Mating-Type Genes and *MAT* Switching in *Saccharomyces cerevisiae*

James E. Haber
Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254-9110

ABSTRACT Mating type in *Saccharomyces cerevisiae* is determined by two nonhomologous alleles, *MAT***a** and *MAT*α. These sequences encode regulators of the two different haploid mating types and of the diploids formed by their conjugation. Analysis of the *MAT***a**1, *MAT*α1, and *MAT*α2 alleles provided one of the earliest models of cell-type specification by transcriptional activators and repressors. Remarkably, homothallic yeast cells can switch their mating type as often as every generation by a highly choreographed, site-specific homologous recombination event that replaces one *MAT* allele with different DNA sequences encoding the opposite *MAT* allele. This replacement process involves the participation of two intact but unexpressed copies of mating-type information at the heterochromatic loci, *HML*α and *HMR***a**, which are located at opposite ends of the same chromosome-encoding *MAT*. The study of *MAT* switching has yielded important insights into the control of cell lineage, the silencing of gene expression, the formation of heterochromatin, and the regulation of accessibility of the donor sequences. Real-time analysis of *MAT* switching has provided the most detailed description of the molecular events that occur during the homologous recombinational repair of a programmed double-strand chromosome break.

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doi: 10.1534/genetics.111.134577
Manuscript received September 10, 2011; accepted for publication November 4, 2011
Address for correspondence: Rosenstiel Center, Brandeis University, 415 South St., Mailstop 029, Waltham, MA 02254-9110. E-mail: haber@brandeis.edu

SACCHAROMYCES cerevisiae is a budding yeast that propagates vegetatively either as *MAT***a** or *MATα* haploids or as *MAT***a**/*MATα* diploids created by conjugation of opposite haploid types (Figure 1). Mating type is determined by two different alleles of the mating-type (*MAT*) locus. Like many other fungi, budding yeast has acquired the capacity to convert some cells in a colony from one haploid mating type to the other (Figure 1). This process is called homothallism. The subsequent mating of cells to the opposite mating type enables these homothallic organisms to self-diploidize. The diploid state provides yeast with a number of evolutionarily advantageous strategies unavailable to haploids, most notably the ability to undergo meiosis and spore formation under nutritionally limiting conditions. Mating-type gene switching in *S. cerevisiae* is a highly choreographed process that has taught us much about many aspects of gene regulation, chromosome structure, and homologous recombination.

The *MAT* locus lies in the middle of the right arm of chromosome III, ~100 kb from both the centromere and the telomere. The two mating-type alleles, *MATα* and *MAT***a**, differ by ~700 bp of sequences designated Yα and Y**a**, respectively (Figure 2). Yα and Y**a** contain the promoters and most of the open reading frames for proteins that regulate many aspects of the cell's sexual activity (for reviews, see Klar 1987; Herskowitz 1988; Strathern 1988; Haber 1992, 1998, 2006, 2007). The *MAT* locus is divided into five regions (W, X, Y, Z1, and Z2) on the basis of sequences that are shared between *MAT* and the two cryptic copies of mating-type sequences located at *HMLα* and *HMR***a** (Figure 1). *HMLα* and *HMR***a** serve as donors during the recombinational process that allows a *MAT***a** cell to switch to *MATα* or vice versa.

Functions of the *MAT* Proteins

MATα encodes two proteins, *MATα1* and *MATα2*. *MATα1*, in conjunction with a constitutively expressed protein, Mcm1, activates a set of α-specific genes (Klar 1987; Hagen *et al.* 1993; Bruhn and Sprague 1994), including those encoding the mating pheromone, α-factor, and Ste2, a *trans*-membrane receptor of the opposite mating pheromone, **a**-factor (Figure 3). As noted above, these mating pheromones trigger G1 arrest of the budding yeast cell cycle and facilitate conjugation, ensuring that the zygote will contain two unreplicated nuclei. *MATα2* encodes a homeodomain helix-turn-helix protein that acts with Mcm1 to form a repressor that binds to a 31-bp symmetric site with Mcm1 in the center and Matα2 at the ends (Smith and Johnson 1992). Matα2-Mcm1 represses **a**-specific genes including those that produce **a**-factor (*MFa1*and *MFa2*) and the Ste3 transmembrane receptor protein that detects the presence of α-factor in the medium. Repression also requires the action of Tup1 and Ssn6 proteins (Keleher *et al.* 1989; Herschbach *et al.* 1994; Patterton and Simpson 1994; Smith and Johnson 2000).

When the bidirectional promoter controlling *MATα1* and *MATα2* is deleted (or if *MAT* is entirely deleted), haploid cells

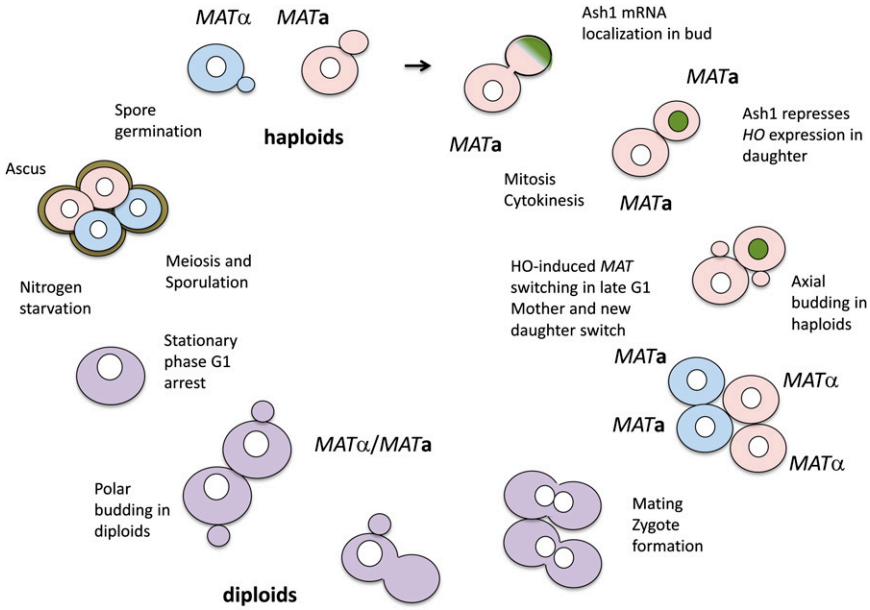

Figure 1 Homothallic life cycle of *Saccharomyce cerevisiae*. A homothallic (HO) *MAT***a** (light red) mother cell and its new daughter can switch to *MAT*α (light blue). This lineage is established by the asymmetric partitioning of the mRNA encoding the Ash1 repressor of HO gene expression in daughter cells (light green). These cells can conjugate to form a zygote that gives rise to *MAT***a**/*MAT*α diploids (purple), in which *HO* gene expression is repressed. Under nitrogen starvation, diploids undergo meiosis and sporulation to produce four haploid spores (two *MAT***a** and two *MAT*α) in an ascus. The spores germinate and grow vegetatively and can repeat the homothallic cycle. Heterothallic (*ho*) cells have stable mating types and grow vegetatively until they exhaust their nutrients and enter stationary phase.

have an **a**-like mating behavior (*e.g.*, they mate identically to *MAT***a** cells), because **a**-specific genes are constitutively expressed in the absence of Matα2 and α-specific genes are not transcribed in the absence of Matα1 (Strathern *et al.* 1981). But although *MAT***a** is not required for **a**-mating, the *MAT***a**1 gene is required, along with *MAT*α2, to extinguish haploid-specific gene expression and to manifest diploid-specific attributes. A *MAT***a**/*MAT*α cell is nonmating, whereas the diploid resulting from mating *mat*Δ and *MAT*α is α-mating. The nonmating phenotype of *MAT***a**/*MAT*α diploids results from the action of a very stable corepressor of Mat**a**1 and Matα2 proteins (Jensen *et al.* 1983; Goutte and Johnson 1988; Strathern 1988; Li *et al.* 1995; Johnson *et al.* 1998; Tan and Richmond 1998). This repressor turns off a set of haploid-specific genes and allows expression of diploid-specific genes.[1] The **a**1-α2 repressor turns off transcription of *MAT*α1, the activator of α-specific genes, but allows expression of *MAT*α2, the repressor of **a**-specific genes; hence the diploid is nonmating.

The *MAT***a** locus actually has two divergently transcribed open reading frames, *MAT***a**1 and *MAT***a**2; but although *MAT***a**2 is remarkably well conserved evolutionarily, it has yet to be assigned a biological function (Tatchell *et al.* 1981). *MAT***a**2 and *MAT*α2 share part of the same protein sequence as the transcript extends into the *MAT-X* region and the evolutionary preservation of *MAT*α2 may account for some of the conservation of *MAT***a**2, despite its lack of apparent function.

Haploid-Specific and Diploid-Specific Genes Under *MAT* Control

There are a number of important mating-type–dependent differences. These distinctions are not simply a question of haploidy *vs.* diploidy: *MAT***a**/*MAT*α diploids are notably different from diploids homozygous for either *MAT***a** or *MAT*α. First of all, they are nonmating. Second, *MAT***a**/*MAT*α cells can initiate meiosis and spore formation, whereas **a**-mating or α-mating diploids cannot. The ability to enter the meiosis and sporulation program is controlled by the repression of *RME1* (repressor of meiosis 1) by the **a**1-α2 repressor. If *RME1* is deleted, then a *MAT***a**/*MAT***a** or a *mat*Δ/*MAT*α diploid can undergo meiosis and produce viable spores.

Another key gene under mating-type control is *NEJ1*, which encodes a necessary component of nonhomologous end joining (NHEJ) (Frank-Vaillant and Marcand 2001; Kegel *et al.* 2001; Ooi and Boeke 2001; Valencia *et al.* 2001). Double-strand breaks (DSBs) in chromosomes can be repaired either by homologous recombination (HR) or by nonhomologous end-joining (NHEJ) (reviewed by Pâques and Haber 1999; Haber 2006). In haploids, both processes are efficient; for example a DSB at *MAT* created by the HO endonuclease is repaired ~90% of the time by HR, using *HML* or *HMR* as the donor, but ~10% of cells use NHEJ to religate the DSB ends, recreating the cleavage site.[2] But if cells are arrested in the G1 phase of the cell cycle, by treating *MAT***a** cells with α-factor, NHEJ is the predominant pathway. These arrested cells fail to activate the critical early

[1]Although haploid-specific genes are turned off in *MAT***a**/*MAT*α diploids, they can also be turned off in haploids if both *MAT***a** and *MAT*α are coexpressed. This can occur in a strain disomic for chromosome III, which carries the *MAT* locus, or if a plasmid expressing the opposite *MAT* locus is transformed into a haploid strain. The repression of haploid-specific genes can also occur in a haploid if the two silent mating-type donor sequences *HML*α and *HMR***a** are expressed, for example in *sir2*Δ mutants that fail to maintain their silencing. In contrast, diploid-specific genes are not turned off simply because the cell has two copies of every chromosome. A *MAT***a**/*MAT***a** or a *mat*Δ/*MAT*α diploid behaves like an **a**- or α-mating haploid.

[2]If HO is expressed only for a short time and the HO protein is then rapidly degraded, then nearly all of the religation events anneal the 4-bp overhanging 3′ ends and regenerate the HO cutting site. If HO is expressed continuously, then perfect religation is futile and most cells die. About 1/500 cells survives by imperfect end joining, destroying the cleavage site. The great majority of these imperfect NHEJ events result from misalignments of the 5′ AACA 3′ and 3′ TGTT 5′ complementary ends either to delete 3 bp or to insert CA or ACA (Kramer *et al.* 1994; Moore and Haber 1996).

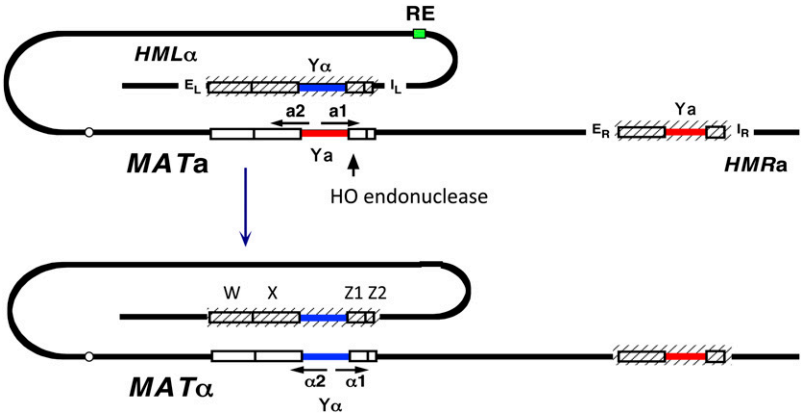

Figure 2 Arrangement of *MAT*, *HML*, and *HMR* on chromosome III. The gene conversion from *MAT***a** to *MAT*α is illustrated. Transcription of **a-** and α-regulatory genes at *MAT* are transcribed from a bidirectional promoter. Both *HML* and *HMR* could be transcribed but are silenced by the creation of short regions of heterochromatin (hatched lines) by the interaction of silencing proteins with flanking *cis*-acting silencer E and I sequences. The recombination enhancer (RE) located 17 kb centromere proximal to *HML* acts to promote the usage of *HML* as the donor in *MAT***a** cells.

steps of HR because the Cdk1 protein kinase is inactive (Ira *et al.* 2004; Aylon and Kupiec 2005). Nevertheless, in *MAT***a**/*MAT*α diploids, NHEJ is turned off by **a**1-α2 repression of the *NEJ1* gene and by the partial repression of another NHEJ component, *LIF1*. The idea that NHEJ is repressed because diploids have two copies of each chromosome and therefore can always repair from a homolog is hard to accept if indeed HR is impaired in G1 cells.[3] Perhaps NHEJ would be a dangerous pathway in meiosis in *MAT***a**/*MAT*α cells, where there are ~100 DSBs to generate meiotic crossovers.

Some other aspects of DNA repair are under mating-type control (Friis and Roman 1968; Heude and Fabre 1993; Nickoloff and Haber 2000; Valencia-Burton *et al.* 2006; Fung *et al.* 2009). *MAT***a**/*MAT*α diploids are substantially more resistant to ionizing radiation than are *MAT***a**/*MAT***a** or *MAT*α/*MAT*α cells. In addition, spontaneous rate of recombination, for example between alleles of various biosynthetic genes, is higher in *MAT***a**/*MAT*α cells. Both *rad55*Δ and *rad57*Δ mutants are partly defective in recombinational repair of DNA damage, being X-ray sensitive at lower temperatures, but this defect is suppressed in haploids in which both *MAT***a** and *MAT*α are expressed (termed mating-type heterozygosity). *rad55*Δ is also sensitive to the topoisomerase inhibitor, camptothecin; this sensitivity is partially suppressed by mating-type heterozygosity and can be mimicked by knocking out NHEJ (Valencia-Burton *et al.* 2006). In a similar fashion, defective alleles of recombination proteins Rad52 (*rad52-327*) and Rad51 (*rad51-K191R*) can be suppressed by mating-type heterozygosity, apparently through repression of other genes including Pst2 or Rfs1, respectively. Why it would be advantageous for diploid cells to overcome some of the defects caused by the Rad mutants is not evident to this reviewer.

[3]Cells arrested at the "start" point of the cell cycle, *e.g.*, by α-factor–mediated arrest, are unable to initiate HR; however, this is not the case for cells that have progressed past start but are still in G1, that is, prior to the initiation of S phase. Repair of a DSB by gene conversion does not depend on Cdc7-Dbf4, which are required to initiate DNA replication (Ira *et al.* 2004). Hence cells arrested by inactivating Cdc7 are competent for HR as well as NHEJ.

Another haploid/diploid distinction is found in the pattern of bud formation (Figure 1): haploids or diploids expressing only one *MAT* allele exhibit an axial pattern of budding that appears to be designed to facilitate efficient mating in homothallic cells (see below), while nonmating *MAT***a**/*MAT*α diploids (or haploids expressing both mating-type genes) have a bipolar budding pattern (Chant 1996). Axial budding depends on the Axl1 protein that is expressed in haploids but not diploids. Axl1 localizes to the constriction at the mother–bud neck and, in conjunction with a number of other gene products (Bud3, Bud4, Bud5, and Bud10), establishes the emergence of a bud in this position (Chant and Pringle 1991; Lord *et al.* 2002).

Finally, *MAT* heterozygosity plays a key role in the switching of mating-type genes. Homothallic strains expressing the HO endonuclease gene, *HO*, are able to switch from *MAT***a** to *MAT*α and vice versa, but once cells of opposite mating type conjugate to form a diploid, *HO* expression is repressed, again by the **a**1-α2 repressor.

The phenotypic switch from *MAT*α to *MAT***a** is quite rapid—within a single cell division. For example if one places *MAT*α cells in the presence of the mating pheromone, α-factor, they will grow because they are insensitive to the pheromone, but if one plates *HO MAT*α spores, then by the time the cell has divided (and switched to *MAT***a**) the mother and new daughter cells are unable to progress beyond the G1 phase of the cell cycle, because they are now arrested by α-factor. Thus the cell must have the capacity to shift rapidly from expressing α-specific genes to expressing the **a**-specific program. Consequently the newly switched *MAT***a** cell should turn off expression of the Ste3 pheromone receptor for **a**-factor and should also stop producing α-factor; at the same time it must quickly be able to respond to α-factor by inserting Ste2 into the cell membrane, while at the same time start pumping out **a**-factor. Hence it is not surprising that Mat**a**1, Matα1, and Matα2 transcription regulators are quite rapidly turned over, degraded by ubiquitin-mediated proteolysis by the proteasome (Laney and Hochstrasser 2003; Laney *et al.* 2006). In contrast, the a1-α2 corepressor is much more stable (Johnson *et al.* 1998).

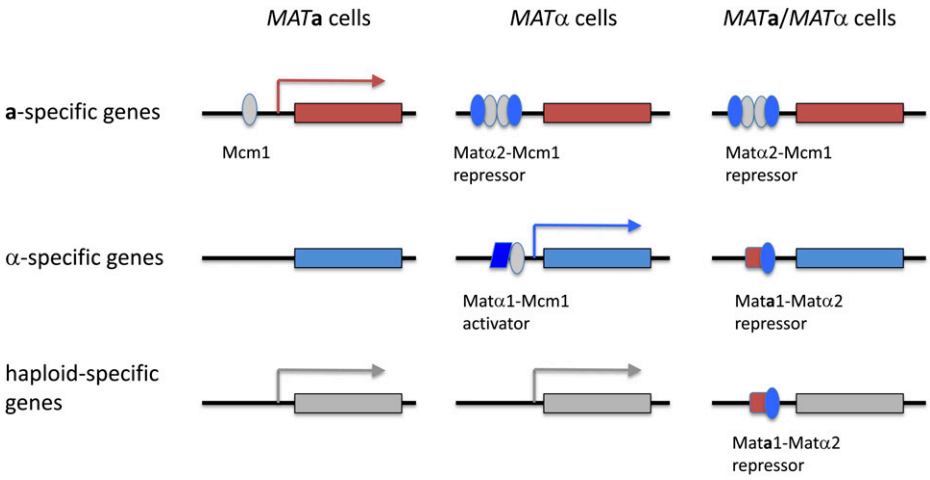

Figure 3 Control of mating-type–specific genes. The Mcm1 protein, in combination with Matα1 and Matα2, activates the transcription of α-specific genes or represses **a**-specific genes, respectively, while a Mat**a**1-Matα2 repressor turns off haploid-specific genes.

Mating-Type Switching: a Model of Cell Lineage, Gene Silencing, and Programmed Genomic Rearrangement

S. cerevisiae has evolved an elaborate set of mechanisms to enable cells to switch their mating types. Learning how these processes work has provided some of the most fascinating observations in eukaryotic cell biology. *MAT* switching depends on four phenomena: (1) the presence of two unexpressed (silenced) copies of mating-type sequences that act as donors during *MAT* switching; (2) the programmed creation of a site-specific double-strand break at *MAT* that results in the replacement of Y**a** or Yα sequences; (3) a cell lineage pattern that ensures that only half of the cells in a population switch at any one time, to ensure that there will be cells of both mating types in close proximity; and (4) a remarkable mechanism that regulates the selective use of the two donors (donor preference). Each of these important mechanisms is reviewed below.

Silencing of *HML* and *HMR*

The presence of intact, but unexpressed copies of mating-type genes at *HML* and *HMR* implied that these two loci had to be maintained in an unusual, silent configuration. The study of the mechanism of silencing of these donors has occupied the attention of many labs and has provided some important insights into the way in which chromatin structure influences gene expression and recombination (see reviews by Laurenson and Rine 1992; Loo and Rine 1994; Sherman and Pillus 1997; Aström and Rine 1998; Rusche *et al.* 2003; Hickman *et al.* 2011). Our current understanding can be summarized as shown in Figure 4A. Both *HML* and *HMR* are surrounded by a pair of related but distinct silencer sequences, designated *HML*-E, *HML*-I, *HMR*-E, and *HMR*-I. These *cis*-acting elements interact, directly or indirectly, with several *trans*-acting factors to repress the transcription of these genes (Figure 4A). Among the *trans*-acting proteins that play critical roles in this process are four Silent Information Regulator (Sir) proteins, a set of silencer binding proteins, histone proteins, the multipurpose Rap1 protein, as well as several chromatin modifiers. Together, these gene products and *cis*-acting sequences create short regions (~3 kb) of heterochromatin, in which the DNA sequences of *HML* and *HMR* are found as a highly ordered nucleosome structure (Nasmyth 1982; Weiss and Simpson 1998; Ravindra *et al.* 1999) (Figure 4B). These heterochromatic regions are transcriptionally silent for both PolII- and PolIII-transcribed genes (Brand *et al.* 1985; Schnell and Rine 1986) and resistant to cleavage by several endogenously expressed endonucleases, including the HO endonuclease (Connolly *et al.* 1988; Loo and Rine 1994).

It should be noted that silencing also occurs adjacent to yeast telomeres and many of the genes involved in *HML*/*HMR* gene silencing also play a role in telomeric silencing (see reviews by Laurenson and Rine 1992; Loo and Rine 1994; Sherman and Pillus 1997; Grunstein 1998; Lustig 1998; Stone and Pillus 1998; Gasser and Cockell 2001; Rusche *et al.* 2003; McConnell *et al.* 2006; Hickman *et al.* 2011). There is a hierarchy of silencing, with *HMR* and *HMR* being more strongly silenced than telomeres. In general, telomeric silencing is less robust; several mutations that strongly affect telomeric silencing (*e.g.*, *yku70*Δ (Moretti *et al.* 1994; Wotton and Shore 1997; Vandre *et al.* 2008) have either no effect on *HM* loci or have an effect only with a partially debilitated *HMR*-E sequence.

Cis-acting silencer sequences

There appear to be some important differences in the silencing of *HML* and *HMR*. Analysis of *HMR*-E (essential) and *HMR*-I (important) sequences showed that *HMR*-E alone was sufficient for silencing of the **a**1 transcript at *HMR***a** or of other PolII- or PolIII-transcribed genes inserted in place of the Y**a** region, whereas *HMR*-I can not silence completely without *HMR*-E (Abraham *et al.* 1984; Hicks *et al.* 1984; Brand *et al.* 1985). Mutations of *HMR*-E that weaken silencing can be "tightened" by the presence of *HMR*-I. In contrast, either *HML*-E or *HML*-I is each sufficient to silence *HML* or other genes placed nearby (Mahoney and Broach 1989).

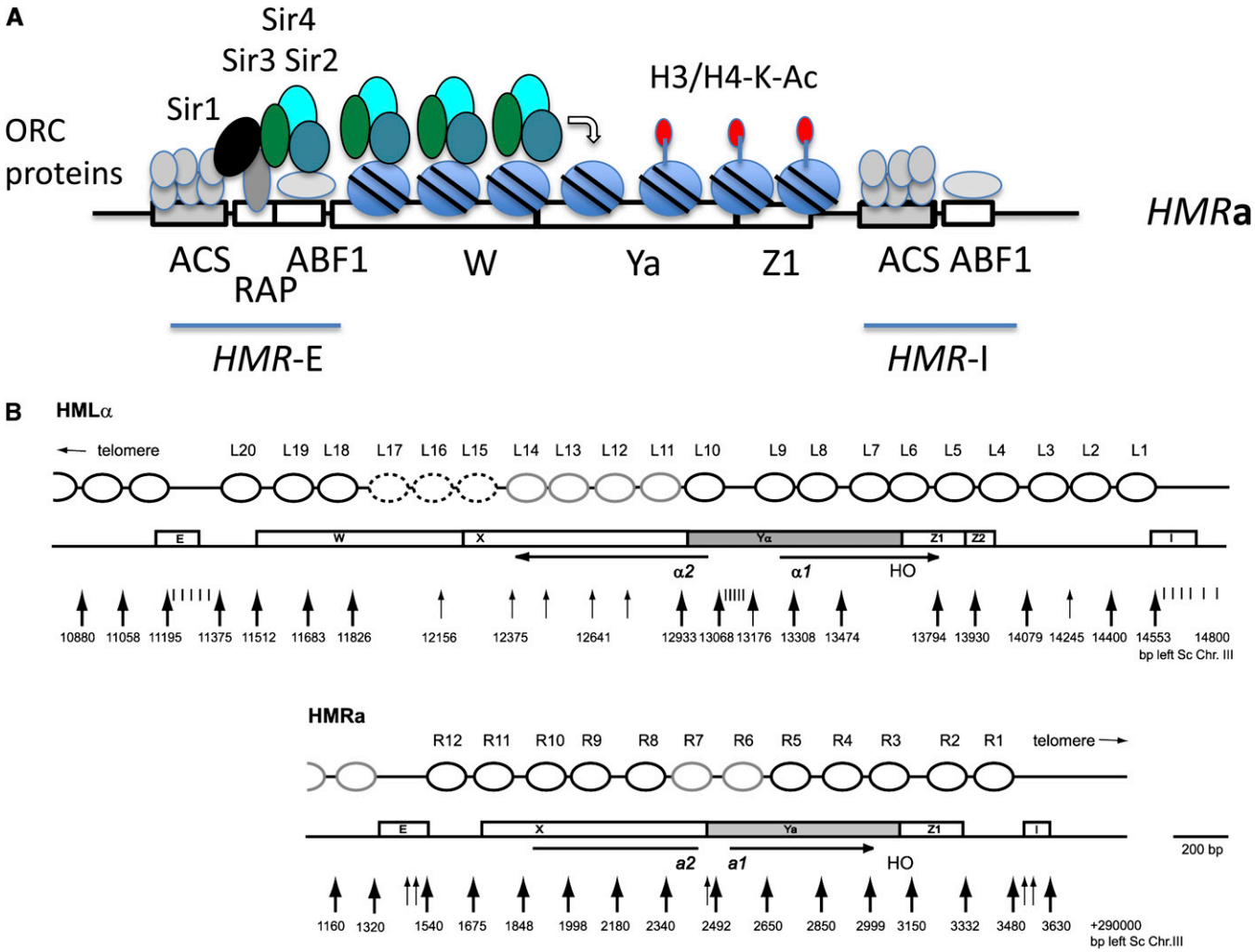

Figure 4 Silencing of *HMR* and *HML*. (A) Establishment of silencing at *HMR*-E. The processive process of silencing is illustrated. Proteins bound to the three elements of the *HMR*-E silencer recruit Sir1 that in turn recruits the Sir2-Sir3-Sir4 complex. The NAD$^+$-dependent HDAC Sir2 deacetylates lysines on histones on the N-terminal tails of H3 and H4, which allows the Sir3-Sir4 to bind and stabilize the position of the nucleosome. Sir2 can then deactylate the next nucleosome and silencing spreads further. Here the spread of silencing is shown progressing in one direction and from one of the two silencing elements. In reality, silencing spreads from both *HMR*-E and *HMR*-I and also spreads in a limited fashion to the flanking regions. (B) Highly positioned nucleosomes in *HML* and *HMR* as determined by the Simpson lab (Weiss and Simpson 1998; Ravindra *et al.* 1999).

Silencing also appears to be enforced by the fact that both *HMR* and *HML* lie relatively near chromosome ends (telomeres) that also exhibit gene silencing. When *HML*-E or *HMR*-E silencer sequences are inserted at other chromosome locations, further from telomeres, their ability to silence various adjacent genes is less strong (Thompson-Stewart *et al.* 1994; Shei and Broach 1995; Maillet *et al.* 1996; Marcand *et al.* 1996). This may explain why a circular chromosomal fusion of *MAT***a** and *HMLα* (*i.e.*, containing *HML*-I but lacking *HML*-E and lacking telomeres) is expressed (Strathern *et al.* 1979).

The distance over which E and I silencers can act to completely silence genes is not very great. Normally the distance between E and I is <3 kb. Silencing is weakened if that distance is increased. For example, if most of the mating-type gene sequences lying between *HMR*-E and *HMR*-I are deleted and replaced by a 2.2 kb *LEU2* gene fragment, *LEU2* is completely silent; however if the same *LEU2* gene is simply inserted into the middle of the mating-type sequences, thus moving *HMR*-E and *HMR*-I further apart, there is sufficient *LEU2* gene expression to allow *leu2* cells to grow (B. Connolly and J. E. Haber, unpublished results). Similarly, Weinstock *et al.* (1990) discovered that tandem insertions of the Ty1 retrotransposon within *HML* unsilenced the locus, with the degree of expression correlated to the size of the array and thus the distance between E and I sites.

At telomeres, there are no specific silencer sequences, but the telomere-associated Rap1 protein interacts with both Sir3 and Sir4 (Moretti *et al.* 1994; Wotton and Shore 1997; Mishra and Shore 1999). Moreover telomere termini are also enriched in yKu70-yKu80, which also recruit Sir4 (Roy *et al.* 2004; Ribes-Zamora *et al.* 2007). Telomeric silencing can extend >10 kb (Strahl-Bolsinger *et al.* 1997), but strong silencing is confined to the first 1–2 kb (Rusche and Lynch 2009). Part of the way silencing is established

seems to be by tethering sequences to the nuclear periphery, as exemplified by Sternglanz' experiment of artificially tethering a partially crippled *HMR* locus to the nuclear membrane by use of a Gal4-fusion protein that contained an integral membrane protein that bound to a Gal4-binding domain as part of the *HMR*-E silencer (Roy *et al.* 2004). However silencing is not dependent on peripheral localization (Gartenberg *et al.* 2004). Overexpression of Sir3 has the surprising effect of causing telomere delocalization toward the center of the nucleus but silencing is stronger (Ruault *et al.* 2011).

A striking feature of all four silencer sequences is that each is capable of acting as an autonomously replicating sequence (ARS) on a plasmid, thus allowing it to replicate (Abraham *et al.* 1984; Feldman *et al.* 1984; Kimmerly *et al.* 1988). Yet when one examines these same sequences on the chromosome by two-dimensional gel electrophoresis, to detect structures characteristic of the presence of an origin of DNA replication, neither *HML*-E nor *HML*-I is active, while *HMR*-E does appear to act as a chromosomal origin, although only in a fraction of cell cycles (Dubey *et al.* 1991; Rivier and Rine 1992; Collins and Newlon 1994). Whether silencing depends on origin activity has been the subject of much debate; current evidence suggests that the binding of the ORC proteins to silencer regions is a key step in establishing silencing, but it is not necessary that replication be initiated at that site (Fox *et al.* 1993; Ehrenhofer-Murray *et al.* 1995; Fox *et al.* 1997; Li *et al.* 2001). Indeed mutations of Orc5 were isolated that were defective in silencing but not in replication (Fox *et al.* 1995). A notable experiment in this regard was the demonstration by Sternglanz' lab that the ORC sequence could be replaced by a Gal4-binding domain to recruit Gal4-Sir1 in the apparent absence of ORC proteins to establish silencing (Chien *et al.* 1993; Fox *et al.* 1997).

Another interesting aspect of silencer sequences is that they confer centromere-like behavior on plasmids, allowing them to segregate properly in most cell divisions (Kimmerly *et al.* 1988). One explanation for this behavior is that silencer sequences may be anchored to some nuclear structure that is involved in chromosome partitioning. Evidence that silencers are anchored has been presented in studies of scaffold-attachment regions by Amati and Gasser (1988) and Towbin *et al.* (2009) and in elegant topological experiments by Ansari and Gartenberg (1997) showing that DNA bound by a lexA-Sir4 fusion protein is anchored within the nucleus. However, whereas deleting yKu partially releases telomeres from their peripheral association, it appears to allow a stronger association of *HML* with the nuclear envelope. Only when both yKu proteins and either Esc1 or Sir4 are deleted does one see a release of both telomeres and *HML* from the periphery (Bystricky *et al.* 2009; Miele *et al.* 2009).

The most detailed dissection of a silencer has been carried out with *HMR*-E. A combination of deletion analysis and protein-binding experiments has demonstrated that *HMR*-E contains three distinct subdomains (Figure 4A): ARS consensus sequence to which ORC proteins bind, a binding site for the ARS-binding factor (ABF1) protein, and a binding site for Rap1, which plays a complex role in both gene activation and in gene silencing (McNally and Rine 1991). Deletion of any one of these three regions still allows substantial silencing, but deletion of any two removes all repression of transcription. As noted above, the ORC-binding domain can be replaced by a lexA-binding domain to serve as a target to localize a lexA-Sir1 fusion protein, suggesting that one important role for ORC binding is in tethering another protein such as Sir1 to facilitate the establishment of silencing (Chien *et al.* 1993; Fox *et al.* 1997). The arrangement of DNA-binding sites varies at each silencer; for example *HMR*-I does not contain a Rap1-binding domain.

Control of the spreading of silencing by boundary elements

Silencing is quite restricted around *HML* and *HMR*, though it extends ~1 kb beyond the E and I sequences. At *HMR*-I the spread of silencing is blocked by a tRNA gene (Donze *et al.* 1999; Dhillon *et al.* 2009), but even when the tRNA is deleted, silencing does not spread much further (Lynch and Rusche 2010). At *HML*-E there is also a nearby sequence but its precise role has not been clarified (Bi 2002). In the case of *HML*-I, it seems that the silencer itself somehow has directionality so that inverting this region promotes longer silencing outside the *HML* locus and weakens silencing inside *HML* (Bi *et al.* 1999). Further study has shown that directionality can be attributed to the asymmetric positioning of nucleosomes around the silencer (Zou *et al.* 2006). A clever and more general search for silencer boundary elements was designed by Laemmli in which *ADE2* and *URA3* genes were placed inside *HMR* but with a pair of Gal4-binding sites flanking *ADE2* (Ishii *et al.* 2002). Initially both genes are silenced but the binding of a putative boundary element to both Gal4 sites should isolate *ADE2*, allowing its expression but leaving *URA3* still silent. The screen found a number of nuclear pore complex proteins and exportins; so, here, tethering to the nuclear periphery seems to prevent an extension of silencing.

Trans-acting silencing proteins

The establishment and maintenance of silencing requires several proteins. The first *trans*-acting silencing gene was identified by Klar *et al.* (1979a) based on the fact that coexpression of both mating types in a haploid cell produced a nonmating phenotype. Eventually four *SIR* genes were identified (Haber and George 1979; Rine *et al.* 1979; Rine and Herskowitz 1987). A deletion of three of these—*SIR2*, *SIR3*, and *SIR4*—completely abolished silencing, while loss of *SIR1* had a less extreme phenotype (see below). The keystone of these silencing proteins is the NAD^+-dependent Sir2 histone deacetylase, which is responsible for deacetylating a number of lysines on the N-terminal tails of histones H3 and H4 (Imai *et al.* 2000). Sir3 exhibits homology with the origin binding protein Orc1 and has a nucleosome

binding BAH (bromo adjacent homology) domain (Wang *et al.* 2004; Gallagher *et al.* 2009; Hickman and Rusche 2010). None of the Sir proteins binds directly to DNA, but they interact with each other, with Sir3 and Sir4 directly interacting and Sir4 binding to Sir2 (Moazed *et al.* 1997). Sir3 and Sir4 bind to deacetylated histone H3 and H4 tails (Johnson *et al.* 1990; Hecht *et al.* 1995, 1996; Grunstein 1997; Gasser and Cockell 2001). The acetylated form of histone H4K16 promotes Sir2-Sir4 binding (which might promote deacetylation), while the acetylated form has a reduced affinity for Sir3 (Oppikofer *et al.* 2011). These interactions set up the basis for a processive silencing mechanism in which Sir2 deacetylates the adjacent histone (Figure 4A), allowing Sir3 to bind, and so on, propagating an array of highly ordered (heterochromatic) nucleosomes (Grunstein 1997; Moazed 2011). Sir4 also interacts with yKu70 (Tsukamoto *et al.* 1996), which is important in telomere associations with the nuclear periphery (Taddei *et al.* 2004).

Sir proteins play a number of other roles

Sir2, but not Sir3 and Sir4, acts to reduce the expression of PolII-transcribed reporter genes embedded in rDNA and reduce recombination in rDNA (Gottlieb and Esposito 1989; Fritze *et al.* 1997; Smith and Boeke 1997; Smith *et al.* 1998). Overexpression of Sir3p or Sir4p also causes a significant increase in chromosome instability (Holmes *et al.* 1997), which might have something to do with its effects on telomeres and their position in the nucleus (Palladino *et al.* 1993). The striking discovery that the NAD^+-regulated *SIR2* HDAC affects yeast lifespan (Kennedy *et al.* 1995; Smeal *et al.* 1996; Gotta *et al.* 1997) was attributed to the formation and asymmetric segregation of extrachromosomal rDNA circles (Sinclair and Guarente 1997), which is unlikely to be a general mechanism of regulating longevity, but, remarkably Sirtuins (proteins homologous to Sir2) have proven to play key roles in longevity in metazoans and to affect many other aspects of cell metabolism, including autophagy (reviewed by Blander and Guarente 2004; Donmez and Guarente 2010; Herranz and Serrano 2010).

Our understanding of Sir2 has been enlivened by the discovery that there are four other genes exhibiting **h**omology to **sir2** (*HST* genes) (Brachmann *et al.* 1995; Derbyshire *et al.* 1996). Individual deletions of these *HST* genes do not affect silencing with an intact *HMR*, but *hst3* and *hst4* mutants partially derepress telomere silencing and overexpression of *HST1* suppresses a *sir2* mutation in *HM* gene silencing. Moreover *hst3* and *hst4* mutations are also radiation sensitive and show significant increases in chromosome instability (Brachmann *et al.* 1995). Recent studies have shown that these two histone deacetylases are required to remove Rtt109-mediated acetylation of histone H3-K56, an event that is normally strongly cell-cycle regulated. Without Hst3 and Hst4, cells fail to replicate properly and have greatly reduced viability. Removing the acetylation site or deleting Rtt109 suppresses the lethality of *hst3*Δ *hst4*Δ (Yang *et al.* 2008).

Establishment and maintenance of silencing

Regulation of acetylation of the N-terminal tails of histones H3 and H4 is directly implicated in silencing, first by mutations that replace the four evolutionarily conserved lysine residues (Megee *et al.* 1990; Park and Szostak 1990; Thompson-Stewart *et al.* 1994; Fisher-Adams and Grunstein 1995; Hecht *et al.* 1995). More direct evidence came from the fractionation of chromatin[4] in terms of the state of acetylation of lysine-16 of histone H4 (Braunstein *et al.* 1996), showing that *HML* and *HMR* are preferentially recovered in the hypoacetylated fraction. A similar analysis, taking advantage of affinity chromatography that detected an alteration in histone H3, had also shown a difference in chromatin structure between *MAT* and the two silent loci (Chen-Cleland *et al.* 1993).

The use of modified histones H3 and H4 has also revealed dramatic differences between *HML* and *HMR* silencing. A single H4-K16A mutation strongly unsilences *HML*α but has little effect at *HMR***a**, and even mutation of four lysines in the H4 tail only weakly affects silencing at *HMR* (Park and Szostak 1990). Some of this difference reflects chromosomal context; however, it seems that silencing at *HMR* is made substantially stronger by its chromosome location near the right end of chromosome III (although *HMR* is 23 kb from its telomere compared to 12 kb for *HML* on the left arm). If both *HML* and *HMR* are inserted ~50 kb from a telomere on chromosome VI, both *HML* and *HMR* show equivalent silencing defects with a histone H4-K16N mutation (Thompson *et al.* 1994). Quite possibly there are "booster" sequences (other Rap1- or Abf1-binding sites or even ARS sequences) in the vicinity of HMR that make it so much more silent. A large deletion of the H4 N-terminal tail does unsilence *HMR* as well as *HML* (Park and Szostak 1990). In contrast, a tail deletion of histone H3 causes a loss of silencing at *HML*α, but the same mutations have no effect on *HMR* silencing, whether *HMR***a** or *HMR*α. For example, in a *hml*Δ *mat*Δ *HMR*α strain that is **a**-like in its mating phenotype, deleting histone H3's N-terminal tail does not change the mating phenotype from **a**-like to α-mating behavior, as would be expected if *HMR*α were expressed even at a low level (J. A. Kim, Q. Wu, and J. E. Haber, unpublished results).

Silencing at *HML* also requires the protein transacetylases encoded by *NAT1* and *ARD1*, whose target appears to be the N terminus of Sir3 (Wang *et al.* 2004). The Nat1/Ard1 complex plays many roles in addition to silencing, including a failure to arrest in G1 after starvation (Whiteway *et al.* 1987; Mullen *et al.* 1989). Deletion of Ard1 (and presumably Nat1) also fails to unsilence *HMR***a** or *HMR*α, while allowing *HML* to be strongly expressed (Whiteway *et al.* 1987). Silencing is influenced by two other protein transacetylases encoded by *SAS2* and *SAS3* (Reifsnyder *et al.* 1996; Ehrenhofer-Murray *et al.* 1997). The pleiotropic

[4]This experiment is probably the first chromatin immunoprecipitation experiment.

deletion of the *RPD3* deacetylase also influences silencing (De Rubertis *et al.* 1996; Rundlett *et al.* 1996).

At least some of the difference in silencing at *HML* and *HMR* comes from the fact that the two cassettes normally carry different Y sequences. As further discussed below, silencing of *HMR* requires passage through S phase (but not replication *per se*). However, "substantial silencing of *HML*α" could be established without passage through S phase (Ren *et al.* 2010). These authors attributed this difference in promoter strength—with the α1-α2 promoter being weaker than **a**1-**a**2—because *HMR*α is partially silenced without S phase, while *HML***a** requires S phase passage.

One key question in silencing is whether the *establishment* of silencing differs from its subsequent *maintenance*. Although tethering Sir1 in place of ORC will indeed establish silencing (Chien *et al.* 1993; Fox *et al.* 1997), in a wild-type cell its role is more subtle. Cells carrying a *sir1* deletion exhibit a striking epigenetic variegation in *HML* silencing (Pillus and Rine 1989). Some cells express *HML* and others do not, but each state is persistent through many cell divisions. A *sir1* cell with *HML* in a silent state gives rise predominantly to silent *HML* for many generations, but occasionally a cell will arise where *HML* is not silenced, and this unsilenced state will also persist for many generations, until a cell reestablishes silencing, and so on. This epigenetic inheritance suggests that the establishment of silencing and its subsequent maintenance are separable, an idea that is strongly supported by other observations (reviewed by Stone and Pillus 1998). The epigenetic nature of silencing has been more directly visualized by Xu *et al.* (2006), who inserted *URA3*::GFP inside *HML* and *URA3*::CFP within *HMR*. Strikingly in a *sir1*Δ cell, the silent states of *HML* and *HMR* were independent of each other (that is, some cells expressed only CFP while others expressed only GFP and other expressed both, or neither). Moreover, there was a significant difference between the silencing of *HML* and *HMR*, as ∼60% of cells expressed *HML*::YFP, whereas ∼90% of all cells expressed *HMR*::CFP. By reducing the strength of *URA3* expression, with a *ppr1*Δ mutation, they found that silencing was nearly normal in *sir1*Δ for *HML*::YFP but still *HMR*::CFP was expressed in most cells. This result is striking in part because, as discussed earlier, many other mutations more strongly unsilence *HML* than *HMR*. It is also curious that *sas2*Δ *sir1*Δ completely loses silencing, whereas *sas2*Δ suppresses *sir1*Δ's defect at *HMR* (Xu *et al.* 1999).

The idea that tethering *HML* or *HMR* to the nuclear periphery enhances silencing has been substantiated by the finding that the silencing that does occur in a *sir1*Δ is dependent on the yKu70 and YKu80 proteins (Patterson and Fox 2008; Vandre *et al.* 2008). The Ku proteins, along with Esc1, redundantly tether telomeres and *HM* loci to the nuclear periphery (Taddei *et al.* 2004, 2005) and this sequestering may place the *HM* locus near a higher concentration of Sir2 and other silencing factors. However Bystricky *et al.* (2009) has reported that *HML*, but not *HMR*, becomes *more* tightly associated with the nuclear envelope, though this was not assessed in *sir1*Δ.

An early study by Miller and Nasmyth (1984) showed that raising a temperature-sensitive *sir3* mutant to its restrictive temperature caused immediate loss of silencing, but returning cells to their permissive temperature did not restore silencing until cells had passed through the next S phase. However this experiment does not mean that DNA replication *per se* is required; silencing may only require an enzyme whose synthesis is confined to the S phase of the cell cycle (Holmes and Broach 1996; Bi and Broach 1997; Kirchmaier and Rine 2001; Li *et al.* 2001). But additional experiments argue that full silencing requires cells to progress through mitosis (Lau *et al.* 2002; Martins-Taylor *et al.* 2004, 2011).

Site-specific recombination has been used to "pop out" a DNA circle from *HML* either with or without a silencer sequence. When silent chromatin was popped out such that the excised circle lacked E and I silencers, silencing could not be maintained, even in G1- or G2-arrested cells (Cheng and Gartenberg 2000). Silencing was lost if cells passed though S phase. Surprisingly, the loss of silencing is apparently not directly caused by the partitioning of nucleosomes to newly synthesized DNA, as the popped-out circle does not contain an origin of replication. This result suggests that some events in maintaining silencing depend on progressing through the S phase part of the cell cycle but are not intimately involved in replication itself. Nevertheless, it is entirely possible that, in normal cells, when replication does occur, the maintenance of silencing is closely connected to chromatin assembly. Enomoto and Berman (1998) further showed that a deletion of the Cac1 subunit of chromatin assembly factor 1 (CAF-1) affects the maintenance of *HML* silencing but apparently not its establishment. It has been suggested that *cac1*Δ and *asf1*Δ have the global effect of reducing histone acetylation, which in turn might attract Sir2 away from the silent loci to many other loci (Rusche *et al.* 2003).

The list of genes affecting silencing continues to grow, although their contributions are relatively minor. Two mutations affecting the ubiquitylation of proteins affect silencing (Moazed and Johnson 1996; Huang *et al.* 1997). The case of Ubp3 protein is especially interesting, as it has been reported to be affinity purified in a complex with Sir4 (Moazed and Johnson 1996). Uls1—a member of the Swi2/Snf2 family of proteins implicated in chromatin remodeling—has been shown to tighten silencing when it is deleted and weaken silencing when a truncated gene is overexpressed (Zhang and Buchman 1997). Similar phenotypes have been found for still another antisilencing factor (*ASF1*) (Sharp *et al.* 2001, 2005). The absence of Asf1 or Rtt109 leads to a loss of histone H3-K56 acetylation and in some way this change alters the efficiency of Sir2-mediated silencing. Similarly, Dot1 methyltransferase, which modifies histone H3-K79, also plays some minor role in *HM* gene silencing (Takahashi *et al.* 2011). In addition temperature-

sensitive mutations in two essential genes, *NLP3* and *YCL054*, also perturb silencing by unknown means (Loo and Rine 1995). The *CDC7* gene, encoding a protein kinase necessary to initiate chromosomal DNA replication, also plays a role in silencing (Axelrod and Rine 1991), though how this relates to the role of ORC proteins remains unknown. Finally, recent studies have shown that the MAP kinase pathway(s) responsible for mating pheromone, starvation, and heat-shock response all cause the hyperphosphorylation of Sir3 (Stone and Pillus 1996), though how phosphorylation affects normal silencing is not yet established.

Further analysis of silencing has suggested that the full establishment is more complex than had been imagined. It takes several generations for the complete silencing of *HMR* (Katan-Khaykovich and Struhl 2005; Kirchmaier and Rine 2006), although 90% of the transcriptional repression is accomplished in the first cell cycle. These results were supported by Xu *et al.* (2006), who used single-cell analysis of strains carrying GFP inserted inside *HML*, allowing them to use microscopic analysis of silencing in individual cells. Their work showed that the establishment of silencing after returning *sir3-ts* cells to their permissive temperature was stochastic, and that silencing of all cells took several generations.

Rine's lab has also studied silencing at the microscopic level in single cells when Sir3 is turned on (Osborne *et al.* 2009). An α-mating *sir3*Δ strain carrying *HML*α *mat*Δ *hmr*Δ is mated to an **a**-like strain lacking all three mating loci, but *SIR3*. When two G1 cells conjugate, a zygote is formed that initially expresses *MAT*α1 and *MAT*α2 (from *HML*α), but as this locus is silenced, the zygote should become inhibited in its growth by α-factor. This clever assay demonstrated that silencing was accomplished in 1–2 generations. In a later assay, Rine's group used GFP embedded in *HML* in a similar assay to ask how rapidly GFP intensity decreased (the assay relies on GFP turning over rapidly) (Osborne *et al.* 2011). These assays revealed "unexpected complexity" in the contributions of a histone acetyltransferase (Sas2), two histone methytransferases (Dot1 and Set1), and one histone demethylase (Jhd2) to the dynamics of silencing and suggested that removal of methyl modifications at histone H3-K4 and -K79 were important steps in silent chromatin formation and that Jhd2 and Set1 had competing roles in the process.

Despite intensive work in this area, there are still many mysteries yet to solve. Moreover, two recent articles have called into question some previously published studies examining silencing at crippled *HM* silencers or at telomeres. First, it seems that in contrast to the most frequently used telomere silencing construct created at a truncation at the left arm of chromosome VII, most subtelomeric regions do not show strong position effect variegation and do not show much change in gene expression when Dot1 is absent (Takahashi *et al.* 2011). Moreover, the effects of some mutations such as alterations of PCNA, may not actually affect normal silencing. In many studies, *URA3* has been used as a reporter gene, but it now appears that its inhibitor, 5-FOA, itself alters nucleotide pools and dNTP ratios and is sensitive to other perturbations of nucleotide metabolism (Rossmann *et al.* 2011). Alterations in dNTP levels appear to account at least in part for the previous finding that mutants of PCNA affected telomere silencing (Li *et al.* 2009).

Silencing in the absence of Sir proteins

Surprisingly a gain-of-function mutation in the *SUM1* gene will establish silencing even in the absence of the *SIR* genes (Klar *et al.* 1985; Laurenson and Rine 1991; Chi and Shore 1996). A single-amino-acid substitution in *SUM1* is sufficient to convert it from a localized transcriptional repressor to a broad repressor of gene expression. At *HML* and *HMR*, *SUM1-1* silencing still depends on histone deacetylation, but now it turns out to be mediated not by Sir2 but by its homolog, Hst1 (Rusche and Rine 2001; Yu *et al.* 2006; Safi *et al.* 2008). Curiously, the *S. cerevisiae SUM1-1* allele behaves like the wild-type allele of *SUM1* in *Kluyveromyces lactis*, where it normally plays a role in *HML* and *HMR* silencing (Hickman and Rusche 2009).

A brief word about unsilencing

One measure of unsilencing is the susceptibility of *HML* or *HMR* to be cleaved by HO endonuclease. As noted before, these loci are normally protected from HO cleavage, except that *HML* will show a small amount of cleavage after several hours of overexpressing HO. When these loci are desilenced by raising a temperature-sensitive allele of *sir3* to 37° or by adding nicotinamide to inhibit Sir2, *HML* is consistently cleaved 1 hr before *HMR* (A. Walther and J. E. Haber, unpublished results). If *HMR*α is inserted in place of *HML*α, then it is cleaved ~30 min after *HML*α; so there seem to be both sequence and position effects on the persistence of silencing.

Cell Lineage and Cell Cycle Control of *HO* Gene Expression

MAT switching provided a powerful early model to study the determination of cell lineage.[5] Only half of the cells in a colony are able to switch mating type in any one cell division (Figure 1). A germinating haploid spore grows, produces a bud, and divides without changing mating type. Then, in the next cell division cycle, the older mother cell and its next (second) daughter change mating type while the first daughter buds and divides without any change (Haber and George 1979). The axial budding pattern of haploids places two *MAT***a** cells immediately adjacent to two *MAT*α cells and they readily conjugate, forming *MAT***a**/*MAT*α diploids in which the HO endonuclease gene is turned off so that further mating-type switching is repressed. If cells are prevented from mating—for example, by micromanipulating cells apart before conjugation—one can establish that the

[5]The subsequent development of a detailed description of cell lineage in *Caenorhabditis elegans* has overshadowed the importance of the analysis of cell fate in budding yeast.

lineage rules persist: any cell that has previously divided once is capable of switching *MAT*, while new daughter cells cannot (Haber and George 1979).

Nasmyth's lab (Breeden and Nasmyth 1987; Nasmyth 1987a; Nasmyth *et al.* 1987) first demonstrated that the control of this lineage pattern depended on the asymmetric expression of the HO endonuclease gene, which is restricted to mother cells that have divided at least once. Control of HO expression depends on the Swi5 transcription factor, which is localized to the mother cell nucleus and not that of her daughter (Nasmyth 1987b). The absence of Swi5 expression in daughters is caused by the Ash1 repressor protein that exhibits a striking localization only in the daughter cell (Figure 1) (Bobola *et al.* 1996; Derbyshire *et al.* 1996; Sil and Herskowitz 1996). Ash1 acts upstream of Swi5 and may directly repress *SWI5* transcription, thus restricting HO expression to the mother cell in the next G1 stage of the cell cycle. The localization of Ash1 occurs not by selective transport of the protein to the daughter but by localizing its mRNA prior to cell division (Long *et al.* 1997). mRNA localization apparently depends on the myosin-like protein Myo4 (Bobola *et al.* 1996), as well as actin (Takizawa *et al.* 1997). A complex of two other RNA-binding proteins, She2 and She3, act to effect cargo binding. Two other proteins have also been shown to be important for efficient *ASH1* localization in yeast: Loc1p, a nuclear protein, and Khd1, which is thought to link translational repression to the localization process (Long *et al.* 2001; Irie *et al.* 2002; Hasegawa *et al.* 2008). Since Ash1 mRNA localization was first discovered, >20 other mRNAs have been shown to show similar localization in *Saccharomyces* (Shepard *et al.* 2003; Jambhekar *et al.* 2005).

Mother–daughter control is only one aspect of the regulation of *HO* expression. *HO* transcription is confined to a narrow window in the cell cycle, after the cell passes "start." Start is the point at which α-factor arrests *MAT***a** cells (or **a**-factor arrests *MAT*α cells) so that cells beginning conjugation should contain unreplicated nuclei that will fuse by karyogamy to create a diploid nucleus. Start is also the point at which the key cell division kinase Cdk1 is inactive. As cells pass start, Cdk1 in conjunction with its G1 cyclins (Cln1, Cln2, and Cln3) becomes active. Cdk1 activates two transcription factors, Swi4-Swi6, which together are called SBF, and which bind to the SCB DNA motif (Swi4,6-regulated cell cycle box). Swi6 also pairs with Mbf1 to form the MBF (Mlu1 cell cycle box) cell cycle regulatory factor that turns on genes prior to initiating new DNA synthesis. The *HO* upstream regulatory region is perhaps the largest of any yeast gene—on the order of 1.4 kb (Nasmyth 1993). The *HO* promoter contains 10 copies of SCB, which is bound by Swi4 and Swi6. There is also a binding site for the Mata1-Matα2 repressor that turns HO off in diploids.

The HO endonuclease protein is quite unstable and is rapidly degraded so that mother cells suffer a brief pulse of endonuclease activity. Were the HO protein to persist, it would be able to cut the switched locus and cause a second recombination event. Studies by Raveh (Kaplun *et al.* 2003, 2006) have shown that HO endonuclease is rapidly degraded by the ubiquitin-mediated SCF protein degradation complex. HO has a half-life of only 10 min.[6] HO is apparently targeted for degradation by its phosphorylation by the Chk1 kinase of the DNA damage response; hence a kinase-dead mutation of the ATR homolog Mec1, as well as *rad9*Δ and *chk1*Δ stabilize HO-LacZ. However, degradation does not depend on the actual induction of a DSB and triggering the DNA damage checkpoint *per se*.

HO is a member of the LAGLIDADG family of site-specific endonucleases (reviewed by Haber and Wolfe 2005); it recognizes a degenerate 24-bp sequence that spans the *MAT*-Y/Z border (Nickoloff *et al.* 1986, 1990). A haploid yeast has three possible targets for HO: the *MAT* locus, *HML*α, and *HMR***a**, but only the *MAT* locus is accessible under normal conditions.[7] So, combining all these controls, there is a single, programmed DSB inflicted on the *MAT* locus only in mother cells and prior to the initiation of DNA replication.

First Models of *MAT* Switching

Early studies of *MAT* switching recognized the existence of two additional key loci that were required for the replacement of *MAT* alleles: *HML* and *HMR* (Takahashi *et al.* 1958; Takano and Oshima 1967; Santa Maria and Vidal 1970). A remarkably insightful hypothesis by Oshima and Takano (1971) suggested that these loci were the seat of controlling elements that could transpose to *MAT* and activate opposite mating-type alleles. Coupled with the key experiments of Hawthorne (1963), these ideas led Herskowitz' lab (Hicks *et al.* 1977; Haber and George 1979) to suggest a specific version of the transposition model known as the "cassette model" in which an unexpressed copy of Yα sequences was located at *HML* (*HML*α) and unexpressed Y**a** sequences were found at *HMR***a**. These sequences could be transposed to the *MAT* locus, where they would be expressed. In these early models, there was no suggestion that *MAT* switching involved homologous recombination; rather a site-specific duplicative transposition imagined. Subsequent studies (Nasmyth and Tatchell 1980; Strathern *et al.* 1980; Astell *et al.* 1981; Tatchell *et al.* 1981) confirmed that there were indeed two additional copies of mating-type information at *HML* and *HMR*. Most laboratory strains carry *HML*α and *HMR***a**, but natural variants exist that carry the opposite configuration: *HML***a** and *HMR*α (Takahashi *et al.* 1958; Naumov and Tolstorukov 1971; Tolstorukov and Naumov 1973). One early surprise in the molecular analysis of *MAT*, *HML*, and *HMR* was that the two donor cassettes did not carry simply the Y**a** and Yα donor sequences that could be "played" in the cassette player of the *MAT* locus, but were in fact intact, complete copies of mating-type genes carrying their own bidirectional promoters (Figure 2). But

[6]The rapid degradation of HO, even after galactose induction, contrasts with the stability of the I-SceI protein, which is also used to study induced DSB repair.
[7]If *HML* and *HMR* are unsilenced, *e.g.*, by ablating a silencing protein such as Sir3, then all three sites are equivalently cleaved (Miyazaki *et al.* 2004).

somehow these genes were not transcribed. The two unexpressed cassettes differ in the extent of homology they share with *MAT*. *HMR*, *HML*, and *MAT* all share two regions flanking the Y sequences, termed X and Z1. *HML* and *MAT* share additional sequences, termed W and Z2 (Figure 2).

We now know that during switching there is no change in either donor sequence; that is, *MAT* switching does not involve a reciprocal exchange of Y**a** and Yα sequences, but rather a copying of the sequences from either *HML*α or *HMR***a** and their insertion at *MAT* in place of the original *MAT* allele (Hicks *et al.* 1979). This asymmetric recombination event is termed a gene conversion. The idea that *HML* and *HMR* repeatedly served as donors during *MAT* switching provided an explanation for an early observation of Hawthorne (1963) that a mutant *MAT*α cell could be replaced by *MAT***a**, which then switched to a wild-type *MAT*α allele. Subsequent "healing" and "wounding" experiments were carried out in which mutations at *MAT* were corrected by recombination with the donor or in which a mutation at the donor was introduced into the *MAT* locus (Hicks and Strathern 1977; Klar *et al.* 1979b; Sprague *et al.* 1981). In some cases, the replacement of *MAT* information included not only the Y region but at least part of the flanking X and Z1 regions as well that were shared by *MAT* and its two donors (Sprague *et al.* 1981; McGill *et al.* 1989).

In the 35 years since the cassette model was articulated, the *MAT* switching system and other HO-induced DSBs have been the object of intense study, to learn both about gene silencing and about the multiple mechanisms of double-strand break repair by homologous recombination, nonhomologous-, and microhomology-mediated end-joining and new telomere addition (Rattray and Symington 1995; Pâques and Haber 1999; Aylon and Kupiec 2004; Krogh and Symington 2004; Daley *et al.* 2005; McEachern and Haber 2006; Sung and Klein 2006; Lydeard *et al.* 2007; Li and Heyer 2008; McVey and Lee 2008; San Filippo *et al.* 2008; Jain *et al.* 2009; Heyer *et al.* 2010; Schwartz and Heyer 2011). Here, we focus more specifically on *MAT* switching.

MAT Switching: a Model for Homologous Recombination

The conversion of one mating type to the other involves the replacement at the *MAT* locus of Y**a** or Yα by a gene conversion induced by a DSB by HO endonuclease (Strathern *et al.* 1982; Kostriken *et al.* 1983). The process is highly directional, in that the sequences at *MAT* are replaced by copying new sequences from either *HML*α or *HMR*a, while the two donor loci remain unchanged by the transaction. Directional gene conversion reflects the fact that HO endonuclease cannot cleave its recognition sequence at either *HML* or *HMR*, as these sites are apparently occluded by nucleosomes in silenced DNA. Thus the *MAT* locus is cleaved and becomes the recipient in this gene conversion process. A very weak cleavage of *HML* has been observed when HO endonuclease is overexpressed (Connolly *et al.* 1988); moreover, rare "illegal" switches, where *MAT* is the donor and the silent locus is switched, have been observed when *MAT* itself cannot be cut because of a change in the HO recognition site (Haber *et al.* 1980a). In Sir$^-$ cells where *HML* or *HMR* is expressed, HO can readily cut these loci and they become recipients (Klar *et al.* 1981; Bressan *et al.* 2004).

Normally the *HO* gene is tightly regulated to be expressed only in haploid mother cells and only at the G1 stage of the cell cycle (Nasmyth 1987b); however, the creation of a galactose-inducible HO gene made it possible to express *HO* at all stages of the cell cycle and in all cells (Jensen and Herskowitz 1984). This made it possible to deliver a DSB to all cells simultaneously and to follow the appearance of intermediates and final products by physical analysis of DNA extracted at times after HO induction (Connolly *et al.* 1988; Raveh *et al.* 1989; White and Haber 1990). An example of Southern blot analysis of *MAT***a** switching to *MAT*α is shown in Figure 5. Physical monitoring of recombination at *MAT* has yielded much of what we know about DSB-induced mitotic recombination (reviewed in Haber 1995, 2006; Pâques and Haber 1999; Krogh and Symington 2004; Hicks *et al.* 2011). Some related studies have been done by inserting small HO endonuclease recognition sites at other locations and from the induction of other site-specific endonucleases, most notably I-SceI (Rudin and Haber 1988; Nickoloff *et al.* 1989; Ray *et al.* 1989; Plessis *et al.* 1992; McGill *et al.* 1993; Liefshitz *et al.* 1995; Weng *et al.* 1996; Inbar and Kupiec 1999; Wilson 2002; Storici *et al.* 2003; Lydeard *et al.* 2007, 2010; Jain *et al.* 2009; Marrero and Symington 2010). Additional information about DSB repair has been gleaned from the analysis of DSB-induced recombination in meiotic cells (reviewed in Kleckner 1996; Borner *et al.* 2004; Keeney and Neale 2006; Longhese *et al.* 2009). By and large the results are sequence independent, though some interesting aspects particular to *MAT* switching are noted below.

The overall process of *MAT***a** switching to *MAT*α is illustrated in Figure 6. Following HO cleavage of *MAT***a**, the ends are resected in a 5′ to 3′ direction, creating a 3′-ended ssDNA tail that assembles a filament of the Rad51 recombinase protein. This protein::DNA complex engages in a search for a homologous sequence (in this case *HML*α) with which repair can be effected. Homology search culminates in strand exchange in which the ssDNA base pairs with the complementary sequence in the donor, creating a displacement loop, or D loop. The 3′ end of the invading strand is then used as a primer to initiate copying of one strand of the donor locus, and the newly copied strand is displaced until it can anneal with homologous sequences on the opposite end of the DSB. The 3′-ended nonhomologous tail is clipped off and the new 3′ end is used to prime a second strand of DNA synthesis, completing the replacement of *MAT***a** by *MAT*α. Each of these steps is discussed in more detail below.

HO cleavage

HO endonuclease cleaves a degenerate recognition site of 24 bp *in vitro* (Nickoloff *et al.* 1986), although sites of 117 bp

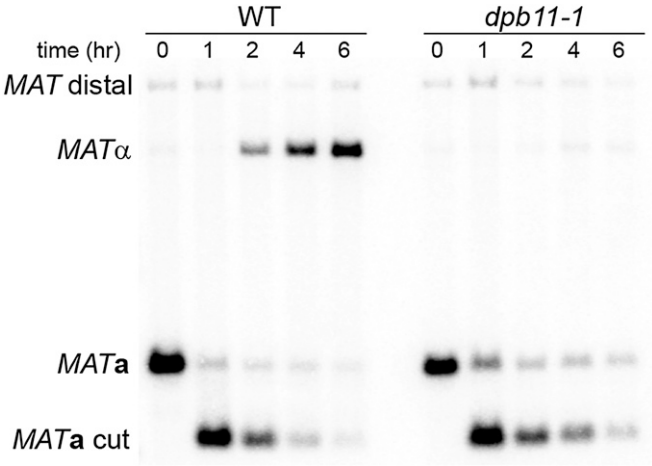

Figure 5 Physical monitoring of *MAT* switching. Southern blot analysis of *Sty*I-digested DNA after galactose induction of HO endonuclease. The probe detects sequences just distal to *MAT*-Z1/Z2 and shows a difference in the size of the *Sty*I restriction fragments of *MAT***a** and *MAT*α. In this experiment, a *ade3*::*GAL*::*HO* strain carrying *HML*α *MAT***a** *hmr*Δ *cdc7-as3* was used. Cells were arrested prior to DNA replication by inhibiting Cdc7 with 1-NMPP1 (Ira *et al.* 2004) and then shifted to 37° to inactivate a temperature-sensitive mutation of the DNA replication factor Dpb11. In Dpb11$^+$ cells, one can see the cleavage of *MAT***a** into a smaller HO-cut segment, followed by the appearance of the *MAT*α product. Switching fails in absence of Dpb11 at the restrictive temperature. Data are from Hicks *et al.* (2011).

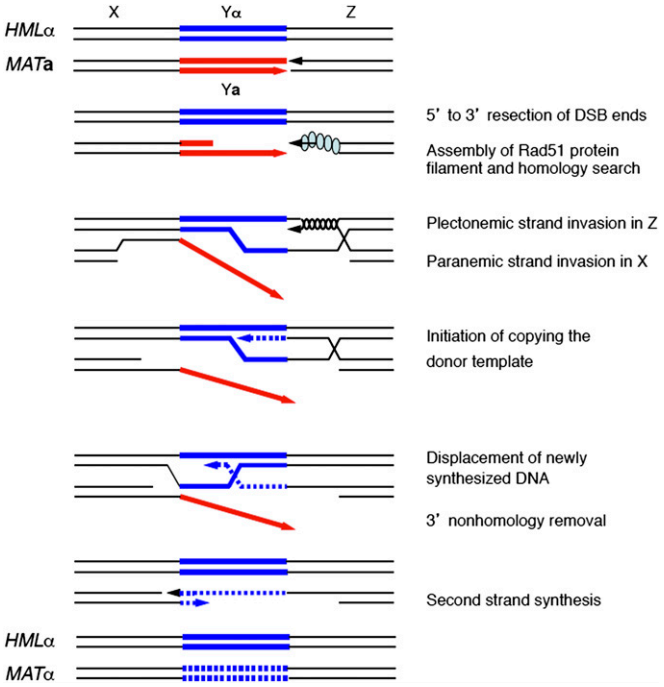

Figure 6 Mechanism of *MAT* switching. Key steps in the switching of *MAT***a** to *MAT*α by a synthesis-dependent strand-annealing (SDSA) mechanism (reviewed by Pâques and Haber 1999). An HO-induced DSB is resected by 5′ to 3′ exonucleases or helicase endonucleases to produce a 3′-ended ssDNA tail, on which assembles a Rad51 filament. The Rad51::ssDNA complex engages in a search for homology. In the *MAT*-Z region, strand invasion can form an interwound (plectonemic) joint molecule that can assemble DNA replication factors to copy the Yα sequences. Unlike normal replication, the newly copied strand is thought to dissociate from the template and, when sufficiently extended, anneal with the second end, still blocked from forming a plectonemic structure by the long nonhomologous single-stranded Y**a** sequences. These sequences are clipped off once strand annealing occurs, by the Rad1-Rad10 flap endonuclease, so that the new 3′ end can be used to primer extend and copy the second strand of the Yα sequences. Consequently all newly synthesized DNA is found at the *MAT* locus while the donor is unaltered. A small fraction of DSB repair events apparently proceed by a different repair mechanism involving the formation of a double Holliday junction (see Pâques and Haber 1999 for details).

down to 33 bp are generally used when the HO recognition site is inserted at other locations. A site with only 21 bp results in inefficient single-strand nicking that, by replication, can be converted to a DSB (Cortes-Ledesma and Aguilera 2006). Single-base-pair *MAT-inc* (inconvertible) or *MAT-stk* (stuck) substitutions in the recognition site abolish or greatly reduce switching[8] (Weiffenbach and Haber 1981; Ray *et al.* 1991). HO cutting generates 4 bp, 3′-overhanging ends, both of which are accessible to exonucleases *in vitro* (Kostriken *et al.* 1983). *In vivo*, however, the DSB is processed almost exclusively by several 5′ to 3′ exonucleases to create long 3′-ended tails (White and Haber 1990). As discussed more fully below, the 3′ end is remarkably resistant to exonucleolytic removal. It is possible that there are no 3′ to 5′ exonucleases that act on a 3′-overhanging end or that the end is protected *in vivo* by the binding of RPA or Rad51 or other proteins.

MAT switching, induced by a galactose-regulated HO endonuclease, is a surprisingly slow process, requiring 1 hr to complete, independent of the time during the cell cycle (Connolly *et al.* 1988; Raveh *et al.* 1989; White and Haber 1990). It is possible that normal *MAT* switching may be more rapid, when HO is expressed in G1, only in mother cells, and in a coordinated fashion with other genes; this may be inferred from the low level of steady-state HO-cleaved *MAT* DNA in cells that can continually switch (*i.e.*, *MAT***a** cells with *HML***a** and *HMR***a** cassettes) (Strathern *et al.* 1982). However, additional experiments using *HO* whose expression is restricted to the G1 phase of the cell cycle (Nasmyth 1987b) shows similar slow kinetics, as does expressing a conditional allele of HO under normal cell cycle control (M. Yamaguchi, M. Gartenberg, and J. E. Haber, unpublished results).

5′ to 3′ resection of the DSB ends

5′ to 3′ resection proceeds rather slowly, at a rate of ~4 kb/hr—about 1 nt/sec—(Fishman-Lobell *et al.* 1992; Zhu *et al.*

[8]One of the annoying aspects of the *Saccharomyces* Genome Database is that—because the original sequence was performed on a *MAT*α strain—*MAT***a** does not exist! However the sequence of *HMR***a** of course carries the same Y**a** region as *MAT***a**, so one can find the sequence. However, it happens that *HMR***a** in the reference strain S288c and its derivatives (*e.g.*, the oft-used BY4741 and BY4742) carries a "stuck" mutation at position Z11, so that this sequence is very poorly cleaved if used as a cleavage site. This is also the case when this *HMR***a** sequence is switched to replace *MAT*α. Worse, it turns out that, whereas *MAT***a**-*stk* is poorly cleaved, *MAT*α-*stk* is not cut at all (Ray *et al.* 1991).

2008) and in a strain where there is no repair of the DSB, resection will continue at roughly this rate for more than 24 hr![9] Physical analysis of the rate of 5′ to 3′ degradation initially implicated the trio of interacting proteins, Rad50, Mre11, and Xrs2 (MRX complex), in this process (Sugawara and Haber 1992; Ivanov *et al.* 1994; Tsubouchi and Ogawa 1998). MRX somehow associates with Sae2, though a direct interaction has not been demonstrated, and together these proteins appear to do the initial 5′ to 3′ resection at HO-induced DSB ends. Mre11 has 3′ to 5′ exonuclease activity and both Mre11 and Sae2 have endonuclease activity (Bressan *et al.* 1998; Lee *et al.* 2002; Nicolette *et al.* 2010); however, mutation of the nuclease activity of Mre11 has very little effect on resection (Bressan *et al.* 1998; Moreau *et al.* 1999; Lee *et al.* 2002). Mre11's nuclease activity is required in mitotic cells to cleave hairpin ends (Lobachev *et al.* 2004; Yu *et al.* 2004) and in meiosis to remove the Spo11 protein from DSB ends (but in both of these cases Sae2 is also necessary). Deleting Sae2 does significantly retard resection (Clerici *et al.* 2005). While MRX-Sae2 appears to get resection started, extensive resection depends on two competing pathways of resection, one comprising the 5′ to 3′ exonuclease Exo1 and the other consisting of the Sgs1-Top3-Rmi1 (STR) helicase complex coupled to the nuclease function of Dna2 (which itself has nuclease activity not relevant for this process) (Huertas *et al.* 2008; Mimitou and Symington 2008; Zhu *et al.* 2008; Niu *et al.* 2010). Exo1 activity is increased when the Ku proteins are ablated (Mimitou and Symington 2010; Shim *et al.* 2010).

When both Sgs1 and Exo1 are deleted, there is very limited resection, which appears to depend on MRX and Sae2. This result suggests that MRX acts first and hands off resection to Exo1 or the STR-Dna2 complex; however, this idea is contradicted by the long-observed fact that deletion of MRX proteins or Sae2 only reduces resection to about half in cycling cells. However in G2-arrested cells deletion of Rad50 eliminates nearly all resection (Diede and Gottschling 2001); possibly an alternative resection activity is absent in G2-arrested cells. Moreover in G1 cells prior to the activation of Cdk1 at the start point of the cell cycle, there is nearly no resection (Aylon *et al.* 2004; Ira *et al.* 2004). Inhibition of Cdk1 at other points in the cell cycle also blocks resection (Aylon *et al.* 2004; Ira *et al.* 2004). Taken together, it seems that the modest inhibition of resection in cycling cells by deleting MRX or Sae2 argues that MRX-Sae2 does not act as the obligate gatekeeper of resection and that in S phase, MRX-Sae2 may have a very minimal role.

Resection of course must plow through chromatin and it is not yet clear how these complexes accomplish the necessary chromatin remodeling. Deleting the Arp8 subunit of the Ino80 complex has—in some hands—a modest effect on resection. But a much more profound inhibition of resection is seen when the Swi2/Snf2 homolog Fun30 is deleted (G. Ira, personal communication; B. Llorente, personal communication; V. Eapen, N. Sugawara, M. Tsabar, and J. E. Haber, unpublished results). Fun30 is an ATPase that has been shown to displace a positioned nucleosome *in vitro* (Neves-Costa *et al.* 2009; Awad *et al.* 2010).

Recruitment of Rad51 recombinase and the search for homology

Once long 3′ tails have been generated, they can associate with the Rad51 recombination protein that facilitates a search for homologous regions, to initiate recombination (Figure 6). Chromatin immunoprecipitation (ChIP) experiments have shown that once ssDNA is generated, it is first bound by the ssDNA-binding protein complex, RPA, which is then displaced by Rad51 (Wang and Haber 2004). The loading of Rad51 depends completely on the Rad52 protein (Sugawara *et al.* 2003; Wolner *et al.* 2003). In the absence of the Rad55 and Rad57 proteins, which are known as Rad51 paralogs, Rad51 filament assembly is slow and apparently incomplete, and *MAT* switching fails to occur. In other DSB-mediated repair events where the donor is not silenced, recombination also fails in the absence of Rad55 or Rad57 (Sugawara *et al.* 2003; Wolner *et al.* 2003).

ChIP, using an anti-Rad51 antibody, allows one to visualize the kinetics of Rad51 loading onto ssDNA (Figure 7). The same approach permits visualization of the synapsis between the *MAT* DSB and the donor, as Rad51 will be associated with both the invading *MAT* strand and the *HMLα* duplex DNA (Figure 7). This step takes ~15 min after appearance of Rad51 assembly at the DSB (Sugawara *et al.* 2003; Wolner *et al.* 2003; Hicks *et al.* 2011). The time to achieve pairing between *MAT* and *HML* has also been seen microscopically by examining GFP-tagged LacO and TetR arrays situated close to *HML* and *MAT*, respectively (Bressan *et al.* 2004; Houston and Broach 2006). It should be noted that the time to pair with donor sequences located interchromosomally is significantly longer than what occurs between *HML* and *MAT*. The relatively rapid encounter between these two loci is undoubtedly aided by the *cis*-acting recombination enhancer (RE), located ~17 kb centromere proximal to *HML*, which will be discussed in detail below.

It is striking that the amount of homology shared by *MAT* and its donors is quite small, especially on the Z side, which seems to initiate copying of the donor. *MAT* and *HMR* only share 230 bp, while *MAT* and *HML* share 327 bp. In contrast, there is much more extensive homology on the W/X side, beyond the Y nonhomologous sequences. But the efficiency of repair is largely dictated by the smaller Z side. We will discuss the mechanism of donor preference in detail below, but suffice it to say that one can set up an experiment in which *MAT* will normally switch with *HMR* as a partner and *HML* is the "wrong donor." By artificially increasing the size of the homology on the Z side of *HML* from 327 to 650 to 1800 bp, one can significantly increase its use as a donor in

[9]Most likely the exonuclease(s) are removed by a more rapidly moving repair DNA polymerase, which is needed to fill in ssDNA regions when the repair is completed. Genetic experiments suggest that the translesion DNA polymerase Polζ acts at this step (Holbeck and Strathern 1997). But there must be alternative polymerases because deleting *REV3* does not impair the completion of repair.

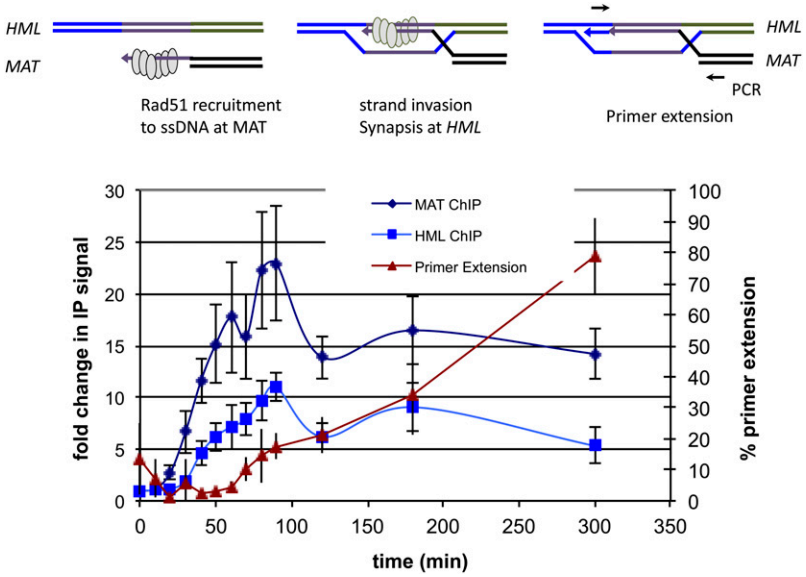

Figure 7 Detection of intermediates of *MAT* switching. Chromatin immunoprecipitation (ChIP) and PCR can be used to detect three early intermediates in *MAT* switching. (Top, from left to right) First, Rad51 assembles on the resected end of the *MAT*-Z region, as detected by ChIP using a pair of PCR primers specific to the *MAT*-distal region. Then Rad51::ss*MAT*-Z DNA engages the homologous sequences of *HML*, detected by PCR primers specific for sequences to the right of *HML*-Z. Finally, the initiation of new DNA synthesis is detected by a PCR assay using one primer in *HML*-Yα and a second primer distal to *MAT*-Z, so that no amplification is possible until at least 50 nt of new DNA synthesis has occurred. (Bottom) Data for these three processes are modified from Hicks *et al.* (2011).

competition with *HMR* (Coïc *et al.* 2011). This is a surprising result because there is always 1400 bp of homology in the W/X region; it suggests that the engagement of the donor is done by the side of the DSB that can initiate new DNA synthesis. The results suggest that although the preferred donor will be encountered in a population of cells 90% of the time, this choice is not irrevocable. On the basis of a model in which an encounter between the DSB end and a donor of normal size is only likely to culminate in a switching event, but that the success of the encounter will depend on the size of the homologous region, it appears that encounters between the Rad51 filament and the preferred donor will happen on average four times before some irreversible step will lead to the completion of recombination (Coïc *et al.* 2011). If the wrong *HML* donor carries a donor that shares much more homology with *MAT* on the right side, then whenever the DSB end encounters *HML* it will have a higher probability to complete repair.

Once resection exposes both the X and Z homologous regions flanking the DSB, they can each synapse with their homologous sites in *HML* (Hicks *et al.* 2011), but until the nonhomologous Y region is removed from the left end of the DSB, it does not seem to participate in the next key step in gene conversion, the initiation of new DNA synthesis, primed by a 3′ end of the strand-invaded DNA. The 3′ end of the invading strand in *HML-Z1* acts as a primer to initiate new DNA synthesis, copying the Y region of the donor (Figure 7). This step can be seen by a PCR reaction using two primers—one complementary to sequences distal to *MAT* and one in the Yα region of the donor (White and Haber 1990). PCR amplification can take place only after the invasion of the 3′-ended single strand into the Z region of the donor locus and the copying of at least 50 nt, primed from the 3′ end, thus creating a recombination intermediate that covers both primers. This step occurs ~15–20 min after synapsis between *MAT* and *HML* is observed by ChIP and 15–30 min prior to the completion of gene conversion, as monitored by both Southern blots and a second PCR assay, detecting the time when the donor Y sequences are joined to the proximal side of *MAT* (White and Haber 1990; Hicks *et al.* 2011).

The region that is replaced during *MAT* switching is substantially longer than the Y region itself. McGill *et al.* (1989) used artificial restriction sites inserted at different places in the X and Z regions to show that replacement of the Y segment often extends well into the flanking homologous regions. They further showed that there was no reciprocal transfer of markers from *MAT* to the donor. This observation was supported by studies of the mismatch repair of a single-bp mutation only 8 bp from the 3′ of the HO cut, in the Z region (Ray *et al.* 1991). In the absence of mismatch repair, this mutation was most often retained during switching (thus confirming physical studies showing that there was almost no 3′ to 5′ removal of the 3′-ended tail). Usually only one of the two daughter cells carried the mutation. This type of postswitching segregation is analogous to postmeiotic segregation observed among meiotic segregants when the DNA inherited into one spore is heteroduplex (mutant/wild type) in the absence of mismatch correction. A kinetic analysis (Haber *et al.* 1993) further demonstrated that, in repair-proficient cells, mismatch correction occurred very rapidly (as quickly as the PCR-amplified intermediate could be detected), suggesting that correction occurred soon after the strand invaded the donor locus. Moreover the heteroduplex DNA was corrected in a highly biased way, such that mutant sequence in the invading Z DNA was corrected to the genotype of the donor. This observation is probably the most direct *in vivo* demonstration of the idea that mismatch repair will preferentially correct a mismatch adjacent to a nick (in this case, the 3′ end of an invading strand) (Porter *et al.* 1993; Leung *et al.* 1997).

Recently, it has been possible to examine strand invasion at the level of chromatin, using a PCR-based nucleosome

protection assay after treating chromatin with micrococcal nuclease (Hicks *et al.* 2011). As noted before, *HML* has very highly positioned nucleosomes, whereas there is little order to nucleosomes at *MAT*. At the time of synapsis, there is a transient reduction in protection of several nucleosomes at the site of strand invasion. By delaying the initiation of new DNA synthesis, it was possible to see a longer period of change in nucleosome organization. This study produced two interesting results. First, the region of nucleosome protection extended several nucleosomes beyond the point where strand invasion occurred. (Recall that *MAT* and *HML* share only 327 bp on the right side of the break where strand invasion is seen.) This observation suggests that the D loop is extended—perhaps by helicases—prior to the initiation of new DNA synthesis. It has not been determined yet whether the extended and apparently open region would bind RPA.

The second important finding was that nucleosome rearrangement at *HML* did not occur in a *rad54*Δ mutant. Rad54 is a Swi2/Snf2 homolog that has been shown to engage in chromatin remodeling *in vitro* (Jaskelioff *et al.* 2003). The surprising finding here is that some sort of strand invasion occurs in the absence of Rad54, as seen by ChIP for Rad51 associating with *HML*-adjacent sequences (Sugawara *et al.* 2003); but this association apparently is distinct from the full chromatin rearrangement necessary to complete DSB repair. Without Rad54, there is no primer extension and new DNA synthesis. One possible explanation for this result is that the kind of association of *MAT* and *HML* strands mediated by Rad51 is a *paranemic* joint in which the invading strand does not intertwine with the donor duplex, whereas with Rad54 a *plectonemic*, interwound structure is formed with the displaced strand in an extended D loop (see Figure 5).

The synthesis-dependent strand-annealing (SDSA) mechanism that seems to be used predominantly differs from normal DNA replication in that the newly synthesized strand is displaced from the template and is subsequently used by the second end as the template for completing gene conversion. This model predicts that all the newly copied DNA should be found at the recipient locus while the donor remains unaltered. Indeed this is the case, as measured by a heavy $^{15}N/^{14}N$ density-shift experiment analogous to that used by Meselson and Stahl to show that bacterial replication was semiconservative. Here the outcome is "conservative" (Ira *et al.* 2006).

For the second strand's end to act as a primer, the nonhomologous Y**a** sequences have to be removed. One strand is of course removed by 5′ to 3′ resection, but the second one is clipped off—apparently only after the elongated first strand anneals in *MAT*-X region—by the Rad1-Rad10 endonuclease (Fishman-Lobell and Haber 1992; Lyndaker *et al.* 2008). In other HO-induced events (and probably in *MAT* switching) removal of the nonhomologous tail also requires a number of other proteins, including Msh2-Msh3, Saw1, and Slx4 (Colaiacovo *et al.* 1999; Li *et al.* 2008). This "clippase" acts apparently only on branched, annealed structure with a 3′-ended tail, which is also produced during single-strand annealing. This step also requires the action of either of the two DNA damage-responsive protein kinases, Mec1 or Tel1 to phosphorylate Slx4 (Toh *et al.* 2010).

Copying the donor sequences

Physical analysis has also made it possible to analyze conditional lethal mutants to ask which DNA replication enzymes are required for *MAT* switching. In contrast to another HO-induced repair event, break-induced replication,[10] only a fraction of the proteins necessary for origin-dependent DNA replication are also required for *MAT* switching (Lydeard *et al.* 2010). In this process, which appears to involve the elongation of one strand and then the elongation of the second strand, DNA polymerase α is not required, while both DNA polymerases δ and ε appear to act either sequentially or redundantly. The PCNA clamp is required, but the GINS-Cdc45-Mcm helicase complex is dispensable. A mutation of the largest subunit of RPA, *rfa1-t11* (L45E) is also required at or after strand invasion, as Rad51 can load and engage in synapsis with *HML* but there is no new DNA synthesis. In contrast, DNA synthesis during *MAT* switching does not need most of the loading factors required at an origin for normal replication, including the ORC proteins, Cdt1 and Cdc6. The exception is that the Dpb11-Sld2-Sld3 proteins are required. These proteins have been shown to be part of a preloading complex at origins (Muramatsu *et al.* 2010), but how they would work when DNA copying is not dependent on an ARS or on other early-functioning proteins is unknown.

The fact that DNA synthesis during gene conversion does not use all the processivity factors employed in normal replication may explain why gene conversion is much more susceptible to mutation of the replicated sequences. Taking advantage of several features of *MAT* switching, it was possible to select for mutations that arose during gene conversion (Hicks *et al.* 2010). First, the normal **a**1 ORF of *HMR***a** was replaced by the *K. lactis URA3* ORF, also removing part of the HO cleavage site (Figure 8). Because *HMR* is silenced, the cells remain Ura$^-$. With *HML* deleted, when *MAT*α is induced to switch with GAL::HO, the Y**a**::*Kl-URA3* sequences are gene converted into *MAT*, allowing the ORF to be expressed. Thus nearly all cells become Ura$^+$. However, if a mutation arose during switching, then the cells would be Ura$^-$ and thus resistant to selection by 5-FOA, which kills Ura$^+$ cells. Such mutations arose at a rate of $\sim 1 \times 10^{-5}$, ~1000 times higher than the spontaneous rate of mutation for a *MAT* locus carrying the same Y**a**::*Kl-URA3*. That these were *de novo* mutations could be demonstrated by showing that these cells still carried a wild-type *Kl-URA3* allele at

[10]When only one end of the DSB shares homology with a donor sequence, gene conversion is not possible. Instead, the strand invasion leads to the assembly of a complete leading- and lagging-strand replicative fork, which can process down the entire chromosome arm to the telomere, in some cases copying at least 100 kb (reviewed by McEachern and Haber 2006).

HMR. After inhibiting Sir2, with the addition of nicotinamide to the medium, the cells became Ura$^+$, evidence that the Y**a**::*Kl-URA3* at *HMR* had not been altered. The majority of mutations were base-pair substitutions but ~40% represented template jumps, as if the DNA polymerase was less processive than would be found during normal replication. There were three types of mutations: −1 frameshifts in homonucleotide runs, complex mutations explained by the use of quasipalindromes, and remarkable jumps from the *Kl-URA3* sequences to the 73% homeologous *S. cerevisiae ura3-52* sequences located on a different chromosome. In the last case, there had to be a second jump, back to *Kl-URA3* sequences, to complete the "switch." Even more surprising is that all three types of mutations were eliminated in a strain with a proofreading defect in DNA polymerase δ. We surmised, on the basis of some *in vitro* studies of a similar proofreading mutation (Stith *et al.* 2008), that the proofreading-defective mutant enzyme is in fact less prone to dissociate from the template. This result argues strongly that DNA Polδ is a major player in *MAT* switching. However, there was also evidence that DNA polymerase ε was active, since a proofreading-defective mutant of Polε resulted in the appearance of +1 frameshifts. The appearance of these mutations in gene conversion was apparently independent of the mismatch repair system and insensitive to the error-prone DNA polymerase Polζ or another translesion DNA polymerase, Polη.

Completion of switching

One of the other striking aspects of *MAT* switching is that it is very rarely accompanied by crossing over. Such exchanges produce lethal outcomes: a *MAT-HML* centromeric deficiency chromosome that would lack essential genes distal to *MAT* or a large internal and equally lethal *MAT-HMR* deletion (Haber *et al.* 1980b; Klar and Strathern 1984). Crossovers are not expected when the SDSA mechanism is used, because there is no stable single or double Holliday junction that would be cleaved to produce crossovers. On the basis of ectopic recombination studies in which HO induces a gene conversion between *MAT***a** on chromosome V and an uncuttable *MAT***a**-*inc* allele on chromosome III (and where the normal donors are deleted), it seems that crossovers are prevented predominantly by the action of two helicases, Sgs1 (with its partners Top3 and Rmi1) and Mph1 (Ira *et al.* 2003; Prakash *et al.* 2009). The Sgs1 complex acts as a dissolvase to remove double Holliday junctions that would otherwise become crossovers. Mph1 appears to ensure that the SDSA pathway is used rather than the alternative double-strand break repair mechanism that is much more prevalent in meiosis.

Finally, it is worth noting that *MAT* switching represents a case of gap repair rather than strictly break repair; that is, the regions of homology located by the two DSB ends are separated on the donor template by ~700 bp. In a related study, using HO-cleaved *LEU2* sequences, we found that break repair and gap repair, when the gap was larger than ~2 kb, are surprisingly different repair processes (Jain *et al.* 2009). Break repair occurs with relatively rapid kinetics, whereas there is a delay of hours before new DNA synthesis is initiated when there is a long gap. This delay is quite similar to that seen when only one end of the DSB is homologous to a template and repair can only occur by assembling a complete replication fork, resulting in break-induced replication (BIR). Consequently long gap repair and BIR depend on Pol32, a nonessential subunit of DNA Polδ, while break repair (and *MAT* switching) is Pol32 independent. Apparently the two ends of the DSB need to be in contact with each other at the time of strand invasion; they must pair close enough to each other, and in the proper orientation, to permit some signal to be propagated. We termed the assessment of the nature of the strand invasion—the difference between break repair and gap repair—as a manifestation of a recombination execution checkpoint. *MAT* switching appears to have a small enough gap to be treated as break repair.

Donor preference: On top of the inherent directionality of switching, namely, that *HML* or *HMR* are not cut by HO and therefore only donate sequences to *MAT*, there is an elaborate mechanism that gives yeast the ability to choose between its two donors. It makes sense that *MAT***a** should seek out and recombine with *HML*α rather than *HMR***a**, so that the recombinational repair of the DSB will lead to a switch to the opposite mating type. Donor selection is however not dictated by the Y**a** or Yα content of the donors: a strain with reversed silent information (*HML***a** *MAT***a** *HMR*α) still chooses *HML* ~85–90% of the time (Klar *et al.* 1982; Weiler and Broach 1992). Weiler and Broach showed that replacing the entire *HML* region including its silencers with a cloned *HMR* locus did not change donor preference, so it is the *location* of the donor, not the sequence differences between *HML* and *HMR*, that direct donor selection. There must therefore be one or more *cis*-acting sequences, outside of the donors themselves that activate or repress one or both donors, depending on mating type.

*MAT*α's choice of *HMR* over *HML* occurs independently of the *MAT*α1 gene, but is strongly dependent on *MAT*α2, the gene that acts as a repressor of **a**-specific genes (Hicks *et al.* 1977; Tanaka *et al.* 1984; Weiler *et al.* 1995; Wu *et al.* 1996; Szeto and Broach 1997; Szeto *et al.* 1997). *MAT***a** donor preference does not depend on a functional *MAT***a**1 gene (Wu and Haber 1995). These observations might suggest that *MAT***a** cells activate *HML* for switching through one or more **a**-specific gene products that are turned off in *MAT*α cells, while α-specific proteins might activate *HMR*. However, as we will see, *HMR* appears to be used as the default locus and all the active regulation is in making *HML* more accessible (Figure 9). Thus a *MAT***a** cell deleted for *HML* can easily use *HMR*, but 10–20% of *MAT*α cells die when their only choice of a donor is *HML* (Wu and Haber 1996; Wu *et al.* 1996, 1997). The failure of many *MAT*α cells to use the "wrong" donor occurs despite the fact that cells experiencing an unrepaired DSB become arrested at a G2/M checkpoint

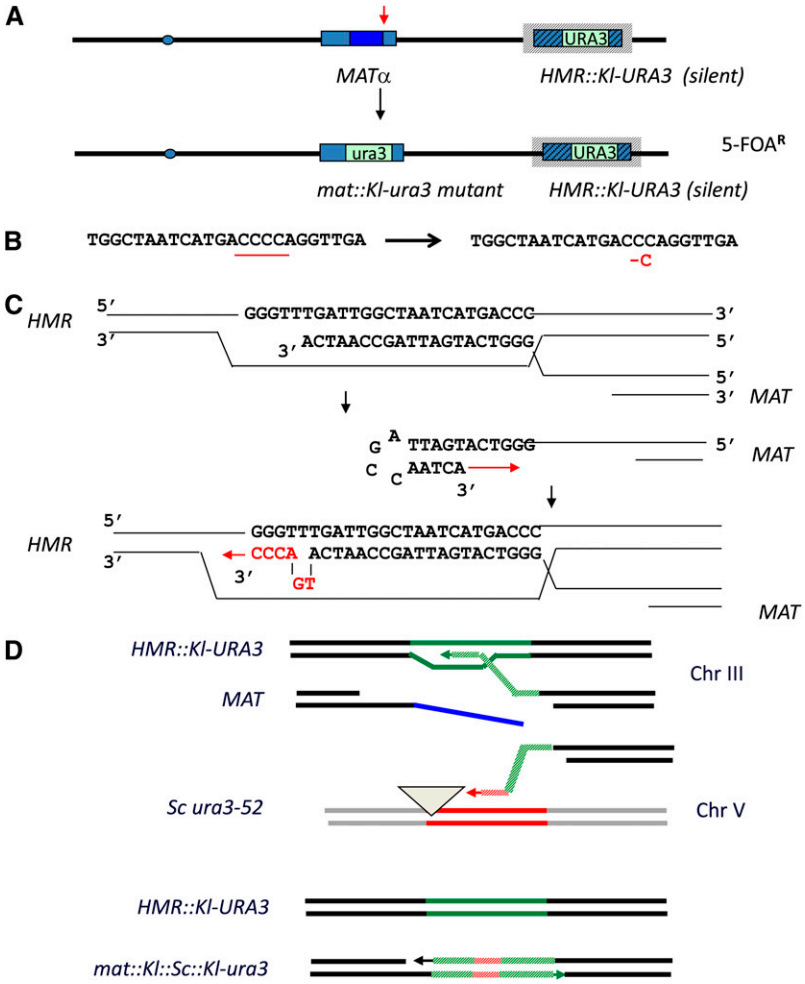

Figure 8 Mutations arising during *MAT* switching. The Y**a** sequences of *HMR***a** were replaced by *K. lactis URA3* (*Kl-URA3*) sequences such that the normal HO cleavage site at the Y**a**-Z border was ablated (A). *HML* was also deleted, so that induction of HO endonuclease resulted in the switching of *MAT*α to *mat*::*Kl-URA3*. At a rate of ~1 in 10^5, the switched sequences were Ura3$^-$ and 5-FOA resistant. About half of the mutant events were single-base-pair substitutions, but the rest apparently resulted from template switching during repair, resulting in −1 frameshifts in homonucleotide runs (B), frameshifts by copying quasi-palindromes (C), and interchromosomal template jumps using the homeologous *ura3-52* sequences on a different chromosome (D).

(Sandell and Zakian 1993); this checkpoint should theoretically have allowed cells time to locate a donor and repair the DSB by gene conversion, but as DSB ends are resected, alternative, lethal recombination events—for example, between several Ty retrotransposons located 30 kb proximal to *MAT* and Tys on other chromosomes—may lead to the death of cells that cannot easily repair the DSB (VanHulle *et al.* 2007).

Identification of a recombination enhancer

To locate a *cis*-acting element (now called the recombination enhancer) on the left arm of chromosome III, Wu and Haber (1996) deleted *HML* at its normal location, 12 kb from the telomere, and inserted either *HML*α or *HMR*α at a site 41 kb from the left end of the chromosome. At this position the donor was still strongly preferred in *MAT***a** cells. By a series of truncation deletions they identified a region that was required to activate the donor at 41 kb. A further series of internal deletions was created to pinpoint the key *cis*-acting element. A 2.5-kb deletion located 17 kb proximal to *HML* completely reversed donor preference, so that a *MAT***a** cell now used *HML* only 10% of the time instead of 90% (Figure 9). Deletion of this sequence also abolished *MAT***a** donor preference for donors located 41, 62, and 92 kb from the left end. In the absence of this sequence, donors at these positions were not used 50:50 with *HMR*; rather they were found only 10% of the time. Deletion of this region had no effect on *MAT*α cells, which continued to use *HMR* most of the time.

Further mapping of RE was accomplished by inserting subfragments of the smallest deletion back into the chromosome. This led initially to the identification of a 700-bp RE that restored *MAT***a** donor preference almost to wild-type levels. Subsequent analysis has narrowed down the most important sequences to ~250 bp, although full activity resides in a region of ~400 bp. This further refinement was accomplished by showing that a syntenic region in *S. carlsbergensis* and in *S. bayanus* (but not in more distant species such as *S. servazzii*) contained an active RE that would substitute for the *S. cerevisiae* RE (Wu *et al.* 1998; Sun *et al.* 2002). By comparing the divergent sequences of these REs, we defined five well-conserved subdomains, named A–E (Figure 9B). Of these, domain B appears to be unimportant, because it can be deleted without significant effect on *MAT***a** or *MAT*α donor preference. In a minimum enhancer of 250 bp lacking region E, deletions of subdomains A, C, and D all abolish *MAT***a** donor preference, causing cells to use *HMR* 90% of the time. Subdomains C and D

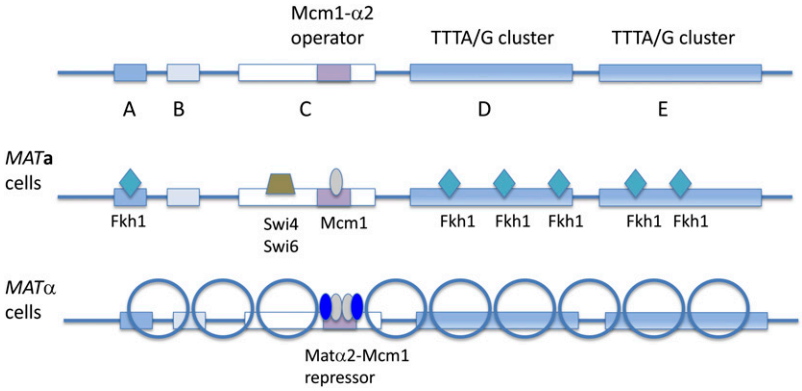

Figure 9 Consensus elements in the RE and protein binding. (Top) DNA sequences shared by evolutionarily conserved and functional RE elements in ~250 bp from *S. cerevisiae*, *S. bayanus*, and *S. carlsbergensis*. (Middle) In *MAT***a** cells, Mcm1 binding facilitates the binding of Swi4-Swi6 and multiple copies of Fkh1. (Bottom) In *MAT*α cells, the Matα2-Mcm1 repressor binds to a 31-bp conserved operator that is shared by **a**-specific genes.

can be inverted relative to A and B and still function properly. It was startling to discover that subdomain A was completely missing from the 700-bp RE defined by Wu and Haber (1996). Clearly, other sequences in the larger region must carry out redundant functions that subdomain performs in the minimum RE.

Subdomains D and E are intriguing because of their unusual sequence. There are 10 or 15 perfect repeats of TTT (G/A). Truncations of this region, leaving only 8 repeats, reduces donor preference by ~50% and further truncations have no RE activity (Wu *et al.* 1998). However, further analysis has revealed that what domains A, D, and E have in common is that they each contain one or more binding sites for the Fkh1 transcription regulator (Wu *et al.* 1998; Sun *et al.* 2002). As we will see below, this protein plays a central role in the activity of RE. A key finding was that one could replace the entire RE with multimers of only region A, or similarly with multimers of D or E, and retain RE activity. Most strikingly, four copies of the 21-bp region A was sufficient to raise *HML* usage from ~10% in the absence of any RE to 65%. A 2-bp site-directed mutation in the A sequence abolished this activity. Region A, but not the 2-bp mutant form, binds Fhk1. The activity of the 4xA construct is abolished in a *fkh1*Δ mutant.

Role of an Matα2-Mcm1 operator in turning RE off in MATα cells

RE lies in perhaps the largest "empty" region of the yeast genome, ~2.5 kb with no open reading frames or regulatory sequences associated with the flanking genes, *KAR4* and *SPB1*. Although RE regulates the usage of *HML*, it does not do this through any global change in apparent chromosome condensation or transcriptional activity, as measured by the level of expression of genes along the left arm of chromosome III. However, there are dramatic chromatin changes in the 2.5-kp intergenic region harboring RE. In *MAT***a**, the region is "open" and binds a number of proteins, whereas in *MAT*α cells, the region is covered with highly positioned nucleosomes that cover the entire RE and extend between, but not into, two flanking open reading frames (Weiss and Simpson 1997; Wu *et al.* 1998). This change is mediated by the Matα2-Mcm1 repressor complex that represses transcription at all **a**-specific genes (Smith and Johnson 1994; Tan and Richmond 1998).

The 90-bp domain C harbors a conserved 31-bp consensus Matα2-Mcm1 binding site. This same repressor binding turns off the RE in *MAT*α cells, in conjunction with the corepressor Tup1p (Szeto and Broach 1997). Mutations of the Matα2-binding sites is sufficient to alter donor preference in *MAT*α, so that *HML* usage is increased from ~10% to >50% (Szeto and Broach 1997; Wu *et al.* 1998). These results also suggest that any **a**-specific gene products are unlikely to play an essential role in activating RE, since these genes should still be repressed by Matα2-Mcm1 in the *MAT*α cell. However, the difference between *HML* use in *MAT***a** (80%) and *MAT*α (55%) in this mutant RE could be attributed to **a**-specific genes.

With the exception of the RE, all Matα2-Mcm1 binding sites are located just upstream of **a**-specific genes. The RE contains no open reading frame, but indeed there are two "sterile" transcripts of the RE region that are transcribed in *MAT***a**, but not *MAT*α (Szeto *et al.* 1997). Despite the increasing evidence of the role of noncoding RNAs in regulating chromatin structure in organisms that also have RNAi silencing, it is unlikely that the sequence of the RNA transcript is important for RE activity, as truncations of RE that remove all of this transcribed region have full activity (Wu and Haber 1996; Wu *et al.* 1998; Li *et al.* 2012). Indeed, as we will see below, the entire RE can be replaced by completely foreign, LexA-binding domains and RE activity can be mimicked by the binding of a LexA-Fkh1 protein.

Activation of RE in MATa cells depends on Mcm1 protein

The Mcm1 protein is not only a corepressor; it can also act as a coactivator of transcription for both **a**-specific (Elble and Tye 1991) and α-specific genes (Hagen *et al.* 1993; Bruhn and Sprague 1994). In the case of α-specific genes, Mcm1 acts as a heterodimer with Matα1p (Figure 3); no possible coactivator with Mcm1 in *MAT***a** cells has been identified. A 2-bp mutation that eliminates Mcm1 binding in the Matα2-Mcm1 operator sequence abolishes *MAT***a** donor preference (Wu *et al.* 1998); *HML* is used only 10–20% of the time, even in the case where the 2-bp mutation is introduced into an otherwise unmodified chromosome III. Thus, although

the minimum RE is ~250 bp long and apparently contains some regions of redundant function, the elimination of Mcm1p binding is sufficient to completely inactivate RE. A single-amino-acid-substitution mutation in *MCM1* (*mcm1-R89A*) also reduces Mcm1 binding and had a similar reduction in *HML* usage (Wu *et al.* 1998).

The analysis of Mcm1 binding has led to another surprising finding concerning the role of Matα2 and Mcm1 protein binding in the establishment of repression. The 2-bp operator mutation that prevents Mcm1 binding also causes a dramatic change in the chromatin structure of the RE. Even in *MAT***a** cells, where there is no Matα2 protein, the mutant RE has an array of highly positioned nucleosomes that is very similar to what is seen in normal *MAT*α cells (Wu *et al.* 1998). Apparently other sequences within RE can organize a phased nucleosome structure in the absence of Matα2-Mcm1 binding, although the repressor proteins seem to more precisely position and "lock in" the repressing chromatin structure. The idea that the sequences surrounding the Mcm1-binding site play important roles in both activating and inactivating RE finds support in the otherwise paradoxical observations that a deletion of nearly the entire 31-bp Matα2-Mcm1 operator has a less profound effect on reducing *HML* usage in *MAT***a** cells than does the simple 2-bp deletion of the Mcm1p-binding site (Szeto and Broach 1997; Wu *et al.* 1998). The adjacent sequences may be important in determining how "cold" the left arm of the chromosome is in the absence of RE activation.

Clearly Mcm1 binding is critical in the activation of the normal RE sequence, but it is also evident that since multimers of domain A, D, or E (which lack Mcm1-binding sites) are sufficient to promote preferential use of *HML*; Mcm1's importance is in regulating other chromatin features of the normal RE.

The RE affects other recombination events

The regulation of *HML* is not specific to these particular donor sequences nor to HO-mediated recombination. If *HML* is replaced with an allele of the *leu2* gene and a second *leu2* allele was placed elsewhere on chromosome III—or even on another chromosome—the rate of Leu^+ spontaneous recombination was ≥10 times higher in *MAT***a** cells than in *MAT*α (Wu *et al.* 1996). This difference is lost when the RE is deleted. There is no significant mating-type–dependent difference in mRNA levels for the *leu2* gene inserted in place of *HML* despite a dramatic recombination difference. That RE should stimulate recombination between sequences unrelated to *MAT*, and even between chromosomes, ruled out the idea that there were specific pairing sequences that would bring *MAT* and a left-arm donor together. There was no significant mating-type–dependent difference when a similar experiment was done with one *leu2* allele in place of *HMR*, thus supporting the conclusion that donor preference was effected through changes in the left arm of the chromosome, with *HMR* being a more passive participant.

RE acts over a long distance and is portable

When *HML* was deleted and either *HML* or *HMR* was inserted at other chromosome locations along chromosome III, the donor could be activated at several sites along the entire left arm of chromosome III in *MAT***a** cells, though the efficiency decreased as the donor was moved further from the telomere (Wu and Haber 1995, 1996). Conversely, RE itself can be moved to sites closer to the centromere and still stimulate the use of *HML* (Coïc *et al.* 2006a). RE can also stimulate *HML* usage when *MAT* is moved to a different chromosome (Wu *et al.* 1997). Finally, we have established a competition assay in which the *LEU2* gene is placed near RE and used as a donor to repair an HO-induced DSB in a *leu2* sequence on chromosome V. A second donor, carrying *leu2-K*, is located ~100 kb centromere proximal on chromosome V. When RE is active, the use of the adjacent interchromosomal *LEU2* sequence is ~50% of all repair events, but when RE is deleted, the use of the interchromosomal *LEU2* donor falls to ~15%.

Evidence that RE is portable has come from two additional experiments. First, if a copy of RE is placed near *HMR* in a *MAT***a** strain that also has its normal RE, then the usage of *HMR* increases from ~10–50%, suggesting that RE can activate a nearby *HMR* in its normal location (Coïc *et al.* 2006a). A second approach was to remove *MAT*, *HML*, and *HMR* from chromosome III and to insert them in roughly the same configuration on the larger chromosome V. Here the use of the more distant *HML*, on the opposite side of the centromere, was ~40%, but when RE was inserted near *HML*, its use increased to >90% (Coïc *et al.* 2006a). So RE can work in a similar fashion in an entirely different chromosome context.

It should be emphasized that the "coldness" of the left arm in *MAT*α cells does not depend on the presence of RE; in RE-deleted strains both *MAT***a** and *MAT*α cells use *HML* only 10% of the time (Wu and Haber 1996). Whether there are sequences that intrinsically restrict the motion of the left arm without RE remains a question to be addressed.

RE binds Fkh1 and Swi4/Swi6

As indicated above, we found that several domains of RE should bind one or more copies of Fkh1. Actual binding was confirmed by ChIP, using a functional epitope-tagged Fkh1-HA construct. As expected, Fkh1 bound only in *MAT***a** and not in *MAT*α, where the Matα2-Mcm1 repressor precludes such binding (Coïc *et al.* 2006b). Deletion of Fkh1 markedly reduces *HML* donor preference in *MAT***a** without affecting *HMR*'s use in *MAT*α; however, the reduction is only from ~85 to ~35%, suggesting that there must be other proteins involved in the action of the complete RE (Coïc *et al.* 2006b). When a 4xA construct was used in place of the whole RE, *fkh1*Δ dropped *HML* usage to the same level as deleting RE (Coïc *et al.* 2006b).

Domain C is much larger than the Matα2-Mcm1 operator region. Further inspection revealed that it contains a SCB

that binds the cell cycle regulators Swi4-Swi6, known together as SBF. When the SCB was mutated or if Swi6 was deleted, donor preference dropped, again to ~35% (Coïc *et al.* 2006b). That both Swi4-Swi6 and Fkh1 play nonoverlapping roles in RE activity was shown by the fact that a deletion of SCB, coupled with *fkh1*Δ further reduced *HML* usage to ~15%. This still leaves a small amount of *HML* usage greater than the 10% found in REΔ apparently contributed by several proteins.

Other donor preference mutations: Two other *trans*-acting factors have been shown to play less decisive roles in donor preference. Screening directly for donor preference mutations has not been very productive, largely because mutations that affect HO expression and *cis*-acting mutations that reduce HO cleavage tend to interfere with the evaluation of donor choice scored at the colony level. Only *chl1*Δ has emerged in this way. A deletion of the *CHL1* gene reduces donor preference in *MAT***a** switching from 80 to 60%; but it has no effect on *MAT*α (Weiler *et al.* 1995). *CHL1* is not a donor preference-specific gene; it was identified >20 years ago because *chl1*Δ causes a large increase in both the loss and gain of chromosomes and is therefore most likely a nondisjunction mutation (Liras *et al.* 1978; Gerring *et al.* 1990). Subsequent studies have shown that *CHL1* encodes a nuclear protein with presumed but undemonstrated helicase activity that is implicated in the establishment and maintenance of sister-chromatid cohesion (Petronczki *et al.* 2004). It is possible that if sister-chromatid cohesion is reduced, some cells in which *MAT***a** is repaired after DNA replication might allow the two sisters to search independently for a donor.

The other factors implicated in donor preference are the yKu70 and yKu80 proteins. The Ku complex plays many roles in chromosome architecture and in DSB repair. They are required for the predominant mechanism of nonhomologous end joining that can rejoin the 4-bp 3′-overhanging DSB ends created by HO. They are also critical in associating telomeres with the nuclear periphery and in ensuring the full activity of telomerase. When Ku proteins are deleted, telomeric regions are delocalized from the periphery. But, paradoxically, it seems that *HML* is more frequently associated with the nuclear periphery in a *yku70*Δ mutant. This might explain why, when either Ku protein is deleted, there is an ~10% reduction in *HML* usage in *MAT***a** but no discernible effect on *MAT*α.

It seems that Swi4-Swi6, Yku70-yKu80, and Chl1 may all act in a common pathway, as double mutants among this set continue to use *HML* ~30% of the time, whereas *fkh1*Δ *yku80*Δ and *fkh1*Δ *chl1*Δ both resemble *fkh1*Δ SCBΔ (Coïc *et al.* 2006b). There is some additional contribution of these genes, however, because *fkh1*Δ SCBΔ *chl1*Δ *yku80*Δ reaches to the same low level as REΔ.

How does RE work?

RE does not alter the silencing of *HML*. One way that *HML* might be used more efficiently in *MAT***a** would be if *HML* were much more accessible in *MAT***a**, but this does not seem to be the case. First, there is no obvious difference in the positioning of nucleosomes over *HML* in *MAT***a** *vs.* *MAT*α strains (Weiss and Simpson 1998). Second, whereas *HML* is fully accessible to HO endonuclease in a *sir3*Δ mutant that abolishes silencing, there is no evident change in the trace of HO cleavage in **a** *vs.* α strains when GAL::HO is overexpressed. Finally, if *HML* and its adjacent silencer sequences are replaced by a similar-sized segment of *MAT*α*-inc* (which HO cannot cut), it is not a better donor than the normally silenced *HML*α locus in a RE-deleted *MAT***a** strain (Coïc *et al.* 2011).

One attractive idea to explain RE's role is that it changes the localization or the higher-order folding of the entire left arm of chromosome III to make it more flexible in locating and pairing with the recipient site in *MAT***a** cells. In this view, the chromosome arm would be sequestered or immobilized (perhaps by being bound to the nuclear envelope) in such a way that *HML* is unavailable in *MAT*α or REΔ *MAT***a** cells, even though the chromatin structure at *HML* itself was unchanged. Several approaches suggest that there are differences in chromosome arrangement in the two mating types, but these changes, prior to creation of a DSB, do not seem to explain donor preference. First, chromosome segments near *MAT*, *HML*, and *HMR* can be fluorescently tagged by binding LacI-GFP or TetR-GFP (or some other color) to LacO or TetO arrays. In one such study of fixed cells, there was a small difference in *MAT***a** *vs.* *MAT*α (Bressan *et al.* 2004), but in *MAT***a**, *HML* is not closer than *HMR*. A more detailed study (I. Lassadi and K. Bystricky, personal communication) suggests that in *MAT***a** cells, *HML* is being drawn closer to the centromere. Both fluorescent and chromosome conformation capture (3C) techniques have suggested that *HML* and *HMR* are relatively closer together than either is to *MAT* (Miele *et al.* 2009), but again recent sequencing-based chromosome conformation capture (termed 5C) finds *HML* being more strongly associated with the centromere (J. Dekker, personal communication).

Of course for switching to take place, *HML* and *MAT* or *HMR* and *MAT* must come into contact. Although *HML* and *HMR* have been shown to preferentially reside near the nuclear periphery, it appears that this tethering does not prevent the donors from engaging *MAT* away from the periphery, since Bystricky has observed among cells undergoing switching that *MAT* remains in the center of the nucleus (Bystricky *et al.* 2009).[11]

RE can be mimicked by tethering Fkh1 to the LexA operators

As noted before, RE can be replaced by as few as four copies of the A subdomain of the RE. The strong use of *HML* in using 4xA is eliminated by deleting Fkh1. To explore how

[11]A number of studies have shown that when a DSB cannot be repaired, it becomes associated with the nuclear periphery (Gartenberg 2009). But this pathological state is only seen after several hours and does not seem to reflect what happens during a successful DSB repair event.

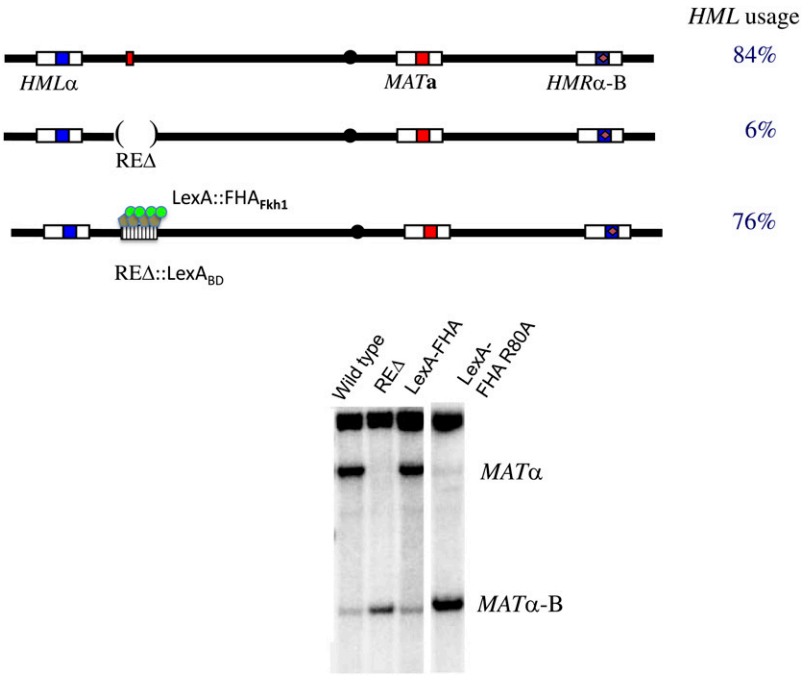

Figure 10 Role of the recombination enhancer in *MAT***a** donor preference. (A) Arrangement of *HML*α, *MAT***a**, and *HMR*α-*Bam*HI (*HMR*α-B) in wild type, REΔ, and when the RE is replaced by four LexA-binding domains to which a LexA-FHA$_{Fkh1}$ fusion protein can bind. (B) Southern blot data after induction of switching showing the proportion of *Bam*HI-digested *MAT*α or *MAT*α-B DNA in the strains depicted above. A strain in which the LexA-FHA$_{Fkh1}$ domain carries a R80A mutation that prevents phosphothreonine binding fails to enhance the usage of *HML* (Li *et al.* 2012)

Fkh1 might act, we took the approach of replacing RE with four copies of the lexA operator and expressing lexA-Fkh1 (J. Lin, E. Coïc, and J. E. Haber, unpublished results). This construct significantly increased the use of *HML* in comparison to expressing LexA or LexA-Swi4. Subsequently, LexA-Fkh1 was further truncated so that it contained only the first 120 amino acids, comprising the forkhead-associated (FHA) domain. This construct proved to have even stronger *HML* usage, whereas other fusions carrying the transcription regulatory domain near the C terminus had no activity (Figure 10). FHA domains have been shown to bind phosphothreonines. Taken together these data suggested that RE might act by binding to phosphothreonines that would only be created or exposed after induction of a DSB. There are a number of proteins that exhibit DSB-induced post-translational modification of both series and threonines, including histones, proteins bound near the DSB end such as Sae2 and RPA, and which might serve as the target for RE's bound FHA domains. Supporting this hypothesis, we showed that mutation of conserved histidine and arginine residues, which are found in all FHA domains, abolished LexA-FHA activity (Figure 10).

If Fkh1's FHA domains bound to RE are responsible for moving *HML* close to *MAT*, then it should be possible to show that FHA associates with *MAT* only after a DSB is induced; indeed ChIP experiments have shown this to be the case, even in strains where *HML* itself is deleted (J. Lin, E. Coïc, and J. E. Haber, unpublished results). Conversely, a DNA damage-dependent chromatin modification that spreads around the *MAT* locus, the phosphorylation of S129 of histone H2A (called γ-H2AX) also spreads to surround the normal RE region (K. Lee and J. E. Haber, unpublished observation). However this Mec1/Tel1-dependent modification is only seen in *MAT***a** cells when RE is able to bind Fkh1.

Neither γ-H2AX nor the DSB-dependent phosphorylation of histone H4-S1 is responsible for donor preference. What remains to be determined is the phosphothreonine-binding partner of the Fkh1-FHA domain and the identity of the damage-dependent protein kinase that is employed. Neither Mec1 nor Tel1 appear to play a role, but strong donor preference is dependent on casein kinase II (J. Lin, E. Coïc, and J. E. Haber, unpublished results).

So, at present we are coming close to understanding the mechanism that underlies donor preference. A simple model to explain donor preference is presented in Figure 11. The cluster of Fkh1 proteins bound at RE in *MAT***a** (but not *MAT*α) comes into contact with DSB-induced, casein kinase II-dependent phosphorylated threonines in proteins that bind near the DSB ends. This association effectively tethers the nearby *HML* locus close to *MAT* and facilitates its use in *MAT* switching. It is still not clear whether there are any other constraints preventing *HML* use in *MAT*α cells or whether there are any facilitating sequences that aid HMR usage. Of course this is hardly the entire story since RE also binds Swi4/Swi6 in domain C, in a cell-cycle–dependent fashion. The activity of the entire RE is likely to be substantially more elaborate.

Mating-type switching and donor preference in other yeasts

Although other distantly related yeasts switch mating type and have silent donor cassettes and even donor preference, these processes are surprisingly different. I once quipped that *MAT* switching in *Schizosaccharomyces pombe* is the same as that in *S. cerevisiae* except in every detail.

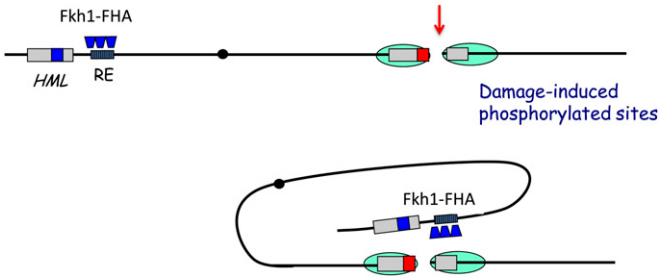

Figure 11 Model for donor preference. A cluster of Fkh1-FHA domains bound to RE in *MAT***a** cells can associate with phosphothreonine residues that are located near the DSB and created by casein kinase II, and possibly other kinases, in response to the DSB. This association tethers *HML*α within ~20 kb of the DSN ends and facilitates its use over *HMR*, located 100 kb away.

Mating-type gene switching in fission yeast, *S. pombe* (Klar 1993; Thon and Klar 1993) also has an expressed mating-type locus, *mat1* locus, which can carry either nonhomologous P or M alleles. Switching from one allele to the other uses one of two silent donors (*mat2P* and *mat3M*), which are close together and located only a short distance away from *mat1* on the same chromosome arm. *mat1*, *mat2*, and *mat3* share regions of homology flanking the P or M region, but they are unrelated to those in budding yeast. The two donors are silenced by heterochromatin established in small part by Sir2 but mostly by a silencing system that is absent in yeast, using a homolog of the Su(var)9 histone methyltransferase, Clr4, and the HP-1 relative, Swi6 (Nonaka *et al.* 2002). And there is no HO-like enzyme. Instead, a persistent single-strand nick is created at *mat1*, which is converted to a DSB when cells enter S phase (Arcangioli 1998; Kaykov and Arcangioli 2004). Thus only one of the two daughter cells can switch, but the repair is directed to *mat2* or *mat3* rather than to repair from the intact sister. Repair therefore takes place in the context of normal replication and the repair is conservative in that both the strands at the *mat1* locus are newly copied (Arcangioli and De Lahondes 2000). Mutations that alter donor preference also alter silencing, which is not the case for *S. cerevisiae* (Thon *et al.* 1994; Thon and Friis 1997). Donor preference involves dramatic changes in chromatin modification and structure (Jia *et al.* 2004). Thus, although the two systems seem to share some common features, they seem to be the consequence of convergent evolution.

The switching system of the more closely related *K. lactis* is substantially more similar to *S. cerevisiae*, in having sequences that are recognizably similar to *MAT***a***1*, *MAT*α*1*, and *MAT*α*2*, but the shared flanking sequences are not closely related to *S. cerevisisae*'s *MAT*-W, X, or Z1/Z2. *HML* carries not only α1 and α2 but also a novel gene, α3, while *HMR* has **a**1 and **a**2; both *HML* and *HMR* are silenced by a Sir2-dependent mechanism (Sjostrand *et al.* 2002). Switching is dependent on the Mts1 protein that is the homolog of the *S. cerevisiae* repressor *RME1*; but here it is required to *activate* switching and is turned off in *MAT***a**/*MAT*α cells by an **a**1-α2 repressor. But the big surprise is that there is no functional *HO* gene, although there is evidence of an eroded, ancient *HO* gene. In its place—at least for *MAT*α to *MAT***a** switching—is the α3 gene, which proves to be a transposable element that can excise from the DNA as a circle and somehow catalyze switching, dependent on Mts1 (Barsoum *et al.* 2010). *MAT***a** to *MAT*α switching proceeds without the α3 gene, but it proceeds by the formation of a DSB with hairpin intermediates, reminiscent of transposon excision. Like the repair of hairpin intermediates in *S. cerevisiae* generated by excision of the plant Ds transposon (Yu *et al.* 2004), the hairpin ends require Mre11 to be cleaved for further steps in repair (Barsoum *et al.* 2010). *MAT***a** switching seems to be under the control of a different transposable element (S. Aström, personal communication).

Postscript

In reviewing what we have learned about mating-type genes and the recombination process leading to *MAT* switching, one tends to focus on recent experiments that have dramatically illuminated the subject. Inevitably some of the pioneering work that laid the foundation tends to become obscured. In some cases, gene names have been changed to reflect a more comprehensive understanding of their function (*e.g.*, *MAR1* is now *SIR2*, *HM***a** is now *HML*α, *HDF1* is now *YKU70*). We owe particular debts of gratitude to: Donald Hawthorne (1963), who published little but inspired many with his recounting the first pedigree analysis of *MAT* switching and the creation of fusions of *MAT* and *HMR* (before *HMR* had been defined), which provided essential keys to the model proposed by Hicks, Strathern, and Herskowitz (1977); Isamu Takano and Yasuji Oshima, whose early studies of *MAT* switching in the early 1970s included the formulation of their seminal transposable "controlling element" model (Takano and Oshima 1970; Oshima and Takano 1971); Vivian MacKay and Thomas Manney, whose sterile mutations, including those in *MAT*α1 and *MAT*α2 provided not only insights about signal transduction but also most of the genetic reagents that were used to demonstrate the way *MAT* alleles were replaced during homothallic switching (Mackay and Manney 1974a,b); Robert Mortimer, whose lab identified most of the key *RAD* genes needed for recombination (Rodarte-Ramon and Mortimer 1972; Game and Mortimer 1974) and who, along with Seymour Fogel, established most of the basic rules about gene conversion (Fogel *et al.* 1979). Michael Resnick also was the first to propose DSB repair mechanisms that fundamentally resemble SDSA and the double Holliday junction mechanisms that we continue to invoke (Resnick 1976).

Acknowledgments

I am grateful for the helpful comments of Laura Rusche, Scott Holmes, and members of my lab. The part of this work that derives from my own lab has been carried out for more than 30 years by an exceptional contingent of graduate

students, postdoctoral fellows, technicians, and Brandeis University undergraduates and has been generously supported, primarily by National Institutes of Health (NIH) grant GM20056, but also by NIH grants GM61766 and GM76020, as well as previous support from the National Science Foundation and the Department of Energy.

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Communicating editor: J. Thorner