Haploinsufficiency of the sex-determining genes at MATα restricts genome expansion in Saccharomyces cerevisiae

Sex-determining Matα levels

Low Matα levels

↓

Sex fluctuation

↓

Pheromone signaling ↑↑

↓

Mortality ↑↑ & Severe morphological change like “Barbapapa”

Four copy of Matα

4N

MATα loss

One copy of Matα

4N

No copy of Matα

4N

Highlights

Polyplloid cells with reduced MATα copies show abnormal morphology and increased death

These phenotypes are due to hyperactivation of the mating pheromone response pathway

Quantity of the MATα products is important for stable sexual phenotype

This property may be involved in the suppression of speciation via polyploidization
Haploinsufficiency of the sex-determining genes at MATα restricts genome expansion in Saccharomyces cerevisiae

Kazumasa Oya1 and Akira Matsuura2,3,*

SUMMARY

In Saccharomyces cerevisiae, mating type of haploid cells is determined by the presence or absence of the MATα idiotype containing MATα 1 and MATα 2, which encode the transcription factors. These proteins are characterized by rapid turnover, but the physiological relevance of this property remains unclear. Here, we show a direct link between their intracellular levels and sexual stability. Polyploid cells with fewer MATα copies had unstable sexual phenotypes, causing morphological changes and an increase in cell death; these effects were mediated by hyperactivation of the mating pheromone response pathway. Thus, the MATα 1 and MATα 2 genes are haploinsufficient genes, and the reduction in their product levels causes sex fluctuation. Chromosome III harboring the mating type locus is the most prone to loss in diploids. We propose that the haploinsufficiency of MATα compensates for the drop-out prone nature of chromosome III, thereby suppressing speciation through increased genome size via polyploidization.

INTRODUCTION

Because of its relevance to development, behavior, evolution, and diversity, the mechanism of sex determination has been studied extensively. Sexual dimorphism controlled by the sex-determination system is ubiquitous among eukaryotic organisms from yeasts to humans. Genetic analysis has allowed extensive analysis of the regulation of dichotomous sex and reproduction in the budding yeast Saccharomyces cerevisiae (Herskowitz, 1988). S. cerevisiae haploid cells show either an α- or ρ-mating type; mating two cells of different mating types produces an α/ρ diploid. Yeast mating types are determined by the mating type (MAT) locus, which is located on chromosome III and contains either the MATa or MATα idiotype (Klar, 1987). Mata1, which is expressed from MATa1, and Mata1 and Mata2, which are expressed from MATa2, are transcription factors with multiple targets that give rise to the differences among these three distinct cell types (Galatro et al., 2004). MATα contributes to determining haploid mating type, with Mata1 promoting expression of α-specific genes (asg) and Mata2 suppressing α-specific genes (asg) (Strathern et al., 1981). In contrast, MATa is involved in the repression of haploid-specific genes (hsg) in diploid cells but not in the determination of the haploid mating type (Strathern et al., 1981). Therefore, the sexual phenotype (α or ρ) in haploid yeast is determined by the presence or absence of the MATa (Hanson and Wolfe, 2017).

Because chromosome III is equivalent to sex chromosomes in other eukaryotes, the MAT locus and chromosome III may be involved in speciation, analogous to sex chromosomes in higher eukaryotes (Payseur et al., 2018). Chromosome III is among one of the shortest of 16 chromosomes in S. cerevisiae, and the most prone to loss in diploid yeast (Kumaran et al., 2013). Although an α/ρ diploid is intrinsically non-mating, it acquires mating ability upon loss of chromosome III, or when a MAT idiotype is mutated or deleted (Haber, 1974). In artificial hybridization by the rare mating of a diploid S. cerevisiae cell with a haploid Saccharomyces kudriavzevii spore/cell, the complete loss of one copy of chromosome III confers mating ability to S. cerevisiae (Morard et al., 2020). It has been speculated that the deletion of one idiotype at the MAT locus is also responsible for whole-genome duplication (WGD), as has occurred in the history of the Saccharomycetales lineage (Marcet-Houben and Gabaldón, 2015).

Previous studies have shown that the sex-determining MATα products show constant and rapid turnover during vegetative growth, so as not to interfere with mating type switching (Lane and Hochstrasser, 2003; Nixon et al., 2010). Therefore, we speculated that such MATα regulation is also involved in the
expression of the sexual phenotype. In this study, we devised a method of controlling the levels of intracellular sex-determining transcription factors using polyploid yeast cells and addressed the relationship between their intracellular levels and sexual stability.

RESULTS

The copy number of sex-determining MATα genes affects polyploid cell morphology and death rate

To examine the effects of decreased levels of Matα proteins on the sexual phenotype in polyploid cells, we generated a series of mutants with altered ratios of the MATα copy number to genome set number (M/G ratio). We then examined the effect of the M/G ratio on mortality and morphological parameters such as size, roundness, and slenderness. In MATα polyploid strains with a normal M/G ratio of 1, cell size tended to increase, and cell shape tended to elongate, with increasing ploidy (αα, ααα, and αααα; Figure 1A). The ααα and αααα strains were significantly larger and longer (increased by 1.47 and 2.17 times in size and 1.08 and 1.18 times in slenderness, respectively) than the αα strain (Figure 1B), consistent with the findings of previous polyploidy studies (Galitski et al., 1999). Interestingly, we found that a decrease in the M/G ratio in MATα polyploid cells caused highly elongated, amorphous Barbapapa-like cell morphologies (ΔΔΔα; Figure 1A), which is typical of cells under prolonged exposure to mating pheromones or those that display activation of the abnormal/unsuppressed mating pheromone response pathway (mating pathway) (Siekhause and Drubin, 2003; Tanaka and Yi, 2009; Udden and Finkelstein, 1978). The ΔΔΔα cells showed reduced circularity (0.89-0.77) and were 1.61 times larger and 1.23 times longer than αααα (Figure 1B). Diseased cells were apparent in the ΔΔΔα culture, as confirmed by selective propidium iodide (PI) staining of dead cells. The PI-positive fraction increased with ploidy, and as the M/G ratio decreased, the latter effect was independent of the ploidy level (Figure 1C). In comparison, tetraploid cells with complete loss of MATα (ΔΔΔΔ) and only one copy of MATα (αΔΔΔ; 6.13 ± 2.88%) had a lower percentage of dead cells compared to ΔΔΔα cells (Figures 1A and 1C). These cells were not significantly different from αααα cells in the morphological parameters (Figures 1A and 1B). These results are consistent with the observation that matΔ cells with no MATα behave as an α-mating type (Strathem et al., 1981).

Since a high concentration of mating pheromones can induce rapid cell death (Zhang et al., 2006), we examined the relationship between the mating pathway and mortality in M/G ratio mutants. STE4 encodes the beta-subunit of a heterotrimeric G protein downstream of mating pheromone receptors involved in the mating pathway; receptor signaling is blocked by the loss of Ste4 protein (Hartwell, 1980). In mutants lacking STE4, the morphological changes induced by a reduced M/G ratio were suppressed regardless of ploidy; steΔ M/G ratio mutant strains were comparable to the ploidy-matched MATα polyploid strains in the three parameters (Figures 1B and 1D), and showed a decreased proportion of PI-positive cells (Figure 1C), indicating that the phenotypes induced by the decreased M/G ratio are dependent on the mating pathway. This result suggests that the ΔΔΔα Barbapapa-like morphology is caused by the activation of the mating pathway (Figure 1A).

Because Matα1 promotes MATα-specific gene expression, whereas Matα2 represses MATα-specific gene expression, we expected that mata1ΔΔΔα and mata2ΔΔΔα would show phenotypes similar to ΔΔΔΔ and ΔΔΔα, respectively. Unexpectedly, however, the abnormal morphologies were suppressed in the single mutants (Figures 1B and 1E). In particular, the induction of mata1Δ or mata2Δ resulted in the recovery of circularity (0.77 in ΔΔΔα to 0.92 or 0.90, respectively), which was close to the ΔΔΔΔ circularity (0.89). The increased cell death was also suppressed completely by mata1Δ and partially by mata2Δ (Figure 1C). These results indicate that the reduced expression of these two genes is involved in the synthesis of the ΔΔΔα phenotype.

Figure 1. A decrease in the M/G ratio affects cell morphology and mortality

(A) Microscopic observation to assess the effect of the M/G ratio on cell morphology. Scale bar = 20 μm.
(B) Bee swarm plots show the quantitative results of the images shown in Figures 1A, 1D, and 1E. Red bars represent mean values. The calculation of each parameter is described in Quantification and statistical analysis. Different letters represent significant differences, determined by one-way ANOVA and Tukey’s HSD comparisons.
(C) Fractions of dead cells. The numbers above the graphs are mean values and the error bars indicate the SD (n = 3 experiments). To calculate the p value for M/G ratio mutants with one copy of MATα relative to WT MATα haploid, we applied the Dunnett’s test; ***p < 0.001, **p < 0.01.
(D) Effect of mata1Δ and mata2Δ on the ΔΔΔα cell morphology. Scale bar = 20 μm.
(E) Effect of steΔ on the cell morphology of M/G ratio mutants. Scale bar = 20 μm.
We then quantified the amounts of Mat\textsubscript{a}1 and Mat\textsubscript{a}2 proteins in M/G ratio mutants by Western blotting. Both proteins decreased with the M/G ratio, with Mat\textsubscript{a}2 exhibiting greater depletion (Figures 2A, 2B, and S1). Previous studies estimated the half-lives of Mat\textsubscript{a}1 and Mat\textsubscript{a}2 as 15 and 5 min, respectively (Hochstrasser and Varshavsky, 1990; Nixon et al., 2010). Therefore, we speculated that the different effects of the M/G ratio on the amounts of Mat\textsubscript{a}1 and Mat\textsubscript{a}2 were due to the differences in their respective half-lives.

Finally, we examined the effect of the M/G ratio using a series of ectopic MAT\textsubscript{a} expressed from loci other than the native MAT. Aberrant morphologies were observed at an M/G ratio of <1, with a lower M/G ratio associated with greater severity of aberrant morphology; the introduction of ectopic MAT\textsubscript{a} reversed the abnormal morphology of M/G ratio mutants (Figure 3A). For each ploidy, a decrease in roundness and an increase in size and slenderness were observed when the M/G ratio was less than 1 (Figure 3B). Complementation with ectopic MAT\textsubscript{a} also normalized mortality to the same rate as in the wild type (MAT\textsubscript{a}+3 ectopic MAT\textsubscript{a} tetraploid; 4.92 ± 0.299%). These results confirmed that the phenotype of M/G ratio mutants was dependent on the relative copy number of MAT\textsubscript{a}, and indicated that reduced expression of the sex-determining MAT\textsubscript{a} has adverse effects on S. cerevisiae cells.

Cells with a decreased M/G ratio cannot maintain a stable sexual phenotype

As described above, the morphology of M/G ratio mutants is associated with the mating pathway. Activation of the mating pathway requires the presence of both mating type cells, or at least the simultaneous expression of a pheromone specific to one mating type cell and its receptor normally expressed in cells of the other mating type. Since the phenotypes of the M/G ratio mutants suggest an abnormality in the sex-determination system, we generated fluorescent reporters that facilitated monitoring the expression of mating type-specific genes. These reporters express green fluorescent protein (GFP) and mCherry downstream of the promoters of MAT\textsubscript{a}-specific MFA1 and MAT\textsubscript{a}-specific MFALPHA1, respectively, which enables mating type discrimination by fluorescence observation. As expected, GFP fluorescence was observed in wild-type (WT) MAT\textsubscript{a} cells, mCherry fluorescence was observed in WT MAT\textsubscript{a} cells, and no fluorescence was observed in WT MAT\textsubscript{a}/MAT\textsubscript{a} diploid cells (Figure 4A). Moreover, no fluorescence was observed in haploid mat\textsubscript{a1Δ} cells that lost the expression of both a\textsubscript{sg} and a\textsubscript{sg} (Bender and Sprague, 1989), and fluorescence of both was observed in mat\textsubscript{a2Δ} cells (which express both a\textsubscript{sg} and a\textsubscript{sg}) (Bender and Sprague, 1989), confirming the efficacy of our fluorescence mating type reporters (Figure 4A). We found that ΔΔΔ\textalpha emitted not only red fluorescence but also some green fluorescence, although it is genetically MAT\textalpha (Figure 4A). This suggests that the M/G ratio mutants cannot stably express either the a- or \textalpha-mating type, leading to sex fluctuation.

Our observations with the mating type reporters suggested that not only the pheromone-encoding genes used to make the reporters but also other a- and \textalpha-specific genes, such as receptors, were expressed abnormally. We noticed that the mCherry fluorescence of ΔΔΔ\textalpha cells was markedly higher than that of \textalpha cells (Figure 4B), suggesting that the activity of the MFALPHA1 promoter is enhanced in ΔΔΔ\textalpha cells. As the expression of mating pheromone genes was activated by the mating pathway (Roberts et al., 2000;
**A**

|          | WT | aa  | aaa | aaaa |
|----------|----|-----|-----|------|
| M/G ratio| 1  | 1   | 1   | 1    |
| Haploid  | 1  | 1/2 | 1/3 | 1/4  |
| Diploid  | 2  | 1   | 2/3 | 1/2  |
| Triploid | 3  | 3/2 | 1   | 3/4  |
| Tetraploid| 4 | 2   | 4/3 | 1    |

**B**

![Graph showing Size, Roundness, and Slenderness](image)

**Table for Size**

| MATa copy number | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
|------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Area (a.u.)      | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

**Table for Roundness**

| MATa copy number | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
|------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Circularity (a.u.)| n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |

**Table for Slenderness**

| MATa copy number | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
|------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Major axis/Minor axis (a.u.)| n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. | n.s. |
The mating pathway is hyperactivated in cells with a lower M/G ratio

Since mating type instability is thought to activate the mating pathway via the binding of mating pheromones to their receptors, we verified the fluorescence intensity of FUS1pr-GFP, using flow cytometry to evaluate the activation level of the mating pathway in the M/G ratio mutants. The FUS1 promoter is commonly used to measure the transcriptional response of the mating pathway (Bandera et al., 2016; Hagen et al., 1991; Paliwal et al., 2007; Takahashi and Pryciak, 2008). Indeed, the M/G ratio mutants had greater FUS1pr-GFP fluorescence, indicating mating pathway activation (Figure 4C). However, the activity of our FUS1pr-GFP reporter was saturated in mutants with M/G ratios of 1/3 and 1/4. The reporter activity in these mutants was much higher than that of the mata2Δ and sst2Δ mutants, which are constitutively activated mating pathway mutants (Figure 4C) (Bender and Sprague, 1989; Siekhaus and Drubin, 2003). Thus, hyperactivation of the mating pathway explains the high death rate (Figure 1C) in M/G ratio mutants.

Morphological changes and a high percentage of dead cells in the M/G ratio mutants are not observed after mating with MATα cells

Consistent with the mating type instability phenotype (Figures 4A–4C), M/G ratio mutants showed substantial mating with MATα cells, in addition to normal mating with MATα cells (Figure 5A). However, no octaploid cells formed when ΔΔx was cultured alone, under conditions where triploid cells formed by mating in a co-culture of haploid matΔ cells and haploid MATα cells (Figure S2), suggesting that the phenotype of M/G ratio mutants mating with MATα cells is unstable. This indicates that the bimater phenotype of M/G ratio mutants is caused by mating type fluctuation, and not solely by chromosome loss, which was reported to occur in polyploid mitosis (Comai, 2005; Kumaran et al., 2013; Mayer and Aguilera, 1990).

Interestingly, M/G ratio mutants showed no morphological changes; despite the increase in ploidy, the ΔΔx and ΔΔΔx strains were smaller and shorter (reduced by 0.81 and 0.75 times in size and 0.93 and 0.79 times in slenderness, respectively) after mating with MATα cells (Figures 5B and S3). Moreover, mating with MATα cells resulted in fewer P1-positive in ΔΔΔx cells (tetraploid ΔΔΔx; 17.8% ± 2.92% to pentaploid aΔΔΔx; 10.81% ± 3.71%). The Mata1, Matα1, and Matα2 transcription regulators, which are involved in controlling the expression of mating type-specific genes, are rapidly turned over by degradation of control of the ubiquitin proteasome system (Johnson et al., 1998; Laney and Hochstrasser, 2003; Nixon et al., 2010). By contrast, in the a/α diploid with both MATα and MATα idiootypes formed by mating, Mata1 and Matα2 are stabilized by binding to each other and forming an a1-α2 repressor (Johnson et al., 1998). This supports our model, in which the phenotypes of M/G ratio mutants are due to rapid turnover of Matα proteins normally found in MATα haploid cells, and shows that the expression of Matα proteins with a reduced number of MATα copies is sufficient for function if the product is stabilized after mating.

DISCUSSION

The S. cerevisiae mating type determination system harbors instability

Although it was previously thought that the S. cerevisiae mating type was determined by the presence or absence of MATα (Hanson and Wolfe, 2017), we show that the amount of MATα products is also important for sex determination; furthermore, we found that an insufficient quantity of MATα products induces unstable sex-specific gene expression (Figures 4A and 4B). We speculate that this is due to the rapid turnover of MATα products. In addition to the regulation of Matα proteins turnover described above, it is interesting to note that the expression of MATα 1 is repressed in the presence of the a1-α2 repressor (Johnson, 1995). In fact, we observed that, in the a/α-mating type state, a decrease in MATα products was not problematic (Figures 5B and S3), which supports the idea that this phenomenon is caused by rapid turnover. Nixon et al. (2010) discussed that the rapid turnover prevents simultaneous display of both a- and α-mating...
Figure 4. M/G ratio mutants are unable to maintain a stable sexual phenotype
(A and B) Fluorescence of the mating type reporters, MFA1pr-GFP and MFALPHA1pr-mCherry acquired by fluorescence microscopy (A) and flow cytometry (B) show the sexual phenotype and its intensity. Scale bar = 10 µm. Histograms and plots show each fluorescence intensity and its variation (n = 10,000).
(C) Violin plots show the activation level of the mating pathway, determined by measuring the fluorescence of FUS1pr-GFP with flow cytometry (n = 10,000). Letters represent significant differences, determined by one-way ANOVA and Tukey’s HSD comparisons.

Type phenotypes during mating type switching, and does not interfere with the definition of the new mating type (Nixon et al., 2010). Consistently, pheromone receptors, which are the most upstream factors in the mating pathway, are constitutively taken up by endocytosis (Davis et al., 1993; Jenness and Spatrick, 1986).

In this study, we demonstrated the impact of decreased MATα products on sexual identity and mating using polyploid cells. We previously observed Barbapapa-like cells when Matα proteins were expressed from MATα on a centromeric plasmid in haploid matα cells (unpublished observation). Since plasmid-borne gene expression is highly variable from cell to cell (Chou et al., 2015), we assume that this phenotype is caused by a shortage of Matα proteins in a fraction of cells with low expression of MATα genes from the plasmid. Thus, we speculate that the ratio of MATα-derived protein amounts to genome size is critical for the stable expression of the sexual phenotype regulated by MATα, irrespective of the ploidy.

Low levels of Matα proteins result in mating pathway confusion
In homothallic strains of S. cerevisiae, switching between the α- and α-mating type phenotypes is completed within the span of a single division cycle, and pheromones and receptors, for example, must also be replaced within this span (Herskowitz, 1988). The mating type reporters used in this study were based on free GFP and mCherry, and their half-lives in yeast cells are longer than the duration of the cell cycle (Mateus and Avery, 2000; Natarajan et al., 1998). This property allows the respective fluorescence emission to continue even after the corresponding gene is repressed. Therefore, our reporters represent sexual history rather than the sex at the moment. Taking advantage of this character, we demonstrated that a decrease in MATα copy number in polyploid cells caused sex fluctuation, as manifested by the expression of αsg and ααg (Figures 4A and 4B), and hyperactivation of the mating pathway (Figure 4B). In addition, hyperactivation was also manifested by strong expression of FUS1pr-GFP (Figure 4C).

Although ΔΔΔαα cells exhibited abnormal αsg gene expression, they still behaved as the α-mating type, as judged by rare mating with an α-mating type tester (Figure 5A) and no conspicuous mating in ΔΔΔαα culture (Figure S2). Our reporters, which reflect the expression of mating pheromones, provide insight into the specific phenotype of ΔΔΔαα and the underlying mechanisms by revealing the sexual history. ΔΔΔαα showed a bias toward mCherry over GFP, i.e., stronger expression of αsg than ααg (Figure 4B). Furthermore, our reporter analysis revealed intriguing differences in the expression levels of ααg and αsg between mata2Δ and ΔΔΔαα, although the expression of αsg was derepressed in both mutants (Figures 4A–4C).

When a and α cells are fused to form a zygote, activity of the mating pathway is attenuated through the interaction of MATα-specific Asg7 and MATα-specific Ste3 (Roth et al., 2000). Our flow cytometry data showed that the expression levels of GFP and mCherry in mata2Δ were comparable to those in MATα and MATα, respectively (Figure 4B), despite activation of the mating pathway (Figure 4C). This was presumably mediated by the attenuation of the mating pathway through the interaction of MATα-specific Asg7 and MATα-specific Ste3, which is thought to be important in the zygotic fusion event (Roth et al., 2000) and was proposed to occur in mata2Δ cells (Rivers and Sprague, 2003). By contrast, ΔΔΔαα expressed less αsg than ααg, which would result in insufficient attenuation by Asg7-Ste3, causing hyperactivation of the mating pathway. In conclusion, we assume that ΔΔΔαα, which is genetically MATα, causes pheromone-receptor-mediated activation of the mating pathway due to transient expression of the α-mating type phenotype caused by a quantitative deficiency of MATα products.

MATα is composed of haploinsufficient genes encoding transcription factors
The above-described properties are related to the fact that the two proteins expressed from MATα, Matα1 and Matα2, are transcription factors with multiple targets. In cells expressing MATα, Matα1 promotes αsg expression and Matα2 represses αsg expression, so that the cells stably express the α-mating type (Strathern et al., 1981). It is conceivable that, if the amount of each MATα product is insufficient, the transcriptional
state of all target genes cannot be maintained. In other words, MAT α products have a threshold for the sexual phenotype, and the morphological changes and increased mortality in M/G ratio mutants occurred (Figures 1A–1C, 3A, and 3B) because it was below this threshold (Figures 2A and 2B).

For Matα2 to act as a transcriptional repressor, it needs to form a complex with Mcm1, which is also involved in cell cycle regulation (Herskowitz, 1989; Keleher et al., 1988). Apart from the prediction that the overproduction of MATα products interferes with mating type switching (Laney and Hochstrasser, 2003; Nixon et al., 2010), we confirmed that MATα2 overexpression causes phenotypes such as growth defects (Figure S4). Since loss-of-function MCM1 mutants have a similar phenotype (Lim et al., 2003), this result may be due to the depletion of Mcm1 by preferential complex formation by excessive Matα2. Taken together with our finding that the phenotype of a MATα polyploid depends on the amount of intracellular Matα (Figures 1A–1C, 2A, 2B, 3A, 3B, and 4A–4C), these data argue for the need to balance the expression of MATα, neither too much nor too little. This is in line with the dosage-stabilizing hypothesis explaining haploinsufficient (HI) genes, such that a decrease in expression of their products causes a decrease in fitness, while an increase in expression is toxic (Morrill and Amon, 2019). We speculate that MATα has evolved as an HI idiotype.

Figure 5. M/G ratio mutants still maintain the α-mating type, and their phenotypes are normalized after mating
(A) Confirmation of mating type through mating with α- and α-tester strains. As selection markers, tester strains have the LEU2 gene and sample strains have the HIS3 gene. On the SC -Leu -His plate, only zygotes could grow.
(B) Effect of mating with a MATα cell on the cell morphology of M/G ratio mutants. Scale bar = 20 μm.
HI genes are thought to be "stuck" in evolutionary terms, i.e., are unable to regulate gene expression in response to gene dosage variation (Morrill and Amon, 2019). The narrow range of expression of HI genes and significantly reduced fitness when they are outside this range indicate that HI genes are highly sensitive to changes in genome size. The accumulation of HI genes on chromosome III is thought to be the result of selection pressure that prevents diploids from losing this chromosome (de Clare et al., 2011; Delneri et al., 2008). We hypothesize that the haploinsufficiency of MATa, which we discovered, also reduces the fitness of diploids with loss of function of MATa or loss of chromosome III. The loss of a MATa locus in diploids would lead to the production of triploid cells incapable of normal meiosis; S. cerevisiae must have evolved such a mechanism to suppress the emergence of such diploids. In other words, we speculate that the haploinsufficiency of the sex-determining transcription factor genes at MATa prevents diploids from re-entering the mating stage.

**The haploinsufficiency in MATa idiootype suppresses genome expansion**

Significant events in the genome evolution of S. cerevisiae include WGD and alterations to the sex-determination mechanism (Dujon and Louis, 2017; Hanson and Wolfe, 2017). WGD provides the potential for new functional divergence between paralogous genes and creates a reproductive barrier to ancestral species, leading to speciation (Otto, 2007). WGD is involved in speciation of S. cerevisiae, as indicated by genome comparison between S. cerevisiae and Lachancea waltii (Kellis et al., 2004). As for the mechanism of mating type determination, the transcriptional circuit regulating asg expression of the ancestral species was altered in S. cerevisiae (Hanson and Wolfe, 2017). Mata2, which activates asg expression in ancestral yeast a-mating type cells, was lost in the S. cerevisiae lineage, and S. cerevisiae now uses a Mata2 repressing mechanism of asg expression (i.e., expression asg by default) (Sorrells et al., 2015; Tsong et al., 2003). We speculate that MATa haploinsufficiency evolved after the loss of MATa2 because it depends on the mating type determination mechanism that exhibits an a-mating type by default. It should be noted that the evolution of the asg regulatory circuit is assumed to occur at the same time as WGD, about 100 million years ago (Tsong et al., 2006; Wolfe and Shields, 1997).

Despite experiencing WGD, the current genome of S. cerevisiae is comparable in size to the genome of the pre-WGD yeast L. waltii (approximately 12 and 11 Mb, respectively) (Goffeau et al., 1996; Kellis et al., 2004). This suggests a size-limiting mechanism in the genome of S. cerevisiae, which may be the haploinsufficiency of MATa identified in this study. In conclusion, we speculate that the haploinsufficiency of MATa cooperates with HI genes accumulation on chromosome III to compensate for the loss-prone nature of chromosome III and to suppress speciation with an increased genome size.

**Limitations of the study**

This study demonstrated that the MATa idiootype is composed of HI genes and that the concentration of their products is involved in the stability of mating type in S. cerevisiae, suggesting that the haploinsufficiency of MATa may affect genome evolution of S. cerevisiae. Although spontaneous diploidization is a relatively common phenomenon under certain stressful conditions and polyploid cells can be easily produced (Galitski et al., 1999; Harari et al., 2018), WGD occurred only once, about 100 million years ago, in the Saccharomycetales lineage (Wolfe and Shields, 1997). We hypothesize that the accumulation of HI genes on the mating type chromosome and the haploinsufficiency of MATa may explain why WGD was an unusual event in the history of the order Saccharomycetales. However, since our research has so far focused exclusively on S. cerevisiae, we have not been able to discuss the evolution of Saccharomycetales. It would be interesting to address the haploinsufficiency of sex-determining genes in species other than S. cerevisiae in a future study.

**STAR Methods**

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104783.

ACKNOWLEDGMENTS
We thank Drs. Koei Okazaki, Takashi Tsuchimatsu, and Eigo Takeda, and members of Matsuura laboratory for valuable discussions, and Textcheck for editing the manuscript. We also thank anonymous reviewers for critically reading the manuscript and suggesting substantial improvements. This work was supported by JST SPRING, Grant Number JPMJSP2109.

AUTHOR CONTRIBUTIONS
Conceptualization, K.O. and A.M.; Methodology, K.O. and A.M.; Investigation, K.O.; Resources, K.O. and A.M.; Writing - Original Draft, K.O.; Writing - Review and editing, A.M.; Funding Acquisition, K.O. and A.M.

DECLARATION OF INTERESTS
The authors declare no competing interests.

Received: February 8, 2022
Revised: May 18, 2022
Accepted: July 13, 2022
Published: August 19, 2022

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-Myc | MBL | Cat#M047-3; RRID: AB_592470 |
| Mouse monoclonal anti-GFP | Wako | Cat#012-22541; RRID: AB_2922809 |
| Anti-mouse IgG, HRP-linked Antibody | Cell Signaling technology | Cat#7076; RRID: AB_330924 |
| **Chemicals, peptides, and recombinant proteins** | | |
| 5-fluoroorotic acid | Apollo Scientific | Cat#PC4054 |
| 4,18 Disulfate | nacalai tesque | Cat#16512-94 |
| Hygromycin B | nacalai tesque | Cat#07296-24 |
| Nourseothricin | JENABI0 | Cat#AB-102L |
| Propidium iodite | Wako | Cat#164-16721 |
| Immunostar Zeta | Wako | Cat#297-72403 |
| RNase A | nacalai tesque | Cat#30142-04 |
| Proteinase K | nacalai tesque | Cat#29442-85 |
| **Deposited data** | | |
| Raw data of images | This paper | Mendeley data: https://doi.org/10.17632/kfg8k65w7w.2 |
| **Experimental models: Organisms/strains** | | |
| All Saccharomyces cerevisiae strains used in this study are | Matsuura Lab | N/A |
| the BY4742 strain background and are listed in Table S1 | |
| **Oligonucleotides** | | |
| See Table S2 for a list of oligonucleotides. | This study | N/A |
| **Recombinant DNA** | | |
| See Table S3 for a list of Recombinant DNA. | See Table S3 | N/A |
| **Software and algorithms** | | |
| BZ-X800 Viewer | Keyence | N/A |
| Deltavision softWoRx | Applied Precision | N/A |
| CytExpert software | Beckman Coulter | N/A |
| R | R | N/A |
| ImageJ | Fiji team | N/A |
| Fiji | Fiji team | N/A |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Akira Matsuura (amatsuur@faculty.chiba-u.jp).

Materials availability
Strains generated in this study will be deposited to National BioResource Project-Yeast.

Data and code availability
- Original western blot images have been deposited at Mendeley and are publicly available as of the date of publication. The DOI is listed in the key resources table. Microscopy data reported in this paper will be shared by the lead contact upon request.
This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The yeast strains used in this study are listed in Table S1. All strains were derived from BY4742. Gene disruption and tagging were conducted using the conventional one-step PCR-mediated method (Longtine et al., 1998). Polyploid strains were created by mating, and their ploidy was confirmed by DNA content measurement as described below. Yeast cells were grown in YPD medium, consisting of 1% yeast extract (BD Biosciences, San Jose, CA), 2% hipolypepton (Nihon Pharmaceutical Corporation, Tokyo, Japan), and 2% glucose (Nacalai Tesque, Kyoto, Japan), SD consisting of 0.17% yeast nitrogen base without amino acids and ammonium sulfate (BD Biosciences), 0.5% (NH₄)₂SO₄ (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 2% glucose, SC supplementing amino acids and nucleobases to SD medium at the concentrations described previously (Maruyama et al., 2016), and SDCA supplementing 0.5% casamino acid (BD Biosciences) to SD medium. KanMX, hphMX and natMX strain selections were performed in YPD plates containing 250 μg/mL G418, 300 μg/mL Hygromycin B and 100 μg/mL Nourseothricin, respectively. Yeast strains used in the presented experiments are as follows:

Figure 1
(A) OYA1320, OYA1321, OYA1322, OYA228, OYA1343, OYA1344, OYA1332, OYA1334; (B) OYA1320, OYA1321, OYA1322, OYA228, OYA1343, OYA1344, OYA1332, OYA1334, OYA1334, OYA1334, OYA1352, OYA1352, OYA1352, OYA1398, OYA569; (C) BY4742, OYA1, OYA1320, OYA28, OYA1343, OYA1351, OYA1321, OYA356, OYA1344, OYA1352, OYA1322, OYA228, OYA1332, OYA1335, OYA1398, OYA569; (D) OYA1351, OYA1352, OYA1353; (E) OYA1398, OYA569.

Figure 2
(A, B) OYA449, OYA798, OYA799, OYA800.

Figure 3
(A) OYA1320, OYA1321, OYA1322, YOM22, OYA254, OYA255, OYA412, OYA126, OYA369, OYA370, OYA415, OYA280, OYA377, OYA378, OYA418, OYA281, OYA349, OYA350, OYA421; (B) YOM22, OYA126, OYA280, OYA281, OYA254, OYA369, OYA377, OYA349, OYA255, OYA370, OYA378, OYA350, OYA412, OYA415, OYA418, OYA421.

Figure 4.
(A) OYA121, OYA115, OYA809, OYA1395, OYA103, OYA777; (B) OYA121, OYA115, OYA103, OYA777; (C) OYA868, OYA1192, OYA961, OYA962, OYA963, OYA1242, OYA1250.

Figure 5
(A) OYA503, YOM22, OYA1039, OYA254, OYA255, OYA412, OYA233, YOM37; (B) OYA1366, OYA1367, OYA1368.

Figure S1
OYA800, OYA228.

Figure S2
BY4742, OYA1, OYA1322, OYA1332.
METHOD DETAILS

Yeast strains, plasmids, and media
To construct a completely isogenic strain, the mating type of BY4742 was artificially converted from α to a to create OYA38 (BY4742 MATa). First, BY4742 was transformed with YCp50-GAL-HO (Herskowitz and Jensen, 1991), then HO was induced by culturing in medium containing galactose (0.17% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% (NH₄)₂SO₄, 0.5% casamino acid and 3% galactose (Nacalai Tesque, Kyoto, Japan)) at 30°C for 6 h. Second, the culture was spread on a SDCA plate and formed a single colony. Third, those responsive to α-factor were selected, and then those losing the plasmid were selected in a medium containing 5-fluoroorotic acid.

OYA1 (BY4742 mat::kanMX) was created by transformation of matΔ with a PCR-based mat::kanMX deletion cassette generated by amplifying kanMX from pFA6a-kanMX (Wach et al., 1994) using primers, MAT del F and MAT del R. Transformants were confirmed by PCR using primers, MAT check F and MAT check R, and sensitivity to α-factor.

MATα/MATα diploid strains were created by rare-mating. To construct a MATα/MATα diploid strain, MATα haploid strains were transformed with plasmids with different auxotrophic markers (pRS313, pRS315) (Baccarini et al., 1983), mixed and cultured overnight on YPD medium plate, and then selected on a SC -Leu -His medium plate in which only the zygote could grow. Then, plasmids were naturally lost by incubation in YPD medium. In the same way, MATα/MATα/MATα tripl oid strains were constructed by mating a MATα haploid strain and a MATα/MATα diploid strain, and MATα/MATα/MATα/MATα tetraploid strains were constructed by mating MATα/MATα/MATα diploid strains with each other. These strains were confirmed to be α-mating type by assessing the formation of sexual agglutination in a co-culture with the MATα tester but not with the MATα tester.

matΔ/matΔ diploid strains were created by mating by complementation of matΔ with MATα-harbored YCpMATα or MATα-harbored YCpMATα plasmid, restoring fertility as an intact α- or α-mating type, respectively. To construct a matΔ/matΔ diploid strain, matΔ strains were transformed with YCpMATα or YCpMATα (Shin et al., 1997) and plasmids with different auxotrophic markers (pRS313, pRS315), mixed and cultured overnight on YPD medium plate, selected on a SC -Leu –His -Ura medium plate in which only the zygote could grow, and then selected for loss of YCpMATα and YCpMATα on 5-FOA medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% (NH₄)₂SO₄, 0.5% casamino acid, 0.1% 5-fluoroorotic acid, 50 µg/mL uracil and 2% galactose) plate. Then, cells that lost auxotrophic marker plasmids by incubation in YPD medium were picked up. Similarly, matΔ/matΔ/matΔ tripl oid strains were created by mating a matΔ haploid strain and a matΔ/matΔ diploid strain, and a matΔ/matΔ/matΔ/matΔ tetraploid strain were created by mating matΔ/matΔ diploid strains with each other. These strains were confirmed to be α-mating type by assessing the formation of sexual agglutination in a co-culture with the MATα tester but not with the MATα tester.

matΔ/MATα diploid strains (M/G ratio mutants) were created by mating. To construct a matΔ/MATα diploid strain, a matΔ strain with YCpMATα and a MATα haploid strain with pRS313 mixed and cultured overnight on YPD medium plate and selected on a SC -His -Ura medium plate in which only the zygote could grow. Then selected for loss of YCpMATα on 5-FOA medium plate. Then, cells that lost pRS313 by incubation in YPD medium were picked up. Similarly, matΔ/matΔ/MATα tripl oid strains were created by mating a matΔ/matΔ diploid strain and a MATα haploid strain, and matΔ/matΔ/matΔ/MATα tetraploid strains were created by mating a matΔ/matΔ/matΔ tripl oid strain and a MATα haploid strain. These strains were confirmed by PCR using primers, MAT check F and MAT check R, to have two distinct products corresponding to MATα and matΔ.
Similarly, the M/G ratio mutant strains shown in Figure 3 were created by mating. First, strains with M/G ratio = 2 (OYA126 MATα ho::MATα), = 3 (OYA280 MATα ho::MATα Chr3::MATα) and = 4 (OYA281 MATα ho::MATα Chr3::MATα Chr14::MATα) were created by sequential introduction of ectopic MATα constructs. Then, the M/G ratio mutant strains were constructed by mating strains having ectopic MATα with each ploidy strain of MATα.

In the mating of M/G ratio mutant strains and MATα strain, the strains were also each transformed with plasmids with different auxotrophic markers (pRS313, pRS315), mixed and cultured overnight on YPD medium plate, selected on a SC-Leu-His medium plate in which only the zygote could grow. Then, auxotrophic marker plasmids were naturally lost by incubation in YPD medium. Since ste4Δ strains were not capable of mating, pRS317-STE4 was transformed to restore fertility, and ste4Δ M/G ratio mutants were produced using the method for producing M/G ratio mutants described above.

The mating type reporters are two constructs expressing MFA1 promoter-driven GFP and MFALPHA1 promoter-driven mCherry introduced into intergenic region between SAM50 and SSN8 on Chromosome XIV and HO locus on Chromosome IV, respectively.

To construct cells harboring Chr14::MFA1pr-GFP-kanMX, two PCR fragments were isolated, one amplified with the primers M13U-MFA1P F and NLS-MFA1P R using genomic DNA of BY4742 strain as a template, and the other amplified with the primers NLS-F1 F and Chr14 R using the plasmid pFA6a-GFP(S65T)-kanMX6 (Wach et al., 1997) as a template, resulting the two PCR products were MFA1 promoter and GFP-kanMX sequence. Then, the two resulting fragments were amplified using overlapping PCR with the primers Chr14-M13U F and Chr14 R. The final PCR product was transformed into Chromosome XIV (Location: Chromosome XIV 583,958^583,959) and correct insertion was confirmed by PCR.

Cells harboring ho::MFALPHA1pr-mCherry-hphMX was constructed in the same way using the corresponding primers (M13U-MFALPHA1P F, NLS-MFALPHA1P R, HO-R1 R and HO-M13U F), and the plasmid pBS35 (Hailey et al., 2002) as a template.

The FUS1pr-GFP reporter is a construct expressing FUS1 promoter-driven GFP into HO locus. To construct cells harboring ho::FUS1pr-GFP-kanMX, a fus1::FUS1pr-GFP strain was first constructed through the conventional one-step PCR-based gene replacement. The fus1::FUS1pr-GFP strain was created by replacing the FUS1 ORF of BY4742 with PCR product containing the GFP gene and the kanMX marker gene, using amplified with the primers FUS1pr-F1 F and FUS1 del R and the plasmid pFA6a-GFP(S65T)-kanMX6 as a template. Transformants expressing the GFP from the FUS1 promoter were selected on plates containing G418. Next, to construct a strain in which the HO gene was replaced with FUS1pr-GFP, a fragment was amplified with the primers M13U-FUS1pr F and HO-R1 R using genomic DNA of the fus1::FUS1pr-GFP strain as a template, and then using that fragment as a template, ho::FUS1pr-GFP-kanMX fragment was amplified with the primers HO-M13U F and HO-R1 R. Transformants were selected on plates containing G418 and correct insertion of the fragment was confirmed by PCR.

To quantify Matα1 and Matα2 by Western blotting, Myc and GFP were tagged, respectively. Strains expressing Matα1-13Myc were generated by the conventional one-step PCR-based transformation, using the primers MATα1 Ctag F, MATα1 Ctag R, and the plasmid pFA6a-13Myc-kanMX6 (Longtine et al., 1998). Transformants expressing MATα1-13Myc were selected on plates containing G418. Strains expressing GFP-Matα2 were generated by PCR-based transformation and subsequent marker excision described previously (Khmelinskii et al., 2011), using the primers MATα2 Ntag F and MATα2 Ntag R, and the plasmid pMaM189. First, cells in which the amplified fragment was introduced to express GFP-Matα2 from the NOP1 promoter were selected on SDCA plates. Then, cells which express GFP-MATα2 from the MATα2 promoter were selected in a medium containing 5-fluoroorotic acid. Those selected were cells in which the URA3-NOP1 promoter sequence was popped out by homologous recombination. Correct insertion of the fragment was confirmed by PCR.

Ectopic MATα is defined as MATα (MATα1 and MATα2) genes introduced at different locus from the native MAT locus. In this study, constructs ho::MATα, Chr3::MATα, and Chr14::MATα were generated to introduce MATα.
genes at three different locus, HO on Chromosome IV, intergenic region between YCRWdelta11 and FEN2 on Chromosome III, and intergenic region between SAM50 and SSN8 on Chromosome XIV, respectively. To construct ectopic MATα strains, three strains, each with a selection marker gene near MAT locus, were constructed through PCR-based gene insertion. The downstream region of MATα was replaced with PCR products containing the selection marker gene and homologous flanking regions downstream of MATα.

To construct the ho::MATα-His3MX strain, the PCR products were amplified with the primers MATα down marker F and MATα down marker R using the plasmid pFA6a-His3MX6 (Wach et al., 1997) as a template. The strain transformed with this PCR product is the MATα-His3MX strain, which has His3MX downstream of their MAT locus. Next, the fragment was amplified using genomic DNA of the MATα-His3MX strain as a template with the primers M13U-MATα up F and MATα check R. Then, using that resulting fragment as a template, ho::MATα-His3MX construct was further amplified using the primers HO-M13U F and HO-R1 R, resulting to replace HO gene with MATα-His3MX.

Chr 3::MATα-kanMX strain and Chr 14::MATα-natMX strain were constructed in the same way using the corresponding primers (Chr3-M13U F, Chr3R, Chr14-M13U F, Chr14R) and plasmids (pFA6a-kanMX6 and pFA6a-natMX6 (Goldstein and McCusker, 1999)). The final PCR products were transformed into Chromosome III (Location: Chromosome III 170,287^170,288) and Chromosome XIV (Location: Chromosome XIV 583,958^583,959), and correct insertion was confirmed by PCR.

To construct MATα1 overexpression strains, native MATα1 promoter was replaced with a PCR product containing homologous flanking regions upstream of MATα1 ORF and TEF1 promoter or NOP1 promoter. The PCR products were amplified with the primers MATα1 Ntag F and MATα1 Ntag R, and the plasmid pMaM173 or pMaM189 (Khmelinskii et al., 2011) as a template. Correct insertion of the fragment was confirmed by PCR.

MATα2 overexpression strains were constructed in the same way using the corresponding primers (MATα2 Ntag F and MATα2 Ntag R).

Auxotrophic mutations were rescued by sequential integration of the corresponding wild-type genes as described previously (Maruyama et al., 2016). ura3 and lys2 mutations were complemented by PCR fragments amplified from genomic DNA; URA3 fragments was amplified with the primers URA3 up and URA3 down using genomic DNA of X2180-1A, and LYS2 fragments was amplified with the primers LYS2 up and LYS2 down using genomic DNA of BY4741. his3 and leu2 mutations were complemented by fragments cloned on plasmids; HIS3 fragment was cut out by BamHI and XhoI from pJJ215 (Jones and Prakash, 1990), and LEU2 fragment was cut out by Hpal and SalI from pJJ283 (pJJ252) (Jones and Prakash, 1990). Transformants were then selected by SDCA, SC –Lys, SC –His, or SC –Leu plates.

To construct the plasmid pRS317-STE4, the STE4 fragment was amplified with the primers STE4 GRC F and STE4 GRC R using genomic DNA of BY4742 strain as a template. The fragment was cotransformed into the ste4Δ strain with HindIII and SalI-digested pRS317 (Sikorski and Boeke, 1991), and the gap-repaired plasmid was then isolated from the transformant.

**Microscopy**

Yeast cells grown to late log phase in overnight culture were collected by centrifugation (3,000 rpm for 30 s), and then the cells were observed. Fluorescence microscopy was performed by DeltaVision (Applied Precision, Issaquah, WA) and Bright field microscopy was performed by BZ-X810 (Keyence, Osaka, Japan). Images taken in DeltaVision were captured using softWoRx image acquisition and analysis software.

For quantification of cell morphology, overnight culture was stained with FITC-ConA for 30 min, washed with PBS, imaged with BZ-X810, and analyzed with ImageJ.

**Analysis of mating type reporters**

Yeast cells were grown to log phase and resuspended in PBS. Flow cytometric analysis was conducted with the CytoFLEX S flow cytometer with 488-nm (for GFP) and 561–nm (for mCherry) lasers (Beckman Coulter, Brea, CA). Approximately 10,000 events were acquired for each sample.
Analysis of FUS1pr-GFP reporter

Yeast cells were grown to log phase and resuspended in PBS containing 1 μg/mL Propidium iodide (PI). Flow cytometric analysis was conducted with the CytoFLEX S flow cytometer with 488-nm (for GFP) and 561-nm (for PI) lasers. Dead cells detected by PI staining were excluded, and approximately 10,000 events were acquired for each sample.

Quantification of live/dead cells

Yeast cells were grown to log phase and resuspended in PBS containing 1 μg/mL PI at room temperature for 10 min. Microscopic analysis was conducted with the BZ-X810. At least 200 cells were scored for every strain and the percentage of dead cells stained with PI was calculated.

Mating test

A single colony of each sample strain was picked up with a sterile toothpick and spread as a separate thick vertical strip. On a separate YPD plate, tester strains were spread in the same way. The two plates were incubated at 30°C overnight. By replica plating, strips from the plate of the sample strain were stamped onto a new YPD plate through a sterile velvet cloth. Then, tester strains were transferred to another velvet cloth in the same way, and stamped so that tester strains and sample strains were oriented perpendicular to each other onto the YPD plate that already had sample strains. The plate was incubated overnight at 30°C, then stamped through a sterile velvet cloth into a selective medium in which only zygotes could grow, and incubated at 30°C for 3 days.

Western blot analysis

Protein extraction was performed based on alkaline-trichloroacetic acid method with some modifications. 1.0 OD600 unit of cells was removed from culture, pelleted and stored at −80°C. The pellets were suspended in 200 μL of an alkaline solution (1.85 M NaOH-7.4% 2-mercaptoethanol) and incubated on ice for 10 min. Then, 200 μL of 50% trichloroacetic acid was added and incubated on ice for 10 min. Samples were boiled for 5 min prior to SDS/polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Immunoblot analysis was performed with the indicated antibodies, and the immunoreactive proteins were visualized using ImmunoStar Zeta (Wako Pure Chemical Industries, Ltd.).

DNA content measurement

Flow cytometry analysis method were modified from a previous study (Matsui et al., 2013). Cells were grown overnight and fixed with 70% ethanol at 4°C overnight. Then the cells were washed and suspended in 50 mM sodium citrate, treated with 250 μg/mL RNase A at 50°C for 1 h, and then treated with 1 mg/mL proteinase K at 50°C for 1 h. The resuspended cells were stained with 16 μg/mL propidium iodide at room temperature for 30 min. The DNA content of cells was measured on a CytoFLEX S flow cytometer.

Spot assay

Yeast cells grown to log phase were counted on a haemocytometer and diluted with DW to 1 × 10^7 cells/ml. The diluted solutions were serially diluted 10-fold and then spotted onto SDCA plate medium. Colonies were photographed after 2 days.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis were performed using statistical software R (Figures 1B, 1C, 3B, 4C, and S3).

Microscopy image analysis

For analysis of cell size, roundness, and slenderness, images of cells stained with FITC-ConA were analyzed with ImageJ. Each parameter calculated as Area, Circularity, and Major axis/Minor axis (Fit Ellipse) in Analyze Particles, respectively. For each strain, 30 unbudded cells were measured.