yEvo: experimental evolution in high school classrooms selects for novel mutations that impact clotrimazole resistance in *Saccharomyces cerevisiae*

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Abstract

Antifungal resistance in pathogenic fungi is a growing global health concern. Nonpathogenic laboratory strains of *Saccharomyces cerevisiae* are an important model for studying mechanisms of antifungal resistance that are relevant to understanding the same processes in pathogenic fungi. We have developed a series of laboratory modules in which high school students used experimental evolution to study antifungal resistance by isolating azole-resistant *S. cerevisiae* mutants and examining the genetic basis of resistance. We have sequenced 99 clones from these experiments and found that all possessed mutations previously shown to impact azole resistance, validating our approach. We additionally found recurrent mutations in an mRNA degradation pathway and an uncharacterized mitochondrial protein (Csf1) that have possible mechanistic connections to azole resistance. The scale of replication in this initiative allowed us to identify candidate epistatic interactions, as evidenced by pairs of mutations that occur in the same clone more frequently than expected by chance (positive epistasis) or less frequently (negative epistasis). We validated one of these pairs, a negative epistatic interaction between gain-of-function mutations in the multidrug resistance transcription factors Pdr1 and Pdr3. This high school–university collaboration can serve as a model for involving members of the broader public in the scientific process to make meaningful discoveries in biomedical research.

Keywords: yeast; azole resistance; experimental evolution; genome sequencing; science education; course-based research experience

Introduction

Azoles are the primary class of antifungals used in medicine and agriculture. Azole resistance in fungi is an emerging problem that is impacting human health and food security (Fisher et al. 2018). Characterizing the genetic basis of azole resistance provides an opportunity to predict whether a newly observed clinical isolate will have resistance to commonly utilized antifungals and may reveal candidates for new treatment paradigms (Cowan et al. 2009; Usher and Haynes 2019; Song et al. 2020).

Like many antifungal drugs, azoles target a component of cell membrane biogenesis. Azoles competitively bind the active site of the enzyme Erg11, preventing a key rate-limiting step in sterol biosynthesis (Veen et al. 2003). Ergosterol in yeasts is functionally equivalent to human cholesterol and the production pathway is highly conserved between humans and yeast (Kachroo et al. 2015). Like cholesterol, ergosterol is a key component of the cell membrane, influencing its fluidity, permeability, and organization (Dufourc 2008; Hannich et al. 2011). Depletion of membrane ergosterol by inhibiting Erg11 leads to the accumulation of toxic intermediates of sterol biosynthesis causing growth arrest, but not cell death (Kelly et al. 1995; Allen et al. 2015).

Due to the widespread use of azoles as therapeutics and prophylactics, azole drug resistance has been studied in detail in various species of fungi for decades. Azole resistance mutations fall into two overarching classes. The first class compensates for Erg11 inhibition through the increased production of the Erg11 enzyme (to overcome the competitive inhibition by azoles) or by alterations to the Erg11 active site (to prevent enzyme inhibition by azoles). This can be accomplished through ERG11 gene duplication, altered activity of regulators of ERG11 expression, or point
mutations in the enzyme itself (Berkow and Lockhart 2017). The second class of mutations, referred to as pleiotropic drug resistance or multidrug resistance mutations (Balzi and Goffeau 1991; Gulshan and Moye-Rowie 2007), lead to increased production or activity of efflux pumps such as Pdr5 (Wolger et al. 2001; Kumari et al. 2021). This can be accomplished through point mutations in the pleiotropic drug response transcription factors Pdr1 (Balzi 1987) and Pdr3 (Delaveau 1994), loss of PDR5 repression, or point mutations in PDR5 itself (Balzi and Goffeau 1991; Gulshan and Moye-Rowie 2007).

Though the budding yeast *Saccharomyces cerevisiae* is an opportunistic pathogen only under rare circumstances (Clemens et al. 1994), it shares drug resistance pathways with pathogenic fungi and has been a useful model for clarifying drug resistance mechanisms beyond the major mutations described above (Paul and Moye-Rowie 2007; Demuyser and Van Dijck 2019).

The majority of genetic studies of azole resistance have utilized traditional mutant selection or classical isolate screening approaches that tend to focus on single, strong-effect mutations. Experimental evolution provides an opportunity for the identification of mutations with a small or background-dependent effect. Previous evolution experiments selecting for azole resistance have demonstrated that this paradigm can identify new resistance factors and can validate candidate secondary antifungals that prevent resistance evolution (e.g. Cowen et al. 2000; Anderson et al. 2003; Selmecki et al. 2009; Hill et al. 2013; Boyer et al. 2021; Carolus 2021; Ksiezopolska 2021; Ottilie 2022).

We anticipated that additional replicates would lead to the identification of novel resistance factors.

The wealth of information available on azole resistance mechanisms and the possibility of identifying new resistance factors make this system particularly attractive for incorporating authentic research into a high school classroom. To leverage this, we developed protocols suitable for the experimental evolution of *S. cerevisiae* to select for azole resistance in high school classrooms as a course-based research experience called yEvo (available at yevo.org; Taylor 2022). These experiments utilized the drug clotrimazole, which can be found in over-the-counter antifungal treatments. We developed partnerships with teachers at two high schools to ensure these protocols were compatible with their classrooms and learning objectives. The laboratory activities explicitly connect evolution to underlying molecular biology in an open-ended inquiry framework and provide a powerful demonstration of how evolution occurs at the molecular level. Connecting these topics is a key goal of the Next Generation Science Standards (NGSS Lead States 2013), a current benchmark for K-12 science education in the United States.

Our pedagogical aims and evaluation will be reported in detail elsewhere (Taylor 2022). In this paper, we characterize 99 evolved clones from these experiments (performed by 203 students) using phenotyping assays and whole-genome sequencing. We were able to isolate clotrimazole-resistant clones as early as two weeks into the evolution protocol, though the majority of experiments reported here were continued for longer, and in some cases for an entire school year (30–34 weeks), which allowed time for multiple mutations to arise in most clones. Evolved clones were enriched for mutations impacting known azole resistance factors, as well as in genes that had not previously been associated with azole resistance. We provide evidence that these mutations impact azole resistance, expanding our understanding of the genetics of this important trait. We further show that epistatic interactions between evolved mutations can impact the evolution of resistance.

## Materials and methods

### Yeast strains

Evolution experiments were performed with lab-derived strains of *Saccharomyces cerevisiae*. Haploid replicates utilized the MATa S288C derivative BY4741 and the MATu S288C derivative BY4742. Five replicates used a diploid S288C derived by mating BY4741 and BY4742. All strain genotypes are listed in Supplementary Table 1 in Supplementary File 1. Strains used in evolution experiments carried a 2 μm plasmid with KanMX, which provides resistance to the general antibiotic G418, and a pigmentation pathway that gives each strain a unique color (Supplementary Table 2 in Supplementary File 1; courtesy of the Boeke lab at New York University). G418 was added to the media (200 mg/L) to select for maintenance of these plasmids and reduce the risk of contamination.

### Classroom protocols

#### Westridge School evolution protocol

Evolution experiments were carried out via batch transfer. Every 2–4 days yeast were transferred on a sterile cotton swab from a saturated culture to a tube of fresh media containing clotrimazole. Clotrimazole was obtained from an over-the-counter 1% solution dissolved in 70% isopropanol. Clotrimazole was added directly to culture tubes in the desired concentration prior to the introduction of yeast. Cultures were maintained in 5 mL of YPD medium with 200 mg/mL G418 and clotrimazole without aeration. Most were grown at room temperature, though some were grown at 30°C. Student groups maintained three replicates of an assigned strain throughout their AP Biology course (grades 11–12; their second biology course). Yeasts were initially exposed to a low dose of clotrimazole (2.25 μM), which slowed but did not prevent ancestor growth. The dose of clotrimazole was increased at student discretion at 2x intervals (e.g. 4.5, 9, 18 μM, etc.). Cultures were maintained for up to 34 weeks.

#### Moscow high school evolution protocol

Evolution experiments were carried out via batch transfer. Every seven days, yeast were transferred on a sterile cotton swab from a saturated culture to a tube of fresh media containing clotrimazole. Clotrimazole was obtained from an over-the-counter 1% solution dissolved in 70% isopropanol. Cultures were maintained in 5 mL of YPD medium with G418 (200 mg/L) and clotrimazole at room temperature without aeration. Student groups maintained one replicate of an assigned strain throughout their Honors Biology course (grade 10; first biology course). Yeasts were initially exposed to a much higher dose of clotrimazole (10 μM), which prevented visible growth of the ancestor over short time scales (Fig. 1b), but permitted growth over the extended time between transfers. The dose of clotrimazole was increased at student discretion at 2x intervals (e.g. 20, 40, 80 μM, etc.). Cultures were maintained for up to 12 weeks.

#### Strain storage

Freezer stocks of student populations at Westridge School were generated weekly by adding a 1:1 mixture of 50% glycerol solution in a cryovac vial and stored in a nonfrost-free −20°C freezer until transfer to −80°C at the University of Washington. Populations from Moscow High School were stored at 4°C until the end of the evolution protocol. Researchers at the University of Idaho isolated clones from these populations by a streak purification method and stored them at −80°C in YPD with 25% glycerol added.
University lab protocols

Phenotyping of evolved clones

Clones from Westridge School were isolated and colony purified from each experiment at two timepoints (early and final, approximately seven and 30–34 weeks, respectively) and at weekly intervals from Moscow High School experiments. One clone was chosen from each student group for whole-genome sequencing. These were assayed to determine their maximum tolerated concentration of clotrimazole as follows: clones were grown for 48 h at 30°C in 200 μL of YPD + G418 medium in a 96-well plate without aeration. Cultures were resuspended, and 2 μL of each culture was transferred to a new 96-well plate containing YPD medium with 200 μg/mL G418 and 2.25, 9, 18, 36, or 144 μM clotrimazole (which correspond to 0.00078, 0.0031, 0.0062, 0.012, or 0.050 mg/L, respectively) and monitored for growth over two days. To assay petite status, clones were additionally transferred to YPG (glycerol as carbon source) + G418 medium and monitored for growth over three days.

Whole-genome sequencing

Whole-genome sequencing libraries were generated using a modified version of the Illumina Nextera protocol based on (Baym et al. 2015). Briefly, genomic DNA is fragmented using an Illumina Tagmentation enzyme that adds Illumina adaptor sequences to ends. “Tagmented” samples are then PCR amplified using oligonucleotide primers that add unique barcodes to each sample so that samples can be multiplexed on a single Illumina sequencing run. Coverage of all clones described can be found in Supplementary Table 5 in Supplementary File 1. Sequencing reads are deposited in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA742704.

Mutation calling

Briefly, reads were aligned to an S288C reference genome using Burrows–Wheeler Aligner (BWA; Li and Durbin 2009) and mutations were called with Samtools. Mutations within 200 bases of a start codon were listed as putative promoter mutations. Single nucleotide polymorphisms with a quality score lower than 100 (out of 228) were discarded. All mutations with a quality score less than 200, as well as all indels, were manually inspected in the Integrative Genomics Viewer (IGV). This was to ensure variants were present in a majority of reads from each mutant and were not present in reads from the respective ancestor. Common mutation targets were manually inspected in IGV to look for evidence of mutations that were missed by our mutation calling pipeline. We identified one such instance in the gene PDR1 in strain: Westridge_C_final_1_2017-2018.

Copy number variants

The average genome-wide coverage was determined in 1,000-bp windows using the program IGVtools and plotted in R. Continuous regions with coverage that exceeded the genome-wide average by ≥2× in haploids were considered increased copy number variants (CNVs). Regions that deviated from the genome-wide average by 0.5× in diploids were considered decreased CNVs. Regions that deviated from the genome-wide average by 1.5× in diploids were considered increased CNVs.

Transposable element calling

Clone sequencing data were examined for evidence of mobilization events of transposable elements vs the reference genome using the McClintock pipeline (Nelson et al. 2017). Comparisons were made between the ancestral strain and the clones to find exclusively de novo events within our experiments that were at least 1,000-bp away from existing transposon insertions with the software Bedtools. Subsequently, the transposition events were manually inspected in IGV to look for evidence of mutations that were missed by our mutation calling pipeline. One mobilization in the gene PDR3 was confirmed by PCR and Sanger sequencing using primers PDR3_genotype_F and PDR3_genotype_R (Supplementary Table 3 in Supplementary File 1).

Lineage determination

Clones from the same group that shared at least one point mutation were considered as originating from the same lineage. Shared mutations are denoted as nonindependent in Supplementary Table 6 in Supplementary File 1. Mutations that were shared by multiple members of a lineage were considered as single mutation events for the purpose of identifying recurrently mutated genes.

CRISPR/Cas9 allele replacements

A PAM (polyspacer adjacent motif) near CSF1_A2913P was targeted for cutting by the Cas9 enzyme. Oligonucleotides for guide RNA design and repair donors can be found in Supplementary Table 3 in Supplementary File 1. Guide RNA oligonucleotides were introduced into pML104 backbone by Gibson assembly (Supplementary...
Table 2 in Supplementary File 1, Laughery et al. 2015). A mutation substituting G to C was introduced to recreate the CSF1<sub>A2913P</sub> allele detected in one evolved clone. To prevent recutting, a synonymous mutation that altered the PAM was introduced in codon A2913 on its own (control) or in combination with the A2913P mutation. A stationary yeast culture of MA7a haploid S288C (Supplementary Table 1 in Supplementary File 1) was transformed with 100 ng Cas9 and gRNA expression vector (Supplementary Table 2 in Supplementary File 1) and 1 µg donor DNA with the lithium acetate protocol. The genotype of transformed clones at all sites was determined by Sanger sequencing with primers CSF1_genotype<sub>F</sub> + CSF1_genotype<sub>R</sub> (Supplementary Table 3 in Supplementary File 1).

**CSF1 allele replacement competitions**

Stationary phase cultures of wild-type, a synonymous mutant, and a nonsynonymous mutant were mixed in equal proportions. Fifty microliters of this mixture was inoculated into 5 mL of either YPD or YPD plus 9 µM clotrimazole. These cultures were grown at 30°C in a roller drum until they reached stationary phase after ~2 days. Fifty microliters of stationary culture was transferred to 5 mL of respective media. This backdiluting was performed twice for a total of three outgrowths. The frequency of CSF1<sub>A2913P</sub> was determined at initial and final time points by Sanger sequencing. The frequency of CSF1<sub>A2913P</sub> allele at each sequenced time point was determined with the program QSVAnalyzer (Carr et al. 2009). Frequencies depicted are averages of three replicates. Error bars are one standard deviation in each direction (Supplementary Table 1 in Supplementary File 1).

**Tetrad dissections**

Diploids were grown overnight in 5 mL YPD in a roller drum at 30°C. One milliliter of stationary-phase culture was pelleted and resuspended in 5 mL sporulation medium as in (Dunham et al. 2015). These sporulation cultures were grown on a roller drum at room temperature. After 3–5 days, 50-µL culture was pelleted and resuspended in 15 µL Yeast Lytic Enzyme (YLE) for 22 min at 30°C. Five microliters of water were slowly added to this mixture to digest cells. Fifty microliters of digested spore mixture were dripped down a YPD agar plate and allowed to dry. Tetrad s were dissected with a micromanipulator microscope.

**Restriction enzyme genotyping**

Cleaved Amplified Polymorphic Sequence (CAPS) markers were identified through manual examination of mutant and wild-type sequences in SnapGene. Polymorphisms were amplified with Phusion polymerase with kit protocol. Primer sequences can be found in Supplementary Table 3 in Supplementary File 1. PCR product (8.5 µL) was mixed with 1 µL 10× cutsmart buffer, 0.25 µL enzyme, 0.25 µL water, and digested for 1 h at 37°C. Restriction enzymes Rsal, MboI, HaellI, BplI, KpnI, and BamHI were obtained from New England Biolabs.

**Generation of ERG25<sub>T202P</sub>/ATP2<sub>D382H</sub> and ERG25<sub>T202P</sub>/ROX1<sub>S46A</sub> strains**

Evolved clones Westridge_W_final_3_2018-2019 (ERG25<sub>T202P</sub> ATP2<sub>D382H</sub> PDR1<sub>I1042E</sub> HAP1<sub>C84F</sub> TA03<sub>A2283P</sub>) and Westridge_W_early_1_2018-2019 (ERG25<sub>T202P</sub> ROX1<sub>S46A</sub> PDR1<sub>1G1042S</sub>) were backcrossed again to YMD895 to remove all unwanted mutations and produce combinations of desired mutations. Spores were genotyped by restriction enzyme digestion or Sanger sequencing by Genewiz/Azenta (Supplementary Table 4 in Supplementary File 1). Clones from Westridge School tended to have more mutations than clones from Moscow High School, and clones from later timepoints tended to have more mutations than earlier timepoints (Fig. 1c). Based on these data, we hereafter refer to these clones as resistant to azoles.

**Whole-genome sequencing of evolved clones identified de novo mutations connected toazole resistance**

The genome sequence of each confirmed drug-resistant clone was determined using short-read next-generation sequencing.

**Plate reader experiments**

Genotyped spores were grown on 200 µL YPD medium in 96-well plates for 48 h. Cultures were resuspended and 2 µL of each culture was transferred to 198 µL YPD media with clotrimazole added. The growth of these cultures was monitored in a plate reader for 36–48 h at 30°C with orbital shaking. The average growth rate of all strains with the same genotype was calculated by linear fit to logarithmic growth phase and plotted in R; script is available on GitHub (github.com/reneegeck/DunhamLab/blob/main/plate reader growthplotter.R).

**Results**

Isolation of clotrimazole-resistant clones from a course-based research module

We implemented an experimental evolution lab module at two high schools in the United States. Protocols for these experiments were developed in close collaboration with each teacher to best align with their time, resources, and learning objectives (Fig. 1a; Taylor 2022). Students used a simple serial transfer protocol to evolve yeast populations to grow in inhibitory concentrations of azole. In this way, students were able to compare the growth rates of different yeast strains in the presence and absence of clotrimazole. The evolved yeast populations were collected by partnering research laboratories at the University of Washington and the University of Idaho (Materials and Methods).

Clones were isolated from evolved populations at early time points (7 weeks at Westridge School and 2–12 weeks at Moscow High School), as well as from later time points (30–34 weeks at Westridge School). These evolved clones were capable of growing in higher concentrations of clotrimazole than the unevolved ancestors, which had a maximum tolerated dose of 2.25 µM (Fig. 1b). Clones from later timepoints were able to grow in high concentrations of azole with 53 of 57 late timepoint clones able to tolerate 144 µM clotrimazole, compared to 14 of 42 early timepoint clones (Fig. 1b; Supplementary Table 4 in Supplementary File 1). Clones from Westridge School tended to have more mutations than clones from Moscow High School, and clones from later timepoints tended to have more mutations than earlier timepoints (Fig. 1c). Based on these data, we hereafter refer to these clones as resistant to azoles.
The genome sequences of the azole-resistant clones and their ancestors were aligned to the *S. cerevisiae* reference genome to identify sequence differences unique to evolved clones (*Materials and Methods*). We looked for point mutations (Supplementary Table 6 in Supplementary File 1; Table 1), loss of mitochondrial DNA (ρ0 petite mutations) (Supplementary Table 8 in Supplementary File 1), copy number changes (Supplementary Table 9 in Supplementary File 1; Fig. 2), and transposable element mobilizations (Supplementary Table 10 in Supplementary File 1; Supplementary File 1).

As a quality check on both the evolution procedure and the sequencing analysis, we first looked in our list of genes found to have repeatedly independent mutations for genes known to confer azole resistance (Table 1; Supplementary Fig. 1, b and c). We found every sequenced clotrimazole-resistant clone had at least one of the following: (1) a nonsynonymous mutation in PDR1 or PDR3, known transcriptional regulators of the pleiotropic drug response; (2) a loss of the mitochondrial genome leading to a petite phenotype, which has also been previously associated with azole resistance; and/or (3) a DNA copy number increase involving ERG11, the target of azoles (see below). We then expanded our analysis to more completely catalog all mutations in the evolved clones.

### CNVs were common and may impact azole resistance factors

The majority of clones (61 of 99) had at least one CNV. The most common CNVs involved chromosomes I (7 clones), II (4 clones), IV (4 clones), VIII (49 clones), and XV (11 clones) and were most often full chromosome amplifications or segmental duplications (Fig. 2; Supplementary Table 9 in Supplementary File 1). In addition, two of the five sequenced diploid clones had decreased coverage of chromosome I, indicating a loss of one copy of that chromosome. ERG11 is on *S. cerevisiae* chromosome VIII, the most frequently amplified chromosome across sequenced clones, making this gene an attractive candidate for providing the observed fitness benefit. This result is supported by the fact that all of our segmental duplications include ERG11. Candidate resistance genes are included within all segmental duplications in Fig. 2 and Supplementary Table 9 in Supplementary File 1.
Loss of mitochondrial genome was common in evolved clones

The loss of the mitochondrial genome (aka petite or \( \rho^0 \) status) occurred in 73% (72 of 99) of drug-resistant clones. These observations were made based on an examination of sequence alignments to the reference mitochondrial genome. Petite mutants have been shown to have an increased resistance to azole drugs (Hallstrom and Moye-Rowley 2000; Ferrari et al. 2011), with the tradeoff that they are unable to grow using nonfermentable carbon sources. To confirm that these were petite mutants, all clones were cultured using a medium containing glycerol, a nonfermentable carbon source (Supplementary Table 8 in Supplementary File 1). We identified one clone (Westridge_M_final_1_2018-2019) with a wild-type mitochondrial genome that did not grow in glycerol medium, indicating it had lost respiratory activity due to a nuclear mutation. This clone possessed unique nonsynonymous mutations in the genes ERG25, TDA9, and VPS501, as well as a noncoding mutation 276 bases upstream of the gene SLP1. None of these genes have previously been associated with respiratory deficiency, though TDA9 regulates acetate production and may be an appealing candidate (see Discussion).

Transposable element mobilizations biased toward azole resistance factors

We identified only 14 transposable element mobilizations across all 99 sequenced clones. Only one gene had multiple (four) independent mobilization events (all in independent clones) that interrupted its sequence: SUR1, an enzyme involved in sphingolipid production, which impacts membrane composition, a known mechanism that influencesazole resistance (François et al. 2009). Another mobilization event was detected in the 3' end of PDR3, which encodes a transcription factor regulating the pleiotropic drug response. Both of these genes were found to harbor point mutations in other evolved clones (see below).

Point mutations in well-characterized azole resistance factors

One goal of the evolution experiments was to isolate clones with multiple mutations that could impact azole resistance. Clones from Moscow High School had an average of 4.8 point mutations, Westridge School early clones had 5.0, and late clones had 11.6. One clone, from a late timepoint at Westridge School had a particularly high number of mutations (48, compared to the next highest with 23). Since clones from Westridge School were isolated at multiple time points, some clones shared mutations. We have denoted shared mutations as nonindependent events, which can be found in Supplementary Table 6 in Supplementary File 1.

In total, 575 unique point mutations were detected across all 99 clotrimazole resistant clones. Of these, 466 were nonsynonymous, indel, or nonsense mutations. A GO term enrichment analysis on this subset (using the GO term finder tool at https://yeastgenome.org/goTermFinder last accessed April 12 2021) found clusters of genes related to drug binding, DNA binding, and regulation of metabolic processes, but with diverse cellular functions (Supplementary Table 7 in Supplementary File 1). Even among these nonsynonymous mutations, we anticipate that a nontrivial fraction will be neutral. In other experimental evolution contexts only 20% (Buskirk et al. 2017) to 35% (Payen et al. 2016) of all mutations found in evolved clones were estimated to be beneficial. To gain further insight into mechanisms of resistance, attention was focused on genes with three or more independent nonsynonymous or nonsense point mutations, since these are more likely to be causative as opposed to passenger mutations, which should be more randomly distributed (Table 1). Mutations in this subset of genes accounted for 33.9% of all detected mutations. Many of these genes (11/16) or their associated pathways (13/16) have well-characterized connections toazole resistance, which we discuss below and summarize in Table 1 and Supplementary Fig. 1c.

Half of the azole-resistant clones (49 of 99) possessed missense mutations in ERG25. None of the mutations in ERG25 in the experiment were clear null alleles (nonsense or frameshift), as expected since ERG25 is essential unless media is supplemented with ergosterol. We also identified mutations in, and copy number increases centered around, UPC2 and PDR5 (Fig. 2; Supplementary Table 9 in Supplementary File 1). UPC2 encodes a master regulator of ergosterol synthesis and PDR5 encodes a major drug efflux pump regulated by Pdr1 and Pdr3. Gain-of-function mutations in both these genes have been shown to impact azole resistance (Flowers et al. 2012, UPC2; The Uniprot Consortium 2021, PDR5).

We also detected mutations in genes that impact sphingolipid production such as SRO1, CSG2, and SIT4 (Baudry et al. 2001; Gulshan and Moye-Rowley 2007; François et al. 2009), in addition to the four transposable element mobilizations interrupting SUR1 (Supplementary Table 10 in Supplementary File 1). Sphingolipids interact with ergosterol to produce lipid rafts and perturbation of sphingolipid levels is thought to impact azole accumulation (François et al. 2009). SIT4 has been shown to impact the expression of the multidrug resistance pathway as well (Miranda et al. 2010).

We detected six independent point mutations in TAO3, which encodes a component of the conserved RAM kinase signaling network (Nelson et al. 2003). In addition, we detected three independent mutations in RAM network genes HYM1 and CBK1 and two
Each in KCl and SOG2. To our knowledge, none of these genes have a reported azole phenotype in S. cerevisiae (SGD, accessed 2019 August 30). However, zinc finger transcription factor Ace2, which is regulated by the RAM network (specifically by phosphorylation by Cbk1), has been implicated in both increased azole susceptibility (miconazole) andazole resistance (fluconazole; Kapitzy et al. 2010; Vandenbosch et al. 2013). In S. cerevisiae, null mutations of ACE2 confer an increasedazole resistance when yeast are grown on agar media (Kapitzy et al. 2010), and a decreased resistance when grown as biofilms (Vandenbosch et al. 2013). Also, deletion of MOB2 of the RAM network has been shown to cause increased susceptibility to conazoles (Guan et al. 2020).

Evolved clones were enriched for mutations in the heme-regulated transcription factor genes HAP1 and ROX1. These genes are regulated by oxygen (Kwaast et al. 1998) and in turn, regulate a variety of cellular processes including expression of genes involved in ergosterol synthesis (Serratore et al. 2018). The lab strain we utilized for our evolution experiments (S288C) has a transposable element insertion near the 3’ end of HAP1 that reduces the functionality of the Hap1 protein (Gaisne et al. 1999). Nine of the HAP1 mutations we detected are frameshift and 16 are nonsense, indicating that further loss-of-function leads to the resistance phenotype. Two of the six detected ROX1 mutations are an early stop and a single-base deletion leading to a frameshift (Y204* and I39indel), suggesting that these are null mutations. Indeed, deletions of ROX1 have been shown to increaseazole resistance (Henry et al. 2002).

We detected four unique missense mutations in each of the genes ATP1 and ATP2 (Table 1), which function in the mitochondrial F1F0 ATP synthase (Saltzgaber-Muller et al. 1983; Takeda et al. 1986). This complex plays an important role in cellular respiration by synthesizing ATP from the electrochemical gradient generated by the electron transport chain (Nilsson and Nielsen 2016), so it is possible that these mutations impact metabolism in a way that is similar to or synergistic with petite status. Indeed, deletions of MOB2 of the RAM network has been shown to cause increased susceptibility to conazoles (Guan et al. 2020).

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Evidence of epistatic interactions between adaptive mutations

The large number of replicates available allowed us to look for patterns of mutation exclusivity and co-occurrence in our evolved clones. These instances can be due to epistatic interactions, which often indicate a functional connection between genes (Lehner 2011; Costanzo et al. 2019).
Almost all (91 of 99) clones possessed a mutation in either PDR1 or PDR3, which encode paralogous transcription factors that activate the pleiotropic drug response. We found mutations in these genes in both haploids and diploids, suggesting that they are gain of function (Balzi and Goffeau 1991).

Despite the prevalence of these mutations and the length of our selection protocol, no evolved clone possessed mutations in both PDR1 and PDR3. This may indicate that once a gain-of-function mutation has occurred in one of these paralogs, there is no benefit (or even a negative consequence) to having a second in the context of these experiments. To test this hypothesis, we designed a crossing scheme to generate recombinant progeny in which mutations in both PDR1 and PDR3 segregated. To aid in this effort, we examined our list of mutants for clones with (1) opposite mating types, (2) minimal other mutations, and (3) PDR1 and PDR3 mutations that could be genotyped by restriction enzyme digestion. Clones Westridge_T_early_1_2017-2018 (PDR3T949A) and Westridge_S_early_1_2017-2018 (PDR1F749I and HBT1T202I) fit these characteristics. These clones were mated to form a diploid, sporulated, and 16 tetrads were dissected; all segregants were then genotyped and monitored for growth in the presence of 0, 9, and 18 μM clotrimazole (Materials and Methods; Fig. 4 and Supplementary Fig. 2). Strains with mutations in both PDR1 and PDR3 showed very similar growth rates to strains with a mutation in only one, suggesting that a second mutation does not increase or decrease fitness in the presence (or absence) of clotrimazole. This result may explain the absence of double mutants among sequenced clones.

We also observed that mutations in HAP1, ROX1, ATP1, and ATP2 co-occurred with mutations in ERG25 (Fig. 5a). Specifically, ERG25 mutants seemed more likely to have secondary mutations in either HAP1 or ROX1 and either ATP1 or ATP2. Though approximately half of the sequenced clones have a mutation in ERG25 (49 of 99), only five mutations in HAP1, ROX1, ATP1, and ATP2 occurred in an ERG25 wild-type background, compared to 35 that had a mutation in ERG25. In the presence of clotrimazole, ERG25 mutations generally occurred by early time points, followed by mutations in HAP1, ROX1, ATP1, or ATP2 which were primarily identified at late time points. This ordering is supported by the higher prevalence of clones with mutations in only ERG25, and that clones from lineages with the same ERG25 mutation have different mutations in HAP1, ROX1, ATP1, or ATP2 (Fig. 5b). To experimentally test the hypothesis that these combinations are evidence of epistatic interactions, we backcrossed these mutants to a wild-type ancestor to generate panels of mutant strains that possessed combinations of mutations (Materials and Methods). We grew these strains in media containing clotrimazole and monitored their growth to calculate a doubling time (Fig. 5, d and e; Supplementary Fig. 3; Materials and Methods). Strains with mutations in ERG25 doubled faster than wild-type strains at a low dose of clotrimazole, but not significantly so at a higher dose. Surprisingly, ATP2 and ROX1 single mutants grew more slowly than any other mutant class or the wild-type strains. In addition, mutations in ATP2 or ROX1 in combination with ERG25 mutations did not have a large effect on doubling time compared to ERG25 single mutants. However, ATP2 in combination with ERG25 mutation led to a significantly faster doubling time at a low dose of clotrimazole. Taken together, these results indicate that mutations in ATP2 and ROX1 do not provide a direct benefit toward doubling time at the tested concentrations of clotrimazole, and may be selected for a benefit that we did not measure.

Discussion

In this article, we demonstrated how course-based research experiments with high school students can yield insights into mechanisms of azole resistance. In addition to furthering our science research goals, student involvement advanced their understanding of how genotype and phenotype intersect, which is critical to understanding the process of evolution (NRC 2012; NGSS Lead States 2013). We expand on our pedagogical goals and outcomes from this project in (Taylor 2022).

Laboratory evolution experiments provide time for multiple mutations to occur in a single lineage. This may allow for mutations with smaller or epistatic effects to occur. As evidence of these advantages, all of the novel CSF1 mutations we identified were found in clones isolated from later time points, as well as much of the evidence implicating P-body mutations and candidate epistatic interactions. Together, our findings underscore the utility of long-term selection to isolate diverse mutations that
can impact azole resistance. Increasing the replication of these experiments can improve their power to detect novel adaptive mutations and candidate epistatic interactions, making them ideal for large-scale replication in a classroom setting.

Concordance with pathogenic isolate sequencing and prior genetic screens

Many of the mutations we identified are in-line with findings from sequencing of drug-resistant pathogenic species of yeast isolated in clinical or agricultural settings (e.g. Ford et al. 2015; Paul and Moye-Rowley 2014). Like the clones from our experiments, drug-resistant fungi frequently possess gain-of-function mutations in orthologs of PDR1 and PDR3, copy number variations that lead to increased copy number of the ERG11 gene, and/or mutations in regulators of ergosterol production such as UPC2. CNVs akin to what we observed in S. cerevisiae have been shown to impact azole resistance in experimental evolution and clinical isolates of the pathogenic yeast Candida albicans (Selmecki et al. 2006, 2008, 2009). For instance, aneuploidy has been shown to impact azole resistance in C. albicans due to increased copy number of the genes ERG11 and TAC1. Since azoles inhibit the function of ERG11, additional copies of this gene may lead to increased expression and thus compensate for the drug’s impact. Clinical and environmental isolates of pathogenic fungi frequently have point mutations in ERG11 (Fisher et al. 2018) that alter the interaction of the enzyme’s active site with azoles, which we did not detect in our experiments.

Surprisingly, we have not observed mutations in ERG3, which are frequently identified in pathogenic yeasts with azole resistance. Mutations in ERG3 are thought to prevent the accumulation of the fungistatic azole intermediate 14 alpha methylergosta 8-24 (28) dienol, produced as the result of Erg11 inhibition by

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**Fig. 5.** Signatures of epistasis involving ERG25. a) Number of clones with genotypes related to the genes ERG25, HAP1, ROX1, ATP1, and ATP2. Frequency is biased toward double mutant groupings in the bottom right of each square (P-values by t-test listed below). Clones were sequenced at multiple timepoints; therefore, not all clones represent independent mutation events (Materials and Methods). b) Lineages within individual replicates were identified by shared mutations in clones from that replicate. Several lineages are made up of clones with ERG25 mutations that later acquire mutations in HAP1, ROX1, ATP1, or ATP2, as evidenced by multiple individuals with the same ERG25 mutation but different mutations in HAP1, ROX1, ATP1, or ATP2. No lineages were detected in which a HAP1, ROX1, ATP1, or ATP2 mutant later acquired an ERG25 mutation. c) Blue lines between ROX1-HAP1 and ATP1-ATP2 are known mechanistic relationships (Hap1 regulates Rox1; Atp1 and Atp2 are in the same complex). Orange lines between HAP1-ATP1 and HAP1-ATP2 are previously identified genetic interactions (van Leeuwen et al. 2016). The six yellow dashed lines are putative novel genetic interactions supported by presented data. d) Doubling time of segregants from backcrossing of strain containing mutations in ERG25 and ATP2 or e) ERG25 and ROX1 (Materials and Methods) grown in a 96-well plate in 4.5 or 9 mM clotrimazole media at 30°C. Doubling time calculated by linear fit of logarithmic growth phase (Supplementary Fig. S3, Materials and Methods). P-value by t-test, *P < 0.05; **P < 0.01; ***P < 0.001.
azoles. Instead, we saw mutations in the enzyme Erg25, which is involved downstream of Erg11 in ergosterol biosynthesis. Mutations in Erg25 may act to decrease activity of the enzyme in some way, as decreased expression has been shown to increase azole resistance (Smith et al. 2016). Loss of Erg11 and Erg25 function is thought to enable the incorporation of lanosterol as an alternative to ergosterol in the fungal membrane. Similar to Erg3, loss of Erg25 function has been shown to suppress toxicity associated with null mutations in Erg11 or the inhibition of Erg11 by azoles. Importantly, it was shown that reduced heme biosynthesis suppressed this toxicity (Gachotte et al. 1997), which is consistent with the appearance of nonfunctional alleles of HAPI and ROX1 that encode regulators of heme biosynthesis.

Another class of known pathogenic mutations that are common among our clones are respiratory deficiencies. Petite mutants of Candida glabrata have been isolated in clinical settings (Bouchara et al. 2000) and may have increased in vivo virulence (Ferrari et al. 2011). These mutants are unable to undergo cellular respiration due to mutations that impact mitochondrial function, which has been shown to increase the activity of Pdr1 and Pdr3 through an as yet unknown post-translational mechanism [Traven et al. 2001; possible mechanisms in Shahi et al. (2007) and (2010)]. These mutants are thus unable to grow on nonfermentable carbon sources. $p^0$ (loss of mitochondrial genome) petite mutants were common across the clones we sequenced (72 of 99 clones). The one petite clone from our experiments that still possessed its wild-type mitochondrial genome possessed several mutations, including a nonsynonymous mutation in the transcription factor TDA9. TDA9 regulates acetate production, and TDA9 null mutants produce less acetate acid during wine fermentation (Walkey et al. 2012). TDA9 has a paralog, RSF2, which encodes a gene that regulates growth in glycerol-containing medium (Lu et al. 2005). These details make it an attractive candidate for causing the observed petite phenotype.

The relevance of RAM mutations in azole resistance

We additionally observed recurrent mutations in genes related to the RAM signaling network. The RAM network is required for the control of the localization of the transcription factor Ace2 by phosphorylation and also has an important role in polarized growth (Nelson et al. 2005). Disruption of RAM network genes results in growth defects and aberrant cell morphologies, as well as sensitivity to cell wall and membrane damaging agents due to changes in cell wall and membrane organization and composition (Nelson et al. 2003). Null mutations in proteins of the RAM network cause severe growth defects and lethality in S. cerevisiae that is dependent on the genetic background (Xu et al. 2019). Literature from other species of yeast indicates that RAM network component knockouts can impact azole resistance in a species or growth phase-dependent manner (Mulhern et al. 2006; Walton et al. 2006; Song et al. 2008; Homann et al. 2009; Saputo et al. 2012). Surprisingly, in C. albicans, null mutations in the RAM network are viable and result in the downregulation of ERG genes and hypersusceptibility to azoles (Song et al. 2008). All of the RAM network mutations selected by our experiments are either missense or nonsense. The RAM network premature stop codons are all close to the C-terminal end of each protein resulting in truncations that remove less than 10% of the protein (two of six mutations in TAO3; one of three in HVM1; one of two in SOG2). The lack of obvious null mutations suggests that these mutations allow some degree of RAM network functionality. However, we cannot completely rule out secondary site suppressors of lethality, as the RAM mutants appeared late in the evolution experiments in the presence of other mutations. In CBK1 most missense mutations were identified outside of the conserved AGC kinase domain and known phosphorylation sites (Bidlingmaier et al. 2001) and TAO3 mutations are found at either the far C-terminus (A2283P) or at sites 228 or 229. In both CBK1 and TAO3 all mutations overlap with the interaction interfaces that mediate Cbk1 and Tao3 interaction, and C-terminal truncations of Tao3 would also remove a Cbk1-binding site (Nelson et al. 2003). This could indicate that evolved mutations are potentially altering the interaction between these RAM network proteins, but how this alteration of RAM network signaling is impacting azole resistance remains to be explored.

Connection between P-bodies and azole resistance

Three recurrently mutated genes function in an mRNA degradation pathway related to P-bodies. P-bodies have not explicitly been implicated in azole resistance. However, two of our candidate genes (XRN1 and DHH1) have been previously shown to impact azole resistance in separate genome-wide deletion collection screens, and a genome-wide mutagenesis study (Gao et al. 2018) found an enrichment for the P-body GO term. It is unclear how these mutations would impact azole resistance at a mechanistic level, though intriguing candidates exist. Evidence suggests that gain-of-function mutations in CaCDR1 (PDR1/PDR3 ortholog) impact the stability of its transcripts (Manoharaj et al. 2010; Khakhina et al. 2018), and that mitochondrial activity regulates these genes through a post-transcriptional mechanism (Shahi et al. 2007; Shahi et al. 2010). Perturbation of mitochondrial function and application of clotrimazole alters the frequency and morphology of P-bodies (Buchan et al. 2011). Loss-of-function mutations in P-body components such as Dh1, Dcp2, and Xrn1 may then prevent degradation of PDR1 and PDR3 transcripts. Such a mechanism would likely have pleiotropic effects on other traits, since P-bodies accumulate many mRNAs.

Connection between CSF1 and azole resistance

We observed recurrent mutations in CSF1, which encodes a protein of unknown function that localizes to the mitochondria (Reinders et al. 2006; Dubreuil et al. 2019). Mutations in CSF1 impact fermentation, specifically at low temperatures (Tokai et al. 2000). In a genome-wide screen, CSF1 was implicated in matura-

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so clarifying its mechanism of action and role in cellular function will be of interest.

### Large number of replicates reveals epistatic interactions

We also found novel combinations of mutations that indicate potential epistatic interactions between resistance mutations. Epistatic interactions tend to reflect an underlying mechanistic connection between involved genes (Lehner 2011; Costanzo et al. 2019). These interactions can thus provide insight into the basic molecular biology of gene networks involved in drug resistance phenotypes. Knowledge of epistatic interactions can allow researchers to predict genes that are essential for resistance evolution (Lukačíšková et al. 2020).

Elucidating the mechanism of the interaction between mutations in PDR1 and PDR3 may provide interesting new insights into azole resistance. Single gain-of-function mutations or overexpression of each of these genes individually leads to a dramatic increase in expression of the drug efflux pump PDR5, among other resistance factors. It is thus surprising that a second mutation does not provide some additional benefit. This may be due to a ceiling on the benefit possible via these mutations and/or a tradeoff that overrides the beneficial impact of the second mutation, though we did not observe evidence of such a tradeoff when double mutants were grown in media without clotrimazole (Supplementary Fig. 2).

Pdr1 and Pdr3 are paralogs (products of an ancient whole genome duplication) that regulate an overlapping but distinct set of genes related to multidrug resistance and iron metabolism (DeRisi et al. 2000; Tuttle et al. 2003). They are additionally regulated in overlapping but distinct ways: for instance, loss of mitochondrial genome impacts PDR3 but not PDR1 (Hallstrom and Moye-Rowley 2000; Zhang and Moye-Rowley 2001). Many pathogenic yeasts have only a single copy of these transcription factors, but many of the regulatory associations with these genes are conserved (Khakhina et al. 2018). Clarifying the mechanism of this epistatic interaction may thus provide insight into resistance mechanisms in pathogenic species. It may additionally provide insight into the forces that shaped the evolution of these paralogs after the ancestral gene was duplicated.

The co-occurrence of mutations in the genes ERG25, HAP1, ROX1, ATP1, and ATP2 suggests that these mutations interact in some way. Based on their frequency and evidence from lineages with identical ERG25 mutations (Fig. 5b), it is likely that ERG25 mutations occur first, followed by mutations in HAP1 or ROX1 and ATP1 or ATP2. It is thus possible that ERG25 mutations have a stronger effect on their own, or that mutations in these other genes have a stronger effect when in an ERG25 background. These points are supported, though not proven, by the finding that individual ROX1 or ATP2 mutations seem to lead to a decrease in growth rate, which is not observed in strains that additionally possess mutations in ERG25 (Fig. 5, d and e).

Hap1 regulates ROX1 expression (Keng 1992), and they can act as activators or repressors of ERG gene expression under different conditions (Serratore et al. 2018). HAP1 mutations have previously been shown to co-occur with mutations in ATP1 and ATP2, and have been speculated to be suppressors of a fitness defect from perturbation of the F1F0 ATP synthase (Leeuwen et al. 2016). Together, these observations support that these patterns of co-occurrence are indeed due to epistatic interactions representing an underlying mechanistic connection (Fig. 5c). The mutants isolated from these experiments will be of value for investigating the mechanisms of these interactions. The small effect sizes of ERG25 mutations, alone or in combination with ATP2 and ROX1 mutations (Fig. 5, d and e), suggest that mutations in other genes are important for increased growth rates at higher concentration of azoles; characterizing these relationships could elucidate additional interactions or parallel mechanisms of resistance.

### Effectiveness of yEvo as a scalable course-based research experience

Recent decreases in the cost of whole-genome sequencing have made evolve-and-sequencing paradigms more accessible in a broader range of settings. Our findings here demonstrate the power and robustness of this approach in high school classrooms, which could also be applicable to undergraduate courses (Govindan et al. 2020). These experiments provide a compelling demonstration of the process of evolution in a pedagogical setting, since they can be explored at many levels of biological organization. Other research areas may also benefit from this type of a student–teacher–scientist partnership in advancing both pedagogical and biomedical research goals.

### Data availability

All sequencing data are available at the NCBI Sequence Read Archive (SRA) under BioProject PRJNA742704. Strains and plasmids are available upon request.

Supplemental material is available at G3 online.

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**Conflicts of interest**

None declared.

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