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Dynamics of transcriptional silencing in *Saccharomyces cerevisiae*

By

Anne Elizabeth Dodson

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Committee in charge:

Professor Jasper Rine, Chair
Professor Abby Dernburg
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Abstract

Dynamics of transcriptional silencing in *Saccharomyces cerevisiae*

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Jasper Rine, Chair

Eukaryotic cells package designated regions of their DNA into a condensed structure referred to as heterochromatin. By doing so, they preserve integrity of both the genome and the epigenome, as heterochromatin represses recombination and transcription at loci where such activities would be undesirable. In *Saccharomyces cerevisiae*, heterochromatin represses transcription at the silent mating-type loci, *HML* and *HMR*. Although the heterochromatin structure is dynamic and subject to perturbations such as DNA replication, all analyses to date have classified *HML* and *HMR* as transcriptionally inert. Therefore, either the mechanism of silencing compensates for fluctuations in the heterochromatin structure and flawlessly reassembles each cell cycle, or there are transient losses of silencing that underlie a hidden, dynamic dimension to heterochromatic repression.

To test whether RNA polymerase ever gains access to the silent mating-type loci, I used Cre-*loxP* recombination technology to devise a genetic assay whereby transient transcription of a cre reporter at *HML* would trigger a permanent, recombination-based switch from RFP expression to GFP expression. By recording short-lived events of *HML::cre* expression with the Cre-Reported Altered States of Heterochromatin (CRASH) assay, I found that approximately 1/1000 cells lost silencing per cell division. Consistent with this observation, measurements I made by single-molecule RNA FISH indicated that transcription of *HML* was rare, yet detectable, and limited in the number of RNA molecules that could be synthesized before silencing was re-established. These approaches revealed dynamics of silencing that had escaped detection by all previous measurements.

I used the CRASH assay to identify several genetic and environmental factors that modify the dynamics of heterochromatic repression. Whereas Sir2 is the only member of the sirtuin family of NAD\(^+\)-dependent deacetylases previously shown to have a role in silencing at *HML*, I uncovered roles for two additional sirtuins. I showed that one of these sirtuins, Hst3, helped stabilize silencing through the deacetylation of acetylated lysine 56 on histone H3. In addition, I identified effects of histone gene dosage, *SIR* gene dosage, ploidy, and various
environmental conditions on the stability of silencing. Quantitative analyses of these phenotypes were streamlined through the development of software that detects and compares patterns of differential gene expression in yeast colonies.

The sensitivity of the CRASH assay also revealed that the stability of silencing at \textit{HML} differed between the two mating types of haploid cells. This mating-type effect depended on the activity of the recombination enhancer, a DNA element centromere-proximal to \textit{HML} that was previously described for its role in directing the pattern of mating-type switching. I showed that this locus acted in cis to destabilize silencing at \textit{HML} in a mating-type specific manner. Thus, the recombination enhancer was moonlighting as a long-range regulator of gene expression, the first such element identified in yeast.
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Chapter 1. An introduction to heterochromatin in *Saccharomyces cerevisiae*

In eukaryotes, nuclear DNA winds around octamers of histone proteins to form chromatin, a structure that contributes to both the maintenance of genome integrity and the regulation of gene expression. Genetic and environmental cues shape the landscape of chromatin through the activity of chromatin modifiers and remodelers and through the binding of accessory proteins. As a result, chromatin comes in many flavors.

One such flavor is heterochromatin. In 1928, Emil Heitz coined the term 'heterochromatin' to describe cytologically distinct regions of chromosomes that remain condensed throughout the cell cycle (Heitz 1928). Other defining features of heterochromatin include its inaccessibility to several DNA-interacting proteins, as well as its ability to silence transcription. Heterochromatin-mediated gene silencing is often heritable and thus provides a means for genetically identical cells to differentiate into distinct and stable cell types.

Heterochromatin formation typically involves the addition or removal of modifications on certain histone residues (Grewal & Moazed 2003). Depending on the organism and the type of heterochromatin, the exact mechanism through which this occurs may involve sequence-specific DNA-binding proteins, RNA interference, DNA methylation, or long non-coding RNA. In budding yeast, heterochromatin assembly depends on specific DNA elements that recruit the Silent information regulator (Sir) proteins, including the conserved histone deacetylase Sir2 (Rusche et al. 2003; Grunstein & Gasser 2013). This chapter reviews the function and formation of Sir-mediated heterochromatin in the budding yeast *Saccharomyces cerevisiae*.

1.1 Regulation of yeast mating types

To fully appreciate heterochromatin in *S. cerevisiae*, one should acquire a basic understanding of the different mating types. Heterochromatic repression serves an important role in mating-type determination. In fact, the discovery of Sir-mediated heterochromatin arose from the desire to understand certain curiosities of mating-type biology. Therefore, this review begins with an introduction to the three mating types: \( _a \), \( \alpha \) and \( _a/\alpha \).

1.1.1 Mating-type determination

The sexual life cycle of *Saccharomyces* requires the mating of two distinct cell types, referred to as \( _a \) and \( \alpha \). Haploid cells exhibit either the \( _a \) or \( \alpha \) mating type and can mate with haploids of the opposite mating type to form an \( _a/\alpha \) diploid. The \( _a/\alpha \) status of the diploid permits the induction of meiosis, which completes the cycle by producing four spores, two that mate as \( _a \) and two that mate as \( \alpha \).
The mating-type (MAT) locus is the master controller of mating type (Strathern et al. 1981). The two different wild-type alleles of this locus, MATa and MATa, code for proteins that regulate the expression of mating-type specific genes (Figure 1.1). MATa specifies the α mating type by encoding both α1, an activator of α-specific genes, and α2, a repressor of α-specific genes. In the absence of α2 protein, α-specific genes are constitutively active. Therefore, MATa cells (and matΔ cells) mate as α by default. In MATa/MATa diploids, the α1 protein encoded by MATa interacts with α2 to repress the expression of haploid-specific genes.

Two additional loci, HML and HMR, each contain an exact copy of either the a or α mating-type allele (Figure 1.1). Unlike MAT, however, HML and HMR are transcriptionally silent. How do genetically identical loci adopt different states of expression? The answer lies within the flanking regions of HML and HMR. These regions contain cis-regulatory sites referred to as silencers that nucleate heterochromatin formation and thereby render HML and HMR transcriptionally inactive (Figure 1.1). Importantly, the heterochromatic repression of HML and HMR ensures that MAT is the sole source of mating-type information.

1.1.2 Mating-type switching

HML and HMR serve a critical role in mating-type switching, a process whereby haploids convert the MAT genotype from MATa to MATα or vice versa (Haber 2012). In the first step of mating-type switching, the site-specific endonuclease HO generates a double-strand break at the MAT locus (Strathern et al. 1982; Kostriken et al. 1983). Then, the homologous recombination machinery repairs the double-strand break using either HML or HMR as a donor template, as both loci share some sequence identity with MAT. The repair is typically a gene conversion event that copies the information stored at HML or HMR into the MAT locus, leaving the donor sequence unchanged.

In the event that the donor allele differs from the original MAT allele (for example, cleavage of MATa is repaired using HMLα as the donor), the cell will switch mating types. Interestingly, selection of the donor template is nonrandom—a cells prefer to use HML, whereas α cells prefer to use HMR (Figure 1.1). These preferences increase the probability that double-strand break repair will result in a mating-type switch, as most strains store α information at HML and a information at HMR.

Donor preference is controlled in part by an extraordinary cis-acting element referred to as the recombination enhancer (RE) (Wu & Haber 1996). The RE locus resides on the left arm of chromosome III, approximately 17kb away from HML (Figure 1.1). In a cells, the RE activates recombination throughout the entire left arm of chromosome III and thereby promotes the selection of HML as a donor template. When the RE is absent or inactive, cells prefer to use HMR by default. For example, α cells prefer to use HMR as a donor because the α2 protein encoded by MATα represses RE activity (Szeto & Broach 1997; Szeto et al. 1997). Hence, HMR is always available for
Figure 1.1 MATα and MATα versions of chromosome III in *S. cerevisiae*. The centromere (black circle) demarcates the left and right arms. The silencers (red boxes) mediate the repression of *HML* and *HMR*. Percentages indicate how often *HML* or *HMR* is used as a donor template during repair of a HO-induced double-strand break at MAT. Donor preference differs between mating types due to the activity of the recombination enhancer (*RE*). Features are not drawn to scale.
recombination, whereas *HML* is only made available through the action of the *RE*.

A few points regarding HO are worth mentioning here. First, *HO* expression, and therefore mating-type switching, occurs only in haploid mother cells during the G1 phase of the cell cycle (Nasmyth 1993). Second, HO recognition sites also reside at *HML* and *HMR*, but the heterochromatin-dependent positioning of nucleosomes over these sites protects them from HO cleavage (Strathern et al. 1982; Weiss & Simpson 1998; Ravindra et al. 1999). Finally, most laboratory strains do not contain a functional HO and thus do not readily switch mating types. The recombination enhancer is still functional in laboratory strains, however.

### 1.2 Heterochromatin assembly

The identification of *HML* and *HMR* in early studies of mating-type biology marks the beginning of the quest to understand position-effect silencing in budding yeast. Today, a wealth of literature exists on this topic; yet, the mechanisms underlying certain fundamental aspects of silencing remain unclear.

#### 1.2.1 Silencers

Transcriptional repression of *HML* and *HMR* depends on the silencers, short DNA elements that border the left and right sides of each locus (Abraham et al. 1984; Feldman et al. 1984). Each silencer contains a binding site for the origin recognition complex (ORC), as well as a binding site for either Abf1 or Rap1, or both. Paradoxically, Abf1 and Rap1 often function individually in the transcriptional activation of many genes. In the context of a silencer, however, these proteins cooperate with ORC to recruit the Sir proteins and thereby silence transcription.

#### 1.2.2 Sir proteins

The four *SIR* genes were identified in a forward genetic screen designed to isolate mutants that aberrantly express *HML* and *HMR* (Rine & Herskowitz 1987). Loss-of-function mutations in *SIR2*, *SIR3* or *SIR4* result in full derepression, meaning that the genes present at *HML* and *HMR* are expressed at the same level as their counterparts at *MAT*. By contrast, loss-of-function mutations in *SIR1* result in an extraordinary bistable phenotype—some cells exhibit full repression, other cells exhibit full derepression, and each state of expression is heritable for multiple cell divisions (Pillus & Rine 1989; Xu et al. 2006). The existence of *sir1* cells in the silenced state demonstrates that Sir1, unlike the other Sir proteins, is not essential for silencing.

In addition to silencing transcription at *HML* and *HMR*, the Sir proteins also silence a subset of subtelomeric genes. Placing a reporter gene next to
certain telomere constructs results in the variegated expression of that gene—a fraction of cells silence the reporter, and that silencing depends on Sir2, Sir3 and Sir4 (Gottschling et al. 1990; Aparicio et al. 1991; Renauld et al. 1993). Given that Sir1 does not contribute to this effect (Aparicio et al. 1991), it is interesting to note that telomere-proximal reporter genes exhibit two distinct, semistable states of expression, much like the genes at HML and HMR in a sir1Δ background. Most studies of telomeric position effect were performed using truncated telomeres that contain the terminal TG1-3 repeats but lack more internal features such as the X and Y’ elements. In their native context, telomeres vary in the degree to which they mediate heterochromatic repression (Pryde & Louis 1999; Fourel et al. 1999; Wyrick et al. 1999; Ellahi et al. 2015). In fact, the Sir proteins silence less than 10% of subtelomeric genes (Wyrick et al. 1999; Ellahi et al. 2015).

Sir2, the only Sir protein with known enzymatic activity, is a highly conserved protein deacetylase. As a member of the sirtuin family of NAD⁺-dependent deacetylases, Sir2 couples protein deacetylation with the breakdown of NAD⁺ (Tanner et al. 2000; Tanny & Moazed 2001). Specifically, Sir2 cleaves NAD⁺ into nicotinamide and ADP-ribose and transfers the acetyl group on the substrate to ADP-ribose to produce 2’-O-acetyl-ADP-ribose. Sir2 preferentially targets acetylated lysine 16 of histone H4 (H4 K16-ac), as well as acetylated lysines 9 and 14 of histone H3 (H3 K9-ac and H3 K14-ac) (Imai et al. 2000; Landry, Sutton, et al. 2000; Smith et al. 2000; Tanny et al. 2004; Borra et al. 2004). Independent of the other Sir proteins, Sir2 suppresses intrachromosomal recombination between ribosomal DNA repeats as a component of the RENT (regulator of nucleolar silencing and telophase exit) complex (Gottlieb & Esposito 1989).

1.2.3 Establishment of the silenced state

Since wild-type cells are programmed to constitutively silence HML and HMR, they rarely encounter the need to establish silencing de novo. However, analyses of the establishment process have greatly informed our understanding of heterochromatin assembly. To study the de novo establishment of silencing, one must be able to conditionally inactivate and reactivate silencing. Therefore, temperature-sensitive SIR alleles, small-molecule inhibitors of Sir2, and cleverly designed assays that control the state of silencing have all been particularly useful for dissecting the individual steps of heterochromatin assembly.

The establishment of silencing begins with the loading of Sir proteins onto the silencers (Figure 1.2A). Sir1 localizes to the silencers through a direct interaction with the ORC subunit Orc1 and assists in recruiting the other Sir proteins (Triolo & Sternglanz 1996; Rusche et al. 2002; Zhang et al. 2002; Hou et al. 2005). The Sir1-Orc1 interaction is stabilized by the presence of Sir4, which binds Sir1, Sir2, Sir3 and Rap1 (Triolo & Sternglanz 1996; Bose et al. 2004; Rusche et al. 2002; Moazed et al. 1997; Ghidelli et al. 2001; Hecht et al. 1996; Moretti et al. 1994; Moretti & Shore 2001; Cockell et al. 1995). Sir3, in turn, interacts with Rap1 and possibly Abf1 (Moretti et al. 1994; Moretti & Shore 2001;
Cockell et al. 1995; Chen et al. 2011). Thus, the silencers weave a web of interactions between Sir proteins and silencer-binding proteins. Although most of these interactions are weak in isolation, they stabilize one another when combined.

Once the Sir proteins assemble at the silencers, they recruit additional Sir proteins that associate throughout the locus (Figure 1.2A). This step requires the deacetylation of histone residues by Sir2 and the binding of nucleosomes by Sir3 and Sir4 (Hecht et al. 1995; Rusche et al. 2002; Hoppe et al. 2002). Sir3 preferentially binds histones in the hypoacetylated state (Carmen et al. 2002; Liou et al. 2005); therefore, Sir2 helps create high-affinity binding sites for Sir3. Deacetylation of H4 K16-ac by Sir2 seems particularly important, as a crystal structure of the Sir3-nucleosome interaction predicts that acetylation of H4 K16 would disrupt several electrostatic contacts (Arma 2011). Chromatin immunoprecipitation (ChIP) analyses show co-enrichment of Sir2, Sir3 and Sir4 at the silencers and at internal sites of HML and HMR, whereas Sir1 associates only with the silencers (Rusche et al. 2002; Hoppe et al. 2002; Thurtle & Rine 2014; Ellahi & Rine 2016). Therefore, Sir1 does not spread across HML and HMR like the other Sir proteins do.

Transcriptional silencing marks the completion of heterochromatin formation. Surprisingly, this last step is separable from the deacetylation and binding of nucleosomes by Sir proteins (Lau et al. 2002; Kirchmaier & Rine 2006). Induction of the establishment process in G1-arrested cells leads to the spreading of Sir proteins across HMR, yet transcription of the a1 gene at HMR persists until the cells are allowed to pass through S phase (Kirchmaier & Rine 2006). Therefore, the establishment of silencing involves an additional, unknown step that occurs during S phase (Miller & Nasmyth 1984). This step does not require the passage of a replication fork through the locus (Kirchmaier & Rine 2001; Li et al. 2001), and it is less important for the silencing of HML than it is for the silencing of HMR (Ren et al. 2010). A second cell-cycle requirement for silencing exists in G2/M under certain conditions, and disruption of sister-chromatid cohesion allows cells to bypass this requirement (Lau et al. 2002).

The de novo establishment of silencing typically occurs within one to two cell divisions, and mutations known to affect certain histone modifications either shorten or lengthen this timeline (Katan-Khaykovich & Struhl 2005; Osborne et al. 2009; Osborne et al. 2011). For example, mutants defective in the methylation of histone H3 at lysine 79 (H3 K79) establish silencing faster than wild type (Katan-Khaykovich & Struhl 2005; Osborne et al. 2009; Osborne et al. 2011). These data suggest that H3 K79 methylation antagonizes one or more steps in the establishment process. Given that H3 K79 methylation reduces the affinity of Sir3 for nucleosomes (Altaf et al. 2007; Onishi et al. 2007; Martino et al. 2009; Ehrentraut et al. 2011; Armache et al. 2011), this modification probably impedes the spreading of Sir proteins throughout HML and HMR. Enzymes that modify H4 K16 and lysine 4 of histone H3 (H3 K4) also contribute to the kinetics of silencing establishment (Katan-Khaykovich & Struhl 2005; Osborne et al. 2009).
Figure 1.2 Establishment and maintenance of silencing. (A) Establishment of silencing. Silencer-binding proteins (yellow) recruit the Sir proteins (blue). Dashed lines indicate direct interactions. Nucleation at the silencers leads to the recruitment of additional Sir3 molecules and Sir2-4 heterodimers, which then deacetylate and bind nucleosomes throughout the target locus. (B) Maintenance of silencing. Once established, the silenced state is continuously reinforced by Sir2-mediated deacetylation and by the incorporation of new Sir proteins.
1.2.4 How does heterochromatin silence transcription?

To succeed in producing messenger RNA, the transcription machinery must complete a series of coordinated events (Li et al. 2007). First, RNA polymerase II and general transcription factors associate with the promoter to form the pre-initiation complex (PIC). Transcriptional activators that bind specific sequences upstream of the promoter facilitate PIC assembly. The PIC unwinds the promoter DNA, allowing polymerase to engage with the template strand and scan for the initiation site. Then, the transcription machinery synthesizes RNA as it proceeds along the template strand. This step coincides with 5’-capping of the nascent RNA. Finally, the termination of transcription, as well as additional RNA processing, results in a mature RNA message.

In principle, Sir-mediated heterochromatin should block one or more of the steps described above. Although our current understanding of transcriptional silencing is incomplete, multiple observations support a model whereby Sir proteins inhibit the binding of the transcription machinery to the promoter. In particular, ChIP analyses indicate that RNA polymerase II and general transcription factors are absent from the promoters at HML and HMR when silencing is intact (Chen & Widom 2005). How, then, do Sir proteins prevent assembly of the PIC? Given that the heterochromatin structure protects DNA from site-specific cleavage and methylation (Strathern et al. 1982; Singh & Klar 1992; Gottschling 1992; Loo & Rine 1994), it may limit interactions between the promoter and the transcription machinery through steric hindrance. Consistent with this possibility, comprehensive ChIP profiling suggests that the Sir proteins bury the promoter regions of HML and HMR within a higher-order chromatin structure (Thurtle & Rine 2014). Alternatively, or in addition, heterochromatin may prevent binding of the transcription machinery by positioning nucleosomes over the promoter region (Weiss & Simpson 1998; Ravindra et al. 1999; Wang et al. 2015). Sir-mediated silencing is not specific to the eukaryotic transcription machinery, as Sir proteins also repress the activity of a single-subunit RNA polymerase derived from bacteriophage T7 (Steakley & Rine 2015).

The exact mechanism of transcriptional silencing has been difficult to resolve due to conflicting data. Whereas one study was unable to detect PIC assembly by ChIP (Chen & Widom 2005), other ChIP-based studies conclude that the Sir proteins do permit PIC assembly and instead block transcription elongation and 5’-capping of the nascent RNA (Sekinger & Gross 2001; Gao & Gross 2008). However, permanganate footprinting does not detect melted DNA at or near the promoter of silenced chromatin, which would be expected if Sir proteins were to block an event downstream of PIC assembly (Steakley & Rine 2015). Regardless of the discrepancy between different ChIP experiments, ChIP-based measurements should be interpreted with caution, as silenced chromatin is resistant to shearing and can also mask antibody recognition sites upon formaldehyde cross-linking (Teytelman et al. 2009; Thurtle & Rine 2014).
1.3 Heterochromatin stability

Because heterochromatin is typically devoid of activities such as transcription and recombination, one might assume that it is fairly static. Every cell cycle, however, processes such as DNA replication and chromosome condensation challenge the integrity of the heterochromatin structure. Even outside of the cell cycle, maintaining the status quo of heterochromatin is an active process.

1.3.1 Maintenance and inheritance of the silenced state

Silencing is often conceptualized as a cycle of three stages: 1) the initial establishment of repression, 2) the maintenance of repression outside of S phase, and 3) the inheritance of repression through S phase. The establishment stage relies heavily on the presence of Sir1, as sir1Δ cells in the derepressed state rarely transition to a state of repression (Pillus & Rine 1989). In the unlikely event that a sir1Δ cell establishes silencing, however, that cell typically remains in the silenced state for multiple cell divisions (Pillus & Rine 1989). Therefore, Sir1 is not required for the maintenance or inheritance of silencing.

Although the sir1Δ phenotype seems to distinguish the establishment of silencing from the maintenance thereafter, these two processes are very similar. Section 1.2.3 describes the steps required for the de novo assembly of heterochromatin—namely, the recruitment of Sir proteins, the deacetylation of histones by Sir2, and the binding of Sir3 and Sir4 to nucleosomes. The maintenance of mature heterochromatin depends on all of these steps, as well (Figure 1.2B). Since Sir2, Sir3 and Sir4 are integral components of the heterochromatin structure, eliminating any of these proteins leads to a loss of silencing. Heterochromatin also disassembles upon the inhibition of Sir2, suggesting that Sir2 must continuously deacetylate histone residues to preserve silencing (Bedalov et al. 2001; Bitterman et al. 2002; Özaydin 2009). In addition, the silencers promote the maintenance of silencing, presumably through the recruitment of new Sir proteins. When heterochromatin is artificially separated from the silencers, it loses its characteristic structure and also loses its ability to silence gene expression (Cheng & Gartenberg 2000).

In light of these findings, one could argue that the only distinction between establishment and maintenance is the initial state of expression. The Sir proteins maintain silencing by targeting chromatin that is already in the silenced state and is therefore hypomethylated and hypoacetylated (Braunstein et al. 1993; Suka et al. 2001; Bernstein et al. 2002; Ng et al. 2003). By contrast, the Sir proteins establish silencing by targeting active chromatin, which may contain modifications known to antagonize heterochromatin formation (see section 1.2.3). In principle, this distinction could explain the sir1Δ phenotype, assuming that the deletion of SIR1 destabilizes heterochromatin to a level where it is sufficient to template its own propagation, but is unable to overcome the roadblocks imposed by active chromatin marks. Two studies propose a similar
model for the bistable expression of telomere-proximal reporter genes (Ng et al. 2003; Kitada et al. 2012).

One aspect of the sir1Δ phenotype is often overlooked—at a low rate, cells in the repressed state transition to a state of expression (Pillus & Rine 1989). What could explain this instability? Perhaps Sir1 serves an unprecedented role in the maintenance of silencing. Alternatively, silencing loss may be a natural occurrence that goes undetected in wild-type cells due to the efficiency of re-establishment. Both explanations seem plausible given the dynamic nature of the heterochromatin structure.

1.3.2 Heterochromatin dynamics: knowns and known unknowns

The structure of heterochromatin fluctuates in both dividing and non-dividing cells. Similar to heterochromatin in other species, Sir-mediated heterochromatin undergoes regular exchange of at least some of its structural components, as G1-arrested cells incorporate newly synthesized Sir3 into silenced chromatin (Figure 1.2B) (Cheng & Gartenberg 2000). In addition, heterochromatin must compensate for events that dramatically alter all forms of chromatin. Such events include DNA replication in S phase, chromosome condensation in G2/M phase, and homologous recombination. Note that mating-type switching requires the recombination machinery to invade heterochromatin at either HML or HMR.

In principle, any of these fluctuations could transiently expose silenced chromatin to RNA polymerase and factors that promote its activity. The observation that G1-arrested cells lose silencing upon the inhibition of Sir2 implies that heterochromatin is permeable to acetyltransferases, at the least (Bedalov et al. 2001; Bitterman et al. 2002). However, all measurements of silencing to date indicate that HML and HMR are transcriptionally inert. Even at the single-cell level, no assay has detected a loss of mating-type identity or the expression of a reporter gene in a wild-type cell. Taken together, these observations imply one of two scenarios: either the mechanism of silencing is immune to fluctuations in the heterochromatin structure, or wild-type cells undergo transient lapses in silencing, followed by rapid re-establishment of silencing, that have escaped detection thus far.

To test whether dynamics of the heterochromatin structure ever compromise its ability to silence transcription, I designed and implemented a genetic strategy to capture transient events of expression. By preserving a historical record of loss-of-silencing events, this approach revealed that Sir-mediated heterochromatin fails to completely block transcription at HML or HMR approximately once every 1,000 cell divisions. Due to the combination of their rarity and transience, these loss-of-silencing events escaped detection by all other assays. Hence, I unmasked a dynamic dimension to heterochromatic repression. I also showed a definitive role for Sir1 in the maintenance of silencing and thereby conclude that Sir1 functions in wild-type cells to prevent losses of silencing from occurring in the first place, as well as to re-establish
silencing in the event that a loss does occur. Several other factors modify the stability of heterochromatin, as well, and in collaboration with Yun Song and his lab members, we developed a method to quantify the contributions of these factors. Among this list of modifiers is a cis element located approximately 17kb away from HML that destabilizes silencing in a mating-type-specific manner. Thus, akin to transcriptional enhancers in other eukaryotes, this cis element mediates a long-distance effect on gene expression.

By revealing the dynamic nature of heterochromatic repression, the work presented in this thesis opens up a long line of questions. Namely, what causes silencing to fail in wild-type cells? The observation that telomeric silencing is particularly unstable in the G₂/M phase suggests that one or more cell-cycle components contributes to the dynamics of silencing (Aparicio & Gottschling 1994). It remains unclear, however, whether losses of silencing at HML and HMR are a function of time or of progression through the cell cycle, or both. It is worth noting that I observed at least one instance of silencing loss at HML in G₁-arrested cells (data not shown). Another question that merits further investigation is whether the descendants of cells that lose silencing are particularly prone to losing silencing themselves. In other words, is instability a heritable feature of heterochromatic repression? In support of this possibility, one study shows that switches between two semistable states of expression are coordinated in closely related cells (Kaufmann et al. 2007). Finally, because I often measured heterochromatin dynamics in the form of colony sectoring, I (and others in the lab) noticed some surprising effects of colony physiology on the stability of repression. Although budding yeast is a single-celled organism, yeast colonies are complex structures consisting of physiologically diverse strata. Therefore, following up on these observations will inform our understanding of how specific environments can influence the stability of an epigenetic state.
Chapter 2. Heritable capture of heterochromatin dynamics in *Saccharomyces cerevisiae*

(This chapter is an adaptation of Dodson AE and Rine, J (2015). *eLife* 4:e05007)

2.1 Abstract

Heterochromatin exerts a heritable form of eukaryotic gene repression and contributes to chromosome segregation fidelity and genome stability. However, to date there has been no quantitative evaluation of the stability of heterochromatic gene repression. We designed a genetic strategy to capture transient losses of gene silencing in *Saccharomyces* as permanent, heritable changes in genotype and phenotype. This approach revealed rare transcription within heterochromatin that occurred in approximately 1/1000 cell divisions. In concordance with multiple lines of evidence suggesting these events were rare and transient, single-molecule RNA FISH showed that transcription was limited. The ability to monitor fluctuations in heterochromatic repression uncovered previously unappreciated roles for Sir1, a silencing establishment factor, in the maintenance and/or inheritance of silencing. In addition, we identified the sirtuin Hst3 and its histone target as contributors to the stability of the silenced state. These approaches revealed dynamics of a heterochromatin function that have been heretofore inaccessible.

2.2 Introduction

Heterochromatin is a heritable, condensed chromatin structure that silences the expression of most genes within or near it. Phenomena such as clonal inheritance of inactivated X-chromosomes in female mammals, as well as position-effect variegation in *Drosophila* and yeast, demonstrate the remarkable ability of cells to propagate heterochromatic repression through mitosis. As an epigenetic state, heterochromatic gene repression provides a means for genetically identical cells to differentiate into stable, distinct cell types. However, despite its significance, little is known about the dynamics of heterochromatic repression and which factors contribute to or disrupt its stability.

In *Saccharomyces cerevisiae*, heterochromatin forms at the silent mating-type loci, *HML* and *HMR*, through the recruitment and subsequent spreading of Sir proteins (Grunstein & Gasser 2013). DNA elements known as the *E* and *I* silencers flank each locus and nucleate complexes of Sir2, Sir3 and Sir4. Sir complexes then deacetylate histones and bind nucleosomes throughout the region, thereby rendering *HML* and *HMR* transcriptionally silenced and largely inaccessible to DNA-interacting proteins. Since each locus contains either a or α mating-type information, as does the *MAT* locus, heterochromatic repression of
HML and HMR ensures that the MAT genotype is the only determinant of whether haploids mate as a or α cells.

Sir-mediated heterochromatin can be thought of as having a cycle consisting of its initial establishment, its maintenance through the G₁, G₂ and M phases, and its inheritance through S phase. Sir2, Sir3 and Sir4 are essential for all aspects of silencing (Rine & Herskowitz 1987). Thus, mutants lacking any of these proteins express HML and HMR to the level of the transcriptionally active MAT locus. By contrast, mutants lacking Sir1 exhibit a bistable silencing phenotype (Pillus & Rine 1989; Xu et al. 2006). Within a population of sir1 cells, HML and HMR exist in one of two phenotypic states: silenced or expressed. Each state is heritable for multiple cell divisions, demonstrating the epigenetic nature of Sir-mediated heterochromatin and inspiring the notion that Sir1 functions in the establishment of silencing, but not the maintenance or inheritance thereafter.

Notably, rare switches occur between the two expression states of HML and HMR in sir1 mutants, during which silencing is either lost or established. If Sir1 functioned exclusively in establishment, then losses of silencing should also occur in wild-type cells, yet no such event has been detected. Wild-type expression levels of genes at HML and HMR are 1000-fold lower than the expression levels of the same genes when at the MAT locus, and efforts to detect expression of HML and HMR by any molecular method have shown the expression signal is indistinguishable from background noise. Moreover, 100% of MATα cells respond to α-factor, and diploids homozygous at the MAT locus are completely unable to sporulate. Thus, by all previous molecular criteria, the silent mating-type loci are transcriptionally inert. However, heterochromatin undergoes regular exchange of at least some of its structural components with newly synthesized molecules of the same proteins (Cheng & Gartenberg 2000; Festenstein et al. 2003; Cheutin et al. 2003; Ficz et al. 2005) and is subject to perturbations, such as its replication in S phase. These fluctuations in heterochromatin structure imply that either the mechanism of silencing compensates for these changes and flawlessly reassembles each cell cycle, or that there are rare, as yet undetected losses of silencing resulting from heterochromatin dynamics.

To address whether RNA polymerase ever succeeds in transcribing silent chromatin at HML and HMR, we designed an assay capable of detecting short-lived gene expression with single-cell resolution. By capturing the consequences of transcription with a permanent, heritable mark, we detected transient losses of silencing at HML and HMR in wild-type cells, characterized the nature of these losses, and identified genetic determinants of heterochromatin stability.

2.3 Materials and methods

Yeast strains. The strains, plasmids, and oligonucleotides used in this chapter are listed in Table 2.1, Table 2.2 and Table 2.3, respectively. All strains were
derived from the W303 background. Deletions were made using one-step integration of gene disruption cassettes (Longtine et al. 1998; Goldstein & McCusker 1999; Gueldener et al. 2002) and confirmed by PCR of the 5' and 3' junctions. For analysis of HHT2 (histone H3) mutations, the URA3-marked plasmid (pJR2657) in JRY9638 was replaced with a TRP1-marked plasmid (either pJR2759, pJR3212 or pJR3213) by plasmid shuffle to produce JRY9639, JRY9640 and JRY9641. Using the hst3Δ::K.I.LEU2 fwd/rev primers and pUG73 (Gueldener et al. 2002), HST3 was deleted in each of these strains to create JRY9736, JRY9737 and JRY9738.

The cre gene from bacteriophage P1 was integrated at HML by transformation of a strain containing HMLα::K.I.URA3 and counter-selection on medium containing 5-fluoroorotic acid (5-FOA). First, the K.I.URA3 gene was amplified by PCR from pUG72 (Gueldener et al. 2002) using the HMLα::K.I.URA3 fwd/rev primers and integrated at HMLα, replacing the coding sequence of the a2 gene. Then, the cre gene was amplified from pSH47 (Güldener et al. 1996) using the HMLα::cre fwd/rev primers, and the resulting PCR product was transformed into the strain containing HMLα::K.I.URA3. 5-FOA was used to select for HMLα::cre cells, which no longer contained K.I.URA3. Integration of cre was confirmed by sequencing.

To construct the HMRα::cre allele, the K.I.URA3 gene was amplified by PCR from pUG72 (Gueldener et al. 2002) using the HMRα::K.I.URA3 fwd/rev primers and integrated at HMRα, replacing the coding sequence of the a1 gene. Then, the HMRα::cre fwd/rev primers were used to amplify a fragment of HMLα::cre from JRY9628 genomic DNA that spanned from the X region to the Z1 region (see Figure 2.1A), and the resulting PCR product was transformed into the strain containing HMRα::K.I.URA3. 5-FOA was used to select for HMRα::cre cells, which no longer contained K.I.URA3. Integration of cre was confirmed by sequencing.

For integration of the RFP-GFP cassette at the URA3 locus, the P_{GPD-loxP-yEmRFP-T_{CYC1}}-kanMX-loxP-yEGFP-T_{ADH1} sequence in pJR3214 was amplified by PCR using the ura3Δ::RFP-GFP fwd/rev primers. pJR3214 contains the following features: the ampR-containing fragment of pUG73 (Gueldener et al. 2002) resulting from digestion with restriction enzymes Ndel and NotI (2301bp); the GPD promoter from p413-GPD (Mumberg et al. 1995); a loxP site (5'-ATAACTTCGTATAGCATACATACGAAGTTAT-3'), separated from P_{GPD} by a Sall restriction site (GTCGAC); immediately downstream of the first loxP site is the yEmRFP gene (Kepper-Ross et al. 2008), followed by a BglII restriction site (AGATCT) and the CYC1 terminator from pSH63 (Gueldener et al. 2002); the kanMX cassette from pUG6 (Güldener et al. 1996), separated from T_{CYC1} by a Stul restriction site (AGGCCT); adjacent to the kanMX cassette is a second loxP site (5'-ATAACTTCGTATAGCATACATACGAAGTTAT-3'); and immediately following the second loxP site is an EcoRV restriction site (GATATC) and the yEGFP-T_{ADH1} sequence from pKT127 (Sheff & Thorn 2004).

The version of the RFP-GFP cassette marked with hphMX instead of kanMX (in strains JRY9729 and JRY9730) was constructed by transforming
strains containing the kanMX-marked RFP-GFP cassette with PCR product amplified from pAG32 (Goldstein & McCusker 1999) using the P_{TEF} fwd and T_{TEF} rev primers.

**Colony growth and imaging.** All colonies were imaged using a Zeiss Axio Zoom.V16 microscope equipped with ZEN software (Zeiss), a Zeiss AxioCam MRm camera and a PlanApo Z 0.5× objective. The colonies shown in Figures 2.2, 2.7, 2.8 and 2.9 were imaged on day 6 of growth, whereas the colonies shown in Figures 2.3 and 2.6 were imaged on day 3 of growth. Images were assembled using Photoshop (Adobe Systems).

In preparation for colony analysis, RFP-expressing cells were selected using medium containing G418 (Geneticin; Life technologies). Due to the complete loss of silencing in sir2Δ cells, JRY9633 was unable to grow in the presence of G418 and was therefore grown on nonselective medium. Cells were then grown to log phase in Complete Supplement Mixture (CSM) –Trp (Sunrise Science Products), and serial dilutions were performed to spread approximately 10 cells/plate (CSM –Trp, 1% agar). To inhibit sirtuin activity, 200 µl of 0.5M nicotinamide was spread onto CSM –Trp, 1% agar plates prior to plating cells.

For Figure 2.6, RFP-expressing cells of JRY9729 and JRY9730 were selected using medium containing both G418 and Hygromycin B (Sigma-Aldrich), and then sporulated for 4-5 days at room temperature on 1% potassium acetate. The resulting tetrads were dissected on CSM –Trp plates and grown into colonies for imaging.

For half-sector analysis, RFP-expressing cells were selected using G418 and then grown to log phase in CSM –Trp. Serial dilutions were performed to plate approximately 100 cells/plate (CSM –Trp). On day 3 of growth, plates were scanned, face up, with a Typhoon Trio (GE Healthcare Life Sciences) using the 488-nm laser and 520-nm emission filter to detect GFP fluorescence. Since colonies of cells containing the H3 K56R substitution grew notably slower than colonies of other genotypes, plates were scanned on day 4 of growth for JRY9640 and JRY9737. Colonies that were half green or completely green were manually counted. The total number of colonies was determined with Matlab (MathWorks) by applying a threshold and converting scans to a binary image. Then, the following equation was used to calculate the half-sector frequency:

\[
\frac{\text{# of half-sectored colonies}}{\text{(# of total colonies - # of fully green colonies)}}
\]

Three independent experiments were performed for each genotype.

**Flow cytometry.** Colonies were grown as described above, scraped off the agar surface and resuspended in synthetic complete (SC) medium (Sunrise Science Products). After growth overnight, cultures were diluted back to 0.01 OD_{600} and then grown in SC to approximately 0.2 OD_{600}. Cells were harvested by centrifugation, and then washed and resuspended in PBS, pH 7.4 on ice. A FC-500 flow cytometer (Beckman-Coulter) was used to measure the GFP fluorescence intensity of 10^5 cells/sample. Using FlowJo software (Tree Star),
cells were gated based on forward and side scatter, and the data were exported as FCS files. Data analysis was performed with Matlab.

**RNA preparation for quantitative RT-PCR.** Total RNA was isolated from log-phase cells using hot acidic phenol (Collart & Oliviero 2001). RNA was digested with DNase I (Roche Diagnostics) and then purified by phenol-chloroform extraction and precipitation with isopropanol. cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo(dT) primers. Quantitative PCR of cDNA was performed using the Thermo Scientific DyNAmo HS SYBR Green qPCR Kit (Fisher Scientific) and a Mx3000P machine (Stratagene). Samples were analyzed in technical triplicate for three independent RNA preparations.

**Single-molecule RNA FISH.** RNA FISH was performed as described (Raj et al. 2008) using JRY9630, JRY9631, JRY9632 and JRY4012. All probes (see Table 2.4 for probe sequences) were designed, synthesized and labeled by Stellaris (Biosearch Technologies). Probes targeting cre were coupled to Quasar 670 dye (Biosearch Technologies) and probes targeting KAP104 were coupled to CAL Fluor Red 590 dye (Biosearch Technologies). Each probe mix (5 nmol) was dissolved in 100 µl of TE buffer, pH 8.0. For hybridization, 1:10 dilutions of the cre and KAP104 probe stocks were made, and 1 µl of each probe dilution was added to 50 µl of hybridization buffer.

Cells were grown in CSM to 0.2-0.3 OD600 and then fixed and hybridized using the protocol as described (Youk et al. 2010) with additional guidance (Raj 2013) and the following modification to the spheroplasting procedure: cells were digested with 3 µl of zymolyase (2.5 mg/ml) at 30˚C and monitored by phase contrast microscopy until approximately 80% of cells appeared to be digested (35-50 minutes) (L Teytelman, personal communication, January 2013). The cre and KAP104 probes were hybridized to cells overnight at 30˚C in 10% formamide buffer. Samples were then washed, stained with DAPI, washed again and resuspended in 10 µl of glucose-oxygen-scavenging (GLOX) solution without enzymes. Prior to imaging, 10 µl of GLOX solution with enzymes (1% [v/v] catalase [Sigma-C3515], 1% [v/v] glucose oxidase [Sigma-G2133], 2 mM Trolox [Sigma-238813]) was added to the sample.

Images were acquired with a DeltaVision RT microscope and softWoRx software (Applied Precision) using a 60×/1.40 oil-immersion objective (Olympus), a CoolSNAP HQ CCD camera (Photometrics) and the following filters from Chroma Technology: ET402/15x, ET455/50m (DAPI); ET555/25x, ET605/52m (CAL Fluor Red 590); and ET645/30x, ET705/72m (Quasar 670). Series of z-stacks were acquired with a step size of 0.2 µm. Cell boundaries were hand-drawn using ImageJ software (NIH). Spots were detected and analyzed using Matlab. First, a three-dimensional Laplacian of Gaussian filter (Raj et al. 2008) was applied to raw images of cre and KAP104 RNA. Then, a fluorescence intensity threshold was selected to identify spots within an expected range of sizes. Given that some sir4A and sir1A cells contained a relatively large number of cre transcripts, some of which appeared to overlap each other in a maximum-
intensity projection of z-stacks, spot detection was performed in three dimensions to resolve individual spots. Within each experiment (three independent experiments were performed), the same threshold values (one for cre RNA images and one for KAP104 RNA images) were applied across all samples.

**Live-cell imaging.** Cells containing *HML*α::*cre* and the RFP-GFP cassette (JRY9628) were grown to mid-log phase in CSM–Trp, sonicated once for 5 seconds at a power output of 10%, and then imaged over time using the CellASIC ONIX Microfluidic Platform (EMD Millipore). Growth was restricted to a single focal plane within chambers of the Y04C Microfluidic Plate for Haploid Yeast (EMD Millipore). CSM–Trp flowed through the chambers continuously at a pressure of 3 psi. Brightfield and fluorescence images of 46 different fields were taken every 10 minutes for 13 hours using MetaMorph software (Molecular Devices) and an Eclipse Ti microscope (Nikon Instruments) equipped with a Clara Interline CCD camera (Andor Technology) and a CFI Apo TIRF 60×/1.49 oil-immersion objective (Nikon Instruments), which was heated to 30°C using an objective heater (Bioptechs). Exposure times for yEGFP and yEmRFP fluorescence were each 100 ms. Image analysis was performed using ImageJ software.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tr>
<td>JRY9623</td>
<td>matΔ::kanMX lys2 his3-11,15 leu2-3,112 can1-100 trp1-1 ura3-1 hmrΔ::hphMX</td>
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<td>JRY9624</td>
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<tr>
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<td>JRY9638</td>
<td>MATa lys2 his3-11,15 leu2-3,112 can1-100 trp1-1 HMLa-q2Δ::cre</td>
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pJR2657 [HHT2-HHF2 URA3]
JRY9639   MATa lys2 his3-11,15 leu2-3,112 can1-100 trp1-1 HMLα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{CYC1}\)-kanMX-loxP-yEGFP\(_{-}\text{TRP1}\)
           hht1-hhf1Δ::hphMX hht2-hhf2Δ::natMX
           pJR2759 [HHT2-HHF2 TRP1]

JRY9640   MATa lys2 his3-11,15 leu2-3,112 can1-100 trp1-1 HMLα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{CYC1}\)-kanMX-loxP-yEGFP\(_{-}\text{TRP1}\)
           hht1-hhf1Δ::hphMX hht2-hhf2Δ::natMX
           pJR3212 [hht2(K56R)-HHF2 TRP1]

JRY9641   MATa lys2 his3-11,15 leu2-3,112 can1-100 trp1-1 HMLα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{CYC1}\)-kanMX-loxP-yEGFP\(_{-}\text{TRP1}\)
           hht1-hhf1Δ::hphMX hht2-hhf2Δ::natMX
           pJR3213 [hht2(K56Q)-HHF2 TRP1]

JRY9736   MATa lys2 his3-11,15 leu2-3,112 can1-100 trp1-1 HMLα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{CYC1}\)-kanMX-loxP-yEGFP\(_{-}\text{TRP1}\)
           hst3A::K.I LEU2 hht1-hhf1Δ::hphMX hht2-hhf2Δ::natMX
           pJR2759 [HHT2-HHF2 TRP1]

JRY9737   MATa lys2 his3-11,15 leu2-3,112 can1-100 trp1-1 HMLα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{CYC1}\)-kanMX-loxP-yEGFP\(_{-}\text{TRP1}\)
           hst3A::K.I LEU2 hht1-hhf1Δ::hphMX hht2-hhf2Δ::natMX
           pJR3212 [hht2(K56R)-HHF2 TRP1]

JRY9738   MATa lys2 his3-11,15 leu2-3,112 can1-100 trp1-1 HMLα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{CYC1}\)-kanMX-loxP-yEGFP\(_{-}\text{TRP1}\)
           hst3A::K.I LEU2 hht1-hhf1Δ::hphMX hht2-hhf2Δ::natMX
           pJR3213 [hht2(K56Q)-HHF2 TRP1]

JRY9739   MATa lys2 his3-11,15 leu2-3,112 can1-100 sir1Δ::LEU2
           HMLα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{CYC1}\)-kanMX-loxP-yEGFP\(_{-}\text{TRP1}\)
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{TRP1}\)

JRY9740   MATa lys2 his3-11,15 leu2-3,112 can1-100 sir1Δ::LEU2
           HMRα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{CYC1}\)-kanMX-loxP-yEGFP\(_{-}\text{TRP1}\)

JRY9741   MATa MATa lys2/lys2 his3-11,15 his3-11,15 leu2-3,112 can1-100/can1-100
           sir1Δ::LEU2/SIR1 HMLα/HMLα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{TRP1}\)
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{TRP1}\)

JRY9742   MATa MATa lys2/lys2 his3-11,15 his3-11,15 leu2-3,112 can1-100/can1-100
           sir1Δ::LEU2/SIR1 HMLα/HMLα-α2Δ::cre
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{TRP1}\)
           ura3Δ::P\(_{GPD}\)-loxP-yEmRFP\(_{-}\text{TRP1}\)
Table 2.1 Strains used in Chapter 2.

K.I., *Kluyveromyces lactis*
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**Table 2.2** Plasmids used in Chapter 2.
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Table 2.3 Oligonucleotides used in Chapter 2.
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**Table 2.4** FISH probes used in Chapter 2.
2.4 Results

To determine the stability of gene silencing in heterochromatin, we placed the gene encoding the Cre recombinase under control of the a2 promoter at either HMLα or HMRα (Figure 2.1A). RNA measurements made by quantitative RT-PCR showed that cre was as repressed as the native a2 gene at this location (Figure 2.1B). On chromosome V of both the HMLα::cre and HMRα::cre strains, we integrated a sequence in which two loxP sites flank the RFP gene and the selectable drug marker kanMX (Figure 2.1A). The loxP–RFP-kanMX-loxP sequence resided downstream of a strong promoter and upstream of a promoterless GFP gene. Therefore, cells carrying this RFP-GFP cassette were RFP-positive, drug resistant, and GFP-negative. However, in the event that cre repression were lost, the resulting Cre protein could mediate recombination at the loxP sites, thereby excising the RFP and kanMX genes and positioning the GFP gene adjacent to the promoter (Figure 2.1A). It should be noted that the recombination event would be essentially irreversible in that the excised DNA lacked an origin of replication and would thereby be lost upon cell division. Thus, transient expression of cre would trigger a permanent switch to GFP expression and drug sensitivity in that cell and in all of its descendants. By convention, we refer to cells expressing RFP as red, and cells expressing GFP as green.

2.4.1 Transient losses of silencing detected at HML and HMR

After plating cells with the cre gene at either HMLα or HMRα on non-selective medium, we imaged the fluorescence of the resulting colonies. We first tested whether expression of cre from the a2 promoter would produce enough recombinase to cause an efficient RFP-to-GFP switch by adding the Sir2 inhibitor nicotinamide (NAM) to the medium. The NAM-induced derepression of either HMLα::cre or HMRα::cre resulted in entirely green colonies (Figure 2.2A). By contrast, cells that contained the RFP-GFP cassette but lacked any source of the cre gene formed entirely red colonies (Figure 2.2A). These observations were consistent with the quantification of GFP fluorescence by flow cytometry (Figure 2.2B). Therefore, cre expression was both necessary and sufficient for the switch from RFP to GFP expression. We then plated HMLα::cre and HMRα::cre cells on medium lacking NAM to determine the stability of silencing under normal conditions. The resulting colonies were predominantly red, indicating that most cells maintained the repression of cre throughout growth of the colony (Figure 2.2A). Within these colonies, however, we observed discrete green sectors, each of which represented a loss-of-silencing event that occurred in a cell at the vertex of the sector (Figure 2.2A). Although the switch to GFP expression was irreversible, some of the smaller sectors did not extend to the edge of the colony, likely due to a combination of genetic drift (Hallatschek et al. 2007) and the three-dimensional nature of colony growth. GFP expression was also detectable by flow cytometry in a fraction of HMLα::cre and HMRα::cre cells (Figure 2.2B). Thus, silent chromatin at HML and HMR was transcriptionally dynamic, exhibiting losses of silencing below the level of detection by all previous assays.
Figure 2.1 Cre-based assay. (A) Design of assay to detect short-lived gene expression. The gene coding for the Cre recombinase was integrated at either HMLα or HMRα, replacing the α2 coding region, and was expressed from the native α2 promoter. Black rectangles denote the E and I silencers of HML and HMR. The W, X, Ya, Z1 and Z2 regions of the mating-type loci are indicated by brackets. Loss of cre silencing induces Cre-mediated recombination of the loxP sites (arrowheads) in the RFP-GFP reporter cassette, causing a switch from RFP to GFP expression and a loss of G418 resistance. (B) Quantitative RT-PCR analysis of α1 and α2 mRNA levels in a strain containing wild-type HMLα (JRY9623), and α1 and cre mRNA levels in a strain containing HMLα::cre (JRY9625). To determine the fold repression, mRNA levels were also measured in HMLα and HMLα::cre strains lacking Sir3 (JRY9624 and JRY9626). All strains carried mata and hmrΔ mutations to relieve the repression of α1 by the a1-α2 heterodimer in sir3Δ cells (Strathern et al. 1981). mRNA values were normalized to ACT1 mRNA values. Expression levels are shown relative to the corresponding sir3Δ values (set to 1) (red dotted line). The fold repression of α2 and cre did not significantly differ (p = 0.12; Student’s t-test). Data are means ± standard deviation (SD) (n = 3).
Figure 2.2 Transient transcription of HML and HMR captured by Cre-based assay. (A) Colonies derived from single cells containing the RFP-GFP cassette in addition to either HMLα::cre, HMRα::cre, or no source of the cre gene (No cre). Colonies of each genotype were grown in either the presence or absence of 5mM nicotinamide (NAM). Scale bar, 2 mm. (B) Flow cytometry measurements of GFP fluorescence intensity in individual cells from within a single colony. Cells containing the RFP-GFP cassette and either HMLα::cre, HMRα::cre or no cre.
gene were plated on Complete Supplement Mixture (CSM) - Trp in the presence (red) or absence (blue) of 5mM NAM. On day 6 of growth, representative colonies of each genotype and condition were resuspended in synthetic complete (SC) medium and grown to early log phase for analysis by flow cytometry. Each distribution represents approximately $10^5$ cells. The percentage of GFP-positive cells shown for each sample was determined by a gate (dotted line) that was set based on the intensity profile of the $HML\alpha::cre$ (+NAM) sample. (C) Quantitative RT-PCR measurements of $HML\alpha::cre$ transcription in red and green sectors. To determine whether silencing loss was transient, $cre$ and $GFP$ mRNA levels were quantified in the GFP-expressing (green) and RFP-expressing (red) regions of a colony of cells containing $HML\alpha::cre$ and the RFP-GFP cassette (JRY9628). Three independent experiments were performed, each on a different colony with a large green sector. Cells from the red and green regions of each colony were grown to log phase in YPD and harvested for RNA isolation. As a positive control for $HML\alpha::cre$ expression, mRNA levels were also measured in a $sir2\Delta$ mutant (JRY9633). All mRNA values were normalized to $ACT1$ mRNA values. Expression levels are shown relative to the corresponding $sir2\Delta$ values (set to 1) (red dotted line). The fold repression of $HML\alpha::cre$ did not significantly differ between the red and green samples ($p = 0.43$; Student’s $t$-test). Data are means ± SD ($n = 3$).
At least two lines of evidence indicated that these losses of silencing were transient and did not arise from mutations in SIR genes. First, cre and GFP mRNA levels were measured in both red and green regions from within the same colony. If the loss-of-silencing event that resulted in the green sector were temporary, then the level of HMLα:cre repression would be indistinguishable between red and green sectors. Alternatively, if the loss of silencing were permanent, then cre mRNA would be detectable in cells from the green sector. Consistent with the efficiency of heterochromatin formation, which occurs de novo in approximately 1-2 cell divisions (Osborne et al. 2009), HMLα::cre repression was fully restored in the descendants of a cell that experienced a loss of silencing (Figure 2C). The slight difference in GFP expression between wild-type cells from the green sector and sir2Δ cells may have been due to the constitutive presence of Cre protein in the sir2Δ mutant, which could have potentially disrupted transcription by binding the loxP site between the promoter and the GFP gene. In addition, the GFP mRNA values in the red population likely reflected a low level of GFP expression since colonies were grown in the absence of G418 selection and therefore RFP-to-GFP switches occurred at a low rate. As secondary confirmation that losses of silencing were transient, we have never detected green sectors in colonies of a wild-type strain containing the GFP gene under control of the a2 promoter at HMLα (data not shown).

The pattern of colony fluorescence revealed the history of individual lapses in silencing, with the size of each green sector corresponding to how early the silencing loss occurred during colony growth. Colonies that were half red/half green reflected a loss of silencing in either the mother or daughter cell of the first division that gave rise to the colony (Figure 2.3A). Therefore, the frequency of half-sectored colonies equaled the rate of RFP-to-GFP switches per cell division. In the HMLα::cre strain, the frequency of half-sectored colonies was 1.6×10^-3 (Table 2.5 and Figure 2.3B). Thus, for every thousand cells, one to two cells temporarily failed to repress HMLα. Silencing of HMRα was slightly more stable than HMLα, with a lower rate of 7×10^-4 losses per division (p = 0.003; Student’s t-test) (Table 2.5 and Figure 2.3B).

2.4.2 Transient transcription of silent chromatin was restricted to low levels and was captured effectively by the Cre-based assay

In principle, the rates of RFP-to-GFP switches could be compatible with at least three possible distributions of cre expression. In one model, cre is transcribed at a low level in many cells, but the resulting level of Cre protein is sufficient to catalyze recombination of the RFP-GFP cassette in only a subset of those cells. Alternatively, cre transcription could be completely absent in most cells, but in a low fraction of cells, occur at the same level as in a sir- mutant. Finally, cre transcription may be absent in most cells, and limited even in the small fraction of cells that switch from RFP to GFP expression.

Thus far, efforts to detect transcription at HML and HMR have relied on quantitative RT-PCR and other population-based assays that report the average level of RNA for all the cells in a sample. However, advances in RNA imaging
Figure 2.3 Rates of silencing loss as measured by half-sector frequency. (A) Example of a half-sectored colony derived from a single cell containing HMLα::cre and the RFP-GFP cassette. Scale bar, 1 mm. (B) Frequency of half-sectored colonies. The half-sector frequency of a strain containing the cre gene at HMLα (JRY9628) was significantly different (p = 0.003; Student’s t-test) from the half-sector frequency of a strain containing the cre gene at HMRα (JRY9629). Data are means ± SD (n = 3).
can now measure the absolute number of transcripts in individual cells (Itzkovitz & van Oudenaarden 2011). Using a version of fluorescence **in situ** hybridization (FISH) with single-molecule sensitivity (Raj et al. 2008), we hybridized fixed cells with two sets of probes distinguished by their fluorophore label (Figure 2.4A). One probe set was specific to the cre RNA expressed from HMLα::cre. As an internal control to account for sample variation, the other probe set was specific to KAP104 RNA, which encodes a protein involved in nuclear transport. We measured cre and KAP104 transcripts by imaging and quantifying fluorescent spots, each representative of a single RNA molecule.

As a positive control, we examined cre transcription in a mutant lacking Sir4, a protein integral to heterochromatin structure. As expected, HML was transcriptionally active in the sir4Δ mutant (Figure 2.4B,C), with a mean of 12 cre transcripts per cell (±2 standard error of the mean [SEM]), and 98% (±1 SEM) of cells containing at least one RNA molecule (Table 2.6). Transcripts of the constitutively active KAP104 gene were present in 99.0% (±0.3 SEM) of cells (Table 2.6, Figure 2.4B,C). In concordance with previous studies (Zenklusen et al. 2008; Gandhi et al. 2011), KAP104 expression approximated a Poisson distribution, suggesting that most of the cell-to-cell variation in KAP104 RNA levels could be explained by stochastic, constitutive transcription. By contrast, the variance of cre RNA abundance was large in relation to the mean (Table 2.6), perhaps reflecting an alternative mode of transcription.

In a wild-type strain with HMLα::cre, cre transcripts were absent in nearly every cell, whereas KAP104 transcripts were present at a level comparable to that in sir4Δ cells (Figure 2.4B,C). The distribution of KAP104 expression in wild-type cells was slightly lower than the distribution in the sir4Δ mutant, which may be explained by our observation that sir4Δ cells were slightly larger than wild type (Figure 2.5), as noted before for sir2Δ mutants (Moretto et al. 2013). Therefore, HML was completely silent in the vast majority of cells, indicating the Cre-based assay was sensitive to rare events of transcription. Although we detected an apparent cre signal in 4.2×10⁻³ (±0.7×10⁻³ SEM) wild-type cells, we also detected an apparent cre signal at a frequency of 3×10⁻³ (±1×10⁻³ SEM) in cells that lacked the cre gene, which were imaged to control for hybridization specificity (Table 2.6, Figure 2.4B,C). The wild-type strain and the negative control strain were distinguishable, however, by the frequency of cells containing more than one spot of cre signal (Table 2.6). We observed five wild-type cells across all three replicates (13,695 wild-type cells total) that contained more than one cre spot, ranging from 2 to 4 spots per cell, whereas we never detected more than one cre spot per cell in the negative control strain (13,722 No-cre cells total). Together, the RNA FISH analysis and the Cre-based assay converged on the same striking conclusion: the low rate of RFP-to-GFP switches reflected the complete absence of HMLα::cre transcription in the vast majority of cells.

There was no evidence of any wild-type cells containing cre RNA levels typical of sir4Δ cells, suggesting that in the rare cells that lost silencing, the duration of transcription was short, presumably due to continuous nucleation of silent chromatin by Sir1 and other factors. Silencing in individual sir1 cells was
Figure 2.4 Single-molecule imaging of HMLα::cre transcription. (A) Schematic of cre and KAP104 coding sequences (black arrows), with colored lines showing sites targeted by the 20-nucleotide FISH probes (Table 2.4). To detect both RNA sequences in the same cell, the cre-specific and KAP104-specific probes were differentially labeled. (B) Single-molecule RNA FISH of cre (yellow) and KAP104 (red) transcripts shown as maximum-intensity projections of z-stacks. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Merged images were superimposed on the corresponding brightfield (BF) micrographs. Scale bar, 5 µm. (C) Distributions of cre and KAP104 RNA abundance determined by FISH. Each violin plot was normalized so that maximum bin width was the same across all samples. Data shown are from a representative replicate.
Figure 2.5 Boxplots of cell widths measured using brightfield images from the RNA FISH replicate shown in Figure 2.4C. The widths of thousands of hand-drawn cell boundaries were quantified using Matlab (MathWorks). Red lines represent the median values. The whiskers (dashed lines) extend to the values within 1.5 times the interquartile range (box). Outliers are shown as red points.
previously measured by indirect bioassays (Pillus & Rine 1989; Xu et al. 2006), and with respect to transcription, the expression levels of HML and HMR underlying the two phenotypically distinct cell types in a sir1 population have never been established. RNA FISH allowed us to evaluate sir1Δ cells at the level of transcription on a per cell basis to determine whether these two states were molecularly equivalent to the levels of HML expression seen in sir4Δ and wild-type cells. In the absence of Sir1, 46% (±7 SEM) of cells expressed HML (Table 2.6), exhibiting a range in cre RNA levels that resembled the sir4Δ mutant (Figure 2.4B,C). The remaining 54% of sir1Δ cells lacked detectable cre RNA molecules, but expressed KAP104 at normal levels (Figure 2.4B,C). Therefore, in approximately half of all cells lacking Sir1, HML was fully repressed, whereas the other half expressed HML at levels indistinguishable from those in a sir4Δ mutant.

2.4.3 Effects of Sir protein availability on the dynamics of silencing

Although Sir1 was defined by its role in the establishment of silencing, and is not required for maintenance or inheritance, the possibility that it contributes to these other processes has never been directly tested. Such a test would require a comparison of the rate of silencing loss in wild-type cells to the rate of silencing loss in the population of sir1 cells in which HML was silenced. Whereas past measurements in wild-type cells were confounded by the role of Sir1 in establishment (Xu et al. 2006), the Cre-based assay is sensitive enough to record losses of silencing before re-establishment occurs.

To determine whether Sir1 contributes to the maintenance or inheritance of silencing, we sporulated a sir1 hemizygote containing HMLα::cre and the RFP-GFP cassette and then imaged the fluorescence of colonies derived from the meiotic products. If the rate of silencing loss were unaffected by the absence of Sir1, then the sectoring patterns would be indistinguishable between colonies arising from SIR1 cells and colonies arising from sir1Δ cells in which HMLα::cre was repressed. However, green sectors were notably more abundant in the colonies of spores lacking Sir1 (Figure 2.6A). Moreover, the frequency of half-sectoring colonies in a sir1Δ haploid was 35-fold above the frequency of half-sectoring colonies in a wild-type haploid (Table 2.5 and Figure 2.6B), indicating that cells lost silencing at HML at a strikingly higher rate when Sir1 was absent. In strains containing HMRα::cre instead of HMLα::cre, sir1Δ colonies showed elevated sectoring and a higher abundance of half sectors in comparison to wild-type colonies, as well (Table 2.5 and Figure 2.6). Thus, beyond its role in establishment, Sir1 contributed to either the maintenance or inheritance of silencing at both HMLα and HMRα. Consistent with the RNA FISH measurements, which revealed a large population of sir1Δ cells that lacked detectable cre transcripts (Table 2.6 and Figure 2.4C), many of the cells within the sir1Δ colonies remained red (Figure 2.6A). Therefore, sir1Δ cells that were in the silenced state exhibited the same level of repression as wild-type cells, but transitioned to a transcriptionally active state more often than wild-type cells.
Figure 2.6 Increased silencing loss in cells lacking Sir1. (A) Fluorescence of colonies derived from SIR1 and sir1Δ cells containing the RFP-GFP cassette and either HMLα::cre or HMRα::cre. All colonies shown were grown from MATa spores of JRY9729 and JRY9730 tetrad dissections. Scale bar, 1 mm. (B) Frequencies of half-sectored colonies for haploid strains carrying the sir1Δ mutation and either HMLα::cre (JRY9739) or HMRα::cre (JRY9740). For both HMLα::cre- and HMRα::cre-containing strains, the half-sector frequency of the sir1Δ mutant was significantly higher (p = 2×10^{-4} for HMLα::cre, p = 7×10^{-7} for HMRα::cre; Student’s t-test) than the half-sector frequency of wild type (red dotted lines represent the rates shown in Figure 2.3B). Data are means ± SD (n = 3).
Sir1 facilitates the recruitment of Sir2, Sir3 and Sir4 and therefore helps maintain a local concentration of heterochromatin components at HML and HMR. To test whether the dynamics of silencing were sensitive to the availability of Sir proteins, HMLα::cre expression was monitored in diploids hemizygous for individual SIR genes. While measuring silencing loss in diploids, we noticed that a wild-type diploid with one copy of HMLα::cre and the RFP-GFP cassette showed considerably reduced colony sectoring and a 4-fold decrease (p = 1×10⁻⁵; Student’s t-test) in the frequency of half-sector colonies in comparison to an isogenic haploid (Table 2.5), indicating a ploidy effect on the rate of RFP-to-GFP switching. Compared to the wild-type diploid, colonies of the sir1, sir3 and sir4 hemizygotes showed a modest yet consistent increase in the number of green sectors (Figure 2.7A) and a higher occurrence of half sectors (Table 2.5 and Figure 2.7B). Therefore, reducing the dose of the genes encoding Sir3 or Sir4, two structural components of heterochromatin, or Sir1, a protein that recruits these components, rendered silencing at HML less stable. Altered gene dosage of SIR1 and SIR4 also affects the silencing of a sensitized HMR::ADE2 allele (Sussel et al. 1993). Hemizygosity for SIR2 had no perceivable impact on colony sectoring (Figure 2.7A).

### 2.4.4 A novel role for the sirtuin Hst3 in the stabilization of silencing at HML

Sir2 is the only member of the sirtuin family of NAD⁺-dependent deacetylases previously shown to have a role in silencing at HML or HMR. As measured by mating efficiency, silencing is indistinguishable between wild type and mutants lacking any of the four HST genes (Brachmann et al. 1995; Yang et al. 2008). However, Hst3 and Hst4 have been shown to contribute to the silencing of subtelomeric genes (Brachmann et al. 1995; Yang et al. 2008), as well as silencing of a URA3 reporter in a plasmid-borne HMR cassette (Grünweller & Ehrenhofer-Murray 2002). Since NAM inhibits all five sirtuins in yeast (Landry, Slama, et al. 2000; Smith et al. 2000; Imai et al. 2000; Landry, Sutton, et al. 2000; Tanny & Moazed 2001; Tanner et al. 2000), we tested whether other sirtuins, in addition to Sir2, were involved in the NAM-induced loss of silencing observed in Figure 2.2. As expected, colonies of cells with HMLα::cre and the RFP-GFP cassette turned completely green in the absence of Sir2, a protein essential for the nucleation and maintenance of heterochromatin (Figure 2.8A). The colonies of hst1Δ, hst2Δ and hst4Δ mutants exhibited sectoring patterns that were indistinguishable from wild-type colonies (Figure 2.8A). In contrast, the hst3Δ mutant showed a striking increase in colony sectoring and a 7-fold greater frequency of half-sector colonies compared to wild type (Table 2.5, Figure 2.8A,B). Thus, the Hst3 deacetylase contributed to the stability of silenced chromatin at HML. Whereas silencing defects have not been detected at subtelomeric genes in either the hst3Δ or hst4Δ single mutant, the hst3Δ hst4Δ double mutant shows a measurable phenotype (Brachmann et al. 1995; Yang et al. 2008). Therefore, it is likely that the deletion of HST4 would enhance the sectoring phenotype of the hst3Δ mutant.
Figure 2.7 Stability of silencing at *HML* in diploids hemizygous for individual *SIR* genes. (A) Fluorescence of colonies derived from single diploid cells containing one copy of *HMLα::cre*, one copy of the RFP-GFP cassette, and the indicated *SIR* genotypes. Scale bar, 2 mm. (B) Frequencies of half-sectored colonies for a wild-type diploid and different *sir* hemizygotes. The half-sector frequencies of the *sir1*, *sir3* and *sir4* hemizygotes were significantly higher (*p < 0.05, **p < 0.01; Student’s t-test) than the half-sector frequency of the wild-type diploid. Data are means ± SD (n = 3). The frequency of half-sectored colonies was not determined for the *sir2* hemizygote.
Hst3 regulates the deacetylation of acetylated lysine 56 on histone H3 (H3 K56-ac) (Celic et al. 2006; Maas et al. 2006; Yang et al. 2008). To determine whether Hst3 stabilized silencing through deacetylation of H3 K56-ac or through deacetylation of yet undiscovered substrates, we measured the effects of amino acid substitutions that either mimic (K56Q) or prevent (K56R) the acetylation of H3 K56. Although these histone gene mutations were previously analyzed for their effects on silencing, the outcome varies depending on the locus and the assay (Hyland et al. 2005; Xu et al. 2007; Miller et al. 2008; Yu et al. 2011). In our strains, with cre under the native a2 promoter at HML, colonies of the K56Q mutant showed a dramatic increase in sectoring, whereas the K56R mutant was similar to wild type (Figure 2.8C). Consistent with the possibility that the hst3Δ silencing defects were due to an increase in acetylated histone H3 K56, the sectoring phenotypes suggested that neutralizing the positive charge at this position on histone H3 impaired the stability of repression. By half-sector analysis, however, both the K56Q and K56R mutants showed a significant increase ($p = 3 \times 10^{-3}$ and $p = 9 \times 10^{-5}$, respectively; Student’s t-test) in the rate of silencing loss compared to wild type (Table 2.5 and Figure 2.8D). The relatively high frequency of half-sector colonies in the histone H3 K56R mutant was unexpected due to the similarity in sectoring patterns between histone H3 K56R mutant colonies and wild-type colonies (Figure 2.8C). However, the histone H3 K56R substitution has previously been shown to decrease silencing under various conditions (Xu et al. 2007; Yu et al. 2011). Differences between sectoring patterns and half-sector frequencies are considered further in the Discussion.

Cells expressing the H3 K56Q mutant produced similar patterns of colony sectoring regardless of whether Hst3 was present (Figure 2.8C). This phenotype was slightly less severe than that of hst3Δ cells expressing wild-type H3 K56 (Figure 2.8C), perhaps because glutamine was not quite as disruptive to silencing as acetylated lysine at this residue. In addition, the H3 K56R substitution in hst3Δ cells restored silencing to wild-type levels, as determined by overall colony sectoring (Figure 2.8C). Therefore, blocking the acetylation of residue 56 suppressed the hst3Δ sectoring phenotype. Collectively, the sectoring patterns suggested that Hst3 promoted the stability of silencing through the deacetylation of histone H3 K56-ac. Whereas the deletion of HST3 caused a 3-fold increase in the half-sector frequency of wild type ($p = 3 \times 10^{-4}$; Student’s t-test), it did not significantly affect the half-sector frequency of the H3 K56Q mutant ($p = 0.3$; Student’s t-test) or the surprisingly high half-sector frequency of the H3 K56R mutant ($p = 0.06$; Student’s t-test) (Table 2.5 and Figure 2.8D). Thus, the state of histone H3 K56 affected the stability of silencing at HML, and amino acid substitutions of this residue were epistatic to the hst3Δ phenotype.

Colonies derived from cells in which the only source of histone H3-H4 genes was a plasmid-borne copy of HHT2-HHF2 exhibited more sectoring than colonies of cells containing both HHT1-HHF1 and HHT2-HHF2 at their native chromosomal loci (Figure 2.9). Assuming the decrease in gene copy number led to a decrease in protein abundance, silencing was likely affected by levels of histones H3 and H4 that were either limiting or improperly balanced with the
Figure 2.8 Effects of sirtuin gene deletions and histone H3 K56R or K56Q substitutions on the stability of silencing at HML. (A) Fluorescence of colonies derived from single cells containing HMLα::cre, the RFP-GFP cassette, and the indicated deletions. Scale bar, 2 mm. (B) Frequency of half-sectored colonies for the hst3Δ mutant shown in panel A. The half-sector frequency of the hst3Δ mutant was significantly greater (p = 6×10⁻⁶; Student's t-test) than the half-sector frequency of wild type (red dotted line represents the rate shown in Figure 2.3B). Data are means ± SD (n = 3). The frequency of half-sectored colonies was not determined for the hst1Δ, hst2Δ or hst4Δ mutant. (C) Fluorescence of colonies derived from single cells containing HMLα::cre, the RFP-GFP cassette, hht1-hhf1Δ and hht2-hhf2Δ mutations, and a plasmid-borne copy of either HHT2-HHF2, hht2(K56R)-HHF2 or hht2(K56Q)-HHF2. Scale bar, 2 mm. (D) Frequency of half-sectored colonies for the genotypes shown in panel C. The half-sector frequency of the wild-type strain containing only one copy of the histone H3-H4 gene pair was significantly higher (p = 0.001; Student’s t-test) than the half-sector frequency of the strain shown in Figure 2.3B containing both copies of histone H3-H4 gene pair (red dotted line) (see Figure 2.9). Data are means ± SD (n = 3).
**Figure 2.9** Histone H3-H4 gene dosage effect on silencing at HML. GFP fluorescence of colonies derived from single cells containing HMLα::cre, the RFP-GFP cassette, and either the native histone H3-H4 gene pairs (JRY9628), or deletions of both histone H3-H4 gene pairs and a plasmid-borne copy of HHT2-HHF2 (JRY9639). Scale bar, 2 mm.
levels of histones H2A and H2B. Consistent with these possibilities, histone dosage has previously been shown to affect various functions of heterochromatin (Moore et al. 1979; Moore et al. 1983; Venditti et al. 1999).

In principle, the effect of histone H3-H4 gene dosage should sensitize silencing in all genetic backgrounds. Whereas the reduction from two copies of the histone H3-H4 gene pair to one copy did enhance the sectoring phenotype of hst3∆ colonies (compare panels A and C in Figure 2.8), it did not increase the half-sector frequency in the hst3∆ background as it did in the wild-type background (Table 2.5 and Figure 2.8B,D). Therefore, the degree to which histone H3-H4 gene dosage affected silencing, at least during the first cell division of colony growth, depended on the genetic background.

2.4.5 Live-cell imaging revealed a variety of switching patterns

Whereas colony sectors revealed the history of heterochromatin dynamics, we sought to also capture these events in real time at single-cell resolution to determine whether observable losses of silencing exhibited a fixed pattern or were more stochastic. Therefore, we monitored the fluorescence of cells containing HMLα::cre and the RFP-GFP cassette as they divided in a chamber supplied with fresh medium. Most cells remained red throughout the duration of the time course. Consistent with the sectors observed within colonies, rare RFP-to-GFP switches occurred in a subset of the lineages. The rate of switching observed with live-cell imaging was within 10% of the rate of switching determined by the half-sector assay.

The time lag between the expression of cre and the ultimate maturation of GFP limits the temporal resolution from such analyses. Nonetheless, a survey of the pattern of switching events allowed some useful inferences. For each RFP-to-GFP switch, we determined whether cells were unbudded, small-budded, or large-budded at the moment when GFP expression was initially detected. We observed a fluorescent switch in 24 different cells, 9 of which were unbudded and thus in the G1 phase during the onset of GFP expression (Figure 2.10A,D). Multiple occurrences of both patterns 1 and 2 (Figure 2.10D) indicated that silencing loss followed by recombination was not restricted to either the mother or daughter cell (given that pattern 3 was supported by only one example, we refrained from offering an interpretation of this event).

Of the remaining cells that switched, 3 cells had small buds when GFP fluorescence was first detected and 12 had large buds (Figure 2.10D). All 3 small-budded cells gave rise to mothers and daughters that both remained green (pattern 6) (Figure 2.10B,D). Since the bud emerges during early S phase, and there were no cases of only the mother or daughter cell remaining green following the onset of GFP expression in a small-budded cell (patterns 4 and 5), it was likely that recombination of the loxP sites occurred prior to DNA replication. Alternatively, recombination may have occurred shortly after DNA replication at both newly synthesized copies of the RFP-GFP cassette. In contrast, 8 of the 12 cells that expressed detectable GFP as large-budded cells gave rise to mother-
daughter pairs in which only the mother or daughter remained green (patterns 7 and 8) (Figure 2.10C,D), indicating that Cre catalyzed recombination after S phase and acted on only one of the two RFP-GFP cassettes. Switches that followed patterns 7 and 8 also demonstrated that the time between recombination, which occurred some point after DNA replication, and GFP detection, which occurred when a large bud was present, spanned only a fraction of a complete cell cycle.

For 3 of the RFP-to-GFP switches, we observed a second switch in the daughter cell that arose from the immediately preceding cell division (Figure 2.10B,D and Figure 2.11). These paired switches, which occurred either simultaneously or within 40 minutes of each other, could have reflected either two loss-of-silencing events in directly related cells, or rather one loss-of-silencing event that happened when the mother cell and its daughter still shared a cytoplasm, assuming there was perdurance of the Cre protein in both cells and a delay in recombination. These patterns are considered further in Figure 2.11 and in Section 2.5.6.
Figure 2.10 Patterns of RFP-to-GFP switches observed in real time. (A-C) Brightfield (BF) and GFP fluorescence montages (shown in 20-minute intervals, 140 minutes total) showing different switching patterns of wild-type cells containing \textit{HMLa::cre} and the RFP-GFP cassette. Labeled arrows track the mother (m) and daughter (d) cells of interest. (A) GFP expression first detected in an unbudded daughter cell. (B) GFP expression first detected in a small-budded cell. Both the mother (m) and daughter (d2) cells remained GFP-positive (pattern 6). *A second RFP-to-GFP switch occurred in the daughter (d1) of the preceding cell division (see Figure 2.11, pedigree B). (C) GFP expression first detected in a
large-budded cell. Only the mother (m) cell remained GFP-positive. (D) Distribution of different switch patterns. RFP-to-GFP switches were initially categorized by bud morphology at the time that GFP expression was first detected (depicted as yellow cells in the cartoons since RFP was still present). Events in each bud category were then classified according to whether the mother cell, daughter cell, or both underwent recombination of the RFP-GFP cassette, as determined by whether they continued to express GFP (depicted as green cells in the last phase of the pedigree cartoons). Asterisks denote the number of events within a particular category that were associated with a second switch in a directly related cell (see Figure 2.11).
Figure 2.11 Cartoons of lineages that showed two RFP-to-GFP switches in directly related cells. Switches are labeled with the numbers 1 and 2. In pedigree A, onset of GFP expression (depicted as yellow) was detected in a large-budded cell (1). At the same time, GFP also appeared in the daughter cell that arose from the previous cell division (2). Due to the assymetry of switch 1 (only the mother cell remained green, implying that recombination occurred after DNA replication), switches 1 and 2 must have arose from independent recombination events. It is unknown, however, whether the two recombination events resulted from two losses of silencing, or rather one loss of silencing that occurred when the two cells shared a cytoplasm (earliest stage shown for the pedigree). In pedigree B (montage shown in Figure 2.10), GFP fluorescence was first detected in a small-budded cell that gave rise to two cells that remained green (1). Approximately 40 minutes later, the daughter cell of the preceding cell division also began to show GFP fluorescence as a small-budded cell. In pedigree C, GFP expression was first detected in a mother-daughter pair subsequent to cytokinesis, but prior to both cells budding. Whereas the mother cell became green, however, the daughter cell developed a large bud without ever reaching a similar level of GFP expression, at which point the experiment ended. This pattern could be explained by two switching events, one early assymetric switch where only the mother inherited a recombined RFP-GFP cassette and the daughter cell inherited GFP molecules along with an intact RFP-GFP cassette, followed by a later switch that occurred in the daughter cell. Alternatively, there could have been only one early switch where the RFP-GFP cassette inherited by the daughter cell either did not undergo recombination and GFP perdurance was especially long, or the cassette did undergo recombination, but GFP expression was weak, perhaps due to an error in recombination.
<table>
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<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Half-sector frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>JRY9628</td>
<td>matΔ HMLα::cre</td>
<td>0.00158 ± 0.00007</td>
</tr>
<tr>
<td>JRY9629</td>
<td>matΔ HMRα::cre</td>
<td>0.0006 ± 0.0002</td>
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<tr>
<td>JRY9739</td>
<td>MATa HMLα::cre sir1Δ</td>
<td>0.055 ± 0.007</td>
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<td>MATa HMRα::cre sir1Δ</td>
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<tr>
<td>JRY9731</td>
<td>MATα/matΔ HMLα/HMLα::cre</td>
<td>0.00037 ± 0.00004</td>
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<tr>
<td>JRY9732</td>
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<td>0.0007 ± 0.0002</td>
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<tr>
<td>JRY9734</td>
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<tr>
<td>JRY9735</td>
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<td>JRY9639</td>
<td>MATα HMLα::cre hht1-hhf1Δ hht2-hhf2Δ [HHT2-HHF2]</td>
<td>0.0033 ± 0.0004</td>
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<tr>
<td>JRY9640</td>
<td>MATα HMLα::cre hht1-hhf1Δ hht2-hhf2Δ [hht2(K56R)-HHF2]</td>
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<tr>
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**Table 2.5** Frequency of half-sectored colonies for each strain that showed a sectoring phenotype. All values represent the mean of three independent experiments ± standard deviation. All strains contained a copy of the RFP-GFP cassette. See Table 2.1 for full genotypes.
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<th>Mean spots/cell</th>
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<td>cre</td>
<td>HMLα::cre, sir4Δ</td>
<td>12 ± 2</td>
<td>53 ± 9</td>
<td>98 ± 1</td>
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<td>-</td>
<td>0.3 ± 0.1</td>
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<td>KAP104</td>
<td>HMLα::cre, sir4Δ</td>
<td>7.3 ± 0.4</td>
<td>11 ± 1</td>
<td>99.0 ± 0.3</td>
<td>97.2 ± 0.8</td>
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<td>10.8 ± 0.9</td>
<td>99.28 ± 0.04</td>
<td>97.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>HMLα::cre, SIR+</td>
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<td>7.8 ± 0.3</td>
<td>99.1 ± 0.2</td>
<td>96.5 ± 0.8</td>
</tr>
<tr>
<td>No cre, SIR+</td>
<td>5.9 ± 0.5</td>
<td>7.1 ± 0.5</td>
<td>99.0 ± 0.7</td>
<td>96 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6 Summary of single-molecule RNA FISH statistics. All values represent the mean of three independent experiments ± standard error of the mean.
2.5 Discussion

Several lines of evidence establish the dynamic nature of the heterochromatin structure. The incorporation of newly synthesized Sir3 into silenced chromatin during G1-phase arrest suggests that at least some Sir3 binding to nucleosomes is transient (Cheng & Gartenberg 2000). Similarly, fluorescence-recovery-after-photobleaching experiments have shown that heterochromatin protein 1 (HP1), a structural component of heterochromatin in other eukaryotes, can rapidly associate and dissociate from heterochromatic loci (Cheutin et al. 2003; Festenstein et al. 2003). However, the consequences of these structural fluctuations on the underlying functions of heterochromatin remain poorly understood. Here, we showed that heterochromatin dynamics had a functional impact on the transcriptional repression imparted by Sir proteins. Moreover, we have established a route to a comprehensive understanding of all factors influencing heterochromatin dynamics.

2.5.1 Inherent dynamics of heterochromatic gene silencing

At a low rate, the heterochromatic loci HML and HMR underwent losses of silencing that had escaped detection by all previous efforts due to the combination of their rarity and transience. Independent observations indicated that silencing was promptly re-established following its loss. First, HMLα::cre was fully silenced in the descendants of cells that switched to GFP expression, showing that HMLα::cre expression was only temporary. Secondly, single-molecule RNA FISH did not detect any wild-type cells that expressed HMLα::cre to the average level of sir4Δ cells, implying that when silencing was lost in wild-type cells, it was restored before cre transcription could reach that level. These data, taken together with the inability of less sensitive assays to capture expression of HML or HMR, suggested that losses of silencing were rare and short-lived.

The detection of silencing loss in a subset of wild-type cells supported a model where Sir proteins and the transcriptional machinery constantly compete for access to the silent mating-type loci. Such a concept is consistent with the dynamic exchange of structural components in heterochromatin, the continuous recruitment of Sir proteins by the silencers (Cheng & Gartenberg 2000), and the ability of increased levels of a transcriptional activator to overcome the silenced state (Aparicio & Gottschling 1994; Ahmad & Henikoff 2001; Xu et al. 2006). This model could explain why losses of silencing were more frequent when cells contained only half the normal gene dosage of SIR1, SIR3 or SIR4. Perhaps losses of silencing at HML and HMR resulted from stochastic fluctuations in the local concentration of silencing factors. Alternatively, specific perturbations such as DNA replication may have disrupted the binding of Sir proteins to nucleosomes, thereby creating an opportunity for RNA polymerase to access heterochromatin.
2.5.2 Defining the roles of Sir1 in silencing

The contribution of Sir1 to silencing in wild-type cells was previously underappreciated. Heritability of the repressed state in a subset of sir1Δ cells showed that Sir1 was not absolutely required for the maintenance or inheritance of silenced chromatin, and suggested that Sir1 primarily functioned in establishment (Pillus & Rine 1989). However, the presumed role of Sir1 seemed paradoxical under the assumption that wild-type cells never lose silencing and thus do not undergo events requiring re-establishment. This point of confusion can now be resolved by two key findings. First, the Cre-based assay revealed that transient transcription of HML and HMR occurred at a low rate in wild-type cells. Therefore, Sir1 may serve a purpose in the recovery of silencing on the rare occasion that it is lost. Secondly, losses of silencing occurred more often in cells lacking Sir1, indicating that Sir1 contributed to either the maintenance or inheritance of silencing, or both.

Previous measurements of silencing established that two phenotypic states exist within population of sir1 cells (Pillus & Rine 1989; Xu et al. 2006), yet the levels of expression in each of the two subpopulations remained unresolved. For example, in sir1Δ cells containing a fluorescent reporter under control of the URA3 promoter at HML, the fluorescence intensity profile of each subpopulation shifted in relation to the profiles of wild-type cells and sir3Δ cells depending on whether the URA3 trans-activator Ppr1 was present (Xu et al. 2006). By directly measuring the transcription of a gene from the native α2 promoter at HML with single-cell and single-molecule resolution, we showed that the silenced subset of sir1Δ cells was fully repressed, whereas the expressed subset of sir1Δ cells transcribed cre to the level of sir4Δ cells. Together, the Cre-based assay and the RNA FISH measurements resolved two separable aspects of silencing: the level of repression, and the rate at which it is lost. Deletion of SIR1 affected the rate of silencing loss, but not the level of repression since approximately half of all sir1 cells completely lacked cre transcripts. Moreover, the persistence of RFP expression within regions of sir1Δ colonies containing either HMLa::cre or HMRa::cre and the RFP-GFP cassette underscored the remarkable ability of heterochromatin to template its own replication for multiple cell divisions.

2.5.3 Is there a role for a dynamic dimension to heterochromatic gene silencing?

In principle, the dynamic nature of silenced chromatin could result from stochastic processes intrinsic to, for example, the binding constants of Sir proteins for nucleosomes and serve no useful role. Alternatively, the stability of silencing at HML and HMR could be tuned high enough to achieve cell-type specificity, yet just low enough to allow for events such as DNA replication or mating-type interconversion. Haploid cells of a homothallic strain can switch mating types through repair of a double-stranded DNA break at MAT using HML or HMR as a template. Therefore, successful switching requires that the
invading MAT strand, along with the accessory proteins involved in recombinational repair, gain access to the heterochromatic donor sequence. We speculate that the dynamics of silenced chromatin may contribute to the efficiency of mating-type interconversion.

### 2.5.4 Role of the Hst3 sirtuin and its histone H3 target in heterochromatin stability

The stability of silencing at HML depended in part on Hst3 and the deacetylation of H3 K56-ac. H3 K56 localizes to the DNA entry/exit region of the nucleosome (Luger et al. 1997), and in the acetylated state it is thought to promote transient DNA “breathing”, during which the DNA partially unwraps from the nucleosome (Neumann et al. 2009; North et al. 2012; Simon et al. 2011). Furthermore, H3 K56-ac is important for transcriptional activation (Williams et al. 2008; Värv et al. 2010), and telomeric heterochromatin is more accessible to the bacterial dam DNA methylase in various H3 K56 mutants (Xu et al. 2007). Collectively, these observations suggest that H3 K56-ac destabilizes the nucleosome conformation and thereby renders DNA more accessible to RNA polymerase.

H3 K56 acetylation and Hst3 expression are both regulated by the cell cycle. Whereas H3 K56 acetylation peaks during S phase (Masumoto et al. 2005), Hst3 expression does not peak until G2/M phase (Spellman et al. 1998; Celic et al. 2006; Maas et al. 2006). Therefore, heterochromatin may be especially susceptible to transcription during the time after K56-acetylated histone H3 is deposited into chromatin, but before Hst3 removes the modification.

### 2.5.5 A tractable tool for measuring the dynamics of heterochromatic repression

As previous measurements of HML silencing were unable to distinguish hst3 mutants from wild type, our detection of a stability phenotype in this mutant underscored the importance of measuring fluctuations in repression for a fuller understanding of heterochromatin. The ability of this assay to capture the existence of transient events through their conversion to permanent, heritable marks can now allow a comprehensive evaluation of the contribution of any and all genes to heterochromatin dynamics. By preserving a historical record of transcription, the Cre-based assay also provides the opportunity to measure the effects of transient environmental or cellular stresses on silencing subsequent to their occurrence. Such analyses will inform our understanding of the mechanisms by which cells maintain integrity of the epigenome.

Measuring the frequency of half-sectored colonies allowed for quantification of the rate at which silencing was lost. In general, half-sector frequencies were consistent with the overall patterns of colony sectoring. However, strains bearing the histone H3 K56R substitution, for example, were indistinguishable from wild type by sectoring pattern, yet showed a significant
increase in the frequency of half-sectored colonies. While we do not fully understand why silencing in histone H3 K56R mutants appeared to be less stable at the two-cell stage of colony growth than at later stages, the complexities of colony development may offer a simple explanation. The physiology of cells during first cell division of colony growth, at which point silencing was measured by half-sector analysis, would be expected to differ from the physiology of cells during later divisions. Therefore, it is possible that the histone H3 K56R mutation rendered heterochromatin more sensitive to the physiological state of cells. Overall, both assays were informative, and sectoring patterns in particular provided the opportunity to survey heterochromatin dynamics throughout the various microenvironments and metabolic states of colonies.

A direct comparison could not be made between the rate of RFP-to-GFP switching, as determined by half-sector analysis, and the frequency of wild-type cells expressing HMLα::cre, as determined by FISH. Whereas half-sector analysis measured events throughout the duration of an entire cell cycle, the FISH method measured expression in a snapshot of time. Furthermore, the background levels of FISH signal in the negative control strain, though very low, limited quantitative interpretation of the low cre RNA levels in wild-type cells. The FISH measurements did show, however, that any detectable transcription of HMLα::cre in wild-type cells was both rare and restricted to relatively low levels. These observations implied that a small number of cre transcripts produced enough recombinase to catalyze the RFP-to-GFP switch. Further experiments would be necessary to determine the number of cre RNAs sufficient for recombination.

2.5.6 Capturing losses of silencing in real time

The live-cell imaging of pedigrees of wild-type cells endowed with the Cre-based assay reflected our first efforts to visualize in real time the silencing loss events whose history is written in the number and size of green colony sectors. In principle, RNA FISH by itself could provide better time resolution, but morphological classification of fixed spheroplasts using brightfield images was unreliable, especially with the high density of cells needed to capture rare events. Despite the time lag between loss-of-silencing events and the production of the GFP chromophore, several points were clear. First, there was no obvious mother-daughter bias as to which cell contained the more labile HML locus. Secondly, the expression of cre and the subsequent switches from red to green could occur within the same cell cycle. Overall, the variety of patterns we observed were consistent with the possibility that losses of silencing occur at multiple points during the cell cycle, but such conclusions rely on assumptions for which we have no independent verification. Knowing the extent of variation in the time lag between HMLα::cre expression and recombination would help solidify further interpretation. Intriguingly, there were three pedigrees in which a red daughter, born from a mother that switched to green, itself switched to green. Given the low probability of a switch, two switches in such closely related cells implied there was either perdurance of the Cre protein following a single loss-of-
silencing event, or some “heritable” instability shared between these two cells, as has been previously suggested in a different context (Kaufmann et al. 2007). Future experiments such as live-cell imaging of transcription should reveal the exact timing of silencing loss and address whether the history of a lineage affects stability.
Chapter 3. Riches of phenotype computationally extracted from microbial colonies

(This chapter is an adaptation of Liu T-Y*, Dodson AE*, Terhorst J, Song YS and Rine, J (2016). Proceedings of the National Academy of Sciences of the United States of America, in press. *These authors contributed equally to this work)

3.1 Abstract

The genetic, epigenetic and physiological differences among cells in clonal microbial colonies are under-explored opportunities for discovery. A recently developed genetic assay reveals that transient losses of heterochromatic repression, a heritable form of gene silencing, occur throughout the growth of Saccharomyces colonies. This assay requires analyzing two-color fluorescence patterns in yeast colonies, which is qualitatively appealing but quantitatively challenging. In this paper, we developed a suite of automated image processing, visualization, and classification algorithms (MORPHE) that facilitated the analysis of heterochromatin dynamics in the context of colonial growth and that can be broadly adapted to many colony-based assays in Saccharomyces and other microbes. Using the features that were automatically extracted from fluorescence images, our classification method distinguished loss-of-silencing patterns between mutants and wild type with unprecedented precision. Application of MORPHE revealed subtle but significant differences in the stability of heterochromatic repression between various environmental conditions, revealed that haploid cells experienced higher rates of silencing loss than diploids, and uncovered the unexpected contribution of a sirtuin to heterochromatin dynamics.

3.2 Introduction

Microbial colonies arising from single cells have been a workhorse of molecular genetics for decades, yet the genetic and physiological complexity of the population of cells within a colony is often overlooked. For most microbes, the number of cells in a colony is sufficiently large to contain, in some fraction of cells, a loss-of-function mutation in every gene in the genome, and even the majority of possible base-pair changes. The physiology of cells located in different regions of the colony can also vary widely due to limitations of oxygen and nutrient diffusion through the colony (Meunier & Choder 1999; Váchová et al. 2009). Reporter-gene fusions have revealed some of the remarkable differences between cells in the same colony (Shapiro 1984). To date, analyzing colony-wide patterns of reporter-gene expression and how they change in response to mutations has been limited to qualitative approaches. For these patterns to serve as a reliable phenotype, however, rigorous quantitation is necessary. In
In this work, colonial patterns resulting from the dynamic nature of heterochromatin formed the basis upon which to develop a quantitatively robust pattern classifier. Heterochromatin is a tightly packed state of chromatin that represses, or silences, the expression of genes within it. Furthermore, heterochromatin is an epigenetically heritable form of chromatin structure that helps maintain chromosome segregation fidelity and genome stability. Repression of gene expression in heterochromatin is an important form of gene regulation, but currently little is understood about its dynamics or stability. In Saccharomyces cerevisiae, heterochromatin plays an important role in stabilizing the highly repetitive telomeres and ribosomal DNA repeats (Gottlieb & Esposito 1989). Heterochromatin also mediates silencing of the cryptic mating-type loci (HML and HMR) so that only the mating-type allele at the MAT locus is expressed (Grunstein & Gasser 2013). HML and HMR are silenced by the Silent information regulator (Sir) proteins Sir1, Sir2, Sir3 and Sir4 (Rine & Herskowitz 1987), which are the structural components of heterochromatin at these loci.

Our recent genetic assay, based on Cre-loxP recombination, captures transient losses of gene silencing in Saccharomyces cerevisiae by converting these transient events into a permanent and heritable feature (Chapter 2). In this assay, transient expression of HML::cre catalyzes a recombination event that removes a RFP gene and substitutes a GFP gene in such a way that cells that were red are now green, as are all of their descendants. The Cre-catalyzed changes in genotype and phenotype are permanent and heritable, leading to characteristic two-color fluorescence patterns in yeast colonies. We hereinafter refer to this method as the Cre-Reported Altered States of Heterochromatin (CRASH) assay.

To date, the only method available for quantifying the dynamics of heterochromatic repression is half-sector analysis (Hieter et al. 1985), whereby the rate of RFP-to-GFP switches per cell division is determined by measuring the frequency of half-sectored colonies. However, half-sector analysis of rare events is laborious and, because it is based upon events confined to the first cell division of colony growth, potentially misses information reflected in the patterns of green spots and sectors throughout colonies. In this paper, we developed a suite of automated image processing, visualization, and classification algorithms to facilitate the analysis of heritable and clonal red-to-green transitions that occurred during the growth of a colony. This suite of programs was built on the basis of mathematical morphological operations, and we refer to it as MORphological PHenotype Extraction (MORPHE). It is freely available at https://sourceforge.net/projects/morphe.

Using MORPHE, we automatically extracted a set of useful features from the observed patterns produced by GFP-expressing cells in a colony (also referred to below as switching patterns) and performed classification on patterns of colonies from various yeast strains and from wild-type strains grown under various environmental conditions. MORPHE enabled multiple discoveries that had eluded all previous methods by applying quantitative image analysis to the classification of phenotype.
3.3 Materials and methods

**Yeast strains.** All strains used in this study were derived from W303 and were previously described (see Chapter 2). See Table 3.1 for a description of each genotype.

**Colony growth and imaging.** Strains were initially streaked onto solid medium containing G418 (Geneticin; Life Technologies) to select for cells expressing RFP, which were then grown to mid-log phase in liquid Complete Supplement Mixture (CSM)–Trp (Sunrise Science Products) under nonselective conditions (no G418). Following 10-fold serial dilutions in 1× phosphate buffered saline, cells were spread onto CSM–Trp, 1% agar plates at a density of approximately 10 cells/plate and were grown for 6 days at 30˚C.

The resulting colonies were imaged with a Zeiss Axio Zoom.V16 microscope equipped with ZEN software (Zeiss), a Zeiss AxioCam MRm camera, and a PlanApo Z 0.5× objective. For each experiment, the magnification and exposure times remained constant across all genotypes or conditions. Micrographs were assembled using Photoshop (Adobe Systems).

All experiments testing the effects of environment on the stability of silencing at *HML* were performed using JRY9628. For the comparison of different carbon sources, cells were grown on CSM–Trp, 1% agar plates containing either D-glucose (Fisher Scientific), D-galactose (Sigma-Aldrich), or D-raffinose pentahydrate (Sigma-Aldrich) at a concentration of 2% (w/v). To test the effects of other metabolites on the stability of silencing, aqueous stock solutions were first made as follows: L-ascorbic acid 2-phosphate (Sigma-Aldrich) was at 0.1 M, 30% hydrogen peroxide (BDH Chemicals) was at 0.1 M, and nickel(II) chloride hexahydrate (Sigma-Aldrich) was at 0.01 M. Each stock solution was filter-sterilized and then mixed in with freshly autoclaved CSM–Trp, 1% agar to achieve the specified concentrations.

**Algorithm.** We developed the analysis package MORPHE to extract informative features for characterizing the sectoring pattern resulting from heterochromatin dynamics in *Saccharomyces cerevisiae*. The method was divided into five sections: (i) colony segmentation; (ii) switching events detection; (iii) onset detection; (iv) onset-frequency estimation; and (v) colony classification based on these features.

i. Colony segmentation.

The first step was to segment the raw colony images. Motivated by the segmentation examples in (Gonzalez et al. 2004; Mathworks 2015), we developed a pipeline tailored to our application: a Canny edge detector (Canny 1986) was first applied to the raw image, and then the detected edges were dilated to form a closed boundary surrounding a given colony (Figure 3.1). By restricting all subsequent analyses to this enclosed region, we could remove background noise and detect the switching events specific to the colony of interest. Our method was a combination of feature detection and morphological
Figure 3.1 Segmentation of the colonies. (A) Edge detection was applied to the raw image. (B) The detected edges were dilated to form a closed boundary surrounding the colony of interest. (C) The interior pixels of the colonies were detected. (D) Identification of the colony with the largest area by counting the number of connected components found in panel C and the area of each connected component. The boundary of the colony of interest is outlined with the red curve.
filtering, both of which are widely used for other purposes (Meijering 2012; Pham et al. 2000). The underlying assumption that the petri-dish background was homogeneous compared to the object of interest (i.e., the colonies) held, and our tailored method outperformed classic methods based on normalized cuts (Shi & Malik 2000) or energy minimization (Delong et al. 2012).

ii. Switching events detection

After segmenting the colonies, we detected the bands and dots within each colony for subsequent featurization. Separating features from the colony background by intensity thresholding was precluded by the lack of a universal intensity threshold that worked for all images, since the pixel intensity distribution varied widely between images. To eliminate the need for manual tuning, we processed the data using the aforementioned edge detection and dilation approach restricted to each colony found by the segmentation procedure described earlier. Then, for each connected component detected (i.e., a region of pixels in which any two pixels are connected to each other by the edge detection and edge dilation), we applied the Moore-Neighbor tracing algorithm (Moore 1962; Gonzalez et al. 2004) modified by Jacob's stopping criteria (Gonzalez et al. 2004) to extract the boundary contour. The pixel intensities in the enclosed area of a contour were compared to those of the exterior region. If the inner pixels had intensity values larger than the exterior, we labeled the enclosed area as a feature. This step enabled us to detect the boundaries of the features of interest, overcoming the sensitivity of edge detection to the change of pixel intensities. However, the interior pixels of the features may be homogeneous and hence could be missed by edge detection. By comparing the inner and outer pixels, we successfully detected the band and dot features.

iii. Onset detection

Next, we determined the distance from the colony centroid to the vertex (i.e., the point closest to the origin in Euclidean distance) of each connected component. (In what follows, we translated each colony centroid to the origin of a Cartesian coordinate system.) This step provided a measure of the timing of the genetic switching event that gave rise to each feature.

iv. Onset-frequency estimation

Next, we estimated the frequency of feature formation (termed “onset frequency”) per unit area using the learned onset features. For a fixed radius \( r \), a naive estimate of this frequency was simply the number of observed switching events divided by the total area (excluding GFP-expressing regions) in the annulus of width \( \Delta r \) (i.e., the radii of the outer ring being \( r + \Delta r \) and the radii of the inner ring being \( r \)). However, because switching events are rare, this estimator had high variance, inspiring further processing of these raw onset-frequency estimates.

We applied kernel smoothing to recover the shape of the underlying probability density function from the discrete events observed. This method is
widely used in time-series analysis that extracts an underlying continuous function from limited discrete events, e.g., estimating the firing rate from the train of action potentials in neuroscience (Dayan & Abbott 2001). We let the sequence of onset frequencies of the \(i_{th}\) colony be \(\{r_{i,j}, q_{i,j}^f\}_{j=1}^R\), in which \(r_{i,j}\) is the distance from the origin of the Cartesian coordinate system, \(q_{i,j}^f\) is the corresponding onset frequency of switching events, and \(R\) is the radius of the colony in terms of number of pixels. Then, the estimated smoothed onset frequency function of switching events was

\[
s_i^f(r) = \frac{\sum_{j=1}^R k_\lambda(r, r_{i,j})q_{i,j}^f}{\sum_{j=1}^R k_\lambda(r, r_{i,j})}.
\]

The kernel \(k_\lambda\) has a window parameter \(\lambda\). We adopted the sliding-window averaging method as the kernel function with a window size of \(\lambda=50\) pixels for illustration.

v. Colony classification

In addition to the onset of switching events, we also introduced the area of GFP fluorescence, denoted as \(s_i^a(r)\), as an additional feature. These derived features provided an efficient way of visualizing heterochromatin dynamics and could also be used as discriminative features in classification, which could provide insights into the different switching patterns that were not obvious by visual inspection. We let the extracted features and labels be \(\{x_i, y_i\}_{i=1}^N\), where \(x_i = [s_i(r_1), s_i(r_2), ..., s_i(r_R)]\) and \(s_i(r) = [s_i^f(r), s_i^a(r)]\). That is, \(x_i \in \mathbb{R}^{1 \times 2R}\) is a vector consisting of the smoothed onset frequencies of switching events and area of GFP fluorescence evaluated at \(R\) radii; and \(y_i \in \{1, 2, ..., K\}\) represents the class label, which could be the specific yeast strain or the environmental condition. For convenience, we denoted \(X = [x_1; x_2; ...; x_N]\) and \(Y = [y_1; y_2; ...; y_N]\). We tested the performance of decision trees and ensemble learning methods, including AdaBoost and random forest.

The decision tree is a greedy algorithm that recruits splitting nodes to the tree by defining half planes \(P_1 = \{z_j \leq s\}\) and \(P_2 = \{z_j > s\}\), in which \(z_j\) is the splitting variable from one of the features, and \(s\) is the splitting point. At each candidate node, one computes an error measure such as the misclassification rate, Gini index, or the cross-entropy (Hastie et al. 2003). Then, the splitting nodes are selected and added sequentially to improve performance. Each terminal node (or a leaf) represents a class label and provides a prediction if one follows the path from the root to that leaf, and a decision is made by taking the majority vote among the leaf nodes.

AdaBoost (Freund & Schapire 1999) and random forest (Breiman 2001) are common ensemble methods, in which a committee is formed by combining the outputs of many weak learners (Hastie et al. 2003), and the committee performs a majority vote to decide the predicted class labels. All of the
classification results were obtained with the leave-one-out test, and we applied stratified sampling such that the number of samples for each class was the same in the training set. The AdaBoost or AdaBoost.M2 for multi-class was applied with 200 learning cycles; the random forest used the square root of the total number of features at random at each split, and 200 trees were generated. When the colonies had different sizes, we limited all the samples to the smallest size observed. All confusion matrices presented in this chapter were performed using random forest.
<table>
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</tr>
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</tr>
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</tr>
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**Table 3.1** Strains used in Chapter 3.
3.4 Results

In a microbial colony of cells containing a cre gene silenced by heterochromatin and a fluorescent reporter cassette, transient failures of silencing produce GFP-expressing sectors or dots (Figure 3.2A,B; Chapter 2). Multiple features of a GFP sector, such as its size and position within the colony, as well as the overall pattern of colony sectoring, inform our understanding of heterochromatin dynamics. To quantitatively analyze these features, we developed an algorithm that detects the sectoring pattern (Figure 3.2C). Large regions of GFP expression, referred to as bands, typically extended to the periphery of a colony, whereas most small regions of GFP expression, referred to as dots, did not extend to the colony edge, most likely due to neighboring RFP-positive cells randomly taking over the local population (Figure 3.2D,E). As both bands and dots arise from a loss of silencing, we combined these two features to determine the overall number of switching events from RFP to GFP expression, which was a direct measure of transient loss-of-silencing (transient failures of heterochromatin) events.

The frequency of switching events was computed in terms of the distance of bands and dots from the center of the colony (see Section 3.3 for a description of the algorithm). We first labeled the vertex of each band and dot (Figure 3.3A), as the vertex marks the location of the cell that experienced a loss of silencing at HML::cre and therefore marks the origin, or onset, of GFP expression. Next, we determined onset frequencies by confining the analysis to a specific distance from the colony center and quantifying the proportion of vertices within that ring, or annulus (Figure 3.3B). Since the switch from RFP expression to GFP expression is irreversible and thus GFP-expressing cells do not have the potential to undergo a second switch, GFP-expressing regions other than vertices were excluded from measurements of the total area. This step was repeated for a series of concentric circles with increasing radii, resulting in plots of onset frequency as a function of distance from the colony center (Figure 3.3C). A summary statistic was obtained by taking the average of the frequency function, and was denoted as the mean onset frequency.

In addition to onset frequency, we also extracted a feature defined as the area of GFP fluorescence (Figure 3.3C). This measurement corresponded to the number of pixels containing GFP signal within the annulus of interest. Together, the onset frequency and area of GFP fluorescence at each given radius provided a set of features by which to compare colonial patterns of GFP expression.

3.4.1 Feature extraction detected obvious phenotypes, in agreement with previous analyses, as well as less obvious phenotypes that escaped previous analyses

To test whether the computational method could distinguish patterns that markedly differ by visual inspection, we performed image analysis on both wild-type colonies and hst3Δ colonies. Deletion of the HST3 gene, which encodes a
Figure 3.2 Design of the CRASH assay and detection of switching events by MORPHE. (A) The CRASH assay captures transient losses of silencing at HML::cre through a permanent, red-to-green switch in fluorescence. (B) Fluorescence of a colony of haploid cells containing HML::cre and the fluorescent reporter construct. Scale bar, 2 mm. (C) Close-up of colony shown in panel B (orange box) following colony detection (left) and segmentation (right). For each contour of a connected component (i.e., the boundary of the connected component, found by edge detection and dilation), we compared the pixel intensities of the interior versus the pixel intensities on the contour. If the interior pixels had higher intensities, the area enclosed by the contour was labeled as a bright region, shown in green. Once each contour was traced, we found the connected components within the colony and computed the area of each connected component. (D) Most of the detected connected components had an area of less than 500 pixels. (E) Band and dot features, both of which originated from loss-of-silencing events, were classified by thresholding the area of each connected component.
Figure 3.3 Features extracted from the fluorescence pattern of a colony. (A) The origin of the colony is shown as a red dot. The vertex of each detected connected component is represented by a green circle. Each vertex records a point in time when a loss-of-silencing event occurred. (B) The onset frequency was defined as the number of switching events divided by the area in the white ring at each given radius. The difference between the outer radius and inner radius is denoted as $\Delta r$ (pixels). Since the switch to GFP expression is irreversible, we excluded the area of GFP-expressing regions from the calculation. (C) The smoothed onset frequencies were obtained by applying a sliding window across the onset-frequency spike trains and taking the average within the window. The window size was fixed to 50 pixels in this example. We also computed the area of GFP fluorescence in the white ring at each radius.
NAD$^+$-dependent histone deacetylase known to target histone H3 K56-ac, reduced the stability of heterochromatic repression and thus caused a dramatic increase in the frequency of sectors (Figure 3.4A), as previously described (Chapter 2). Consistent with qualitative analysis and half-sector analysis (Chapter 2), the computational analysis distinguished wild-type colonies from $hst3\Delta$ colonies with regard to all extracted features (Figure 3.4). MORPHE successfully captured the early onsets and the large regions expressing GFP in the $hst3\Delta$ colonies relative to wild type.

The colonies of cells lacking the NAD$^+$-dependent deacetylase Hst1, Hst2, or Hst4 exhibited sectoring patterns that were indistinguishable from that of wild type by visual inspection (Figure 3.4). However, feature extraction revealed that the mean onset frequency in $hst1\Delta$ colonies was slightly yet significantly lower than the mean onset frequency in wild-type colonies (Figure 3.4D and Table 3.2), suggesting that in wild-type cells, Hst1 destabilized heterochromatic repression to a limited extent. To provide an independent test of the results of the computational analysis, we turned to the traditional half-sector analysis to determine rates of switching events in microbial colonies (Hieter et al. 1985). Consistent with the feature extraction, half-sector measurements showed that deletion of $HST1$ caused a subtle reduction ($p = 0.004$; Student’s t-test) in the rate of silencing loss. Whereas wild-type cells lose silencing at a rate of $1.58 \times 10^{-3}$ ($\pm 7 \times 10^{-5}$) per cell division (Chapter 2), $hst1\Delta$ cells lost silencing at a rate of $1.2 \times 10^{-3}$ ($\pm 1 \times 10^{-4}$) per cell division. Thus, MORPHE uncovered a novel silencing phenotype in $hst1\Delta$ mutants that previously escaped detection by visual inspection and that could be confirmed, rather laboriously, by the traditional method.

### 3.4.2 Haploid cells exhibited a lower switching rate relative to diploid cells

MORPHE provided a convenient way of visualizing the switching pattern for multiple colonies and was therefore applied to micrographs of diploids lacking one copy of individual $SIR$ genes (Figure 3.5A). In concordance with measurements acquired by traditional half-sector analysis (Chapter 2), feature extraction showed that diploids containing only one copy of either the $SIR1$, $SIR3$ or $SIR4$ gene had higher onset frequencies of switching events and larger GFP-expressing regions compared to wild type (Figure 3.5 and Table 3.3).

A direct comparison of the wild-type haploid and wild-type diploid revealed that haploid colonies exhibited higher onset frequencies of GFP-expressing regions and larger areas of overall GFP expression than diploid colonies (Figure 3.6 and Table 3.4). This observation was consistent with the four-fold increase in the frequency of half-sector colonies in haploids relative to diploids (Chapter 2). The diploid strains used in this study were pseudo-haploids due to the deletion of one copy of the $MAT$ locus. Therefore, the difference between haploids and diploids in red-to-green switching frequencies could not be attributed to any changes in the expression of haploid-specific genes.
Figure 3.4 Feature extraction of haploid colonies. (A) GFP fluorescence of representative colonies for haploid strains containing individual deletions of sirtuin genes. Scale bar, 2 mm. (B) Smoothed onset frequencies of switching events for each genotype. The horizontal axis represents the distance from the origin in pixels, and each row represents a colony. The color bar indicates the natural logarithm of smoothed onset frequencies. (C) The area of GFP fluorescence. The color bar indicates the natural logarithm of the area of GFP fluorescence. (D) Boxplot of mean onset frequencies. Hereinafter, the red line represents the median, the whiskers extend to the most extreme values that lie within 1.5 times the interquartile range (box edges), and plus signs represent outliers.

<table>
<thead>
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<th>Relevant genotype</th>
<th>Mean onset frequency</th>
<th>Student’s t-test with JRY9628</th>
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</thead>
<tbody>
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</tr>
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<td>JRY9364</td>
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<td>4.33e-04</td>
<td>8.13e-06</td>
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<td>JRY9636</td>
<td>hst3Δ</td>
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<td>8.15e-10</td>
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<td>JRY9637</td>
<td>hst4Δ</td>
<td>7.31e-04</td>
<td>2.64e-01</td>
</tr>
</tbody>
</table>

Table 3.2 Average of the mean onset frequencies and significance test of haploid cells. The mean onset frequencies were averaged over colonies of the same class.
Figure 3.5 Feature extraction of diploid colonies. (A) GFP fluorescence of representative colonies for diploid strains hemizygous for individual SIR genes. Scale bar, 2 mm. (B) The smoothed onset frequencies of switching events for each genotype. The horizontal axis represents the distance from the origin in pixels, and each row represents a colony. The color bar indicates the natural logarithm of smoothed onset frequencies. (C) The area of GFP fluorescence. The color bar indicates the natural logarithm of the area of GFP fluorescence. (D) Boxplot of mean onset frequencies.

<table>
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</tr>
</thead>
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<td>1.33e-02</td>
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<td>JRY9735</td>
<td>sir4Δ/SIR4</td>
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</tbody>
</table>

Table 3.3 Average of the mean onset frequencies and significance test of diploid cells. The mean onset frequencies were averaged over colonies of the same class.
Figure 3.6 Feature extraction of colonies containing various copy numbers of \textit{HML::cre} and the RFP-GFP cassette. (A) GFP fluorescence of representative colonies for strains containing the specified number of chromosome sets (1n denotes haploidy, 2n denotes diploidy), \textit{HML::cre} alleles, and RFP-GFP cassettes. Scale bar, 2 mm. (B) The smoothed onset frequencies of switching events for each genotype. The horizontal axis represents the distance from the origin in pixels, and each row represents a colony. The color bar indicates the natural logarithm of smoothed onset frequencies. (C) The area of GFP fluorescence. The color bar indicates the natural logarithm of the area of GFP fluorescence. (D) Boxplot of mean onset frequencies.

Table 3.4 Average of the mean onset frequencies and significance test of cells with the indicated copy numbers of \textit{HML::cre} and the RFP-GFP cassette. The mean onset frequencies were averaged over colonies of the same class.
In principle, a change in the frequency of red-to-green switches could arise from either a change in the stability of silencing or a change in the efficiency of Cre-loxP recombination. Given that the diploid was hemizygous for the RFP-GFP cassette and therefore contained twice the ratio of DNA content to loxP sites in comparison to the haploid, we considered the possibility that Cre was less efficient at targeting the loxP sites in the diploid. Indeed, increasing the number of RFP-GFP cassettes in the diploid from one copy (JRY10639) to two copies (JRY10640) increased the mean onset frequency, albeit not up to the level of the haploid (Figure 3.6 and Table 3.4). Therefore, the dosage of RFP-GFP cassettes affected the efficiency of Cre-loxP recombination in the diploid and contributed in part to the difference in switching frequencies between haploids and diploids.

It is unknown whether transient losses of silencing reflect a local disturbance in heterochromatin or rather a systemic failure in the repression of all heterochromatic loci. To distinguish between these two possibilities, we used MORPHE to compare the frequency of silencing loss between a diploid that was hemizygous for HML::cre (JRY10639) and a diploid that was homozygous for HML::cre (JRY10641). If instability arose from a locus-specific event, then the two HML::cre loci in the homozygote would lose silencing independently of each other and thus double the frequency of red-to-green switches relative to the hemizygote. Alternatively, a cell-wide disruption to heterochromatin would cause a concurrent loss of silencing at both HML::cre loci and thus trigger red-to-green switches at the same rate as the hemizygote. MORPHE revealed that the mean frequency of switching events was approximately two-fold higher in the HML::cre homozygote than in the HML::cre hemizygote (Figure 3.6 and Table 3.4). This trend occurred between diploids containing one copy of the RFP-GFP cassette, as well as between diploids containing two copies of the RFP-GFP cassette (Figure 3.6). These results, which suggested that the majority of loss-of-silencing events were locus-specific, were consistent with the observation that a sir1Δ diploid containing a unique reporter gene at each HML locus shows no correlation in expression state between the two HML alleles (Xu et al. 2006). In principle, however, loci that lose silencing in concert could increase sectoring if the level of Cre protein produced during a typical loss-of-silencing event were limiting for recombination efficiency. That is, concurrent losses of silencing could produce higher levels of Cre, which could increase the probability of loxP recombination. We cannot rule out this alternative explanation, especially in light of previous studies suggesting that silencing states are a property of the cell, rather than a property of the locus (Pillus & Rine 1989; Sussel et al. 1993).

3.4.3 The classifiers distinguished genotypes that appeared similar by visual inspection

Beyond uses of the feature extraction method as a visualization tool, we applied classification methods to the extracted features, including the onset frequencies of switching events and the area of GFP fluorescence to distinguish the classes from one another. Classification, an active area of research in
machine learning, has been fruitfully applied in biomedical research (Chen et al. 2006; Segal et al. 2007; Held et al. 2010; Jones et al. 2008; Sommer et al. 2011). Briefly, a classifier can be trained on the distribution of labeled feature data to minimize the probability of classification errors. The trained classifier can then be applied to a new sample to predict its label. To prevent differences in colony size from confounding the classification, analysis was restricted to switching events located within a distance from the colony center that was equal to the radius of the smallest colony tested. Classification of haploid and diploid colonies was obtained using random forest, an ensemble statistical learning method (Figure 3.7A). Each row of the confusion matrix represents the true class, and each column represents the predicted class. Hence, the \((i,j)\) entry of the confusion matrix corresponds to the proportion of colonies of type \(i\) that got classified by our method as type \(j\). The confusion matrix showed that the similarity values of the various genotypes resolved into a two-block structure. One block corresponded to haploid colonies and the other corresponded to diploid colonies. Thus, the classifier distinguished haploids from diploids, consistent with the observation that haploid cells exhibited higher levels of sectoring than diploid cells.

We also applied binary classification to test whether each mutant type could be differentiated from wild type (Figure 3.7B). The classification performance reached more than 95% accuracy differentiating wild type from the \(hst3\Delta\) mutant, which was the most distinct mutant. The second most distinct mutant was \(hst1\Delta\), with accuracy over 86%. The \(hst2\Delta\) and \(hst4\Delta\) mutants did not show distinct patterns from wild type. Most of the diploid mutant types could also be differentiated from the wild-type diploid, with accuracy over 75%. This included the \(sir4\Delta/SIR4\) mutants, which exhibited relatively large variation in sectoring patterns (Figure 3.5). The only exception was the \(sir2\Delta/SIR2\) mutant, which was indistinguishable from wild type.

### 3.4.4 The classifiers distinguished subtle differences between colonies grown under various environmental conditions

Treatment of various eukaryotic cells with either ascorbate, more commonly known as vitamin C, or nickel causes changes in gene expression that are thought to occur through modifications to the state of chromatin, reviewed in (Sun et al. 2013; Young et al. 2015). Therefore, we tested whether varying levels of either agent influenced the stability of heterochromatic repression at \(HML\). Whereas visual inspection did not reliably detect a difference in the patterns of GFP expression between colonies grown in the presence versus absence of vitamin C (Figure 3.8A), MORPHE revealed a slight reduction in onset frequency with increasing concentrations of vitamin C (Figure 3.8 and Table 3.5). At sublethal concentrations, nickel also caused a decrease in onset frequency (Figure 3.9 and Table 3.6). The classifier distinguished each condition (0 mM, 0.05 mM, and 0.1 mM NiCl\(_2\)) from the other conditions with at least 80% accuracy. Consistent with the nickel-induced stabilization of silencing at \(HML\), NiCl\(_2\) treatment of *S. cerevisiae* cells also improves Sir-mediated silencing of a subtelomeric reporter gene (Broday et al. 1999).
Figure 3.7 Classification of genotypes based on the extracted features. (A) Confusion matrix by random forest on the multi-class classification of wild type and mutants, including both the haploid and diploid strains. Each row of the confusion matrix represents a different genotype (actual class), and the values within a row show the proportion of colonies that were predicted by the classifier to belong to the genotype specified by each column (predicted class). The color intensity, ranging from 0 to 1, corresponds to the fraction of colonies that were assigned to a particular predicted class. Successful classification results in high values along the diagonal, where each actual genotype intersects with its corresponding predicted genotype. The confusion matrix showed a block corresponding to the haploid strains and another block corresponding to the diploid strains, indicating that the classifier was able to distinguish the two different ploidies. (B) Confusion matrices by random forest on the binary classification of wild type versus each mutant. The color intensity, ranging from 0 to 1, corresponds to the fraction of colonies that were assigned to a particular predicted class.
Figure 3.8 Feature extraction and classification of colonies grown under various levels of vitamin C. (A) GFP fluorescence of representative colonies for haploid strains grown in the presence of vitamin C. Scale bar, 2 mm. (B) The smoothed onset frequencies of switching, derived by applying a sliding window average. The color bar indicates the natural logarithm of smoothed onset frequencies. (C) The area of GFP fluorescence. The color bar indicates the natural logarithm of the area of GFP fluorescence. (D) Boxplot of mean onset frequencies. (E) Confusion matrix by random forest on classification of colonies grown with different levels of vitamin C. The lower concentrations of vitamin C tested (0.1 mM and 1 mM) were grouped together with the colonies grown without vitamin C. The color intensity, ranging from 0 to 1, corresponds to the fraction of colonies that were assigned to a particular predicted class.

<table>
<thead>
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<th>[vitamin C]</th>
<th>Mean onset frequency</th>
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<tbody>
<tr>
<td>0 mM</td>
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<td>0.1 mM</td>
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<tr>
<td>10 mM</td>
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</table>

Table 3.5 Average of the mean onset frequencies and significance test of colonies grown under varying levels of vitamin C. The mean onset frequencies were averaged over colonies of the same class.
Figure 3.9 Feature extraction and classification of colonies grown under various levels of NiCl₂. (A) GFP fluorescence of representative colonies for haploid strains grown in the presence of NiCl₂. Scale bar, 2 mm. (B) The smoothed onset frequencies of switching, derived by applying a sliding window average. The color bar indicates the natural logarithm of smoothed onset frequencies. (C) The area of GFP fluorescence. The color bar indicates the natural logarithm of the area of GFP fluorescence. (D) Boxplot of mean onset frequencies. (E) Confusion matrix by random forest on classification of colonies grown with the specified doses of NiCl₂. The color intensity, ranging from 0 to 1, corresponds to the fraction of colonies that were assigned to a particular predicted class.

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<td>0.1 mM</td>
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Table 3.6 Average of the mean onset frequencies and significance test of colonies grown under varying levels of NiCl₂. The mean onset frequencies were averaged over colonies of the same class.
Physiological differentiation during colony development leads to microenvironments within the same colony that differ in metabolite levels. One such metabolite is H$_2$O$_2$ (Váchová & Palková 2005; Čáp et al. 2009), a reactive oxygen species that accumulates in cells undergoing respiration. To test whether H$_2$O$_2$-induced oxidative stress affects heterochromatin, we extracted features of GFP expression in colonies grown with increasing concentrations of H$_2$O$_2$. At the highest concentration tested, H$_2$O$_2$ caused a reduction in the onset frequencies, suggesting that H$_2$O$_2$ improved the stability of silencing (Figure 3.10 and Table 3.7).

In the laboratory, most experiments are performed with medium containing glucose as a carbon source. In nature, however, *S. cerevisiae* encounters and metabolizes a wide variety of sugars. To determine whether alternative carbon sources affect the dynamics of heterochromatin, we compared the patterns of GFP expression between colonies grown on medium containing 2% glucose and colonies grown on medium containing either 2% galactose or 2% raffinose. In comparison to glucose-grown colonies, colonies grown on the alternative carbon sources exhibited higher onset frequencies and therefore a destabilization of silencing at *HML* (Figure 3.11 and Table 3.8). In addition, silencing was slightly less stable in cells grown on raffinose than in cells grown on galactose. Collectively, these examples indicated that a variety of environmental inputs have the capacity to modify the dynamics of heterochromatin.

## 3.5 Discussion

The sensitivity of the CRASH assay has enabled the discovery of genetic and environmental factors that contribute to the stability of heterochromatic repression. However, phenotypic analysis has been limited by the lack of methods available to quantify and distinguish patterns of differential GFP expression in colonies. Here, we present a robust, automated approach to extract the features of GFP expression that inform our understanding of when and how often losses of silencing occur throughout the growth of a colony. The MORPHE software suite allowed quantitative comparisons between known genotypes or conditions, and also has the capacity to identify distinct patterns in colonies containing unknown mutations that could arise naturally or from random mutagenesis.

### 3.5.1 MORPHE revealed genetic and environmental effects on the dynamics of silencing

The classification algorithm was reliably able to categorize different patterns of fluorescence that were deemed indistinguishable by visual inspection. Notably, the classifier uncovered a novel role for the sirtuin Hst1 in antagonizing the stability of silencing. Hst1 is a paralog of the NAD$^+$-dependent histone deacetylase Sir2, an essential component of heterochromatin at *HML* and *HMR*.Interestingly, Hst1 represses the expression of genes involved in de novo
Figure 3.10 Feature extraction and classification of colonies grown under various levels of H$_2$O$_2$. (A) GFP fluorescence of representative colonies for haploid strains grown in the presence of H$_2$O$_2$. Scale bar, 2 mm. (B) The smoothed onset frequencies of switching events, derived by applying a sliding window average. The color bar indicates the natural logarithm of smoothed onset frequencies. (C) The area of GFP fluorescence. The color bar indicates the natural logarithm of the area of GFP fluorescence. (D) Boxplot of mean onset frequencies. (E) Confusion matrix by random forest on classification of colonies grown with the specified doses of H$_2$O$_2$. The lowest concentration of H$_2$O$_2$ tested (0.1 mM) was grouped together with the colonies grown without H$_2$O$_2$. The color intensity, ranging from 0 to 1, corresponds to the fraction of colonies that were assigned to a particular predicted class.

<table>
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<th>[H$_2$O$_2$]</th>
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Table 3.7 Average of the mean onset frequencies and significance test of colonies grown under varying levels of H$_2$O$_2$. The mean onset frequencies were averaged over colonies of the same class.
Figure 3.11 Feature extraction and classification of colonies grown with different sugars. (A) GFP fluorescence of representative colonies for haploid strains grown in the presence of the indicated carbon sources. Scale bar, 2 mm. (B) The smoothed onset frequencies of switching events, derived by applying a sliding window average. The color bar indicates the natural logarithm of smoothed onset frequencies. (C) The area of GFP fluorescence. The color bar indicates the natural logarithm of the area of GFP fluorescence. (D) Boxplot of mean onset frequencies. (E) Confusion matrices by random forest on classification of colonies grown with the specified sugar supply. The color intensity, ranging from 0 to 1, corresponds to the fraction of colonies that were assigned to a particular predicted class.

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Table 3.8 Average of the mean onset frequencies and significance test of colonies grown with different sugars. The mean onset frequencies were averaged over colonies of the same class.
synthesis of NAD⁺, and deletion of HST1 causes a slight increase in cellular levels of NAD⁺ (Bedalov et al. 2003). One implication of this result was that NAD⁺ levels may be limiting for Sir2 activity in wild-type cells, such that deletion of HST1 would improve the capacity of Sir2 to catalyze the deacetylation reactions necessary for silencing.

The classification algorithm also successfully differentiated cells grown under various environmental conditions by comparing the frequencies of switching events and the area of the GFP-expressing regions. Vitamin C, for example, was shown to slightly increase the stability of heterochromatic repression at HML. Interestingly, vitamin C can stimulate in vitro activity of human and murine demethylases that target histones (Tsukada et al. 2006; Wang et al. 2011), possibly through controlling the oxidation state of Fe located in the active site. Given that S. cerevisiae contains homologous histone demethylases (Tsukada et al. 2006; Tu et al. 2007) and that the methylation status of histones affects silencing at HML, vitamin C may stabilize silencing through the enhancement of histone demethylation. Less-direct mechanisms are also possible, however, as vitamin C has the potential to serve as a cofactor for other related enzymes such as Jlp1 and Tpa1 of yeast (Hogan et al. 1999; Kim et al. 2009) and also functions as a reducing agent.

3.5.2 A new tool for the quantification of colony sectoring

Given that certain environmental factors influence silencing and that colonies are environmentally complex, the stability of silencing could, in principle, fluctuate throughout different stages of colony growth or even vary between different microenvironments within the same colony. In certain genetic backgrounds, the rate of silencing loss during the initial stage of colony growth does not correspond to the overall sectoring phenotype of mature colonies (Chapter 2), suggesting that processes inherent to later stages of colonial growth can modify the stability of silencing. Whereas half-sector analysis is confined to measuring silencing loss during the first cell division of colony growth, the image analysis suite presented here has the potential to quantify heterochromatin dynamics as a function of colony development.

GFP-expressing regions within colonies vary in size and shape due to multiple factors. For example, losses of silencing that occur during the early stages of colony growth have the potential to produce large sectors, whereas losses of silencing that occur during the late stages of colony growth do not. In addition, genetic drift could randomly stunt the growth of a GFP-expressing subclone. In this study, we used MORPHE to quantify the onset frequencies of all GFP-expressing regions, regardless of size; however, the MORPHE software also contains the option to analyze smaller regions (dots) separately from larger regions (bands, or sectors). This option could be useful for the analysis of mutants or conditions that skew the ratio of bands to dots, either through changes in the general growth pattern of colonies, or through effects on the stability of silencing that act during a specific window of colony development.
Just as MORPHE complements the limitations of half-sector analysis, half-sector analysis also complements the limitations of MORPHE. In principle, high levels of sectoring would impair the ability of MORPHE to distinguish individual GFP-expressing regions. Given that MORPHE successfully analyzed the variety of sectoring patterns presented here, we anticipate that the software will perform well on colonies of cells that lose silencing at a rate ranging anywhere from that of wild-type diploids ($3.7 \times 10^{-4}$ per cell division) to that of $hst3\Delta$ mutants ($1.1 \times 10^{-2}$ per cell division) (Chapter 2). For rates of silencing loss that occur above this range, however, half-sector analysis may be a more suitable method of quantification. In addition, half-sector analysis provides a measurement of the absolute number of red-to-green switches per cell division, whereas the measurements made by MORPHE are relative.

Notably, the application of MORPHE extends beyond the measurement of heterochromatin dynamics. Analyses of several other phenomena leading to differential gene expression in colonies, such as telomeric position effect (Gottschling et al. 1990), will benefit from the quantitative method described here. Moreover, this approach may be applicable to the study of any generator of diversity within microbial colonies, from phase variation (Henderson et al. 1999) and antigen switching (Vink et al. 2012) to genome rearrangements and mutagenesis.
4.1 Abstract

In budding yeast, a small, intergenic region known as the recombination enhancer regulates donor selection during mating-type switching and also shapes the conformation of chromosome III. I found that this extraordinary locus also acted at a distance to modify the levels of gene expression. In a mating-type specific manner, the recombination enhancer destabilized the heterochromatic repression of a gene located approximately 17 kb away. This effect depended on a subregion of the recombination enhancer that is largely sufficient to determine donor preference. Therefore, this subregion affects both recombination and transcription from a distance. These observations identified a rare example of long-range transcriptional regulation in yeast and raise the question of whether other cis elements also mediate dual effects.

4.2 Introduction

Cis-acting elements regulate several processes in the nucleus, including gene expression, recombination, and DNA replication. Some elements act locally, whereas others act in some eukaryotes at distances ranging from several kb to remarkable lengths of 1 or 2 Mb (Pfeifer et al. 1999; Lettice et al. 2003; Smemo et al. 2014) or even an entire chromosome (Clemson et al. 1996; Lee & Jaenisch 1997). In budding yeast, a 2-kb region referred to as the recombination enhancer (RE) acts in cis to promote the use of sites located throughout the left arm of chromosome III as donor templates during homologous recombination (Wu & Haber 1996). Through a separate, unknown mechanism, RE also regulates the spatial conformation of chromosome III (Belton et al. 2015). Hence, this extraordinary locus is enriched for features that mediate long-range effects.

RE functions with other loci on chromosome III to facilitate mating-type switching (Haber 2012). The genotype of the mating-type (MAT) locus, located on the right arm of chromosome III, specifies mating type; MATa haploids mate as a, whereas MATa haploids mate as α. In addition, silent copies of the mating-type alleles reside on opposite ends of chromosome III—on the left end, the HML locus typically contains a silent copy of the α information, and on the right end, the HMR locus typically contains a silent copy of the a information. To switch mating types, haploids induce expression of the HO site-specific endonuclease, which creates a double-strand break at the MAT locus. Then, the homologous recombination machinery orchestrates a gene conversion event that replaces the original MAT sequence with a copy of the sequence stored at either HML or HMR. HMR serves as the default donor. In a cells, however, RE somehow
promotes the use of HML as the donor template. Therefore, \( a \) cells prefer to use HML\( a \) as a donor and \( \alpha \) cells prefer to use HMR\( a \) as a donor.

Similar to transcriptional enhancers in metazoans, \( RE \) interacts with transcription factors in a cell-type-specific manner. The left portion of \( RE \) contains several binding sites recognized by the forkhead transcription factor Fkh1 (Sun et al. 2002). Fkh1 binds \( RE \) in \( a \) cells, but not \( \alpha \) cells, and is important for \( RE \) activity (Sun et al. 2002; Ercan et al. 2005; Coïc, Sun, et al. 2006; Li et al. 2012). \( RE \) also contains an SCB (Swi4/Swi6 cell cycle box) sequence that binds the SBF (SCB binding factor) transcription factor (Coïc, Sun, et al. 2006). The SCB contributes to \( RE \) activity independently of Fkh1 (Coïc, Sun, et al. 2006). In \( \alpha \) cells, the \( \alpha 2 \) protein encoded by \( MAT\alpha \) functions with Mcm1 to bind to and inactivate \( RE \) and also repress the transcription of \( a \)-specific genes (Johnson 1995; Szeto & Broach 1997; Szeto et al. 1997). The \( \alpha 2 \)-Mcm1 repressor complex binds two sites in \( RE \) and positions highly ordered nucleosomes over the locus that presumably inhibit the binding of transcription factors (Weiss & Simpson 1997). Hence, the effect of \( RE \) on recombination (and therefore donor preference) is specific to \( a \) cells.

\( RE \) promotes recombination at sites located throughout the entire left arm of chromosome III, which is approximately 110 kb, and the efficacy of \( RE \) is roughly proportional to the proximity of the target (Wu & Haber 1995; Wu & Haber 1996; Coïc, Richard, et al. 2006). Given that \( a \) cells and \( \alpha \) cells show similar patterns of DNase-I sensitivity throughout this chromosome arm, \( RE \) does not seem to alter the local chromatin structure (Ercan & Simpson 2004). \( RE \) does effect a change in the higher-order folding of chromosome III; however, this function is genetically separable from the recombination function (Lassadi et al. 2015; Belton et al. 2015). Therefore, the mechanism by which \( RE \) acts at a distance remains unresolved.

The \( RE \) locus is atypical in that its effects extend across a large region. Other cis elements in budding yeast tend to act locally. In contrast to metazoan enhancers, which often activate promoters at a distance of several kb (Smith & Shilatifard 2014), upstream activation sequences in yeast typically reside within the promoter region (Hahn & Young 2011) and are limited in their ability to activate transcription when moved further away from the TATA box (Dobi & Winston 2007; Reavey et al. 2015). It is possible to overcome distance limitations in yeast, however, by strategically placing the upstream activation sequence at a site predicted to interact with the promoter through looping (de Bruin et al. 2001). Silencer elements in yeast also have the potential to act distally (up to approximately 12 kb) (Reauld et al. 1993), although boundary elements typically prevent them from doing so in their native context (Pryde & Louis 1999; Fourel et al. 1999; Donze & Kamakaka 2001; Meneghini et al. 2003). In other words, long-range effects on gene expression are possible in yeast, but are rarely observed.
4.3 Materials and methods

**Yeast strains.** The strains used in this chapter (Table 4.1) were derived from the W303 background. The plasmids and oligonucleotides used in this chapter are listed in Table 4.2 and Table 4.3, respectively. Deletions were made using one-step integration of gene-disruption cassettes (Goldstein & McCusker 1999; Gueldener et al. 2002) and were confirmed by PCR of the 5' and 3' junctions. For the deletion of SAS2, the natMX cassette was amplified from pAG25 (Goldstein & McCusker 1999) using the sas2∆::natMX fwd/rev primers. For the deletion of MAT, the K.I.URA3 sequence was amplified from pUG72 (Gueldener et al. 2002) using the mat∆::K.I.URA3 fwd/rev primers. For the deletion of the entire RE locus (chr III: 28,961-31,213), the hphMX cassette was amplified from pAG32 (Goldstein & McCusker 1999) using the re∆::hphMX fwd/rev primers. For the deletion of the left portion of RE (chr III: 28,961-29,852), the hphMX cassette was amplified using the re∆::hphMX fwd primer and re-left∆::hphMX rev primer, and for the deletion of the right portion of RE (chr III: 29,853-31,213), the hphMX cassette was amplified using the re-right∆::hphMX fwd primer and re∆::hphMX rev primer. The coordinates defining the entire RE locus were based on a recombination study (Houston et al. 2004), and the coordinate splitting RE into the left and right regions was based on a chromosome conformation study (Belton et al. 2015). The minimal RE shown in Figure 4.3 corresponds to the 700-bp region defined in the original identification of RE (Wu & Haber 1996). For the construction of pJR3389, the pJR3389 fwd/rev primers were first used to amplify RE from genomic DNA (W303). The RE amplicon was then inserted into pJR734 following digestion of both the insert and backbone with EagI and XhoI.

**Colony growth and imaging.** For tetrad analysis, JRY10656 was streaked out onto YPD containing both G418 (Geneticin; Life technologies) and Hygromycin B (Sigma-Aldrich) to select for RFP-expressing cells, which were then sporulated for 4-5 days on 1% potassium acetate. Tetrads were dissected on Complete Supplement Mixture (CSM) –Trp (Sunrise Science Products) plates and imaged after 3 days of growth at 30°C. For all other image analyses, strains were first streaked out onto YPD +G418 (or CSM –His +G418 for strains containing HIS3-marked plasmids) to select for RFP-expressing cells. Cells were then grown to log phase in CSM –Trp and plated onto CSM –Trp, 1% agar at a density of approximately 10 cells/plate. Cells containing HIS3-marked plasmids (JRY10668-JRY10673) were grown to log phase in CSM –His –Trp and plated onto CSM –His –Trp, 1% agar at a density of approximately 10 cells/plate. Colonies were imaged after 6 days of growth at 30°C. Images were acquired with a Zeiss Axio Zoom.V16 microscope equipped with ZEN software (Zeiss), a Zeiss AxioCam MRm camera and a PlanApo Z 0.5× objective. Images were assembled in Photoshop (Adobe Systems).

**RNA preparation for quantitative RT-PCR.** Cells were grown to log phase in YPD and then harvested for RNA isolation. Total RNA was isolated using hot acidic phenol and chloroform (Collart & Oliviero 2001). Following treatment with
DNase I (Roche Diagnostics), RNA was purified by phenol-chloroform extraction. Complementary DNA (cDNA) was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo(dT) primers. Quantitative PCR of cDNA was performed using the primers listed in Table 4.3, the Thermo Scientific DyNAmo HS SYBR Green qPCR Kit (Fisher Scientific), and an Mx3000P machine (Stratagene). Standard curves were generated from the cDNA of JRY10663. Three independent RNA preparations were performed for each sample. Two-tailed Student’s t-test was used to determine p values.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<td>JRY10656</td>
<td>MATα/MATα lys2/lys2 his3-11,15/his3-11,15 leu2-3,112 can1-100/can1-100 sas2Δ::natMX/SAS2 HMLα-a2Δ::cre/HMLα-a2Δ::cre ura3Δ::P_{GPD-}loxP-yEmRFP-TCYC1-kanMX-loxP-yEGFP-T_{ADH1}/ura3Δ::P_{GPD-}loxP-yEmRFP-TCYC1-hphMX-loxP-yEGFP-T_{ADH1}</td>
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<td>JRY10657</td>
<td>MATα lys2 his3-11,15 leu2-3,112 can1-100 sas2Δ::natMX HMLα-a2Δ::cre ura3Δ::P_{GPD-}loxP-yEmRFP-TCYC1-kanMX-loxP-yEGFP-T_{ADH1}</td>
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Table 4.1 Strains used in Chapter 4.
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<th>Plasmid</th>
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<th>Description</th>
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<tr>
<td>pJR734</td>
<td>pRS313</td>
<td>HIS3 ampR CEN/ARS</td>
</tr>
<tr>
<td>pJR3389</td>
<td>pRS313</td>
<td>RE (chr III: 28,961-31,213) His3 ampR CEN/ARS</td>
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Table 4.2 Plasmids used in Chapter 4.
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<tr>
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</tr>
<tr>
<td>sas2Δ::natMX rev</td>
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<tr>
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</tr>
<tr>
<td>matΔ::K.l.URA3 rev</td>
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</tr>
<tr>
<td>reΔ::hphMX fwd</td>
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</tr>
<tr>
<td>reΔ::hphMX rev</td>
<td>AAAATTAGCAAAAAAGATAAATAAAGTGAAATGAAAATG</td>
</tr>
<tr>
<td>re-leftΔ::hphMX rev</td>
<td>TATGTCACCTGGGACAAATAAATTTGGAAAAACGTCTCTA</td>
</tr>
<tr>
<td>re-rightΔ::hphMX fwd</td>
<td>GTTTATATCTAAGTTAAAAATTATAATCCAATTTTAAATTC</td>
</tr>
<tr>
<td>pJR3389 fwd</td>
<td>CTAGACGGCTAGCTCAATATATATATAC</td>
</tr>
<tr>
<td>pJR3389 rev</td>
<td>GTCAGCTCTCGATTAATACATAGTGTGAGG</td>
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<tr>
<td><strong>Oligonucleotides used for quantitative RT-PCR</strong></td>
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</tr>
<tr>
<td>ACT1 fwd</td>
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<tr>
<td>ACT1 rev</td>
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<tr>
<td>cre fwd</td>
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<td>cre rev</td>
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<td>GCTAGTTCTGCGTATATGC</td>
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<tr>
<td>PRD1 fwd</td>
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</tr>
<tr>
<td>PRD1 rev</td>
<td>GTTTCCCTCGTCAAGTCTCA</td>
</tr>
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</table>

*K.l., Kluyveromyces lactis*

**Table 4.3** Oligonucleotides used in Chapter 4.
4.4 Results

An assay designed to capture short-lived events of transcription recently revealed a dynamic dimension to heterochromatin at the silent mating-type loci, HML and HMR (Chapter 2). On rare occasion, a cre reporter gene under control of the native α2 promoter at either HMLα or HMRα escapes heterochromatin-mediated gene silencing and triggers a recombination-based switch from RFP expression to GFP expression. By converting transient transcription into permanent changes in genotype and phenotype, the Cre-Reported Altered States of Heterochromatin (CRASH) assay preserves an historical record of silencing loss that manifests as GFP-expressing sectors within a colony.

4.4.1 Sas2 stabilized the silencing of HML

With the ability to now monitor the dynamics of silencing, I set out to identify factors that modify these dynamics. Sas2 was a particularly interesting candidate, as it both promotes and antagonizes the silenced state. Sas2 catalyzes the addition of an acetyl group on lysine 16 of histone H4 (H4 K16), which Sir2 must remove to establish and maintain silencing (Tanny et al. 1999; Imai et al. 2000; Shia et al. 2005). Consistent with the anti-silencing role of H4 K16 acetylation, deletion of SAS2 improves silencing at HMR and the telomeres (Ehrenhofer-Murray et al. 1997; Kimura et al. 2002; Suka et al. 2002). At HML, however, sas2 mutations delay the de novo establishment of silencing and enhance the silencing defect of sir1 mutants (Reifsnyder et al. 1996; Xu et al. 2006; Osborne et al. 2009).

To determine the effects of Sas2 on the stability of silencing, I deleted one copy of SAS2 in a diploid homozygous for HML::cre and the RFP-GFP cassette, induced sporulation, dissected tetrads and then imaged the fluorescence of the resulting colonies. Within each tetrad, two of the four colonies showed sectoring patterns indicative of frequent silencing loss (Figure 4.1). Genotyping of each tetrad revealed that the high-sectoring phenotype co-segregated consistently with the sas2∆ mutation. Therefore, silencing of HML was less stable in colonies derived from the sas2∆ spores than in colonies derived from the SAS2 spores.

4.4.2 Silencing of HML was less stable in a cells than in α cells

Curiously, sas2∆ colonies seemed to fall into one of two phenotypic classes. Approximately half of the sas2∆ colonies exhibited high rates of silencing loss compared to wild type, whereas the other half exhibited even higher rates of silencing loss (Figure 4.1). The 1:1 ratio of the two sas2∆ phenotypes suggested that a second modifier of silencing (the first being SAS2) was segregating in the cross. Surprisingly, sas2∆ colonies with the highest levels of sectoring contained the MATa mating-type allele, whereas sas2∆ colonies with relatively lower sectoring levels contained the MATα mating-type allele (Figure 4.1). This observation strongly suggested that the MAT genotype affected the stability of silencing at HML.
Figure 4.1 Tetrad analysis of a sas2 hemizygote containing \textit{HML::cre} and the RFP-GFP cassette. Images show GFP expression within colonies derived from the four spores (a-d) of individual tetrads (1-5). Boxes indicate spores that inherited the \textit{sas2}\textDelta\textit{MATa} mutation. Scale bar, 1 mm.
To test whether the apparent MAT effect was due to the MAT genotype or rather to a de novo mutation that was closely linked to one of the MAT copies, I measured silencing in sas2Δ MATa cells and sas2Δ MATα cells in a different strain background. Consistent with the tetrad analysis, MATa colonies showed higher levels of sectoring than MATα colonies (Figure 4.2A). The MAT effect on silencing showed incomplete penetrance, as a small fraction of MATa colonies exhibited sectoring levels comparable to the majority of MATα colonies and vice versa (data not shown).

### 4.4.3 Mating type affected silencing through the RE locus

Only a few features distinguish a cells from α cells. MATα encodes α1, an activator of α-specific gene expression, as well as α2, a repressor of both a-specific gene expression and RE activity. By contrast, MATa encodes α1, which functions with α2 in diploids to repress haploid-specific gene expression, as well as a2, a protein of unknown function. In principle, any of the MAT products or any of the loci regulated by a MAT product could be responsible for the effect on silencing.

Given that most mating-type specific genes encode components of the pheromone signaling pathway and therefore seemed unlikely to affect heterochromatin, I instead turned my attention to the RE locus. I deleted the full RE region (chr III: 28,961-31,213) in both sas2Δ MATα and sas2Δ MATα strains and analyzed the stability of silencing. Deletion of RE had no detectable effect on silencing in MATα cells (Figure 4.2A). In MATa cells, however, deletion of RE reduced the frequency of silencing loss to a level indistinguishable from the levels observed in MATα RE and MATα reΔ cells. Thus, in the absence of RE, the stability of silencing was similar between a and α cells.

Although the strains used in this study do not contain a functional HO gene and therefore do not induce mating-type switching, the cre gene at HML could, in principle, be copied into the transcriptionally active MAT locus at a very low rate. If RE were to promote the occurrence of this event as it normally does during mating-type switching, then RE could potentially increase the frequency of RFP-to-GFP switches in a cells without affecting the stability of silencing. To test this possibility, I removed the sequences shared between HML and MAT by deleting the entire MAT locus. Since a-specific genes are constitutively expressed in the absence of α2, matΔ cells mate as a. Sectoring levels were similar between MATα and matΔ colonies, suggesting that the majority of RFP-to-GFP switches observed in MATα colonies did not arise from a transposition of cre into the MAT locus (Figure 4.2A). Furthermore, the reΔ mutation reduced sectoring levels in matΔ colonies as it did in MATα colonies and therefore acted independent of the MAT locus (Figure 4.2A).

RE could conceivably increase the frequency of RFP-to-GFP switches by enhancing recombination between the two loxP sites in the RFP-GFP cassette rather than by modifying the expression of HML::cre. However, RE promotes recombination in cis, and the RFP-GFP cassette was located on a different
Figure 4.2 Effects of mating type and RE on HML::cre expression. (A) GFP images of colonies. All strains contained the sas2Δ mutation. Scale bar, 2 mm. (B) Quantitative RT-PCR of cre mRNA levels normalized to ACT1 mRNA levels. Horizontal lines represent the means (n = 3). All strains contained the sas2Δ mutation.
chromosome (chromosome V). To test whether RE directly affected silencing, I quantified the levels of cre mRNA in a sas2Δ background using quantitative RT-PCR. Consistent with the sectoring phenotypes in the CRASH assay, cre mRNA levels were significantly higher in MATa RE cells than in MATα RE cells (p = 2×10^{-4}) (Figure 4.2B). In the absence of RE, cre mRNA levels were not significantly different between MATa cells and MATα cells (p = 0.1) (Figure 4.2B). Therefore, mating type directly affected HML::cre expression, and this effect depended on RE.

4.4.4 The left region of RE mediated the effect on silencing

The RE locus contains several features of interest. A 700-bp sequence in the left portion of RE, often referred to as the minimal RE, is necessary and largely sufficient for the enhancement of recombination (Wu & Haber 1996). The minimal RE contains an SCB site, multiple arrays of Fkh1 binding sites, and one of the two α2-Mcm1 operators located within RE (Figure 4.3A). The right portion of RE contains the second α2-Mcm1 operator, an origin of replication, and a putative open reading frame (Figure 4.3A). In addition, α cells transcribe multiple RNA species from the central and right portions of RE (Szeto et al. 1997; Ercan et al. 2005). A recent study showed that the conformation of chromosome III differs between α and α cells, and that this difference depends on the right portion of RE, but not on the left portion containing the minimal RE (Belton et al. 2015). Given that the right portion makes only a small contribution to the effect on recombination, RE is roughly divisible into two subregions with separate functions.

To determine which features were required for the mating-type effect on silencing, I made partial deletions that removed either the left (chr III: 28,961-29,852) or right (chr III: 29,853-31,213) portion of RE (Figure 4.3). Deletion of the left portion, which contained the minimal RE, eliminated the difference in sectoring between MATa and MATα colonies (Figure 4.3B). By contrast, MATα colonies lacking the right RE portion still exhibited high levels of sectoring relative to their MATa counterparts (Figure 4.3B). Therefore, the mating-type effect on silencing depended only on the left region of the RE locus. Interestingly, deletion of the right side seemed to stabilize silencing in α cells, suggesting that RE affected silencing in more than one way (Figure 4.3B).

4.4.5 RE destabilized the silencing of HML in cis

Since RE activates recombination in cis and resides approximately 17 kb away from HML (Wu & Haber 1996), I tested whether RE affected silencing in cis by introducing a plasmid-borne copy of RE into reΔ mutants. If RE acted in cis, then the plasmid-borne RE should not be sufficient to restore the difference in silencing between a and α cells. As expected, MATa reΔ cells containing an empty plasmid showed fewer losses of silencing than MATα RE cells (Figure 4.4). MATα reΔ cells containing RE on a plasmid phenocopied the empty plasmid control and therefore also showed fewer losses of silencing than MATα.
Figure 4.3 RE features and their effects on HML::cre expression. (A) Map of the RE locus. The left region contains the minimal RE and several protein binding sites, whereas the right region contains an origin of replication (ARS304) and a putative open reading frame (RDT1). (B) GFP images of colonies. All strains contained the sas2Δ mutation. Scale bar, 2 mm.
Figure 4.4 Comparison between native $RE$ and plasmid-borne $RE$. Images show GFP expression within colonies. Brackets indicate plasmid contents. All strains contained $HML::cre$ and the $sas2\Delta$ mutation. Scale bar, 2 mm.
cells containing $RE$ at its native locus (Figure 4.4). Assuming that $RE$ is sufficient for the mating-type effect on silencing, these observations suggested that $RE$ must be on the same molecule of DNA as $HML$ to affect $HML$ expression.

4.4.6 The effect of $RE$ on gene expression was not widespread

Approximately 10 genes lie between $HML$ and $RE$ (Figure 4.5). If $RE$ were to activate a large domain for transcription as it does for recombination, then $RE$ should increase the expression levels of these genes, as well. Since quantitative RT-PCR was sensitive enough to detect a difference in $HML::cre$ expression between $MATa RE$ cells and $MATa re\Delta$ cells ($p = 4 \times 10^{-4}$) (Figure 4.2B), I used this approach to sample a subset of the other nearby genes. $CHA1$ mRNA levels were slightly higher in $RE$ cells than in $re\Delta$ cells, whereas $KRR1$ and $PRD1$ mRNA levels were not significantly different between $RE$ and $re\Delta$ cells ($p_{CHA1} = 0.03, p_{KRR1} = 0.9, p_{PRD1} = 0.7$) (Figure 4.5). Collectively, these measurements suggested that the effect of $RE$ on gene expression was locus-specific rather than widespread.
Figure 4.5 RNA measurements corresponding to genes that lie between HML and RE. CHA1, KRR1, and PRD1 mRNA levels were normalized to ACT1 mRNA levels. Horizontal lines represent the means (n = 3). All strains contained the sas2Δ mutation.
4.5 Discussion

An evaluation of Sas2 and its contributions to heterochromatin dynamics led to the unexpected identification of a long-range effect on gene expression. I found that the recombination enhancer, a 2-kb intergenic region located on chromosome III, acted in cis at a distance of approximately 17 kb to antagonize the silencing of \textit{HML}. Partial deletions of the \textit{RE} locus revealed that this effect depended on an 891-bp subregion that contains an unusually high density of transcription factor binding sites. This subregion is also critical for the role of \textit{RE} in donor preference (Wu & Haber 1996). Similar to the effect of \textit{RE} on recombination, the effect of \textit{RE} on gene expression was specific to \textit{a} cells. Thus, the \textit{RE} locus, originally defined for its role in enhancing recombination, moonlighted as a cell-type-specific modifier of heterochromatin dynamics.

4.5.1 \textit{RE}: transcriptional enhancer or silencing suppressor?

Whereas \textit{RE} promotes recombination throughout the entire left arm of chromosome III, the effects of \textit{RE} on gene expression were locus-specific. That is, \textit{RE} increased the expression of \textit{HML}, but did not generally affect other genes in the vicinity. At least two explanations could account for this specificity. In the first scenario, \textit{RE} would somehow interfere with the ability of Sir proteins to silence transcription. \textit{KRR1} and \textit{PRD1} are euchromatic loci and would therefore be immune to any effect mediated by \textit{RE}. In support of this model, \textit{RE} showed a very modest effect on the expression of \textit{CHA1}, which sits adjacent to \textit{HML} and is partially repressed by the Sir proteins (Moreira & Holmberg 1998; Ellahi et al. 2015). In the second scenario, there would be something specific about the \textit{a2} promoter at \textit{HML} that renders it sensitive to \textit{RE} activity. For example, \textit{RE} could physically interact with the \textit{a2} promoter and thereby activate transcription. Of these two models, I favor the one in which \textit{RE} antagonizes the silencing machinery, particularly because I was unable to detect a reproducible effect of \textit{RE} on \textit{HML} expression in the absence of silencing (data not shown).

4.5.2 Action at a distance

One noteworthy difference between \textit{a} cells and \textit{a} cells is the spatial conformation of chromosome III (Lassadi et al. 2015; Belton et al. 2015). In comparison to \textit{a} cells, \textit{a} cells show more frequent interactions between the left arm of chromosome III, which contains \textit{HML} and \textit{RE}, and a region on the right arm that extends from the centromere to the \textit{MAT} locus. Upon deletion of the right portion of the \textit{RE} locus, chromosome III adopts a configuration that is indistinguishable between the two mating types (Belton et al. 2015). This configuration is distinct from that of both \textit{MATa RE} cells and \textit{MATa RE} cells, suggesting that \textit{RE} regulates chromosome folding in both mating types.

Since \textit{RE} controls the three-dimensional organization of chromosome III, it is tempting to speculate that \textit{RE} antagonized silencing by either facilitating or disrupting certain long-range contacts involving \textit{HML}. However, the role of \textit{RE} in
chromosome folding depends largely on the right portion of the RE locus (Belton et al. 2015), whereas the difference in silencing between $a$ and $\alpha$ cells depended only on the left portion of RE. Deletion of the right portion slightly stabilized silencing in $\alpha$ cells, but this effect only heightened the overall difference between the two mating types. Therefore, the major effect of RE on silencing could not be attributed to its role in chromosome folding.

Alternatively, RE could relay a message to HML by establishing a particular chromatin state that then spreads in cis across the left arm of chromosome III. At face value, this model seems to conflict with the observation that RE did not detectably affect the expression of more proximal loci such as KRR1 and PRD1. However, the nature of the chromatin state could be such that it only affects loci subject to silencing. This model predicts that a large, continuous domain on the left arm of chromosome III contains a unique chromatin signature. Although no such domain is evident to date, a comprehensive comparison of chromatin modifications between $a$ and $\alpha$ cells remains to be tested.

4.5.3 At the intersection of heterochromatin and recombination

Given that the left portion of RE is largely responsible for the effect on recombination (Wu & Haber 1996), it is possible that RE antagonized silencing through the same mechanism by which it enhances recombination. The factors known to facilitate donor preference in $a$ cells include Mcm1, Fkh1, SBF (Swi4/Swi6), the DNA helicase-like protein Chl1, and the DNA repair proteins yKu70 and yKu80 (Wu et al. 1998; Sun et al. 2002; Coïc, Sun, et al. 2006; Weiler et al. 1995; Ruan et al. 2005; Bystricky et al. 2009). Interestingly, most of these factors have roles in silencing, as well (Hollenhorst et al. 2000; Sussel et al. 1995; Laman et al. 1995; Das & Sinha 2005; Vandre et al. 2008; Patterson & Fox 2008). It is unclear, however, how these roles could explain the effects described in this study. For example, Fkh1 and the Ku proteins help strengthen silencing (Hollenhorst et al. 2000; Vandre et al. 2008; Patterson & Fox 2008), whereas RE acted to destabilize silencing. Assuming the effect of RE on gene expression is somehow tied to the effect of RE on recombination, then future insight into the mechanism of donor preference should provide additional clues. Determining which factors affect RE activity upstream of a double-strand break should be particularly informative given that RE destabilized silencing in the absence of a programmed double-strand break.

4.5.4 A newfound appreciation of mating type

Sir proteins preserve the mating-type identity of cells by silencing the transcription of HML and HMR. I found that mating type, in turn, affected the ability of Sir proteins to silence transcription. Specifically, cells that mated as $a$ lost silencing at HML more often than cells that mated as $\alpha$. This distinction had gone unnoticed until now, presumably because the effects of RE are rare and transient. Deletion of SAS2 sensitized silencing to a level where the difference
between a and α cells became apparent. The mating-type effect was not specific to the sas2Δ background, as I observed a similar phenotype in sir1Δ mutants (data not shown). Overall, these observations highlight both the power of segregation analysis and the importance of maintaining consistent MAT genotypes when comparing the stability of silencing between strains.
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Appendix A. Stability of promoter-specific gene repression in *Saccharomyces cerevisiae*

A.1 Introduction

Multiple mechanisms of transcriptional repression exist in *Saccharomyces cerevisiae*. As described in Chapter 1, silencer elements recruit the Sir proteins to repress transcription in a gene-nonspecific manner. In addition, several genes are subject to regulation by sequences located in the promoter region. Some promoters contain Upstream Repression Sequences (URSs), which recruit proteins that inhibit transcription by modifying chromatin or by interfering directly with the transcription machinery. Promoters also contain Upstream Activation Sequences (UASs) that bind transcriptional activators. Gene expression driven by a particular activator can be repressed through the inhibition of that activator.

Glucose-mediated repression of the *GAL1* promoter is a classic example of promoter-specific repression and is subject to several layers of regulation (Figure A.1). In cells grown with galactose, the transcriptional activator Gal4 drives the expression of *GAL1* and other *GAL* genes (Bram & Kornberg 1985; Keegan et al. 1986). In non-inducing conditions when galactose is absent, Gal80 binds to and inhibits the activity of Gal4 (Johnston et al. 1987; Ma & Ptashne 1987). Glucose adds another layer of repression by importing Mig1 into the nucleus (De Vit et al. 1997). Mig1 binds a URS in the *GAL1* promoter and recruits the Ssn6-Tup1 repressor complex to repress transcription (Nehlin et al. 1991; Treitel & Carlson 1995). In addition, Mig1 reduces the expression of *GAL4* (Griggs & Johnston 1991; Nehlin et al. 1991). Therefore, several mechanisms contribute to the 1,000-fold repression of *GAL1* expression in glucose conditions (Lamphier & Ptashne 1992; Johnston et al. 1994).

A.2 Materials and methods

**Yeast strains.** The strains and plasmids used in this appendix are listed in Table A.1 and Table A.2, respectively. All strains were derived from the W303 background. Plasmid pJR2537 is also known as pSH47 (Güldener et al. 1996).

**Colony growth and imaging.** To grow colonies for imaging, JRY9628 was grown to log phase in Complete Supplement Mixture (CSM) –Trp (Sunrise Science Products) and plated onto CSM –Trp plates containing 1% agar and 2% (w/v) D-glucose (Fisher Scientific) at a density of approximately 10 cells/plate. JRY10674 and JRY10675 were grown to log phase in CSM –Trp –Ura and plated onto CSM –Trp –Ura plates containing 1% agar and either 2% (w/v) D-glucose or 2% (w/v) D-galactose (Sigma-Aldrich) at a density of approximately 10 cells/plate. Colonies were imaged after 6 days of growth at 30°C using a Zeiss Axio Zoom.V16 microscope equipped with ZEN software (Zeiss), a Zeiss.
Figure A.1 Regulation of the GAL1 promoter in various conditions. In galactose conditions, Gal4 binds the UAS and activates transcription. When cells are grown with other carbon sources, such as raffinose, Gal80 binds to and inhibits Gal4. In glucose conditions, Mig1 further represses GAL1 expression by binding to URSs in the promoters of both GAL1 and GAL4 and recruiting repressors.
AxioCam MRm camera and a PlanApo Z 0.5× objective. Images were assembled in Photoshop (Adobe Systems).

**Flow cytometry.** Colonies of JRY9628 grown as described above were scraped off the agar surface and resuspended in synthetic complete (SC) medium (Sunrise Science Products) containing 2% (w/v) D-glucose. To grow colonies of JRY9628 in the presence of nicotinamide, 200 µl of 0.5M nicotinamide was spread onto CSM –Trp, 1% agar plates prior to plating the cells. Colonies of JRY10674 and JRY10675 grown as described above were scraped off the agar surface and resuspended in SC –Ura medium containing 2% (w/v) D-glucose. The resuspensions were grown overnight, diluted back to 0.01 OD<sub>600</sub> the next morning and then harvested by centrifugation after reaching a density of approximately 0.2 OD<sub>600</sub>. Cells were washed and resuspended in PBS, pH 7.4 on ice. The intensity of GFP fluorescence was measured for 10<sup>5</sup> cells per sample using a FC-500 flow cytometer (Beckman-Coulter). Cells were gated based on forward scatter and side scatter using FlowJo software (Tree Star) and the remaining measurements were exported as FCS files and plotted using Matlab (MathWorks).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>JRY9628</td>
<td>matΔ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HMLα-α2Δ::cre</td>
</tr>
<tr>
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<td>ura3Δ::P_{GPD}::loxP-yEmRFP-T_{CYC1}::kanMX-loxP-yEGFP-T_{ADH1}</td>
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</tr>
<tr>
<td></td>
<td>pJR1112 [URA3]</td>
</tr>
<tr>
<td>JRY10675</td>
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</tr>
<tr>
<td></td>
<td>pJR2537 [P_{GAL1}::cre, URA3]</td>
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**Table A.1** Strains used in Appendix A.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Backbone</th>
<th>Description</th>
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</thead>
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<tr>
<td>pJR1112</td>
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<td>URA3 ampR CEN/ARS</td>
</tr>
<tr>
<td>pJR2537</td>
<td>pRS416</td>
<td>P_{GAL1}::cre URA3 ampR CEN/ARS</td>
</tr>
</tbody>
</table>

**Table A.2** Plasmids used in Appendix A.
A.3 Results

At a low rate, genes at the heterochromatic loci HML and HMR temporarily escape repression mediated by the Sir proteins (Chapter 2). To compare these dynamics to another form of repression, I fused the cre gene to the GAL1 promoter and tested whether pGAL1::cre ever escapes glucose-mediated repression. If the cre reporter is expressed even for a short time, the resulting Cre protein will recombine the loxP sites in the RFP-GFP cassette (Chapter 2) and trigger a permanent switch from RFP expression to GFP expression.

A.3.1 Glucose-mediated repression of the GAL1 promoter was highly stable in comparison to Sir-mediated silencing

To determine whether the expression of pGAL1::cre is sufficient for loxP recombination, cells containing pGAL1::cre and the RFP-GFP cassette were grown on galactose medium and the resulting colonies were analyzed by fluorescence imaging. As expected, galactose-mediated induction of the GAL1 promoter resulted in uniformly green colonies (Figure A.2A). Cells containing pGAL1::cre and the RFP-GFP cassette were then grown on glucose to determine the stability of glucose-mediated repression. Colonies containing the pGAL1::cre construct were largely devoid of GFP-expressing sectors, whereas colonies containing the HML::cre construct described in Chapter 2 showed multiple GFP-expressing sectors indicative of a loss of cre repression (Figure A.2A). Therefore, glucose-mediated repression of the GAL1 promoter was more stringent than Sir-mediated repression of HML.

A.3.2 Glucose-mediated repression was sensitive to specific microenvironments within a colony

Curiously, the centers of pGAL1::cre colonies grown in glucose appeared to contain a mixture of RFP-expressing and GFP-expressing cells (Figure A.2A). GFP expression was restricted to cells located in the top layer of the colony, as cells located near the agar surface did not express GFP except when rare sectors formed (data not shown). To quantify GFP expression, colonies were resuspended in liquid and analyzed by flow cytometry. These measurements revealed that, although pGAL1::cre colonies rarely formed GFP-expressing sectors, they contained a substantial fraction of GFP-positive cells in comparison to HML::cre colonies (Figure A.2B). Presumably, most of the GFP-positive cells in the pGAL1::cre sample were derived from the top of the colony, where GFP expression was noticeable by imaging (Figure A.2A). Switches to GFP expression depended on the cre gene, as GFP expression was never detected in cells lacking a source of Cre (Figure A.2). Therefore, cells located in the topmost region of colonies escaped glucose-mediated repression at a low rate.
Figure A.2 Transient expression of a cre reporter gene repressed through different mechanisms. (A) Fluorescence images of representative colonies. Scale bar, 2 mm. (B) GFP fluorescence intensity, as measured by flow cytometry. Each population was derived from a single, 6-day-old colony. For each condition, two additional colonies were analyzed and showed similar results. HML::cre colonies were grown either in the presence or absence of 5 mM nicotinamide (NAM), which inhibits Sir2. pGAL1::cre colonies were grown on either 2% glucose (Glu) to repress cre expression or on 2% galactose (Gal) to induce cre expression. Percentages indicate the fraction of cells with intensity values that fell within the range indicated by the dashed line.
A.4 Discussion

Measurements of transient expression of a cre gene subjected to different forms of repression revealed that the stability of repression varied according to the mechanism. Glucose-mediated repression of the GAL1 promoter was much tighter than Sir-mediated repression of HML. The marked stability of pGAL1::cre repression was due in part to the repressive action of Mig1, as pGAL1::cre colonies grown in raffinose, a non-inducing condition where Mig1 localizes to the cytoplasm, showed sectoring patterns comparable to the patterns of HML::cre colonies (data not shown). Identifying factors that contribute to the stability of promoter-specific repression should inform our understanding of the different ways in which yeast fine-tune the repression of gene expression.

Glucose-mediated repression of pGAL1::cre was very stable in all cells except those located near the apex of the colony. Given that the apex contained a mixture of RFP-expressing cells and GFP-expressing cells rather than a uniformly green patch, the green cells located in this region presumably reflected multiple RFP-to-GFP switches that occurred during the late stages of colony growth. In principle, this unique fluorescence pattern could result from the poor diffusion of glucose to the top layer of mature colonies. Cells located in the top layer are separated from the agar medium by millions of cells in the bottom layer and may therefore be limited for glucose. These observations highlight the complexity of colony physiology and reveal the potential impact of colony physiology on the regulation of gene expression.