530 is detected in the input sample, which should contain properly folded and purified Sum1p 1-530. Surprisingly, Sum1p binding to histone H4 was not detected in the immunoblot despite a robust band at 67 KDa on the Coomassie stained gel. This result questions whether the protein binding to histone H4 is Sum1p, since it cannot be detected using an anti-HIS antibody.

To determine the identity of the 67 KDa protein, mass spectrometry was performed on a gel slice of 67 KDa protein from a Sum1p 1-530 purification by the Duke Proteomics Facility. The sample was run on a 10% SDS-PAGE gel to allow for separation from other proteins running close to this size. Binding assays with Sum1p 1-530 show the presence of two bands around 67 KDa, with the lower band binding to histone peptides. Therefore, the lower portion of the gel band at 67 KDa was isolated and submitted for mass spectrometry. With a peptide confidence of 99% and a two peptide minimum, the presence of Sum1p 1-530 was confirmed, along with contaminant E. coli
protein glucosamine-fructose-6-phosphate aminotransferase which is also 67 KDa. The 
*E. coli* protein is twice as abundant as Sum1p, which may explain the absence of 
detectable HIS-Sum1p 1-530 in the immunoblot. Induction of empty pET15b vector 
confirmed the presence of *E. coli* proteins at 67 KD and 25 KDa that bind to the Ni 
column under the purification conditions used (Figure 21 E, Ni column fractions 1-11).

Based on the control experiments showing an absence of detectable Sum1p in the 
histone binding samples and the presence of contaminant *E. coli* proteins in the Ni 
column and purified Sum1p samples, it is unlikely that Sum1p is the histone binding 
protein. As these experiments were being conducted, I obtained encouraging data that 
Orc1p, which is required for Sum1-1p recruitment to *HMR*, might possess histone 
binding properties (discussed in Chapter 2). As a result, the search for a histone binding 
property within Sum1p was not pursued further.

### 3.4 Discussion

This study was designed to identify a histone binding domain in Sum1p and 
determine if there is a role for histone binding in Sum1 repression. However, without a 
solved structure of Sum1p, it is hard to design deletions and mutations and know how 
these local changes will affect the overall functioning of Sum1p. Using conservation and 
secondary structure predictions, a region of interest containing a high degree of 
structure and conservation was found in the N-terminus of Sum1p. This region was the 
focus of *in vitro* experiments testing binding of Sum1p and histone H3 and H4 peptides.
I also studied the role of Sum1p 85-179 in vivo. Mutations to this region did not cause disruptions, while deletion of this region abolished all tested functions of the Sum1 complex. As a result, meaningful conclusions about a role for this region in binding histones were hard to determine.

The complete deletion of this region most likely led to structural changes and improper folding of Sum1p, which would explain its decreased expression as a result of increased protein degradation. The decrease in Sum1p binding at Sum1p-repressed promoters may be attributed to misfolding that affects the C-terminal DNA binding domain, or the proximity of the deletion to the AT-hook binding motifs located in Sum1p. AT-hook motifs are found in many eukaryotic chromatin and DNA-binding proteins (ARAVIND and LANDSMAN 1998) and bind the minor groove of DNA, and this AT-hook may no longer be properly positioned to assist with the DNA binding ability of Sum1p.

In addition to the complete deletion, conserved residues within this region were mutated to disrupt favorable interactions with positively charged, unacetylated lysine residues in the H3 and H4 tails. These mutations were not disruptive to Sum1 repression or other functions of the Sum1 complex. The mutated residues may not be the critical residues in this region that are important for histone binding ability and Sum1 repression, or there may be redundant residues involved in interactions with histone
tails. It is also possible that these mutations may cause slight disruptions to Sum1 repression that are not detected with this reporter assay.

These results do not rule out the possibility that residues 85-179 contain a histone binding domain, and additional mutagenesis to this region may uncover residues that disrupt histone binding and/or Sum1 repression. However, these residues within Sum1p may not be important for binding histones and the mechanism of Sum1 repression may not involve binding to histones. Data suggesting a biologically significant role for histones in Sum1 repression has not been uncovered, despite attempts to mutate Sum1p and histones H3 and H4. In Chapter 2 I discussed a genetic screen for histone mutations that disrupt Sum1 repression, in which no mutations were recovered, despite screening over 17,000 colonies. A similarly conducted screen uncovered 22 histone mutations that disrupt Sum1-1p silencing at HMR, suggesting that histone mutations are important to silencing and not repression. Additional attempts to mutate histone H3 and H4 at key modifiable lysine residues or complete deletions of the histone tails did not disrupt Sum1 repression either (Chapter 2, Table 2). Given these results, it does not appear that an interaction between Sum1p and histones H3 and H4 is critical for maintaining repression.

Several Sum1p N-terminal truncations were designed and tested for in vitro binding to histone tails to define a potential histone binding domain. These truncations did not help to narrow down a potential histone binding domain located within Sum1p
1-530, and led to concerns over the validity of the *in vitro* binding results. Specifically, several results suggest that the binding observed between Sum1p and histone peptides was not due to the presence of Sum1p and was nonspecific.

The inability to detect HIS-Sum1p 1-530 bound to histone peptides using an anti-HIS antibody adds support to the argument for nonspecific interactions. HIS-Sum1p 1-530 was detected in the starting protein fraction, but not in the sample that exhibits binding to histone H4 peptide. This result, combined with mass spectrometry data indicating the presence of contaminant *E. coli* protein at 67 KDa, suggest that the majority of protein bound to histone H4 peptide is not Sum1p. It is possible that binding to histone H4 peptide affects the ability of the antibody to recognize the HIS tag, which may explain why Sum1p is not detected in the bound sample. However, the presence of an *E. coli* protein at the exact size of Sum1p does cause concern for the validity of the results, and it is hard to quantify the amount of Sum1p versus *E. coli* protein present in the 67 KDa band.

I confirmed the presence of *E. coli* proteins at 67 KDa in a mock induction with vector alone, and should have purified the mock induction and tested it for binding to histone tails. If binding is due to the presence of Sum1p, then binding would not be observed using protein from the mock induction. Alternatively, if the binding observed is due to the presence of *E. coli* protein, then binding to histones H3 and H4 would still occur in the absence of Sum1p. Although this experiment was not conducted, the
experiments that were performed support nonspecific interactions between *E. coli* proteins and histone peptides.

The presence of contaminant *E. coli* protein may have been avoided with an optimized purification scheme. While the C-terminus of Sum1p has been purified using similar conditions (PIERCE *et al.* 2003; SAFI *et al.* 2008), these conditions did not work as well for the purification of the N-terminus of Sum1p. During the purification, Sum1p experienced insolubility, aggregation, and degradation. The N-terminus of *K. lactis* Sum1p behaved similarly using these conditions, and changes to induction time, temperature, and concentration of IPTG did not yield noticeable improvements in Sum1p solubility and purification. The use of insect cells rather than bacteria may have improved the expression and purification conditions, as it may more closely mimic the native eukaryotic environment and assist in folding of Sum1p. However, optimal purification conditions for N-terminal Sum1p were not thoroughly investigated.

While the conserved N-terminal region of Sum1p does not appear to bind histone tails, the conservation in this region suggests that it plays an important role. An alternative, biologically significant role for this conserved N-terminal region is as an interaction domain with Rfm1p. An interaction domain between Sum1p and Rfm1p has not been identified in the literature, and is likely to be globular, structured, and conserved. Rfm1p is required for tethering Sum1p and Hst1p to form the complex, as they do not directly interact (MCCORD *et al.* 2003). Hst1p can then deacetylate histones or
other proteins at the promoter region bound by Sum1p, resulting in repression of the target genes. Deletion of Sum1p residues 85-179 abolished co-immunoprecipitation with Hst1p, consistent with a role for this region in binding Rfm1p, although this also disrupted Sum1p binding to the DNA as well. In any case, it is interesting to think about this region as a potential interaction domain with Rfm1p, and further studies could be performed to directly test this idea.

The Tup1-Ssn6 repression complex in *S. cerevisiae* works to repress over 180 genes and utilizes multiple DNA binding proteins and histone deacetylases to customize recruitment and repression of several gene families. The N-terminus of Tup1p preferentially associates with hypoacetylated histone H3 and H4 tails (EDMONDSON et al. 1996), and histone mutations that disrupt the association of Tup1p with histone tails or increase histone acetylation disrupt repression. The Sum1 complex also utilizes a DNA binding protein and deacetylase to achieve repression of about 50 target genes. However, there does not appear to be a conserved role for histones H3 and H4 in facilitating Sum1 repression. The N-terminus of Sum1p does not bind to hypoacetylated histone H3 and H4 tails *in vitro*, and mutations and deletions of the histone H3 and H4 tails did not disrupt Sum1 repression (discussed in Chapter 2).

One explanation for this difference in utilization of the histone tails may be due to the extreme versatility in the Tup1-Ssn6 complex. The Tup1-Ssn6 repression complex functions to repress a much larger subset of genes than the Sum1 complex, and
flexibility and versatility plays an integral role in the adaptability of the complex for its targets in many diverse gene families. The interaction of Tup1p with N-terminal histone tails may allow for increased stability of the complex at gene promoters and its capability to spread across the coding regions of a subset of the genes it regulates. While Hst1p is thought to be a histone deacetylase, its target may not be histones H3 and H4, since histone mutations do not disrupt Sum1 repression. Sum1 repression has not been studied as thoroughly as Tup1-Ssn6 repression, and future studies are needed to understand the crucial interaction surfaces between these complex components and the target of Hst1p deacetylation, which will help to decipher the mechanism of Sum1 repression.
4. Sir contributions to Sum1-1 silencing

4.1 Introduction

The Sum1-1 complex can restore silencing of the mating type loci in sir2Δ, sir3Δ, or sir4Δ strains (CHI and SHORE 1996; LAURENSON and RINE 1991; LIVI et al. 1990; RUSCHE and RINE 2001). This has been taken to indicate that Sir proteins do not participate in Sum1-1 silencing. However, the observation that the LRS/H4 tail region of the nucleosome is important for Sum1-1 silencing raises the possibility that Sir3p, which also binds to this region of the nucleosome, could be involved in Sum1-1 silencing. Sir3p contains a BAH domain that interacts with the LRS/H4 tail region of the nucleosome and is critical for Sir silencing and propagation (BUCHBERGER et al. 2008; NORRIS et al. 2008; ONISHI et al. 2007; SAMPATH et al. 2009). The importance of histones and the BAH domain of Orc1p in Sum1-1 silencing could be due to similar interactions. However, Orc1p does not appear to function exactly like Sir3p and does not spread with Sum1-1p (Chapter 2), suggesting that its contributions are unique.

While Sum1-1 silencing does not require Sir proteins, they may still be recruited to the silencers and contributing to Sum1-1 silencing. Most studies of Sum1-1 silencing have been conducted in sir2Δ strains where Sir3p and Sir4p are present. Sir4p can be recruited to the silencers in the absence of the other Sir proteins (MOAZED et al. 2004; RUSCHE et al. 2002), allowing for Sir3p recruitment. The BAH domain of Sir3p is capable of interactions with nucleosomes and its presence may contribute to Sum1-1 silencing.
Therefore, studying Sum1-1 silencing in \( \textit{sir3} \Delta \) and \( \textit{sir4} \Delta \) strain backgrounds will allow for a more accurate assessment of the Sum1-1 silencing complex and its capabilities in the absence of Sir proteins.

While doing some control experiments, I discovered that the requirement for histone residues in the LRS/H4 tail domain is dependent on the absence of \( \textit{SIR2} \). Additionally, Sir3p is present in Sum1-1p-silenced chromatin, and the involvement of Sir3p in Sum1-1 silencing may explain why the LRS/H4 tail region is important for silencing and the ability of Sum1-1p to spread.

\textbf{4.2 Materials and methods}

\textit{Yeast strains}

Strains used in this study were derived from W303-1b. The \( \textit{SUM1-1}, 7\text{myc-SUM1-1}, \textit{sir2} \Delta::\textit{HIS3}, \textit{sir3} \Delta::\textit{LEU2} \) alleles were previously described (RUSCHE and RINE 2001). The \( \textit{hht1-hhf1} \Delta::\textit{NatMX} \) and \( \textit{hht2-hhf2} \Delta::\textit{HygMX} \) alleles are complete deletions of the open reading frames generated by one step gene replacement. These alleles were moved into the desired genetic backgrounds through standard genetic crosses to generate the strains listed in Table 4.

\textit{Plasmids}

Plasmids used in this study are described in Table 5. pDM18 (\( \textit{HHT2 HHF2 TRP1} \)) was previously described (DUINA and WINSTON 2004). pLR615 (\( \textit{HHT2 E73G HHF2} \)) and pLR619 (\( \textit{HHT2 K4I HHF2} \)) were constructed using site directed mutagenesis of pDM18.
**Reporter mating assay**

For the semi-quantitative mating assay, one optical density (OD) equivalent of cells was collected from logarithmically growing cultures by centrifugation and resuspended in 100 μl yeast minimal medium (YM). Tenfold serial dilutions were prepared, and 3 μl of each dilution was spotted onto rich medium (YPD) to monitor growth. For mating, an equal volume of a tester strain of the opposite mating type (LRY1021 MATa his4) resuspended at 10 OD equivalents per ml in YPD was mixed with each sample in the dilution series. 3 μl of this mixture was spotted onto minimal medium to select for the growth of prototrophic diploids. Yeast were grown at 30°C for 3 days before imaging. For yeast containing mutated histone genes on plasmids, 3 μl of each sample in the dilution series was spotted onto medium containing 5-FOA and lacking tryptophan to monitor growth in the presence of mutant histone plasmids.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation was performed as previously described (RUSCHE and RINE 2001). Two independent logarithmically growing cultures of each strain were grown and 50 OD equivalents of cells were cross-linked with 1% formaldehyde for 30 min. Lysis and subsequent steps were as described. 3 μl of antibody was used; anti-Sir2p, anti-Sir3p, and anti-Sir4p (Jasper Rine). Samples were quantified using real-time PCR with SYBR Green on a Bio-Rad iCycler. A standard curve was prepared from input DNA, and input DNA and samples were amplified using primers
for a control locus and the locus of interest in separate reactions using real time PCR cycling parameters as described (SAFI et al. 2008). Oligonucleotides used are listed in Table 6. Data represent the average of two independent IPs for each strain, each analyzed in two independent PCR reactions. Error bars represent the standard error.

4.3 Results

The observation that the LRS/H4 tail region of the nucleosome is important for Sum1-1 silencing raises the possibility that Sir3p could be binding to this region and contributing to Sum1-1 silencing. Studying Sum1-1 silencing in the absence of Sir3p would alleviate this possibility, and a MATα sir3Δ SUM1-1 strain background was tested for mating ability in the presence of histone mutations. Surprisingly, mutations to the LRS domain in the H3 core and the H4 tail were not disruptive to Sum1-1 silencing in sir3Δ strains (Figure 22 A). This result is not due to differences in the ability of SUM1-1 to restore mating in sir2Δ compared to sir3Δ strains, as the strains mated equivalently in the presence of wild-type histones (Figure 22 B). Consistent with a unique role for the H3 tail in Sum1-1 silencing, H3 tail mutations were still disruptive to silencing in a sir3Δ background. Therefore, this suggests that histone residue requirements in the LRS/H4 tail region are specific to the sir2Δ strain background, and these differences could be a result of the absence of Sir2p or the presence of Sir3p.

The absence of Sir2p may weaken the ability of the Sum1-1 complex to fully silence HMRa in the presence of histone mutations. Alternatively, Sir3p may bind the
LRS/H4 tail region, and histone mutations would disrupt these interactions and may create an unfavorable chromatin structure. To further investigate the contributions of Sir2p and Sir3p in Sum1-1 silencing, a strain lacking \textit{SIR2} and \textit{SIR3} was tested for mating ability in the presence of histone mutations. If the disruption of Sum1-1 silencing is due to the absence of Sir2p and histone mutations, then histone mutations should be disruptive in a strain lacking \textit{SIR2} and \textit{SIR3}. Alternatively, if the disruption of Sum1-1 silencing in histone mutants is due to the presence of Sir3p, then Sum1-1 silencing should be maintained in a strain lacking \textit{SIR2} and \textit{SIR3}.

Similar to the results obtained in a \textit{sir2Δ} strain, histone mutations in the H3 tail, H3 LRS region, and H4 tail were all disruptive to silencing in a \textit{MATα sir2Δ sir3Δ SUM1-1} strain (Figure 22 A). H3 R72G, H3 A75V and H4 G28P were the exception and maintained silencing. The presence of Sir3p does not appear to be disruptive to Sum1-1 silencing, as histone mutations are still disruptive in \textit{sir2Δ sir3Δ} strains. Therefore, the disruption of Sum1-1 silencing appears to be due to the absence of Sir2p in the presence of histone mutations, suggesting that Sir2p and the LRS/H4 region contribute to silencing in parallel pathways.
Figure 22: Histone H3 LRS and H4 tail mutations contribute to Sum1-1 silencing only in the absence of Sir2p.

A. Sum1-1 silencing of *HMRa* was tested in *MATα SUM1-1* strains with *sir2Δ* (LRY1450), *sir3Δ* (LRY2505), or *sir2Δ sir3Δ* (LRY2506) using a reporter mating assay. These histone mutations were previously described in Figure 7. Mutations causing more than a 10-fold loss in mating were scored as defective (-).

B. Reporter spot mating assay of the above strains containing wild-type histones.

Histone mutations disrupt Sum1-1 silencing in the absence of Sir2p, suggesting that Sir2p contributes to Sum1-1 silencing. Chromatin immunoprecipitation of Sir2p was performed to determine if Sir2p acts at *HMRa* and directly contributes to Sum1-1 silencing.
silencing. As expected, Sir silenced control strains were significantly enriched for Sir2p across HMRa (Figure 23). Deletion of SIR3 abolished Sir silencing and severely diminished the enrichment of Sir2p. Some association of Sir2p above background still occurred at the E silencer in sir3Δ SUM1 strains, consistent with the ability of Sir proteins to be recruited in the presence of Sir4p. While the genetic data suggest a role for Sir2p in Sum1-1 silencing, recruitment of Sir2p to HMR-E is not Sum1-1p-dependent, and sir3Δ SUM1-1 strains have equivalent enrichment of Sir2p at the E silencer. These data suggest that the small amount of Sir2p at the E silencer may not specifically contribute to Sum1-1 silencing. Alternatively, the stable association of Sir2p across HMRa may not be necessary, and Sir2p may contribute to Sum1-1 silencing without direct interactions at HMRa.

A similar pattern of enrichment was observed in Sir4p chromatin immunoprecipitations, suggesting that the small amount of Sir4p at the E silencer may not specifically contribute to Sum1-1 silencing. While Sir2p and Sir4p enrichment levels were assessed in SIR2 sir3Δ strain backgrounds, a sir2Δ SIR3 strain background was used to investigate the enrichment of Sir3p across HMRa. The BAH domain of Sir3p is capable of interactions with the LRS/H4 region of the nucleosome and its presence may contribute to Sum1-1 silencing. In fact, Sir3p is significantly enriched in sir2Δ SUM1-1 strains as compared to sir2Δ SUM1 strains, suggesting that Sir3p is associating across HMRa in a Sum1-1p-dependent manner. Therefore, Sir3p may spread across HMRa
through interactions between the BAH domain and nucleosomes and may contribute to Sum1-1p silencing.

Figure 23: Sir3p is enriched across HMRa in Sum1-1p-silenced chromatin.

ChIP of Sir2p, Sir3p, and Sir4p at HMRa in wild-type MATα yeast (LRY1007) and yeast containing sir2Δ SUM1 (LRY0164), sir2Δ SUM1-1 (LRY1363), sir3Δ SUM1 (LRY341) and sir3Δ SUM1-1 (LRY344). Plus sign indicates addition of antibody (+ anti-Sir2, anti-Sir3, or anti-Sir4). Primers are spaced ~1kb across HMRa, and enrichment values are relative to PHO5, which is not associated with Sum1-1p. Significance values (p<0.0001) and (p<0.001) were calculated relative to sir2Δ SUM1 and sir3Δ SUM1 strains which are defective for Sir silencing.
To determine if mutations in the LRS region of the nucleosome disrupt Sir3p enrichment across HMRα, chromatin immunoprecipitation was performed in sir2Δ SUM1-1 strains containing wild-type H3, H3 E73G, or H3 K4I. The presence of these histone mutations decreased the enrichment of Sir3p across HMRα (Figure 24), and the LRS mutant H3 E73G was more detrimental than tail mutant H3 K4I. These results are consistent with a role for Sir3p in binding the LRS region of the nucleosome, and Sir3p may spread across the locus when interactions between the Sir3p BAH domain and nucleosomes are favorable.

Figure 24: Histone mutations disrupt Sir3p enrichment across HMRα in Sum1-1-silenced chromatin.

ChIP of Sir3p at HMRα in MATα sir2Δ h3Δ h4Δ SUM1-1 yeast (LRY1450) transformed with plasmids containing WT H3 (pDM18), H3 E73G (pLR615), and H3 K4I (pLR619). Plus sign indicates addition of antibody (+ anti-Sir3). Primers are spaced ~1kb across HMRα, and enrichment values are relative to PHO5, which is not associated with Sum1-1p. Significance values (p<0.006) were calculated relative to +H3 strains.
4.4 Discussion

The observation that the LRS/H4 tail region of the nucleosome is important for Sum1-1 silencing raises the possibility that Sir3p could be involved in Sum1-1 silencing since it binds to this region. In \textit{sir3}\textendash \textit{SUM1-1} strains, histone residues in the LRS/H4 tail region are not disruptive to Sum1-1 silencing. Additionally, in \textit{sir2}\textendash \textit{SUM1-1} strains, Sir3p is enriched across \textit{HMRa} and a LRS mutation decreases Sir3p enrichment across \textit{HMRa}. These results are consistent with Sir3p binding this region of the nucleosome in the context of Sum1-1 silencing, which may or may not contribute to silencing.

Sir3p may be beneficial when nucleosomes are favorable for interaction, but detrimental when histone mutations are present. In this model, histone mutations are disruptive to \textit{sir2}\textendash strains because Sir3p is present but cannot bind the nucleosome properly, which may cause disruption to the chromatin structure. Histone mutations do not disrupt \textit{sir3}\textendash strains because Sir3p is not present to participate in binding the nucleosome, so the histone residues are not as critical to Sum1-1 silencing. However, this model does not hold true in \textit{sir2}\textendash \textit{sir3}\textendash strains, which lack Sir3p yet show disruption of Sum1-1 silencing in the presence of histone mutations. Therefore, Sir3p does not appear to be detrimental to Sum1-1 silencing, but its contribution to Sum1-1 silencing remains to be determined.

Studying the effect of histone mutations on \textit{sir2}\textendash and \textit{sir2}\textendash \textit{sir3}\textendash \textit{SUM1-1} strains demonstrated that histone mutations are detrimental in the absence of \textit{SIR2}. Sir2p may
be making contributions to Sum1-1 silencing that bypass the requirement for interactions with histone residues, and the small amount of Sir2p observed at HMR-E may be enough to contribute to Sum1-1 silencing. Sir2p may interact with Orc1p at HMR-E and assist in the formation of a specialized chromatin structure important for silencing. Alternatively, Sir2p may assist Hst1p in deacetylation at HMRa. The exact target of Hst1p deacetylation is not known, and deacetylation of histones or proteins within the Sum1-1 complex may be required for silencing. Further experiments with catalytically inactive SIR2 could be conducted to determine if the catalytic activity of Sir2p contributes to Sum1-1 silencing. Sir2p may also act at a distance, and direct association of Sir2p at HMRa may not be necessary for its contribution to Sum1-1 silencing. This may explain why Sir2p is not significantly enriched at HMRa, yet its deletion appears to have an effect on Sum1-1 silencing.

While these results are consistent with a possible role for Sir2p and Sir3p in Sum1-1 silencing, they do not prove or disprove their involvement. Experiments in sir4Δ SUM1-1 strains were planned to alleviate the recruitment of Sir proteins to the silencers and test the effect of histone mutations on Sum1-1 silencing. Despite several attempts to generate a sir4Δ SUM1-1 background in which the effect of histone mutations could be examined, I was not successful. These strains had trouble mating as a result of the Sir deletions and utilized all available markers, so my options for successful crosses were more limited and complicated than usual. Studying Sum1-1 silencing in a sir2Δ sir3Δ
sir4Δ SUM1-1 strain would be another way to further investigate these models and
determine whether Sir proteins are required for, utilized in, or disruptive to Sum1-1
silencing. Sum1-1 silencing could be studied at a basic level in the absence of all Sir
proteins, and plasmids containing the Sir proteins could be added to address Sir protein
involvement in Sum1-1 silencing.
5. Conclusions and perspectives

These findings identified a role for histones H3 and H4 in Sum1-1 silencing. Mutations in the H3 LRS and H4 tail regions of the nucleosome are also disruptive to silencing at the mating type loci, telomeres and rDNA, which suggests a common role for the LRS/H4 tail region of the nucleosome in long-range silencing. While this region may facilitate the formation of a specialized chromatin structure through nucleosome-nucleosome interactions or serve as a common binding site for chromatin remodeling complexes, my work favors the idea that it serves as a binding site for silencing proteins.

The BAH domain of Sir3p interacts with the LRS/H4 tail region of the nucleosome to facilitate Sir silencing (Buchberger et al. 2008; Norris et al. 2008; Onishi et al. 2007; Sampath et al. 2009). I investigated if the primary role of the LRS/H4 tail region in Sum1-1 silencing is to interact with the BAH domain of Orc1p, which has been shown to be required for Sum1-1p silencing (Rusche and Rine 2001). When Sum1-1p was recruited to HMRa independently of ORC, the BAH domain of Orc1p was still required for silencing. Additionally, mutations in ORC1 predicted to decrease the affinity of the BAH domain for nucleosomes also disrupted silencing. Therefore, Orc1p contributes to Sum1-1 silencing at a step other than recruitment and may interact with the LRS/H4 tail region of the nucleosome. Studies in the yeast K. lactis, which lacks a distinct Sir3p, also support a role for Orc1p acting in a Sir3p-like manner to facilitate silencing through interactions with the nucleosome (Hickman and Rusche 2010).
Therefore, the LRS/H4 tail region of the nucleosome appears to serve as a common interaction surface with silencing proteins.

Orc1p could contribute to Sum1-1 silencing in a similar manner to Sir3p, where it facilitates spreading through interactions with the LRS/H4 tail region of the nucleosome. However, the data are not consistent with this model. I found that the enrichment of Sum1-1p was only moderately decreased in strains containing mutations in the Orc1p BAH domain or a LRS histone mutant. Additionally, Orc1p was not uniformly distributed across \textit{HMRa} and was localized to the E silencer. Therefore, Orc1p does not appear to spread across \textit{HMRa} in Sum1-1 silencing.

The presence of Orc1p at the E silencer may promote transcriptional silencing by recruiting factors that make the region less permissive for transcription. Based on the role of ORC in Sir silencing in \textit{S. cerevisiae}, it is thought to serve as a platform for recruiting heterochromatin proteins and is associated with heterochromatin in a variety of species. In \textit{Drosophila} and humans, ORC interacts with HP1 (AUTH \textit{et al.} 2006; LIDONNICI \textit{et al.} 2004; PAK \textit{et al.} 1997; PRASANTH \textit{et al.} 2004) and is enriched in telomeric and pericentromeric heterochromatin (DENG \textit{et al.} 2007; DENG \textit{et al.} 2009; PRASANTH \textit{et al.} 2010; SHEN \textit{et al.} 2010). ORC may also help to organize chromatin into a specialized structure through its ability to wrap DNA (CLAREY \textit{et al.} 2008). This property may facilitate a more compacted chromatin structure that favors the silenced state.
The current model of Sum1-1 silencing suggests that ORC binding sites at the E and I silencers recruit Sum1-1p to HMRa (Sutton et al. 2001). However, in the absence of RFM1 and HST1, Sum1-1p is only enriched at HMR-E (Lynch et al. 2005). Additionally, I have shown that Orc1p is recruited to HMRa in a Sum1-1p-dependent manner in the absence of ORC binding sites, but Orc1p is only enriched at HMR-E. I propose a new model for Sum1-1 silencing that utilizes HMR-E as the primary recruitment site of Sum1-1p. HMR-I may stabilize the formation of a specialized chromatin structure or serve to recruit other factors that block transcription, but it does not appear to directly recruit silencing proteins.

The ability of Sum1-1p to spread across HMRa is still unclear. Orc1p does not appear to contribute to the spreading ability of Sum1-1p, and my results were not consistent with a role for Sum1p as a histone binding protein. An important interaction between Sum1p and histones would likely participate in Sum1p repression and Sum1-1 silencing, but histone mutations only disrupt Sum1-1 silencing. Given that the distance across the locus is relatively small, Sum1-1p enrichment across HMRa may not be due to a linear spreading of Sum1-1p through interactions with nucleosomes. Sum1-1p has a higher affinity for interactions with ORC and itself (Rusche and Rine 2001; Safi et al. 2008; Sutton et al. 2001). Self-interaction of Sum1-1p in a specialized chromatin structure could facilitate Sum1-1p association across HMRa. Sum1-1p AT hook motifs and a compacted chromatin structure could stabilize these associations.
While Sum1-1p is enriched across HMRa, the presence of Sum1-1p is not enough to allow for transcriptional silencing. In addition to the assembly of silencing proteins, the formation of a specialized chromatin structure is probably needed to achieve full silencing of the mating type loci. Recent studies suggest that the H3 tail contributes to the formation of this specialized chromatin in Sir silencing (Sperling and Grunstein 2009), since Sir proteins can assemble in the absence of the H3 tail, but silencing is disrupted. I have shown that the H3 tail makes important contributions to Sum1-1 silencing, as mutations in the H3 tail disrupt Sum1-1 silencing even though robust enrichment of Sum1-1 is seen in H3-K4I strains. Additionally, Sir2p appears to restore Sum1-1 silencing in the presence of H3 LRS and H4 tail mutations, while H3 tail mutations are always disruptive to Sum1-1 silencing. These results are consistent with a role for the H3 tail in higher order chromatin compaction after the proper assembly of silencing proteins to achieve complete silencing.

These studies have shown that histones H3 and H4 contribute to Sir and Sum1-1 silencing complexes in a similar fashion, and do not contribute to Sum1 repression. This result is in contrast to the Tup1-Ssn6 co-repressor complex in S. cerevisiae, which is disrupted by histone mutations. Unlike Tup1-Ssn6, the Sum1 complex may not require a stabilizing interaction with histones to achieve repression. However, the deacetylase activity of Hst1p is required for Sum1 repression, and it has been assumed that histones are the relevant substrate of Hst1p. Given that mutations mimicking the acetylated state
did not disrupt repression, the key substrate of Hst1p may not be histone H3 or H4, and Hst1p may deacetylate other proteins such as histones H2A and H2B or the Sum1 complex.

In conclusion, I have shown that the LRS/H4 tail region of the nucleosome serves as a common interaction surface for silencing proteins, while the H3 tail may facilitate the formation of a specialized chromatin structure required to achieve complete silencing.
## Appendix A

Table 4: Strains used in this study

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Table 6: Oligonucleotides used in this study

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Table 7: Plasmids obtained in the genetic screen for Sum1-1 silencing

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<td><strong>71</strong></td>
<td><strong>31</strong></td>
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*a H3 K4R was isolated 3 independent times
*b H3 E73G was isolated 2 independent times
Plasmids containing 1 to 6 amino acid mutations were recovered in a screen. For each plasmid, individual histone mutations were tested until a mutation or combination of mutations could be identified that contributed a greater than 10-fold loss of silencing.
Table 8: Histone mutations that disrupt Sum1-1 silencing

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<th>Plasmid</th>
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<th>Phenotype</th>
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14 histone H3 and 8 histone H4 mutations were found to disrupt Sum1-1 silencing by more than 10-fold. The observed decrease in mating is shown, along with the method of identification for each mutation.
### Table 9: Histone H3 mutations that do not disrupt Sum1-1 silencing

<table>
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<th>Plasmid</th>
<th>Mutation</th>
<th>Phenotype</th>
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</tr>
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<td>WT</td>
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<tr>
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<td>JD-2A4</td>
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<td>JD-2A7</td>
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</table>

49 histone H3 residues were found to maintain Sum1-1 silencing. The observed decrease in mating as determined using a reporter mating assay is shown, along with the method of identification for each mutation.
Table 10: Histone H4 mutations that do not disrupt Sum1-1 silencing

<table>
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<tr>
<th>Plasmid</th>
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25 histone H4 residues were found to maintain Sum1-1 silencing. The observed decrease in mating as determined using a reporter mating assay is shown, along with the method of identification for each mutation.
Figure 25: Summary of Sum1p secondary structure and globular predictions.

The protein sequence of Sum1p identifies regions likely to form alpha helicies (red) and beta sheets (green) based on secondary structure prediction from eight programs. The program Globplot was used to identify regions likely to be disordered (dashed lines) and globular (solid lines). Numbers indicate the amino acid sequence and arrowheads indicate sites of Sum1p N-terminal truncation designed with conservation of predicted structure in mind. Yellow areas highlight the conserved AT hooks.
Figure 26: Multiple sequence alignment of *S. cerevisiae* and 10 related yeast species.

Clustal X2 was used to align Sum1p regions of conservation in 10 yeast species.
References


DENG, Z., J. NORSEEN, A. WIEDMER, H. RIETHMAN and P. M. LIEBERMAN, 2009 TERRA RNA binding to TRF2 facilitates heterochromatin formation and ORC recruitment at telomeres. Mol Cell 35: 403-413.


DUCKER, C. E., and R. T. SIMPSON, 2000 The organized chromatin domain of the repressed yeast a cell-specific gene STE6 contains two molecules of the corepressor Tup1p per nucleosome. EMBO J 19: 400-409.


KIM, J., J. DANIEL, A. ESPEJO, A. LAKE, M. KRISHNA et al., 2006 Tudor, MBT and chromo domains gauge the degree of lysine methylation. EMBO Rep 7: 397-403.


LENFANT, F., R. K. MANN, B. THOMSEN, X. LING and M. GRUNSTEIN, 1996 All four core
histone N-termini contain sequences required for the repression of basal

LI, B., M. CAREY and J. L. WORKMAN, 2007 The role of chromatin during transcription.

LI, B., and J. C. REESE, 2001 Ssn6-Tup1 regulates RNR3 by positioning nucleosomes and
affecting the chromatin structure at the upstream repression sequence. J Biol
Chem 276: 33788-33797.

LI, S. S., 2000 Structure and function of the Groucho gene family and encoded
transcriptional corepressor proteins from human, mouse, rat, Xenopus,

LIDONNICI, M. R., R. ROSSI, S. PAIXAO, R. MENDOZA-MALDONADO, R. PAOLINELLI et al.,
2004 Subnuclear distribution of the largest subunit of the human origin

LIEB, J. D., X. LIU, D. BOTSTEIN and P. O. BROWN, 2001 Promoter-specific binding of Rap1
revealed by genome-wide maps of protein-DNA association. Nat Genet 28: 327-
334.

Yeast histone H3 and H4 amino termini are important for nucleosome assembly
in vivo and in vitro: redundant and position-independent functions in assembly

LIOU, G. G., J. C. TANNY, R. G. KRUGER, T. WALZ and D. MOAZED, 2005 Assembly of the
SIR complex and its regulation by O-acetyl-ADP-ribose, a product of NAD-

LIVI, G. P., J. B. HICKS and A. J. KLAR, 1990 The sum1-1 mutation affects silent mating-


POKHOLOK, D. K., C. T. HARBISON, S. LEVINE, M. COLE, N. M. HANNETT et al., 2005
Genome-wide map of nucleosome acetylation and methylation in yeast. Cell 122:
517-527.

Human Orc2 localizes to centrosomes, centromeres and heterochromatin during
chromosome inheritance. EMBO J 23: 2651-2663.

PRASANTH, S. G., Z. SHEN, K. V. PRASANTH and B. STILLMAN, 2010 Human origin
recognition complex is essential for HP1 binding to chromatin and

RAISNER, R. M., P. D. HARTLEY, M. D. MENEGHINI, M. Z. BAO, C. L. LIU et al., 2005 Histone
variant H2A.Z marks the 5’ ends of both active and inactive genes in

RECHT, J., B. DUNN, A. RAFF and M. A. OSLEY, 1996 Functional analysis of histones H2A
and H2B in transcriptional repression in Saccharomyces cerevisiae. Mol Cell Biol
16: 2545-2553.

REDD, M. J., M. B. ARNAUD and A. D. JOHNSON, 1997 A complex composed of tup1 and


RINE, J., and I. HERSKOWITZ, 1987 Four genes responsible for a position effect on

RIPPE, K., A. SCHRADER, P. RIEDE, R. STROHNER, E. LEHMANN et al., 2007 DNA sequence-
and conformation-directed positioning of nucleosomes by chromatin-remodeling
ROBERT, F., D. K. POKHOLOK, N. M. HANNETT, N. J. RINALDI, M. CHANDY et al., 2004
Global position and recruitment of HATs and HDACs in the yeast genome. Mol Cell 16: 199-209.


VARANASI, U. S., M. KLIS, P. B. MIKESSELL and R. J. TRUMBLY, 1996 The Cyc8 (Ssn6)-Tup1 corepressor complex is composed of one Cyc8 and four Tup1 subunits. Mol Cell Biol 16: 6707-6714.


Biography

Eugenia Christine Tsamis Prescott was born in Ft. Lauderdale, FL on March 19, where she was raised until moving to Cedar Rapids, IA at the age of 13. Upon graduation as a valedictorian of John F. Kennedy High School in 2001, she attended the University of Northern Iowa as a recipient of the James W. Kercheval Scholarship for her achievements in Chemistry. Eugenia was a member of the American Chemical Society, National Society of Collegiate Scholars and Phi Eta Sigma during her time at UNI, and graduated Summa Cum Laude with a Bachelor of Science in Chemistry, a Biochemistry emphasis and a minor in Biology in 2005.

Following graduation, she pursued a degree in Biochemistry at Duke University. She joined the laboratory of Dr. Laura Rusche in the Department of Biochemistry and Institute for Genome Sciences and Policy. Eugenia has presented numerous posters and talks throughout her graduate career, winning outstanding poster presentations in 2006 and 2009 at the Duke Biochemistry Departmental Retreat. In addition to completion of the Doctor of Philosophy in Biochemistry at Duke University, she completed a Certificate in Teaching College Biology and the Preparing Future Faculty Program.