

Interspecies variation reveals a conserved repressor of α -specific genes in *Saccharomyces* yeasts

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The mating-type determination circuit in *Saccharomyces* yeast serves as a classic paradigm for the genetic control of cell type in all eukaryotes. Using comparative genetics, we discovered a central and conserved, yet previously undetected, component of this genetic circuit: active repression of α -specific genes in a cells. Upon inactivation of the *SUM1* gene in *Saccharomyces bayanus*, a close relative of *Saccharomyces cerevisiae*, a cells acquired mating characteristics of α cells and displayed autocrine activation of their mating response pathway. Sum1 protein bound to the promoters of α -specific genes, repressing their transcription. In contrast to the standard model, $\alpha 1$ was important but not required for α -specific gene activation and mating of α cells in the absence of Sum1. Neither Sum1 protein expression, nor its association with target promoters was mating-type-regulated. Thus, the $\alpha 1$ /Mcm1 coactivators did not overcome repression by occluding Sum1 binding to DNA. Surprisingly, the mating-type regulatory function of Sum1 was conserved in *S. cerevisiae*. We suggest that a comprehensive understanding of some genetic pathways may be best attained through the expanded phenotypic space provided by study of those pathways in multiple related organisms.

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

The mating-type-specific patterns of gene expression are dictated by the allele present at the mating-type (*MAT*) locus. The *MAT α* allele encodes two transcription factors: $\alpha 1$, which activates α -specific genes, and $\alpha 2$, which represses **a**-specific genes. The *MAT a* allele encodes only the **a1** protein, which forms a heterodimer with $\alpha 2$ in *MAT a* / α diploids to repress a third set of genes, the haploid-specific genes. In the standard model, known as the $\alpha 1$ - $\alpha 2$ hypothesis (Strathern et al. 1981), expression of **a**-specific genes in **a** cells is a default state, resulting merely from the absence of the $\alpha 1$ and $\alpha 2$ pro-

teins. Two transcription factors common to both **a** and α cells, Ste12 and Mcm1, are also necessary for proper expression of mating-type genes. Ste12 works at two regulatory levels in both mating types to activate transcription: It is required for basal transcription of **a**-specific and α -specific genes in the absence of the mating pheromones, **a**-factor and α -factor, and for their further induction in response to pheromones (Fields and Herskowitz 1985; Kirkman-Correia et al. 1993). Mcm1, a MADS-box transcription factor similar to mammalian Serum Response Factor, is required for the activation of α -specific genes and for both the activation and the repression of **a**-specific genes (Jarvis et al. 1989; Elble and Tye 1991; Hwang-Shum et al. 1991; Bruhn and Sprague 1994). In α cells, Mcm1 homodimers interact directly with $\alpha 1$ to activate α -specific genes; similarly, Mcm1 homodimers interact with $\alpha 2$ to repress **a**-specific genes (Smith and Johnson 1992). Ste12 and Mcm1 themselves interact to activate transcription, forming complexes with $\alpha 1$ at α -specific gene promoters, or acting on their own at **a**-specific gene promoters in **a** cells (Fig. 1A; Sengupta and Cochran 1990; Yuan et al. 1993; Bruhn and Sprague 1994).

Together, Mcm1 and Ste12 can activate **a**-specific genes, yet they require $\alpha 1$ to activate α -specific genes. This additional coactivator requirement has been ex-

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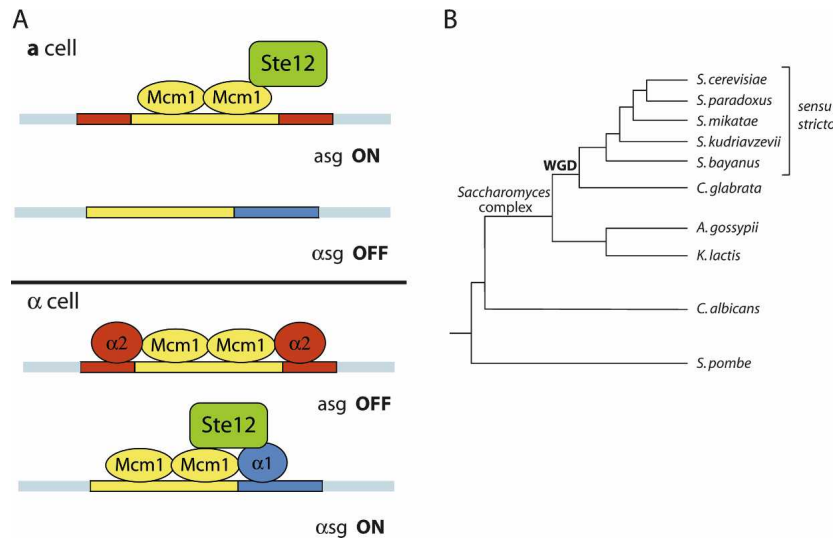


Figure 1. (A) The standard model for the transcriptional control of haploid mating-type genes in *S. cerevisiae*. The operator elements, contained within *a*-specific gene (*asg*) and α -specific gene (α sg) promoters, are schematized as colored boxes, which correspond to binding sites for $\alpha 1$ (blue), $\alpha 2$ (red), and Mcm1 (yellow). (B) Simplified phylogenetic tree, based on concatenated sequences of 153 genes present in all species shown, depicts the sensu stricto yeasts and some related yeast species. "WGD" denotes the whole-genome duplication that occurred along the branch leading to *C. glabrata* and the *Saccharomyces* species. Modified from Fitzpatrick et al. (2006).

plained solely by differential activator–DNA affinities encoded in distinct classes of Mcm1-binding sites. Whereas the highly conserved, palindromic sequence found in *a*-specific gene promoters, known as the *P* element, binds Mcm1 tightly, the related sequence known as the *P'Q* element, which confers α -cell specificity, binds Mcm1 with reduced affinity. Thus, the standard model holds that cooperative DNA binding by $\alpha 1$ and Mcm1 is necessary for α -specific gene expression. Despite extensive mutational and in vitro binding analyses of *P'Q* elements, no evidence for repression of α -specific gene expression has emerged (Bender and Sprague 1987; Flessel et al. 1989; Ganter et al. 1993; Hagen et al. 1993).

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

Interspecies genetic analysis may allow deeper probing into gene circuits because of mutations that strengthen or weaken known genetic interactions, or that bring about novel interactions. Other comparative studies have attempted to understand how genetic variation between species creates phenotypic variation. For example, the varying shapes and sizes of beaks in Galapagos finches can be explained by changes in BMP4 expression during development (Abzhanov et al. 2004). Here, we attempt to harness the natural variation present in related species to identify components of gene regulatory pathways that have eluded phenotypic detection in single-species analyses. Indeed, the distinction between

these goals can sometimes blur: This study was inspired by differences in phenotype caused by the same mutation in different species, yet led to discovery of a common regulatory component of both species.

The power of comparative genetic analysis is greatly expanded in taxa in which the genomes of multiple related species have been sequenced (Stark et al. 2007). The sensu stricto yeasts comprise several closely related *Saccharomyces* species—five of which have published genome sequences—that afford an excellent opportunity for studying gene regulatory pathways and their evolution (Cliften et al. 2003; Kellis et al. 2003). *S. bayanus* is the most evolutionarily distant of the sensu stricto yeasts from *S. cerevisiae* (Fig. 1B). Thus, comparisons between these two species are useful for making inferences about the sensu stricto ancestral state. The nucleotide substitution level between *S. bayanus* and *S. cerevisiae* is approximately equivalent to that between mouse and human (Kellis et al. 2003), offering a calibration for how that amount of variation affects function.

Analysis of the *Saccharomyces bayanus* genome sequence suggested that transcriptional repression by the Silent Information Regulator (Sir) proteins had changed in that lineage (Kellis et al. 2003; J. Babiarz and L. Teytelman, unpubl.). While examining transcriptional silencing in *S. bayanus*, we discovered an unexpected species-specific and mating-type-specific colony-wrinkling phenotype in *a* cells lacking the *SUM1* gene. *SUM1* encodes a transcriptional repressor of several dozen genes, many of which are expressed only during meiosis. Similar to silencing by the Sir proteins, Sum1 uses a NAD⁺-dependent histone deacetylase, called Hst1, to effect transcriptional repression. Sum1 recruits Hst1 via a protein called Rfm1, with these three proteins constituting a transcriptional repressor complex (McCord et al. 2003). A gain-of-function allele, *SUM1-1*, can restore silencing to the *HMRa* mating type locus in *sir* mutants (Klar et al. 1985; Laurenson and Rine 1991; Rusche and Rine 2001). The fortuitous phenotype of *S. bayanus sum1* Δ mutants suggested either that some dimension of mating type con-

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

Results

S. bayanus MATa sum1Δ had mating-type-specific and species-specific phenotypes

Upon targeted inactivation of the *SUM1* gene in *S. bayanus*, we observed a dramatic mating-type-specific change in colony morphology. *MATa sum1Δ* colonies were distinctly wrinkled, whereas *MATα sum1Δ* colonies were smooth and identical to wild type (Fig. 2A; see Fig. 4B, below, for wild-type comparison). Microscopic inspection of *MATa sum1Δ* cells revealed that some cells had elongated cell morphology reminiscent of the shmoo (plural of shmoo) formed in response to mating pheromone (Fig. 2B). The mutant cells also formed lightly flocculent clumps in liquid culture (indicated by their faster sedimentation than wild type), suggesting that up-regulation of adhesion molecules in *sum1Δ* mutants altered colony morphology (Supplemental Fig. S1). These phenotypes were especially intriguing given that *S. cerevisiae sum1Δ* mutants of both mating types have no discernible cell or colony phenotypes (Fig. 2A) and their mating behavior is identical to wild type (Klar et al. 1985; Laurenson and Rine 1991). Given the shmoo-like cells, we asked whether the mating response in *S. bayanus MATa sum1Δ* cells was activated even in the absence of a mating partner. Indeed, the pheromone-induced genes *FIG1* and *FIG2* were highly up-regulated in *MATa sum1Δ* cells relative to wild type (Fig. 2C).

Mating tests on *sum1Δ* mutants of both mating types

revealed that the activated mating response indeed reflected a change in mating behavior. A subset of *MATa sum1Δ* mutant cells mated with the *MATa* tester strain, with more cells retaining the ability to mate with the *MATα* strain (Fig. 2D). This bimating ability of the population suggested that the expression of mating genes normally expressed only in α cells was not properly regulated. This prediction was born out in quantitative RT-PCR analysis: The expression of *MFα1* and *MFα2*, the two genes encoding α -factor, and *STE3*, the **a**-factor receptor gene, were increased 90-fold, 10-fold, and 30-fold, respectively, in *MATa sum1Δ* cells compared with wild type (Fig. 3A,B). The expression of *YLR040c*, an α -specific gene of unknown function, also was increased by threefold. However, expression of *SAG1*, which encodes the α -specific agglutinin involved in cell–cell adhesion during mating, was unaffected in mutant **a** cells but decreased threefold in *MATα sum1Δ* mutants relative to wild-type α cells (Fig. 3B; discussed further below).

Two other features of α -specific gene expression were noteworthy. First, for *STE3* and *YLR040c*, a small increase in gene expression was observed in *MATα sum1Δ* mutants compared with wild type (discussed further below). Second, although substantial α -specific gene expression occurred in *MATa sum1Δ* cells, the levels reached only 10%–30% of those in wild-type α cells. This difference in expression was potentially due to the absence of the $\alpha 1$ activator in **a** cells. Nonetheless, the expression of most α -specific genes in *MATa sum1Δ* cells did not fit the standard model for mating type determination developed in *S. cerevisiae*, and implied that Sum1 was a novel repressor of α -specific genes in *S. bayanus*.

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

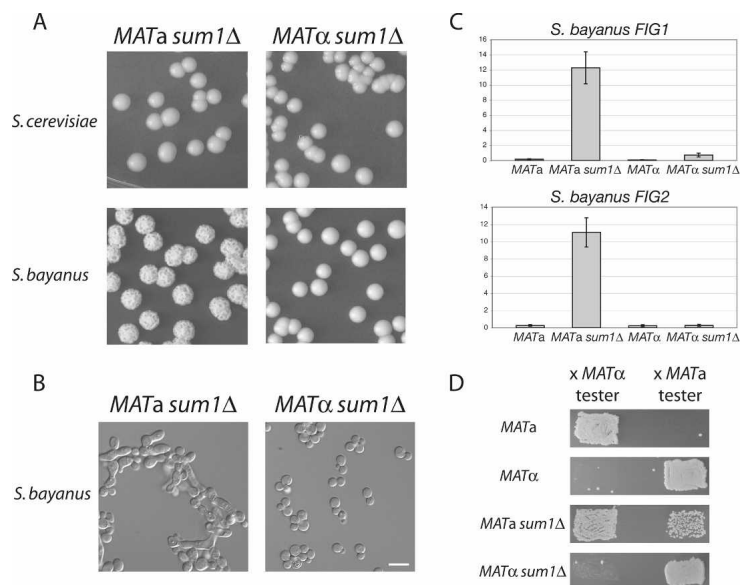


Figure 2. Species-specific and *MATa*-specific phenotypes in *sum1Δ* mutants. (A, bottom panels) *S. bayanus MATa sum1Δ* and *MATα sum1Δ* colonies grown for 5 d on YPD (for wild-type comparison see Fig. 4B). (Top panels) *S. cerevisiae sum1Δ* colonies (W303 background) of both mating types grown for 5 d on YPD. (B) DIC microscopy of *S. bayanus sum1Δ* cells from colonies grown on YPD. Bar, 10 μ m. (C) Quantitative RT-PCR analysis of the *S. bayanus* mating pheromone-induced genes *FIG1* and *FIG2* in wild-type and *sum1Δ* cells of both mating types. (D) Patch mating assays of *S. bayanus* wild-type and *sum1Δ* cells of both mating types. Growth of a patch is approximately proportional to the mating ability of the strain indicated to the left of the picture. Note that a subset of *MATa sum1Δ* cells mated with **a** cells, while most cells mated with α cells.

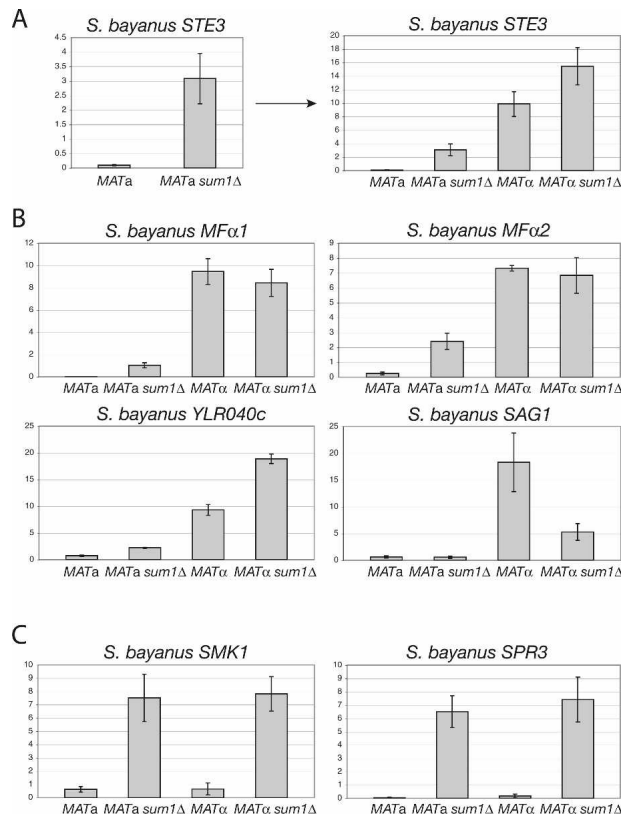


Figure 3. α -Specific genes were up-regulated in *S. bayanus* *MATa sum1Δ* cells analysis compared with wild type. (A) Expression of the α -specific gene *STE3* was assayed by quantitative RT-PCR. *MATa* wild type versus *sum1Δ* are shown on separate plot at left as an example of this two-way comparison for all α -specific genes. These same data are replotted at the right for four-way comparison with wild-type and mutant α cells. (B) Expression of the α -specific genes *MFα1*, *MFα2*, *YLR040c*, and *SAG1* in wild-type and *sum1Δ* cells of both mating types. (C) Expression of the meiotic genes *SMK1* and *SPR3*, which are targets of Sum1 repression in *S. cerevisiae*, in wild-type and *sum1Δ* cells of both mating types.

repressed by Sum1 in *S. cerevisiae*, *SMK1* and *SPR3*, was analyzed in *S. bayanus sum1Δ* cells. The increase in expression observed for these genes in *sum1Δ* mutants of both mating types (Fig. 3C) indicated that Sum1 retained its role as a repressor of meiotic genes in *S. bayanus*.

Sum1 prevented autostimulation of a cells by α -factor

The gene expression data suggested that *S. bayanus MATa sum1Δ* mutants expressed and responded to α -factor, producing shmoo and the bimating phenotype. However, it remained possible that stimulation by α -factor through Ste3, the α -factor receptor, also contributed to the mutant phenotypes, as loss of Sum1 led to expression of *STE3* in α cells. To distinguish between autostimulation of *MATa sum1Δ* cells by misexpression of α -factor or by misexpression of the α -factor receptor, we generated double mutants in the *MATa sum1Δ* back-

ground with genes required for pheromone signaling, inactivating different steps in the mating pathway. Strikingly, inactivation of *STE2*, which encodes the α -factor receptor and is expressed only in α cells, completely suppressed *sum1Δ* colony wrinkling, cell elongation, and flocculation phenotypes (Fig. 4A). *MFα1* produces the majority of α -factor, whereas *MFα2* produces a smaller fraction (Kurjan 1985). Consistent with these studies, the *S. bayanus MATa sum1Δ mfα1Δ* double mutants had very little flocculence and no apparent cell elongation (Fig. 4A), whereas *MATa sum1Δ mfα2Δ* double mutants had phenotypes indistinguishable from *MATa sum1Δ* single mutants (data not shown). Similar to *STE2* deletion, inactivation of *STE12* completely suppressed the *MATa sum1Δ* phenotypes (Fig. 4A). In contrast, deletion of *STE3* enhanced wrinkling, bimating, and expression of *FIG2* (data not shown). This result was consistent with expression of *STE3* in α cells dampening the pheromone-signaling response (Roth et al. 2000; Rivers and Sprague 2003). Taken together, these data established that the cell and colony morphology defects in *MATa sum1Δ* mutants were caused by production of α -factor and subsequent stimulation by the same or neighboring cells within a growing colony.

To test whether the α -factor response was sufficient for the *MATa sum1Δ* phenotypes, the galactose-inducible *GAL1* promoter was introduced into the genome of an otherwise wild-type *S. bayanus MATa* strain so as to drive expression of the *MFα1* gene. In contrast to wild-type α cells, *MATa GAL1pro-MFα1* cells grown on galactose-containing medium formed wrinkled colonies that contained cells with enhanced flocculence and a high percentage of shmoo (Fig. 4B). Additionally, when assayed on galactose medium, the inducible *MFα1*-expressing cells mated robustly with both α cells and α cells (Fig. 4B). Thus, expression of *MFα1* in *S. bayanus a* cells was both necessary and sufficient to produce the flocculation, shmooing, and bimating phenotypes observed in the *MATa sum1Δ* mutant. Furthermore, the cell elongation was classic "shmooing" behavior caused by derepression of α -specific genes in α cells that, by definition, lack the $\alpha 1$ activator protein.

As Sum1 had been implicated in repression of the silent mating type loci, *HML* and *HMR*, it was possible that induction of α -specific genes and associated phenotypes in the absence of Sum1 were indirect effects of derepression of the silenced $\alpha 1$ gene at *HMLα* (Irlbacher et al. 2005). However, *MATa sum1Δ hmlΔ* double mutants possessed phenotypes identical to those of *MATa sum1Δ* single mutants (Fig. 5A). Therefore, the *sum1Δ* phenotypes did not depend on *HMLα*, ruling out this indirect-effect explanation.

Sum1 bound to and repressed α -specific gene promoters

In the standard model of mating-type determination, no repressor of α -specific genes exists; merely the absence of the $\alpha 1$ activator protein prevents their expression. To explore the possibility that Sum1 directly repressed α -

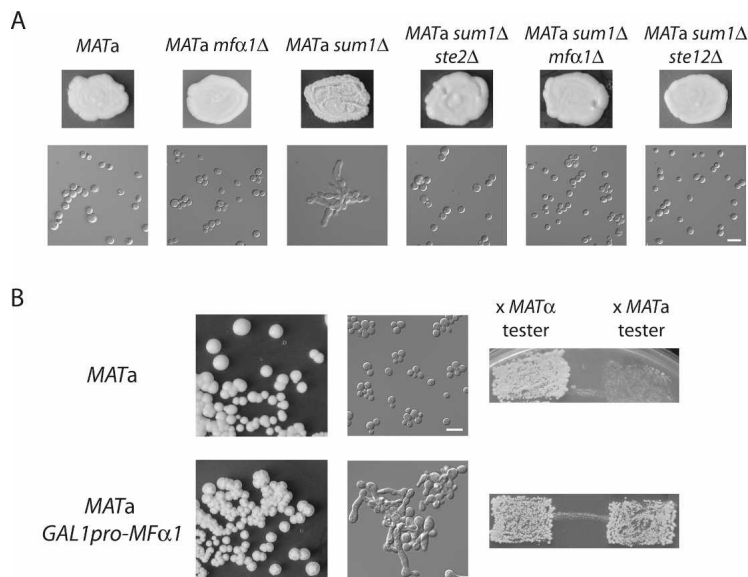


Figure 4. Sum1 prevented autostimulation of **a** cells by α -factor. (A) Inactivation of the mating pheromone signaling pathway suppressed *MATa sum1 Δ* phenotypes. *Top* panels depict patches of each indicated genotype growing on YPD. *Bottom* panels show DIC microscopy of cells taken from the above patches. (B, *left* panels) *MATa* wild-type and *GAL1pro-MF α 1* colonies grown for 5 d on YPG. Cells taken from the same colonies at *left* were photographed (*middle* panels) and tested in patch mating assays (*right* panels). On YPD, the *GAL1pro-MF α 1* strain behaved identically to the wild-type *MATa* strain (data not shown). Bars, 10 μ m.

specific genes, we scanned their promoters (defined here as 1 kb of sequence 5' of the start codon) for consensus Sum1-binding sites derived from detailed analyses of Sum1's specific DNA sequence binding in vitro and its repression activity in vivo (Pierce et al. 2003). Using the consensus sequence AGYGWCAACAAA, a near-perfect match in the *STE3* promoter was detected that was 100% conserved across the sensu stricto yeast species (Fig. 5B). With a less stringent consensus, DSYGWCA YWDW, at least one match was found in the *STE3*, *MF α 1*, and *YLR040c* promoters, each of which was highly conserved across the sensu stricto. Intriguingly, the sequence matches in the *STE3* and *MF α 1* promoters occurred within the previously characterized *P'Q* elements, which in *S. cerevisiae* direct their α -specific expression through binding α 1 and Mcm1 (for the *YLR040c* promoter, the match occurred 100 base pairs [bp] 5' of the predicted *P'Q* box). None of the matches found in the *SAG1* promoter were well conserved; however, this promoter was less conserved than the other α -specific gene promoters.

Sum1 was Flag-tagged at its C terminus in *S. bayanus* haploid strains and shown to provide Sum1 function in a *sum1 Δ* mutant. Chromatin immunoprecipitation (ChIP) was used to test whether Sum1 protein bound the promoters of α -specific genes. Quantitative PCR analysis of Sum1-Flag precipitates revealed two- to fourfold enrichment of the *STE3*, *MF α 1*, *MF α 2*, and *YLR040c* promoters relative to precipitates from no-tag control strains (Fig. 5C). A negative control primer set amplified a region within the *SEN1* ORF. As positive controls, we examined *S. bayanus* Sum1 binding at three loci to which the *S. cerevisiae* Sum1 protein binds (Lee et al. 2002). Strong enrichment (more than eightfold) of both the *SMK1* and *DAL1* promoters was observed (Fig. 5C), but only 2.5-fold enrichment of the *SPR3* promoter was observed (comparable with the *MF α 1* level), despite this gene being a confirmed Sum1 target in *S. cerevisiae* (Xie et al.

1999). A simple model consistent with the ChIP and genetic analysis was that, at least in *S. bayanus*, Sum1 bound directly to α -specific gene promoters to repress their transcription.

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

The conserved Sum1-binding site in the *S. cerevisiae* *STE3* promoter suggested that similar repression might occur throughout the *Saccharomyces* clade, although Sum1 had not previously been implicated in control of mating type in *S. cerevisiae*. Data from genome-wide localization studies (Lee et al. 2002; Harbison et al. 2004; Robert et al. 2004) identified highly reproducible binding of both Sum1 and Hst1 at the *STE3* promoter in *S. cerevisiae* **a** cells. Furthermore, microarray data indicated that the expression of *STE3*, *YLR040c*, and *FIG1* increased in *S. cerevisiae* *MATa sum1 Δ* cells compared with wild-type **a** cells (Pierce et al. 2003), although the biological impact of this increase was unknown. Thus, we tested whether Sum1 repressed α -specific genes in *S. cerevisiae* despite the absence of gross phenotypes in *MATa sum1 Δ* mutants of this species. In fact, quantitative RT-PCR analysis of α -specific genes revealed an expression profile similar to that of *S. bayanus*. Expression of *S. cerevisiae* *STE3* and *MF α 1* increased 10-fold and 50-fold, respectively, in *MATa sum1 Δ* cells compared with wild type (Fig. 6A).

These data presented a conundrum: If Sum1 repressed α -specific genes in *S. cerevisiae*, then why did *MATa sum1 Δ* mutants of this species appear normal? We inspected the *S. bayanus* orthologs of *S. cerevisiae* mating genes to ask whether changes in mating genes might contribute to the *S. bayanus* *MATa sum1 Δ* phenotype. Pairwise alignment of the *S. cerevisiae* and *S. bayanus* **a**-specific gene *BAR1*, which encodes a protease that degrades α -factor, revealed a single base-pair deletion

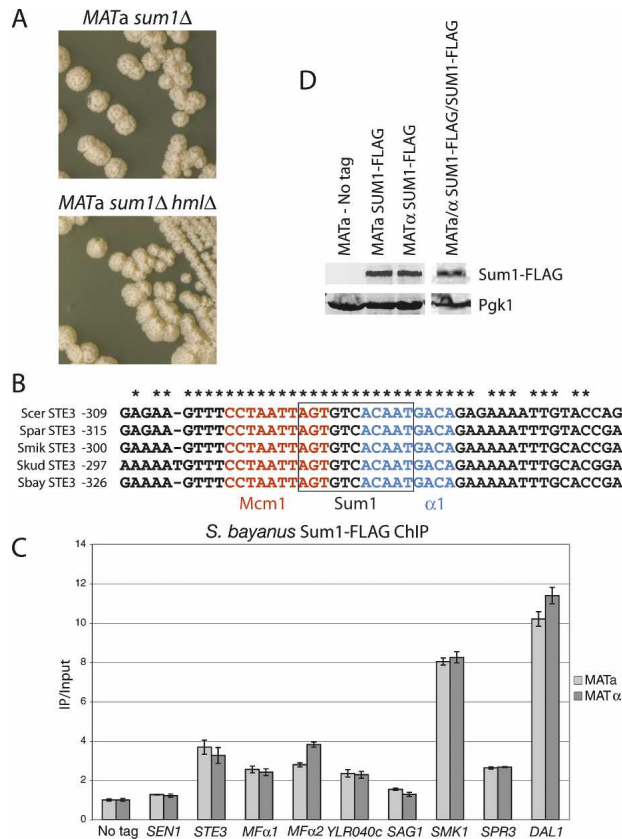


Figure 5. Sum1 repressed α -specific genes directly by binding to their promoters. (A) *S. bayanus* *MATa sum1Δ* and *MATa sum1Δ hmlΔ* colonies grown on YPD for 5 d. (B) Sum1-binding site predictions for the *STE3* promoters from five sensu stricto yeasts. A predicted Sum1 site partially overlapped the known Mcm1 and α 1 sites in the *P'Q* element of the *STE3* promoter. The sequence encompassing all three binding sites was 100% conserved across all five species. (Scer) *S. cerevisiae*; (Spar) *S. paradoxus*; (Smik) *S. mikatae*; (Skud) *S. kudriavzevii*; (Sbay) *S. bayanus*. Numbers indicate the nucleotide position relative to the start codon. (C) ChIP of Sum1-Flag was performed, followed by quantitative PCR using primers corresponding to select promoter regions in the *S. bayanus* genome. IP amplification values were first normalized to input amplification values. These data were then normalized to a representative “No Tag” amplification value. (D) Sum1 protein levels were assayed by immunoblotting for Flag-tagged Sum1p (top row) and phosphoglucokinase (Pgc1p, bottom row), a loading control, using *S. bayanus* whole-cell extracts. Lanes from two separate blots are shown, with protein levels in haploid cells shown in lanes 1–3, and in diploid cells in lane 4. Lysate from the same haploid cells’ samples was run on both gels.

(Δ T741) in the *S. bayanus* ortholog. This frameshift mutation was predicted to generate an inactive protein lacking the C-terminal catalytic site. Although this mutation had been annotated as a sequencing error (Kellis et al. 2003), we confirmed its presence by resequencing. This loss-of-function *bar1* allele likely rendered *S. bayanus a* cells hypersensitive to α -factor. Mating pheromone halo assays confirmed that *S. bayanus a* cells were, in fact, hypersensitive to α -factor, but α cells were not hy-

persensitive to **a**-factor (data not shown). Transformation of *S. bayanus* *MATa sum1Δ* cells with a plasmid bearing the *S. cerevisiae* *BAR1* gene suppressed the colony wrinkling phenotype, confirming that the *bar1* mutation contributed to the phenotypic difference between species (Supplemental Fig. S2).

To test whether Bar1 function masked some of the phenotypic consequences of loss of Sum1, *BAR1* was deleted in a *S. cerevisiae* *MATa sum1Δ* strain (W303 background). Intriguingly, *MATa sum1Δ bar1Δ* double-mutant cells had readily observable shmooing behavior, recapitulating much of the *S. bayanus sum1Δ* phenotype in *S. cerevisiae*. However, the *S. cerevisiae* mutant colonies were not wrinkled and the cells were not flocculent (Fig. 6B,C), suggesting that interspecies differences in adhesion molecules, or in their ability to be induced by α -factor, might account for the rest of the phenotype. A clue to this discrepancy is that *S. cerevisiae a* cells undergo pseudohyphal-like growth upon low-level stimu-

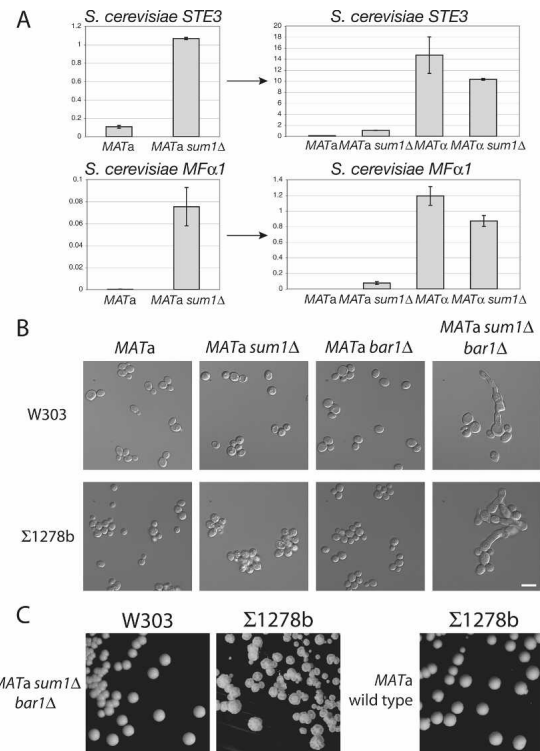


Figure 6. Sum1-mediated repression of α -specific genes was conserved in *S. cerevisiae*. (A) Expression of *STE3* and *MFα1* in *S. cerevisiae* was assayed by quantitative RT-PCR. *MATa* wild-type versus *sum1Δ* cells are shown on separate plots at left to allow two-way comparison. These same data are replotted at right for four-way comparison with wild-type and mutant α cells. (B) DIC microscopy of *S. cerevisiae* *MATa sum1Δ bar1Δ* double-mutant cells plus wild-type and single mutant controls of W303 (top row) and Σ 1278b (bottom row) backgrounds. Bar, 10 μ m. (C) Σ 1278b *MATa sum1Δ bar1Δ* colonies grown on YPD for 5 d (middle panel); compare with wild-type colonies (right panel). W303 *sum1Δ bar1Δ* colonies, which had only a subtle change in morphology, are shown at the left for comparison (cf. Fig. 2A).

lation by α -factor (Erdman and Snyder 2001). In the *S. cerevisiae* $\Sigma 1278b$ strain, which is competent to form pseudohyphae, the *MAT α sum1 Δ bar1 Δ* double mutant fully recapitulated the shmooing and colony wrinkling phenotypes seen in the orthologous *S. bayanus* mutant (Fig. 6C). Thus, some difference between W303 and $\Sigma 1278b$ affected the ability of pheromone signaling to trigger flocculence (Liu et al. 1996; Guo et al. 2000).

$\alpha 1$ was required to overcome repression by Sum1 in α cells, but Mcm1 and Ste12 could activate transcription in the absence of Sum1 and $\alpha 1$

As Sum1 blocked α -specific gene expression, some mechanism must prevent it from repressing these genes in α cells. Sum1 protein was present at equivalent levels in both **a** cells and α cells (Fig. 5D). Sum1 bound equivalently in vivo to all promoters assayed in **a** cells and α cells (Fig. 5C). Therefore, neither the synthesis of nor the DNA-binding activity of Sum1 was mating-type-regulated.

A simple model to explain how α -specific genes were

expressed despite the presence of a repressor on their promoters was that $\alpha 1$ protein somehow counteracted Sum1's repressive function, and contributed to transcription activation together with Mcm1 and Ste12. If $\alpha 1$ were required to overcome Sum1 repression in α cells, and were not completely required for α -specific gene expression, then deletion of *SUM1* should suppress the nonmating phenotype of *mata1 Δ* mutants. Indeed, whereas both *S. cerevisiae* and *S. bayanus* *mata1 Δ* mutants were almost completely sterile, *mata1 Δ sum1 Δ* double mutants of both species mated substantially better (Fig. 7A). Furthermore, *mata1 Δ sum1 Δ hml Δ* triple mutants mated equivalently to *mata1 Δ sum1 Δ* double mutants, demonstrating that removing Sum1 restored mating ability to α cells that were otherwise sterile because they lacked the $\alpha 1$ activator. Thus, Sum1 was active in both mating types of both species, and $\alpha 1$ was required to overcome Sum1 repression, in addition to providing a transcriptional activation function.

The restoration of mating in *mata1 Δ sum1 Δ* double mutants argued that Mcm1 and Ste12 could activate α -specific genes on their own, in contrast to the need for $\alpha 1$

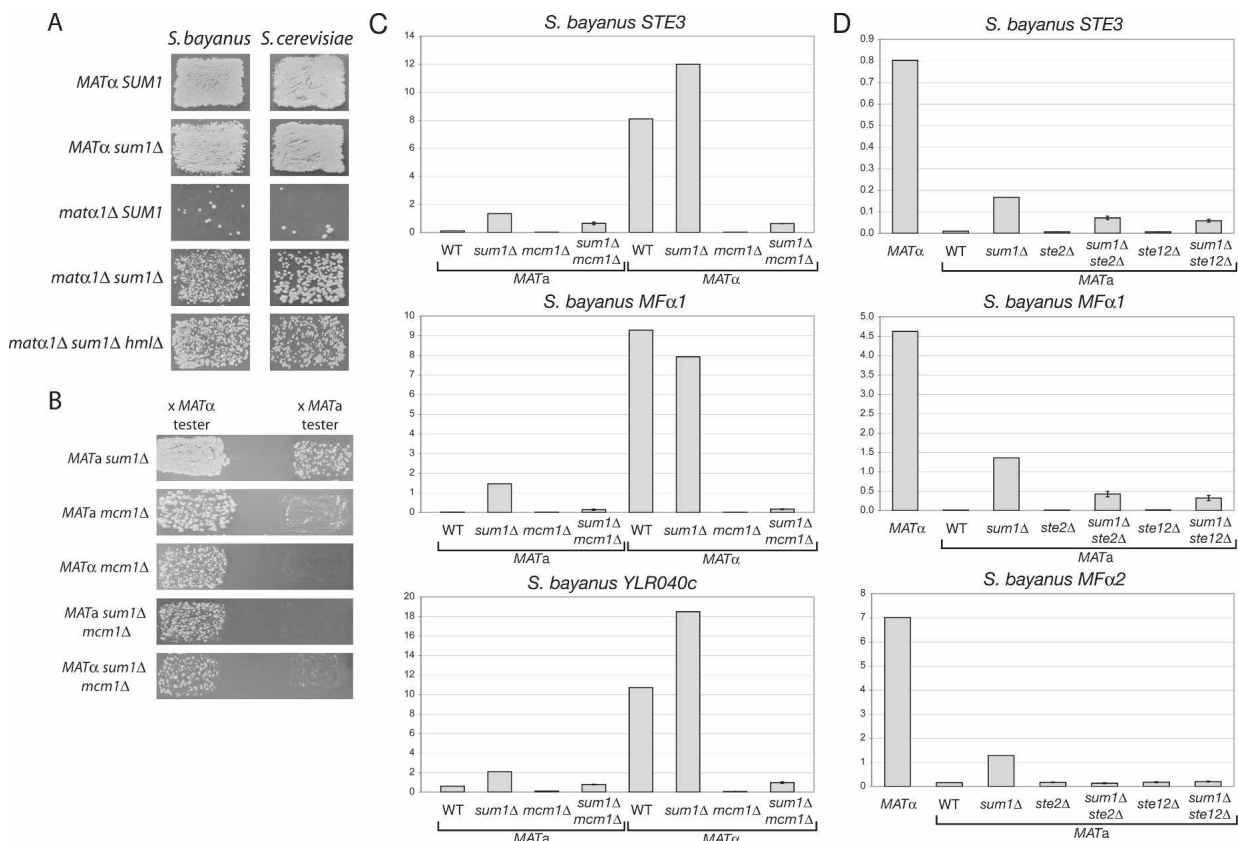


Figure 7. Sum1 was a general repressor of mating-type-specific genes. (A) Patch mating assays (using only the *MAT α* tester) were performed on *MAT α* , *MAT α sum1 Δ* , *mata1 Δ* , *mata1 Δ sum1 Δ* , and *mata1 Δ sum1 Δ hml Δ* strains in both *S. bayanus* and *S. cerevisiae* (W303). (B) Patch mating assays were performed on *S. bayanus* *sum1 Δ* , *mcm1 Δ* , and *sum1 Δ mcm1 Δ* strains of the indicated mating types. (C) Expression of α -specific genes in cells of indicated genotypes was assayed by quantitative RT-PCR (WT, wild type). Expression of *STE3* is reduced 10-fold in *mcm1 Δ* cells (columns 3 and 7) compared with wild-type α cells (column 1). It should be noted that *ACT1* (actin) RNA levels in *mcm1 Δ* mutant cells were reduced twofold, causing the normalized expression values for *sum1 Δ mcm1 Δ* cells to be inflated. (D) Expression of α -specific genes in cells of indicated genotypes was assayed by quantitative RT-PCR (WT, wild type).

as in the $\alpha 1$ – $\alpha 2$ hypothesis. To test whether Mcm1 and Ste12 were necessary for α -specific gene expression in the absence of Sum1, mating ability and α -specific gene expression were assayed in *sum1 Δ mcm1 Δ* and *sum1 Δ ste12 Δ* double mutants. (The *MCM1* gene was not essential in *S. bayanus*, in contrast to *S. cerevisiae*, although some lethality was observed in germinating spores from *mcm1 Δ /+* diploids and some viable cells had a growth defect [data not shown].) *sum1 Δ ste12 Δ* mutant cells of both mating types were completely sterile (data not shown); likewise, *MAT α mcm1 Δ* strains were unable to mate as α cells (Fig. 7B). The complete loss of mating with **a** cells by *MAT α mcm1 Δ* cells was not surprising, given that Mcm1 is required for activation of α -specific genes and repression of **a**-specific genes (Passmore et al. 1988; Elble and Tye 1991). However, unexpectedly, an equivalent small fraction of both *mcm1 Δ* and *sum1 Δ mcm1 Δ* mutant cells mated as **a** cells, regardless of the allele present at *MAT*. Although most *mcm1 Δ* mutant cells in the population were unable to mate, the **a**-like mating ability in a subset of cells demonstrated that at least some **a**-specific gene expression was Mcm1-independent (Kronstad et al. 1987). As suggested by the mating assays, Mcm1 was necessary for α -specific gene expression in the presence or absence of Sum1 (Fig. 7C). Indeed, in *MAT α mcm1 Δ* cells, *STE3* expression was down 10-fold from its level in wild-type **a** cells.

Ste12, on the other hand, was partially required for α -specific gene expression, with the exception of *MF α 2* (Fig. 7D). The expression of *STE3* and *MF α 1* remained severalfold increased in both *MAT α sum1 Δ ste12 Δ* cells and *MAT α sum1 Δ ste2 Δ* cells compared with wild-type, *ste2 Δ* , or *ste12 Δ* controls, yet neither gene was expressed in the double mutants to the level of the *sum1 Δ* single mutant. It appeared that the level of expression of α -specific genes was a composite of two effects: derepression in the absence of Sum1, and autostimulation by α -factor. Specifically, expression of *MF α 2* in both *MAT α sum1 Δ ste12 Δ* and *MAT α sum1 Δ ste2 Δ* cells was equivalent to that in wild-type **a** cells, consistent with this gene being completely Ste12-dependent (Fields et al. 1988). The remaining *STE3* and *MF α 1* expression in the absence of Sum1 and Ste12 implied that Mcm1 could activate some α -specific genes, albeit weakly, without other known coactivators. Interestingly, purified recombinant Mcm1 can bind *P'Q* elements in vitro, although it does

so with low affinity relative to its binding of *P* elements from **a**-specific genes (Grayhack 1992).

The mechanism of repression of α -specific genes

The above results pointed to a simple mechanism for repression of α -specific genes by Sum1: preventing Mcm1 from binding to DNA. To test this model, ChIP assays were performed on Myc-tagged Mcm1 in wild-type and *sum1 Δ* cells. Consistent with this model, ChIP revealed enhanced association of Mcm1 with all five α -specific gene promoters in *MAT α sum1 Δ* cells compared with *MAT α* wild type (Table 1). *STE2*, an **a**-specific gene, served as a control, as Mcm1 bound this gene's promoter in both cell types, and its binding was unaffected by deletion of *SUM1* (as predicted) (see Fig. 1). The greater enrichment of *STE2* in α cells versus **a** cells may reflect conformational changes in Mcm1's association with DNA introduced by the $\alpha 2$ protein. Intriguingly, Mcm1 precipitates from *MAT α sum1 Δ* cells did not show a substantial enrichment of α -specific gene promoter sequences compared with *MAT α* wild type. However, although the standard model suggests that Mcm1 does not bind to α -specific gene promoters in the absence of $\alpha 1$ protein, we observed association of Mcm1 with the *MF α 1*, *MF α 2*, and *YLR040c* promoters in *MAT α* cells. Thus, models to explain the mechanism of α -specific gene activation in *sum1 Δ* cells need not require enhanced occupancy by Mcm1 (discussed further below).

To explore whether the mechanism of repression of α -specific genes involved the proposed Sum1–Hst1–Rfm1 repressor complex, the *HST1* and *RFM1* genes were inactivated. *S. bayanus* *MAT α hst1 Δ* and *MAT α rfm1 Δ* mutants had colony and cellular phenotypes similar, although not identical, to *MAT α sum1 Δ* mutants (Supplemental Fig. S3). These results suggested that histone deacetylation by Hst1 was required for repression of α -specific genes, and that the interactions between Sum1, Rfm1, and Hst1 were conserved between *S. bayanus* and *S. cerevisiae*.

Discussion

The standard model of mating-type control in *Saccharomyces cerevisiae* posits that **a** is the default mating type,

Table 1. *S. bayanus* Mcm1 ChIP assay

	<i>MATα</i>	<i>MATα</i>	<i>MATα MCM1-myc</i>	<i>MATα MCM1-myc</i>	<i>MATα sum1Δ MCM1-myc</i>	<i>MATα sum1Δ MCM1-myc</i>
<i>STE3</i>	0.60 (0.16)	0.60 (0.18)	0.64 (0.16)	3.49 (0.73)	0.78 (0.20)	5.83 (0.63)
<i>MFα1</i>	0.68 (0.08)	0.67 (0.12)	1.51 (0.38)	3.46 (0.83)	1.89 (0.34)	6.51 (1.44)
<i>MFα2</i>	0.73 (0.08)	0.66 (0.09)	1.32 (0.26)	3.07 (0.86)	1.54 (0.27)	4.73 (0.92)
<i>YLR040c</i>	0.79 (0.20)	0.68 (0.13)	1.15 (0.37)	10.56 (1.33)	1.28 (0.21)	20.96 (1.68)
<i>SAG1</i>	0.97 (0.25)	0.65 (0.15)	0.81 (0.16)	3.15 (0.95)	0.85 (0.20)	4.54 (0.87)
<i>STE2</i>	0.59 (0.08)	0.54 (0.07)	3.25 (0.14)	13.88 (2.62)	4.02 (0.32)	14.41 (0.93)
<i>SEN1</i>	0.62 (0.18)	0.58 (0.14)	0.46 (0.04)	0.54 (0.09)	0.57 (0.15)	0.56 (0.05)

IP/input amplification ratios for each primer set were normalized to the *ACT1* IP/input ratio. *SEN1* served as a negative control locus. Numbers in parentheses indicate the standard error of the mean.

with the α mating type requiring the activation of α -specific gene expression by $\alpha 1$ (in combination with Mcm1 and Ste12), and the repression of α -specific gene expression by $\alpha 2$ (also in combination with Mcm1) (Strathern et al. 1981; Sprague 1990). We found that the Sum1 protein acted as a direct repressor of α -specific genes in both *S. bayanus* and *S. cerevisiae* (Fig. 8A). At least two differences between these two species conferred phenotypic differences that allowed the discovery of this conserved aspect of mating type determination, which has been overlooked for 26 years. Part of the phenotypic difference was explained by *S. bayanus* α cell hypersensitivity to α -factor due to a *bar1* mutation. (This mutation was probably a polymorphism among *S. bayanus* strains [M. Dunham, A. Tsong, pers. comm.].) However, another factor, present in both *S. bayanus* and *S. cerevisiae* strain $\Sigma 1278b$, caused *sum1 Δ bar1* mutant α cells to flocculate in response to α -factor. A good candidate for this "flocculation factor" was *FLO8*, which is required for flocculation in $\Sigma 1278b$ and has inactivating mutations in both W303 and S288C strains of *S. cerevisiae* (Liu et al. 1996).

Modifying the model for control of α -specific gene expression

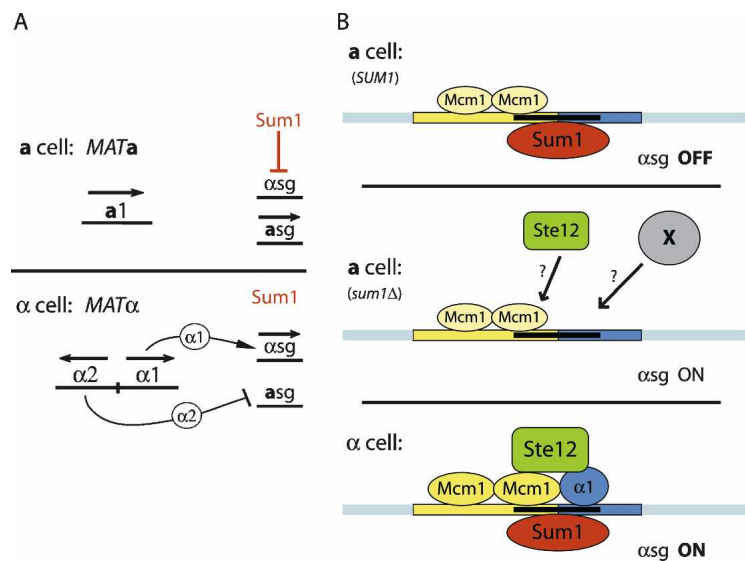
Our results demonstrated two roles for the $\alpha 1$ protein: counteracting Sum1 repression, and inducing the maximal expression of α -specific genes (Fig. 3A,B). *MAT α sum1 Δ* and *mata1 Δ sum1 Δ* cells, both of which lack $\alpha 1$, were capable of α cell mating behavior (Fig. 7A,B). Consistent with the standard model, Mcm1 and Ste12 were necessary for mating of α cells and for robust α -specific gene expression in the absence of Sum1 (Fig. 7B–D). As Mcm1 and Ste12 were able to activate transcription in the absence of $\alpha 1$ and Sum1, the $\alpha 1$ /Mcm1 cooperative binding model cannot fully explain α -specific gene activation (Fig. 8). Indeed, Mcm1 was associated with the *MF $\alpha 1$* , *MF $\alpha 2$* , and *YLR040c* promoters in *MAT α* cells

(Table 1). Mcm1's occupancy of α -specific gene promoters was enhanced in the absence of Sum1, but only in *MAT α* cells (when $\alpha 1$ is present). Surprisingly, Mcm1 ChIP of α -specific gene promoters was not substantially enhanced in *MAT α sum1 Δ* cells compared with *MAT α* wild-type cells. This result forced us to consider two additional models for the interplay between Sum1 and the coactivator proteins at α -specific genes: (1) Sum1 interfered with transcriptional activation at a step downstream from Mcm1 binding, or (2) a novel activator was recruited to α -specific genes in the absence of Sum1. Formally, Sum1 might have blocked DNA binding by Ste12 in *MAT α* cells. However, some expression of α -specific genes was observed in *MAT α sum1 Δ ste12 Δ* cells (Fig. 7D), and Ste12-binding sites in most α -specific gene promoters did not overlap predicted Sum1-binding sites, making increased promoter occupancy by Ste12 in *sum1 Δ* cells seem a less likely explanation.

Our data also highlighted gene-specific aspects of α -specific gene regulation. One exception to the model that Sum1 repressed α -specific genes was *SAG1*, whose expression in α cells was promoted by *SUM1* (Fig. 3B). These data raised the possibility that some other factor represses *SAG1*, implying that there may be yet another dimension to α -specific gene regulation. The expression of *MF $\alpha 2$* increased in α cells lacking Sum1, however, this increase was completely abolished in *sum1 Δ ste2 Δ* and *sum1 Δ ste12 Δ* double mutants (Fig. 7D). *SAG1* and *MF $\alpha 2$* thus appear to be regulated differently than *MF $\alpha 1$* , *STE3*, and *YLR040c*, which may represent unanticipated complexity in α -cell mating behavior.

Sum1 repressed α -specific genes yet was expressed and active in both α cells and α cells (Fig. 8B). Therefore, some mechanism involving $\alpha 1$, Mcm1, and Ste12 must exist to prevent Sum1 from repressing α -specific genes in α cells. One possible mechanism is DNA bending by $\alpha 1$ and Mcm1 (Hagen et al. 1993; Carr et al. 2004), which may alter the physical conformation of the promoter, preventing Sum1 from making protein–DNA or protein–

Figure 8. Models for Sum1-mediated repression of α -specific genes. (A) Model for Sum1 role in repression of α -specific genes and mating type determination. In contrast to the standard model, active repression of α -specific genes by Sum1 was required for proper α cell identity. (B) Molecular mechanism for α -specific gene regulation. In α cells, Sum1 repressed α -specific genes by binding either to sites directly overlapping the $\alpha 1$ and Mcm1-binding sites, or to other nearby sites. Mcm1 bound some, but not all, α -specific gene promoters in α cells. Without repression by Sum1, Mcm1 and Ste12 were able to activate α -specific genes in α cells to a level that permitted α -like mating. In α cells, $\alpha 1$ induced high levels of α -specific gene expression and was required to overcome the block imposed by Sum1. Despite their high levels of transcription, Sum1 remained associated with α -specific genes in α cells.



protein interactions critical for repression. It is also possible that $\alpha 1$ and Mcm1 out-compete Sum1 for contacts with general transcription factors, Mediator, or RNA Polymerase holoenzyme. Finally, it is possible that α -specific gene activators directly contact Sum1 to prevent repression. Notably, some *mcm1* and *ste12* alleles are specifically defective in activating expression of α -specific genes in α cells (Passmore et al. 1988; La Roche et al. 1995). These mutant proteins may fail to oppose Sum1 repression, or they may be unable to interact with $\alpha 1$.

Sum1 binding to α -specific gene promoters in their active state could be explained if this repressor bound to the minor groove of DNA while the $\alpha 1$ and Mcm1 activators bound the major groove. This scenario seemed plausible given that Sum1 contains two "AT-hooks"—short, positively charged motifs of thirteen amino acids—in its N-terminal half. These moieties often mediate binding to the minor groove of AT-rich DNA sequences (Reeves and Nissen 1990; Huth et al. 1997). Regardless of the specific mechanism involved, our results suggest a more complex model of α -specific promoter function than previously appreciated.

Control of cell-type-determining genes during differentiation

Why should α -specific genes have required repression by Sum1? As in the development of distinct cell types in multicellular eukaryotes, proper yeast cell identity must be ensured as soon as a differentiation event occurs; in this case, a mating-type switch. The most straightforward role for mating-type regulation by Sum1 was preventing α -mating behavior in **a** cells, particularly when cells switch from *MAT α* to *MATa*. In such a cell, α -specific genes would continue to be expressed if there were any residual $\alpha 1$ protein. Without active repression of α -specific genes, this newly formed **a** cell might mate with another **a** cell, generating a *MATa/a* diploid unable to undergo meiosis and destined for meiotic oblivion should it mate with an α cell to form a triploid. A complementary role for Sum1 was maintenance of robust **a** cell mating ability, as expression of α -specific genes in **a** cells causes decreased mating ability (Roth et al. 2000; Rivers and Sprague 2003). Furthermore, our results show that α -specific genes can be expressed at a substantial level even in the absence of their primary activator. For **a** cells that secreted even a low level of α -factor, their adaptation and subsequent desensitization (Dietzel and Kurjan 1987) would likely make them less sensitive to signals from bona fide mating partners. (Indeed, Sum1 was necessary for preventing autostimulation of **a** cells with α -factor. We note that our data did not formally distinguish between autocrine stimulation of an **a** cell by α -factor produced by that same **a** cell versus stimulation occurring primarily in *trans*, by α -factor secreted from neighboring cells. As a substantial amount of α -factor was likely present within growing *MATa sum1 Δ* colonies, some level of *trans*-stimulation undoubtedly occurred.)

Further insight into why this function of Sum1 evolved might be gained by asking when it evolved. Given the evolutionary distance between *S. cerevisiae* and *S. bayanus*, our results suggest that the Sum1 protein likely performed at least two functions in the sensu stricto ancestor: repression of α -specific genes and repression of meiotic genes. The conservation of the Sum1 protein and predicted Sum1-binding sites in α -specific gene promoters in *Saccharomyces paradoxus*, *Saccharomyces mikatae*, and *Saccharomyces kudriavzevii* support this idea. α -Specific gene activation by $\alpha 1$ appears to be conserved throughout Ascomycete fungi (Tsong et al. 2003). However, Sum1 orthologs are present in *Ashbya gossypii* and *Kluyveromyces lactis*, but are not in species more distant from *S. cerevisiae*. Therefore, it would be interesting to ask how α -specific genes were regulated in ancestral Ascomycetes. It is possible either that a different repression mechanism is used outside of the *Saccharomyces* complex, or that Sum1-based repression is unique to these species, possibly to accomplish a unique aspect of their mating behavior (Fig. 1B).

Advantages of comparative genetic analysis

In retrospect, active repression of α -specific genes has evaded detection for so long largely because conventional biochemical and single-species genetic approaches were not sufficiently sensitive. One apparent complication was overlap of the $\alpha 1$ /Mcm1 activators' binding sites by the putative Sum1-binding site in the promoters of some α -specific genes. Previously characterized mutations in the *STE3* promoter that abolished activator binding (Hagen et al. 1993) also ablated the Sum1-binding site, obscuring the contribution of Sum1 to repression of *STE3* expression. Similarly, gel-shift analyses of the *STE3* and *MF $\alpha 1$* operators largely failed to detect a complex containing anything other than $\alpha 1$ and Mcm1 (Bender and Sprague 1987; Jarvis et al. 1988). However, reaction conditions optimized for $\alpha 1$ /Mcm1 binding or low Sum1 protein abundance relative to that of $\alpha 1$ and Mcm1 may have confounded the ability of such experiments to detect Sum1 binding. Sum1 recruitment to α -specific genes might involve chromatin or other protein-protein interactions, rather than simply site-specific DNA binding.

The perspective of evolutionary biology offers the ability to infer conservation of functions between organisms, and the ability to ask how alterations in genetic circuits generate novel forms. Current large-scale biochemical, genetic, and computational efforts seek to identify novel genetic pathways and novel components of known pathways (Tong et al. 2004; Krogan et al. 2006; Collins et al. 2007). However, the recognition of a genetic pathway's components may depend on the range of phenotypes produced by common perturbations in multiple species. As we have shown, comparative genetic analysis in closely related species can provide the phenotypic depth sometimes needed for ascribing novel functions to specific genes. In this case, adventitious variation in phenotypes between species revealed a regu-

latory circuit conserved among species. Because nature provides variation not captured by model organisms, more detailed pictures of shared and unique regulatory pathways should emerge from a montage of multiple species' genetic interaction networks.

Materials and methods

Yeast strains, culture, and genetic manipulations

Yeast strains are listed in Table 1. Heterothallic *S. bayanus* wild-type strains were derived from CB54001, obtained from Ed Louis. The *HO* gene was inactivated in this diploid strain and haploid *MAT α* and *MAT α ho Δ* strains were isolated by sporulation and tetrad dissection of the *HO/ho Δ* heterozygotes. Auxotrophic markers *ade2-1* and *his3-1* were generated in haploid prototrophic strains by EMS mutagenesis followed by screening for colonies unable to grow on minimal media. For *lys2-5* and *ura3-1*, mutagenized haploids were plated on α -aminoadipate and 5-FOA media, respectively, to select for resistant mutants. Strains JRY8729 and JRY8730 were generated by sequential crosses of individual auxotrophic mutants and dissecting tetrads for multiple-marker mutants. All growth of *S. bayanus* was performed using standard conditions for *S. cerevisiae*, except that plate and liquid culturing was performed at 25°C for both species. *S. cerevisiae* wild-type strain W303-1a has been described previously (Thomas and Rothstein 1989). The *MAT α Σ 1278b* strain, JRY6896, was originally designated 10560-4A (G. Fink), and was obtained from Jeremy Thorner (JTY2560). One-step gene replacement, C-terminal 13xMyc tag integration (Longtine et al. 1998; Goldstein and McCusker 1999) and C-terminal 3xFlag tag integration (Gelbart et al. 2001) have been described previously, and these genetic manipulations were performed identically for both *S. bayanus* and *S. cerevisiae*. All gene disruptions in both species (*sum1 Δ ::Hyg*, etc.) were confirmed using PCR to examine the 5' and 3' ends of targeted ORFs. To construct strain JRY8736, the *GAL1* promoter (Longtine et al. 1998) was integrated by homologous recombination immediately upstream of the ATG of the *MF α 1* gene, removing 50 bp of the native promoter.

Microscopy and mating assays

DIC microscopy was performed using a Nikon Eclipse E600 microscope (100 \times objective). Cells were gently dispersed in complete synthetic media and spotted onto 2% agarose pads mounted on slides. Multiple fields were observed to score cellular morphology. Patch-mating assays were performed by mixing approximately equal amounts of query strain and tester strain, each obtained from individual colonies, on YPD (glucose) or YPG (galactose) and incubating overnight. The following day, patches were replica plated onto minimal media to select for diploids. Patch mating assays shown in Figure 4 (galactose induction of *MAT α* wild-type and *GAL1pro-MF α 1* strains) were performed on rich medium containing 2% galactose as the carbon source before replica plating to minimal media containing 2% glucose and no galactose. The tester strains used in all mating assays were JRY2726 (*MAT α*) and JRY2728 (*MAT α*), both of which are *S. cerevisiae his4*. All *S. bayanus* strains described in the text mated equivalently with these tester strains and with *S. bayanus* tester strains.

RNA and protein analysis

RNA isolation was performed using the hot-phenol method (Schmitt et al. 1990). Total RNA was digested with Amplifica-

tion-grade DNase I (Invitrogen) and purified using the RNeasy Minelute kit (Qiagen). cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR and oligo(dT) primer (Invitrogen). Quantitative PCR on cDNA was performed using an MX3000P machine (Stratagene) and the DyNAmo HS SYBR Green qPCR kit (NEB). Amplification values for all primer sets were normalized to actin (*ACT1*) cDNA amplification values. Samples were analyzed in triplicate for two or three independent RNA preparations. For Figure 7, C and D, analysis, duplicate *mcm1 Δ* , *sum1 Δ mcm1 Δ* , *ste2 Δ* , *ste12 Δ* , *sum1 Δ ste2 Δ* , and *sum1 Δ ste12 Δ* samples were processed in parallel to single wild-type and *sum1 Δ* control samples from identical strains to those analyzed in Figure 3.

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

ChIP analysis

ChIPs were performed as described (Davies et al. 2005) using formaldehyde cross-linking of log-phase cultures for 1 h at room temperature. IPs were performed using anti-Flag M2-agarose from mouse and anti-c-Myc agarose from rabbit (Sigma). Quantitative PCR was performed on precipitated DNA fragments as described above. The "Reference" primer set, which amplifies a region within the *SEN1* ORF, corresponded to a locus with a minimal level of Sum1 binding in the *S. cerevisiae* genome. Amplification values for the precipitated DNA were normalized to the values for the input DNA for all primer sets. Samples were analyzed in triplicate for three independent ChIPs and one no-tag control for each mating type.

Binding site predictions

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

Acknowledgments

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