Directed evolution for cell separation in budding yeast

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Abstract

We evolved haploid and diploid natural isolates of the yeast *Saccharomyces cerevisiae* under a daily transfer protocol that selected against clumpy growth and for cell separation. For the haploid populations, whole genome sequencing revealed strong selection to deactivate *AMN1*, a known regulator of cell separation, recapitulating in part the perhaps-unintended evolution that occurred during the domestication of common lab strains. We also observed multiple instances of evolution on the Rim101 pathway, which was not expected, and the gene *STE11*. In the diploid populations, we observed repeated selection for a large partial deletion of chrIII (the so-called Hawthorn's deletion), the understanding of which had an important role in the working out of the triple-locus mating-type system, but the positive selection of which, in this or indeed in any context, is unexpected.

Introduction

Cell aggregation phenotypes in the yeast *S. cerevisiae* phenotypes include flocculation, pseudohyphal growth, invasive growth, biofilms, and clumpy growth (Soares, 2011). Clumpy growth (sometimes called chain formation (Stewart, 2009)), is a phenomenon in which growing cells do not fully separate after budding, leading to clusters of related cells. In contrast to clumpy growth, flocculation is reversible, fast-acting, and not dependent on relatedness. Typical laboratory strains of yeast are not clumpy, with much of the variation in clumpiness across strains explained by different alleles of the cell separation regulator *AMN1* (Li *et al.*, 2013). In contrast to laboratory strains, wild isolates of *S. cerevisiae* are often clumpy growers, especially as haploids (Fig. 1). Wild strains tend to be more robust than lab strains in many respects, including faster growth rate, higher sporulation efficiency, and lower rate of petite production (Dimitrov *et al.*, 2009). One barrier to the use of wild strains in laboratory work is that their clumpiness can make some otherwise routine assays—for example dilution plating to obtain colonies and flow cytometry of single cells—challenging or complicated. Evolution experiments in which selection pressure is applied to an aggregation phenotype have been carried out (e.g. Ratcliff *et al.*, 2015; Hope *et al.*, 2017) but so far as we are aware all such experiments to date have selected for increased aggregation. Here we report the results of an evolution experiment in which we selected for *non*-clumpy growth. We evolved replicate populations of haploid and diploid strains, derived from wild isolates, for 57 days under a daily transfer protocol that selected against large clumps and for separated cells. Across populations, we found genetic parallelisms at the level of gene, pathway, and macromutation.

Methods

Strains

The diploid strain YPS602 is a monosporic isolate of YPS133, which was isolated from Tyler Arboretum, Pennsylvania, as described previously (Sniegowski, Dombrowski and Fingerman, 2002); similarly, the closely related diploid strain YPS602 is a monosporic isolate of YPS142, also isolated from Tyler

Arboretum. Both strains are related to the more widely known YPS 128, and are members of the North American Oak clade (Peter *et al.*, 2018). YPS2070 is a haploid *MATα* heterothallic *(ho::kanMX)* derivative of YPS602 and YPS2055 is a haploid *MATa ho::kanMX* derivative of YPS606.

Media and growth conditions

In the evolution experiment, growth occurred in glass 50 mL Erlenmeyer-type flasks containing 10 mL synthetic dextrose (SD) medium supplemented with ampicillin and tetracycline, at 30 degrees C and shaken at 200 rpm. SD was chosen as the media for the experiment because the ancestral diploid strain was not clumpy in YPD after the exponential growth phase.

Evolution experiment

4 replicate cultures of each of the strains YPS602 (diploid), YPS 606 (diploid), YPS2055 (*MATa* haploid), and YPS2070 (*MATα* haploid) were founded and transferred daily according to the following protocol: a 2 mL aliquot of well-mixed culture were removed to a small glass tube and allowed to settle undisturbed for 10 (Day 1 – Day 8), 20 (Day 9 – Day 14), or 30 (Day 15 – Day 66) minutes. After the waiting period, 100 uL of culture was carefully removed from near the top of the culture aliquot and transferred to 9.9 fresh media in a new flask.

Daily transfers were performed for 57 days. At regular intervals, the number of cells transferred was estimated by hemocytometer count. Also at regular intervals, 3 mL of culture was frozen in 30 % glycerol and stored at -80 C. Clonal isolates were obtained from frozen population samples by streaking. For the control (no selection against clumping) evolution experiment, conditions were identical except that 50 ul of well-mixed culture was added to 9.95 mL fresh media, and the evolution was carried out for 66 days.

Populations analyzed

After completion of the experiment, we discovered that the diploid YPS606-founded populations been inadvertently discontinued and replaced with YPS2055-founded populations at some point after Day 29 and before Day 35; that is say, the 4 YPS2055 populations were each accidentally duplicated. We therefore adopted the following numbering scheme to distinguish populations. The haploid populations H1, H2, H3, and H4 were founded from YPS 2070 and the haploid populations H5a, H5b, H6a, H6b, H7a, H7b, H8a, H8b were founded from YPS 2055, with the a/b scheme signifying the culture duplication event. Population H4 was not analyzed due to a separate technical error; therefore after 57 days of evolution there were 11 haploid populations available for analysis. The diploid populations D1, D2, D3, and D4 were founded from YPS 602 and evolved for 57 days. The diploid populations D5, D6, D7, and D8 were founded from YPS 606 and evolved for 29 days.

Whole-genome sequencing

We used the genome assembly of YPS128 (also a member of the North American Oak clade) as provided by (Yue *et al.*, 2017) as the reference genome for all sequencing described here. For clones, a culture started from a frozen chunk, while for frozen populations, the entire frozen sample was thawed and 10 ul used to inoculate a new culture. After growth overnight, DNA was extracted from 1 mL of culture using kit D7005 from Zymo Research. Reads were mapped to the reference genome using `bwa`, and duplicate reads removed by `PicardTools MarkDuplicates`. Mutations were called using `freebayes` on the default settings, with all potential variants reviewed manually and examined in IGV. Plots of read depth were created in R from the output of `samtools -depth` and used to locate chromosomal deletions. Results from this pipeline were spot-checked using `breseq`.

Settling and sedimentation assays

To produce indirect measurements of the degree of clumpy growth, we quantified the settling of cultures. When a culture of yeast is allowed to settle, two features are naturally apparent to an observer: (1) sediment collects at the bottom of the culture vessel and (2) the culture suspension moves visibly downwards, replaced by clear media. We measured both of these manifestations of settling. Clones were started from a frozen chunk and grown overnight in YPD, and then grown for two 24h cycles in SD (diluted 1/100 at each transfer) before assay. In the sedimentation assay, 5 mL of well-mixed culture was placed into a 15 mL conical tube suspended vertically; after two hours, the amount of sediment at the bottom was measured. In the settling assay, 900 ul of culture was briefly spun down (90 s at 10,000 rcf) and resuspended in 900 ul sterile water, 700 ul of this resuspension was pipetted into a narrow-profile cuvette and allowed to settle. The resuspension step was necessary because cultures in SD do not settle evenly. After two hours the position of the point at which the liquid visible transitioned from clear to cloudy was measured.

While diploid strains are far less clumpy in YPD than in SD, for haploid strains the reverse is true: they are clumpier in YPD than SD. Coupled with the fact that cultures are a good deal denser in YPD, this makes changes to settling and sedimentation easier to observe in YPD than SD. Thus, although the evolution experiment was carried out in SD, for haploid strains we carried out settling and sedimentation assays in YPD. For these assays, both sedimentation and settling measurements were taken from the same 5 mL of culture in a 15 mL conical tube; the settling line in these cases was estimated as the midpoint between clear media and cloudiest culture.

Microscopy and cluster size analysis

To produce direct measurements of the distribution of cluster sizes in diploid strains, we employed the following procedure. A frozen chunk of the strain of interested inoculated an overnight culture in YPD; the following day this culture was transferred to SD diluting 1/100 and allowed to grow for 24 h; then another 1/100 dilution and growth cycle in SD was performed; and finally on the 4th day 200 ul of culture was transferred to 12 mL fresh SD and grown until A600=0.6, at which point photographs of the culture were taken at 400x magnification. Subsequently, the number of cells in each cluster was manually labelled (see Supplemental Fig. 5) and recorded.

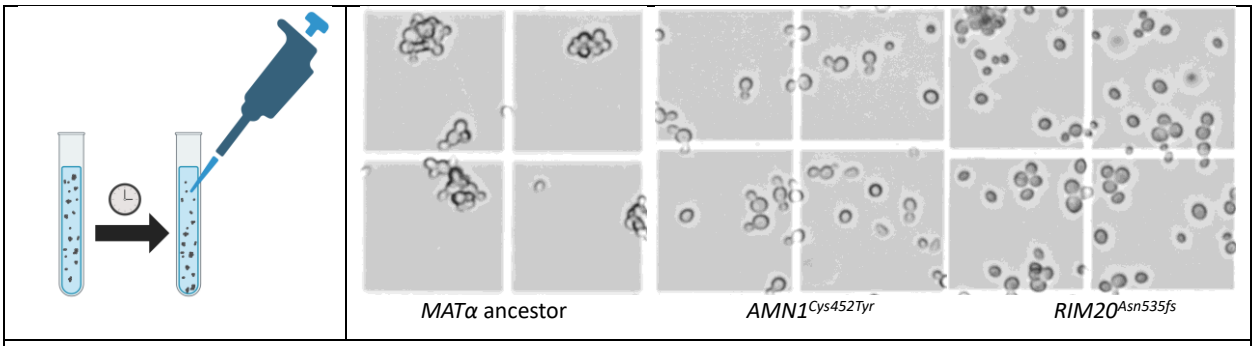
Sporulation and tetrad dissection

Sporulation and tetrad dissection were carried out by standard methods.

Results

Evolution in haploid populations produced parallel changes at the gene and pathway level

All clones isolated from haploid Day 57 populations were clearly less clumpy when examined microscopically (illustrative examples in Fig. 1B), and displayed changes of large magnitude in our bulk property assays (Fig. 2). Whole-genome sequencing revealed that all clones had either a mutation to *AMN1*, a known regulator of cell separation, or a gene in the Rim101p pathway: *RIM8, RIM13, RIM20, or RIM101*. There were no *AMN1* / Rim101p double mutants. Of 6 independent *AMN1* mutations, 4 seemed to be obvious loss-of-function mutations (nonsense or frameshift). While there was no suggestion in the data that the substitutions to *AMN1* had a lesser effect than the frameshifts and nonsense mutations, we performed allele replacement assays on these isolated, replacing AMNC452Y and AMN1L472P with the ancestral allele. These replacements completely restored the clumpy phenotype (Supplemental Fig. 1). Similarly, the Rim101 pathway mutations include one substitution and the rest are frameshift or nonsense mutations.



	Haploid population										
Gene	1	2	3	5	5b	6	6b	7	7b	8	8b
AMN1	C452Y	S498fs	K315*		L472P	R446*		D373*	D373*		
RIM8										S354fs	
RIM13							Q110*				
RIM20				N535fs							
RIM101											C184F
STE11									C430*	K236fs	S630Y

Figure 1. (A) The transfer regime selected for well-separated cells, as large clusters tend to settling out. (B) The ancestral haploid strain is a clumpy grower; evolved clones with mutations to AMN1 and RIM20 are far less clumpy. (C) Across 11 haploid populations, parallel changes at the gene (*AMN1*, *STE11*) or pathway (Rim101) level were observed.

We also observed three independent mutations to *STE11*. These all appeared in conjunction with an *AMN1* or Rim101p pathway mutation. We confirmed that these clones are sterile (do not mate), as expected. Because populations H7a and H7b were once a single population and share the mutation $AMN1^{D373*}$, we can infer with relative confidence that the $ste11^{C430*}$ mutation arose in a background that already had $AMN1^{D373*}$. The *ste11* mutations appear then to always be second mutations. Our settling and sedimentation assays do not pick up an effect of knocking out *STE11* (Supplemental Fig. 2); however, there is a subtle visual difference in settling appearance (data not shown).

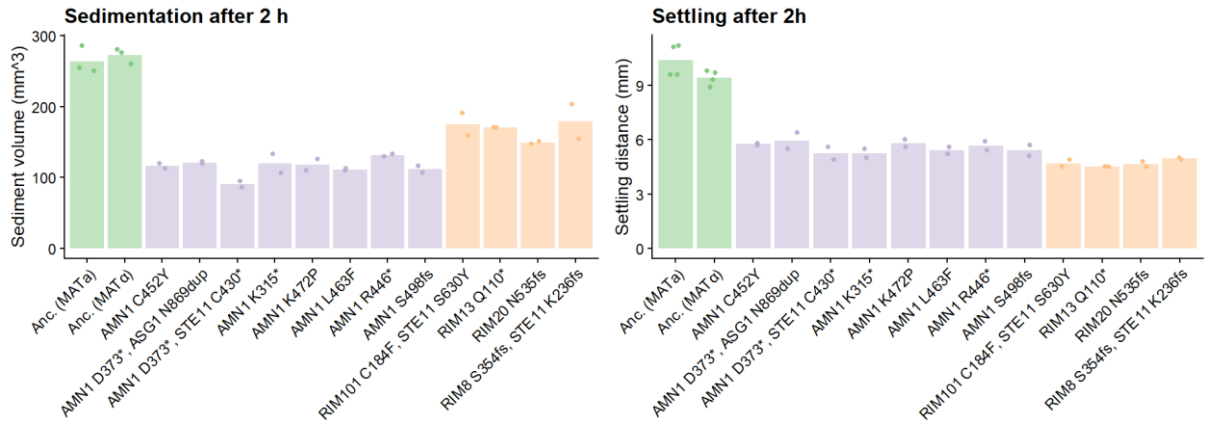


Figure 2. Evolved haploid strains have bulk properties consistent with more cell separation: (A) less sediment at the bottom of a tube after a defined period of settling; and (B) for those cells still in suspension, less visible progress downwards.

Haploid populations were diverse and experienced soft sweeps

We noticed that most pairs of clones isolated from populations that were originally a single population do not share the same mutation (true for H5a and H5b; H6a and H6b; H8a and H8b; but not for H7a and H7b). This suggested that populations were diverse, with many contending lineages. To confirm this we performed whole-population sequencing of two haploid populations. The results (Table 1) confirmed our hypothesis; we detected at least 4 independent *AMN1* or Rim101p mutant lineages in each population. We suspect that the multiple competing *AMN1* mutations are essentially neutral with respect to one another, and that one or another would eventually fix only by drift; however it seems more probable that there is a fitness difference between *AMN1* and Rim101p that would cause one or the other to deterministically fix, given enough evolution time. However, we have been unable to detect any such difference to date (data not shown).

Haploid pop. H3		Haploid pop. H5a	
Mutation	**Freq**	**Mutation**	**Freq**
AMN1 K315*	0.60	RIM20 N535fs	0.43
AMN1 L463F	0.12	AMN1 Q224*	0.24
AMN1 R162*	0.10	RIM8 A>C 409,048	0.08
AMN1 E476*	0.06	RIM8 T>C 409,518	0.04
AMN1 L473P (TG>CT)	0.04		
AMN1 L391S	0.03		

Table 1. Whole population sequencing of two Day 57 haploid populations.

A dominant mutation produces less clumpy growth in diploids

In one of the four Day 57 diploid populations, the representative clone that we isolated had only one apparent mutation, a nonsynonymous heterozygous mutation to the voltage-gated calcium channel gene *CCH1*. This isolate sedimented significantly less than the ancestor and was quantitatively less clumpy (Fig. 4). We sporulated and dissected tetrads of this isolate to produce F994L/F994L and F994F/F994F homozygotes, the genotypes of which were confirmed by Sanger sequencing.

A large deletion of chr III evolved multiple times in diploid populations

We found that a large deletion on chromosome III evolved repeatedly in diploid populations. Whole-population sequencing of population D1 revealed that this macromutation was fixed or nearly fixed. We also found evidence for this deletion in 3 of 4 Day 29 populations (D5, D6, and D8; Supplemental Fig. X), meaning that this deletion was observed in 6 of 8 diploid populations. This deletion spans the region from MAT to HMR, or, in at least one case, the region from MAT to HML (Fig. 3).

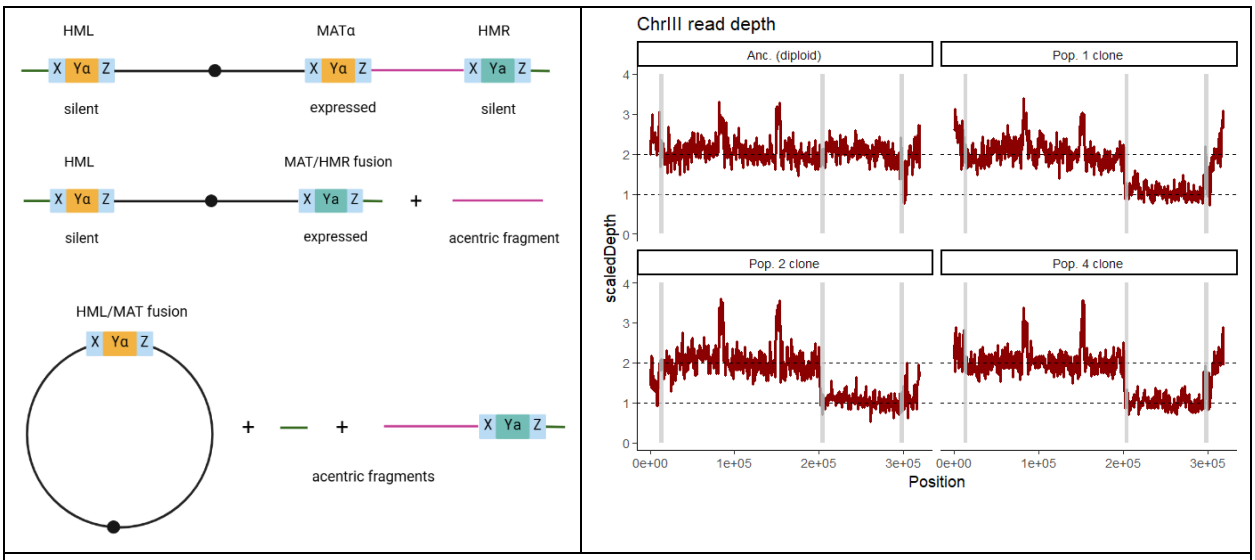


Figure 3. (A) The X and Z regions of homology flank each component of the three-cassette mating type system. These regions of homology can lead to the formation of aberrant fusions. The MAT-HMR fusion causes a large deletion; the MAT-HML fusion causes an even larger deletion and leads to a ring chromosome. (B) Representative clones from 3 of the 4 diploid cultures evolved for 57 days. Two are heterozygous for a MAT-HML fusion and one is heterozygous for a MAT-HMR fusion.

This deletion is historically known as Hawthrone's deletion and its observation had an important role in the working out of the *S. cerevisiae* mating-type switching system. It is surprising to find evidence for its positive selection. Clones with one of these deletions displayed substantially less sedimentation (Fig. 4a) and a quantitative imaging assay showed that they are less clumpy than the ancestor (Fig. 4b). For example, the ancestral proportion of cells occurring in clumps of 4 cells or fewer was 43%; for the Hawthorne's deletion strains, this estimate ranged from 65% to 77%.

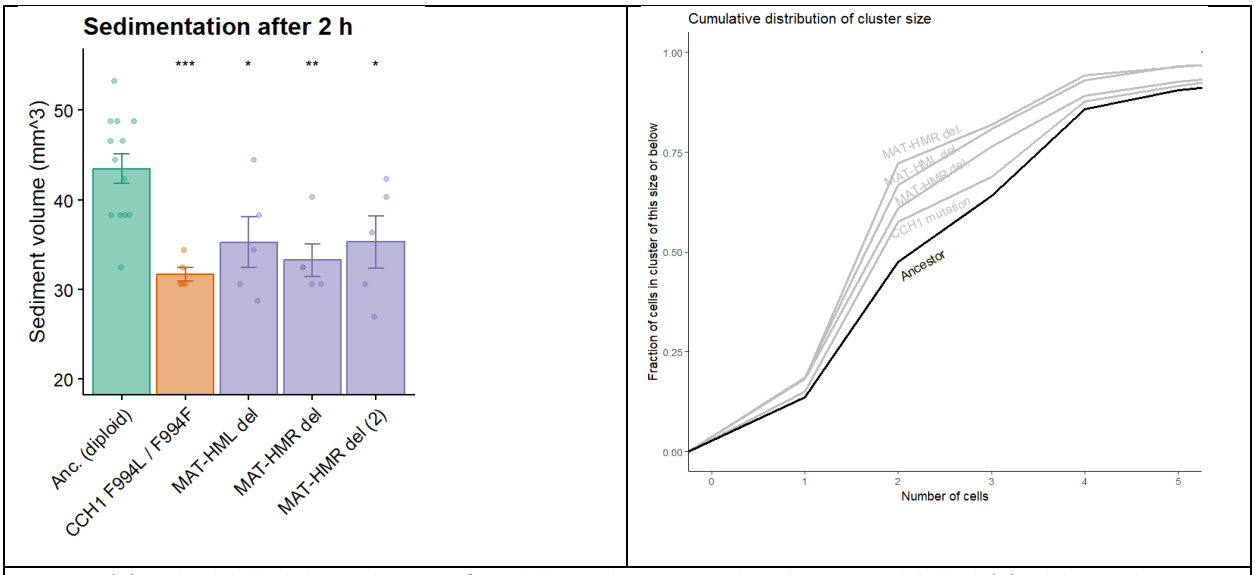


Figure 4. (A) Evolved diploid clones show significantly less sedimentation than the ancestral diploid. (B) While visual microscopic inspection of evolved diploid clones does not show an obvious phenotype, a quantitative investigation revealed that the distribution of cell cluster size is markedly altered.

The MAT-HMR region includes several essential genes. Thus, we expected the deletion strains to segregate 2:2 live:dead when sporulated and dissected, and this was the case (Supplemental Table 2). One curiosity was that the isolate with the MAT-HML fusion would be expected to have a ring chromosome III (Fig. 3a); and we would expect this ring to further depress spore viability before 50%, since crossovers during meiosis between the ring and the linear homolog would run the risk of generating aberrant products. Yet, we found no evidence of a depression in spore viability, which led us to question whether there truly was a ring. Therefore we performed long-read sequencing. A substantial fraction of long reads mapped to regions left of MAT *and* right of HMR (Supplemental Fig. 4), indicating that the ring-generating fusion occurred as suspected; and we saw no evidence of a breakage anywhere else, leaving the lack of depression in spore viability as something of a mystery.

Discussion

This evolution experiment yielded outcomes both predictable and surprising. How *AMN1* regulates (prevents) cell separation in haploids was nicely dissected by Fang *et al.* (2018). Semi-inadvertent selection for *AMN1* mutants is likely part of the evolutionary history of common lab strains. This experiment has perhaps recapitulated that selection; thus it is not surprising that we recovered many *AMN1* mutants. It is a demonstration of the power of experimental evolution to aid in genotype-to-phenotype mapping (given that a selection gradient relevant to the phenotype of interest can be applied) that had we *not* known about *AMN1*'s role in governing cell separation, this experiment would have clearly pointed towards it.

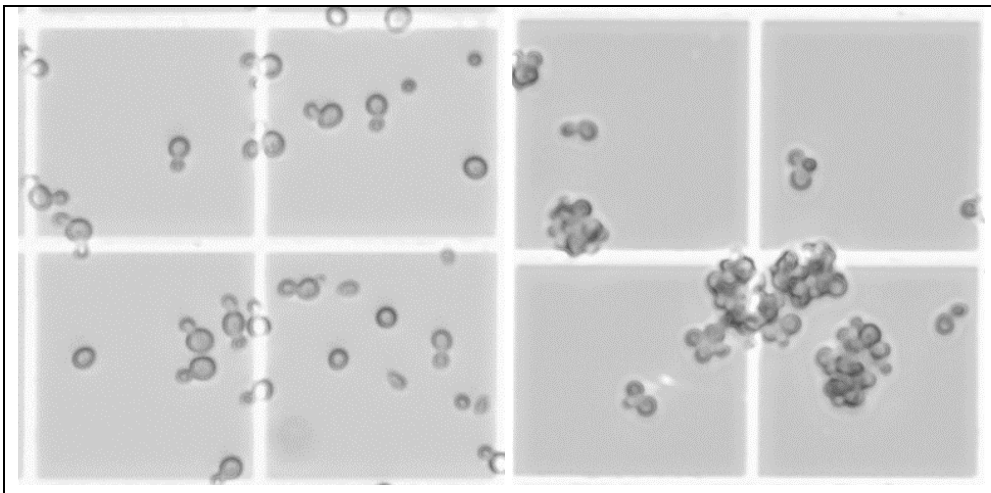
The multiple hits to the Rim101 pathway are less expected. That pathway is known to have a role in pH sensing (Yan *et al.*, 2020) and regulation of cell size (Shimasawa *et al.*, 2023), and to connect to pathways regulating filamentous growth (Cullen and Sprague, 2012). Yet a role in cell separation during normal physiological growth conditions has not previously been elucidated—perhaps because many lab strains are already well-separated.

One motivation for this experiment was to test for differences in the genetic architecture of adaptation between diploids and haploids. We hypothesized that haploids would evolve mainly by loss-of-function mutations, while diploids would tend to evolve by gain-of-function mutations. Indeed, all causative mutations that we found in haploids appear to be loss-of-function, disabling the function of *AMN1*, the Rim101 pathway, and *STE11*. We found only one nonsynonymous mutation, in *CCH1*, that putatively causes more cell separation in diploids, and it does indeed appear to be dominant which at least suggests that it is a gain-of-function mutation, although we have not yet established that as fact.

The most surprising outcome was repeated selection for MAT-HMR or MAT-HML deletions on chromosome III. Such deletions, historically known as Hawthorne's deletion, were instrumental in the long and distinguished effort to work out the mating type switching system (Hawthorne, 1963; Haber, Rogers and McCusker, 1980; Herskowitz, 1988; Haber, 1998). It is interesting that we have observed these deletions in completely wild-type diploids, separately from any context of mating type *switching*, as the ancestral diploid was *MATa/MATα* and the deletion strains remain *MATa/MATα*. It is possible that the initial deletion event caused a switch that led to a transient *MATa/MATa* or *MATα/MATα* state. We are not sure why these deletions cause more cell separation, but we guess that it may be related to apparent haploinsufficiency of *BUD23* and/or *BUD31*. Mutants of both of these genes are known to display random budding patterns, and we find that our deletion strains, which have only one copy of each, have a more disordered budding pattern than their ancestor (Supplemental Table 1). However, this could be an unrelated effect of the deletion.

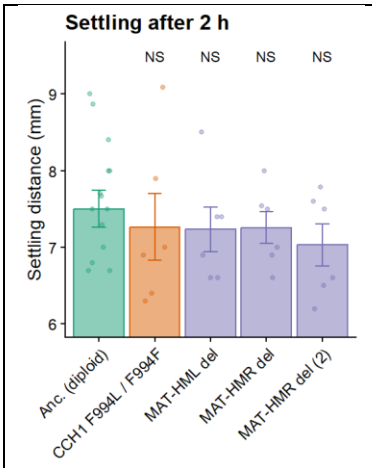
The repeatability of the deletion suggests that it is always happening at some low rate. This points to a previously unknown additional cost or load of maintaining the three-cassette mating type system. Such a load would join a rather long list of the costs of sexual that are rather mysteriously tolerated by eukaryotic populations.

Supplemental Figures and Tables

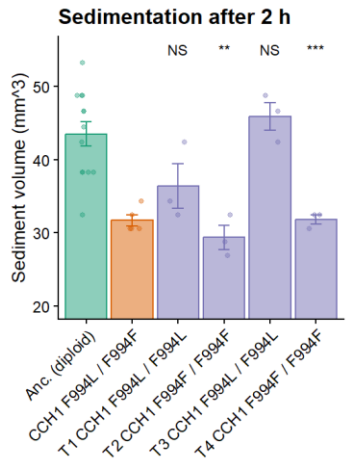


AMN1^{Cys452Tyr} *ΔAMN1*^{Cys452Ty}*r::AMN1*^{Anc}

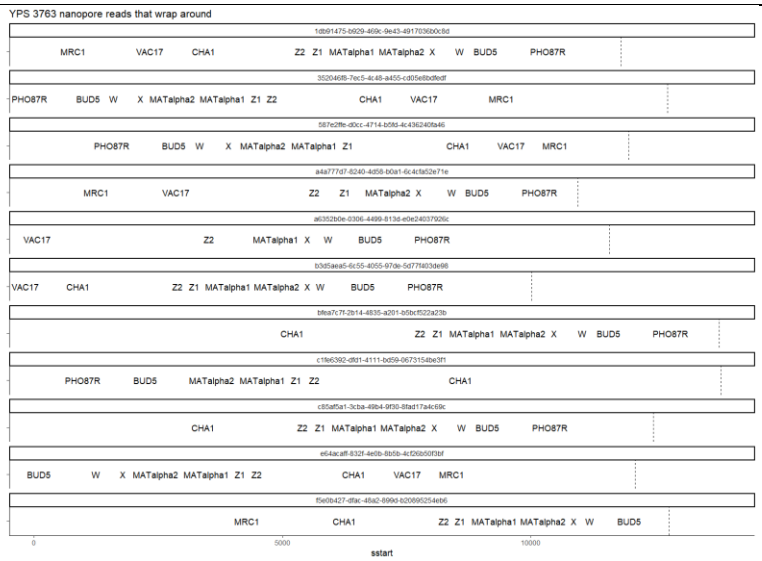
Supplemental Figure 1. Replacement of the evolved AMN1 allele with the ancestral allele completely restores the clumpy phenotype.



Supplemental Figure 2. Settling (as opposed to sedimentation; see Fig. 3) assays in the diploid were noisy and no significant effects were found, although the mean effect was in the expected direction.

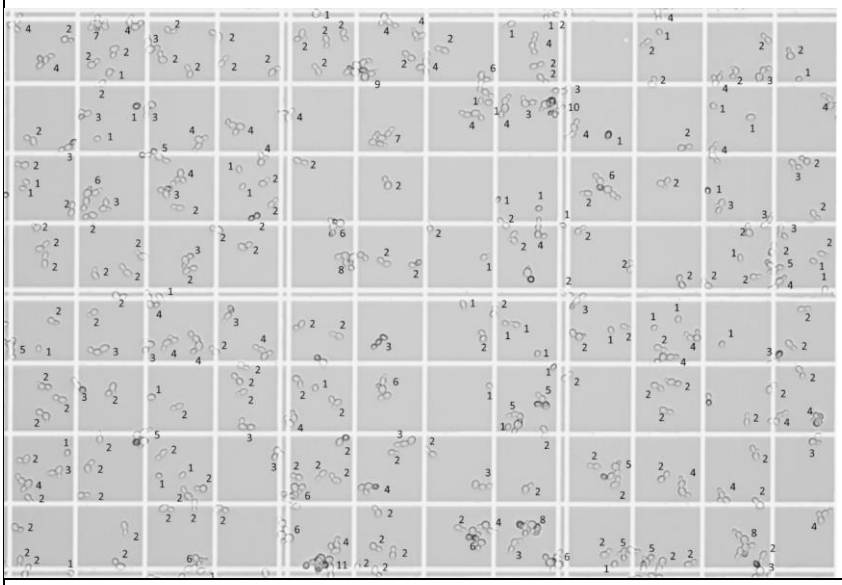


Supplemental Figure 3. Sedimentation assay of dissected sporulation products of the diploid that acquired the *CCH1* F994L mutation; this shows that (1) the effect goes with the mutation and (2) the effect appears to be dominant.

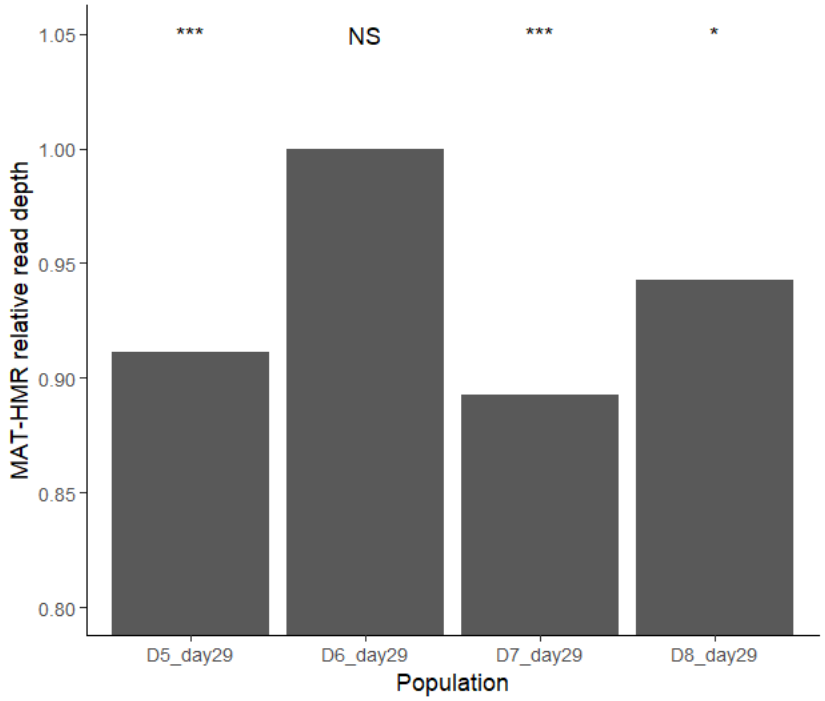


Reads >= 10 kb	
Spanning HML	7
Spanning MAT	18
HML-MAT fusion	11

Supplemental Figure 4. Long-read (Nanopore) sequencing evidence of a ring chromosome III.



Supplemental Figure 5. An example of labelling for cluster size.



Supplemental Figure 6. Three of the four diploid populations halted at Day 29 show evidence of a low=frequency lineage with a chrIII deletion.

Budding polarity			
	Alternating pole	Same pole	Ratio
Diploid ancestor	48	23	2.1
MAT-HML fusion	31	29	1.1 (p <0.01)
MAT-HML fusion (2)	32	25	1.3 (p <0.05)
MAT-HMR fusion	32	27	1.2 (p <0.01)

Supplemental Table 1. The chrIII deletions affect budding polarity. From photographs taken at 400x magnification, diploid mothers were scored for the site of emergence of their last two daughters.

	Surviving spores per tetrad				
	1	**2**	**3**	**4**	**Total viability**
MAT-HMR fusion	1	9	0	0	0.48
MAT-HMR fusion (2)	0	13	0	8	0.69
MAT-HML fusion	1	18	2	0	0.51

Supplemental Table 2. Spore viability of the chrIII deletion isolates.

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