An Evolutionary Perspective on Yeast Mating-Type Switching

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ABSTRACT Cell differentiation in yeast species is controlled by a reversible, programmed DNA-rearrangement process called mating-type switching. Switching is achieved by two functionally similar but structurally distinct processes in the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. In both species, haploid cells possess one active and two silent copies of the mating-type locus (a three-cassette structure), the active locus is cleaved, and synthesis-dependent strand annealing is used to replace it with a copy of a silent locus encoding the opposite mating-type information. Each species has its own set of components responsible for regulating these processes. In this review, we summarize knowledge about the function and evolution of mating-type switching components in these species, including mechanisms of heterochromatin formation, *MAT* locus cleavage, donor bias, lineage tracking, and environmental regulation of switching. We compare switching in these well-studied species to others such as *Kluyveromyces lactis* and the methylotrophic yeasts *Ogataea polymorpha* and *Komagataella phaffii*. We focus on some key questions: Which cells switch mating type? What molecular apparatus is required for switching? Where did it come from? And what is the evolutionary purpose of switching?

KEYWORDS mating-type switching; yeast genetics; evolution; sporulation; homothallism

SACCHAROMYCES cerevisiae is a single-celled organism whose cells come in three types, called **a**, α, and **a**/α. Two principles of cellular differentiation that are almost universal in multicellular eukaryotes are violated in this yeast. First, instead of differentiated cells being genetically identical and varying only at the level of gene expression, in *S. cerevisiae* the three cell types differ in their DNA content at the genetic locus (*MAT*) that specifies cell type. Second, whereas determination of cell type in multicellular organisms is a largely irreversible process in which cells cannot regain pluripotency after progressing to a differentiated state, the two haploid cell types of yeast (**a** and α) are able to interconvert in a reversible manner by means of a programmed DNA-rearrangement process called mating-type switching. Indeed, in a unicellular organism such as yeast it is essential that any DNA rearrangements that occur during differentiation must be reversible, because there is no distinction between the germline and somatic cells. Every cell must retain the capacity to produce every other type of cell.

Mating-type switching was the subject of early studies in *S. cerevisiae* genetics and molecular biology (Oshima 1993; Barnett 2007; Klar 2010). Its mechanism of switching is complex, involving multiple components and multiple levels of regulation (Haber 2012). Dissection of how cell-type specification and mating-type switching are controlled in *S. cerevisiae* led to breakthroughs in our understanding of many other fundamental cellular processes including homologous recombination, cell signaling pathways, gene silencing, and mechanisms of transcriptional regulation (Herskowitz 1989; Rusche *et al.* 2003; Bardwell 2005; Li and Johnson 2010; Haber 2012). In fact, the idea of using arrows and T-bar symbols in network diagrams to symbolize gene activation and repression, respectively, is attributable to Ira Herskowitz (Botstein 2004) whose laboratory discovered the cassette mechanism of switching in *S. cerevisiae*.

Despite our detailed knowledge of the switching mechanism in *S. cerevisiae*, there has been little investigation of the evolutionary origins of this process. Switching seemed to appear abruptly within the family Saccharomycetaceae (Butler *et al.* 2004), with a similar but independently arising

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process also occurring in the very distantly related fission yeast *Schizosaccharomyces pombe* (Klar 2007; Nielsen and Egel 2007; Ni *et al.* 2011). However, insights into the evolution of switching have recently come from studies of methylotrophic yeasts (Hanson *et al.* 2014; Maekawa and Kaneko 2014; Riley *et al.* 2016) and of *Kluyveromyces lactis* (Barsoum *et al.* 2010a; Rajaei *et al.* 2014). The goal of this review is to summarize our current knowledge of the evolution of switching in yeasts, its components and regulation, and the evolutionary forces that underlie its maintenance in nature. Due to space limitations, we have concentrated on topics of particular evolutionary relevance, and on more recent publications. For aspects of mating and mating-type switching not covered here, and for a view of the historical context, we refer readers to several excellent review articles and books (Herskowitz 1989; Heitman *et al.* 2007; Madhani 2007; Haber 2012; Klar *et al.* 2014).

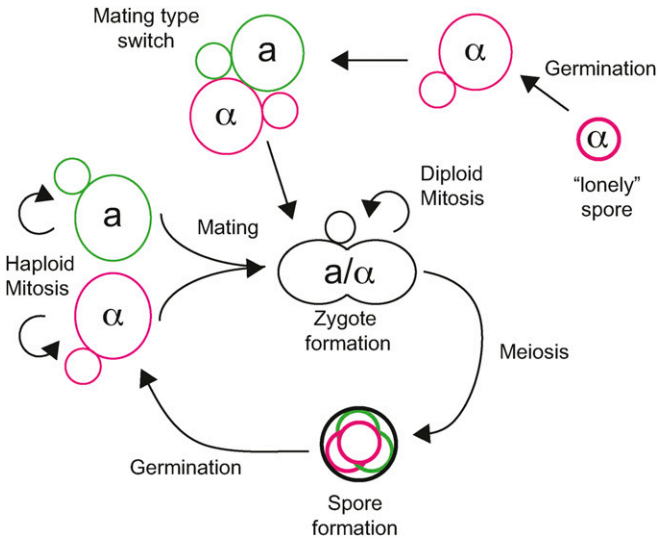

Figure 1 Schematic life cycle of *S. cerevisiae*.

Cell-Type Specification in Saccharomycotina

The life cycle of budding yeasts (Figure 1) is primarily comprised of three cell types: haploids of two isogamous mating types, **a** and α, and **a**/α diploids (Herskowitz 1988; Madhani 2007). The two types of haploid are often called mating types because they describe mating behavior: mating occurs only between **a** cells and α cells. Mating-type switching is the process by which a haploid **a** cell can become a haploid α cell, by changing its genotype at the mating-type (*MAT*) locus from *MAT***a** to *MAT*α, or vice versa. Although it is historically called mating-type switching, the process could also be called cell-type switching.

All three cell types can divide mitotically given favorable environmental conditions, but, in *S. cerevisiae*, vegetatively growing haploid cells of opposite mating types will mate readily if they meet (Merlini *et al.* 2013). Haploid **a** cells express the G protein-coupled receptor Ste2, which detects the α-factor mating pheromone expressed by haploid α cells. Reciprocally, haploid α cells express the receptor Ste3, which binds the **a**-factor pheromone expressed by haploid **a** cells. Interaction between a pheromone and its receptor in either haploid cell type triggers a MAP-kinase signaling cascade resulting in G_1-phase arrest of mitotic proliferation, formation of a mating projection (shmoo) polarized toward the pheromone source, and finally mating by cell and nuclear fusion to generate a diploid zygote (Bardwell 2005; Jones and Bennett 2011; Merlini *et al.* 2013). Diploids are induced to undergo meiosis and sporulation by nutrient-limiting conditions in the environment (specifically starvation for nitrogen in the presence of a nonfermentable carbon source), resulting in the formation of an ascus. The ascus normally contains four haploid spores (two **a**'s and two α's) that germinate upon restoration of favorable conditions (Honigberg and Purnapatre 2003; Piekarska *et al.* 2010; Neiman 2011).

Species within the fungal phylum Ascomycota vary as to whether they prefer to grow vegetatively as haploids ("haplontic") or as diploids ("diplontic") (Phaff *et al.* 1966). Whereas natural isolates of *S. cerevisiae* are primarily diploid, many other yeast species are primarily haploid, including *K. lactis*, *S. pombe*, and the methylotrophic yeasts such as *Ogataea* (*Hansenula*) *polymorpha* (Dujon 2010). Consistent with these ploidy preferences, *S. cerevisiae* mates spontaneously (even in rich media) and uses an environmental cue only for sporulation. In contrast, in haplontic yeasts, mating and sporulation are co-induced by poor environments and usually occur in succession without intervening diploid mitotic cell divisions, for example in *Zygosaccharomyces*, *Kluyveromyces*, *Ogataea*, *Clavispora*, and *Schizosaccharomyces* (Herman and Roman 1966; Gleeson and Sudbery 1988; Booth *et al.* 2010; Merlini *et al.* 2013; Sherwood *et al.* 2014). Sporulation of the zygote immediately after nuclear fusion results in a characteristic "dumbbell-shaped" ascus that retains the outline of the two shmooing haploid cells that formed it (Kurtzman *et al.* 2011).

The *MAT* locus controls processes dictating cell-type identity (Herskowitz 1989; Johnson 1995). For haploid cells (both **a** and α), cell type-specific processes include the induction of competence to mate and the repression of sporulation, whereas diploid cells require repression of mating and the ability to initiate meiosis and sporulation. Other processes also differ between haploid and diploid cells, such as the choice of location for formation of the next bud (axial *vs.* bipolar patterns; Chant and Pringle 1995), and the preferred mechanism for double-strand DNA (dsDNA) break repair (homologous recombination in diploids *vs.* nonhomologous end joining in haploids; Kegel *et al.* 2001; Valencia *et al.* 2001).

The *MAT***a** and *MAT*α alleles (sometimes called idiomorphs) of the *MAT* locus are completely dissimilar in sequence. In *S. cerevisiae* the *MAT*α allele contains two genes, *MAT*α1 and *MAT*α2, and the *MAT***a** allele contains a single gene, *MAT***a**1 (Figure 2). These three genes code for transcription regulators. They determine the cell type of the haploid by activating or repressing expression of **a**-specific (**a**sg)

and α-specific (αsg) genes (Johnson 1995; Galgoczy *et al.* 2004; Haber 2012). There are ~5–12 **a**sg's and αsg's, depending on the species (Sorrells *et al.* 2015). In addition to these, a shared set of haploid-specific genes (hsg's) (~12–16 in number) that facilitate mating is constitutively expressed in both **a** and α cells but not in **a**/α diploids (Booth *et al.* 2010), and a larger group of ~100 general pheromone-activated genes is induced in haploids of both types once a pheromone signal from the opposite type of haploid is detected (Sorrells *et al.* 2015). For example, in *S. cerevisiae*, the pheromone genes *MFA1* and *MFα1* are an **a**sg and an αsg, respectively; the pheromone signaling pathway G protein-subunit genes *GPA1*, *STE4*, and *STE18* are hsg's, and the MAP kinase *FUS3* is a general pheromone-activated gene (Sorrells *et al.* 2015).

In *S. cerevisiae* haploid α cells, the *MAT*α1 gene codes for the HMG-domain transcription activator α1 (previously referred to as an "α-domain" protein but now recognized as a divergent HMG domain; Martin *et al.* 2010), and the *MAT*α2 gene for the homeodomain-transcription repressor α2. The α1 and α2 proteins can both individually form complexes with the constitutively expressed Mcm1 (MADS domain) protein which binds upstream of **a**sg's and αsg's. In α cells, transcription of αsg's is activated because the α1-Mcm1 complex recruits the transcription factor Ste12 to their promoters, while transcription of **a**sg's is repressed because the α2-Mcm1 complex recruits the Tup1-Ssn6 corepressor (Figure 3).

In *S. cerevisiae* haploid **a** cells, the *MAT* locus contains only the *MAT***a**1 gene coding for the homeodomain protein **a**1, but this protein is not required for **a** cell-type identity. The identity of **a** cells is instead defined by the absence of both α1, the activator of αsg's; and α2, the repressor of **a**sg's. Instead of requiring an **a**-specific activator, **a**sg's are activated by Mcm1 and Ste12, which are constitutively expressed in all cell types (Figure 3). Thus in *S. cerevisiae*, the **a** cell type is the default type, and yeast cells lacking a *MAT* locus will mate with haploid α cells.

In **a**/α diploid cells of *S. cerevisiae*, αsg's, **a**sg's, and hsg's are all repressed. These cells have *MAT*α1 and *MAT*α2 genes at the *MAT* locus on one chromosome, and *MAT***a**1 on the other (Figure 2), which results in formation of the **a**1-α2 heterodimer of the two homeodomain proteins. The **a**1-α2 dimer directly represses transcription of hsg's, and indirectly suppresses αsg's through repression of *MAT*α1 (Figure 3). Transcription of **a**sg's in diploids is repressed by α2-Mcm1 as in haploid α cells. Because *S. cerevisiae* uses the formation of a heterodimer to sense heterozygosity of its *MAT* locus, and because this heterodimer is a repressor, there are no "diploid-specific" genes in *S. cerevisiae* (Galgoczy *et al.* 2004). Instead, diploid-specific processes such as meiosis and sporulation are repressed in haploids. This repression is achieved via the hsg *RME1*, a haploid-specific activator that transcribes *IRT1*, a noncoding RNA which in turn represses *IME1*, the master inducer of meiosis (van Werven *et al.* 2012). Thus the combined action of *RME1* and *IRT1* inverts the output of the hsg regulatory logic to restrict *IME1* expression to diploids (Figure 3).

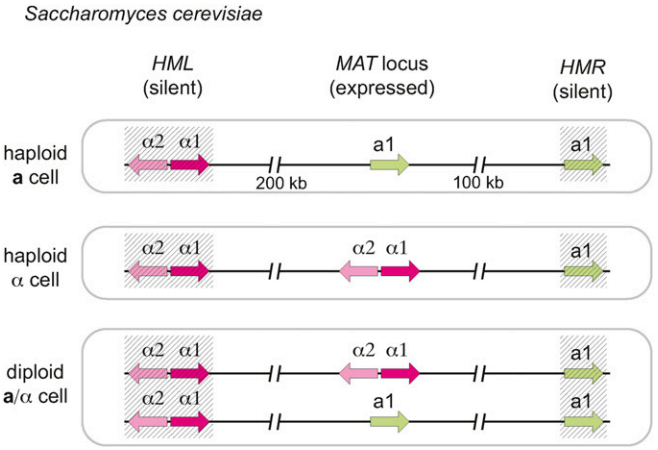

Figure 2 Gene organization in the *MAT*, *HML*, and *HMR* loci on *S. cerevisiae* chromosome *III*. Shading indicates genes whose transcription is repressed.

IME1 expression also requires the environmental signals of nitrogen and glucose depletion that initiate meiosis (Neiman 2011). No genes have constitutive diploid (**a**/α) specific expression in the same way that hsg's, αsg's, and **a**sg's have constitutive cell type-specific expression in haploids.

The **a**sg's and αsg's regulated by the *MAT* locus primarily include genes for pheromones, their receptors, and signaling proteins required for recognition of cells with the opposite mating type (Johnson 1995; Galgoczy *et al.* 2004). Binding of the pheromones to their receptors triggers a signaling cascade called the pheromone response pathway, which induces further gametic differentiation toward mating competence in haploids (Wittenberg and La Valle 2003; Merlini *et al.* 2013). This cascade culminates in the activation of the transcription factor Ste12, which is required for the expression of a large number of genes responsible for mating—the general pheromone-activated genes (Roberts *et al.* 2000; Sorrells *et al.* 2015). Ste12 is expressed in all cell types but when haploids detect a pheromone, Ste12 becomes substantially more active at pheromone-responsive promoters because the Fus3 MAP kinase inactivates two proteins, Dig1 and Dig2, that inhibit Ste12 (van Drogen *et al.* 2001). Ste12 is required for expression of all **a**sg's, αsg's, and hsg's. It binds to hsg promoters as a dimer that can be occluded by the **a**1-α2 complex in diploids, and it is brought to αsg promoters by α1-Mcm1. Ste12 also activates **a**sg promoters in conjunction with Mcm1, although **a**sg regulation has undergone recent dramatic evolutionary change (Sorrells *et al.* 2015).

Rewiring of the Logic Circuit After Whole-Genome Duplication in Saccharomycetaceae

The cell type-specification circuit of *S. cerevisiae* has undergone extensive reorganization since it diverged from other species in the yeast family Saccharomycetaceae, such as *Candida albicans* and *K. lactis*. The reorganization involved three distinct steps: the gain of α2 binding sites upstream of **a**sg's to

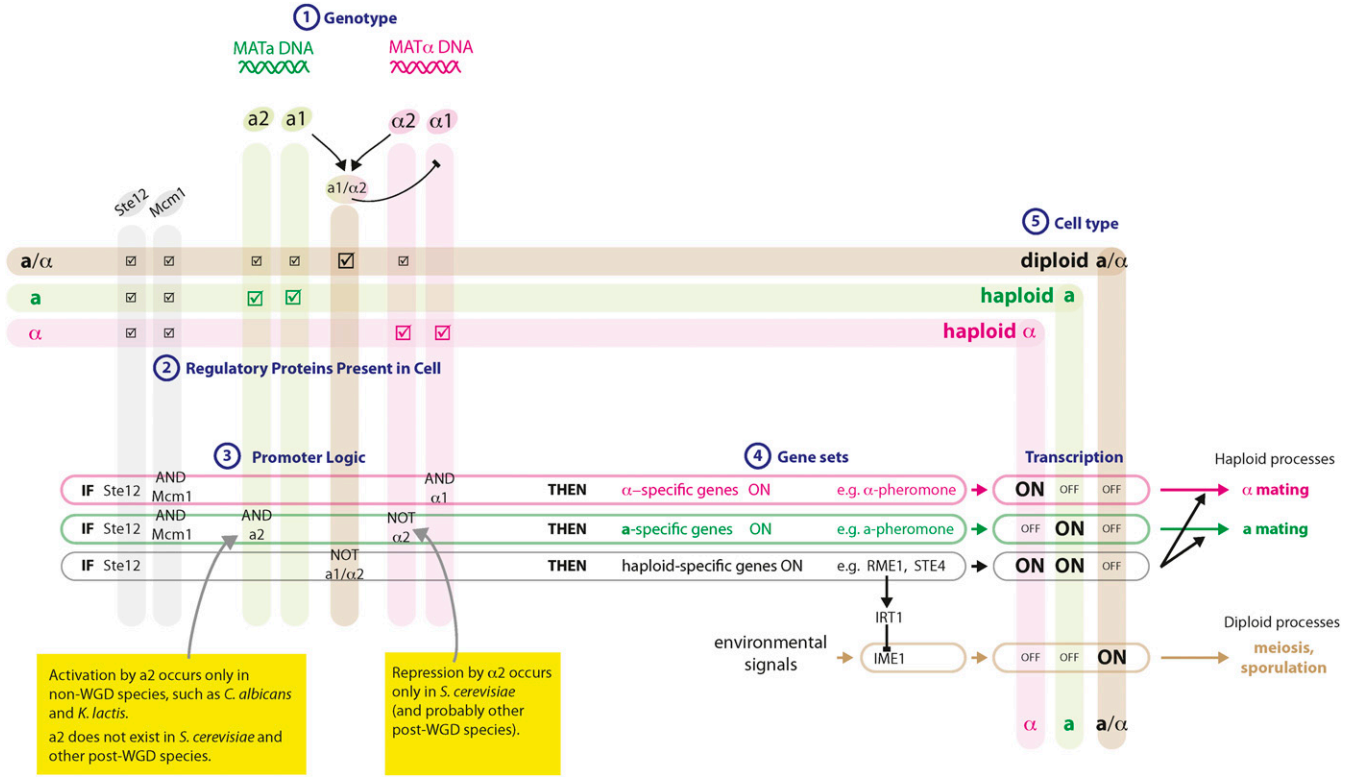

Figure 3 Logic of the cell type-specification circuits in Saccharomycetaceae species. Solid colors represent cell types (green, **a**; pink, α; brown, **a**/α), and outline colors represent gene sets. The genotype (1) of a cell's *MAT* locus specifies the regulatory proteins present in that cell (2; checkboxes), which act at promoters (3) to generate appropriate transcription of the three gene sets (4; **a**sg's, αsg's, and hsg's) and determine the cell type (5). The yellow boxes describe the rewiring event that occurred when *MAT***a**2 was lost, coinciding with the WGD. The diagram summarizes information from the post-WGD species *S. cerevisiae* and the non-WGD species *K. lactis*, *L. kluyveri*, and *C. albicans* (Tsong *et al.* 2003, 2006; Booth *et al.* 2010; Baker *et al.* 2012; Sorrells *et al.* 2015).

repress them in α haploids (Tsong *et al.* 2006), the gain of Ste12 binding sites upstream of **a**sg's so that they are expressed by default (Sorrells *et al.* 2015), and the complete loss of the *MAT***a**2 gene. *MAT***a**2 codes for an HMG-domain transcription activator[1] called **a**2, which acts as an activator of **a**sg's in *C. albicans* and *K. lactis* (Tsong *et al.* 2003, 2006; Baker *et al.* 2012; Sorrells *et al.* 2015). Interestingly, the second and third of these steps occurred on the same branch of the phylogeny as the whole-genome duplication (WGD) (Sorrells *et al.* 2015), but whether they predated or postdated the WGD is not known. Recent phylogenomic analysis shows that the WGD was in fact an interspecies hybridization between two yeast lineages: one related to *Zygosaccharomyces/Torulaspora*, and one related to *Kluyveromyces/Lachancea/Eremothecium* (Marcet-Houben and Gabaldon 2015). *MAT***a**2 is present in most non-WGD species including these two parental lineages, but is absent in *S. cerevisiae* and all other post-WGD lineages including early diverging ones such as *Vanderwaltozyma polyspora* (Scannell *et al.* 2007; Wolfe *et al.* 2015).

Figure 3 summarizes the logic of the cell type-specification circuit in multiple yeast species, and how the output of this logic circuit remained unchanged by the rewiring event (Tsong *et al.* 2006; Sorrells *et al.* 2015). In species such as *C. albicans* and *K. lactis* that retain the *MAT***a**2 gene, repression of **a**sg expression is not required in α cells, because **a**sg's are not on by default, so the repression of **a**sg's by α2 occurs only in *S. cerevisiae*. Therefore *C. albicans MAT*-deletion strains are sterile rather than defaulting to haploid **a** mating behavior (Tsong *et al.* 2003). The phylogenetic relationship (Figure 4) suggests that the cell specification circuit in *C. albicans* and *K. lactis* (Figure 3) represents the ancestral state of the network. Loss of **a**2 in the post-WGD lineage was only possible because, prior to this event, the promoters of **a**sg's in this lineage gained direct DNA-binding sites for Ste12 (Sorrells *et al.* 2015). In the ancestral situation, Ste12 was brought to **a**sg promoters indirectly by a protein-protein interaction with **a**2, as occurs in *K. lactis*. An intermediate state survives in *K. wickerhamii* and *Lachancea kluyveri*, where some **a**sg's have "hybrid" promoters that are both repressed by α2 in α cells and activated by **a**2 in **a** cells (Baker *et al.* 2012). In addition to the loss of *MAT***a**2 in the post-WGD

[1]Confusingly, the gene name *MAT***a**2 was used previously for an ORF located upstream of *MAT***a**1 in the *MAT***a** allele in *S. cerevisiae* (Astell *et al.* 1981). There is no evidence that this *S. cerevisiae* ORF codes for a functional protein. It is actually a copy of part of the 3′ end of the *MAT*α2 homeodomain gene, due to *MAT*α2's partial overlap with the X-repeat region. This ORF is unrelated to the *MAT***a**2 HMG-domain transcription activator gene discussed here, which is present only in non-WGD species.

clade of Saccharomycetaceae, other variations in *MAT* gene content can be found in the CUG-Ser (*Candida*) clade, in which multiple species lack the homeodomain genes *MAT***a**1 and/or *MAT*α2, and one species appears to have no *MAT* genes at all (Butler *et al.* 2009; Butler 2010).

Cell-type specification in yeasts is achieved by combinatorial regulatory gene presence or absence (Figure 3). The requirement for one copy of each *MAT* allele (*MAT***a** or *MAT*α) to form the heterodimeric repressor acts as a sensor of ploidy, ensuring that only diploid cells are competent for meiotic entry (Haag 2007), and that diploid cells are incompetent for additional mating events that would result in aneuploidy. Furthermore, the presence of only one active *MAT* allele in haploid cells prevents the expression and recognition of self-pheromone that would otherwise induce mating processes in the absence of a mating partner. It has therefore been suggested that the structure of the *MAT* locus acts as a "developmental switch" that triggers mating and sporulation responses at the appropriate stages of the yeast life cycle (Perrin 2012).

Heterothallism and Two Types of Homothallism

Classical mycology makes a distinction between homothallic (self-fertile) and heterothallic (self-sterile) species of fungi. In homothallic species, any strain can mate with any other strain. In heterothallic species, strains fall into mating types (usually two of them) such that mating is only possible between strains of different types. Mating types are not the same as genders. For example, a single mycelium of the heterothallic filamentous ascomycete *Podospora anserina* can produce gametes with both male and female morphology, but each gamete is only able to fuse with a gamete that has the opposite morphology and that comes from a thallus (vegetative body) of the opposite mating type (Coppin *et al.* 1997).

The definition of homothallism becomes more finessed when applied to cells instead of strains, which is crucial for yeasts because they are unicellular organisms. At the cellular level there are two very different forms of homothallism: primary and secondary (Lin and Heitman 2007; Almeida *et al.* 2015; Inderbitzin and Turgeon 2015; Wilson *et al.* 2015). In primary homothallic species, any cell can mate with any other cell. That is, any two haploid cells can go through cell fusion and nuclear fusion to form a diploid. In contrast, in secondary homothallic species, mating occurs only between two cells with opposite mating types, just like in heterothallic species. However, cells of secondary homothallic species can switch their mating types quite easily. Consequently in these species, a strain, which is a population of cells that has been grown from a single progenitor cell, does not have a permanent mating type. Any strain can mate with any other strain because, even if the two progenitors originally had the same mating type, some cells in the cultures grown from them will switch their mating types, enabling them to mate with unswitched cells from the other strain. *S. cerevisiae* is a familiar example of a secondary homothallic species, although most laboratory strains and many natural isolates of this species have become heterothallic due to mutations in the *HO* gene (Mortimer 2000; Katz Ezov *et al.* 2010). Indeed, mating-type switching was one of the first discoveries in *S. cerevisiae* genetics because the isolates used by Carl Lindegren and Øjvind Winge in the 1930s were, respectively, mutant and wild type for this gene (Winge and Roberts 1949; Mortimer 2000).

Secondary homothallism by mating-type switching has been rigorously examined in Saccharomycotina (*S. cerevisiae* and its relatives) and Taphrinomycotina (*S. pombe*) (Klar 2007; Haber 2012; Klar *et al.* 2014; Lee and Haber 2015). In contrast, surprisingly little is known about the molecular basis of primary homothallism. In Pezizomycotina, primary homothallics such as some *Cochliobolus* species have a single type of *MAT* locus containing genes normally found in both alleles, whose structures indicate that they were formed by recombination between *MAT* chromosomes of heterothallic ancestors (Yun *et al.* 1999; Inderbitzin and Turgeon 2015). In Taphrinomycotina, it has recently been proposed that the pathogen *Pneumocystis* is a primary homothallic because it too has *MAT* genes from both alleles in close proximity on the same chromosome (Almeida *et al.* 2015). In Saccharomycotina, in our opinion, the only known strong candidates for primary homothallism are *Debaryomyces hansenii* and *Scheffersomyces* (*Pichia*) *stipitis* (Riley *et al.* 2016). They both have *MAT***a**1, *MAT***a**2, and *MAT*α1 genes in proximity (Butler 2010), and meiotic recombination has been reported in *S. stipitis* crosses (Melake *et al.* 1996; Bajwa *et al.* 2010). However, the functions of their *MAT* genes and the details of what pheromone/receptor interactions occur in primary homothallic yeasts remain almost completely uninvestigated. Most fundamentally, it is unclear if and how cells of these yeasts avoid responding to their own pheromones. It has been suggested that primary homothallic filamentous fungi possibly sidestep this problem by expressing different pheromones and receptors in male and female tissues (Coppin *et al.* 1997; Martin *et al.* 2013), but this proposal has not been investigated experimentally and this solution is not available to unicellular yeasts (Billiard *et al.* 2012).

Multiple Mechanisms of Yeast Mating-type Switching

Programmed differentiation processes mediated by genomic DNA rearrangement are rare, with only a handful of examples known among all living organisms, and can affect single genes or entire genomes (Zufall *et al.* 2005). For organisms in which the germline genome is sequestered from the somatic genome, changes to somatic DNA can be irreversible and involve the loss of genes in differentiated tissues. For example, rearrangements during lymphocyte differentiation in vertebrates involve recombination between separate loci, resulting in antibody diversity and a robust immune response (Jung *et al.* 2006; Saha *et al.* 2010). Chromatin diminution during somatic tissue development in nematodes results in a more streamlined somatic genome by removal of heterochromatic regions (Muller *et al.* 1996; Muller and Tobler 2000). Ciliates,

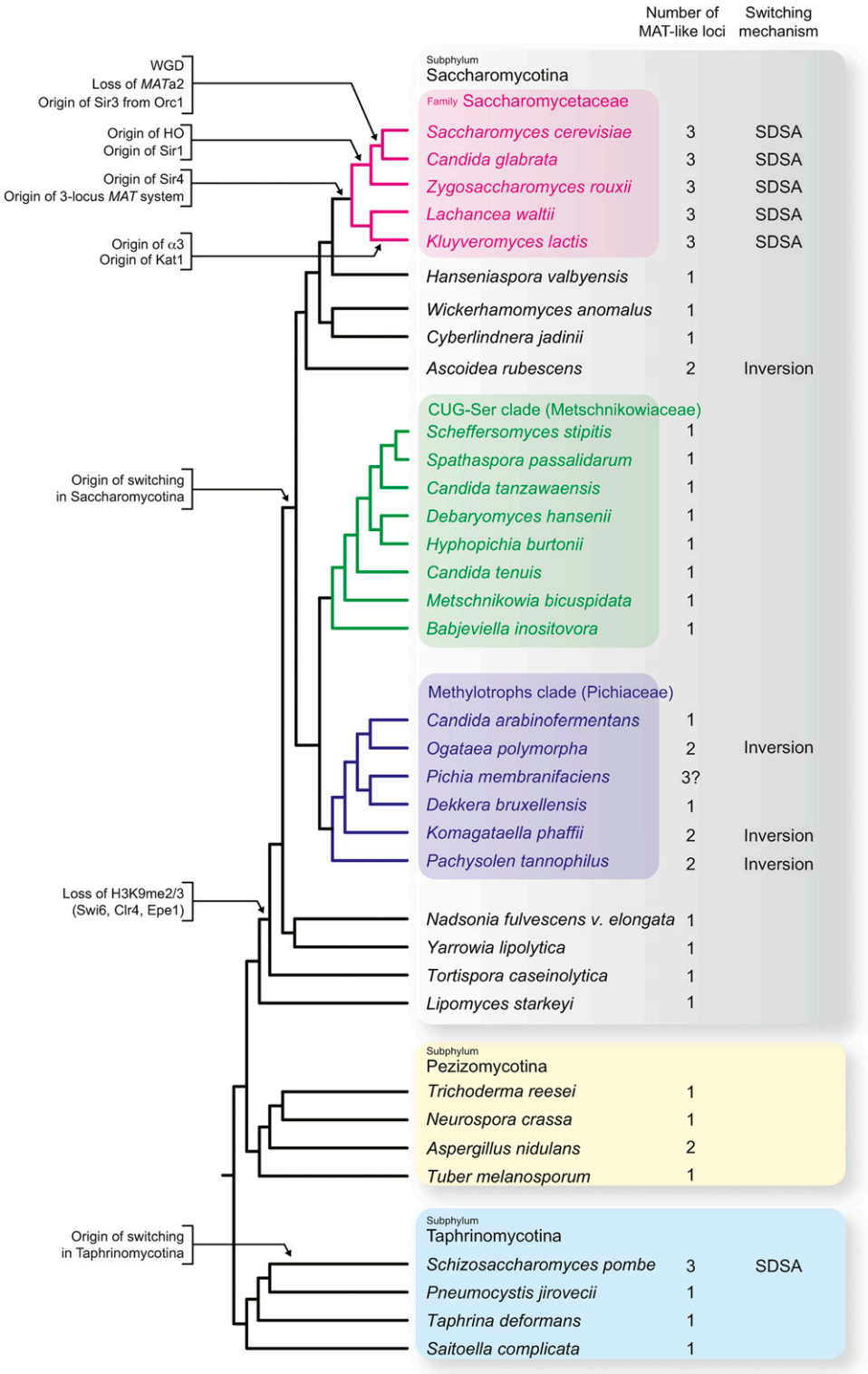

Figure 4 Phylogenetic tree of phylum Ascomycota showing major clades, *MAT*-locus organization, and known or inferred mating-type switching mechanisms. Based on Riley *et al.* (2016), with placement of *A. rubescens* as in Shen *et al.* (2016). Mating-type switching does not occur in species with only one *MAT*-like locus or in *Aspergillus nidulans*, which is a primary homothallic species.

unicellular eukaryotes whose germline DNA is harbored in a micronucleus, dramatically fragment and amplify somatic DNA during development of the macronucleus (Chen *et al.* 2014). Unicellular organisms that do not contain separate germline and somatic DNA cannot make permanent changes to their genomes during development, as they will be transmitted to offspring. Instead, programmed DNA rearrangements underlying cell-type specification in these organisms must be reversible (Nieuwenhuis and Immler 2016). In addition to mating-type switching in yeasts, examples of reversible rearrangements include the shuffling of variant surface glycoprotein genes in kinetoplastids (Li 2015) and phase variation in *Salmonella* (Simon *et al.* 1980), both of which facilitate evasion of the host immune system.

Switching in *S. cerevisiae* involves unidirectional DNA replacement. The current gene content at the *MAT* locus of a haploid cell is replaced by copying a reserve version of the *MAT* genes of the opposite allele, stored at a transcriptionally silent location elsewhere in the genome (Haber 2012; Lee and Haber 2015). This process requires the genome to have three copies of mating-type sequence information, all of which are on chromosome *III* in *S. cerevisiae*: the active *MAT* locus (either *MAT***a** or *MAT*α), two silent loci termed *HML* (containing the reserve copy of *MAT*α sequence information), and *HMR* (containing the reserve copy of *MAT***a** sequence information). All three loci are flanked by identical sequence regions called X and Z (Figure 5). The Y region in the center comes in two forms, Y**a** and Yα, that are allelic but completely different in sequence. During switching, the actively expressed *MAT* locus is cleaved by the endonuclease HO. Guided by homology at the X and Z regions, the cleaved *MAT* locus uses *HML* or *HMR* as a template for DNA repair with a strong preference for the silent locus containing the mating-type information opposite to the current *MAT* genotype (Haber 2012). This mechanism of switching was named the cassette model (Hicks and Herskowitz 1977; Hicks *et al.* 1977) because the sequences at *HML* and *HMR* become inserted into the *MAT* locus for playback like cassette tapes in a player, and the three loci (*MAT*, *HML*, and *HMR*) are often described as cassettes.

Although mating-type switching in *S. cerevisiae* is often called gene conversion, it is more accurately described as a synthesis-dependent strand annealing (SDSA) process because of the nonhomology of the Y regions between the outgoing and incoming alleles (Ira *et al.* 2006). Switching is initiated when the HO endonuclease makes a dsDNA break at the Y-Z junction of *MAT*. The 3′ end of a DNA strand from the Z region beside *MAT* then invades the donor (*HMR* or *HML*) locus and is extended by DNA polymerase through the donor Y region and into the X region, after which it reinvades the *MAT* locus. Finally, the second strand of the new Y region at *MAT* is synthesized in the direction from X to Z. Switching is slow, taking ∼70 min, and is >1000 times more error prone than normal DNA replication (Hicks *et al.* 2010, 2011). Even though the newly synthesized *MAT* DNA will generally be replaced the next time the cell switches mating type, the high error rate nevertheless imposes an evolutionary cost because sometimes the errors will render the *MAT* genes nonfunctional.

Mating-type switching in the fission yeast *S. pombe* uses an analogous, but structurally unrelated, means to accomplish the same goal (Arcangioli and Thon 2004; Nielsen 2004; Egel 2005; Klar 2007; Klar *et al.* 2014). The *S. pombe* genome also contains one active (*mat1*) and two silenced (*mat2* and *mat3*) mating-type loci (Figure 5). The proteins specifying the two mating types *P* (plus) and *M* (minus) include two HMG-domain transcription factors (Pc and Mc) and a homeodomain transcription factor (Pi), and thus share similarities with the *S. cerevisiae* proteins, but the mechanism of switching is different from that in *S. cerevisiae*. Instead of cleavage by an endonuclease, a fragile chromosomal site consisting of an epigenetic mark at the *mat1* locus in switching-competent cells leads to a dsDNA break during replication. Repair of the break by SDSA using *mat2* or *mat3* as a donor is facilitated by homologous flanking regions, H1 and H2, which are analogous to the X and Z regions of *S. cerevisiae* but do not share sequence similarity or synteny with them (Beach and Klar 1984). The mechanisms used by *S. pombe* to silence expression of *mat2* and *mat3*, and to bias the SDSA event to the locus containing the opposite mating type, are very different from those in *S. cerevisiae* (see below). The difference in mechanisms used to create the dsDNA break means that only one of the four mitotic grandchildren of a cell switches mating type in homothallic *S. pombe* strains, whereas two of the four switch in *S. cerevisiae* (Klar 2007; Hanson *et al.* 2014).

The lack of homology in every aspect of mating-type switching between *S. cerevisiae* and *S. pombe* has led to the remarkable conclusion that these distantly related species acquired their complex switching processes independently (Egel 2005; Lee *et al.* 2010; Rusche and Rine 2010). In this regard, it should be noted that *S. cerevisiae* and *S. pombe* underwent independent evolutionary transitions to unicellularity from a multicellular common ancestor (Nagy *et al.* 2014). Secondary homothallism by reversible changing of cell type is unknown in multicellular fungi and would not appear to serve any purpose in a multicellular context, so it is unlikely to have existed in the common ancestor of *S. cerevisiae* and *S. pombe*. A process known as mating-type switching has been described in some Pezizomycotina, in which half the progeny obtained by self-fertilization of a homothallic parent are heterothallic (Perkins 1987). This process has not been well characterized at the molecular level in any species, but it is consistent with models in which a recurrent inversion toggles between heterothallic and primary homothallic forms of a *MAT* locus (Chitrampalam *et al.* 2013), or in which a recurrent deletion converts a primary homothallic *MAT* locus into a heterothallic one (Witthuhn *et al.* 2000). There are no well-documented examples in Pezizomycotina of yeast-style secondary homothallism by a reversible switch between two mating types, each of which is self-sterile (Nieuwenhuis and Immler 2016).

In 2014, mating-type switching was reported in a clade of Saccharomycotina containing methylotrophic yeasts (family Pichiaceae; Figure 4) (Hanson *et al.* 2014; Maekawa and Kaneko 2014). These haploid yeasts contain one copy each of *MAT***a** and *MAT*α loci, flanked by a pair of sequences forming an inverted repeat (IR) that are orthologous to the X or Z regions of the Saccharomycetaceae (Figure 5). One of the *MAT* loci is proximal to a heterochromatic region of the genome, a centromere in the case of *O. polymorpha* and a telomere in the case of *Komagataella phaffii* (these methylotrophic species are often called by their obsolete names *H. polymorpha* and *P. pastoris*, respectively). This arrangement confers repression of transcription on one *MAT* locus, while allowing active expression of the other locus, to specify cell type. Mating-type switching in these species occurs by

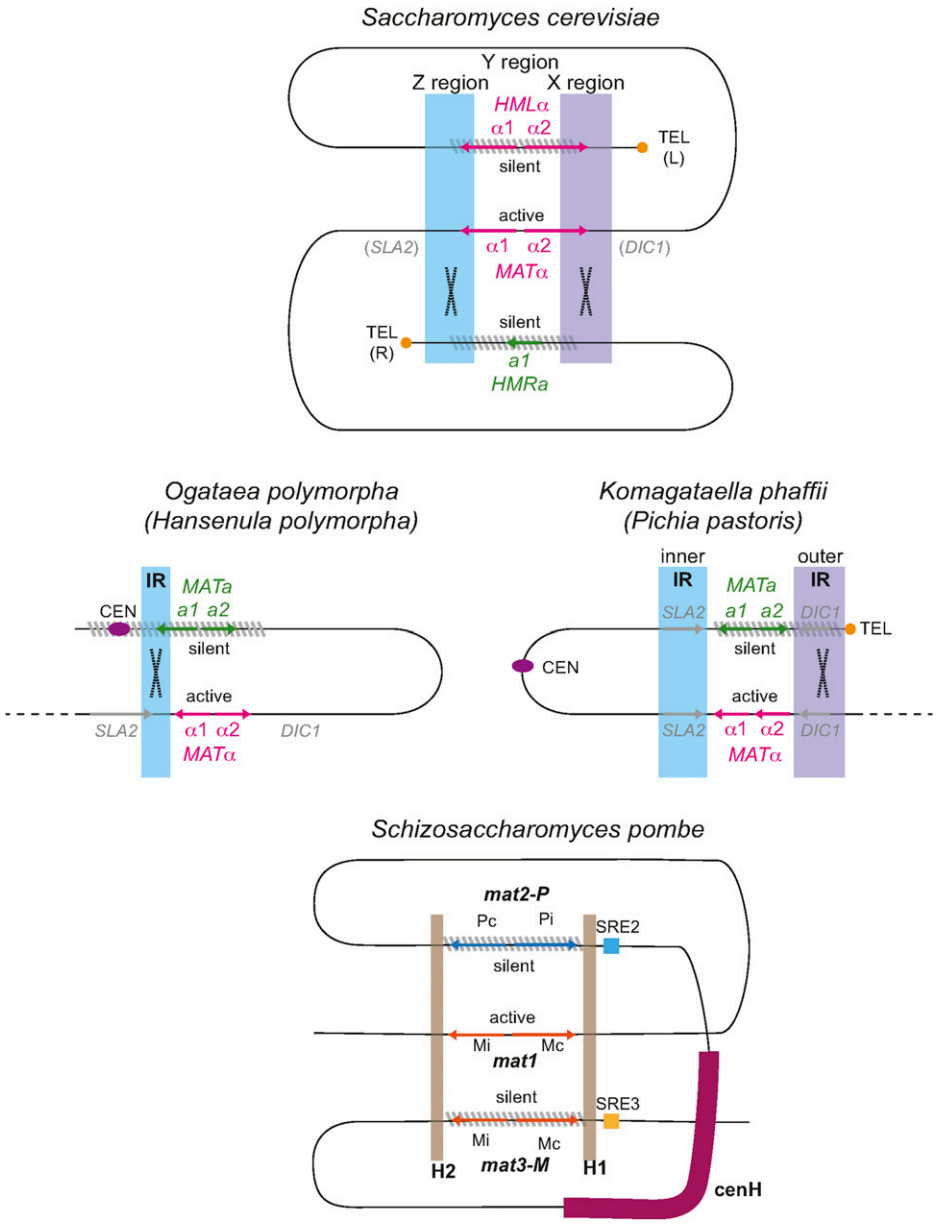

Figure 5 Organization of repeat sequences flanking the *MAT* loci in four species (Klar 2007; Hanson *et al.* 2014). In *K. phaffii*, the region that becomes inverted during mating-type switching is 138-kb long, and was recently discovered to contain a centromere at its approximate center (Coughlan *et al.* 2016). CEN, centromere; TEL, telomere.

recombination between the IRs, leading to inversion of the entire *MAT* region including all the genes located between *MAT***a** and *MAT*α (19 kb in *O. polymorpha*, 138 kb in *K. phaffii*), swapping the positions of the active and repressed *MAT* loci relative to the centromere or telomere. It is important to note that switching in these species involves a reciprocal exchange of DNA: the previously expressed *MAT* genes become silenced, the previously silenced *MAT* genes become activated, and there is no new DNA synthesis. By contrast, in *S. cerevisiae* and *S. pombe*, the previously expressed *MAT* genes are degraded by exonucleases and replaced by newly synthesized DNA copied from the silent loci.

In recent work, we found evidence for similar flip-flop, mating-type switching mechanisms in two other yeasts (Riley *et al.* 2016). One is *Pachysolen tannophilus*, a haploid species in the methylotrophs clade (Figure 4). In response to nitrogen limitation, it inverts a 9-kb genomic region flanked by two identical sequences that form a 2-kb IR, similar to *O. polymorpha*. This 9-kb region has *MAT***a**1 and *MAT***a**2 genes at one end and *MAT*α1 and *MAT*α2 at the other end, and these loci are separated by ~4 kb of noncoding DNA. While this process strongly resembles mating-type switching in *O. polymorpha*, its regulatory consequences have not been investigated. More significantly, a similar switching mechanism appears to operate in *Ascoidea rubescens*. Phylogenetically, *A. rubescens* was previously placed as deep lineage of Saccharomycotina that is an outgroup to all three major clades of this subphylum (Riley *et al.* 2016), although a recent phylogenomic study (Shen *et al.* 2016) placed it closer to family Saccharomycetaceae, as shown in Figure 4. Strains of *A. rubescens* were found to be polymorphic for the orientation of a 50-kb chromosomal region beside a

telomere (Riley *et al.* 2016). Again, the invertible region is completely noncoding except for *MAT***a**1/**a**2 genes at one end and *MAT*α1/α2 genes at the other, and the region is flanked by a 2-kb IR. Although it has not yet been shown that the orientation of the region can be induced to change, or that orientation affects expression of the *MAT* genes, the structure of the *A. rubescens* locus points to mating-type switching by inversion of a section of chromosome by recombination in the IR, placing either *MAT***a** or *MAT*α genes beside the telomere.

The conservation of local gene order (synteny) around the *MAT* locus between the methylotrophs and the Saccharomycetaceae (Hanson *et al.* 2014) indicates that the two-locus inversion mechanism as seen in the methylotrophs and the three-locus (*MAT*, *HML*, and *HMR*) SDSA switching system as seen in *S. cerevisiae* share a common ancestor. It is likely that the two-locus system corresponds to a simpler ancestral mechanism of switching (Hanson *et al.* 2014), and this conclusion is supported by the existence of switching by inversion in both *A. rubescens* and the methylotrophs, regardless of which of the proposed phylogenetic positions of *A. rubescens* is correct (Riley *et al.* 2016; Shen *et al.* 2016).

More broadly, despite the variations in gene content, synteny around the *MAT* locus is reasonably well conserved among all ascomycetes. Although there have been many rearrangements in its vicinity, physical linkage of *MAT* to one or more neighboring genes (*SLA2*, *SUI1*, *NVJ2*, *APC5*, and *APN2*; none of which have known roles in mating) is widely conserved among the three subphyla: Saccharomycotina (budding yeasts), Pezizomycotina (filamentous ascomycetes), and Taphrinomycotina (fission yeasts) (Figure 4; Butler *et al.* 2004; Gordon *et al.* 2011; Riley *et al.* 2016). This conservation indicates that cell type has been specified by the same genetic locus throughout all of ascomycete evolution, even though there has been extensive turnover of the homeodomain and HMG-domain genes contained at *MAT* itself.

An ancestral mating-type switching system based on inversion of two *MAT* loci at least partly resolves the previously perplexing observation that two nonhomologous switching systems, both highly complex, appeared to have arisen independently and abruptly in the *S. cerevisiae* and *S. pombe* clades. We can hypothesize that a relatively simple ancestral inversion system has been made progressively more complex in the *S. cerevisiae* lineage through the addition of structural components and regulatory mechanisms, as discussed below.

Evolution of Mating-Type Switching Components

MAT-locus cassettes

The most obvious difference in complexity between the switching mechanisms of methylotrophs and those of *S. cerevisiae* and *S. pombe* is the number of *MAT*-locus copies each mechanism uses. The SDSA mechanism of *S. cerevisiae* and *S. pombe* requires a reserve copy of each *MAT*-locus allele as well as the active locus (three cassettes total), whereas the inversion mechanism of methylotrophs only requires one copy of each allele (two cassettes total). To guide the DNA recombination steps, the three-cassette system also requires two different sequences each to be present in triplicate (the X and Z regions; Figure 5), whereas a minimal two-cassette system requires only one sequence in duplicate (the IR) as seen in *O. polymorpha*, *P. tannophilus*, and *A. rubescens*. However, it must be noted that the methylotroph *K. phaffii* contains two sets of IRs in the *MAT* region and thus more closely resembles the X and Z structure of *S. cerevisiae* (Figure 5). In this species, mating-type switching occurs by recombination between the outer set of IRs. The function of its inner IRs is unclear, but exchange between them may act to restore collinearity between homologous chromosomes in diploids, enabling meiotic recombination to occur in the large (123-kb) interval between these IRs (Hanson *et al.* 2014).

In *S. cerevisiae*, four regions of sequence identity between *MAT* and the *HM* loci have traditionally been defined: W, X, Z1, and Z2 (Astell *et al.* 1981; Haber 2012), each a few hundred bp long. X and Z1 occur in three copies in the genome; whereas W and Z2 are regions that extend the similarity between *MAT* and *HML*, but not *HMR*, and so occur in two copies. In comparisons of other Saccharomycetaceae species, we found that in some cases the W and Z2 regions have negative length, *i.e.*, the regions of similarity between *MAT* and *HMR* are longer than between *MAT* and *HML* (Gordon *et al.* 2011). Some species even have two *HMR* loci on different chromosomes, with different lengths of flanking sequence identity to *MAT*. We therefore do not think that W and Z2 have any functional significance separate from the roles of X and Z1, so for simplicity we use the names X and Z (instead of Z1) to refer to the triplicated regions, and ignore any extensions not shared by all the silent loci (Figure 5). We also usually draw the *MAT* locus in the order Z, Y, X, because in most Saccharomycetaceae species other than the genus *Saccharomyces*, the Z region is closest to *HML* and the telomere of the chromosome (Gordon *et al.* 2011). *Saccharomyces* sustained a bizarre trio of rearrangements, wherein *MAT*, *HML*, and *HMR* each became inverted by separate events (Fabre *et al.* 2005). The net effect of these three inversions, which are unique to the genus *Saccharomyces*, was to keep the orientations of *HML*, *MAT*, and *HMR* parallel, but with their X regions now closest to the left end of the chromosome.

Among Saccharomycetaceae species with the three-cassette system, the X and Z regions show very unusual evolutionary dynamics. To guide the DNA strand exchanges that occur during SDSA, the cell needs the sequences on each side of *MAT* to be identical to those beside *HML* and *HMR*, but the actual sequences that are triplicated vary enormously among species (Figure 6; Gordon *et al.* 2011; Wolfe *et al.* 2015). They usually consist of parts of some *MAT* genes and/or parts of the neighboring chromosomal genes. For example, in *S. cerevisiae* the X region contains the 3′ end of the *MAT*α2 gene, and the Z region contains the 3′ end of the *MAT*α1 gene. Switching from *MAT*α to *MAT***a** replaces the 5′ ends of the two *MAT*α genes (on Yα) with the whole *MAT***a**1 gene (on Y**a**), while switching from *MAT***a** to *MAT*α does the

opposite. Comparison among Saccharomycetaceae species reveals a remarkable diversity of ways that the X and Z repeats are organized relative to the four *MAT* genes (Figure 6). The primary evolutionary constraints on X and Z appear to be (1) to maintain homogeneity of the three copies so that DNA repair is efficient (they have a very low rate of nucleotide substitution; Kellis *et al.* 2003); and (2) to avoid containing any complete *MAT* genes within X or Z, so that the only intact genes at the *MAT* locus are ones that can be formed or destroyed by replacement of the Y region during switching.

The diversity of organization of X and Z regions and their nonhomology among species is consistent with evidence that these regions have repeatedly been deleted and recreated during yeast evolution (Gordon *et al.* 2011). Comparative genomics shows that chromosomal DNA flanking the *MAT* locus has been progressively deleted during Saccharomycetaceae evolution, with the result that the chromosomal genes neighboring *MAT* differ among species. These progressive deletions have been attributed to recovery from occasional errors that occurred during attempted mating-type switching over evolutionary timescales (Gordon *et al.* 2011). Each time a deletion occurs, the X and Z regions need to be replaced, which must require retriplication (by copying *MAT*-flanking DNA to *HML* and *HMR*) to maintain the switching system. We only see the chromosomes that have successfully recovered from these accidents, because the others have gone extinct.

Gene silencing

Gene silencing mechanisms in the Ascomycota are highly diverse and these processes appear to be very rapidly evolving, particularly within the Saccharomycetaceae. In *S. pombe*, assembly of heterochromatic regions, including centromeres, telomeres, and the silent *MAT*-locus cassettes, requires many components conserved with multicellular eukaryotes including humans and fruit flies; making it a popular model for studying the mechanisms of heterochromatin formation and maintenance (Perrod and Gasser 2003). The two silent cassettes are contained within a 20-kb heterochromatic region bordered by 2-kb IR sequences (Singh and Klar 2002). Heterochromatin formation in the 20-kb region initiates at a 4.3-kb sequence (*cenH*, resembling the outer repeat units of *S. pombe* centromeres) located between the silent *MAT* cassettes (Grewal and Jia 2007), where the RNA-induced transcriptional silencing (RITS) complex, which includes RNA-interference (RNAi) machinery, is recruited by small interfering RNA expressed from repeat sequences present within *cenH* (Hall *et al.* 2002; Noma *et al.* 2004). RITS-complex association with *cenH* is required for Clr4-mediated methylation of lysine 9 of histone H3 (H3K9me2/3). H3K9 hypoacetylation and methylation is necessary for recruitment of the chromodomain protein Swi6, which is in turn needed for recruitment of chromatin-modifying factors that propagate heterochromatin formation across the silent cassettes (Nakayama *et al.* 2001; Yamada *et al.* 2005; Grewal and Jia 2007; Allshire and Ekwall 2015). The fact that a centromere-like sequence is involved in silencing the silent *MAT* loci of *S. pombe* may be significant in terms of how this silencing system evolved. The *S. pombe MAT* locus is not linked to the centromere, and the *cenH* repeat at *mat2,3* is the only noncentromeric copy of this repeat in the *S. pombe* genome.

In contrast, heterochromatin formation in *S. cerevisiae* differs dramatically from that in other eukaryotes (Hickman *et al.* 2011). Although components of the RNAi pathway can be found in the Taphrinomycotina, Pezizomycotina, and in some Saccharomycotina species, it is absent in *S. cerevisiae* (Drinnenberg *et al.* 2009). *S. cerevisiae* also lacks homologs of Swi6 and Clr4, and thus does not use H3K9 methylation for chromatin modification. Instead, *S. cerevisiae* has replaced this mechanism of transcriptional repression with one directed in large part by the histone deacetylase Sir2 (Rusche *et al.* 2003; Hickman *et al.* 2011; Moazed 2011; Haber 2012). Transcriptionally repressed regions of the *S. cerevisiae* genome include the silent *MAT* cassettes and the telomeres. The mechanisms of repression in these two genomic regions share many components (Wellinger and Zakian 2012), but transcriptional silencing at the *MAT* cassettes is stronger and more robust than that at the telomeres (Haber 2012). *HMR* and *HML* are flanked by the *cis*-acting E and I silencer sequences that serve as the sites of recruitment for silencing proteins. Silencer sequences are directly bound by Orc1, Rap1, and Abf1, which recruit Sir1 and the SIR complex (Sir2/Sir3/Sir4). Sir2 is an NAD^+ histone deacetylase, and Sir3 and Sir4 bind deacetylated histones H3 and H4. This has led to a model for progressive spreading of heterochromatin across the *MAT* cassettes by Sir2 histone deacetylation, followed by SIR-complex recruitment. As well as silencing transcription at *HML* and *HMR*, the SIR complex also protects the Y/Z junctions in these loci from cleavage by HO endonuclease during switching in *S. cerevisiae*. This protection mechanism seems to have been lost in *C. glabrata*, a close relative of *S. cerevisiae* that has lost the *SIR1* gene; overexpression of *HO* in *C. glabrata* is lethal, probably due to cleavage of *HML* (Boisnard *et al.* 2015).

Examination of the silencing components required for transcriptional repression of *HMR* and *HML* in other Saccharomycetaceae species has demonstrated the rapid evolution of this process. The silencer sequences that nucleate the formation of heterochromatin across the silent loci have a high nucleotide-substitution rate within the *Saccharomyces* genus (Teytelman *et al.* 2008), and the binding sites for Orc1, Rap1, and Abf1 are absent in the non-WGD yeast *K. lactis* (Astrom *et al.* 2000; Sjostrand *et al.* 2002). Sir1, which is present only in post-WGD species and the *Zygosaccharomyces/ Torulaspora* clade (Figure 4), has undergone expansion and contraction in copy number within these species. Four members of a Sir1 family have been identified in *S. uvarum* (syn. *S. bayanus*), all of which contribute to *HMR* and *HML* silencing (Gallagher *et al.* 2009). In addition, Sir1, Sir4, and the silencer sequences are incompatible in interspecies hybrids between *S. cerevisiae* and *S. uvarum*, further demonstrating the rapid evolution of these components (Zill *et al.* 2010, 2012).

Several important differences in the silencing mechanisms in *K. lactis* relative to *S. cerevisiae* have been identified,

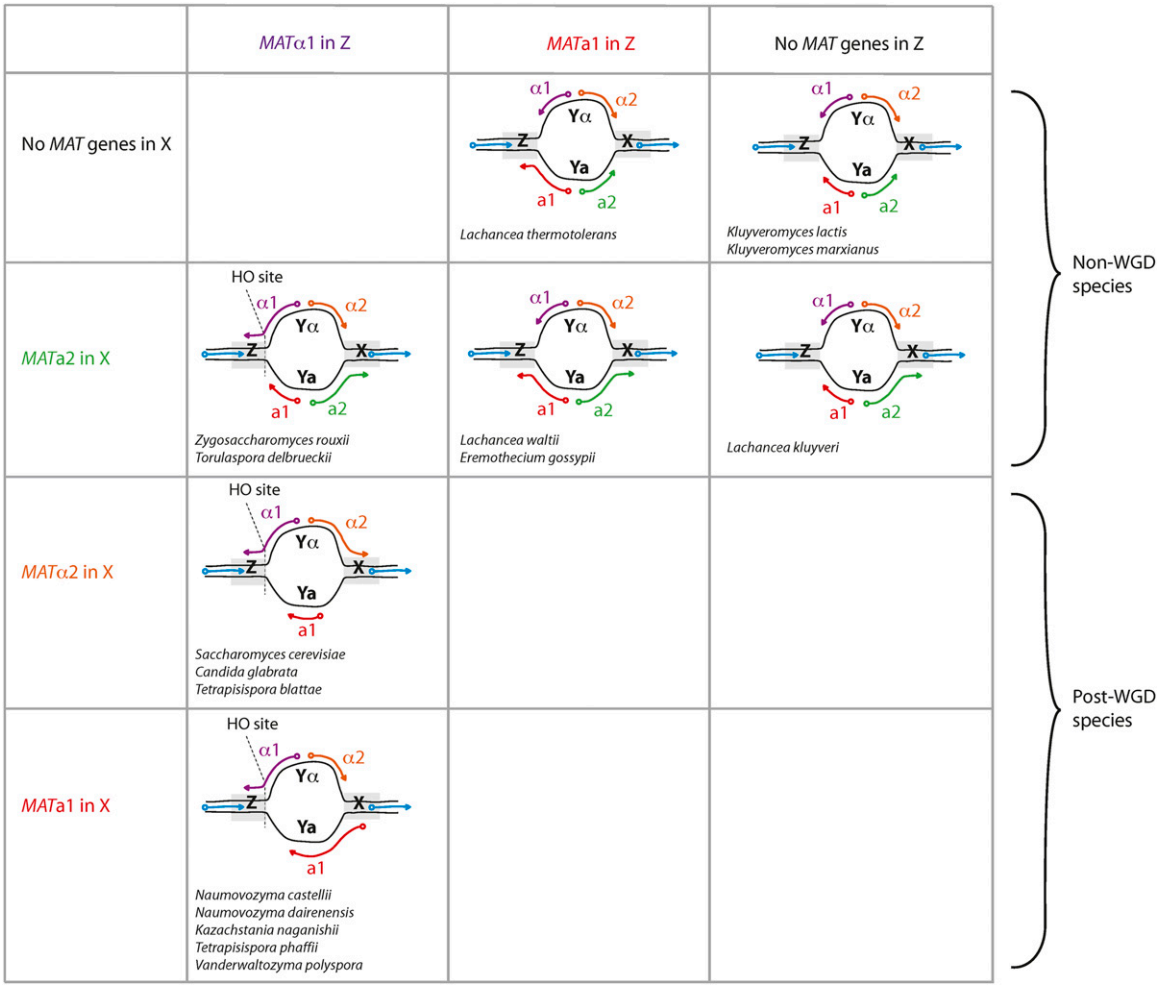

Figure 6 Seven ways to organize a *MAT* locus in family Saccharomycetaceae. The X and Z repeats, which occur in three copies in the genome (at *MAT*, *HML*, and *HMR*), overlap with parts of different *MAT* genes in different species. Each cartoon illustrates how the *MAT* genes are arranged, relative to the X and Z regions, in a group of species. The horizontal lines in each cartoon represent sequence that is shared between the *MAT*α and *MAT***a** alleles, while the bubbles represent the divergence between the Yα and Y**a** regions. Blue arrows represent the neighboring chromosomal genes, which also vary among species (Gordon *et al.* 2011).

including a requirement of Sum1 for repression of *HMR* and *HML* (Hickman and Rusche 2009). The SUM1 complex, which represses αsg's as well as meiotic genes in *S. cerevisiae* (Zill and Rine 2008), localizes with Sir2 to repress both *HMR* and *HML* in *K. lactis*. Intriguingly, although Sir4 also localizes to *HML*, it is absent from *HMR*. Since *K. lactis* also lacks Sir3, because Sir3 is a paralog of Orc1 that arose from the WGD (Hickman and Rusche 2009; Hickman *et al.* 2011), the histone-associating factors that silence *HMR* in *K. lactis* are still unknown. Additional roles for Ume6, which is required for meiotic gene repression in *S. cerevisiae*, have been described for repression of *HMR* and *HML* in *K. lactis* (Barsoum *et al.* 2010b).

In methylotrophic yeasts, the silencing of one copy of the *MAT* locus is conferred by its proximity to a heterochromatic region of the genome, but the components required for transcriptional repression are unknown. In *O. polymorpha*, one copy of the *MAT* locus is located next to a centromere, which structurally resembles the regional centromeres of *S. pombe*, *Neurospora crassa*, and *C. albicans* rather than the point centromeres of the Saccharomycetaceae family (Roy and Sanyal 2011; Coughlan *et al.* 2016). The *O. polymorpha* centromere is bound by the centromeric histone variant Cse4 (CenH3), and this binding extends into the proximal *MAT* locus cassette (Hanson *et al.* 2014). However, the direct or indirect contribution of Cse4 to transcriptional repression of this *MAT* locus has not been determined.

In *K. phaffii*, one copy of the *MAT* locus is adjacent to a telomere. Intriguingly, expression of the *MAT* genes from this locus is reduced rather than completely silenced (Hanson *et al.* 2014), similar to the variegated expression observed in subtelomeric regions in *S. cerevisiae* (telomere position effect) (Gottschling *et al.* 1990). This mechanism for silencing *MAT*-locus cassettes has previously been described for the *HML* (*MTL3*) locus in *C. glabrata*, which has lost Sir1 and does not use silencer sequences for initiation of heterochromatin formation (Ramirez-Zavaleta *et al.* 2010). The implications of concurrent expression of *MAT* genes in haploid cells for the expression of **a**sg's and αsg's are unknown.

The genes required for *S. pombe*-like transcriptional silencing, including Clr4 (H3K9 methyltransferase), Epe1 (H3K9me demethylase), and Swi6 (H3K9me-binding chromodomain protein) were lost at a very early stage of the evolution of the Saccharomycotina subphylum, before the divergence between the Saccharomycetaceae, methylotrophs, and CUG-Ser clades (Figure 4; Riley *et al.* 2016). RNAi components were also lost in many lineages, including the methylotrophs and many Saccharomycetaceae. These losses predate the inferred emergence of mating-type switching in Saccharomycotina (Figure 4). In contrast, the SIR silencing system appears to be relatively young because the genes *SIR1*, *SIR3*, and *SIR4* are only found in the family Saccharomycetaceae. Because all switching systems require a mechanism to repress transcription of the silent *MAT* loci, these observations indicate that, prior to the origin of the SIR proteins, another mechanism must have existed to silence the silent *MAT* genes. It is possible that this mechanism is connected to the centromeric and/or telomeric locations of *MAT* genes. Elucidation of the silencing mechanisms in methylotrophic species is likely to provide valuable insight into this evolutionary transition from RNAi/Swi6-mediated to Sir2-mediated silencing (Hickman *et al.* 2011).

MAT-locus cleavage

In species that switch mating types by SDSA, such as *S. cerevisiae* and *S. pombe*, the first step in the process is the formation of a dsDNA break in the old *MAT* locus. This break will subsequently be repaired by using a silent *MAT* gene (from *HML/HMR* or *mat2/mat3*) as the template for new DNA synthesis. In contrast, in methylotrophs that switch by a flip-flop mechanism, the first step is the initiation of nonallelic homologous recombination (NAHR) between the two copies of the IR. Whether the methylotrophs employ an endonuclease to initiate the NAHR (analogous to Spo11 in meiotic recombination) is not currently known.

The mechanisms of dsDNA-break formation differ completely among the three species in which it has been studied in detail: *S. cerevisiae*, *K. lactis*, and *S. pombe*. The *S. pombe* mechanism is not fully understood, but it does not involve an endonuclease. Instead, the dsDNA break arises from an imprint (epigenetic mark) on one strand of the *MAT*-locus DNA (Arcangioli and Thon 2004; Klar *et al.* 2014). The nature of this mark has been controversial, but it involves a replication pause that leaves either a single-strand nick or two ribonucleotides (probably from an incompletely removed Okazaki fragment primer) at a specific site on one DNA strand (Holmes *et al.* 2005; Dalgaard 2012). When the imprinted *MAT* locus is replicated, the imprint gives rise to a double-strand break which is then repaired by switching. Notably, this process is not regulated: mating-type switching occurs in one of the four grandchildren of every *S. pombe* cell (in homothallic strains), regardless of environmental or other signals. In this regard, switching in *S. pombe* resembles switching in *S. cerevisiae* and differs from the inducible switching seen in methylotrophs.

The agent of *MAT*-locus cleavage in *S. cerevisiae* is the HO endonuclease, whose mechanism and evolution have been studied extensively. HO cleaves the *S. cerevisiae MAT* locus at an ~18-bp recognition sequence that spans the junction between the Y and Z regions (Nickoloff *et al.* 1990). The recognition sequence lies within the *MAT*α1 gene, because the 3′ end of this gene extends from Yα into the Z region (Figure 6), and coincides with a short conserved amino acid motif (FAQQ) in the α1 protein. The recognition-site specificity of HO endonuclease in species other than *S. cerevisiae* has not been investigated experimentally, but the *HO* gene is known to catalyze switching in the *C. glabrata* clade (Edskes and Wickner 2013; Boisnard *et al.* 2015) and in *Naumovozyma castellii* (Drinnenberg *et al.* 2009). Moreover, the Y-Z junction occurs at or near the FAQQ motif in the *MAT*α1 gene of all Saccharomycetaceae species that have *HO*, so HO is likely to cut at this site in all these species (Butler *et al.* 2004; Gordon *et al.* 2011).

Phylogenetic analysis of the HO endonuclease has established its relationship to a selfish genetic element, an intein found in some alleles of the *VMA1* gene coding for a subunit of vacuolar H^+-ATPase in *S. cerevisiae* (Hirata *et al.* 1990; Gimble and Thorner 1992; Haber and Wolfe 2005; Koufopanou and Burt 2005). Inteins are insertions in protein sequences analogous to introns in gene sequences (Dalgaard *et al.* 1997; Gogarten *et al.* 2002). The intein in the Vma1 protein is called VDE or PI-SceI (Gimble and Thorner 1992). The initial translation product from *VMA1* alleles that contain VDE is a chimeric precursor polypeptide in which VDE interrupts the mature Vma1 protein. VDE has two domains. Its protein-splicing domain enables VDE to autoexcise from the precursor polypeptide, making Vma1 functional by ligating its N- and C-terminal halves together with a new peptide bond. Its DNA-endonuclease domain enables VDE to "home," that is, it enables the VDE-encoding sequence to spread selfishly through a yeast population. Homing occurs during meiosis in diploids that are heterozygotes for *VMA1* alleles with and without the intein (Gimble and Thorner 1992; Fukuda *et al.* 2006). The endonuclease domain of VDE cleaves the intein-less allele of *VMA1* in a site-specific manner; its recognition sequence spans the intein insertion site, so it only recognizes *VMA1* alleles that lack the VDE coding region. The cleaved intein-less allele is then repaired by gene conversion from the intein-containing allele, leading to super-Mendelian inheritance of the element. The endonuclease domain of VDE is a member of the large LAGLIDADG family of homing endonucleases, named after a conserved and semipronounceable peptide sequence motif (Gimble 2000). *HO* does not self-propagate in this manner, but instead appears to be a former intein that has been adapted to function in mating-type switching (Gimble and Thorner 1992; Keeling and Roger 1995).

The *HO* gene is significantly more closely related to the VDE intein of *VMA1*, which is one of the very few inteins found in fungi (most are in bacteria), than to other inteins. *HO* does not exist in Saccharomycotina outside the family Saccharomycetaceae, and on the phylogenetic tree it first appears on the branch leading to the common ancestor of *Zygosaccharomyces/Torulaspora* and the post-WGD clade (Figure 4). Even though HO does not undergo protein

splicing, motifs that are conserved among the protein-splicing domains of inteins are also conserved in HO (Pietrokovski 1994); in fact, these motifs are more strongly conserved in HO than in VDE (Haber and Wolfe 2005). The details of how an intein, an ancient mobile genetic element found in all domains of life, turned into HO are still unknown. The DNA manipulations initiated by the HO and VDE proteins are similar: cleavage of dsDNA at a specific site in the recipient locus, leading to copy-and-paste repair by SDSA from a donor that does not contain the cleavage site, guided by flanking sequences that are identical between recipient and donor. The key evolutionary innovation during the evolution of HO was that the endonuclease became redeployed to cleave a recipient site that is not the *HO* locus itself, but a completely different place in the genome (*MAT*). Relative to VDE and other inteins, HO has acquired an extra domain at its C terminus, a zinc finger DNA-binding domain that is required for cleavage of *MAT* (Nahon and Raveh 1998; Bakhrat *et al.* 2004). The gain of this domain is likely to have been important for the evolutionary retargeting of HO to the *MAT* locus (Bakhrat *et al.* 2004; Butler *et al.* 2004).

Importantly, the *HO* gene was acquired after the three-cassette, *MAT*-locus system had emerged in Saccharomycetaceae (Figure 4; Butler *et al.* 2004). Orthologs of HO are not found in clades that branched off before the point marked in Figure 4. *K. lactis* contains a sequence that was initially proposed to be an *HO* pseudogene (Fabre *et al.* 2005), but does not share synteny with *HO* (Butler 2007). *HO* appears to be a member of a small family of paralogous intein-zinc finger fusion genes in Saccharomycetaceae, many of which are pseudogenes. In addition, the acquisition of *HO* coincides with the gain of *SIR1* (Hickman *et al.* 2011), which may relate to the role of Sir1 in preventing HO from cleaving *HMR* and *HML* by changes to chromatin structure (Haber and Wolfe 2005).

Instead of HO, *K. lactis* employs two other domesticated selfish genetic elements to induce cleavage of its *MAT* locus. *K. lactis* differs from *S. cerevisiae* by having two separate mechanisms for *MAT*α → *MAT***a** switching and *MAT***a** → *MAT*α switching (Barsoum *et al.* 2010a; Rajaei *et al.* 2014). Both of these mechanisms involve making a dsDNA break in the outgoing *MAT* locus by processes that resemble the first steps of mobilization of DNA transposons. Cleavage of the *MAT*α locus for switching to *MAT***a** is induced by α3, a gene present at both *MAT*α and *HML* (Barsoum *et al.* 2010a). This gene was named α3 because it is a third gene located in the Yα region of the *K. lactis MAT*α allele (Astrom *et al.* 2000), but the name is somewhat misleading because α3 is not a regulator of transcription like α1 and α2. Rather, it is part of an arcane mechanism for generating a double-strand break in *MAT*α during the *MAT*α → *MAT***a** switch. The α3 protein is similar to the DNA transposase of Mutator-like elements (MULEs), a family within the Mutator superfamily of DNA transposons (class II mobile elements) (Neuveglise *et al.* 2005; Wicker *et al.* 2007). The α3 protein is brought to the *MAT*α locus by Rme1 (also called Mts1 in *K. lactis*), where it cuts at two sites on either side of the *MAT*α3 gene, excising the gene, and leaving behind a double-strand break. These steps are similar to the "cut" part of the cut-and-paste mechanism that MULE elements use to transpose. Surprisingly, it is the copy of the α3 gene located in the *HML* locus, rather than *MAT*α3, that is expressed and translated into the α3 protein necessary for successful cleavage of the *MAT* locus (Barsoum *et al.* 2010a). It is perhaps for this reason that the dynamics of the silencer elements flanking *HML* in *K. lactis* are different from those in *S. cerevisiae* (Hickman and Rusche 2009).

When *K. lactis* switches in the opposite direction, from *MAT***a** to *MAT*α, the outgoing *MAT***a** locus is cleaved by Kat1, a member of the Roamer family of hobo/Activator/Tam3 (hAT) DNA transposases (Rajaei *et al.* 2014). Kat1 cuts between the *MAT***a**1 and *MAT***a**2 genes to create the double-strand break needed for SDSA with *HML*. The ends of the break are covalently closed into hairpin caps, a characteristic feature of the breaks made when hAT family elements transpose, which are subsequently resolved by Mre11 nuclease (Barsoum *et al.* 2010a). The *KAT1* gene is not located near *MAT* or *HML/HMR*, but its expression is activated by Rme1. It is interesting that Rme1 stimulates mating-type switching in both directions, but its role in one direction is as a transcription factor, whereas its role in the other direction seems to be only as a DNA- and protein-binding factor (it binds to the *MAT*α3 gene and probably interacts with the α3 protein) (Barsoum *et al.* 2010a). Kat1-protein expression is also modulated by a natural frameshift in the *KAT1* gene that requires ribosomal slippage for correct translation. Syntenic orthologs of the α*3* and *KAT1* genes are present only within the genus *Kluyveromyces*, suggesting that this switching mechanism is genus specific (Figure 4; Barsoum *et al.* 2010a; Rajaei *et al.* 2014). The order of evolutionary recruitment of α3 and Kat1 into the mating-type switching process is unknown, as is the mechanism of dsDNA-break formation in the three-cassette system that preceded it in the common ancestor of *Saccharomyces* and *Kluyveromyces*. Some other species of Saccharomycetaceae have genes similar to MULE or Roamer transposases that are distant paralogs of α*3* and *KAT1* (Sarilar *et al.* 2015; Wolfe *et al.* 2015), but these have not been implicated in mating-type switching.

Mobile elements as endonucleases

The discovery that HO, α3, and Kat1 are all domesticated versions of selfish genetic elements is intriguing. Inteins and DNA transposons take advantage of the cell's repair system for broken chromosomes to spread through the population at a rate faster than expected under genetic drift (Burt and Trivers 2008). Super-Mendelian inheritance of inteins is achieved by homing in diploids as described above, enabling the intein-containing allele at a particular locus to spread vertically through the population. DNA transposons that mobilize by a cut-and-paste mechanism, such as MULE and hAT elements, can also increase their numbers in a population at a faster rate than expected under drift. However, for these elements, the increase occurs horizontally by transposition to additional sites in the genome. The copy number of the

element will increase if the double-strand break formed during excision from the old site is repaired using a second (transposon-containing) copy of the old site as a donor. In diploids, the second allele of the old site can act as a donor in this way. Thus a diploid cell that is homozygous for a transposon at one insertion site can give rise to a mitotic descendant that is still homozygous at the original site as well as heterozygous at a new insertion site, increasing the copy number of the transposon from two to three (Burt and Trivers 2008). Alternatively, in either haploids or diploids, if the chromosomal region of the old site replicates before excision occurs, then the sister chromatid formed by replication can act as a donor for repair (Burt and Trivers 2008). However, the latter mechanism will only increase the transposon's copy number if mobilization occurs in an interval of S phase when the old site has replicated but the new site has not.

It has been hypothesized that sexual reproduction may be driven by selfish elements as a means to spread themselves more efficiently in a population (Hickey 1982; Rose 1983; Keeling and Roger 1995). Homing endonucleases in particular are proposed to have a cycle of vertical proliferation within a population until a 100% allele frequency is reached, which will then inevitably be followed by degeneration of the element because it can proliferate no further, unless it is transmitted horizontally to another population or its site specificity drifts to a new target (Gimble 2000; Burt and Koufopanou 2004). This cycle of degradation might be escaped if the element were to integrate into a process that increases the probability of outcrossing. The domestication of HO for mating-type switching has been suggested as an example of a selfish gene driving sexual reproduction; if mating-type switching was initially a passive process that occurred at a low frequency, then a selfish element that increased the frequency of switching (and therefore the frequency of sexual reproduction) could spread more rapidly in a population (Keeling and Roger 1995). Experimental studies in yeast have demonstrated the increased spread of selfish elements via sexual reproduction, even at a fitness cost to the cell, and have presented evidence that selfish elements can increase the rate of sporulation in *S. cerevisiae* (Futcher 1988; Goddard *et al.* 2001; Kelly *et al.* 2012; Giraldo-Perez and Goddard 2013; Harrison *et al.* 2014). An important caveat to this hypothesis, however, is that in *S. cerevisiae* mating-type switching increases the rate of haplo-selfing rather than outcrossing (see below), which would not facilitate the proliferation of a selfish element in a population.

For a DNA transposon in a haplontic yeast such as *K. lactis*, gaining control of mating-type switching could nevertheless be a desirable goal in some circumstances. Specifically, it would be advantageous to any type of transposon for which repairing the site of excision by interhomolog repair (in diploid cells) leads to more efficient spreading than intersister chromatid repair (in haploid cells after DNA replication). For example, if a transposon excised early in the cell cycle before replication began, intersister repair would not be possible, but the interhomolog repair pathway would be available if the cell were diploid. Therefore, a transposon of this type requires its haploid host cell to mate if it is to spread selfishly. It would be advantageous for the transposon to put its transposase under the same regulatory signals (*e.g.*, nitrogen starvation) that induce mating or meiosis so that it only attempted to mobilize in diploids. For a transposon in a "lonely" isolated haploid cell (lacking a partner of the opposite mating type) it would be a master stroke if induction of the cut phase of the transposon's mobilization cycle also induced cutting of the haploid's *MAT* locus; so that mating-type switching occurs, a diploid is formed by mating, and the transposon can complete its mobilization—becoming homozygous at the old site and heterozygous at a new site. This hypothesis provides a rationale for a link between cut-and-paste DNA transposons and the control of switching, but it cannot be applied to inteins unless homing is frequently off target.

The connection between *MAT*-locus cleavage mechanisms and selfish elements raises many questions about how these mechanisms evolved (Rusche and Rine 2010). Was an ancestral cleavage mechanism supplanted on two separate occasions, by HO and α3/Kat1, suggesting rapid turnover of mechanisms? Was switching ever a passive process that did not require an induced DNA break? *HO*-deficient strains of *S. cerevisiae* are still capable of switching mating types, albeit at a frequency ~1,000,000-fold lower than in wild-type strains (Herskowitz 1988); so an ancestral mechanism that relied only on spontaneous breakage and homologous recombination is perhaps plausible. Some other yeasts, such as *L. waltii*, have a three-cassette *MAT*-locus structure but lack both *HO* and *α3/KAT1* genes (Di Rienzi *et al.* 2011). *L. waltii* contains several hAT transposons (Rover family) (Souciet *et al.* 2009; Bleykasten-Grosshans and Neuveglise 2011; Sarilar *et al.* 2015) and has been shown to switch mating types (Di Rienzi *et al.* 2011). The two-cassette system in methylotrophs may also be informative in this respect, because in these species mating-type switching is inducible under nutrient-limiting conditions (Tolstorukov *et al.* 1982; Hanson *et al.* 2014; Maekawa and Kaneko 2014). The inducibility of switching suggests that recombination between the IRs is not a passive process, but no candidates for the endonuclease or recombinase responsible have been identified. Furthermore, in *K. phaffii*, nutrient-limiting conditions induce recombination only between the outer set of IRs at its *MAT* loci. Neither the inner IRs nor other sets of IRs present at its centromeres recombine during nutrient limitation (Hanson *et al.* 2014; Coughlan *et al.* 2016). This specificity suggests that switching in methylotrophs involves targeted recombination rather than induction of a general mechanism for NAHR.

Evolution of Mating-Type Switching Regulation

Mating-type switching is inherently risky due to the need to make a double-strand break in a haploid genome. It is therefore tightly regulated both in direction, to ensure that it produces a cell of the required mating type, and in timing,

to ensure that switching only occurs when it is most likely to result in successful mating. As discussed below, regulation is effected through multiple controls: on the choice of donor locus, by tracking the cell lineage and controlling the point in the cell cycle when switching occurs, and in some species by regulating switching in response to environmental conditions.

Donor bias

Because the three-cassette systems of *S. cerevisiae* and *S. pombe* include silent copies of both *MAT* alleles, the choice of template for repair of the double-strand break at *MAT* cannot be random. Random choice of a donor would result in a successful ("productive") mating-type switch only 50% of the time, the others being futile *MAT***a** → *MAT***a** or *MAT*α → *MAT*α switches. Both species have overcome this problem and bias the choice of donor to the opposite allele in 80–90% of switching events by mechanisms that are independent of the sequences present in the silent loci themselves (Klar *et al.* 1982).

In *S. cerevisiae*, a recombination enhancer (RE) sequence present on the left arm of chromosome *III* biases repair of the *MAT* locus in *MAT***a** cells toward *HML* as the donor, leading to a high frequency of use of *HML* which normally contains the silent α cassette and hence productive switching (Wu and Haber 1996; Wu *et al.* 1997). In *MAT*α cells, the RE is bound by the α2-Mcm1 complex, inhibiting the use of *HML* which is ~17 kb away. This complex is not present in *MAT***a** cells. The presence of an RE located between *MAT* and *HML* may explain why these two loci are physically linked on the same chromosome in all known Saccharomycetaceae species, and why (apart from in some exceptional *S. cerevisiae* strains) the genotype of *HML* is always *HML*α, whereas *HMR***a** is usually found on a separate chromosome (Oshima 1993; Gordon *et al.* 2011; Vakirlis *et al.* 2016). Synteny is exceptionally well conserved in the genomic regions between *MAT* and *HML* including the RE, suggesting that the linkage between *MAT* and RE is constrained, even though the DNA sequence of RE itself is not strongly conserved (Zhou *et al.* 2001). In most species other than *S. cerevisiae*, the fact that using *HML*α as a donor for *MAT* repair is an intramolecular reaction, whereas the use of *HMR***a** is an intermolecular reaction with a different chromosome, may create a bias toward using *HML*α in *MAT***a** cells where the RE is not bound (Coic *et al.* 2006; Agmon *et al.* 2009).

S. pombe contains two RE sequences, SRE2 and SRE3, located proximally to *mat2* and *mat3*, respectively (Figure 5; Jia *et al.* 2004; Jakociunas *et al.* 2013). The recombination promoting complex (Swi2/Swi5) localizes differentially across the entire silenced region, including the SRE sequences, in a cell-type specific manner (Jia *et al.* 2004). Swi2 expression is in part regulated by the Mat-Mc protein (Matsuda *et al.* 2011; Yu *et al.* 2012), and the levels of Swi2 influence the biased repair to the appropriate cassette, with higher expression in M cells resulting in preferential repair by *mat2*, and lower expression in P cells resulting in preferential repair by *mat3*.

The need for a donor-bias mechanism is unique to species with three-cassette switching systems. The two-cassette system in methylotrophs does not encounter this problem because there is no choice of donor to be made: switching always swaps the single expressed *MAT* locus with the single silent one. However, if inversion of the *MAT* region in methylotrophs occurs by mitotic NAHR between the two copies of the IR, then resolution of the Holliday junction is likely to result in only 50% of the attempted switching events being productive, with the other 50% being resolved as noncrossovers that fail to invert the *MAT* region (Hanson *et al.* 2014). Switching may therefore be only 50% productive in methylotrophs as compared to 80–90% in *S. cerevisiae* and *S. pombe*. This hypothesis has not been directly tested experimentally, but is supported by experiments with *P. methanolica* (formerly *P. pinus*) in which Tolstorukov *et al.* (1982) found that the proportion of switched cells appearing in a liquid culture after induction reached a plateau of 50%. Although this observation suggests that methylotroph cells take on a great amount of risk with a relatively small probability of reward, the NAHR mechanism may be less susceptible to failure leading to chromosome breakage than the SDSA mechanism, and it may function to maintain approximately equal numbers of each mating type within a population.

Lineage tracking and cell-cycle regulation

The developmental timing of mating-type switching is also strictly regulated in *S. cerevisiae*. Switching can occur only during G_1 phase in haploid cells that have divided at least once (Strathern and Herskowitz 1979). This tight regulation is primarily due to control of expression of *HO*, which has one of the largest and most complex promoters in the *S. cerevisiae* genome, and to the rapid degradation of the HO protein. HO expression is restricted to haploids but not diploids, to mothers but not daughters, and to the G_1-S transition point in the cell cycle (Stillman 2013). *HO* expression is haploid specific because, like other hsg's, it is repressed in diploids by the **a**1-α2 heterodimer. *HO* expression is confined to the G_1 phase of the cell cycle because it requires the G_1-specific SBF complex, comprised of Swi4 and Swi6. *HO* expression also shows cell-lineage tracking, an unusual form of gene regulation that operates via the Ash1 protein. Ash1 binds to the *HO* promoter and represses it by recruiting the Rpd3(L) histone deacetylation complex, but Ash1 protein is predominantly confined to daughter cells (Bobola *et al.* 1996; Stillman 2013). This differential protein localization is achieved by a mechanism that transports *ASH1* messenger RNA into daughter cells, resulting in daughter-specific *ASH1* translation, and hence repression of *HO* (Cosma 2004; Haber 2012). In contrast, mother cells lack Ash1 and express *HO*. Because *HO* expression occurs just prior to DNA replication (S phase), both the mother cell and its next (second) daughter cell will inherit the switched *MAT* genotype. The first daughter cell, having not yet budded, will produce a daughter of the original mating type. Thus, in *S. cerevisiae*, switched cells appear in pairs alongside two unswitched cells. This process of cell-lineage tracking, in combination with the axial budding pattern of haploid cells, ensures that switched cells are in close physical

proximity to a potential mating partner (Nasmyth 1982; Gimeno and Fink 1992; Knop 2011).

In *S. pombe*, which divides by fission and does not have morphologically distinct mother and daughter cells, the inheritance of the epigenetic mark responsible for double-strand break induction dictates which cells are competent for switching (Egel 2005; Klar 2007). A cell that generates the epigenetic mark during DNA replication will pass the mark to one of the two daughter cells it makes after fission, which will then be able to produce one switched granddaughter cell (Arcangioli and Thon 2004).

The possibility of cell-lineage tracking in *O. polymorpha* or *K. phaffii* has not yet been investigated. A study in *P. methanolica* found no evidence for such a mechanism, as both mother and daughter cells were capable of switching, but as in *S. cerevisiae* switching seemed to occur only in cells that have already budded once (Tolstorukov and Benevolenskii 1981; Tolstorukov *et al.* 1982). As discussed above, it is possible that instead of lineage tracking, the availability of mating partners in methylotrophs is ensured by the low productivity of switching (50% success) due to unbiased resolution of Holliday junctions formed in the IR. If inversion of the *MAT* region instead occurs by a pathway independent of homologous recombination such as a site-specific recombinase, or if proteins that bias the resolution of Holliday junctions in favor of crossovers (analogous to *S. cerevisiae* Zip3 in meiosis; Oke *et al.* 2014) are involved, then a bias toward >50% productive switching could be achieved in methylotrophs.

Response to environmental conditions

In most yeast species, processes related to mating and sporulation, including mating-type switching, are coordinated with nutrient availability. It is unsurprising that this is the case, given the likely benefits of recombination under conditions of environmental stress. However, *S. cerevisiae* is an exception to this generalization. In *S. cerevisiae*, which is diplontic, only meiosis and sporulation are controlled by nutrient availability. Mating-type switching and mating occur spontaneously in vegetatively growing haploid *S. cerevisiae*, even in rich media. In poor conditions, diploid *S. cerevisiae* cells make a complex decision between sporulation, quiescence, and foraging by pseudohyphal growth (Honigberg 2016). Four conditions must be met for diploid *S. cerevisiae* to enter into meiosis (Freese *et al.* 1982; Piekarska *et al.* 2010; Neiman 2011; Broach 2012): (1) The cells must be starving for nitrogen (or possibly other nutrients), which results in arrest of the cells in G_1 phase. (2) Glucose must be absent from the environment, (3) while a nonfermentable carbon source is present, stimulating respiration, which is essential for sporulation. Finally, (4) the cells must have the **a**/α genotype at the *MAT* locus. These conditions result in the integration of several signaling pathways that converge on the large and complex promoter of the transcription factor *IME1*. *IME1* activates the expression of early meiosis genes, including *IME2* and *NDT80*, which in turn lead to induction of the later stages of meiosis (Brar *et al.* 2012).

In haplontic yeast species, switching, mating, meiosis, and sporulation are coordinated and coregulated processes (Gleeson and Sudbery 1988; Barsoum *et al.* 2011; Sherwood *et al.* 2014). In some, induction is even required for expression of the mating-type genes and pheromones (Kelly *et al.* 1988; Sherwood *et al.* 2014), which are constitutive in *S. cerevisiae*. For many haplontic yeasts, such as *O. polymorpha* (Gleeson and Sudbery 1988) and *K. lactis* (Barsoum *et al.* 2010a), environmental nutrient levels act as a signal that normally induces a suite of steps comprising switching, mating, meiosis, and sporulation. These species have a "fused" sexual cycle (Sherwood *et al.* 2014) in which meiosis follows immediately after mating without requiring a separate trigger. However, examination of the molecular mechanisms regulating these processes in several haplontic species has revealed substantial variation in how they are coordinated, demonstrating that extensive changes in the regulatory pathways have occurred during Ascomycota evolution.

In *K. lactis*, Rme1 (Mts1) plays a central role in switching, mating, and meiosis, and is under environmental regulation. Haploid *K. lactis* cells repress switching and mating under vegetative growth conditions (Barsoum *et al.* 2010a). Rme1 is required for activation of both of these processes, in addition to having a role conserved with *S. cerevisiae* Rme1 in suppressing meiosis genes (Barsoum *et al.* 2010a; Booth *et al.* 2010). Activation of *K. lactis RME1* by phosphate and glucose depletion is required for expression of hsg's to facilitate mating, in contrast to the constitutive expression of hsg's in vegetative *S. cerevisiae* haploids. In addition, although repression of *RME1* in diploids is mediated by **a**1-α2 in *K. lactis* and *S. cerevisiae*, all other hsg's in *K. lactis* have lost **a**1-α2 repression and instead are activated by Rme1 (Booth *et al.* 2010). Vegetative expression of hsg's in *L. kluyveri* and their lack of regulation by Rme1 suggest that repression of hsg's by **a**1-α2 (as in *S. cerevisiae*) is the ancestral condition, and that hsg regulation in the *K. lactis* lineage has both lost **a**1-α2 repression and gained Rme1 activation (Booth *et al.* 2010).

Clavispora (*Candida*) *lusitaniae*, a haploid species in the CUG-Ser clade, also has a fused sexual cycle in which mating proceeds directly into meiosis (Sherwood *et al.* 2014). These processes are induced by starvation, which in the laboratory is achieved by growth on dilute potato dextrose agar (Reedy *et al.* 2009), a medium deficient in nitrogen (Sholberg 1981). *C. lusitaniae* does not undergo mating-type switching, but it has reassigned the functions of two key regulators of mating and meiosis. The transcription factor Ste12 and the kinase Ime2 are both required for both mating and meiosis in *C. lusitaniae*, whereas in *S. cerevisiae* they have specific roles in mating and meiosis, respectively (Sherwood *et al.* 2014). This regulatory rewiring is limited to the *C. lusitaniae* lineage, as other haplontic species including *K. lactis*, *Yarrowia lipolytica*, and *K. phaffii* do not require *STE12* for meiosis (Sherwood *et al.* 2014). Notably, *C. lusitaniae* has lost *MAT*α2 and therefore lacks the **a**1-α2 repressor necessary for meiosis in *S. cerevisiae* (Reedy *et al.* 2009). This loss of *MAT*α2 may be related to the rewiring of other regulatory pathways. In addition, Ime2 has known nonmeiotic functions in *S. cerevisiae* (Strudwick *et al.* 2010), and is involved in other developmental

processes outside of the Saccharomycotina (Hutchison and Glass 2010; Irniger 2011; Hutchison *et al.* 2012).

Induction of switching, mating, and sporulation in *S. pombe* is analogous to that in Saccharomycotina. As in other haploid yeasts, induction of these processes is mediated by environmental conditions: nitrogen starvation and the induction of stress response and mating-pheromone-activated signaling pathways (Davey 1998; Otsubo and Yamamoto 2012). These signals converge on the activation of *S. pombe STE11*, an HMG-domain transcription factor analogous in response and function to *S. cerevisiae STE12* but not homologous to it (Sugimoto *et al.* 1991). Ste11 activates genes required for cell and nuclear fusion, as well as mating-type genes and pheromones (Otsubo and Yamamoto 2012; Merlini *et al.* 2013). It is further needed for activation of Mei2, an RNA-binding protein required for entry into meiosis.

Methylotrophic yeasts also require nutrient conditional activation of mating-type switching, mating, and sporulation (Gleeson and Sudbery 1988; Lahtchev 2002; Hanson *et al.* 2014; Maekawa and Kaneko 2014), but the regulatory genes involved have not yet been identified. *RME1*, *STE12*, and *IME2* are present in the genomes of both *O. polymorpha* and *K. phaffii*, although *IME1* could not be identified. Analysis of the upstream regions of transcriptionally coregulated mating genes in *K. phaffii* did not identify the Rme1 binding motif conserved between *S. cerevisiae* and *K. lactis* (Rebnegger *et al.* 2014), but this does not exclude the possibility of direct or indirect Rme1 regulation of these genes. Given the diversity of regulatory mechanisms for the coordinated activation of switching, mating, and sporulation in Saccharomycotina, the regulatory mechanisms in methylotrophs may be informative as to the ancestral state.

Resporulation as the Purpose of Mating-Type Switching

Although the mechanisms of mating-type switching have been explored in detail in several species, the evolutionary *raison d'être* of this process has received much less consideration. Switching is an error-prone process, as is clear from the elevated point-mutation rate at the newly synthesized *MAT* locus (Hicks *et al.* 2010) and the litany of gene deletions and transpositions that have occurred beside *MAT* during the evolution of the Saccharomycetaceae family (Gordon *et al.* 2011). From an evolutionary perspective, switching must therefore serve a function or provide a benefit to the host species that outweighs its mutagenic cost; otherwise natural selection would not have maintained the switching apparatus in so many species.

When thinking about the evolutionary advantage of mating-type switching, we need to disentangle the advantage of switching from the advantage of diploidy over haploidy as the primary mitotic growth form. Some yeast species that can switch mating types, such as *K. lactis* and *O. polymorpha*, are haplontic. Switching in these species normally leads immediately to mating and sporulation, without intervening diploid mitoses, so they appear to shun the often-cited advantages of a diploid mitotic lifecycle, such as the masking of recessive deleterious alleles or the ability to repair DNA by homologous recombination (Herskowitz 1988). *S. cerevisiae* is diplontic because it mates without requiring a nutritional signal, not because it can switch mating types. The phylogenetic distribution of diplonty *vs.* haplonty as the primary mitotic growth form of yeast species has not been studied in detail and is complicated by the existence of hybrids and species with cryptic sexual cycles (Dujon 2010). As a generalization, many post-WGD Saccharomycetaceae are diplontic and many outgroups to the WGD are haplontic (*i.e.*, non-WGD Saccharomycetaceae, methylotrophs, and fully sexual CUG-Ser clade species), but there are several exceptions to this rule of thumb. For species that have the option of either haploid or diploid mitosis, the relative advantages of each may depend on the environmental context (Zorgo *et al.* 2013).

It could be argued that because mating-type switching increases the frequency of mating, the benefits of mating-type switching are the same as the benefits of sexual reproduction—that is, the ability to reassort beneficial alleles and purge deleterious ones (McDonald *et al.* 2016)—but this argument only applies if switching leads to outcrossing. The argument is consistent with the link between switching and transposable elements. Following spore germination, a diploid can be formed in one of three ways: by outcrossing (amphimixis), by intratetrad mating (automixis), or by haplo-selfing (mating between a mother and daughter cell after mating-type switching) (Greig and Leu 2009). Population genomic studies of *S. paradoxus* have led to estimates of the rates of these methods of diploidization in natural populations, with 94% of sexual generations resulting in intratetrad mating, 5% in haplo-selfing, and 1% in outcrossing (Tsai *et al.* 2008). Outcrossing has also been estimated to be as infrequent as 1 in every 50,000 generations in *S. cerevisiae* (Ruderfer *et al.* 2006), and the rate of outcrossing in *S. cerevisiae* is of the same order of magnitude as in *L. kluyveri* which cannot switch mating types (Friedrich *et al.* 2015). Thus mating-type switching appears not to be a significant factor contributing to the frequency of outcrossing in *Saccharomyces*.

Switching has the net effect of converting haploid cells into diploids. Yeast cells are not motile, so a haploid cell can only mate if it detects a haploid of the opposite type within shmooing distance. The effect of mating-type switching is that a haploid can create a mating partner for itself when no other potential partners are nearby. Several aspects of *S. cerevisiae*'s lifecycle indicate that the formation of diploids is a trait that has been strongly selected for in this species, leading to diplonty. First, in tetrad asci, the four haploid spores are arranged in a tetrahedral pyramid shape that ensures that every spore is positioned beside spores of the opposite mating type (Nasmyth 1982). Second, in circumstances where the sporulating diploid cell does not have sufficient resources to form a tetrad, the most common outcome is that a dyad (two-spored ascus) is formed instead, and a phenomenon termed spore number control results in the preferential production of dyads containing two nonsister spores that have compatible

MAT loci to facilitate mating (Taxis *et al.* 2005; Lacefield and Ingolia 2006; Knight and Goddard 2016). Third, the axial budding pattern of haploid cells, in combination with *S. cerevisiae*'s restriction of mating-type switching to mother cells, ensures that when a spore germinates, *MAT***a** and *MAT*α cells will be as close to one another as possible in the developing colony and therefore able to mate (Gimeno and Fink 1992). Fourth, asci of *S. cerevisiae* are "persistent," meaning that the four spores are likely to remain together before germination (Knop 2006), and scanning electron microscopy of asci reveals interspore bridges that may promote intratetrad mating (Coluccio and Neiman 2004). Notably, these features of *S. cerevisiae* asci are not found in asci of haplontic species such as *S. pombe*, consistent with selection for features facilitating rapid diploidization in *S. cerevisiae* (Coluccio and Neiman 2004; Taxis *et al.* 2005; Knop 2006). Fifth and perhaps foremost, mating ability in *S. cerevisiae* is constitutive and does not require induction.

The maintenance of mating-type switching is simpler to rationalize in haplontic species that have fused sexual cycles with only transient diploid states. Cells that require an environmental signal to diploidize may grow for several haploid generations before mating conditions are encountered. This increases the probability that such a cell may not find a mating partner. The simultaneous induction of mating-type switching and mating would therefore be more likely to result in successful diploidization and subsequent sporulation. However, if a mating partner is present when mating is induced, mating-type switching will be unnecessary and should be repressed (such as by pheromone-signaling pathways) (Barsoum *et al.* 2011), although this hypothesis has not been tested in haplontic species.

What evolutionary pressures might act to maintain mating-type switching in diplontic species like *S. cerevisiae*? Mortimer proposed that it could be maintained because occasional haplo-selfing events are necessary to purge recessive deleterious alleles that accumulate during generations of asexual reproduction or intratetrad mating in the population (Mortimer *et al.* 1994; Mortimer 2000; Magwene 2014). This problem is exclusive to diplontic species, because in haplontic species the recessive alleles will be exposed during haploid mitotic growth. In fact, *S. cerevisiae* populations are polymorphic for the ability to switch, with null alleles of the *HO* locus being present in 25% of natural isolates in one study (Katz Ezov *et al.* 2010). Losses of switching can also be inferred to have occurred in two species in the family Saccharomycetaceae (*L. kluyveri* and *Kazachstania africana*) (Gordon *et al.* 2011), as well as in the large CUG-Ser clade of species related to *C. albicans* (Butler *et al.* 2009). One could therefore argue that although there has been evolutionary pressure to maintain switching in most yeast species, this pressure can sometimes disappear.

Our laboratory has proposed an alternative hypothesis, that switching is maintained by selection for the ability of a cell to resporulate after germinating (Gordon *et al.* 2011; Hanson *et al.* 2014). This hypothesis can be applied to both diplontic and haplontic species, and is based on the concept of a "lonely spore," first proposed by Herskowitz (1988). A lonely spore is one that is physically isolated from any other spores of the same species, so that when it germinates it will give rise to a haploid colony unless it is capable of mating-type switching, in which case some cells in the colony will be diploid. The factors determining when a spore germinates are not well understood, but it seems likely that the control of germination timing is under strong selection. Spores are survival structures that can protect a cell during times when vegetative growth is not possible. In environmental conditions that are improving (changing from inhospitable to hospitable), a spore that germinates earlier than its peers will have a competitive advantage. However, if it germinates too early, the environment may not be sufficient and it could die. Because only diploid cells can form spores, we have argued that a mating-type switching system enables spores to try germinating earlier (to test the environment); because if the environment is inhospitable they can form new spores after as few as two rounds of cell division. In contrast, germination is an all-or-nothing commitment for a yeast strain that cannot switch mating types, because once it germinates it cannot make new spores unless it finds a mating partner. By computer simulation, we have shown that features such as donor bias and lineage tracking increase the proportion of diploid cells (or haploid cells with a potential mating partner) in the microcolony formed from a germinating spore, so the emergence of these features can be explained by natural selection for an increased ability of germinating cells to resporulate (Hanson *et al.* 2014). The change from a two-cassette to a three-cassette switching system can also be explained by this model, because donor bias is only possible in a three-cassette system.

If the purpose of mating-type switching systems is to maximize the ability of microcolonies to form new spores, the benefit of switching could be a direct one related to cell survival as envisaged above, or an indirect one related to cell dispersal. *S. cerevisiae* is not carried by wind and instead relies on vectors such as insects for its introduction into new environments (Mortimer and Polsinelli 1999; Goddard *et al.* 2010; Stefanini *et al.* 2012). The spore wall enables spores to survive digestion by *Drosophila melanogaster* (Coluccio *et al.* 2008). Thus, for *S. cerevisiae*, the induction of sporulation by a poor environment may be a means to disperse into a new and perhaps more favorable environment after meiotic recombination and the generation of potentially advantageous genotypic variation (Neiman 2011). The passage of spores through the gut of *D. melanogaster* has also been shown to increase the rate of outcrossing in *S. cerevisiae* (Reuter *et al.* 2007), suggesting that spores in asci become separated from one another during dispersal. Whether this spore separation also results in an increased rate of haplo-selfing is not known.

Mating-type switching can be viewed as a form of reproductive assurance (Nieuwenhuis and Immler 2016): it enables haploid cells to mate, even if they are immotile and dispersed at low density in the environment. Secondary homothallism by switching thus guarantees that most cells can

mate, without losing the benefits of having separate mating types (such as increased genetic diversity through outcrossing) that are absent in primary homothallism. While we agree with this view, we would suggest that in yeasts, which have the option of long-term mitotic reproduction without sex, the purpose of assuring mating is not to assure reproduction but to assure that spores can be formed.

Perspectives

Recent advances (Hanson *et al.* 2014; Maekawa and Kaneko 2014; Riley *et al.* 2016) allow us to postulate that the ancestral mating-type switching system in the fungal subphylum Saccharomycotina consisted of a two-cassette, flip-flop system, which later developed into the more complex three-cassette SDSA system of family Saccharomycetaceae. The ancestral flip-flop system originated prior to the divergence between *A. rubescens*, the methylotrophs, and the Saccharomycetaceae (Figure 4). We do not know what mechanism was used to repress transcription of the silent *MAT* locus in this ancestor, but it cannot have been either the H3K9me2/3 system (which was already lost) or the SIR system (which had not yet been invented), so centromeric or telomeric silencing seems likely. One possible, albeit speculative, way that the first two-locus system might have originated is that a diploid cell of a heterothallic species could have sustained a chromosomal rearrangement that moved one of the *MAT* alleles onto the same chromosome as the other allele, but in a heterochromatic region (centromeric or telomeric). Recombination between repetitive elements such as transposons could then have allowed flip-flop switching to emerge, inverting the region containing the two *MAT* alleles.

One of the most intriguing aspects of mating-type switching is the extent of parallels between the switching systems of subphyla Taphrinomycotina (*S. pombe*) and Saccharomycotina (*S. cerevisiae*, *K. lactis*, the methylotrophs, and *A. rubescens*). Since these subphyla share a multicellular common ancestor, and mating-type switching does not occur in multicellular fungi, it appears beyond doubt that the Saccharomycotina and Taphrinomycotina switching systems are a dramatic example of convergent genomic evolution in which each system independently developed silent cassettes and donor bias. At present we have little information about what the ancestors of the *S. pombe* system looked like. The unicellular (yeast) lifestyle emerged from multicellular ancestors on at least five separate occasions during fungal evolution (Nagy *et al.* 2014). Taphrinomycotina and Saccharomycotina are two of these clades, but we can also wonder whether switching may have emerged in the other three clades, or why it did not. These three clades are all in Basidiomycota. One, which includes the genus *Cryptococcus*, has been studied extensively and shows heterothallism with no evidence of switching, but the others which include the genera *Sporobolomyces* and *Malessezia* are much less investigated.

A final more philosophical question concerns why cell-type specification in secondary homothallic yeasts like *S. cerevisiae* includes a DNA-rearrangement mechanism at all. Cell differentiation in most other eukaryotes is achieved using normal transcription factor networks, so why do yeasts use a chromosomal rearrangement to change mating types? It is interesting to compare the *MAT* system to the white/opaque switching system of *C. albicans*. This species has two yeast cell morphologies that are specified by the two stable states of a gene regulatory circuit with feed-forward loops (Zordan *et al.* 2007). In the opaque state, the Wor1 transcription factor is active and Efg1 is repressed; while in the white state, Efg1 is active and Wor1 is repressed; but the DNA content of white and opaque cells is identical. Switching between the two states occurs at a frequency of 5–16% (Zordan *et al.* 2007). Why can yeast mating type not be controlled by a simple network like this? The answer is that the states of a regulatory network cannot segregate in meiosis. If cell type were specified by the state of a network, a network in the **a**/α state would need to be able to go through meiosis and produce spores with only **a** or α networks, which seems impossible. Cell type needs to have Mendelian inheritance, so it needs to be controlled by alleles of a chromosomal locus. Therefore, in turn, if haploids are to switch mating types, they must be able to alter the DNA of that locus to exchange the alleles in a reversible manner.

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