Gene Duplication and the Evolution of Silenced Chromatin in Yeasts

by

Meleah A. Hickman

University Program in Genetics and Genomics
Duke University

Date:_____
Approved:

_____
Laura Rusche, Advisor

_____
Paul Magwene, Chair

_____
Fred Dietrich

_____
Joe Heitman

_____
Beth Sullivan

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the
University Program in Genetics and Genomics in the
Graduate School of Duke University

2010

ABSTRACT

Gene Duplication and the Evolution of Silenced Chromatin in Yeasts

by

Meleah A. Hickman

University Program in Genetics and Genomics
Duke University

Date:_____
Approved:

_____
Laura Rusche, Advisor

_____
Paul Magwene, Chair

_____
Fred Dietrich

_____
Joe Heitman

_____
Beth Sullivan

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics and Genomics in the Graduate School of Duke University

2010

Abstract

In *Saccharomyces cerevisiae*, proper maintenance of haploid cell identity requires the SIR complex to mediate the silenced chromatin found at the cryptic mating-type loci, *HML* and *HMR*. This complex consists of Sir2, a histone deacetylase, and the histone binding proteins Sir3 and Sir4. Interestingly, both Sir2 and Sir3 have paralogs from a genome duplication that occurred after the divergence of *Saccharomyces* and *Kluyveromyces* species, approximately 100 million years ago. The histone deacetylase *HST1* is the paralog of *SIR2* and works with the promoter-specific *SUM1* complex to repress sporulation and alpha-specific genes. *ORC1* is the paralog of *SIR3* and is an essential subunit of the Origin Recognition Complex and also recruits SIR proteins to the *HM* loci. I have investigated the functions of these proteins in the non-duplicated species *Kluyveromyces lactis* and compared these functions to those found in *S. cerevisiae*.

I have shown that *SIR2* and *HST1* subfunctionalized post-duplication via the duplication, degeneration and complementation mechanism. In *S. cerevisiae*, Sir2 and Hst1 have non-overlapping functions in a wild-type strain background, yet Sir2 has retained the ability to function like Hst1 and contribute to the repression of sporulation genes in an *hst1*Δ strain. I have also shown, with a chimeric Sir2-Hst1 protein, that distinct specificity domains have diverged between Sir2 and Hst1 that specify a Sir2 interaction with the SIR complex and an Hst1 interaction with the SUM1 complex. Trans-species complementation assays show that the non-duplicated Sir2 from *K. lactis* can interact with both SIR and SUM1 complexes in *S. cerevisiae*.

Further analysis into the non-duplicated experimental system of *K. lactis* has revealed that KlSir2 functions like both ScSir2 and ScHst1 as deletion of *KlSIR2* de-represses the *HM* loci as well as sporulation and cell-type specific genes. A physical

interaction between KlSir2 and the histone binding protein KlSir4 is conserved in *K. lactis*, and both proteins spread across the *HML* locus and associate with telomeres in a manner similar to *S. cerevisiae*. KlSir2 also physically interacts with the DNA-binding protein, KlSum1, to repress sporulation and cell-type specific genes in a promoter-specific manner and recruitment of KlSir2 to these loci is dependent on KlSum1. Surprisingly, deletion of *KlSUM1* also de-represses *HML* and *HMR*, a phenotype not observed in *S. cerevisiae*. I show by chromatin immunoprecipitation that KlSum1 directly regulates the *HM* loci in *K. lactis* by spreading across these regions in a mechanism that is distinct from its role in repressing sporulation-specific genes. This result indicates that KlSum1 is a key regulator of not only meiotic, but also mating-type transcriptional programming.

The *SIR3-ORC1* gene pair has previously been used as an example of neofunctionalization based on accelerated rates of evolution. However, my studies of KlOrc1 show it is distributed across *HML* and associates with Sir2 and Sir4 at telomeres, indicative of it having Sir3-like capabilities to spread across chromatin. This ability of KlOrc1 to spread is distinct from its functions with ORC, and is entirely dependent on it's BAH domain, a domain which is capable of binding histones in *S. cerevisiae* both in vivo and in vitro. These findings demonstrate that prior to the genome duplication there was a silencing complex that contained both KlSir2 and KlOrc1. In addition to their functions at *HML* and the telomeres, KlOrc1 associates with replication origins and KlSir2 and KlSum1 work in complex to repress sporulation genes in a promoter-specific manner. The multiple functions of both KlOrc1 and KlSir2 in *K. lactis* indicate that after duplication, these properties were divided among paralogs and subsequently specialized to perform the functions that have been characterized in *S. cerevisiae*.

Contents

List of Tables

List of Figures

1. Introduction

1.1 Overview of heterochromatin

Heterochromatin was originally described cytologically in Drosophila as regions of highly condensed chromatin that were maintained throughout the cell cycle (Schultz 1936). Heterochromatin, or silenced chromatin, has since been identified in eukaryotes ranging from yeast, maize, nematodes, and vertebrates and has important functions in transcriptional gene repression and chromosome structure. Extensive studies investigating centromeres, telomeres and sex chromosomes have greatly contributed to the current understanding of heterochromatin.

At the molecular level, heterochromatin is comprised of close associations between nucleosomes and histone-binding proteins. A common theme in heterochromatin across diverse groups of organisms is the coupling of post-translational histone modifications with proteins that specifically bind to nucleosomes bearing those particular modifications (reviewed in (Moazed 2001)). Heterochromatic histone binding proteins often form a complex with a histone-modifying enzyme such that a self-reinforcing loop is established to maintain the heterochromatic state. Among eukaryotes there are two unrelated groups of proteins that comprise such complexes. In most eukaryotes, including the fission yeast, fruit fly and mammals, heterochromatin is defined by histone H3 lysine 9 methylation, and binding by HP1 chromodomain proteins (Elgin and Grewal 2003). However, in budding yeasts, including *Saccharomyces cerevisiae*, silenced chromatin is achieved through histone H4 lysine 16 deacetylation and binding by the SIR proteins (Rusche et al. 2003). Understanding how the SIR proteins have evolved to participate in silenced chromatin formation is a central theme of this thesis.

1.1.1 SIR silencing in S. cerevisiae

1.1.1.1 Genomic regions subject to silencing

SIR silencing is responsible for repressing the *HM* loci and telomeres (reviewed in (Rusche et al. 2003)). A specialized chromatin structure at the *HM* loci is required to maintain cell identity in a haploid cell. *S. cerevisiae* has a three locus mating system: one active *MAT* locus and two cryptic *HM* loci, *HMLα* and *HMR***a**. The *MAT* locus can contain either **a** or *α* information and dictates haploid cell identity whereas *HMLα* and *HMR***a** serve as repositories for mating type information and were first identified as loci conferring homothallism (Takahashi 1958). These repositories need to be transcriptionally inactive to maintain a specific haploid cell identity and ability to mate. In order to silence the alleles at the *HM* loci, without inactivating the identical *MAT* allele, a sequence independent mechanism of repression is required and in *S. cerevisiae* is achieved through the SIR complex. The role of SIR silencing at the telomeres is thought to serve a more structural role, rather than transcriptional, although reporter genes as well as a few endogenous genes in the subtelomeric regions of the genome are subjected to SIR-mediated transcriptional repression (Gottschling et al. 1990).

In addition to the *HM* loci and the telomeres, silencing also occurs in the rDNA array, however it is achieved through a distinct mechanism, involving the RENT complex. Sir2 is also part of the RENT complex, which modulates chromatin structure in the rDNA repeats (Straight et al. 1999). The RENT complex does not contain the other Sir proteins and its mechanism of action is less well understood and not the focus of this work.

1.1.1.2 Establishment of silencing

The establishment of SIR-mediated silencing requires cis-acting elements. At the *HM* loci, these elements are termed silencers and include DNA binding sites for ORC (Origin Replication Complex), Rap1 and Abf1. ORC, specifically Orc1, interacts with Sir1, which also interacts with Sir4 to recruit Sir2 and Sir3 to the *HM* loci (Chien et al. 1993; Triolo and Sternglanz 1996; Gardner et al. 1999). Rap1 interacts with both Sir3 and Sir4 (Moretti et al. 1994) and Abf1 is thought to interact with Sir3 (Gasser and Cockell 2001). These interactions are summarized in Figure 1 (top panel). The telomeres recruit SIR proteins via multiple molecules of Rap1 binding to sites generated by the degenerate $TG_{1\text{-}3}$ repeat sequence (Longtine et al. 1989) but do not have "silencers" as found at the *HM* loci. Sir1 does not associate with telomeres (Aparicio et al. 1991). Elsewhere in the genome, single Rap1 binding sites not associated with ORC or independent ORC binding sites do not recruit SIR proteins, and SIR protein association is restricted to the *HM* loci and the telomeres.

1.1.1.3 Mechanism of silencing

Once established, the mechanism behind regional, spreading silencing in *S. cerevisiae* is considered to be a process of sequential deacetylation as illustrated in Figure 1 (Rusché et al. 2002). Only Sir2, Sir3, and Sir4 proteins spread along the chromosome (Hoppe et al. 2002a; Luo et al. 2002a; Rusché et al. 2002). Sir2 is a NAD^+-dependent deacetylase, and its enzymatic activity is required for the spreading of all three SIR proteins. Sir3 and Sir4 bind preferentially to deacetylated tails of histones H3 and H4 (Hecht et al. 1995; Carmen et al. 2002). It is thought that Sir2 deacetylates the neighboring nucleosome, creating new high affinity binding sites for Sir3 and Sir4, which in turn recruit additional Sir2 to the newly deacetylated nucleosome. Sir2 and

Sir4 form a close association, independent of Sir3 (Moazed et al. 1997; Cockell et al. 2000; Hoppe et al. 2002a). Furthermore, both Sir3 and Sir4 self-associate (Chien et al. 1993; Moretti et al. 1994; Enomoto et al. 2000), which is a property, that facilitates spreading. As the Sir proteins spread, they generate a specialized chromatin structure that is restrictive to transcription and independent of DNA sequence. Mutations in *SIR2*, *SIR3* or *SIR4*, while not lethal, will abolish silencing, lead to the loss of haploid identity and mating ability (Ivy et al. 1986; Rine and Herskowitz 1987). The consequences of disrupting SIR silencing at the telomeres are less well understood.

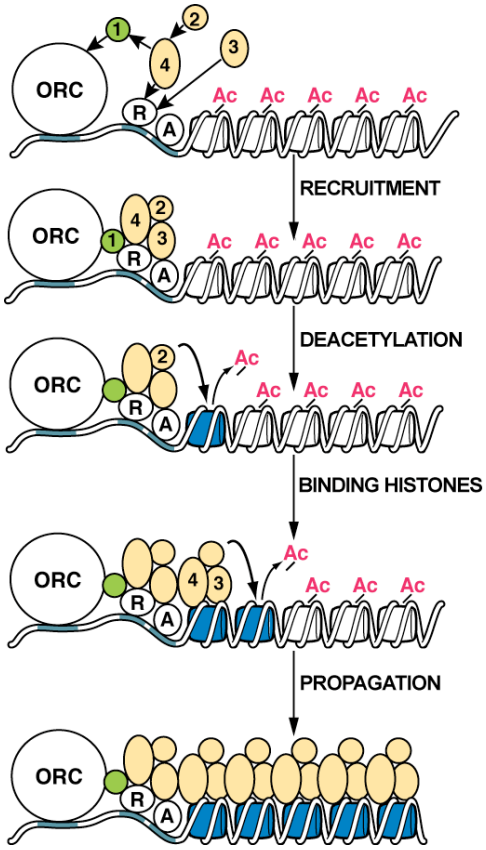


Figure 1: Mechanism of SIR silencing.
ORC: Origin Recognition Complex, R: Rap1, A: Abf1, 1-4: Sir1, Sir2, Sir3 and Sir4, respectively.

1.1.2 "Classical" silencing in *S. pombe*

The fission yeast, *Schizosaccharomyces pombe*, also contains a three-locus mating-type architecture, and must silence two of the three loci to maintain haploid cell identity (Egel 1981). While, the mechanism of silencing is well conserved between these two species, in that a histone-modifying enzyme coupled to a histone-binding protein facilitates spreading, the specific silencing factors do not overlap. Likewise, the establishment of silencing is quite different in the two species.

The silencer element that is responsible for recruiting silencing factors to the extra MAT loci consists of binding sites for the silencer binding proteins, atf1 and pcr1, (Jia et al. 2004) and are not related to the silencer binding proteins, ORC, Abf1 and Rap1, found in *S. cerevisiae*. There is another type of sequence that is responsible for recruiting silencing factors in *S. pombe*, cenH, which is found in outer centromeric repeat sequences (Ayoub et al. 2000; Volpe et al. 2003). Recruitment of silencing factors through cenH involves a protein-RNA complex known as RITS (RNA-induced transcriptional silencing) that is related to the RISC post-translational silencing complex (Noma et al. 2004; Verdel et al. 2004). RITS contain siRNA derived from the centromeric repeat sequences as well as an argonaute protein (ago1), the key protein in RISC complexes.

Two important silencing factors in *S. pombe* are a methyltransferase, clr4, which specifically methylates lysine 9 of histone H3, and a chromodomain protein, swi6, which binds preferentially to H3 tails that are methylated on lysine 9 (Ekwall et al. 1996; Nakayama et al. 2000). These silencing proteins are thought to spread by a sequential modification mechanism akin to that proposed for *S. cerevisiae* SIR proteins. Specifically, clr4 is recruited through silencer and cenH sequences and targeted to methylate lysine 9 of histone H3 in adjacent nucleosomes (Ivanova et al. 1998). These methylated

nucleosomes are then bound by swi6, which in turn recruits additional clr4. In addition, clr4 and swi6 form silenced chromatin at the telomeres and centromeres by a similar mechanism and the pericentric heterochromatin is important for proper centromere function.

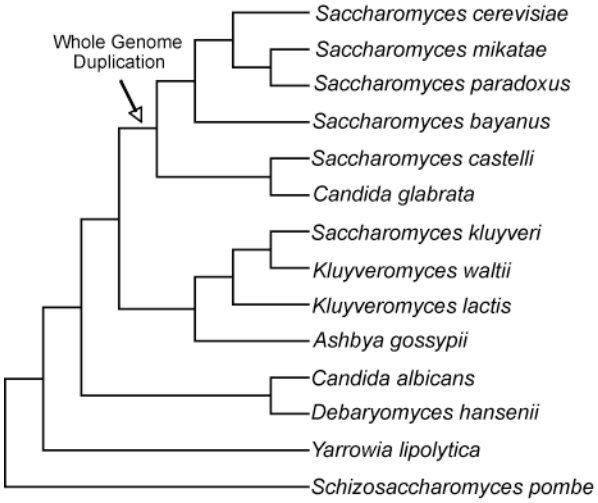
1.1.3 Conservation of silencing factors

Most of the factors known to contribute to the silencing in *S. pombe* are well conserved among eukaryotes and also contribute to the formation of heterochromatic structures, notably at the pericentromeric regions. The homolog of swi6 is HP1 (heterochromatin protein 1) in Drosophila and mammals, and clr4 is homologous to SuVar3-9 (reviewed in (Huang 2002)). The RNAi proteins that act in silencing, argonaute and dicer, are also well conserved in Drosophila and mammals. However, none of these heterochromatin proteins have identifiable homologs in *S. cerevisiae*, although homologs of the RNAi protein, argonaute, have been identified in the related budding yeast species, *C. albicans* and *S. castellii* (Drinnenberg et al. 2009). Additionally, *SIR1*, *SIR3* and *SIR4* do not have distinct homologs beyond the close relative of *S. cerevisiae*. Thus, the "classical" heterochromatin, as defined by HP1 and H3K9 methylation does not exist in *S. cerevisiae* and an alternative type of silenced chromatin involving the SIR proteins has evolved. My thesis centers around the evolution of the SIR-mediated silenced chromatin in *S. cerevisiae* and other budding yeast species.

1.2 Genomic features of S. cerevisiae and other hemiascomycete yeasts

S. cerevisiae belongs to the hemiascomycete class of fungi, which consists primarily of unicellular yeasts (Figure 2). Due to their relatively small genome sizes and relationship to the well-studied *S. cerevisiae*, many of these yeasts have been have been

sequenced, and a few are even being investigated experimentally, providing an exceptional opportunity for comparative genomics. Complete genome sequences for distantly related hemiascomycete species *Candida glabrata, Ashbya gossypii, Kluyveromyces lactis, Debaryomyces hansenii* and *Yarrowia lipolytica* and partial genome sequences for many other hemiascomycete fungi have made comparative genetics and genomics available to look at broad evolutionary patterns of genes and protein complexes, in addition to genomic architecture (Dujon et al. 2004).



Mating Loci	Sir2-Hst1	Sir4	Sir3-Orc1	Sir1	Sum1	Rfm1
3	++	+	++	+	+	+
3	++	+	++	+	+	+
3	++	+	++	+	+	+
3	++	+	++	+	+	+
3	++	+	++	-	++	++
3	++	+	++	-	+	+
1	+	+	+	-	+	+
2	+	+	+	-	+	+
3	+	+	+	-	+	+
3	+	+	+	-	+	+
1	+	-	+	-	-	-
1	+	-	+	-	-	-
1	+	-	+	-	-	-
3	+	-	+	-	-	-

Figure 2: Phylogeny of selected hemiascomycetes and conservation of SIR and SUM1 complexes.

Conservation of silencing factors in budding yeasts. (+) indicates if a gene is present, (-) indicates if a gene is absent and (++) indicates if both paralogs were retained after the whole genome duplication.

1.2.1 Whole-genome duplication

An important event in the evolution of the hemiascomycete clade is a whole genome duplication, which took place after the divergence of *Kluyveromyces* and

Saccharomyces species, approximately 100 million years ago, as illustrated in Figure 2. Hemiascomycete genomes display conserved gene order, or synteny, that has been widely observed between post-duplication species such as *S. cerevisiae* and *Candida glabrata* and non-duplication species, like *Ashbya gossypii* and *Kluyveromyces* species. A robust and widely accepted phylogeny has been determined, aided by the addition of several sequenced fungal genomes (Wong et al. 2002). It is unclear whether this polyploidization resulted from an allo- or auto-tetraploidy event (Liti and Louis 2005). Regardless of the mechanism of polyploidization, subsequent to this whole-genome duplication, most of the duplicated genes returned to a single-copy status through degeneration and loss of one copy. As a result, only about twelve percent of duplicated pairs have been retained. This massive loss of genetic material is thought to have occurred before the divergence of *S. castellii* and *S. cerevisiae* (Cliften et al. 2006). Interestingly, and central to this thesis, two of the SIR proteins, Sir2 and Sir3, have retained paralogs resulting from this genome duplication, Hst1 and Orc1, respectively.

1.2.1.1 Fates of duplicated genes

Gene duplication provides a source of new genetic material that is free of selective constraint and can evolve novel functions, but, at the same time, gene duplication provides genetic robustness against deleterious mutations through redundant function. Many studies have looked on a global scale on the impact of the whole-genome duplication, but few have taken an experimental approach to investigate how specific duplicated gene pairs have diverged over the course of evolution. Furthermore, such studies have generally looked at the role of the duplicated genes in overall fitness and have not taken into consideration the specific biological process those genes govern.

Duplicated genes have two ultimate fates: degeneration or preservation in the genome. Degeneration can occur when one of the duplicates acquires an inactivating mutation, degenerates into a pseudogene, and subsequently is lost, resulting in a return to a single-copy status. Preservation of a duplicated gene pair can result in one of three functional outcomes: conservation, neo-functionalization and sub-functionalization (Ohno 1970). Conservation, i.e. having multiple copies with no functional divergence, is important for gene dosage and genetic redundancy. Neo-functionalization occurs when one duplicate gene evolves a new function by acquiring new, beneficial mutations, while the other gene retains the original function. In such a case, it is predicted that the gene with the new function will experience a more rapid change in sequence, i.e. "accelerated evolution," compared with the duplicate retaining the original function.

An alternative paradigm in the preservation of duplicated genes has been proposed to account for the much larger retention of duplicate genes than the neo-functionalization model would predict. Simply stated, if the ancestral gene had multiple, independent functions, then upon duplication, those functions can be partitioned between the duplicates, in a model termed sub-functionalization (reviewed in (Conant and Wolfe 2008; Hahn 2009)). There are a variety of ways a duplicated gene can sub-functionalize. The model of duplication degeneration and complementation (DDC), states that duplicated genes could each lose one of the original functions and together retain the entire set of ancestral functions (Force et al. 1999; Lynch and Force 2000). This is a popular model because it does not require any adaptive mutations to occur for selection to act upon. This model has been used not only to study divergence in protein functions, but also patterns of gene expression among gene duplicates.

Another type of sub-functionalization, specialization, is often difficult to distinguish from neo-functionalization (Conant and Wolfe 2008; Hahn 2009). Specialization occurs after the divergence of functions among paralogs and is the result of accumulation of advantageous mutations in at least one of the duplicated genes that allows it to out-perform the ancestral function. Also discussed as escape from adaptive conflict (Lynch and Katju 2004) or sub-neo-functionalization (He and Zhang 2005), the signature of positive selection at the sequence level makes this model difficult to distinguish from neo-functionalization and requires experimental investigation, of which there have been few (Hittinger and Carroll 2007; Des Marais and Rausher 2008).

It has been difficult to resolve these models experimentally because determining "non-duplicated" function is often elusive. Looking experimentally at *both* non-duplicated and duplicated functions within existing biological systems provides a unique opportunity to gain a comprehensive understanding of duplicate gene preservation. To investigate how the duplicated gene pairs, Sir2-Hst1 and Sir3-Orc1 have diverged, I have characterized the function of a representative non-duplicated Sir2 and Orc1 from the yeast species, *Kluyveromyces lactis*.

1.2.2 Three locus mating-type architecture

All of the hemiascomycetes contain MAT alleles that determine mating type. However, only some possess additional, silenced copies of mating-type cassettes, which allow for switching of mating type (Figure 2). *Y. lipolytica* and *C. albicans* contain only a single mating-type locus. *D. hansenii* also has a single mating type locus, although this locus contains both **a**- and α-genes (Fabre et al. 2005). It is not known if these genes are alternatively expressed, which would seem to be necessary for haploid-identity and mating. *K. waltii* has only two identified mating-type loci on one chromosome. *A.*

gossypii, *K. lactis*, *C. glabrata* and all of the closely related *sensu stricto Saccharomyces* species all have three mating-type loci. Only in th*e sensu stricto* species are all three mating-type loci on the same chromosome. However, in the hemiascomycete species with three mating-type loci, two of the three loci are located near telomeres (Fabre et al 2004).

1.3 Silencing proteins

1.3.1 Sir2

Deacetylases of the Sir2 family are key regulators of lifespan and stress resistance in many organisms ranging from yeast to humans. These enzymes couple deacetylation with hydrolysis of NAD^+ and consequently their activity is linked to the metabolic state of the cell. These enzymes have a highly conserved catalytic core domain and variable terminal extensions. The distinct and variable functions of Sir2 family members are a result of multiple duplication events with subsequent diversification of substrates and functions. In *S. cerevisiae*, there are five NAD+ dependent deacetylases: Sir2, the founding member of the entire family, Hst1, Hst2, Hst3 and Hst4 (Brachmann et al. 1995; Derbyshire et al. 1996; Smith et al. 2000). Hst2 is a predominantly cytoplasmic protein (Perrod et al. 2001), but may have a cell cycle specific nuclear localization (Vaquero et al. 2006; Wilson et al. 2006). Hst3 and Hst4 deacetylate lysine 56 on histone H3 and are involved in cell cycle and DNA damage checkpoints that modulate chromatin, enabling replication and condensation to occur properly (Brachmann et al. 1995; Celic et al. 2006; Maas et al. 2006). Of these five NAD+ dependent deacetylases in *S. cerevisiae*, *HST1* is the most closely related to *SIR2*.

1.3.1.1 Sir2 and silencing

Sir2 has been most extensively studied in regards to its function in forming silenced chromatin with the SIR complex. The NAD^+-dependent enzymatic activity is essential for SIR associated silenced chromatin (Hoppe et al. 2002b; Luo et al. 2002b; Rusché et al. 2002). In vitro, Sir2 deacetylates histone H4 lysine 16 and lysines 9 and 14 of H3 (Imai et al. 2000). The requirement for Sir2 deacetylase activity can be partially bypassed if histone H4 lysine 16 is mutated to arginine, which mimics the hypoacetylated state (Yang and Kirchmaier 2006). However, a byproduct of the Sir2 NAD+-dependent deacetylase reaction, 2'-O-acetyl-ADP ribose, may be important for silenced chromatin, as it induces a conformational change in the Sir2-Sir3-Sir4 complex in vitro (Liou et al. 2005), although, others have shown that histone deacetylation, but not 2'-O-acetyl-ADP ribose, is necessary for SIR-mediated silent chromatin (Chou et al. 2008).

Sir2 also interacts with the RENT complex, via an interaction with Net1 (Straight et al. 1999; Ghidelli et al. 2001). As part of this complex, Sir2 functions to reduce the level of recombination between rDNA repeats (Gottlieb and Esposito 1989) although this result has been challenged (Kobayashi et al. 2004) and it has been asserted that Sir2 is important for maintaining cohesin association with the rDNA and suppressing unequal sister chromatid exchange (Kobayashi et al. 2004; Kobayashi and Ganley 2005). Sir2 also represses reporter genes inserted into the rDNA array (Smith and Boeke 1997).

1.3.1.2 The Sir2-Hst1 duplicated gene pair

As mentioned previously, Sir2 and Hst1 arose in the whole genome duplication. Overall sequence conservation between Sir2 and Hst1 is 63% (76% similar) (Brachmann et al. 1995) with three conserved regions: the well conserved catalytic core domain with

82% sequence identity (92% similarity), and lesser-conserved regions in the N-terminus and the extreme C-terminal tail (Cockell et al. 2000). Despite their sequence similarity, Hst1 and Sir2 have non-overlapping functions in wild-type strains (Brachmann et al. 1995; Derbyshire et al. 1996; McCord et al. 2003). However, in the absence of one deacetylase, the other can fulfill both functions (Derbyshire et al. 1996; Hickman and Rusche 2007) and Sir2 can acquire Hst1 function with the mutation of just two amino acids in its C-terminus (Mead et al. 2007).

1.3.1.3 Hst1 and SUM1 repression

In contrast to Sir2 and its role in SIR-mediated silencing, Hst1 is found in the promoter-specific SUM1 complex, which contains two other proteins, Sum1 and Rfm1. The SUM1 complex represses over fifty genes that are involved in sporulation, NAD^+ biosynthesis, and α-cell identity . Sum1 is a DNA binding protein that associates with a conserved sequence, the middle sporulation element (MSE), found in the promoters of target genes (Xie et al. 1999; Bedalov et al. 2003; Pierce et al. 2003). Hst1 deacetylates histones H3 and H4, and this deacetylation is thought to be important for its repressive function (Robert et al. 2004; Hickman and Rusche 2007). Rfm1 is a small protein considered to serve an architectural role by associating with both Sum1 and Hst1 (McCord et al. 2003). There are homologs of both Sum1 and Rfm1 in most hemiascomycete species, except in the more distantly related *Candida* species (Figure 2). Both Sum1 and Rfm1 returned to single copy status after the WGD in most species except for in *S. castellii* (Byrne and Wolfe 2005), whereas the Hst1-Sir2 duplicated gene pair has persisted in all sequenced post-WGD species.

There are two noteworthy differences among phenotypes of *sum1*Δ, *rfm1*Δ and *hst1*Δ null mutations in *S. cerevisiae*. First, the subset of mid-sporulation genes

derepressed in these backgrounds differs. One group of genes requires Rfm1 and Hst1 in addition to Sum1; the other group requires only Sum1 for repression. Second, for genes that are repressed by both Sum1 and Hst1, there is a difference in the level of derepression of target loci between *sum1*Δ and *hst1*Δ strain backgrounds. Deletion of Sum1 results in a strong derepression of target mid-sporulation genes, whereas deletion of Hst1 results in a modest derepression of the same target mid-sporulation genes. It has been unclear what contributes to this difference in phenotypes, and is the subject of experimental investigation in presented in Chapter 2.

1.3.1.4 Sum1 and silencing

Sum1 was initially identified in a genetic screen searching for mutations that restore silencing in *sir*Δ strains (Klar et al. 1985). The mutant, Sum1-1, is a dominant allele, with a single amino acid substitution T988I and restores mating ability in *sir*Δ strains by forming an extended, repressive chromatin structure (Rusche and Rine 2001; Sutton et al. 2001). Sum1-1, like wild-type Sum1, works in complex with Rfm1 and Hst1 (Rusche and Rine 2001; Lynch et al. 2005), but is relocalized to the *HM* loci. The T988I mutation is simultaneously a gain-of-function and loss-of-function mutation, because it no longer associates with sporulation gene promoters and has a decreased affinity for the MSE and has acquired the ability to self-associate and to interact with ORC (Sutton et al. 2001; Lynch et al. 2005; Safi et al. 2008). Recently, wild-type Sum1 has been shown to bind at the *HML-E* silencer and perhaps plays a role in establishment of silencing at this locus (Irlbacher et al. 2005). In this work I show that wild-type Sum1, in *K. lactis*, is a key regulator of both sporulation specific genes as well the cryptic mating-type loci and participates with KlSir2 in both promoter-specific and long-range silencing mechanisms, accounting for the ability of a single amino acid change to confer silencing

1.3.2 Sir3

1.3.2.1 Sir3 and silencing

Sir3 is a structural component of the SIR complex and binds histones (Hecht et al. 1995; Carmen et al. 2002). This property likely facilitates SIR spreading along the chromosome, because in a *sir3*Δ strain, Sir2 and Sir4 are recruited to the silencers but do not spread (Hoppe et al. 2002b; Rusché et al. 2002). Sir3 is proposed to be the limiting factor of SIR silencing since overexpression of Sir3 leads to improvement of telomeric repression (Renauld et al. 1993). The N-terminus of Sir3 contains a BAH domain (bromo-adjacent homology, amino acids 1-214) that is essential for its silencing ability (Bell et al. 1995). Overexpression of this region is sufficient to improve telomeric silencing (Gotta et al. 1998). The BAH domain has recently been shown to interact with nucleosomes with a degree of specificity in vivo, and is important to forming higher-order SIR-nucleosome filaments in vitro (Onishi et al. 2007; Buchberger et al. 2008; Sampath et al. 2009). Histone H4 lysine 16 is required for Sir3 BAH-mediated interactions with nucleosomes (Onishi et al. 2007). The C-terminus of Sir3 mediates interactions with Sir4 (Moretti et al. 1994; Moazed et al. 1997; Park et al. 1998; Enomoto et al. 2000), Rap1 (Moretti et al. 1994) and histones H3 and H4 (Hecht et al. 1995).

1.3.2.2 The Sir3-Orc1 duplicated gene pair

Like Sir2, Sir3 has retained its paralog from the WGD, Orc1 (Kellis et al. 2004). Orc1 is an essential gene and the largest subunit of ORC (Origin Recognition Complex) (Bell et al. 1995), a multi-protein complex that associates with DNA replication origins. Unlike the Sir2-Hst1 duplicated gene pair, Sir3 and Orc1 are not well conserved. The most conserved region between these two proteins is the N-terminal BAH domain displaying 50% identity and 65% similarity and is structurally almost identical (Bell et al.

1995; Zhang et al. 2002; Connelly et al. 2006). These proteins have diverged to the extent that they cannot complement each other, even when overexpressed, although the BAH domains of Sir3 and Orc1 are interchangeable (Bell et al. 1995). Non-duplicated homologs of Sir3-Orc1 show more sequence similarity to Orc1 than to Sir3. This accelerated sequence divergence in Sir3 has led to the hypothesis that Sir3 function is the result of neo-functionalization (Kellis et al. 2004), although others have argued that the Orc1-Sir3 duplicated gene pair sub-functionalized via the DDC mechanism (van Hoof 2005).

1.3.2.3 Orc1 and silencing

Orc1 also has distinct functions in the formation of silenced chromatin in *S. cerevisiae*, in addition to its evolutionary connection to Sir3. Orc1, as part of ORC, binds to all four silencers found at *HML* and *HMR* (Rusche et al. 2003). The BAH domain of Orc1 is not vital for its functions in DNA replication, but is essential for its role in silencing (Bell et al. 1995). The BAH domain of Orc1 interacts with Sir1 (Triolo and Sternglanz 1996) to recruit the other Sir proteins. Recruitment of Sir1 to the silencers is thought to be the only function of ORC at the *HM* loci because when Sir1 is tethered to the silencer, the need for ORC at the silencers is bypassed (Triolo and Sternglanz 1996; Fox et al. 1997).

Interestingly, Orc1 has been implicated in the formation of heterochromatin in other species. Human and Drosophila Orc1 bind to HP1 (Pak et al. 1997; Lidonnici et al. 2004; Prasanth et al. 2004; Auth et al. 2006) and ORC has been shown to associate with telomeres (Deng et al. 2007; Deng et al. 2009), although the precise role that it has in heterochromatin is still unclear. In *Plasmodium falciparum,* the protozoan that causes

malaria, Orc1 and Sir2 work together to repress genes near the telomeres (Mancio-Silva et al. 2008). These studies indicate that Orc1 has a widely conserved role in silencing.

1.3.3 Sir4

Sir4 is perhaps the least understood of the spreading proteins. It is a structural component of the SIR complex that binds histones (Hecht et al. 1995). Sir4 also functions to bring Sir2 to the silent mating-type loci and telomeres through its interactions with Sir1 (Bose et al. 2004) and Rap1 (Moretti et al. 1994). Sir4 has experienced dramatic changes in its protein coding sequence. The one conserved feature of Sir4 is a C-terminal coiled-coil domain (Astrom and Rine 1998; Fabre et al. 2005), which is essential for silencing in *S. cerevisiae* (Chang et al. 2003; Murphy et al. 2003). Like Sir2 and Sir3, Sir4 is also a result of a duplication event. However, this duplication was not from the WGD, but is an ancient tandem duplication that occurred prior to the divergence of *Saccharomyces* and *Kluyveromyces* species (Fabre et al. 2005). The paralog of Sir4 is Asf2 (Anti-Silencing Factor 2), which is antagonistic to silencing when overexpressed (Le et al. 1997). The relationship between Sir4 and Asf2 has not been carefully investigated.

Homologs of Sir4 are found among some of the hemiascomycetes (Figure 2). However, unlike Sir2, the Sir4 identification is based solely on conserved synteny. The species most distant from *S. cerevisiae* in which Sir4 has been identified are *Kluyveromyces* and *Ashbya* species (Fabre et al. 2005). Sir4 could not be identified in *D. hansenii* based on synteny or homology (Fabre et al. 2005). However, given the poor sequence conservation, it is hard to be certain that an ortholog of Sir4 does not exist in *D. hansenii* or other, more distant species.

1.3.4 Sir1

Sir1 is the only non-spreading Sir protein and functions to recruit Sir2, Sir3 and Sir4 to the *HM* loci. Deletion of Sir1 does not completely abolish mating ability, as is observed with other Sir deletions, although there is a mating defect in a sub-population of cells (Pillus and Rine 1989). Sir1 interacts with both Sir4 and Orc1 (Triolo and Sternglanz 1996). The Orc1 interacting region (OIR) in the C-terminus of Sir1 has been extensively mapped and has been shown to interact with the BAH domain of Orc1 (Gardner et al. 1999; Hou et al. 2005; Hou et al. 2009). The N-terminus of Sir1 is highly similar to the OIR, but does not interact with the BAH domain of either Orc1 or Sir3, yet is important for silencing (Hou et al. 2009). Sir1 has the most limited distribution of homologs among the hemiascomycetes (Figure 2), only being present in the *Saccharomyces sensu stricto* species and *Zygosaccharomyces rouxii* (Gallagher et al. 2009). Interestingly, in some sensu stricto species, there are as many as four homologs of Sir1, all of which participate in silencing and whose open reading frames are located near the telomeres (Gallagher et al. 2009).

1.4 Experimental studies of SIR silencing proteins in non-Saccharomyces species

1.4.1 Candida glabrata

The only post-duplication species other than *S. cerevisiae* in which silencing has been examined experimentally is *Candida glabrata*. *C. glabrata* is the second most common cause of yeast infection in humans, after *Candida albicans* (Fidel et al. 1999; Ghidelli et al. 2001). Although both of these pathogenic species bear the name "Candida" they are phylogenetically distant (Figure 2). *C. albicans*, which clusters with most other *Candida* species, is a diploid, non-WGD species, with a single mating type

locus. In contrast, *C. glabrata* is more closely related to *S. cerevisiae*, and is a haploid, post-WGD species with three mating-type loci (Brockert et al. 2003; Srikantha et al. 2003).

Silencing in *C. glabrata* has been studied primarily at the telomeres. As in *S. cerevisiae*, CgSir2, CgSir3, CgSir4 and CgRap1 are all required for telomeric silencing (De Las Peñas et al. 2003; Domergue et al. 2005; Iraqui et al. 2005) and by analogy these proteins are likely to function at the mating-type loci. However there is differential regulation of the two *HM* loci. The *CgHMLα* locus is silenced, whereas the *CgHMR**a*** locus is not effectively silenced, such that in a *MATa* cell, *α1*, *α2* and ***a1*** transcripts are present (Muller et al. 2008). In these cells, *MATα* identity is maintained through defective splicing of the ***a1*** transcript, rendering the ***a1*** gene product inactive.

Interestingly, telomeric silencing in *C. glabrata* is regulatable (Domergue et al. 2005), whereas in *S. cerevisiae* silencing at both *HM* loci and telomeres is constitutive. The regulation of silencing in *C. glabrata* is made possible by an auxotrophy for nicotinic acid, the precursor of NAD^+. It is thought that in nicotinic acid-poor environments, the cellular level of NAD^+ drops, reducing the function of the NAD^+-dependent deacetylase, CgSir2 and hence silencing is disrupted. The ability to regulate silencing in this way may facilitate the infection of the urinary tract, which is poor in nicotinic acid. It is thought that subtelomeric silencing is reduced in the urinary tract, leading to the induction of several subtelomeric EPA genes, which encode adhesion proteins found in the cell wall. The expression of these genes enables the cells to adhere to epithelial cells and colonize the urinary tract (Kaur et al. 2005).

C. glabrata reproduces mitotically and is not known to have a sexual stage in its lifecycle. Nevertheless, examination of the sequenced genome has revealed that in

addition to possessing three mating-type loci (Srikantha et al. 2003), *C. glabrata* also has genes involved in meiosis and producing and responding to mating pheromones (Wong et al. 2003; Muller et al. 2008) and likely has an undiscovered sexual cycle.

1.4.2 Kluyveromyces lactis

The only other hemiascomycete in which silencing has been examined experimentally is the non-WGD species, *K. lactis*. K. lactis is a budding yeast that was originally isolated from milk-derived products, although it grows on a wide range of carbon sources. Interest in cultivating *K. lactis* for biotechnology led to the development of its genetics, and its phylogenetic position as a non-WGD species makes *K. lactis* an important alternative model system to *S. cerevisiae* and is the focus of much of the work presented in this thesis.

K. lactis strains have both *α*- and **a**- mating types. Mating does occur and the resulting diploids sporulate spontaneously (Herman and Roman 1966). Several elements of the *S. cerevisiae* pheromone response pathway have been identified in *K. lactis*, and their role in regulation of the mating response differs from *S. cerevisiae* (reviewed in (Coria et al. 2006)). Silent mating-type alleles in *K. lactis* were first identified in 1966 by Herman and Roman (Herman and Roman 1966), who showed that there are two independent, unlinked loci and that mating-type switching occurred at a very low frequency. The mechanism for switching has only been recently identified, and involves *a3*, a *Kluyveromyces* specific gene encoding a MULE-like transposase, that is found at *HMLα* and *MATα* mating-type cassettes (Barsoum et al. 2010a).

K. lactis has homologs of Sir2 and Sir4 (Chen and Clark-Walker 1994; Astrom and Rine 1998), and deletion of either *KlSIR2* or *KlSIR4* leads to increased expression of *α1* and *α3* mRNA at *HML* (Chen and Clark-Walker 1994; Astrom and Rine 1998; Aström et

al. 2000). In addition, Both KlSir2 and KlSir4 can partially complement mating defects of *sir2*Δ or *sir4*Δ *S. cerevisiae* strains (Chen and Clark-Walker 1994; Astrom and Rine 1998), illustrating their conserved function. Curiously, deletions of *KlSIR2* and *KlSIR4* have much less profound affects on mating efficiency than do *sir* deletions in *S. cerevisiae*, and mating ability varies substantially from strain to strain (Chen and Clark-Walker 1994; Astrom and Rine 1998). As described previously, Sir3 and Orc1 are paralogs and the nearest equivalent of Sir3 in *K. lactis* is KlOrc1. The potential role of KlOrc1 in silencing has not been studied until now, which I present in Chapter 4.

Examination of the *HM* loci in *K. lactis* has revealed several intriguing differences compared to *S. cerevisiae*. First, *HMLα* in *K. lactis* contains three genes, *α1*, *α2* and *α3*, whereas there are only two genes in *S. cerevisiae*, resulting in an *HMLα* locus that is more than twice as large as *HMLα* in *S. cerevisiae* (Aström et al. 2000). Secondly, the ***a2*** gene found at *HMR**a*** is a functional gene, similar to the ***a2*** gene in *C. albicans* (Butler et al. 2004).

Another interesting observation is that the silencers and silencer-binding proteins differ significantly between *K. lactis* and *S. cerevisiae* (Sjöstrand et al. 2002). Three DNA elements, named A, B and C boxes, have been identified as important for silencer function at *HMLα* in *K. lactis*. *HMR**a*** also contains A and B boxes. None of these DNA elements correspond to ORC or Rap1 binding sites, which are required for silencer function in *S. cerevisiae*. The B-box binds the essential protein KlReb1, which is required for silencing and mating (Sjöstrand et al. 2002). KlReb1 contains two Myb domains, which are related to a helix-loop-helix motif. Other Myb-containing proteins from *S. cerevisiae* (Rap1) and *S. pombe* (Taz1) have been shown to be important for telomeric silencing (Kyrion et al. 1993; Kanoh et al. 2005; Hansen et al. 2006). The A-box contains

an exact consensus-binding site for ScUme6. KlUme6 has recently been shown to bind to the silencers at *HMLα* and *HMR***a** and is required for the transcriptional silencing of these loci (Barsoum et al. 2010b). The C-box contains a sequence with weak similarity to the ScAbf1 binding site, but rigorous testing of this possibility or its implications have not been conducted. Thus, the silencers in *K. lactis* and *S. cerevisiae* are remarkably divergent.

In this thesis, I have developed *K. lactis* as a model system to study the non-duplicated Sir2 and Orc1 proteins and characterize the functions they serve in long-range silencing, promoter-specific repression and DNA replication.

2. Substitution as a mechanism for genetic robustness: The duplicated deacetylases Hst1 and Sir2 in Saccharomyces cerevisiae[1]

2.1 Introduction

Gene duplication is an important force in evolution, as it provides a source of new genetic material. However, the mechanisms by which duplicated genes are retained and diverge are understudied at the experimental level. I have examined the pair of duplicated histone deacetylases, Hst1 and Sir2 from *S. cerevisiae* that are important for distinct types of gene repression. In this study, I show that before the duplication the ancestral histone deacetylase contained both Hst1- and Sir2- like functions and after the duplication, Sir2 and Hst1 subfunctionalized via the duplication, degeneration, complementation model, giving rise to two distinct proteins with non-overlapping functions under standard conditions. Despite having partitioned the ancestral functions after the duplication, Sir2 can substitute for Hst1 in its absence by interacting with the normal partner of Hst1, Sum1. This study suggests that the evolutionary path of duplicate gene preservation may be an important indicator for the ability of duplicated genes to substitute for one another and hence protect the organism against deleterious mutations.

2.2 Results

Sir2 substitutes for Hst1 in an hst1Δ background

Gene expression data indicate that deletion of *HST1* derepresses target genes modestly, compared to the level of derepression observed in a *sum1*Δ background (Figure 3) (McCord et al. 2003). These results suggest that either deacetylation is not

[1] Adapted from Hickman, MA and Rusche, LN (2007) Substitution as a mechanism for genetic robustness: The duplicated deacetylases Hst1p and Sir2p in *Saccharomyces cerevisiae*. *PLoS Genetics* (**3**) e126.

critical for gene repression or another deacetylase acts at these promoters in the absence of Hst1. To identify other deacetylases that may function in the absence of Hst1, the four other known NAD+ dependent deacetylases, *SIR2*, *HST2*, *HST3* and *HST4* were deleted in combination with *HST1*. To assay levels of expression in these double deletion backgrounds, a p_{GAS2}-*HIS3* reporter was used. The *GAS2* promoter is not strongly induced in the absence of Hst1 but is greatly induced in the absence of Sum1 (McCord et al. 2003). In addition, the promoter contains a middle sporulation element and is reported to bind Sum1 (Harbison et al. 2004). If another deacetylase contributes to repression at this promoter in the absence of Hst1, then deletion of both deacetylases should derepress the p_{GAS2}-*HIS3* reporter to a greater extent than deletion of Hst1 alone. Increased expression was observed in the *hst1*Δ *sir2*Δ double deletion strain compared to the *hst1*Δ strain (Figure 3). The other double deletions, *hst1*Δ *hst2*Δ, *hst1*Δ *hst3*Δ and *hst1*Δ *hst4*Δ, did not display any difference in derepression compared to the single *hst1*Δ background.

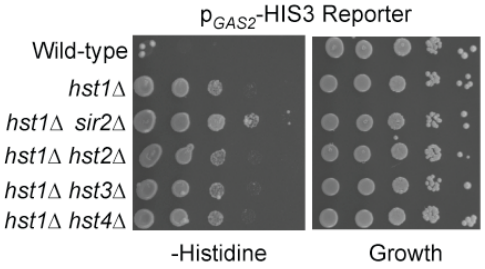


Figure 3: Sir2, but not other NAD+-dependent deacetylases, contributes to repression of Sum1-repressed genes in the absence of Hst1.

Expression of the Sum1-repressed p_{GAS2}-*HIS3* reporter was tested in wild-type (LRY1453), *hst1*Δ (LRY1454), *hst1*Δ *sir2*Δ (LRY1422), *hst1*Δ *hst2*Δ (LRY1686), *hst1*Δ *hst3*Δ (LRY1704) and *hst1*Δ *hst4*Δ (LRY1687) strain backgrounds. Ten-fold serial dilutions were plated on rich medium (growth) or medium lacking histidine (-histidine) and photographed after three days of growth at 30°. The most concentrated samples on rich medium and the most dilute samples on selective medium were omitted. Thus, the first spot on complete medium is equivalent to the second spot on medium lacking histidine.

To extend this observation and examine more quantitatively the difference between *hst1*Δ and *hst1*Δ *sir2*Δ derepression phenotypes, gene expression levels of *DTR1* and *SPS1*, two mid-sporulation genes repressed by Sum1 and Hst1 (McCord et al. 2003), were measured by quantitative RT-PCR in wild-type, *hst1*Δ and *hst1*Δ *sir2*Δ strains. *DTR1* and *SPS1* were modestly induced in an *hst1*Δ background (Figure 4), in accordance with previous observations (McCord et al. 2003). Consistent with the results of the p_{GAS2}-*HIS3* reporter, the induction of *DTR1* and *SPS1* in an *hst1*Δ *sir2*Δ strain was dramatically greater than in an *hst1*Δ strain (Figure 4). It should be noted that although derepression of mid-sporulation genes in an *hst1*Δ *sir2*Δ background is greater than is observed in an *hst1*Δ background, this derepression is not to the level observed in a *sum1*Δ strain (data not shown). These results indicated that Sir2 contributed to the repression of mid-sporulation genes in the absence of Hst1.

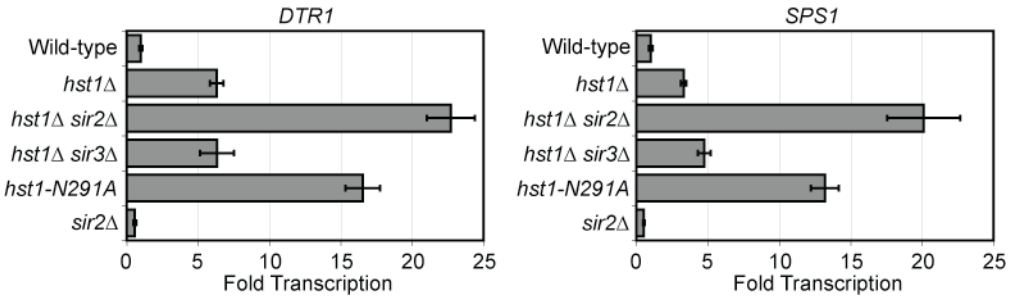


Figure 4: Sir2 contributes to the repression of *DTR1* and *SPS1* in the absence of Hst1.

Expression of the Sum1-repressed genes *DTR1* and *SPS1*. RNA was extracted from the following strains and analyzed by quantitative RT-PCR: wild-type (W3031-a), *hst1*Δ (LRY198), *hst1*Δ *sir2*Δ (LRY333), *hst1-N291A* (LRY1306), *sir2*Δ (LRY1079) and *hst1*Δ *sir3*Δ (LRY345). *DTR1* and *SPS1* transcript levels were normalized to *ACT1* transcript levels and then compared to wild-type to measure gene induction. A value of one corresponds to no induction.

To determine whether the increased expression of Hst1-repressed loci in an *hst1*Δ *sir2*Δ background resulted specifically from the loss of Sir2 or is an indirect effect due to the disruption of Sir-mediated silencing, the induction of *DTR1* and *SPS1* was examined in an *hst1*Δ *sir3*Δ background. If the observed increased expression resulted from the loss of Sir-mediated silencing, then the *hst1*Δ *sir3*Δ strain should have the same level of *DTR1* and *SPS1* induction as the *hst1*Δ *sir2*Δ strain. On the other hand, if the increased gene expression observed in the *hst1*Δ *sir2*Δ strain resulted specifically from the loss of Sir2, then retaining Sir2 while disrupting Sir-mediated silencing should resemble the *hst1*Δ phenotype rather than the *hst1*Δ *sir2*Δ phenotype. The level of *DTR1* and *SPS1* induction in the *hst1*Δ *sir3*Δ strain was comparable to the *hst1*Δ strain and dramatically less than the *hst1*Δ *sir2*Δ strain (Figure 4). I conclude that it was the absence of the Sir2 deacetylase and not disruption of Sir-mediated silencing that contributed to the elevated level of *DTR1* and *SPS1* gene expression in the *hst1*Δ *sir2*Δ background.

It is possible that Sir2 always contributes to the repression of the mid-sporulation genes. Alternatively, the absence of Hst1 could provide an opportunity for Sir2 to associate with the Sum1 complex, such that Sir2 only contributes to this repression in the absence of Hst1. To test the latter hypothesis, I characterized *DTR1* and *SPS1* expression in a strain in which Hst1 is enzymatically inactive such that the mutant Hst1 cannot contribute to deacetylation yet is present and should physically block Sir2 from acting in its place. To inactivate Hst1, a single amino acid substitution, N291A (described in (Lynch et al. 2005)) was used, analogous to a characterized substitution in Sir2 (N345A), which has been shown to be enzymatically inactive (Imai et al. 2000) but structurally intact (Min et al. 2001). This point mutation in Hst1 reduced deacetylation *in vivo* (see ahead to Figure 7). The *hst1-N291A* strain displayed significantly greater induction of *DTR1* and *SPS1* compared to the *hst1*Δ strain (Figure 4). This observation suggests that

Sir2 may be acting in the absence of Hst1, but not when the mutated Hst1-N291A protein is present. Furthermore, the increased induction in the *hst1-N291A* strain compared to the *hst1*Δ strain indicates that the main function of Hst1 in repression is deacetylation.

To examine whether *SIR2* normally contributes to repression of *DTR1* and *SPS1*, the expression profiles at *DTR1* and *SPS1* in a *sir2*Δ background were analyzed. If Sir2 has no role in Hst1-mediated repression when Hst1 is present, then deleting *SIR2* alone should have no discernable phenotype compared to wild-type yeast and repression of *DTR1* and *SPS1* will be maintained. Gene expression analysis of a *sir2*Δ strain revealed that repression of *DTR1* and *SPS1* was maintained in a *sir2*Δ background (Figure 4) and the p_{GAS2}-*HIS3* reporter also remained repressed in a *sir2*Δ (data not shown). These results suggest that Sir2 does not normally play a role in Hst1-mediated repression when Hst1 is present.

Sir2 associates with Sum1 at DTR1 and SPS1 promoters

To further test the hypothesis that Sir2 substitutes for Hst1 but does not normally act with Sum1, the association of Sir2 with repressed promoters in the presence and absence of Hst1 was examined. If the substitution model is correct, Sir2 should not be enriched at *DTR1* and *SPS1* promoters when Hst1 is present (in wild-type or *hst1-N291A* strains) but should be recruited to these promoters in an *hst1*Δ background. Chromatin immunoprecipitation was used to detect Sir2 or Hst1 at *DTR1* and *SPS1* promoters in wild-type and *hst1*Δ mutant strains. In a wild-type background, there was a high level of Hst1 enrichment but no detectable enrichment of Sir2 at the promoters of *DTR1* and *SPS1* (Figure 5). There was also no enrichment of Sir2 observed in the *hst1-N291A* background (Figure 5). These results are consistent with the model that Sir2 is absent

from these promoters when Hst1 is present. However, when Hst1 was absent, there was a modest enrichment of Sir2 at *DTR1* and *SPS1* promoters (Figure 5). The enrichment of Sir2 was not as robust as wild-type Hst1 at these loci, suggestive of a weaker interaction between Sir2 and the Sum1 complex.

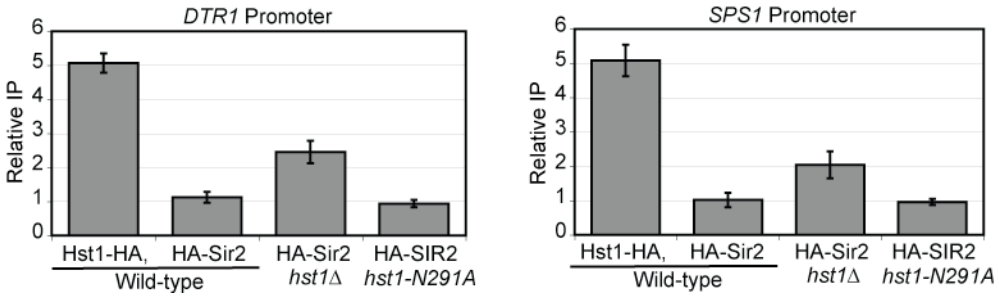


Figure 5: Sir2 associates with the promoters of *DTR1* and *SPS1*.

Association of HA-*SIR2* (pRO298) with *DTR1* and *SPS1* promoters in wild-type (W3031-a), *hst1-N291A* (LRY1306) and *hst1*Δ (LRY198) strain backgrounds was assessed with ChIP followed by quantitative real-time PCR. Also shown is the association of Hst1-HA (LRY558) with the same promoters in a wild-type background. Association at *DTR1* (B primers) and *SPS1* promoters were normalized to an internal control, the *ATS1* promoter, which is not regulated by Hst1 or Sir2. The y-axis represents the enrichment of *DTR1* or *SPS1* relative to *ATS1*. A value of one corresponds to no enrichment.

To examine directly whether the recruitment of Sir2 to repressed promoters is due to an interaction with the Sum1 complex, co-immunoprecipitation experiments between Sum1 and Sir2 in an *hst1*Δ background were performed. Hst1 and Sum1 are part of a stable complex that co-precipitates (Pijnappel et al. 2001; Rusche and Rine 2001; McCord et al. 2003) (and Figure 6). If Sir2 substitutes for Hst1 via a similar interaction with the Sum1 complex, then a physical association between these two proteins should be detectable. Sum1 was immunoprecipitated, and the IP samples were probed for Sir2 by immunoblotting. Consistent with the substitution model, Sir2 associated with Sum1 in the *hst1*Δ background (Figure 6). This co-precipitation of Sir2 with Sum1 was weaker

than the precipitation observed for Hst1 from equivalent amount of cell extract. This qualitative comparison is consistent with the Hst1-Sum1 interaction being more robust than the Sir2-Sum1 interaction and in accordance with the reduced enrichment of Sir2 compared to Hst1 observed at the promoters of *DTR1* and *SPS1* (Figure 5).

To test the hypothesis that the presence of Hst1 physically blocks the association of Sir2 with the Sum1 complex, the Sir2-Sum1 interactions in wild-type and *hst1-N291A* backgrounds were examined. In the presence of Hst1, Sir2 would not be expected to interact with the Sum1 complex, and indeed Sir2 was not observed to co-precipitate with Sum1 in wild-type yeast. There was a faint band in the *hst1-N291A* background that could be indicative of Sir2 interaction with the Sum1 complex, however this band was considerably less robust than that observed in the *hst1*Δ strain (Figure 6). Therefore, I conclude that Sir2 is recruited to Hst1-repressed loci through an interaction with the Sum1 complex and this recruitment only occurs in the absence of Hst1. Presumably, Hst1 outcompetes Sir2 for association with the Sum1 complex because Hst1 has a higher affinity for the Sum1 complex.

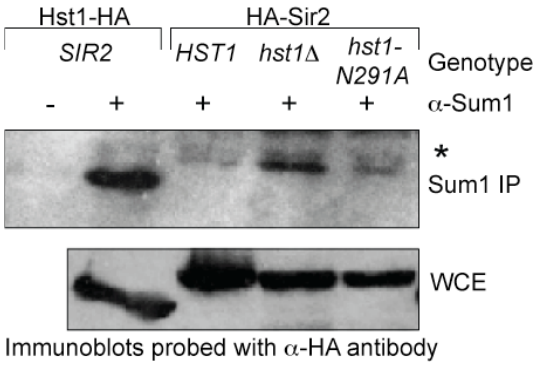


Figure 6: Sir2 interacts with Sum1.

Association of HA-Sir2 or Hst1-HA with Sum1. Sum1 was immunoprecipitated from whole cell extracts from the same strains used in Figure 5, and the precipitated material was examined by immunoblotting with an α-mouse HA antibody to detect Hst1-HA or HA-Sir2.

The results in the previous section suggested that Sir2 can substitute for Hst1 but does not normally act with Sum1. Gene expression data (Figures 3 and 4) in addition to the physical interactions described above (Figure 6) do not support the hypothesis that Sir2 plays a role in Sum1-mediated repression when Hst1 is present. Instead, these results support the hypothesis that Sir2 and Hst1 have non-overlapping functions in wild-type backgrounds (Brachmann et al. 1995; Derbyshire et al. 1996; McCord et al. 2003).

Sir2 acts as a histone deacetylase at Sum1-repressed loci

To investigate whether Sir2 acts as a deacetylase at these repressed promoters, chromatin immunoprecipitation experiments with two different histone H4 antibodies, one specific for acetylated lysine 8 (K8) and the other specific for acetylated lysine 16 (K16), were performed. The changes in acetylation of K8 or K16 at the *DTR1* promoter in *hst1*Δ, *hst1-N291A*, *hst1*Δ *sir2*Δ, and *sir2*Δ strains relative to wild-type were analyzed. Loss of deacetylation by Hst1 and Sir2 at *DTR1*, such as in *hst1-N291A* and *hst1*Δ *sir2*Δ backgrounds, should result in increased acetylation of K8 and K16. Indeed, increased levels of acetylation of both K8 and K16 were observed in both *hst1-N291A* and *hst1*Δ *sir2*Δ backgrounds (Figure 7). These results parallel the patterns observed in the gene expression profiles. The single *sir2*Δ deletion did not display elevated levels of acetylated K8 or acetylated K16 at *DTR1*, providing further support for the model that Sir2 does not normally act at Hst1-repressed loci when Hst1 is present.

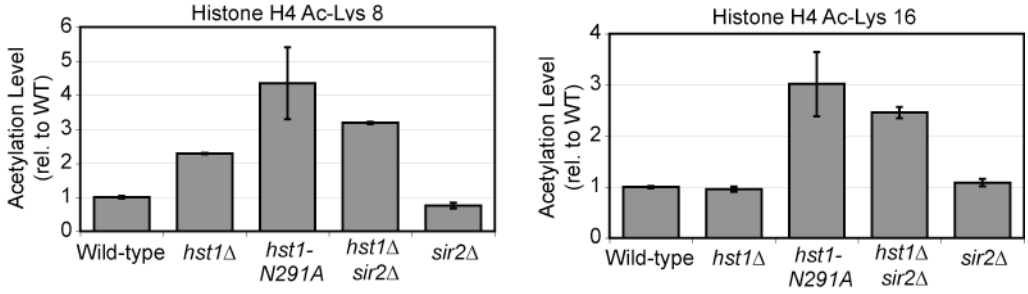


Figure 7: Histone H4 K8 and K16 acetylation at the *DTR1* promoter increases in the absence of deacetylase activity.

ChIP of H4 K8-Ac and H4 K16-Ac in wild-type (W3031-a), *hst1*Δ (LRY198), *hst1-N291A* (LRY1306), *hst1*Δ *sir2*Δ (LRY333) and *sir2*Δ (LRY1079) backgrounds at the *DTR1* promoter (B primers, see Figure 10). Histone H4 K8-Ac and K16-Ac enrichment at *DTR1* was normalized to the repressed promoter of *PHO5* as well as for total histone H4 occupancy and quantified relative to the wild-type strain.

Interestingly, when the *hst1*Δ and wild-type strains were compared, changes in acetylation were different for K8 and K16 (Figure 7). A modest increase of acetylation at K8 was observed in the *hst1*Δ background, whereas no detectable change (compared to wild-type) in acetylation of K16 was noted. These results suggest that Sir2 more efficiently deacetylates K16 than K8 because changes in K16 acetylation were only revealed when both Hst1 and Sir2 are absent (*hst1*Δ *sir2*Δ). These data are consistent with published reports that Sir2 preferentially deacetylates H4 K16 *in vitro* (Imai et al. 2000; Tanny and Moazed 2001). Nevertheless, K16 must also be a target for deacetylation by Hst1 because K16 acetylation increases when Hst1 is non-functional (*hst1-N291A*) (Figure 7). In conclusion, these results indicate that Sir2 acts as a deacetylase at Sum1-repressed promoters in the absence of Hst1.

Sir2 substitution for Hst1 is limited by dosage

To determine whether there is still sufficient Sir2 available to silence the mating type loci (a primary function of Sir2) when Sir2 is substituting for Hst1, the ability of

wild-type, *sir2*Δ and *hst1*Δ strains to mate was assessed. If Sir2 recruitment to Hst1-repressed loci in an *hst1*Δ background reduces the pool of available Sir2, then silencing at the mating type loci might be reduced, leading to diminished mating. Alternatively, if the preferred function of Sir2 is to silence the mating type loci, then there should be no defect in mating ability in an *hst1*Δ background, even though Sir2 is substituting for Hst1. There was no observable defect in mating ability in an *hst1*Δ background compared to wild-type (Figure 8 and (Brachmann et al. 1995; Derbyshire et al. 1996)). Therefore, Sir2 is more likely to silence the mating type loci than to substitute for Hst1. Furthermore, these results suggest that Sir2 has a higher affinity for the Sir complex than the Sum1 complex, because the ability to mate is not perturbed in the absence of Hst1, whereas repression of mid-sporulation genes is not complete when Sir2 is substituting for Hst1.

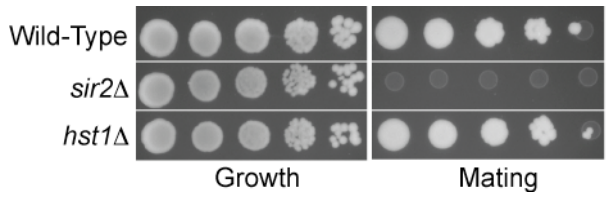


Figure 8: Mating is not disrupted with Sir2 substitution.

Mating ability was assayed using 10-fold serial dilutions of wild-type (W3031-a), *sir2*Δ (LRY1079) and *hst1*Δ (LRY198) strains mated against a MATα tester strain (LRY1022). Prototrophic diploids were selected on minimal plates.

If the majority of Sir2 is involved in silencing the mating type loci (and telomeres), and only a few molecules of Sir2 are available for recruitment to Hst1-repressed loci in the absence of Hst1, then additional copies of Sir2 may enhance repression of Hst1-repressed loci. Overexpression of Sir2 has been reported to reduce β-Galactosidase activity from an MSE containing promoter driving *lacZ* expression in an

*hst1*Δ background (Xie et al. 1999). To further characterize this observation, the amount of Sir2 in the cell was varied to determine whether overexpression of Sir2 enhanced its ability to substitute for Hst1. To assay repression, a reporter construct consisting of the Sum1-repressed *PES4* promoter fused to the open reading frame of *HIS3* was utilized. In the absence of Hst1, the *PES4* promoter is derepressed to a greater extent than the *GAS2* promoter described previously (Figure 3), enabling an enhancement of repression to be detected. *hst1*Δ cells were transformed with low copy plasmids expressing *HST1-HA* or *HA-SIR2*, and a 2 micron overexpression plasmid expressing *SIR2*. The relative levels of Sir2 are shown in Figure 9B. Expression of the p_{PES4}-*HIS3* reporter was monitored on medium lacking histidine and uracil (to ensure plasmid retention). A wild-type strain displayed no growth on selective medium, indicating that the *PES4* promoter was repressed as expected (Figure 9A). In an *hst1*Δ background, cells were able to grow on selective medium, as a result of derepression of the p_{PES4}-*HIS3* reporter (Figure 9A); demonstrating that the reporter assay is functional. Note that endogenous levels of Sir2 are present in all strains (Figure 9B). The addition of Sir2 on a low copy plasmid resulted in an enhancement of repression of p_{PES4}-*HIS3*, and overexpression of Sir2 from a high copy plasmid enhanced repression of p_{PES4}-*HIS3* to an even greater extent. Despite the enhancement in repression observed upon overexpression of Sir2, repression of p_{PES4}-*HIS3* was not complete in the absence of Hst1. This incomplete suppression probably results from the relatively weaker interaction of the Sum1 complex with Sir2 compared to Hst1.

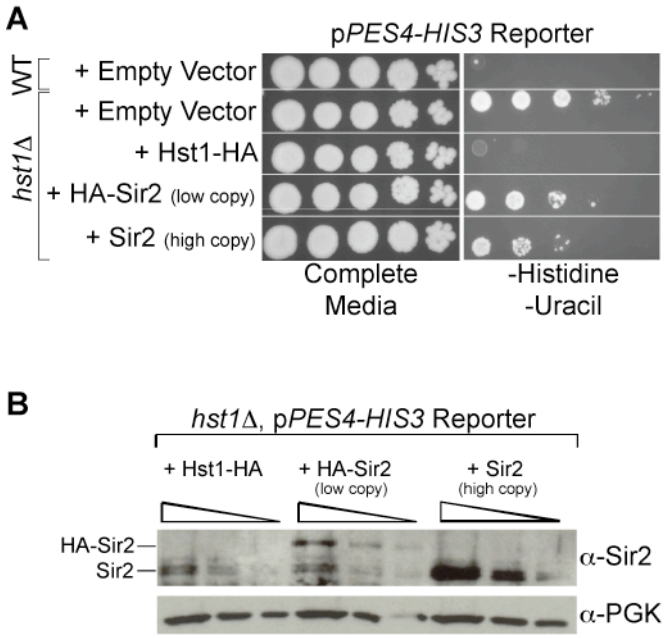


Figure 9: Sir2 substitution for Hst1 is limited by dosage.

A) Repression was assayed with the p_{PES4}-*HIS3* reporter. Wild-type yeast (LRY1593) was transformed with an empty vector (pRS416) and *hst1*Δ yeast (LRY1545) was transformed with an empty vector (pRS416), *HST1-HA* (pLR30), *HA-SIR2* (pRO298 (low copy)) or *SIR2* (pLP317 (high copy)). 5-fold serial dilutions were plated on rich medium and medium lacking histidine and uracil (to maintain plasmids) and photographed after 3 days growth at 30°. **B)** Immunoblot analysis showing relative amounts of Sir2 protein in these strains. Endogenous Sir2 (lower band) is present in all strains. The HA-Sir2 is slightly shifted due to the epitope tag. 3-phosphoglycerate kinase was used a loading control.

It is thought that Sir2 associates directly with Sir4 but not Sir3 (Moazed et al. 1997). Therefore, additional Sir2 might become available by deleting Sir4, which would result in a stronger repression phenotype than observed in an *hst1*Δ background. However, gene expression analysis of *DTR1* in an *hst1*Δ *sir4*Δ strain showed roughly equivalent levels of *DTR1* induction to an *hst1*Δ strain (data not shown).

The Sir2-Sum1 complex does not spread

A key distinguishing feature between Hst1 and Sir2 is that Sir2 is normally part of the Sir silencing complex that spreads along the chromosome (Rusche et al. 2003) whereas the Hst1-Sum1 complex does not spread (Lynch et al. 2005). I was interested to determine whether the Sir2-Sum1 complex was able to spread although the Hst1-Sum1 complex does not spread, indicating some intrinsic property in Sir2 to promote spreading. To assess the ability of Sum1 to spread, the distribution of myc-Sum1 across the *DTR1* locus was analyzed by chromatin IP when the Sum1 complex was interacting with Hst1 (wild-type) or Sir2 (*hst1*Δ). There is a probable MSE sequence in the promoter of *DTR1* to which Sum1 is thought to bind (Harbison et al. 2004) (indicated in Figure 10). When the Sum1-Hst1 complex is present, myc-Sum1 should associate most strongly with the MSE DNA sequence and should have reduced association with the surrounding sequences (approximately 200 bp upstream and downstream of the MSE). Due to technical limitations of shearing DNA by sonication, sequences near the binding site are also enriched in immunoprecipitated material, even if the protein does not spread. If Sir2 causes Sum1 to spread when it substitutes for Hst1, Sum1 should be more broadly distributed across the *DTR1* promoter and into the open reading frame. However, if Sir2 does not confer the ability to spread, then the distribution of Sum1 across *DTR1* should not be appreciably different in *HST1* and *hst1*Δ strains. The distribution of Sum1 across the *DTR1* locus remained the same regardless of which deacetylase was interacting with Sum1 (Figure 10). Therefore, Sir2 did not cause noticeable spreading of Sum1 when substituting for Hst1, and Sum1 continued to act as a promoter-specific repressor.

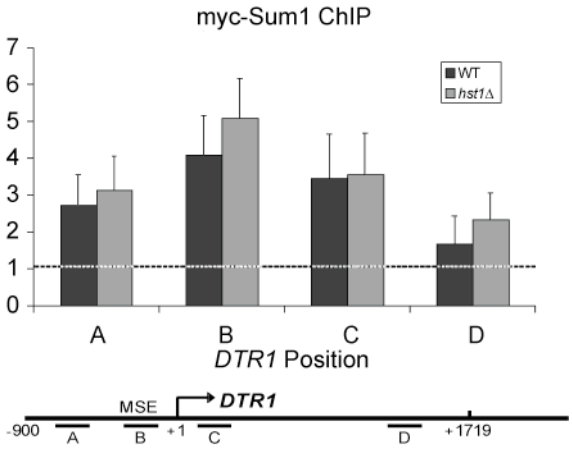


Figure 10: Sir2 does not facilitate Sum1 spreading at *DTR1*.

Distribution of myc-Sum1 with the *DTR1* promoter in wild-type (LRY523) and *hst1*Δ (LRY521) strain backgrounds. Also shown is the *DTR1* locus indicating the positions of qPCR amplicons.

I extended this analysis to examine whether Sir2 itself can spread across the *DTR1* locus, even though Sum1 does not spread. The distribution of Hst1 (in a wild-type background) and Sir2 (in an *hst1*Δ background) across the *DTR1* locus was assessed by chromatin immunoprecipitation. As expected, Hst1 had a distribution centering on the MSE and did not extend into the open reading frame (Figure 11), indicating that Hst1 is not spreading at repressed mid-sporulation genes. The localization of Sir2 had a similar distribution that was centered at the MSE and did not extend out into the open reading frame (Figure 11). These results demonstrate that Sir2 can act in a promoter-specific manner to repress gene expression when associated with the Sum1 complex.

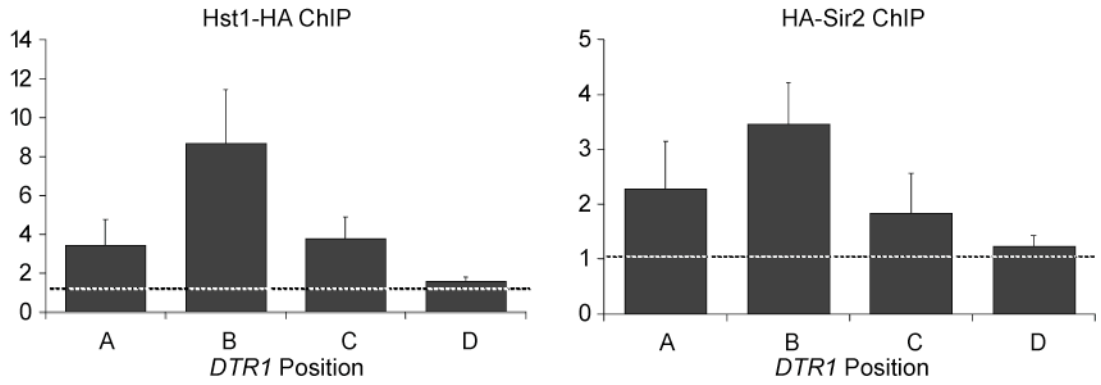


Figure 11: Hst1 and Sir2 do not spread at *DTR1*.

Distributions of Hst1-HA in a wild-type (LRY558) background and of HA-Sir2 in an *hst1*Δ (LRY198 with pRO298) background across the *DTR1* locus were assessed by ChIP followed by qPCR. Association at the *DTR1* promoter was normalized to the *ATS1* promoter and the same primer sets were assessed as in Figure 10.

Hst1 and Sir2 have different protein interaction domains

How do Hst1 and Sir2 maintain non-overlapping functions when both deacetylases are present, despite considerable sequence identity and the ability of Sir2 to substitute for Hst1? One possibility is that Hst1 and Sir2 have unique determinants that confer specificity for the Sum1 complex and the Sir complex, respectively. Because the N-terminus is less conserved than the catalytic core, (Figure 12), this region may have evolved distinct specificities for either the Sir or Sum1 complex. To determine whether such determinants exist, a chimeric Sir2-Hst1 molecule was constructed, in which the N-terminus of Sir2 was fused to the catalytic core of Hst1. The junction of the Sir2-Hst1 chimera was at the start of the catalytic core domain, such that amino acids 1-255 of Sir2 are fused to amino acids 201-503 from Hst1 (Figure 12), generating HA-Sir2$_{1\text{-}255}$-Hst1$_{201\text{-}503}$. This chimeric gene was expressed from the *SIR2* promoter. I also constructed the reverse chimera, HA-Hst1$_{1\text{-}200}$-Sir2$_{256\text{-}562}$, but was not able to detect protein expression by immunoblotting (data not shown) and continued analysis only with HA-Sir2$_{1\text{-}255}$-Hst1$_{201\text{-}503}$.

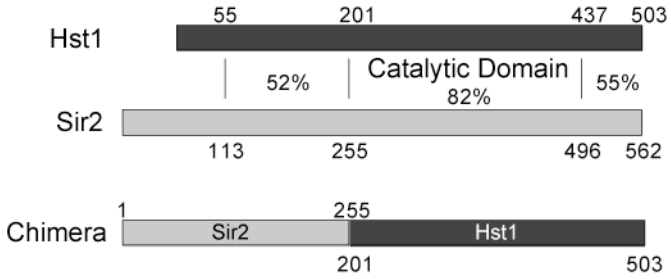


Figure 12: Sir2 and Hst1 protein domains and conservation.

Schematic overview of conserved domains of Sir2 and Hst1 (adapted from (Cockell et al. 2000)) as well as the architecture of the $Sir2_{1-255}$-$Hst1_{201-503}$ chimera.

This chimeric protein was tested for its ability to function like Sir2 and Hst1. If specificity for the Sir complex (in Sir2) and specificity for the Sum1 complex (in Hst1) is established with the N-terminus, then HA-$Sir2_{1-255}$-$Hst1_{201-503}$ should only function like Sir2. Alternatively, if specificity for the Sir or Sum1 complex is determined by the C-terminus, then HA-$Sir2_{1-255}$-$Hst1_{201-503}$ should associate with the Sum1 complex and function like Hst1. Finally, it may be that specificity for the Sir and Sum1 complex may be determined in entirely different regions of Sir2 and Hst1. If this were true, then it may be possible that HA-$Sir2_{1-255}$-$Hst1_{201-503}$ can associate with both the Sir and Sum1 complexes and function in an Hst1- and Sir2-like manner or interact with neither complex and HA-$Sir2_{1-255}$-$Hst1_{201-503}$ would be non-functional in both Sir- and Sum1-mediated repression.

To determine whether HA-$Sir2_{1-255}$-$Hst1_{201-503}$ has Hst1-like function, the ability of this chimera to repress p_{PES4}-*HIS3* was examined. HA-$Sir2_{1-255}$-$Hst1_{201-503}$ completely suppressed an *hst1*Δ mutation (Figure 13). In fact, the chimeric protein was more effective than Sir2 at repressing p_{PES4}-*HIS3* (Figure 13A compared to Figure 9A). However, as previously discussed results suggest, Sir2 can substitute for Hst1. It is possible that suppression of the *hst1*Δ mutation is due to Sir2 substitution and not $Sir2_{1-255}$-$Hst1_{201-503}$. To directly compare the abilities of $Sir2_{1-255}$-$Hst1_{201-503}$, Hst1 and Sir2 to

function in an Hst1-like manner, each of these proteins was expressed in an *hst1*Δ *sir2*Δ strain, and *DTR1* expression was assayed by quantitative RT-PCR. The chimera was better at repressing *DTR1* expression than Sir2 and was equally able to repress *DTR1* expression as wild-type Hst1 (Figure 13B).

To determine whether this repression mediated by HA-Sir2$_{1\text{-}255}$-Hst1$_{201\text{-}503}$ resulted from a strong interaction with the Sum1 complex, co-immunoprecipitation studies were performed. When Sum1 was immunoprecipitated, Sir2$_{1\text{-}255}$-Hst1$_{201\text{-}503}$ co-precipitated to an extent comparable to, if not greater than, wild-type Hst1 and was much greater than Sir2 (Figure 13C). From these results I propose that unique features in the C-terminus of Hst1 specify an interaction with Sum1.

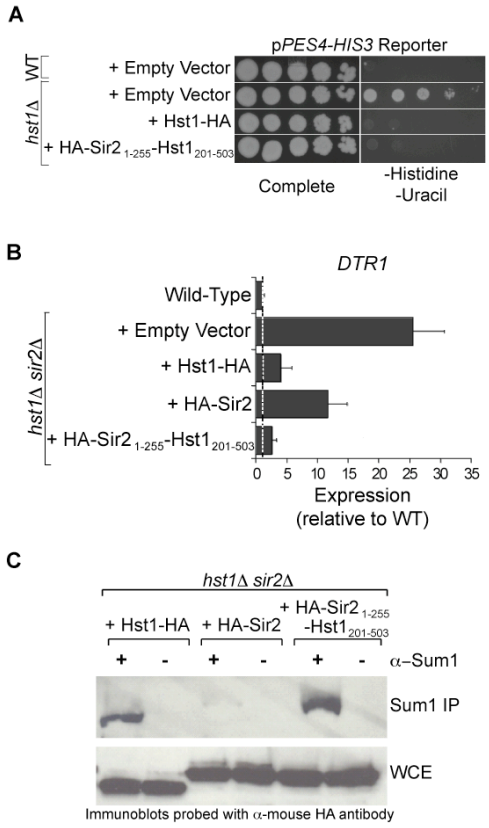


Figure 13: The Sir2$_{1\text{-}255}$-Hst1$_{201\text{-}503}$ chimera functions like Hst1.

A) The p_{PES4}-*HIS3* reporter was used to assay repression by the HA-$Sir2_{1-255}$-$Hst1_{201-503}$ chimera (pLR488) and *HST1*-HA (pRO298) in an *hst1*Δ strain (LRY1545). 10-fold serial dilutions were plated on rich medium and medium lacking histidine and uracil (to maintain plasmid) and photographed after 3 days growth at 30°. **B)** *DTR1* gene expression of *hst1*Δ *sir2*Δ (LRY333) cells transformed with an empty vector (pRS416), *HST1*-HA (pLR30), HA-*SIR2* (pRO298) or HA-$Sir2_{1-255}$-$Hst1_{201-503}$ (pLR488) relative to wild-type (W303-1a) expression. **C)** Association of HA-$Sir2_{1-255}$-$Hst1_{201-503}$, HA-Sir2 and Hst1-HA with Sum1. Sum1 was immunoprecipitated from whole cell extracts from the same strains used in part **B**, and the precipitated material was examined by immunoblotting with an α-mouse HA antibody to detect Hst1-HA, HA-Sir2 or HA-$Sir2_{1-255}$-$Hst1_{201-503}$.

To determine whether $Sir2_{1-255}$-$Hst1_{201-503}$ has Sir2-like function, the ability to silence the mating type loci was examined by mating assays. $Sir2_{1-255}$-$Hst1_{201-503}$ enabled the cells to mate in the absence of Sir2 to a level comparable to that seen with wild-type Sir2 (Figure 14A). The extent of mating was greater with $Sir2_{1-255}$-$Hst1_{201-503}$ than in cells expressing only wild-type Hst1 (Figure 14A). To test if the mating ability of $Sir2_{1-255}$-$Hst1_{201-503}$ resulted from an association with the Sir complex, I co-immunoprecipitated Sir4 with Sir2, Hst1 and $Sir2_{1-255}$-$Hst1_{201-503}$. Results from these experiments showed an interaction between $Sir2_{1-255}$-$Hst1_{201-503}$ and Sir4 comparable to that of wild-type Sir2 (Figure 14B). I conclude that there is a critical component in the N-terminus of Sir2 that specifies Sir2 to interact with the Sir complex. The ability of the chimeric Sir2-Hst1 protein to suppress both *hst1*Δ and *sir2*Δ mutations suggests that different regions of the protein are involved in conferring specificity for the Sir and Sum1 complexes.

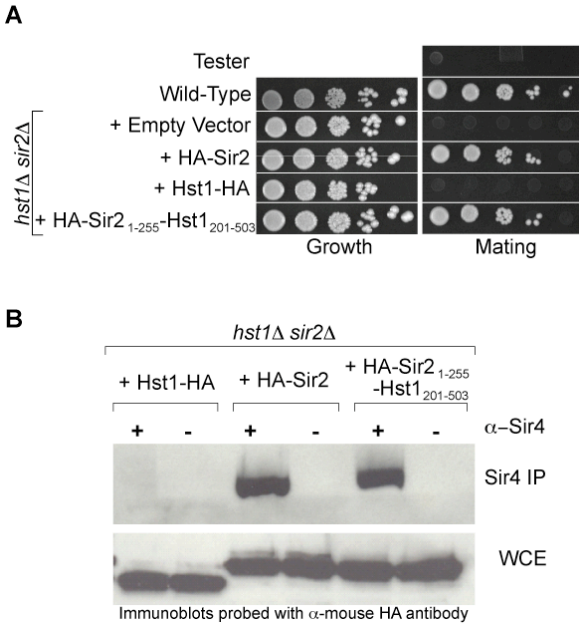


Figure 14: The $Sir2_{1-255}$-$Hst1_{201-503}$ chimera functions like Sir2.

A) Ability of Sir2, Hst1 and HA-$Sir2_{1-255}$-$Hst1_{201-503}$ to silence *HML* was assessed by a mating assay. 10-fold serial dilutions of an *hst1*Δ *sir2*Δ (LRY333) strain transformed with an empty vector (pRS416), *HA-SIR2* (pRO298), *HST1*-HA (pLR30) and HA-$Sir2_{1-255}$-$Hst1_{201-503}$ (pLR488) were mated against a MATα tester strain (LRY1022). Prototrophic diploids were selected on minimal plates. A ten-fold dilution of the tester strain was plated on minimal plates as a negative control. **B**) Association of HA-$Sir2_{1-255}$-$Hst1_{201-503}$ with Sir4. Sir4 was immunoprecipitated from whole cell extracts from the strains used in part A, and the precipitated material was examined by immunoblotting with an α-mouse HA antibody to detect HA-Sir2, Hst1-HA or HA-$Sir2_{1-255}$-$Hst1_{201-503}$.

A recent study (Mead et al. 2007) also analyzed Sir2 and Hst1 interaction domains by using chimeric molecules and obtained similar results. This study determined that amino acids 12-155 in the N-terminus of Sir2 were important for Sir-mediated silencing at the mating type loci, telomeres and rDNA, consistent with the chimera analysis. Additionally, it was shown that two non-conserved amino acids in the catalytic core of Hst1, N324 and L325, were critical for Hst1-mediated repression. Together, these results strongly indicate the presence of two different domains in Sir2 and Hst1 that confer substrate specificity for the Sir or Sum1 complex.

The non-duplicated KlSIR2 functions in SIR2- and HST1-like repression

As outlined in the introduction, *SIR2* and *HST1* arose by gene duplication, and it is possible that the ancestral deacetylase interacted with both the Sum1 and Sir complexes. To test this model, I examined the function of the single *SIR2*/ *HST1* gene from *Kluyveromyces lactis*, a species known to have diverged from *S. cerevisiae* before the whole genome duplication (Dietrich et al. 2004; Kellis et al. 2004). If the ancestral *SIR2*/*HST1* gene possessed only the function of *ScSIR2* or *ScHST1* and the other function evolved after the duplication, the gene having the new function would be expected to have experienced accelerated evolution compared with the gene retaining the original function. However, there appears to have been no accelerated evolution of either ScSir2 or ScHst1 compared to KlSir2 ((Kellis et al. 2004) and Figure 15), an observation more consistent with a partitioning of functions after the duplication.

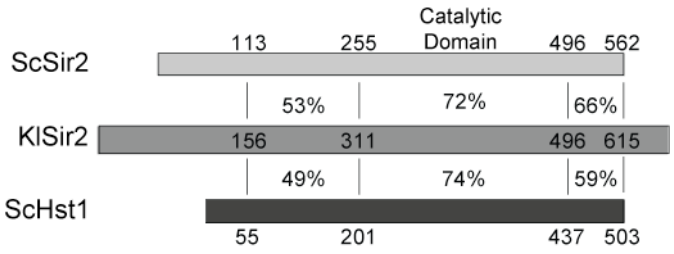


Figure 15: Conservation of Sir2 deacetylases between *S. cerevisiae* and *K. lactis*.

Pairwise sequence identity between domains of KlSir2, ScSir2 and ScHst1. FASTA alignment software was used to calculate percent identity and percent similarity (in parentheses) for each pairwise comparison.

The initial identification of Kl*SIR2*/*HST1* (referred to hereafter as Kl*SIR2*) reported that overexpression of Kl*SIR2* in *S. cerevisiae* was able to partially suppress a *sir2*Δ mating defect (Chen and Clark-Walker 1994). I did not observe suppression of a *sir2*Δ mating defect by KlSir2 (data not shown), however, this could be due to

differences in expression between this work and previously reported findings. Nevertheless, I could detect a weak interaction between KlSir2 and ScSir4 in co-immunoprecipitation experiments (Figure 16). Subsequent studies of Kl*SIR2* revealed a role in silencing the mating type loci in *K. lactis* (Aström et al. 2000). Therefore, it has clearly been demonstrated that Kl*SIR2* has *SIR2*-like function both in *K. lactis* as well as in *S. cerevisiae*.

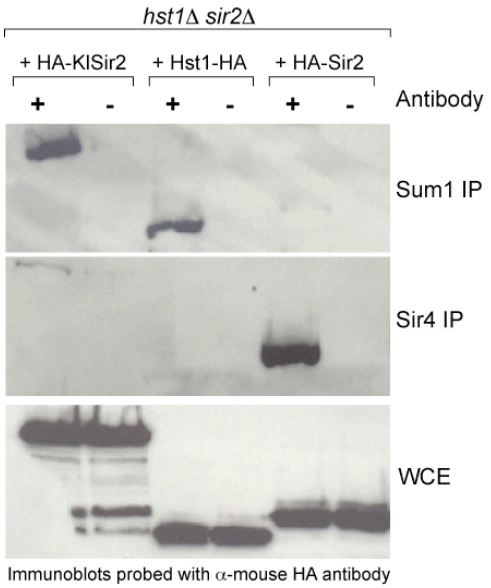


Figure 16: KlSir2 interacts with Sum1 and Sir4 in *S. cerevisiae*.

Association of HA-KlSir2 with Sir4 and Sum1. Sir4 and Sum1 were immunoprecipitated from whole cell extracts from *hst1*Δ *sir2*Δ (LRY333) cells transformed with HA-Kl*SIR2* (pLR490), and the precipitated material was examined by immunoblotting with an α-mouse HA antibody to detect HA-Sir2, Hst1-HA or HA-KlSir2. The Hst1-Sum1 and Sir2-Sir4 co-immunoprecipitation samples are the same as those shown in Figures 13 and 14, respectively.

To test whether Kl*SIR2* is able to function in Hst1-mediated repression in *S. cerevisiae,* Kl*SIR2* was cloned into an ARS-CEN plasmid such that Kl*SIR2* has an N-terminal HA epitope tag and is expressed from the Sc*SIR2* promoter. This plasmid was

used to transform the *hst1*Δ p_{PES4}-*HIS3* strain of *S. cerevisiae*. Immunoblot analysis showed that Kl*SIR2* was stably expressed in *S. cerevisiae* (Figure 16). There was complete repression of the p_{PES4}-*HIS3* reporter by Kl*SIR2*, with no observed difference from the wild-type level of repression (Figure 17A). I also examined the ability to KlSir2 to repress mid-sporulation genes in an *hst1*Δ *sir2*Δ background by analyzing *DTR1* expression levels and found that KlSir2 repressed *DTR1* to a level comparable to ScHst1 and better than ScSir2 (Figure 17B).

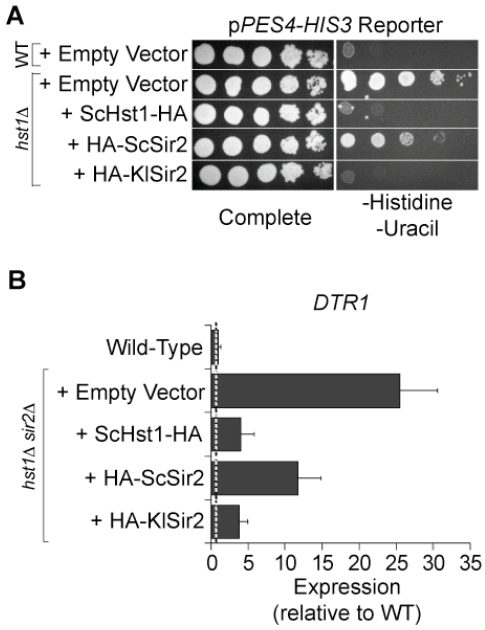


Figure 17: Hst1 function is conserved in KlSir2.

A) The p_{PES4}-*HIS3* reporter was used to assay repression by *HA*-Kl*SIR2* (pLR490) and *HST1-HA* (pLR30) in an *hst1*Δ strain (LRY1545). 10-fold serial dilutions were plated on rich medium and medium lacking histidine and uracil (to maintain plasmid) and photographed after 3 days growth at 30°. **B)** *DTR1* gene expression of *hst1*Δ *sir2*Δ (LRY333) cells transformed with HA-Kl*SIR2* (pLR490) compared to wild-type (W303-1A) expression. Data for the empty vector, Hst1-HA and HA-Sir2 are the same as shown in Figure 13.

To test if this repression was a result of an interaction with the *S. cerevisiae* Sum1 complex, I immunoprecipitated ScSum1 and found that KlSir2 co-precipitated (Figure 16). From this data, as well as studies described previously (Chen and Clark-Walker 1994; Aström et al. 2000), I conclude that the pre-duplicated Kl*SIR2* carries out both *SIR2* and *HST1* like functions. These data provide further evidence that the ancestral *SIR2*/ *HST1* had dual functions that diverged after the genome duplication.

2.3 Discussion

In this study I provide evidence that in *S. cerevisiae*, the deacetylase Sir2 substitutes for Hst1 in its absence. Deletion of both *HST1* and *SIR2* results in dramatically greater derepression of Sum1-repressed genes than observed in an *hst1*Δ background. This additional derepression is not observed when other related NAD^+-dependent deacetylases or other silencing factors are deleted (Figure 3). Furthermore, Sir2 localizes to the promoters of Hst1-repressed loci through an interaction with the Sum1 complex (Figure 6) and acts as a histone deacetylase at these loci in the absence of Hst1 (Figure 7). On the basis of these results, I propose a substitution model rather than genetic redundancy to explain the overlapping roles of this duplicate gene pair. I consider this phenomenon as substitution and not redundancy because Sir2-mediated repression at Hst1-regulated genes is not as proficient as wild-type Hst1-mediated repression and only occurs when Hst1 is absent. I propose that this substitution by Sir2 in an *hst1*Δ background accounts for some of the difference in derepression observed between *hst1*Δ and *sum1*Δ strains, although Sir2 substitution did not account for the entire difference in derepression phenotypes between *hst1*Δ and *sum1*Δ strains. It is possible that another deacetylase may also have the limited ability to substitute, or it could be that the Sum1 has repressive properties that are independent of a deacetylase.

It has also been observed by others that Hst1 substitutes for Sir2 in a *sir2*Δ background. Overexpression of Hst1 from a high copy plasmid partially suppresses *sir2*Δ mating defects in *MATα* cells (Brachmann et al. 1995). However, this suppression does not completely restore function, as genetic redundancy would predict, because mating efficiency is still about 30-fold lower than wild-type. Given that overexpression of Hst1 is required to observe this effect, Hst1 may be less capable of substituting for Sir2 than Sir2 is capable of substituting for Hst1. The requirement for multiple Sir2-Sir4 complexes to silence a single *HMR* locus may also reduce the ability of Hst1 to substitute for Sir2. This is in contrast to what is considered to be a single Hst1-Sum1 complex required for repression, which would make it easier for Sir2 to substitute for Hst1, even if the affinity of Hst1 for the Sir complex were comparable to the affinity of Sir2 for the Sum1 complex. Regardless, this previously published result is consistent with the model of the duplicated *SIR2-HST1* gene pair acting as substitutes for each other. This type of biological phenomenon has been proposed previously as the imposter model, with some controversy, for the MAP kinases Fus3 and Kss1 in *S. cerevisiae* (Madhani et al. 1997; Breitkreutz et al. 2001). However, this study has further developed this model to consider the evolutionary relationships between substituting proteins.

This substitution is likely a consequence of *SIR2* and *HST1* originating by duplication. Duplication has been proposed to be a strong evolutionary force because it generates a source of new genetic material that is free of selective constraint (Ohno 1970). Duplicated genes have two ultimate fates: degeneration or preservation in the genome. Clearly *SIR2* and *HST1* have been retained. Two models have been proposed outlining the steps towards preservation. The classical model proposed that the only way to preserve duplicated genes is through neofunctionalization, in which one of the duplicate genes evolves a new function by acquiring beneficial mutations, while the

other gene retains the original function. In such a case, it is predicted that the gene with the new function will experience a more rapid change in sequence, i.e. "accelerated evolution," compared with the duplicate retaining the original function. A more recent paradigm for the preservation of duplicated genes has been proposed (Force et al. 1999; Lynch and Force 2000) to account for the much larger retention of duplicate genes than the classical model would predict. This new model of duplication, degeneration, and complementation (DDC) states that if the ancestral gene had multiple functions, duplicate genes can each lose one of the original functions by degenerative mutations, while still retaining a different ancestral function. The DDC mechanism was originally proposed in the context of cis-regulatory elements of duplicated gene pairs. However, my work suggests that the DDC mechanism can also act on protein coding sequences.

This study provides evidence to suggest that the *SIR2*-*HST1* duplicate gene pair had an ancestral function that provided both *SIR2*- and *HST1*-like functions. After the duplication, *SIR2* and *HST1* subfunctionalized to evolve into distinct *SIR2* and *HST1* genes with non-overlapping functions. By using *K. lactis* as a representative non-duplicated species, I found that the single *HST1*/*SIR2* gene completely suppressed an *hst1*Δ mutation in *S. cerevisiae* (Figure 17). Previous studies have reported that Kl*SIR2* contributes to silencing the *HM* loci in *K. lactis* (Aström et al. 2000) and can partially suppress a *sir2*Δ mating defect in *S. cerevisiae* (Chen and Clark-Walker 1994). I have extended this analysis to show that KlSir2 can interact with Sir4 in *S. cerevisiae* (Figure 16). Together, these results indicate that the non-duplicated *HST1*/*SIR2* is likely to have had both functions. It is probable that the Sir2 family has diversified by this type of mechanism.

Results from my functional characterization of the chimeric $Sir2_{1\text{-}255}$-$Hst1_{201\text{-}503}$ as well as those reported elsewhere (Mead et al. 2007) provide preliminary evidence that

the evolution of *SIR2* and *HST1* may have followed a DDC mechanism. Two different specificity determinants in Sir2 and Hst1 have been found - a domain specific for determining an interaction with the Sum1 complex residing in the C-terminus of Hst1 (Figure 13), specifically N378 and L379 (Mead et al. 2007), and a domain specific for conferring an interaction with the Sir complex in amino acids 12-155 of the N-terminus of Sir2 (Figure 14 and (Mead et al. 2007)). These interaction domains have likely been conserved subsequent to the duplication. I propose a scenario in which, after the duplication, Hst1 acquired degenerative mutations in the N-terminal domain that interacts with the Sir complex, leading to the loss of affinity for Sir4, yet maintaining its ability to interact with the Sum1 complex. Sir2, on the other hand, acquired degenerative mutations in the C-terminal domain required for interaction with the Sum1 complex, leading to a reduced affinity for the Sum1 complex, while maintaining a strong interaction with the Sir complex. Nevertheless, Sir2 has retained an interaction domain for the Sum1 complex, although it has a weaker affinity for this complex than Hst1 (Figure 6). Interestingly, of the two amino acids Q324 and I325 important for Hst1 with the Sum1 complex in *S. cerevisiae*, only the isoleucine is conserved in *K. lactis* (K434 and I435). However, KlSir2 can fully suppress an *hst1*Δ mutation in *S. cerevisiae* (Figure 17). There may be additional residues in Hst1 that confer an interaction with the Sum1 complex but are conserved between ScHst1, ScSir2 and KlSir2.

In this study I suggest that the particular evolutionary path taken as duplicated genes diverge from one another may be an important indicator of their potential contribution to genetic robustness. Duplicates that have subfunctionalized through a DDC mechanism may be more likely to substitute for each other than duplicates that display accelerated evolution or neofunctionalizion. *SIR3* and *ORC1* represent a pair of duplicated genes arising from the whole genome duplication that, in contrast to *SIR2*

and *HST1*, experienced accelerated evolution (Kellis et al. 2004). Orc1 is an essential component of the origin recognition complex. Deletion of *ORC1* results in lethality, and Sir3 cannot complement an *orc1* mutation. Likewise, Orc1 cannot suppress a *sir3*Δ mating defect (Bell et al. 1995). *ORC1* and *SIR3* are clearly an example of a duplicated gene pair that does not provide genetic robustness.

These results illustrate how gene duplication can provide genetic robustness against null mutations. It has been shown in *S. cerevisiae* that genes with duplicates are significantly more likely to have a weaker fitness defect phenotype compared to non-duplicated genes (Gu et al. 2003; Pasek et al. 2006). Here I present data revealing that duplication provides genetic robustness through substitution not redundancy. This is an important distinction because about 550 duplicated gene pairs in *S. cerevisiae* were retained after the genome duplication (Dietrich et al. 2004; Kellis et al. 2004), and many of these duplicates have diverged from each other (Kellis et al. 2004). It is quite likely that there are other duplicate genes, in addition to *SIR2* and *HST1*, which in wild-type backgrounds have non-overlapping functions, yet, are able to substitute for one another in the event of a deletion. The biological significance of this phenomenon will be reflected in a null phenotype that underestimates or masks the real function of the deleted gene. Thus, one should apply caution in interpreting deletion phenotypes, particularly if it is known that the gene of interest has a retained duplicate. This study also demonstrates that, in the case of an enzyme, the use of an inactivating mutation that abolishes enzymatic activity may be more useful in characterizing protein function than a complete deletion because such inactivating mutations retain the protein in the cell and thereby prevent an alternative protein from taking its place.

Finally, I can draw some conclusions about the relationship between different transcriptional repression mechanisms. It is clear from this study that deacetylation is

an important component of Sum1-mediated repression, as it is in Sir-mediated silencing. However, there is no intrinsic property of the deacetylase that determines whether it will act in a promoter-specific or regional manner (Figures 10 and 11). The results described here are consistent with previous results indicating that a mutant form of Sum1 does spread and that this spreading requires the deacetylase activity of Hst1 rather than Sir2 (Lynch et al. 2005). Therefore, the tendency for a repressor complex to spread or not to spread is probably a function of the DNA or histone binding proteins with which the histone deacetylase associates. Sir2 is able to spread because its partners, Sir3 and Sir4 are able to spread. In fact, Sir3 and Sir4 can spread in the absence of Sir2 deacetylase activity when the histone tails mimic a deacetylated state (Yang and Kirchmaier 2006), supporting the model that the role of Sir2 is to provide a substrate for its partners to bind. In contrast to Sir2, Hst1 does not spread because its partner, Sum1, normally does not spread (Lynch et al. 2005). This model is consistent with the hypothesis that the single ancestral histone deacetylase associated with both spreading (Sir) and non-spreading (Sum1) complexes.

3. The Sir2-Sum1 complex represses transcription using both promoter-specific and long-range mechanisms to regulate cell identity and sexual cycle in the yeast Kluyveromyces lactis[1]

3.1 Introduction

Deacetylases of the Sir2 family regulate lifespan and response to stress. I have examined the evolutionary history of Sir2 and Hst1, which arose by gene duplication in budding yeast and participate in distinct mechanisms of gene repression. In *Saccharomyces cerevisiae*, Sir2 interacts with the SIR complex to generate long-range silenced chromatin at the cryptic mating-type loci, *HMLα* and *HMR**a***. Hst1 interacts with the SUM1 complex to repress sporulation genes through a promoter-specific mechanism. I examined the functions of the non-duplicated Sir2 and its partners, Sir4 and Sum1, in the yeast *Kluyveromyces lactis*, a species that diverged from *Saccharomyces* prior to the duplication of Sir2 and Hst1. KlSir2 interacts with both KlSir4 and KlSum1 and represses the same sets of target genes as ScSir2 and ScHst1, indicating that Sir2 and Hst1 subfunctionalized after duplication.

However, the KlSir4-KlSir2 and KlSum1-KlSir2 complexes do not function as the analogous complexes do in *S. cerevisiae*. KlSir4 contributes to an extended repressive chromatin only at *HMLα* and not at *HMR**a***. In contrast, the role of KlSum1 is broader. It employs both long-range and promoter-specific mechanisms to repress cryptic mating-type loci, cell-type specific genes and sporulation genes and represents an important regulator of cell identity and the sexual cycle. This study reveals that a single repressive complex can act through two distinct mechanisms to regulate gene expression and

[1] Adapted from Hickman, MA and Rusche, LN (2009) The Sir2-Sum1 complex represses transcription using both promoter-specific and long-range mechanisms to regulate cell identity and sexual cycle in the yeast *Kluyveromyces lactis*. *PLoS Genetics* **5**(11): e1000710

illustrates how mechanisms by which regulatory proteins act can change over evolutionary time.

3.2 Results

KlSir2 physically associates with both KlSir4 and KlSum1

To determine whether the non-duplicated KlSir2 has functions analogous to both ScSir2 and ScHst1, I first identified its binding partners in *K. lactis* (described in Table 1). If KlSir2 functions similarly to ScSir2, it should associate with KlSir4, and if it has a function analogous to ScHst1 it should associate with KlSum1. Trans-species complementation experiments previously demonstrated that KlSir2 associates with both ScSir4 and ScSum1 in *S. cerevisiae* (Hickman and Rusche 2007), suggesting that analogous interactions occur in *K. lactis*. I created a *K. lactis* strain with alleles of *KlSIR2*-HA, *KlSIR4*-Flag and myc-*KlSUM1* integrated at their chromosomal locations. All three tagged proteins were detectable by immunoblotting (Figure 18) and maintained wild-type function, as assessed by RT-PCR analysis of genes repressed by these proteins (data not shown).

Table 1: Overview of *K. lactis* genes described in this study.

Common Name	*K. lactis* systematic name	*S. cerevisiae* homolog	Conservation[1]	Biological Function in *S. cerevisiae*
KlSIR2	*KLLA0F14663g*	*ScSIR2*[2]	**56** (78)	silences *HML* , *HMR* , telomeres, and the rDNA locus, in complex with Sir4 and Sir3
		ScHST1[2]	**63** (84)	repressor of middle sporulation-specific genes, in complex with Rfm1 and Sum1
KlSUM1	*KLLA0C14696g*	*ScSUM1*	**33** (59)	repressor of middle sporulation-specific genes, in complex with Rfm1 and Hst1
KlRFM1	*KLLA0C07062g*	*ScRFM1*	**36** (63)	repressor of middle sporulation-specific genes, in complex with Hst1 and Sum1
KlSIR4[3]	*KLLA0F14320g*	See Figure 20		silences *HML* , *HMR* and telomeres, in complex with Sir2 and Sir3
KlASF2[3]	*KLLA0F13998g*	See Figure 20		Anti-silencing protein that causes derepression of silent loci when overexpressed

1) **Percent identity** (percent similar), calculated from FASTA sequence alignments
2) *SIR2* and *HST1* are a duplicate gene pair, duplicated in the whole-genome duplication
3) *SIR4* and *ASF2* are a tandem duplicate gene pair, duplicated prior to the whole-genome duplication

If KlSir2 associates with both KlSir4 and KlSum1, it should co-precipitate with these proteins, and indeed, KlSir2 did co-precipitate with both KlSir4 and KlSum1 (Figure 18A). In *S. cerevisiae*, the association of ScSum1 with ScHst1 requires ScRfm1 (McCord et al. 2003). To determine if Rfm1 mediates the interaction between Sum1 and Sir2 in *K. lactis,* I examined whether the co-precipitation between KlSir2 and KlSum1 persisted in the absence of KlRfm1. There was no observable co-precipitation between KlSir2 and KlSum1 in an *rfm1*Δ strain (Figure 18A), suggesting that the architecture of the SUM1 complex is conserved between *S. cerevisiae* and *K. lactis*.

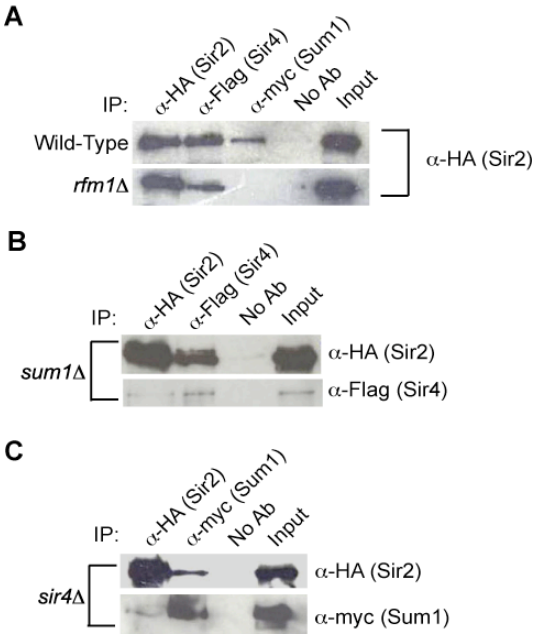


Figure 18: KlSir2 co-precipitates with KlSir4 and KlSum1.

A) KlSir2-HA, KlSir4-Flag or myc-KlSum1 was precipitated from a lysate prepared from wild-type (LRY2285) or *rfm1*Δ (LRY2528) strains, and the precipitated material was examined by immunoblotting with an antibody against the HA tag to detect KlSir2-HA. The input represents 33% of the IP. **B**) KlSir2-HA or KlSir4-Flag was immunoprecipitated from a *sum1*Δ strain (LRY2158), and the precipitated material was examined with an antibody against the HA tag to detect KlSir2-HA or the Flag tag to detect KlSir4-Flag. **C**) KlSir2-HA or myc-KlSum1 was immunoprecipitated from a *sir4*Δ strain (LRY2282), and the precipitated material was examined with an antibody against the HA tag to detect KlSir2-HA or the myc tag to detect myc-KlSum1.

Given the association of KlSir2 with both KlSir4 and KlSum1, all three proteins might be part of a stable complex. However, a co-precipitation between KlSir4 and KlSum1 was not detected (data not shown), although we could not distinguish whether this result reflected the absence of a complex containing KlSir4 and KlSum1 or simply its instability. Nevertheless, if this complex does exist, the components are not mutually dependent on one another for association, as KlSir2 and KlSir4 still co-precipitated in the absence of KlSum1 (Figure 18B) and KlSir2 and KlSum1 co-precipitated in the absence of KlSir4 (Figure 18C). Therefore, KlSir2 forms independent associations with both KlSir4 and KlSum1, a finding consistent with KlSir2 having functions analogous to those of both ScSir2 and ScHst1.

KlSir2, KlSir4 and KlSum1 repress HMLα

I next investigated whether the Sir4-Sir2 and Sum1-Sir2 complexes have the same repressive functions in *K. lactis* as they do in *S. cerevisiae*. If these functions are conserved, deletion of *KlSIR4* should derepress the *HM* loci, deletion of *KlSUM1* should derepress mid-sporulation genes, and deletion of *KlSIR2* should derepress both *HM* loci and mid-sporulation genes. I first examined silencing at *HMLα*, which is known to be repressed by KlSir2 and KlSir4 (Astrom and Rine 1998; Aström et al. 2000). To extend this previous result and address the role of KlSum1 at *HMLα*, I isolated RNA from *MAT**a*** wild-type, *sir2*Δ, *sir4*Δ, *sum1*Δ, and *rfm1*Δ strains and examined the expression of *HMLα1*, *HMLα2* and *HMLα3* by quantitative RT-PCR. All three genes were significantly derepressed in the absence of KlSir2 and modestly derepressed in the absence of KlSir4 (Figure 19), consistent with previous reports. Surprisingly, deletion of KlSum1 resulted in derepression of *HMLα* to a similar extent as observed in the *sir2*Δ strain. In contrast to KlSum1, deletion of KlRfm1 had very little effect on the transcription of *HMLα*. This

result suggests that KlSir2 does not require KlRfm1 to act at *HMLα* and therefore may act independently of KlSum1. In this case, a *sir2*Δ *sum1*Δ double deletion might disrupt silencing to a greater extent than either single deletion. However, there was no difference in transcription of *HMLα* in a *sir2*Δ *sum1*Δ strain compared to a *sir2*Δ or *sum1*Δ strain (Figure 19).

To confirm that these phenotypes resulted from the deletions of the intended genes, plasmids expressing the wild-type *KlSIR2*, *KlSIR4* and *KlSUM1* genes were introduced into the corresponding deletion strains. In all cases, repression was restored (data not shown). These results reveal that KlSum1, in addition to KlSir2 and KlSir4, contributes to the silencing of *HMLα*. Thus, KlSum1 behaves differently than its ortholog in *S. cerevisiae*, as the deletion of ScSum1 does not alter the expression of *ScHMLα* (Chi and Shore 1996).

It is interesting to note that in both the *sir2*Δ and *sum1*Δ strains the induction of *HMLα3* was modest compared to *HMLα1* or *HMLα2*, suggesting that *HMLα3* may be regulated differently than the other two genes at *HMLα*. The *α3* gene, which is specific to *Kluyveromyces*, is proposed to be a MULE family DNA transposase (Babu et al. 2006) and is required for mating and mating type-switching in MAT*α* cells (Aström et al. 2000; Barsoum et al. 2010a).

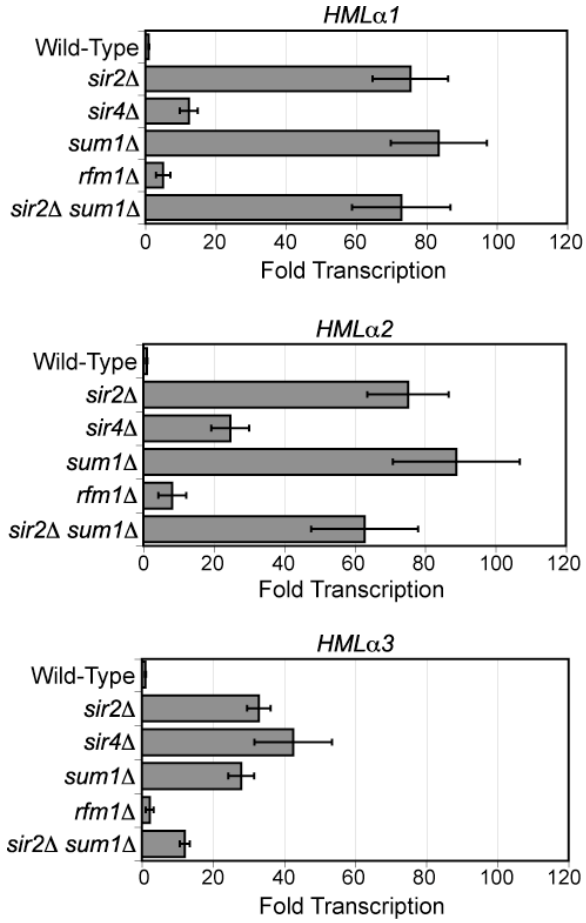


Figure 19: KlSir2, KlSir4 and KlSum1 silence the cryptic mating-type locus *HML*.

Quantitative RT-PCR analysis of *HMLα1*, *HMLα2* and *HMLα3* mRNA in wild-type (CK213), *sir2*Δ (SAY569), *sir4*Δ (LRY2038), *sum1*Δ (LRY2035), *rfm1*Δ (LRY2528) and *sir2*Δ *sum1*Δ (LRY2533) strains. The amount of cDNA was first normalized to the control locus *ACT1*. The values shown here represent the relative amount of cDNA for each deletion strain compared to the wild-type strain. Error bars represent the SEM.

The modest derepression of the *HMLα* locus observed in the *sir4*Δ strain suggested that another protein might compensate for KlSir4 in its absence. The *SIR4* gene was duplicated in tandem prior to the whole-genome duplication, and each of the tandem duplicates was retained as a single gene after the whole-genome duplication (Byrne and Wolfe 2005). This ancient duplicate of Sir4, Asf2 (Anti-Silencing Factor 2), reduces silencing when over-expressed in *S. cerevisiae* (Le et al. 1997). The *SIR4* and *ASF2*

genes are rapidly evolving, making it difficult to determine which *K. lactis* gene is orthologous to which *S. cerevisiae* gene (Figure 20). Gene *KLLAOF14320g* has been designated *KlSIR4* based on functional studies (Astrom and Rine 1998), and therefore I refer to the other gene (*KLLA0F13398g*) as *KlASF2*.

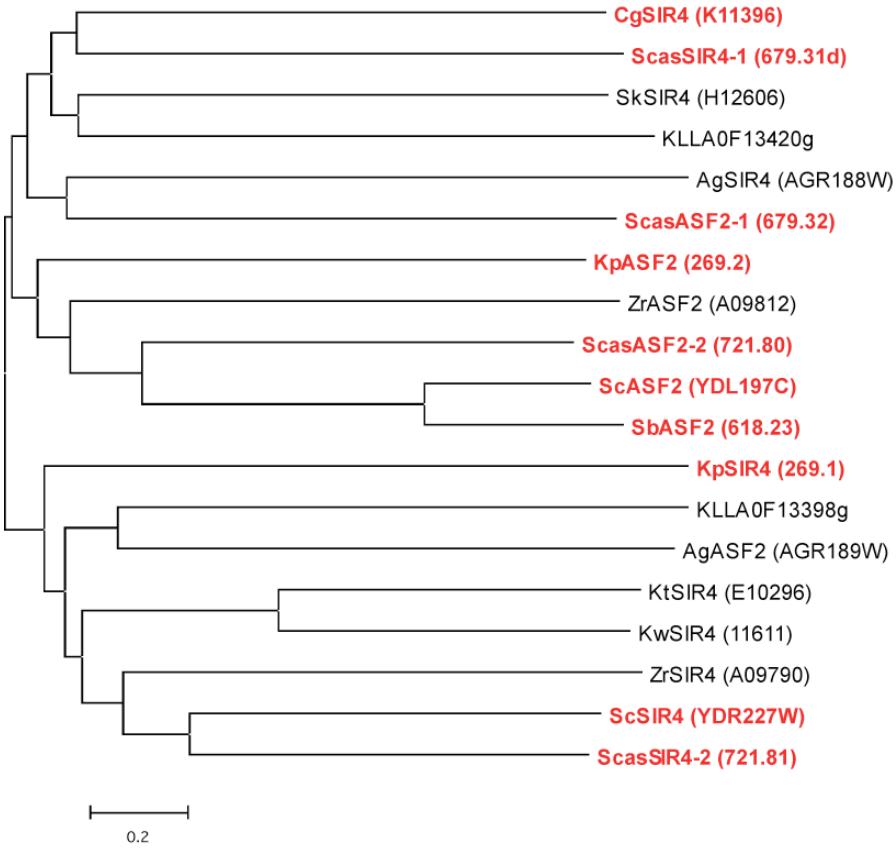


Figure 20: Phylogenetic gene tree of *SIR4* and *ASF2* orthologs from several hemiascomycete species.

Sequences and nomenclature were obtained from the Yeast Gene Order Browser (Byrne and Wolfe 2005) and analyzed using MEGA (Tamura et al 2007) to construct the neighbor joining gene tree. Bold, red fonts indicate species that underwent the whole genome duplication. ***Sc=S. cerevisiae, Sb=S. bayanus, Cg=C. glabrata, Scas=S. castellii, Kp=K. polysporus***, *Zr=Z. rouxii, Ag=A. gossypii, Sk=S. kluyveri, Kt=K. thermotolerans, Kw=Kwaltii.* Common names, as notated in the YGOB, are given along with the systematic names in parentheses. *K. lactis* common gene names are not given to illustrate how *KLLA0F1430g* and *KLLA0F13998g* cluster.

To determine whether its common ancestry with KlSir4 enables KlAsf2 to silence *HMLα* in the absence of KlSir4, I constructed both *asf2*Δ and *asf2*Δ *sir4*Δ strains and examined expression of the *HMLα* genes. The lack of KlAsf2 resulted in the further repression of all three genes to less than one-tenth the level of the wild-type strain, and the double deletion of *asf2*Δ and *sir4*Δ resembled the single *sir4*Δ deletion (Figure 21). Therefore, *KlASF2* does not have a *SIR4*-like function. In fact, *KlASF2*, like *ScASF2,* is antagonistic to silencing.

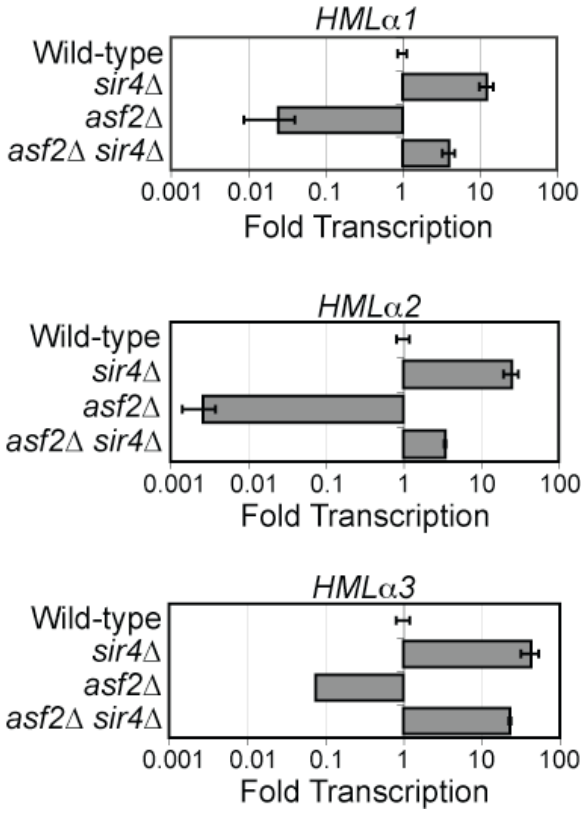


Figure 21: KlAsf2 antagonizes silencing at *HML*.

Quantitative RT-PCR analysis of *HMLα1*, *HMLα2* and *HMLα3* mRNA in wild-type (CK213), *sir2*Δ (SAY569), *sir4*Δ (LRY2038), *asf2*Δ (LRY2377), *asf2*Δ *sir4*Δ (LRY2374) and *sir2*Δ *asf2*Δ (LRY2523) strains. The data for wild-type, *sir2*Δ and *sir4*Δ strains is the same as Figure 19.

KlSir2, KlSir4 and KlSum1 spread across HMLα but not MATα

Given the surprising result that KlSum1 affects the expression of *HMLα*, it was important to investigate whether KlSum1 acts directly at *HMLα* to silence transcription. I also examined the association of KlSir2 and KlSir4 with *HMLα*, as the association of these proteins with *HMLα* had not been assessed previously. I used chromatin immunoprecipitation to map the distributions of KlSir2, KlSir4, KlSum1 and KlRfm1 across *HMLα*. I observed a robust enrichment of all four proteins across the entire *HMLα* locus (Figure 22), demonstrating that not only KlSir2 and KlSir4, but also the components of the SUM1 complex, KlSum1 and KlRfm1, spread across this locus. Therefore, KlSum1 contributes directly to transcriptional silencing at *HMLα*.

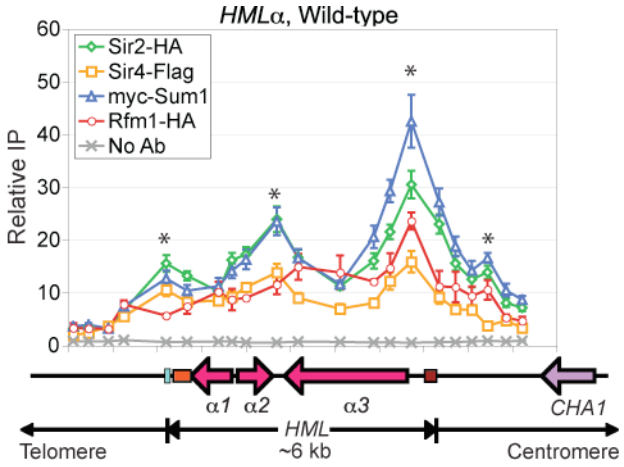


Figure 22: KlSir2, KlSir4, KlSum1 and KlRfm1 spread across *HML*.

The association of KlSir2-HA, KlSir4-Flag, myc-KlSum1 (LRY2239) and KlRfm1-HA (LRY2327) with *HMLα* as assessed by chromatin IP followed by quantitative PCR. The y-axis represents the relative enrichment normalized to a control locus, *RRP7*, which is not detectably associated with KlSir2, KlSir4 or KlSum1. A diagram of the *HMLα* locus is shown under the x-axis. The aqua bar represents the characterized silencer and the orange and brown boxes represent sequences found at *HMLα*, *MAT*, and *HMRa* loci. Asterisks indicate the peaks of enrichment.

The enrichment of KlSir2, KlSir4 and KlSum1 peaked at a previously identified silencer ((Sjöstrand et al. 2002), represented as an aqua bar in Figure 22), suggesting that this sequence may stabilize the association of silencing proteins with chromatin. Three other peaks were also observed (indicated by asterisks in Figure 22): one in the intergenic region in which the *α2* and *α3* genes converge, one in the *α3* promoter, and a smaller peak on the centromere proximal side of *HMLα*. These peaks could represent additional silencers or proto-silencers. Curiously, two of the peaks coincided with sequences that are conserved between the transcriptionally silent *HMLα* locus and the transcriptionally active *MATα* locus. If these peaks represent binding sites for silencing factors, then these factors might be recruited to *MATα*. To examine this possibility, I constructed a strain in which the *α*-cassette at *HML* was replaced with an **a**-cassette, so that the only *α*-cassette in the genome was at the *MAT* locus. Using this strain, I investigated whether KlSir2, KlSir4 or KlSum1 associated with the *MAT* locus. All three proteins associated with control loci (data not shown). However, I observed no significant enrichment of KlSum1, KlSir2 or KlSir4 anywhere along the *MATα* locus (Figure 23). Therefore, the peaks of silencing proteins at the *α3* promoter and the *α2*- *α3* intergenic regions are specific to the *HMLα* locus, and these sequences cannot recruit silencing proteins independently.

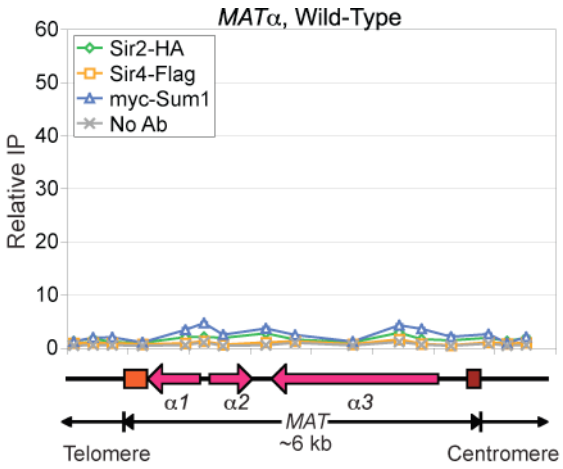


Figure 23: KlSir2, KlSir4 and KlSum1 do not associate with *MATα*.

The association of KlSir2-HA, KlSir4-Flag and myc-KlSum1 with the *MATα* locus in a strain in which the *α*-cassette is only found at *MAT* (LRY2398) as assessed by chromatin IP.

Both KlSir4 and KlSum1 recruit KlSir2 to HMLα

Sir2 deacetylases lack DNA-binding and histone-binding domains and consequently are recruited to chromatin through adaptor proteins such as Sum1, a DNA binding protein, or Sir4, a histone binding protein. To determine whether KlSir4 and/or KlSum1 recruit KlSir2 to *HMLα*, I examined the association of KlSir2 with *HMLα* in strains lacking these proteins. In a *sir4*Δ strain, the enrichments of KlSir2 and KlSum1 were significantly reduced over the silencer and across the open reading frames of *α1*, *α2*, and *α3* (Figure 24). However, the associations of KlSir2 and KlSum1 with the promoter of *α3* and centromere-proximal side of *HMLα* were unchanged. Thus, there may be different requirements for the assembly of silenced chromatin on the two sides of the *HMLα* locus. On the telomere-proximal side, containing the known silencer, KlSir4 is important for the recruitment and spreading of silencing proteins. However, on the centromere-proximal side, the recruitment of KlSum1 and KlSir2 is independent of KlSir4.

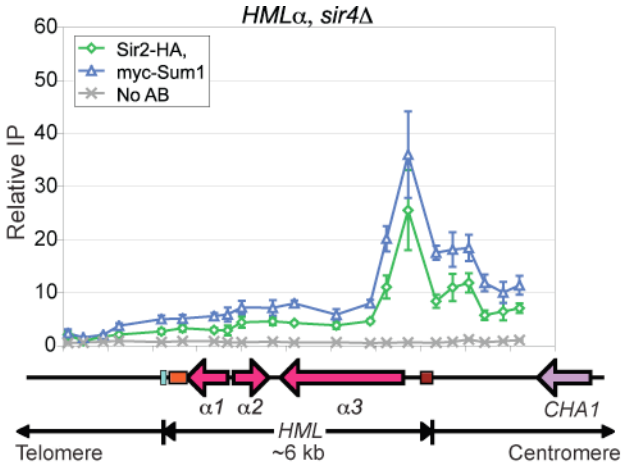


Figure 24: KlSir2 and KlSum1 association with *HML* in a *sir4*Δ strain.

The association of KlSir2-HA and myc-KlSum1 with *HMLα* in a *sir4*Δ strain (LRY2281) as assessed by chromatin IP.

The ability of KlSir2 to associate with the centromere-proximal side *HMLα* in the absence of KlSir4 suggests that another protein is recruiting KlSir2 to this region. To determine whether KlSum1 is required for the recruitment or spreading of KlSir2 and KlSir4, I examined the associations of these proteins with *HMLα* in a *sum1*Δ strain. The deletion of KlSum1 caused a reduction in the association of KlSir2 at the *α*2-*α*3 intergenic region, the *α*3 promoter and on the centromere-proximal side of the *HMLα* locus. There was no observable difference in the association of KlSir4 with *HMLα* (Figure 25). These results suggest that KlSum1 is important for stabilizing the association of KlSir2 with the *HMLα* locus, particularly at the *α*3 promoter and centromere-proximal regions, but that it is not absolutely required for the recruitment or spreading of either KlSir2 or KlSir4. Together, these results indicate that neither KlSir4 nor KlSum1 is solely responsible for the recruitment of KlSir2 to *HMLα*. This finding is consistent with the independent interactions of KlSir2 with KlSir4 and KlSum1 (Figure 18).

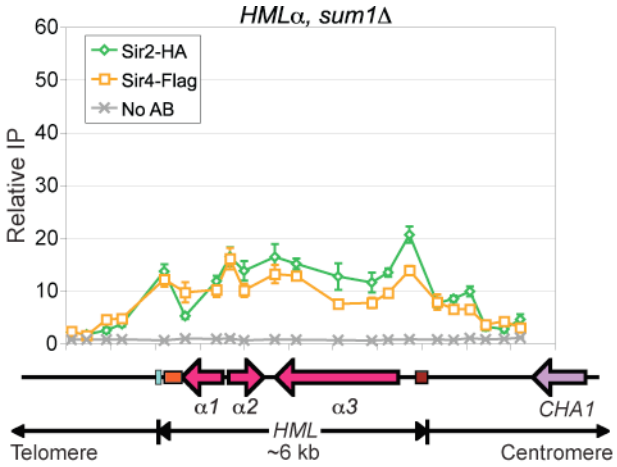


Figure 25: KlSir2 and KlSir4 association with *HML* in a *sum1*Δ strain.

The association of KlSir2-HA and KlSir4-Flag with *HMLα* in a *sum1*Δ strain (LRY2158) as assessed by chromatin IP.

The greater level of transcription of *HMLα* in a *sir2*Δ strain compared to an *rfm1*Δ strain (Figure 19) suggests that KlRfm1 is not critical for the recruitment of KlSir2 or other silencing proteins. In fact, in the absence of KlRfm1, all three silencing proteins, KlSir2, KlSir4 and KlSum1, still associated with the entire *HMLα* locus (Figure 26). The enrichment of KlSum1 was indistinguishable between the wild-type and *rfm1*Δ strains, indicating that its association with *HMLα* does not require KlRfm1 and may be an inherent property of the Sum1 protein. Interestingly, the enrichments of both KlSir2 and KlSir4 were significantly enhanced in the *rfm1*Δ strain compared to the wild-type strain, although the overall pattern, with peaks of association at the silencer, *α2-α3* intergenic region, *α3* promoter and centromere-proximal side of *HMLα,* was maintained. Perhaps in the absence of KlRfm1, KlSir2 is better able to associate with KlSir4.

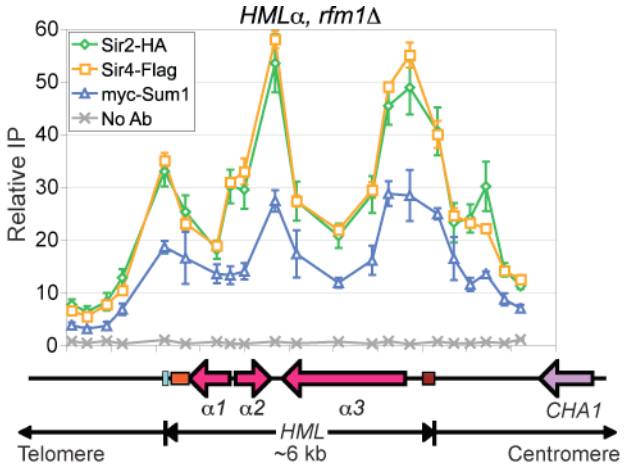


Figure 26: KlSir2, KlSir4 and KlSum1 association with *HML* in an *rfm1*Δ strain.

The association of KlSir2-HA, KlSir4-Flag and myc-KlSum1 with *HMLα* in an *rfm1*Δ strain (LRY2528) as assessed by chromatin IP.

In *S. cerevisiae*, the deacetylase activity of Sir2 is required for the spreading of Sir3 and Sir4 (Hoppe et al. 2002b; Luo et al. 2002b; Rusché et al. 2002). To determine whether a similar requirement exists in *K. lactis*, I examined the associations of KlSir4 and KlSum1 with *HMLα* in a *sir2*Δ strain. KlSir4 and KlSum1 were reduced over the silencer and the three open reading frames (Figure 27). However, both silencing proteins remained strongly associated with the *α3* promoter, and KlSir4 displayed a more robust enrichment with this region in the absence of KlSir2. This pattern of association is similar to the distribution of KlSum1 and KlSir2 in the *sir4*Δ strain (Figure 24). Therefore, KlSir2 may contribute to the assembly of silenced chromatin on the telomere-proximal side of *HMLα*, but it is not required to assemble these factors at the *α3* promoter.

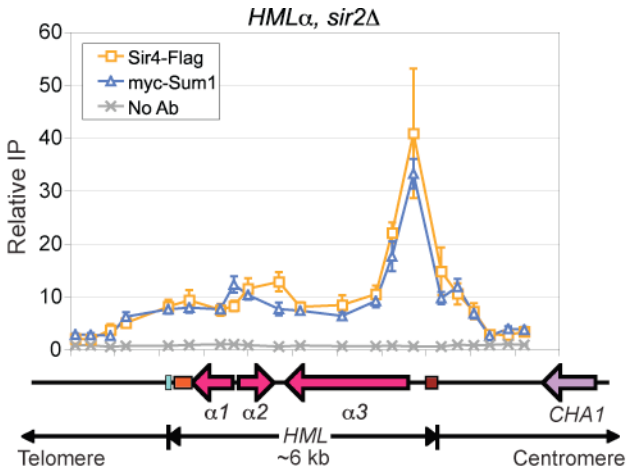


Figure 27: KlSir4 and KlSum1 association with *HML* in a *sir2*Δ strain.

The association of KlSir4-Flag and myc-KlSum1 with *HMLα* in a *sir2*Δ strain (LRY2388) as assessed by chromatin IP.

KlSum1 associates with HMLα independently of KlSir2 and KlSir4

Given that KlSum1 is a DNA-binding protein, I was curious whether it binds directly to a sequence at *HMLα*. The mid-sporulation element (MSE) consensus sequence, to which Sum1 binds in *S. cerevisiae*, appears to be conserved in *K. lactis*, as it occurs at the promoters of a number of sporulation genes (data not shown). However, a match to the MSE consensus sequence was not found in the known telomere-proximal silencer (aqua box) or the rest of the *HMLα* locus. Moreover, the observation that the enrichment of KlSum1 was significantly reduced on the telomere-proximal side of *HMLα* in the absence of KlSir4 or KlSir2 (Figures 24 and 27) makes it unlikely that KlSum1 binds directly to this side of the locus. Furthermore, KlSum1 did not associate with the *MATα* locus (Figure 23), indicating that the sequences conserved between *MATα* and *HMLα* are unable to recruit KlSum1 directly. It remains possible that KlSum1 binds directly to a non-MSE sequence on the centromere-proximal side of the *HMLα*, and KlSum1 did associate with this region of *HMLα* in the absence of both KlSir2 and

KlSir4 (Figure 28), indicating that the recruitment of KlSum1 to *HMLα* is independent of KlSir2 and KlSir4. However, it is also possible that another, unidentified protein recruits KlSum1 to this region.

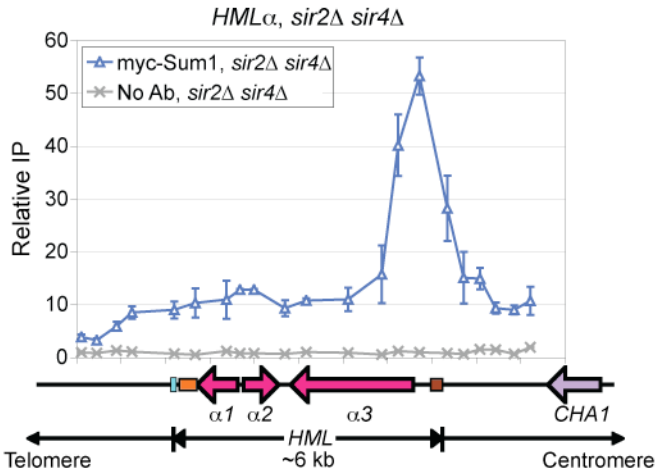


Figure 28: KlSum1 association with *HML* in a *sir2Δ sir4Δ* strain.

The association of myc-KlSum1 with *HMLα* as assessed by chromatin IP followed by quantitative PCR in a *sir2Δ sir4Δ* strain (LRY2530).

*KlSir2 and KlSum1, but not KlSir4, repress HMR**a***

I next investigated the roles KlSir2, KlSir4, KlSum1 and KlRfm1 have in regulating the other cryptic mating-type locus, *HMR**a***. In *S. cerevisiae*, both *HM* loci are silenced by the same set of Sir proteins. However, in *K. lactis*, deletion of KlSir4 had little effect on the expression of the ***a1*** or ***a2*** genes found at *HMR**a*** (Figure 29). Furthermore, deletion of KlAsf2, the paralog of KlSir4, either singly or in conjunction with KlSir4 did not result in derepression of *HMR**a*** (Figure 29). In contrast, deletion of KlSir2 or KlSum1 resulted in a substantial derepression of *HMR**a1*** and *HMR**a2***, whereas deletion of KlRfm1 resulted in very little change in *HMR**a1*** or *HMR**a2*** expression (Figure 29). These

results suggest that only a subset of the proteins that contribute to the silencing of *HMLα* also repress *HMR**a***.

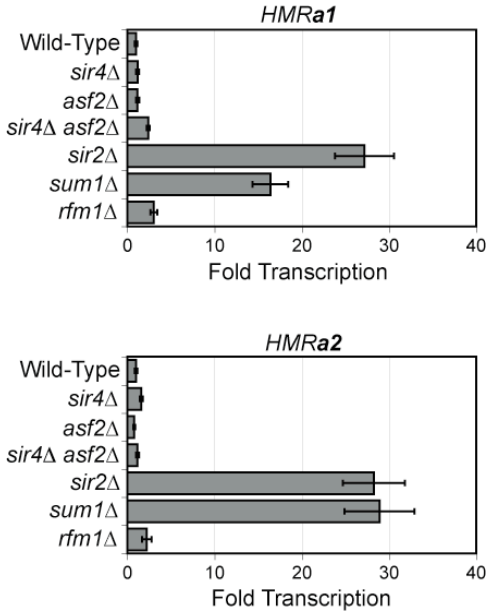


Figure 29: KlSir2 and KlSum1, but not KlSir4, silence *HMR*.

Quantitative RT-PCR analysis of *HMRa1* and *HMRa2* in wild-type (SAY538), *sir4*Δ (LRY1946), *asf2*Δ (LRY1856), and *asf2*Δ *sir4*Δ (LRY1948), *sir2*Δ (SAY544), *sum1*Δ (LRY1947) and *rfm1*Δ (LRY2529) strains.

To determine whether KlSir2 and KlSum1 act directly at *HMR**a***, I examined their association by chromatin immunoprecipitation. I observed an asymmetric distribution of KlSir2 and KlSum1, as well as KlRfm1, with the *HMR**a*** locus. A substantial peak of enrichment was observed on the centromere-proximal side of *HMR**a***, and a shoulder extended across the open reading frames (Figure 30). The peak likely indicates the location of a silencer element. In contrast to KlSir2 and KlSum1, there was no significant association of KlSir4 with any part of *HMR**a***, consistent with the deletion of *SIR4* resulting in no change in the transcription of *HMR**a1*** and *HMR**a2***. These results indicate

that KlSum1 and KlSir2, but not KlSir4, are responsible for repressing *HMR**a***. Thus, the mechanisms of silencing at *HMR**a*** and *HML**α*** are distinct.

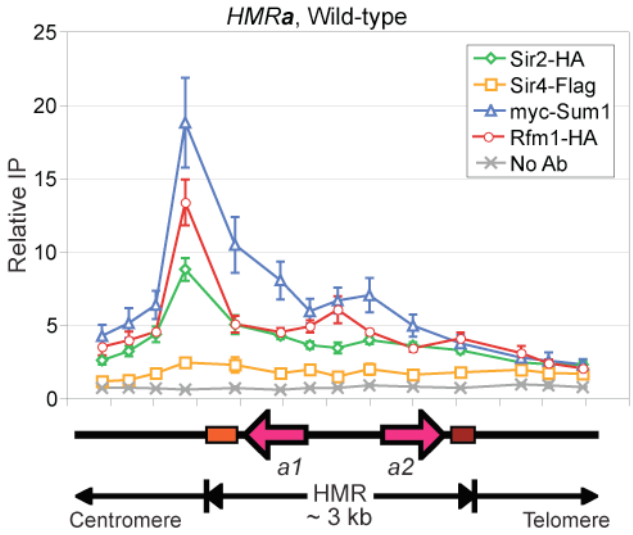


Figure 30: KlSir2 and KlSum1, but not KlSir4, associate with *HMR*.

The association of KlSir2-HA, KlSir4-Flag, myc-KlSum1 (LRY2285) and KlRfm1-HA (LRY2328) with *HMR**a*** as assessed by chromatin IP followed by quantitative PCR.

Curiously, KlRfm1 associated with *HMR**a*** (Figure 30), yet was not required for repression of the *HMR**a1*** and *HMR**a2*** genes (Figure 29). I examined the association of KlSum1 and KlSir2 with *HMR**a*** in an *rfm1*Δ strain and found that KlSum1 was only slightly reduced at the proposed silencer (Figure 31). Intriguingly, KlSir2 was still able to associate with *HMR**a*** in the absence of KlRfm1, despite the fact that it no longer co-precipitated with KlSum1 (Figure 18). I propose that the absence of KlRfm1 may enable KlSir4 to interact with KlSir2 and KlSum1, thereby stabilizing the association of KlSir2 with *HMR**a***. To test this hypothesis, I assessed whether KlSir4 associated with *HMR**a*** in an *rfm1*Δ strain, and indeed, KlSir4 associated with *HMR**a*** (Figure 31). This result is reminiscent of the increase in KlSir4 at *HML**α*** in the absence of KlRfm1 (Figure 26).

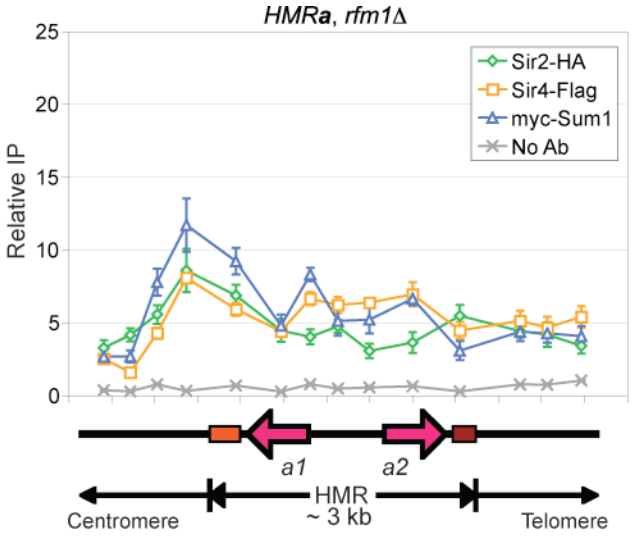


Figure 31: KlSir2 and KlSum1 association with HMR in an *rfm1*Δ strain.

The association of KlSir2-HA (LRY2528), myc-KlSum1 and KlSir4-Flag (LRY2529) with *HMR**a*** in an *rfm1*Δ strain as assessed by chromatin IP.

To determine whether KlSum1 and KlSir2 depended on one another for association with *HMR**a***, I performed chromatin immunoprecipitation experiments in the absence of KlSum1 or KlSir2. In the absence of KlSum1, KlSir2 no longer associated with any region of the *HMR**a*** locus (Figure 32), and therefore KlSum1 was required for recruitment of KlSir2 to *HMR**a***. This result contrasts with what was observed in the absence of KlRfm1 (Figure 31). Deletion of KlSir2, like deletion of KlRfm1, resulted in a reduced association of KlSum1 with the proposed silencer at *HMR**a***. Despite this reduction, KlSum1 still spread across *HMR**a*** (Figure 32). Thus, the association and spreading of KlSum1 does not require KlSir2 or KlRfm1.

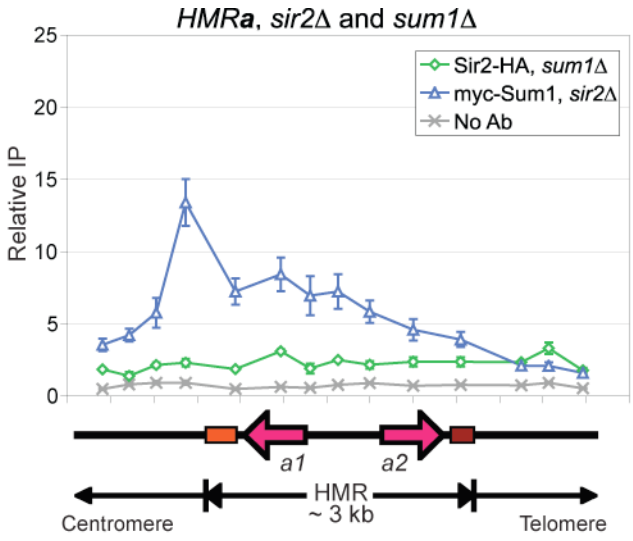


Figure 32: KlSir2 and KlSum1 association with HMR in *sir2*Δ and *sum1*Δ strains.

The association of KlSir2-HA with *HMR**a*** in a *sum1*Δ strain (LRY2126) and the association of myc-KlSum1 with *HMR**a*** in a *sir2*Δ strain (LRY2390) as assessed by chromatin IP.

KlSir2 and KlSum1 repress mid-sporulation genes in a promoter-specific manner

In *S. cerevisiae*, the Sum1-Hst1 complex represses mid-sporulation genes. To assess whether KlSir2 regulates mid-sporulation genes in a manner similar to ScHst1, I isolated RNA from wild-type, *sir2*Δ, *sum1*Δ and *rfm1*Δ strains and examined expression of the *K. lactis* orthologs of the mid-sporulation genes *CDA2*, *SPR3*, *SPS4*, and *SPS2* that are repressed by ScHst1 in *S. cerevisiae* (Xie et al. 1999). Deletion of KlSir2, KlSum1 and KlRfm1 all resulted in derepression of *CDA2*, *SPS4*, and *SPR3*, but not *SPS2* (Figure 33, note the different scales of the x-axes). I also examined whether KlSir4 has a role in regulating transcription of these genes, as KlSir2 and KlSum1 functioned with KlSir4 to regulate *HMLα*. However, the *sir4*Δ strain had no effect on the expression of *CDA2*, *SPS4*, *SPR3* or *SPS2* (Figure 33). Therefore, KlSum1, KlSir2 and KlRfm1, repress sporulation genes independently of KlSir4. In addition, many (*CDA2*, *SPS4* and *SPR3*),

but not all (*SPS2*) of the targets of the Sum1-Hst1 complex in *S. cerevisiae* are also targets in *K. lactis*.

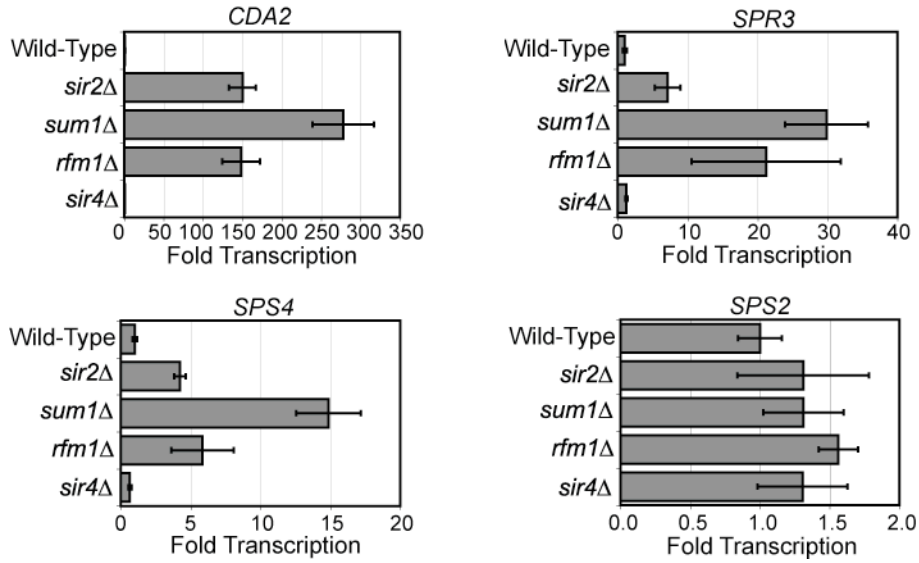


Figure 33: KlSir2, KlSum1 and KlRfm1 repress sporulation genes.

Quantitative RT-PCR analysis of *CDA2* (*KLLA0C17226g*), *SPS4* (*KLLA0F08679g*), *SPR3* (*KLLA0B08129g*) and *SPS2* (*KLLA0C01001g*) mRNA in wild-type (SAY538), *sir2*Δ (SAY544), *sum1*Δ (LRY1947), *rfm1*Δ (LRY2529) and *sir4*Δ (LRY1946) strains.

To determine if KlSir2, KlSum1, and KlRfm1 repress mid-sporulation genes directly, I used chromatin immunoprecipitation to assess the association of KlSir2, KlSum1, KlRfm1 and KlSir4 with the promoters of these genes. KlSir2, KlSum1 and KlRfm1 were enriched at the promoters of *CDA2*, *SPS4* and *SPR3* (Figure 34A), suggesting that these proteins repress these genes directly, presumably as a complex. In contrast, KlSir4 did not associate with mid-sporulation genes, consistent with the *sir4*Δ strain having no effect on transcription. To address whether KlSir2, KlSum1 and KlRfm1 spread at sporulation genes, as they do at *HMLα* and *HMR**a***, I examined a 3-kb region around the *CDA2* promoter and open reading frame. A relatively narrow peak of

KlSum1, KlRfm1 and KlSir2 coincided with an MSE consensus sequence at the promoter of *CDA2* (indicated by the blue bar in the schematic), and the association of these proteins diminished significantly in both directions (Figure 34B), suggesting that these proteins do not spread at the *CDA2* locus. Therefore, the ability of the SUM1 complex to spread differs between the *HM* loci and mid-sporulation genes.

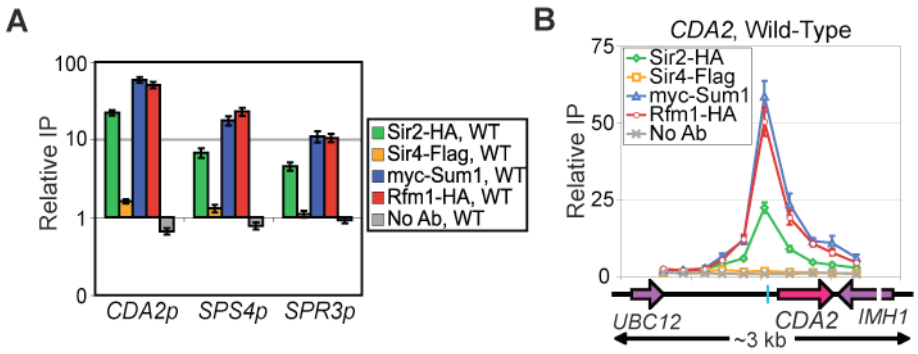


Figure 34: KlSir2, KlSum1 and KlRfm1 associate with sporulation gene promoters.

A) The association of KlSir2-HA, KlSir4-Flag, myc-KlSum1 (LRY2285) and KlRfm1-HA (LRY2328) with the promoters of *CDA2*, *SPS4* and *SPR3* was assessed by chromatin IP followed by quantitative PCR. The y-axis is a log-scale. **B**) Distribution of KlSir2-HA, KlSir4-Flag, myc-KlSum1 (LRY2285) and KlRfm1-HA (LRY2328) across the *CDA2* locus. The blue bar in the schematic represents the conserved MSE sequence.

I had observed at *HMLα* that KlAsf2 was antagonistic to silencing (Figure 21), and it was possible that KlAsf2 restricts the spreading of the Sum1-Sir2 complex at sporulation genes and therefore accounts for the difference in spreading at *HMR**a*** compared to sporulation genes. To test this hypothesis, I assessed the distribution of KlSum1 and KlSir2 at the sporulation gene *CDA2* in an *asf2*Δ strain. I observed no changes in the distribution of KlSir2 and KlSum1 across the *CDA2* locus (Figure 35A). Furthermore, the transcription of several mid-sporulation genes was not altered (Figure 35B). Therefore, KlAsf2 only antagonized silencing at *HMLα.*

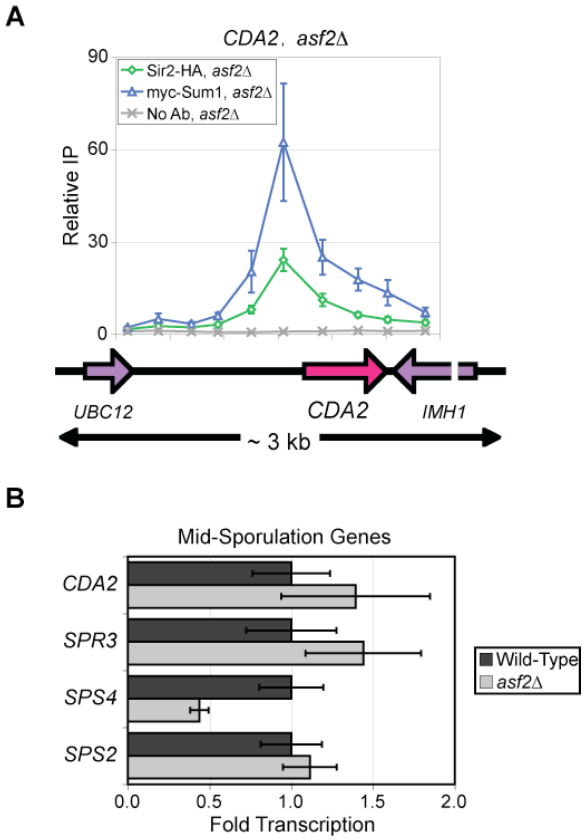


Figure 35: KlAsf2 does not affect the regulation of sporulation genes.

A) Association of KlSir2-HA and myc-KlSum1 with *CDA2* as assessed by chromatin IP followed by quantitative PCR in an *asf2*Δ strain (LRY2525). **B)** Quantitative RT-PCR analysis of *CDA2*, *SPS4*, *SPR3* and *SPS2* mRNA in wild-type (SAY538) and *asf2*Δ (LRY1856) strains.

I discovered that KlSir2 was more dependent on KlRfm1 for recruitment to *CDA2* as compared to *HMR**a***. At *HMR**a***, KlSir2 required KlSum1 but not KlRfm1 for recruitment (Figures 31 and 32). In contrast, the association of KlSir2 with *CDA2* was greatly reduced in both *sum1*Δ and *rfm1*Δ strains (Figure 36). This dependence was similar to what has been observed for the *S. cerevisiae* SUM1 complex at mid-sporulation genes. One potential explanation for the reduced role of KlRfm1 at the *HM* loci is the ability of KlSir4 to compensate for the loss of KlRfm1. For example, at both *HMLα* and

HMRa, the association of KlSir4 increased in the absence of KlRfm1 (Figures 26 and 31). In keeping with the greater role of KlRfm1 at *CDA2*, I observed only a modest increase in the association of KlSir4 (Figure 36) in the absence of KlRfm1. I also found that the ability of KlSum1 to associate with the promoter of *CDA2* was unaltered in the absence of KlSir2 (Figure 36), and was reduced, but not abolished, in the absence of KlRfm1 (Figure 36). Thus, KlRfm1 contributes to the ability of the SUM1 complex to associate with DNA. I conclude that the promoter-specific mechanism by which the SUM1 complex represses mid-sporulation genes is conserved between *K. lactis* and *S. cerevisiae*.

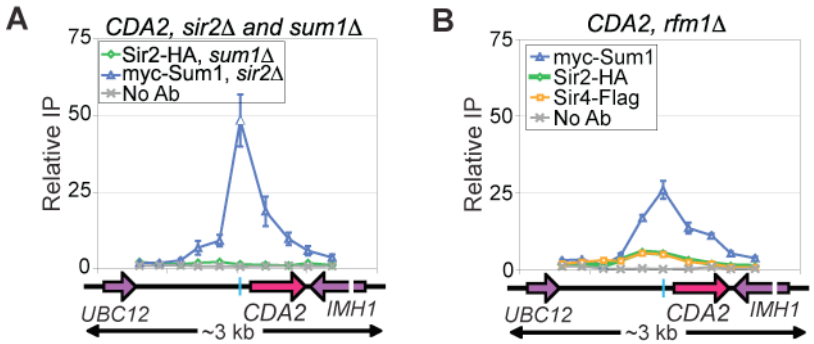


Figure 36: KlSir2 and KlSum1 association with *CDA2* in *sir2Δ*, *sum1Δ* and *rfm1Δ* strains.

A) The association of KlSir2-HA with *CDA2* in a *sum1*Δ strain (LRY2126) and association of myc-KlSum1 with *CDA2* in a *sir2*Δ strain (LRY2390). **B)** The association of KlSir2-HA and myc-KlSum1 with *CDA2* in an *rfm1*Δ strain (LRY2529). All y-axes are set to the same scale to compare changes in protein association across experiments.

KlSum1 and KlSir2 also repress cell-type specific genes

The KlSum1-KlSir2 complex is clearly critical to the regulation of sexual identity and the sexual cycle as it represses both the *HM* loci and sporulation genes. However, the Sum1-Sir2 complex may have an even broader role in controlling sexual identity. It has recently been shown in both *Saccharomyces bayanus* and *S. cerevisiae* that Sum1

represses *α*-specific genes (Zill and Rine 2008). To investigate whether the Sum1-Sir2 complex in *K. lactis* also represses *α*-specific genes or other cell-type specific genes, I examined whether promoters of cell-type specific genes were associated with KlSir2. Remarkably some, but not all, *α*-specific, **a**-specific and haploid-specific genes were associated with KlSir2 (Figure 37 and data not shown). For example, the *α*-specific gene *MFα1*, the **a**-specific gene *BAR1*, and the haploid-specific gene *STE18* were associated with KlSir2, KlSum1, and KlRfm1, but not KlSir4 (Figure 37).

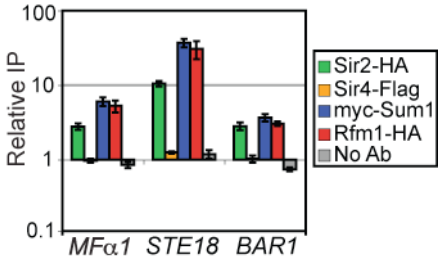


Figure 37: KlSir2 and KlSum1 associate with cell-type specific genes.

Association of KlSir2-HA, KlSir4-Flag, myc-KlSum1 (LRY2285) and KlRfm1-HA (LRY2328) at the *MFα1* (*KLLA0E19173g*), *STE18* (*KLLA0E06138g*) and *BAR1* (*KLLA0D15917g*) promoters in a *MATα* strain as assessed by chromatin IP followed by quantitative PCR. The y-axis is a log-scale.

To determine whether the Sum1-Sir2 complex represses these genes, RNA was isolated from both *MAT**a*** and *MATα* cells and expression of *MFα1*, *STE18*, and *BAR1* was examined by quantitative RT-PCR. *MFα1* encodes *α*-pheromone and in *S. cerevisiae* is expressed in *MATα* cells but not in *MAT**a*** cells. However in *K. lactis*, deletion of KlSum1 or KlSir2 resulted in the derepression of *MFα1* in both cell types to a comparable extent (Figure 38B). Quantification of cDNA from wild-type cells revealed that *MFα1* was repressed to a similar degree in both *MAT**a*** and *MATα* cells (Figure 38A). These findings suggest that during vegetative growth, haploid *K. lactis* cells are not

transcribing or producing α-pheromone, regardless of their mating-type identity, and that the Sum1-Sir2 complex contributes to the repression of this gene.

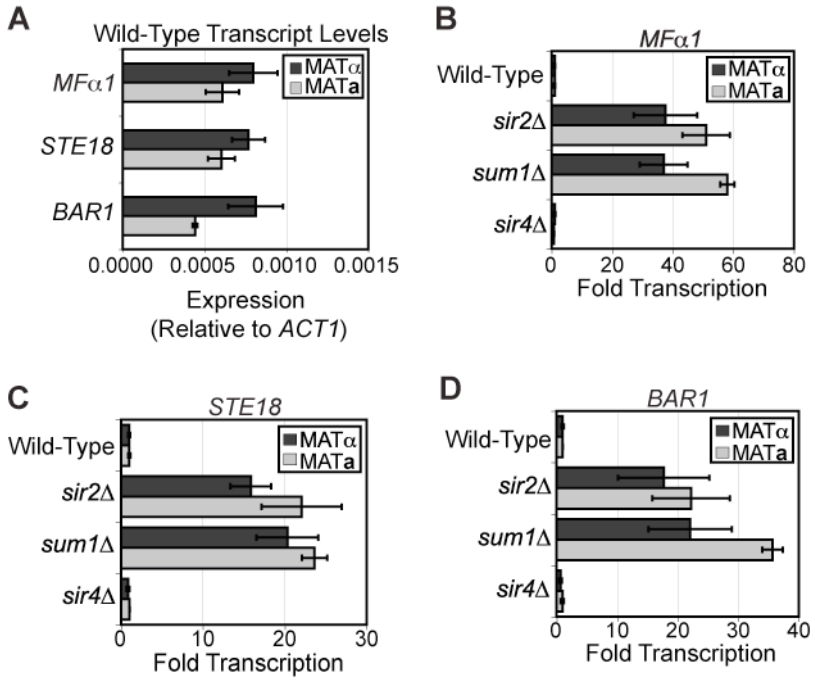


Figure 38: KlSir2 and KlSum1 repress cell-type specific genes.

A) Quantitative RT-PCR analysis of the cryptic mating-type loci, mid-sporulation genes and cell-type specific genes in wild-type strains of *MAT**a*** and *MATα* cells. The amount of cDNA was normalized to *ACT1*. **B)** Quantitative RT-PCR analysis of *MFα1* mRNA in *MATα* wild-type (SAY538), *sir2*Δ (SAY544), *sum1*Δ (LRY1947), and *sir4*Δ (LRY1946) strains and *MAT**a*** wild-type (CK213), *sir2*Δ (SAY569), *sum1*Δ (LRY2035) and *sir4*Δ (LRY2038) strains. **C)** Quantitative RT-PCR analysis of *STE18* mRNA in the same strains analyzed in panel B. **D)** Quantitative RT-PCR analysis of *BAR1* mRNA in the same strains analyzed in panel B. Error bars represent the SEM.

STE18 encodes the G protein gamma subunit in the mating signaling pathway and in *S. cerevisiae* is expressed in both *MATα* and *MAT**a*** haploid cells. In *K. lactis*, *STE18*, like *MFα1*, was repressed in both *MATα* and *MAT**a*** cells (Figure 38A), and deletion of either KlSir2 or KlSum1 resulted in derepression of *STE18* in both cell types

(Figure 38C). *BAR1* encodes an α-pheromone protease that in *S. cerevisiae* is expressed to a greater extent in *MAT**a*** than *MATα* cells. This pattern of gene expression was also found in *K. lactis* (Figure 38A). However, as for *MFα1* and *STE18*, deletion of KlSum1 or KlSir2 resulted in the derepression of *BAR1* in both *MAT**a*** and *MATα* cells (Figure 38D). To verify that I had correctly identified the mating-type of the strains used for these experiments, I analyzed a segment of the *MAT* locus using mating-type specific PCR primers that yield different sized products in *MAT**a*** and *MATα* strains. All strains had the expected genotypes (data not shown). Together, these results suggest that the KlSum1-KlSir2 complex represses a variety of cell-type specific genes as well as mid-sporulation genes and the *HM* loci. Therefore, this complex represents an important regulator of yeast sexual identity and activity.

3.3 Discussion

This study has made the striking discovery that the Sum1-Sir2 complex in *K. lactis* achieves repression through several distinct mechanisms. In *S. cerevisiae*, the Sum1-Hst1 complex functions primarily as a promoter-specific repressor of mid-sporulation, α-specific, and NAD^+-biosynthetic genes, and loss of ScSum1 or ScHst1 do not alter the expression of the *HM* loci (Chi and Shore 1996; Derbyshire et al. 1996). In contrast, in *K. lactis*, the Sum1-Sir2 complex not only uses a promoter-specific mechanism to repress the same sets of genes as in *S. cerevisiae*, it also has a major role in silencing the *HM* loci by forming extended chromatin structures.

Interestingly, the KlSum1-KlSir2 complex acts differently at *HMLα*, where it works in conjunction with KlSir4, compared to *HMR**a***, where KlSir4 is not normally present. Thus, the mechanism by which *HMR**a*** is silenced is unlike the mechanism employed at *HMLα*. The absence of KlSir4 at *HMR**a*** is surprising, as the spreading of

silencing proteins is thought to require a histone-binding protein, such as KlSir4, and neither KlSum1 nor KlSir2 is known to have this capacity. An important subject for future studies will be to determine how the spreading capacity of the KlSum1-KlSir2 complex is modulated at different genomic locations. It is possible that factors associated with the *HM* loci promote the spreading of KlSum1-KlSir2. For example, silencers may recruit additional proteins that facilitate the spreading process. It has recently been shown that the *HMR-E* silencer in *S. cerevisiae* can promote the assembly of silenced chromatin through a mechanism that is independent of recruitment (Lynch and Rusche 2009), and it is possible that silencers in *K. lactis* have similar properties. Alternatively, factors associated with the promoters of mid-sporulation genes may limit or disable the spreading of KlSum1-KlSir2.

This study also revealed that, although the KlSum1-KlSir2 and KlSir4-KlSir2 complexes cooperate at *HMLα*, they have distinct contributions to chromatin assembly and transcriptional repression. For example, the KlSir4-KlSir2 complex was critical for assembly of silencing proteins on the telomere proximal side of *HMLα*. However, silenced chromatin on the centromere-proximal side did not depend on KlSir2 or KlSir4, but was affected by the loss of KlSum1. These results suggest that the chromatin structure differs on the two sides of *HMLα*, perhaps due to different types of silencer elements. Another indication that the KlSum1-KlSir2 and KlSir4-KlSir2 complexes have independent properties is the observation that the associations of KlSir4 and KlSir2 increased at *HMLα* and *HMR**a*** in the absence of KlRfm1. This result suggests that KlSir4 and KlRfm1 may compete for association with KlSir2.

One puzzling observation was that the absence of KlSir4 resulted in a relatively modest induction of the *HMLα1* and *HMLα2* genes despite a significant decrease in the associations of both KlSir2 and KlSum1 with the α1-α2 promoter. Conversely, the

absence of KlSum1 resulted in a large increase of transcriptional activity yet had seemingly little effect on the associations of KlSir2 and KlSir4 with *HMLα*. These results are reminiscent of observations that, in some situations, Sir proteins in *S. cerevisiae* associate with *HM* loci but do not achieve repression (Lau et al. 2002; Kirchmaier and Rine 2006). I speculate that the presence of the KlSum1-KlSir2 complex at *HMLα* is more critical for repression than is the presence of KlSir4. Moreover, KlSum1 and KlSir2 must be able to achieve repression over a distance, because their presence at the *HMLα3* promoter is sufficient to repress the *HMLα1* and *HMLα2* genes. Similarly, KlSum1 and KlSir2 may act at distance at *HMR**a***, as their greatest enrichment is some distance from the promoter. In contrast, the KlSir4-KlSir2 complex appears to be somewhat permissive to transcription in the absence of KlSum1. Perhaps this chromatin structure serves another biological function, such as preventing illegitimate mating-type switching. While *K. lactis* is considered to be a homothallic yeast species (Herman and Roman 1966), an ortholog of the HO endonuclease, which initiates switching in *S. cerevisiae*, has not been identified (Butler et al. 2004) however, *α3* and *MTS1*, a homolog of Rme1, have recently been implicated in mating-type switching (Barsoum et al. 2010a). These switching events are relatively rare and have not been studied recently (Herman and Roman 1966).

This study was initiated to investigate how the deacetylases *SIR2* and *HST1* diverged after duplication. Two models, subfunctionalization and neofunctionalization, have been proposed to explain how duplicated genes diverge. I used the non-duplicated KlSir2 as a proxy for the ancestral protein and found that it interacted with both KlSir4 and KlSum1 (Figure 18), the partners of ScSir2 and ScHst1, respectively. Furthermore, KlSir2 functioned as a promoter-specific repressor of sporulation genes (similar to ScHst1; Figures 33) and also as a silencing factor that spreads across the *HM* loci (similar

to ScSir2; Figures 19 and 22). Therefore, KlSir2 has both Hst1- and Sir2-like functions. The most parsimonious interpretation of these results is that the ancestral deacetylase also had both functions and that subfunctionalization occurred after duplication. This conclusion is supported by the observation that ScSir2 has retained the ability to substitute for ScHst1 in its absence (Hickman and Rusche 2007). This is an important contribution to the understanding of the evolution of duplicated genes, as it provides an example of subfunctionalization of protein-protein interactions as opposed to partitioning of expression patterns, which have previously been documented (Lynch and Force 2000).

Previous work provides insight into how the subfunctionalization of *SIR2* and *HST1* occurred. A chimeric protein consisting of the N-terminus of ScSir2 and the C-terminus of ScHst1 has both Sir2- and Hst1-like functions in *S. cerevisiae* (Hickman and Rusche 2007; Mead et al. 2007). This observation suggests that different regions of the deacetylases are important for specifying interactions with the SIR and SUM1 complexes. It is likely that the ancestral deacetylase used these same domains to interact with the SIR and SUM1 complexes. After *SIR2* was duplicated, the two copies likely acquired mutations that reduced their affinities for either the SIR or SUM1 complexes, leading to subfunctionalization.

Over the course of evolution it was not simply the deacetylase that subfunctionalized. The proteins associated with Sir2 and Hst1 are used in different ways to achieve repression of essentially the same sets of genes in *S. cerevisiae* and *K. lactis*. Other studies have revealed changes in the transcriptional regulatory circuits of yeasts (Wong and Wolfe 2005; Tsong et al. 2006; Hittinger and Carroll 2007). However in previous examples, evidence suggested that promoter elements have changed to bring genes under the control of different regulators or alter their expression patterns. This

study expands the scope of adaptations that can lead to modifications in transcriptional networks, as it reveals that the molecular mechanisms by which regulatory proteins act can also change over evolutionary time.

In addition to the paralogs *SIR2* and *HST1*, I investigated a second duplicated gene pair, *SIR4* and *ASF2*. *SIR4* and *ASF2* were tandemly duplicated prior to the whole genome duplication and to the divergence of *Kluyveromyces* and *Saccharomyces* species. Due to their tandem arrangement and rapid rate of sequence change, it has been difficult to determine which gene is the ortholog of *ScSIR4* or *ScASF2*. Functional analysis shows that *KLLA0F14320g* silences *HMLα* (Figures 2, 3, and (Astrom and Rine 1998)) and thus has a Sir4-like function, whereas *KLLA0F13998g* antagonizes silencing at *HMLα* (Figure 21) and thus has Asf2-like function. This experimental evidence seems to contradict phylogenetic analyses implying that *KLLA0F13998g* is the ortholog of *ScSIR4*, as it clusters with *SIR4* genes from other yeast species, and that *KLLA0F13420g* is an ortholog of *ScASF2*, as it clusters with *ASF2* genes as well as *SIR4* genes from *Candida glabrata, S. castellii*, *S. kluyveri* and *Ashbya gossypii* (Figure 20 and (Byrne and Wolfe 2005)). However, this gene tree does not match the species phylogeny, perhaps due to the rapid rate of sequence change and consequently may not accurately reflect the evolutionary relationships among these genes.

The observation that KlSum1 spreads at the *HM* loci provides a new perspective on the perplexing *SUM1-1* mutation identified in *S. cerevisiae*. This mutation was originally isolated as a suppressor of a *sir2*Δ mutation (Klar et al. 1985) and results from a single point mutation, T988I. It causes Sum1 to re-localize from mid-sporulation promoters to the *HM* loci and form an extended chromatin structure (Rusche and Rine 2001; Sutton et al. 2001). It had been thought that the *SUM1-1* mutation is a gain-of-function mutation that creates the ability to spread *de novo*, and it was surprising that a

single amino acid change could have such a profound effect. However, this study suggests a new interpretation. The ability of both KlSum1 and ScSum1-1 to spread at *HM* loci suggests that the ancestral Sum1 also had this ability, which was subsequently lost in the *Saccharomyces* lineage. Consequently, wild-type ScSum1 probably retains most of the properties necessary to spread, and the T988I mutation unmasks this latent potential.

Current knowledge of the mechanism of the *SUM1-1* mutation may provide insights into how the spreading of KlSum1 is controlled. Residue T988 of ScSum1 is conserved in KlSum1, as well as in many other budding yeasts, and is located in the DNA-binding domain. Mutating this residue reduces the affinity of Sum1 for DNA (Safi et al. 2008) and replacing threonine 988 with isoleucine enables the protein to associate with new partners - ORC (the Origin Recognition Complex) and itself (Sutton et al. 2001; Lynch et al. 2005; Safi et al. 2008). These observations led to the hypothesis that the *SUM1-1* mutation occurs in an interaction domain, and the switch between threonine and isoleucine causes the protein to interact with different partners (Safi et al. 2008). Perhaps this domain of KlSum1 also has the capacity to interact with multiple partners, and the genomic context dictates whether this surface functions as a DNA-binding domain to recruit the Sum1-Sir2 complex to mid-sporulation genes or as a self-associating surface to enable KlSum1 to propagate along the chromatin at the *HM* loci.

The *K. lactis* Sum1-Sir2 complex plays a critical role as a regulator of sexual identity because it regulates some cell-type specific genes (Figures 33 and 34). Within budding yeasts there has been a transition from positive to negative regulation of **a**-specific genes. *Candida albicans* requires an activator to turn on **a**-specific genes in *MAT**a*** cells, whereas in *S. cerevisiae*, **a**-specific genes are on by default and must be turned off in *MATα* cells (Tsong et al. 2003). *K. lactis* has been proposed to have an intermediate

circuitry in regulating cell-type identity (Tsong et al. 2006), as **a**-specific gene promoters share features of both *C. albicans* and *S. cerevisiae* promoters. In this study we have demonstrated that many cell-type specific genes, including **a**- and α-specific genes are repressed by the KlSum1-KlSir2 complex in both haploid cell types providing an additional level of regulation to sexual identity.

Differences between the life cycles of *K. lactis* and *S. cerevisiae* may heighten the importance of the Sum1-Sir2 complex in *K. lactis*. Vegetative growth of *K. lactis* occurs predominantly in the haploid phase, and mating occurs in response to nutrient deprivation, leading almost immediately to sporulation (Herman and Halvorson 1963; Herman and Roman 1966; Wesolowski-Louvel and Wolf 1996). In contrast, *S. cerevisiae* propagates primarily in the diploid phase. Mating occurs shortly after germination in rich nutrient conditions, but sporulation of the resulting diploid cells is delayed until nutrients become scarce. Thus, unlike *S. cerevisiae*, *K. lactis* requires a mechanism to suppress mating of haploid cells under nutrient-rich conditions, and perhaps the Sum1-Sir2 complex contributes to this regulation by repressing some of the α-specific, **a**-specific, and haploid-specific genes required for mating. The use of a repressive complex containing a NAD^+-dependent deacetylase may help connect the sexual cycle of *K. lactis* with nutrient availability.

4. A novel role for Orc1 in the formation of silent chromatin: A case study in the yeast Kluyveromyces lactis

4.1 Introduction

As discussed in Chapter 1, the histone-binding protein Sir3 has a paralog, Orc1, as result of the genome duplication that occurred after the divergence of *Saccharomyces* and *Kluyveromyces* species. Orc1 is an essential subunit of the Origin Recognition Complex and functions in DNA replication and also recruits SIR proteins to the *HM* loci. The most conserved region between Sir3 and Orc1 is the N-terminal BAH domain displaying 50% identity and 65% similarity and is structurally almost identical (Bell et al. 1995; Connelly et al. 2006), yet have distinct silencing functions in Orc1 and Sir3. These proteins have diverged to the extent that they cannot complement each other (Bell et al. 1995). I have investigated the functions of the non-duplicated Orc1 in *K. lactis* and compared these functions to those found in *S. cerevisiae*.

The *SIR3-ORC1* gene pair has previously been used as an example of neo-functionalization based on accelerated rates of evolution. However, my studies investigating KlOrc1 function show that is distributed across *HMLα* and associates with telomeres, in addition to associating with ORC at DNA replication origins. Association with *HMLα* co-occurs with Sir2 and Sir4, but not other subunits of ORC. These results indicate that KlOrc1 has a Sir3-like capability to spread across chromatin and that Sir3 and Orc1 sub-functionalized after duplication. The BAH domain of KlOrc1 is critical for its silencing function at *HMLα*, as KlOrc1-Δbah no longer associates with *HMLα* and results in derepression of *HMLα* genes, yet this region is not essential for Orc1's function in DNA replication because KlOrc1-Δbah continues to bind to replication origins.

4.2 Results

KlOrc1 associates with replication origins and HMLα

To determine whether KlOrc1 functions strictly as a replication factor or also has additional functions in the formation of silenced chromatin in *K. lactis*, I assessed KlOrc1 association with replication origins and with *HMLα* by chromatin immunoprecipitation. There was a strong association of Orc1 at *KARS12*, a known replication origin (Irene et al. 2007), with a single, narrow peak of association coinciding with the ARS sequence (Figure 39A). Surprisingly, Orc1 also associates extensively with *HMLα*, spreading across the entire 6-kb locus (Figure 39B). This association is similar to what I have observed with other silencing factors that spread across this locus (Hickman and Rusche 2009), with peaks of enrichment at the silencer element, the *α2-α3* intergenic region and the *α3* promoter.

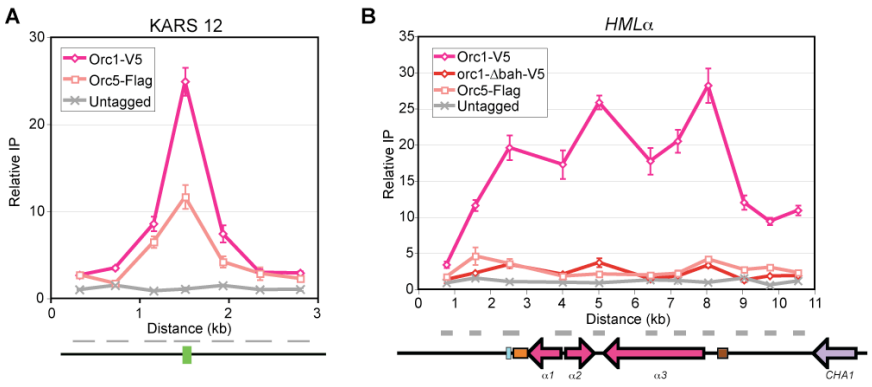


Figure 39: KlOrc1 associates with replication origins and *HML*.

A) The association of KlOrc1-V5 (LRY2261), KlOrc5-Flag (LRY2235), KlOrc1 and KlOrc5 (CK213) with the replication origin KARS12 as assessed by chromatin IP followed by quantitative PCR. The green bar denotes the ARS sequence The y-axis represents the relative enrichment normalized to a control locus, *RRP7*, which is not detectably associated with KlOrc1 or KlOrc5. **B)** The association of KlOrc1-V5 (LRY2261), KlOrc1-Δbah-V5 (LRY2562), KlOrc5-Flag (LRY2235), KlOrc1 and KlOrc5 (CK213) with *HMLα* as assessed by chromatin IP followed by quantitative PCR. A diagram of the *HMLα* locus is shown under the x-axis.

There are two possibilities for KlOrc1 association with *HMLα*. The first is KlOrc1, as part of ORC, is associating with *HMLα*, and this region could represent a very large replication origin. The second possibility is that the spreading ability of KlOrc1 is independent of ORC and reflects a unique property of KlOrc1. To distinguish between these two possibilities I assessed the association of another subunit of ORC, KlOrc5, with *KARS12* and *HMLα*. If the latter possibility is correct, KlOrc5 will associate with replication orgins, but not with *HMLα*. KlOrc5 associated with *KARS12* in a manner similar to KlOrc1 (Figure 39A), but not associating with *HMLα* (Figure 39B). This result indicates that KlOrc1, and not ORC, has a role in forming a specialized chromatin structure at *HMLα* in *K. lactis* and this property of KlOrc1 likely gave rise to Sir3-like function post-duplication.

KlOrc1 BAH domain is necessary for silencing, but not for replication

I wanted to determine how KlOrc1 contributes to silenced chromatin at *HMLα* and if this ability could be separated from its functions in DNA replication. I chose to focus on the BAH domain for several reasons. This domain is the most conserved region between Orc1 and Sir3 in *S. cerevisiae* with a high degree of both sequence and structural similarity (Bell et al. 1995; Connelly et al. 2006). Furthermore, this region is critical for the silencing function of Sir3. However, the BAH domain is dispensable for Orc1 function in DNA replication, but is important for the interaction between Orc1 and Sir1 in *S. cerevisiae* (Bell et al. 1995; Hou et al. 2005; Hou et al. 2009). I truncated the genomic copy of Orc1 in *K. lactis*, removing the first 217 amino acids corresponding to the BAH domain. Cells harboring the *orc1-Δbah* allele as the only copy of KlOrc1 are viable and display no growth defects, indicating that the ability of KlOrc1 to function in DNA

replication is not disturbed. Indeed KlOrc1-Δbah still associates with replication origins (data not shown). However, KlOrc1-Δbah no longer associates with any region of *HMLα* (Figure 39B). This result suggests that not only is the BAH domain of KlOrc1 important for recruitment to *HMLα*, it also is crucial for its ability to spread across the locus. To determine if the loss of KlOrc1 with *HMLα* has any transcriptional consequences, I isolated RNA from wild-type and *orc1-Δbah* strains and looked for changes in transcription of the *HMLα1*, *HMLα2* and *HMLα3* genes by quantitative RT-PCR. In the absence of the BAH domain of KlOrc1, all three genes are modestly de-repressed (Figure 40), demonstrating that KlOrc1 contributes to the silenced state of *HMLα*.

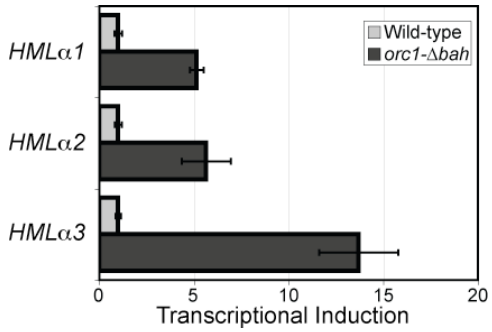


Figure 40: KlOrc1 contributes to the repression of *HML*.

Quantitative RT-PCR analysis of *HMLα1*, *HMLα2* and *HMLα3* mRNA in wild-type (CK213) and *orc1-Δbah* (LRY2562) strains. The amount of cDNA was first normalized to the control locus *ACT1*. The values shown here represent the relative amount of cDNA for each deletion strain compared to the wild-type strain.

The KlOrc1 BAH domain affects KlSir2 and KlSir4 association across HMLα

I was curious as to what effect the BAH domain of KlOrc1 contributes to the ability of KlSir2, KlSir4 and KlSum1 to associate with *HMLα.* Others and I have previously reported that KlSir2 interacts with KlSir4 and with KlSum1 (Astrom and Rine

1998; Aström et al. 2000; Hickman and Rusche 2009) to silence *HMLα* and form a specialized chromatin structure. I immunoprecipitated KlSir2, KlSir4 and KlSum1 from both *ORC1* and *orc1-Δbah* strains and assessed the association of each protein with *HMLα*. The association of KlSum1, KlSir2 and KlSir4 with *HMLα* in the wild-type strain is consistent with my previously published results (Hickman and Rusche 2009) with peaks of enrichment at the silencer, the *α2-α3* intergenic region and the *α3* promoter. In the absence of the KlOrc1 BAH domain, both KlSir2 and KlSir4 association is greatly reduced across the entire *HMLα* locus, with the maximal peak of association at the *α3* promoter (Figure 41A, B). However, there is very little change in the association of KlSum1 with *HMLα* in the absence of the Orc1 BAH domain (Figure 41C), with the only significant reduction in association occurring at the *α2-α3* intergenic region. This results indicates that the BAH domain of KlOrc1 does not recruit KlSir2, KlSir4 or KlSum1 to *HMLα*, as all three proteins are still able to associate with the *α3* promoter, the BAH domain is important for the ability of KlSir2 and KlSir4, but not KlSum1, to spread across this locus.

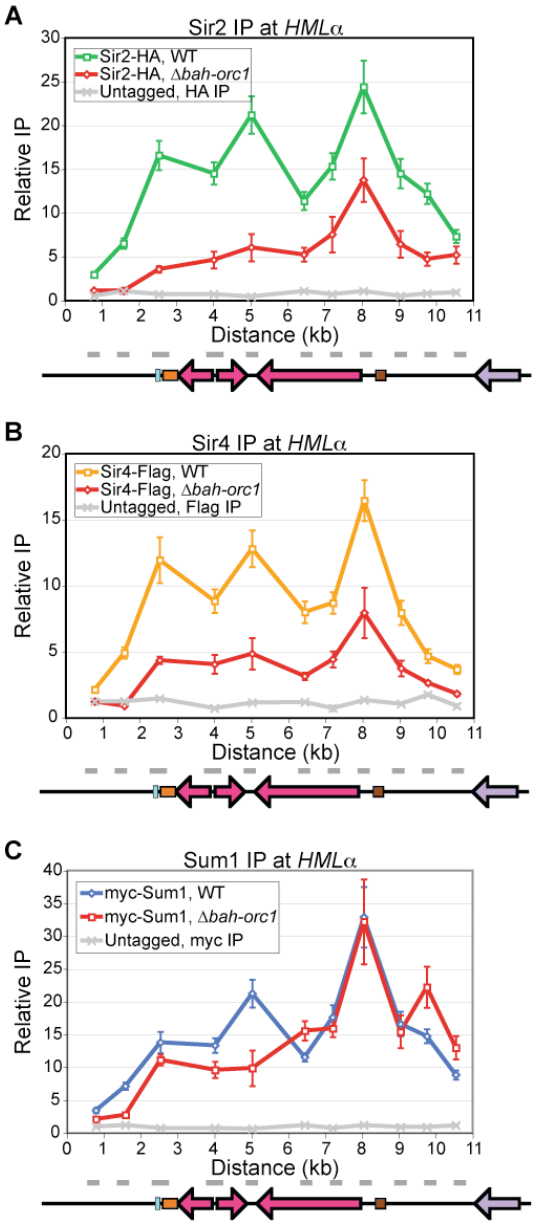


Figure 41: KlSir2, KlSir4 and KlSum1 association with *HML* in an *orc1-Δbah* strain.

A) The association of KlSir2-HA with *HMLα* in wild-type (LRY2239) and *orc1-Δbah* (LRY2563) strains as assessed by chromatin IP followed by quantitative PCR. **B)** The association of KlSir4-Flag with *HMLα* in wild-type (LRY2239) and *orc1-Δbah* (LRY2563) strains. **C)** The association of myc-KlSum1 with *HMLα* in wild-type (LRY2239) and *orc1-Δbah* (LRY2563) strains. The data for the wild-type IPs are the same as Figure 22.

KlSir2 and KlSir4 are important for KlOrc1 spreading at HMLa

I have shown that the BAH domain of KlOrc1 is critical for KlOrc1 association with *HMLα* and is also important for the spreading of the silencing factors KlSir2 and KlSir4, but not KlSum1. To determine how the silencing factors KlSir2 and KlSir4 affect KlOrc1 association with *HMLα*, I immunoprecipitated KlOrc1 from *sir2*Δ and *sir4*Δ strains and compared them to wild-type KlOrc1 association. KlOrc1 association with *HMLα* was restricted to just the *α3* promoter region when the histone deacetylase KlSir2 was absent. There was a severe reduction of KlOrc1 from the silencer and open reading frames of *α1*, *α2* and *α3*, as well as on the centromere-proximal side beyond the *α3* promoter (Figure 42). Deletion of KlSir4 resulted in a very similar distribution of KlOrc1 association with *HMLα*. However, unlike in the *sir2*Δ strain, there was an accumulation of KlOrc1 on the centromere proximal side of *HMLα* (Figure 42). These results suggest that both KlSir2 and KlSir4 are important for the spreading ability of KlOrc1 at the *HMLα* locus, but neither KlSir2 nor KlSir4 are singly responsible for the recruitment of KlOrc1.

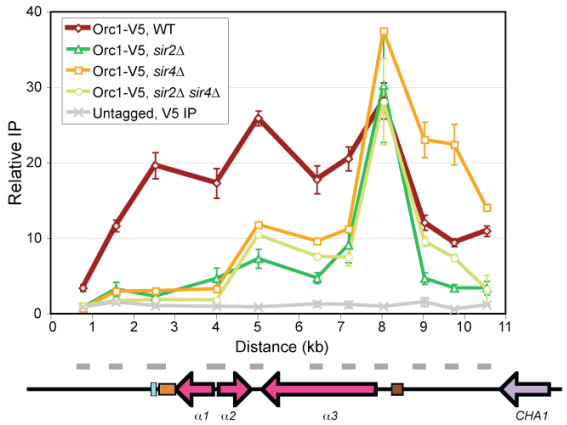


Figure 42: KlSir2 and KlSir4 facilitate KlOrc1 spreading across *HML*.

The association of KlOrc1-V5 with *HMLα* in wild-type (LRY2261), *sir2*Δ (LRY2572), *sir4*Δ (LRY2573) and *sir2*Δ *sir4*Δ (LRY2577) strains as assessed by chromatin IP followed by quantitative PCR. The wild-type IP data is the same as Figure 39.

There are two possible hypotheses to explain KlOrc1 recruitment to the *α3* promoter. One possibility is that KlSir2 and KlSir4 each have the capacity to recruit KlOrc1 to *HMLα*, such that in the absence of KlSir2, KlOrc1 can still be recruited to *HMLα* via KlSir4 and vice versa. Alternatively, the *α3* promoter region could represent a silencer element that recruits KlOrc1 independently of both KlSir2 and KlSir4. To distinguish between these hypotheses, I assessed KlOrc1 association with *HMLα* in a *sir2*Δ *sir4*Δ double deletion strain background. I observed a very similar KlOrc1 association pattern with *HMLα* in a *sir2*Δ *sir4*Δ background as compared to either *sir2*Δ or *sir4*Δ single deletion background (Figure 42). This result indicates that that KlOrc1 is recruited to *HMLα* independently of both KlSir2 and KlSir4.

I next turned my attention to the transcription factor KlSum1, which I have recently shown to be important for silencing *HMLα*. It is possible that KlOrc1 is recruited to *HMLα* via an interaction with KlSum1. KlSum1 maintains its association with *α3* promoter in the absence of both Sir2 and Sir4 (Hickman and Rusche 2009) and would be able to recruit KlOrc1 under these conditions. Surprisingly, deletion of Sum1 did not result in a reduction of KlOrc1 association with *HMLα*. Wild-type levels of KlOrc1 were maintained across the locus and were even enriched at the *α2*-*α3* intergenic region in the absence on KlSum1 (Figure 43). It was possible that KlSir2/KlSir4 and KlSum1 were acting "redundantly" to recruit KlOrc1 to *HMLα*. To test this I looked at KlOrc1 association with *HMLα* in *sum1*Δ *sir2*Δ and *sum1*Δ *sir4*Δ double deletion strains. In both of these strains there is a severe reduction of KlOrc1 association with *HMLα* over the *α3* promoter and open reading frame and a complete abolishment of KlOrc1 across the silencer, *α1* and *α2* open reading frames (Figure 43). This data illustrates the complexity of the interaction of all of these silencing factors. While a single deletion of

KlSum1 as no affect on KlOrc1 association, KlSum1 still cooperates with KlSir2 and KlSir4 to recruit KlOrc1 to *HMLα*, because the absence of either KlSir2 or KlSir4 in conjunction with KlSum1 results in a more severe reduction of KlOrc1 association with *HMLα* than in either of the single deletion or *sir2*Δ *sir4*Δ double deletion strains.

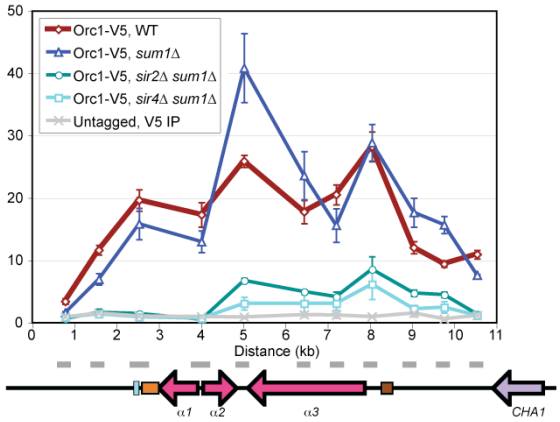


Figure 43: KlOrc1 association with *HML* in *sum1*Δ strains.

The association of KlOrc1-V5 with *HMLα* in wild-type (LRY2261), *sum1*Δ (LRY2574), *sum1*Δ *sir2*Δ (LRY2578) and *sum1*Δ *sir4*Δ (LRY2576) as assessed by chromatin IP followed by quantitative PCR. The wild-type IP data is the same as Figure 39.

*KlOrc1 associates with telomeres, but not the other cryptic mating-type locus, HMR**a**.*

I was interested in evaluating other genomic loci where KlOrc1 might function in a silencing context and I investigated whether KlOrc1 associated with *HMR***a** and telomeres, as these are silenced regions in *S. cerevisiae*. I have previously shown that KlSir2 and KlSum1 silence the ***a1*** and ***a2*** genes found at *HMR***a**, yet KlSir4 does not associate with or silence *HMR***a** (Hickman and Rusche 2009). KlOrc1, like KlSir4, does not significantly associate with *HMR***a** (Figure 44) as compared to KlSir2 or KlSum1 association. Unlike in *S. cerevisiae*, there is differential regulation of the cryptic mating

type loci in *K. lactis*, with a Sir2-Sum1 complex regulating both *HMR***a** and *HML*α and the additional silencing factors, KlSir4 and KlOrc1, associate only with *HML*α.

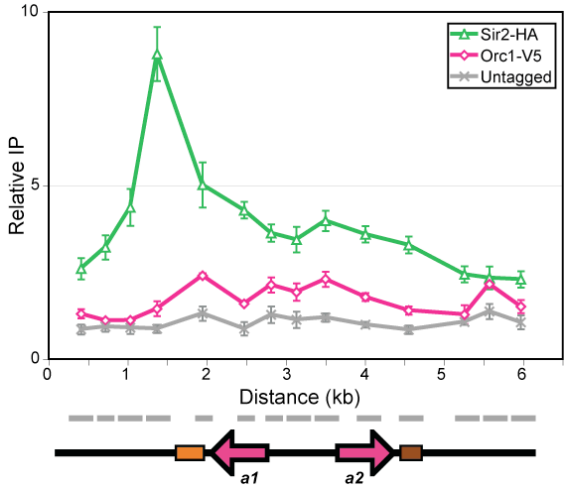


Figure 44: KlOrc1 does not associate with *HMR*.

The association of KlOrc1-V5 (LRY2281), KlSir2-HA (LRY2285) and KlOrc1 (SAY69) with *HML*α as assessed by chromatin IP followed by quantitative PCR. A diagram of the *HMR***a** locus is shown under the x-axis. The orange and brown boxes represent sequences found at *HML*α, *MAT*, and *HMR*a loci. Data for the KlSir2-HA IP is the same as in Figure 30.

I also examined the telomeres for the association of KlOrc1 and other silencing factors. I first looked at the last 5 kb of the right arm of chromosome B (notated as Tel-BR). All telomeres in *K. lactis* contain a 1.5 - 2 kb region of sequence that has a strong purine/pyrimidine strand bias immediately adjacent to the telomeric repeat (Nickles and McEachern 2004) termed the R element. I observed a strong association of KlOrc1 with the most telomere-proximal side of the R element of the subtelomere (Figure 45A). Similar to *HML*α, this association of KlOrc1 is independent of ORC, as KlOrc5 did not associate with this region. KlSir2 and KlSir4 association was also observed in a similar pattern to what was seen for KlOrc1 (Figure 45A). Interestingly, there was no detection

of KlSum1 at Tel-BR (Figure 45A). Extending this analysis to look at the ends of other chromosomes, I observed strong KlSir2 association at all telomeres examined, as well as KlSir4 and KlOrc1 association at most telomeres (Figure 45B). All of the telomeres show some variation in the absolute levels of KlOrc1, KlSir2 and KlSir4 association, although Tel-EL is deviates the most from the others, with KlSir2, but not KlSir4 or KlOrc1 association. This data supports the model that Orc1-Sir2-Sir4 associates with chromatin to form a highly structured domain.

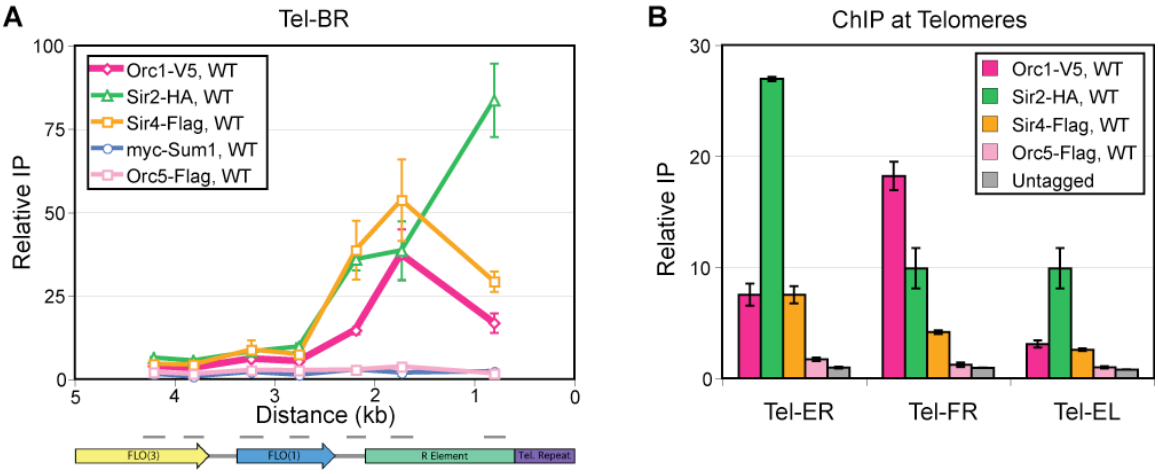


Figure 45: KlOrc1, KlSir2 and KlSir4 associate with telomeres.

A) The association of KlOrc1-V5 (LRY2261), KlOrc5-Flag (LRY2235), KlSir2-HA (LRY2239), KlSir4-Flag (LRY2239), and myc-KlSum1 (LRY2239) with Tel-BR as assessed by chromatin IP followed by quantitative PCR. A diagram of Tel-BR locus is shown under the x-axis. The purple region represents the telomeric repeat sequence, the green region represents the R element and the yellow and blue arrows represent the open reading frames FLO(3) and FLO(1), respectively. Although both ORFs are annotated as FLO, they belong to different families of duplicated genes found at the telomeres (Fairhead and Dujon 2006). **B)** Association of KlOrc1-V5 (LRY2261), KlOrc5-Flag (LRY2235), KlSir2-HA (LRY2239), KlSir4-Flag (LRY2239), and an untagged strain (CK213) with Tel-ER, Tel-FR and Tel-EL as assessed by chromatin IP followed by quantitative PCR.

4.3 Discussion

I have shown in this study that the essential subunit of the origin recognition complex, KlOrc1, not only has the ability to associate with replication origins but also functions to form silenced chromatin with KlSir2 and KlSir4 at *HML* and telomeres. Unlike in *S. cerevisiae*, KlOrc1 associates with *HML* independently and not as a subunit of ORC, as other subunits of ORC do not associate with *HML* (Figure 39). Furthermore, KlOrc1 does not function as a silencing binding protein, as it does in *S. cerevisiae*, because the other silencing factors, KlSir2, KlSir4 and KlSum1 are still recruited to *HML* in an *orc1*- Δ*bah* strain (Figure 41). Finally, KlOrc1 is distributed across the entire locus of *HML* and is not restricted to a single binding site (Figure 39), indicating that KlOrc1 has the capacity to spread. This spreading ability of KlOrc1 is likely the progenitor function of Sir3 and indicates that post-duplication the Orc1-Sir3 duplicated gene pair subfunctionalized.

The silencing function of KlOrc1 is distinct from its function in DNA replication. First, only KlOrc1, and not other subunits of ORC, associated with *HML* (Figure 39). Secondly, I was able to disrupt silencing function while maintaining cell viability and DNA replicative function by the BAH domain of KlOrc1. This mutation resulted in transcriptional derepression of the *α1*, *α2* and *α3* genes found at *HMLα*, as well as a complete disassociation of KlOrc1 with *HMLα*. The truncation of KlOrc1 also affected the association of KlSir2 and KlSir4 with *HMLα*. Both KlSir2 and KlSir4 are still recruited to the *α3* promoter at *HMLa* in the absence of the BAH domain of KlOrc1, yet are no longer able to spread across the open reading frames of *α1*, *α2* and *α3*. This data indicates that this domain of KlOrc1 is absolutely critical for its silencing functions.

Work done in *S. cerevisiae* investigating the BAH domain of both Sir3 and Orc1 provides clues to how the BAH domain of KlOrc1 may function in silencing. Recent

biochemical work has demonstrated that the BAH domain of Sir3 is necessary and sufficient to bind nucleosomes and will co-precipitate with histone H3 (Onishi et al. 2007; Buchberger et al. 2008; Sampath et al. 2009). This property is conserved in the BAH domain of ScOrc1 (Onishi et al. 2007) and presumably KlOrc1 also has this property, which allows it to associate directly with chromatin, independent of DNA sequence.

The data presented in this study demonstrates the dual functions of the non-duplicated KlOrc1 to participate with the ORC complex in DNA replication, as well as with KlSir2 and KlSir4 in forming a specialized chromatin structure. However, there has been significant sequence divergence between Orc1 and Sir3 post-duplication, leading some to postulate that Sir3 function arose after the duplication event or neo-functionalized (Kellis et al. 2004). Clearly, studies that go beyond sequence analysis are critical for understanding the evolution of duplicated gene pairs, as discussed previously.

An earlier study investigating the non-duplicated Orc1 from *Saccharomyces kluyveri*, showed that Orc1 had sub-functionalized (van Hoof 2005), but concluded that it evolved under a duplication, degeneration, complementation mechanism (Force et al. 1999). I would suggest that the evolution of the Sir3-Orc1 duplicated gene pair, while undergoing sub-functionalization, did not diverge via the DDC mechanism. Instead, it is more likely a case specialization. Specialization states that the ancestral gene is constrained by its multiple functions and is unable to fully optimize any single function that it performs. A prediction from this model is that the ancestral gene will not perform the individualized functions as well as the duplicated genes will. Indeed, the van Hoof study showed that while SkOrc1 does complement a *sir3*Δ mating defect in *S. cerevisiae*, this mating ability is much weaker than when ScSir3 is present (van Hoof 2005) and I

have not been able to observe KlOrc1 suppressing a *sir3*Δ mating defect in *S. cerevisiae* (data not shown). This data, in conjunction with the accelerated sequence divergence, would suggest that Sir3 has continued to evolve, post-duplication. There is also evidence that Orc1 has continued to evolve post-duplication. The BAH domain of Orc1 in *S. cerevisiae* is critical for interacting with Sir1 (Triolo and Sternglanz 1996; Gardner et al. 1999; Hou et al. 2005), thereby recruiting the SIR complex to the *HM* loci. However, Sir1, is a relatively young gene and is not present in species beyond the *Saccharomyces sensu stricto* complex (Gallagher et al. 2009; Hou et al. 2009) and therefore this interaction must have evolved after Sir1 was acquired.

5. Materials and Methods

5.1 Strain and plasmid construction

5.1.1 S. cerevisiae strains

Strains used in this study were all derived from W303-1a (Table 2). The *hst1*Δ*::KanMX*, *HST1*-HA, myc-*SUM1* (Rusche and Rine 2001) and *hst1-N291A* (Lynch et al. 2005) alleles were described previously. The *sir3*Δ*::LEU2* and *sir2*Δ*::URA3* alleles were obtained from J. Rine (unpublished). The *sir2*Δ*::TRP1*, *hst2*Δ*::TRP1*, *hst3*Δ*::TRP1* and *hst4*Δ*::TRP1* alleles were complete deletions of the open reading frames generated by one-step gene replacement. The open reading frames of *GAS2* and *PES4* were replaced precisely with the *HIS3* open reading frame to generate the p_{GAS2}-*HIS3* and p_{PES4}-*HIS3* reporter alleles. The correct integration was confirmed by PCR using primers flanking the sites of recombination. These alleles were moved into various genetic backgrounds (as described in Table 2) through standard genetic crosses.

5.1.2 S. cerevisiae plasmids

Plasmids used in this study are described in Table 3. The plasmid containing *HST1*-HA (pLR30) has been previously described (Rusche and Rine 2001). To generate plasmid pLR488 expressing the chimeric *SIR2-HST1* protein, the N-terminus of *SIR2* (amino acids 1-255) was amplified from genomic DNA, with the 5′ primer containing the recognition site for *Eco*RI and the 3′ primer containing 20 base pairs of overlapping homology to the start of the catalytic core of *HST1*. The C-terminus of *HST1* (amino acids 201-503) was amplified from genomic DNA, with the 5′ primer containing 20 base pairs of overlapping homology to the *SIR2* sequence just upstream of the catalytic core sequence and the 3′ primer containing the recognition sequence for *Age*I restriction endonuclease. A second PCR reaction was performed in which equimolar amounts of

the *SIR2* N-terminus amplicon and the *HST1* C-terminus amplicon were pooled in a 25 μl PCR reaction and allowed to run in the thermocycler for five cycles, after which an additional 25 μl of reaction mix containing the 5′ oligonucleotide used previously for the *SIR2* amplification and the 3′ oligonucleotide used previously for the *HST1* amplification were added to the initial PCR reaction and allowed to cycle for 25 more rounds. The PCR product was cloned into the *Eco*RI site, between the multermerized HA tag and the start of *SIR2* and into the *Age*I site at the 3′ end of the *SIR2* ORF of pRO298, thereby replacing the *SIR2* open reading frame with the chimeric *SIR2-HST1* gene while retaining the N-terminal HA tag. The correct plasmid was verified by restriction enzyme analysis and sequencing. Expression of the HA-Sir2$_{1\text{-}255}$-Hst1$_{201\text{-}503}$ chimeric molecule was confirmed by immunoblotting. To generate plasmid pLR490 containing Kl*SIR2*, Kl*SIR2* was amplified from genomic DNA from a wild-type *K. lactis* strain (SAY45, from Stephan Astrom). The 5′ primer contained an *Mfe*I site and the 3′ primer contained an *Age*I site. The resulting PCR product was cloned into the *Eco*RI and *Age*I sites of pRO298, thereby replacing the Sc*SIR2* sequence with Kl*SIR2*. The correct plasmid was verified by restriction enzyme analysis and sequencing. Expression of HA-KlSir2 in *S. cerevisiae* was confirmed by immunoblotting.

5.1.3 K. lactis strains

Strains used in the course of this study were derived from SAY538 (Table 4). The *sir2*Δ::*KanMX* allele was obtained from S. Astrom. The *sir2*Δ::*NatMX*, *sir4*Δ::*URA3*, *asf2*Δ::*NatMX*, *sir4*Δ *asf2*Δ::*URA3*, *sum1*Δ::*NatMX* and *rfm1*Δ::*URA3* alleles were complete deletions of the open reading frames generated by one-step gene replacement. The replacement markers *NatMX* and *URA3* were derived from pAGT100 (Kaufmann and Philippsen 2009) and pRS316 (Sikorski and Hieter 1989), respectively. The *orc1-*Δ*bah* mutation was obtained by first cloning the complete *ORC1*-V5 open reading frame plus

surrounding DNA into a plasmid, followed by PCR-mediated deletion the first 651 nucleotides (corresponding to the first 217 amino acids). The deletion was confirmed by sequencing. The entire *orc1-Δbah-V5* cassette was amplified and transformed into a wild-type *K. lactis* strain and integrated at the endogenous *ORC1* locus. The *HML**a*** allele was a fortuitous gene conversion event that occurred during the course of crossing a *sir2*Δ strain. The *SIR2*-HA, *RFM1*-HA, *SIR4*-Flag, *ORC1*-V5 and *ORC5*-Flag alleles were constructed by integrating the tag plus a selectable marker at the end of the open reading frame. Tagging cassettes were generated from pAGT105 (Kaufmann and Philippsen 2009) containing the HA-epitope tag along with the entire open reading frame of *NatMX*, p3FLAG-KanMX, (Gelbart et al. 2001) containing the Flag tag plus *KanMX* or pFA6a-6xGLY-V5-hphMX4 containing the V5 tag plus *HphMX* (Funakoshi and Hochstrasser 2009). The myc-*SUM1* allele was generated in two steps. First, a myc-*URA3*-myc-*SUM1* construct, derived from p3MPY-3xMyc, (Schneider et al. 1995) was integrated into the *K. lactis* genome. After correct integration was confirmed by PCR, cells were grown in non-selective media to allow for recombination between the identical myc-tags and cells were plated on 5-FOA to select for the loss of the *URA3* marker. In all cases, the correct integration was confirmed by PCR using primers flanking the sites of recombination. To confirm that the tagged proteins were functional, expression of genes regulated by these factors was examined by quantitative RT-PCR. Alleles were moved into various genetic backgrounds through genetic crosses.

All *K. lactis* strains used in this study were grown at 30° in YPD medium containing 1% yeast extract, 2% peptone and 2% glucose. Antibiotic supplements were added to YPD medium at 50 μg/ml of clonNAT, 50 μg/ml hygromycin B and 200 μg/ml of geneticin. Electroporation conditions were as described (Sanchez et al. 1993) with the following changes. Cells were washed with LiAc buffer (10 mM Tris pH 7.5, 270 mM

sucrose, 1 mM lithium acetate) after initial centrifugation. After treatment with the pre-treating buffer (YPD, 20 mM HEPES pH 8.0, 25 mM DTT), cells were resuspended in LiAc buffer to a final concentration of 2 x 10^9 cells/ml and electroporation was performed in a 0.2 cm cuvette, with a final at volume between 50 and 55 µl. The settings for electroporation were 1,000 V, 25 µF and 300 Ω. Cells transformed with antibiotic resistance markers were grown at 30° in YPD for 3-5 hours before being plated on selective medium.

Mating was carried out by patching cells from the two parental strains together on malt extract (ME) medium (2% malt extract, 2% agar) and incubating at 30° for 2-3 days. Cells were then streaked on media to select for diploids and subsequently transferred to ME plates for sporulation. After 3-4 days, the sporulated culture was suspended in 500 µl water, incubated at 56° for 15 minutes, serially diluted and plated on media to select for alleles of interest. Genotypes were confirmed by PCR.

5.2 Gene expression analysis

RNA was isolated from two independent logarithmically growing cultures of each strain as previously described (Schmitt et al. 1990). To remove DNA, 3 µg of RNA was treated with DNAseI in a final volume of 30 µl. To verify that there was no contaminating DNA, 1 µl of DNAse-treated material was used in a PCR reaction containing primers to amplify the *ACT1* transcript. A lack of product indicated successful removal of DNA. 1 µg of DNA-free RNA was used for cDNA synthesis by addition of 1 µl 10 mM dNTPs and 1 µl oligo dT_{16} [500 ng/µl] and incubation at 65° for 5 minutes, followed by a quick chill on ice. A master mix of 4 µl 5x 1st strand buffer (Invitrogen), 2 µl 0.1 M DTT and 1 µl RNAseOUT (Invitrogen) was added to the samples. The resulting reaction was incubated at 37° for 2 minutes at which point 1 µl of M-MLV-RT (Invitrogen) is added to the reaction and incubated for 50 minutes at 37°

followed by a 15 minutes incubation at 70° to inactivate the enzyme. 1/40 of the resulting cDNA was subsequently analyzed by real time PCR in the presence of SYBR Green on a Bio-rad iCycler to quantify the relative amounts of mRNA transcripts. Duplicate qPCR reactions were performed to ensure consistency. Data were analyzed with iCycler iQ Optical System Software.

For *S. cerevisiae*, the standard curve was generated with genomic DNA isolated from the wild-type strain (W303-1a). Oligonucleotide sequences are provided in Table 4. *DTR1* and *SPS1* transcript levels were normalized to *ACT1* transcript levels. To determine fold-induction, *DTR1* and *SPS1* transcript levels were normalized to the wild-type strain. Results reflect the average fold induction (relative to wild-type) of at least two independent cultures for each strain background, each analyzed in duplicate qPCR reactions. The standard deviation was calculated from the difference in fold induction of the two independent cultures from the mean.

For *K. lactis*, the standard curve was generated with genomic DNA isolated from the wild-type strain (SAY538). Oligonucleotide sequences are provided in 4. Transcript levels of queried genes were first normalized to the *KlACT1* mRNA for each genetic background. The fold-induction was calculated by normalizing to the wild-type strain. Results represent the average fold induction (relative to wild-type) of at least two independent cultures of each strain background. The standard error measurement (SEM) was calculated from the differences in fold induction of two or more independent cultures from the mean.

5.3 Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as previously described (Rusche and Rine 2001) using 10 optical density equivalents of cells and 2-4 μl anti-HA tag antibody (Upstate Biotechnology 05-902), 2 μl of antibodies against acetyl-lysine 8 or

acetyl-lysine 16 of histone H4 (Upstate Biotechnology 07-328 and 06-762) or 3 μl anti-myc tag antibody (Upstate Biotechnology 06-549). For chromatin immunoprecipitation of HA-Sir2 and Hst1-HA, a second crosslinking agent was used (Kurdistani et al. 2002). 50 optical density units of cells were harvested by centrifuging at 2700 rpm for 5 minutes and resuspended in 1x ice cold DMA (10 mM dimethyl adipimidate, 0.1% DMSO, 1x PBS) and crosslinked for 45-60 minutes at room temperature. After crosslinking with DMA, cells were washed twice with cold 1x PBS, resuspended in 50 ml 1x PBS and treated with 1% formaldehyde for 45-60 minutes at room temperature. The DNA was sheared by sonication to an average size between 500 to 1000 bp in all experiments. Chromatin IP samples were analyzed by qPCR using a standard curve prepared from input DNA.

For *S. cerevisiae*, the amounts of the immunoprecipitated DNA at experimental promoters (*DTR1* or *SPS1*) and a control promoter (*ATS1*) were determined relative to the input DNA, and then the enrichment of the *DTR1* or *SPS1* promoter was determined relative to the control locus, *ATS1*. Oligonucleotide sequences are provided in Table 5. Enrichment is considered significant if the ratio of experimental to control region equals two or higher. Oligonucleotide sequences are provided in Table 6. Results reflect the relative immunoprecipitation of two independent cultures for each strain background, and the standard deviation was calculated from the difference in fold induction of the two independent cultures from the mean. To determine the relative acetylation level of Lys8 and Lys16 of H4 (Figure 7) in various strain backgrounds, normalized *DTR1* and *SPS1* IP levels were quantified relative to the wild-type strain. To determine differences in nucleosome occupancy in the various strain backgrounds, an independent ChIP using an antibody against the H4 core domain (Upstate Biotechnology 07-108) was performed.

Results in Figure 7 depict relative acetylation levels of Lys8 and Lys16 of H4 for each strain that accounts for strain differences in nucleosome occupancy.

For *K. lactis*, the amounts of the immunoprecipitated DNA at experimental loci and a control locus, *KlRRP7*, were determined relative to the input DNA, and the relative enrichment of the experimental loci compared to the control locus was calculated. Oligonucleotide sequences are provided in Table 6 and schematics indicating primer position for *HMLa*, *MATa*, *HMR**a***, *CDA2* and *KARS12* are provided in Figures 46-50. Results represent the relative immunoprecipitation of two or more independent cultures of each strain background, and the SEM was calculated from differences in the relative enrichment from the mean. No antibody control data represent the average values from multiple chromatin IP experiments using different strains.

5.4 Co-immunoprecipitations

Co-immunoprecipitations were performed as previously described (Rusche and Rine 2001) using 30 optical density equivalents of cells grown in media lacking uracil to ensure plasmid retention. The whole-cell lysates were incubated for four hours at 4° C with 5 μl of antibody. Subsequently, 60 μl of Protein A agarose beads were added and samples were rotated at 4° overnight and protein was eluted in 75 μl 3× protein sample buffer (30% glycerol, 15% β-mercaptoethanol, 0.006% bromophenol blue, 0.1875 M Tris pH 6.8) for 3 minutes at 95°. 20 μl of IP samples and 7.5 μl of whole-cell extracts were electrophoretically fractionated on 7.5% polyacrylamide-SDS gels, transferred to nitro-cellulose membranes, and probed with either mouse polyclonal α-HA antibody (Sigma H-3663), mouse polyclonal α-myc antibody (Calbiochem OP10), rabbit (Sigma F-7425) or mouse (Sigma F-3165) α-Flag antibodies and detected by chemiluminescence (GE RPN2135).

In *S. cerevisiae*, Sum1 immunoprecipitations, serum from a guinea pig inoculated with a C-terminal fragment of Sum1 was used (Pocono Rabbit Farm). For Sir4 immunoprecipitations, serum from a rabbit inoculated with Sir4 was used (Axelrod 1991).

In *K. lactis*, antibodies used for immunoprecipitation and immunoblotting include: a-HA (Sigma H-6908) for Sir2-HA, a-Flag (Sigma F-7425) for Sir4-Flag and a-myc (Millipore 06-549) for Sum1 antibody.

5.5 Immunoblotting

Whole cell protein samples were prepared from three optical density equivalents of cells grown in media lacking uracil to ensure plasmid retention. 0.1 volumes of 100% trichloroacetic acid (TCA) was added to the culture medium and incubated on ice for 20 minutes. Cells were pelleted, washed with Tris pH 8.0, and resuspended in 75 μl 3X protein sample buffer. Cells were lysed by vortexing in the presence of glass beads and subsequently incubated at 95°. Whole cell protein extracts were electrophoretically fractionated on 7.5% polyacrylamide-SDS gels, transferred to nitro-cellulose membranes (Amersham), and probed with rabbit α-Sir2 (from J. Rine), rabbit α-HA antibody (Upstate Biotech 05-902) or 3-phosphoglycerate kinase (Molecular Probes A-6457).

5.6 Mating assays

One optical density equivalent of cells was collected from logarithmically growing cultures by centrifugation and resuspended in 100 μl YM (yeast minimal) medium. 10-fold serial dilutions were made for each strain, and 3 μl of each sample in the dilution series was spotted onto a YPD plate to monitor growth. To assay mating, an equal volume of the tester strain LRY1022 (MATα *his4*) at 10 OD unit cells/ml in YPD was mixed with each sample in the dilution series. 3 μl of this mixture was spotted onto

YM plates to select for the growth of prototrophic diploids. Yeast were grown at 30° for 3-4 days and subsequently photographed.

5.7 Reporter assays

One optical density equivalent of logarithmically growing cells was collected by centrifugation in a microcentrifuge and subsequently resuspended in 100 μl YM medium. 10-fold or 5-fold serial dilutions were made, and 3 μl of each sample in the dilution series was spotted onto complete medium to monitor overall growth and medium lacking histidine or lacking histidine and uracil to monitor Hst1-mediated repression. Uracil was omitted to maintain the presence of the plasmids in the p_{PES4}-*HIS3* reporter assays. Yeast were grown at 30° for 3-4 days and subsequently photographed.

6. Concluding Remarks

These findings demonstrate that prior to the genome duplication there was a silencing complex that contained both KlSir2 and KlOrc1. In addition to their functions at *HML* and the telomeres, KlOrc1 associates with replication origins and KlSir2 and KlSum1 work in complex to repress sporulation genes in a promoter-specific manner. The multiple functions of both KlOrc1 and KlSir2 in *K. lactis* indicate that after duplication, these properties were divided among paralogs and subsequently specialized to perform the functions that have been characterized in *S. cerevisiae*.

Surprisingly, I found a novel role for the DNA-binding protein, Sum1, to repress *HML* and *HMR* in *K. lactis*, a phenotype not observed in *S. cerevisiae*. KlSum1 directly regulates the *HM* loci by spreading across these regions in a mechanism that is distinct from its role in repressing sporulation-specific genes. This result indicates that KlSum1 is a key regulator of not only meiotic, but also mating-type transcriptional programming.

In the course of this work, the distinction between a specialized chromatin structure and transcriptional repression has been prominent. Deletion of KlSum1 dramatically disrupts the repression of genes found at *HML*, but the other silencing factors, KlSir2, KlSir4 and KlOrc1 continue to associate strongly with this locus. Conversely, mutations in KlSir4 and KlOrc1, have only modest defects in transcriptional repression, but the association of other silencing factors are significantly reduced across *HML*. Deletion of KlSir2 results in striking defects in both transcriptional repression and chromatin structure. These results suggest that there are two complexes regulating the *HML* locus, both of which contain KlSir2: a Sir2-Sum1 complex responsible for maintaining transcriptional repression and a Sir2-Sir4-Orc1 complex that is important for maintaining a specialized chromatin structure.

This model predicts that there would be genomic locations where transcriptional repression, but not a specialized chromatin structure, is critical. Indeed, this is observed at cell-type specific and sporulation genes, where the KlSir2-KlSum1 complex functions as a promoter-specific repressor and there is no evidence for KlSir4 or KlOrc1 association with these loci. This model also predicts that there would be genomic locations where a specialized chromatin structure is important, but transcriptional repression is dispensable. The telomeres are good examples of this type of genomic region and I have shown that in *K. lactis*, KlSir2, KlSir4, KlOrc1, but not KlSum1, associate with telomeres. Due to the wide conservation of Sir2 and Orc1 proteins among eukaryotes and from the work presented here, I suggest that Sir2 and Orc1 have ancient silencing capabilities and that their duplication presented an opportunity for specialization of function.

Appendix A

Table 2: *S. cerevisiae* strains used in this study.

Strain	Genotype	Source
W303-1a	*MATa ade2-1 can1-100 his3-11 leu2-3,112 trp1-1 ura3-1*	R. Rothstein
LRY198	W303-1a *MATa hst1Δ::KanMX*	
LRY333	W303-1a *MATa hst1Δ::KanMX sir2Δ::TRP1 ADE2 lysΔ*	
LRY345	W303-1a *MATa hst1Δ::KanMX sir3Δ::TRP1 ADE2 lysΔ*	
LRY521	W303-1a *MATa* 6myc-*SUM1 hst1Δ::KanMX*	
LRY523	W303-1a *MATa* 6myc-*SUM1*	
LRY558	W303-1a *MATα HST1-5HA*	
LRY1022	*MATα his4*	P. Schatz
LRY1079	W303-1a *MATa sir2Δ::URA3*	J. Rine
LRY1306	W303-1a *MATa hst1-N291A*	
LRY1422	W303-1a *MATα pGAS2-HIS3 pYJL038C-URA3 hst1Δ::KanMX sir2Δ::TRP1*	
LRY1453	W303-1a *MATα pGAS2-HIS3 pYJL038C-URA3*	
LRY1454	W303-1a *MATα pGAS2-HIS3 pYJL038C-URA3 hst1Δ::KanMX*	
LRY1545	W303-1a *MATα pPES4-HIS3 hst1Δ::KanMX ADE2 lysΔ*	
LRY1593	W303-1a *MATa pPES4-HIS3 ADE2*	
LRY1686	W303-1a *MATα pGAS2-HIS3 pYJL038C-URA3 hst1Δ::KanMX hst2Δ::TRP1*	
LRY1687	W303-1a *MATα pGAS2-HIS3 pYJL038C-URA3 hst1Δ::KanMX hst4Δ::TRP1*	
LRY1704	W303-1a *MATα pGAS2-HIS3 pYJL038C-URA3 hst1Δ::KanMX hst3Δ::TRP1*	

Table 3: *S. cerevisiae* plasmids used in this study.

Plasmid	Description	Vector Description	Source
pRS416		CEN/ARS *URA3*	Sikorski & Hieter
pLP317	*SIR2*	2μm *URA3*	L. Pillus
pJR2298	HA-*SIR2*	CEN/ARS *URA3*	J. Rine
pLR30	*HST1* -5HA	CEN/ARS *URA3*	
pLR488	HA-*SIR2* $_{1\text{-}201}$ ::*HST1* $_{255\text{-}503}$	CEN/ARS *URA3*	
pLR490	HA-KI *SIR2*	CEN/ARS *URA3*	

Table 4: *K. lactis* strains used in this study.

	Genotype
CK213	*MAT**a** lysA1 leu2 trp1 uraA1*
SAY538	*MATα nej1Δ::LEU2 ade1 leu2 trp1 uraA1*
SAY544	*MATα sir2Δ::KanMX nej1Δ::LEU2 ade1 leu2 trp1 uraA1*
SAY569	*MAT**a** sir2Δ::KanMX leu2 trp1 uraA1*
LRY1856	*MATα asf2Δ::NatMX nej1Δ::LEU2 ade1 leu2 trp1 uraA1*
LRY1946	*MATα sir4:Δ:URA3 nej1Δ::LEU2 ade1 leu2 trp1 uraA1*
LRY1947	*MATα sum1Δ::NatMX nej1Δ::LEU2 ade1 leu2 trp1 uraA1*
LRY1948	*MATα sir4Δ, asf2Δ::URA3 nej1Δ::LEU2 ade1 leu2 trp1 uraA1*
LRY2035	*MAT**a** sum1Δ::NatMX nej1Δ::LEU2 ade1 lysA1 leu2 trp1 uraA1*
LRY2038	*MAT**a** sir4Δ::URA3 nej1Δ::LEU2 lysA1 leu2 trp1 uraA1*
LRY2126	*MATα SIR2-HA::NatMX SIR4-Flag::KanMX sum1Δ::NatMX nej1Δ::LEU2 lysA1 ade1 leu2 trp1 uraA1*
LRY2158	*MAT**a** SIR2-HA::NatMX SIR4-Flag::KanMX sum1Δ::NatMX nej1Δ::LEU2 lysA1 leu2 trp1 uraA1*
LRY2235	*MAT**a** ORC5-Flag::KanMX nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2239	*MAT**a** SIR2-HA::NatMX SIR4-Flag::KanMX myc-SUM1 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2281	*MAT**a** SIR2-HA::NatMX sir4Δ::URA3 myc-SUM1 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2285	*MATα SIR2-HA::NatMX SIR4-Flag::KanMX myc-SUM1 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2327	*MAT**a** SIR4-Flag::KanMX myc-SUM1 RFM1-HA::NatMX nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2328	*MATα SIR4-Flag::KanMX myc-SUM1 RFM1-HA::NatMX nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2374	*MAT**a** sir4Δ, asf2Δ::URA3 nej1Δ::LEU2 lysA1 ade1 leu2 trp1 uraA1*
LRY2377	*MAT**a** asf2Δ::NatMX nej1Δ::LEU2 ade1 leu2 trp1 uraA1*
LRY2388	*MAT**a** sir2Δ::NatMX SIR4-Flag::KanMX myc-SUM1 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2390	*MATα sir2Δ::NatMX SIR4-Flag::KanMX myc-SUM1 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2398	*MATα HML**a** HMR**a** SIR2-HA::NatMX SIR4-Flag::KanMX myc-SUM1 nej1Δ::LEU2 ade1 leu2 trp1 uraA1*
LRY2523	*MAT**a** sir2Δ::KanMX asf2Δ::NatMX lysA1 ade1 leu2 trp1 uraA1*
LRY2525	*MAT**a** SIR2-HA::NatMX asf2Δ::NatMX myc-SUM1 leu2 trp1 uraA1*
LRY2528	*MAT**a** SIR2-HA::NatMX SIR4-Flag::KanMX myc-SUM1 rfm1Δ::URA3 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2529	*MATα SIR4-Flag::KanMX myc-SUM1 rfm1Δ::URA3 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2530	*MAT**a** sir2Δ::NatMX sir4Δ::URA3 myc-SUM1 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2533	*MAT**a** sir2Δ::NatMX SIR4-Flag::KanMX sum1Δ::NatMX nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2561	*MAT**a** ORC1-V5::HphMX lysA1 leu2 trp1 uraA1*
LRY2562	*MAT**a** orc1-Δbah-V5::HphMX lysA1 leu2 trp1 uraA1*
LRY2563	*MAT**a** orc1-Δbah::HphMX SIR2-HA::NatMX SIR4-Flag::KanMX myc-SUM1 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2572	*MAT**a** ORC1-V5::HphMX sir2Δ::NatMX SIR4-Flag::KanMX myc-SUM1 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2573	*MAT**a** ORC1-V5::HphMX sir4Δ::URA3 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2574	*MAT**a** ORC1-V5::HphMX SIR2-HA::NatMX SIR4-Flag::KanMX sum1Δ::NatMX nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2576	*MAT**a** ORC1-V5::HphMX sir4Δ::URA3 sum1Δ::NatMX nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2577	*MAT**a** ORC1-V5::HphMX sir2Δ::NatMX sir4Δ::URA3 myc-SUM1 nej1Δ::LEU2 leu2 trp1 uraA1*
LRY2587	*MAT**a** ORC1-V5::HphMX sir2Δ::NatMX SIR4-Flag::KanMX sum1Δ::NatMX nej1Δ::LEU2 leu2 trp1 uraA1*
LRY3581	*MATα ORC1-V5::HphMX nej1Δ::LEU2 ade1 leu2 trp1 uraA1*

Table 5: Oligonucleotides used for cDNA analysis.

Gene	Sequence 5' - 3'
	S. cerevisiae
ACT1	GCCTTCTACGTTTCCATCCA GGCCAAATCGATTCTCAAA
DTR1	GCCGCCTAAAGCAGGTTTGAC CATACCAAAGGCAGTGAGAGCG
SPS1	TTTCATCGTCGCGCGCAC AAGGTCCCTTTTCGGATGCAG
	K. lactis
ACT1	CGTCGCTTTGGACTTCGAACAA GTGGTACCACCGGACATGAC
HMLα1	GAATGCTCCAACCTTTAAAGTGG GTAAGAATTCCATTCAGTAGCCTGG
HMLα2	CCCATACACTCATTGCTAAACCC CAAAGTTCTTAGAGGATTGTCGG
HMLα3	GATACTCTGAGACTTTCTCTTGG CAGTGACTGGTCATTAGCCGAG
HMRa 1	CGCCTTCTTCACAAAACTAC GATGAACCTGCACCTACA
HMRa 2	CGCCTTCTTCACAAAACTAC GGAACGAATGGTCACCGGAC
CDA2	CGGATCTTAGGAAAGGATTAGAG GTACACATACTTGGTCACATCC
SPS4	CCTCCTGGTTGTCCAAATTTACG GAGGTTCGTTGGATCCACTTG
SPS2	CAGCTCAGTGTCTCTGCAAACG CCGAGCGTACCACCAACCG
SPR3	GCGCTTTGAAGAGTCTAGATGTG CCATCATGGTGAATTGAACACCG
MFα1	GCTGAAGCATCCCCATGGAG CTCGGCCTCAGGGTTGGC
STE18	CCTCGTGAGATCTAGCAAAATTG CTAGCAAGGGAAAGAATGACTG
BAR1	GCCCAGGTAAAAACCACGAGC CCGGACATGTCTTAGAGGGTG

Table 6: Oligonucleotides used for ChIP analysis.

S. cerevisiae

PHO5	GCACGTTTTCGCATAGAACG
	CATTGGCCAAAGAAGCGG
ATS1	GGTAACGCAGCCGTTTGAGC
	CCTCATCGTGCCCCAGTCC
SPS1	CAGGAACGTTTGCCTCAG
	CCAAATGCTCAAGCAGAGAC
DTR1	
A	CCTTCAGACATCGTTAAATGAGC
	GCGCAGTCCGGGTAAATAC
B	GTAGCCAAAGCTGCCTGTTG
	CTTACTACCATCCTTCTAGCC
C	GGAATATGTGCTTGAGTATCAGG
	GGTGGGCACCTCTCAGATTATC
D	CATACCAAAGGCAGTGAGAGCG
	GGTGGGCACCTCTCAGATTATC

K. lactis

RRP7	GCAACAACAGATACTGTGGG
	CCTACTACTAATGTGAAACCATC
SPS4	CGGCCTACAGAAATGACTACTG
	CTCGCTTAATATCGGTTGACAC
SPR3	CGTTCGAAGCCTCCTGGTAAAC
	CTGGCATATGAAATATCTGTAGTGAC
MFα1	CAATATGAATGAGAACTGTAACTGC
	GGAAAGAGCACTCATTTCATACC
BAR1	CACGATGATGATAGGTACAGGG
	GGCCGTCTTGAAGATGTCACT
STE18	CCAAGGAAGAGAGCTTATCTTTAC
	CCACAACCAGACAAATATTGACTG
CDA2	
A	GGATCCCTTGAATAAAGATGCAG
	GCTCTCTTCTCTTTTAGGTTAGAC
B	GACGGACTTCATCATCATAAAACAG
	CCTCTCTGCTTATGACTGCTAC
C	GGAATGTAGCACTTAGAAGAAGTG
	GGAGCTGGTTCATAGTTGAGTG
D	CGTCCAGACCAATAATTAGTAAGG
	GGATCAGAGATAATCACGTAACG
E	CCACAGAAACAACGGTGAGTTC
	CCGGCCGATTAACATAGCTGC
F	CGATGTATCGGCTAGTAATATTTCG
	GGATCAATGGGGAGGCTGTAG
G	GTAGCTAAAGACAACGGTTTGGC
	CACGATTCCCTCGGGTTCAC
H	CGGATCTTAGGAAAGGATTAGAG
	GTACACATACTTGGTCACATCC
I	CGACATTTGAACCAGGAAAAAGAG
	GAACACAGAGATCAGCGTGATC
J	GAGTTCAACGTTAGTGTTCATAATC
	CACGCAACTGCAATCTGTTAAC

K. lactis

HML

A	GGATATTTACTAGCCCAAATGAAGC
	CTAGGCCAGTCACATTCTACTTC
B	GTTCATCGTTGTCATAACTTTCTCG
	GATGATGGGGTGCCCGAAAG
C	GAGTGAATTGATTATGGTCACGG
	GTAGGGGTAAACTTCCTCAATTTC
D	CGTATTATACATGACAGCCAAAGG
	GTTGGGTGAATAAACCATCTCAC
E	CCTGTACAATTGCTATGCCTACAG
	CATGCCGCTTCTGTCTTTAGC
P	CACTCAAGTATTCCCCATCC
	CAAACGAATTCAATTCGGTGTG
Q	GGCTGAAGTTCGATGAACTAGG
	GGTGCTCGGCATACAAACTTC
R	CAAGCCCCTACTACCAAACATG
	GTCACTCTGACTTCGTGGCC
S	GAAGGGATTTAGTGGTATCTCAAC
	CCAACATAAGGTATCTCTTCTTATG
T	CTCTCAATGGCATCCAGTCC
	GTCTCACTGGTATAAGTCACTC
U	GCATATATTTCAGTACGGTGAGC
	CTTTCAAAAAGAATAGTGCTTACTGG

MAT

AA	CGAGCAATGTGCACGTTTAAATG
	CAGGAGACGGATCACGTAAATTC
BB	GTGATGCCTGTTGTTACAGGG
	GCTTGCTGTTCTTGTTGAAGCTG
CC	CAGTACGAACGAGACCAAGTTATG
	CACCTCAGATTCAGCTTCGGC
PP	GTATTCCCCATCCTTTTAATATACTATG
	GTGAAGGTATTGATGTATGTGCTG
QQ	GTGTGGGATCCTTCAGTAGAC
	CATCTTCCTTATCCACCATCTCG
RR	GTTCCTCTCTACCACGATAATAC
	GTGAATTGGCAAAGCTTCAGG

HML/MAT

F	CAGGAGATGCAGACCATAGC
	CTTGATGAACTCAGTCTGAGTAAC
G	GAATGCTCCAACCTTTAAAGTGG
	GTAAGAATTCCATTCAGTAGCCTGG
H	CCACTTTAAAGGTTGGAGCATTC
	GGGTTTAGCAATGAGTGTATGGG
I	CCCATACACTCATTGCTAAACCC
	CAAAGTTCTTAGAGGATTGTCGG
J	GGGTCCGTTTCTTGGGATTAATG
	CCGCTGCCGTTCTAAGTCTG
K	CCCTCACTCTCATCGTCAATC
	GGAAAACGAAATAACGGAGTTCC
L	GATACTCTGAGACTTTCTCTTGG
	CAGTGACTGGTCATTAGCCGAG
M	GTACTGTCTAAGGGAAGCTGG
	GGACAGGATACCGTGTAAATACC
N	GGTGTCGGAGAATATGAGCAAG
	CTAAAAAACTGATTGTCTTGGTGTC
O	GCTGTTGAATATTGGATTGGGCTG
	GTACTTGCTTTGTTCCATTAACGTG

K. lactis

HMR	
A	CTACTACCACTGCCTCTGAAAAC CTGGAATCGTTGTTAGAGCCTG
B	CTGGTGCGTCATCAACTAGC GGGATAAATACGGATAGGGTATC
C	CCAGTAAAGAACACACACCGC GAGCGAAGACCCTTTTCTGAG
D	GCGACAACAAGTGGAAGAGTTG CTCTAGACATGCATGTCCGAC
E	CAGGAGATGCAGACCATAGC CGAGCTAAAATAGCTCGGGTC
F	CGCCTTCTTCACAAAACTAC CGCCTTCTTCACAAAACTAC
G	CCACATAGATACGTCCCTGTTTTAG GACTCCGGTCAATGCTGAGG
H	CCATCAAATGGTGTGAATTGAATCTC CCTGAATATTAACGATTGCTCACC
I	GACCAACCATGTCTTCCTTTCC GGTTGGTCGATGGATTTTCGTG
J	GGAACGAATGGTCACCGGAC GGAACGAATGGTCACCGGAC
K	CAAGGCAGTATCTCTCCGAAC GGCATTCTACCGACCTCCG
L	CCCGTATCTGCCATACCAAAC GTCTTCGTGGCTGCTAATAGGC
M	GGGCGTATCGCATCACGTAG GGTCGCTCTTACGTGAGCTG
N	GGCCAGGTTGAAATACGCAAG CAGCAACAAGAACTGTAGACATC

KARS12	
A	CACAGCATTAAGCACCACTTC GATCGACCTTCGATCCGTC
B	CCA GCA ATG CGA ACA TAA CAC GGA TTT CAC CAT GGT TCT TGA AG
C	CTATTCTCTGCCAAGCTATCC GGTTCTGTCTACAGATTTCCAC
D	GGGCCTCTATTCAAATTACCTATG GTTGGTGTTCAAGTAACGACTAC
E	GGAAGAGCTTCAGGGTC GCGATCGAAATACGTAGATTGC
F	CAAACACTCGGCTTGGCTAG GAAAGACTGTAGATTAGTAGACC
G	GAGCATGCGGTTCTCTTCC GTTGCGATTTGGGCAGCG

TEL-BR	
A	CCCGCTATATTTGGTCCATCATC AACAAAGAGAATGCAGGGAGAGT
B	CATCCCCAGCATAAATTCATCA ACAGGAAAGAAAGGAGTAGAGGTG
C	TTGCAACGATTCGAACATGCTGT ACTCACGTGACTGGAAGTCGAGT
D	CTTCTGGGGTATTAATGCTGCTG AGCTCTAGTGTTGTTGTTGGCTC
E	GTGAACGAATCCGATGTCTGTG TGGAGAGTTCTATTACTTCCGCC
F	GAGTAAACACCGTTGTGGTAGGA CAAACACCAGAAATTGAAACTGCC
G	ATATACGGTACCGGTCCAAGGA TCGAGACCCCAGAGTTTAAGAC
Tel-EL	GTCCTTGCATAGGATACACACGTT AGGTGAAGAGAAGCAGGTGATG
Tel-ER	CCAAGCATTGGCCATGGCAAG CTCGAAACGGATTTCCTCTTTCG
Tel-FR	GGTGAAAAACAGTCAAGAATACGC CTCTAGACGGATTTCCTCTTCTG

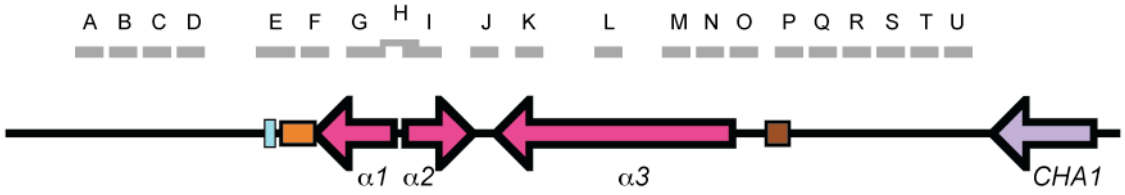


Figure 46: Schematic of *HML* locus in *K. lactis* with positioned primer sets.

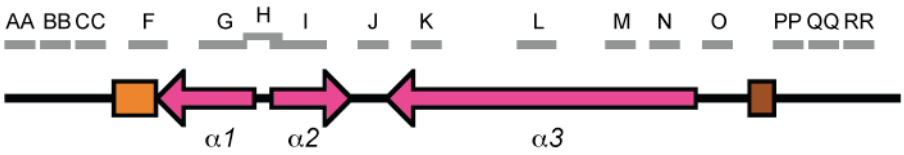


Figure 47: Schematic of MAT locus in K. lactis with positioned primer sets.

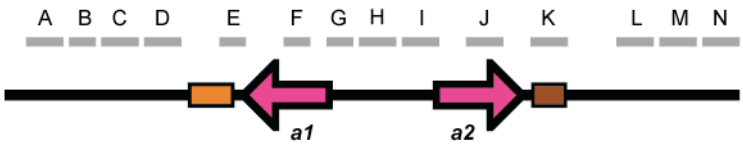


Figure 48: Schematic of *HMR* locus in *K. lactis* with positioned primer sets.

Figure 49: Schematic of *CDA2* locus in *K. lactis* with positioned primer sets.

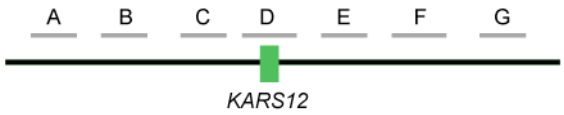


Figure 50: Schematic of *KARS12* replication origin in *K. lactis* with positioned primer sets.

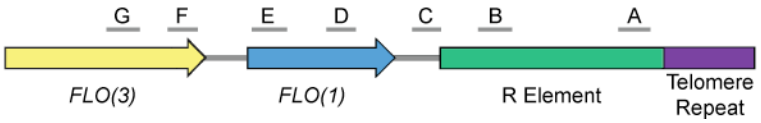


Figure 51: Schematic of Telomere-BR in *K. lactis* with positioned primer sets.

Works Cited

Aparicio, O.M., Billington, B.L., and Gottschling, D.E. 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. *Cell* **66**(6): 1279-1287.

Astrom, S. and Rine, J. 1998. Theme and variation among silencing proteins in Saccharomyces cerevisiae and Kluyveromyces lactis. *Genetics* **148**(3): 1021-1029.

Aström, S.U., Kegel, A., Sjöstrand, J.O., and Rine, J. 2000. Kluyveromyces lactis Sir2p regulates cation sensitivity and maintains a specialized chromatin structure at the cryptic alpha-locus. *Genetics* **156**(1): 81-91.

Auth, T., Kunkel, E., and Grummt, F. 2006. Interaction between HP1alpha and replication proteins in mammalian cells. *Exp Cell Res* **312**(17): 3349-3359.

Axelrod, A. 1991. Role of a cell-cycle gene in transcriptional silencing. In. University of California, Berkely, Berkeley.

Ayoub, N., Goldshmidt, I., Lyakhovetsky, R., and Cohen, A. 2000. A fission yeast repression element cooperates with centromere-like sequences and defines a mat silent domain boundary. *Genetics* **156**(3): 983-994.

Babu, M., Iyer, L., Balaji, S., and Aravind, L. 2006. The natural history of the WRKY-GCM1 zinc fingers and the relationship between transcription factors and transposons. *Nucleic Acids Res* **34**(22): 6505-6520.

Barsoum, E., Martinez, P., and Aström, S.U. 2010a. Alpha3, a transposable element that promotes host sexual reproduction. *Genes Dev* **24**(1): 33-44.

Barsoum, E., Sjöstrand, J.O.O., and Aström, S.U. 2010b. Ume6 is required for the MATa/MAT{alpha}-cellular identity and transcriptional silencing in Kluyveromyces lactis. *Genetics*.

Bedalov, A., Hirao, M., Posakony, J., Nelson, M., and Simon, J.A. 2003. NAD+-dependent deacetylase Hst1p controls biosynthesis and cellular NAD+ levels in Saccharomyces cerevisiae. *Mol Cell Biol* **23**(19): 7044-7054.

Bell, S.P., Mitchell, J., Leber, J., Kobayashi, R., and Stillman, B. 1995. The multidomain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. *Cell* **83**(4): 563-568.

Bose, M.E., McConnell, K.H., Gardner-Aukema, K.A., Müller, U., Weinreich, M., Keck, J.L., and Fox, C.A. 2004. The origin recognition complex and Sir4 protein recruit Sir1p to yeast silent chromatin through independent interactions requiring a common Sir1p domain. *Mol Cell Biol* **24**(2): 774-786.

Brachmann, C., Sherman, J., Devine, S., Cameron, E., Pillus, L., and Boeke, J. 1995. The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. *Genes Dev* **9**(23): 2888-2902.

Breitkreutz, A., Boucher, L., and Tyers, M. 2001. MAPK specificity in the yeast pheromone response independent of transcriptional activation. *Curr Biol* **11**(16): 1266-1271.

Brockert, P.J., Lachke, S.A., Srikantha, T., Pujol, C., Galask, R., and Soll, D.R. 2003. Phenotypic switching and mating type switching of Candida glabrata at sites of colonization. *Infect Immun* **71**(12): 7109-7118.

Buchberger, J.R., Onishi, M., Li, G., Seebacher, J., Rudner, A.D., Gygi, S.P., and Moazed, D. 2008. Sir3-nucleosome interactions in spreading of silent chromatin in Saccharomyces cerevisiae. *Mol Cell Biol* **28**(22): 6903-6918.

Butler, G., Kenny, C., Fagan, A., Kurischko, C., Gaillardin, C., and Wolfe, K.H. 2004. Evolution of the MAT locus and its Ho endonuclease in yeast species. *Proc Natl Acad Sci USA* **101**(6): 1632-1637.

Byrne, K.P. and Wolfe, K.H. 2005. The Yeast Gene Order Browser: combining curated homology and syntenic context reveals gene fate in polyploid species. *Genome Res* **15**(10): 1456-1461.

Carmen, A., Milne, L., and Grunstein, M. 2002. Acetylation of the yeast histone H4 N terminus regulates its binding to heterochromatin protein SIR3. *J Biol Chem* **277**(7): 4778-4781.

Celic, I., Masumoto, H., Griffith, W.P., Meluh, P., Cotter, R.J., Boeke, J.D., and Verreault, A. 2006. The sirtuins hst3 and Hst4p preserve genome integrity by controlling histone h3 lysine 56 deacetylation. *Curr Biol* **16**(13): 1280-1289.

Chang, J.-F., Hall, B.E., Tanny, J.C., Moazed, D., Filman, D., and Ellenberger, T. 2003. Structure of the coiled-coil dimerization motif of Sir4 and its interaction with Sir3. *Structure* **11**(6): 637-649.

Chen, X.J. and Clark-Walker, G.D. 1994. sir2 mutants of Kluyveromyces lactis are hypersensitive to DNA-targeting drugs. *Mol Cell Biol* **14**(7): 4501-4508.

Chi, M.H. and Shore, D. 1996. SUM1-1, a dominant suppressor of SIR mutations in Saccharomyces cerevisiae, increases transcriptional silencing at telomeres and HM mating-type loci and decreases chromosome stability. *Mol Cell Biol* **16**(8): 4281-4294.

Chien, C.T., Buck, S., Sternglanz, R., and Shore, D. 1993. Targeting of SIR1 protein establishes transcriptional silencing at HM loci and telomeres in yeast. *Cell* **75**(3): 531-541.

Chou, C.-C., Li, Y.-C., and Gartenberg, M.R. 2008. Bypassing Sir2 and O-acetyl-ADP-ribose in transcriptional silencing. *Molecular Cell* **31**(5): 650-659.

Cliften, P.F., Fulton, R.S., Wilson, R.K., and Johnston, M. 2006. After the duplication: gene loss and adaptation in Saccharomyces genomes. *Genetics* **172**(2): 863-872.

Cockell, M.M., Perrod, S., and Gasser, S.M. 2000. Analysis of Sir2p domains required for rDNA and telomeric silencing in Saccharomyces cerevisiae. *Genetics* **154**(3): 1069-1083.

Conant, G. and Wolfe, K. 2008. Turning a hobby into a job: how duplicated genes find new functions. *Nature Rev Genet* **9**(12): 938-950.

Connelly, J.J., Yuan, P., Hsu, H.-C., Li, Z., Xu, R.-M., and Sternglanz, R. 2006. Structure and function of the Saccharomyces cerevisiae Sir3 BAH domain. *Mol Cell Biol* **26**(8): 3256-3265.

Coria, R., Kawasaki, L., Torres-Quiroz, F., Ongay-Larios, L., Sánchez-Paredes, E., Velázquez-Zavala, N., Navarro-Olmos, R., Rodríguez-González, M., Aguilar-Corachán, R., and Coello, G. 2006. The pheromone response pathway of Kluyveromyces lactis. *FEMS Yeast Res* **6**(3): 336-344.

De Las Peñas, A., Pan, S.-J., Castaño, I., Alder, J., Cregg, R., and Cormack, B.P. 2003. Virulence-related surface glycoproteins in the yeast pathogen Candida glabrata are encoded in subtelomeric clusters and subject to RAP1- and SIR-dependent transcriptional silencing. *Genes Dev* **17**(18): 2245-2258.

Deng, Z., Dheekollu, J., Broccoli, D., Dutta, A., and Lieberman, P.M. 2007. The origin recognition complex localizes to telomere repeats and prevents telomere-circle formation. *Curr Biol* **17**(22): 1989-1995.

Deng, Z., Norseen, J., Wiedmer, A., Riethman, H., and Lieberman, P.M. 2009. TERRA RNA Binding to TRF2 Facilitates Heterochromatin Formation and ORC Recruitment at Telomeres. *Molecular Cell* **35**(4): 403-413.

Derbyshire, M., Weinstock, K., and Strathern, J. 1996. HST1, a new member of the SIR2 family of genes. *Yeast* **12**(7): 631-640.

Des Marais, D. and Rausher, M. 2008. Escape from adaptive conflict after duplication in an anthocyanin pathway gene. *Nature* **454**(7205): 762-765.

Dietrich, F.S., Voegeli, S., Brachat, S., Lerch, A., Gates, K., Steiner, S., Mohr, C., Pöhlmann, R., Luedi, P., Choi, S. et al. 2004. The Ashbya gossypii genome as a tool for mapping the ancient Saccharomyces cerevisiae genome. *Science* **304**(5668): 304-307.

Domergue, R., Castaño, I., De Las Peñas, A., Zupancic, M., Lockatell, V., Hebel, J.R., Johnson, D., and Cormack, B.P. 2005. Nicotinic acid limitation regulates silencing of Candida adhesins during UTI. *Science* **308**(5723): 866-870.

Drinnenberg, I.A., Weinberg, D.E., Xie, K.T., Mower, J.P., Wolfe, K.H., Fink, G.R., and Bartel, D.P. 2009. RNAi in budding yeast. *Science* **326**(5952): 544-550.

Dujon, B., Sherman, D., Fischer, G., Durrens, P., Casaregola, S., Lafontaine, I., De Montigny, J., Marck, C., Neuvéglise, C., Talla, E. et al. 2004. Genome evolution in yeasts. *Nature* **430**(6995): 35-44.

Egel, R. 1981. Mating-type switching and mitotic crossing-over at the mating-type locus in fission yeast. *Cold Spring Harb Symp Quant Biol* **45 Pt 2**: 1003-1007.

Ekwall, K., Nimmo, E.R., Javerzat, J.P., Borgstrøm, B., Egel, R., Cranston, G., and Allshire, R. 1996. Mutations in the fission yeast silencing factors clr4+ and rik1+ disrupt the localisation of the chromo domain protein Swi6p and impair centromere function. *J Cell Sci* **109 (Pt 11)**: 2637-2648.

Elgin, S.C.R. and Grewal, S.I.S. 2003. Heterochromatin: silence is golden. *Curr Biol* **13**(23): R895-898.

Enomoto, S., Johnston, S.D., and Berman, J. 2000. Identification of a novel allele of SIR3 defective in the maintenance, but not the establishment, of silencing in Saccharomyces cerevisiae. *Genetics* **155**(2): 523-538.

Fabre, E., Muller, H., Therizols, P., Lafontaine, I., Dujon, B., and Fairhead, C. 2005. Comparative genomics in hemiascomycete yeasts: evolution of sex, silencing, and subtelomeres. *Mol Biol Evol* **22**(4): 856-873.

Fairhead, C. and Dujon, B. 2006. Structure of Kluyveromyces lactis subtelomeres: duplications and gene content. *FEMS Yeast Res* **6**(3): 428-441.

Fidel, P.L., Vazquez, J.A., and Sobel, J.D. 1999. Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to C. albicans. *Clin Microbiol Rev* **12**(1): 80-96.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L., and Postlethwait, J. 1999. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* **151**(4): 1531-1545.

Fox, C.A., Ehrenhofer-Murray, A.E., Loo, S., and Rine, J. 1997. The origin recognition complex, SIR1, and the S phase requirement for silencing. *Science* **276**(5318): 1547-1551.

Funakoshi, M. and Hochstrasser, M. 2009. Small epitope-linker modules for PCR-based C-terminal tagging in Saccharomyces cerevisiae. *Yeast* **26**(3): 185-192.

Gallagher, J.E.G., Babiarz, J.E., Teytelman, L., Wolfe, K.H., and Rine, J. 2009. Elaboration, diversification and regulation of the Sir1 family of silencing proteins in Saccharomyces. *Genetics* **181**(4): 1477-1491.

Gardner, K.A., Rine, J., and Fox, C.A. 1999. A region of the Sir1 protein dedicated to recognition of a silencer and required for interaction with the Orc1 protein in saccharomyces cerevisiae. *Genetics* **151**(1): 31-44.

Gasser, S.M. and Cockell, M.M. 2001. The molecular biology of the SIR proteins. *Gene* **279**(1): 1-16.

Gelbart, M.E., Rechsteiner, T., Richmond, T.J., and Tsukiyama, T. 2001. Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates. *Mol Cell Biol* **21**(6): 2098-2106.

Ghidelli, S., Donze, D., Dhillon, N., and Kamakaka, R.T. 2001. Sir2p exists in two nucleosome-binding complexes with distinct deacetylase activities. *EMBO J* **20**(16): 4522-4535.

Gotta, M., Palladino, F., and Gasser, S.M. 1998. Functional characterization of the N terminus of Sir3p. *Mol Cell Biol* **18**(10): 6110-6120.

Gottlieb, S. and Esposito, R.E. 1989. A new role for a yeast transcriptional silencer gene, SIR2, in regulation of recombination in ribosomal DNA. *Cell* **56**(5): 771-776.

Gottschling, D.E., Aparicio, O.M., Billington, B.L., and Zakian, V.A. 1990. Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. *Cell* **63**(4): 751-762.

Gu, Z., Steinmetz, L.M., Gu, X., Scharfe, C., Davis, R.W., and Li, W.-H. 2003. Role of duplicate genes in genetic robustness against null mutations. *Nature* **421**(6918): 63-66.

Hahn, M.W. 2009. Distinguishing among evolutionary models for the maintenance of gene duplicates. *J Hered* **100**(5): 605-617.

Hansen, K.R., Ibarra, P.T., and Thon, G. 2006. Evolutionary-conserved telomere-linked helicase genes of fission yeast are repressed by silencing factors, RNAi components and the telomere-binding protein Taz1. *Nucleic Acids Res* **34**(1): 78-88.

Harbison, C.T., Gordon, D.B., Lee, T.I., Rinaldi, N.J., Macisaac, K.D., Danford, T.W., Hannett, N.M., Tagne, J.-B., Reynolds, D.B., Yoo, J. et al. 2004. Transcriptional regulatory code of a eukaryotic genome. *Nature* **431**(7004): 99-104.

He, X. and Zhang, J. 2005. Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution. *Genetics* **169**(2): 1157-1164.

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S., and Grunstein, M. 1995. Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. *Cell* **80**(4): 583-592.

Herman, A. and Halvorson, H. 1963. Genetic Control of Beta-Glucosidase Synthesis in Saccharomyces Lactis. *J Bacteriol* **85**: 901-910.

Herman, A. and Roman, H. 1966. Allele specific determinants of homothallism in *Saccharomyces lactis*. *Genetics* **53**(4): 727-740.

Hickman, M.A. and Rusche, L.N. 2007. Substitution as a mechanism for genetic robustness: the duplicated deacetylases Hst1p and Sir2p in Saccharomyces cerevisiae. *PLoS Genet* **3**(8): e126.

-. 2009. The Sir2-Sum1 complex represses transcription using both promoter-specific and long-range mechanisms to regulate cell identity and sexual cycle in the yeast Kluyveromyces lactis. *PLoS Genet* **5**(11): e1000710.

Hittinger, C. and Carroll, S. 2007. Gene duplication and the adaptive evolution of a classic genetic switch. *Nature* **449**(7163): 677-681.

Hoppe, G., Tanny, J., Rudner, A., Gerber, S., Danaie, S., Gygi, S., and Moazed, D. 2002a. Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. *Mol Cell Biol* **22**(12): 4167-4180.

Hoppe, G.J., Tanny, J.C., Rudner, A.D., Gerber, S.A., Danaie, S., Gygi, S.P., and Moazed, D. 2002b. Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. *Mol Cell Biol* **22**(12): 4167-4180.

Hou, Z., Bernstein, D.A., Fox, C.A., and Keck, J.L. 2005. Structural basis of the Sir1-origin recognition complex interaction in transcriptional silencing. *Proc Natl Acad Sci USA* **102**(24): 8489-8494.

Hou, Z., Danzer, J.R., Mendoza, L., Bose, M.E., Müller, U., Williams, B., and Fox, C.A. 2009. Phylogenetic conservation and homology modeling help reveal a novel domain within the budding yeast heterochromatin protein Sir1. *Mol Cell Biol* **29**(3): 687-702.

Huang, Y. 2002. Transcriptional silencing in Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Nucleic Acids Res* **30**(7): 1465-1482.

Imai, S., Armstrong, C.M., Kaeberlein, M., and Guarente, L. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**(6771): 795-800.

Iraqui, I., Garcia-Sanchez, S., Aubert, S., Dromer, F., Ghigo, J.-M., d'Enfert, C., and Janbon, G. 2005. The Yak1p kinase controls expression of adhesins and biofilm formation in Candida glabrata in a Sir4p-dependent pathway. *Mol Microbiol* **55**(4): 1259-1271.

Irene, C., Maciariello, C., Micheli, G., Theis, J.F., Newlon, C.S., and Fabiani, L. 2007. DNA elements modulating the KARS12 chromosomal replicator in Kluyveromyces lactis. *Mol Genet Genomics* **277**(3): 287-299.

Irlbacher, H., Franke, J., Manke, T., Vingron, M., and Ehrenhofer-Murray, A. 2005. Control of replication initiation and heterochromatin formation in Saccharomyces cerevisiae by a regulator of meiotic gene expression. *Genes Dev* **19**(15): 1811-1822.

Ivanova, A.V., Bonaduce, M.J., Ivanov, S.V., and Klar, A.J. 1998. The chromo and SET domains of the Clr4 protein are essential for silencing in fission yeast. *Nat Genet* **19**(2): 192-195.

Ivy, J.M., Klar, A.J., and Hicks, J.B. 1986. Cloning and characterization of four SIR genes of Saccharomyces cerevisiae. *Mol Cell Biol* **6**(2): 688-702.

Jia, S., Noma, K.-i., and Grewal, S.I.S. 2004. RNAi-independent heterochromatin nucleation by the stress-activated ATF/CREB family proteins. *Science* **304**(5679): 1971-1976.

Kanoh, J., Sadaie, M., Urano, T., and Ishikawa, F. 2005. Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. *Curr Biol* **15**(20): 1808-1819.

Kaufmann, A. and Philippsen, P. 2009. Of bars and rings: Hof1-dependent cytokinesis in multiseptated hyphae of *Ashbya gossypii*. *Mol Cell Biol* **29**(3): 771-783.

Kaur, R., Domergue, R., Zupancic, M.L., and Cormack, B.P. 2005. A yeast by any other name: Candida glabrata and its interaction with the host. *Curr Opin Microbiol* **8**(4): 378-384.

Kellis, M., Birren, B.W., and Lander, E.S. 2004. Proof and evolutionary analysis of ancient genome duplication in the yeast Saccharomyces cerevisiae. *Nature* **428**(6983): 617-624.

Kirchmaier, A. and Rine, J. 2006. Cell cycle requirements in assembling silent chromatin in Saccharomyces cerevisiae. *Mol Cell Biol* **26**(3): 852-862.

Klar, A., Kakar, S., Ivy, J., Hicks, J., Livi, G., and Miglio, L. 1985. SUM1, an apparent positive regulator of the cryptic mating-type loci in Saccharomyces cerevisiae. *Genetics* **111**(4): 745-758.

Kobayashi, T. and Ganley, A.R.D. 2005. Recombination regulation by transcription-induced cohesin dissociation in rDNA repeats. *Science* **309**(5740): 1581-1584.

Kobayashi, T., Horiuchi, T., Tongaonkar, P., Vu, L., and Nomura, M. 2004. SIR2 regulates recombination between different rDNA repeats, but not recombination within individual rRNA genes in yeast. *Cell* **117**(4): 441-453.

Kurdistani, S.K., Robyr, D., Tavazoie, S., and Grunstein, M. 2002. Genome-wide binding map of the histone deacetylase Rpd3 in yeast. *Nat Genet* **31**(3): 248-254.

Kyrion, G., Liu, K., Liu, C., and Lustig, A.J. 1993. RAP1 and telomere structure regulate telomere position effects in Saccharomyces cerevisiae. *Genes Dev* **7**(7A): 1146-1159.

Lau, A., Blitzblau, H., and Bell, S. 2002. Cell-cycle control of the establishment of mating-type silencing in S. cerevisiae. *Genes Dev* **16**(22): 2935-2945.

Le, S., Davis, C., Konopka, J., and Sternglanz, R. 1997. Two new S-phase-specific genes from Saccharomyces cerevisiae. *Yeast* **13**(11): 1029-1042.

Lidonnici, M.R., Rossi, R., Paixão, S., Mendoza-Maldonado, R., Paolinelli, R., Arcangeli, C., Giacca, M., Biamonti, G., and Montecucco, A. 2004. Subnuclear distribution of the largest subunit of the human origin recognition complex during the cell cycle. *J Cell Sci* **117**(Pt 22): 5221-5231.

Liou, G.-G., Tanny, J.C., Kruger, R.G., Walz, T., and Moazed, D. 2005. Assembly of the SIR complex and its regulation by O-acetyl-ADP-ribose, a product of NAD-dependent histone deacetylation. *Cell* **121**(4): 515-527.

Liti, G. and Louis, E.J. 2005. Yeast evolution and comparative genomics. *Annu Rev Microbiol* **59**: 135-153.

Longtine, M.S., Wilson, N.M., Petracek, M.E., and Berman, J. 1989. A yeast telomere binding activity binds to two related telomere sequence motifs and is indistinguishable from RAP1. *Curr Genet* **16**(4): 225-239.

Luo, K., Vega-Palas, M., and Grunstein, M. 2002a. Rap1-Sir4 binding independent of other Sir, yKu, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. *Genes Dev* **16**(12): 1528-1539.

Luo, K., Vega-Palas, M.A., and Grunstein, M. 2002b. Rap1-Sir4 binding independent of other Sir, yKu, or histone interactions initiates the assembly of telomeric heterochromatin in yeast. *Genes Dev* **16**(12): 1528-1539.

Lynch, M. and Force, A. 2000. The probability of duplicate gene preservation by subfunctionalization. *Genetics* **154**(1): 459-473.

Lynch, M. and Katju, V. 2004. The altered evolutionary trajectories of gene duplicates. *Trends Genet* **20**(11): 544-549.

Lynch, P. and Rusche, L. 2009. A silencer promotes the assembly of silenced chromatin independently of recruitment. *Mol Cell Biol* **29**(1): 43-56.

Lynch, P.J., Fraser, H.B., Sevastopoulos, E., Rine, J., and Rusche, L.N. 2005. Sum1p, the origin recognition complex, and the spreading of a promoter-specific repressor in Saccharomyces cerevisiae. *Mol Cell Biol* **25**(14): 5920-5932.

Maas, N.L., Miller, K.M., DeFazio, L.G., and Toczyski, D.P. 2006. Cell cycle and checkpoint regulation of histone H3 K56 acetylation by Hst3 and Hst4. *Mol Cell* **23**(1): 109-119.

Madhani, H.D., Styles, C.A., and Fink, G.R. 1997. MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. *Cell* **91**(5): 673-684.

Mancio-Silva, L., Rojas-Meza, A.P., Vargas, M., Scherf, A., and Hernandez-Rivas, R. 2008. Differential association of Orc1 and Sir2 proteins to telomeric domains in Plasmodium falciparum. *J Cell Sci* **121**(Pt 12): 2046-2053.

McCord, R., Pierce, M., Xie, J., Wonkatal, S., Mickel, C., and Vershon, A.K. 2003. Rfm1, a novel tethering factor required to recruit the Hst1 histone deacetylase for repression of middle sporulation genes. *Mol Cell Biol* **23**(6): 2009-2016.

Mead, J., McCord, R., Youngster, L., Sharma, M., Gartenberg, M.R., and Vershon, A.K. 2007. Swapping the gene-specific and regional silencing specificities of the Hst1 and Sir2 histone deacetylases. *Mol Cell Biol* **27**(7): 2466-2475.

Min, J., Landry, J., Sternglanz, R., and Xu, R.M. 2001. Crystal structure of a SIR2 homolog-NAD complex. *Cell* **105**(2): 269-279.

Moazed, D. 2001. Common themes in mechanisms of gene silencing. *Mol Cell* **8**(3): 489-498.

Moazed, D., Kistler, A., Axelrod, A., Rine, J., and Johnson, A.D. 1997. Silent information regulator protein complexes in Saccharomyces cerevisiae: a SIR2/SIR4 complex and evidence for a regulatory domain in SIR4 that inhibits its interaction with SIR3. *Proc Natl Acad Sci USA* **94**(6): 2186-2191.

Moretti, P., Freeman, K., Coodly, L., and Shore, D. 1994. Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. *Genes Dev* **8**(19): 2257-2269.

Muller, H., Hennequin, C., Gallaud, J., Dujon, B., and Fairhead, C. 2008. The asexual yeast Candida glabrata maintains distinct a and alpha haploid mating types. *Eukaryot Cell* **7**(5): 848-858.

Murphy, G.A., Spedale, E.J., Powell, S.T., Pillus, L., Schultz, S.C., and Chen, L. 2003. The Sir4 C-terminal coiled coil is required for telomeric and mating type silencing in Saccharomyces cerevisiae. *J Mol Biol* **334**(4): 769-780.

Nakayama, J., Klar, A.J., and Grewal, S.I. 2000. A chromodomain protein, Swi6, performs imprinting functions in fission yeast during mitosis and meiosis. *Cell* **101**(3): 307-317.

Nickles, K. and McEachern, M.J. 2004. Characterization of Kluyveromyces lactis subtelomeric sequences including a distal element with strong purine/pyrimidine strand bias. *Yeast* **21**(10): 813-830.

Noma, K.-i., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S.I.S. 2004. RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* **36**(11): 1174-1180.

Ohno, S. 1970. *Evolution by Gene Duplication*.

Onishi, M., Liou, G.-G., Buchberger, J.R., Walz, T., and Moazed, D. 2007. Role of the conserved Sir3-BAH domain in nucleosome binding and silent chromatin assembly. *Mol Cell* **28**(6): 1015-1028.

Pak, D.T., Pflumm, M., Chesnokov, I., Huang, D.W., Kellum, R., Marr, J., Romanowski, P., and Botchan, M.R. 1997. Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* **91**(3): 311-323.

Park, Y., Hanish, J., and Lustig, A.J. 1998. Sir3p domains involved in the initiation of telomeric silencing in Saccharomyces cerevisiae. *Genetics* **150**(3): 977-986.

Pasek, S., Risler, J.-L., and Brézellec, P. 2006. The role of domain redundancy in genetic robustness against null mutations. *J Mol Biol* **362**(2): 184-191.

Perrod, S., Cockell, M.M., Laroche, T., Renauld, H., Ducrest, A.L., Bonnard, C., and Gasser, S.M. 2001. A cytosolic NAD-dependent deacetylase, Hst2p, can modulate nucleolar and telomeric silencing in yeast. *EMBO J* **20**(1-2): 197-209.

Pierce, M., Benjamin, K.R., Montano, S.P., Georgiadis, M.M., Winter, E., and Vershon, A.K. 2003. Sum1 and Ndt80 proteins compete for binding to middle sporulation element sequences that control meiotic gene expression. *Mol Cell Biol* **23**(14): 4814-4825.

Pijnappel, W., Schaft, D., Roguev, A., Shevchenko, A., Tekotte, H., Wilm, M., Rigaut, G., Seraphin, B., Aasland, R., and Stewart, A. 2001. The S. cerevisiae SET3 complex includes two histone deacetylases, Hos2 and Hst1, and is a meiotic-specific repressor of the sporulation gene program. *Genes Dev* **15**(22): 2991-3004.

Pillus, L. and Rine, J. 1989. Epigenetic inheritance of transcriptional states in S. cerevisiae. *Cell* **59**(4): 637-647.

Prasanth, S.G., Prasanth, K.V., Siddiqui, K., Spector, D.L., and Stillman, B. 2004. Human Orc2 localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance. *EMBO J* **23**(13): 2651-2663.

Renauld, H., Aparicio, O.M., Zierath, P.D., Billington, B.L., Chhablani, S.K., and Gottschling, D.E. 1993. Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. *Genes Dev* **7**(7A): 1133-1145.

Rine, J. and Herskowitz, I. 1987. Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae. *Genetics* **116**(1): 9-22.

Robert, F., Pokholok, D.K., Hannett, N.M., Rinaldi, N.J., Chandy, M., Rolfe, A., Workman, J.L., Gifford, D.K., and Young, R.A. 2004. Global position and recruitment of HATs and HDACs in the yeast genome. *Mol Cell* **16**(2): 199-209.

Rusche, L. and Rine, J. 2001. Conversion of a gene-specific repressor to a regional silencer. *Genes Dev* **15**(8): 955-967.

Rusche, L.N., Kirchmaier, A.L., and Rine, J. 2003. The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. *Annu Rev Biochem* **72**: 481-516.

Rusché, L.N., Kirchmaier, A.L., and Rine, J. 2002. Ordered nucleation and spreading of silenced chromatin in Saccharomyces cerevisiae. *Mol Biol Cell* **13**(7): 2207-2222.

Safi, A., Wallace, K.A., and Rusche, L.N. 2008. Evolution of new function through a single amino acid change in the yeast repressor Sum1p. *Mol Cell Biol* **28**(8): 2567-2578.

Sampath, V., Yuan, P., Wang, I.X., Prugar, E., van Leeuwen, F., and Sternglanz, R. 2009. Mutational analysis of the Sir3 BAH domain reveals multiple points of interaction with nucleosomes. *Mol Cell Biol* **29**(10): 2532-2545.

Sanchez, M., Iglesias, F.J., Santamaria, C., and Dominguez, A. 1993. Transformation of *Kluyveromyces lactis* by Electroporation. *Appl Environ Microbiol* **59**(7): 2087-2092.

Schmitt, M., Brown, T., and Trumpower, B. 1990. A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. *Nucleic Acids Res* **18**(10): 3091-3092.

Schneider, B.L., Seufert, W., Steiner, B., Yang, Q.H., and Futcher, A.B. 1995. Use of polymerase chain reaction epitope tagging for protein tagging in Saccharomyces cerevisiae. *Yeast* **11**(13): 1265-1274.

Schultz, J. 1936. Variegation in Drosophila and the Inert Chromosome Regions. *Proc Natl Acad Sci USA* **22**(1): 27-33.

Sikorski, R. and Hieter, P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122**(1): 19-27.

Sjöstrand, J.O.O., Kegel, A., and Aström, S.U. 2002. Functional diversity of silencers in budding yeasts. *Eukaryotic Cell* **1**(4): 548-557.

Smith, J., Brachmann, C., Celic, I., Kenna, M., Muhammad, S., Starai, V., Avalos, J., Escalante-Semerena, J., Grubmeyer, C., Wolberger, C. et al. 2000. A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. *Proc Natl Acad Sci U S A* **97**(12): 6658-6663.

Smith, J.S. and Boeke, J.D. 1997. An unusual form of transcriptional silencing in yeast ribosomal DNA. *Genes Dev* **11**(2): 241-254.

Srikantha, T., Lachke, S.A., and Soll, D.R. 2003. Three mating type-like loci in Candida glabrata. *Eukaryotic Cell* **2**(2): 328-340.

Straight, A.F., Shou, W., Dowd, G.J., Turck, C.W., Deshaies, R.J., Johnson, A.D., and Moazed, D. 1999. Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity. *Cell* **97**(2): 245-256.

Sutton, A., Heller, R.C., Landry, J., Choy, J.S., Sirko, A., and Sternglanz, R. 2001. A novel form of transcriptional silencing by Sum1-1 requires Hst1 and the origin recognition complex. *Mol Cell Biol* **21**(10): 3514-3522.

Takahashi, T. 1958. Complementary Genes Controlling Homothallism in Saccharomyces. *Genetics* **43**(4): 705-714.

Tanny, J.C. and Moazed, D. 2001. Coupling of histone deacetylation to NAD breakdown by the yeast silencing protein Sir2: Evidence for acetyl transfer from substrate to an NAD breakdown product. *Proc Natl Acad Sci USA* **98**(2): 415-420.

Triolo, T. and Sternglanz, R. 1996. Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. *Nature* **381**(6579): 251-253.

Tsong, A.E., Miller, M.G., Raisner, R.M., and Johnson, A.D. 2003. Evolution of a combinatorial transcriptional circuit: a case study in yeasts. *Cell* **115**(4): 389-399.

Tsong, A.E., Tuch, B.B., Li, H., and Johnson, A.D. 2006. Evolution of alternative transcriptional circuits with identical logic. *Nature* **443**(7110): 415-420.

van Hoof, A. 2005. Conserved functions of yeast genes support the duplication, degeneration and complementation model for gene duplication. *Genetics* **171**(4): 1455-1461.

Vaquero, A., Scher, M.B., Lee, D.H., Sutton, A., Cheng, H.-L., Alt, F.W., Serrano, L., Sternglanz, R., and Reinberg, D. 2006. SirT2 is a histone deacetylase with preference for histone H4 Lys 16 during mitosis. *Genes Dev* **20**(10): 1256-1261.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I.S., and Moazed, D. 2004. RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**(5658): 672-676.

Volpe, T., Schramke, V., Hamilton, G.L., White, S.A., Teng, G., Martienssen, R.A., and Allshire, R.C. 2003. RNA interference is required for normal centromere function in fission yeast. *Chromosome Res* **11**(2): 137-146.

Wesolowski-Louvel, M. and Wolf, K. 1996. Kluyveromyces lactis. in *Nonconventional yeasts in Biotechnology: A Handbook*, pp. 139-201.

Wilson, J.M., Le, V.Q., Zimmerman, C., Marmorstein, R., and Pillus, L. 2006. Nuclear export modulates the cytoplasmic Sir2 homologue Hst2. *EMBO Rep* **7**(12): 1247-1251.

Wong, S., Butler, G., and Wolfe, K.H. 2002. Gene order evolution and paleopolyploidy in hemiascomycete yeasts. *Proc Natl Acad Sci USA* **99**(14): 9272-9277.

Wong, S., Fares, M.A., Zimmermann, W., Butler, G., and Wolfe, K.H. 2003. Evidence from comparative genomics for a complete sexual cycle in the 'asexual' pathogenic yeast Candida glabrata. *Genome Biol* **4**(2): R10.

Wong, S. and Wolfe, K. 2005. Birth of a metabolic gene cluster in yeast by adaptive gene relocation. *Nat Genet* **37**(7): 777-782.

Xie, J., Pierce, M., Gailus-Durner, V., Wagner, M., Winter, E., and Vershon, A.K. 1999. Sum1 and Hst1 repress middle sporulation-specific gene expression during mitosis in Saccharomyces cerevisiae. *EMBO J* **18**(22): 6448-6454.

Yang, B. and Kirchmaier, A.L. 2006. Bypassing the catalytic activity of SIR2 for SIR protein spreading in Saccharomyces cerevisiae. *Mol Biol Cell* **17**(12): 5287-5297.

Zhang, Z., Hayashi, M.K., Merkel, O., Stillman, B., and Xu, R.-M. 2002. Structure and function of the BAH-containing domain of Orc1p in epigenetic silencing. *EMBO J* **21**(17): 4600-4611.

Zill, O.A. and Rine, J. 2008. Interspecies variation reveals a conserved repressor of alpha-specific genes in Saccharomyces yeasts. *Genes Dev* **22**(12): 1704-1716.

Biography

Meleah Hickman was born on March 2, 1981 in Olympia, Washington, where she was raised. After graduating from Capitol High School in 1999, she attended South Puget Sound Community College, where she received her Associate of Arts degree in 2001. She went on to graduate with her Bachelors of Science and Bachelors of Arts degrees in 2003 from The Evergreen State College, where she studied biology.

In 2004, she began is graduate studies at Duke University in Durham, North Carolina with the Cell and Molecular Biology program. She went on to join the laboratory of Dr. Laura Rusche in the Department of Biochemistry and Institute for Genome Sciences and Policy, as well as the University Program in Genetics and Genomics. She has published multiple research articles and has been selected to present her work at several research conferences, including the Federation of American Societies for Experimental Biology, the Genetics Society of America Yeast Genetics and Molecular Biology and the European Molecular Biology Organization Workshop on the Evolutionary and Environmental Genomics of Yeasts.

PUBLICATIONS:

Hickman, M.A. and Rusche, L.N. (2009). The Sir-Sum1 complex represses transcription using both promoter-specific and long-range mechanisms to regulate cell identity and sexual cycle in the yeast *Kluyveromyces lactis*. *PLoS Genetics*, **5**(11):e1000710.

Hickman, M.A. and Rusche, L.N. (2007). Substitution as a mechanism for genetic robustness: The duplicated deacetylases Hst1 and Sir2 in *Saccharomyces cerevisiae*. *PLoS Genetics*, **3**(8):e126.

Hickman, M.A. and Rusche, L.N. (2007). "Evolution of Silencing at the Mating-Type Loci in Hemiascomycetes" J. Heitman, JW Taylor, JW Kronstad and LA Casselton (eds) Sex in Fungi: Molecular Determination and Evolutionary Implications. *ASM Press.*