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Evolutionary Genetic Studies of Mating Type and Silencing in *Saccharomyces*

by

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University of California, Berkeley

Committee in charge:

Professor Jasper Rine, Chair

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Abstract

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This thesis describes studies exploring the evolution of the genetic circuits regulating yeast mating-type and silencing by Sir (Silent Information Regulator) proteins in the budding yeast *Saccharomyces bayanus*, a close relative of the laboratory workhorse *S. cerevisiae* (a.k.a., budding yeast, or brewer's yeast). The two central subjects of these studies, mating type and silencing, are textbook examples of "well understood" mechanisms of eukaryotic gene regulation: the former serves as a model for understanding the genetic control of cell-type differentiation, the latter serves as a model for understanding physically condensed, transcriptionally repressed portions of the genome, often referred to as "heterochromatin". The two subjects are intimately connected in the biology of the budding yeast life cycle, as explained below, and I argue that a deeper appreciation of this connection is necessary for further progress in the study of either subject. My thesis brings a critical evolutionary perspective to certain assumptions underlying current knowledge of mating-type regulation and silencing—in short, an appreciation of organismal biology that has been marginalized in the pursuit of understanding molecular mechanisms. The value of this perspective is in attempting to understand the purpose of a biological process—*why* is there such a thing as silencing, and why does it require the particular proteins and DNA elements that it does? To ask what silencing does for a yeast cell, we can start by asking how the silencing mechanism is constrained over evolutionary time. One of the surprising findings of my thesis is how unconstrained some elements of the silencing machinery are during evolution.

At least three major findings arise from the comparative genetics studies described here: First, I describe the first new branch of the mating-type control circuit in almost 25 years. Although α-specific genes were previously thought to be "off" in *MAT***a** cells due to the absence of the α1 activator protein (i.e., by default), I show that these genes are, in fact, actively repressed by the Sum1 protein. This novel regulatory branch highlights the sophisticated control mechanisms necessary to coordinate the mating and mating-type switching processes. This finding has additional implications, including questioning the extent to which the "absence of activator" model is sufficient to explain the absence of a particular gene's expression; and that at least one subset of mating genes may be under environmental or metabolic regulation via the Sum1-associated NAD^+-dependent histone deacetylase Hst1.

Second, I show that at least two major genetic alterations to the Sir-based silencing machinery occurred in the recent ancestry of *S. cerevisiae* and its closest relative species. These changes reveal that our understanding of the silencing mechanism has been limited by the relative lack of comparative genetic sampling of the silencing process. That is, our understanding can improve via functional studies of silencing in close relatives of *S. cerevisiae* with variant silencing machinery, fueling new hypotheses about how silencing works. Although the identities of the major players (Sir1-4) largely remain the same, my discovery that certain silencing proteins are incompatible across closely related *Saccharomyces* species suggests evolutionary alterations in the genetic network of silencing—variation that could be tapped in future studies to understand better the way that silencing works. Of particular note are the rapid sequence evolution of *SIR4*, and the changes in copy number and sequence of *SIR1*, between *S. bayanus* and *S. cerevisiae*. *SIR4* and *SIR1* appear to rapidly evolve for interesting, though not completely overlapping, reasons. *SIR4* appears to be under diversifying selection in modern yeast populations, and its coding sequence evolves rapidly across two rather distant clades spanning the *Saccharomyces* complex—the *sensu stricto* clade, and the *Torulaspora* clade.

Third, I show that Sir4 and silencers are engaged in a remarkable pattern of co-evolution in *Saccharomyces* yeasts. I used a novel combination of classical genetic techniques in *S. cerevisiae/S. bayanus* hybrids to test *cis* versus *trans* contributions to a genetic incompatibility between *S. cerevisiae SIR4* and the *S. bayanus HMR* locus. Comparative ChIP-Seq of Sir4 in these hybrids helped identify the molecular basis for this incompatibility. Critically, I show that the *S. bayanus HMR* locus, when transferred into *S. cerevisiae*, can be silenced only by the specific combination of *S. bayanus* Sir4 and Kos3 proteins, with potential contributions by *S. bayanus* ORC and the other Sir1 paralogs. A striking asymmetry in cross-species compatibility of *S. bayanus* versus *S. cerevisiae SIR4* genes, and in each species' Sir4 ChIP-Seq profile, suggests that compensatory changes have occurred in *SIR4* and in silencers along the *S. cerevisiae* lineage. Although the initial evolutionary pressure(s) driving these rapid changes remains uncertain, my results point to some pressure driving either the silencers' or Sir4's rapid sequence change, with the other factor subsequently changing to maintain compatibility within a species. From a practical standpoint, these results suggest that molecular studies of silencing using only *S. cerevisiae* suffer from a previously unrecognized bias. That *S. bayanus* has four Sir1-like proteins, each important for silencing, suggests additional dimensions (i.e., temporal and/or spatial components) to the interactions occurring at silencers between Sir1, Sir4, ORC, and Rap1.

An interesting consequence of the comparative Sir4 ChIP-Seq experiments was the generation of a high-resolution picture of the architecture of silent chromatin in yeast. The unexpected non-uniform distributions of Sir4 protein across *HML* and *HMR* bring into question the standard "spreading" model for yeast silent chromatin formation, and will fuel future experiments to determine how Sir-based chromatin structures determine gene silencing and the epigenetic inheritance of gene expression states. I describe the novel ChIP-Seq picture of Sir protein association with silenced loci in Appendix A.

Finally, in addition to these specific biological insights, my comparative genetic studies provide guidelines for using the genetic variation between *S. bayanus* and *S. cerevisiae* as a tool to learn more about conserved genetic circuits and gene regulation mechanisms in general. Two substantial advances in evolutionary genetic techniques are presented in Chapters 3 and 4, which involve the use of yeast hybrids. First, I show that the genetic facility of *S. cerevisiae/S. bayanus* hybrids can be used to tease apart interspecies genetic variation of functional consequence that resides in *cis*-regulatory DNA elements from that in *trans*-acting transcriptional regulatory proteins. Second, in the case of silencing, the very act of re-introducing genetic factors that have been independently evolving for millions of years leads to unexpected, emergent phenotypes in the hybrids that can be used to understand the silencing mechanism itself. Lessons from my work should inform principles of comparative genetics using organisms closely related to classical "model organism" species such as *S. cerevisiae*.

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Acknowledgments

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Chapter 1

An Introduction to Comparative Genetics, Mating Type, and Silencing in Budding Yeast

This thesis describes the discoveries of novel dimensions to the regulation of mating-type and the evolution of Sir silencing in *Saccharomyces* (a.k.a., budding yeast). The genetic control of mating-type requires functional silencing due to the genetic underpinnings of the mating-type switching process that has evolved in budding yeast. Because understanding mating-type regulation and silencing requires an appreciation of certain intricacies of the budding yeast life cycle, I first introduce mating-type control in the context of the life cycle. I then introduce silencing and its connection to mating type.

The life cycle of S. cerevisiae and the genetic control of mating type

The budding yeast *S. cerevisiae* has a haplo-diploid life cycle (Herskowitz 1988). Haploid yeast cells, equivalent to gametes in plants and animals, come in two mating types, termed **a** and α. Cells of each mating type secrete a specific peptide pheromone that stimulates cells of the opposite mating type to initiate the mating process. The production and response to these pheromones requires the specific expression of seven "**a**-specific genes" in **a** cells and five "α-specific genes" in α cells (see Chapter 2). The proper expression of these genes is primarily determined by the mating-type (*MAT*) locus. In *S. cerevisaie*, haploid cells harbor in the middle of Chromosome III either a *MAT***a** allele, bearing only the **a**1 gene, or a *MAT*α allele, bearing the α1 and α2 genes. The genetic circuit controlling mating type in haploid cells is described at length in the introduction to Chapter 2. An **a** cell and an α cell will fuse to form an **a**/α diploid zygote, which is competent either to divide mitotically (in nutrient-rich conditions) or to sporulate (in the presence of poor nitrogen and non-fermentable carbon sources), regenerating haploid cells through meiosis. Competency to undergo sporulation is controlled by a heterodimer of the **a**1 and α2 transcription factors produced from the two *MAT* alleles (Rine et al. 1981). This heterodimeric repressor down-regulates the *RME1* gene (Mitchell and Herskowitz 1986), a master regulator at the head of the meiosis initiation cascade, and also down-regulates an anti-sense transcript that normally represses the *IME4* gene in haploids (Hongay et al. 2006).

The non-mating phenotype of diploid cells is also controlled by **a**1-α2 repression of haploid-specific genes. Notably, haploid cells that lack *SIR* gene function have a "pseudo-diploid" non-mating phenotype due to co-expression of **a**1 and α2 from the "**a**" and "α" alleles from *HML*, *MAT*, and *HMR*. (*HML* normally bears a silent copy of the "α" allele, while *HMR* normally bears a silent copy of the "**a**" allele.) Thus, Sir function effectively protects the ability of haploid cells to mate. It is presumed that mating provides a selective advantage for yeast cells in the wild through the genetic protections afforded by the diploid state, and the ability to make recombinant offspring through meiosis, although this has not been carefully tested.

Haploid *S. cerevisiae* cells are able to switch mating type as frequently as each cell division (Strathern and Herskowitz 1979), allowing yeast to regain rapidly the diploid state by mating of a mother cell with a daughter cell of opposite mating type. The genetic regulatory mechanisms involved in mating-type switching are among the most complex of those found in budding yeast (Haber 1998). The *HO* gene encodes an

endonuclease that binds to and cuts a specific sequence at the *MAT* locus, and the resulting DNA double-strand break is healed by non-reciprocal recombination between *MAT* and either the *HML* or *HMR* locus. (Note that *HO* is repressed in diploid cells by **a**1-α2 (Jensen et al. 1983).) Typically, a *MAT***a** allele will be gene converted to a *MAT*α allele via sequence copied from *HML*α, or vice versa via *HMR***a**. Switching achieves this directionality via at least two "donor preference" mechanisms that take advantage of the unique structure of chromosome III, on which *HML*, *MAT*, and *HMR* reside (Haber 1998). As the Ho cleavage site is present at both *HML* and *HMR*, Sir proteins serve the additional purpose of preventing Ho from cutting these loci. Such an inappropriate cut would be detrimental by eliminating via gene conversion either the "**a**" or the "α" allele from a normally silent locus. Notably, the Ho-directed mating-type switching mechanism is limited to a subset of *Saccharomyces* complex species, although the mating-type switching phenomenon is more widely distributed in Ascomycete fungi (Barsoum et al. ; Butler et al. 2004). Thus, many of the specific genetic mechanisms for regulating mating in *S. cerevisiae*, including *HO*, several mating-type specific genes, and the Sir1 and Sir4 proteins, have evolved recently within Hemiascomycete fungi. To a first approximation, however, all of the general life cycle features of *S. cerevisiae* are conserved across the *Saccharomyces sensu stricto* species (introduced below).

Transcriptional silencing by Sir proteins

Chromatin-based regional repression of gene expression can define cell identity and contribute to phenotypic diversity across all eukaryotes, yet it involves distinct mechanisms in different taxa (Grewal and Moazed 2003). For example, plants and animals use small RNAs and a conserved histone methylation machinery to silence transposons and repetitive elements near centromeres. In contrast, budding yeast silence their telomeres and cryptic mating-type loci, *HML* and *HMR*, using Sir proteins. These proteins can generate heritable states of transcription of certain loci and under certain conditions. Thanks to three decades of genetic and biochemical studies in *S. cerevisiae*, Sir silencing is now known to involve dozens of genes, including essential DNA replication proteins, and has been linked to cell-cycle regulation, and to centromere and telomere integrity (Rusche et al. 2003). Indeed, because of their tractability, budding yeasts provide an excellent opportunity to understand deeply the connection between epigenetic inheritance of gene expression (or position-effect variegation) and genome integrity. However, the molecular details of the yeast silencing mechanism have remained debatable in part because the sort of comparative analyses readily available to RNAi-and-HP-1-dependent heterochromatin researchers have been lacking. In this section, I summarize current knowledge of the silencing mechanism, and then describe the conditions under which Sir silencing can create epigenetic states of gene expression.

In *S. cerevisiae*, DNA elements termed "silencers" flank both *HML* and *HMR* and recruit the site-specific DNA-binding proteins ORC (the Origin Recognition Complex), Rap1, and Abf1 (Figure 1.1). Although it is assumed that all three DNA-binding proteins are continuously bound to silencers *in vivo*, ORC is differentially modified during the cell cycle, especially during S phase. Additionally, the Rap1 binding site differs substantially

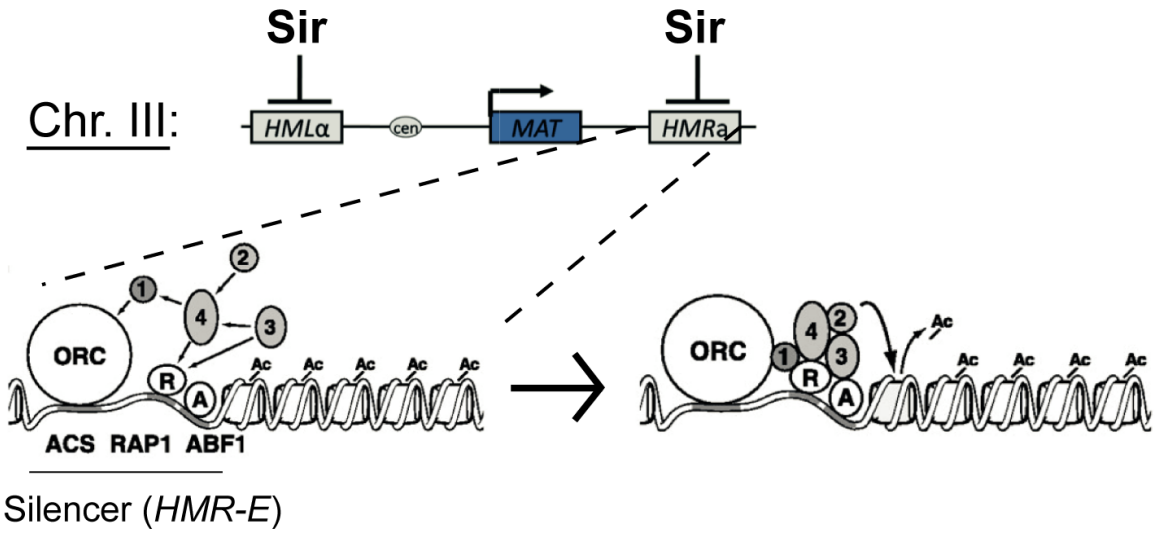

Figure 1.1. Silencing by Sir proteins in *S. cerevisiae*. A schematic of Chromosome III is shown at the top, with the three mating-type cassettes (*HML*, *MAT*, *HMR*) shown. *MAT* is the active locus that determines mating-type; Sir proteins repress transcription of *HML* and *HMR*. A zoomed-in view of the left half of the silent *HMR***a** locus (~1kb) is shown at the bottom. The process of silencing establishment is depicted, focusing on the initial assembly of the four Sir proteins (represented by numbers) and an initial nucleosome deacetylation catalyzed by Sir2. The *HMR-E* silencer is shown, with the Origin Recognition Complex (ORC), and the Rap1 (R) and Abf1 (A) transcription factors bound to a ~100bp stretch of DNA. Upon the assembly of Sir1-4 proteins at the silencers, Sir2/3/4 will "spread" inward through sequential deacetylation and binding events, as described in the text. (Adopted in part from Rusche et al. 2002).

from the Rap1 site consensus sequence, and may not be a high-affinity site. Notably, Rap1 has tandem DNA-binding domains, and thus the single site found in silencers likely binds only one of these domains, in contrast to telomeres (see below). Thus, the specific assemblage of these three DNA-binding proteins may vary somewhat across the cell cycle and may be affected by protein-protein interactions between themselves, or with Sir proteins. Indeed, recent work suggests that the recruitment of ORC to silencers depends at least partially on the presence of Sir1 and Sir2 (Ozaydin and Rine). Although truly cooperative binding to silencers has not been formally demonstrated, the initial nucleation of silencing complexes may involve multiple cooperative interactions between Sir1, ORC, Sir4, and Rap1, and potentially between Sir3 and Abf1.

ORC, Rap1, and Abf1 in turn recruit the four Sir proteins, Sir1-4, which were first identified by a mutational screen (Rine and Herskowitz 1987) (Figure 1.1). Sir2 is a highly conserved NAD^+-dependent histone deacetylase that binds tightly to Sir4 (Imai et al. 2000; Landry et al. 2000; Tanny and Moazed 2001; Hoppe et al. 2002). Sir3 contains a degenerate, catalytically inactive AAA-ATPase domain and an N-terminal BAH (bromo-adjacent homology) domain, and has been the subject of extensive biochemical and structural studies (Georgel et al. 2001; Connelly et al. 2006; Onishi et al. 2007). Sir3 binds to unmodified histone tails via its BAH domain, and to DNA but without sequence specificity (Hecht et al. 1995; Onishi et al. 2007). Sir3 is recruited to silencers via interactions with both Sir4 and Abf1 (Moazed et al. 1997; Rusche et al. 2003). Sir4 interacts with Sir2 and with several other proteins both *in vitro* and *in vivo*, and emerges as perhaps the central player in the order of events that establish silencing. Sir4 binds the site-specific silencer-binding protein Rap1, and to Sir1-ORC, and it interacts with Sir2 and Sir3, bringing them to silencers. Thus, Sir4 serves as a bridge between the "recruitment" proteins bound to DNA and the biochemical effectors of silencing. Additionally, Sir4 can bind unmodified histone tails to allow spreading of Sir2/Sir3/Sir4 complexes from silencers, which is thought to be an intermediate step in silencing establishment (Rusche et al. 2002; Liou et al. 2005). Despite its lynchpin role in silencing complexes, Sir4 has no well-defined features, other than a conserved ~100-amino acid coiled-coil region at the C-terminus (Chang et al. 2003), and a conserved 300-amino acid region just N-terminal to the coiled-coil, referred to as the PAD domain (Ansari and Gartenberg 1997; Taddei et al. 2004). Indeed, as described below, the rapid divergence of Sir4 may be pivotal in the evolution of silencing mechanisms. Finally, Sir1 is thought to help recruit a Sir2/Sir3/Sir4 complex to silencers via interactions with both ORC and Sir4 (Bose et al. 2004).

The current model for the formation of silent chromatin in *S. cerevisiae* is referred to as the "spreading" model (Hecht et al. 1995; Rusche et al. 2002). Upon the initial recruitment, or nucleation, of a Sir2/3/4 complex to a silencer, Sir2 deacetylates a neighboring nucleosome (Figure 1.1). The newly deacetylated histone tails bind another Sir2/3/4 complex by providing high-affinity binding sites for second molecules of Sir3 and Sir4. Another deacetylation by Sir2 then leads to another Sir2/3/4 binding event on the next nucleosome, and this cycle repeats until all the nucleosomes in a silenced region are deacetylated and bound by Sir2/3/4 complexes. Whether the most recent Sir protein binds to silencer-bound proteins and then "oozes" onto neighboring nucleosomes (analogous to how a nascent polypeptide chain grows during translation), or is added to a

growing end of a polymer of Sir2/3/4 proteins has not been resolved. Indeed, in Chapter 4 and Appendix A, I present ChIP-Seq data of Sir proteins that challenges the spreading model, and forces consideration of variant models, even beyond "spreading" and "oozing".

Telomeres are also subject to Sir-dependent position effects (Gottschling et al. 1990). Sir2/3/4 complexes are recruited to telomeres via DNA-bound arrays of the Rap1 protein, which binds both to Sir4 and to the telomeric terminal repeat sequence, $TG_{1\text{-}3}$ (Strahl-Bolsinger et al. 1997). Sir proteins can spread from the ends of telomeres inward, thereby repressing the expression neighboring genes. Over-expression of Sir3 leads to increased spreading and enhanced repression of telomere-linked reporter genes (Hecht et al. 1996). Notably, Sir1 is not thought to associate with telomere ends, although one study has found a role for Sir1 in silencing by a native telomere (Pryde and Louis 1999). Recently, evidence has emerged that suggests that additional subtelomeric elements are involved in recruitment or stabilization of Sir proteins to these regions (Lynch and Rusche ; Rusche and Lynch 2009; Sperling and Grunstein 2009). In Chapter 4, I provide evidence, via ChIP-Seq of Sir4, supporting a role for X elements in recruiting Sir proteins, and of the exclusion of Sir proteins by Y′ elements. These data also paint a substantially more nuanced picture of the architecture of silent chromatin at budding yeast telomeres than the simple "spreading-from-ends" model (Hecht et al. 1996).

The sensu stricto yeasts as a tool for comparative genetic and genomic analyses

The *sensu stricto* clade is composed of five sequenced species (Figure 1.2), and a sixth, *S. arboricolus*, has recently been isolated in China (Wang and Bai 2008). Two other species, *S. pastorianus* and *S. cariocanus*, arose recently via natural hybridizations between *S. cerevisiae* and *S. bayanus* (*S. pastorianus*), and between *S. cerevisiae* and *S. paradoxus* (*S. cariocanus*). *S. cerevisiae* and *S. paradoxus* are sister species, and share approximately 90% identity in coding regions and 80% identity in intergenic regions (Cliften et al. 2003; Kellis et al. 2003). *S. cerevisiae* and *S. bayanus* are the most diverged species in the *sensu stricto*, with 80% identity in coding regions and 62% identity in intergenic regions. Species in this clade are post-zygotically isolated from one another—haploid cells of one species can mate with haploids of another to form hybrid diploids that are mitotically stable (Greig 2009). Although the hybrid diploids will sporulate with high efficiency, the resulting spores are largely inviable (generally <1% viability). The mechanism of this reproductive barrier is not fully understood, but it appears not to be due to classic Dobzhansky-Muller genetic incompatibilities. Rather, it appears that the different species' homologous chromosomes may pair during meiosis I but fail to recombine properly, leading to random segregation, vast aneuploidy, and the subsequent inviability of hybrid spores.

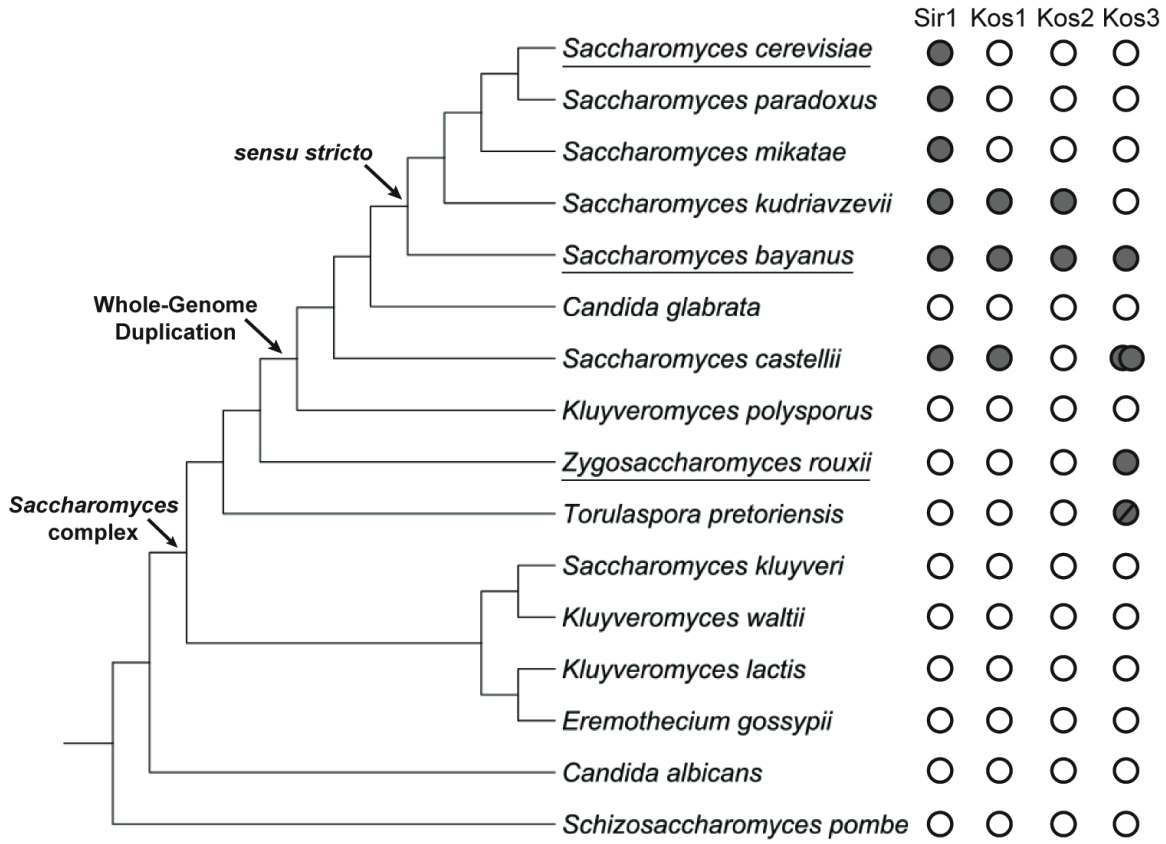

Figure 1.2. Cladogram of selected ascomycete yeasts. Cladogram is based on a Maximum-Likelihood tree derived from the following sequences: 18S, 5.8S/alignable ITS, and 26S rDNAs, EF-1K, mitochondrial small-subunit rDNA, and COX II (adopted from (Hedtke et al. 2006)). Arrows indicate the *Saccharomyces* complex of species, the *sensu stricto* clade, and the branch along which the whole-genome duplication occurred. Presence (gray shaded circle) or absence (open circle) of each Sir1 paralog is indicated for each species. Overlapping gray circles indicate the presence of two highly similar Kos3 paralogs in *S. castellii*, which also has a more divergent paralog called Kos4 (Gallagher, et al. 2009). (Note that Kos4 may in fact be a divergent ortholog of Kos1, as show in the figure.) Gray circle with diagonal line indicates that *T. pretoriensis* Kos3 appears to lack an OIR domain.

Two principal lines of evidence support this view. First, although diploid hybrids produce essentially no viable haploid spores through meiosis, tetraploid hybrids (2n from each species) produce diploid spores with >95% viability (Greig et al. 2002). These experiments used hybrids generated with *S. cerevisiae* and any of the other four *sensu stricto* species, with each tetraploid hybrid showing similarly high spore viability. Thus, it appears that dominant genetic incompatibilities do not interfere with meiosis in hybrids. Second, interspecies hybrids between *S. cerevisiae* and its sister species *S. paradoxus*, which normally have very low spore viability (~1%), show a 6-9-fold increase in spore viability when either of the Msh2 or Pms1 mismatch repair proteins is inactivated (Hunter et al. 1996). The interpretation of this result was that the interspecies homologous chromosomes, which are on average ~10% diverged, can pair and initiate recombination, but that the nucleotide mismatches are then recognized by the mismatch repair machinery, triggering an "anti-recombination" activity. Indeed, the hybrid diploids show 10-70-fold reductions in recombination frequency (Hunter et al. 1996). The failure to recombine successfully appears to lead to non-disjunction during meiosis I (observed as high levels of disomy in the rare viable spores), and thereby to subsequent spore aneuploidy and inviability. Spores generated from *msh2* mutant *S. cerevisiae/S. paradoxus* hybrids show a 3-fold decrease in disomy, suggesting that non-disjunction is decreased. Further, these hybrids show a 6-16-fold increase in recombination frequency (Hunter et al. 1996). A similar, but more modest, increase in spore viability is seen in *msh2* mutant hybrids made between divergent *S. cerevisiae* strains or between divergent *S. paradoxus* strains (Greig et al. 2003). These divergent strains are presumed to represent "incipient species" because the hybrid diploids show reduced spore viability (40-70%). Thus, successful meioses can occur in hybrids given homologous chromosomes of sufficient nucleotide identity to allow proper recombination and disjunction.

Chromosome translocations may also contribute to the post-zygotic isolation of *sensu stricto* species. *S. cerevisiae* diploid strains heterozygous for naturally or artificially generated translocations show approximately 2-fold reductions in spore viability per translocation (Delneri et al. 2003). In certain *S. cerevisiae/S. mikatae* hybrids, hybrid spore viability is increased 10-30% by artificially reversing one (out of three) natural translocation between these species, partially restoring co-linearity to the interspecies homologous chromosomes (Delneri et al. 2003). However, despite these substantial increases in spore viability, the resulting hybrid spores are vastly aneuploid with striking disomy patterns. Although it would seem unusual, it is possible that rampant chromosome amplification in these particular hybrids may have effectively suppressed non-disjunction independent of the engineered single "reverse translocation". It is perhaps noteworthy that certain *S. mikatae* strains appear to have somewhat unstable karyotypes (Naumov et al. 2000).

Large translocations do not exist between *S. cerevisiae* and *S. paradoxus* chromosomes, and the genetic isolation of these species is presumed to result from simple nucleotide divergence and the subsequent problems with meiotic recombination created by nucleotide mismatches. However, five translocations large enough to be detected by pulse-field gel electrophoresis exist between *S. cerevisiae* and *S. bayanus* (Fischer et al.

2000). Thus, both nucleotide divergence and chromosome rearrangements are presumed to contribute to the post-zygotic barrier separating these two species.

The evolution of silencing in Saccharomyces

Comparisons between *S. cerevisiae* and *S. bayanus* are particularly useful because the nucleotide divergence between these species approximates the divergence between human and mouse (Cliften et al. 2003; Kellis et al. 2003). Thus, comparative genetics in these species should allow calibration of the extent to which that level of divergence affects function. A particularly intriguing observation with regard to silencing is that *S. bayanus* harbors four paralogs of the *S. cerevisiae SIR1* gene in its genome. More extensive sequencing of yeast species outside of *Saccharomyces* revealed that the origin of *SIR1* in fact pre-dated the Whole-Genome Duplication (WGD), and that *SIR1*-related genes have duplicated and been lost independently multiple times (Gallagher et al. 2009). The ancestral *SIR1*-like gene has been dubbed *KOS3* (Kin of *SIR1*), and it differs notably from the other *SIR1* paralogs by having only a single C-terminal OIR (ORC-Interacting Region). Interestingly, *S. kudriavzevii* appears to have lost *KOS3*, and thus harbors only three *SIR1* paralogs. *S. mikatae*, *S. paradoxus*, and *S. cerevisiae* have lost *KOS1*, *KOS2*, and *KOS3*, leaving only the *SIR1* gene in these three species. This pattern of loss of *SIR1*-like genes suggests that some evolutionary forces have caused rapid change in *SIR1* gene number. As all four *SIR1* paralogs are important for silencing in *S. bayanus*, and *SIR1* expression has not dramatically increased in *S. cerevisiae* (Gallagher et al. 2009), some other mechanistic aspect of silencing must have changed during the evolution of *S. cerevisiae* such that only one *SIR1* gene is now doing the job of four. This notion forms the basis for the study in Chapter 3.

Another notable evolutionary feature of Sir silencing is that silenced regions are among the fastest evolving sequences in the genomes of *Saccharomyces* yeasts (Teytelman et al. 2008). Although the *HML* and *HMR* "cassettes" (defined as the coding regions, promoters, and flanking regions of homology needed for gene conversion into *MAT*) are highly conserved, several kilobases of flanking sequence, including the silencers, diverge rapidly between species. Additionally, silenced regions have a higher-than-average frequency of SNPs within wild *S. cerevisiae* and *S. paradoxus* populations, suggesting that DNA repair mechanisms may be somewhat inhibited in these regions, perhaps due to occlusion by Sir proteins (Teytelman et al. 2008). However, analyses that account for the considerable, geographically defined population structure of *S. paradoxus* (Liti et al. 2009) suggest that silencer sequences may undergo periodic bursts of rapid evolution, but are otherwise highly conserved within this species (O. Zill, unpublished observations). In a notable parallel, the sequences of heterochromatin in flies and subtelomeric DNA in humans are also found to evolve rapidly (Linardopoulou et al. 2005; Diaz-Castillo and Golic 2007).

Although the Rap1 and Abf1 binding sites in silencers are very highly conserved between *Saccharomyces* species, all of the surrounding sequence is as different as can be (Teytelman et al. 2008). Although the ORC-binding sites appear not to be conserved, the ORC-binding site consensus (a.k.a., ARS consensus sequence, or ACS) is quite

degenerate in *S. cerevisiae*. Thus, other species' silencers may indeed contain functional ORC sites, which are not readily detected in alignments due to this degeneracy. In Chapter 4, I show that the rapidly evolving silencers are in fact co-evolving with the Sir1 and Sir4 proteins.

Sum1 and its connection to silencing

The *S. cerevisiae* gene *SUM1* was first defined genetically by the *SUM1-1* (Suppressor of *mar1-1*) mutation that restored silencing to a *sir2* (*mar1*) mutant (Klar et al. 1985). It was subsequently shown that the *SUM1-1* mutation was dominant (Laurenson and Rine 1991) and capable of restoring silencing to cells lacking all four *SIR* genes (Rusche and Rine 2001). The Sum1-1 protein contains a T988I mutation, which re-distributes Sum1 protein from multiple loci, where it normally acts as a gene-specific repressor, to the *HMR* locus, where it assembles into silent chromatin in a "spreading" fashion. It appears that the Sum1-1 mutant protein has an increased ability to interact with ORC relative to the native Sum1 protein (Sutton et al. 2001; Irlbacher et al. 2005). Sum1-1 can silence a *TRP1* gene integrated at the *HMR* locus, suggesting that it is capable of Sir-like regional repression (Laurenson and Rine 1991). However, by the phenotypic criterion of α-factor resistance, Sum1-1-mediated silencing appears weaker than Sir-mediated silencing (Valenzuela et al. 2006). Potential complications with interpreting these experiments are discussed in Chapter 2. Native Sum1 may also be involved in silencing of *HML* (Irlbacher et al. 2005), but there is some disagreement on this point.

Sum1 normally represses the transcription of several dozen genes, including many mid-sporulation genes, NAD^+ salvage pathway genes (e.g., *BNA* genes), and genes necessary for the utilization of alternative nitrogren sources (e.g., *DAL* genes) (Pierce et al. 2003). Sum1 interacts with a closely related paralog of Sir2, called Hst1, which deacetylates histones to repress transcription. Sum1 and Hst1 form a trimeric complex with Rfm1, which bridges the Sum1-Hst1 interaction (McCord et al. 2003). Both Hst1 and Rfm1 are important for repression of *HMR* by Sum1-1 (Sutton et al. 2001; Lynch et al. 2005). Although the *SUM1* gene arose prior to the Whole-Genome Duplication (Figure 1.2), *HST1* arose as a paralog of *SIR2* during this genomic duplication event. Indeed, in *S. cerevisiae*, Sir2 appears capable of "filling in" for Hst1 in the repression of some, but not all, genes repressed by Sum1 (Hickman and Rusche 2007b; Mead et al. 2007). However, Hst1 has little ability to substitute for Sir2 for silencing the *HML* and *HMR* loci.

Interestingly, *SUM1* is found only within the *Saccharomyces* complex, as are *SIR1* and *SIR4*. In *K. lactis*, a species whose origin preceded the WGD and which lacks Sir3 and Hst1, Sum1 and Sir2 repress both the *HML* and *HMR* loci and appear capable of spreading across them in analogous fashion to Sir proteins in *S. cerevisiae* (Hickman and Rusche 2009). *K. lactis* Sir4, in contrast, is important for silencing *HML* alongside Sum1, but appears to have only a limited role in silencing *HMR*. From these observations, a picture emerges of ancestral shared roles for Sum1 in silencing and gene-specific repression of multiple targets, which were subsequently partitioned into separate

roles played by Sir2/3/4 and Sum1-Hst1 in *S. cerevisiae* (Hickman and Rusche 2009). In an evolutionary genetic sense, then, the *S. cerevisiae SUM1-1* mutation appears to represent a "re-functionalization" mutation, rather than its previous and somewhat mysterious "neo-functionalization" categorization.

An early surprise from comparative genetics in Saccharomyces

In my early work in the Rine lab, I found that *S. bayanus sir3Δ* mutants retain a surprising level of silencing at *HMR* (O. Zill, unpublished observations), given the absolute requirement for *SIR3* in *S. cerevisiae HMR* silencing. Indeed, the presence of four *SIR1* paralogs in *S. bayanus* had suggested that silencing might operate differently in this species. It was possible that *S. bayanus* Sum1 retained some ancestral *HMR*-silencing activity (a la *K. lactis* Sum1), allowing for the observed "Sir3-independent silencing". Thus, I decided to investigate whether native Sum1 was involved in silencing in *S. bayanus*. Although that question remains unresolved, I found that Sum1 plays a surprising role in mating-type regulation in both *S. bayanus* and *S. cerevisiae*, a finding which is described in Chapter 2.

Chapter 2

Interspecies Variation Reveals a Conserved Repressor of α-Specific Genes in *Saccharomyces* Yeasts

Abstract

The mating type determination circuit in *Saccharomyces* yeast serves as a classic paradigm for the genetic control of cell type in all eukaryotes. Using comparative genetics, we discovered a central and conserved, yet previously undetected, component of this genetic circuit: active repression of α-specific genes in **a** cells. Upon inactivation of the *SUM1* gene in *S. bayanus*, a close relative of *S. cerevisiae*, **a** cells acquired mating characteristics of α cells and displayed autocrine activation of their mating-response pathway. Sum1 protein bound to the promoters of α-specific genes, repressing their transcription. In contrast to the standard model, α1 was important but not required for α-specific gene activation and mating of α cells in the absence of Sum1. Neither Sum1 protein expression, nor its association with target promoters was mating-type regulated. Thus, the α1/Mcm1 co-activators did not overcome repression by occluding Sum1 binding to DNA. Surprisingly, the mating-type regulatory function of Sum1 was conserved in *S. cerevisiae*. We suggest that a comprehensive understanding of some genetic pathways may be best attained through the expanded phenotypic space provided by study of those pathways in multiple related organisms.

Introduction

Owing to three decades of intensive genetic, molecular, and biochemical analysis, the genetic circuit responsible for determining mating type in S. cerevisiae stands as the most thoroughly characterized cell-type-regulatory pathway in eukaryotes. The two haploid yeast mating types, **a** and α, are distinguished by their ability to mate with each other to form the third cell type, the **a**/α diploid. Two sets of genes, the **a**-specific genes and the α-specific genes, are differentially transcribed in **a** cells and α cells, respectively, to determine these distinct mating phenotypes.

The mating-type-specific patterns of gene expression are dictated by the allele present at the mating type (*MAT*) locus. The *MAT*α allele encodes two transcription factors: α1, which activates α-specific genes, and α2, which represses a-specific genes. The *MAT*a allele encodes only the a1 protein, which forms a heterodimer with α2 in *MAT*a/α diploids to repress a third set of genes, the haploid-specific genes. In the standard model, known as the α1-α2 hypothesis (Strathern et al. 1981), expression of a-specific genes in a cells is a default state resulting merely from the absence of the α1 and α2 proteins. Two transcription factors common to both a and α cells, Ste12 and Mcm1, are also necessary for proper expression of mating-type genes. Ste12 works at two regulatory levels in both mating types to activate transcription: it is required for basal transcription of a-specific and α-specific genes in the absence of the mating pheromones, a-factor and α-factor, and for their further induction in response to pheromones (Fields and Herskowitz 1985; Kirkman-Correia et al. 1993). Mcm1, a MADS-box transcription factor similar to mammalian Serum Response Factor, is required for the activation of α-specific genes and for both the activation and the repression of a-specific genes (Jarvis et al. 1989; Elble and Tye 1991; Hwang-Shum et al. 1991; Bruhn and Sprague 1994). In α cells, Mcm1 homodimers interact directly with α1 to activate α-specific genes; similarly, Mcm1 homodimers interact with α2 to repress a-specific genes (Smith and Johnson 1992). Ste12 and Mcm1 themselves interact to activate transcription, forming complexes with α1 at α-specific gene promoters, or acting on their own at a-specific gene promoters in a cells (Figure 2.1) (Sengupta and Cochran 1990; Yuan et al. 1993; Bruhn and Sprague 1994).

Together, Mcm1 and Ste12 can activate **a**-specific genes, yet they require α1 to activate α-specific genes. This additional co-activator requirement has been explained solely by differential activator-DNA affinities encoded in distinct classes of Mcm1 binding sites. Whereas the highly conserved, palindromic sequence found in **a**-specific gene promoters, known as the *P* element, binds Mcm1 tightly, the related sequence known as the *P′Q* element, which confers α cell specificity, binds Mcm1 with reduced affinity. Thus, the standard model holds that cooperative DNA binding by α1 and Mcm1 is necessary for α-specific gene expression. Despite extensive mutational and *in vitro* binding analyses of *P′Q* elements, no evidence for repression of α-specific gene expression has emerged (Bender and Sprague 1987; Flessel et al. 1989; Ganter et al. 1993; Hagen et al. 1993).

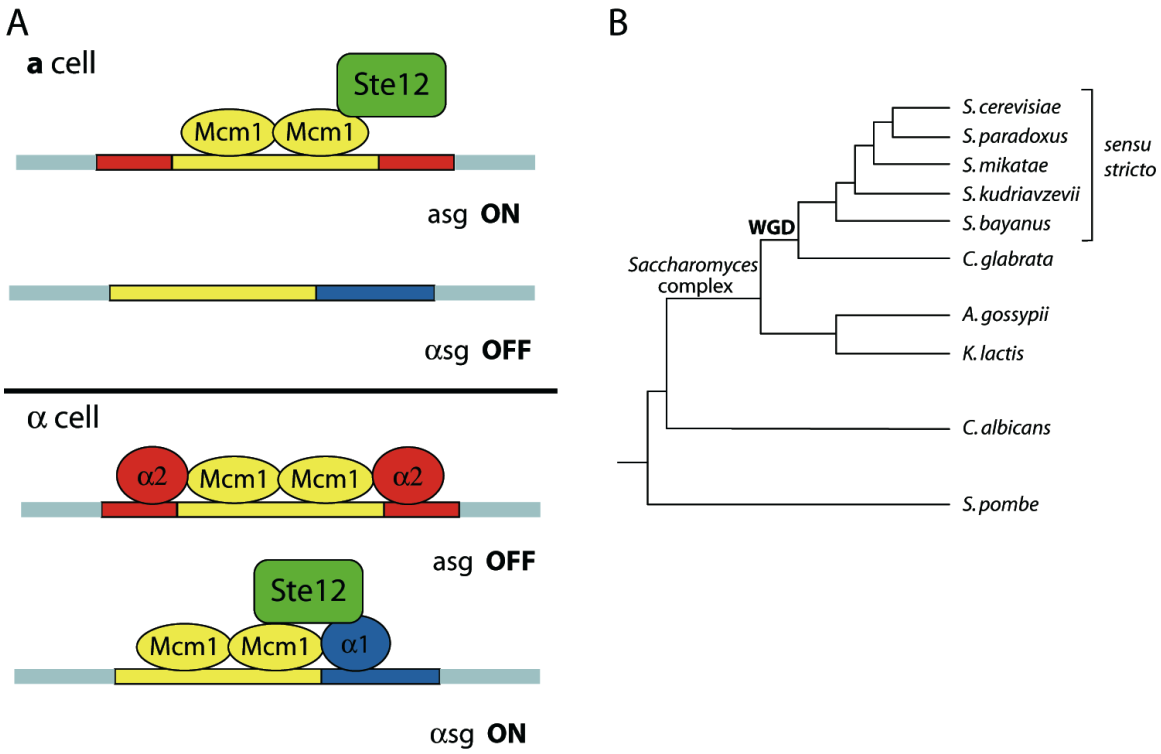

Figure 2.1. Mating-type-specific gene control and cladogram of select yeast species. (A) The standard model for the transcriptional control of haploid mating type genes in *S. cerevisiae*. The operator elements, contained within **a**-specific gene (**a**sg) and α-specific gene (αsg) promoters, are schematized as colored boxes, which correspond to binding sites for α1 (blue), α2 (red), and Mcm1 (yellow). (B) Simplified cladogram, based on concatenated sequences of 153 genes present in all species shown, depicts the *sensu stricto* yeasts and some related yeast species (branch lengths are not accurate). "WGD" denotes the whole genome duplication that occurred along the branch leading to *C. glabrata* and the *Saccharomyces* species. Modified from (Fitzpatrick et al. 2006).

Recently, the full force of modern genomics analysis has been brought to bear on the yeast mating type determination circuit, including microarray analyses of mating-type-specific gene expression, genome-wide location assays of mating-type regulatory proteins, and computational analysis of DNA regulatory sites (Zeitlinger et al. 2003; Galgoczy et al. 2004). These studies revealed additional mating-type regulated genes and a novel mating-type regulated dimension to osmolarity tolerance, but no substantial alterations were made to the mating-type regulatory circuit itself. However, these efforts, and similar studies in other organisms, are potentially limited by the amount of phenotypic information accessible within a single species.

Interspecies genetic analysis may allow deeper probing into gene circuits because of mutations that strengthen or weaken known genetic interactions, or that bring about novel interactions. Other comparative studies have attempted to understand how genetic variation between species creates phenotypic variation. For example, the varying shapes and sizes of beaks in Galapagos finches can be explained by changes in BMP4 expression during development (Abzhanov et al. 2004). Here, we attempt to harness the natural variation present in related species to identify components of gene regulatory pathways that have eluded phenotypic detection in single-species analyses. Indeed, the distinction between these goals can sometimes blur: this study was inspired by differences in phenotype caused by the same mutation in different species, yet led to discovery of a common regulatory component of both species.

The power of comparative genetic analysis is greatly expanded in taxa in which the genomes of multiple related species have been sequenced (Stark et al. 2007). The *sensu stricto* yeasts comprise several closely-related *Saccharomyces* species, five of which have published genome sequences, that afford an excellent opportunity for studying gene regulatory pathways and their evolution (Cliften et al. 2003; Kellis et al. 2003). *S. bayanus* is the most evolutionarily distant of the *sensu stricto* yeasts from *S. cerevisiae* (Figure 2.1). Thus, comparisons between these two species are useful for making inferences about the *sensu stricto* ancestral state. The nucleotide substitution level between *S. bayanus* and *S. cerevisiae* is approximately equivalent to that between mouse and human (Kellis et al. 2003), offering a calibration for how that amount of variation affects function.

Analysis of the *S. bayanus* genome sequence suggested that transcriptional repression by the Silent Information Regulator (Sir) proteins had changed in that lineage (Kellis et al. 2003); (J. Babiarz and L. Teytelman, unpublished observations). While examining transcriptional silencing in *S. bayanus*, we discovered an unexpected species-specific and mating-type-specific colony-wrinkling phenotype in a cells lacking the *SUM1* gene. *SUM1* encodes a transcriptional repressor of several dozen genes, many of which are expressed only during meiosis. Similar to silencing by the Sir proteins, Sum1 uses a NAD^+-dependent histone deacetylase, called Hst1, to effect transcriptional repression. Sum1 recruits Hst1 via a protein called Rfm1, with these three proteins constituting a transcriptional repressor complex (McCord et al. 2003). A gain-of-function allele, *SUM1-1*, can restore silencing to the *HMR*a mating type locus in *sir* mutants (Klar et al. 1985; Laurenson and Rine 1991; Rusche and Rine 2001). The fortuitous phenotype of *S. bayanus sum1Δ* mutants suggested either that some dimension

of mating type control had changed in *S. bayanus*, or that a conserved regulatory mechanism had been missed in studies of in *S. cerevisiae*. The results presented here revealed the existence of a conserved repression mechanism operating on α-specific genes in a cells of both species, uncovering the first new central component of the mating type circuit in 20 years.

Materials and Methods

Yeast strains, culture, and genetic manipulations. Yeast strains are listed in Table 2.1. Heterothallic *S. bayanus* wild-type strains were derived from CBS 7001, obtained from Ed Louis. The *HO* gene was inactivated in this diploid strain and haploid *MAT***a** and *MAT*α *ho*Δ strains were isolated by sporulation and tetrad dissection of the *HO/ho*Δ heterozygotes. Auxotrophic markers *ade2-1* and *his3-1* were generated in haploid prototrophic strains by EMS mutagenesis followed by screening for colonies unable to grow on minimal media. For *lys2-5* and *ura3-1*, mutagenized haploids were plated on α-aminoadipate and 5-FOA media, respectively, to select for resistant mutants. Strains JRY8729 and JRY8730 were generated by sequential crosses of individual auxotrophic mutants and dissecting tetrads for multiple-marker mutants. All growth of *S. bayanus* was performed using standard conditions for *S. cerevisiae*, except that plate and liquid culturing was performed at 25°C for both species. *S. cerevisiae* wild-type strain W303-1a has been described previously (Thomas and Rothstein 1989). The *MAT***a** Σ1278b strain, JRY6896, was originally designated 10560-4A (G. Fink) and was obtained from Jeremy Thorner (JTY2560). One-step gene replacement, C-terminal 13xMyc tag integration (Longtine et al. 1998; Goldstein and McCusker 1999), and C-terminal 3xFLAG tag integration (Gelbart et al. 2001) have been described previously, and these genetic manipulations were performed identically for both *S. bayanus* and *S. cerevisiae*. All gene disruptions in both species (*sum1*Δ*::Hyg*, etc.) were confirmed using PCR to examine the 5′ and 3′ ends of targeted open reading frames. To construct strain JRY8736, the *GAL1* promoter (Longtine et al. 1998) was integrated by homologous recombination immediately upstream of the ATG of the *MF*α*1* gene, removing 50bp of the native promoter.

Microscopy and mating assays. DIC microscopy was performed using a Nikkon Eclipse E600 microscope (100x objective). Cells were gently dispersed in complete synthetic media and spotted onto 2% agarose pads mounted on slides. Multiple fields were observed to score cellular morphology. Patch mating assays were performed by mixing approximately equal amounts of query strain and tester strain, each obtained from individual colonies, on YPD (glucose) or YPG (galactose) and incubating overnight. The following day, patches were replica plated onto minimal media to select for diploids. Patch mating assays shown in Figure 2.5 (galactose induction of *MAT***a** wild-type and *GAL1pro-MF*α*1* strains) were performed on rich medium containing 2% galactose as the carbon source before replica plating to minimal media containing 2% glucose and no galactose. The tester strains used in all mating assays were JRY2726 (*MAT***a**) and JRY2728 (*MAT*α), both of which are *S. cerevisiae his4*. All *S. bayanus* strains described in the text mated equivalently with these tester strains and with *S. bayanus* tester strains.

Table 2.1. Yeast strains used in Chapter 2.

Strain	Species	Genotype	Source
JRY8729	*S. bayanus*	*MAT***a** *hoΔ::Nat ade2-2 his3-1 lys2-5*	This study
JRY8730	*S. bayanus*	*MATα hoΔ::Nat ade2-2 his3-1 lys2-5*	This study
JRY8731	*S. bayanus*	*MAT***a** *sum1Δ::Hyg ade2-2 his3-1 lys2-5*	This study
JRY8732	*S. bayanus*	*MATα sum1Δ::Hyg ade2-2 his3-1 lys2-5*	This study
JRY8600	*S. bayanus*	*MAT***a** *hst1Δ::Kan ade2-2 his3-1 lys2-5*	This study
JRY8762	*S. bayanus*	*MAT***a** *rfm1Δ::Kan ade2-2 his3-1 lys2-5*	This study
JRY8763	*S. bayanus*	*MAT***a** *mfα1Δ::Kan ade2-2 lys2-5 ura3-1*	This study
JRY8764	*S. bayanus*	*MAT***a** *ste2Δ::Kan ade2-2 his3-1 lys2-5*	This study
JRY8765	*S. bayanus*	*MAT***a** *ste12Δ::Kan ade2-2 his3-1 lys2-5*	This study
JRY8733	*S. bayanus*	*MAT***a** *sum1Δ::Hyg ste2Δ::Kan ade2-2 his3-1 lys2-5*	This study
JRY8734	*S. bayanus*	*MAT***a** *sum1Δ::Hyg mfα1Δ::Kan ade2-2 his3-1 lys2-5*	This study
JRY8735	*S. bayanus*	*MAT***a** *sum1Δ::Hyg ste12Δ::Kan ade2-2*	This study
JRY7881	*S. bayanus*	*MAT***a** *ura3-1*	This study
JRY8736	*S. bayanus*	*MAT***a** *Kan::GAL1pro-MFα1 ura3-1*	This study
JRY8737	*S. bayanus*	*MAT***a** *hmlΔ::HIS3 sum1Δ::Hyg his3-1 lys2-5 trp ura3-1*	This study
JRY8738	*S. bayanus*	*MAT***a** *SUM1-3xFLAG::Kan ade2-2 his3-1 lys2-5*	This study
JRY8739	*S. bayanus*	*MATα SUM1-3xFLAG::Kan ade2-2 his3-1 lys2-5*	This study
JRY8740	*S. bayanus*	*MAT***a**/α *SUM1/SUM1-3xFLAG::Kan*	This study
JRY8741	*S. bayanus*	*MAT***a**/α *SUM1-3xFLAG::Kan/SUM1-3xFLAG::Kan*	This study
JRY8742	*S. bayanus*	*matα1Δ::Kan ade2-2 his3-1 lys2-5*	This study
JRY8743	*S. bayanus*	*matα1Δ::Kan sum1Δ::Hyg ade2-2 his3-1 lys2-5*	This study
JRY8766	*S. bayanus*	*matα1Δ::Kan sum1Δ::Hyg hmlΔ::HIS3 ade2-2 his3-1 lys2-5*	This study
JRY8744	*S. bayanus*	*MAT***a** *mcm1Δ::Kan ade2-1 his3-1*	This study
JRY8745	*S. bayanus*	*MATα mcm1Δ::Kan ade2-1 his3-1*	This study
JRY8746	*S. bayanus*	*MAT***a** *mcm1Δ::Kan sum1Δ::Hyg his3-1*	This study
JRY8747	*S. bayanus*	*MATα mcm1Δ::Kan sum1Δ::Hyg his3-1*	This study
JRY8767	*S. bayanus*	*MAT***a** *MCM1-13xMYC::Kan ade2-2 his3-1 lys2-5*	This study
JRY8768	*S. bayanus*	*MATα MCM1-13xMYC::Kan ade2-2 his3-1 lys2-5*	This study
JRY8769	*S. bayanus*	*MAT***a** *sum1Δ::Hyg MCM1-13xMYC::Kan ade2-2 his3-1 lys2-5*	This study
JRY8770	*S. bayanus*	*MATα sum1Δ::Hyg MCM1-13xMYC::Kan ade2-2 his3-1 lys2-5*	This study

JRY2334	*S. cerevisiae* (W303)	*MAT***a** *ade2-1 his3-11 leu2-3, 112 trp1-1 ura3-52 can1-100*	R. Rothstein
JRY3009	*S. cerevisiae* (W303)	*MAT*α *ade2-1 his3-11 leu2-3, 112 trp1-1 ura3-52 can1-100*	R. Rothstein
JRY8748	*S. cerevisiae* (W303)	*MAT***a** *sum1Δ::Kan*	This study
JRY8749	*S. cerevisiae* (W303)	*MAT*α *sum1Δ::Kan*	This study
JRY8750	*S. cerevisiae* (W303)	*MAT***a** *bar1Δ::Hyg*	This study
JRY8751	*S. cerevisiae* (W303)	*MAT***a** *sum1Δ::Kan bar1Δ::Hyg*	This study
JRY8752	*S. cerevisiae* (W303)	*mat*α*1Δ::Kan*	This study
JRY8753	*S. cerevisiae* (W303)	*mat*α*1Δ::Kan sum1Δ::LEU2*	This study
JRY8771	*S. cerevisiae* (W303)	*mat*α*1Δ::Kan sum1Δ::LEU2 hmlΔ::Hyg*	This study
JRY6896	*S. cerevisiae* (Σ1278b)	*MAT***a** *his3Δ::hisG leu2Δ::hisG trp1Δ::hisG ura3-52*	J. Thorner
JRY8754	*S. cerevisiae*	*MAT***a** *sum1Δ::Kan*	This study
JRY8755	*S. cerevisiae*	*MAT***a** *bar1Δ::Hyg*	This study
JRY8756	*S. cerevisiae*	*MAT***a** *sum1Δ::Kan bar1Δ::Hyg*	This study
JRY2726	*S. cerevisiae*	*MAT***a** *his4*	D. Botstein
JRY2728	*S. cerevisiae*	*MAT*α *his4*	D. Botstein

RNA and protein analysis. RNA isolation was performed using the hot-phenol method (Schmitt et al. 1990). Total RNA was digested with Amplification grade DNase I (Invitrogen) and purified using the RNeasy Minelute kit (Qiagen). cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR and oligo(dT) primer (Invitrogen). Quantitative PCR on cDNA was performed using an MX3000P machine (Stratagene) and the DyNAmo HS SYBR Green qPCR kit (NEB). Amplification values for all primer sets were normalized to actin (*ACT1*) cDNA amplification values. Samples were analyzed in triplicate for two or three independent RNA preparations. For Figure 2.9 (C and D) analysis, duplicate *mcm1Δ*, *sum1Δ mcm1Δ*, *ste2Δ*, *ste12Δ*, *sum1Δ ste2Δ*, and *sum1Δ ste12Δ* samples were processed in parallel to single wild-type and *sum1Δ* control samples from identical strains to those analyzed in Figure 2.4.

Yeast whole cell extracts were prepared using 20% TCA and solubilization in SDS loading buffer plus 100mM Tris base. SDS-PAGE and immunoblotting were performed using standard procedures and the LiCOR imaging system. Anti-FLAG M2 antibody from mouse (Sigma) was used to detect FLAG-tagged Sum1 protein. Rabbit anti-Pgk1 antibody (Baum et al. 1978), a kind gift of Jeremy Thorner, was used to verify equal loading.

ChIP analysis. Chromatin immunoprecipitations were performed as described (Davies et al. 2005) using formaldehyde cross-linking of log phase cultures for one hour at room temperature. IPs were performed using Anti-FLAG M2-Agarose from mouse and Anti-c-Myc Agarose from rabbit (Sigma). Quantitative PCR was performed on precipitated DNA fragments as described above. The "Reference" primer set, which amplifies a region within the *SEN1* ORF, corresponded to a locus with a minimal level of Sum1 binding in the *S. cerevisiae* genome. Amplification values for the precipitated DNA were normalized to the values for the input DNA for all primer sets. Samples were analyzed in triplicate for three independent ChIPs and one no-tag control for each mating type.

Binding site predictions. To detect putative Sum1 binding sites, 1kb of sequence 5′ of the ATG of each α-specific gene and pre-defined consensus binding sites (described in the text) were submitted to the SCPD database (M. Zhang lab, Cold Spring Harbor Labs; http://rulai.cshl.edu/SCPD/). To account for site degeneracy, sequential consensus searches that permitted zero or one mismatch were performed.

Results

*S. bayanus MAT***a** *sum1Δ had mating-type-specific and species-specific phenotypes*

Upon targeted inactivation of the *SUM1* gene in *S. bayanus*, we observed a dramatic mating-type-specific change in colony morphology. *MAT***a** *sum1Δ* colonies were distinctly wrinkled, whereas *MAT*α *sum1Δ* colonies were smooth and identical to wild type (Figure 2.2; see Figure 2.5 for wild-type comparison). Microscopic inspection

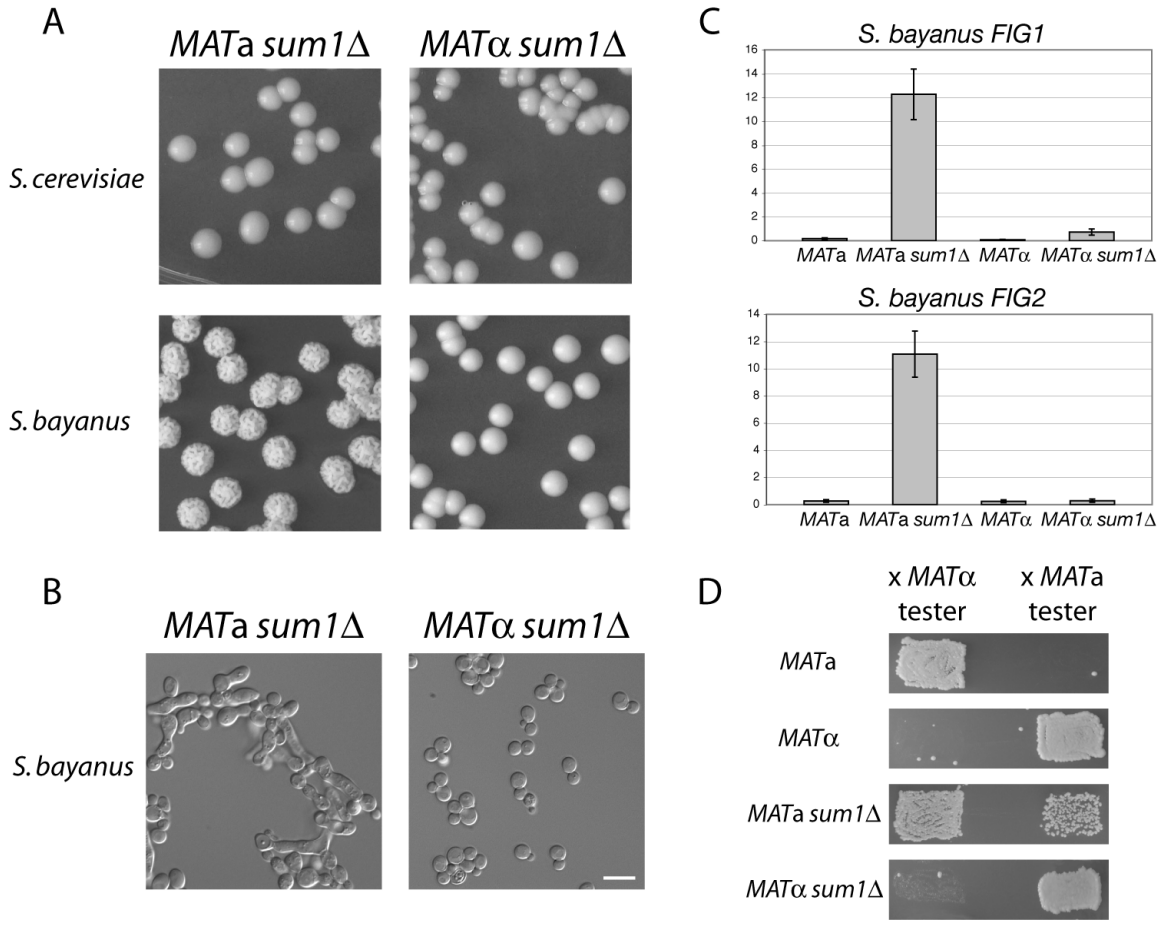

Figure 2.2. Species-specific and *MAT*a-specific phenotypes in *sum1Δ* mutants. (A) *S. bayanus MAT***a** *sum1Δ* and *MATα sum1Δ* colonies grown for five days on YPD (bottom panels; for wild-type comparison see Fig. 4B). *S. cerevisiae sum1Δ* colonies (W303 background) of both mating types grown for five days on YPD (top panels). (B) DIC microscopy of *S. bayanus sum1Δ* cells from colonies grown on YPD. Scale bar indicates 10μm. (C) Quantitative RT-PCR analysis of the *S. bayanus* mating pheromone-induced genes *FIG1* and *FIG2* in wild-type and *sum1Δ* cells of both mating types. (D) Patch mating assays of *S. bayanus* wild-type and *sum1Δ* cells of both mating types. Growth of a patch is approximately proportional to the mating ability of the strain indicated to the left of the picture. Note that a subset of *MAT***a** *sum1Δ* cells mated with **a** cells, while most cells mated with α cells.

of *MAT***a** *sum1Δ* cells revealed that some cells had elongated cell morphology reminiscent of the shmoon (plural of shmoo) formed in response to mating pheromone (Figure 2.2). The mutant cells also formed lightly flocculent clumps in liquid culture (indicated by their faster sedimentation than wild type), suggesting that up-regulation of adhesion molecules in *sum1Δ* mutants altered colony morphology (Figure 2.3). These phenotypes were especially intriguing given that *S. cerevisiae sum1Δ* mutants of both mating types have no discernable cell or colony phenotypes (Figure 2.2) and their mating behavior is identical to wild type (Klar et al. 1985; Laurenson and Rine 1991). Given the shmoon-like cells, we asked whether the mating response in *S. bayanus MAT***a** *sum1Δ* cells was activated even in the absence of a mating partner. Indeed, the pheromone-induced genes *FIG1* and *FIG2* were highly up-regulated in *MAT***a** *sum1Δ* cells relative to wild type (Figure 2.2).

Mating tests on *sum1Δ* mutants of both mating types revealed that the activated mating response indeed reflected a change in mating behavior. A subset of *MAT***a** *sum1Δ* mutant cells mated with the *MAT***a** tester strain, with more cells retaining the ability to mate with the *MAT*α strain (Figure 2.2). This bi-mating ability of the population suggested that the expression of mating genes normally expressed only in α cells was not properly regulated. This prediction was born out in quantitative RT-PCR analysis: the expression of *MF*α*1* and *MF*α*2*, the two genes encoding α-factor, and *STE3*, the **a**-factor receptor gene, were increased 90-fold, 10-fold, and 30-fold, respectively, in *MAT***a** *sum1Δ* cells compared with wild-type (Figure 2.4). The expression of *YLR040c*, an α-specific gene of unknown function, also was increased by 3-fold. However, expression of *SAG1*, which encodes the α-specific agglutinin involved in cell-cell adhesion during mating, was unaffected in mutant **a** cells but decreased 3-fold in *MAT*α *sum1Δ* mutants relative to wild-type α cells (Figure 2.4; discussed further below).

Two other features of α-specific gene expression were noteworthy. First, for *STE3* and *YLR040c,* a small increase in gene expression was observed in *MAT*α *sum1Δ* mutants compared with wild type (discussed further below). Second, although substantial α-specific gene expression occurred in *MAT***a** *sum1Δ* cells, the levels reached only 10-30% of those in wild-type α cells. This difference in expression was potentially due to the absence of the α1 activator in **a** cells. Nonetheless, the expression of most α-specific genes in *MAT***a** *sum1Δ* cells did not fit the standard model for mating type determination developed in *S. cerevisiae*, and implied that Sum1 was a novel repressor of α-specific genes in *S. bayanus*.

In principle, the gene sets repressed by Sum1 could have completely changed—from meiotic genes to α-specific genes—between the two species. To ask whether Sum1 repression of meiotic genes was conserved in *S. bayanus*, the expression of two meiotic

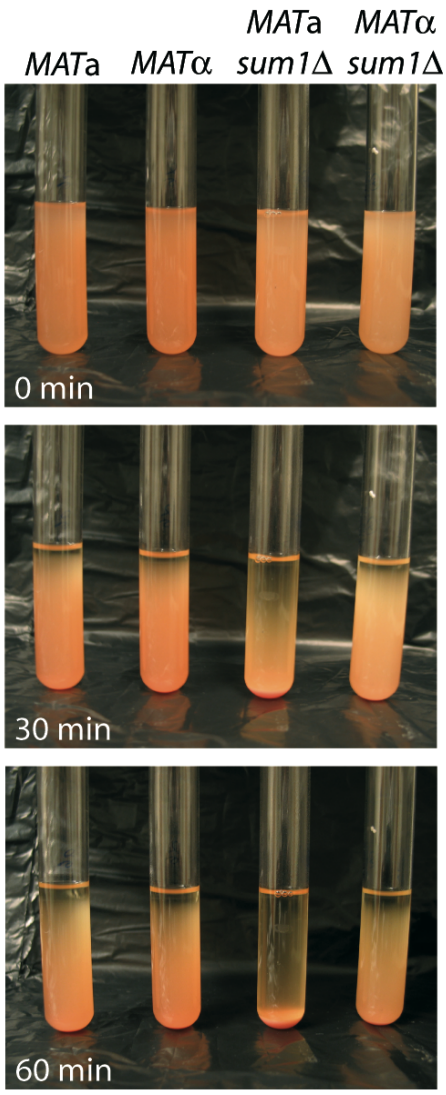

Figure 2.3. *S. bayanus MAT*a *sum1Δ* sedimented rapidly in liquid culture. 10mL YPDA cultures grown for 48 hours (stationary phase) were taken off of the rotating incubator and allowed to settle for one hour. Photographs were taken every thirty minutes. Note the more rapid sedimentation of the flocculent *MAT***a** *sum1Δ* cells in comparison with wild type and *MATα sum1Δ*.

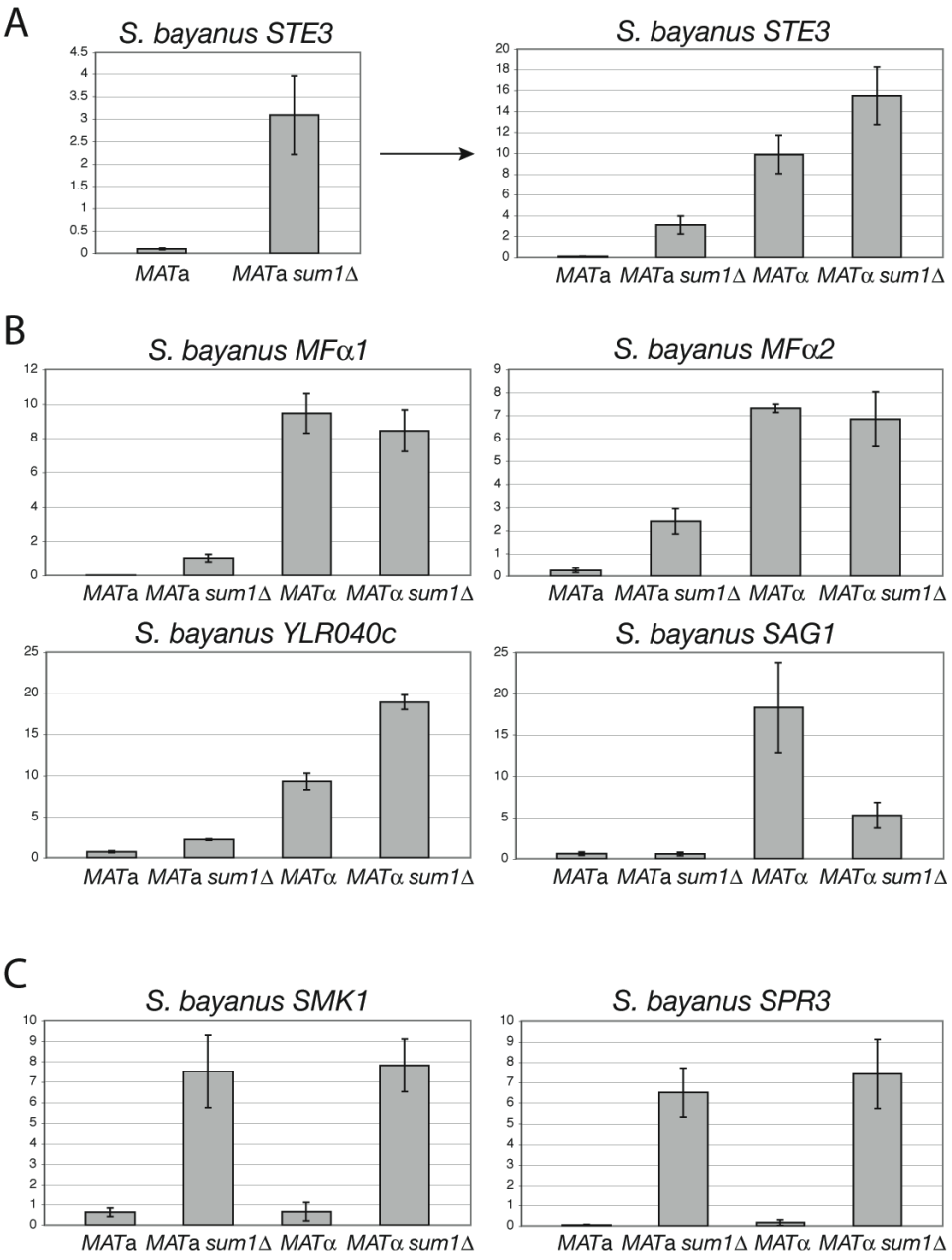

Figure 2.4. α-specific genes were up-regulated in *S. bayanus MAT*a *sum1Δ* cells analysis compared with wild type. (A) Expression of the α-specific gene *STE3* was assayed by quantitative RT-PCR. *MAT***a** wild-type versus *sum1Δ* are shown on separate plot at left as an example of this two-way comparison for all α-specific genes. These same data are re-plotted at right for four-way comparison with wild-type and mutant α cells. (B) Expression of the α-specific genes *MFα1*, *MFα2*, *YLR040c*, and *SAG1* in wild-type and *sum1Δ* cells of both mating types. (C) Expression of the meiotic genes *SMK1* and *SPR3*, which are targets of Sum1 repression in *S. cerevisiae*, in wild-type and *sum1Δ* cells of both mating types.

genes that are repressed by Sum1 in *S. cerevisiae*, *SMK1* and *SPR3*, was analyzed in *S. bayanus sum1Δ* cells. The increase in expression observed for these genes in *sum1Δ* mutants of both mating types (Figure 2.4) indicated that Sum1 retained its role as a repressor of meiotic genes in *S. bayanus*.

Sum1 prevented auto-stimulation of **a** *cells by α-factor*

The gene expression data suggested that *S. bayanus MAT***a** *sum1Δ* mutants expressed and responded to α-factor, producing shmoon and the bi-mating phenotype. However, it remained possible that stimulation by **a**-factor through Ste3, the **a**-factor receptor, also contributed to the mutant phenotypes, as loss of Sum1 led to expression of *STE3* in **a** cells. To distinguish between auto-stimulation of *MAT***a** *sum1Δ* cells by mis-expression of α-factor or by mis-expression of the **a**-factor receptor, we generated double mutants in the *MAT***a** *sum1Δ* background with genes required for pheromone signaling, inactivating different steps in the mating pathway. Strikingly, inactivation of *STE2*, which encodes the α-factor receptor and is expressed only in **a** cells, completely suppressed *sum1Δ* colony wrinkling, cell elongation, and flocculation phenotypes (Figure 2.5). *MFα1* produces the majority of α-factor, whereas *MFα2* produces a smaller fraction (Kurjan 1985). Consistent with these studies, the *S. bayanus MAT***a** *sum1Δ mfα1Δ* double mutants had very little flocculence and no apparent cell elongation (Figure 2.5), whereas *MAT***a** *sum1Δ mfα2Δ* double mutants had phenotypes indistinguishable from *MAT***a** *sum1Δ* single mutants (data not shown). Similar to *STE2* deletion, inactivation of *STE12* completely suppressed the *MAT***a** *sum1Δ* phenotypes (Figure 2.5). In contrast, deletion of *STE3* enhanced wrinkling, bi-mating, and expression of *FIG2* (data not shown). This result was consistent with expression of *STE3* in **a** cells dampening the pheromone-signaling response (Roth et al. 2000; Rivers and Sprague 2003). Taken together, these data established that the cell and colony morphology defects in *MAT***a** *sum1Δ* mutants were caused by production of α-factor and subsequent stimulation by the same or neighboring cells within a growing colony.

To test whether the α-factor response was sufficient for the *MAT***a** *sum1Δ* phenotypes, the galactose-inducible *GAL1* promoter was introduced into the genome of an otherwise wild-type *S. bayanus MAT***a** strain so as to drive expression of the *MFα1* gene. In contrast to wild-type **a** cells, *MAT***a** *GAL1pro-MFα1* cells grown on galactose-containing medium formed wrinkled colonies that contained cells with enhanced flocculence and a high percentage of shmoon (Figure 2.5). Additionally, when assayed on galactose medium, the inducible *MFα1*-expressing cells mated robustly with both **a** cells and α cells (Figure 2.5). Thus, expression of *MFα1* in *S. bayanus* **a** cells was both necessary and sufficient to produce the flocculation, shmooing, and bi-mating phenotypes observed in the *MAT***a** *sum1Δ* mutant. Furthermore, the cell elongation was classic "shmooing" behavior caused by derepression of α-specific genes in **a** cells that, by definition, lack the α1 activator protein.

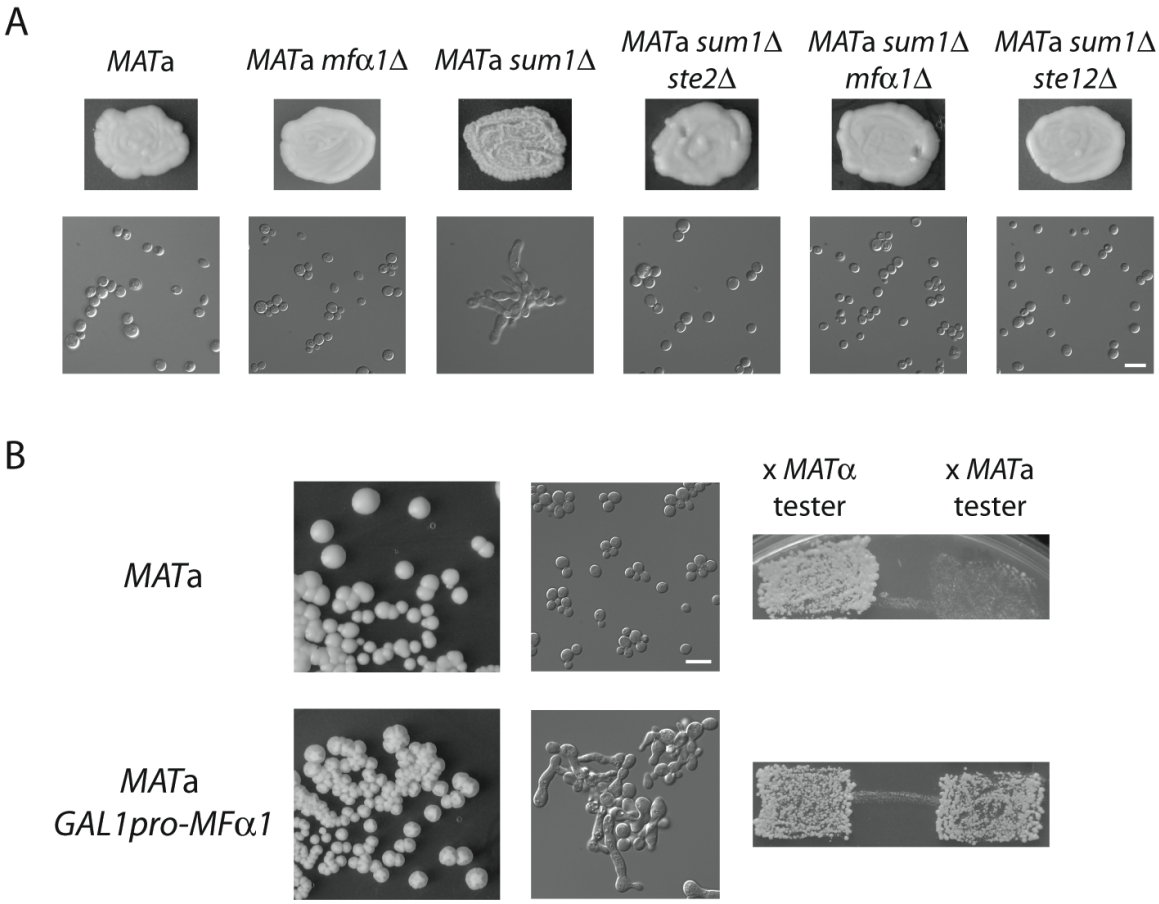

Figure 2.5. Sum1 prevented auto-stimulation of a cells by α-factor. (A) Inactivation of the mating pheromone signaling pathway suppressed *MAT***a** *sum1Δ* phenotypes. Top panels depict patches of each indicated genotype growing on YPD. Bottom panels show DIC microscopy of cells taken from the above patches. (B) *MAT***a** wild-type and *GAL1pro-MFα1* colonies grown for five days on YPG (left panels). Cells taken from the same colonies at left were photographed (middle panels), and tested in patch mating assays (right panels). On YPD, the *GAL1pro-MFα1* strain behaved identically to the wild-type *MAT***a** strain (data not shown). Scale bars indicate 10μm.

As Sum1 had been implicated in repression of the silent mating type loci, *HML* and *HMR*, it was possible that induction of α-specific genes and associated phenotypes in the absence of Sum1 were indirect effects of derepression of the silenced α1 gene at *HML*α (Irlbacher et al. 2005). However, *MAT***a** *sum1Δ hmlΔ* double mutants possessed phenotypes identical to those of *MAT***a** *sum1Δ* single mutants (Figure 2.6). Therefore, the *sum1Δ* phenotypes did not depend on *HML*α, ruling out this indirect-effect explanation.

Sum1 bound to and repressed α-specific gene promoters

In the standard model of mating type determination, no repressor of α-specific genes exists; merely the absence of the α1 activator protein prevents their expression. To explore the possibility that Sum1 directly repressed α-specific genes, we scanned their promoters (defined here as 1 kb of sequence 5′ of the start codon) for consensus Sum1 binding sites derived from detailed analyses of Sum1's specific DNA sequence binding *in vitro* and its repression activity *in vivo* (Pierce et al. 2003). Using the consensus sequence AGYGWCACAAA, a near-perfect match in the *STE3* promoter was detected that was 100% conserved across the *sensu stricto* yeast species (Figure 2.6). With a less stringent consensus, DSYGWCAYWDW, at least one match was found in the *STE3*, *MFα1*, and *YLR040c* promoters, each of which was highly conserved across the *sensu stricto*. Intriguingly, the sequence matches in the *STE3* and *MFα1* promoters occurred within the previously characterized *P′Q* elements, which in *S. cerevisiae* direct their α-specific expression through binding α1 and Mcm1 (for the *YLR040c* promoter, the match occurred 100bp 5′ of the predicted *P′Q* box). None of the matches found in the *SAG1* promoter were well conserved, however, this promoter was less conserved than the other α-specific gene promoters.

Sum1 was FLAG-tagged at its C-terminus in *S. bayanus* haploid strains and shown to provide Sum1 function in a *sum1Δ* mutant. Chromatin immunoprecipitation (ChIP) was used to test whether Sum1 protein bound the promoters of α-specific genes. Quantitative PCR analysis of Sum1-FLAG precipitates revealed 2-4-fold enrichment of the *STE3, MFα1*, *MFα2*, and *YLR040c* promoters relative to precipitates from no-tag control strains (Figure 2.6). A negative control primer set amplified a region within the *SEN1* ORF. As positive controls, we examined *S. bayanus* Sum1 binding at three loci to which the *S. cerevisiae* Sum1 protein binds (Lee et al. 2002). Strong enrichment (>8-fold) of both the *SMK1* and *DAL1* promoters was observed (Figure 2.6), but only 2.5-fold enrichment of the *SPR3* promoter was observed (comparable to the *MFα1* level), despite this gene being a confirmed Sum1 target in *S. cerevisiae* (Xie et al. 1999). A simple model consistent with the ChIP and genetic analysis was that, at least in *S. bayanus*, Sum1 bound directly to α-specific gene promoters to repress their transcription.

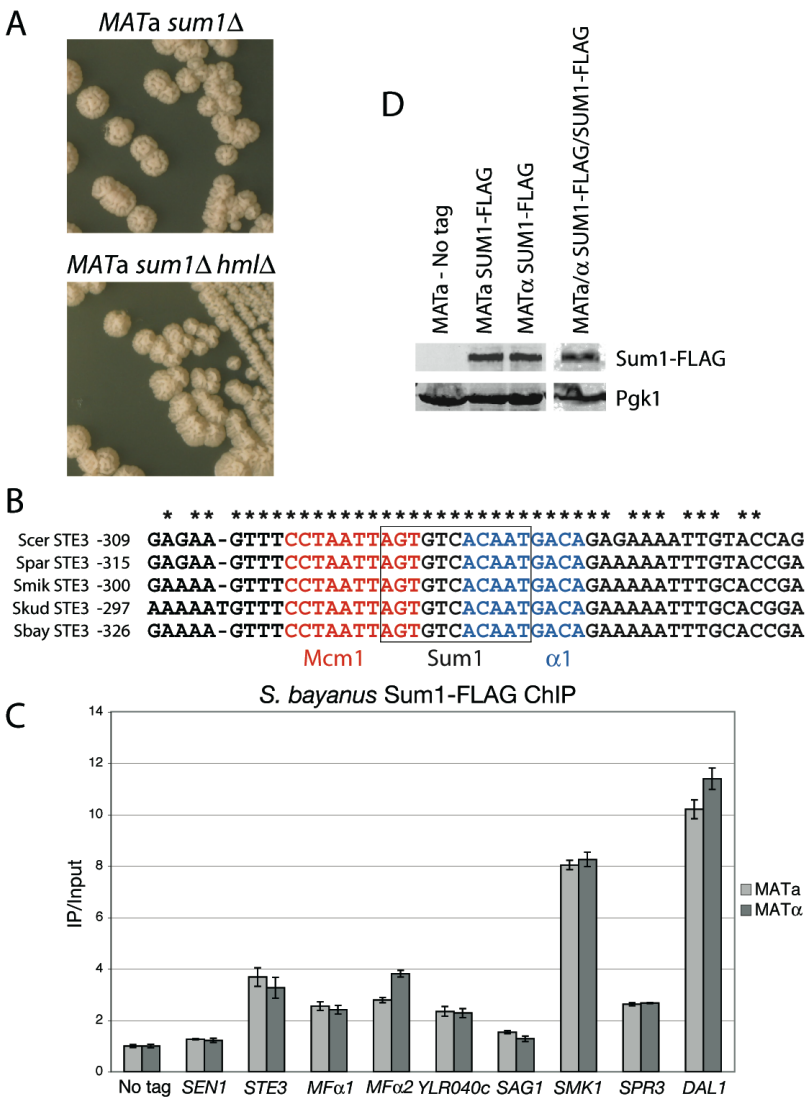

Figure 2.6. Sum1 repressed α-specific genes directly by binding to their promoters. (A) *S. bayanus MAT***a** *sum1Δ* and *MAT***a** *sum1Δ hmlΔ* colonies grown on YPD for five days. (B) Sum1 binding site predictions for *STE3* promoters from *sensu stricto* yeasts. A predicted Sum1 site partially overlapped the known Mcm1 and α1 sites in the *P′Q* element of the *STE3* promoter. The sequence encompassing all three binding sites was 100% conserved across all five species. Yeast species are abbreviated as follows: Scer, *S. cerevisiae*; Spar, *S. paradoxus*; Smik, *S. mikatae*; Skud, *S. kudriavzevii*; Sbay, *S. bayanus*. Numbers indicate the nucleotide position relative to the start codon. (C) Chromatin immunoprecipitation (ChIP) of Sum1-FLAG was performed, followed by quantitative PCR using primers to select promoter regions in the *S. bayanus* genome. IP amplification values were normalized to input values. These data were then normalized to a representative "No Tag" amplification value. (D) Sum1 protein levels were assayed by immunoblot for FLAG-tagged Sum1p (top row) and phosphoglucokinase (Pgk1p, bottom row), a loading control, using *S. bayanus* whole cell extracts. Lanes from two separate blots are shown (haploid cells in lanes 1-3, diploid cells in lane 4).

Sum1 repression of α-specific genes was conserved in S. cerevisiae

The conserved Sum1 binding site in the *S. cerevisiae STE3* promoter suggested that similar repression might occur throughout the *Saccharomyces* clade, although Sum1 had not previously been implicated in control of mating type in *S. cerevisiae*. Data from genome-wide localization studies (Lee et al. 2002; Harbison et al. 2004; Robert et al. 2004) identified highly reproducible binding of both Sum1 and Hst1 at the *STE3* promoter in *S. cerevisiae* **a** cells. Furthermore, microarray data indicated that the expression of *STE3*, *YLR040c*, and *FIG1* increased in *S. cerevisiae MAT***a** *sum1Δ* cells compared with wild-type **a** cells (Pierce et al. 2003), though the biological impact of this increase was unknown. Thus, we tested whether Sum1 repressed α-specific genes in *S. cerevisiae* despite the absence of gross phenotypes in *MAT***a** *sum1Δ* mutants of this species. In fact, quantitative RT-PCR analysis of α-specific genes revealed an expression profile similar to that of *S. bayanus*. Expression of *S. cerevisiae STE3* and *MFα1* increased 10-fold and 50-fold, respectively, in *MAT***a** *sum1Δ* cells compared with wild-type (Figure 2.7).

These data presented a conundrum: if Sum1 repressed α-specific genes in *S. cerevisiae*, then why did *MAT***a** *sum1Δ* mutants of this species appear normal? We inspected the *S. bayanus* orthologs of *S. cerevisiae* mating genes to ask whether changes in mating genes might contribute to the *S. bayanus MAT***a** *sum1Δ* phenotype. Pair-wise alignment of the *S. cerevisiae* and *S. bayanus* **a**-specific gene *BAR1*, which encodes a protease that degrades α-factor, revealed a single base-pair deletion (ΔT741) in the *S. bayanus* ortholog. This frame-shift mutation was predicted to generate an inactive protein lacking the C-terminal catalytic site. Although this mutation had been annotated as a sequencing error (Kellis et al. 2003), we confirmed its presence by re-sequencing. This loss-of-function *bar1* allele likely rendered *S. bayanus* **a** cells hypersensitive to α-factor. Mating pheromone halo assays confirmed that *S. bayanus* **a** cells were in fact hypersensitive to α-factor, but α cells were not hypersensitive to **a**-factor (data not shown). Transformation of *S. bayanus MAT***a** *sum1Δ* cells with a plasmid bearing the *S. cerevisiae BAR1* gene suppressed the colony wrinkling phenotype, confirming that the *bar1* mutation contributed to the phenotypic difference between species (Figure 2.8).

To test whether Bar1 function masked some of the phenotypic consequences of loss of Sum1, *BAR1* was deleted in a *S. cerevisiae MAT***a** *sum1Δ* strain (W303 background). Intriguingly, *MAT***a** *sum1Δ bar1Δ* double-mutant cells had readily observable shmooing behavior, recapitulating much of the *S. bayanus sum1Δ* phenotype in *S. cerevisiae*. However, the *S. cerevisiae* mutant colonies were not wrinkled and the cells were not flocculent (Figure 2.7), suggesting that interspecies differences in adhesion molecules, or in their ability to be induced by α-factor, might account for the rest of the phenotype. A clue to this discrepancy is that *S. cerevisiae* **a** cells undergo pseudohyphal-like growth upon low-level stimulation by α-factor (Erdman and Snyder 2001). In the *S. cerevisiae* Σ1278b strain, which is competent to form pseudohyphae, the *MAT***a** *sum1Δ bar1Δ* double mutant fully recapitulated the shmooing and colony wrinkling phenotypes seen in the orthologous *S. bayanus* mutant (Figure 2.7). Thus, some difference between

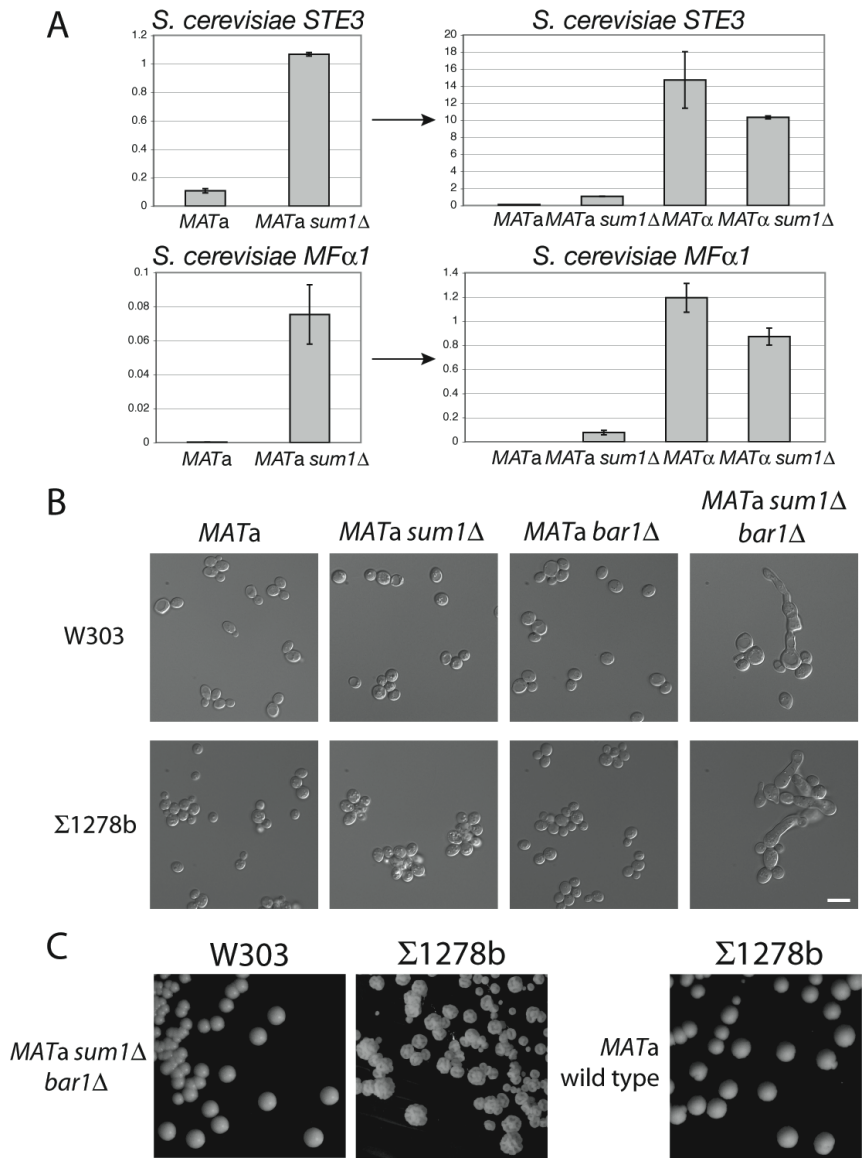

Figure 2.7. Sum1-mediated repression of α-specific genes was conserved in *S. cerevisiae*. (A) Expression of *STE3* and *MFα1* in *S. cerevisiae* was assayed by quantitative RT-PCR. *MAT***a** wild-type versus *sum1Δ* cells are shown on separate plots at left to allow two-way comparison. These same data are re-plotted at right for four-way comparison with wild-type and mutant α cells. (B) DIC microscopy of *S. cerevisiae MAT***a** *sum1Δ bar1Δ* double mutant cells plus wild-type and single mutant controls of W303 (top row) and Σ1278b (bottom row) backgrounds. Scale bar indicates 10μm. (C) Σ1278b *MAT***a** *sum1Δ bar1Δ* colonies grown on YPD for five days (middle panel); compare with wild-type colonies (right panel). W303 *sum1Δ bar1Δ* colonies, which had only a subtle change in morphology, are shown at left for comparison (compare with Fig. 2A).

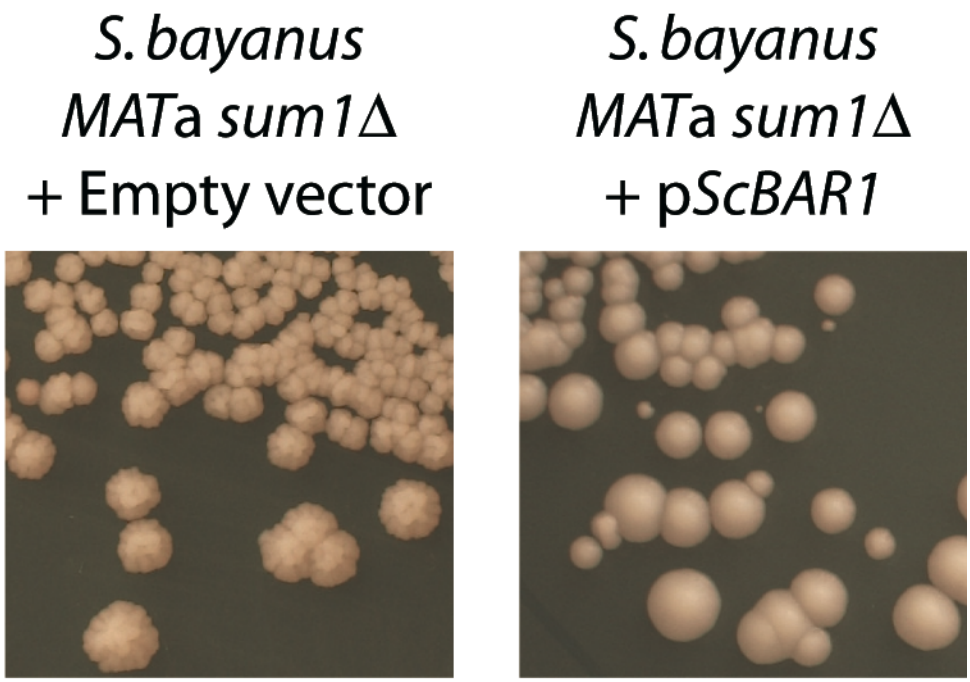

Figure 2.8. *S. cerevisiae BAR1* suppressed colony wrinkling of *S. bayanus MAT*a *sum1Δ*. The pictures show colonies of *S. bayanus MAT***a** *sum1Δ* transformed with empty vector or a plasmid bearing *S. cerevisiae BAR1* (*ScBAR1*). Individual transformants were re-streaked on selective media and allowed to form colonies over five days.

W303 and Σ1278b affected the ability of pheromone signaling to trigger flocculence (Liu et al. 1996; Guo et al. 2000).

α1 was required to overcome repression by Sum1 in α cells, but Mcm1 and Ste12 could activate transcription in the absence of Sum1 and α1

As Sum1 blocked α-specific gene expression, some mechanism must prevent it from repressing these genes in α cells. Sum1 protein was present at equivalent levels in both **a** cells and α cells (Figure 2.6). Sum1 bound equivalently *in vivo* to all promoters assayed in **a** cells and α cells (Figure 2.6). Therefore, neither the synthesis of nor the DNA-binding activity of Sum1 was mating-type regulated.

A simple model to explain how α-specific genes were expressed despite the presence of a repressor on their promoters was that α1 protein somehow counteracted Sum1's repressive function, and contributed to transcription activation together with Mcm1 and Ste12. If α1 were required to overcome Sum1 repression in α cells, and were not completely required for α-specific gene expression, then deletion of *SUM1* should suppress the non-mating phenotype of *matα1Δ* mutants. Indeed, whereas both *S. cerevisiae* and *S. bayanus matα1Δ* mutants were almost completely sterile, *matα1Δ sum1Δ* double mutants of both species mated substantially better (Figure 2.9). Furthermore, *matα1Δ sum1Δ hmlΔ* triple mutants mated equivalently to *matα1Δ sum1Δ* double mutants, demonstrating that removing Sum1 restored mating ability to α cells that were otherwise sterile because they lacked the α1 activator. Thus, Sum1 was active in both mating types of both species, and α1 was required to overcome Sum1 repression, in addition to providing a transcriptional activation function.

The restoration of mating in *matα1Δ sum1Δ* double mutants argued that Mcm1 and Ste12 could activate α-specific genes on their own, in contrast to the need for α1 as in the α1-α2 hypothesis. To test whether Mcm1 and Ste12 were necessary for α-specific gene expression in the absence of Sum1, mating ability and α-specific gene expression were assayed in *sum1Δ mcm1Δ* and *sum1Δ ste12Δ* double mutants. (The *MCM1* gene was not essential in *S. bayanus*, in contrast to *S. cerevisiae*, although some lethality was observed in germinating spores from *mcm1Δ*/+ diploids and some viable cells had a growth defect; data not shown). *sum1Δ ste12Δ* mutant cells of both mating types were completely sterile (data not shown); likewise, *MATα mcm1Δ* strains were unable to mate as α cells (Figure 2.9). The complete loss of mating with **a** cells by *MATα mcm1Δ* cells was not surprising, given that Mcm1 is required for activation of α-specific genes and repression of **a**-specific genes (Passmore et al. 1988; Elble and Tye 1991). However, unexpectedly, an equivalent small fraction of both *mcm1Δ* and *sum1Δ mcm1Δ* mutant cells mated as **a** cells, regardless of the allele present at *MAT*. Although most *mcm1Δ* mutant cells in the population were unable to mate, the **a**-like mating ability in a subset of cells demonstrated that at least some **a**-specific gene expression was Mcm1 independent (Kronstad et al. 1987). As suggested by the mating assays, Mcm1 was necessary for α-specific gene expression in the presence or absence of Sum1 (Figure 2.9). Indeed, in

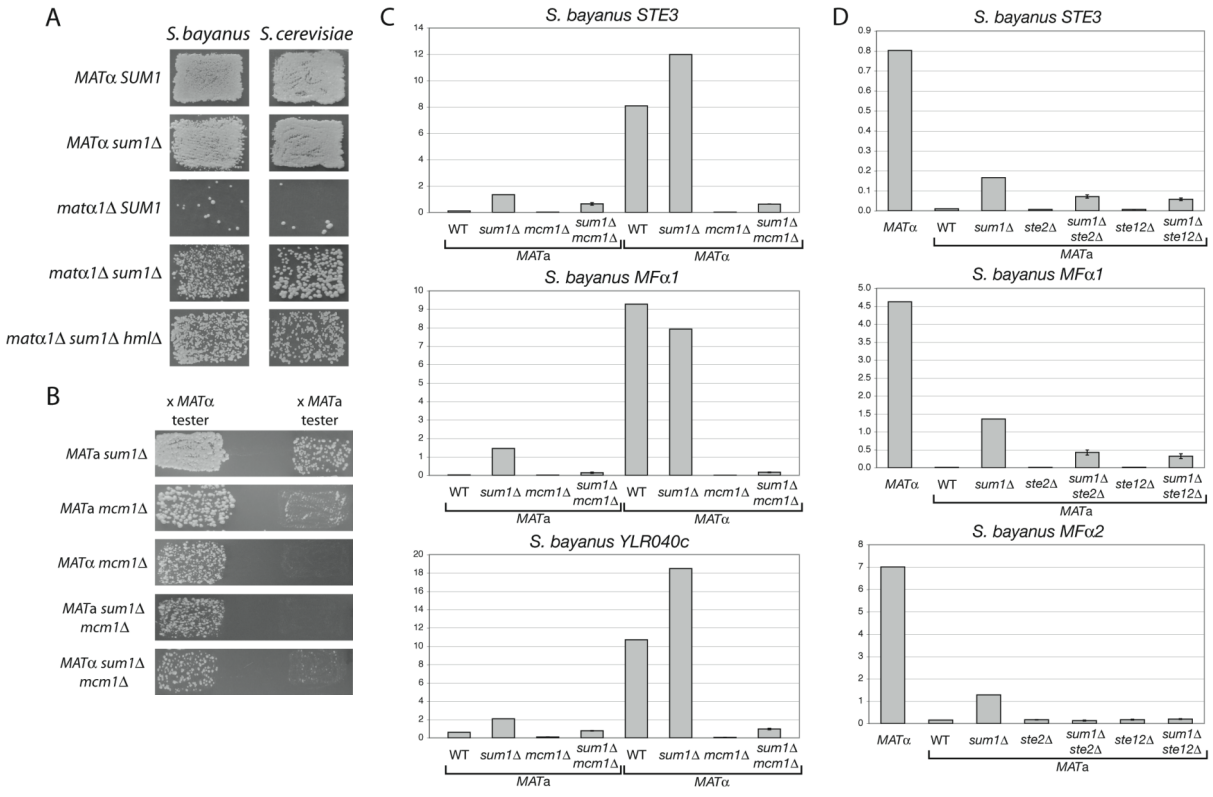

Figure 2.9. Sum1 was a general repressor of mating-type-specific genes. (A) Patch mating assays (using only the *MAT***a** tester) were performed on *MAT*α, *MAT*α *sum1Δ*, *mat*α*1Δ*, *mat*α*1Δ sum1Δ*, and *mat*α*1Δ sum1Δ hmlΔ* strains in both *S. bayanus* and *S. cerevisiae* (W303). (B) Patch mating assays were performed on *S. bayanus sum1Δ*, *mcm1Δ*, and *sum1Δ mcm1Δ* strains of the indicated mating types. (C) Expression of α-specific genes in cells of indicated genotypes was assayed by quantitative RT-PCR (WT, wild type). Expression of *STE3* is reduced 10-fold in *mcm1Δ* cells (columns 3 and 7) compared with wild-type **a** cells (column 1). It should be noted that *ACT1* (actin) RNA levels in *mcm1Δ* mutant cells were reduced 2-fold, causing the normalized expression values for *sum1Δ mcm1Δ* cells to be inflated. (D) Expression of α-specific genes in cells of indicated genotypes was assayed by quantitative RT-PCR (WT, wild type).

*MAT***a** *mcm1Δ* cells, *STE3* expression was down 10-fold from its level in wild-type **a** cells.

Ste12, on the other hand, was partially required for α-specific gene expression, with the exception of *MFα2* (Figure 2.9). The expression of *STE3* and *MFα1* remained several-fold increased in both *MAT***a** *sum1Δ ste12Δ* cells and *MAT***a** *sum1Δ ste2Δ* cells compared with wild type, *ste2Δ*, or *ste12Δ* controls, yet neither gene was expressed in the double mutants to the level of the *sum1Δ* single mutant. It appeared that the level of expression of α-specific genes was a composite of two effects: derepression in the absence of Sum1, and auto-stimulation by α-factor. Specifically, expression of *MFα2* in both *MAT***a** *sum1Δ ste12Δ* and *MAT***a** *sum1Δ ste2Δ* cells was equivalent to that in wild-type **a** cells, consistent with this gene being completely Ste12 dependent (Fields et al. 1988). The remaining *STE3* and *MFα1* expression in the absence of Sum1 and Ste12 implied that Mcm1 could activate some α-specific genes, albeit weakly, without other known co-activators. Interestingly, purified recombinant Mcm1 can bind *P′Q* elements *in vitro*, although it does so with low affinity relative to its binding of *P* elements from **a**-specific genes (Grayhack 1992).

The mechanism of repression of α-specific genes

The above results pointed to a simple mechanism for repression of α-specific genes by Sum1: preventing Mcm1 from binding to DNA. To test this model, ChIP assays were performed on Myc-tagged Mcm1 in wild-type and *sum1Δ* cells. Consistent with this model, ChIP revealed enhanced association of Mcm1 with all five α-specific gene promoters in *MATα sum1Δ* cells compared with *MATα* wild type (Table 2.2). *STE2*, an **a**-specific gene, served as a control, as Mcm1 bound this gene's promoter in both cell types, and its binding was unaffected by deletion of *SUM1* (as predicted; see Figure 2.1). The greater enrichment of *STE2* in α cells versus **a** cells may reflect conformational changes in Mcm1's association with DNA introduced by the α2 protein. Intriguingly, Mcm1 precipitates from *MAT***a** *sum1Δ* cells did not show a substantial enrichment of α-specific gene promoter sequences compared with *MAT***a** wild type. However, although the standard model suggests that Mcm1 does not bind to α-specific gene promoters in the absence of α1 protein, we observed association of Mcm1 with the *MFα1*, *MFα2*, and *YLR040c* promoters in *MAT***a** cells. Thus, models to explain the mechanism of α-specific gene activation in *sum1Δ* cells need not require enhanced occupancy by Mcm1 (discussed further below).

To explore whether the mechanism of repression of α-specific genes involved the proposed Sum1-Hst1-Rfm1 repressor complex, the *HST1* and *RFM1* genes were inactivated. *S. bayanus MAT***a** *hst1Δ* and *MAT***a** *rfm1Δ* mutants had colony and cellular phenotypes similar, though not identical, to *MAT***a** *sum1Δ* mutants (Figure 2.10). These results suggested that histone deacetylation by Hst1 was required for repression of α-specific genes, and that the interactions between Sum1, Rfm1, and Hst1 were conserved between *S. bayanus* and *S. cerevisiae*.

Table 2.2. *S. bayanus* Mcm1 ChIP assay. IP/input amplification ratios for each primer set were normalized to the *ACT1* IP/input ratio. *SEN1* served as a negative control locus. Numbers in parantheses indicate the Standard Error of the Mean.

	*MAT***a** (No tag)	*MATα* (No tag)	*MAT***a** *MCM1-myc*	*MATα MCM1-myc*	*MAT***a** *sum1Δ MCM1-myc*	*MATα sum1Δ MCM1-myc*
STE3	0.60 (0.16)	0.60 (0.18)	0.64 (0.16)	3.49 (0.73)	0.78 (0.20)	5.83 (0.63)
MFα1	0.68 (0.08)	0.67 (0.12)	1.51 (0.38)	3.46 (0.83)	1.89 (0.34)	6.51 (1.44)
MFα2	0.73 (0.08)	0.66 (0.09)	1.32 (0.26)	3.07 (0.86)	1.54 (0.27)	4.73 (0.92)
YLR040c	0.79 (0.20)	0.68 (0.13)	1.15 (0.37)	10.56 (1.33)	1.28 (0.21)	20.96 (1.68)
SAG1	0.97 (0.25)	0.65 (0.15)	0.81 (0.16)	3.15 (0.95)	0.85 (0.20)	4.54 (0.87)
STE2	0.59 (0.08)	0.54 (0.07)	3.25 (0.14)	13.88 (2.62)	4.02 (0.32)	14.41 (0.93)
SEN1	0.62 (0.18)	0.58 (0.14)	0.46 (0.04)	0.54 (0.09)	0.57 (0.15)	0.56 (0.05)

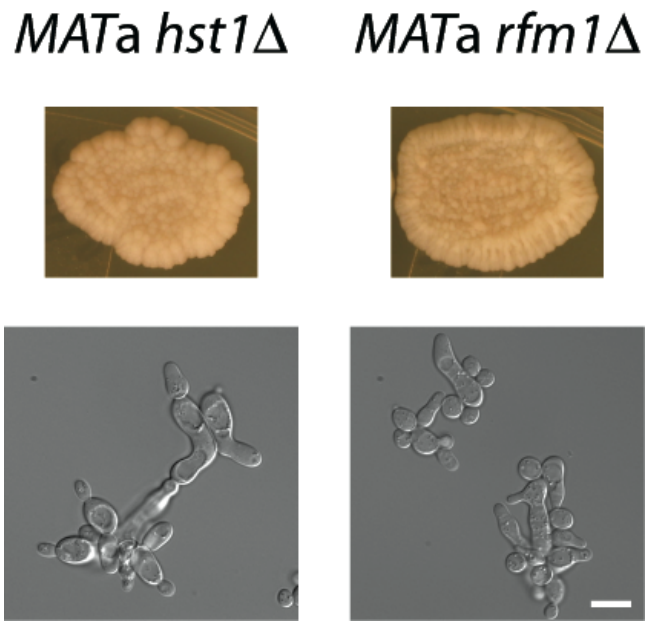

Figure 2.10. ***S. bayanus MATa hst1Δ*** **and** ***rfm1Δ*** **phenocopied** ***sum1Δ*****.** Top panels depict patches of each indicated strain grown for five days on YPD. Bottom panels show DIC microscopy of cells taken from the above patches. Compare these panels with those in Fig. 4A. Scale bar indicates 10μm.

Discussion

The standard model of mating-type control in *Saccharomyces cerevisiae* posits that **a** is the default mating type, with the α mating type requiring the activation of α-specific gene expression by α1 (in combination with Mcm1 and Ste12), and therepression of **a**-specific gene expression by α2 (also in combination with Mcm1) (Strathern et al. 1981; Sprague 1990). We found that the Sum1 protein acted as a direct repressor of α-specific genes in both *S. bayanus* and *S. cerevisiae* (Figure 2.11). At least two differences between these two species conferred phenotypic differences that allowed the discovery of this conserved aspect of mating type determination, which has been overlooked for 26 years. Part of the phenotypic difference was explained by *S. bayanus* **a** cell hypersensitivity to α-factor due to a *bar1* mutation. (This mutation was probably a polymorphism among *S. bayanus* strains; M. Dunham, A. Tsong, personal communications). However, another factor, present in both *S. bayanus* and *S. cerevisiae* strain Σ1278b, caused *sum1Δ bar1* mutant **a** cells to flocculate in response to α-factor. A good candidate for this "flocculence factor" was *FLO8*, which is required for flocculation in Σ1278b and has inactivating mutations in both W303 and S288C strains of *S. cerevisiae* (Liu et al. 1996).

Modifying the model for control of α-specific gene expression

Our results demonstrated two roles for the α1 protein: counteracting Sum1 repression, and inducing the maximal expression of α-specific genes (Figure 2.4). *MAT***a** *sum1Δ* and *matα1Δ sum1Δ* cells, both of which lack α1, were capable of α cell mating behavior (Figure 2.9). Consistent with the standard model, Mcm1 and Ste12 were necessary for mating of α cells and for robust α-specific gene expression in the absence of Sum1 (Figure 2.9). As Mcm1 and Ste12 were able to activate transcription in the absence of α1 and Sum1, the α1/Mcm1 cooperative binding model cannot fully explain α-specific gene activation (Figure 2.11). Indeed, Mcm1 was associated with the *MFα1*, *MFα2*, and *YLR040c* promoters in *MAT***a** cells (Table 2.2). Mcm1's occupancy of α-specific gene promoters was enhanced in the absence of Sum1, but only in *MATα* cells (when α1 is present). Surprisingly, Mcm1 ChIP of α-specific gene promoters was not substantially enhanced in *MAT***a** *sum1Δ* cells compared with *MAT***a** wild-type cells. This result forced us to consider two additional models for the interplay between Sum1 and the co-activator proteins at α-specific genes: (1) Sum1 interfered with transcriptional activation at a step downstream of Mcm1 binding, or (2) a novel activator was recruited to α-specific genes in the absence of Sum1. Formally, Sum1 might have blocked DNA binding by Ste12 in *MAT***a** cells. However, some expression of α-specific genes was observed in *MAT***a** *sum1Δ ste12Δ* cells (Figure 2.9), and Ste12 binding sites in most α-specific gene promoters did not overlap predicted Sum1 binding sites, making increased promoter occupancy by Ste12 in *sum1Δ* cells seem a less likely explanation.

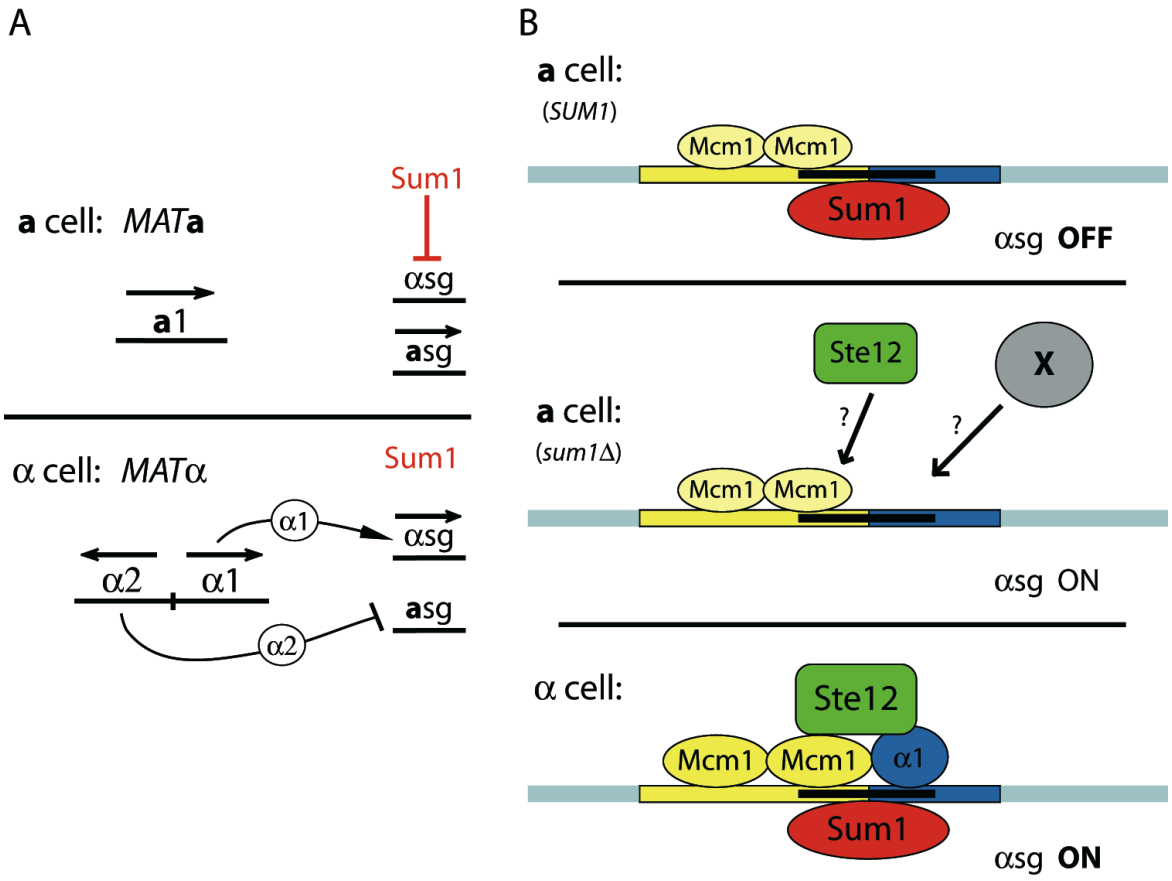

Figure 2.11. Models for Sum1-mediated repression of α-specific genes. (A) Model for Sum1 role in repression of α-specific genes and mating type determination. In contrast to the standard model, active repression of α-specific genes by Sum1 was required for proper **a** cell identity. (B) Molecular mechanism for α-specific gene regulation. In **a** cells, Sum1 repressed α-specific genes by binding either to sites directly overlapping the α1 and Mcm1 binding sites, or to other nearby sites. Mcm1 bound some, but not all, α-specific gene promoters in **a** cells. Without repression by Sum1, Mcm1 and Ste12 were able to activate α-specific genes in **a** cells to a level that permitted α-like mating. In α cells, α1 induced high levels of α-specific gene expression and was required to overcome the block imposed by Sum1. Despite their high levels of transcription, Sum1 remained associated with α-specific genes in α cells.

Our data also highlighted gene-specific aspects of α-specific gene regulation. One exception to the model that Sum1 repressed α-specific genes was *SAG1*, whose expression in α cells was promoted by *SUM1* (Figure 2.4). These data raised the possibility that some other factor represses *SAG1*, implying that there may be yet another dimension to α-specific gene regulation. The expression of *MFα2* increased in **a** cells lacking Sum1, however, this increase was completely abolished in *sum1Δ ste2Δ* and *sum1Δ ste12Δ* double mutants (Figure 2.9). *SAG1* and *MFα2* thus appear to be regulated differently than *MFα1*, *STE3*, and *YLR040c*, which may represent unanticipated complexity in α cell mating behavior.

Sum1 repressed α-specific genes yet was expressed and active in both **a** cells and α cells (Figure 2.11). Therefore, some mechanism involving α1, Mcm1, and Ste12 must exist to prevent Sum1 from repressing α-specific genes in α cells. One possible mechanism is DNA bending by α1 and Mcm1 (Hagen et al. 1993; Carr et al. 2004), which may alter the physical conformation of the promoter, preventing Sum1 from making protein-DNA or protein-protein interactions critical for repression. It is also possible that α1 and Mcm1 out-compete Sum1 for contacts with general transcription factors, Mediator, or RNA Polymerase holoenzyme. Finally, it is possible that α-specific gene activators directly contact Sum1 to prevent repression. Notably, some *mcm1* and *ste12* alleles are specifically defective in activating expression of α-specific genes in α cells (Passmore et al. 1988; La Roche et al. 1995). These mutant proteins may fail to oppose Sum1 repression, or they may be unable to interact with α1.

Sum1 binding of α-specific gene promoters in their active state could be explained if this repressor bound to the minor groove of DNA while the α1 and Mcm1 activators bound the major groove. This scenario seemed plausible given that Sum1 contains two "AT-hooks"—short, positively charged motifs of thirteen amino acids—in its N-terminal half. These moieties often mediate binding to the minor groove of AT-rich DNA sequences (Reeves and Nissen 1990; Huth et al. 1997). Regardless of the specific mechanism involved, our results suggest a more complex model of α-specific promoter function than previously appreciated.

Control of cell-type-determining genes during differentiation

Why should α-specific genes have required repression by Sum1? As in the development of distinct cell types in multicellular eukaryotes, proper yeast cell identity must be ensured as soon as a differentiation event occurs, in this case a mating type switch. The most straightforward role for mating type regulation by Sum1 was preventing α-mating behavior in **a** cells, particularly when cells switch from *MAT*α to *MAT***a**. In such a cell, α-specific genes would continue to be expressed if there were any residual α1 protein. Without active repression of α-specific genes, this newly-formed **a** cell might mate with another **a** cell, generating a *MAT***a**/**a** diploid unable to undergo meiosis and destined for meiotic oblivion should it mate with an α cell to form a triploid. A complementary role for Sum1 was maintenance of robust **a** cell mating ability, as

expression of α-specific genes in **a** cells causes decreased mating ability (Roth et al. 2000; Rivers and Sprague 2003). Furthermore, our results show that α-specific genes can be expressed at a substantial level even in the absence of their primary activator. For **a** cells that secreted even a low level of α-factor, their adaptation and subsequent desensitization (Dietzel and Kurjan 1987) would likely make them less sensitive to signals from bona fide mating partners. (Indeed, Sum1 was necessary for preventing auto-stimulation of **a** cells with α-factor. We note that our data did not formally distinguish between autocrine stimulation of an **a** cell by α-factor produced by that same **a** cell versus stimulation occurring primarily in *trans*, by α-factor secreted from neighboring cells. As a substantial amount of α-factor was likely present within growing *MAT***a** *sum1Δ* colonies, some level of *trans*-stimulation undoubtedly occurred.)

Further insight into why this function of Sum1 evolved might be gained by asking when it evolved. Given the evolutionary distance between *S. cerevisiae* and *S. bayanus*, our results suggest that the Sum1 protein likely performed at least two functions in the *sensu stricto* ancestor: repression of α-specific genes and repression of meiotic genes. The conservation of the Sum1 protein and predicted Sum1 binding sites in α-specific gene promoters in *S. paradoxus*, *S. mikatae*, and *S. kudriavzevii* support this idea. α-specific gene activation by α1 appears to be conserved throughout Ascomycete fungi (Tsong et al. 2003). However, Sum1 orthologs are present in *A. gossypii* and *K. lactis*, but are not in species more distant from *S. cerevisiae*. Therefore, it would be interesting to ask how α-specific genes were regulated in ancestral Ascomycetes. It is possible either that a different repression mechanism is used outside of the *Saccharomyces* complex, or that Sum1-based repression is unique to these species, possibly to accomplish a unique aspect of their mating behavior (Figure 2.1).

The Sum1-ORC connection

In light of our findings, the repression of α-specific genes and silencing of the *HMR* mating type locus bear intriguing similarities. In addition to histone deacetylation by Hst1, which is highly similar to Sir2, repression of α-specific genes may require association of the repressive complex with ORC. The Sum1-1 protein, like the Sir complex, interacts with and requires ORC for silencing *HMR* (Sutton et al. 2001; Irlbacher et al. 2005). Wild-type Sum1 also requires ORC for repression of certain target genes (Lynch et al. 2005; Ramachandran et al. 2006). Intriguingly, replication origins reside near *SAG1* and *YLR040c*, and Mcm protein components of the pre-Replication Complex assembly reportedly bind at or near *STE3* and *MFα1* (Wyrick et al. 2001; Xu et al. 2006). Understanding the role of origins in repression of α-specific genes might explain the seemingly bizarre ability of a point mutation to convert Sum1, a promoter-specific repressor of several dozen loci, to a regional silencing factor capable of repressing the *HMR* locus and reporter genes inserted into it (Laurenson and Rine 1991; Rusche and Rine 2001). Sum1 may mediate long-range repression similar to Sir-based silencing at multiple sites in the yeast genome, possibly to coordinate the execution of specific gene expression programs with cell cycle events during differentiation.

Additionally, our results may help to explain the mating-type specificity of the *SUM1-1* allele, which can suppress the mating defect of *MAT*α *sir* mutants, but not *MAT***a** *sir* mutants. If this variant protein moves from α-specific gene promoters to the *HMR* locus, as the current model for Sum1-1 holds, the net effect would be to remove two sources of repression from α-specific genes: the **a**1 protein, which is encoded at *HMR*, and Sum1 itself. It would be interesting to test whether mating restoration in *MAT*α *sir- SUM1-1* cells depends on Sum1-1 gaining the ability to silence *HMR***a** or losing the ability to repress α-specific genes. A *matα1Δ SUM1-1* double mutant could be informative in distinguishing between these scenarios.

Advantages of comparative genetic analysis

In retrospect, active repression of α-specific genes has evaded detection for so long largely because conventional biochemical and single-species genetic approaches were not sufficiently sensitive. One apparent complication was overlap of the α1/Mcm1 activators' binding sites by the putative Sum1 binding site in the promoters of some α-specific genes. Previously characterized mutations in the *STE3* promoter that abolished activator binding (Hagen et al. 1993) also ablated the Sum1 binding site, obscuring the contribution of Sum1 to repression of *STE3* expression. Similarly, gel-shift analyses of the *STE3* and *MFα1* operators largely failed to detect a complex containing anything other than α1 and Mcm1 (Bender and Sprague 1987; Jarvis et al. 1988). However, reaction conditions optimized for α1/Mcm1 binding or low Sum1 protein abundance relative to that of α1 and Mcm1 may have confounded the ability of such experiments to detect Sum1 binding. Sum1 recruitment to α-specific genes might involve chromatin or other protein-protein interactions, rather than simply site-specific DNA binding.

The perspective of evolutionary biology offers the ability to infer conservation of functions between organisms, and the ability to ask how alterations in genetic circuits generate novel forms. Current large-scale biochemical, genetic, and computational efforts seek to identify novel genetic pathways and novel components of known pathways (Tong et al. 2004; Krogan et al. 2006; Collins et al. 2007). However, the recognition of a genetic pathway's components may depend on the range of phenotypes produced by common perturbations in multiple species. As we have shown, comparative genetic analysis in closely-related species can provide the phenotypic depth sometimes needed for ascribing novel functions to specific genes. In this case, adventitious variation in phenotypes between species revealed a regulatory circuit conserved among species. Because nature provides variation not captured by model organisms, more detailed pictures of shared and unique regulatory pathways should emerge from a montage of multiple species' genetic interaction networks.

Chapter 3

Rapid evolution of Sir4 in budding yeast

The work in this chapter was carried out in collaboration with Devin Scannell, a postdoc in Mike Eisen's lab, and Jeffrey Kuei, a former undergraduate in the Rine lab. Devin Scannell performed all the evolutionary and population genetics analyses. Devin made Figures 3.6-3.10, and wrote the relevant portions of the Results and Methods. Jeff Kuei conducted the screen for silencing-defective mutants in *S. bayanus*, and is responsible for much of the work that is behind Figures 3.1-3.3 and Table 3.2. I am extremely grateful to both Devin and Jeff for their efforts.

Abstract

Transcriptional silencing at cryptic mating-type loci and telomeres in *Saccharomyces cerevisiae* requires Sir (Silent Information Regulator) proteins. The genome sequence of the closely related yeast, *S. bayanus*, has orthologs of the four *SIR* genes of *S. cerevisiae* as well as three additional Sir1-related proteins. The elaboration of the Sir1 family in *S. bayanus* suggested that silencing may operate in a somewhat distinct fashion in this species. A screen for mutations in genes required for silencing in *S. bayanus* identified a set of core components—*SIR2*, *SIR3*, and *SIR4*—that were shared with *S. cerevisiae*. However, cross-species complementation analysis revealed a genetic incompatibility between *S. cerevisiae SIR4* and the *S. bayanus HML* and *HMR* loci normally silenced by Sir4. Although *S. bayanus* Sir4 could silence in either species, *S. cerevisiae* Sir4 could silence only in S. *cerevisiae*. *SIR4* was one of the most rapidly evolving genes in the genomes of both the *Saccharomyces sensu stricto* and *Torulaspora* clades. Within *Saccharomyces*, positive selection of several residues in Sir4's PAD and N-terminal regulatory regions likely contributed to Sir4's functional divergence between species. Additionally, ongoing diversifying selection of *SIR4* was evident in modern yeast populations. Major functional changes in *SIR4* occurred after the divergence of *S. bayanus* and *S. kudriavzevii*, coincident with the loss of multiple *SIR1* paralogs from the yeast genome. The functionally distinct silencing machineries found in two groups of *Saccharomyces* species may represent adaptation that occurred during an evolutionary "arms race" with the Ty5 retrotransposon.

Introduction

Transcriptional silencing by Sir proteins in *Saccharomyces cerevisiae* serves as a paradigm for epigenetic control of gene expression in eukaryotes, but relies on an entirely RNAi-independent mechanism (Grewal and Moazed 2003). Sir-mediated position effects on gene expression at the silent mating type loci and telomeres have led to analogies between the repressive chromatin structures created by Sir proteins and the cytologically defined heterochromatin of animals (Moazed 2001; Pirrotta and Gross 2005). However, three of the original four Sir proteins (Sir1, Sir3, and Sir4) are genetic features unique to a group of Ascomycete yeasts called the *Saccharomyces* complex (Butler et al. 2004; Hickman and Rusche 2007a). The vast majority of studies on the Sir silencing mechanism have been conducted in *S. cerevisiae*, but it is unclear how representative this "model" organism's silencing mechanism is of those of the broader *Saccharomyces* clade.

How did Sir silencing evolve, and can the patterns of Sir protein evolution reveal variations on the silencing mechanism known in *S. cerevisiae*? Sir2, the only Sir protein that is also an enzyme, is one of the most highly conserved histone deacetylases in nature (Frye 2000), with additional roles in regulating aging and cellular metabolism (Imai et al. 2000). Sir3 is a non-catalytic paralog of Orc1, a highly conserved member of the Origin Recognition Complex (ORC). The Sir3/Orc1 pair as well as the Sir2/Hst1 pair serve as examples of the duplication, degeneration, and complementation (DDC, also know as "subfunctionalization") model of paralogous protein evolution (van Hoof 2005). In contrast, *SIR1* is a member of a rapidly evolving gene family, and some yeast species have multiple *SIR1* paralogs (Gallagher et al. 2009). Alignments of *SIR4* across budding yeast species reveals that this gene is very rapidly evolving (Fabre et al. 2005); O. Zill and D. Scannell, unpublished observations). Our understanding of Sir silencing may therefore suffer from "phylogenetic under-sampling" due to the lack of comparative functional analyses of silencing within *Saccharomyces* yeasts.

During the establishment of silencing in *S. cerevisiae*, Sir proteins are recruited to silencer elements that flank the *HML* and *HMR* silent mating-type loci via protein-protein interactions with the site-specific DNA-binding proteins ORC, Rap1, and Abf1 (Rusche et al. 2003). Sir2/3/4 complexes nucleate at silencers via interactions between Sir1 and ORC, Sir4 and Rap1, and Sir1 and Sir4. Sir2 then deacetylates neighboring nucleosomes, creating additional interaction surfaces for Sir3, which binds deacetylated histone tails via its Bromo-Adjacent Homology (BAH) domain (Onishi et al. 2007). Silent chromatin structures are then generated at *HML* and *HMR* via iterative nucleosome deacetylation and binding by Sir2/3/4 complexes (Hecht et al. 1995; Rusche et al. 2002).

The *sensu stricto* clade of *Saccharomyces* yeasts is defined by the ability of haploids from a given species to hybridize with haploids of another. The interspecies hybrid diploids can propagate mitotically, but largely fail to produce viable progeny through meiosis (Greig, 2009). To gain insight into how conserved the *S. cerevisiae* Sir-based silencing mechanism is across budding yeasts, we undertook a genetic screen for silencing-defective mutants in *S. bayanus*, a species with four paralogs of *S. cerevisiae* Sir1 (Gallagher et al. 2009) (Figure 3.1, A). *S. bayanus* is the most diverged species from *S. cerevisiae* within the *sensu stricto* clade; the level of nucleotide divergence between

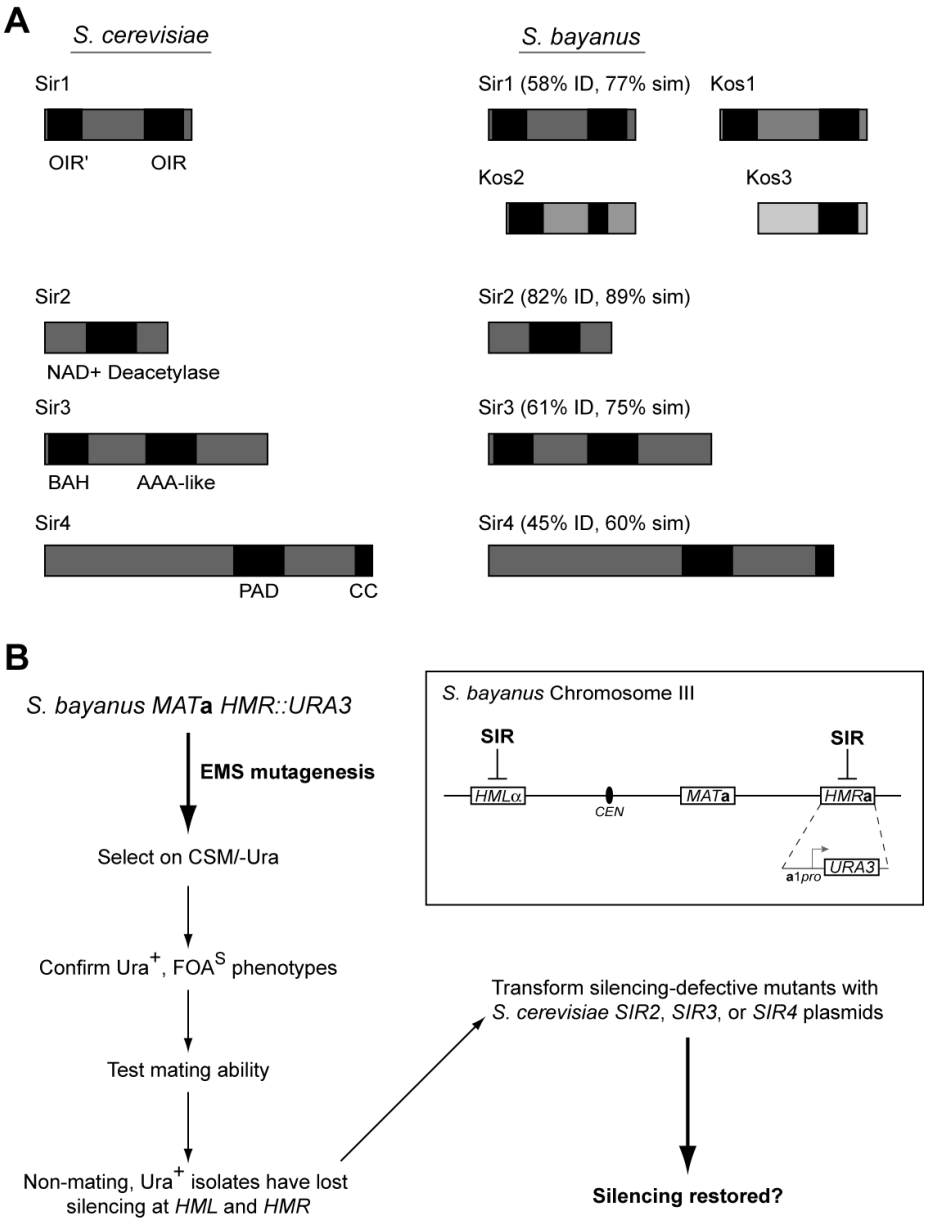

Figure 3.1. Comparative analysis of *SIR* genes in *S. cerevisiae* and *S. bayanus*. (A) Comparison of known Sir proteins in *S. cerevisiae* and *S. bayanus*. Percent identity (ID) and similarity (sim) for each orthologous pair of proteins, as determined by BLASTP alignments, is indicated above each *S. bayanus* ortholog. Black boxes indicate known domains within each protein, with domain names indicated below the *S. cerevisiae* orthologs. **OIR**, ORC-Interacting Region; **BAH**, Bromo-Adjacent Homology domain; **PAD**, Partitioning and Anchoring Domain; **CC**, Coiled-Coil. (B) Flowchart for a screen for *S. bayanus* silencing-defective mutants, and schematic of *S. bayanus* chromosome III configuration in the starting strain. The *S. bayanus HML* and *HMR* mating-type loci were hypothesized to be silenced by Sir proteins, as in *S. cerevisiae*. The strain used for the screen carried an *HMR::URA3* silencing reporter gene that was generated by replacing the *HMR***a**1 ORF with the *K. lactis URA3* ORF.

S. cerevisiae and *S. bayanus* approximates that between human and mouse (Cliften et al. 2003; Kellis et al. 2003). Given the greater diversity of Sir1 proteins present in *S. bayanus*, we reasoned that the silencing mechanism had undergone functional changes in the recent ancestry of *Saccharomyces*.

Materials and Methods

Yeast strains, culture, and genetic manipulations

Yeast strains used in this chapter are listed in Table 3.1. Growth of *S. bayanus* strains was performed using standard conditions for *S. cerevisiae*, except that plate and liquid culturing was performed at 25°C (for both species). One-step gene replacement by homologous recombination was performed using the standard PCR-based method. All gene disruptions in both species were confirmed using PCR to examine the 5′ and 3′ ends of targeted open reading frames.

Two *S. bayanus MAT***a** *hmra1Δ::K.l.URA3* strains (JRY8788 and JRY8789) were the starting strains for the mutagenesis. The construction of these strains has been described (see Chapter 4 Methods; Zill et al., *submitted*). Briefly, the *S. bayanus HMR***a**1 open reading frame was replaced with the *Kluyveromyces lactis URA3* coding sequence by homologous recombination, leaving the *HMR***a**1 promoter intact.

Hybrid diploids were created by patch mating *S. cerevisiae* strains to *S. bayanus* strains of the opposite mating type. Diploids were selected using complementation of auxotrophic markers. In cases where marker complementation was not possible, hybrids were generated by mating single cells of each species together, using a micromanipulator to position the cells adjacent to one another.

Screen for silencing-defective mutants

Two independent cultures of *S. bayanus MAT***a** *HMR::URA3* (JRY8788 and 8789) were mutagenized with EMS using a standard mutagenesis protocol (Amberg, et al. 2005). Briefly, 1mL of each stationary-phase overnight culture was harvested, washed and resuspended in 1mL of 0.1M sodium phosphate buffer (pH 7) with 30µL of ethyl methanesulfonate (EMS). After incubation at 30ºC for one hour, the cells were plated at low density (approximately 500 cells per plate) onto YPD, CSM/-Ura, and CSM/+Canavanine plates. Canavanine resistance was used to estimate mutagenesis efficacy. Colonies were allowed to form over 3 days at 25°C.

52 colonies that grew on CSM/-Ura were streaked onto another CSM/-Ura plate for single colonies, which were then patched on 5-FOA plates to confirm stable *HMR::URA3* expression. To test for loss of silencing at *HML*, we attempted to mate the mutants that were stably expressing *URA3* to an *S. cerevisiae MAT*α strain (JRY2728). Mutants that were both unable to mate and expressed *URA3* were considered silencing defective (Figure 3.1, B). For six mutants (two per complementation group), *HML*α was

Table 3.1. Yeast strains used in chapter 3. All strains were derived in the Rine lab, except JRY2726 and JRY2728, which originated in David Botstein's lab; JRY9141-9143, from Ed Louis; JRY9144, from Daniela Delneri; and JRY9145, from Chris Hittinger and Mark Johnston. The W303 wild-type strains are originally from Rodney Rothstein's lab (e.g., JRY2334 and JRY4012). For all loci in *Species X / Species Y* hybrids, allele configurations are given as *Species-X (gene) / Species-Y (gene)*.

Strain	Species	Genotype
JRY4012	*S. cerevisiae* (W303)	*MAT***a** *his3 leu2 lys2 trp1 ura3 can1*
JRY4013	*S. cerevisiae*	*MAT*α *his3 leu2 lys2 trp1 ura3 can1*
JRY2334	*S. cerevisiae*	*MAT***a** *ade2 his3 leu2 trp1 ura3 can1*
JRY3009	*S. cerevisiae*	*MAT*α *ade2 his3 leu2 trp1 ura3 can1*
JRY2726	*S. cerevisiae*	*MAT***a** *his4*
JRY2728	*S. cerevisiae*	*MAT*α *his4*
JRY8821	*S. cerevisiae*	*MAT*α *HMR::URA3 ade2 his3 leu2 lys2 trp1 ura3*
JRY8676	*S. cerevisiae*	*MAT*α *HMR::URA3 sir4Δ::HIS3 ade2 his3 leu2 trp1 ura3*
JRY9025	*S. cerevisiae*	*MAT***a** *hmlΔ::KanMX sir4Δ::HIS3 his3 leu2 lys2 trp1 ura3*
JRY4587	*S. cerevisiae*	*MAT***a** *sir2Δ::TRP1 his3 leu2 lys2 trp1 ura3*
JRY4604	*S. cerevisiae*	*MAT***a** *sir3Δ::TRP1 his3 leu2 lys2 trp1 ura3*
JRY3411	*S. cerevisiae*	*MAT***a** *sir4Δ::HIS3 ade2 his3 leu2 trp1 ura3*
JRY5323	*S. cerevisiae*	*MAT*α *hmrΔ::HIS3 ade2 his3 leu2 trp1 ura3*
JRY6754	*S. cerevisiae*	*MAT*α *HML*α *HMR*α *sir2Δ::LEU2 his3 leu2 lys2Δ trp1 ura3*
JRY8826	*S. cerevisiae*	*MAT*α *hml::URA3pr::GFP hmr::URA3pr::mCherry sir3Δ::LEU2 his3 leu2 trp1 ura3*
JRY7374	*S. cerevisiae*	*MAT*α *hmrΔ::TRP1-HMG2 sir4Δ:::LEU2 ade2 his3 leu2 lys2 trp1 ura3*
JRY8822	*S. bayanus* (CBS 7001)	*MAT***a** *hoΔ::NatMX lys2 ura3*
JRY8788	*S. bayanus*	*MAT***a** *HMR::URA3 his3 leu2Δ::NatMX lys2 ura3Δ::NatMX*
JRY8789	*S. bayanus*	*MAT***a** *HMR::URA3 his3 leu2Δ lys2 ura3Δ*
JRY8819	*S. bayanus*	*MAT*α *HMR::URA3 ade2 his3 lys2 ura3*
JRY8802	*S. bayanus*	*MAT***a** *sir2*$^{-}$ *HMR::URA3 hmlΔ::HygMX his3 leu2Δ lys2 ura3Δ*
JRY8803	*S. bayanus*	*MAT***a** *sir3*$^{-}$ *HMR::URA3 hmlΔ::HygMX his3 leu2Δ lys2 ura3Δ*
JRY8810	*S. bayanus*	*MAT***a** *sir4*$^{-}$ *HMR::URA3 hmlΔ::HygMX his3 leu2Δ lys2 ura3Δ*
JRY9136	*S. bayanus*	*MAT***a** *sir2Δ::KanMX his3 leu2Δ lys2 ura3Δ*
JRY9137	*S. bayanus*	*MAT***a** *sir3Δ::HygMX leu2Δ ura3*
JRY9138	*S. bayanus*	*MAT***a** *sir4Δ::KanMX his3 ura3*
JRY9043	*S. bayanus*	*MAT*α *HMR::URA3 sir4Δ::KanMX ade2 ura3*
JRY8820	*S. bayanus*	*MAT***a** *hmlΔ::S.p.his5 sir4Δ::KanMX his3 lys2 ura3*
JRY9046	*S. bayanus*	*MAT***a**/α *HMR/HMR::URA3 SIR4/SIR4* (JRY8822 x JRY8819)
JRY9047	*S. bayanus*	*MAT***a**/α *HMR/HMR::URA3 SIR4/sir4Δ::KanMX* (JRY8822 x JRY9043)
JRY9048	*S. bayanus*	*MAT***a**/α *hmlΔ::S.p.his5/HML HMR/HMR::URA3 sir4Δ::KanMX/sir4Δ::KanMX* (JRY8820 x JRY9043)
JRY9135	*S. paradoxus* (CBS 432)	*MAT***a** *hoΔ::NatMX lys2 ura3*
JRY9134	*S. paradoxus*	*MAT*α *hoΔ::NatMX lys2 ura3*
JRY9139	*S. paradoxus*	*MAT***a** *sir4Δ::KanMX lys2 ura3*

JRY9140	*S. paradoxus*	*MATα sir4Δ::KanMX lys2 ura3*
JRY9141	*S. paradoxus* (N-44)	*MAT***a** *hoΔ::HygMX ura3Δ::NatMX*
JRY9142	*S. paradoxus* (YPS 138)	*MAT***a** *hoΔ::HygMX ura3Δ::NatMX*
JRY9143	*S. paradoxus* (CBS 432)	*MAT***a** *hoΔ::HygMX ura3Δ::NatMX*
JRY9144	*S. mikatae* (IFO 1815)	*MAT***a** *hoΔ::loxP-Kan-loxP ura3Δ::HygMX*
JRY9145	*S. kudriavzevii* (IFO 1802)	*MAT***a** *hoΔ::NatMX trp1Δ0 ura3Δ0*
JRY9054	*S. cerevisiae*/ *S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-SIR4/Sb-SIR4* (JRY4012 x JRY8819)
JRY9146	*S. cerevisiae*/ *S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-sir2Δ::HygMX/Sb-SIR2* (JRY9054)
JRY9147	*S. cerevisiae*/ *S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-SIR2/Sb-sir2Δ::HygMX* (JRY9054)
JRY9148	*S. cerevisiae*/ *S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-sir3Δ::HygMX/Sb-SIR3* (JRY9054)
JRY9149	*S. cerevisiae*/ *S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-SIR3/Sb-sir3Δ::HygMX* (JRY9054)
JRY9055	*S. cerevisiae*/ *S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-SIR4/Sb-sir4Δ::KanMX* (JRY4012 x JRY9043)
JRY9057	*S. cerevisiae*/ *S. bayanus*	*MAT***a**/α *Sc-hmlΔ::KanMX/Sb-HML Sc-HMR/Sb-HMR::URA3 Sc-sir4Δ::HIS3/Sb-sir4Δ::KanMX* (JRY9025 x JRY9043)
JRY9058	*S. cerevisiae*/ *S. bayanus*	*MATα*/**a** *Sc-HMR::URA3/Sb-HMR Sc-SIR4/Sb-SIR4* (JRY8821 x JRY8822)
JRY9150	*S. cerevisiae*/ *S. bayanus*	*MATα*/**a** *Sc-HMR::URA3/Sb-HMR Sc-sir2Δ::HygMX/Sb-SIR2* (JRY9058)
JRY9151	*S. cerevisiae*/ *S. bayanus*	*MATα*/**a** *Sc-HMR::URA3/Sb-HMR Sc-SIR2/Sb-sir2Δ::HygMX* (JRY9058)
JRY9152	*S. cerevisiae*/ *S. bayanus*	*MATα*/**a** *Sc-HMR::URA3/Sb-HMR Sc-sir3Δ::HygMX/Sb-SIR3* (JRY9058)
JRY9153	*S. cerevisiae*/ *S. bayanus*	*MATα*/**a** *Sc-HMR::URA3/Sb-HMR Sc-SIR3/Sb-sir3Δ::HygMX* (JRY9058)
JRY9059	*S. cerevisiae*/ *S. bayanus*	*MATα*/**a** *Sc-HML/Sb-hmlΔ::S.p.his5 Sc-HMR::URA3/Sb-HMR Sc-SIR4/Sb-sir4Δ::KanMX* (JRY8821 x JRY8820)
JRY9061	*S. cerevisiae*/ *S. bayanus*	*MATα*/**a** *Sc-HML/Sb-hmlΔ::S.p.his5 Sc-HMR::URA3/Sb-HMR Sc-sir4Δ::HIS3/Sb-sir4Δ::KanMX* (JRY8676 x JRY8820)
JRY9154	*S. paradoxus*/ *S. bayanus*	*MAT***a**/α *Sp-HMR/Sb-HMR::URA3 Sp-SIR4/Sb-SIR4* (JRY9135 x JRY8819)
JRY9155	*S. paradoxus*/ *S. bayanus*	*MAT***a**/α *Sp-HMR/Sb-HMR::URA3 Sp-SIR4/Sb-sir4Δ::KanMX* (JRY9135 x JRY9043)
JRY9156	*S. paradoxus*/ *S. bayanus*	*MAT***a**/α *Sp-HMR/Sb-HMR::URA3 Sp-SIR4/Sb-sir4Δ::KanMX* (JRY9141 x JRY9043)
JRY9157	*S. paradoxus*/ *S. bayanus*	*MAT***a**/α *Sp-HMR/Sb-HMR::URA3 Sp-SIR4/Sb-sir4Δ::KanMX* (JRY9142 x JRY9043)
JRY9158	*S. paradoxus*/ *S. bayanus*	*MAT***a**/α *Sp-HMR/Sb-HMR::URA3 Sp-SIR4/Sb-sir4Δ::KanMX* (JRY9143 x JRY9043)

JRY9159	*S. mikatae*/ *S. bayanus*	*MAT***a**/α *Sm-HMR/Sb-HMR::URA3 Sm-SIR4/Sb-SIR4* (JRY9144 x JRY8819)
JRY9160	*S. mikatae*/ *S. bayanus*	*MAT***a**/α *Sm-HMR/Sb-HMR::URA3 Sm-SIR4/Sb-sir4Δ::KanMX* (JRY9144 x JRY9043)
JRY9161	*S. kudriavzevii*/ *S. bayanus*	*MAT***a**/α *Sk-HMR/Sb-HMR::URA3 Sk-SIR4/Sb-SIR4* (JRY9145 x JRY8819)
JRY9162	*S. kudriavzevii*/ *S. bayanus*	*MAT***a**/α *Sk-HMR/Sb-HMR::URA3 Sk-SIR4/Sb-sir4Δ::KanMX* (JRY9144 x JRY9043)
JRY9163	*S. cerevisiae*/ *S. paradoxus*	*MAT*α/**a** *Sc-HMR::URA3/Sp-HMR Sc-sir4Δ::HIS3/Sp-SIR4* (JRY8676 x JRY9135)

inactivated by gene targeting, and restoration of mating was verified by patch mating tests. These mutants were each mated to a wild-type *S. bayanus MAT*α strain to test dominance. The resulting diploids were sporulated, and tetrads were dissected to assay segregation of the silencing-defective phenotype (by Ura+ or non-mating phenotypes). For select *sir2*, *sir3*, and *sir4* mutants (as defined by complementation tests), the putatively mutated *SIR* gene's coding sequence was PCR-amplified using Phusion high-fidelity DNA polymerase (NEB) from genomic DNA isolated from each mutant. The resulting PCR products were sequenced to identify causative mutations.

Cross-species complementation tests

Plasmids. Rescue of mating ability in the silencing defective mutants isolated from the mutagenesis was attempted by transforming them with CEN-ARS plasmids containing either *S. cerevisiae SIR2* (pJR2025), *SIR3* (pJR2026), *SIR4* (pJR2027), *S. bayanus SIR4*, or a vector control (pRS315). After transformation, we performed patch mating tests on two or three transformants of each mutant. Restoration of mating ability after transformation of a plasmid suggested that silencing ability was restored at *HML*. In mutants that were unable to be rescued by plasmid complementation, the *HML* locus was deleted by homologous recombination. The resulting *MAT***a** *sir*$^-$ *hml*Δ strains were similarly tested for restoration of mating ability.

Interspecies hybrids. To test complementation of the EMS-induced silencing mutants, we mated *S. bayanus MAT***a** *sir*$^-$ *HMR::URA3 hmlΔ* strains to *S. cerevisiae MAT*α *hmrΔ* (JRY5323) or *MAT*α *hmrΔ sirΔ* (JRY6754, 8826, or 7374) strains of the other species. (All strains used for these analyses are listed in Table 3.1.) A similar procedure was used for the comparison to silencing in hybrids made between *S. bayanus sirΔ* strains and *S. cerevisiae* (Figure 3.3). *URA3* expression from either *Sc-HMR* or *Sb-HMR* could be readily assayed by growth of the hybrids on selective (CSM/-Ura) and counter-selective (5-FOA) media. For the panel of interspecies hybrids (Figure 3.4), a *MAT*α *HMR::URA3 sir4Δ* strain (JRY9043) was mated to a *MAT***a** *ura3* strain of each species.

Evolutionary Analyses

Orthologs were identified using HMMER3 and syntenic context (D. Scannell and M. Eisen, unpublished data). Percent identities between orthologous *S. cerevisiae* and *S. bayanus* proteins were obtained by running BLASTP with default parameters, imposing an E-value cutoff of 10^{-5}, harvesting percent identities for each high-scoring segment pair and calculating a length-weighted average. Protein alignments were produced using FSA with default parameters (Bradley et al. 2009), and DNA alignments were obtained by back translation with RevTrans (Wernersson and Pedersen 2003). All site and branch models were fit using codeml in the PAML package (Yang 2007). To test for positive selection we compared model M2a to M1a or model M8 to M7 using a Chisq-test with

two degrees-of-freedom. Posterior probabilities of $\omega > 1$ for individual codons were obtained from the Bayes Empirical Bayes output of M8 only.

For the sliding window (102bp) analyses a step-size of 3bp was used for the *sensu stricto* and 30bp for the *Torulaspora.* For each window we used codeml to estimate a single ω using model M0 implemented in codeml. Results are reported in the coordinates of the *S. cerevisiae* and *T. delbrueckii* sequences respectively.

Population genetic analyses

Polymorphism data for *S. cerevisiae* and *S. paradoxus* were downloaded from http://www.sanger.ac.uk/Teams/Team118/sgrp/. For each gene we discarded all strains with coverage at <50% of bases and discarded codons with coverage (all three positions) in <50% of strains. We also discarded codons at which the reference strain (S288c in the case of *S. cerevisae*) was aligned to its ortholog in *S. paradoxus* with an FSA accuracy score of <5. Finally, codons with SNPs at multiple positions, SNPs with a Phred score of <30 and low frequency SNPs (<10% of accepted strains) were discarded. We then counted synonymous and nonsynonymous SNPs using custom PERL scripts.

Results

A screen for silencing-defective mutants in S. bayanus

To determine which genes were required for silencing in *S. bayanus*, we performed a genetic screen using two independent phenotypic criteria to identify mutants. An *HMR::URA3* silencing reporter was generated by replacing the *HMR***a**1 ORF with the *K. lactis URA3* ORF, such that *URA3* expression would be driven by the normally silent **a**1 promoter (Figure 3.1, B). An *S. bayanus MAT***a** *HMR::URA3* strain was treated with EMS, and *URA3* expression was selected by plating the mutagenized cells on solid medium lacking uracil (Figure 3.1, B). 52 Ura+ colonies were obtained from two independent mutageneses, and these were subsequently screened for FOA sensitivity and their ability to mate. Of the 52 Ura+ isolates, 26 were FOA sensitive. The FOA-resistant colonies were likely to include mutants with weak silencing defects, and were not studied further. In contrast, we reasoned that Ura+, FOA-sensitive mutants that could not mate likely had derepressed *HML*α in addition to *HMR::URA3*, as expected from the behavior of *sir* mutants in *S. cerevisiae*. Although the Ura+ isolates that could still mate might have had *HMR*-specific silencing mutations, they were not studied further since we anticipated that cells that had undergone a heterothallic mating-type switch leading to expression of *URA3* from *MAT* would have the same phenotype. 23 of the 26 FOA-sensitive mutants showed strong mating defects, and these 23 were selected for complementation analysis, as described below and summarized in Table 3.2.

To evaluate whether the screen had specifically identified silencing-defective mutants, we performed additional tests on a subset of the mutants. To test whether loss of mating depended on expression of the α1 and α2 genes from *HML*, this locus was

Table 3.2. Statistics of a screen for silencing-defective mutants in *S. bayanus*.
(*) Note that six mutants were complemented only very weakly by a plasmid bearing *S. cerevisiae SIR4*.

Phenotype criteria	Number of putative mutants		*S. cerevisiae SIR* gene on plasmid	Number of mutants complemented
Ura+	52		*SIR2*	6
Ura+, FOA^S	26		*SIR3*	5
Ura+, FOA^S, Non-mating	23		*SIR4*	6*
			(Not complemented)	6

deleted in six mutants representing all three complementation groups (see below). In all six cases, mating was restored. The six *MAT***a** *HMR::URA3 hmlΔ sir-* strains were mated to a wild-type *MAT*α strain for dominance tests and then sporulated for segregation analysis. All six mutants were recessive, as the resulting diploids were FOA-resistant and Ura-. Tetrad analysis on the resulting meiotic products showed 2 Sir+ : 2 Sir- segregation in at least 20 tetrads analyzed from each cross, with no apparent linkage to *MAT* or *HMR* (data not shown). Thus, single genes had likely been inactivated in each of these mutants.

Cross-species complementation to identify mutant SIR genes

Given the stringent selective criteria imposed, the leading candidates for genes identified by the screen were *S. bayanus SIR2*, *SIR3*, and *SIR4*. *S. cerevisiae* and *S. bayanus* share ~99% of genes, and most orthologs have high sequence identity (83% genome-wide average; (Cliften et al. 2006)). Thus, it is generally assumed that the vast majority of these species' orthologs perform identical functions. We attempted to complement the *S. bayanus sir* mutants with plasmids bearing *S. cerevisiae SIR* genes, using restoration of mating ability to assay complementation. To a first approximation, the plasmids bearing origins of replication and centromeres from *S. cerevisiae* work sufficiently well in *S. bayanus* for such purposes. (We distinguish between the two species' orthologs using the nomenclature *Sc-SIR* and *Sb-SIR*.) As most of the EMS-induced silencing-defective mutants behaved identically to "clean deletion" *S. bayanus sirΔ* mutants in these complementation assays, for simplicity I show only the data for the *sirΔ* mutants in Figure 3.2.

Of the 23 mutants, six were complemented by *Sc-SIR2* and five by *Sc-SIR3* (Table 3.2). The remaining twelve mutants showed either no mating when transformed with any *Sc-SIR* gene, or very slight but reproducible improvements in mating when transformed with *Sc-SIR4*. Importantly, mating was restored upon deletion of *HML* for two mutants selected from the twelve that were not complemented by *S. cerevisiae SIR* genes (data not shown), suggesting either that silencing in *S. bayanus* had a novel requirement, or that there was an incompatibility in cross-species complementation by Sir4.

The ability of the *sensu stricto* species to mate and form mitotically stable hybrid diploids offers a convenient way to use the well-developed genetics of *S. cerevisiae* to assign mutations in *S. bayanus* to genes by complementation tests in interspecies hybrids. Therefore, we mated the *S. bayanus* silencing-defective mutants with *S. cerevisiae sirΔ* mutants to attempt to identify the mutations that had not been complemented by *Sc-SIR* plasmids. The *S. bayanus* mutants fell into three groups, based on their patterns of complementation by *S. cerevisiae sirΔ* strains, as inferred from *Sb-HMR::URA3* silencing ability in the resulting hybrids (Figure 3.2, C). In the first mutant group, the resulting hybrids had robust *Sb-HMR::URA3* silencing except those made using a *S. cerevisiae sir2Δ* strain. In the second group, all hybrids had robust *Sb-HMR::URA3* silencing except those made using a *sir3Δ* strain. In the third group, which contained the 12

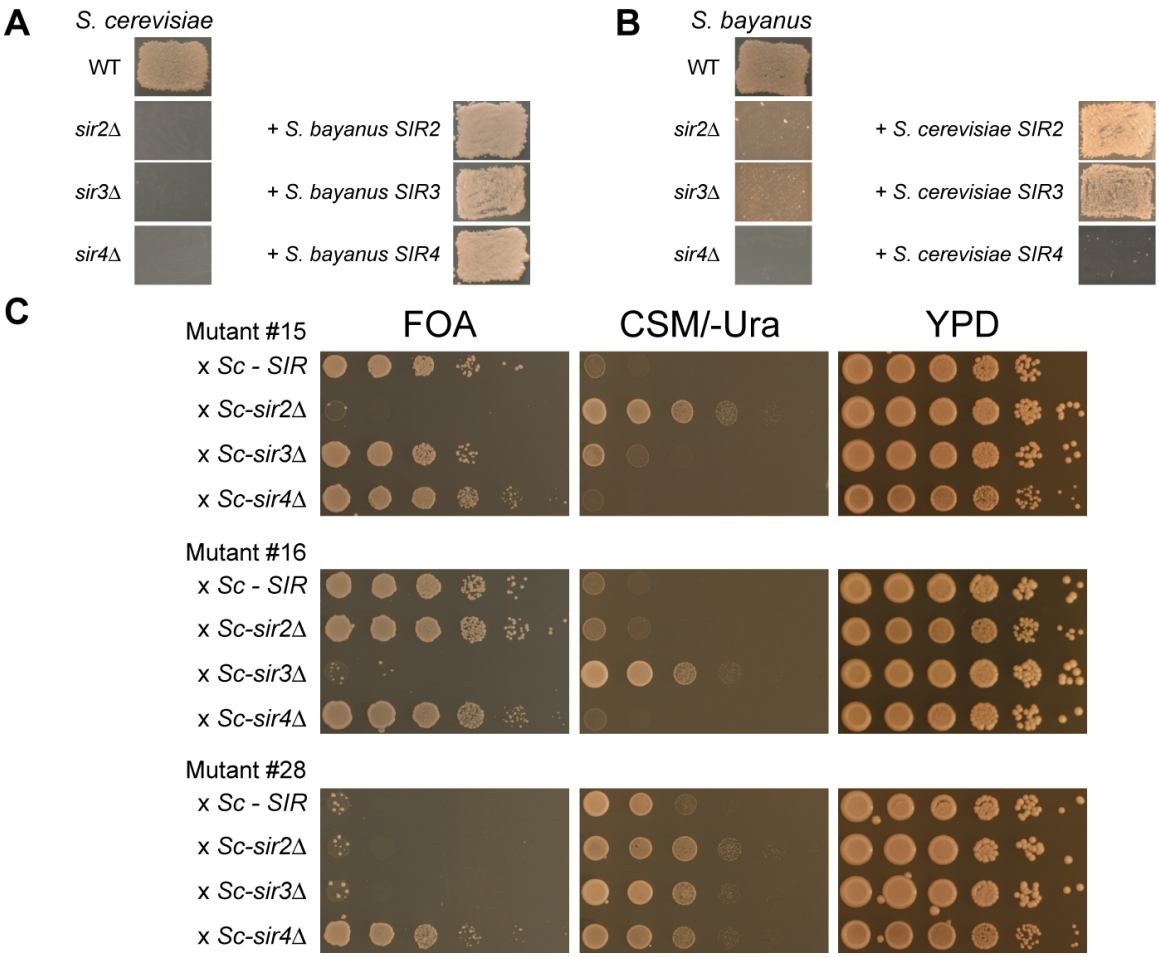

Figure 3.2. Cross-species complementation analysis of *SIR2*, *SIR3*, and *SIR4* between *S. cerevisiae* and *S. bayanus*. (A) Conservation of silencing function across species was measured by patch mating assays. Growth of a patch of yeast cells is proportional to the level of silencing. Single-copy plasmids bearing *S. bayanus SIR* genes were introduced into *S. cerevisiae MAT***a** strains of the genotypes given at left (WT, wild type). (B) Plasmids bearing *S. cerevisiae SIR* genes were introduced into *S. bayanus MAT***a** strains of the genotypes given at left. (C) **Cross-species complementation tests to define mutant silencing genes in *S. bayanus*.** Silencing-defective *S. bayanus* mutants (having the *Sb-HMR::URA3* reporter) were mated to *S. cerevisiae* wild-type (*Sc-SIR*), *sir2Δ*, *sir3Δ*, or *sir4Δ* strains. The resulting hybrid diploids were then plated onto various media in ten-fold dilutions to assay silencing ability. Growth on FOA but not on CSM/-Ura indicates robust silencing ability. The three mutants shown are representative of the three types of complementation behaviors that were observed for all mutants. Note the general non-complementation of Mutant #28 (*sir4*$^{-}$). The modest FOA resistance observed for the (Mutant #28 x *Sc-sir4Δ*) hybrid diploid is as yet unexplained.

mutants that were weakly complemented by the *Sc-SIR4* plasmid or not complemented at all, the resulting hybrids—even those made with *S. cerevisiae* wild-type strains—showed no silencing ability. Thus, although these 12 (presumably independent) mutations appeared recessive in *S. bayanus* diploids, they appeared dominant in *S. cerevisiae/S. bayanus* interspecies hybrid diploids. Notably, all 12 of these mutants behaved similarly in the interspecies hybrid complementation test.

We next compared *Sb-HMR::URA3* silencing ability in hybrids made with *S. bayanus sirΔ* mutants to the EMS-induced silencing-defective mutants. When *Sb-sir2Δ* and *Sb-sir3Δ* mutants were crossed to an *S. cerevisiae* wild-type strain, the resulting hybrids were FOA-resistant and Ura-, demonstrating that genomic *Sc-SIR2* and *Sc-SIR3* could silence *Sb-HMR::URA3* in the hybrids, as expected from the plasmid complementation analysis (Figure 3.3). When these same mutants were crossed to *S. cerevisiae sir2Δ* and *sir3Δ* strains, respectively, the resulting hybrids were FOA-sensitive and Ura+. Similarly, *Sb-sir2Δ* and *Sb-sir3Δ* mutations were not complemented by *Sc-sir2*Δ and *Sc-sir3*Δ mutations, respectively (data not shown).

Given the weak complementation of six of the 12 unknown *S. bayanus* silencing-defctive mutants by the *Sc-SIR4* plasmid, it was possible that these strains harbored mutations in *Sb-SIR4* that, for some reason, could not be complemented by *Sc-SIR4*. Thus, we cloned the *Sb-SIR4* gene and found that a plasmid carrying *Sb-SIR4* restored mating to all 12 remaining *sir* mutants (data not shown). Thus, these mutants were presumed to carry *sir4* mutations, as established below, yet their silencing defects could not be complemented by *Sc-SIR4*. To provide an independent evaluation of the mutations assigned to *S. bayanus SIR* genes by complementation studies, we isolated genomic DNA and sequenced the *SIR2*, *SIR3*, or *SIR4* gene from two independent isolates in each complementation group. In each of these mutants, a single nonsense mutation was identified in the *SIR* gene that had been deduced to be inactivated (Table 3.2). Thus, the results of the genetic screen suggested that *S. bayanus* repressed *HML* and *HMR* using the same core silencing proteins—Sir2, Sir3, and Sir4—as *S. cerevisiae*. Remarkably, however, *Sc-SIR4* could not complement any of the twelve *S. bayanus sir4* mutations identified by our screen. A genetic analysis of the contribution of *Sb-SIR1* and its paralogs has been presented elsewhere (Gallagher et al. 2009).

A genetic incompatibility between S. cerevisiae SIR4 and S. bayanus HML and HMR

To understand the genetic basis for *Sc-SIR4's* failure to complement *Sb-sir4* mutants, I extended the plasmid and hybrid complementation tests using *S. bayanus sir4Δ* null mutants. The *Sc-SIR4* plasmid failed to restore silencing to *S. bayanus sir4Δ* mutants (Figure 3.2, A; data not shown for *Sb-HMR*). Therefore Sc-Sir4 could not silence *Sb-HML* and *Sb-HMR*, and was not simply unable to complement the *Sb-sir4* point mutants, eliminating the unlikely possibility that the point mutants were somehow dominant antimorphs for Sc-Sir4. The *SIR4 – HML/HMR* incompatibility showed an interesting evolutionary asymmetry, as *Sb-SIR4* restored silencing to *HML* and *HMR* in *S. cerevisiae* (Figure 3.2, B; data not shown for *Sc-HMR*). Likewise, plasmids bearing *Sb-SIR2* or *Sb-*

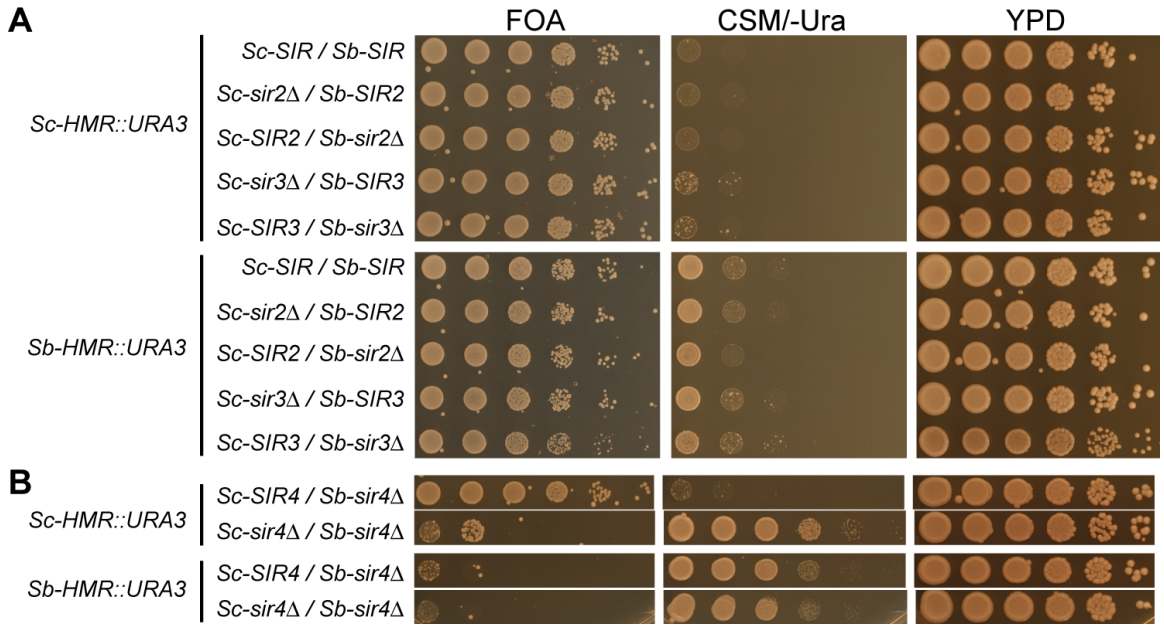

Figure 3.3. Cross-species complementation analysis of *sir2Δ*, *sir3Δ*, and *sir4Δ* deletion mutants in *S. cerevisiae*/*S. bayanus* interspecies hybrids. (A) Cross-species complementation analysis of *sir2Δ* and *sir3Δ* mutations in *S. cerevisiae/S. bayanus* hybrid diploids. **Top panel:** Ten-fold serial dilutions of hybrid strains bearing a *URA3* reporter gene at the *S. cerevisiae HMR* locus (*Sc-HMR::URA3*) were grown on medium counter-selective for *URA3* expression (FOA), selective for *URA3* expression (CSM/-Ura), or rich medium (YPD). Genotypes of both species' *SIR2* or *SIR3* genes are indicated at left. **Bottom panel:** Hybrid strains bearing a *URA3* reporter gene at the *S. bayanus HMR* locus (*Sb-HMR::URA3*), with genotypes of both species' *SIR2* or *SIR3* genes indicated at left. (B) Cross-species complementation analysis of *sir4Δ* mutations in *S. cerevisiae/S. bayanus* hybrid diploids. Top and bottom panels show *HMR* reporters as in (A), with *Sc-sir4Δ/Sb-sir4Δ* mutants shown as complete-loss-of-silencing controls.

SIR3 restored silencing to the *S. cerevisiae sir2Δ* or *sir3Δ* mutants, respectively (Figure 3.2, A).

We constructed two interspecies hybrid strains in which the *URA3* reporter gene was expressed from either the *Sc-HMR***a**1 or *Sb-HMR***a**1 promoter, allowing us to compare the effects on both species' silencing in the absence of either *Sc-SIR4* or *Sb-SIR4*. In *Sc-SIR4/Sb-sir4Δ* hybrids, *Sb-HMR* was not silenced, whereas *Sc-HMR* was silenced (Figure 3.3, B). Importantly, the silencing of *Sc-HMR* in these hybrids established that the hybrid diploid environment did not inhibit Sc-Sir4 expression or function. Thus, in both *S. bayanus* and *S. cerevisiae/S. bayanus* hybrids, Sc-Sir4 was strongly defective in performing its silencing functions at *Sb-HML* and *Sb-HMR*, whereas Sb-Sir4 was capable of silencing *Sc-HML* and *Sc-HMR*.

A trivial possible explanation of the silencing incompatibility was that *Sb-HML* and *Sb-HMR* silencing required a higher level of Sir4 protein than did *Sc-HML* and *Sc-HMR*. The levels of *SIR4* transcripts and Sir4 proteins were equivalent in *S. cerevisiae* and *S. bayanus* (see Chapter 4, Figure 4.1; Zill, et al., *submitted*). Thus the incompatibility was likely due either to an activity of Sir4 that differed between the two species, or to a restriction of Sc-Sir4 function at *Sb-HML* and *HMR*, presumably due to some difference in silencer function. The incompatibility could not be explained by a dysfunction of interspecific heterotypic Sir2/3/4 complexes formed at *Sb-HML* and *HMR*, as it was specific to Sc-Sir4 in both the plasmid and hybrid complementation assays (Figure 3.2, B; Figure 3.3, A and B). Similar *Sb-HMR* silencing defects were observed in hybrids made between *S. bayanus sir4Δ* and *S. cerevisiae* strains W303 (our standard laboratory strain), S288c, Σ1278b, or RM11-1A (data not shown). I have recently shown that the asymmetric complementation of Sir4 across species is due to co-evolution of Sir4 with silencers, such that the *S. bayanus* silencers have a restrictive property that permits only certain species' Sir4 proteins to associate with them, whereas the *S. cerevisiae* silencers are more permissive (see Chapter 4; Zill et al., *submitted*). In the remainder of this chapter, I focus on the origin of this species-specific restriction to Sir4 function.

Phylogenetic mapping of the SIR4 – HMR incompatibility

The two-species comparative genetic analysis identified a substantial alteration in the silencing mechanism, but did not address how and when this difference evolved. To determine the lineage in which the changes in *SIR4* resulting in the incompatibility occurred, I asked whether the ancestral *SIR4* was capable of silencing *Sb-HML* and *Sb-HMR*. The *K. lactis SIR4* gene is able to complement *S. cerevisiae sir4Δ* mutants, and was used as a proxy for the ancestral *SIR4* of the *Saccharomyces* complex species (Astrom and Rine 1998; Hickman and Rusche 2009). A plasmid bearing *K. lactis SIR4* was capable of restoring silencing to *Sb-HML* and *Sb-HMR*, although it did so to a slightly lesser extent than a plasmid bearing *Sb-SIR4* (Figure 3.4, A). *Sc-SIR4* is not able to complement *K. lactis sir4Δ* mutants (Astrom and Rine 1998). Thus, the relevant functional changes in *SIR4* occurred along the branch leading to *S. cerevisiae*, after its divergence from *S. bayanus*.

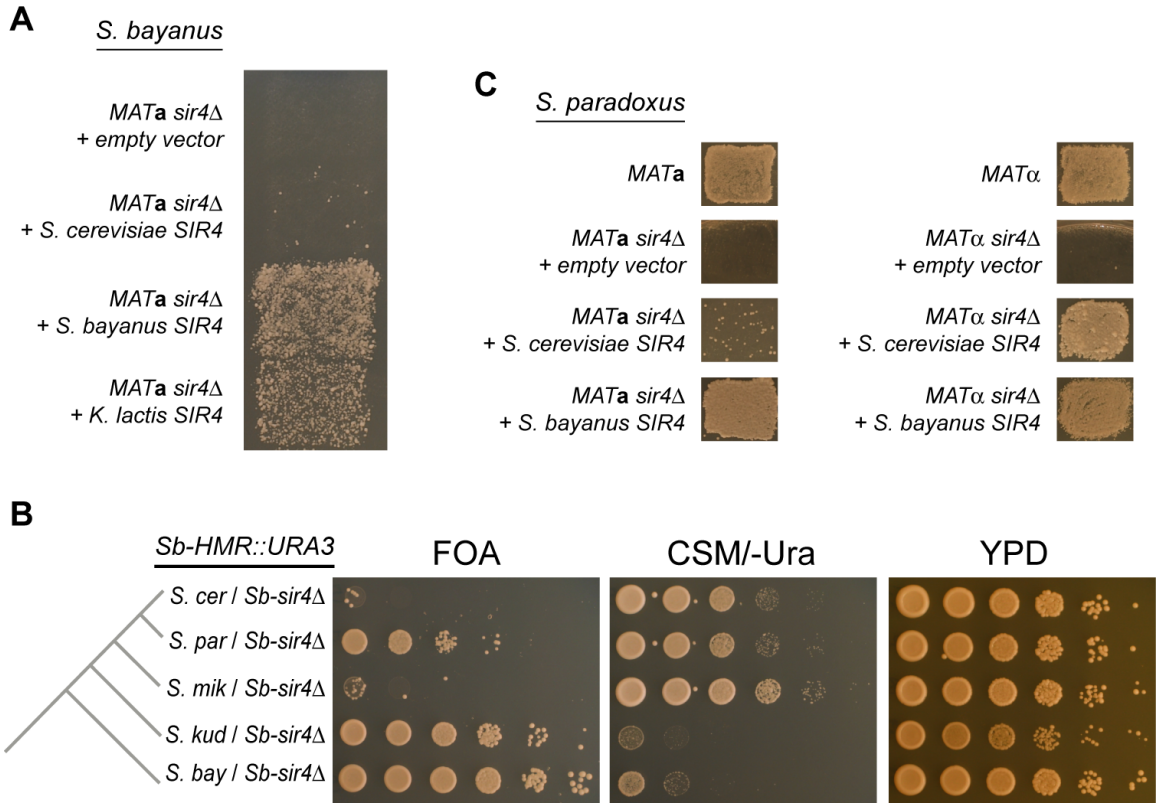

Figure 3.4. Evolutionary genetic analysis of functional changes in *SIR4*. (A) Cross-species complementation analysis of *S. bayanus sir4Δ*. Single-copy plasmids, each bearing a *SIR4* gene from the indicated species, were introduced into an *S. bayanus MAT***a** *sir4Δ* strain, which was then assayed for mating ability. Growth of a patch of yeast cells is proportional to the level of silencing at *S. bayanus HML*. (B) Functional character mapping of *S. bayanus sir4Δ* complementation using *Saccharomyces* interspecies hybrid diploids. Ten-fold serial dilutions of hybrid strains were grown on various media as in Figure 3.2(C). Genotypes of the five hybrid strains are shown at the left, with each strain having *S. bayanus HMR::URA3 sir4Δ* as one parent, and a wild-type strain of another species as the other. Species definitions: *S. cer*, *S. cerevisiae*; *S. par*, *S. paradoxus*; *S. mik*, *S. mikatae*; *S. kud*, *S. kudriavzevii*; *S. bay, S. bayanus*. (C) Cross-species complementation analysis of *S. paradoxus sir4Δ* strains. Single-copy plasmids, either empty vector or bearing a *SIR4* gene from the indicated species, were introduced into *S. paradoxus MAT***a** *sir4Δ* or *MATα sir4Δ* strains, which were then assayed for mating ability. Growth of a patch of *MAT***a** or *MATα* cells is proportional to the level of silencing at *S. paradoxus HML* or *HMR*, respectively.

To map the changes more finely onto the *sensu stricto* tree, we generated a panel of hybrid diploids by mating the *S. bayanus HMR::URA3 sir4Δ* strain to each of the four other *sensu stricto* species, and measuring the ability of the other species' Sir4 proteins (and potentially additional silencing proteins) to silence *Sb-HMR* in the hybrids. The *S. mikatae*/*S. bayanus* hybrid showed a strong silencing defect, suggesting that the *S. mikatae* Sir4 protein could not function on *S. bayanus* silencers (Figure 3.4, B). In all cases, robust silencing was observed in hybrids with both species' *SIR4* alleles intact (Figure 3.5, A). In contrast, the *S. kudriavzevii*/*S. bayanus* hybrid showed robust silencing, equivalent to the *S. bayanus SIR4*/*sir4Δ* diploid. Thus, a species that diverged from *S. bayanus* as long ago as did *S. cerevisiae* maintained the ability to complement *S. bayanus sir4Δ*. These results suggested that a significant evolutionary event occurred along the branch leading to *S. mikatae*, *S. paradoxus*, and *S. cerevisiae*, generating striking functional divergence of silencing mechanisms within the *sensu stricto* clade.

Interestingly, *S. paradoxus*/*S. bayanus* hybrids showed an intermediate level of *Sb-HMR* silencing (Figure 3.4, B; Figure 3.5, B). This result suggested either that part of the ability to silence *Sb-HMR* had been independently lost in *S. cerevisiae* Sir4 and *S. mikatae* Sir4, or that *S. paradoxus* Sir4 had re-gained, in part, the ability to silence *Sb-HMR*. If *S. paradoxus* had retained some *S. bayanus*-like silencing features, an intriguing prediction of the partial compatibility of *S. paradoxus* silencing proteins with*Sb-HMR* was that there might exist an incompatibility between *S. cerevisiae* silencing proteins and *Sp-HML* and *Sp-HMR*. (The underlying logic is that the *S. bayanus sir4* hybrid complementation data reflect the character of intra-species genetic interactions between *SIR4* and silencers for each species.) To test this idea, we deleted the *S. paradoxus SIR4* gene and tested the ability of plasmids bearing either *Sc-SIR4* or *Sb-SIR4* to complement this mutant. Although the *Sb-SIR4* plasmid restored full silencing to both *Sp-HML* and *Sp-HMR*, the *Sc-SIR4* plasmid restored silencing to *Sp-HMR* but only very weakly to *Sp-HML* (Figure 3.4, C). In a reciprocal experiment using an *S. cerevisiae/S. paradoxus* hybrid diploid, we determined that *Sp-SIR4* was able to complement the *S. cerevisiae sir4Δ* mutant for *Sc-HMR* silencing (Figure 3.5, C). Thus, the Sp-Sir4 protein appeared to have an interspecies compatibility range that was intermediate between those of Sc-Sir4 and Sb-Sir4. In correlation with the "intermediate" character of Sp-Sir4, the *Sp-HML* locus retained the *S. bayanus*-like character of incompatibility with Sc-Sir4, whereas *Sp-HMR* was fully compatible with Sc-Sir4.

Rapid evolution of SIR4 by two distinct selection regimes

To better understand the *SIR4* functional divergence, we examined in more detail the sequence and evolution of *SIR4*. By multiple measures, the Sir4 protein was among the most rapidly evolving proteins in *sensu stricto* yeasts. In BLASTP alignments, Sc-Sir4 and Sb-Sir4 were only 45% identical compared to a genome-wide average of 83% (Figure 3.6, A). Of 4981 *S. cerevisiae/S. bayanus* ortholog pairs examined, only 19 showed lower sequence identity than Sir4.

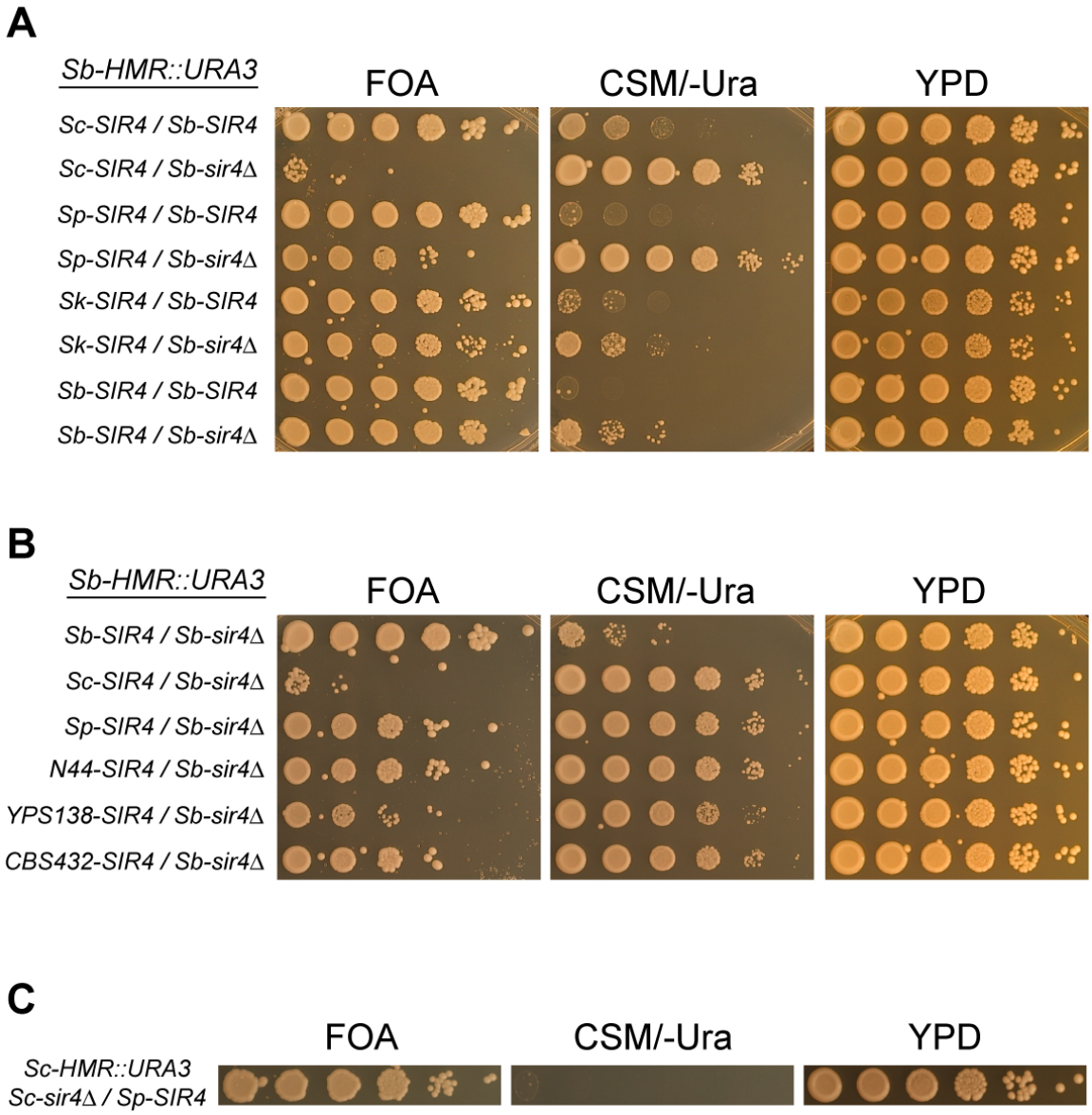

Figure 3.5. Interspecies hybrid complementation analysis of *Sb-HMR* and *Sc-HMR* silencing. (A) *Sb-HMR* remained silenced in all *SIR4/SIR4* interspecies hybrids (species designations as in Figure 3.3, B). (B) Intermediate levels of *Sb-HMR* silencing in hybrids made between *S. bayanus sir4Δ* and wild-type *S. paradoxus* strains representing the three major subspecies (Cubillos et al. 2009; Liti et al. 2009). Subspecies representative strains and places of origin are: N44, Far East; YPS138, Pennsylvania; CBS432, Russia. (C) Robust silencing of *Sc-HMR* by *Sp-SIR4* in an *S. cerevisiae/S. paradoxus* hybrid.

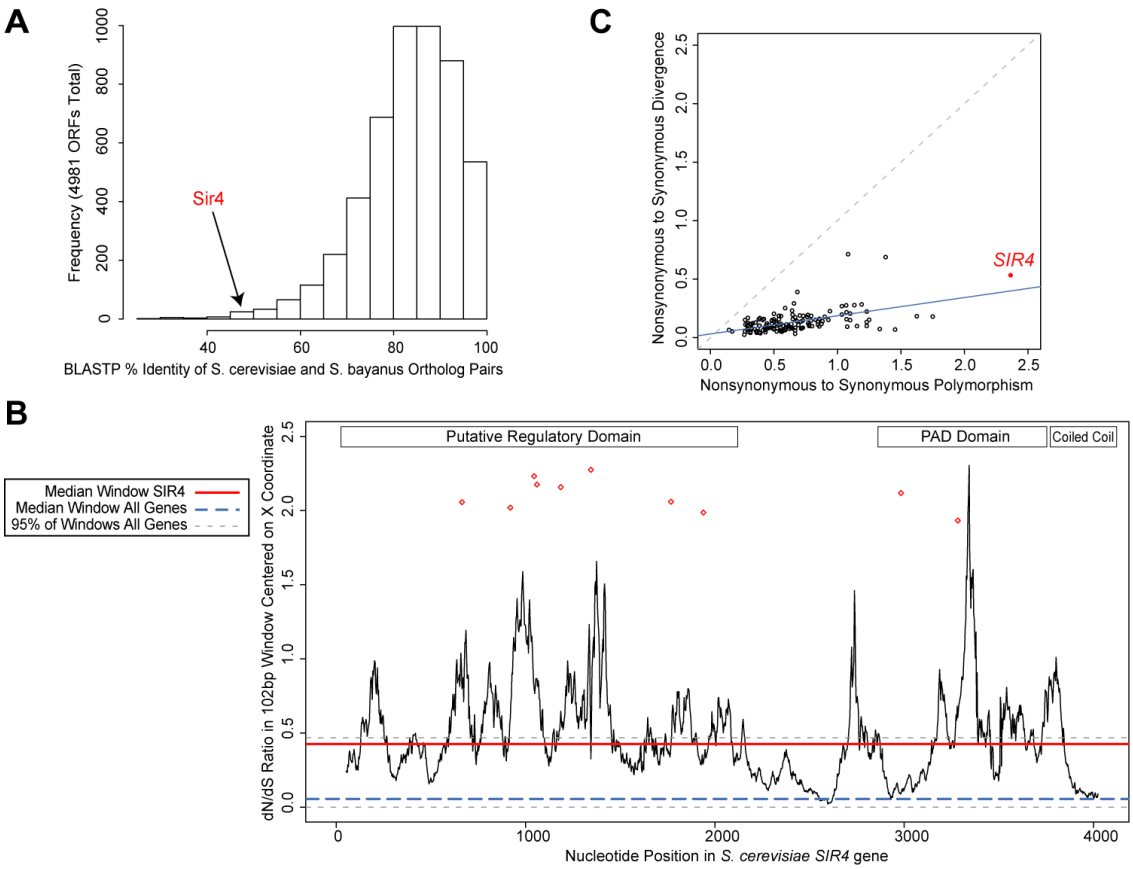

Figure 3.6. Elevated nonsynonymous divergence and polymorphism at the *SIR4* locus in *Saccharomyces* species. (A) Histogram shows percent identities of orthologous *S. cerevisiae* and *S. bayanus* proteins based on BLASTP alignments. The distribution of percent identity of orthologous protein pairs, in bins of five-percent increments, is plotted versus the number of orthologous pairs in each bin. The bin containing Sir4 (45% identity) is indicated with an arrow. (B) Ratios of nonsynonymous to synonymous divergence (ω) computed in 102bp windows every 3bp along the *S. cerevisiae SIR4* gene. Horizontal lines show the value of ω for the median *SIR4* window (thick solid line), the median window of all genes (thick dashed line) and the limits within which 95% of all windows fall (thin dashed line). Diamonds indicate codons having a probability of (ω >1) ≥ 70%. Labelled boxes indicate the locations of functional domains. (C) Plot of the ratio of nonsynonymous to synonymous divergence (ω) against the ratio of nonsynonymous to synonymous polymorphism for *SIR4* and 150 genes with lengths similar to *SIR4*. Low frequency polymorphisms (< 0.1) were excluded. The solid line shows a linear regression of *y* on *x* and the dashed line shows a slope of 1.

Rapid sequence divergence can be driven by mutation pressure (Mower et al. 2007), inefficient repair (Teytelman et al. 2008), biased gene conversion (Galtier and Duret 2007), or selection. To distinguish among these possible explanations we aligned *SIR4* coding sequences from *S. cerevisae, S. bayanus, S. mikatae* and *S. paradoxus* and computed the ratio of nonsynonymous to synonymous divergence (henceforth ω) across the whole gene. Because ω normalizes the rate of nonsynonymous substitution to the (presumed neutral) rate of synonymous substitution, it controls for variation in mutation rate and repair and can be considered a measure of selective constraint. The value of ω for *SIR4* was 0.44, substantially higher than the genomic average of 0.10. Only 16 of 4975 loci we analyzed had a higher ω, indicating that the rapid change at the *SIR4* locus was not simply a consequence of an elevated mutation rate or inefficient repair. Biased gene conversion usually involves preferential A-T → G-C base-pair substitutions (Birdsell 2002). Biased gene conversion was unlikely to be responsible for the rapid *SIR4* sequence change because the rates of strong-to-weak (G-C → A-T) and weak-to-strong (A-T → G-C) substitutions at *SIR4* were similar to those at other loci (data not shown). Taken together, these data indicated that the sequence and functional divergence at the *SIR4* locus was the result of either relaxed purifying selection or positive selection.

A value of ω significantly greater than 1 is evidence of positive selection (Yang 1998). Therefore, a value of 0.44 might suggest that the *SIR4* coding region did not evolve under positive selection. However, because Sir4 is a large protein we investigated whether sub-regions or individual codons might have $\omega > 1$. To determine whether rapidly evolving Sir4 residues might lie within known functional regions of the protein, we computed ω in 102bp (34-codon) windows throughout the *SIR4* open reading frame (Figure 3.6, B). Consistent with our previous whole-gene estimate, the median ω value for all windows in *SIR4* was 0.43 (solid horizontal line in Figure 3.6, B) with a range from 0.02 to 2.30. Because ω estimates calculated in short windows are subject to stochastic noise, we compared the results of this analysis to a sample of 10,000 102bp windows drawn from other *S. cerevisiae* coding regions. The median of these ω values was 0.06 and 95% of windows lie between 0.0001 and 0.47 (dashed lines in Figure 3.6, B). These comparisons supported two conclusions. First, because the median ω for *SIR4* was comparable to the most extreme values in other genes, the unusual molecular evolution of this gene extended over a large fraction of the gene. Second, the non-random distribution of windows with high ω suggested that the rapid evolution of certain residues was connected to functional changes within specific regions of the Sir4 protein. Although the Rap1- and Sir3-binding coiled-coil domain was largely protected from the rapid evolution of *SIR4*, residues within the PAD (Partioning And Anchoring of plasmids) domain and especially the putative N-terminal regulatory domain showed striking signatures of rapid evolution (Figure 3.6, B) (Moazed et al. 1997).

To provide an independent, statistically robust analysis of *SIR4* evolution in this clade, we used a likelihood ratio test to compare nested models of sequence evolution that either allowed or did not allow a subset of codons to have a value of $\omega > 1$. The model allowing $\omega > 1$ fit the data significantly better than the alternative model ($P = 7 \times 10^{-5}$; see Methods), indicating that some codons were likely to be evolving under positive selection. Posterior probabilities indicated that 27 codons may have had $\omega > 1$ and for

seven of these the best estimate of ω was > 2. However, for no single codon did the posterior probability exceed the nominal significance level of 95%. Inclusion of additional *SIR4* orthologs did not alter these results; inclusion of *SIR4* sequences from species outside the *sensu stricto* was not possible because of poor alignment quality. In summary, although we were not able to identify specific codons that were unambiguously under positive selection, these data suggested that multiple codons within *SIR4*, some of which were within the PAD and N-terminal regulatory domains, were targets of positive selection in the *Saccharomyces sensu stricto* clade.

To determine the time period over which *SIR4* was subject to strong selection pressures, we divided the problem into three parts: individual branches of the *sensu stricto* tree, extant populations (*i.e.*, modern *S. cerevisiae*), and deep evolution. To examine variation in selection pressures across the *sensu stricto* tree, we fit models that allowed different branches to have different values of ω. Although increased estimates of ω were obtained for some branches (notably the shared *S. cerevisiae/S. paradoxus* branch; ω = 0.63) none were statistically supported, suggesting that there have been no dramatic shifts in the selection pressures operating on *SIR4* since the divergence of the *sensu stricto*. We caution however that a change in selection pressure that affected only a subset of codons could have gone undetected.

We next examined population genetic data for the *SIR4* locus generated by the *Saccharomyces* Genome Resequencing Project (Liti et al. 2009). Following the McDonald-Kreitman test (McDonald and Kreitman 1991), we compared ω (the ratio of nonsynonymous to synonymous divergence) to the ratio of nonsynonymous to synonymous polymorphism. The ratio of nonsynonymous to synonymous polymorphism for *SIR4* (~ 2.4) was both more extreme than ω and more extreme than that of length-matched control genes (Figure 3.6, C). We also compared *SIR4* to other loci from the same syntenic region of the genome (right arm of chromosome IV) and to other rapidly evolving loci (Figure 3.7). In all cases *SIR4* was an outlier. Note that we excluded low frequency alleles when counting SNPs, but that the same qualitative result was obtained when all SNPs were accepted. In addition, we obtained the same results when performing these analyses with population genetic data from *S. paradoxus* (Figure 3.8). These results suggested that *SIR4* may be under diversifying selection in modern populations of *S. cerevisiae* and *S. paradoxus*. (Note that "positive selection" refers to a situation in which one phenotype is favored (e.g., increased height is beneficial). "Diversifying selection" refers to the case where multiple extreme phenotypes but not their intermediates are favored (e.g., tall and short, but not average height, people have an advantage).) We noticed that there was a modest overlap between sites that are polymorphic within *S. cerevisiae* and sites that are diverged between *S. cerevisiae* and *S. bayanus* (P = 0.05 by Hypergeometric; Figure 3.9). Thus, it was possible that during the evolution of the *sensu stricto* some portions of *SIR4* have been consistent targets of diversifying selection.

Finally, to investigate *SIR4* evolution over a long time scale, we examined an alignment of *SIR4* sequences from three *Torulaspora* species (D. Scannell and M. Eisen unpublished). The sequences of the *Torulaspora SIR4* genes were so diverged from the *Saccharomyces SIR4* genes that robust alignments were not possible. Nevertheless, the

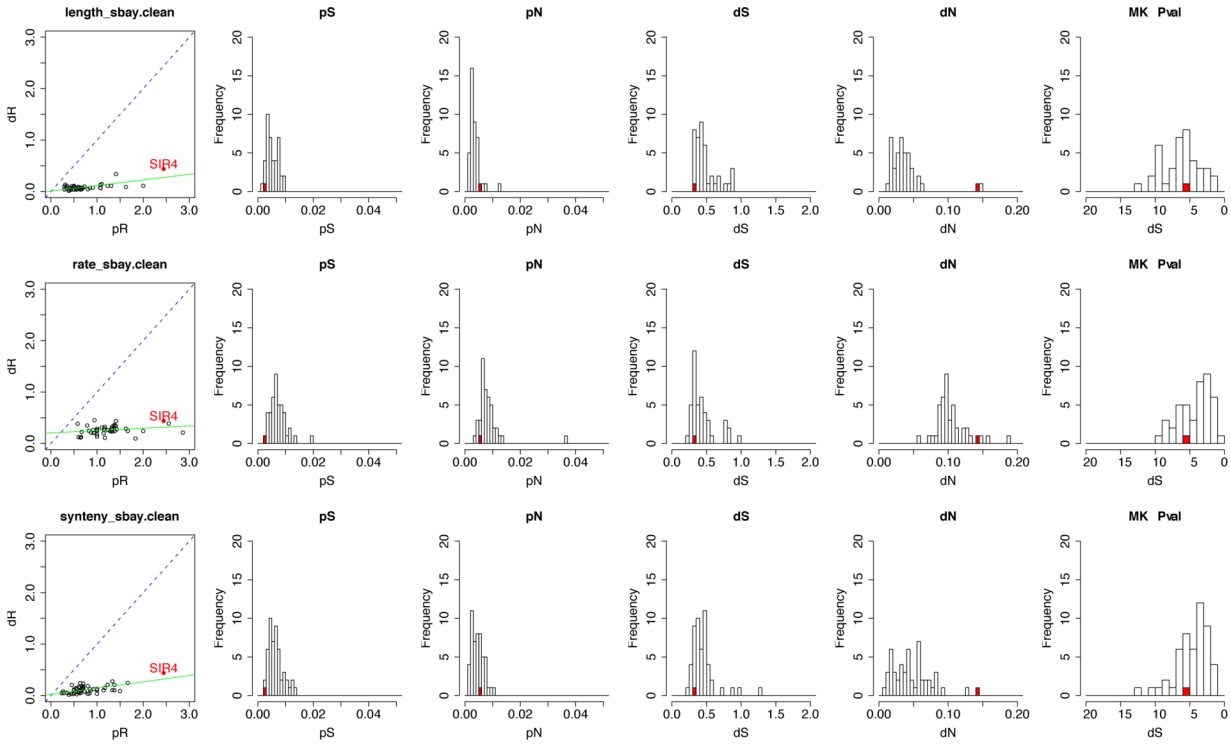

Figure 3.7. McDonald-Kreitman analyses of *SIR4* and control loci in *S. cerevisiae* populations. Nonsynonymous and synonymous divergence and polymorphism for *SIR4* (red) and three sets of matched control genes in *S. cerevisiae*: genes with length similar to *SIR4* (top), genes with similar rate of nonsynonymous divergence to *SIR4* (middle) and genes from the same genomic region as *SIR4* (bottom). From left to right plots are: **(a)** ratio of nonsynonymous to synonymous divergence plotted against the ratio of nonsynonymous to synonymous polymorphism. Solid lines show a linear regression of *y* on *x* and the dashed lines show a slope of 1. **(b)** Histogram of synonymous polymorphism. **(c)** Histogram of nonsynonymous polymorphism. **(d)** Histogram of synonymous divergence. **(e)** Histogram of nonsynonymous divergence. **(f)** Histogram of McDonald-Kreitman Test P-values plotted on a $\log_{10}$ scale. Low frequency polymorphisms (< 0.1) were excluded.

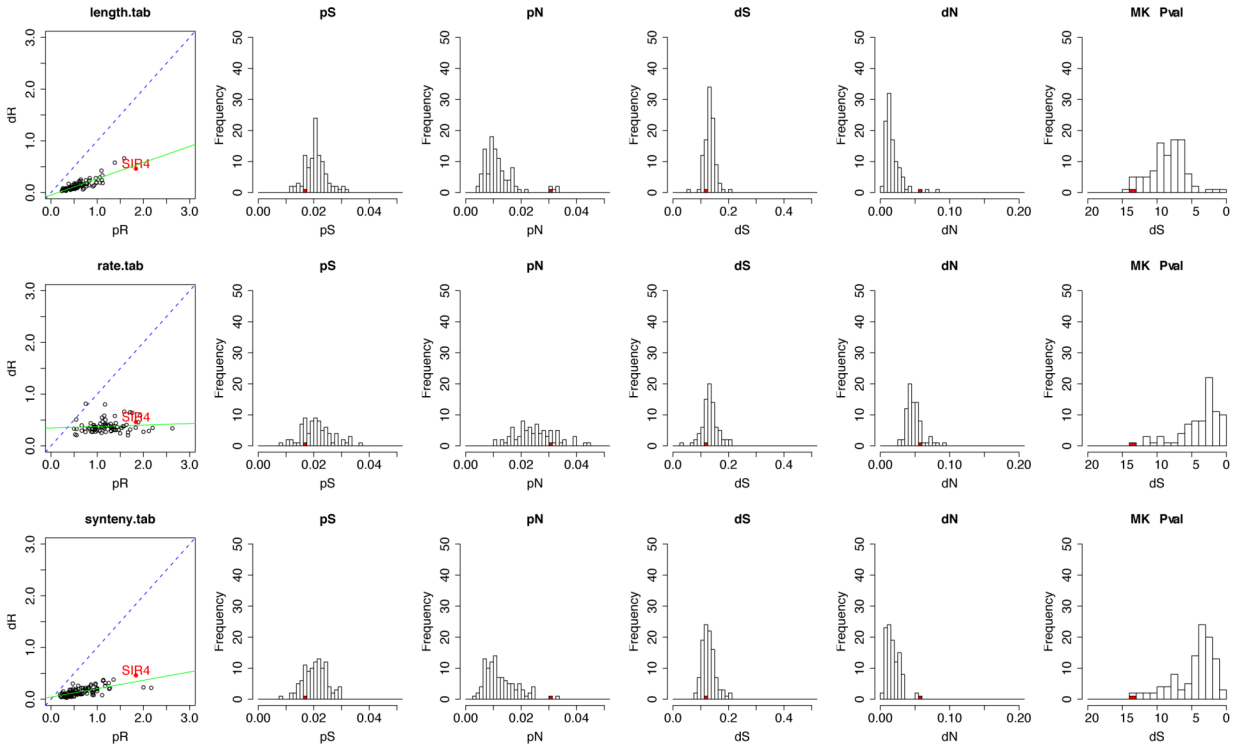

Figure 3.8. McDonald-Kreitman analyses of *SIR4* and control loci in *S. paradoxus* populations. Nonsynonymous and synonymous divergence and polymorphism for *SIR4* (red) and three sets of matched control genes in *S. paradoxus*, presented as in Figure 3.6.

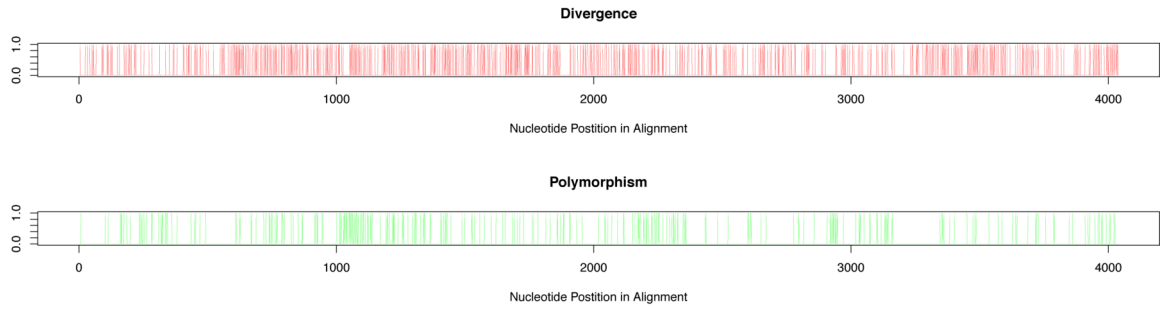

Figure 3.9. Correlation analysis of divergence versus polymorphism in *SIR4* in *sensu stricto* species. SNPs in *SIR4* within *S. cerevisiae* and *S. paradoxus* (green, lower panel) were compared with rapidly diverging sites in *SIR4* (red, upper panel) across all five *sensu stricto* species. The probability that the correlation of polymorphic with divergent sites is greater than that expected by chance was calculated, as described in the text. Y-axes are binary (0 or 1), with 1 indicating the presence of a divergent or polymorphic nucleotide, and 0 indicating a conserved or non-polymorphic nucleotide.

high value of ω for the *SIR4* gene among the *Torulaspora* species (*SIR4* ω = 0.23; median ω = 0.06; 95% of ω values lie between 0.011 and 0.20) suggested that ω may have been high in the ancestor of the *Torulaspora* and *sensu stricto* yeasts and that this property has been inherited by both lineages (Figure 3.10). Taken together, our sequence analyses suggested that *SIR4* has been evolving rapidly for a long period of time and that a subset of codons have been targets of positive selection. Such a scenario is consistent with a co-evolutionary "arms-race", raising the question of what forces drive *SIR4* evolution.

Discussion

The Sir2/Sir3/Sir4 silencing machinery was conserved across Saccharomyces species, but Sir4 had diverged in function

In Ascomycete fungi, there exists an apparent evolutionary dichotomy of epigenetic silencing mechanisms: *S. pombe*, *N. crassa*, and their neighbors use an RNAi-based silencing mechanism, whereas *S. cerevisiae*, *K. lactis*, and their neighbors use an RNAi-independent Sir silencing mechanism. Recently, RNAi components have been found in *Saccharomyces castellii* (Drinnenberg, et al., 2009), an outgroup of the *sensu stricto* clade, raising the question of how broadly the Sir silencing mechanism known from *S. cerevisiae* operates within the set of yeast species in which Sir proteins are found. The screen described in this study was designed as a pilot to determine, in broad outline, whether the Sir-based silencing mechanism persisted throughout the *Saccharomyces sensu stricto* clade, and indeed identified *SIR2*, *SIR3*, and *SIR4* as the primary genes required to silence *HML* and *HMR* in *S. bayanus* (Table 3.2). We anticipate that a deeper screen will reveal species-specific contributions of other genes to silencing in *S. bayanus*. Indeed the multiple Sir1 paralogs that contribute to silencing in *S. bayanus* (Gallagher et al. 2009) underscore the differences in silencing between *S. cerevisiae* and *S. bayanus*. However, even with a limited screen, we established that *S. cerevisiae* mutations in most cases could be used to assign *S. bayanus* mutations to complementation groups in interspecies hybrids. Importantly, the single exception allowed us to identify unexpected functional divergence in Sir4 based on the inability of *S. cerevisiae SIR4* to complement recessive mutations in *S. bayanus SIR4* (Figure 3.2, C). Our evolutionary analysis explained how the recessive, loss-of-function *S. bayanus sir4* mutations appeared dominant in *S. cerevisiae/S. bayanus* hybrids (Figure 3.3, Table 3.2).

Our complementation analyses indicated that Sir2 and Sir3 were cross-species compatible (Figure 3.3), suggesting that changes in interactions made by the Sir2/3/4 complex had diverged mainly with respect to Sir4-mediated interactions. As Sir2 and Sir3 deacetylate and interact with highly conserved histone tails, and considering the extraordinary conservation of histone sequences throughout eukaryotes, it was not surprising that these components of silent chromatin had not substantially diverged between *S. cerevisiae* and *S. bayanus*. What was the molecular basis of the asymmetry in Sir4 interspecies compatibility? Neither the expression, nor the activity *per se*, of Sc-Sir4 was reduced in *S. cerevisiae/S. bayanus* hybrids (Zill et al., *submitted*; Figure 3.3, B). The presence of four Sir1 paralogs in *S. bayanus*, all of which contribute to silencing

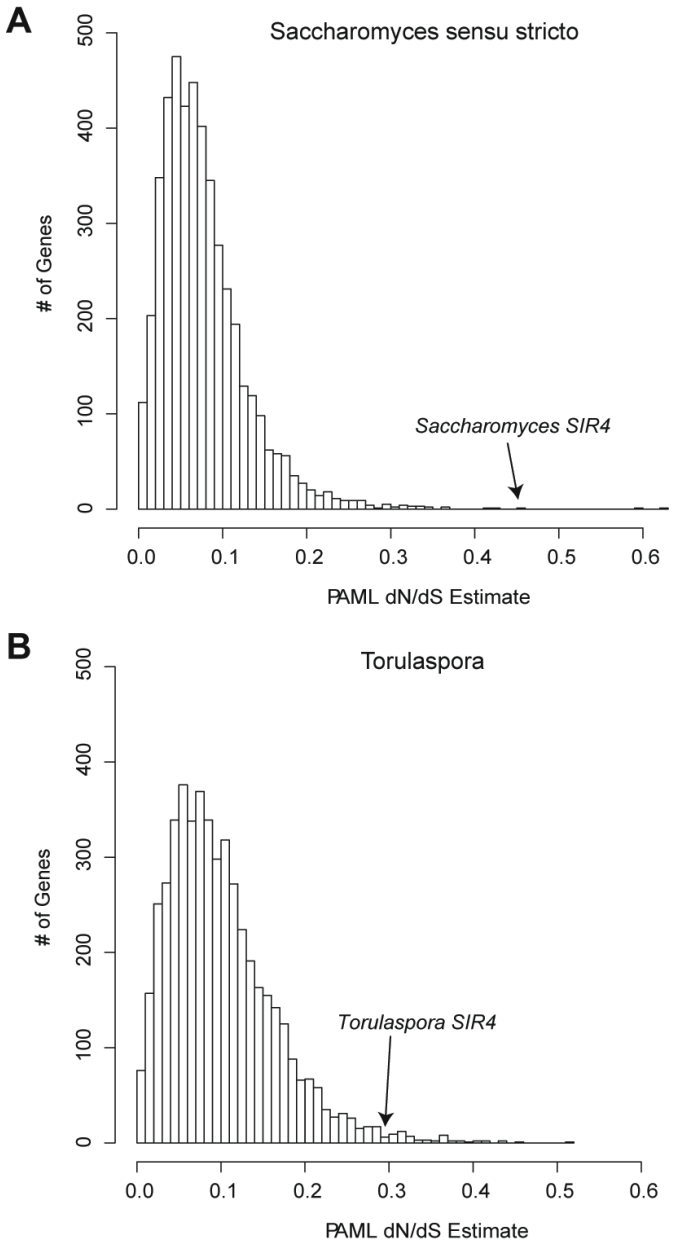

Figure 3.10. *SIR4* was rapidly evolving in the distantly related *Saccharomyces* and *Torulaspora* clades. Histograms of *dN/dS* ratios calculated for 4910 genes among (A) four *Saccharomyces sensu stricto* or (B) three *Torulaspora* species. Arrows indicate bins containing *SIR4* for each clade.

(Gallagher et al. 2009), suggested that factors involved in the initial association of Sir4 with silencers (including Sir1 and/or factors that associate with Sir1) contributed to the incompatibility. Notably, the silencers are among the most rapidly evolving non-coding regions in the yeast genome (Teytelman et al. 2008). It was possible that Sc-Sir4 activity was inhibited specifically at *S. bayanus* silent loci, or that the factors associated with *S. bayanus* silencers were unable to recruit Sc-Sir4, as suggested by recent results (Zill et al., *submitted*). Thus, *S. cerevisiae* and *S. bayanus* share three core components of the silencing machinery, but Sir4 has diverged substantially in sequence between these two species, rendering Sc-Sir4 incapable of silencing *S. bayanus HML* and *HMR*.

The twelve *Sb-sir4* mutants could be parsed into two categories based on the extent of their complementation by *Sc-SIR4*: five that could not be complemented, and seven that were weakly complemented (Table 3.2). This differential cross-species complementation did not correlate with the strength of the *sir4* alleles, as multiple mutants in both classes showed a total loss of silencing at *HML* by mating assays (data not shown). A previous study in *S. cerevisiae* showed that an N-terminal portion of Sir4 and a C-terminal portion of Sir4, neither of which could support silencing on its own, could in combination provide silencing function (Marshall et al. 1987). This observation inspired two possible explanations of the differential ability of *Sc-SIR4* to partially complement *Sb-sir4* alleles. Truncated mutant Sb-sir4 proteins might have dominantly interfered with Sc-Sir4 function, despite Sc-Sir4's inherently weak ability to silence *Sb-HML* and *Sb-HMR*. (Note that *Sc-SIR4* does in fact weakly complement an *Sb-sir4Δ* (O. Zill, unpublished observations).) This explanation predicted that all the weakly complemented mutants made no Sb-Sir4 protein, whereas all non-complemented strains made truncated Sb-Sir4 proteins. Alternatively, it was possible that only certain truncated forms of Sb-Sir4 allowed weak complementation by Sc-Sir4. This explanation predicted that most mutants made truncated, inactive Sb-Sir4 proteins, but only certain forms could collaborate with Sc-Sir4. We sequenced three *sir4* mutants and found that each mutant contained a premature stop codon. The one mutant weakly complemented by *Sc-SIR4* had (G738→STOP); two mutants that were not complemented by Sc-SIR4 had (S812→STOP, Q1378→STOP). However, two of the predicted truncated Sir4 proteins did not differ substantially in length or known functional motifs.

Multiple changes to the silencing machinery happened in quick succession in the S. cerevisiae lineage

Complementation of *S. bayanus sir4Δ* by *K. lactis SIR4* established that functional changes in *SIR4* occurred in the *S. cerevisiae* lineage (Figure 3.4, A). Our evolutionary genetic analysis of *S. bayanus sir4Δ* complementation in interspecies hybrids suggested that one or more critical changes in the Sir4 protein occurred along the branch leading to *S. mikatae*, *S. paradoxus*, and *S. cerevisiae* (Figure 3.4, B). Along this same branch, two *SIR1* paralogs, *KOS1* and *KOS2*, were lost from the yeast genome (Figure 3.11). However, the presence of multiple Sir1 proteins in a species did not necessarily indicate an incompatibility with Sc-Sir4, as Sc-Sir4 was not able to silence *S. paradoxus HML* (Figure 3.4, C). Surprisingly, Sc-Sir4 could silence *S. paradoxus HMR*,

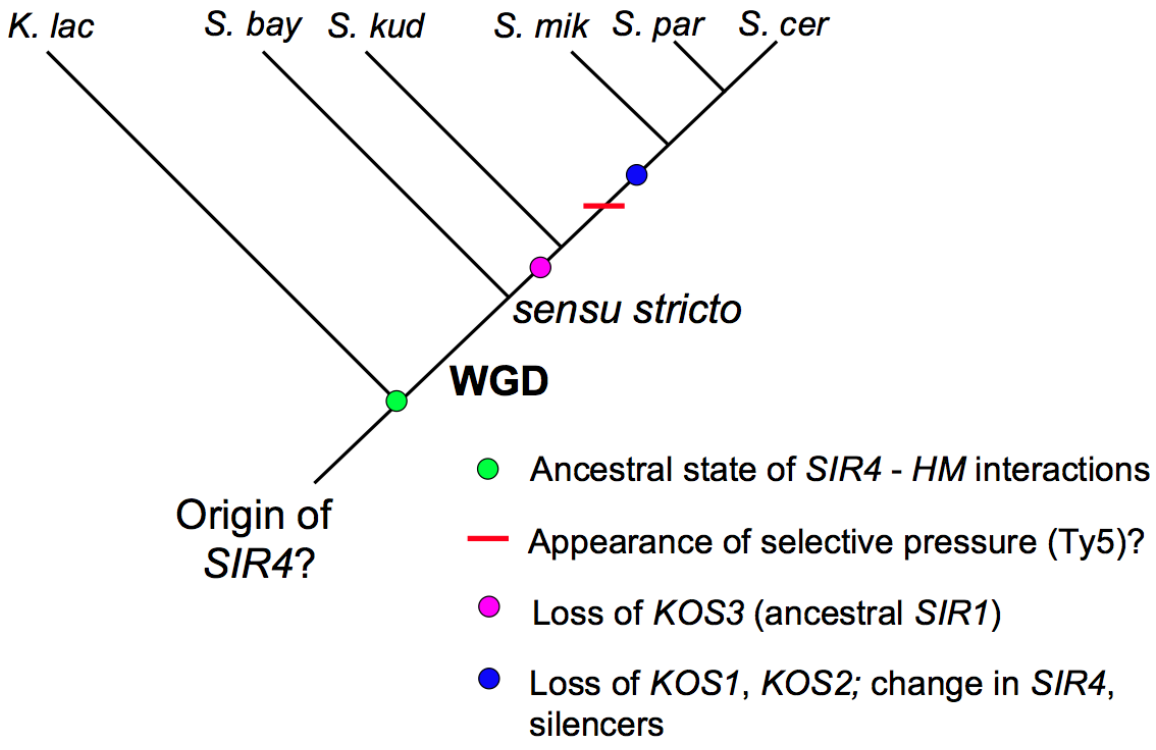

Figure 3.11. Evolutionary model of major changes in the Sir silencing machinery of *Saccharomyces* species. The losses of *SIR1* paralogs and appearance of the Ty5 retrotransposon within the *sensu stricto* clade are described in the text of the Discussion. Note that the placement of the "change in *SIR4*" (blue circle) is based on the cross-species complementation analyses (Figure 3.4). Species abbreviations: *K. lac*, *Kluyveromyces lactis*; *S. bay*, *S. bayanus*; *S. kud*, *S. kudriavzevii*; *S. mik*, *S. miaktae*; *S. par*, *S. paradoxus*; *S. cer*, *S. cerevisiae*.

suggesting that its incompatibility with *Sp-HML* was a function of the *HML* silencers and/or proteins acting specifically at *HML*. In work submitted elsewhere, we mapped the incompatibility of *Sc-SIR4* in silencing *Sb-HMR* to the co-evolution of *Sb-SIR4* with the *HMR* silencers themselves. Alternatively, *Sp-HML* may simply have been more sensitive than *Sp-HMR* to a general functional difference between Sc-Sir4 and Sp-Sir4. In *S. cerevisiae*, for example, loss of *SIR1* leads to a stronger defect in *HML* silencing than in *HMR* silencing (Stone et al. 2000). Quantitative analyses of silencing by Sc-Sir4 in *S. paradoxus* could help distinguish between these possibilities.

It appeared that *S. paradoxus* Sir4 retained some ancestral character that allowed it to partially silence *Sb-HMR*. Thus, *S. cerevisiae* and *S. mikatae* may have independently experienced change in a second genetic feature related to the cross-species incompatibility of Sir4. The multiple functional changes occurring within the closely related species of the *sensu stricto* clade suggested that studies in *S. cerevisiae* have provided an evolutionarily biased view of the silencing mechanism. Comparative mechanistic studies of silencing in *S. bayanus* and outgroup species should reveal new dimensions to this shared biological process. It will be interesting to determine how *S. bayanus* and *S. kudriavzevii* Sir4 proteins spatially and temporally coordinate the functions of multiple Sir1 proteins.

The rapid evolution of SIR4 involved positive selection and ongoing diversifying selection

At least one protein coding change was responsible for the functional divergence of Sir4 between *S. cerevisiae* and *S. bayanus*, as Sir4 expression was equivalent between the two species (Zill et al., *submitted*). Sequence alignments of all *S. cerevisiae/S. bayanus* orthologs revealed that *SIR4* was among the most rapidly evolving genes in the yeast genome (Figure 3.6, A). Sliding window and Bayesian likelihood analyses revealed that multiple sites within the N-terminal regulatory and PAD domains had ω values ≥ 2, suggesting that positive selection altered Sir4 function via changes in these regions (Figure 3.6, B). In contrast, the C-terminal coiled-coil region evolved much more slowly, suggesting its function was highly conserved. However, the high rate of evolution across the *SIR4* gene (Figure 3.6, B, and Figure 3.10) prevented the clear identification of residues responsible for the functional change that led to the incompatibility between Sc-Sir4 and *Sb-HML* and *Sb-HMR*.

Population genetic data from *S. cerevisiae* and *S. paradoxus* revealed an unusually high level of non-synonymous polymorphism within species (Figure 3.6, C, Figure 3.7 and 3.8), suggesting that diversifying selection on *SIR4* operates in modern yeast populations (Figure 3.6, C, Figure 3.7 and 3.8). Due to the correlation—albeit weak—between sites that were polymorphic among *S. cerevisiae* isolates and sites that were divergent between *sensu stricto* species (Figure 3.9), it is possible that *SIR4* may be a target of continuous adaptive pressure. This makes sense, if, as discussed in the next section, *SIR4* is involved in an evolutionary "arms race" with the Ty5 retrotransposon. In either case, the rapid evolution of *SIR4* orthologs in the *Torulaspora* (which diverged from the *Saccharomyces* clades ~100Mya) suggests that the changes that gave rise to the incompatibility between *S. cerevisiae* and *S. bayanus* occurred on a background of rapid

evolution at the *SIR4* locus. Thus it may be useful to view *SIR4* evolution as the product of two distinct selection regimes: a pattern of long-term rapid evolution across the gene and the recent fixation of incompatible substitutions at a more limited number of sites, perhaps as a consequence of Ty5 invasion.

Obtaining multiple *SIR4* sequences from independent populations of other *sensu stricto* species, particularly *S. bayanus*, could test whether diversifying selection operates across most modern *Saccharomyces* species. Some of the rapid evolution in *SIR4* may have been due to neutral processes such as drift, and much of its coding sequence may not be acutely important for the survival of yeast species. Indeed, one contribution of *SIR4* to the fitness of yeast cells, maintenance of mating ability, should be manifested only in the haploid phase of the natural yeast life cycle. It will be of interest to determine *SIR4*'s mutational dynamics in species that spend different proportions of their life cycles in haploid versus diploid phases.

An arms race with the Ty5 retrotransposon as a possible driver of past adaptive evolution at SIR4

The functional divergence in Sir4, together with the phylogenetic signature of positive selection, suggested that at least one major evolutionary event occurred in the *sensu stricto* ancestry that drove changes in Sir4. What selective pressure might have forced yeast cells to adapt by altering the silencing machinery? The Ty5 retrotransposon, whose integrase protein binds Sc-Sir4, targeting Ty5 integration into silent chromatin (Zou et al. 1996; Zou and Voytas 1997), is an obvious candidate for such a pressure. Its appearance in the *sensu stricto* ancestry (Liti et al. 2005) precisely coincided with the change of *SIR4* function observed in our cross-species complementation assays (Figure 3.4, B and C; Figure 3.11). By this model, in the *S. cerevisiae* lineage, Sir4 traded an ancestral function for an ability to protect the genome from harmful Ty5 integrations (Boeke and Devine 1998; Dai et al. 2007). This hypothesis predicts that the Ty5 hopping pattern should change if *S. cerevisiae SIR4* is replaced by a Ty5-naive *SIR4* allele (from *S. bayanus* or *S. kudriavzevii*) in an *S. cerevisiae* strain in which Ty5 is mobilized, or analogous experiments in other species.

Interestingly, Ty5 mobilization has never been observed in native yeast strains. *S. cerevisiae* and *S. mikatae* harbor only truncated or mutant Ty5 elements that are non-functional (Zou et al. 1996; Liti et al. 2005). Full-length Ty5 elements have been found only in *S. paradoxus*, although these do not appear to be expressed under normal lab conditions (Zou et al. 1995; Zhu et al. 1999). If Ty5 indeed presented an adaptive challenge, then these extant yeast species (at least the sequenced isolates, or "type strains") may be the surviving offspring of ancestors victorious in historic battles with this genetic parasite. However, it is possible that current sequencing coverage of *Saccharomyces* populations (Liti et al. 2009) is not sufficient to identify Ty5 that may persist at a relatively low level in some yeast strains. Additional population genomic surveys of all *Saccharomyces* species could determine whether Ty5 can account for our observations of ongoing diversifying selection in *SIR4*.

Using cross-species complementation to identify differences in ortholog function

Most protein homologs between *S. cerevisiae* and *S. bayanus* have highly similar sequences, and it is generally assumed that the vast majority of these orthologs perform identical functions. Indeed, human orthologs of yeast genes often complement the corresponding yeast mutant, suggesting that even distantly related orthologs with extensive sequence divergence have conserved functions. Furthermore, highly diverged *SIR4* orthologs—those of *S. bayanus* and *K. lactis*—complement the *S. cerevisiae sir4Δ* mutant. The surprising and specific failure of *S. cerevisiae SIR4* to complement *S. bayanus sir4Δ* was key to the identification of interesting evolutionary events in the *sensu stricto* ancestry that had functional impact on silencing.

Asymmetric non-complementation across species, in general, may reveal significant evolutionary events that have shaped the biology of individual species or clades. Functional studies in model organisms could help to derive guidelines for reliably predicting true functional divergence of orthologs in closely related species. The case of *SIR4* highlights a general consideration: although rapidly evolving clade-specific genes, such as *SIR1* and *SIR4*, might provide a good vantage point onto key evolutionary genetic interactions that determine the "exceptional biology" of a particular species (Eichler 2001), rapid sequence evolution *per se* is not sufficient to infer functional divergence. *S. kudriavzevii SIR4* was as diverged from *S. bayanus SIR4* as was *S. cerevisiae SIR4*, yet one complemented *S. bayanus sir4Δ* and the other did not. The present study suggests that yeast hybrids might be a powerful tool for determining which sequence changes are functionally significant.

Chapter 4

Co-evolution of transcriptional silencing proteins and the DNA elements specifying their assembly

The work in this chapter was conducted in collaboration with Lenny Teytelman, a former graduate student in the Rine lab. Lenny conducted analyses of the Sir4 ChIP-Seq data, and trained me in how to perform these analyses myself. Lenny contributed greatly to Figure 4.4 and Table 4.2. I am extremely grateful for Lenny's efforts and excellent teaching skills.

Abstract

Co-evolution of transcriptional regulatory proteins and their sites of action has been often hypothesized, but rarely demonstrated. Here we provide experimental evidence of such co-evolution in yeast silent chromatin, a finding that emerged from studies of hybrids formed between two closely related *Saccharomyces* species. A unidirectional silencing incompatibility between *S. cerevisiae* and *S. bayanus* led to a key discovery: asymmetrical complementation of divergent orthologs of the silent chromatin component Sir4. In *S. cerevisiae*/*S. bayanus* interspecies hybrids, ChIP-Seq analysis revealed a restriction against *S. cerevisiae* Sir4 associating with most *S. bayanus* silenced regions; in contrast, *S. bayanus* Sir4 associated with *S. cerevisiae* silenced loci to a greater degree than did *S. cerevisiae*'s own Sir4. Functional changes in multiple silencer DNA elements across the genome paralleled changes in Sir4 sequence and a reduction in Sir1 family members. Critically, species-specific silencing of the *S. bayanus HMR* locus could be reconstituted in *S. cerevisiae* by co-transfer of the *S. bayanus* Sir4 and Kos3 (the ancestral relative of Sir1) proteins. As Sir1/Kos3 and Sir4 are structural chromatin proteins that bind conserved transcription factors, but not specific DNA sequences, these rapidly evolving proteins served to interpret differences in the two species' silencers presumably involving emergent features created by the transcription factors. The results presented here, and in particular the high resolution ChIP-Seq localization of the Sir4 protein, provided unanticipated insights into the mechanism of silent chromatin assembly in yeast.

Introduction

Among all specialized chromatin structures, the difference between heterochromatin and euchromatin is perhaps the most fundamental, motivating intense study of the structural differences between these two states. DNA sequences within heterochromatic regions evolve rapidly in animals (Linardopoulou et al. 2005; Diaz-Castillo and Golic 2007), plants (Hall et al. 2003; Hall et al. 2004), and fungi (Teytelman et al. 2008), presenting a paradox of how the specification of heterochromatin structure persists despite rapid changes in the underlying sequence (Henikoff et al. 2001). In *Saccharomyces* the biology of heterochromatin has proven eminently accessible to genetic studies through its role in gene silencing (Rusche et al. 2003), and comparative studies of silencing now seem poised to illuminate key processes underlying heterochromatin evolution.

Molecular co-evolution of transcriptional regulatory proteins with their sites of action has been proposed to maintain regulatory functions across species divergence (Dover and Flavell 1984; Simpson 2002). In this context, "co-evolution" is typically understood as compensatory changes in a DNA sequence motif and the DNA-binding domain of the cognate transcription factor. Although it has been suggested that such co-evolution is prevalent in nature (Dover and Flavell 1984), in only a few instances has it been directly tested (Evers and Grummt 1995; Shaw et al. 2002; Gasch et al. 2004). In Dipteran insects, for example, co-evolution of *bicoid* binding sites in the *hunchback* promoter and the *bicoid* homeodomain has been proposed to maintain *hunchback*-mediated developmental patterning along the anterior/posterior axis in *Musca* and *Drosophila* (Bonneton et al. 1997; McGregor et al. 2001). However, the large size and complexity of animal regulatory elements, and the difficulty of performing cross-species complementation tests in animal model organisms, have precluded clear distinction between regulatory divergence and bona fide co-evolution.

Transcriptional silencing by Sir (Silent Information Regulator) proteins is necessary for the specialized haploid mating-type system found in *Saccharomyces* (Rine and Herskowitz 1987; Haber 1998). DNA regulatory elements termed "silencers" contain binding sites for the Origin Recognition Complex (ORC), Rap1, and Abf1, which in turn direct the assembly of silent chromatin structures at the *HML* and *HMR* loci and telomeres. The current model for the establishment of silencing holds that a Sir2/Sir3/Sir4 complex is brought to silencers by protein-protein interactions between ORC and Sir1, and between Rap1 and Sir4 (Rusche et al. 2003). Upon nucleation of these complexes, silent chromatin formation is catalyzed by the histone deacetylase activity of Sir2, and propagated, at least in part, through interactions between Sir3 and newly deacetylated histone tails (Hecht et al. 1995; Hoppe et al. 2002; Rusche et al. 2002). Sir proteins are not thought to bind specific DNA sites; instead, efficient nucleation of silencing complexes at silencers requires interactions between Sir1 and Sir4, bridging the ORC-Sir1 and Rap1-Sir4 interactions (Bose et al. 2004).

We have recently shown that silencer elements are among the most rapidly evolving regulatory sequences in *Saccharomyces* genomes (Teytelman et al. 2008); however, the regulatory proteins that directly bind silencers are highly conserved, essential proteins. Intriguingly, the Sir1 and Sir4 proteins parallel the silencers in their

rapid evolution (Zill, et al. *in preparation*), but these proteins show distinct patterns of evolution. *SIR1*-related genes have undergone multiple duplication and loss events: for example, *S. bayanus* has four functional paralogs of the single *S. cerevisiae SIR1* gene, including the ancestral *KOS3* (Kin Of Sir1) paralog, which *S. cerevisiae* has lost along with two other paralogs (Gallagher et al. 2009). In contrast, the Sir4 protein is among the 40 most diverged proteins between *S. cerevisiae* and *S. bayanus* (Zill, et al. *in preparation*), with 45% identity between its orthologs relative to a genome-wide average of 83% identity (Cliften et al. 2003; Kellis et al. 2003).

S. cerevisiae and *S. bayanus* are post-zygotically isolated—haploids of these two species can mate to form mitotically stable hybrid diploids, but meiotic spores derived from these diploids are usually inviable (Liti et al. 2006; Greig 2009). The rapid evolution of the silencers, the Sir4 protein sequence, and the elaboration of Sir1 paralogs make these two species an excellent phylogenetic context for comparative studies of silencing. Here, we describe functional studies in *S. cerevisiae/S. bayanus* interspecies hybrids that demonstrated how co-evolution among two heterochromatin nucleation proteins, Sir1 and Sir4, and multiple silencer DNA elements, allowed two divergent lineages to maintain robust silencing despite these rapid genetic changes. This example of regulatory co-evolution is of particular interest because the co-evolving proteins are not the agents that directly bind the sequence of the divergent regulatory sites.

Materials and Methods

Yeast strain construction and genetic manipulations

Yeast strains used in this chapter are listed in Table 4.1. All *S. cerevisiae* strains were of the W303 background. Generation of marked *S. bayanus* strains from type strain CBS 7001 has been described (Zill and Rine 2008). All yeast strains were cultured at 25^{o}C in standard yeast media. One-step gene replacement and C-terminal 13xMyc tag integration have been described previously (Longtine et al. 1998; Goldstein and McCusker 1999), and these genetic manipulations were performed identically for *S. bayanus*, *S. cerevisiae*, and *S. cerevisiae/S. bayanus* hybrids. The *HMR::URA3* reporter strains were constructed independently in *S. cerevisiae sir4Δ* and *S. bayanus sir4Δ* haploid strains, wherein the *HMR***a**1 ORF was replaced with the *K. lactis URA3* ORF by PCR-based gene targeting, leaving the *HMR***a**1 promoter intact. For most experiments, interspecies hybrids were made by crossing *S. bayanus MAT*α *HMR::URA3* strains (wild-type, *sir4Δ*, or *SIR4-13xMyc*) to *S. cerevisiae MAT***a** strains (*wild-type*, *sir4Δ*, or *SIR4-13xMyc*). For *ORC1*, *RAP1*, and *ABF1* heterozygote analysis, gene targeting was performed directly in hybrid diploids or *S. bayanus* diploids. Three independent transformants were analyzed in all cases. *Sc-SIR4-13xMyc* and *Sb-SIR4-13xMyc* alleles were shown to be functional by two independent silencing assays in each case: by mating ability in *S. cerevisiae SIR4-13xMyc* and *S. bayanus SIR4-13xMyc* haploid strains, and by FOA resistance in hybrid diploids bearing the appropriate *HMR::URA3* reporter (data not shown).

Table 4.1. Yeast strains used in chapter 4. Unless otherwise indicated, all strains originated from this study. For all loci in *S. cerevisiae/S. bayanus* hybrids, allele configurations are given as *S. cerevisiae (gene) / S. bayanus (gene)*.

Strain	Species	Genotype
JRY4012	*S. cerevisiae* (W303)	*MAT***a** *his3 leu2 lys2 trp1 ura3 can1* (Source: R. Rothstein)
JRY4013	*S. cerevisiae*	*MAT*α *his3 leu2 lys2 trp1 ura3 can1* (Source: R. Rothstein)
JRY8821	*S. cerevisiae*	*MAT*α *HMR::URA3 ade2 his3 leu2 lys2 trp1 ura3*
JRY8676	*S. cerevisiae*	*MAT*α *HMR::URA3 sir4Δ::HIS3 ade2 his3 leu2 trp1 ura3*
JRY9025	*S. cerevisiae*	*MAT***a** *hmlΔ::KanMX sir4Δ::HIS3 his3 leu2 lys2 trp1 ura3*
JRY9026	*S. cerevisiae*	*MAT***a** *SIR4-13xMyc::KanMX his3 leu2 lys2 trp1 ura3*
JRY9027	*S. cerevisiae*	*MAT***a** *sir4Δ::LEU2-Sb-SIR4 his3 leu2 lys2 trp1 ura3*
JRY9028	*S. cerevisiae*	*MAT*α *sir4Δ::LEU2-Sb-SIR4 his3 leu2 lys2 trp1 ura3*
JRY9029	*S. cerevisiae*	*MAT*α *Sc::(Sb-HMR::URA3) sir4Δ::KanMX ade2 his3 leu2 trp1 ura3*
JRY9030	*S. cerevisiae*	*MAT***a**/α *HMR/Sc::(Sb-HMR::URA3) SIR4/sir4Δ::KanMX* (JRY4012 x JRY9029)
JRY9031	*S. cerevisiae*	*MAT*α *Sc::(Sb-HMR::URA3) Sc-SIR4 his3 leu2 trp1 ura3*
JRY9032	*S. cerevisiae*	*MAT*α *Sc::(Sb-HMR::URA3) Sc-sir4Δ::LEU2-Sb-SIR4 his3 leu2 lys2 trp1 ura3*
JRY9033	*S. cerevisiae*	*MAT*α *Sc::(Sb-HMR::URA3) Sc-SIR4 sir1Δ::TRP1 his3 leu2 lys2 trp1 ura3*
JRY9034	*S. cerevisiae*	*MAT*α *Sc::(Sb-HMR::URA3) Sc-sir4Δ::LEU2-Sb-SIR4 sir1Δ::TRP1 his3 leu2 lys2 trp1 ura3*
JRY9035	*S. cerevisiae*	*MAT*α *ORC5-HA::KanMX his3 leu2 trp1 ura3*
JRY9036	*S. cerevisiae*	*MAT*α *Sc-sir4Δ::LEU2-SbSIR4 ORC5-HA::KanMX his3 leu2 lys2 trp1 ura3*
JRY9037	*S. cerevisiae*	*MAT*α *Sc::(Sb-HMR::URA3) ORC5-HA::KanMX his3 leu2 trp1 ura3*
JRY9038	*S. cerevisiae*	*MAT*α *Sc::(Sb-HMR::URA3) Sc-sir4Δ::LEU2-SbSIR4 ORC5-HA::KanMX his3 leu2 trp1 ura3*
JRY9039	*S. cerevisiae*	*MAT*α *ABF1-13xMyc::KanMX* (JRY4013)
JRY9040	*S. cerevisiae*	*MAT*α *sir4Δ::LEU2-SbSIR4 ABF1-13xMyc::KanMX* (JRY9029)
JRY9041	*S. cerevisiae*	*MAT*α *Sc::(Sb-HMR::URA3) ABF1-13xMyc::KanMX* (JRY9031)
JRY9042	*S. cerevisiae*	*MAT*α *Sc::(Sb-HMR::URA3) sir4Δ::LEU2-SbSIR4 ABF1-13xMyc::KanMX* (JRY9032)
JRY8822	*S. bayanus* (CBS 7001)	*MAT***a** *hoΔ::NatMX lys2 ura3*
JRY8819	*S. bayanus*	*MAT*α *HMR::URA3 ade2 his3 lys2 ura3*
JRY9043	*S. bayanus*	*MAT*α *HMR::URA3 sir4Δ::KanMX ade2 ura3*
JRY8820	*S. bayanus*	*MAT***a** *hmlΔ::S.p.his5 sir4Δ::KanMX his3 lys2 ura3*
JRY9044	*S. bayanus*	*MAT***a** *SIR4-13xMyc::KanMX lys2 ura3*
JRY9045	*S. bayanus*	*MAT*α *HMR::URA3 SIR4-13xMyc::KanMX ade2 his3 lys2 ura3*
JRY9046	*S. bayanus*	*MAT***a**/α *HMR/HMR::URA3 SIR4/SIR4* (JRY8822 x JRY8819)
JRY9047	*S. bayanus*	*MAT***a**/α *HMR/HMR::URA3 SIR4/sir4Δ::KanMX* (JRY8822 x JRY9043)
JRY9048	*S. bayanus*	*MAT***a**/α *hmlΔ::S.p.his5/HML HMR/HMR::URA3 sir4Δ::KanMX/sir4Δ::KanMX* (JRY8820 x JRY9043)
JRY9049	*S. bayanus*	*MAT***a** *sir4Δ::LEU2-Sc-SIR4 hoΔ::KanMX leu2 lys2 trp1 ura3*
JRY9050	*S. bayanus*	*MAT*α *HMR::URA3 sir4Δ::LEU2-Sc-SIR4 leu2 lys2 trp1 ura3*
JRY9051	*S. bayanus*	*MAT*α *HMR::URA3 sir4Δ::LEU2-Sc-SIR4 ade2 his3 leu2 lys2 ura3*
JRY9052	*S. bayanus*	*MAT***a**/α *HMR/HMR::URA3 RAP1/rap1Δ::HygMX* (JRY9046)
JRY9053	*S. bayanus*	*MAT***a**/α *HMR/HMR::URA3 ORC1/orc1Δ::HygMX*
JRY9054	*S. cerevisiae*/ *S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-SIR4/Sb-SIR4* (JRY4012 x JRY8819)

JRY9055	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-SIR4/Sb-sir4Δ::KanMX* (JRY4012 x JRY9043)
JRY9056	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-hmlΔ::KanMX/Sb-HML Sc-HMR/Sb-HMR::URA3 Sc-sir4Δ::HIS3/Sb-SIR4* (JRY9025 x JRY8819)
JRY9057	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-hmlΔ::KanMX/Sb-HML Sc-HMR/Sb-HMR::URA3 Sc-sir4Δ::HIS3/Sb-sir4Δ::KanMX* (JRY9025 x JRY9043)
JRY9058	*S. cerevisiae/ S. bayanus*	*MAT*α/**a** *Sc-HMR::URA3/Sb-HMR Sc-SIR4/Sb-SIR4* (JRY8821 x JRY8822)
JRY9059	*S. cerevisiae/ S. bayanus*	*MAT*α/**a** *Sc-HML/Sb-hmlΔ::S.p.his5 Sc-HMR::URA3/Sb-HMR Sc-SIR4/Sb-sir4Δ::KanMX* (JRY8821 x JRY8820)
JRY9060	*S. cerevisiae/ S. bayanus*	*MAT*α/**a** *Sc-HMR::URA3/Sb-HMR Sc-sir4Δ::HIS3/Sb-SIR4* (JRY8676 x JRY8822)
JRY9061	*S. cerevisiae/ S. bayanus*	*MAT*α/**a** *Sc-HML/Sb-hmlΔ::S.p.his5 Sc-HMR::URA3/Sb-HMR Sc-sir4Δ::HIS3/Sb-sir4Δ::KanMX* (JRY8676 x JRY8820)
JRY9062	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-SIR4-13xMyc::KanMX/Sb-sir4Δ::LEU2-Sc-SIR4* (JRY9026 x JRY9051)
JRY9063	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-sir4Δ::LEU2-Sb-SIR4/Sb-SIR4-13xMyc::KanMX* (JRY9027 x JRY9045)
JRY9064	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-SIR4-13xMyc::KanMX/Sb-SIR4* (JRY9026 x JRY8819)
JRY9065	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-SIR4/Sb-SIR4-13xMyc::KanMX* (JRY4012 x JRY9045)
JRY9066	*S. cerevisiae/ S. bayanus*	*MAT*α/**a** *Sc-HMR::URA3/Sb-HMR Sc-sir4Δ::HIS3/sir4Δ::LEU2-Sc-SIR4* (JRY8676 x JRY9049)
JRY9067	*S. cerevisiae/ S. bayanus*	*MAT*α/**a** *Sc::(Sb-HMR::URA3)/Sb-HMR sir4Δ::KanMX/Sb-SIR4* (JRY9029 x JRY8822)
JRY9068	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-sir4Δ::LEU2-Sb-SIR4/Sb-sir4Δ::KanMX* (JRY9027 x JRY9043)
JRY9069	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-rap1Δ::HygMX/Sb-RAP1* (JRY9054)
JRY9070	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-RAP1/Sb-rap1Δ::HygMX*
JRY9071	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-orc1Δ::HygMX/Sb-ORC1*
JRY9072	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-ORC1/Sb-orc1Δ::HygMX*
JRY9073	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-abf1Δ::HygMX/Sb-ABF1*
JRY9074	*S. cerevisiae/ S. bayanus*	*MAT***a**/α *Sc-HMR/Sb-HMR::URA3 Sc-ABF1/Sb-abf1Δ::HygMX*

The *Sc::(Sb-HMR::URA3)* replacement allele (Figure 4.6, A) was generated in two steps. The *Sb-HMR::URA3* cassette plus 1kb of leftward-flanking sequence was PCR-amplified out of the *S. bayanus* genome, and the PCR product was used to replace the syntenic portion of *Sc-HMR* (including the *E* silencer) in *S. cerevisiae sir4Δ* strains. A *HygMX* marker was then targeted into the *S. bayanus* genome 3kb to the right of *Sb-HMR*. The entire rightward-flanking 3kb region plus the *HygMX* marker was PCR-amplified out of the *S. bayanus* genome, and the PCR product was used to replace the syntenic portion of *Sc-HMR* in the *S. cerevisiae* genome (including the *I* silencer). The *Sc::(Sb-HMR::URA3)* replacement allele therefore included a total of 5.5kb of *Sb-HMR* sequence, plus the 1.7kb *HygMX* marker.

To construct the *SIR4* replacement alleles, the *Sc-SIR4* and *Sb-SIR4* genes were separately cloned into the yeast plasmid pRS315 (Sikorski and Hieter 1989) such that the *LEU2* marker was 5′ of, and in opposite orientation to, each *SIR4* gene. Each *SIR4* gene plus the *LEU2* marker was PCR-amplified from each plasmid. The *LEU2-Sc-SIR4* PCR product was used to replace the *URA3* marker at the *Sb-SIR4* locus in an *S. bayanus sir4Δ::URA3 leu2* strain; likewise, the *LEU2-Sb-SIR4* PCR product was targeted into the *Sc-SIR4* locus in an *S. cerevisiae sir4Δ::URA3 leu2* strain. The integrated *Sc-SIR4* gene was shown to silence *Sc-HMR::URA3* in hybrids (Figure 4.3, A); and the integrated *Sb-SIR4* gene was shown to silence *Sb-HMR::URA3* in hybrids (Figure 4.6, C), and *Sc-HML* and *Sc-HMR* in *S. cerevisiae* strains (Zill, et al., *in preparation*). The expression level of each *SIR4* replacement allele was determined by quantitative RT-PCR (Figure 4.1).

Silencing reporter assays

Assays of yeast strain growth on FOA and CSM/-Ura media were performed using standard "frogging" techniques. Briefly, for each strain, a ten-fold dilution series of yeast cells at an approximate density of $4x10^7$/mL was spotted onto each plate. For Figures 4.2, 4.3(A), 4.6, and 4.7(A) plates were photographed after two days for YPD, and after three days for FOA and CSM/-Ura. For Figure 4.7(B), plates were photographed after three days for all media. For Figure 4.8(B), plates were photographed after three days for FOA and YPD, and after five days for CSM/-Ura. We note that some changes in silencing could be seen only on FOA and not on CSM/-Ura. Incomplete silencing of the *HMR***a**1 promoter likely led to heterogeneous expression states within the population of cells, with some remaining silent while others were expressed (Pillus and Rine 1989).

RNA and protein analysis

RNA isolation was performed using the hot-phenol method (Schmitt et al. 1990). Total RNA was digested with Amplification grade DNase I (Invitrogen) and purified using the RNeasy MinElute kit (Qiagen). cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR and oligo(dT) primer (Invitrogen). Quantitative PCR on cDNA was performed using an MX3000P machine (Stratagene) and

the DyNAmo HS SYBR Green qPCR kit (NEB). Amplification values for all primer sets were normalized to actin (*ACT1*) cDNA amplification values. Samples were analyzed in triplicate from two or three independent RNA preparations.

Yeast whole cell extracts were prepared using 20% TCA, and solubilized in SDS loading buffer plus 100mM Tris base. SDS-PAGE and immunoblotting were performed using standard procedures and the LiCOR imaging system. Anti-c-Myc antibody from rabbit (Sigma, Cat. No. C3956) was used to detect Myc-tagged Sir4 and Abf1 proteins. Mouse anti-Pgk1 antibody (Invitrogen, Cat. No. 459250) was used to verify equal loading. The *S. cerevisiae* Orc5-HA strain derivation has been described (Ozaydin and Rine).

All chromatin immunoprecipitations (Sir4-Myc, Orc5-HA, Abf1-Myc) were performed as described (Davies et al. 2005), using formaldehyde cross-linking of log phase cultures for one hour at room temperature. IPs were performed overnight at 4^{o}C using Anti-c-Myc-Agarose (Sigma, Cat. No. A7470) and Anti-HA-Agarose (Sigma, Cat. No. A2095). Quantitative PCR was performed as described above.

ChIP-Seq analysis of Sir4 in S. cerevisiae/S. bayanus hybrids

Library preparation and sequencing. ChIP-sequencing libraries were prepared from chromatin input and Sir4 precipitate fractions as per the Illumina paired-end and ChIP-Seq protocols, with modifications as per (Quail et al. 2008; Lefrancois et al. 2009). Specifically, for both input and IP chromatin fractions, 1μg of DNA was used for library construction; melting of gel slices for size selection was performed at room temperature to prevent loss of AT-rich sequences; and 18 cycles of PCR were performed to enrich adapter-ligated fragments. Library size and quality were verified by Bioanalyzer and quantitative PCR analysis. Insert size, excluding adapter sequences, averaged 320bp for all libraries. Each input or IP library was loaded onto a single lane on a flow cell, and was sequenced by 36bp paired-end reads on the Illumina Genome Analyzer II. All sequencing reads will be deposited in the Short Read Archive at NCBI upon publication.

Data analysis. Paired-end reads were mapped to an *S. cerevisiae/S. bayanus* hybrid genome, made by concatenating the two species' genomes into a single FASTA file, using the MAQ software (Li et al. 2008). Every base of the hybrid genome was assigned the total number of sequence reads mapping to it, done separately for the input and IP reads. Median read counts were calculated for each set of reads in 100bp windows, sliding along each chromosome or contig in 50bp steps (Teytelman et al. 2009). Median genome-wide coverage ranged 25-41x across all input samples and 8-13x across all IP samples. Correlation coefficients of median coverage values for each 100bp window were determined for all pairs of datasets using R. Correlation coefficients were R=0.96-0.98 for all possible pairs of input samples, and R=0.98-0.99 for all IP samples.

For select telomeric regions, the IP/input ratio of read counts was determined for each base and subsequently plotted versus chromosome position (Figures 4.4, B, and 4.5, B). Binding of Sc-Sir4 and Sb-Sir4 to specific *S. cerevisiae* telomeres, and to putative *S.*

bayanus subtelomeric regions, was confirmed using the peak-calling software MACS (Zhang et al. 2008), which allowed determination of statistical confidence by modeling IP/Input background noise across the genome. For all other analyses, IP/Input ratios were calculated for all 100bp sliding windows covering select 600bp silenced regions: the *HMR-E* and *HML-E* silencers of both species, the *Sc-HMR***a**1 and *Sb-HMR::URA3* ORFs, and control regions. The mean IP/Input ratio across these windows was determined for each 600bp region (Table 4.2). For *Sc-HMR-E*, *Sb-HMR-E*, and *Sb-HMR::URA3*, the normalized (mean IP/Input, *query region*):(mean IP/Input, *control region*) ratio was plotted (Figure 4.4, A).

Results

An incompatibility between S. cerevisiae SIR4 and S. bayanus HMR revealed by genetic analysis of interspecies hybrids

In the course of a genetic screen for *S. bayanus* silencing mutants, we discovered that *S. cerevisiae SIR4* failed to complement *S. bayanus sir4Δ* mutants for silencing of both *HML* and *HMR*, but *S. cerevisiae SIR2* and *SIR3* complemented mutations in *S. bayanus* orthologs (Zill, et al. *in preparation*). This result was unanticipated as there are many cases of human proteins that can replace their yeast counterparts, even for proteins that function in large complexes and have considerably more sequence divergence than that seen between *S. cerevisiae* and *S. bayanus* proteins (Kataoka et al. 1985; Lee and Nurse 1987; Basson et al. 1988; Wang et al. 2009). The incompatibility was unidirectional as *S. bayanus SIR2*, *SIR3*, and *SIR4* complemented *S. cerevisiae sir2Δ*, *sir3Δ*, and *sir4Δ*, respectively. Importantly, *SIR4* functional divergence was due to one or more coding changes, as the level of expression of the two Sir4 orthologs, measured at either the RNA or protein level, was equivalent (Figure 4.1). To assay the function of both species' silencing machineries in the same cellular milieu, we developed a highly sensitive transcriptional reporter assay in *S. cerevisiae*/*S. bayanus* interspecies hybrid diploids that allowed us to monitor silencing of each species' *HMR* locus (hereafter referred to as *Sc-HMR* or *Sb-HMR*). The reporter consisted of the *K. lactis URA3* open reading frame placed under the control of the endogenous *HMR***a**1 promoter of each species, in two separate, but otherwise isogenic, hybrid strains (Figure 4.2).

In these hybrids the *S. cerevisiae SIR4* (*Sc-SIR4*) allele could not, on its own, silence *Sb-HMR* (Figure 4.2, A, row 2). Reduced dosage of Sir4 *per se* did not cause loss of silencing at *Sb-HMR*, as *S. bayanus* diploids with only one copy of *Sb-SIR4* showed no silencing defect (Figure 4.2, B, row 2), nor did *S. cerevisiae* diploids with only one copy of *Sc-SIR4* (data not shown). Furthermore, a hybrid diploid containing two copies of *Sc-SIR4* (the *Sb-SIR4* gene was replaced by *Sc-SIR4*) also failed to silence *Sb-HMR* (Figure 4.2, A, row 5). In contrast, one *Sc-SIR4* gene was able to silence *Sc-HMR* in all hybrid strains tested (Figure 4.2, A, bottom panel; Figure 4.3, A). Thus, the hybrid cellular environment did not interfere with Sc-Sir4 function, and within a species, *SIR4* was not

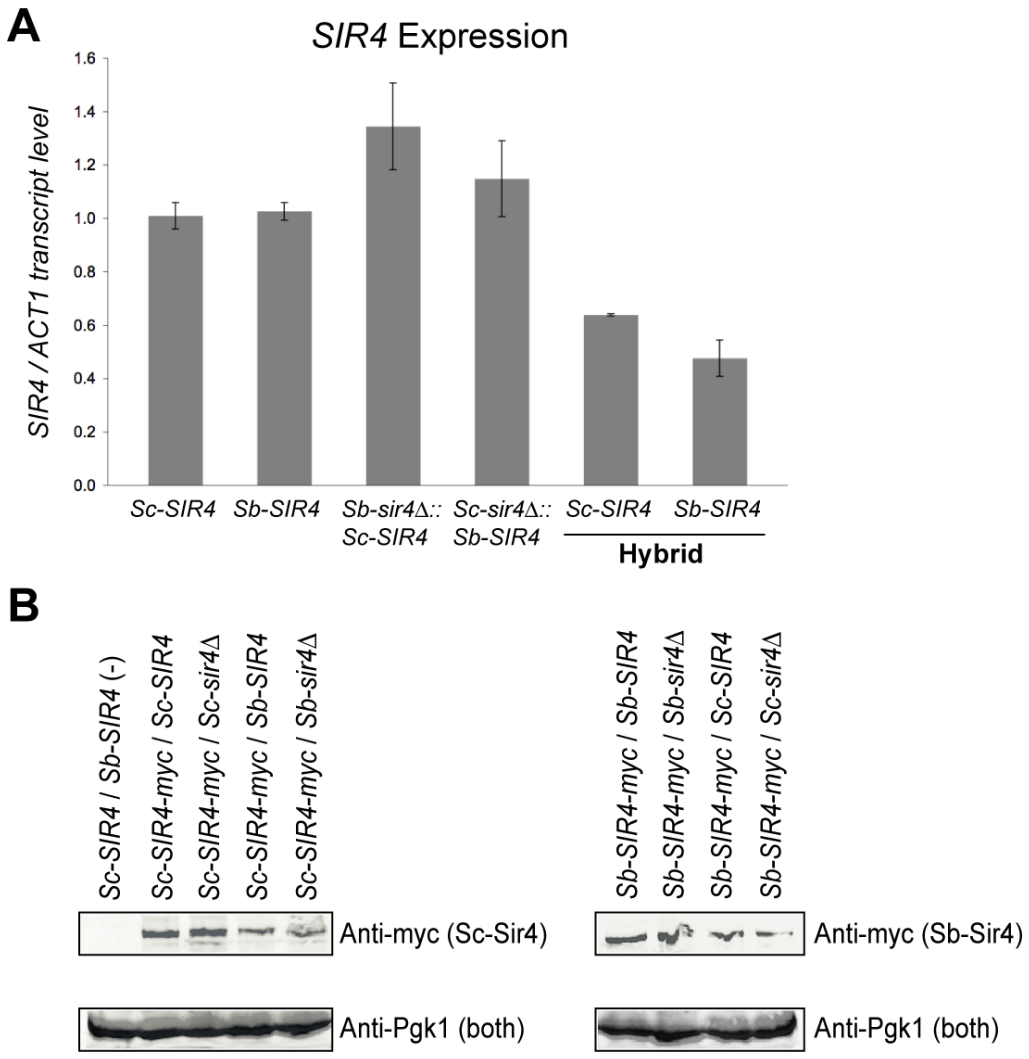

Figure 4.1. *SIR4* expression analysis in *S. cerevisiae*, *S. bayanus*, and *S. cerevisiae/S. bayanus* interspecies hybrids. (A) *Sc-SIR4* and *Sb-SIR4 RNA* analysis by quantitative RT-PCR. Amplification values for *SIR4* cDNA were normalized to those of actin (*ACT1*), as indicated in Methods. **Left to right:** *Sc-SIR4* expression in *S. cerevisiae* haploid (JRY4012); *Sb-SIR4* expression in *S. bayanus* haploid (JRY8822); expression of *Sc-SIR4* replacement allele in *S. bayanus* haploid (JRY9049); expression of *Sb-SIR4* replacement allele in *S. cerevisiae* haploid (JRY9027); expression of either the *Sc-SIR4* or *Sb-SIR4* allele in a hybrid diploid (JRY9054). Note that because equivalent amounts of total cDNA were added to all qRT-PCR reactions, the apparent expression levels of *Sc-SIR4* and *Sb-SIR4* in this hybrid diploid were expected to be 50% of their levels in haploids. Error bars show standard deviations (n = 3). (B) Sc-Sir4 and Sb-Sir4 protein expression analysis by immunoblot. **Left panel:** A hybrid diploid with no Myc tag (lane 1), and Sc-Sir4-myc expression in *S. cerevisiae* diploids (lanes 2 and 3) and hybrid diploids (lanes 4 and 5). **Right panel:** Sb-Sir4-myc expression in *S. bayanus* diploids (lanes 6 and 7) and hybrid diploids (lanes 8 and 9). Phosphoglucokinase (Pgk1) expression is shown as a loading control.

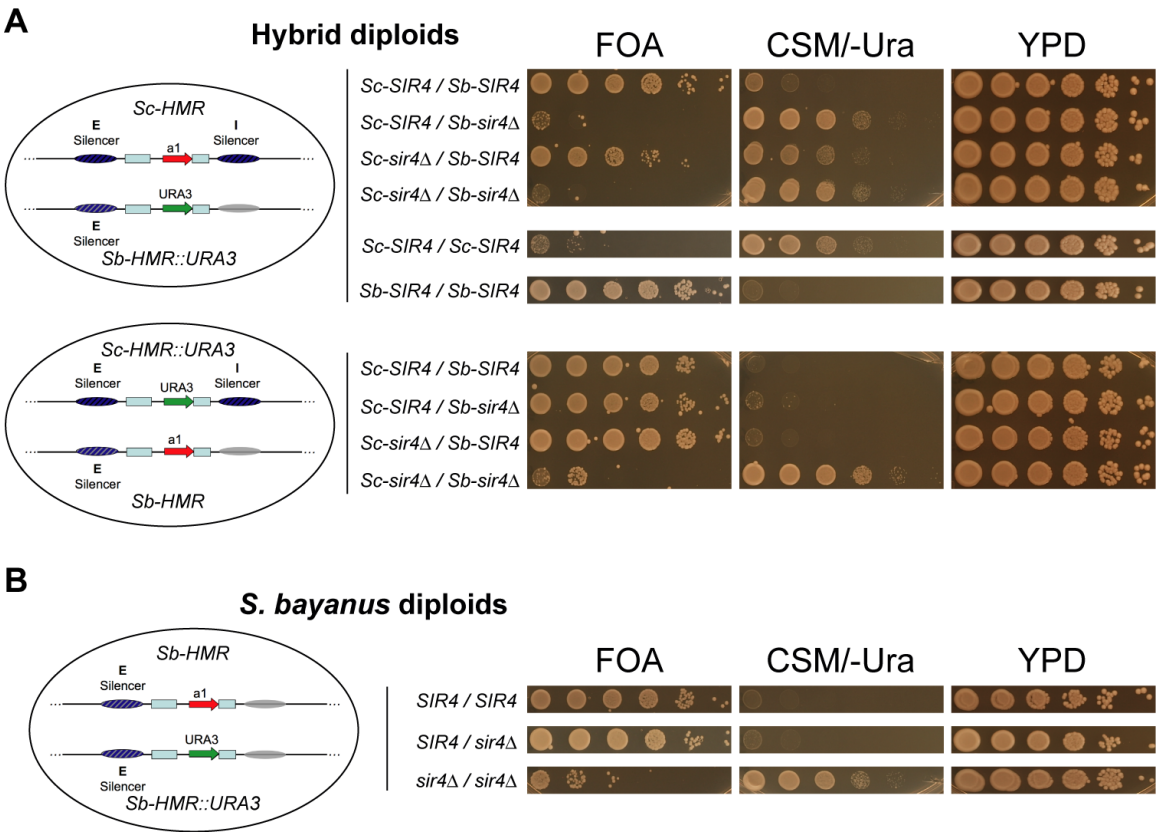

Figure 4.2. Incompatibility between *S. cerevisiae SIR4* and *S. bayanus HMR* in *S. cerevisiae/S. bayanus* interspecies hybrids. (A) Silencing of the *Sb-HMR::URA3* reporter gene (top panel) or the *Sc-HMR::URA3* reporter gene (bottom panel) in *S. cerevisiae/S. bayanus* hybrids was assayed by growth on selective media. For each strain, a ten-fold dilution series of yeast cells was spotted onto medium counter-selective for *URA3* expression (FOA), selective for *URA3* expression (CSM/-Ura), or rich medium (YPD). Schematics at left show the configurations of the salient features of two species' *HMR* loci in each hybrid strain: silencers (ovals), mating-type cassette homology regions (blue boxes), *HMR***a**1 ORF (red arrow), *URA3* ORF (green arrow). Differential shading of silencers indicates their overall poor conservation (except the Rap1 and Abf1 binding sites) between species. Gray oval indicates the presumed location of the *Sb-HMR-I* silencer. Presence or absence (*Δ*) of the *S. cerevisiae* (*Sc*) and *S. bayanus* (*Sb*) *SIR4* alleles are indicated to the right of schematics. See Table 4.1 for complete strain genotypes. (B) Silencing of the *Sb-HMR::URA3* reporter gene in wild-type, *SIR4/sir4Δ*, or *sir4Δ/sir4Δ S. bayanus* diploids.

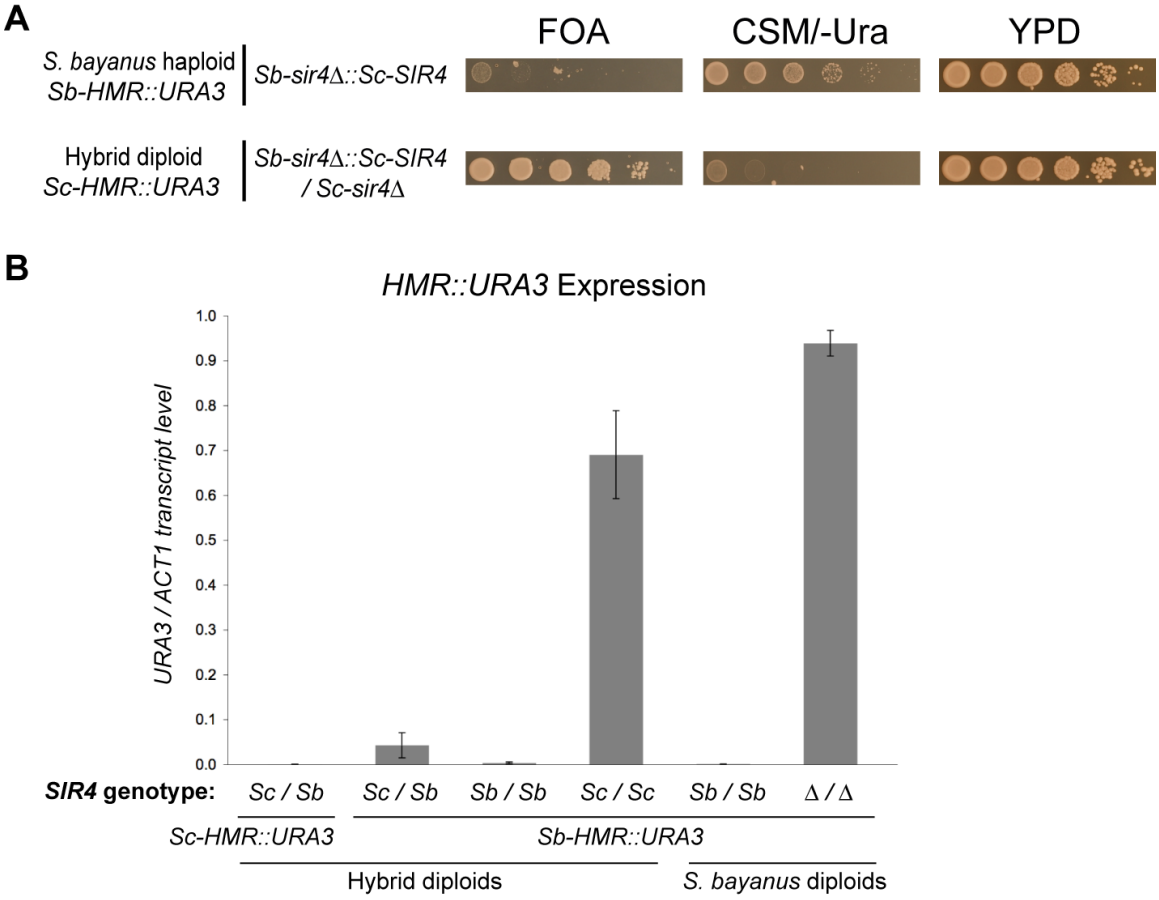

Figure 4.3. Further characterization of the silencing incompatibility. (A) *Sc-SIR4* was unable to silence *Sb-HMR* in *S. bayanus*. **Top row:** Silencing of *Sb-HMR::URA3* in an *S. bayanus* haploid strain bearing *Sc-SIR4* integrated in place of *Sb-SIR4*. **Bottom row:** Control showing that the *Sb-sir4Δ::Sc-SIR4* replacement allele could supply silencing function to *Sc-HMR* in an *S. cerevisiae/S. bayanus* hybrid. (B) RNA analysis of *HMR::URA3* reporters in *S. cerevisiae/S. bayanus* hybrids and *S. bayanus* diploids. *URA3* amplification values were normalized to those of actin (*ACT1*) for each strain. Error bars show standard deviations (n = 3).

haplo-insufficient. It appeared that Sc-Sir4 was either inhibited specifically at *Sb-HMR*, or somehow failed to interact with proteins that promoted *Sb-HMR* silencing.

Transcription analysis of a critical set of the hybrid strains showed good correspondence between expression of the *HMR::URA3* reporter and growth patterns observed on FOA and CSM/-Ura media (Figure 4.3, B).

We note that in the interspecies hybrids with native *SIR4* allele configuration (*Sc-SIR4*/*Sb-SIR4*), *Sb-HMR* silencing appeared weakly defective relative to the complete silencing of *Sb-HMR* in *S. bayanus* diploids by both the reporter assay and direct RNA measurement (compare Figure 4.2, A, row 1 with Figure 4.2, B, row 1; Figure 4.3, B). In addition, *Sb-HMR* silencing was further weakened in hybrids lacking Sc-Sir4 (Figure 4.2, A, compare row 3 with row 1). This result was paradoxical because Sc-Sir4 appeared to have no ability to silence *Sb-HMR* in hybrids lacking Sb-Sir4. As explained below, these weak *Sb-HMR* silencing defects were likely due to an emergent property of the hybrids, resulting from unusually strong interactions between Sb-Sir4 and *S. cerevisiae* silent loci that effectively reduced Sb-Sir4 associations with *Sb-HMR*. The presence of Sc-Sir4 limited the competition for Sb-Sir4.

Conditional association of Sc-Sir4 with S. bayanus HML and HMR

The inability of Sc-Sir4 to function at *Sb-HML* and *Sb-HMR* could have been manifested either during its recruitment, or after its assembly into chromatin (Kirchmaier and Rine 2006). To determine where in the assembly of *S. bayanus* silenced chromatin Sc-Sir4 protein was blocked, we compared the ability of Sc-Sir4 and Sb-Sir4 proteins to associate with all silent loci of both species at high resolution using chromatin-immunoprecipitation followed by deep-sequencing of the precipitate (ChIP-Seq). Sir4 ChIP-Seq was performed using hybrid diploids expressing Sc-Sir4 only, Sb-Sir4 only, or both Sc-Sir4 and Sb-Sir4, allowing the occupancy of each species' *HML* and *HMR* loci to be evaluated simultaneously. In each strain, only one *SIR4* allele carried a 13xMyc epitope tag (Longtine et al. 1998). In hybrids expressing Sc-Sir4 only, robust enrichment of *Sc-HML* and *Sc-HMR* silencers was observed as expected, with very weak enrichment of *Sb-HML* and *Sb-HMR* silencers (Figure 4.4, A; Table 4.2). Strikingly, Sc-Sir4 association with an internal region of *Sb-HMR* was indistinguishable from non-silenced regions. In contrast, as predicted from the genetic results, Sb-Sir4 associated robustly with *HML* and *HMR* loci from both species, and did so most robustly at *S. cerevisiae* silencers (Figure 4.4, A; Table 4.2). The ChIP-Seq results were validated at *Sc-HMR*, *Sb-HMR*, and control loci using standard ChIP-qPCR analysis (Figure 4.5, A). Thus, Sc-Sir4 showed strongly reduced association with *Sb-HML* and *Sb-HMR* silencers, and no detectable association with their internal regions. The relative absence of Sc-Sir4 from these normally silenced regions of the *S. bayanus* genome was consistent with two possibilities. Perhaps Sc-Sir4 could not interact properly with Rap1, ORC, or the *S. bayanus* Sir1 paralogs assembling on their silencers, or perhaps an *S. bayanus* protein was preventing stable association between Sc-Sir4 and *S. bayanus* silencers.

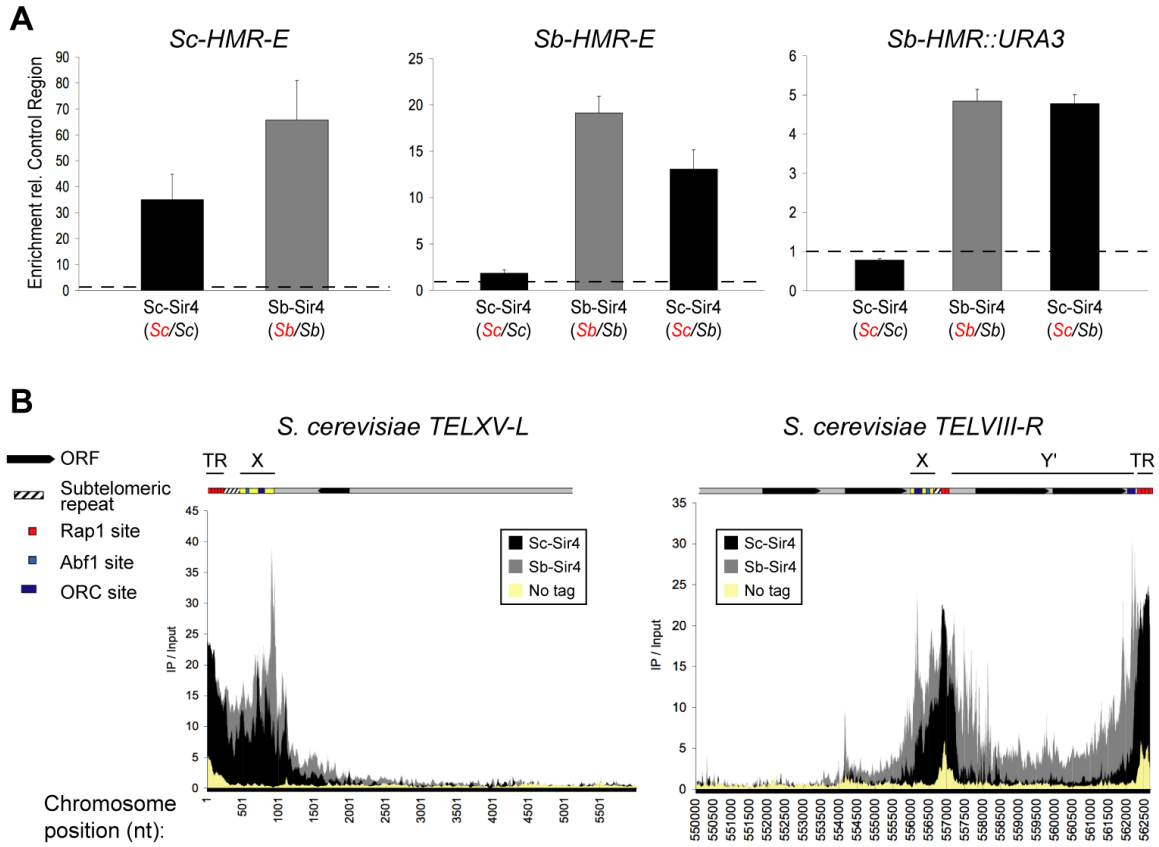

Figure 4.4. Sc-Sir4 versus Sb-Sir4 ChIP-Seq analysis in *S. cerevisiae/S. bayanus* hybrids. (A) **Left:** Sir4 IP/Input ratios, normalized to control regions within each experiment, for the *Sc-HMR-E* silencer. **Center:** Normalized IP/Input ratios for the *Sb-HMR-E* silencer. **Right:** Normalized IP/Input ratios for the *Sb-HMR::URA3* ORF. "Sc-Sir4" or "Sb-Sir4" labels indicate which species' Sir4 protein was examined by ChIP. Species' identities of both *SIR4* alleles in each strain are given in parentheses, with the allele bearing the 13x-Myc tag indicated in red: (*Sc/Sc*), JRY9062; (*Sb/Sb*), JRY9063; (*Sc/Sb*), JRY9064 (see Table 4.1 for complete strain genotypes). Dashed lines indicated IP/Input ratio of non-silenced control regions. Error bars indicate the standard error of the mean of all 100bp windows covering a region. See Table 4.2 for non-normalized IP/Input ratios, and Methods for a description of data processing. (B) ChIP-Seq profiles of Sc-Sir4 (JRY9062), Sb-Sir4 (JRY9063), and the "No tag" control (JRY9054) at two *S. cerevisiae* telomere regions. The ratio of IP/Input read counts for each base of a telomeric region is plotted. Diagrams indicate salient genetic features of two telomeres (see key at left) with X elements (yellow boxes), Y′ elements, and terminal repeats (TR) containing Rap1 binding sites, labeled above. *TELXV-L* (left panel) has an X-element-only end, whereas *TELVIII-R* (right panel) has an X-Y′ end. The *TELVIII-R* Y′ element spans nucleotide positions 556986-562456, with two helicase-encoding ORFs located between positions 558014-562047 (www.yeastgenome.org). For the ORFs within this Y′ element, Sc-Sir4 had a mean IP/Input ratio of 1.2, and the "No tag" control had a mean IP/Input ratio of 0.9 (the mean IP/Input ratio for all non-silenced regions, genome wide, was approximately 0.7 for both Sc-Sir4 and Sb-Sir4 ChIPs).

Table 4.2. Average IP/Input signals for selected regions of the *S. cerevisiae/S. bayanus* hybrid genome. Each genetic element indicated at left represents a 600bp region containing a silencer, an ORF inside an *HMR* locus, or a non-silenced control region. These control regions were located 3kb to the left of *HMR-E* in either species' genome, and correspond to syntenic regions of both species' *YCR095c* genes. Strains used in this analysis, from left to right: JRY9062, JRY9063, JRY9064, JRY9065.

Region	Sc-Sir4 (*Sc*/*Sc*)	Sb-Sir4 (*Sb*/*Sb*)	Sc-Sir4 (*Sc*/*Sb*)	Sb-Sir4 (*Sc*/*Sb*)
Sc-HML-E	12.49	17.99	8.59	10.07
Sb-HML-E	2.75	13.78	6.11	8.28
Sc-HMR-E	18.58	45.98	12.44	14.51
Sb-HMR-E	1.04	7.33	2.85	4.83
Sc-HMRa1	1.76	3.33	1.16	1.39
Sb-HMR::URA3	0.44	1.86	1.04	1.11
Sc-Control	0.54	0.68	0.47	0.40
Sb-Control	0.56	0.38	0.22	0.21

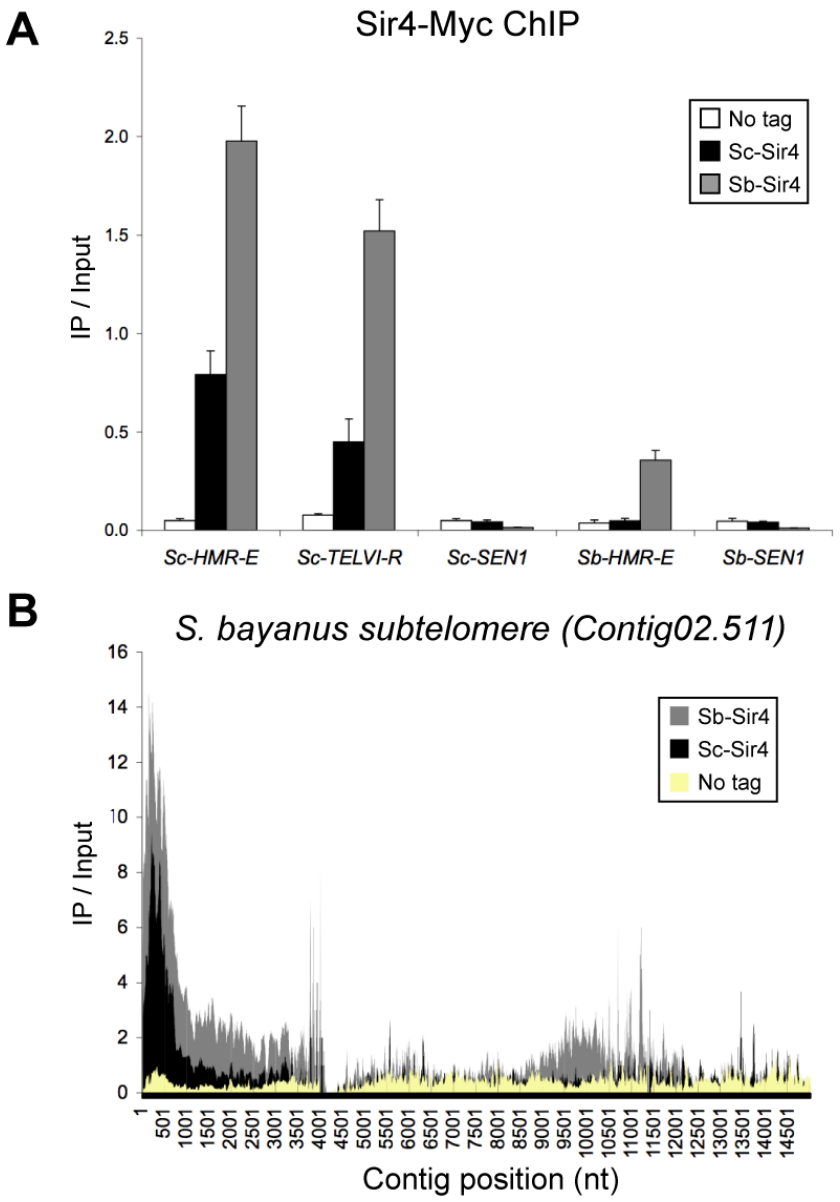

Figure 4.5. Additional comparative Sir4 ChIP analyses. (A) ChIP-qPCR analysis of Sc-Sir4 versus Sb-Sir4. For each primer set, the IP/Input ratios for Sc-Sir4 (JRY9062), Sb-Sir4 (JRY9063), and the "No-tag" control (JRY9054) are shown. Error bars show standard deviations (n = 3). (B) ChIP-Seq analysis of Sc-Sir4 versus Sb-Sir4 association on an *S. bayanus* contig containing subtelomeric sequence (GenBank accession number AACG02000166). Hybrid strains used in this analysis were identical to those used in Figure 4.4(B) and 4.5(A): **Sc-Sir4**, JRY9062; **Sb-Sir4**, JRY9063; **No tag**, JRY9054. Per-base IP/Input ratios, determined as in Figure 4.4(B), are plotted versus contig position. We note that the terminal $TG_{1\text{-}3}$ repeats are not present in the current *S. bayanus* genome assembly (Cliften, et al. 2006).

The comparative Sir4 ChIP-Seq data provided a surprising insight into the mechanism of Sir4 incorporation into silent chromatin. Although Sc-Sir4 binding to *Sb-HML* and *Sb-HMR* loci was barely detectable in hybrids expressing Sc-Sir4 only, in hybrids expressing both Sc-Sir4 and Sb-Sir4, Sc-Sir4 binding increased substantially at *Sb-HML* and *Sb-HMR* silencers and internal regions (Figure 4.4, A, compare center panel with right panel; Table 4.2). Thus, despite the poor ability of Sc-Sir4 to associate with *Sb-HML* and *Sb-HMR* on its own, Sb-Sir4 somehow provided Sc-Sir4 access to them. It appeared that Sir4 association with *S. bayanus HML* and *HMR* involved two distinguishable modes of interaction. Sb-Sir4 was capable of both interaction modes, but Sc-Sir4 was capable of only one. Moreover, the divergent mode was apparently critical only for the initial association of Sir4 with a silencer, and not for subsequent associations with the silenced region.

Sb-Sir4-assisted incorporation of Sc-Sir4 into *Sb-HML* and *Sb-HMR* was consistent with Sc-Sir4 contributing to silencing at these loci, as suggested by the decreased *Sb-HMR* silencing in hybrids lacking Sc-Sir4 (Figure 4.2, A, row 3). However, this hypothesis *per se* could not explain the sensitivity of *Sb-HMR* silencing to reduced *Sb-SIR4* dosage that was observed in interspecies hybrids, but not in *S. bayanus* diploids (compare Figure 4.2, A, rows 1 and 3, with Figure 4.2, B, row 2; Figure 4.3, B). Further analysis of Sir4 localization on the *S. cerevisiae/S. bayanus* hybrid genome by ChIP-Seq provided an explanation of this hybrid-specific *Sb-SIR4* dosage sensitivity, as describe next.

Differential association of the two Sir4 proteins with native telomeric regions: Sb-Sir4 sequestration by S. cerevisiae subtelomeres

Given the differential association of Sc-Sir4 and Sb-Sir4 with the two species' *HML* and *HMR* loci, we asked if any other loci, genome-wide, also showed a dramatic discrepancy. In *S. cerevisiae,* silencing by Sir proteins occurs at telomeres and subtelomeres, in addition to *HML* and *HMR* (Gottschling et al. 1990; Palladino et al. 1993; Hecht et al. 1995). A comparison of the interspecies hybrids expressing Sc-Sir4 only versus Sb-Sir4 only showed that all *S. cerevisiae* $TG_{1\text{-}3}$ terminal repeats (which contain embedded Rap1 binding sites), including those present on the centromere-proximal side of some Y′ elements, were comparably occupied by both species' Sir4 proteins (Figure 4.4, B). This result was not surprising as the telomerase-replicated repeated sequence, templated by the *TLC1* RNA, is identical in the two species (O. Zill and J. Rine, unpublished observations). Thus, it appeared that Sir4 association with the *S. cerevisiae* genome, as promoted by Rap1, was not substantially different between Sc-Sir4 and Sb-Sir4. Indeed, the C-terminal residues of Sc-Sir4 critical for its interaction with Rap1 are conserved in Sb-Sir4 (O. Zill, unpublished observations).

Unexpectedly, *S. cerevisiae* subtelomeres had two types of regions notably more enriched by Sb-Sir4 ChIP than by Sc-Sir4 ChIP. These regions corresponded to X elements and the helicase-encoding ORFs of Y′ elements. For X elements, ChIP-Seq of Sc-Sir4 showed an average of 7-fold enrichment, whereas Sb-Sir4 showed an average of 14-fold enrichment, with even greater disparity often evident immediately adjacent to X

elements (Figure 4.4, B). Therefore, Sb-Sir4 either associated more robustly with factors bound to X elements than did Sc-Sir4, or conceivably was excluded less effectively. X element core sequences contain ORC and Abf1 binding sites, and are bordered on the telomere-proximal side by X element combinatorial repeats (formerly known as subtelomeric repeats or STRs; (Louis 1995)) and the terminal repeats (see http://www.yeastgenome.org/images/yeastendsfigure.html for schematics depicting X-only and X-Y′ telomere ends). The differential pattern of Sir4 association with X elements was consistent with Sb-Sir4 associating more robustly than Sc-Sir4 with sequences at, and immediately adjacent to, the ORC binding sites, presumably via ORC-mediated interactions (Figure 4.4, B). Other *S. bayanus* proteins produced in the hybrids, such as the Sir1 paralogs, may have been involved in the enhanced association of Sb-Sir4 with X elements, as discussed below.

We observed weak Sc-Sir4 association with Y′ elements despite its strong association with neighboring terminal repeats (Figure 4.4, B, right panel), consistent with earlier observations using ChIP-chip and transcription reporter analyses (Pryde and Louis 1999; Sperling and Grunstein 2009). Surprisingly, Sb-Sir4 associated considerably better than Sc-Sir4 with all Y′ elements, which showed an average of 5-fold enrichment across their coding regions by Sb-Sir4 ChIP versus 1.2-fold enrichment by Sc-Sir4 ChIP. We note that the *S. bayanus* genome lacks Y′ elements, and thus *S. bayanus* subtelomeres may have reduced Sir4 recruitment potential relative to *S. cerevisiae* subtelomeres (Liti et al. 2005; Martin et al. 2009). The enhanced associations of the Sb-Sir4 protein with Y′ elements suggested that, in the hybrid strains, *S. cerevisiae* telomeres might have limited Sb-Sir4 association with its cognate silent loci, leading to the somewhat weakened *Sb-HMR* silencing observed in hybrids with only one copy of Sb-Sir4 (Figure 4.2, A, rows 1 and 3; Figure 4.3, B). Sb-Sir4 association was indeed reduced at *Sb-HMR* and *Sb-HML* silencers in a hybrid expressing both Sc-Sir4 and Sb-Sir4, relative to Sb-Sir4 association with these silencers in the Sb-Sir4-only hybrid, which had two copies of *Sb-SIR4* (Table 4.2, compare columns 2 and 3). Thus, Sc-Sir4 may have, in effect, protected *Sb-HMR* silencing in hybrids when Sb-Sir4 was present (Figure 4.2, A, compare rows 1 and 3) by occupying highly attractive sites for Sb-Sir4 at *S. cerevisiae* telomeres.

The ChIP-Seq data allowed us to determine whether the species restriction to Sc-Sir4 association, evident at *Sb-HML* and *HMR*, also applied to *S. bayanus* telomeres. Although subtelomeric regions of the *S. bayanus* genome are presently incompletely assembled and annotated (see *Saccharomyces* Genome Database, www.yeastgenome.org), we identified several candidate subtelomeric contigs based on homology to *S. cerevisiae* subtelomeric genes and X elements. Contigs from the *S. bayanus* genome assembly that contained Sir4-bound regions (as determined by peak-calling software, see Methods) and putative subtelomeric sequence were further examined for Sir4 ChIP enrichment (an example is shown in Figure 4.5, B). Sb-Sir4 associated with one end of each of these contigs and usually with an internal region as well, typically within 10kb of the contig end. Interestingly, in the Sc-Sir4-only hybrid, Sc-Sir4 association was observed at the contigs' ends, but not at the internal regions that bound Sb-Sir4. This result suggested that Sc-Sir4, even in the absence of Sb-Sir4, was capable of associating with *S. bayanus* telomere ends, presumably via the conserved

Rap1 protein, but could not make some additional contacts necessary to associate with internal sequences.

The Sb-HMR silencers mediated the species restriction of Sc-Sir4

The cross-species complementation and ChIP analyses suggested that the incompatibility between *Sc-SIR4* and *Sb-HML* and *HMR* was caused by the failure of one or more physical interactions occurring at *S. bayanus* silencers. In principle, the lack of productive Sc-Sir4 association with *Sb-HML* and *Sb-HMR* could have resulted either from an *S. bayanus*-specific inhibitor of silencing that Sc-Sir4 could not overcome, or an *S. bayanus*-specific positive regulator of silencing (e.g., Sb-Rap1 or Sb-Sir1) with which Sc-Sir4 could not interact. To distinguish between these models, in an *S. cerevisiae* strain, we replaced the *Sc-HMR* locus with *Sb-HMR* containing the *URA3* reporter, including the flanking silencer elements (Figure 4.6, A). If *S. bayanus* encoded an inhibitor of silencing that Sc-Sir4 could not overcome, *Sb-HMR* should be silenced in *S. cerevisiae*, given the strong conservation of ORC, Rap1, and Abf1 proteins, and the Rap1 and Abf1-binding sites in the *HMR-E* silencer (Teytelman et al. 2008). If, however, Sc-Sir4 failed to be recruited to *S. bayanus* silencers, we would expect little or no silencing of *Sb-HMR* in *S. cerevisiae*.

Upon transfer into *S. cerevisiae*, *Sb-HMR* was silenced extremely poorly (Figure 4.6, B, row 1). However, the transplanted *Sb-HMR* locus could still be silenced in the context of the *S. cerevisiae* chromosome: in hybrids made by mating the *S. cerevisiae Sb-HMR* strain to wild-type *S. bayanus*, the transplanted *Sb-HMR* locus was silenced to approximately the same degree as the native *Sb-HMR* locus in hybrids (Figure 4.6, B, row 2, compare with Figure 4.6, C, rows 1 and 2). The incomplete nature of *Sb-HMR* silencing in this hybrid was likely due to the *Sb-SIR4* dosage sensitivity observed in the original set of hybrids (Figure 4.2, A, row 3). Thus, the lack of silencing of *Sb-HMR* in hybrids expressing only Sc-Sir4 (Figure 4.2, A, rows 2 and 5) was not due to an inhibitor of silencing encoded elsewhere in the *S. bayanus* genome. Rather, the incompatibility was encoded in the *Sb-HMR* locus itself, requiring *S. bayanus*-specific silencing proteins to interpret *Sb-HMR*-specific sequence information. These "interpreter" proteins potentially included DNA-binding proteins such as ORC, Rap1, and Abf1, or proteins indirectly associated with silencers, such as Sir1 and Sir4, or both.

Alignments of *Sc-HMR* and *Sb-HMR* suggested that their functional divergence was due to changes in the silencer sequences between the two species. The *HMR***a**1 promoter was 93% identical between *S. cerevisiae* and *S. bayanus*, well above the genome-wide average of 62% identity for all intergenic regions, and the mating-type cassette-homology sequences (shared with *MAT* and *HML*) approached 100% identity (Figure 4.6, A). Notably, the silencer sequences share well below the genome-wide average identity for intergenic regions, and are difficult to align outside of the conserved Rap1 and Abf1 sites (Teytelman et al. 2008).

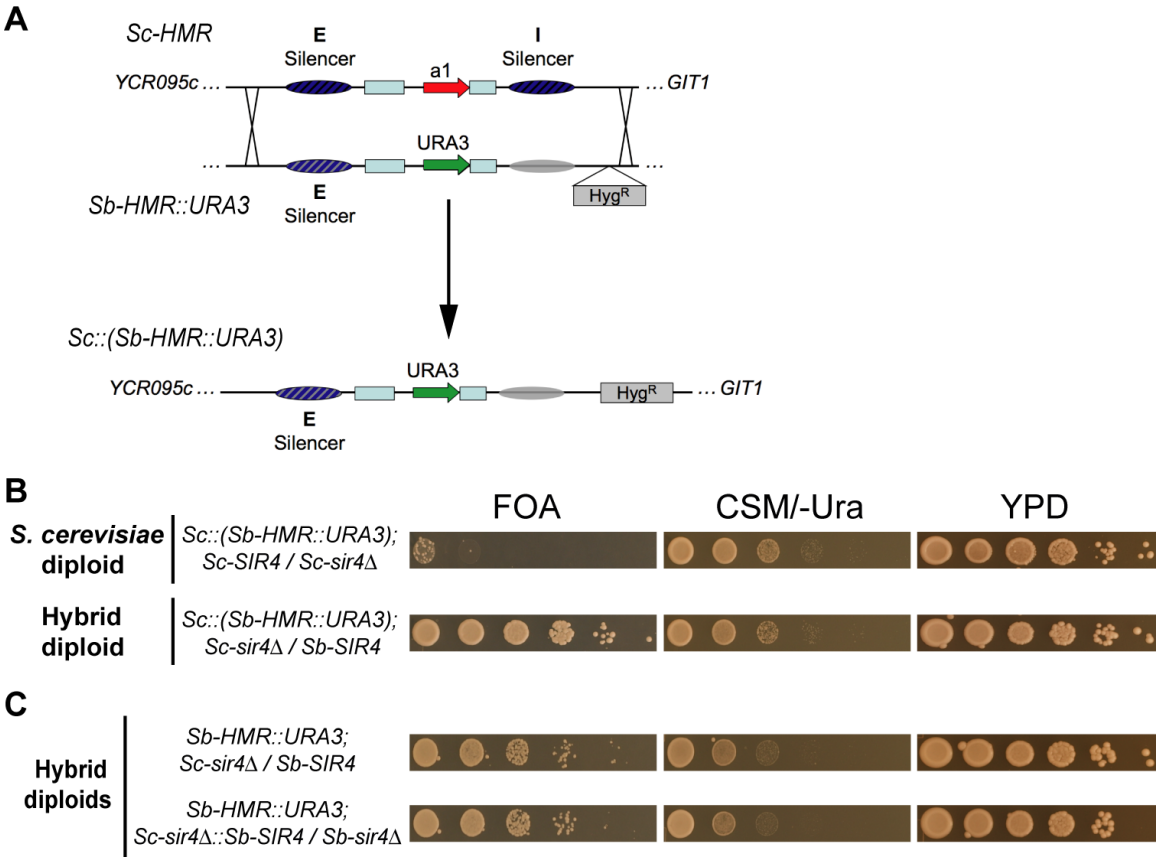

Figure 4.6. Transfer of *Sb-HMR* into *S. cerevisiae*, identifying *cis*-component of cross-species silencing incompatibility. (A) Schematic diagram depicts replacement of *Sc-HMR* by *Sb-HMR::URA3* in *S. cerevisiae*, creating the *Sc::(Sb-HMR::URA3)* allele. Diagonal lines depict cross-overs for the *HMR* allele swap, with other genetic features of the two *HMR* loci as in Figure 4.2. A hygromycin-resistance marker (Hyg^R) was inserted 3kb to the right of *Sb-HMR* to allow targeted recombination. (B) Silencing of the *Sc::(Sb-HMR::URA3)* reporter in *SIR4/sir4Δ S. cerevisiae* diploids (first row), and in *Sc-sir4Δ/Sb-SIR4 S. cerevisiae/S. bayanus* hybrids (second row). (C) Control strains showing expected silencing functions of *Sc::(Sb-HMR::URA3)* and *Sc-sir4Δ::Sb-SIR4* replacement alleles in interspecies hybrids. Silencing of the *Sb-HMR::URA3* reporter gene, located in its native *S. bayanus* chromosomal context, in *Sc-sir4Δ/Sb-SIR4* hybrids (top row), and in *Sc-sir4Δ::Sb-SIR4/Sb-sir4Δ* hybrids (bottom row). Note that silencing of *Sb-HMR::URA3* in these hybrids was equivalent to *Sc::(Sb-HMR::URA3)* silencing in (B), indicating that the functions of *Sb-HMR* and *Sb-SIR4* were largely unaffected by *S. cerevisiae* chromosomal context.

Reconstitution of S. bayanus silencing in S. cerevisiae with Sb-SIR4 and Sb-KOS3

The simplest model consistent with the results so far was that the silencing incompatibility was limited to Sir4, with Sc-Sir4 having a more restricted range of interactions than Sb-Sir4. To test this possibility, we replaced *Sc-SIR4* with *Sb-SIR4* in the *S. cerevisiae* strain bearing *Sb-HMR*. If the incompatibility involved only *SIR4* and silencers, *Sb-SIR4* should restore silencing to *Sb-HMR*. The *S. cerevisiae* strain with *Sb-SIR4* and *Sb-HMR* indeed showed an increase in silencing relative to the *Sc-SIR4 Sb-HMR* strain, confirming that changes in Sir4 itself contributed to the silencing incompatibility. However, this silencing increase—a modest five-fold change—was detectable only as an increase in FOA resistance, and was still at least 100-fold below the level of *HMR* silencing seen in the hybrids (Figure 4.7, A, row 2; compare with Figure 4.6, B, row 2). Thus, although a portion of the incompatibility could be explained by *SIR4* and silencer co-evolution, one or more additional *S. bayanus* proteins were likely required to recruit Sb-Sir4 efficiently or to stabilize its association with *S. bayanus* silencers.

Interestingly, Sc-Sir4's very weak ability to silence the transplanted *Sb-HMR* locus resulted in the low-frequency appearance of FOA-resistant colonies (occurring at an approximate frequency of $5x10^{-5}$; Figure 4.7, A, row 1). Within these colonies, the cells were able to grow under conditions that killed the majority of cells that did not form colonies. Hence this silencing occurred at low frequency, but was nonetheless heritable. Indeed, *Sb-HMR* silencing by either Sb-Sir4 or Sc-Sir4 was fully dependent on *S. cerevisiae* Sir1 (Figure 4.7, A), whose role is to promote the establishment of heritable silencing. That *Sb-HMR* could be silenced at all in *S. cerevisiae* suggested that a critical subset of Sc-ORC, Rap1, and Abf1 bound productively to *Sb-HMR* silencers. It was therefore possible that providing additional *S. bayanus* silencing proteins could stabilize interactions between the *S. cerevisiae* DNA-binding proteins and *S. bayanus* silencers. Likely candidates to provide this presumptive function were the *S. bayanus* Sir1 paralogs, Kos1, Kos2, and Kos3, with Kos3 being the most closely related to the ancestral member of the Sir1 family (Gallagher et al. 2009). Interestingly, *Sb-KOS3* enhanced *Sb-HMR* silencing synergistically with *Sb-SIR4*, but not with *Sc-SIR4* (Figure 4.7, B; compare rows 1, 5, and 10). None of the other Sir1 paralogs of *S. bayanus* provided a dramatic enhancement of *Sb-HMR* silencing. The *Sb-HMR Sb-SIR4 +Sb-KOS3* strain showed 100-fold better silencing than the *Sb-HMR Sc-SIR4* strain (Figure 4.5, B, compare rows 1 and 10). This result was particularly interesting because Sir4 interacts weakly and non-specifically with DNA (Martino et al. 2009), and Kos3 is not thought to bind DNA at all. Thus, the "interpretation" of differences between the *Sb-HMR* and *Sc-HMR* silencers by Sb-Kos3 and Sb-Sir4 presumably required some sort of *HMR*-allele-specific collaboration with silencer-binding proteins that could be interpreted by Sb-Kos3 and Sb-Sir4 in a species-specific way.

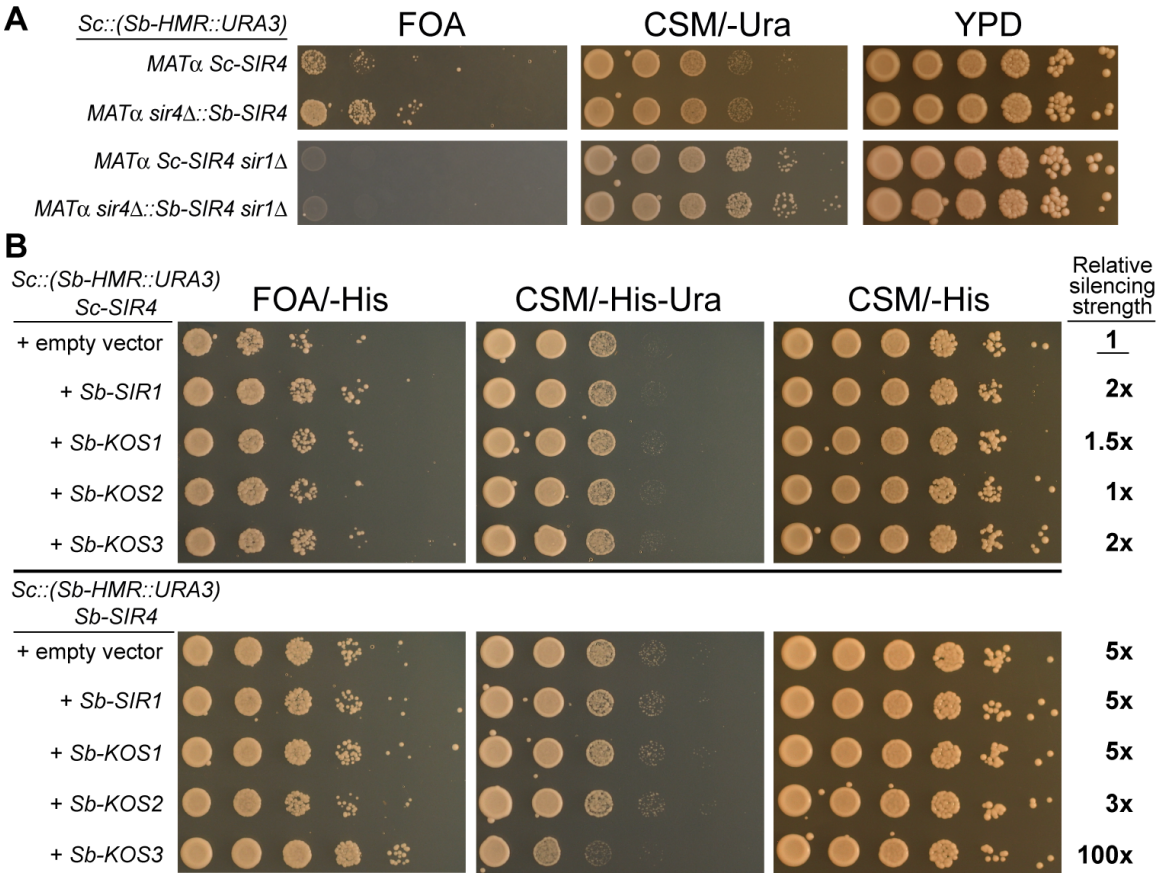

Figure 4.7. Partial reconstitution of *Sb-HMR* silencing in *S. cerevisiae* by transfer of *S. bayanus* Sir4 and Kos3 proteins. (A) **Top panel:** Silencing of the *Sc::(Sb-HMR::URA3)* replacement allele in *S. cerevisiae MAT*α haploids bearing either the endogenous *Sc-SIR4* gene or an integrated *Sb-SIR4* gene (top panel). **Bottom panel:** Silencing of the *Sc::(Sb-HMR::URA3)* replacement allele in the absence of *Sc-SIR1*. (B) *S. cerevisiae* strains bearing the *Sc::(Sb-HMR::URA3)* replacement allele, and either the endogenous *Sc-SIR4* gene or an integrated *Sb-SIR4* gene, were transformed with plasmids encoding individual *S. bayanus* Sir1 paralogs and assayed for silencing function (FOA/-His, CSM/-His-Ura, or CSM/-His indicate silencing reporter media also selective for maintenance of plasmids bearing the *HIS3* marker). Quantification of relative silencing function, based on growth on FOA/-His, is indicated at right. Fold-change comparisons were made relative to the *Sc::(Sb-HMR::URA3) Sc-SIR4* strain bearing an empty vector (row 1). We note that the *CEN/ARS* plasmid itself appeared to enhance *Sb-HMR* silencing relative to the untransformed strains (compare Figure 4.5, B, rows 1 and 6, to Figure 4.5, A, rows 1 and 2). However, relative comparisons among transformed strains were still possible.

Differential ORC utilization by S. bayanus silencers

By sequence conservation, Rap1 and Abf1 binding sites can be detected in the *Sb-HMR-E* silencer, but the ORC binding site is not readily identified (Teytelman et al. 2008). Given Sb-Sir4's dependence on Sir1 and Kos3, and their dependence on ORC (Triolo and Sternglanz 1996; Fox et al. 1997; Bose et al. 2004), our results suggested two likely explanations for why *Sb-HMR* was not silenced in *S. cerevisiae*: either Sc-ORC bound *S. bayanus* silencers less well than *S. cerevisiae* silencers, or Sc-ORC bound equivalently, but failed to promote silencing because it was in a suboptimal conformation or context with respect to other silencer binding proteins. In either case, the subsequent interactions with Sc-Sir1 and Sc-Sir4 might suffer. To test whether Sc-ORC indeed bound to *S. bayanus* silencers, we performed ChIP analysis on HA-tagged Sc-Orc5 in *S. cerevisiae* bearing *Sb-HMR*. Sc-Orc5 associated with the *Sb-HMR-E* silencer, albeit at a level several-fold below its association with *Sc-HMR-E* (Figure 4.8, A, left panel; note log scale on y-axis). A parallel analysis with Sc-Abf1 ChIP showed robust association of this protein with both *Sc-HMR-E* and *Sb-HMR-E* silencers (Figure 4.8, A, right panel). We note that both Sc-Orc5 and Sc-Abf1 associations with *Sb-HMR-E* showed small alterations in the *Sb-SIR4* strain relative to the *Sc-SIR4* strain, however, these changes did not correlate with *Sb-HMR* silencing levels (Figure 4.7, A, rows 1 and 2). These ChIP data were consistent with *Sb-HMR* silencers having conserved functional binding sites for ORC and Abf1.

To test whether Sc-ORC, Rap1, and Abf1 indeed participated in *S. bayanus* silencing, we monitored silencing of *Sb-HMR* in hybrids lacking either species' complement of each of these proteins (out of the six ORC subunits, we focused on Orc1 because it directly interacts with Sir1). Because *RAP1*, *ABF1*, and *ORC1* are essential, we assayed silencing in hybrids heterozygous for each gene. *S. cerevisiae* diploids sensitized to detect silencing defects at *HMR* show strong silencing defects if either *SIR1* or *SIR4* dosage is also reduced (Sussel et al. 1993). Similarly, *Sb-HMR* silencing was weakly compromised in hybrids (Figure 4.2, A), potentially providing a sensitized background to uncover similar types of genetic interactions. For this reason, any such silencing defects in heterozygous hybrids were expected to affect silencing of *Sb-HMR* but not *Sc-HMR*. Indeed, *Sb-HMR*, but not *Sc-HMR*, was further derepressed in hybrids lacking either *Sc-RAP1* or *Sb-RAP1* (Figure 4.8, B, top panel; data not shown). Note that *Sb-HMR* was fully silenced in *S. bayanus RAP1/rap1Δ* diploids; therefore, reduced *RAP1* dosage *per se* did not cause the loss of silencing observed in the hybrid (Figure 4.8, B, bottom panel). Thus, Sc-Rap1 participated in *Sb-HMR* silencing in hybrids, likely by direct binding to *S. bayanus* silencers. In contrast to the analysis with *RAP1*, *Sb-HMR* was derepressed to a greater extent in hybrids lacking *Sb-ORC1*, but not in hybrids lacking *Sc-ORC1* (Figure 4.8, B, top panel). Again, *Sb-HMR* was fully silenced in *S. bayanus ORC1/orc1Δ* diploids (Figure 4.8, B, bottom panel), ruling out simple dosage explanations. Hence, Sb-Orc1 was more important for *Sb-HMR* silencing in hybrids than Sc-Orc1, suggesting that a partial species restriction existed with respect to ORC binding or activity at *Sb-HMR* silencers. Heterozygosity of *ABF1* had no effect on either *Sb-HMR* or *Sc-HMR* silencing (Figure 4.8, B, top panel; data not shown).

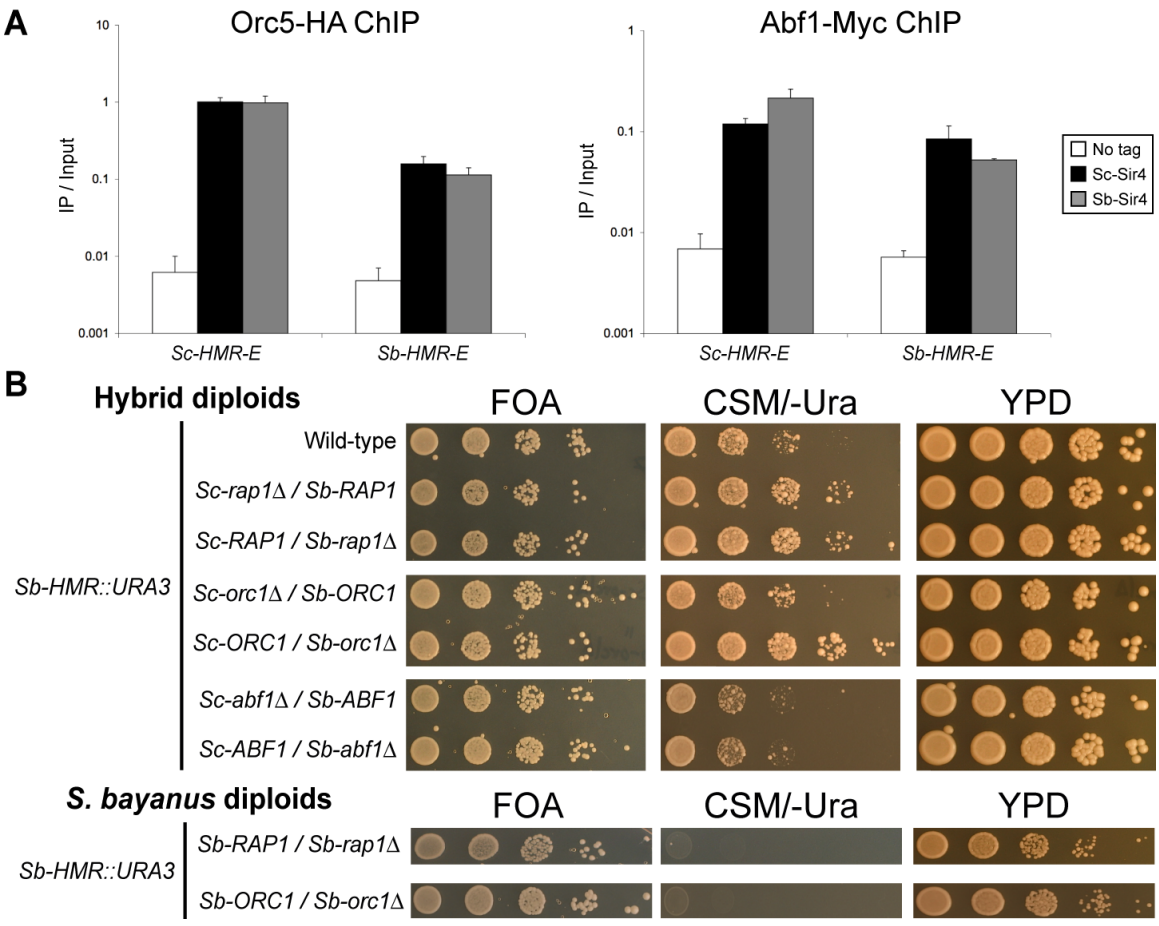

Figure 4.8. ChIP and genetic interaction analysis of ORC, Rap1, and Abf1 silencing functions. (A) ChIP analysis of Sc-Orc5 and Sc-Abf1 in *S. cerevisiae* at *Sc-HMR-E* versus *Sb-HMR-E*. Relative enrichment of silencer sequences was verified by comparison to amplification values for a positive control region, the *ARS1* replication origin, and a negative control region in the *SEN1* gene (data not shown). Note log scale on y-axis. Error bars show standard deviations (n = 3). (B) **Top panel:** Silencing of the *Sb-HMR::URA3* reporter gene in *S. cerevisiae/S. bayanus* hybrids each lacking a single allele of the *RAP1*, *ORC1*, or *ABF1* genes. **Bottom panel:** Silencing of the *Sb-HMR::URA3* reporter gene in *S. bayanus* diploids lacking one allele of *RAP1* or *ORC1*.

Discussion

Using interspecies hybrids, we have shown by three functional criteria—cross-species complementation assays, cross-species *cis-trans* tests, and genome-wide localization by ChIP-Seq—that the functions of both the Sir4 protein and multiple silencer elements have strikingly diverged over the short divergence time between closely related yeast species. Cross-species complementation assays revealed an incompatibility between Sc-Sir4 and *Sb-HML* and *Sb-HMR* (Figure 4.2, A). The inability of Sc-Sir4 to silence *Sb-HML* and *Sb-HMR* was due to a difference in the protein sequence of Sir4 between the two species rather than a difference in expression level (Figure 4.1). This incompatibility likely resulted from the coordinated divergence of multiple heterochromatin determinants: Sir1, Sir4, and silencers. Two pieces of evidence implicated *cis*-acting changes in silencer sequences as being key to the incompatibility. First, comparative ChIP-Seq analysis of Sir4 pinpointed an inability of Sc-Sir4 to associate stably with *S. bayanus* silencers (Figure 4.4, A; Table 4.2). Second, and most definitively, transfer of the *Sb-HMR* locus into *S. cerevisiae* demonstrated that this locus was inherently restrictive to Sc-Sir4 function (Figure 4.6, B). This result established that *S. bayanus* did not produce an inhibitor of Sc-Sir4 function, and mapped the locus of the species restriction to Sc-Sir4 function to the *Sb-HMR* silencers. As silencing of the transplanted *Sb-HMR* locus was largely restored in an *S. cerevisiae/S. bayanus* hybrid (Figure 4.6, B), *S. bayanus*-specific proteins were required to assemble silent chromatin at *Sb-HMR* in the manner dictated by the *Sb-HMR* silencers, with Sb-Sir4 and the Sb-Sir1 paralogs being the most likely candidates for species-specific "interpreter" proteins.

Co-evolution of silencer elements and heterochromatin proteins in budding yeast

The Sir4 protein and silencers diverged rapidly in concert, a process that was accompanied by loss of three Sir1 paralogs in the *S. cerevisiae* lineage (Gallagher, et al.). As silencing was robustly maintained in each species, it was likely that these factors had co-evolved such that coding changes in Sir4 and a reduction in Sir1 family members led to compensatory changes in silencers, or vice versa. The asymmetrical complementation of *SIR4* alleles (Figure 4.2, A), and the enhanced ability of Sb-Sir4 to bind *S. cerevisiae* silent loci compared to its cognate silent loci (Figure 4.4, B), suggested that *S. cerevisiae* silencer elements had become stronger than those of *S. bayanus*, while *S. cerevisiae* Sir1 and Sir4 proteins had become weaker (based on an operational definition) than *S. bayanus* Sir4 and its four Sir1 paralogs. The intra-species combinations of Sir1 and Sir4 proteins and silencers allowed efficient nucleation of silencing complexes at *HML* and *HMR* in each species.

Broadly speaking, we imagine two possible evolutionary paths for this co-evolution, with variations on either pathway possible. In an "adaptive" model, hypothetical selective pressure(s) induced coding changes in Sir4 and reduction in Sir1 family members (Zill, et al. *in preparation*), which then required "strengthening" mutations (for example, a change that increased the affinity of ORC for a silencer) in the silencers to maintain robust silencing. In a "constructive neutral" model (Stoltzfus 1999), strengthening mutations accumulated in silencers at random, thus relaxing the selective

constraints to maintain Sir1 paralogs and certain Sir4 residues. Once Sir1 paralogs were lost, the "stronger" silencers would need to be maintained by purifying selection.

An important question relevant to these models was in which lineage did the observed changes in Sir4 and silencer function occur relative to the common ancestor of *S. cerevisiae* and *S. bayanus*. Although accurate determination of the ancestral state of the silencing mechanism will require extensive evolutionary analyses, it appears that *S. bayanus* has retained at least two ancestral characters that *S. cerevisiae* has lost. First, Kos3, the ancestral Sir1-related protein, has been lost in *S. cerevisiae*. Second, the *SIR4* gene from *K. lactis*, an outgroup to the *Saccharomyces* clade, was able to complement silencing function in *S. bayanus sir4Δ* mutants (Zill, et al., *in preparation*). That a Sir4 protein from a species outside of *Saccharomyces* is compatible with *S. bayanus* silencers suggests that these elements did not "gain" a restrictive property in the *S. bayanus* lineage. The more likely scenario is that Sir4 changed in the *S. cerevisiae* lineage such that its range of interactions with other species' silencers has become restricted, consistent with earlier observations of cross-species function of Sir4 (Astrom and Rine, 1998). It will therefore be of interest to understand in detail the mechanism of silencing in *S. bayanus*, and to determine what forces caused the dramatic shift in Sir1 and Sir4 functionality in the *S. cerevisiae* lineage.

Perhaps the most striking finding of this study was that the heterochromatin proteins that showed the most dramatic evidence of co-evolution with silencers, Sir1 and Sir4, were not the ones that bind specific DNA sites, but rather associate with DNA indirectly via the conserved transcription factors Rap1, Abf1, and ORC. The key evidence demonstrating functional co-evolution between Sir4 and the Sir1 family and silencers came from attempts to reconstitute *Sb-HMR* silencing in *S. cerevisiae*. The changes in Sir4 sequence were not sufficient to explain the inability of Sc-Sir4 to function at *S. bayanus* silencers: expression of Sb-Sir4 in an *S. cerevisiae* strain was only modestly effective in silencing an *Sb-HMR* locus transplanted into that strain (Figure 4.7, A). The Sir1-dependence of the rare but heritable silencing events mediated by Sb-Sir4 at *Sb-HMR* in *S.cerevisiae* suggested that the limitation involved proteins dedicated to establishing silencing. Indeed, adding Sb-Kos3, the ancestral member of the Sir1 family, together with Sb-Sir4, enhanced silencing of *Sb-HMR* in *S. cerevisiae* by 100-fold (Figure 4.7, B), although not completely. It was possible that the site-specific DNA-binding proteins ORC, Rap1, and Abf1 had also co-evolved with silencer sequences. If this were the case, we would expect hybrids lacking the Sb-ORC, Sb-Rap1, or Sb-Abf1 proteins to have shown defective *Sb-HMR* silencing. However, only Sb-Orc1 inactivation (and by inference, inactivation of the entire Sb-ORC complex) showed the expected *S. bayanus* allele-specific effect on *Sb-HMR* silencing (Figure 4.8, B). This effect of *Sb-ORC1* deletion on *Sb-HMR* silencing was relatively modest, and the addition of *Sb-ORC1* (together with *Sb-SIR4*) had no effect on *Sb-HMR* silencing in *S. cerevisiae* reconstitution experiments (data not shown). Because Sc-Orc1, Sc-Rap1, and Sc-Abf1 were capable of supporting *Sb-HMR* silencing in hybrids (Figure 4.8, B), and in *S. cerevisiae* (Figure 4.7, B), their DNA-binding domains' interactions with silencers were largely conserved across species and hence were not engaged in notable co-evolution with silencers or with Sir4. Indeed, we were able to ChIP Sc-Orc5 and Sc-Abf1 on the *Sb-HMR-E* silencer in *S. cerevisiae* (Figure 4.8, A). Together, these results suggested

that the *cis*-acting differences between the two species' silencers were interpreted largely indirectly, via interactions between ORC, Sir1/Kos3, and Sir4, with a somewhat lesser contribution of differences in ORC-silencer DNA interactions.

Asymmetrical interactions of heterochromatin determinants in interspecies hybrids yielded insights into the silencing mechanism

Why did Sc-Sir4 not bind efficiently to *S. bayanus* silencers? In principle, the simplest explanation might be that the sequence divergence between *S. cerevisiae* and *S. bayanus* silencers precluded some contacts that Sir4 would make with specific DNA sequence. However, biochemical data on Sir4 point to a lack of sequence-specific binding to DNA (Martino et al. 2009), and are instead consistent with Sir4 being recruited to silencers solely via protein-protein interactions (Moazed et al. 1997; Moretti and Shore 2001; Bose et al. 2004). The next level of potential explanations might include differences in the identities of proteins that directly bind silencers in the two species. However, the preponderance of evidence points to ORC, Rap1, and Abf1 as the critical silencer-binding proteins in both species (Figures 4.7, B, and 4.8). Further, the residues mediating Sc-Orc1 interaction with Sc-Sir1 (Hou et al. 2005; Hsu et al. 2005) are conserved in Sb-Orc1 (J. Gallagher and O. Zill, unpublished observations). Hence we are forced to consider models in which it is something special about how ORC, Rap1, and Abf1 bind *S. bayanus* silencers that restricts the ability of Sc-Sir4 to interact with them. One class of explanations would involve qualitative models, in which the precise juxtaposition or conformation of these site-specific DNA-binding proteins allow or restrict interactions with a particular species of Sir4. Alternative possibilities involve quantitative models, wherein reduced affinity of ORC or Rap1, or the ensemble of nucleation proteins, for *S. bayanus* silencers is compensated by binding energy provided by Sb-Kos3 and Sb-Sir4, but not by Sc-Sir4. Distinguishing among these various models will require quantitative assessments of the interaction capabilities of all of the proteins and specific binding sites involved in establishing silencing at the two species' silencers.

An unexpected finding of the Sir4 comparative ChIP-Seq experiment provided insight into the mechanism of silent chromatin assembly. The Sb-Sir4-assisted Sc-Sir4 incorporation into *Sb-HML* and *HMR* (Figure 4.4, A) suggested two distinct types of interactions made by Sir4 proteins at these loci: only Sb-Sir4 was capable of making stable contacts either with the Sir1 paralogs, or with Rap1. In addition, there was a second and qualitatively distinct mode of Sir4 protein association that was species-independent, but occurred only if the species-specific interaction occurred. Two types of interactions might account for the secondary mode of Sc-Sir4 association with *Sb-HML* and *Sb-HMR*: direct Sb-Sir4-Sc-Sir4 interaction via a conserved dimerization surface (Chang et al. 2003), or Sc-Sir4 interaction with deacetylated histone tails (Hecht et al. 1995). We note that Sc-Sir4 association with the *Sb-HMR-E* silencer increased in the presence of Sb-Sir4 at least as much as did its association with internal regions of *Sb-HMR* (Figure 4.4, A). Thus, this secondary mode of Sir4 interaction did not appear to be restricted to regions of *Sb-HMR* where the deacetylated histones reside. Further studies will resolve whether Sb-Sir4-assisted Sc-Sir4 incorporation involves contacts with

multiple silencing proteins versus simple Sir4-Sir4 dimerization, and whether it requires Sir2 catalytic activity.

Additionally, the enhanced interaction of Sb-Sir4 across Y′ elements at *S. cerevisiae* telomeres (Figure 4.4, B) suggested that novel interactions in the hybrids somehow led to enhanced Sir4 occupancy of neighboring regions. This differential long-range occupancy by Sir complexes presents an opportunity to ask whether Sir1 and Sir4-mediated interactions during Sir complex nucleation regulate the "strength" of silent chromatin over a distance. Alternatively, Sb-Sir4 (and potentially other *S. bayanus* silencing proteins) may have been less sensitive to factors that exclude Sc-Sir4 from the Y′ elements. The species-specific Sir4 distributions occurring in these interspecies hybrids should be further dissected to understand the determinants limiting silent chromatin formation across subtelomeric regions.

Another unusual property of the interspecies hybrids led to a weak, genetically dominant silencing defect affecting *Sb-HMR*, but not *Sc-HMR* (Figure 4.2, A, row 1; Figure 4.3, B). In hybrids lacking Sc-Sir4 this defect was more evident (Figure 4.2, A, row 3), which paradoxically suggested that Sc-Sir4 protected *Sb-HMR* silencing in the presence of Sb-Sir4, despite having no ability to silence *Sb-HMR* on its own. How might Sc-Sir4 have "enhanced" Sb-Sir4 function at *S. bayanus* silent loci in hybrids? Because Sc-Sir4 bound efficiently to the *Sb-HMR* locus in the presence of Sb-Sir4 (Figure 4.4, A), perhaps Sc-Sir4 enhanced *Sb-HMR* silencing through this direct association. However, that hypothesis could not explain the hybrid-specific sensitivity of *Sb-HMR* silencing to reduced *Sb-SIR4* dosage (compare Figure 4.2, A, rows 1 and 3 with Figure 4.2, B, row 2). One potential explanation of the novel dosage sensitivity was that Sc-Sir4 occupied positions in the hybrid genome that would otherwise attract Sb-Sir4, were Sc-Sir4 not to occupy those sites. Strong evidence compatible with this model was the ability of both Sb-Sir4 and Sc-Sir4 to bind extensively to *S. cerevisiae* telomeres. Moreover, Y′ elements present at many *S. cerevisiae* telomeres, but missing from *S. bayanus* telomeres, bound Sb-Sir4 more extensively than Sc-Sir4 (Figure 4.4, B), and Sb-Sir4 was partially depleted from *Sb-HMR-E* and *Sb-HML-E* silencers in hybrids with both Sb-Sir4 and Sc-Sir4 relative to Sb-Sir4-only hybrids (Table 4.2). Hence, the hybrid state may result in dosage sensitivity to *Sb-SIR4* not evident in *S. bayanus SIR4/sir4Δ* intra-species diploids due to additional binding sites provided by the Sc-Y′ elements, and potentially other elements. We note the resemblance of this "Sb-Sir4 sequestration" model to the "Circe effect" proposed to explain Sc-Sir4-mediated clustering of *S. cerevisiae* telomeres (Gasser et al. 2004).

On the special properties of interspecies hybrids with regard to heterochromatin

Gregor Mendel's studies were motivated by a desire to understand the emergent properties of interspecies hybrids, such as hybrid vigor, that were of great practical significance at the time. Although he became famously distracted by discovering two fundamental laws of genetics, his original interest in the processes by which hybrid species are not necessarily the "average" of the two parental species remains as

interesting today as it was practically important in Mendel's day. Indeed, the striking asymmetry in the ability of Sb-Sir4 to silence *Sc-HMR*, but inability of Sc-Sir4 to silence *Sb-HMR* (Figure 4.2, A), was the seminal observation that inspired this study. By and large, however, in interspecies hybrids of *S. cerevisiae* and *S. bayanus*, a protein from either species was fully capable of providing all of that protein's function to hybrids. Although this result could be anticipated from the ability to "clone by complementation" genes of one species by their function in another, this study established that symmetry of complementation is an important general consideration. For example, the essential proteins Rap1 and Abf1 from either species had all the functions necessary to support viability of the hybrids and, in work in preparation, we established that Sir2 and Sir3 of both species were fully interchangeable, despite being members of a complex in which another member of that complex, Sir4, has extraordinary divergence. By extrapolation, asymmetrical deviations from a general expectation of cross-species compatibility, such as in the case of Sir4, may signal situations of uncommon interest.

The studies presented here capitalized on the extraordinary genetic properties of interspecies hybrids to tease out important dimensions to the evolution and structure of silent chromatin in yeast. Although silencing behavior in these yeast hybrids was rather unusual, some type of defect might have been anticipated from recent studies of hybrid sterility genes in *Drosophila*, which have implicated rapidly evolving heterochromatin proteins as key factors contributing to interspecies genetic incompatibility (Brideau et al. 2006; Bayes and Malik 2009). It is notable that in budding yeast multiple regulatory sites mediating silencing have rapidly evolved in a phylogenetically asymmetrical fashion along with a set of divergent silencing proteins, paralleling observations of rapid evolution in *Drosophila* heterochromatin (Vermaak et al. 2005; Bayes and Malik 2009). It will be of great interest to determine whether the similar patterns of heterochromatin evolution in these distant taxa reflect similar underlying evolutionary processes.

References

Abzhanov, A., Protas, M., Grant, B.R., Grant, P.R., and Tabin, C.J. 2004. Bmp4 and morphological variation of beaks in Darwin's finches. *Science* **305**(5689): 1462-1465.

Ansari, A. and Gartenberg, M.R. 1997. The yeast silent information regulator Sir4p anchors and partitions plasmids. *Mol Cell Biol* **17**(12): 7061-7068.

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

Auerbach, R.K., Euskirchen, G., Rozowsky, J., Lamarre-Vincent, N., Moqtaderi, Z., Lefrancois, P., Struhl, K., Gerstein, M., and Snyder, M. 2009. Mapping accessible chromatin regions using Sono-Seq. *Proc Natl Acad Sci U S A* **106**(35): 14926-14931.

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

Basson, M.E., Thorsness, M., Finer-Moore, J., Stroud, R.M., and Rine, J. 1988. Structural and functional conservation between yeast and human 3-hydroxy-3-methylglutaryl coenzyme A reductases, the rate-limiting enzyme of sterol biosynthesis. *Mol Cell Biol* **8**(9): 3797-3808.

Baum, P., Thorner, J., and Honig, L. 1978. Identification of tubulin from the yeast Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **75**(10): 4962-4966.

Bayes, J.J. and Malik, H.S. 2009. Altered heterochromatin binding by a hybrid sterility protein in Drosophila sibling species. *Science* **326**(5959): 1538-1541.

Bender, A. and Sprague, G.F., Jr. 1987. MAT alpha 1 protein, a yeast transcription activator, binds synergistically with a second protein to a set of cell-type-specific genes. *Cell* **50**(5): 681-691.

Birdsell, J.A. 2002. Integrating genomics, bioinformatics, and classical genetics to study the effects of recombination on genome evolution. *Mol Biol Evol* **19**(7): 1181-1197.

Boeke, J.D. and Devine, S.E. 1998. Yeast retrotransposons: finding a nice quiet neighborhood. *Cell* **93**(7): 1087-1089.

Bonneton, F., Shaw, P.J., Fazakerley, C., Shi, M., and Dover, G.A. 1997. Comparison of bicoid-dependent regulation of hunchback between Musca domestica and Drosophila melanogaster. *Mech Dev* **66**(1-2): 143-156.

Bose, M.E., McConnell, K.H., Gardner-Aukema, K.A., Muller, 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.

Bradley, R.K., Roberts, A., Smoot, M., Juvekar, S., Do, J., Dewey, C., Holmes, I., and Pachter, L. 2009. Fast statistical alignment. *PLoS Comput Biol* **5**(5): e1000392.

Braunstein, M., Sobel, R.E., Allis, C.D., Turner, B.M., and Broach, J.R. 1996. Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern. *Mol Cell Biol* **16**(8): 4349-4356.

Brideau, N.J., Flores, H.A., Wang, J., Maheshwari, S., Wang, X., and Barbash, D.A. 2006. Two Dobzhansky-Muller genes interact to cause hybrid lethality in Drosophila. *Science* **314**(5803): 1292-1295.

Bruhn, L. and Sprague, G.F., Jr. 1994. MCM1 point mutants deficient in expression of alpha-specific genes: residues important for interaction with alpha 1. *Mol Cell Biol* **14**(4): 2534-2544.

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 U S A* **101**(6): 1632-1637.

Carr, E.A., Mead, J., and Vershon, A.K. 2004. Alpha1-induced DNA bending is required for transcriptional activation by the Mcm1-alpha1 complex. *Nucleic Acids Res* **32**(8): 2298-2305.

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.

Cheng, T.H. and Gartenberg, M.R. 2000. Yeast heterochromatin is a dynamic structure that requires silencers continuously. *Genes Dev* **14**(4): 452-463.

Cliften, P., Sudarsanam, P., Desikan, A., Fulton, L., Fulton, B., Majors, J., Waterston, R., Cohen, B.A., and Johnston, M. 2003. Finding functional features in Saccharomyces genomes by phylogenetic footprinting. *Science* **301**(5629): 71-76.

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.

Collins, S.R., Miller, K.M., Maas, N.L., Roguev, A., Fillingham, J., Chu, C.S., Schuldiner, M., Gebbia, M., Recht, J., Shales, M., Ding, H., Xu, H., Han, J., Ingvarsdottir, K., Cheng, B., Andrews, B., Boone, C., Berger, S.L., Hieter, P., Zhang, Z., Brown, G.W., Ingles, C.J., Emili, A., Allis, C.D., Toczyski, D.P., Weissman, J.S., Greenblatt, J.F., and Krogan, N.J. 2007. Functional dissection of

protein complexes involved in yeast chromosome biology using a genetic interaction map. *Nature* **446**(7137): 806-810.

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.

Cubillos, F.A., Louis, E.J., and Liti, G. 2009. Generation of a large set of genetically tractable haploid and diploid Saccharomyces strains. *FEMS Yeast Res* **9**(8): 1217-1225.

Dai, J., Xie, W., Brady, T.L., Gao, J., and Voytas, D.F. 2007. Phosphorylation regulates integration of the yeast Ty5 retrotransposon into heterochromatin. *Mol Cell* **27**(2): 289-299.

Davies, B.S., Wang, H.S., and Rine, J. 2005. Dual activators of the sterol biosynthetic pathway of Saccharomyces cerevisiae: similar activation/regulatory domains but different response mechanisms. *Mol Cell Biol* **25**(16): 7375-7385.

Delneri, D., Colson, I., Grammenoudi, S., Roberts, I.N., Louis, E.J., and Oliver, S.G. 2003. Engineering evolution to study speciation in yeasts. *Nature* **422**(6927): 68-72.

Diaz-Castillo, C. and Golic, K.G. 2007. Evolution of gene sequence in response to chromosomal location. *Genetics* **177**(1): 359-374.

Dietzel, C. and Kurjan, J. 1987. Pheromonal regulation and sequence of the Saccharomyces cerevisiae SST2 gene: a model for desensitization to pheromone. *Mol Cell Biol* **7**(12): 4169-4177.

Dover, G.A. and Flavell, R.B. 1984. Molecular coevolution: DNA divergence and the maintenance of function. *Cell* **38**(3): 622-623.

Eichler, E.E. 2001. Segmental duplications: what's missing, misassigned, and misassembled--and should we care? *Genome Res* **11**(5): 653-656.

Elble, R. and Tye, B.K. 1991. Both activation and repression of a-mating-type-specific genes in yeast require transcription factor Mcm1. *Proc Natl Acad Sci U S A* **88**(23): 10966-10970.

Erdman, S. and Snyder, M. 2001. A filamentous growth response mediated by the yeast mating pathway. *Genetics* **159**(3): 919-928.

Evers, R. and Grummt, I. 1995. Molecular coevolution of mammalian ribosomal gene terminator sequences and the transcription termination factor TTF-I. *Proc Natl Acad Sci U S A* **92**(13): 5827-5831.

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.

Fan, X., Lamarre-Vincent, N., Wang, Q., and Struhl, K. 2008. Extensive chromatin fragmentation improves enrichment of protein binding sites in chromatin immunoprecipitation experiments. *Nucleic Acids Res* **36**(19): e125.

Fields, S., Chaleff, D.T., and Sprague, G.F., Jr. 1988. Yeast STE7, STE11, and STE12 genes are required for expression of cell-type-specific genes. *Mol Cell Biol* **8**(2): 551-556.

Fields, S. and Herskowitz, I. 1985. The yeast STE12 product is required for expression of two sets of cell-type specific genes. *Cell* **42**(3): 923-930.

Fischer, G., James, S.A., Roberts, I.N., Oliver, S.G., and Louis, E.J. 2000. Chromosomal evolution in Saccharomyces. *Nature* **405**(6785): 451-454.

Fitzpatrick, D.A., Logue, M.E., Stajich, J.E., and Butler, G. 2006. A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. *BMC Evol Biol* **6**: 99.

Flessel, M.C., Brake, A.J., and Thorner, J. 1989. The MF alpha 1 gene of Saccharomyces cerevisiae: genetic mapping and mutational analysis of promoter elements. *Genetics* **121**(2): 223-236.

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.

Frye, R.A. 2000. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun* **273**(2): 793-798.

Galgoczy, D.J., Cassidy-Stone, A., Llinas, M., O'Rourke, S.M., Herskowitz, I., DeRisi, J.L., and Johnson, A.D. 2004. Genomic dissection of the cell-type-specification circuit in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **101**(52): 18069-18074.

Gallagher, J.E., 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.

Galtier, N. and Duret, L. 2007. Adaptation or biased gene conversion? Extending the null hypothesis of molecular evolution. *Trends Genet* **23**(6): 273-277.

Ganter, B., Tan, S., and Richmond, T.J. 1993. Genomic footprinting of the promoter regions of STE2 and STE3 genes in the yeast Saccharomyces cerevisiae. *J Mol Biol* **234**(4): 975-987.

Gasch, A.P., Moses, A.M., Chiang, D.Y., Fraser, H.B., Berardini, M., and Eisen, M.B. 2004. Conservation and evolution of cis-regulatory systems in ascomycete fungi. *PLoS Biol* **2**(12): e398.

Gasser, S.M., Hediger, F., Taddei, A., Neumann, F.R., and Gartenberg, M.R. 2004. The function of telomere clustering in yeast: the circe effect. *Cold Spring Harb Symp Quant Biol* **69**: 327-337.

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.

Georgel, P.T., Palacios DeBeer, M.A., Pietz, G., Fox, C.A., and Hansen, J.C. 2001. Sir3-dependent assembly of supramolecular chromatin structures in vitro. *Proc Natl Acad Sci U S A* **98**(15): 8584-8589.

Goldstein, A.L. and McCusker, J.H. 1999. Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. *Yeast* **15**(14): 1541-1553.

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.

Grayhack, E.J. 1992. The yeast alpha 1 and MCM1 proteins bind a single strand of their duplex DNA recognition site. *Mol Cell Biol* **12**(8): 3573-3582.

Greig, D. 2009. Reproductive isolation in Saccharomyces. *Heredity* **102**(1): 39-44.

Greig, D., Borts, R.H., Louis, E.J., and Travisano, M. 2002. Epistasis and hybrid sterility in Saccharomyces. *Proc Biol Sci* **269**(1496): 1167-1171.

Greig, D., Travisano, M., Louis, E.J., and Borts, R.H. 2003. A role for the mismatch repair system during incipient speciation in Saccharomyces. *J Evol Biol* **16**(3): 429-437.

Grewal, S.I. and Moazed, D. 2003. Heterochromatin and epigenetic control of gene expression. *Science* **301**(5634): 798-802.

Guo, B., Styles, C.A., Feng, Q., and Fink, G.R. 2000. A Saccharomyces gene family involved in invasive growth, cell-cell adhesion, and mating. *Proc Natl Acad Sci U S A* **97**(22): 12158-12163.

Haber, J.E. 1998. Mating-type gene switching in Saccharomyces cerevisiae. *Annu Rev Genet* **32**: 561-599.

Hagen, D.C., Bruhn, L., Westby, C.A., and Sprague, G.F., Jr. 1993. Transcription of alpha-specific genes in Saccharomyces cerevisiae: DNA sequence requirements for activity of the coregulator alpha 1. *Mol Cell Biol* **13**(11): 6866-6875.

Hall, A.E., Keith, K.C., Hall, S.E., Copenhaver, G.P., and Preuss, D. 2004. The rapidly evolving field of plant centromeres. *Curr Opin Plant Biol* **7**(2): 108-114.

Hall, S.E., Kettler, G., and Preuss, D. 2003. Centromere satellites from Arabidopsis populations: maintenance of conserved and variable domains. *Genome Res* **13**(2): 195-205.

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., Jennings, E.G., Zeitlinger, J., Pokholok, D.K., Kellis, M., Rolfe, P.A., Takusagawa, K.T., Lander, E.S., Gifford, D.K., Fraenkel, E., and Young, R.A. 2004. Transcriptional regulatory code of a eukaryotic genome. *Nature* **431**(7004): 99-104.

Hecht, A., Laroche, T., Strahl-Bolsinger, S., Gasser, S.M., 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.

Hecht, A., Strahl-Bolsinger, S., and Grunstein, M. 1996. Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. *Nature* **383**(6595): 92-96.

Hedtke, S.M., Townsend, T.M., and Hillis, D.M. 2006. Resolution of phylogenetic conflict in large data sets by increased taxon sampling. *Syst Biol* **55**(3): 522-529.

Henikoff, S., Ahmad, K., and Malik, H.S. 2001. The centromere paradox: stable inheritance with rapidly evolving DNA. *Science* **293**(5532): 1098-1102.

Herskowitz, I. 1988. Life cycle of the budding yeast Saccharomyces cerevisiae. *Microbiol Rev* **52**(4): 536-553.

Hickman, M. and Rusche, L.N. 2007a. Evolution of Silencing at the Mating-Type Loci in Hemiascomycetes. in *Sex in fungi: molecular determination and evolutionary implications* (ed. J. Heitman, J. Kronstad, J. Taylor, and L. Casselton). ASM Press, Washington, D.C.

Hickman, M.A. and Rusche, L.N. 2007b. 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.

Hongay, C.F., Grisafi, P.L., Galitski, T., and Fink, G.R. 2006. Antisense transcription controls cell fate in Saccharomyces cerevisiae. *Cell* **127**(4): 735-745.

Hoppe, G.J., Tanny, J.C., Rudner, A.D., Gerber, S.A., Danaie, S., Gygi, S.P., and Moazed, D. 2002. 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 U S A* **102**(24): 8489-8494.

Hsu, H.C., Stillman, B., and Xu, R.M. 2005. Structural basis for origin recognition complex 1 protein-silence information regulator 1 protein interaction in epigenetic silencing. *Proc Natl Acad Sci U S A* **102**(24): 8519-8524.

Hunter, N., Chambers, S.R., Louis, E.J., and Borts, R.H. 1996. The mismatch repair system contributes to meiotic sterility in an interspecific yeast hybrid. *EMBO J* **15**(7): 1726-1733.

Huth, J.R., Bewley, C.A., Nissen, M.S., Evans, J.N., Reeves, R., Gronenborn, A.M., and Clore, G.M. 1997. The solution structure of an HMG-I(Y)-DNA complex defines a new architectural minor groove binding motif. *Nat Struct Biol* **4**(8): 657-665.

Hwang-Shum, J.J., Hagen, D.C., Jarvis, E.E., Westby, C.A., and Sprague, G.F., Jr. 1991. Relative contributions of MCM1 and STE12 to transcriptional activation of a- and alpha-specific genes from Saccharomyces cerevisiae. *Mol Gen Genet* **227**(2): 197-204.

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.

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

Jarvis, E.E., Clark, K.L., and Sprague, G.F., Jr. 1989. The yeast transcription activator PRTF, a homolog of the mammalian serum response factor, is encoded by the MCM1 gene. *Genes Dev* **3**(7): 936-945.

Jarvis, E.E., Hagen, D.C., and Sprague, G.F., Jr. 1988. Identification of a DNA segment that is necessary and sufficient for alpha-specific gene control in Saccharomyces cerevisiae: implications for regulation of alpha-specific and a-specific genes. *Mol Cell Biol* **8**(1): 309-320.

Jensen, R., Sprague, G.F., Jr., and Herskowitz, I. 1983. Regulation of yeast mating-type interconversion: feedback control of HO gene expression by the mating-type locus. *Proc Natl Acad Sci U S A* **80**(10): 3035-3039.

Kataoka, T., Powers, S., Cameron, S., Fasano, O., Goldfarb, M., Broach, J., and Wigler, M. 1985. Functional homology of mammalian and yeast RAS genes. *Cell* **40**(1): 19-26.

Kellis, M., Patterson, N., Endrizzi, M., Birren, B., and Lander, E.S. 2003. Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* **423**(6937): 241-254.

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

Kirkman-Correia, C., Stroke, I.L., and Fields, S. 1993. Functional domains of the yeast STE12 protein, a pheromone-responsive transcriptional activator. *Mol Cell Biol* **13**(6): 3765-3772.

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

Krogan, N.J., Cagney, G., Yu, H., Zhong, G., Guo, X., Ignatchenko, A., Li, J., Pu, S., Datta, N., Tikuisis, A.P., Punna, T., Peregrin-Alvarez, J.M., Shales, M., Zhang, X., Davey, M., Robinson, M.D., Paccanaro, A., Bray, J.E., Sheung, A., Beattie, B., Richards, D.P., Canadien, V., Lalev, A., Mena, F., Wong, P., Starostine, A., Canete, M.M., Vlasblom, J., Wu, S., Orsi, C., Collins, S.R., Chandran, S., Haw, R., Rilstone, J.J., Gandi, K., Thompson, N.J., Musso, G., St Onge, P., Ghanny, S., Lam, M.H., Butland, G., Altaf-Ul, A.M., Kanaya, S., Shilatifard, A., O'Shea, E., Weissman, J.S., Ingles, C.J., Hughes, T.R., Parkinson, J., Gerstein, M., Wodak, S.J., Emili, A., and Greenblatt, J.F. 2006. Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. *Nature* **440**(7084): 637-643.

Kronstad, J.W., Holly, J.A., and MacKay, V.L. 1987. A yeast operator overlaps an upstream activation site. *Cell* **50**(3): 369-377.

Kurjan, J. 1985. Alpha-factor structural gene mutations in Saccharomyces cerevisiae: effects on alpha-factor production and mating. *Mol Cell Biol* **5**(4): 787-796.

La Roche, S.D., Shafer, B.K., and Strathern, J.N. 1995. A ste12 allele having a differential effect on a versus alpha cells. *Mol Gen Genet* **246**(1): 80-90.

Landry, J., Sutton, A., Tafrov, S.T., Heller, R.C., Stebbins, J., Pillus, L., and Sternglanz, R. 2000. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc Natl Acad Sci U S A* **97**(11): 5807-5811.

Laurenson, P. and Rine, J. 1991. SUM1-1: a suppressor of silencing defects in Saccharomyces cerevisiae. *Genetics* **129**(3): 685-696.

Lee, M.G. and Nurse, P. 1987. Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature* **327**(6117): 31-35.

Lee, T.I., Rinaldi, N.J., Robert, F., Odom, D.T., Bar-Joseph, Z., Gerber, G.K., Hannett, N.M., Harbison, C.T., Thompson, C.M., Simon, I., Zeitlinger, J., Jennings, E.G., Murray, H.L., Gordon, D.B., Ren, B., Wyrick, J.J., Tagne, J.B., Volkert, T.L., Fraenkel, E., Gifford, D.K., and Young, R.A. 2002. Transcriptional regulatory networks in Saccharomyces cerevisiae. *Science* **298**(5594): 799-804.

Lefrancois, P., Euskirchen, G.M., Auerbach, R.K., Rozowsky, J., Gibson, T., Yellman, C.M., Gerstein, M., and Snyder, M. 2009. Efficient yeast ChIP-Seq using multiplex short-read DNA sequencing. *BMC Genomics* **10**: 37.

Li, H., Ruan, J., and Durbin, R. 2008. Mapping short DNA sequencing reads and calling variants using mapping quality scores. *Genome Res* **18**(11): 1851-1858.

Linardopoulou, E.V., Williams, E.M., Fan, Y., Friedman, C., Young, J.M., and Trask, B.J. 2005. Human subtelomeres are hot spots of interchromosomal recombination and segmental duplication. *Nature* **437**(7055): 94-100.

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., Barton, D.B., and Louis, E.J. 2006. Sequence diversity, reproductive isolation and species concepts in Saccharomyces. *Genetics* **174**(2): 839-850.

Liti, G., Carter, D.M., Moses, A.M., Warringer, J., Parts, L., James, S.A., Davey, R.P., Roberts, I.N., Burt, A., Koufopanou, V., Tsai, I.J., Bergman, C.M., Bensasson, D., O'Kelly, M.J., van Oudenaarden, A., Barton, D.B., Bailes, E., Nguyen, A.N., Jones, M., Quail, M.A., Goodhead, I., Sims, S., Smith, F., Blomberg, A., Durbin, R., and Louis, E.J. 2009. Population genomics of domestic and wild yeasts. *Nature* **458**(7236): 337-341.

Liti, G., Peruffo, A., James, S.A., Roberts, I.N., and Louis, E.J. 2005. Inferences of evolutionary relationships from a population survey of LTR-retrotransposons and telomeric-associated sequences in the Saccharomyces sensu stricto complex. *Yeast* **22**(3): 177-192.

Liu, H., Styles, C.A., and Fink, G.R. 1996. Saccharomyces cerevisiae S288C has a mutation in FLO8, a gene required for filamentous growth. *Genetics* **144**(3): 967-978.

Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* **14**(10): 953-961.

Louis, E.J. 1995. The chromosome ends of Saccharomyces cerevisiae. *Yeast* **11**(16): 1553-1573.

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.

Lynch, P.J. and Rusche, L.N. An auxiliary silencer and a boundary element maintain high levels of silencing proteins at HMR in Saccharomyces cerevisiae. *Genetics*.

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

Marshall, M., Mahoney, D., Rose, A., Hicks, J.B., and Broach, J.R. 1987. Functional domains of SIR4, a gene required for position effect regulation in Saccharomyces cerevisiae. *Mol Cell Biol* **7**(12): 4441-4452.

Martin, O.C., DeSevo, C.G., Guo, B.Z., Koshland, D.E., Dunham, M.J., and Zheng, Y. 2009. Telomere behavior in a hybrid yeast. *Cell Res* **19**(7): 910-912.

Martino, F., Kueng, S., Robinson, P., Tsai-Pflugfelder, M., van Leeuwen, F., Ziegler, M., Cubizolles, F., Cockell, M.M., Rhodes, D., and Gasser, S.M. 2009. Reconstitution of yeast silent chromatin: multiple contact sites and O-AADPR binding load SIR complexes onto nucleosomes in vitro. *Mol Cell* **33**(3): 323-334.

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.

McDonald, J.H. and Kreitman, M. 1991. Adaptive protein evolution at the Adh locus in Drosophila. *Nature* **351**(6328): 652-654.

McGregor, A.P., Shaw, P.J., Hancock, J.M., Bopp, D., Hediger, M., Wratten, N.S., and Dover, G.A. 2001. Rapid restructuring of bicoid-dependent hunchback promoters within and between Dipteran species: implications for molecular coevolution. *Evol Dev* **3**(6): 397-407.

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.

Mitchell, A.P. and Herskowitz, I. 1986. Activation of meiosis and sporulation by repression of the RME1 product in yeast. *Nature* **319**(6056): 738-742.

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 U S A* **94**(6): 2186-2191.

Moretti, P. and Shore, D. 2001. Multiple interactions in Sir protein recruitment by Rap1p at silencers and telomeres in yeast. *Mol Cell Biol* **21**(23): 8082-8094.

Mower, J.P., Touzet, P., Gummow, J.S., Delph, L.F., and Palmer, J.D. 2007. Extensive variation in synonymous substitution rates in mitochondrial genes of seed plants. *BMC Evol Biol* **7**: 135.

Naumov, G.I., James, S.A., Naumova, E.S., Louis, E.J., and Roberts, I.N. 2000. Three new species in the Saccharomyces sensu stricto complex: Saccharomyces cariocanus, Saccharomyces kudriavzevii and Saccharomyces mikatae. *Int J Syst Evol Microbiol* **50 Pt 5**: 1931-1942.

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.

Ozaydin, B. and Rine, J. Expanded roles of the origin recognition complex in the architecture and function of silenced chromatin in Saccharomyces cerevisiae. *Mol Cell Biol* **30**(3): 626-639.

Palladino, F., Laroche, T., Gilson, E., Axelrod, A., Pillus, L., and Gasser, S.M. 1993. SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. *Cell* **75**(3): 543-555.

Passmore, S., Maine, G.T., Elble, R., Christ, C., and Tye, B.K. 1988. Saccharomyces cerevisiae protein involved in plasmid maintenance is necessary for mating of MAT alpha cells. *J Mol Biol* **204**(3): 593-606.

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.

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

Pirrotta, V. and Gross, D.S. 2005. Epigenetic silencing mechanisms in budding yeast and fruit fly: different paths, same destinations. *Mol Cell* **18**(4): 395-398.

Pryde, F.E. and Louis, E.J. 1999. Limitations of silencing at native yeast telomeres. *EMBO J* **18**(9): 2538-2550.

Quail, M.A., Kozarewa, I., Smith, F., Scally, A., Stephens, P.J., Durbin, R., Swerdlow, H., and Turner, D.J. 2008. A large genome center's improvements to the Illumina sequencing system. *Nat Methods* **5**(12): 1005-1010.

Ramachandran, L., Burhans, D.T., Laun, P., Wang, J., Liang, P., Weinberger, M., Wissing, S., Jarolim, S., Suter, B., Madeo, F., Breitenbach, M., and Burhans,

W.C. 2006. Evidence for ORC-dependent repression of budding yeast genes induced by starvation and other stresses. *FEMS Yeast Res* **6**(5): 763-776.

Ravindra, A., Weiss, K., and Simpson, R.T. 1999. High-resolution structural analysis of chromatin at specific loci: Saccharomyces cerevisiae silent mating-type locus HMRa. *Mol Cell Biol* **19**(12): 7944-7950.

Reeves, R. and Nissen, M.S. 1990. The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. *J Biol Chem* **265**(15): 8573-8582.

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.

Rine, J., Sprague, G.F., Jr., and Herskowitz, I. 1981. rme1 Mutation of Saccharomyces cerevisiae: map position and bypass of mating type locus control of sporulation. *Mol Cell Biol* **1**(10): 958-960.

Rivers, D.M. and Sprague, G.F., Jr. 2003. Autocrine activation of the pheromone response pathway in matalpha2- cells is attenuated by SST2- and ASG7-dependent mechanisms. *Mol Genet Genomics* **270**(3): 225-233.

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.

Roth, A.F., Nelson, B., Boone, C., and Davis, N.G. 2000. Asg7p-Ste3p inhibition of pheromone signaling: regulation of the zygotic transition to vegetative growth. *Mol Cell Biol* **20**(23): 8815-8825.

Rusche, 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.

-. 2003. The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. *Annu Rev Biochem* **72**: 481-516.

Rusche, L.N. and Lynch, P.J. 2009. Assembling heterochromatin in the appropriate places: A boost is needed. *J Cell Physiol* **219**(3): 525-528.

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

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

Sengupta, P. and Cochran, B.H. 1990. The PRE and PQ box are functionally distinct yeast pheromone response elements. *Mol Cell Biol* **10**(12): 6809-6812.

Shaw, P.J., Wratten, N.S., McGregor, A.P., and Dover, G.A. 2002. Coevolution in bicoid-dependent promoters and the inception of regulatory incompatibilities among species of higher Diptera. *Evol Dev* **4**(4): 265-277.

Sikorski, R.S. 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.

Simpson, P. 2002. Evolution of development in closely related species of flies and worms. *Nat Rev Genet* **3**(12): 907-917.

Smith, D.L. and Johnson, A.D. 1992. A molecular mechanism for combinatorial control in yeast: MCM1 protein sets the spacing and orientation of the homeodomains of an alpha 2 dimer. *Cell* **68**(1): 133-142.

Sperling, A.S. and Grunstein, M. 2009. Histone H3 N-terminus regulates higher order structure of yeast heterochromatin. *Proc Natl Acad Sci U S A* **106**(32): 13153-13159.

Sprague, G.F., Jr. 1990. Combinatorial associations of regulatory proteins and the control of cell type in yeast. *Adv Genet* **27**: 33-62.

Stark, A., Lin, M.F., Kheradpour, P., Pedersen, J.S., Parts, L., Carlson, J.W., Crosby, M.A., Rasmussen, M.D., Roy, S., Deoras, A.N., Ruby, J.G., Brennecke, J., Hodges, E., Hinrichs, A.S., Caspi, A., Paten, B., Park, S.W., Han, M.V., Maeder, M.L., Polansky, B.J., Robson, B.E., Aerts, S., van Helden, J., Hassan, B., Gilbert, D.G., Eastman, D.A., Rice, M., Weir, M., Hahn, M.W., Park, Y., Dewey, C.N., Pachter, L., Kent, W.J., Haussler, D., Lai, E.C., Bartel, D.P., Hannon, G.J., Kaufman, T.C., Eisen, M.B., Clark, A.G., Smith, D., Celniker, S.E., Gelbart, W.M., and Kellis, M. 2007. Discovery of functional elements in 12 Drosophila genomes using evolutionary signatures. *Nature* **450**(7167): 219-232.

Stoltzfus, A. 1999. On the possibility of constructive neutral evolution. *J Mol Evol* **49**(2): 169-181.

Stone, E.M., Reifsnyder, C., McVey, M., Gazo, B., and Pillus, L. 2000. Two classes of sir3 mutants enhance the sir1 mutant mating defect and abolish telomeric silencing in Saccharomyces cerevisiae. *Genetics* **155**(2): 509-522.

Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. 1997. SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. *Genes Dev* **11**(1): 83-93.

Strathern, J., Hicks, J., and Herskowitz, I. 1981. Control of cell type in yeast by the mating type locus. The alpha 1-alpha 2 hypothesis. *J Mol Biol* **147**(3): 357-372.

Strathern, J.N. and Herskowitz, I. 1979. Asymmetry and directionality in production of new cell types during clonal growth: the switching pattern of homothallic yeast. *Cell* **17**(2): 371-381.

Suka, N., Luo, K., and Grunstein, M. 2002. Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. *Nat Genet* **32**(3): 378-383.

Sussel, L., Vannier, D., and Shore, D. 1993. Epigenetic switching of transcriptional states: cis- and trans-acting factors affecting establishment of silencing at the HMR locus in Saccharomyces cerevisiae. *Mol Cell Biol* **13**(7): 3919-3928.

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.

Taddei, A., Hediger, F., Neumann, F.R., Bauer, C., and Gasser, S.M. 2004. Separation of silencing from perinuclear anchoring functions in yeast Ku80, Sir4 and Esc1 proteins. *EMBO J* **23**(6): 1301-1312.

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 U S A* **98**(2): 415-420.

Teytelman, L., Eisen, M.B., and Rine, J. 2008. Silent but not static: accelerated base-pair substitution in silenced chromatin of budding yeasts. *PLoS Genet* **4**(11): e1000247.

Teytelman, L., Ozaydin, B., Zill, O., Lefrancois, P., Snyder, M., Rine, J., and Eisen, M.B. 2009. Impact of chromatin structures on DNA processing for genomic analyses. *PLoS One* **4**(8): e6700.

Thomas, B.J. and Rothstein, R. 1989. Elevated recombination rates in transcriptionally active DNA. *Cell* **56**(4): 619-630.

Tong, A.H., Lesage, G., Bader, G.D., Ding, H., Xu, H., Xin, X., Young, J., Berriz, G.F., Brost, R.L., Chang, M., Chen, Y., Cheng, X., Chua, G., Friesen, H., Goldberg, D.S., Haynes, J., Humphries, C., He, G., Hussein, S., Ke, L., Krogan, N., Li, Z., Levinson, J.N., Lu, H., Menard, P., Munyana, C., Parsons, A.B., Ryan, O., Tonikian, R., Roberts, T., Sdicu, A.M., Shapiro, J., Sheikh, B., Suter, B., Wong, S.L., Zhang, L.V., Zhu, H., Burd, C.G., Munro, S., Sander, C., Rine, J., Greenblatt, J., Peter, M., Bretscher, A., Bell, G., Roth, F.P., Brown, G.W., Andrews, B., Bussey, H., and Boone, C. 2004. Global mapping of the yeast genetic interaction network. *Science* **303**(5659): 808-813.

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.

Valenzuela, L., Gangadharan, S., and Kamakaka, R.T. 2006. Analyses of SUM1-1-mediated long-range repression. *Genetics* **172**(1): 99-112.

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

Vermaak, D., Henikoff, S., and Malik, H.S. 2005. Positive selection drives the evolution of rhino, a member of the heterochromatin protein 1 family in Drosophila. *PLoS Genet* **1**(1): 96-108.

Wang, A.Y., Schulze, J.M., Skordalakes, E., Gin, J.W., Berger, J.M., Rine, J., and Kobor, M.S. 2009. Asf1-like structure of the conserved Yaf9 YEATS domain and role in H2A.Z deposition and acetylation. *Proc Natl Acad Sci U S A* **106**(51): 21573-21578.

Wang, S.A. and Bai, F.Y. 2008. Saccharomyces arboricolus sp. nov., a yeast species from tree bark. *Int J Syst Evol Microbiol* **58**(Pt 2): 510-514.

Weiss, K. and Simpson, R.T. 1998. High-resolution structural analysis of chromatin at specific loci: Saccharomyces cerevisiae silent mating type locus HMLalpha. *Mol Cell Biol* **18**(9): 5392-5403.

Wernersson, R. and Pedersen, A.G. 2003. RevTrans: Multiple alignment of coding DNA from aligned amino acid sequences. *Nucleic Acids Res* **31**(13): 3537-3539.

Wyrick, J.J., Aparicio, J.G., Chen, T., Barnett, J.D., Jennings, E.G., Young, R.A., Bell, S.P., and Aparicio, O.M. 2001. Genome-wide distribution of ORC and MCM proteins in S. cerevisiae: high-resolution mapping of replication origins. *Science* **294**(5550): 2357-2360.

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.

Xu, W., Aparicio, J.G., Aparicio, O.M., and Tavare, S. 2006. Genome-wide mapping of ORC and Mcm2p binding sites on tiling arrays and identification of essential ARS consensus sequences in S. cerevisiae. *BMC Genomics* **7**: 276.

Yang, Z. 1998. Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. *Mol Biol Evol* **15**(5): 568-573.

-. 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Mol Biol Evol* **24**(8): 1586-1591.

Yuan, Y.O., Stroke, I.L., and Fields, S. 1993. Coupling of cell identity to signal response in yeast: interaction between the alpha 1 and STE12 proteins. *Genes Dev* **7**(8): 1584-1597.

Zeitlinger, J., Simon, I., Harbison, C.T., Hannett, N.M., Volkert, T.L., Fink, G.R., and Young, R.A. 2003. Program-specific distribution of a transcription factor dependent on partner transcription factor and MAPK signaling. *Cell* **113**(3): 395-404.

Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nussbaum, C., Myers, R.M., Brown, M., Li, W., and Liu, X.S. 2008. Model-based analysis of ChIP-Seq (MACS). *Genome Biol* **9**(9): R137.

Zhu, Y., Zou, S., Wright, D.A., and Voytas, D.F. 1999. Tagging chromatin with retrotransposons: target specificity of the Saccharomyces Ty5 retrotransposon changes with the chromosomal localization of Sir3p and Sir4p. *Genes Dev* **13**(20): 2738-2749.

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.

Zou, S., Ke, N., Kim, J.M., and Voytas, D.F. 1996. The Saccharomyces retrotransposon Ty5 integrates preferentially into regions of silent chromatin at the telomeres and mating loci. *Genes Dev* **10**(5): 634-645.

Zou, S. and Voytas, D.F. 1997. Silent chromatin determines target preference of the Saccharomyces retrotransposon Ty5. *Proc Natl Acad Sci U S A* **94**(14): 7412-7416.

Zou, S., Wright, D.A., and Voytas, D.F. 1995. The Saccharomyces Ty5 retrotransposon family is associated with origins of DNA replication at the telomeres and the silent mating locus HMR. *Proc Natl Acad Sci U S A* **92**(3): 920-924.

Appendix A

High-resolution studies on the architecture of silent chromatin in *Saccharomyces* using ChIP-Seq technology

Surprises from comparative ChIP-Seq of Sir4 in S. cerevisiae/S. bayanus interspecies hybrids

In the course of conducting a screen for silencing-defective mutants in *S. bayanus* (Chapter 3), Jeffrey Kuei and I performed simple cross-species complementation experiments with the *SIR2*, *SIR3*, and *SIR4* genes from both *S. cerevisiae* and *S. bayanus*. For *SIR2* and *SIR3*, both species' orthologs complemented both *S. cerevisiae* and *S. bayanus sir2Δ* or *sir3Δ* mutants, respectively. As described in Chapters 3 and 4, the surprising result was that while *S. bayanus SIR4* (hereafter, *Sb-SIR4*) complemented *S. cerevisiae sir4Δ* mutants, *S. cerevisiae SIR4* (hereafter, *Sc-SIR4*) failed to complement *S. bayanus sir4Δ* mutants (Zill, et al., *submitted*). A trivial explanation would be that Sir4 is simply expressed at lower levels in *S. cerevisiae* than *S. bayanus*. I have tested this possibility by both quantitative RT-PCR and immunoblot, and concluded that Sir4 RNA and protein are expressed at equivalent levels in both species (Chapter 4; Zill, et al, *submitted*). To determine how and why Sc-Sir4 failed to silence *S. bayanus HML* and *HMR*, I performed ChIP, followed by high-throughput sequencing (ChIP-Seq), using a set of *S. cerevisiae*/*S. bayanus* hybrid diploid strains to compare the binding profile of Sc-Sir4 with that of Sb-Sir4 across both species' genomes. This experiment yielded two important results, which have inspired ongoing and future experiments in the Rine lab (described in next section).

First, the picture of the silencing mechanism was much different from what had been shown using ChIP-PCR with primers tiled across *S. cerevisiae* silent loci (Hecht et al. 1996; Rusche et al. 2002). Earlier data from our lab and others led to the anticipation of plateaus or broad peaks of Sir4 binding across *HML* and *HMR* as a result of Sir proteins spreading inward from the flanking silencers. However, the ChIP-Seq analysis showed Sc-Sir4 association most strongly with the left-side (E) silencers, with shorter, broader peaks over the right-side (I) silencers, and only modest enrichment of associated sequences within *HMR* (Figure A.1, A). These results are consistent with a recent study that showed that Sir proteins are recruited faster and to higher levels at the *HMR* E silencer than at the *HMR* I silencer, and that accumulation of Sir proteins, as measured by ChIP-qPCR after Sir3 induction, is a non-linear process (Lynch and Rusche 2009). Another interesting feature of our data was a peak of Sir4 binding directly over the promoter of *S. cerevisiae HML* (Figure A.1, A), which is consistent with a study showing that this promoter contains a Rap1 binding site that functions in silencing (Cheng and Gartenberg 2000). The strikingly different picture of Sir4 in silent chromatin painted by ChIP-Seq compared with ChIP-PCR likely resulted from the small chromatin fragment size-selection step in the preparation of Illumina sequencing libraries (our libraries had average insert size of 320bp), which appears to have offered unprecedented resolution to Sir4 localization (Auerbach et al. 2009; Teytelman et al. 2009). Future experiments will determine, at the resolution provided by ChIP-Seq technology, a precise picture of the Sir2/Sir3/Sir4 complex's association with chromatin in *S. cerevisiae*.

The second exciting finding of the Sir4 ChIP-Seq experiment has led to a new view of the establishment of silencing, involving a distinction between two types of interactions made by Sir4 at silencers (see Chapter 4). When only Sc-Sir4 was present in the hybrid diploids, it failed to associate with *S. bayanus HMR* (*Sb-HMR*) (Figure A.1, B,

top panel). This suggested that some critical protein-protein interactions could not occur between Sc-Sir4 and *S. bayanus* proteins at silencers (presumably ORC, Rap1, and the Sir1 paralogs). However, when Sb-Sir4 was supplied in the same cell with Sc-Sir4, both species' Sir4 proteins were able to associate with *Sb-HMR* (Figure A.1, B, bottom panel)! (The large difference in Sc-Sir4 ChIP shown in Figure A.1, B, top versus bottom panels, was confirmed by standard ChIP-qPCR.) The conditional cross-species restriction to Sc-Sir4 function offers two insights. First, it would appear that only Sb-Sir4 is capable of making the primary contacts with ORC, Rap1, and Sir1 paralogs at *S. bayanus* silencers. Second, there is a secondary and qualitatively distinct mode of Sir4 protein association with silencers that is species-independent. Although the nature of this secondary mode of association is not yet known, the possibilities fall between two extremes: (1) Sir4 proteins can self-associate across species through an interaction surface that has not changed substantially between the two species, or (2) recruitment of Sir4 to silencers is a two-step process involving a cooperative transition, with secondary binding at an allosteric site at the silencer. In the second model, Sc-Sir4 would be capable of binding ORC, Rap1, and Sir1 paralogs only after an Sb-Sir4-mediated allosteric change has occurred in this complex. This dependence of Sc-Sir4 on Sb-Sir4 can be exploited to test self-association and cooperativity models, and to identify portions of the Sir4 protein that are necessary and sufficient for Sc-Sir4 association with *S. bayanus* silencers.

Using ChIP-Seq to define the molecular architecture of Sir-mediated silencing

Silencing has been thought to involve spreading of Sir4, but our ChIP-Seq results suggest that most Sir4 remains at silencers. The promoters of *HML* and *HMR* are located 1kb or more from the flanking silencers. The current molecular model for how Sir proteins repress transcription at a distance posits that they spread from the silencers to a promoter inside a silent locus via iterative associations with Sir2-deacetylated histone tails and through interactions with each other (Rusche et al. 2003). Traditional ChIP assays interrogate bulk sonicated chromatin, which contains a distribution of fragment sizes up to 1-2kb (Fan et al. 2008). Our lab has recently shown that silent chromatin is relatively resistant to shearing, and thus the size distribution of fragments originating from these regions is shifted upwards relative to euchromatin (Teytelman et al. 2009). As noted above, the selection of small chromatin fragments for ChIP-Seq removes the large-fragment bias in silent chromatin. Importantly, a fraction of the input chromatin was sequenced for each immunoprecipitate, allowing adjustment for the expected under-recovery of reads from *HML* and *HMR*.

The spreading model would predict that even single-nucleosome-size fragments originating from inside *HML* and *HMR* should co-precipitate with Sir4. Our ChIP-Seq results suggest that Sir4 remains largely at the silencers in both *S. cerevisiae* and *S. bayanus*, with the exception of the peak over the promoter of *S. cerevisiae HML* (Figure A.1, A and B). However, these results leave open the possibility that Sir2 and/or Sir3 might spread internally into the locus. Because recombinant Sir3 can bind histone tails, exists in oligomeric states, and spreads further at telomeres than Sir2 or Sir4, Sir3 alone

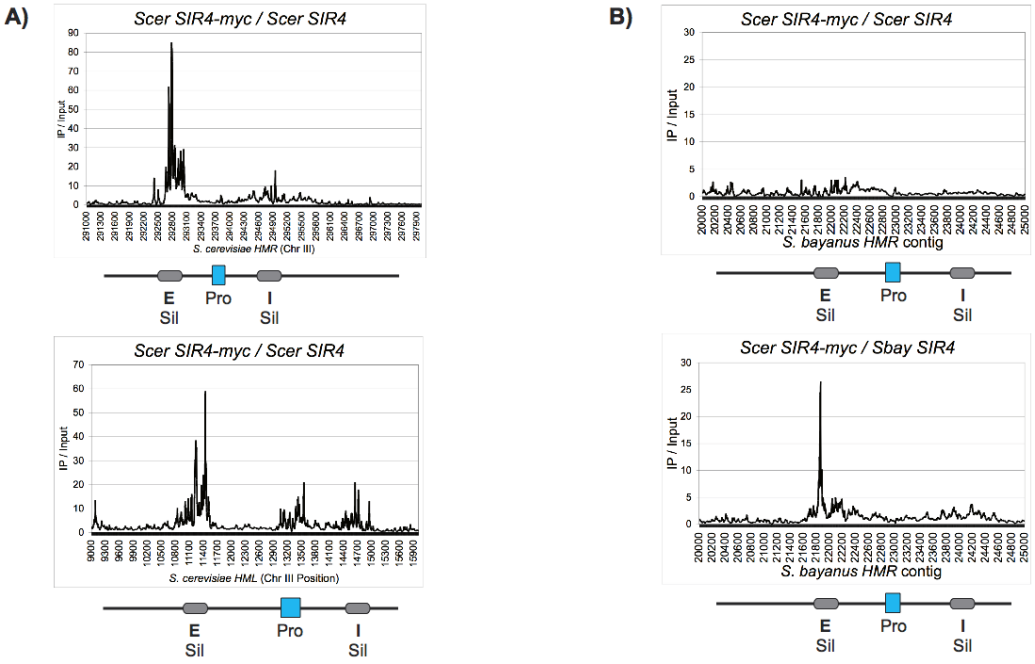

Figure A.1. ChIP-Seq distribution of Sc-Sir4 at *Sc-HML*, *Sc-HMR*, or *Sb-HMR* in *S. cerevisiae/S. bayanus* interspecies hybrids. (A) ChIP-Seq analysis of *S. cerevisiae* Sir4 at *S. cerevisiae HMR* (top panel) and *HML* (bottom panel) in *S. cerevisiae/S. bayanus* hybrid diploids. 7kb of each locus is shown, with the positions of *S. cerevisiae* Chromosome III notated on the x-axis. (B) ChIP-Seq analysis of *S. cerevisiae* Sir4 at *S. bayanus HMR*. The top panel shows a hybrid in which the *S. bayanus SIR4* gene has been replaced by the *S. cerevisiae SIR4* gene (*S. cerevisiae SIR4/S. cerevisiae SIR4*). The bottom panel shows a hybrid with the endogenous *SIR4* configuration (*S. cerevisiae SIR4/S. bayanus SIR4*). Below each panel, schematics show the relative locations of the promoters (Pro), E silencers (E Sil), and I silencers (I Sil) of *HM* loci. Pair-wise correlation coefficients of sequence coverage, calculated in 100bp sliding windows, were R = 0.96-0.98 for all possible pairs of input samples, and R = 0.92-0.94 for all possible pairs of IP samples. Median genome-wide coverage, average of four inputs: 34. Median genome-wide coverage, average of four IPs: 11. Median coverage of *S. cerevisiae* silent loci, including silencers, average of four inputs: *HML*, 16; *HMR*, 18.

may have the capacity to spread, perhaps aided by Sir2 (Hecht et al. 1996; Georgel et al. 2001; Connelly et al. 2006; Onishi et al. 2007). One could test these alternative versions of the spreading model by performing ChIP-Seq on *S. cerevisiae* epitope-tagged Sir2 strains and epitope-tagged Sir3 strains, which have already been subjected to standard ChIP analysis. As I established for Sir4, Illumina paired-end reads allow one to maximize the number of reads uniquely mapping to silent mating-type loci and telomeres, which being partially homologous loci, would present read-mapping problems were the paired-end strategy not available.

There are three likely outcomes of this experiment: Either Sir2 and Sir3 will ChIP inside *HML* and *HMR*; or just Sir3 will be found to spread; or neither will be found to spread. In any of these cases, a substantial reconsideration of how Sir proteins repress transcription at a distance will be in order. The first case would suggest that histone-tail binding by Sir4 throughout *HML* and *HMR* is not critical for silencing. The second case, perhaps the most radical, would suggest that silencing does not function exclusively through heterotrimeric Sir2/Sir3/Sir4 complexes, and that Sir2 deacetylates histones not through spreading, but by the tails being reeled in towards the silencers. I think of this as the "reeling" or "gravitational attractor" model of silencer-associated silencing complex function. (Another macromolecular structure that may act in analogous fashion is the Microtubule Organizing Center (MTOC).) Importantly, this result would argue that Sir3 is the critical repression factor acting at the silent promoters, with Sir2 and Sir4 aiding in its recruitment. The third case, in which no Sir proteins are found to spread, would also implicate histone-tail reeling as an explanation for the hypoacetylated status of H3 and H4 in silenced chromatin. Moreover, such results would disfavor the view of steric occlusion of RNA polymerase by spread Sir complexes. They would instead inspire more silencer-centric models for how events at silencers are transmitted to promoters in their vicinity, such as contractile assembly of a highly structured three-dimensional nucleosome array, or long-range DNA torsional effects. It should be noted that stable repression mechanisms often employ overlapping mechanisms such as the DNA methylation on the inactive mammalian X that comes after X-inactivation, leading to more stable repression. Hence, I do not mean to imply that there might be no effect of Sir protein spreading, and indeed our ChIP-Seq data indicates some Sir4 above background levels throughout *HML* and *HMR*. Rather, the preponderance of Sir4 binding at silencers, and at the promoter in the case of *HML* (Figure A.1, A), are difficult to reconcile with repression purely by spreading models, and demand a fine-grained revisiting of whether all Sir-proteins are distributed throughout these regions.

To determine how histone acetylation status correlates with our new picture of Sir protein distribution, one could perform ChIP-Seq using antibodies to acetylated H4-K16 in Sir+ and Sir- cells. Antibodies to bulk H4 would allow normalization to local nucleosome distribution, and should provide an excellent calibration of ChIP-Seq data against the DNaseI nucleosome mapping data from Robert Simpson's lab (Weiss and Simpson 1998; Ravindra et al. 1999). An even spread of the hypoacetylated state throughout *HML* and *HMR* by Chip-Seq, as indicated by conventional ChIP analysis (Braunstein et al. 1996; Suka et al. 2002), but a largely unequal distribution of Sir-proteins, would provide support for a contribution by a silencer-bound Sir complex attraction model. One could additionally evaluate Sir3 and Sir4 chromatin association in

catalytically defective *sir2* mutants to ask whether there is mutual reinforcement of Sir proteins at silencers with hypoacetylated histone tails throughout the silenced domain.