**Title**

Paths to adaptation under fluctuating nitrogen starvation: The spectrum of adaptive mutations in *Saccharomyces cerevisiae* is shaped by transposons and microhomology-mediated recombination

**Authors**

Michelle Hays[1]*, Katja Schwartz[1]*, Danica T. Schmidtke[2], Dimitra Aggeli[1,3], Gavin Sherlock[1]

*Equal contribution

Corresponding author: G.S. (Email: gsherloc@stanford.edu)

Departments of [1]Genetics and [2]Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305

[3]Current address: Department of Biological Sciences, Lehigh University, Bethlehem, PA 18015

**Abstract**

There are many mechanisms that give rise to genomic change: point mutations are often emphasized in genomic analyses, but evolution acts through many mechanisms that give rise to less subtle perturbations. Changes in chromosome structure, DNA copy number, and novel transposon insertions all create large genomic changes, which can have correspondingly large impacts on phenotypes and fitness. In this study we investigate the spectrum of adaptive mutations that arise in a population under fluctuating nitrogen starvation conditions. We specifically contrast these adaptive alleles and the mutational mechanisms that create them, with mechanisms of adaption under glucose limitation and continuous selection in low, non-fluctuating, nitrogen conditions. We observe that retrotransposon activity accounts for a substantial number of adaptive alleles, along with microhomology-mediated mechanisms of insertion, deletion, and gene conversion. In addition to loss of function alleles, which are often exploited in genetic screens, we identify adaptive gain of function alleles and alleles acting through as-of-yet unclear mechanisms. Taken together our findings emphasize that *how* selection is applied shapes adaptation, just as the selective pressure does itself. Fluctuating environments can activate different mutational mechanisms, shaping adaptive alleles accordingly. Using experimental evolution is thus a complementary approach to both classical genetic screens and natural variant studies as a way to characterize the genotype-to-phenotype-to-fitness map.

## Introduction

Genomic changes underlie phenotypic variation and population evolution. To understand phenotypic variation, genetic screens can identify relevant loci, but are often limited in the types of genomic changes they explore. For example, screens often use mutagens with specific mutational mechanisms, or collections of deletion mutants. Alternatively, comparative genomics can establish which changes have previously arisen and survived, but linking such changes to fitness and adaptation can be challenging. By contrast, experimental evolution can reveal how organisms adapt under specific selective pressures, revealing the most fit and/or frequent beneficial mutations (e.g. Jacquier et al. 2013; Lang et al. 2013; Hong and Gresham 2014; Payen et al. 2016; Venkataram et al. 2016; Good et al. 2017). The mutational types that arise during experimental evolution are not limited to those typically isolated from genetic screens, but rather reflect the mutational mechanisms active in a population experiencing a selective pressure of interest. The relative rates of different mutagenic mechanisms differ greatly in yeast, with single nucleotide polymorphisms (SNPs) arising at ~$10^{-10}$ per nucleotide per generation (Lang and Murray 2008), while aneuploidy, for example, has been observed at ~$10^{-4}$ per generation (Zhu et al. 2014) (though it can vary greatly between chromosomes and genetic backgrounds (Kumaran et al. 2013)) and affects hundreds of genes at a time. Other mutagenic mechanisms, such as transposition, recombination-driven gene conversion, loss of heterozygosity, and structural rearrangements may also give rise to adaptive alleles unique to those mechanisms and can also occur during experimental evolution. Therefore experimental evolution is an especially useful tool to understand how genome changes facilitate adaptation. By profiling the target loci at which adaptation occurs, the mutagenic mechanisms that create those changes, and the specific adaptive alleles that arise, agnostic to gain or loss of function, a more complete view of the genotype-to-phenotype map can emerge. Thus, experimental

evolution complements, rather than simply reproduces, findings obtained through other types of genetic studies.

Some stress conditions are linked to increases in specific mutagenic mechanisms and stress-induced genomic instability may even improve evolvability of organisms (Galhardo et al. 2007). Populations under stress may therefore sample more genotypic and phenotypic states, aiding population survival in environments for which ancestors were ill-adapted. Transposons are mobilized in many species in response to stress (e.g. Chen et al. 2003; Hashida et al. 2003; Hashida et al. 2006; Sehgal et al. 2007; Chenais et al. 2012; Jardim et al. 2015; Miousse et al. 2015; Esnault et al. 2019; Fouche et al. 2020; Roquis et al. 2021), including in *Saccharomyces cerevisiae* (Bradshaw and McEntee 1989; Scholes et al. 2003; Sacerdot et al. 2005; Todeschini et al. 2005; Servant et al. 2008; Stoycheva et al. 2010; Servant et al. 2012), leading to an increase in transposon-driven mutagenesis. *S. cerevisiae* has five families of Long Terminal Repeat (LTR) retrotransposons: Ty1 - Ty5 (Rowley 2017; Bonnet and Lesage 2021). Ty1 and Ty2 are closely related and are the two most abundant families in the reference genome. Ty1 transcription increases under DNA damaging conditions (Bradshaw and McEntee 1989), extreme adenine starvation (Servant et al. 2008), filamentous growth (as a result of carbon or nitrogen starvation; Morillon et al. 2000), and telomere shortening (Scholes et al. 2003). Newly integrated Ty copies can interrupt host gene coding sequences or alter host gene regulation, causing increased, decreased or even constitutive activation of neighboring ORFs. This regulatory capacity arguably makes transposon mutagenesis more powerful for driving host evolution, and possibly even determining evolvability itself, than, for example, point mutations. Genomic variation also arises comparatively frequently through several microhomology mediated mechanisms (Dan et al. 2021). These events, such as microhomology-mediated translocations, duplications, deletions, and gene conversion events, cause variants to arise more frequently specifically at sites of short sequences found tandemly repeated within a

genome (Dan et al. 2021). This increased frequency of site-specific mutation could influence population evolvability and adaptation, disproportionately impacting the evolution of genomic regions containing microhomology.

Laboratory directed microbial evolution experiments allow investigation of how selective pressures shape genome evolution and most often make use of either serial transfer or continuous culture (Long et al. 2015; Voordeckers and Verstrepen 2015; Cooper 2018). The selective pressure can come from nutrient limitation, the presence of an inhibitor (such as an antibiotic) or a restrictive environmental condition (e.g. temperature). In serial batch transfer evolutions, populations are iteratively passaged into fresh media, leading to regular environmental fluctuations. By contrast, in chemostat or turbidostat growth, continuous flow into the culture chamber of fresh medium and outflow of spent medium/cells produces a constant environment (Gresham and Dunham 2014). This fundamental difference in fluctuating vs. constant selective pressure can drive different means of genome evolution: the rates that some classes of mutations arise may differ between these approaches, and the relative fitness of evolved alleles may differ as well. Experimental evolution can thus help us dissect the relative contributions of mutational stochasticity and adaptive determinism under given selective conditions.

*S. cerevisiae's* adaptation to glucose limitation has been well-studied in both chemostat and serial transfer conditions and each results in different adaptive mutational spectra (Gresham et al. 2008; Kao and Sherlock 2008; Kvitek and Sherlock 2013; Lang et al. 2013; Venkataram et al. 2016). Continuous culture in limiting glucose predominantly selects for mutants with more rapid glucose uptake, such as amplifications of the *HXT* glucose transporters, as well as lineages that fail to mount starvation stress responses (Kvitek and Sherlock 2013). By contrast, in fluctuating glucose conditions, self-diploidization and point mutations in the Ras and TOR pathways

predominate (Venkataram et al. 2016), the latter often resulting in a shorter lag phase (Li et al. 2018).

Like carbon, nitrogen is an essential nutrient for yeast survival. Nitrogen sensing and uptake also uses a complex signaling network with several feedback loops and points of self-regulation. It also impacts many other cell states, such as initiation of catabolic programs to scavenge nutrients from within, as well as changes in growth programs such as filamentous growth and biofilm formation. Just as yeast favor glucose as a carbon source, there are preferred and unpreferred nitrogen sources (see Hofman-Bang 1999); the nitrogen catabolite repression (NCR) and retrograde response pathways facilitate regulation and usage of different nitrogen sources (see Cooper 2002; Conrad et al. 2014). Both of these pathways involve TOR signaling, and NCR contains multiple feedback loops for self-regulation. For example, Gat1 is a central regulator of NCR and participates in several feedback loops, including self-activation (Coffman et al. 1997). Gat1 is also required as a coactivator along with Gln3 at many NCR promoters. Additionally, NCR repressors Gzf3 and Dal80 function by interfering with Gat1 binding (including competition at the *GAT1* promoter), *GAT1* expression and Gat1 cellular localization (Georis et al. 2009).

Previously, experimental evolution of *S. cerevisiae* under continuous nitrogen limitation demonstrated adaptive amplification of the *GAP1* transporter gene, via extrachromosomal circle formation which is facilitated by recombination between flanking LTRs (Gresham et al. 2010). We set out to determine if, as was observed in adaption to glucose limitation, different classes of mutational mechanisms give rise to different modes of adaptation under fluctuating nitrogen limitation as compared to continuous limitation. The relative complexity of regulation, pathway feedback and pleiotropy of nitrogen signaling may give rise to a distinct adaptive spectrum under fluctuating conditions that vary between low and zero nitrogen during the growth cycle.

This fluctuating nitrogen condition provides an interesting case for comparison of adaptive mutational spectra, contrasting adaptation to limiting nutrients (glucose vs. nitrogen), and also continuous vs. fluctuating conditions. These comparisons provide insight into how the application of selection (continuous vs. fluctuating) shapes available adaptive routes and their relative fitness, as well as how the genetic architecture of a trait (nitrogen vs. carbon sensing) influences the spectrum of beneficial mutations.

Previously, we analyzed the population dynamics of prototrophic *S. cerevisiae* evolved under nitrogen limited conditions by serial transfer (Blundell et al. 2019). We noted an initial increase in adaptive single-mutants, followed by a crash in population diversity due to clonal interference as a result of rapid expansion of highly beneficial double-mutants. Here, we more deeply explore the specific mechanisms underlying adaptation to nitrogen limitation, focusing on recurrently mutated loci and the different mutational classes that give rise to these adaptive alleles. We identify six loci that are often targets of selection specifically under fluctuating nitrogen limitation where cells experience transient total nitrogen starvation. This suggests that mutation of these loci leads to the greatest fitness increases and/or that these loci are most easily and frequently mutated. In addition to SNPs and small indels, we observe several adaptive alleles that result from microhomology-mediated insertion, deletion or gene conversion, and multiple alleles that result from Ty insertions. This is in contrast to others' results under continuous growth conditions, where these classes of mutations are rarely observed and different loci are found most frequently mutated. While many of our identified adaptive alleles are likely loss of function mutants, we also identify putative gain of function mutations, again emphasizing the utility of experimental evolution for exploring the breadth and depth of adaptive alleles and the mechanisms that birth them.

**Results**:

**Summary of nutrient limited evolution experiments and lineage tracking**

We previously evolved yeast by serial transfer under both glucose and nitrogen limitation (Levy et al. 2015; Blundell et al. 2019), using lineage tracking by barcode sequencing to identify adaptive lineages and to estimate fitness. Evolved clones were previously isolated for fitness validation and whole genome sequencing; briefly, ~5,000 clones were isolated from generations 88 and 192 (from glucose and nitrogen limitation respectively), their barcodes identified, and their ploidy was determined using a benomyl based assay as described (Venkataram et al. 2016; see Methods).

**Sequencing of evolved clones from nitrogen limitation**

To identify the underlying adaptive mutations, we whole-genome sequenced 331 evolved clones with unique barcodes (295 haploids and 36 diploids) isolated from generation 192 of the nitrogen-limited evolution. These clones were selected based on the previously characterized barcode frequencies and their estimated fitness during the evolution itself and were expected to include both neutral and adaptive clones. For diploids, we only sequenced those with estimated fitness greater than diploidy alone would provide. We analyzed the data for SNPs, small indels, and *de novo* transposition events; sequenced clones contained between zero and five SNPs/indels and zero to nine Ty insertions (see Methods). To determine which of the observed mutations are likely adaptive, we looked for genomic loci that were recurrently mutated between different lineages and where these mutations correlated with reproducible fitness increases by fitness remeasurement (see below; Table 1).

| | Number of alleles | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | SNPs | | | Microhomology-mediated mutations | | | Ty insertions | | |
| Gene | Missense | Nonsense | Indels | MHMI | MHMD | MHMR | Ty | 5' Ty | 3' Ty |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***GAT1*** | 12 | 2 | 2 | 1 | 4 | 0 | 42 | 8 | 0 |
| ***PAR32*** | 1 | 6 | 2 | 2 | 2 | 0 | 25 | 4 | 0 |
| ***MEP1*** | 8 | 0 | 0 | 0 | 0 | 0 | 8 | 0 | 17 |
| ***MEP2*** | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ***MEP3*** | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ***FCY2*** | 4 | 4 | 2 | 0 | 0 | 10 | 5 | 1 | 0 |
| *ARO80* | 3 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *TOR1* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *TOR2* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *GPB1* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *GPB2* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *SSK2* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *PDE1* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 1. Genes with recurrent mutations identified in both haploids and diploids, or whose mutation has been previously shown to be adaptive.** Bolded genes are specifically "nitrogen adaptive" (see below).

**Fitness of evolved clones in both limiting nitrogen and glucose**

To validate the increased fitness of unique isolated clones that were whole genome sequenced, fitness remeasurements were performed via pooled competitive fitness assay (Methods) in both limiting glucose and limiting nitrogen conditions. This allowed us to distinguish which lineages were adaptive specifically in limiting nitrogen, versus those that were adaptive in both conditions. The barcoded clones were pooled with 48 confirmed neutral strains from previous experiments (Venkataram et al. 2016), and were competed 1:10 against an unbarcoded ancestor in triplicate. Lineage trajectories were highly reproducible (Figure S1) as were fitnesses calculated from those trajectories (Figure S2).

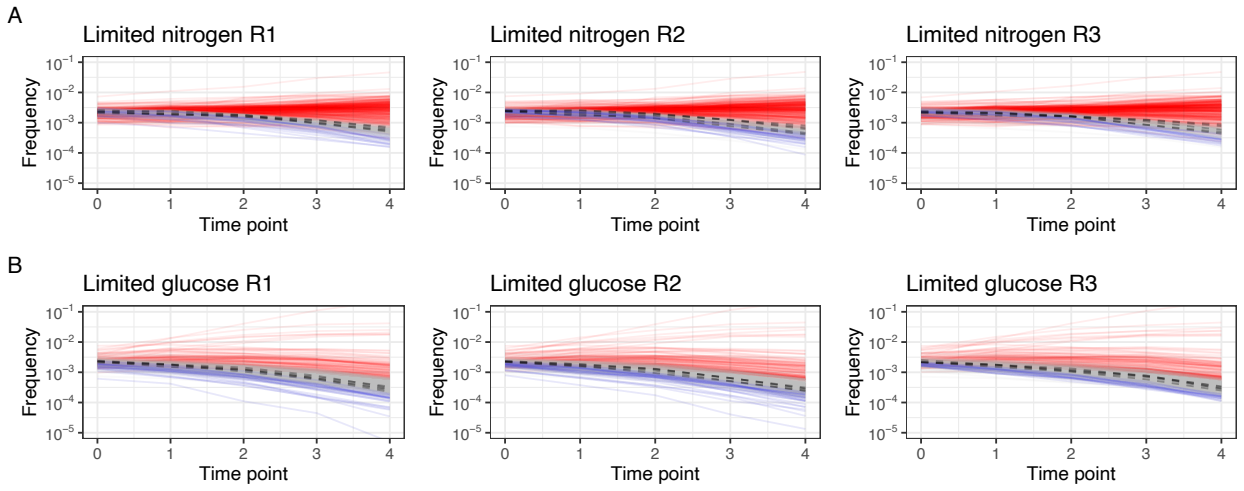


**Figure S1. Barcode trajectories during pooled fitness remeasurement experiments, A) in nitrogen limiting conditions, and B) in glucose limiting conditions**. A subset of known neutral lineages is represented by dotted lines, while lineages in red have an estimated fitness > 0.01. Grey represents evolved strains with fitness between -0.01 and +0.01, and blue is <-0.01.

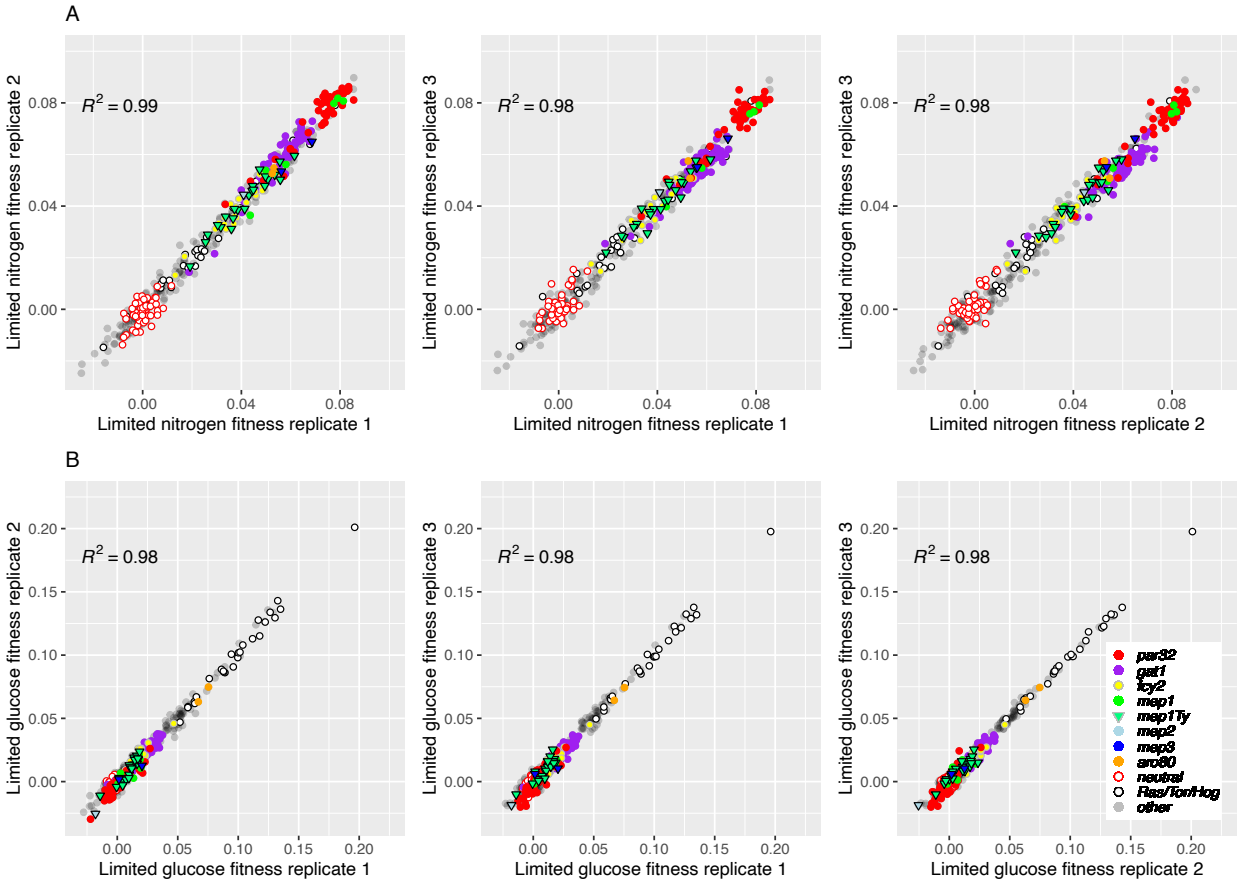


**Figure S2. Fitness of isolated clones is reproducible across biological replicates.** Estimated fitness between replicates for each clone under A) nitrogen limitation, and B) glucose limitation.

Many of the 331 clones isolated from the nitrogen limited evolution were beneficial only under nitrogen limited conditions and were either neutral or even maladaptive in glucose limitation (Figure 1, upper left region), while others were found to be roughly equally beneficial under both glucose limitation and nitrogen limitation conditions (Figure 1, upper right). For the remainder of this manuscript, we focus on genes whose mutation provides substantially greater fitness under nitrogen limitation than under glucose limitation.

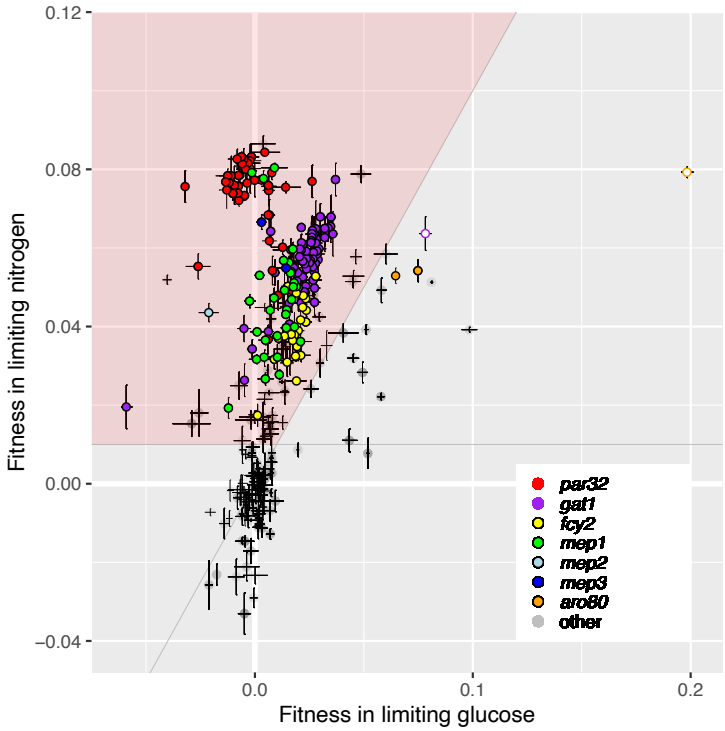


**Figure 1. Fitness of haploid clones isolated from the nitrogen limited evolution in nitrogen- versus glucose-limitation.** Clones in the upper left region (pink) are considered to be "nitrogen adaptive" (see text). The two white-filled circles correspond to double mutants, a *gat1* mutant with an *ssk2* mutation, and an *aro80* mutant with a *gpb2* mutation. Error bars indicate standard deviation of the replicate fitness measurements.

**Loci recurrently mutated across evolution conditions and identification of presumptive beneficial mutations**

To classify mutations as specifically adaptive for nitrogen limitation, we identified loci that were recurrently mutated in independent lineages that were also validated to a fitness effect of >0.01 in nitrogen-limited media and have substantially greater increased fitness in nitrogen-limitation than in glucose-limitation. Independent lineages were designated by the presence of unique barcodes. We identified six such loci that had two or more independent mutations that satisfied these criteria, which we refer to as "nitrogen adaptive" from here forward: *GAT1*, *FCY2*, *PAR32*,

*MEP1*, *MEP2*, and *MEP3* (Table 1). Together, these loci were found independently mutated a total of 176 times across multiple lineages with reproducible fitness increases across multiple alleles. *MEP2* and *MEP3* were only observed as mutated twice each, with one of the *MEP2* mutations occurring heterozygously in a diploid, while we identified 33 adaptive *MEP1* alleles. Notably, and in contrast to prior results from chemostat evolutions, we did not observe recurrent mutations at the *GAP1* locus, the permease previously associated with adaptation to continuous nitrogen limitation (Gresham et al. 2010). We identified an additional six loci that had also acquired multiple independent mutations during this evolution; however, they did not show reproducible fitness gains, making the status of these mutations (adaptive or neutral) inconclusive: *MIT1*, *DAL81*, *VID28*, *PFA4*, *CRP1*, and *FAS1*. While *FAS1* was found mutated three times independently in confirmed adaptive lineages, these mutations were only identified in the context of *PAR32* or *GAT1* mutant backgrounds and seemingly did not increase fitness beyond that of the confirmed adaptive alleles. Finally, some loci were mutated and adaptive in both glucose- and nitrogen-limited conditions, including *ARO80* and members of the TOR and RAS pathways: *TOR1*, *TOR2, LST8, SCH9*, and *GPB1* and *GPB2* (Table 1).

**Fitness gains associated with nitrogen adaptive loci**

The pooled fitness assays showed that the six recurrently mutated loci consistently correlate with increased fitness in nitrogen limitation, and moreover that different alleles show consistent fitness increases (Figures 1 and 3). For example, most of *GAT1* mutations show a reproducible increased fitness of 0.04-0.06/generation, while *MEP1* mutations fall in two clusters with Ty insertions having an average fitness increase of 0.03-0.05 and missense mutations affecting residue Asp262 have a fitness increase of ~0.08. The *MEP2* and *MEP3* alleles showed fitness gains between 0.04 and 0.07. Together, these data show that the evolved alleles at these six loci are indeed adaptive mutations, reproducibly driving 0.03-0.08 increases in fitness in fluctuating nitrogen starvation conditions.

**Further validation of adaptive alleles**

To further confirm these mutations are indeed the drivers of increased fitness (rather than 'hitchhiking' neutral mutations), for five of the nitrogen adaptive loci we generated progeny for one or more alleles of each gene and performed a pooled competitive fitness assay as above. The resulting fitness estimates confirmed that the observed fitness gains are correlated with mutations in *GAT1*, *MEP1, MEP2, MEP3, and PAR32* and that progeny show equivalent fitness gains to the adaptive parent, demonstrating that mutations at these loci likely account for all, or nearly all, of the fitness gain in these lineages (Figure 2). Note, the magnitudes of the measured fitnesses are often somewhat smaller than in the fitness remeasurements in Figure 1, we hypothesize that this is a result of the competing pool composition being different (see Methods).

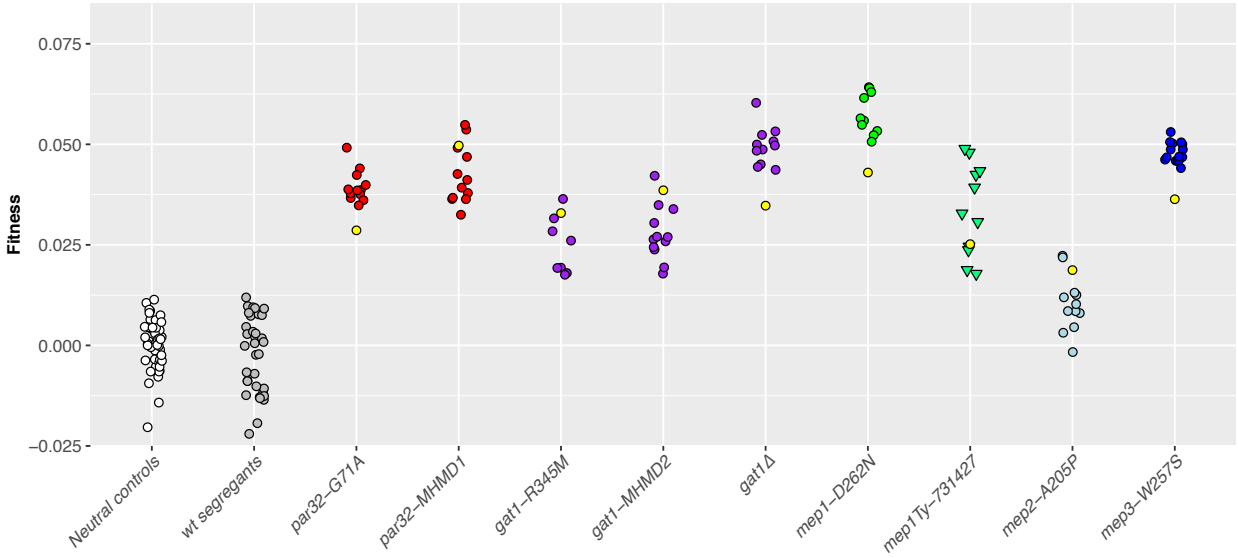


**Figure 2**. **Fitness remeasurements of progeny containing putatively adaptive alleles confirm adaptive mutations.** Neutral controls are the neutral lineages previously isolated from glucose limited evolutions, while wild-type segregants are segregants from the crosses that lack the adaptive alleles of interest. Yellow dots correspond to the parental lineages.

## Targets of adaptation

Lineages with mutations in the six nitrogen-adaptive loci account for 71% of adaptive lineages sequenced in our evolution (162/212 adaptive haploid lineages and 14/29 adaptive diploid lineages). Here we further discuss the mutation types and allele locations observed and their roles as adaptive targets (Figure 3).

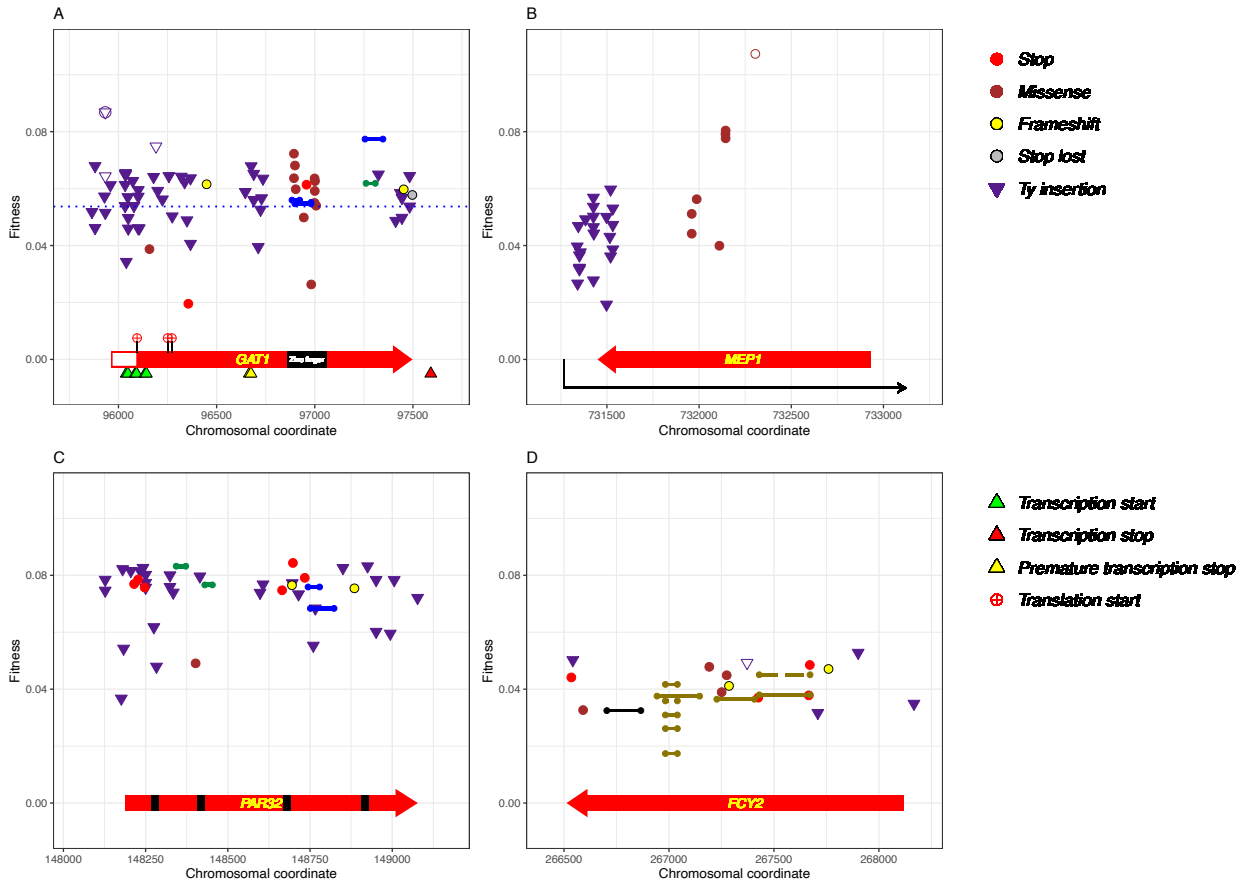


**Figure 3. Targets of adaptation and their fitness effects.** A) *GAT1*, B) *MEP1*, C) *PAR32*, D) *FCY2*. Glyphs indicate mutation type (see key); the dotted line for *GAT1* indicates the fitness of a complete deletion mutant; the black boxes in *PAR32* indicate repeat regions.

*GAT1 locus*

*GAT1* encodes a GATA-type DNA-binding zinc-finger transcription factor which, among other activities, activates nitrogen catabolite repression (NCR) genes such as *GAP1* and *GLN1*. Gat1 binds DNA directly, but also facilitates transcriptional regulation via protein-protein interactions with other transcription factors (Georis et al. 2009). *GAT1* transcription itself is typically upregulated by Gln3 under nitrogen limited conditions, reducing nitrogen utilization within the cell. When a preferred nitrogen source is available, Gat1 and Gln3 are phosphorylated by TOR kinases and are sequestered to the cytoplasm, while *GAT1* transcription is simultaneously repressed by Ure2 and Dal80.

At the *GAT1* locus, novel Ty insertions account for a large proportion (70%) of all haploid adaptive mutations (46/66 lineages) in this evolution, predominantly inserting in the 5' half of the CDS (Figure 3A, purple triangles). However, several other classes of mutations were also observed in *GAT1*, including two nonsense mutations (red circles), two frameshift mutations (beige circles), a lost stop codon (black circle), several short, out-of-frame intragenic rearrangements that likely arose by microhomology mediated recombination (green and blue segments in Figure 3A; also see Figure 4B), and 12 missense mutations (burgundy circles), as well as a whole-locus deletion (3.9 kb, from 722bp upstream to 1.6kb downstream, blue dotted line).

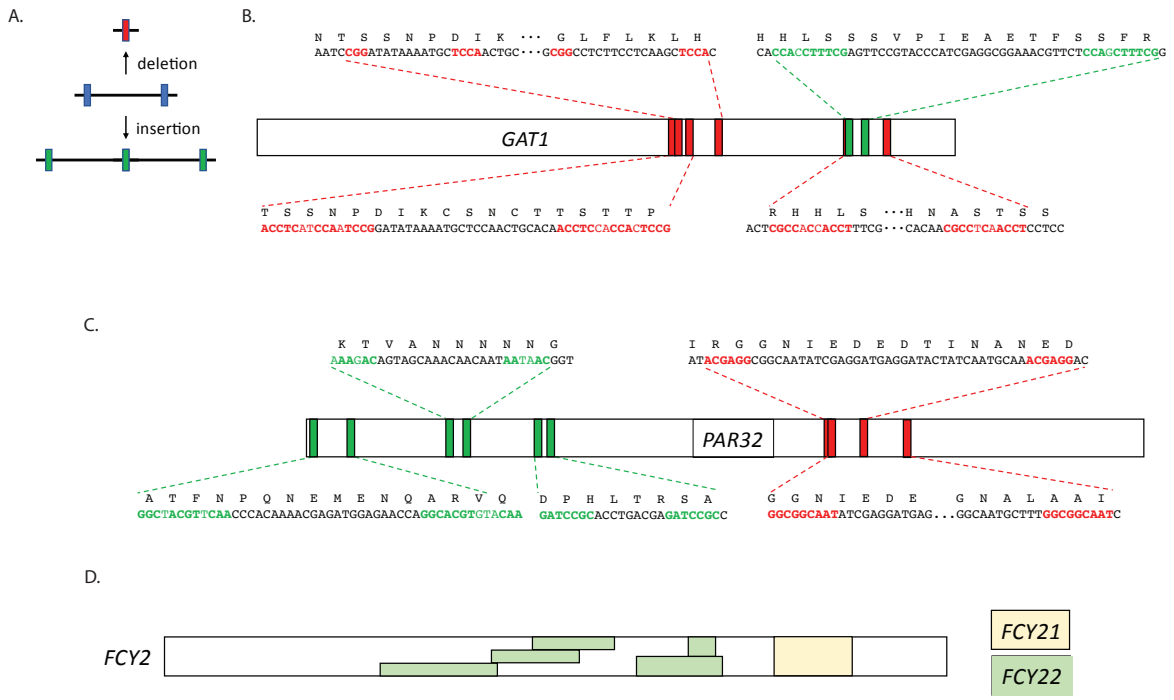


**Figure 4. Microhomology-mediated mutations.** A) diagram of how sequence flanked by regions of microhomology can either be deleted or duplicated; color is used to denote insertion vs. deletion. B) microhomology mediated mutations in *GAT1*, C) microhomology mediated mutations in *PAR32*, and D) regions of *FCY2* replaced by ectopic recombination from either *FCY21* or *FCY22*; one of the *FCY22* gene conversions was observed five independent times.

Across both haploid and diploid clones, we observed 12 independent missense mutations affecting *GAT1* (Figure 3A, burgundy circles). All but one (11/12) of these mutations fall within the zinc-finger domain and show fitness increases of ~0.06, similar to the putative null mutant alleles. We also identified two alleles with small deletions in the zinc finger domain that each result in a frameshift that truncates Gat1.

Several (9/65) adaptive diploid clones were isolated bearing *GAT1* mutations (open shapes, Figure 3A) in addition to the 56 haploids. Diploidization alone gives rise to increased fitness of

~0.03, while diploids with heterozygous missense mutations at *GAT1* saw fitness increases of 0.06-0.07, similar to *GAT1* mutant haploids. Homozygous *GAT1* mutants had even larger fitness gains (0.09). Indeed, a single diploid with a homozygous *GAT1* mutation (Ty insertion, putative null, also observed in a haploid background) was identified for which we measured a fitness increase of greater than 0.08. Heterozygous missense mutations at the *GAT1* locus were also identified in adaptive diploid backgrounds. Additionally, several of these adaptive mutations putatively affect the *GAT1* RNA isoforms differentially (triangles below gene name), either through impacting alternate start (green triangle) or alternate stop sites (maroon and red triangles).

In our study, we observe adaptive *GAT1* alleles with polymorphisms at the DNA-binding site, as well as putative null mutations. These adaptive alleles all show similar fitness increases in our study (0.06±0.01) in haploid isolates. Given the many mechanisms by which Gat1 interacts with NCR regulation, these *GAT1* alleles may act on cell fitness through similar or differing mechanisms: Gat1 DNA binding mutants maybe functionally null, explaining the similarity in fitness increase, or perhaps DNA-binding mutants impact fitness through alternative means, such as modulating Gat1p protein-protein interactions with other transcription factors.

The NCR program allows cells to increase their uptake and usage of nonpreferred nitrogen sources during low nitrogen abundance but is repressed in the presence of preferred nitrogen sources. *GAT1* null mutations may be beneficial under these evolution conditions (serial transfer in ammonium-limited media) as *gat1* cells may be better poised to immediately utilize the nitrogen available upon transfer to new media, relative to *GAT1* competitors that would require repression of NCR factors to utilize the freshly-introduced nitrogen supply. For this reason, *gat1* null alleles may be most likely to be beneficial in cells experiencing fluctuating ammonium concentrations from moderate (3mM) to total nitrogen starvation. While *GAT1* alleles have also

been shown to be beneficial in constant low nitrogen chemostat environments (Abdul-Rahman et al. 2021), only adaptive DNA-binding SNP mutations were identified in that study. No null alleles were observed in adaptive clones or populations evolved in chemostat low ammonium conditions (Hong and Gresham 2014).

*MEP loci*

*S. cerevisiae* has three paralogs, *MEP1, MEP2, and MEP3,* that encode ammonium permeases; they have differing affinities for ammonia and are induced at different levels in response to the quality and quantity of the nitrogen source (Marini et al. 1997). Mep1 forms a heterotrimeric complex with Mep3, while Mep2 forms a homotrimeric complex (see Boeckstaens et al. 2015). The Mep1/3 complex is inactivated by Par32 binding (Boeckstaens et al. 2015; see below), while Mep2 is regulated directly via Npr1 phosphorylation (Boeckstaens et al. 2007). In our evolution conditions, we observe 33 independent mutations affecting the *MEP1* locus, and just two mutations each at both the *MEP2* and *MEP3* loci (Figure 3B; Table 1, Figure S3). The most fit *MEP1* mutants (~0.08 in haploids) have greater fitness increases than the *MEP2* or *MEP3* mutations we measured, which are still comparable to other *MEP1* missense mutants (~0.04 - 0.06) evolved in this study.

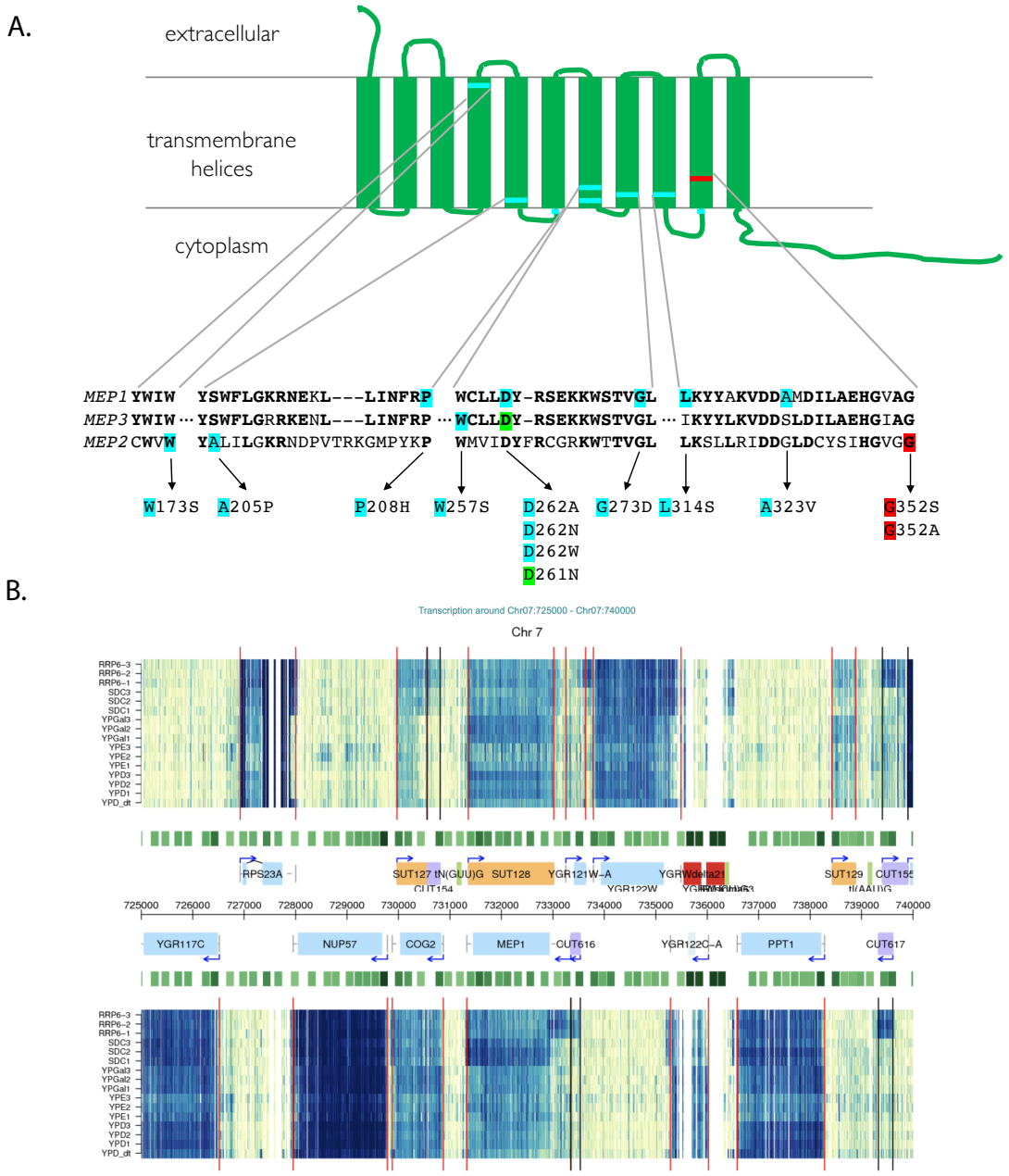


**Figure S3. Locations of *MEP1* mutations and antisense transcript at the *MEP1* locus.** A) Locations of Mep mutations in a multiple alignment, and B) transcription at *MEP1* locus indicates the presence of an antisense transcript downstream of *MEP1*.

Interestingly, one of the *MEP3* missense mutations affects the equivalent residue with the same as an adaptive *MEP1* (Figure S3A). In these alleles, Asp261 is mutated to Asn. In addition to our findings, others have observed *MEP* mutations under nitrogen limited chemostat evolution conditions, interestingly only in *MEP2*, not in *MEP1/3* (Hong and Gresham 2014). It is possible that the missense mutations at these sites give rise to a higher affinity ammonium transporter in all cases, but that differences in stable vs. fluctuating nitrogen starvation environments determine which transporter is most important or active.

In addition to missense mutations, we observed a striking pattern of Ty insertions at the *MEP1* locus (Figure 3B), with 25 novel insertions falling in the 3' UTR and the 3' most region of the gene. These insertions fall close to the 5' end of an antisense transcript (SUT128) that has been observed in RNA-Seq experiments (Xu et al. 2009; Figure S3B).

The bolus of Ty insertions in the 3' region unique to the *MEP1* locus may affect fitness in nitrogen limitation via a mechanism different than the putative gain of function missense mutations in the transporter domain. These alleles may be increasing Mep1p activity (via RNA regulation for example) or affecting NCR regulation independently of Mep1p. *MEP1* expression is normally controlled by nitrogen catabolite repression regulation via TORC1p and Par32p (Boeckstaens et al. 2015). The lack of such insertions downstream of either *MEP2* or *MEP3* suggest that this mode of regulation may be unique to *MEP1*.

Because serial transfer in limited ammonium conditions gave rise to two main classes of adaptive mutation in *MEP1* (missense mutations in the transport domain, and 3' Ty insertions) and similar missense mutations in *MEP2* were observed in chemostat ammonium-limited conditions, we asked whether continuous culture conditions also give rise to Ty insertions near *MEP* genes. We reanalyzed the data from (Hong and Gresham 2014) (see Methods) to look for

Ty insertions at *MEP1/2/3* and found no evidence for adaptive Ty insertions at these loci. This suggests that 1) Ty elements are less active under continuous culture conditions (even when the same nutrient is limiting), or 2) that the mechanism by which Ty insertions are beneficial under fluctuating nitrogen starvation conditions is not equally adaptive in constant low-nitrogen conditions, or 3) a combination of both. We speculate that the adaptive *MEP1* missense alleles observed in our study may in fact be gain of function mutants, capable of transporting ammonium more effectively, while the downstream Ty insertions may increase Mep1 activity through other means.

*PAR32 locus*

We observed 42 independent beneficial *PAR32* mutations, predominantly comprised of putative null alleles [41/42] arising from nonsense mutation, Ty insertion or frameshift mutations (Figure 3C), with just a single adaptive missense allele. Four frameshift alleles all likely arose by microhomology-mediated recombination (Figure 4D). Most of the putative null alleles are associated with a ~0.08 fitness increase, suggesting that lack of Par32 gives rise to similar host fitness benefits regardless of mutation type that creates the null allele. Par32 is an unstructured protein known to inhibit Mep1/Mep3 transporters, so *par32* null alleles likely affect fitness through increasing Mep activity, just as *MEP* loci gain of function alleles would be beneficial in these same evolution conditions (Boeckstaens et al. 2015). We observed only one *PAR32* missense mutation (Figure 3C, burgundy circle). This missense mutation lands in the second of four repeat motifs (Figure 3C, black bars) found in Par32; these repeat regions are essential for direct Par32 interaction with the Mep proteins (Boeckstaens et al. 2015).

Null *par32* alleles are likely to be adaptive by preventing the inactivation of Mep1/3 transporters specifically during fluctuating low-to-no nitrogen conditions. *par32* null alleles may facilitate more rapid uptake of nitrogen when moved to fresh medium containing ammonium relative to wild

type cells that would need to re-activate Mep1/3 expression. Under continuous culture conditions where low ammonium concentrations remain steady and do not drop to zero, others have observed that *par32* null mutants are actually maladaptive (Abdul-Rahman et al. 2021).

*FCY2 locus*

Finally, we found the *FCY2* locus mutated in 26 different unique lineages in our evolution (Figure 3D). *FCY2* is a purine-cytosine permease found at the plasma membrane, important for the import of adenine and other substrates into the cell (Hopkins et al. 1992; Ferreira et al. 1997; Ferreira et al. 1999; Kurtz et al. 1999; Wagner et al. 2001). Ten out of the 26 *FCY2* beneficial mutations are the result of regions of microhomology-mediated gene conversion with other *FCY* genes (*FCY21* and *FYC22*; Figure 4D) that result in a frameshift. Fcy21 and 22 are both nucleobase transmembrane transporters, but cannot functionally replace Fcy2 (Wagner et al. 2001). Such gene conversion events have been observed previously between *FCY2* and *FCY22*, but not involving *FCY21* (Quinto-Alemany et al. 2012). These regions of gene conversion likely decrease or abrogate the transport function of Fcy2 as was seen previously (Quinto-Alemany et al. 2012). The *FCY21/22* converted alleles show fitness increases of ~0.04 on average.

Additionally, we observed several Ty insertions in the 5' region of the CDS of *FCY2* (Figure 3D) as well as both nonsense and frameshift mutations, suggesting that null alleles are also beneficial under nitrogen starvation. These mutations exhibit a similar range of fitness increases, again suggesting that a decrease in *FCY2* activity is likely adaptive in ammonium limited serial transfer.

**Timing and establishment of adaptive mutations**

Through barcode lineage tracking and comparison to neutral fitness lineages, we explored the relative dynamics of adaptive allele establishment in the population (Figure S4). We note that the lower fitness, but still adaptive, alleles (such as mutations of *fcy2,* yellow Figure S4 panel E) established early in the evolution, while single mutations corresponding to larger fitness increases (e.g. *PAR32* and *GAT1*, red (panel A) and purple (panel B) respectively) increased more rapidly but arose later in the evolution. These observations may reflect the 'ease' of acquiring these mutations: for example, *fcy2* appears to be the result of a gene conversion event, which may arise more often than inactivating point mutations in *par32 and gat1*, or the putative gain of function mutants in *MEP* genes. In addition to how frequently some mutation types occur, the relative fitness of a mutation shapes how quickly those adaptive lineages expand in the population. As we have observed previously (Levy et al. 2015) observed lower fitness adaptive mutations arise early during evolution, while higher fitness single mutations can occur later and still reach population frequencies high enough to be sampled in our clonal sequencing (Figure S4 panel F).

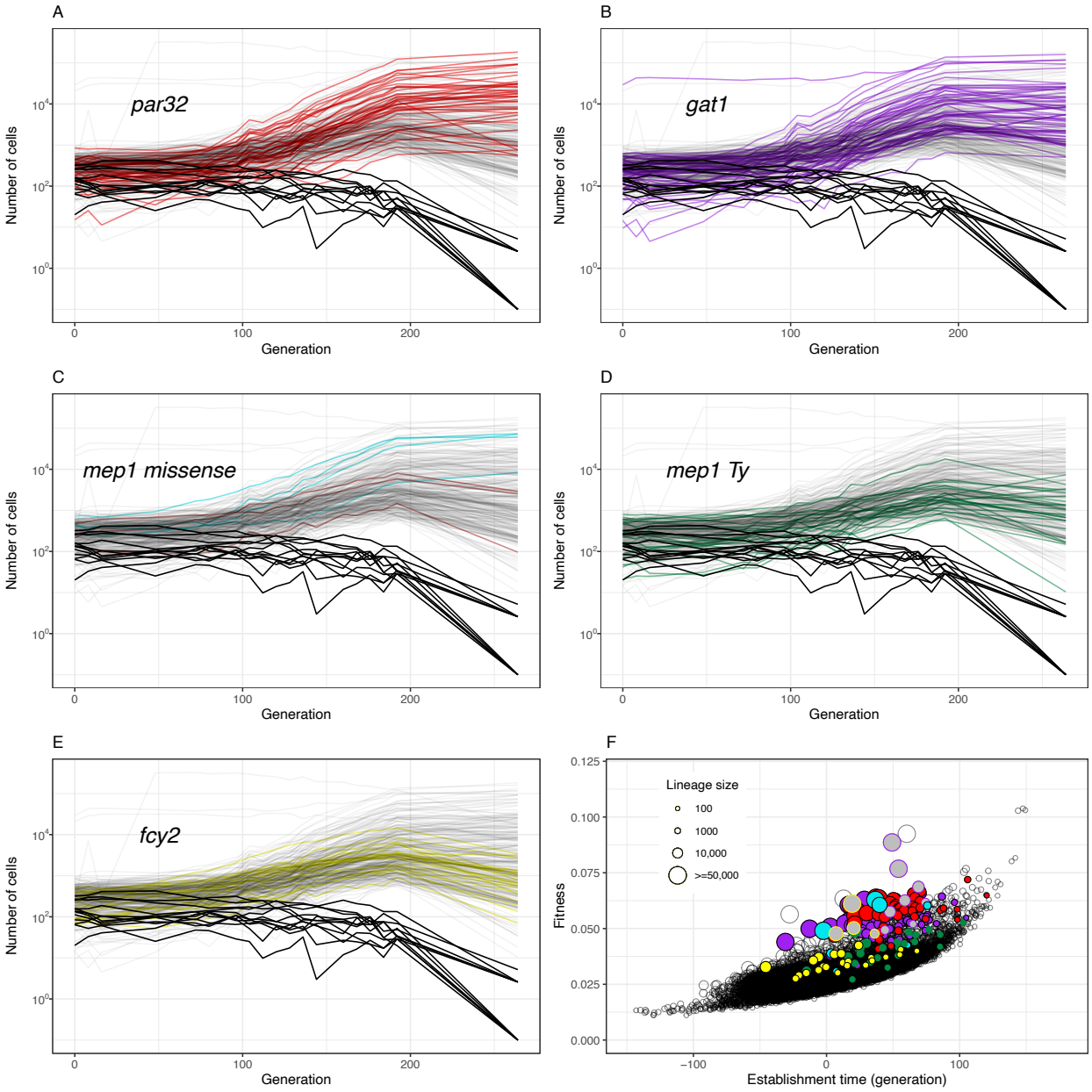


**Figure S4. Dynamics of increase in frequency of lineages with adaptive mutations in specific genes.** A) *PAR32*, B) *GAT1*, C) *MEP1* missense mutations, D) *MEP1* Ty insertions, E) *FCY2*, and F) fitness and lineage size as a function of establishment time.

**Mutational spectra in nitrogen vs. glucose limited populations: Increased Ty transposition in N limitation conditions**

To better understand the classes of adaptive mutations underlying genome evolution, we compared the mutational spectra observed in both neutral and adaptive populations under both nitrogen and glucose limitation. First, we note that autodiploidy itself confers a significant fitness benefit in both nitrogen limitation (Blundell et al. 2019) and glucose limitation (Venkataram et al. 2016). Beyond autodiploidy, we observed that in both neutral and adaptive haploids, SNPs comprise the majority of genome changes under glucose limitation (Figure 5A) and in both glucose and nitrogen limited conditions SNPs were enriched in adaptive lineages relative to neutral lineages (black bars, Figure 5A). By contrast, Ty insertions are most prevalent under nitrogen limitation with approximately ~3 novel insertions per clone compared to only ~1.1 novel Ty insertions in clones from glucose limited evolutions (Figure 5A). Novel Ty insertions were more prevalent in neutral than adaptive lineages (blue bars), however in nitrogen limitation in particular, Ty mutagenesis accounts for a substantial number of adaptive mutations (gold bar).

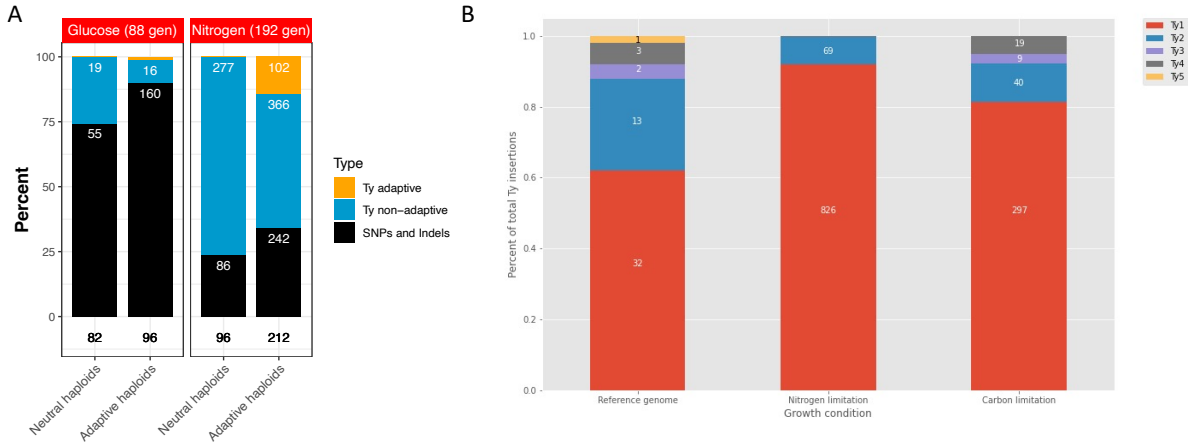


**Figure 5. Ty activity is increased in fluctuating nitrogen-limitation conditions, and gives rise to a significant number of adaptive alleles** (A) Observed mutational types in neutral and adaptive clones isolated under glucose and nitrogen limitation. (B) Ty activity under nitrogen and glucose limited conditions relative to the ancestral strain (reference genome). Stacked bar charts represent the fraction of each Ty type (Ty1-Ty5) present in the reference genome, or of

novel insertions identified in clones evolved in the nitrogen and glucose limited conditions. Numbers on bars represent raw counts of the given Ty type.

**Not all Ty families are equally activated under nitrogen limitation**

To better understand how nitrogen limitation increases transposition, we examined which Ty elements in the ancestral strain were most active under these conditions (Figure 5B). The relative abundance of each Ty class is similar between the ancestral strain and the novel insertions that arose under glucose limitation (Figure 5B; p=.019376, chi-squared test), while the origin of novel insertions under nitrogen limitation is significantly different to the ancestor (p=< 0.00001; chi-squared test), showing an increase in Ty1 activity in particular. Ty1 is the most prevalent class in the ancestral strain (accounting for 32/50 intact Ty insertions). Ty1 accounts for 92% of novel insertions with Ty2 accounting for just the remaining 8% in nitrogen-limited clones, as opposed to the 26% of insertions in the ancestor. Ty1 is also frequently the most activated Ty element in *S. cerevisiae* under other stress conditions (Morillon et al. 2000; Morillon et al. 2002). In the ancestor, Ty3 and Ty4 account for 4% and 6% of insertions respectively, but just 0.1% and 0.2% in nitrogen-limited clones. The single copy of Ty5 in the ancestor is known to be inactive and gives rise to no new insertions under either glucose or nitrogen limited conditions.

**Adaptive Ty insertions enriched in ORF regions**

Ty elements preferentially target gene-poor regions and Ty1 in particular has been observed to most often land upstream of genes transcribed by RNA polymerase III, such as tRNA and snRNA genes (Devine and Boeke 1996). We observed that hundreds of such insertions arose in both nitrogen and glucose limitation and noted a striking periodicity of their insertion locations (Figure S5) suggesting not only tRNA proximity bias, but also preference for nucleosome free regions, consistent with previous observations (Mularoni et al. 2012). A larger proportion of

mutations in neutral haploids were due to Ty element insertions (76%) relative to in adaptive mutants (66%) (Table 1 and Figure 5A); however, the locations of adaptive Ty insertions show striking clusters at some of the nitrogen-adaptive loci (Figures 3 and 5).

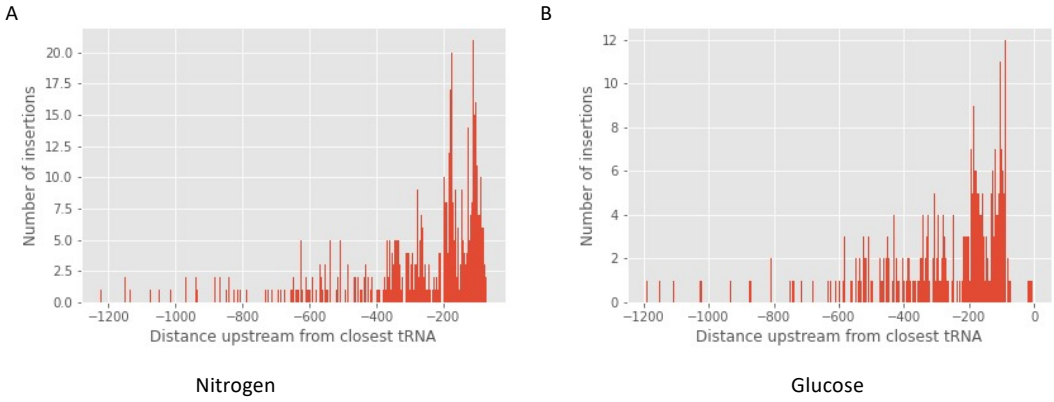


**Figure S5. Insertion locations of Ty elements upstream of tRNA genes in A) nitrogen limitation and B) glucose limitation**

## Discussion

Here we profiled adaptive mutations that arose in nitrogen-limited yeast cultures under conditions fluctuating between low-nitrogen and total nitrogen starvation (Blundell et al. 2019). We observed that frequent Ty transposition and microhomology-mediated recombination events are responsible for a large number of adaptive mutations. This is in contrast to glucose limitation under similarly fluctuating conditions, where self-diploidization and SNPs/short indels gave rise to most adaptive lineages (Levy et al. 2015; Venkataram et al. 2016). Initial adaptive clones arise to high frequency under glucose limitation, showing larger fitness effects than those initially arising under nitrogen limitation (Blundell et al. 2019) – whether this reflects the altered spectrum of mutational types, or is simply a result in the difference of strength of selection and

distance from the fitness optimum is unclear, though these possibilities are not mutually exclusive.

In contrast to our observations in serial transfer experiments under nitrogen limitation, increased Ty activity is not a frequent mechanism of adaptation under nitrogen-limited continuous culture conditions, where cells experience a nutrient poor condition but never experience total starvation (Gresham et al. 2010; Gresham and Hong 2015). Instead, copy number variation of nitrogen transporters, specifically of *GAP1*, most often drive adaptation under continuous low nitrogen (Gresham et al. 2010). This adaptive mechanism bears resemblance to the means of adaptation observed in glucose-limited chemostats, where the copy number of *HXT* hexose transporters is often amplified, facilitating increased glucose uptake (Brown et al. 1998; Gresham et al. 2008; Kao and Sherlock 2008). This suggests that despite different metabolite restrictions, chemostat evolution predominantly favors adaptation via increased abundance of transporters for the limiting nutrient, indicating these mutations arise most often and/or are the most fit. Similarly, in chemostat sulfate limitation amplification of *SUL* transporters is observed to be the most common adaptation (Gresham et al. 2008; Payen et al. 2014; Sanchez et al. 2017). Taken together these results suggest that an adaptive 'winning strategy' under chemostat conditions favors increases in transporter abundance, regardless of the specific nutrient restriction. While these amplifications are not the sole means of adaptation, the prevalence of these amplifications reflects both how easy these changes are to achieve (relative frequency of mutation type) and how beneficial (degree of fitness increase) these changes are specifically in the low, but not zero, nutrient availability conditions of a chemostat. This suggests that under fluctuating conditions that the period during the growth cycle in which additional transporters might be beneficial is insufficient to drive them to high frequency under such conditions – indeed, such amplifications may be deleterious at the beginning of a growth cycle, when the medium is replete with the eventually limiting nutrient (Wenger et al. 2011).

Under serial transfer conditions, cells fluctuate into total starvation for a limiting nutrient. Previous work from our lab and others shows that under fluctuating glucose limitation self-diploidization and SNPs affecting the TOR and Ras pathways arise to high frequency early during such evolutions (Venkataram et al. 2016). These mutations improve fermentation performance and also drive adaptation through decreasing time in lag phase upon reintroduction of fresh glucose (Li et al. 2018). By contrast, here we find that Ty and microhomology facilitated mutation contribute substantially (though not exclusively) to the suite of adaptive mutations observed under nitrogen limited serial transfer. This suggests two things: first that unlike chemostats, where the common 'strategy' for adaptation is frequently transporter copy number amplification, serial transfer does not initially favor a specific class of genomic changes irrespective of the restrictive nutrient. Second, that stably nitrogen poor conditions give rise to different adaptive 'routes' than fluctuating conditions where cells experience total nitrogen starvation. This may be due, at least in part, to some mutagenic mechanisms, such as transposon activity, being more or less active under nitrogen poor vs. starving conditions.

The significant increase of Ty activity under this specific fluctuating nitrogen-starvation condition, relative to glucose limitation and chemostat conditions, raises the question as to what drives this increase, specifically in Ty1 activity. It could be that all Ty1 insertions are derepressed globally within the cell under nitrogen starvation, or that a subset or even a single existing Ty1 insertion becomes active, and alone is driving increased mutagenesis. Investigation of the origin of the new insertions can address these two possibilities. Global derepression could be associated with host cell transcription changes due to stress, accumulation of other mutations (Staleva Staleva and Venkov 2001) or even disruption of the Ty elements themselves, some of which restrict other subclasses of Ty (Czaja et al. 2020). Alternatively, others have shown Ty1 increase in activity is sometimes due to an increase in transcription at

*specific* Ty1 loci, during both severe adenine starvation and when environmental conditions trigger the invasive growth pathways - which includes nitrogen starvation (Company et al. 1988; Laloux et al. 1990; Laloux et al. 1994; Conte et al. 1998; Morillon et al. 2000; Morillon et al. 2002; Lesage and Todeschini 2005; Sacerdot et al. 2005; Todeschini et al. 2005; Rutherford et al. 2008; Servant et al. 2008; Servant et al. 2012). It has also been shown that this transcriptional increase is not equal amongst Ty elements, and that specific transposons were activated more than others. If the observed increase in novel Ty insertions in our results is also due to an increase in Ty transcription from a specific subset of originating loci (possibly the same subset as in (Morillon et al. 2002) it will be interesting to understand the mechanism underlying this insertion-specific activation.

The observed repertoire of beneficial mutations reflects both the underlying rate at which such mutations arise, as well as their resulting fitness. As such, we find it notable that some mutational mechanisms appear to be differentially active under certain environmental conditions, as is the case with Ty activity under fluctuating nitrogen starvation. If some Ty copies are active only under stress conditions such as nitrogen starvation, it could be due to changes in chromatin silencing, or changes in regulatory sequences of that Ty copy. This association of environmental stress with increase in Ty activity could also be a mechanism for stress induced mutagenesis (Galhardo et al. 2007; Ram and Hadany 2014). If cells experience increased mutation rates while under stress conditions, but not under regular growth conditions, this could improve the overall population's chances of survival. Host populations would sample greater genetic and phenotypic variation while experiencing conditions they might not otherwise survive, without the mutational burden during times of high fitness. A Ty insertion that is primarily silenced, but then active during extreme nutrient starvation could provide additional host adaptive potential. Indeed, if Ty activity under nitrogen starvation is a 'happy accident' of a single insertion becoming active, or if global Ty derepression reflects a host-parasite coevolution

that minimizes host cost and maximizes potential for survival of both, the role of transposons in host evolvability is important (Levin and Moran 2011). If some strains are poised to “turn on” mutagenesis under otherwise deadly stress conditions, selection could favor these backgrounds; further evolutionary analysis of Ty1 insertions associated with starvation-induced activity could reveal one facet of selection shaping host-transposon coevolution (Bleykasten-Grosshans et al. 2021).

Alternatively, perhaps rather than being beneficial under stress and the trait being selected for, Ty activity may not have been strongly selected against in starvation conditions, or may be impacted by historical survivor bias. Although competent under lab conditions, Ty elements rarely “hop” (Scholes et al. 2001), so perhaps the correlation of Ty activity to stress response and environmental fluctuations is somewhat analogous to the meiosis-linked transposable element hopping in metazoans, where it is thought to minimize somatic damage and maximize spread in sexual populations (Haig 2016). Perhaps low Ty activity has been selected for during favorable yeast growth conditions, and the relative increase in activity reflects a lack of historical negative selection under nitrogen starvation. If this is the case, however, it would still be interesting to better understand the mechanistic basis of why increased Ty mobilization does occur in total nitrogen starvation and not under low nitrogen conditions. Perhaps exploring the activity of transposons in other yeast species, including those with different lifestyles, such as pathogens, could provide further insight into the complex relationship between host and transposon coevolution (Maxwell 2020).

While Ty activity was notably increased under the evolution conditions we studied here, there were several other mutational mechanisms that were active and gave rise to multiple adaptive mutations. Notably, microhomology-mediated changes gave rise to gene conversion, duplication and deletion events in our evolutions. There are several mechanisms that can result in structural

changes at sites of microhomology including recombination and end joining mechanisms, but also DNA replication based, such as fork stalling and template switching, or microhomology-mediated break-induced replication (Ottaviani et al. 2014). Like Ty activation, these mechanisms may occur more frequently than point mutations in the genome, and because of homologous sequences, can impact some regions of the genome more frequently than others. Homology rich regions could be regions of instability relative to other parts of the genome, shaping host evolvability at those sites in particular, and potentially minimizing deleterious effects elsewhere in the genome.

Several of the adaptive alleles we identified would likely have been missed in a classic loss of function genetic screen, including the Ty and microhomology alleles, but also some SNPs, such as the putative gain of function missense mutations in the *MEP* genes. In other cases, we identified adaptive alleles that we predict are null mutants, and that would be (and in some cases have been) identified in genetic screens. By taking an experimental evolution approach we also learn about the competitive fitness of these alleles – information that is not generated in most genetic screens. This emphasizes both the utility of experimental evolution for understanding the genetics of phenotypes of interest, but also how complementary this approach is to classical genetics.

When exploring a trait of interest, experimental evolution can both expand upon and reinforce lessons learned through other approaches. Natural variation in extant strains and species can help clarify what has happened in naturally evolving populations, but the context of those changes and their fitness consequences may not always be clear. Experimental evolution can leverage large populations and controlled selective pressure to understand how genomes *can* evolve, what mechanisms give rise to novel alleles, and the relative fitness of those alleles. The

alleles sampled will be shaped by how ‘easily’ some mutations are achieved: the frequency is driven by the mutation mechanisms active in those cells, which can be shaped by the cells’ environment, and the ‘best’ alleles will be those that provide the biggest fitness gains under the specific selective pressure. Lower fitness adaptive mutations can be well represented particularly if they arise more readily (such as autodiploids), or if they occur early in an evolution and secondary adaptive mutations were then also acquired. Often in genetic analyses, emphasis is placed on coding SNPs. While this class of mutation undeniably shapes how genomes evolve and organisms adapt, there are many other mutational mechanisms active in driving biological innovation. Studying the full complement of mutational mechanisms, and the spectrum of alleles that they create, provides a deeper understanding of important biological processes.

## Methods

### Isolation and sequencing of evolved clones

Isolation of clones analyzed in this work were described previously (Venkataram et al. 2016; Blundell et al. 2019). Briefly, we isolated evolved clones from Nitrogen- and glucose-limited evolutions from generation 192 and generation 88, respectively, as described (Venkataram et al. 2016; Blundell et al. 2019). Frozen aliquots from the appropriate time point were suspended in water and plated on SD-Ura plates. Single colonies were picked in unbiassed fashion were suspended in 15μl of water and aliquots were saved for barcode PCR, ploidy determination (using a medium throughput benomyl sensitivity test (Venkataram et al. 2016)) and for storage in 25% glycerol at -80°C. Unique haploid isolates with fitness (as estimated from barcode trajectories during the Nitrogen limited evolution experiment) over 0 and self-diploidized isolates with fitness over 0.03, as well as 12 neutral clones were whole genome sequenced as described (Blundell et al. 2019).

**Fitness remeasurement experiments**

The 331 sequenced clones isolated from the Nitrogen-limited evolution were pooled with ~50 strains that were designated as having neutral fitness based on previous experiments. The barcoded pool was then mixed with 90% unbarcoded wild-type (to minimize the change in population mean fitness during the experiment) and propagated via serial transfer for four transfers in either glucose-limited or Nitrogen limited media (as used previously (Levy et al. 2015; Blundell et al. 2019)). Barcodes were sequenced at each transfer, and fitness of clones was estimated from their barcode frequencies as described (Blundell et al. 2019).

**Multiplexed barcode sequencing**

To determine individual barcodes in the selected clones, 5μL of the suspension was lysed in 30μL of lysis buffer (1mg/mL Lyticase (Sigma L4025-250KU), 0.45% Tween20, 0.45% Igepal CA-630, 50mM KCl, 10mM Tris-HCl pH 8.3 (adapted from (Kwiatkowski et al. 1990)). 2.5μL of lysed cells were amplified in a 25μL PCR reaction with 12.5μL of 2xOneTaq mix, 5μM of Forward (F(n) )and Reverse (R(n)) multiplexing primers and water as follows: 94°C x 2', 35 cycles of 94°C x 30", 48°C x 30", 68°C x 30".

52 plates of clones were multiplexed and barcode sequenced in two paired end 300bp MiSeq runs.

**Whole genome sequencing.**

DNA for whole genome sequencing was prepared using YeaStar Genomic DNA kit (ZymoResearch D2002). Sequencing libraries from clones were prepared using the Nextera kit (Illumina FC-121-1031 and FC-121-1012) as described in (Kryazhimskiy et al. 2014), starting with 5-10 ng of genomic DNA. Resulting libraries from each 96-well plate were pooled at an

equal volume. Pooled libraries were analyzed on the Qubit and Bioanalyzer platforms and sequenced paired end 100bp on HiSeq 2000 (one lane per 96 clone pool)

**Variant calling with CLC Genomics Workbench 11**

Short reads from whole genome sequencing were trimmed to remove the adapter sequence and mapped (match score 1, mismatch cost 2, Linear gap cost: insertion 3, deletion 3, length fraction 0.5, similarity fraction 0.8) to *S. cerevisiae* reference genome (R64-2-1_20150113). SNPs and indels were called ignoring broken pairs with 85% minimal frequency in haploids and 35% minimal frequency in diploid strains, with base quality filter 5/20/15, direction frequency filter 5% and significance 1%, and read position filter 1%. Structural variation was called using InDels and Structural Variants identifying novel breakpoints.

**Mutant strain re-barcoding**

Evolved barcoded strains containing mutated alleles of *gat1*, *par32*, *mep1*, *mep2*, *mep3* were crossed to GSY5936-5940 to replace the double barcode with the barcode landing pad able to accept new barcoding. Multiple MATalpha Ura- segregants from the crosses were combined and transformed with pBAR3-L1 to generate double barcodes as described (Levy et al. 2015); multiple transformants were used to collect independently barcoded genotypes subsequently included in fitness remeasurements along with the original evolved strains.

**Fitness re-measurement**

To re-measure fitness, individual barcoded strains were grown in 100µL of YPD in microtiter plates and pooled. Pools were pre-grown in M14 or M3 medium (Levy et al. 2015), mixed with 10 volumes of culture of GSY5929 (wild type strain with digestible barcode) also pre-grown in M14 or M3 medium (time point 0 was taken right after mixing) and then passaged in triplicate through four batch transfers (400ul into 100ml of fresh M14 or M3 medium). 1.5mL of culture

grown for 48 hours (time points 1, 2, 3 and 4) were used for DNA preparation using the YeaStar Genomic DNA kit (ZymoResearch D2002).

75ng of genomic DNA were used for PCR amplification in two 50µl reactions with 25µl 2xQ5 mix (NEB M0492), 2.5µM of each forward (FOS) and reverse (ROS) primers and water (to 50µl) for 22 cycles (care was taken to not overamplify the libraries). After amplification DNA was digested with ApaL1 enzyme overnight, run on a gel to select uncut PCR fragments (approximately 300bp) and purified from the gel using the QIAquick Gel Extraction Kit (Qiagen 28704). Individual libraries were quantified, mixed equimolarly and sequenced in paired end 300bp runs on MiSeq platform. Sequencing results were demultiplexed and resulting barcode frequencies were used to calculate the fitness as in (Blundell et al. 2019).

To measure fitness of the backcrossed multiple individually barcoded derivatives of selected mutations, the strains were pooled (with the original strains that were remeasure above included) and mixed with the GSY145 (wild type strain without an amplifiable barcode). After four batch transfers in M14 medium cells were collected, DNA was prepared and barcodes were amplified as above as, except individual PCR reactions were not digested, but instead were gel size selected in pools of six. Care was taken to not overamplify the libraries adjusting the cycle number for amplification.

**Identification of Ty insertions**

Raw sequence reads were trimmed using Cutadapt, and pairs of reads that shorter than 60bp after trimming were removed. Mitochondrial reads were also removed. McClintock v2.0.0 (Nelson et al. 2017) was run using default parameters. The *S. cerevisiae* reference genome file was downloaded from SGD (Engel et al. 2022). Of the suite of tools available through McClintock we found that RelocaTE2 (Chen et al. 2017) performed best against a subset of manually curated Ty insertions, so RelocaTE2 outputs were used for further analysis. Insertion locations given in the relocate2 output file were then mapped back to the S288C reference

genome to determine what genetic feature the insertion was in (‘gene', 'tRNA_gene', 'snoRNA_gene', 'snRNA_gene', 'long_terminal_repeat', 'transposable_element_gene'). The distance from each insertion location to the closet downstream tRNA was calculated.

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