Cross-feeding affects the target of resistance evolution to an antifungal drug

Short title: Cross-feeding and antifungal resistance evolution

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Abstract

Pathogenic fungi are a cause of growing concern. Developing an efficient and safe antifungal is challenging because of the similar biological properties of fungal and host cells. Consequently, there is an urgent need to better understand the mechanisms underlying antifungal resistance to prolong the efficacy of current molecules. A major step in this direction would be to be able to predict or even prevent the acquisition of resistance. We leverage the power of experimental evolution to quantify the diversity of paths to resistance to the antifungal 5-fluorocytosine (5-FC), commercially known as flucytosine. We generated hundreds of independent 5-FC resistant mutants derived from two genetic backgrounds from wild isolates of *Saccharomyces cerevisiae*. Through automated pin-spotting, whole-genome and amplicon sequencing, we identified the most likely causes of resistance for most strains. Approximately a third of all resistant mutants evolved resistance through the pleiotropic drug response, a potentially novel mechanism in response to 5-FC, marked by cross-resistance to fluconazole. For the majority of the remaining two thirds, resistance was acquired through loss-of-function mutations in *FUR1*, which encodes an important enzyme in the metabolism of 5-FC. We describe conditions in which mutations affecting this particular step of the metabolic pathway are favored over known resistance mutations affecting a step upstream, such as the well-known target cytosine deaminase encoded by *FCY1*. This observation suggests that ecological interactions may dictate the identity of resistance hotspots.

Introduction

The past decades have seen great advances in the medical field, with the death rate from complications of medical and surgical care steadily declining ("QuickStats: Death Rate* From Complications of Medical and Surgical Care Among Adults Aged ≥45 Years, by Age Group — United States, 1999–2009" 2012). Numbers have also gone down for some of the leading causes of death globally, notably deaths from HIV, which have decreased by 51% between 2000 and 2019 ("The Top 10 Causes of Death" n.d.). Unfortunately, some of the treatments which help increase life expectancy also lower immune defenses, which puts patients at risk of potentially life-threatening infections caused by opportunistic pathogens (Robbins, Caplan, and Cowen 2017; Armstrong-James, Meintjes, and Brown 2014; Alcazar-Fuoli and Mellado 2014). Among them, *Candida auris*, several *Candida* species and *Aspergillus fumigatus*, all drug-resistant fungal pathogens, feature on the CDC's list of antimicrobial resistance threats (CDC 2019). Among other factors, the biological similarities between fungal and host cells make it difficult to design efficient antifungals with low toxicity (Vanreppelen et al. 2023). Consequently, there are only five main classes of antifungals (Vanreppelen et al. 2023). One may wonder what makes a 'good' antifungal target.

The role of most antifungals is to prevent proper synthesis of the membrane or cell wall, because they are made of components not found in mammalian cells (ergosterol, chitin-glucan, for example). If we were to look for other molecules, should the antifungal target an essential metabolic end product? An enzyme in a metabolic pathway? If the enzyme catalyzes an early step of a specific pathway, resistance could potentially arise through loss of function downstream, for instance when a toxic molecule is produced as a byproduct or through the conversion of a prodrug. Among the considerations for the development of powerful drugs targeting cell metabolism is the organization of cell communities *in vivo*. For instance, treatment can have drastically different outcomes depending on the microbe lifestyle, fungi in biofilms being notoriously less susceptible to antifungals (Desai, Mitchell, and Andes 2014).

Interestingly, despite the urgent need for better antifungals, the mechanisms of resistance are still poorly understood outside of the direct drug targets (Robbins, Caplan, and Cowen 2017). Particularly in the medical field, it is common practice to sequence as few as a single gene to look for 'common' resistance-conferring mutations, which introduces a bias in databases such as MARDy (Perlin and Wiederhold 2017). In doing so, resistance is too often studied through a keyhole. Even more challenging is deciphering if the mechanisms of resistance to a specific drug are general, or if they depend on the genetic background or the environmental conditions in which they take place. Here, we leveraged the power of experimental evolution coupled with high-scale whole-genome sequencing (WGS) to try and capture a large number of mutations conferring resistance to 5-fluorocytosine (5-FC, also known as flucytosine) using the model yeast *Saccharomyces cerevisiae*. We focused on 5-FC because it is among the oldest antifungals and its metabolism is documented enough that we can interpret this data with respect to the adaptive mechanisms and rationalize the paths to resistance (Delma et al. 2021).

5-FC is a prodrug that is not toxic to fungi before it is metabolized (Bennet 1977). Its metabolism involves several steps, which ultimately lead to cell toxicity (Fig 1). First, it is

imported into the cell by several permeases, typically those involved in the import of exogenous pyrimidines (Fcy2, Fur4, Fcy21, Fcy22) (Paluszynski et al. 2006). Next, 5-FC is deaminated by the cytosine deaminase Fcy1 into the cytotoxic compound 5-fluorouracil (5-FU). 5-FU is a molecule known outside of the field of antifungals that has been used to treat cancers for several decades (Longley, Harkin, and Johnston 2003). Its main activation relies on the addition of a phosphate group by the uracil phosphoribosyltransferase Fur1. Misincorporation of fluoronucleotides into RNA and DNA, as well as inhibition of the thymidylate synthase either cause growth arrest or cell death (Longley, Harkin, and Johnston 2003; Santi, McHenry, and Sommer 1974; Kufe and Major 1981). Resistance to 5-FC is typically conferred by mutations in the genes involved in its metabolism, preventing its import (*FCY2*), its conversion into 5-FU (*FCY1*) or the activation of 5-FU (*FUR1*) (Fig 1). *FCY* genes were named after their phenotypes associated with FluoroCYtosine resistance. Mutations in *FCY1* have regularly been reported as being associated with 5-FC resistance in clinical isolates of a broad range of pathogens such as *Candida albicans*, *Candida dubliniensis*, *Nakaseomyces* (*Candida*) *glabrata* and *Clavispora* (*Candida*) *lusitaniae* (Hope et al. 2004; McManus et al. 2009; Edlind and Katiyar 2010; Florent et al. 2009).

Fig 1. Pyrimidine and 5-FC import and metabolism around some of the known and potential mechanisms of resistance. 5-Fluorocytosine (5-FC), cytosine, uracil and 5-fluorouracil (5-FU) are represented as blue, dark purple, light purple and light blue beads, respectively. For clarity, only two permeases known to import pyrimidines are shown: Fcy2 and Fur4. In prototrophic yeast strains, pyrimidines can be synthesized *de novo* from amino acids such as glutamine and aspartate (Ljungdahl and Daignan-Fornier 2012-3). The steps leading to the obtention of the nucleotide precursors uridine monophosphate (UMP) and uridine diphosphate (UDP) are catalyzed by Ura3 and Ura6, respectively, in the *de novo* pathway (Lacroute 1968; Liljelund and Lacroute 1986). Alternatively, pyrimidines can be imported from the medium (Kurtz et al. 1999). Uracil can also be obtained by deamination of cytosine by Fcy1 (Erbs, Exinger, and Jund 1997). Finally, UMP can be obtained by the addition of a phosphate group by Fur1 (Kern et al. 1990). 5-FC is a prodrug, which is metabolized the same way cytosine is, except its deamination leads to the obtention of the cytotoxic compound 5-FU (Polak and Scholer 1975). Based on work done in laboratory strains or other fungal species, resistance to 5-FC can arise when either of the following three steps are compromised: import, conversion and activation, typically through mutations inactivating Fcy2 (for example), Fcy1 and Fur1, respectively (Fasoli et al. 1990). We also hypothesize an alternative path to resistance in the pleiotropic drug response, wherein loss of mitochondrial function leads to derepression of the transcription factor Pdr3, which in turn overexpresses efflux pumps such as Pdr5. Figure created with BioRender, using the structures of Fcy1 (1P6O), Fur1 from *C. albicans* (7RH8), Ura3 (3GDL), Ura6 (1UKZ) and Pdr5 (7P04) and AlphaFold predictions of Fcy2 (P17064) and Fur4 (P05316) (Jumper et al. 2021; Varadi et al. 2022).

Alternative paths to 5-FC resistance have been shown, however they are still poorly understood. Notably, a study published in 2013 was the first one to report efflux-mediated resistance to 5-FC, in *N. glabrata* (Costa et al. 2013). This path to resistance is far better characterized for azoles. Azole treatment typically favors the formation of *rho*^{0} mutants (also known as *petite* mutants), which are characterized by their loss of mitochondrial DNA. This results in an alleviation of repression of the transcription factors (Pdr1, Pdr3) regulating the expression of efflux pumps (Paul and Moye-Rowley 2014). Some of these efflux pumps, for

example Pdr5, end up overexpressed, conferring cross-resistance to several antifungals (Paul and Moye-Rowley 2014). So far, 5-FC treatment has also been shown to select for *rho*^{0} mutants (Steier et al. 2013). However, contrary to azole resistance, 5-FC resistance does not appear to be associated with transporters belonging to the ATP-binding cassette (ABC) superfamily (Steier et al. 2013; Pais et al. 2016). Instead, it has been shown to be mediated by transporters belonging to major facilitator superfamily (MFS) or aquaglyceroporins (Pais et al. 2016; Costa et al. 2015; Kannan et al. 2019). Also noteworthy is 5-FC resistance caused by the repression of intake permeases at neutral pH in *A. fumigatus* (Gsaller et al. 2018). In summary, it appears that 5-FC resistance could potentially arise through the formation of *rho*^{0} mutants, characterized by their overexpressed efflux pumps. However, this path to resistance, sometimes referred to as the pleiotropic drug response, remains to be shown (Fig 1).

Because of the tight relationship with pyrimidine metabolism, the use of auxotrophic laboratory yeast strains to study the mechanisms of resistance may paint a distorted picture of how resistance to 5-FC and 5-FU comes to evolve outside the lab. Notably, it has been shown that the presence of uracil in the growth medium triggers a negative feedback by repressing the expression of the uracil permease Fur4 (Séron et al. 1999), potentially limiting cell entry by 5-FC. Not only does the use of specific lab strains narrow the number of detectable paths to 5-FC resistance, it also prevents the detection of potential epistatic effects. Indeed, the impact of the genetic background on the evolution of resistance is still poorly understood and has only recently become one of many interesting avenues of research in the field (Gerstein and Berman 2020). Finally, searching a relatively small gene space for resistance-conferring mutations often limits interpretations. With the decreasing costs of WGS, we can now hope to reach saturation, in that sequencing a larger number of mutants would not substantially increase the number of relevant mutations.

For the aforementioned reasons, we set up to generate hundreds of 5-FC resistant mutants, subjecting two wild prototrophic strains of *Saccharomyces cerevisiae* to a fluctuation assay in minimal medium. Their relative fitness was estimated in several conditions to test for respiration capacity, fitness cost and cross-resistance. We show that about a third of all mutants developed generalized resistance through loss of mitochondrial function. The majority of the remaining two thirds evolved resistance to 5-FC and 5-FU. To explain their phenotype, the genomes of 276 of these mutants were analyzed by WGS. In particular, we sought out to determine if the resistance phenotype conferred by a given mutation was dependent on the genetic background, or if there were genetic interactions for a given background. For both backgrounds, virtually all sequenced mutants evolved resistance through loss-of-function mutations in the gene *FUR1*. Interestingly, none of the sequenced genomes showed any mutation in the well-known target *FCY1*, which we show is the result of negative selection imposed by the 5-FU-producing *FUR1* mutants. Ultimately, sequencing a large number of genomes also led to the identification of rare alleles most likely sufficient to confer resistance to 5-FC.

Results

Mutants evolved in 5-FC develop cross-resistance to 5-FU

Two environmental strains of *S. cerevisiae*, LL13-040 and NC-02 were subjected to several rounds of fluctuation assays, which generated 682 5-FC resistant mutants. All candidate resistant strains were streaked and spotted on YPG agar medium to evaluate mitochondrial function. *rho*$^-$ mutants amounted to 83/296 (28 %) and 140/386 (36 %) for LL13-040 and NC-02, respectively. The fitness of 408 5-FC resistant mutants was estimated by measuring colony growth through time on solid media. For both backgrounds, most mutants show an expected increase in relative fitness (relative to the matching WT strain) in minimal medium supplemented with 5-FC compared to control conditions (rich or minimal medium) (Fig 2). *rho*$^-$ mutants show a lower relative fitness in drug-free media, with LL13-040 *rho*$^-$ mutants displaying a larger fitness defect than NC-02 counterparts. For both backgrounds, the relative fitness in minimal medium, the same medium in which the strains were evolved, is generally lower or equivalent to that of the parental strains. Additionally, when we performed the fluctuation assay in the absence of 5-FC, no resistant mutant was generated. Both observations suggest that, if mutants evolved to grow better in minimal medium, none of the evolutionary routes coincidentally conferred resistance to the antifungal. We also measured the growth on minimal medium supplemented with 5-FU, the converted form of 5-FC obtained by Fcy1-mediated deamination in the pyrimidine salvage pathway (Fig 1). *rho*$^-$ and *rho*$^+$ mutants show a similar relative fitness twice that of the parental strains in minimal medium supplemented with either 5-FC or 5-FU. Interestingly, the relative fitness in 5-FC significantly correlates with the one in 5-FU, hinting at a mechanism of resistance that would take place downstream of the conversion of 5-FC into 5-FU or that affects intake of these molecules. We hypothesized this mechanism to be either the pleiotropic drug response in the case of *rho*$^-$ mutants, or the inactivation of *FUR1* in the case of *rho*$^+$ mutants.

Fig 2. Growth of individual mutants relative to the WT strains in various media. Relative growth corresponds to the mean area under the curve (AUC, calculated on 22 h) from four replicate colonies, normalized by the WT for individual strains arrayed on solid media: YPD, SD, SD + 25 µg/mL 5-FC and SD + 6.25 µg/mL 5-FU. A two-way ANOVA followed by Tukey's multiple comparison test was performed to compare the relative fitness of *rho*$^+$ mutants to that of *rho*$^-$ mutants for each background in each condition. Statistical significance is as follows: ****, adj. *p*-value < 0.0001; ***, adj. *p*-value < 0.001; *, adj. *p*-value < 0.05; ns, not significant. Insets show the correlation between relative fitness in SD + 5-FC and relative fitness in SD + 5-FU, with the corresponding Spearman's rank correlation coefficient (S. *rho*) and *p*-value.

rho$^-$ mutants are cross-resistant to fluconazole

To test our hypothesis of pleiotropic drug response caused by the loss of respiration, we first evaluated the fitness of *rho*$^-$ mutants in rich medium, as well as in minimal medium, supplemented or not with several antifungals belonging to distinct classes. We tested growth in the presence of three other classes of clinical antifungals: echinocandins (micafungin and caspofungin) , polyene (nystatin) and azole (fluconazole). Because of the slow growth of *rho*$^-$ mutants in some antifungal conditions, we incubated the plates at 37°C. In these conditions,

rho$^-$ mutants display a higher relative fitness in 5-FC than *rho*$^+$ mutants, while still showing a fitness defect in control media (Fig 3A). Interestingly, *rho*$^-$ mutants of both backgrounds appear to have developed cross-resistance to fluconazole, with a relative growth more than twice that of the parental strain for NC-02 (Fig 3A). Also noteworthy is their fitness in micafungin, especially for LL13-040 mutants, although this is likely the result of a very low growth rate (Supp Fig 1).

Fig 3. Cross-resistance of *rho*$^-$ mutants to 5-FC and fluconazole may be explained by efflux capacity. A) Relative growth corresponds to the mean area under the curve (AUC, calculated for the first 22 h of incubation) from four replicate colonies, normalized by the WT for individual strains arrayed on solid media: YPD, SD, SD + 25 µg/mL 5-FC, SD + 0.5 µg/mL micafungin, SD + 2 µg/mL caspofungin, SD + 16 µg/mL nystatin and SD + 64 µg/mL fluconazole. A two-way ANOVA followed by Tukey's multiple comparison test was performed to compare the relative fitness of *rho*$^+$ mutants to that of *rho*$^-$ mutants for each background in each condition. Statistical significance is as follows: ****, adj. *p*-value < 0.0001; **, adj. *p*-value < 0.01; *, adj. *p*-value < 0.05; ns, not significant. B) Rhodamine retention assay. Blue bars highlight the resulting detection thresholds (one negative control for each background i.e. cells not treated with rhodamine). *abc*Δ (s_012 from Table 1) was used as the positive control. C, D) Rhodamine retention (data from panel B normalized with the WT) and relative growth in C) fluconazole or D) 5-FC (data from panel A) are compared, with the corresponding Spearman's rank correlation coefficient (S. *rho*) and *p*-value.

Table 1. Yeast strains used in this study.

ID	Background	Relevant genotype	Mating type	Reference
s_001	LL13-040	WT	*MAT***a**/*MAT*α	(Leducq et al. 2016)
s_002	LL13-040	*ho*::*NAT*	*MAT***a**	This study
s_003	LL13-040	*ho*::*NAT FCY1*::mEGFP-*HPH*	*MAT***a**	This study
s_004	LL13-040	*ho*::*NAT fcy1*Δ::*HPH*	*MAT***a**	This study
s_005	LL13-040	*ho*::*NAT fcy2*Δ::*HPH*	*MAT***a**	This study
s_006	LL13-040	*ho*::*NAT fur1*Δ::*HPH*	*MAT***a**	This study
s_007	NC-02	WT	*MAT***a**/*MAT*α	(Bleykasten-Grosshans, Friedrich, and Schacherer 2013)
s_008	NC-02	*ho*::*HPH*	*MAT***a**	This study

s_009	NC-02	*ho*::*HPH fcy1*Δ::*NAT*	*MAT***a**	This study
s_010	NC-02	*ho*::*HPH fcy2*Δ::*NAT*	*MAT***a**	This study
s_011	NC-02	*ho*::*HPH fur1*Δ::*NAT*	*MAT***a**	This study
s_012	RY0566 x TWAS21230902	*pdr5*Δ *snq2*Δ *ybt1*Δ *ycf1*Δ *yor1*Δ	*MAT***a**	(Celaj et al. 2020)

Since the loss of mitochondrial function in *rho*$^-$ mutants has been shown to lead to a generalized resistance through the pleiotropic drug response, we hypothesized that cross-resistance to 5-FC and fluconazole is a result of an increased expression or activity of efflux pumps. To test this hypothesis, we performed a rhodamine retention assay. Rhodamine is a known substrate of ABC transporters and its accumulation in cells has been shown to be inversely correlated with efflux capacity (Maesaki et al. 1999). We find that virtually all *rho*$^-$ mutants display a lower count of rhodamine-fluorescent cells compared to their parental strain, confirming they have gained an increased efflux capacity (Fig 3B). Notably, both parental strains accumulate much less rhodamine than a laboratory strain deleted for all ABC transporters, meaning that parental strains were already capable of rhodamine efflux in the conditions of the experiment. Even though the NC-02 strain appears to have a diminished efflux capacity compared to the LL13-040 strain, the *rho*$^-$ mutants of both backgrounds display comparable rhodamine accumulation levels (Fig 3B). Therefore, the net gain of efflux capacity for NC-02 mutants is higher than that of LL13-040 counterparts. This efflux capacity is correlated with the measured growth in fluconazole, however the correlation is statistically significant only for NC-02 (Fig 3C). For either background, the correlation with the measured growth in 5-FC is not significant (Fig 3D). This could be explained by a lack of statistical power, but in all likelihood, a complex regulation drives the expression of multiple transporters involved in resistance to fluconazole, as well as 5-FC.

rho$^+$ mutants display a large number of distinct loss-of-function mutations in *FUR1*

Next, we focused on the mechanisms of resistance at play in *rho*$^+$ mutants. To make sure we identified resistance conferring mutations, including potentially rare ones, we sequenced the genomes of most assayed *rho*$^+$ mutants (149/160 LL13-040 mutants and 127/134 NC-02 mutants). Overall average sequencing coverage was around 60X, with no apparent CNVs (Supp Fig 2). Two computational methods were carried out to detect indels and SNPs, one with stringent criteria (“samtools”), and one with more tolerant criteria (“gatk”). Because of the frequency of mutations observed in *FUR1* in our preliminary analysis, we also directly amplified and Sanger sequenced *FUR1* (including the promoter region) from the same genomic DNA for most of these mutants. We included WT controls as well as *fcy1*Δ and *fcy2*Δ mutants for all three analyses (samtools, gatk and Sanger).

Confirming our hypothesis, most sequenced genomes (258 out of 276 mutants) harbored a mutation in *FUR1* (Fig 4A). Aggregating results from samtools, gatk and Sanger sequencing, we identified 118 distinct SNPs or indels in *FUR1* (Supplemental Data 1). The use of two

computational methods to call mutations proved relevant, since part of the mutations were detected by only one method (Fig 4A). As expected, gatk, with its set of more permissive criteria, detected mutations in around 500 genes in each background, whereas samtools detected mutations in only 150 genes on average (Fig 4A). Mutations in other genes than *FUR1* were rarely detected by both computational methods, which might be linked to the quality of their call (Fig 4A). Overall, approximately 120 genes per individual genome were found to be mutated by either method (Fig 4B). This large number is a result of the filtering criteria, which were set to be more flexible to make sure rare mutations would not be discarded. Ultimately, the number of unique mutations found in a given gene appeared as a good indicator to sort through the noise. Specifically, the number of unique mutations found in *FUR1* was much larger than in any other gene, which is strong evidence that *FUR1* constitutes a hotspot for 5-FC resistance-conferring mutations (Fig 4A).

Fig 4. **Genomic data analysis and validation**. A) Upset plots showing the number of unique mutated genes (# genes) detected by gatk, samtools or both for each background. Categorical plots on top show the number of distinct genomes for which at least one mutation was detected in a given gene (one dot per gene, colored according to the number of distinct mutations which have been detected). B) Distribution of the number of unique mutated genes per genome (as detected either by gatk or samtools) for each background. C) Data from Fig 2, where dots are colored by the mutated/deleted gene most likely to confer resistance. *FCY1* and *FCY2* both refer to their deletion mutant in the appropriate background. D) Growth assay for 35 resistant mutants for which a mutation in *FUR1* was detected.

Out of all strains (n = 268) for which *FUR1* was Sanger sequenced, all six controls (parental strains, *fcy1*Δ and *fcy2*Δ mutants) and only 13 mutants carried the WT allele of *FUR1*. Through a detailed look into our dataset, we found three candidate target genes which could alone explain the resistance phenotype of these 13 strains with a WT *FUR1* sequence: *URA6*, *GFA1* and *ARG2* (Table 2). Interestingly, the corresponding mutants display a relative fitness distinct to that of *FUR1* mutants (Fig 4C). Notably, the mutations detected in *URA6* and *GFA1* are harbored by strains resistant to 5-FU, whereas the *ARG2* mutant is only mildly resistant to 5-FC in our experimental conditions (Fig 4C). All identified mutations in these three genes (n=7) are predicted to impact conserved residues with five out of seven also predicted to impact protein stability.

Table 2. Low frequency mutations putatively conferring resistance to 5-FC.

Background		***URA6***	***GFA1***	***ARG2***
LL13-040	# genomes	2	0	0
	mutations	R52S		
NC-02	# genomes	1	8	1
	mutations	G73S	V478F, N496K, G482R, G492S	K113*

Among the sequenced strains for which no clear candidate resistance mutation was identified, one appears to have been initially miscategorized as resistant to 5-FC, four display the same relative fitness in 5-FC and 5-FU as *FUR1* mutants (and are therefore likely *FUR1* mutants as well) and one appears to be mildly resistant to 5-FC but not 5-FU (Fig 4C). Finally, most unsequenced *rho*$^+$ mutants showed little to no resistance, which suggests they were false positives of the fluctuation assay (Fig 4C).

To further investigate the extent and potential mechanisms of resistance, 35 resistant isolates (across both backgrounds) for which mutations were predicted in *FUR1* were tested in a growth assay, alongside the parental strains, and the deletion mutants for *FCY1*, *FCY2* and *FUR1* (s_004/5/6/9/10/11 from Table 1). All tested mutants showed a relative fitness similar to their null mutant counterpart (Fig 4D). This suggests most mutations inactivate the function of *FUR1* and are sufficient to explain the resistance phenotypes, meaning that mutations in other genes likely play no role (Fig 4C). Importantly, the relative fitness of the *fcy1*Δ mutants was comparable to that of all tested *FUR1* mutants. The reason why inactivating mutations in *FCY1* were not picked up in our experiment is thus not because they provide lower resistance levels than *FUR1* inactivating mutations in these conditions (see below).

Most mutations identified in *FUR1* were confirmed by our three detection methods (samtools not picking up indels), with all Sanger sequenced samples confirming what had been detected by WGS (Fig 5A). One mutation (R110G) stands out as being much more prevalent than others in LL13-040 genomes (Fig 5A). Further examination confirmed that it is shared among 39 strains which arose from the same preculture (Supp Fig 3). This could be explained by a rapid fixation of the mutation that was segregating in the preculture. It is important to note however, that it constitutes a rare case, since we often selected less than 20 mutants per preculture, which generally carried different mutations each (Supp Fig 3).

Fig 5. Mutations observed in Fur1 affect residues impacting stability and conserved positions. (A) Upset plots showing the number of unique mutations identified in Fur1 for each background. Categorical plots on top show the number of distinct genomes for which the same mutation was detected. Only one amino acid change resulted from two adjacent mutations (back-to-back in the same codon), all others corresponding to a single SNP or indel. (B, C) Effect on stability (B) and conservation (C) of all possible amino acid substitutions in Fur1 which are predicted to be impactful by mutfunc (Wagih et al. 2018). Substitutions predicted to be non-deleterious are not shown. The substitutions captured in our dataset are highlighted (bigger colored dots). (B) Side plots indicate the kernel densities for all data points (lightgrey) and substitutions captured in our dataset (black) along the position in Fur1 (top) or the ΔΔG value (right). (C) SIFT scores are represented on an inverted y-axis and are analogous to a *p*-value. Scores < 0.05 (dashed grey line) indicate a predicted deleterious mutation, with a low value (top of the plot) indicating that the amino acid change is very likely to affect protein function based on sequence conservation. For substitutions captured in our dataset, the color indicates the conservation level of the residue at that position. D) Location of substitutions from our dataset on the predicted structure of Fur1, visualized using UCSF ChimeraX (Pettersen et al. 2021). The tetramer (*C. albicans* Fur1 assembly 7RH8) is represented in light gray, with 1 chain in dark grey. Residues found to be mutated at least once in our dataset are colored in pink. UTP molecules are represented in sticks colored by atom.

To understand why all validation mutants behaved like the null mutant, we compared their predicted impact to that of all possible substitutions. Out of 4,104 possible missense mutations in Fur1, 1,279 are predicted to impact stability. Our dataset captured a total of 76 missense mutations, 42 of which are predicted to impact stability. This is significantly more than what can be expected by chance (Fisher's exact test *p*-value = 1.39e-5, Fig 5B). The same observation can be made of mutations predicted to impact function based on conservation, with 67 out of 2,600 being captured in our dataset (Fisher's exact test *p*-value = 4.96e-6, Fig 5C). The results are consistent with loss-of-function mutations. We find that the substitutions in our dataset are predicted to destabilize Fur1 with generally high ΔΔG values, with no apparent bias in terms of position along the sequence (Fig 5B). Finally, we also mapped the corresponding residues on the predicted structure of the Fur1 tetramer from *C. albicans* (PDB: 7RH8). We find that most mutated residues are not located close to the active site, but instead appear to be involved in structurally important folds and helices (Fig 5D). Additionally, some mutated residues reside at the interface between two monomers, which could indicate that the mutation prevents proper assembly of the tetramer (Fig 5D).

FUR1 path to resistance prevents selection of *FCY1* mutations

One of the known resistance genes to 5-FC encodes for the cytosine deaminase Fcy1, which acts upstream of Fur1 in the pyrimidine salvage pathway (Fig 1). We included *fcy1*Δ mutants in our validation experiments as positive controls. However, we were surprised to find no single Fcy1 mutants in our set of sequenced genomes. As loss of function in *FCY1* is sufficient to lead to resistance and many mutations in the gene are destabilizing enough to cause resistance (Després et al. 2022), one would expect *FCY1* mutations to also be frequent in our experiments.

To diagnose the absence of detectable mutations in *FCY1*, we randomly selected mutants (n=14) and performed Sanger sequencing: all carried the wild-type sequence. Next, we confirmed that *FCY1* is well expressed in LL13-040, using a mEGFP protein fusion (Supp Fig 4A). Finally, we evaluated the functionality of Fcy1 in parental strains. A growth assay using cytosine as the sole nitrogen source in minimal medium confirmed that both strains can grow, which would not be possible without the deamination of cytosine performed by Fcy1 (Supp Fig 4B).

Next, we sought out to confirm the fitness of the *fcy1*Δ mutants in liquid medium compared to our 5-FC mutants. We performed growth assays to calculate the relative fitness in three conditions: in minimal medium, in 5-FC and in minimal medium where the only nitrogen source is cytosine. As expected, the *fcy1*Δ mutants fared well compared to our evolved mutants in 5-FC, but displayed the lowest relative fitness among *rho*$^+$ mutants in cytosine (Fig 6A). In comparison, virtually all *rho*$^+$ mutants have little to no fitness tradeoff in cytosine, indicating that *FCY1* is functional.

Fig 6. *FCY1*-mediated resistance is context-dependent. A) Growth assay in liquid medium. Cultures were inoculated from single replicates in a 384-well plate containing SD (control), SD + 25 µg/mL 5-FC (5-FC) or YNB + 2% glucose + 250 µg/mL cytosine

(cytosine). Relative growth corresponds to the area under the curve (AUC, calculated for the first 13 h of incubation) normalized by the WT. B) Relative growth measured in liquid medium (data from panel A) compared to the one measured on solid medium for the corresponding mutants (data from Fig 2) at equal concentrations of 5-FC. C) Predicted cross-feeding interactions between cells with different alleles of *FCY1* and *FUR1*. Uracil (top) and 5-FU (middle) are expected to diffuse in the medium and be converted by Fur1 of neighboring cells, which would allow or inhibit growth, respectively. Notably, the growth of any *FUR1*$^+$ cells, regardless of their *FCY1* allele, will be inhibited because of 5-FU cross-feeding. On the contrary, any *fur1*$^-$ cells would grow despite 5-FU cross-feeding (bottom). Figure created with BioRender, using the structures of Fcy1 (1P6O) and Fur1 from *C. albicans* (7RH8). D) Medium conditioning assay. First, cultures of the deletion mutants were prepared in SD with or without 1.56 µg/mL 5-FC. After reaching an OD_{600} of 0.6, the cultures were filtered and the medium was transferred to a 96-well plate. Cultures were inoculated with either the WT or the other deletion mutant. Relative growth corresponds to the area under the curve (AUC) normalized by the WT and was obtained from biological triplicates. A two-way ANOVA followed by Tukey's multiple comparison test was performed to compare the relative growth in SD with 5-FC to the one obtained in SD without 5-FC in each condition. Statistical significance is as follows: ****, adj. *p*-value < 0.0001; ns, not significant.

Interestingly, when we compared the fitness measured in liquid medium versus the one measured on solid medium, we observed that the *fcy1*Δ mutant shows a significant fitness defect on solid medium when grown alongside other mutants (Fig 6B). A recent study by our group showed that *fcy1*Δ could grow on plates supplemented with cytosine as the only nitrogen source as long as strains with a functional *FCY1* allele (*FCY1*$^+$) were growing in proximity (Després et al. 2022). The auxotrophy of a *fcy1*Δ strain can therefore be rescued by cross-feeding uracil from *FCY1*$^+$ strains. We hypothesized that this could also occur for 5-FU and, through interactions between cells of different genotypes and diffusion in the medium, it would lead to strains with a loss of function of *FUR1* (*fur1*$^-$ strains) taking over strains with a loss of function of *FCY1* (*fcy1*$^-$ strains) during experimental evolution.

Specifically, if a mutant is sensitive to a compound effluxed by its neighbor, its growth will be inhibited. The ultimate toxic compound produced from the prodrug is 5-FUMP, downstream of Fcy1 and Fur1. This means that in a complex community, a *FCY1*$^+$ strain could produce 5-FU, which would inhibit its growth and that of *fcy1*$^-$ neighboring cells too, provided they are *FUR1*$^+$ (Fig 6C). On the other hand, a *fur1*$^-$ strain would be resistant to its own production of 5-FU and to that of neighboring *FCY1*$^+$ cells (Fig 6C). During experimental evolution, both *fcy1*$^-$ and *fur1*$^-$ mutants could occur but because they do so at low frequency at first, only the latter would increase in frequency. Pure cultures of *fcy1*$^-$ and *fur1*$^-$ would however be both equally resistant to 5-FC, as our previous results revealed. If this model is correct, conditioning the medium with a *fcy1*Δ strain should allow growth of a *fur1*Δ strain, whereas the opposite would lead to growth inhibition. We confirmed this prediction (Fig 6D). For both backgrounds, conditioning the medium with the *fcy1*Δ mutant led to the *fur1*Δ mutant growing significantly better than the WT. On the other hand, conditioning the medium with the *fur1*Δ mutant led to growth inhibition of both the WT and the *fcy1*Δ mutant.

In summary, the design of our fluctuation assay, where the mutants are selected by spotting precultures on a plate, negatively selects against spontaneous *FCY1* mutants. This would likely occur as well in a mixed community.

Discussion

Efflux-mediated resistance to 5-FC in *rho*$^-$ mutants

We generated and analyzed the fitness of hundreds of independent 5-FC resistant mutants to identify the most frequent molecular signatures of resistance to this antifungal. Challenging a common assumption of the usual paths to 5-FC resistance, we show that around a third of mutants gained resistance through a previously unknown mechanism involving drug efflux rather than inactivation of key metabolic enzymes. This path to resistance, often referred to as the pleiotropic drug response, is well characterized for azole resistance (Hallstrom and Moye-Rowley 2000; Ferrari et al. 2011; Taylor et al. 2022). Mutants which have lost mitochondrial function are no longer able to respirate and display a specific *petite* morphotype on glucose-containing medium. In turn, they display enhanced efflux activity, which confers generalized resistance. To date, this resistance mechanism has not been described for 5-FC.

Studies have shown that 5-FC treatment does select for *rho*^{0} mutants, but does not significantly affect the expression of transcription factors such as Pdr1 or the related expression of ABC transporters (Steier et al. 2013). Instead, a group has shown that DHA antiporters Aqr1, Flr1 and Flr2, as well as aquaglyceroporins Fps1 and Fps2, can mediate 5-FC resistance in *N. glabrata* (Pais et al. 2016; Costa et al. 2013, 2015). However, in these studies, 5-FC treatment did not affect the expression of the transporters, hinting at a more complex regulation. Reduced susceptibility to azoles has previously been reported in 5-FC resistant isolates of pathogenic fungi (Cuenca-Estrella et al. 2001). A possible explanation could be a single mutation conferring resistance to both classes of antifungals. One study supporting this hypothesis reported that a substitution in the gene *MRR1* was sufficient to confer cross-resistance to 5-FC and azoles in clinical isolates of *C. lusitaniae* (Kannan et al. 2019). This gene encodes a transcriptional regulator, which when mutated upregulates the expression of the multidrug transporter encoding gene *MFS7*.

Here, we confirm that 5-FC treatment at least selects for *rho*$^-$ mutants, which are cross-resistant to 5-FC and fluconazole (Fig 3A). Additionally, *rho*$^-$ mutants appear to be resistant to micafungin, although their growth was slow, despite the low drug concentration (Fig 3A and Supp Fig 1). For one of our two environmental backgrounds, we find the fitness of *rho*$^-$ mutants in fluconazole correlates significantly with their propensity to accumulate rhodamine (Fig 3C), which is likely an indication that resistance is mediated by ABC transporters. However, the correlation between rhodamine accumulation and fitness in 5-FC was not significant for either background (Fig 3D). This corroborates a previous report that 5-FC and rhodamine are imported independently and suggests that resistance is mediated by different types of transporters (Mansfield et al. 2010).

These results suggest that pathogenic fungi can acquire resistance to 5-FC through efflux, in addition to the more commonly described resistance paths in the canonical pathway. The evolution of resistance through this path is likely limited by the strong fitness costs associated with it in conditions that require respiration, which would make this path unfavored in some environments. However, it has notably been reported for a clinical isolate of *N. glabrata* in response to fluconazole (Bouchara et al. 2000).

FUR1-inactivating mutations confer 5-FC resistance in *rho*$^+$ mutants

Using WGS, we found that most *rho*$^+$ mutants gained resistance through *FUR1* inactivation. Interestingly, a large diversity of mutations were detected in this gene. In contrast, the MARDy database only lists two *FUR1* mutations conferring resistance to 5-FC at the time of writing (version 1.1DB:1.3WS, http://mardy.dide.ic.ac.uk/). Even accounting for mutations reported elsewhere, the intersection with the ones captured in our experiment is surprisingly small (Delma et al. 2021). They do correspond however to mutations of clinical relevance, such as F211I and G190D found in clinical isolates of *C. auris* and *N. glabrata*, respectively (Chapeland-Leclerc et al. 2010; Rhodes et al. 2018). Our work shows that the available paths through mutations in *FUR1* are far more diverse than the literature lets appear. Most mutations in *FUR1* appear to be loss-of-function mutations. We observed several indels (deletions and insertions of up to 49 and 39 bp, respectively) and five mutations affecting the translation start site of Fur1 (M1V, M1I, M1L, M1K, M1T) (Supplemental Data 1). Many more are predicted to impact the stability of the protein. This would make resistance through mutations in *FUR1* very likely since many of the possible amino acid changes are predicted to impact the stability of Fur1 and/or conserved regions (Fig 5BC), in addition to all possible stop codons and other possible frameshifting indels. Not only the mutations picked up in our experiments capture both of these phenomena (Fig 5BC), some also appear to likely affect proper assembly of the Fur1 tetramer (Fig 5D).

It is also possible that the drug concentration used to select for resistant mutants was high enough that a partially functional Fur1 would not confer resistance. It would be interesting to study in the future the relationship between the extent of Fur1 inactivation and inhibitory concentrations to formally test this hypothesis. In any case, our results highly suggest that the number of resistance-conferring mutations in fungal pathogens is profoundly underestimated. They do confirm however that resistance emerges rapidly in response to 5-FC monotherapy (Chang et al. 2021). Interestingly, cases have also been reported where resistance through nonsense mutations in *FUR1* was rapidly acquired upon combination therapy with caspofungin (Charlier et al. 2016). Additionally, cross-resistance to fluconazole (and potentially other azoles) also evolves rapidly, an observation which has also been reported in the clinic (Cuenca-Estrella et al. 2001). The fact that resistance to different antifungals is connected through shared resistance mutations despite their apparent lack of similarity in their mode of action is worrisome and stresses the need for more studies on cross-resistance.

Alternative paths to resistance in *rho*$^+$ mutants

Our experiment led to the identification of three other genes potentially conferring resistance to 5-FC when mutated: *URA6*, *GFA1* and *ARG2* (Fig 4C, Table 2).

URA6 encodes the enzyme which catalyzes the step right after the reaction performed by Fur1 (Fig 1), therefore it is highly likely that the detected mutations inactivate *URA6* and are sufficient to confer 5-FC and 5-FU resistance. A temperature-sensitive mutant of this enzyme in a laboratory strain is known to be resistant to 5-FU, confirming that loss of function is responsible for resistance (Liljelund and Lacroute 1986). *GFA1* encodes an

important enzyme involved in the biosynthesis of chitin. Some studies have shown fungal cells can display increased chitin levels in response to fluconazole or echinocandins (Shahi et al. 2022; Lara-Aguilar et al. 2021; Walker et al. 2008), however it appears to be one of many consequences of a generalized stress response. Both *URA6* and *GFA1* are essential in auxotrophic laboratory strains (Liljelund and Lacroute 1986; Giaever et al. 2002). This shows that epistasis can play an important role in the evolution of resistance, and that the use of prototrophic strains in experimental evolution experiments will likely lead to different outcomes.

Finally, it has previously been shown that deletion of *ARG2* can confer resistance to 5-FU, through crosstalks between the ornithine biosynthesis pathway and the pyrimidine biosynthetic pathway (Carlsson et al. 2013). The hypothesized mechanism is that blocking ornithine formation hinders the consumption of carbamoyl-phosphate, both being required for the synthesis of arginine. As a result, carbamoyl-phosphate is used as an early precursor of UTP, which ends up being produced in larger quantities than 5-FUTP (Carlsson et al. 2013). This would corroborate a previous observation that five other genes involved in arginine metabolism are linked with 5-FC resistance (Costa et al. 2015).

Our results highlight the need for studies using prototrophic strains, instead of the popular laboratory strains, as they allow the identification of rare alleles conferring resistance.

FUR1 is the main target to confer 5-FC resistance

Out of all genes involved in the metabolism of 5-FC, we unexpectedly detected mutations only in *FUR1* and *URA6* (Fig 4C). Resistance to 5-FC in clinically relevant fungi typically results from mutations in *FCY2*, *FCY1* and/or *FUR1* (Whelan 1987; Hope et al. 2004; Papon et al. 2007). Since resistance often requires loss of function of those genes, redundant entry routes in *S. cerevisiae* can explain the absence of resistance mutations in genes involved in the import of 5-FC, namely *FCY2*, *FCY21*, *FCY22* and *FUR4* (Paluszynski et al. 2006) as their loss of function would be masked by others. The case of *FCY1* was more difficult to interpret as there is no known redundancy for its function. We hypothesized that depending on the context, *FUR1* could be a more likely target than *FCY1* due to cross-feeding. In a mix of $FCY1^{+}$ and $fcy1^{-}$ cells, 5-FU would be produced and secreted, leading to the death of $FCY1^{+}$ cells, as well as loss-of-function mutants. The benefits of losing the cytosine deaminase function would therefore be highly specific to the ecological context.

Accordingly, we performed a growth-based experiment in which the medium was previously conditioned by the growth of either a *fcy*1Δ mutant or a *fur1*Δ mutant. A medium conditioned by the latter contains 5-FC, as well as 5-FU, as a result of deamination of 5-FC by Fcy1. 5-FU being toxic for any cell that encodes a functional Fur1, it imparts selective pressure upon *FUR1* mutants. For this reason, compound sharing during the selection step dilutes the populations of cells in which mutations impact any step upstream of the conversion of 5-FU. This is of the utmost ecological importance, since the ecological context will define which mutations are more likely to lead to resistance (Das et al. 2020).

No strong background-dependent effect for path to resistance but many background-dependent effects on fitness

Even though we used two distinct environmental isolates as parental strains, we observe strong parallel evolution of resistance. Notably, the main outcome of our evolution experiment did not seem to be affected by the presence of active transposable elements in only one of the two parental strains (LL13-040). Indeed, for both backgrounds, most evolved strains gained resistance through loss-of-function mutations in *FUR1*. However, differences in fitness reflect the different behaviors of each background before and after evolution. For example, even though both parental strains grow at comparable rates in all tested conditions (Supp Fig 1), NC-02 displays more rhodamine accumulation than LL13-040 (Fig 3B). After evolution however, *rho*$^-$ strains of both backgrounds display strikingly similar distributions in rhodamine accumulation (Fig 3B). One background, NC-02, therefore evolved to gain much more efflux capacity than the other. This observation is corroborated by the significant correlation with fitness in fluconazole, which is again markedly higher for NC-02 *rho*$^-$ strains compared to LL13-040 counterparts (Fig 3C). Similarly, the same mutants show a lower fitness tradeoff in drug-free media (Figs 2 and 3A). Together, our results could indicate that NC-02 would potentially evolve along a broader range of paths to resistance. Interestingly, this hypothesis is also supported by the mutations detected in the sequenced genomes. For example, more indels were detected in *FUR1* for NC-02 strains (Fig 5A). Two genes potentially sufficient to confer resistance, *GFA1* and *ARG2*, were found to be mutated only in NC-02 strains (Table 2). On the other hand, a single mutation in Fur1 (R110G) was found to be present in 39 different LL13-040 strains (Supp Fig 3). And despite selection seemingly acting more strongly in one background than the other, we found an identical number of unique mutations in *FUR1* for each background (52 background-specific mutations for each background and 14 shared mutations, Supplemental Data 1).

Experimental design and evolutionary relevance

Our experimental design does not represent a real-world clinical situation in which 5-FC would be used for treatment. However, we revealed a very interesting community interaction that can affect the route of evolution. Upon treatment of an infection, rapid establishment of resistance would arguably be impacted by two main factors: the local concentration of antifungal and the local population size. The first factor determines for instance the strength of selection and the second the size of the mutational supply and the efficiency of selection. Our results indicate that population size or density could also affect the route of evolution.

Drug concentration is rarely homogeneous, but instead exists as a spatiotemporal gradient linked to the inherent pharmacodynamic properties of the drug, such as imperfect drug penetration (Moreno-Gamez et al. 2015). This can have a crucial effect on the establishment of resistance. For example, it can allow (or not) the expansion of a resistance-conferring mutation from a single cell to a whole subpopulation (Alexander and MacLean 2020). Drug concentration has also been shown to impact the nature of beneficial mutations and affect whether a population will develop resistance or tolerance (Todd et al. 2023). Local population size is also heterogeneous. For example, a localized higher density of cells could be the result of a biofilm having developed on a catheter (Ramage, Martínez, and López-

Ribot 2006; Desai, Mitchell, and Andes 2014). Our findings suggest that the benefits of losing either of two consecutive steps in a path, both of which confer resistance on their own, will be dictated by the local conditions and density. In conditions that allow cross-feeding and diffusion of small molecules, the loss of the initial step (Fcy1) would not be advantageous. However, for isolated cells or in conditions where the diffusion of nutrients is highly limited, it might be, hence the discrepancy in the resistance phenotype of the *fcy1*Δ mutant on a shared plate versus in an isolated well (Fig 6B). In comparison, the loss of the second step (Fur1) is advantageous, no matter if cells are isolated or in close contact.

Ultimately, evolution experiments such as our study highlight relatively narrow but still very relevant features of evolution of resistance. And even with the complex challenge that is the accurate reproduction of clinically relevant environments, these studies still manage to capture resistance mutations found in clinical isolates (Taylor et al. 2022).

Materials and methods

Strains, plasmids, primers and culture media

We used two environmental strains of *S. cerevisiae*: LL13-040 (Leducq et al. 2016) and NC-02 (Bleykasten-Grosshans, Friedrich, and Schacherer 2013), both wild isolates collected from trees in North America. Unlike LL13-040, previous whole-genome analysis of NC-02 revealed no active transposable element (Bleykasten-Grosshans, Friedrich, and Schacherer 2013), which could potentially change the types of resistance mutations the strains would have access to. For both strains, only haploids were used to be able to detect recessive mutations. First, the *HO* locus was replaced by a nourseothricin resistance marker in LL13-040 and a hygromycin resistance marker in NC-02. Strains underwent sporulation and dissection. Haploid selection and mating type was confirmed by multiplexed PCR. All subsequent experiments were performed using the corresponding haploid strains of mating type **a**. Strains used in this study are listed in Table 1. Constructions were obtained by standard transformation from competent cells using plasmids and primers listed in Supplemental Data 2. The following culture media were used: YPD (1% yeast extract, 2% bio-tryptone, 2% glucose, with or without 2% agar), YPG agar (1% yeast extract, 2% bio-tryptone, 3% glycerol, 2% agar), SD (MSG) (0.174% yeast nitrogen base without amino acids, 2% glucose, 0.1% monosodium glutamate), SD (NH_4) (0.174% yeast nitrogen base without amino acids, 2% glucose, 0.5% ammonium sulfate), SC (SD with standard drop-out mix). When indicated, the following compounds were added to the medium: 5-FC (Fisher Scientific), 5-FU (Fisher Scientific), cytosine (Fisher Scientific), methylcytosine (Fisher Scientific), micafungin (Toronto Research Chemicals), caspofungin (Cedarlane Labs), nystatin (BioShop Canada), fluconazole (Cedarlane Labs), nourseothricin (Millipore Sigma), hygromycin B (BioShop Canada). Unless specified, most biochemical products were acquired from Fisher Scientific or BioShop Canada.

Fluctuation assay

The fluctuation assay was adapted from (Gerstein, Lo, and Otto 09/2012) to isolate independent mutants. Strains were streaked from glycerol stocks onto YPD agar medium, then incubated three days at 30°C. 10 mL YPD was inoculated with a single colony, then incubated for 24 hours at 30°C with shaking. The corresponding number of mitotic generations was calculated from measurements of cell concentrations before and after incubation using cell counts estimated by flow cytometry for four biological replicates, with a guava easyCyte HT cytometer (Cytek, blue laser): LL13-040, 5.3 ± 0.5 generations; NC-02, 5.4 ± 0.5 generations. Each 24 h pre-culture was used to inoculate a 96-deep-well plate with 1 mL synthetic minimal medium (SD) supplemented with 1.56 µg/mL 5-FC, at a final concentration of 0.1 OD_{600}. Border wells were filled with 1 mL sterile water to prevent evaporation. Plates were sealed with porous adhesive film and incubated for 72 hours at 30°C with shaking. Finally, cultures were spotted on SD agar plates with or without 6.25 µg/mL 5-FC to select 5-FC resistant mutants. All mutants were streaked on YPD agar medium to isolate single colonies, only one of which per mutant was selected for further experiments.

rho$^-$ (*petite*) mutants were identified by their morphotype. Once enough mutants were generated, a 96-well plate with 1 mL YPD was inoculated and incubated overnight at 30°C. The plate was then used to prepare a glycerol stock, as well as spotting on YPG agar medium to confirm *rho* status. Overall, the fluctuation assay was iterated eight times, for a total of 49 pre-cultures (26 for LL13-040 and 23 for NC-02), generating a total of 682 5-FC resistant mutants (296 LL13-040 mutants and 386 NC-02 mutants). Two more iterations without 5-FC selection were performed to evaluate whether acclimation to minimal medium could be enough to acquire resistance. It was not, since neither generated any 5-FC resistant mutant.

Media conditions for the fluctuation assay were initially decided based on preliminary growth assays using the constructed haploid LL13-040 strain. Specifically, we noted that the use of minimal medium greatly increases 5-FC sensitivity, the absence of uracil being partly responsible for this effect (Supp Fig 5A). The final concentration of 5-FC used in the fluctuation assay was chosen based on dose-response curves in SD medium obtained from biological triplicates of both strains. In the conditions of this experiment (see section below), a concentration of 1.56 µg/mL 5-FC corresponds to inhibition coefficients of 93.5 ± 0.8% and 91.8 ± 0.3% for LL13-040 and NC-02, respectively (Supp Fig 5B). In an initial fluctuation trial, this concentration yielded 15 LL13-040 spontaneous mutants, compared to only 3 when using 0.78 µg/mL 5-FC. A 10% cutoff (at least six mutants out of 60 wells inoculated on each plate) ultimately prompted us to use 1.56 µg/mL 5-FC for all iterations of the fluctuation assay.

Growth assays in isolated liquid cultures

For all ‘small-scale’ growth assays, strains were streaked on YPD agar medium supplemented with the appropriate antibiotics. Precultures were prepared from three isolated colonies in 5 mL YPD medium with appropriate selection and incubated overnight at 30°C with shaking. Precultures were diluted at 1 OD_{600} in sterile water. Sterile Greiner 96-well plates were prepared with the indicated culture medium and compounds and cultures were inoculated at a final concentration of 0.1 OD_{600}. OD_{600} measurements were performed at 30°C every 15 min until a plateau was reached in a Tecan Infinite M Nano (Tecan Life Sciences).

For the ‘larger-scale’ growth assays, precultures were prepared in 96-well deep well plates with 1 mL YPD and incubated overnight at 30°C with shaking. Precultures were diluted at 1 OD_{600} in sterile water. For a subset of mutants (as well as WT controls and *fcy1*Δ mutants), a sterile 384-well plate containing four different conditions was used: SD, SD + 25 µg/mL 5-FC, SD + 100 µg/mL 5-FC and YNB + 2% glucose + 250 µg/mL cytosine. The lid was conditioned by incubating 3 min with 5 mL 0.05% Triton X-100 / 20% ethanol and dried under sterile conditions. Cultures were inoculated in a single replicate at a final concentration of 0.1 OD_{600} in a final volume of 80 µL per well. OD_{600} measurements were performed at 30°C every 15 min in a Tecan Spark (Tecan Life Sciences) with an active Tecool temperature control module, until curves showed signs of evaporation (13 h).

Dose-response curve assay

A single 96-well plate was prepared like any ‘small-scale’ growth assay, with cultures of LL13-040 and NC-02 in biological triplicates. 5-FC was serially diluted 1:2 seven times starting from a final concentration of 3.125 µg/mL. The maximum growth rate was transformed into the inhibition coefficient, with an inhibition coefficient of 0 corresponding to the maximum growth rate measured in the absence of 5-FC.

Automated growth measurements on solid medium

The growth of resistant mutants, as well as parental strains and deletion mutants for *FCY1* and *FCY2* was assayed with a BM3-SC robot (S&P Robotics Inc.). First, plates of mutants were printed on YPD OmniTrays. Then, mutants were rearrayed depending on their *rho* status.

rho$^+$ mutants

A liquid culture of strain s_009 was used to print a border on a YPD 384-array. 300 *rho*$^+$ mutants (including controls) were then rearrayed onto the same 384-array from five 96-array sources. The resulting 384-array was expanded into a 1536-array to have four replicates for each mutant. The 1536-array was replicated six times to obtain the source plates for the fitness measurements. Source 1 was replicated onto five destination plates: YPD, SD, SD + 25 µg/mL 5-FC, SD + 1.56 µg/mL 5-FU, SD + 6.25 µg/mL 5-FU, which were incubated for 22 h at 30°C. Sources 2-6 were each replicated onto three destination plates for a total of 15 conditions: YPD, SD, SD + 25 µg/mL 5-FC, SD + 16/32/64 µg/mL fluconazole, SD + 4/8/16 µg/mL nystatin, SD + 0.25/1/2 µg/mL caspofungin and 0.0625/0.25/0.5 µg/mL micafungin. Plates were incubated for 90 h at 37°C. During both incubations, pictures of each plate were taken every 2 h in a spImager custom robotic platform (S&P Robotics Inc.).

rho$^-$ mutants

Similarly, two 96-arrays were prepared, one with only LL13-040 mutants, the other with only NC-02 mutants. For both, a liquid culture of the corresponding *fcy1*Δ mutant was used to print the border. 118 *rho*$^-$ mutants as well as controls were rearrayed from either three (NC-02) or four (LL13-040) 96-array sources. The two resulting 96-arrays were expanded into two 384-arrays to have four replicates for each mutant. For each background, the 384-array was replicated five times to obtain the source plates for the fitness measurements. Similar growth media conditions were tested. Additionally, both plates were replicated onto YPG arrays to confirm *rho* status. Following this QC step, four mutants were detected as being initially misannotated as *rho*$^-$ (three LL13-040 mutants and one NC-02 mutant) and were therefore excluded from all analyses (Supp Fig 6).

Cytometry

Rhodamine accumulation assay

Measurement of intracellular accumulation of rhodamine was adapted from (Revie et al. 2020). The two 96-arrays of *rho*$^-$ mutants for LL13-040 and NC-02 (obtained as described

above) were inoculated in 96-well deep well plates containing 1 mL YPD and incubated overnight with shaking at 30°C. For both plates, two controls were added: the WT strain and a strain with deletions for all ABC transporters (*PDR5 SNQ2 YBT1 YCF1 YOR1*; s_012 in Table 1) (Celaj et al. 2020), therefore incapable of rhodamine efflux. In the morning, both plates were subcultured at 0.15 OD_{600}, then incubated at 30°C with shaking until they reached 0.6 OD_{600}. For this step, one of the mutants was subcultured twice to later have a control without rhodamine. 200 uL was transferred to a sterile V-shaped plate. Rhodamine 6G (Millipore Sigma) was added to a final concentration of 10 µg/mL except for 1 well. Plates were sealed with porous adhesive film and incubated 30 min at 30°C with shaking. Cultures were centrifuged 5 min at 230 g and pellets were washed with sterile PBS. Cultures were centrifuged again and pellets were resuspended in 200 µL sterile PBS with 0.2% glucose. Cultures were incubated an extra 30 min at 30°C to activate energy-dependent efflux, then diluted 1:10 prior to fluorescence measurements by flow cytometry. Fluorescence in the green and orange channels using blue and green lasers, respectively, was acquired for approximately 2,000 events per sample with a guava easyCyte HT cytometer (Cytek). Fluorescence values in both channels were normalized with the cell size using the FSC value. Thresholds were set for both normalized fluorescence values to maximize events with fluorescence below the thresholds for the negative control (sample not treated with rhodamine), as well as maximize events with fluorescence above the thresholds for the positive control (s_012 in Table 1). The percentage of cells with fluorescence above both thresholds is considered to be inversely correlated with the efflux capacity.

Fluorescence reporter assay

Precultures of LL13-040 with or without *FCY1-mEGFP* (s_002 and s_003 in Table 1) were prepared from single colonies in 5 mL YPD medium and incubated overnight at 30°C with shaking. Precultures were diluted at 1.5 OD_{600} in sterile water. 24-well plates containing either SC, SC -ura, SD (MSG) or SD (NH_4) with 0, 0.78 or 1.56 µg/mL 5-FC were inoculated at 0.15 OD_{600}, sealed with porous adhesive film and incubated for 4 h at 30°C with shaking. Cultures were diluted at 0.05 OD_{600}. Fluorescence in the green channel using a blue laser was acquired for 5,000 events. Events with a SSC value below 300 or a FSC value below 2,000 were filtered out. The fluorescence values were normalized by the FSC value.

Colony size analyses

Pictures were cropped, then converted into inverted gray levels using scikit-image (van der Walt et al. 2014). Colony sizes were quantified using pyphe-quantify in batch mode (Kamrad et al. 2020). The area parameter was used to generate growth curves and calculate the corresponding area under the curve (AUC) using the composite trapezoidal rule. For each mutant, the relative fitness was calculated as the ratio of the mean AUC (absolute fitness) divided by the corresponding mean AUC obtained for the WT parental strain.

DNA extraction and sequencing

rho$^+$ mutants were cultured overnight at 30°C in 24-well plates with 2 mL YPD, sealed with porous adhesive film. Cells were pelleted from a total of approximately 20 OD units per sample. DNA was extracted using the MasterPure Yeast DNA Purification kit (Epicentre) following the kit's protocol, except for the following adjustments. Isopropanol precipitation

was performed for 1 h at room temperature. Pellets were dried at 55°C for 25 min, after which they were resuspended in 50 µL 0.2 ng/µL RNase. Samples were incubated an additional 5 min at 55°C, then immediately purified using SPRI beads (1:1 ratio, Axygen). Attempts to extract genomic DNA from *rho*$^-$ mutants proved unsuccessful with this method.

Genomic DNA was stored at -20°C, then quantified in technical triplicates using the AccuClear Ultra High Sensitivity dsDNA Quantitation kit. A total of 282 samples (276 out of 294 rearrayed *rho*$^+$ mutants, as well as the parental strain and the *fcy1*Δ and *fcy2*Δ mutants for each background, s_004/5/9/10) were diluted to 10 ng/µL for a total of 40 ng and arranged on three 96-well plates. For eight samples, whose initial concentration was too low, a volume of 15 µL was pipetted and dried in a SpeedVac, then resuspended in the appropriate volume to the desired final concentration of 10 ng/uL. Sequencing libraries were prepared using the Riptide High Throughput Rapid DNA Library Prep kit (iGenomx), according to the kit's protocol. The three 96-well plates were each pooled separately. For the amplification step, we modified the protocol to use barcoded primers for each pool instead of a single universal PCR primer. The three pools were SPRI-purified in two steps with protocol option 3 (recommended for PE150 sequencing). The pools were checked on agarose gel, quantified with the AccuClear Ultra High Sensitivity dsDNA Quantitation kit and analyzed on BioAnalyzer (Agilent). Finally, they were sequenced in paired-end 150 bp on an Illumina NovaSeq 6000 S4 system at the Centre d'expertise et de services Génome Québec.

FCY1 and *FUR1* were amplified from the same genomic DNA using primers listed in Supplemental Data 2, then sequenced by Sanger amplicon sequencing. For *FUR1* amplicons, all samples for which enough material could be recovered (after preparing WGS libraries) were processed. The reactions were performed at the Centre Hospitalier de l'Université Laval sequencing platform (Université Laval, Québec).

Computational analyses

WGS data

FASTQ reads were demultiplexed (and adapter-trimmed) using DemuxFastqs from the fgbio set of tools. A custom snakemake pipeline was used to process the FASTQ files (Mölder et al. 2021). Reads were aligned using bwa-mem2 on the reference S288C genome (Vasimuddin et al. 2019). Aligned reads were sorted and indexed using samtools (Danecek et al. 2021). Reads that did not map uniquely (SAM FLAG 256) were excluded. PCR duplicates were removed using picard's MarkDuplicates. Reads around indels were realigned using samtools calmd. The pipeline was run on the IBIS servers.

Variant calling

Variants were called with two methods, bcftools/samtools and GATK.

SNP calling with samtools/bcftools ("samtools")

SNP calling was conducted with bcftools v1.9. SNP pileup was generated with bcftools mpileup command with options -C50, -min-MQ 4, -min-BQ 13 and prior removal of reads that were unmapped, not in primary alignment, failing quality checks or were PCR/optical

duplicates. The command was run separately for samples from the two backgrounds (LL13-040 and NC-02). Haploid SNP calling was conducted with bcftools call command with option -mv.

SNP calling with gatk ("gatk")

In the second variant calling method, bam files were edited using picard v2.18 (http://broadinstitute.github.io/picard/), SNP and indel calling was conducted with GATK v4.2.6.1 (Poplin et al. 2017). Prior to variant calling, an RG (read group) tag was added to individual bam files. GVCF files were generated with GATK HaplotypeCaller, with options -ploidy 1 -ERC GVCF and --min-base-quality-score 20. GVCF files of samples coming from the same background (LL13-040 or NC-02) were combined with GATK CombineGVCFs, genotyped with GATK GenotypeGVCFs, and processed separately. SNPs with the following criteria were filtered out: variant quality score (QUAL) < 30, QUAL by depth (QD) < 2, mapping quality (MQ) < 40, Fisher's exact tests of strand bias (FS) > 60, symmetric odds ratio test of strand bias (SOR) > 4, mapping quality rank sum test (MQRankSum) < -12.5, and rank sum test for site position within reads (ReadPosRankSum) < -8. Indels were filtered out if they met the following criteria: QD < 2, QUAL < 30, FS > 200, or ReadPosRankSum < -20.

Further filtering was conducted for all variant sets with bcftools v1.9 and python v3.10 scripts. We applied a set of filtering criteria both to samples, and variants. First we removed samples (marked as missing data), which met the following criteria:

1. Read depth < 4
2. Allelic depth (AD) for the second most common allele > 4
3. Ratio of the AD of the second to the first most common allele > 0.2

Then, variants meeting the following criteria were removed:

1. Mean read depth across all samples < 10
2. Total read depth > 20000
3. Variant quality (QUAL) < 20
4. Mapping quality (MQ) < 40
5. High frequency (allele frequency (AF) > 0.99 or allele count (AC) = 0)

Finally, any variants shared with the parental strain were removed as well. Variants were ultimately annotated with SnpEff v5.0 (Cingolani et al. 2012) using *S. cerevisiae* genome vR64-3-1.

CNVs

Coverage of WGS data was analyzed to look for regions with copy number variations (larger than 5,000 bp and smaller than half of the chromosome length). Read count per position for each sample was output using samtools depth. Mean read count was calculated in non-overlapping windows of 5,000 bp and corrected for mapping bias related to GC content. Namely, the read count in each window ("W") was multiplied by a ratio of median read count over all windows divided by a median read count over all windows with the same GC as "W". End chromosome bias (increasing read count towards chromosome ends) was corrected by fitting a curve for the windows closest to one chromosome end (each half of the chromosome), using the LOWESS smoothing method, in python v3 statsmodels package,

with a default of 2⁄3 of data points to estimate each y value. Windows were then divided by the fitted line to obtain the normalized read count.

Predictions of impact of mutations

Mutations were submitted to the online tool mutfunc to predict their impact, notably on stability and/or conservation in a homology model (Wagih et al. 2018).

Medium conditioning assay

Precultures of LL13-040 and NC-02 with or without deletion of *FCY1* or *FUR1* (s_002/4/6/8/9/11) were prepared from three isolated colonies in 5 mL YPD medium and incubated overnight at 30°C with shaking. Precultures were diluted at 1 OD_{600} in sterile water. Subcultures of 5 mL SD medium with or without 1.56 µg/mL 5-FC in 50 mL Falcon tubes were inoculated at a final concentration of 0.1 OD_{600}. After a 5h incubation at 30°C with shaking, cultures had reached 0.5-0.6 OD_{600}. Cultures were centrifuged 5 min at 500 g and the supernatants were filtered, then transferred to a sterile Greiner 96-well plate. The media "conditioned" by the growth of the *fcy1*Δ mutant were inoculated with either the *FUR1* null mutant or the WT. The media conditioned by the growth of the *FUR1* null mutant were inoculated with either the *fcy1*Δ mutant or the WT. Cultures were inoculated at a final concentration of 0.1 OD_{600}. Growth curves were acquired as described above.

Data availability

All sequencing data are available at the NCBI Sequence Read Archive (SRA) under BioProject PRJNA952138. Details on demultiplexing are provided in Supplemental Data 3. All scripts (analyses and figure generation) are available on the dedicated repository. Strains are available upon request.

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Conflicts of interest

None declared

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Supporting information captions

Supp Fig 1. Growth of individual mutants on solid medium. Growth corresponds to the mean area under the curve (AUC, calculated on 22 h) from four replicate colonies, for individual strains arrayed on solid media: (A) YPD, SD, SD + 25 µg/mL 5-FC and SD + 6.25 µg/mL 5-FU, incubated at 30°C and (B) YPD, SD, SD + 25 µg/mL 5-FC, SD + 0.5 µg/mL micafungin, SD + 2 µg/mL caspofungin, SD + 16 µg/mL nystatin and SD + 64 µg/mL fluconazole, incubated at 37°C. Data correspond to Figs 2 and 3A before normalization with

the WT, here represented as split violin plots. For both growth assays (corresponding to panels A and B), all *rho*$^+$ strains were gathered on a single plate, with WT controls for the two backgrounds (LL13-040 and NC-02). The mean AUC for the WT controls in each condition is indicated by a grey diamond. Similarly, the mean AUC for the WT controls present on plates with *rho*$^-$ strains (in this case, one plate for LL13-040 *rho*$^-$ strains and another for NC-02 *rho*$^-$ strains) is indicated by a red diamond.

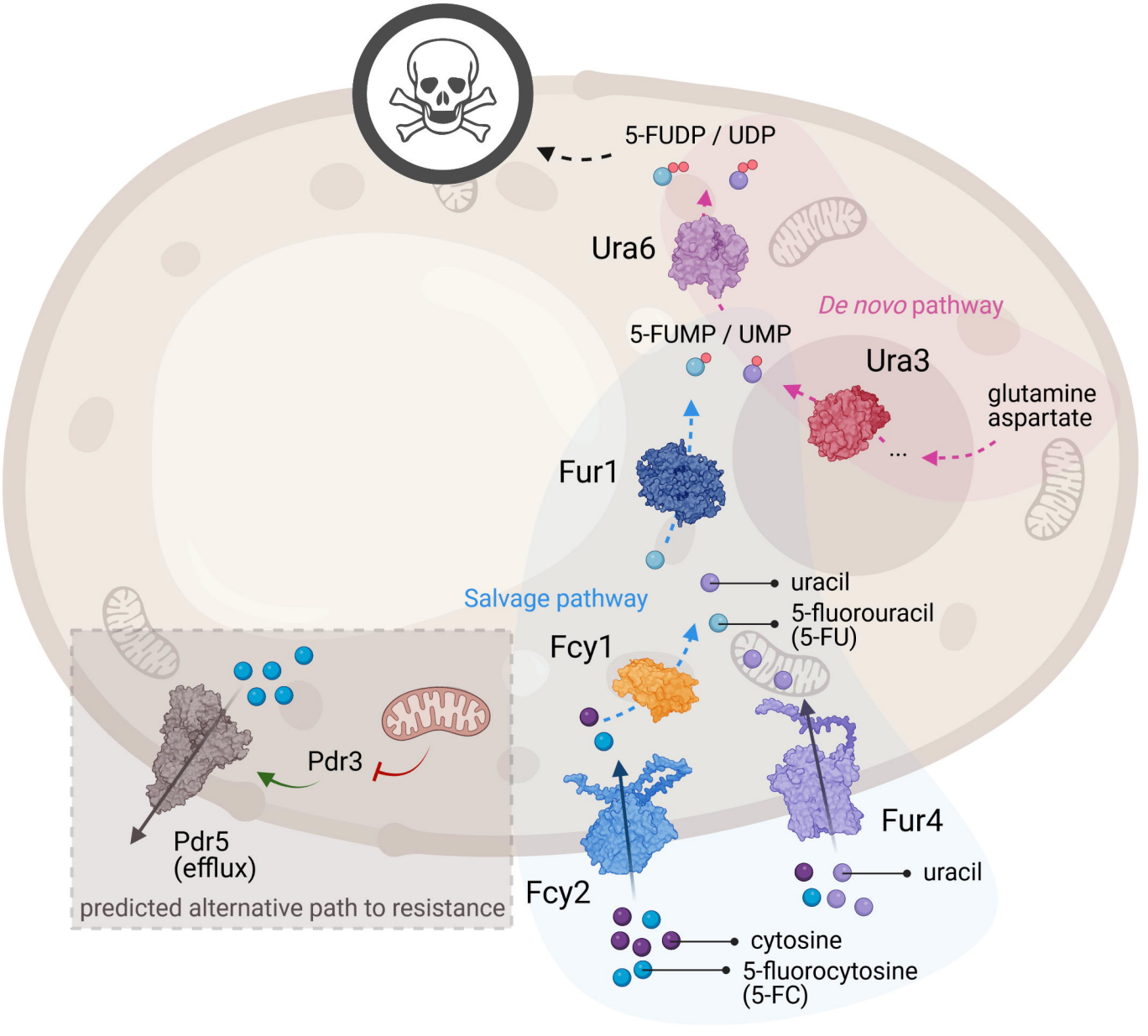
Supp Fig 2. Depth per position. Normalized coverage calculated for 5 kb windows. Each track corresponds to a sequenced genome. Red signal indicates a normalized coverage above 4X. First page corresponds to LL13-040 genomes (B10, B11…) and second page to NC-02 genomes (B14, B15…).

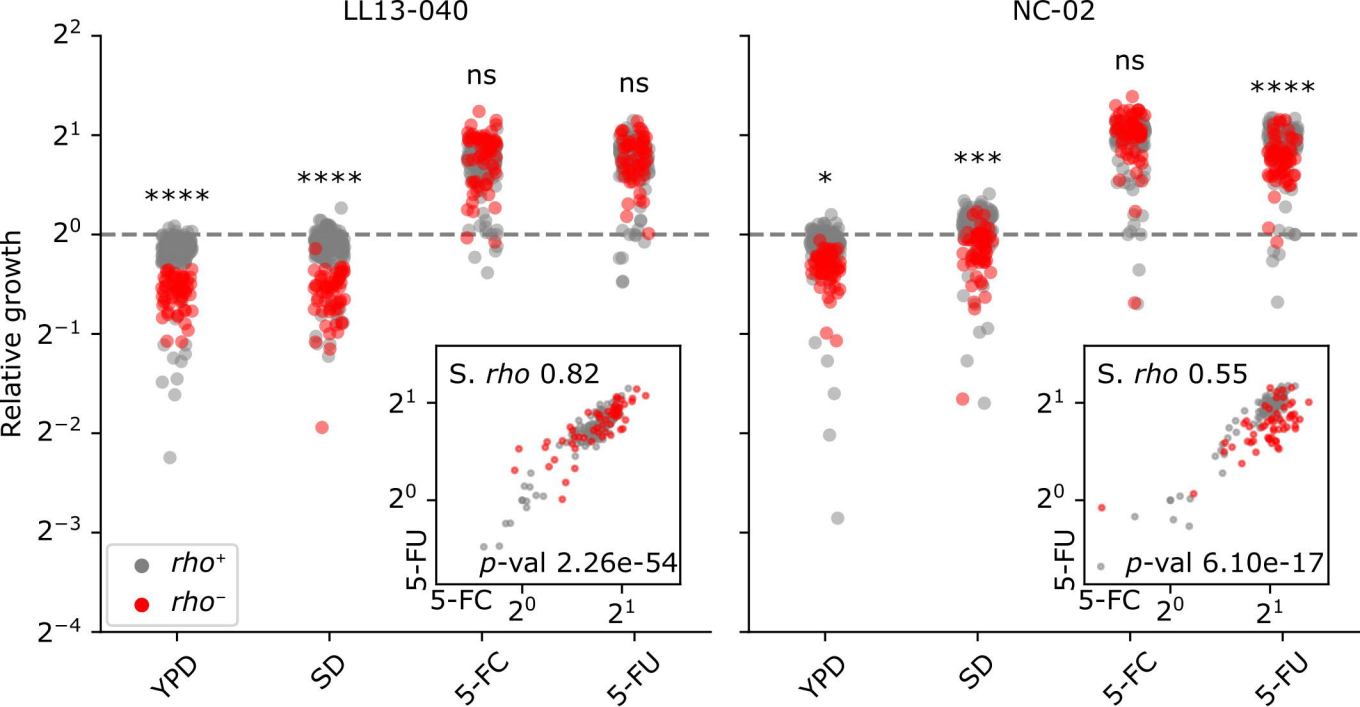
Supp Fig 3. Diversity of Fur1 mutations per preculture. Each marker (dot or triangle) represents a single preculture, from which mutants were selected (# genomes). A dashed gray line indicates if as many mutations have been identified in Fur1 as the number of genomes that carried them. For one outlier (only three mutations found in 41 strains which arose from the same preculture), a pie chart details the corresponding mutations and the number of strains that carried them.

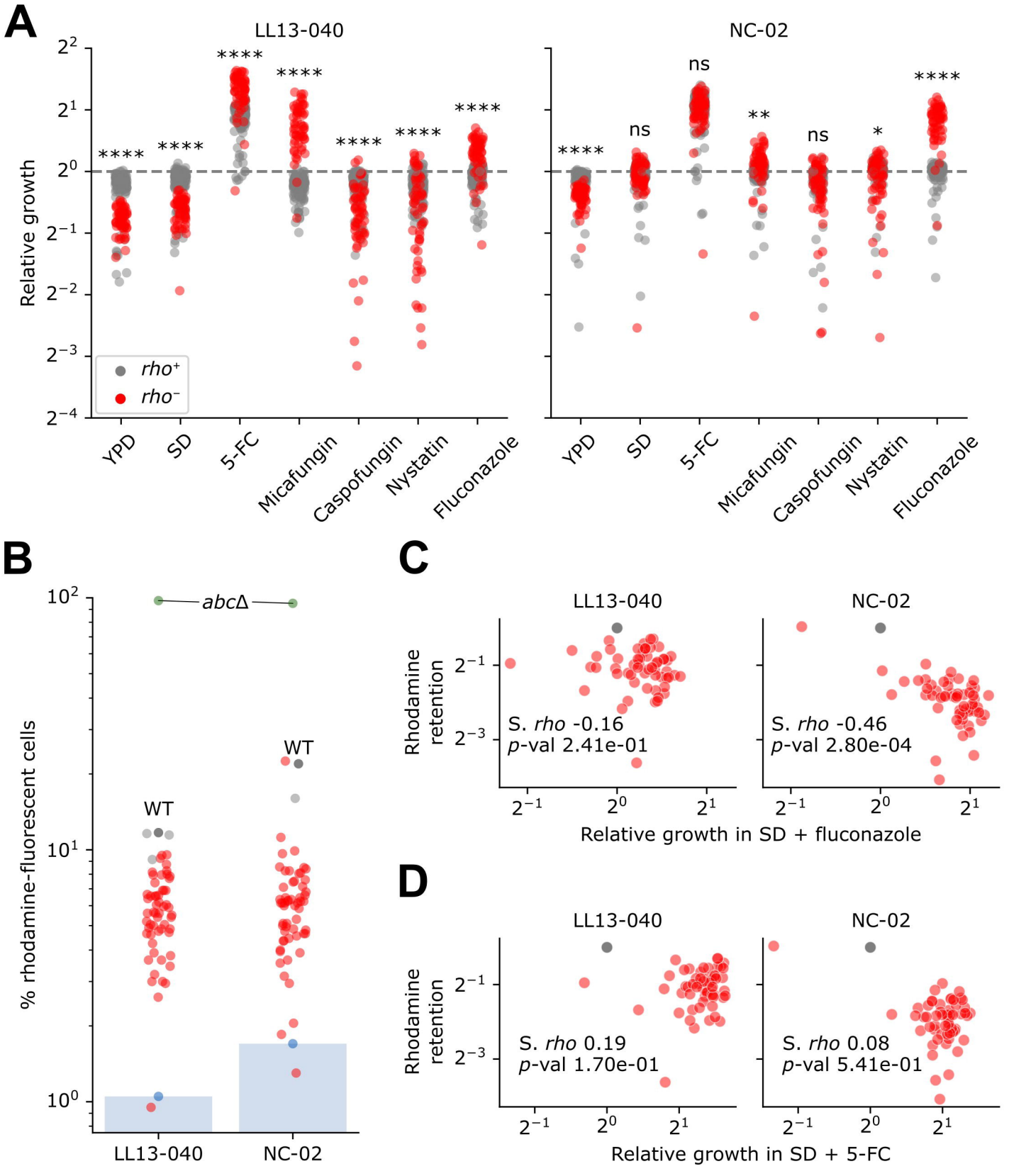
Supp Fig 4. Fcy1 functionality in parental strains. A) Density plots showing the fluorescence signal of *FCY1*-mEGFP (green), compared to the control without fluorescent reporter (gray) in the background LL13-040. Signal was acquired by cytometry for 5,000 events. A single threshold (dashed gray line) was used to indicate relative percentages of events for both strains in each condition. B) Growth was measured for both parental strains in YNB + 2% glucose containing different nitrogen sources.

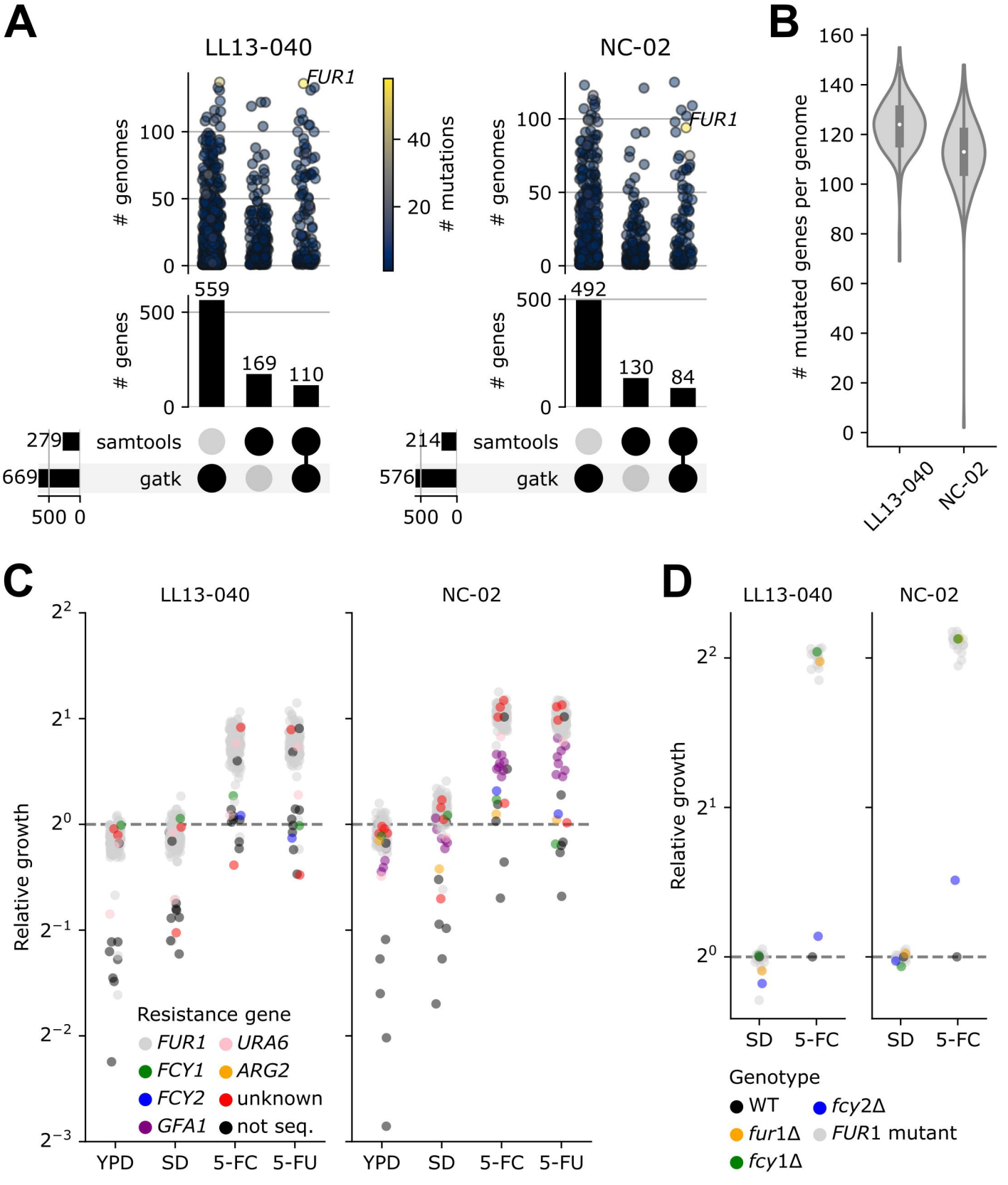
Supp Fig 5. 5-FC dose response. A) Growth assays for LL13-040 in different media supplemented or not with either 1.56 or 3.125 µg/mL 5-FC: synthetic complete medium with standard drop-out mix (SC complete), SC without uracil (SC -ura), SD (MSG) with or without 0.2% aspartate and SD (NH_4) with or without 0.2% aspartate / 0.2% glutamate. The area under the curve (AUC) parameter was calculated from three biological replicates. B) 5-FC dose-response curves in SD (MSG) for LL13-040 and NC-02. The concentration is shown on a log2 scale. 0 µg/mL 5-FC was used to normalize growth values. For each strain, the mean of three biological replicates for each concentration was used to fit the Hill equation. The corresponding IC50 and Hill coefficients are indicated.

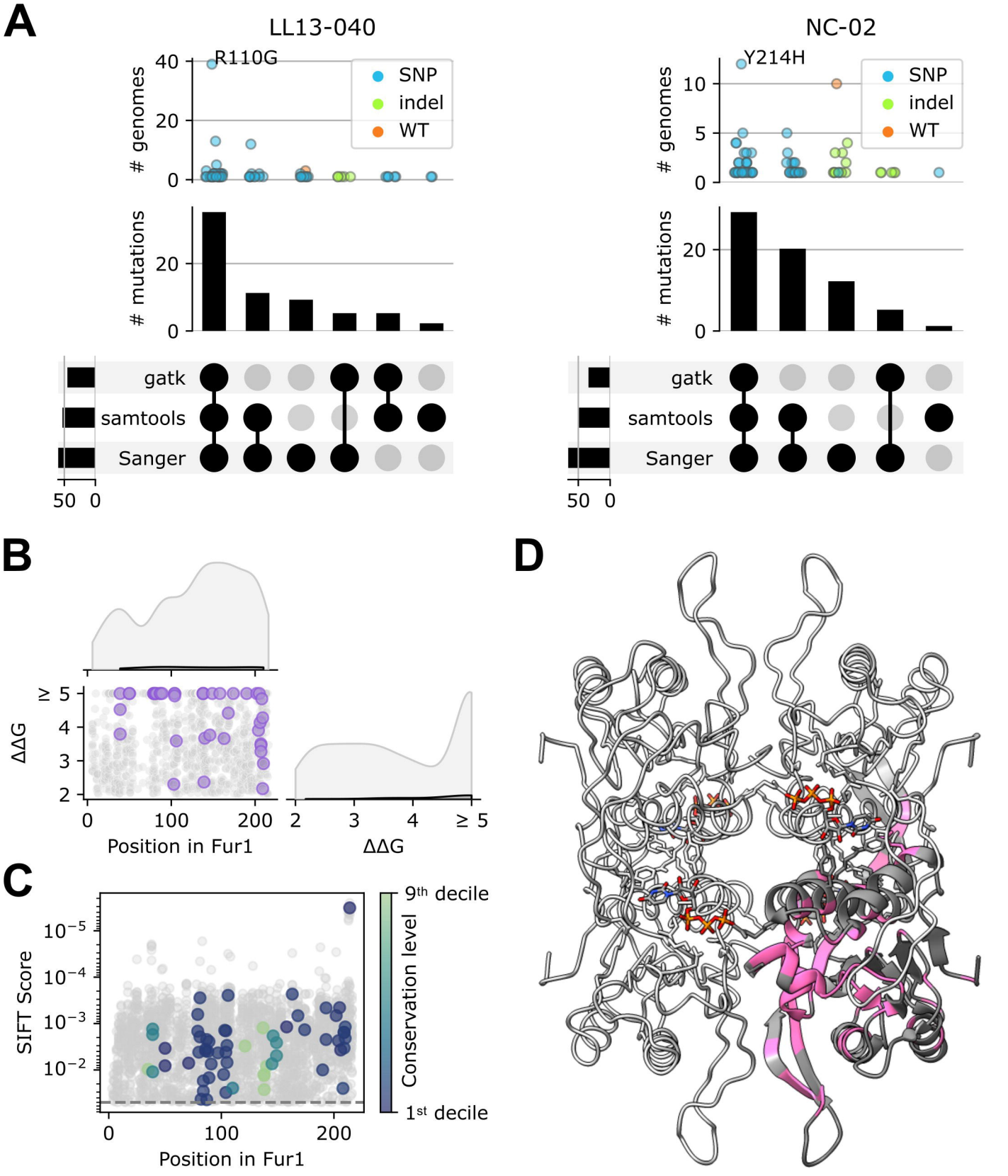
Supp Fig 6. Growth measurements on YPG agar medium. A, B) Pictures of arrays on YPG agar medium after 22 h incubation at 30°C for LL13-040 (A) and NC-02 (B). Each mutant is spotted in four replicates with the *fcy1*Δ mutant occupying the border as positive control. Grey squares highlight mutants initially misannotated as *rho*$^-$. Pictures were cropped and converted into inverted gray levels for clarity and downstream analysis of colony size. C) Growth curves were obtained by automatic detection of colony size on transformed pictures taken every 2 h for 22 h at 30°C. Relative growth corresponds to the mean area under the curve (AUC) normalized by the WT. *rho*$^-$ mutants included in all other figures are colored in red. Grey dots correspond either to the WT control (on the dotted line corresponding to a relative fitness of 1), or to the misannotated mutants mentioned above, which were therefore excluded from all analyses except the rhodamine accumulation experiment.

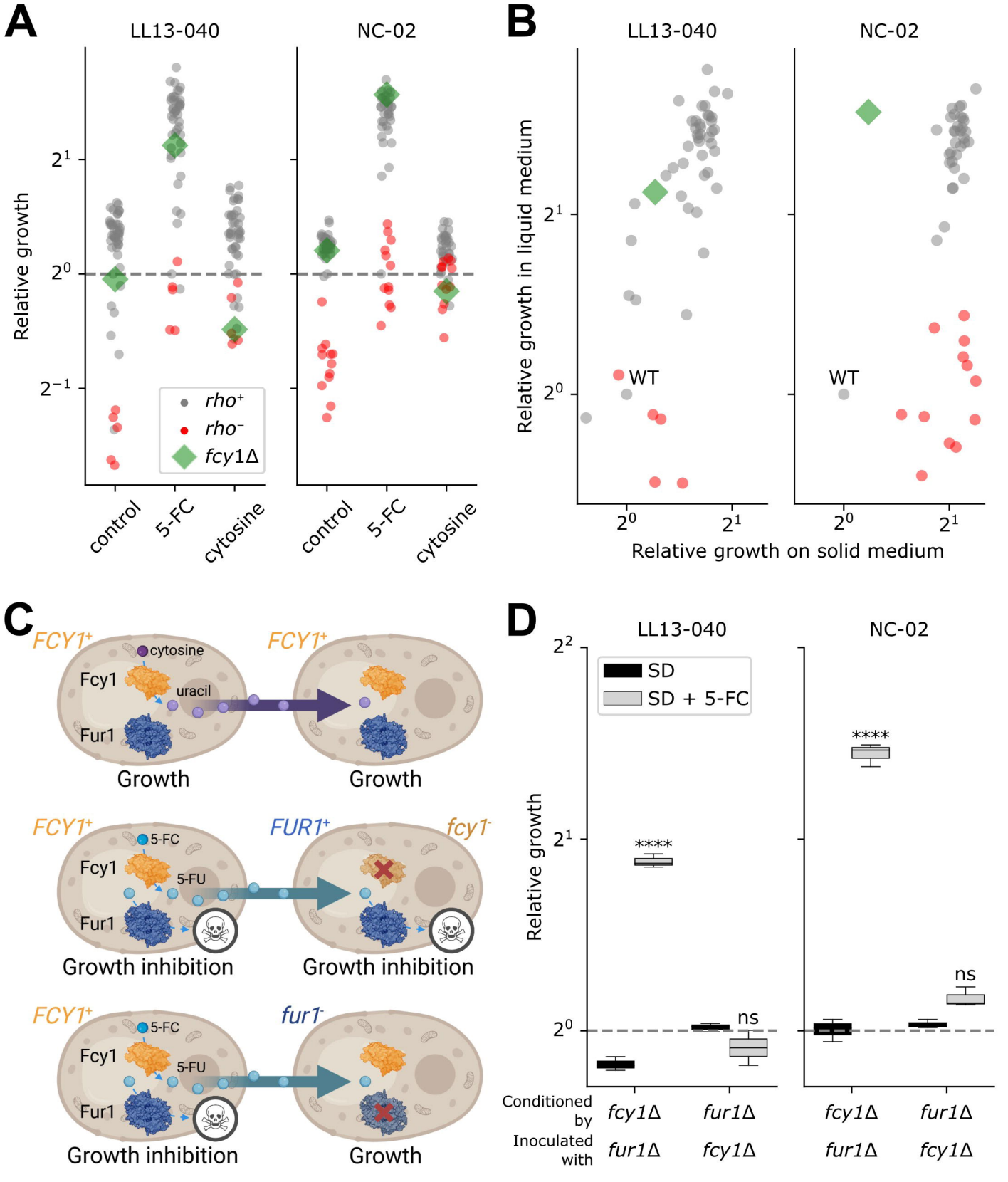

5-FUDP / UDP
Ura6
De novo pathway
5-FUMP / UMP
Ura3
glutamine
aspartate
...
Fur1
uracil
Salvage pathway
5-fluorouracil
(5-FU)
Fcy1
Pdr3
Pdr5
(efflux)
predicted alternative path to resistance
Fur4
uracil
Fcy2
cytosine
5-fluorocytosine
(5-FC)


LL13-040
NC-02
Relative growth
2^2
2^1
2^0
2^{-1}
2^{-2}
2^{-3}
2^{-4}
****
****
ns
ns
*
***
ns
****
S. rho 0.82
p-val 2.26e-54
S. rho 0.55
p-val 6.10e-17
5-FU
5-FC
rho+
rho−
YPD
SD
5-FC
5-FU


A
LL13-040
NC-02
Relative growth
2^2
2^1
2^0
2^{-1}
2^{-2}
2^{-3}
2^{-4}
****
ns
**
*
rho+
rho−
YPD
SD
5-FC
Micafungin
Caspofungin
Nystatin
Fluconazole
B
abcΔ
WT
% rhodamine-fluorescent cells
10^2
10^1
10^0
LL13-040
NC-02
C
LL13-040
NC-02
Rhodamine retention
S. rho -0.16
p-val 2.41e-01
S. rho -0.46
p-val 2.80e-04
Relative growth in SD + fluconazole
D
LL13-040
NC-02
Rhodamine retention
S. rho 0.19
p-val 1.70e-01
S. rho 0.08
p-val 5.41e-01
Relative growth in SD + 5-FC


A
LL13-040
NC-02
FUR1
genomes
mutations
genes
559
169
110
492
130
84
279
669
214
576
samtools
gatk
500 0
B
mutated genes per genome
LL13-040
NC-02
C
Relative growth
Resistance gene
FUR1
URA6
FCY1
ARG2
FCY2
unknown
GFA1
not seq.
YPD
SD
5-FC
5-FU
D
Genotype
WT
fcy2Δ
fur1Δ
FUR1 mutant
fcy1Δ


A
LL13-040
NC-02
R110G
Y214H
SNP
indel
WT
genomes
mutations
gatk
samtools
Sanger
50 0
B
ΔΔG
Position in Fur1
C
SIFT Score
Conservation level
9th decile
1st decile
D


A
LL13-040
NC-02
Relative growth
2^1
2^0
2^{-1}
rho+
rho−
fcy1Δ
control
5-FC
cytosine
B
LL13-040
NC-02
Relative growth in liquid medium
WT
Relative growth on solid medium
C
FCY1+
cytosine
Fcy1
uracil
Fur1
Growth
FUR1+
fcy1-
5-FC
5-FU
Growth inhibition
fur1-
D
SD
SD + 5-FC
****
ns
Conditioned by
Inoculated with
fcy1Δ
fur1Δ