Asymmetrical dose-responses shape the evolutionary trade-off between antifungal resistance and nutrient use

Philippe C Després[1,2,3,4], Angel F Cisneros[1,2,3,4], Emilie MM Alexander[1,2,3,4], Ria Sonigara[1,2,4,5], Cynthia Gagné-Thivierge[1,2,3,4,5], Alexandre K Dubé[1,2,3,4,5], Christian R Landry[1,2,3,4,5]*

1. Département de Biochimie, de Microbiologie et de Bio-informatique, Faculté des Sciences et de Génie, Université Laval, G1V 0A6, Canada

2. Institut de Biologie Intégrative et des Systèmes, Université Laval, G1V 0A6, Canada

3. PROTEO, Le regroupement québécois de recherche sur la fonction, l'ingénierie et les applications des protéines, Université Laval, G1V 0A6, Canada

4. Centre de Recherche sur les Données Massives, Université Laval, G1V 0A6, Canada

5. Département de Biologie, Faculté des Sciences et de Génie, Université Laval, G1V 0A6, Canada

* To whom correspondence should be addressed: PCD: philippe.despres.3@ulaval.ca, CRL: Tel: 1-418-656-3954, Fax 1-418-656-7176, christian.landry@bio.ulaval.ca

Abstract

Antimicrobial resistance is an emerging threat for public health. The success of resistance mutations depends on the trade-off between the benefits and costs they incur. This trade-off is largely unknown and uncharacterized for antifungals. Here, we systematically measure the effect of all amino acid substitutions in the yeast cytosine deaminase Fcy1, the target of the antifungal 5-FC (flucytosine). We identify over 900 missense mutations granting resistance to 5-FC, a large fraction of which appear to act through destabilisation of the protein. The relationship between 5-FC resistance and growth sustained by cytosine deamination is characterized by a sharp trade-off, such that small gains in resistance universally lead to large losses in canonical enzyme function. We show that this steep relationship can be explained by differences in the dose-response functions of 5-FC and cytosine. Finally, we observe the same trade-off shape for the ortholog of *FCY1* in *Cryptoccocus neoformans*, a human pathogen. Our results provide a powerful resource and platform for interpreting drug target variants in fungal pathogens as well as unprecedented insights into resistance-function trade-offs.

Introduction

Pathogenic fungi are an rising threat to human health, agriculture and wildlife[1]. Therapeutic options remain limited and resistance to all antifungals classes has been observed in multiple species[2]. Very little is known about the functional landscape of antifungal targets: the genotype of a strain is rarely enough to infer its phenotype. Recent efforts to catalog mutations associated with resistance have yielded a handful of mutations per species for a few targets, mostly derived from small scale studies and not always experimentally validated[3]. More systematic efforts are being deployed to catalog resistance mutations in a comprehensive manner[4,5], but information remains sparse for most targets.

The benefit of resistance to a given drug is not the only factor that affects the long-term outcome of a mutation: the associated cost is also a key parameter[6]. For instance, a mutation in an essential protein that confers resistance but leads to a decrease in function would rapidly be lost upon treatment interruption. For example, resistance to the antifungal nystatin has been shown to have significant fitness costs in stressful environments[7], which means they would actively be selected against when nystatin concentration drops. In *Candida albicans*, aneuploidies are associated with antifungal resistance but are deleterious in many environments[8]. Both a better inventory of the mutations granting resistance and the trade-offs associated with them are key to improve our ability to predict or interpret clinically relevant genotypes in complex environments. Ideally, we would need approaches that allow us to measure this type of trade-off in a systematic and comprehensive manner for resistance mutations. Beyond their impact on antimicrobial resistance, these relationships can play large roles in shaping which evolutionary trajectories are available in the face of harsh selective pressures, from adaptation to new environments in the wild to cancer evolution.

Most antifungals used in the clinic are drugs that bind their targets and disrupt their activity or impair their structural integrity[9,10]. For instance, azoles bind to the active sites of their targets and inhibit their function. Resistance mutations therefore tend to occur in the substrate binding pocket[11]. An exception to this is 5-fluorocytosine (5-FC, also known as flucytosine), which is administered as a pro-drug that is deaminated into the cytotoxic nucleotide analog 5-fluorouracil (5-FU) inside fungal cells. The accumulation of 5-FU leads to defects in mRNA synthesis and protein translation as well as DNA damage through thymine starvation[12], resulting in dose-dependent fungistatic or fungicidal activity. As one of the oldest antifungals, 5-FC is on the WHO list of essential medicines and is often used to treat cryptococcal meningitis in combination with amphotericin B[13].

In yeast, the cytosine deaminase Fcy1 (Fca1p in *Candida albicans*) forms a homodimeric enzyme that converts 5-FC to 5-FU, and loss of function of this protein leads to 5-FC resistance[14]. Fcy1 is widely conserved throughout fungi, and has a distinct fold compared to the *Escherichia coli* cytosine deaminase[15]. As vertebrates lack a *FCY1* ortholog, 5-FC can be administered as a prodrug that will be selectively converted by fungal cells into 5-FU. *FCY1* deletion was shown to confer full 5-FC resistance to *S. cerevisiae*[14], with little effect on fitness in multiple lab conditions[16,17]. Gene loss of function, for instance through premature stop codons, thus appears to be an easily accessible mechanism of resistance. However, since Fcy1 is a dispensable enzyme in standard growth media, it is unclear if 5-FC

resistance through loss of function leads to any trade-off since enzyme function is generally
decoupled from growth. In contrast, other antifungal targets, such as *ERG11* or *FKS1*, are
considered essential for growth and thus for virulence in some pathogens[18]. Interestingly,
FCY1 can be conditionally essential in yeast strains where the *de novo* uracil synthesis
pathway has been disrupted. In this genetic background, cells must rely on the pyrimidine
salvage pathway, where Fcy1 deaminates cytosine into uracil, complementing the
auxotrophy. For example, in the lab strain BY4743, *FCY1* masks the loss of function of
URA3 in minimal media supplemented with cytosine[19]. By changing environmental
conditions, the interplay between resistance and Fcy1 activity can thus serve as a model for
a common scenario where an essential gene imposes strict constraints on available
evolutionary trajectories because of cell metabolism requirements.
105
An important knowledge gap regarding the emergence of resistance is whether complete
loss of function of Fcy1 is necessary or if partial loss of activity is sufficient, which would
dramatically increase the mutational target size. Because the natural substrate, cytosine,
and the toxic analog 5-FC have very similar structures, it is likely that a loss in the ability to
process 5-FC will also lead to a loss in the ability to metabolize cytosine. Recent work
examining the activity of enzyme variants on different substrates at high throughput have
shown that the level of correlation between mutation effects can vary depending on
substrate pairs and the enzyme itself[20,21]. As such, it is difficult to predict which trade-off
scenario applies in the case of Fcy1. If it is weak, there may be mutations that lead to
resistance while maintaining the ability of the cell to utilize cytosine. These mutations could
in principle lead to resistance without seriously affecting fitness in the absence of the drug.
The relationship could be linear, in which case a 50% increase in resistance would lead to a
50% decrease in cytosine use. At the other end of the spectrum, there may be a strong
trade-off, which means that all mutations leading to 5-FC resistance impede cytosine use.
The emergence of loss of function alleles would entirely be at the expense of Fcy1 activity.
For some types of coding sequence mutations, such as frameshifts or premature stop
codons, loss of function can be easy to predict. However, significant challenges and
uncertainty remain when trying to predict the effect of missense mutations distal from the
active site. Possible consequences could include allosteric effects on enzymatic function, or
simply destabilisation of the protein structure or Fcy1 dimer assembly. These perturbations
would lower the effective protein concentration, potentially leading to loss of function
phenotypes. Sensitivity to destabilisation would then be another key parameter in
determining the mutational target size for resistance mutations.
129
Recent advances in genome editing and DNA sequencing have greatly increased the
throughput at which protein variants can be assayed, allowing to draw a high-resolution map
of fitness effects and potential trade-offs. Here, we use Deep Mutational Scanning (DMS) to
explore the functional landscape of *FCY1* at its endogenous locus in the context of 5-FC
resistance and cytosine deamination. We systematically assess protein variants to
determine which mutations lead to drug resistance and explore which protein properties are
linked to it. We examine how resistance mutations are distributed on the enzyme and we
characterize the trade-off between resistance and cytosine use. We then show that this
trade-off is conserved in the *FCY1* ortholog of *C. neoformans*.
139

Results

Deep mutational scanning of Fcy1

We used the megaprimer method[22] to generate all possible single codon changes along the
FCY1 coding sequence (see Methods). This approach results in the full set of premature
stop codons, silent mutations and single position missense mutants (which result in protein
variants). We integrated the mutations at the native *FCY1* locus by using a "bait and switch"
genome editing strategy[23,24]. Since our approach requires direct sequencing of the coding
sequence and because *FCY1* is too large to sequence with a single read pair using high-
throughput sequencing, we split our mutants into three pools each covering partly
overlapping sections of the coding sequence (Figure 1A). We used two different media
compositions: media with 5-FC to select for resistance, and media without uracil but
enriched with cytosine (cytosine media) to select for cytosine deamination into uracil. In this
cytosine media, the reaction is essential for growth because it complements the uracil
auxotrophy of the strain. Using this approach, growth rate can be used as a reporter for Fcy1
activity for both enzymatic reactions in pooled competition assays (Figure 1B). Comparing
the results in 5-FC and cytosine media allows us to measure the strength of the trade-off
between the two phenotypes across the mutational landscape for all single mutations (Figure
1C). To define the conditions for the pool competitions, we identified which 5-FC or cytosine
concentrations reduce growth rate to ~50% of the maximum (also known as IC_{50}, Figure S1).
This corresponds to roughly 12 µM and 84 µM for 5-FC and cytosine, respectively.
160

During preliminary experiments, we observed growth patterns on solid media suggesting that
fcy1Δ strains could grow on cytosine media if wild-type strains were also present on the
plates (Figure S2). We hypothesized this could be due to cross-feeding between wild-type
and mutant cells, which could potentially impact selection strength during the pooled
competition assay. Previous work has indeed shown that the yeast plasma membrane can
leak uracil in the surrounding media[25]. The main contributor to uracil uptake in yeast is the
transporter *FUR4*[26], the deletion of which is lethal when combined with a non functional
URA3 allele in standard media[27]. As uracil auxotrophy is essential to one of our selection
conditions, we could not disrupt *FUR4* to prevent cross-feeding. Instead, we quantified the
impact of potential uracil leakage on selection intensity in liquid cultures. Using a cytometry
based competition assay, we measured the effect of different cytosine concentrations and
population compositions on the selection coefficient of a *FCY1* deletion. We found that high
cytosine concentrations and a higher initial proportion of wild-type cells tended to lower
selection strength against *fcy1Δ* (Figure S3). Reassuringly, the selection coefficient
remained above 0.3 at the cytosine IC_{50} (84 µM), even in populations with as much as 90%
of wild-type cells. This is well above the threshold required to detect fitness effects in a high-
throughput sequencing based assay[28].
178

We extracted genomic DNA and amplified the appropriate *FCY1* region for each pool and
condition, and measured changes in allele frequencies between the start and the end of the
competition. Variant libraries before selection had high coverage, with over 99% of single
codon mutants with 10 or more read counts (Figure S4). Synonymous codons were used as
internal replicates for individual amino acids: log_2 fold-change for each protein mutant was
calculated as the median of the codon level fold-changes. Spearman's correlation coefficient

for replicate pairs ranged between 0.814 and 0.945 (Figure S5). To adjust for the differences
between the different mutant pools (each covering a third of the coding sequence), we
harmonized the $\log_2$ fold-change to pool 2 using the variants in the overlapping regions of
the pools. The $\log_2$ fold-changes of mutants in these two regions (codons 49 to 67 and 93 to
110 respectively) were well correlated between pools (Figure S6 and S7A-F). We then used
the distribution of $\log_2$ fold changes from the synonymous and nonsense mutants to put both
5-FC and cytosine conditions on the same scale to obtain the DMS score (Figure S7G and
H). This allowed us to obtain a full-length functional landscape of Fcy1 that covered 99.5%
(2968/2983) of possible missense variants in both selection conditions (Figure 2A and B).
194
The distributions of DMS scores are bimodal, with the two modes corresponding to WT
sequences and premature stop codons (Figure S8A-C). Stop codon mutants can have
different effects based on their position in the coding sequence, with generally weaker
effects near the end of the coding sequence[29]. We observed that only the last amino acid of
the C-terminus was partially dispensable. Surprisingly, stop codons that occured before the
methionine in position 6 did not grant 5-FC resistance, suggesting that Fcy1 translation from
an alternative start site, Met 6, remains efficient enough to confer sensitivity. We excluded
stop codon mutants from positions 2-5 and 158 from downstream analyses.
203
Across the mutant library, 32% (940/2968) of missense mutants had 5-FC DMS scores that
fell in the range observed for nonsense mutants (5-FC DMS score ≥ 0.921, 1st percentile of
nonsense scores). Of the resistant mutants, 284 can be recapitulated by a single nucleotide
change in the *S. cerevisiae FCY1* coding sequence, representing a large set of easily
accessible mutations leading to resistance. The mutational target size of resistance is
therefore much larger than stop codon mutations. For mutants in pool 2, which cover most of
the substrate binding and catalytic residues of Fcy1, we also performed the experiments in
an additional condition to investigate whether some mutations could improve cytosine
specificity. To test this, we used cytosine media with 24 µM 5-FC and 113 µM cytosine, in
which wild-type cells grew at a similar rate as in the two other conditions. Mutant scores
were highly correlated between the cytosine and 5-FC+cytosine condition (Figure S7D), with
the only difference being better signal in the latter, probably due to more stringent selection.
Based on this comparison, we did not identify any mutants in our library for which we could
infer increased specificity for cytosine over 5-FC compared to the wild-type. Having
catalogued which amino acid changes conferred resistance or maintained Fcy1 activity, we
next sought to better understand the mechanisms behind mutational effects.

220 5-FC Resistance mutations are distributed across the protein
221 and are amino acids rarely observed at orthologous sites

Mapping the DMS scores on the Fcy1 structure (Figure 2C and 2D), we found that the active
site and the residues in close contact with the substrate were extremely sensitive to
mutations. Our results corroborate what has been observed in small-scale studies on Fcy1
biochemistry. For example, Glu 64 is essential for catalytic activity[30], and we did not observe
any variant at this position that maintained 5-FC sensitivity or growth in cytosine. Focusing
on functional sites known to be involved in the catalytic cycle, we found that the core
components of Fcy1 were strongly intolerant to mutations with respect to cytosine use
(Figure 3A). Surprisingly, most resistance mutations do not reside in the active site of the

enzyme, suggesting that the enzyme is very sensitive to amino acid changes, either by
allosterically affecting the enzymatic activity or perturbing the stability of the complex.
232
To better understand the constraints linked with maintaining Fcy1 function, we assembled a
set of 215 orthologs (Figure S9) and hypothesized that conserved sites would be more
sensitive to mutations. On average, amino acids from *S. cerevisiae* Fcy1 were present in
60% of orthologous sequences at the homologous positions, but the distribution of
occupancy was bimodal (Figure S10A). As much as 18.5% of residues were observed in
more than 95% of sequences, indicating strong structural and functional constraints
maintaining residues for millions of years of evolution. Sites not part of the active site or
involved in the dimer interface accounted for 41% of highly conserved amino acids,
highlighting the importance of distal sites for Fcy1 function.
242
We directly investigated the effect of missense mutants in our library for which an equivalent
wild-type maino acid could be found in orthologs. Variants observed in orthologous
sequences had much lower 5-FC scores and higher cytosine scores compared to those not
observed (Figure 3B). Again the distributions are bimodal, which means for instance that
some amino acids are compatible with the WT phenotype in *S. cerevisiae* but are not seen in
orthologs. Since the number of orthologs is limited and does not cover the entire sequence
space compatible with this enzyme's function, this is not unexpected. There are few cases
where amino acids present in orthologs lead to resistance to 5-FC when introduced in *S.*
cerevisiae but those mainly concern amino acids present in the most distant species, which
means that they could have been compensated for by other amino acid substitutions in the
sequence[31]. Overall, only 56 amino acid changes observed in orthologs conferred
resistance, with most of them being unique to a specific ortholog (median
occupancy=0.47%). These shared functional constraints suggest resistance to 5-FC could
arise by similar mechanisms in *FCY1* orthologs.

257 Protein complex destabilisation is a major mechanism of
258 resistance

Many of the highly conserved residues that lead to resistance when mutated are away from
the active site. This is possibly due to the importance of structural integrity of the complex or
instances of allosteric regulation of enzyme function. Using an *in silico* approach[32], we
predicted the effect on Fcy1 structure and dimer interface stability ($\Delta\Delta G$) of all missense
mutants (see methods). The predicted effects on stability of amino acid mutants were
strongly correlated with their DMS scores, being positively correlated with 5-FC resistance
($\rho=0.523$, $p=1.21\times10^{-206}$, n=2,968) and negatively correlated with cytosine DMS scores ($\rho=-$
0.422, $p=1.1\times10^{-127}$, n=2,968). Protein destabilisation is therefore a key determinant of 5-FC
resistance.
268
The dimer interface also appears to play a role in maintaining Fcy1 function. While protein
engineering efforts have investigated mutations predicted to stabilise the dimer[33], whether
dimerization is essential for activity remains unknown. The alpha-helix at the core of the
interface is highly sensitive to mutations, suggesting that dimerization is important (Figure
2C and D). We found that mutants of residues in contact with the other subunit tended to
have lower cytosine DMS scores compared to those near (within 6 Å of a residue from the

other chain) or distant from the interface (Mood's median test, p= 9.20 x10^{-6} at interface vs
near interface, p=9.15x10^{-15} near interface vs distant). Interestingly, we did not observe the
same relationship for the 5-FC DMS scores, where only the difference in median score
between mutants located near the interface and those distant was significant (Mood's
median test, p= 0.75 at interface vs near interface, p=0.018 near interface vs distant).
Leveraging our set of orthologs, we calculated the evolutionary rate for each position in the
alignment[34] (Figure S10B), which revealed that both the core dimer interface and the
interface rim evolved at a slower rate compared to other regions of the protein (Figure 3D).
While homodimer formation appears to be essential to Fcy1 function, perturbing the
interaction appears insufficient to grant resistance.
285
To validate the role of protein complex depletion on 5-FC resistance in the DMS data as well
as our *in silico* prediction of destabilisation, we decided to investigate Fcy1 dimer formation
in vivo. We used an assay that reports on the amount of protein complex formed in the cell.
Dihydrofolate reductase protein complementation assay (DHFR-PCA) is an *in vivo* assay
that allows quantitative measurement of a protein-protein interaction (PPI) by linking it to the
reconstitution of a methotrexate-resistant DHFR[35]. Growth quantitatively varies as a function
of both the abundance of the proteins interacting and PPI strength[35,36]. This provides
information on the abundance of the Fcy1 dimer in conditions in which *FCY1* is dispensable
so as to not confound the PPI signal with the fitness of the mutants. Starting from a set of
individually reconstructed mutants (see next section), we examined 54 Fcy1 variants to
measure PPI abundance. We measured the interaction between a wild-type copy of Fcy1
and each mutant, which reflects the stability and ability of the WT-mutant complex to form.
Replicate control and methotrexate growth rates of the DHFR-PCA assays were well
correlated between replicates (Figure S11A and B) and did not reveal any bias due to lower
overall growth of some mutants (Figure S11C). As expected if destabilisation were causing
resistance, variants that clustered with silent mutations in the DMS assays generally had
detectable PPI. In contrast, those that clustered with stop codon mutants generally lost the
interaction (Figure 3E, p=0.006, Mood's median test). Importantly, PPI intensity was well
correlated with FoldX predicted effects on protein structure stability (ρ = -0.475, p=0.0003,
Figure S11D), as well as with variant growth in cytosine (ρ=0.375, p=0.009, Figure S11E)
and negatively correlated with 5-FC resistance (ρ =-0.477, p=0.0005, Figure S11F). These
results directly link altered protein complex abundance with mutant phenotypes, and validate
the use of *in silico* tools to predict the effect of mutations on Fcy1 stability. On the other
hand, there was no correlation between PPI intensity and *in silico* changes in PPI stability as
predicted by FoldX (ρ=0.156 and p=0.259 for 54 mutants), limiting the relevance of such
metrics for Fcy1. Based on these observations, structure or subunit destabilisation leading to
lower protein complex abundance appears to be a frequent mechanism driving Fcy1 loss of
function.
314

A strong trade-off links 5-FC resistance and cytosine use

316
At first glance, 5-FC and cytosine DMS scores appear negatively correlated with each other.
This is visible in Figure 2: sites that lead to resistance to 5-FC when mutated generally have
reduced fitness in cytosine media. However, the scores are overall only weakly correlated

(ρ=-0.23, p=5.5x10^{-42}, n=3,272 variants). Directly examining the relationship between scores
(Figure 3F) revealed that a large fraction of mutant were unfit in both conditions, as well
clusters of variants with phenotypes similar to silent or nonsense mutations (green and red
respectively). A potential explanation for this would be a very sharp trade-off resulting in a
front minimum that leads to near vertical and horizontal curves outside of the inflexion point
(i.e. the black line in Figure 1C). Due to the lack of resolution inherent to DMS assays, the
precise nature of this relationship is difficult to determine from this type of data alone. To
verify this hypothesis and validate the DMS scores, we selected 88 missense mutants
across the two-dimensional phenotypic landscape inferred from DMS (Figure S12A) and
generated the corresponding strain using genome editing by inserting the individual
mutations at the *FCY1* locus. We included most mutants that showed large variation from
the main clusters or that appeared to have high scores in both 5-FC and cytosine, as such
outliers could have important implications for the emergence of antifungal resistance by
escaping the trade-off. Based on the similarity of their DMS scores with nonsense or
synonymous mutations, we assigned 46 mutants to clusters with another 42 that were
considered outliers or had not been covered by the DMS assay.
336
For each mutant, we measured growth rates in 5-FC and cytosine DMS conditions (see
methods). Growth rate replicates were highly correlated (Figure S12B and C). The 5-FC
DMS scores were well correlated with validation growth rates (Figure S12G, ρ=0.72,
7.38x10^{-14}), but the correlation was weaker for cytosine scores (Figure S12H, rho=0.30,
0.013), potentially due to noise introduced by uracil leaking in the large-scale experiment.
This is supported by a stronger correlation between growth in cytosine and 5-FC+cytosine
DMS scores for the mutants that were part of pool 2 (Figure S12I, ρ=0.568, p=0.0002,
n=38). We observed a striking pattern in the relationship between a mutant's growth rate in
5-FC and cytosine: all variants, including the outliers from the DMS screen, fell along the
same front (Figure 4A). The relationship was well described by a power law equation with a
negative k, characteristic of a strong trade-off, for which we determined parameters using a
linear regression on the log$_2$ transformed growth rates of individual mutants. In effect, this
strong trade-off means that a Fcy1 mutation increasing growth rate in 5-FC media by ~25%
will incur a cost equivalent to ~67% of growth rate of the wild-type in cytosine media. The
steepness of the relationship generates a front minimum where a large fraction of mutants
are unfit in both 5-FC and cytosine media, as seen in the DMS scores. We found that 45/46
of the mutants to which we had assigned a cluster in the DMS experiments maintained the
same position in the validations. The better signal to noise ratio of the growth curve assay
also allowed us to precisely measure the correlation between phenotypes, which was very
strong (ρ=-0.851, p=1.83x10^{-25}, n=87 variants). Finally, we note that the trade-off escape that
was observed for some outliers of the DMS experiments could not be validated with these
small scale validations.
359
Both the DMS results and our one-by-one growth curves point towards a sharp trade-off
between resistance and cytosine use. We examined what the source of this strong trade-off
might be. First, we examined wether the imbalance in the effect of mutations might be the
result of large differences in the catalytic efficiency of Fcy1 when deaminating cytosine
instead of 5-FC. However, based on reported values in BRENDA[37], this is not the case: the
distributions of catalytic efficiencies reported for the two substrates overlap, and the K_{cat}/K_M
of the median values shows only a small increase in efficiency for 5-FC deamination (111.9
mM^{-1}s^{-1} vs 104.4 mM^{-1}s^{-1}, Figure S13). Furthermore, our dataset also contains some mutants

whose presence in the front minima would be difficult to attribute to change in the catalytic
efficiency of the enzyme. The stop codons mutants from positions 2-5, which did not confer
5-FC resistance, cluster with the front minimum mutants in the DMS data. These mutants
are instead expected to affect phenotype through lower protein levels due to the alternative
translation initiation site and not through changes in the *FCY1* coding sequence. These
observations point toward a difference in the interplay between protein complex abundance
or function and the response to 5-FC and cytosine levels as a way to explain the shape of
the trade-off.
376
As the validations experiments showed, the phenotypes of Fcy1 in 5-FC and cytosine
variants are strongly anticorrelated, but the effects are asymmetric. Additionally, there does
not appear to be any major difference between substrates in terms of metabolic flux for the
wild-type enzyme. Accordingly, we hypothethized that the asymmetry we observe in the
trade-off must be a cellular property linked to pathway flux and how it connects to growth
rate, which should be reflected in the dose-response to each compound. While the dose-
response assays shown in Figure S1 were sufficient to determine IC_{50} in preparation of our
DMS experiments, they did not cover the full range of response to 5-FC and had only
minimal coverage of the range of cytosine concentrations where most changes occurred. We
therefore performed a second round of dose-response curves with a greater emphasis on
precisely determining curve parameters (Figure 4B and 4C). The IC_{50} and Hill coefficient we
had first determined were close to the values we found in our second round of experiments
(5-FC: 12 µM vs 9.14 µM, cytosine: 84 µM vs 71 µM). The most striking difference between
5-FC and cytosine response lies in the steepness of changes around the IC_{50}. Growth
inhibition by 5-FC varies along a gradient that spans a 2,048-fold difference in drug
concentration: for cytosine, variation occurs within a range spanning only a 4-fold difference.
393
The dose-response curves explain the shape of the trade-off between 5-FC resistance and
cytosine use we observe. Because of this difference in steepness around the IC_{50}, the same
level of loss of function leading to lower metabolic flux will result in different outcomes
(Figure 5A). As the level of loss of function due to a mutation increases, the dose-curve
response is expected to shift to reflect the loss of Fcy1 enzymatic activity (shown as the grey
lines in Figure 5B and C), until there is no response to the drug or nutrient anymore (the red
line). For example, a 2-fold decrease in effective 5-FC concentration due to lower Fcy1
activity starting from the IC_{50} would result in 36% (instead of 50%, Figure 5B) growth
inhibition based on the dose-response curve parameters. The same change in effective
cytosine concentration due to loss of activity instead leads to loss of the ability to grow (from
50% to 1.4% relative growth, Figure 5C). This leads to strong asymmetry that explains the
front minimum we observed in the DMS experiment. This difference in dose-response should
result in strong unidirectional effects, so that mutants having a full resistance phenotype
would not recover the ability to use cytosine at higher concentrations. Conversely, some
mutants in the front minima should be able to grow if provided with higher cytosine
concentrations to offset their lower Fcy1 activity.
410
To test our hypothesis, we performed growth curves and measured the area under the curve
(AUC) at 24h for 72 validation mutants in five additional 5-FC and cytosine concentrations
higher than previous conditions (Figure 5D, full growth curves shown in Figures S14 and
S15 and AUC distributions in S16). Large increases in cytosine concentrations (450 µM and
901 µM) could restore growth to levels comparable to the wild-type for multiple mutants that

showed no growth at concentrations closer to the IC_{50}. Conversely, a few mutants showed
some level of sensitivity to higher levels of 5-FC (i.e L37F, T60F, T60R), suggesting residual
levels of Fcy1 activity. Many front minima mutants (i.e. G76H, K80L, D92C) had sharp on/off
dynamics in cytosine, consistent with the shifted dose-response curve our model predicts.
We observed differences in growth between 450 µM and 901 µM cytosine for some mutants,
indicating that the activity of the cytosine permease *FCY2* was not limiting in our assay.
Importantly, the relationship between responses to 5-FC and cytosine was correctly
predicted. No fully resistant mutant recovered the ability to use cytosine, and resistance
gains were always linked to greater losses in cytosine use. The phenotypes of Fcy1 mutants
therefore support our trade-off model based on metabolic flux and shaped by different
response patterns to 5-FC and cytosine.
427
Finally, we also found that the predicted effect of a mutation on structure stability was a
strong predictor of whether it behaved as a complete loss of function (no response to either
5-FC or cytosine), as would be expected in a situation where protein complex abundance is
critical. Mutations with a predicted ΔΔG from 1 to 5 Kcal/mol were significantly more likely to
retain sensitivity or growth at higher compound concentrations compared to those with ΔΔG
> 5 Kcal/mol (19/27 vs 6/18, odds ratio = 7.125, p= 0.002, two-sided Fisher's exact test).
Thus, we find that changes in protein stability, which translates into lower complex
abundance, are an important factor in determining the level of loss of function of Fcy1 and
predicting resistance.
437

438 The resistance-function trade-off is conserved in a *FCY1*
439 ortholog

Having characterized the relationship between 5-FC resistance and cytosine use, we
wondered if our observations were specific to the *S. cerevisiae FCY1* (*scFCY1*) or was
conserved in its orthologs. In clinical settings, 5-FC (flucytosine) is most often used against
Cryptococcus species. We therefore tested whether mutants of the *C. neoformans* ortholog
of *FCY1* (*cnFCY1*) were constrained by a similar trade-off. The two proteins share 41%
residue identity and 58% residue similarity, implying a similar fold and catalytic site
architecture. Since the structure of cnFcy1 has not been solved, we used AlphaFold[38,39] to
predict it. We found it was almost identical to scFcyp (RMSD=0.631 Å over 141 residues,
Figure 6A), with matching sequence alignment positions generally sharing the same position
in the structure.
450
To test *cnFCY1* variants, we codon optimized the protein of the ortholog for expression in
yeast and used it to replace the endogenous *FCY1* coding sequence. We then generated 27
strains with *cnFCY1* alleles matched to *scFCY1* validation mutants from across the
phenotypic space (Figure S17A), prioritizing variants at conserved positions. We then
performed spot dilution assays to compare the 5-FC resistance phenotype of each variant
pair at high 5-FC concentration (194 µM). Yeast expressing *cnFCY1* showed the same level
of sensitivity to 5-FC as either unoptimized or codon optimized *scFCY1* (Figure S17B).
Among variants, we observed three phenotypes: wild-type like sensitivity, slow growth, and
full resistance (Figure S17C). We found the majority of phenotypes were conserved within
orthologous variant pairs (18/27, Figure S18). Interestingly, we did not observe any cases
where a fully resistant mutant was fully sensitive or vice-versa in the ortholog. All changes in

phenotypes were incremental, suggesting the effects of some variants differ just enough
between the two proteins to move them slightly on the trade-off front, which is very steep.
464
Because of uracil leakage, growth using cytosine can not be assayed using spot assays like
5-FC resistance. We therefore selected a subset of 22 variant pairs for which we performed
growth curves in screen conditions to measure both phenotypes. Strinkingly, we found that
variants from both orthologs appear to evolve on the same trade-off front (Figure 6C).
Growth rates in 5-FC and cytosine media are distributed similarly for both orthologs (D=0.23,
p=0.63 for 5-FC and D=0.18, p=0.87 for cytosine, two sample Kolmogorov-Smirnov test). As
in the spot dilution assays, phenotypes were strongly correlated within variant pairs (Figure
S17D and S17E, ρ=0.600 and p=0.003 for 5-FC, ρ=0.461 and p= 0.031 for cytosine, n=22
pairs). Variants with diverging phenotypes did not deviate from the trade-off, as growth rate
changes in the two conditions remained negatively correlated (Figure S17F). These cases of
slight changes in phenotype for the same mutations in the ortholog are potential examples of
epistasis whereby the effects of mutations are strongly contingent on other residues[40]. A
staring phenotypic landscape shared by variants in the two orthologs suggests that the
resistance-function we characterized here might be a generalizable property of cytosine
deaminases from the *FCY1* orthogroup.

Discussion

Systematically characterizing the impact of mutations on drug resistance and normal cellular
function is a key aspect of improving our ability to design new molecules, manage existing
ones and potentially exploit trade-offs associated with resistance mutations. These goals
include interpreting mutations that have never been reported before and understanding the
mutational target sizes of different drug targets. Using genome editing and DMS, we
systematically characterized the impact on 5-FC resistance and canonical enzyme function
of all single amino acid mutations in *S. cerevisiae* Fcy1 and this at its endogenous locus. We
catalogued 940 missense and 151 nonsense mutations that conferred 5-FC resistance,
nearly 33% of all single codon mutations. Resistance mutations were found all across the
enzyme and not only near the active site. No single amino acid mutation was found to
escape the resistance-function trade-off. Our computational work and experimental assays
suggest this is largely due to effects on protein stability. The fact that destabilizing mutations
in Fcy1 can be sufficient to lead to resistance points to the importance of maintaining the
endogenous transcription levels for these assays as overexpression could mask relevant
effects.
496
An important factor influencing the fate of resistance mutations is the cost associated with
them. Understanding the mechanisms that determine the relationship between resistance
and growth in the absence of drugs is important because it could eventually open the door to
using conditions that maximize the cost of mutations. Here we show an example of an
extremely steep resistance-function relationship and provide a mechanistic explanation. Our
results demonstrate that the shape of a trade-off is inherently linked to how steep the
function linking growth to flux through the pathway is compared to the function linking flux
and growth inhibition by the toxic analog. During treatment, the target serum concentration of
5-FC is between 25 and 100 μg/ml (194 to 775 μM)[41]. In our dose-response curve, this
corresponds to the upper plateau. Because of its low steepness, a mutation would have to

reduce Fcy1 flux by over 400-fold to make the effective 5-FC concentration inhibit growth by
less than 10%. At this level of loss of function, the remaining cytosine deaminase activity
would be irrelevant to cell metabolism. The yeast membrane's propensity for uracil leakage[25]
would also be expected to reduce the impact of Fcy1 loss of function, as an initial mutant
would probably be surrounded by wild-type cells that could provide it with enough uracil to
compensate for partial loss of the salvage pathway, alleviating the strength of the trade-off.
The cost of resistance mutations could therefore be frequency dependent. How the shape of
the trade-off we uncover affects evolution *in vivo* therefore needs to be investigated.
515
The characterization of trade-offs may help design better treatment strategies against
pathogens. In the case of 5-FC, combining it with another compound that targets the *de*
novo pyrimidine synthesis pathway could provide a way to reduce or abolish its ability to
buffer loss of functions in the salvage pathways. For example, olorofim is a newly developed
antifungal currently undergoing phase IIb clinical trials that inhibits fungal dihydroorotate
dehydrogenase, which is part of the *de novo* pathway[42,43]. While it has no activity against
Candida and *Cryptococcus* species, olorofim has strong activity against molds such as
Aspergillus species, many of which are also sensitive to 5-FC *in vivo*[44,45]. New compounds
such as these could provide opportunities for combination regimens that take advantage of
the potency of 5-FC while slowing the emergence of resistance during treatment. One key
result of our experiment is that we did not observe any mutants that escaped the strong
resistance-function trade-off. This suggests that gaining resistance inevitably leads to loss in
the ability to use cytosine.
529
The concept of trade-off is ubiquitous in biology[46]. We used a relatively simple model (one
protein, two traits) to explore general questions linked with the evolutionary importance of
trade-offs when conflicts arise between two traits. Our data provide an example of how
whole-organism traits like dose-response dynamics can lead to strong trade-offs at the
molecular level and create scenarios where they are inevitable. Arguably, one limitation is
that we investigated single-step mutations and multiple-step mutations may be able to break
this trade-off. However, in a situation where mutation rate is relatively weak and population
sizes are limited, single-step mutations may be more representative of what could happen
during treatment. Mutations outside of the *FCY1* locus could also provide alternative
evolutionary paths allowing both resistance and nutrient use. Recent work has revealed new
pathways and mechanisms in *C. neoformans* associated with 5-FC resistance outside the
pyrimidine salvage pathway[47,48]. Investigating the role of multiple genes at the same time in
this context will provide insights on how increasing complexity and epistasis can influence
the properties of trade-offs.
544
Antifungal resistance is a growing threat to both human health and food supply, but there is
still limited data on the molecular mechanisms underlying it. By systematically exploring the
effects of all possible single amino acid mutants, we hope to facilitate the interpretation of
new genotypes in the clinical or industrial setting. Numerous efforts have been made in this
sense in the field of antibiotic resistance in bacteria, where antibiotic resistance genes such
as the β-lactamase TEM-1 have been thoroughly dissected and evolutionary pathways to
resistance have been characterized[49]. In human genetics, DMS studies have helped shed
light on the phenotypic effects of thousands of variants of unknown significance in disease or
cancer genes[50]. In effect, the 1,091 mutants we identified will more than quadruple the

number of amino acid substitutions recorded in the MARDy database, which catalogs all
known antifungal resistance mutations[3].
556
Although we focused on 5-FC resistance in a model species, the high correlation between
the phenotypes of *scFCY1* and *cnFCY1* suggests our results could be used to predict
resistance in pathogens. This could prove invaluable for interpreting population genetics of
clinical isolates or mutants isolated in drug resistance studies. For example, genetic variation
within pathogenic lineages sometimes leads to 5-FC resistance even in the absence of
treatment, as observed for *Candida dubliniensis* isolates from the C3 clade, which bear a
S29L substitution in its Fcy1 ortholog, Fca1. In our DMS experiment, the equivalent mutation
in yeast Fcy1 (G35L) confers resistance to 5-FC[51]. Based on a review of known resistance
associated mutation in fungal pathogens, our results correctly predict the resistance
phenotype of 8/9 variants across 5 species (Supplementary table 1). Despite how
widespread *FCY1* orthologs are, not much is known about their contribution to cell survival in
natural or host environments, except that it can provide increased metabolic efficiency in
some conditions[52]. The conservation of the enzyme across fungi suggests it plays a key role,
which contrasts with the fact that it is dispensable in laboratory conditions. Further studies
that examine other orthologs will help determine if the trade-off we observed for *scFCY1* and
cnFCY1 is a general property of the *FCY1* orthogroup. By generating a resistance mutation
catalogue and elucidating the mechanisms between resistance and function, the data we
obtained for the *S. cerevisiae* protein will help interpret genotypes from across fungi. Using
this approach on other antifungal targets could help proactively identify resistance mutations
before they are observed in the wild.

577 Methods

578 Strains, plasmids and culture media

Strains constructed as part of this study are listed in supplementary Table 2, plasmids used
or constructed in supplementary Table 3, and oligonucleotides in supplementary Table 4.
Media recipes can be found in supplementary Table 5. All yeast growth experiments were
performed at 30°C unless stated otherwise and all bacteria were grown at 37°C. Liquid
cultures were incubated with 250 RPM agitation. All Sanger sequencing was performed at
the CHUL sequencing platform (Université Laval, Québec). All high throughput sequencing
was performed using the MiSeq Reagent Kit v3 on an Illumina MiSeq for 600 cycles (IBIS
sequencing platform, Université Laval).
587
All strains were constructed in the BY4741 and BY4742 genetic backgrounds[53] unless stated
otherwise. We used a standard lithium acetate transformation protocol[54] to construct gene
deletions strains. The *FCY1* deletion strains BY4741 *fcy1Δ::NatNT2* (strain s_001) and
BY4742 *fcy1Δ::HphMX4* (strain s_002) were constructed by amplifying the *NatNT2* or
HphMX4 cassettes from pFA-NatNT2[55] or pAG32[56] respectively using oligonucleotides
del_for and del_rev and transforming the product in competent cells of the parental strain.
Gene deletions were verified by PCR using oligonucleotides FCY1_A, Nat_B or Hph_B.

595 Cytometry competition assay

To measure competitive fitness of wild-type vs *fcy1Δ* cells, we constructed a fluorescent
fcy1Δ reporter strain where the endogenous copy of *PDC1* is tagged with *mCherry* (s_003).
We amplified the mCherry tag from a modified version of plasmid pBS35[57] where the
selection marker is *NatMX4* using oligonucleotides PDC1_tag_for and PDC1_tag_rev, and
used a standard lithium acetate protocol to transform the cassette in BY4742
fcy1Δ::HphMX4 and plated the cells on YPD+Nat. Positive transformants were validated by
PCR (using oligonucleotides PDC1_C and mCherry_B) and by fluorescence cytometry using
a Guava® easyCyte 8HT (excitation: 532 nm, detection 609 nm). To perform the competition
assay, four independent pre-cultures of BY4742 and BY4742 *fcy1Δ PDC1-mCherry* were
grown overnight in SC media. The following morning, the cells were diluted to 0.1 OD in SC
media and allowed to reach exponential phase. Cells from each culture were centrifuged and
resuspended in sterile water to a concentration of 1 OD. The culture pairs were then mixed
in 1:9, 1:1, 9:1 (*FCY1:fcy1Δ PDC1-mCherry)* and 20 µL of the mix was used to inoculate 180
µL 1.11X SC-ura media with either 225, 113, 84, or 56 µM cytosine so that the media
reached 1X with the addition of the cells (final concentration: 0.05 OD) in a Greiner flat
bottom transparent 96-well plate. The abundance of each genotype was then measured by
counting the number fluorescent vs non-fluorescent yeast cells in each well, and the initial
cell density was measured by counting the number of cells/µL. After ~16h incubation at
30°C, the final OD for each well was measured, and the cells were diluted to 0.05 OD in
sterile water. The abundance of each genotype and the cell density was measured again for
all wells. The selection coefficient was calculated using the following approach for each
condition and replicate:
618

619 First, we measure the number of doublings for wild-type and *fcy1Δ PDC1-mcherry* by
620 comparing the absolute abundance of each genotype at the start and the end of the
621 competition:
622

$$Doublings_{WT} \ = \ log_2(WT_{start} - WT_{end})$$

$$Doublings_{\Delta} \ = \ log_2(Fluo_{start} - Fluo_{end})$$

624
625 With the endpoint counts adjusted to the final optical density at 16h. We then use the
626 number of doublings of the wild-type and *fcy1Δ PDC1-mcherry* strains to calculate the
627 relative fitness based on the number of doubling of the wild-type:
628

$$w_{WT} \ = \frac{Doublings_{WT}}{Doublings_{WT}} = 1$$

$$w_{\Delta} \ = \frac{Doublings_{\Delta}}{Doublings_{WT}}$$

630
631
632 From there, since the selection coefficient is defined as
633

$$s = 1 - w$$

634 We have

$$s_{\Delta} = 1 - w_{\Delta}$$

635
636 Which gives us the selection coefficient for the *fcy1Δ PDC1-mcherry* strain. As there was
637 little to no overnight growth in the 56 µM cytosine samples, we excluded them from the
638 selection coefficient calculation.

639 Creations and validation of the *FCY1* DMS library

To be able to differentiate it from the wild-type sequence in eventual diploid assays, we
decided to start from a codon optimized *FCY1* sequence to build the DMS library. The yeast
codon optimized sequence was synthesized and cloned into a high copy vector (plasmid
pTWIST-FCY1_opt, Twist Biosciences, San Francisco, USA). We then used the mega-
primer method[22] to generate codon by codon mutant libraries by using oligonucleotides with
three degenerate nucleotides (NNN). In the first PCR step, the degenerate forward
oligonucleotides are paired with a reverse primer to generate a short amplicon where one
codon has been mutated:
648

Reagent	Volume (µL)
Kapa HF buffer 5X	3
dNTPs 10 mM	0.45
Reverse Primer 10 µM	0.45

Codon-specific NNN oligo 10 µM	0.45
Kapa polymerase	0.1
Plasmid DNA 30 ng/µL	0.23
PCR grade water	10.33
Total	15

649
650 With the following PCR cycle:
651
652

95 °C	5 mins	
98 °C	20 s	
60 °C	15 s	35x
72 °C	30 s	
72 °C	50 s	

653
654 For codons 2 to 57, the reverse oligonucleotide was mega_1, mega_2 for codons 58-133
655 and mega_3 for codons 114-158. The resulting mega-primer product was verified on
656 agarose gel and used as a primer for the directed mutagenesis PCR reaction with the
657 following parameters:
658
659

Reagent	Volume (µL)
Kapa HF buffer 5X	3
dNTPs 10 mM	0.45
Mega-primer	0.75
Kapa polymerase	0.1
Plasmid DNA 30 ng/µL	0.23
PCR grade water	10.48
Total	15

660
661 With the following PCR cycle:
662
663

95 °C	5 mins	

98 °C	20 s	
68 °C	15 s	22x
72 °C	3 mins	
72 °C	4.5 mins	

664
665
After amplification, 0.3 µL 20,000 U/ml DpnI (New England Biolabs) was added to the
reaction mix which was then incubated at 37°C for 1 hour to digest the template plasmid. We
then transformed 5 µl of the digested mutagenesis reaction into E. coli MC1061 ($[araD139]_{B/r}$

$\Delta(araA\text{-}leu)7697\ \Delta lacX74\ galK16\ galE15(GalS)\ \lambda\text{-}e14\text{-}mcrA0\ relA1\ rpsL150(strR)\ spoT1$

$mcrB1\ hsdR2)$[58] using a standard chemical transformation method. This was sufficient to

obtain hundreds to thousands of clones per codon, more than enough to cover all 64
possibilities.
673
The mutagenesis was performed in three batches corresponding to the three different
reverse primers in the first PCR steps. For each codon, transformants were recovered by
soaking the transformation plate with 5 mL 2YT media and scraping with a glass rake to
recover cells in a 15 mL falcon tube. The pools were homogenized by vortexing and then
archived by mixing 800 µl bacteria pool and 200 µL 80% glycerol for storage at -80°C.
Finally, plasmid DNA was extracted from each pool with a standard miniprep kit (FroggaBio,
Concord, Canada) using 1.5 ml of bacterial suspension as input and eluting in 50 µL kit
elution buffer (10 mM Tris-HCl pH 8.5). After all extractions were completed, plasmid DNA
concentrations were normalized to 1 ng/µL in two Greiner V-shape 96 well plate by first
diluting to 10 ng/µL in 20 µL PCR grade water and then adding another 180 µL of PCR
grade water. This plasmid DNA dilution was then used as the template for quality control of
the DMS library.
686
To validate the mutant plasmid pools, we used Row-Column (RC) indexing[59] to generate
barcoded position by position libraries for high-throughput sequencing in three PCR steps.
We split the FCY1 coding sequence into three overlapping regions for Illumina paired-end
sequencing. First, we amplified the appropriate FCY1 region with oligonucleotides containing
flanking universal priming sites (PS) at each end using with specific oligonucleotides (F1:
pool_1_for, R1: pool_1_rev, F2: pool_2_for, R2: pool_2_rev, F3: pool_3_for, R3: pool_3_rev
):
694
695

Reagent	Volume (µL)
Kapa HF buffer 5X	3
dNTPs 10 mM	0.45
PS-Forward oligo 10 µM	0.45

PS-Reverse oligo 10 µM	0.45
Kapa polymerase	0.1
DMS plasmid library DNA 1 ng/µL	4.5
PCR grade water	6.05
Total	15

697 With the following PCR cycle:

98 °C	3 mins	
98 °C	30 s	
60 °C	15 s	20X
72 °C	30 s	
72 °C	1 min	

703 After gel verification, the amplicons were diluted 1/2,500 by two consecutive 2 µL PCR :
704 98µL PCR grade water dilutions. This was then used as a template for a second PCR round
705 where plate position specific barcodes were added at both ends of the amplicon (forward:
706 oligonucleotides row_1 to row_8, reverse: oligonucleotides col_1 to col_12).

Reagent	Volume (µL)
Kapa HF buffer 5X	3
dNTPs 10 mM	0.45
Row-Forward oligo 2 µM	2.25
Col-Reverse oligo 10 µM	0.45
Kapa polymerase	0.1
Diluted PS-PCR	2.25
PCR grade water	6.5
Total	15

710 With the following PCR cycle:

98 °C	3 mins	
98 °C	30 s	18X
60 °C	15 s	
72 °C	30 s	
72 °C	1 min	

713
All barcoded amplicons of the same pool were then mixed in roughly equivalent quantities
after gel band intensity quantification. The resulting amplicon mixes were purified using
magnetic beads and used as templates for a final PCR round performed in quadruplicate to
add plate barcodes at each end as well as the Illumina p5 and p7 sequences using a
different combination of oligonucleotides for each pool (pool 1: plate_for_1/plate_rev_3, pool
2: plate_for_2/plate_rev_3, pool 3: plate_for_3/plate_rev_3):
720

Reagent	Volume (µL)
Kapa HF buffer 5X	3
dNTPs 10 mM	0.45
plate_for oligo 10 µM	0.45
plate_rev oligo 10 µM	0.45
Kapa polymerase	0.1
Purified RC-PCR pool 0.01 ng/µL	0.45
PCR grade water	10.1
Total	15

721
722   With the following PCR cycle:
723
724

98 °C	3 mins	
98 °C	30 s	20X
61 °C	15 s	
72 °C	35 s	
72 °C	1 min	

726 The replicate reactions were pooled and then purified on magnetic beads and gel verified to
727 obtain the final libraries.
728
For a few codons (19, 80 and 91), the mutagenesis primers failed systematically and we
therefore used equivalent degenerate oligonucleotides (alt_dms_19, alt_dms_80 and
alt_dms_91) on the reverse strand with a matching forward oligonucleotide (mega_4) to
generate the mutant plasmid pools. We also performed a second round of mutagenesis for
some positions that showed poor coverage in the QC analysis (codons 83, 84, 107 and 108).
These 7 pools were validated by Sanger sequencing.

735 Next-Generation Sequencing data analysis

After sequencing, samples were demultiplexed using custom python scripts
(https://github.com/Landrylab/Despres_et_al_2021) according to plate and RC barcodes
after quality control with FastQC[60] and trimming with Trimmomatic[61]. Data analysis scripts
made use of the following packages: matplotlib[62], numpy[63], pandas[64], seaborn[65] and scipy[66].
After demultiplexing, the forward and reverse reads were merged using the PANDA-Seq
software[67] and collapsed into 100% identity clusters with vsearch[68]. Reads were then aligned
to the appropriate *FCY1* fragment sequences using the Needle software from EMBOSS[69].
All raw sequencing files were deposited on the NCBI SRA, accession number
PRJNA782569.
745

746 CRISPR-Cas9 Knock-in of *FCY1* variants

We used a modified version of the protocol presented in Ryan et al. 2016[70] to generate all
the competent cells required for the DMS yeast transformations in a single batch. Colonies
from a BY4742 *fcy1Δ::HphMX4* plate were used to inoculate a 20 mL YPD + Hyg overnight
pre-culture. The following day, the cells were diluted to 0.2 OD in 300 mL YPD and grown to
~1 OD. The culture was split in 50 ml falcon tubes and the cells were pelleted by
centrifugation at 448 *g* for 2 minutes. The supernatant was removed and the pellet was
washed with 0.5 mL LATE solution (100 mM Lithium acetate, 1mM Tris-Cl, 0.1 mM EDTA,
filter sterilized) before being transferred to a 1.5 ml microcentrifuge tube. The cells were then
pelleted again by centrifugation at 8,600 *g* for 1 minute, and the supernatant was removed.
Finally, the cells were resuspended in 300 µl LATE solution and 300 µl 50% glycerol, mixed
by a quick vortexing, and stored at -80°C until use.
758
To integrate the DMS pools for each codon in yeast, we co-transformed a pCAS[70] variant
bearing a gRNA targeting the *Hph* resistance gene (pCAS-Hph, p_005) as well as donor
DNA amplified from the codon-specific mutant plasmid pool recovered from bacteria. The
modified pCAS vector was generated using the same approach as in Dionne et al. 2021[24],
using oligonucleotides pCAS_hph_for and pCAS_hph_rev. The donor DNA for each codon
was amplified using the following reaction:
765
766

Reagent	Volume (µL)

Kapa HF buffer 5X	3
dNTPs 10 mM	0.45
Oligo knock_in_for 10 µM	0.45
Oligo knock_in_rev 10 µM	0.45
Kapa polymerase	0.1
DMS plasmid library 2 ng/µL	3.5
PCR grade water	7.05
Total	15

767
768   With the following PCR cycle:
769

95 °C	5 mins	
98 °C	20 s	
60 °C	15 s	29X
72 °C	45 s	
72 °C	1.5 mins	

770
We performed one transformation/codon in a 96 well PCR plate using a similar approach to
what is described in Dionne et al 2021[24]. Each well contained 20 µL competent cells, 2 µL
carrier DNA (salmon testes ssDNA 10 mg/mL, boiled for 5 min and then cooled on ice) and
3.75 µL 200 ng/µL pCAS-Hph, prepared as a master mix and dispensed before adding 10 µL
donor DNA. A 200 µL volume of freshly prepared PLATE solution (40% PEG 3350, 100 mM
Lithium acetate, 1mM Tris-Cl, 0.1 mM EDTA, filter sterilized) was then added to each well.
The plates were sealed with sterile aluminum foil, vortex mixed, and incubated at 30°C for 30
minutes before a 15 minutes heat shock at 42°C. The plates were then centrifuged at 448 g
for 6 minutes to pellet the cells, the supernatant was removed, 100 µL YPD was added, and
the plates were sealed with aluminum foil again for a 3h30 outgrowth at 30°C. The cells were
then plated on YPD+G418 with glass beads and incubated at 30°C for ~64 hours.
782
To recover cells, the plates were soaked with 5 ml YPD, scraped with a glass rake and the
cell suspension was transferred to a 15 mL falcon tube. The cell suspensions were
homogenized by vortexing, and the OD was measured for each pool. Based on the cell
concentration, a volume equivalent to 5 OD units of each pool was mixed to generate the
three DMS mutant pools: pool 1 contained codons 2 to 67, pool 2 codons 49 to 110, and
pool 3 codons 93 to 158. The OD of each pool was measured to measure their final cell
concentration, and pool aliquots were archived by mixing 380 µL of cell pool with 180 µL of
80% glycerol. Pool aliquots were stored at -80°C until use. A 500 µL sample of each pool
was spun down and used as input for genomic DNA extraction with the phenol/chloroform

method[71]. Next-generation sequencing libraries were generated and sequenced on illumina
for a final round of quality control to ensure proper coverage in each pool.
794
The same approach was used to build the strain BY4742 *FCY1_codon_opt* (s_004), but
using the wild-type plasmid and oligonucleotides KI_short_for and KI_short_rev to generate
the donor DNA, and recovering single colonies instead of scraping the entire transformation
plate. Transformants were used to inoculate 1 mL YPD cultures, grown overnight, and then
streaked on YPD. One colony from each streak was resuspended in 200 μL water and
spotted on YPD as well as YPD+G418 and YPD+Hyg to check for pCAS loss and successful
loss of the *HphMX4* cassette. The strain was then validated by PCR and Sanger
sequencing.

803 DMS library screen and library preparation

An archived aliquot of each pool was thawed ~30 minutes on ice. From the corresponding
tubes, a volume corresponding to 2 OD units was used to inoculate 20 ml SC media in
duplicate. The cells were grown for 24h and then split in the 5-FC and cytosine conditions:
SC + 12 μM 5-FC or SC-ura + 84 μM Cytosine. Each 12h, 2 OD units of cells were
centrifuged for 5 minutes at 224 *g*, the supernatant was removed, and the pellet was
resuspended in 1 ml sterile water. The cell suspension was then used to inoculate 19 mL of
1.05X media, so that the addition of the cells brought the media to 1X. The competition was
stopped once pools reached ~20 generations based on OD measurements: this took two
passages for the 5-FC condition, and 4 passages for the cytosine condition. Genomic DNA
was extracted from 2 OD pellets (approximately $2x10^7$ cells) of the final passage with the
phenol/chloroform method. The same approach was used for pool2 and the 5-FC+cytosine
condition, but with SC-ura + 24 μM 5-FC + 113 μM cytosine.
816
817 To prepare NGS libraries for deep sequencing, we used a similar method as was used for
818 the plasmid pool QC, but barcoding individual timepoints instead of codons, and with each
819 reaction being performed in duplicate:
820
821

Reagent	Volume (μL)
Kapa HF buffer 5X	3
dNTPs 10 mM	0.45
Pool forward oligo 10 μM	0.45
Pool forward oligo 10 μM	0.45
Kapa polymerase	0.1
DMS screen gDNA 30 ng/μL	5
PCR grade water	5.55
Total	15

822
823 With the following PCR cycle:

98 °C	3 mins	
98 °C	30 s	20X
60 °C	15 s	
72 °C	30 s	
72 °C	1 min	

828   After gel migration, the amplicons were diluted 1/2,500 by two consecutive 2 µL PCR : 98µL

829   PCR grade water dilutions. This was then used as a template for a second PCR round

830   where plate position specific barcodes were added at both ends of the amplicon (primers

831   row_1-3 with primers col_1-6), with each reaction performed in duplicate.

Reagent	Volume (µL)
Kapa HF buffer 5X	3
dNTPs 10 mM	0.45
Row-Forward oligo 2 µM	2.25
Col-Reverse oligo 10 µM	0.45
Kapa polymerase	0.1
Diluted PS-PCR	2.25
PCR grade water	6.5
Total	15

835   With the following PCR cycle:

98 °C	3 mins	
98 °C	30 s	18X
60 °C	15 s	
72 °C	30 s	
72 °C	1 min	

839 Replicate reactions were pooled together, purified using magnetic beads, and quantified
840 using a NanoDrop spectrophotometer. Barcoded amplicons of the same pools were then
841 mixed in equivalent quantities. The resulting pools diluted and used as templates for a final
842 PCR round performed in quadruplicate to add plate barcodes at each end as well as the
843 Illumina p5 and p7 sequences:

Reagent	Volume (µL)
Kapa HF buffer 5X	3
dNTPs 10 mM	0.45
Plate_for-Forward oligo 10 µM	0.45
Plate_rev-Reverse oligo 10 µM	0.45
Kapa polymerase	0.1
Purified RC-PCR pool 0.01 ng/µL	1
PCR grade water	9.55
Total	15

846 With the following PCR cycle:

98 °C	3 mins	
98 °C	30 s	
61 °C	15 s	20X
72 °C	35 s	
72 °C	1 min	

850 The replicate reactions were pooled, purified on magnetic beads and gel verified to obtain
851 the final libraries.

853 DMS score

Data analysis of the DMS screen was performed using custom Python scripts. Briefly,
samples were demultiplexed into individual fastq files containing merged reads using the
same strategy as was used for the plasmid pool QC analysis. Identical reads were then
collapsed using vsearch[68] and aligned on the appropriate *FCY1* region using Needle[69] to
detect mutations. At this stage, we filtered alignments to only keep query sequences that
covered 80% or more of the reference and that had less than 25 differences. Reads with

more than three mutations in the target DMS sequence or with mutations in multiple codons
were discarded. Finally, we applied a stringent filter to avoid including double mutants: only
reads where all differences in the *FCY1* coding sequence occurred within the same codon
were considered when measuring variant abundance. The total number of read counts
obtained per library is shown in Supplementary Figure 4A, and raw read counts for each
codons in each library are provided as Supplementary table 6.
866
When computing log2 fold-change between timepoints, we followed the standard practice of
adding one to all variant counts to make fold-change calculations possible for variants that
dropped out during the competition. Variant read counts were then divided by total read
count (including the WT sequence) to obtain relative abundances. Relative abundances
were then compared between the end of the SC media passage and the end of the selection
conditions (5-FC or cytosine) to obtain log_2 fold-changes for all mutant codons. We
discarded all codon level variants that were covered by less than 10 reads in one of the T0
replicates (prior to selection), which was a rare occurrence (Supplementary Figure 4B). Most
codon level variants were well covered in the initial timepoints (median codon variant
coverage > 60 reads for all pools, Supplementary Figure 4C), which lead to have a high
amino acid level coverage (Supplementary Figure 4D). To convert to amino acid log2 fold-
change, we considered each group of synonymous codons as replicates and calculated the
median fold-change of each group. This allowed us to obtain three (one per pool) amino-acid
level matrices per condition. Finally, we averaged log_2 fold-change between replicates (as
shown in Figure S4). As some *FCY1* positions in pools 1 and 3 overlapped with those in pool
2 (positions 49 to 67 and 93 to 110 respectively), ~400 mutants/pool had their log_2 fold-
change measured in duplicate. As the fold-changes of same mutants were well correlated
between pools and spanned the full range of possible values, we fit a linear regression that
allowed us to adjust all scores to pool 2. For mutants in the overlapping regions, we used the
average of the two log2-fold changes (one from pool 2, and the adjusted value from pool 1 or
3).
888
889 Once we had harmonized log_2-fold changes along our three pools, we wanted the values for
890 both experiments (5-FC and cytosine) to be on the same scale to facilitate comparison. To
891 do this, we scaled values using the median log2-fold change of synonymous mutations and
892 nonsense mutants using the following equations:
893
894 For 5-FC:

$$FC_{score} = \frac{AA_{log_2} - SYN_{median}}{STOP_{median} - SYN_{median}}$$

895 For cytosine

$$CY_{score} = \frac{AA_{log_2} - STOP_{median}}{SYN_{median} - STOP_{median}}$$

896
This scaling relates the log_2 fold-change observed for a DMS mutant with the difference
observed between the values of synonymous (silent) and nonsense (stop) mutants. For
example, a score of 0.5 would represent a log_2 fold-change value that is perfectly in the
middle between the medians of synonymous and nonsense mutations. We chose to set the
score according to the phenotype measured, so that in the 5-FC condition nonsense

mutations would have a median $FC_{score}=1$ and synonymous mutation would have a median
median $CY_{score}=0$. Conversely, in the cytosine condition, synonymous mutations would have
a median $CY_{score}=1$ and nonsense mutations would have median median $FC_{score}=0$.
905

Amino acid and mutation properties analysis

We used the structure from Ireton et al. 2003[15] (PDB: 1P6O) to compute residue relative
solvent accessibility (RSA), distance to the active site and interface, and as baseline for the
FoldX[32] ΔΔG predictions which were performed using the same approach as in Marchant et
al. 2019[72]. Briefly, the biological assembly was generated and energy minimization was
performed using the FoldX Repair function 10 times to ensure convergence[73]. Then, the
FoldX BuildModel and AnalyseComplex functions were used to estimate mutational effects
on ΔG. As mentioned in the text, FoldX predictions were generally comparable between
dimer bearing one or two mutated copies. RSA and temperature measurements of both
chains were averaged to obtain the final value. The distance to the active site was
considered to be the shortest distance between a specific amino acid and any active site
residue. To determine which residues were part of the interface, we used a definition based
on distance to amino acids in the other subunit, where residues whose two closest non-
hydrogen atoms are separated by a distance smaller than the sum of their van der Waals
radii plus 0.5 Å are considered to be at the interface (coded as 1). Residues whose alpha
carbons are within 6 Å of the other chain were considered to be at the interface rim (near the
interface, coded as 0.75)[72].
923

We retrieved a set of 218 high confidence *FCY1* orthologs by combining sequences from
Emsembl[74], MetaPhOrs[75], and the Yeast genome order browser[76]. The sequences were
aligned using MUSCLE[77] with default parameters. After visual inspection, three sequences
were removed as they were discordant with major aligned blocks, yielding a final set of 215
sequences (Supplementary Data 1). The orthologs were aligned with Muscle again, using
the stable.py script to keep the same sequence order in the output. Surprisingly, we found a
higher than expected proportion of orthologs in bacterial and archaeal genomes. Previous
work had hypothesized that cytosine deaminases from the *FCY1* family were exclusive to
fungal species, with bacteria usually having orthologs of *E. coli codA*[15]. We instead found
over 30 orthologs in bacteria and seven in archaea, suggesting a more complex evolutionary
history for this orthogroup. This alignment was then used to calculate normalized
evolutionary rates using Rate4site[34] using default parameters. To determine variant
occupancy, the positions of the alignment corresponding to the *S. cerevisiae* amino acids
were parsed to identify the frequency of each amino acid. The fully annotated dataframe
containing log2 fold-changes, 5-FC and cytosine score, as well as all annotations for
mutants is provided as Supplementary table 7.

Construction of the *FCY1* mutants validation set

The *FCY1* variant strains tested as part of the validation studies were constructed using the
same strategy used to generate the DMS library, starting from BY4742 *fcy1::HphMX4*. We
initially selected 80 variants for validation studies, but not all strain constructions attempts
were successful, explaining why data is not presented for all of them. To generate mutated

donor DNA, we used a fusion PCR strategy where oligonucleotides containing the mutations
of interest are used to generate two overlapping amplicons both bearing a mutation starting
from genomic DNA extracted from strain BY4742 *FCY1_codon_opt*. The first reaction, which
amplified from the five-prime flanking homology arm before the start of the coding sequence
up to the mutated amplicon overlap, used oligonucleotides fusion_for and mutant_rev_1 to
80. The second reaction, which amplified from the mutated overlap to the three-prime
homology arm after the coding sequence, used oligonucleotides mutant_for_1 to 80 and
fusion_rev. Both resulting amplicons are then diluted 1/2,500 and used as template for a
second reaction using oligonucleotides fusion_for and fusion_rev to reconstitute the full
length *FCY1* sequence.
955
We then performed one by one CRISPR knock-ins using the same reaction setup as for the
DMS library, but using the fusion PCR products as donor DNA. Transformants (four/mutant)
were used to inoculate 1.2 mL YPD cultures, grown overnight, and then streaked on YPD.
One colony from each streak was resuspended in 200 μL water and spotted on YPD as well
as YPD+G418 and YPD+Hyg to check for pCAS loss and successful loss of the *HphMX4*
cassette. *FCY1* integration was verified by PCR using oligonucleotides FCY1_alt_A and
fusion_rev on quick DNA extractions[78]. PCR positive strains were then validated by Sanger
sequencing using fusion_for as the sequencing oligonucleotide. We succeeded in
constructing 73/80 mutants. Variant growth rates were measured by performing growth
curves in 384 well plates with a 80 μl sterile water border. Cells from overnight pre-cultures
in SC media were spun down and resuspended in sterile water at a concentration of 0.4 OD.
From these dilutions, 20 μl was used to inoculate either 60 μl 1.33X SC media + 12 μM 5-FC
or 60 μl 1.33X SC-ura media + 84 μM cytosine, for a final media concentration of 1X and 0.1
OD cell density. Each strain was tested in duplicate for both conditions: measurements were
taken every 15 minutes for 48 hours without agitation on a Tecan Spark (Tecan Life
Sciences, Männedorf) with an active Tecool temperature control module. We excluded one
mutant due to a technical problem with the plate cover, leading to the final count of 72
mutants. To further examine variants that appear to escape the trade-off, we constructed an
additional 15 strains with high 5-FC and cytosine DMS scores, as well as some mutants that
could not be measured in the DMS assays. The growth rates of these mutants in SC + 12μM
5-FC or SC -Ura + 84 μM cytosine was measured in triplicate (48 hour, 15 minutes intervals)
using growth curves in 96 well plates in a Tecan Infinite M Nano (Tecan Life Sciences,
Männedorf). A set of 15 mutants present in the first round of validations was used to scale
the growth rates of this experiment to those of the initial validations using a linear regression
(Figure S12 D-F). The data and annotations related to validation mutants is presented in
supplementary table 8.

982 Measurement of mutant-WT dimer interaction

Although the Fcy1-Fcy1 homodimer interaction had already been reported to be detectable
using this DHFR-PCA[79], we reconstructed a full set of DHFR strains (s_005 to s_008) and
validated that we could observe it. The C-terminal DHFR-tagged strains were constructed by
amplifying the DHFR tags from pAG25-DHFR[1,2] and pAG32-DHFR[3] using
oligonucleotides from the DHFR collection (DHFR_tag_F and DHFR_tag_R)[35]. The tagged
strains were validated by PCR and Sanger sequenced.To generate a N-terminal DHFR[1,2]
tagged allele of Fcy1, we used fusion PCR to amplify the DHFR[1,2]-linker fragment from

pGD009[80] (oligonucleotides Nter_DHFR_for and Nter_DHFR_rev) and then fuse it to the
codon optimized sequence (oligonucleotides linker_fcy1_for and knock_in_rev) which was
amplified from strain *FCY1_codon_opt* (s_004) genomic DNA. The fused amplicon was then
used as donor DNA for a CRISPR transformation of *fcy1::HphMX4* with pCAS-Hph. The
positive clone was validated by PCR and Sanger sequencing, yielding strain s_009
(*DHFR[1-2]-FCY1_codon_opt*). Mutant alleles tagged in N-terminal with DHFR[1,2] were
generated using the same strategy, but using genomic DNA from strain s_009 as template
instead. We were able to successfully construct 75/80 N-terminal DHFR[1,2] tagged strains.
998
To measure changes in PPIs with wild-type Fcy1, tagged variants were crossed with
BY4741 *FCY1-DHFR[3]* by mixing 5 µl of a SC-complete DHFR[1,2]-*FCY1* mutant pre-
culture with 5 ul of a YPD+Hyg *FCY1-DHFR[3]* pre-culture in 2 ml YPD and incubating ~8
hours at 30 C. After incubation, 5 µl/mating was spotted on SC-lys-met + Hyg (pH buffered)
media and incubated overnight at 30°C to select for diploids. Parental strains were also
spotted in parallel to control for background growth. Cells from each spot were resuspended
in sterile water and spotted a second time on SC-lys-met+Hyg to perform a second round of
selection. From these spots, we inoculated SC-lys-met pre-cultures that were grown
overnight. The next morning, the cells were spun down and resuspended in sterile water at a
concentration of 0.4 OD. From these dilutions, 8 µl was used to inoculate either 72 µl 1.1X
MTX media or 72 µl 1.1X DMSO media (see supplementary Table 5), for a final media
concentration of 1X and a 0.1 OD cell density. Each strain was tested in duplicate for both
conditions: measurements were taken every 15 minutes for 48 hours without agitation in a
Tecan Spark plate as described above.
1013

1014 Dose-response curves and growth rates measurements in
1015 multiple 5-FC and cytosine concentration

The dose-response growth curves were generated by measuring the maximum growth rate
of BY4742 in 20 different concentrations of 5-FC or cytosine in SC and SC-ura media
respectively. The range of concentrations tested was specific to the compound to ensure
proper coverage of the dynamic range. The growth rates were measured in triplicate, with
each replicate starting from a different colony. The values used to fit the Hill equation are
presented as supplementary Table 9. The shifted dose response curves shown in figure 5B
and C were obtained by adding a term to take into account Fcy1 loss of function in the Hill
equation, so that the 5-FC or cytosine concentration is lowered based on diminished
metabolic flux:

$$Rel\ growth\ = \frac{1}{1 + \left(\frac{IC_{50}}{[drug] \times lof}\right)^n}$$

1026
1027 Where IC_{50} and *n* are the hill equation parameters determined from the dose-response
1028 curve, and lof is a term representing the level of Fcy1 loss of function (e.g. *lof* = 0.5 is a loss
1029 of half the wild-type metabolic flux).
1030

We measured the growth rates of Fcy1 mutants in 96 wells plates that contained the 73
Sanger validated mutants, of which 13 were present in duplicate. The plate also included 4
replicates of *FCY1_codon_opt* and 4 replicates of BY4742, as well as two blank wells with
culture media and no cells. The growth curves were started by diluting overnight pre-cultures
and inoculating 180 µL 1.11X SC or SC -ura media containing different amounts of 5-FC or
cytosine. The optical densities were manually read for each plate using a Tecan Spark with
an active Tecool temperature control module at regular intervals for 24h, at which point the
cultures were fully resuspended and a final datapoint was acquired. The AUC measured for
the wild-type strains (*FCY1_codon_opt* and BY4742) clustered together in all assay
conditions (Figure S16A), and AUCs for replicate Fcy1 mutant cultures were well correlated
across all conditions (ρ=0.8-0.99, n=13 mutants) except in SC media without 5-FC, which is
expected to be non-selective for Fcy1 function (Figure S16B). For mutants present in
duplicate, the values for each replicate were averaged, and the WT AUC was obtained by
taking the median of the 8 WT measurements (the median of 4 replicates of
FCY1_codon_opt and 4 replicates of BY4742). Data from variant T86M was excluded from
Figure 5 because no cytosine growth rate from the validations was available. Raw curves for
all mutants are shown in Figure S14 (5-FC) and S15 (cytosine).The AUC values used to
generate Figure 4D and Figure S16 are available as supplementary Table 10.

1049 Generation of orthologous mutants in the *Cryptococcus*
1050 *neoformans* ortholog

The protein sequence of the *Cryptococcus neoformans* JEC21 ortholog of FCY1 (*CnFCY1*)
was retrieved from NCBI and codon optimised for expression in yeast using the same tool as
for the S. cerevisiae protein. To obtain the structure of cnFcy1, we used Alpha Fold 2[38] via
the ColabFold interactive notebook[39] to predict its structure. We used the cnFcy1 protein
sequence as input with default parameters and following the guidelines for dimer structure
prediction. All output files for the prediction are provided as Supplementary Data 2. The
structures were aligned using the align function of ChimeraX[81], resulting in a pruned
structural alignment with a length of 141 residues with RMSD = 0.631 Å (unpruned
alignment length = 151 aa, RMSD = 3.129 Å). The coding sequence was synthesised and
cloned into a high copy vector (plasmid pTWIST-FCY1_opt_JEC21, Twist Biosciences, San
Francisco, USA) with homology arms for recombination at the *FCY1* genomic locus. We then
used the same CRISPR-Cas9 mediated knock-in strategy to insert the wild-type allele at the
FCY1 locus and generate a *S. cerevisiae* strain bearing *CnFCY1*. We then selected 24
variants from validations that occurred at sites conserved between the two orthologs and
designed oligonucleotides to generate the corresponding mutations in *CnFCY1* by fusion
PCR. Because mutations at conserved sites tended to have more deleterious effects, we
also included 5 mutants at non-conserved sites that had wt-like phenotypes to cover the
whole range of cytosine media growth rates.
1069
We generated the mutants using the same protocol as previously described for *S. cerevisiae*
variants, but using genomic DNA from the CnFCY1 as a template. We were able to generate
and validate 27/29 strain by Sanger sequencing. The spot dilution assays were performed by
growing strains overnight in SC media and then adjusting them to 1 OD in sterile water. For
each strain, we spotted 5 µL of each dilution on SC complete and SC complete + 194 µM 5-
FC. Phenotypes were scored after 48h of growth. Matched growth curves were performed in

duplicates on a randomly chosen subset of variant pairs. Cells from the spot assay pre-
cultures were diluted to 0.1 OD in the same media used for the validations. OD
measurements were taken every 15 minutes for 48h in 96 well plates using a a Tecan
Infinite M Nano (Tecan Life Sciences, Männedorf). The maximum growth rate in both
conditions was then calculated using the same method as for previous validations. Growth
rate values of scFCY1 and cnFCY1 variants used to generate Figure 5C and Figure S17A
and D-F are available as Supplementary Table 11.

Acknowledgements

The authors thank members of the Landry lab for helpful discussions, in particular Daniel Evans-Yamamoto, Mathieu Hénault, Florian Mattenberger and Romain Durand. This work was supported by the Canadian Institutes of Health Research Foundation grant 387697 to C.R.L. and a Vanier graduate scholarship to P.C.D, as well as by the National Science and Engineering Research Council through the EvoFunPath CREATE grant (555337-2021) and by FRQNT through team grant (2022-PR-298169) and a PBEEE scholarship to A.F.C. CRL holds the Canada Research Chair in in Cellular Systems and Synthetic Biology. Figure 1 was generated using Biorender. Molecular graphics and analyses performed with UCSF ChimeraX, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from National Institutes of Health R01-GM129325 and the Office of Cyber Infrastructure and Computational Biology, National Institute of Allergy and Infectious Diseases.

References

1. Fisher, M. C. *et al.* Threats Posed by the Fungal Kingdom to Humans, Wildlife, and Agriculture. *MBio* **11**, (2020).

2. Fisher, M. C., Hawkins, N. J., Sanglard, D. & Gurr, S. J. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science* **360**, 739–742 (2018).

3. Nash, A. *et al.* MARDy: Mycology Antifungal Resistance Database. *Bioinformatics* **34**, 3233–3234 (2018).

4. Ksiezopolska, E. *et al.* Narrow mutational signatures drive acquisition of multidrug resistance in the fungal pathogen Candida glabrata. *Curr. Biol.* **0**, (2021).

5. Bryce Taylor, M. *et al.* yEvo: Experimental evolution in high school classrooms selects for novel mutations and epistatic interactions that impact clotrimazole resistance in S. cerevisiae. *bioRxiv* 2021.05.02.442375 (2021) doi:10.1101/2021.05.02.442375.

6. Andersson, D. I. & Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* **8**, 260–271 (2010).

7. Gerstein, A. C., Lo, D. S. & Otto, S. P. Parallel genetic changes and nonparallel gene-environment interactions characterize the evolution of drug resistance in yeast. *Genetics* **192**, 241–252 (2012).

8. Yang, F. *et al.* The fitness costs and benefits of trisomy of each Candida albicans chromosome. *Genetics* **218**, (2021).

9. Kanafani, Z. A. & Perfect, J. R. Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. *Clin. Infect. Dis.* **46**, 120–128 (2008).

10. Iyer, K. R., Revie, N. M., Fu, C., Robbins, N. & Cowen, L. E. Treatment strategies for cryptococcal infection: challenges, advances and future outlook. *Nat. Rev. Microbiol.* **19**, 454–466 (2021).

11. Flowers, S. A., Colón, B., Whaley, S. G., Schuler, M. A. & Rogers, P. D. Contribution of clinically derived mutations in ERG11 to azole resistance in Candida albicans.

Antimicrob. Agents Chemother. **59**, 450–460 (2015).

12. Longley, D. B., Harkin, D. P. & Johnston, P. G. 5-fluorouracil: mechanisms of action and

clinical strategies. *Nat. Rev. Cancer* **3**, 330–338 (2003).

13. World Health Organization Model List of Essential Medicines – 22nd List, 2021.

Geneva: World Health Organization; 2021 (WHO/MHP/HPS/EML/2021.02). Licence: CC

BY-NC-SA 3.0 IGO.

14. Erbs, P., Exinger, F. & Jund, R. Characterization of the Saccharomyces cerevisiae

FCY1 gene encoding cytosine deaminase and its homologue FCA1 of Candida

albicans. *Curr. Genet.* **31**, 1–6 (1997).

15. Ireton, G. C., Black, M. E. & Stoddard, B. L. The 1.14 A crystal structure of yeast

cytosine deaminase: evolution of nucleotide salvage enzymes and implications for

genetic chemotherapy. *Structure* **11**, 961–972 (2003).

16. Breslow, D. K. *et al.* A comprehensive strategy enabling high-resolution functional

analysis of the yeast genome. *Nat. Methods* **5**, 711–718 (2008).

17. Qian, W., Ma, D., Xiao, C., Wang, Z. & Zhang, J. The genomic landscape and

evolutionary resolution of antagonistic pleiotropy in yeast. *Cell Rep.* **2**, (2012).

18. Becker, J. M. *et al.* Pathway analysis of Candida albicans survival and virulence

determinants in a murine infection model. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 22044–

22049 (2010).

19. Hartzog, P. E., Nicholson, B. P. & McCusker, J. H. Cytosine deaminase MX cassettes

as positive/negative selectable markers in Saccharomyces cerevisiae. *Yeast* **22**, 789–

798 (2005).

20. Wrenbeck, E. E., Azouz, L. R. & Whitehead, T. A. Single-mutation fitness landscapes

for an enzyme on multiple substrates reveal specificity is globally encoded. *Nat.*

Commun. **8**, 15695 (2017).

21. Chen, J. Z., Fowler, D. M. & Tokuriki, N. Comprehensive exploration of the

translocation, stability and substrate recognition requirements in VIM-2 lactamase. *Elife*

9, (2020).

22. Li, A., Acevedo-Rocha, C. G. & Reetz, M. T. Boosting the efficiency of site-saturation

mutagenesis for a difficult-to-randomize gene by a two-step PCR strategy. *Appl.*

Microbiol. Biotechnol. **102**, 6095–6103 (2018).

23. Biot-Pelletier, D. & Martin, V. J. J. Seamless site-directed mutagenesis of the

Saccharomyces cerevisiae genome using CRISPR-Cas9. *J. Biol. Eng.* **10**, 6 (2016).

24. Dionne, U. *et al.* Protein context shapes the specificity of SH3 domain-mediated

interactions in vivo. *Nat. Commun.* **12**, 1597 (2021).

25. Eddy, A. A. Expulsion of uracil and thymine from the yeast Saccharomyces cerevisiae:

contrasting responses to changes in the proton electrochemical gradient. *Microbiology*

143 (Pt 1), 219–229 (1997).

26. Kurtz, J. E., Exinger, F., Erbs, P. & Jund, R. New insights into the pyrimidine salvage

pathway of Saccharomyces cerevisiae: requirement of six genes for cytidine

metabolism. *Curr. Genet.* **36**, 130–136 (1999).

27. Fujimura, H. Growth inhibition of Saccharomyces cerevisiae by the immunosuppressant

leflunomide is due to the inhibition of uracil uptake via Fur4p. *Mol. Gen. Genet.* **260**,

102–107 (1998).

28. Després, P. C., Dubé, A. K., Nielly-Thibault, L., Yachie, N. & Landry, C. R. Double

Selection Enhances the Efficiency of Target-AID and Cas9-Based Genome Editing in

Yeast. *G3* **8**, 3163–3171 (2018).

29. Sadhu, M. J. *et al.* Highly parallel genome variant engineering with CRISPR-Cas9. *Nat.*

Genet. **50**, 510–514 (2018).

30. Wang, J. *et al.* Role of glutamate 64 in the activation of the prodrug 5-fluorocytosine by

yeast cytosine deaminase. *Biochemistry* **51**, 475–486 (2012).

31. Ivankov, D. N., Finkelstein, A. V. & Kondrashov, F. A. A structural perspective of

compensatory evolution. *Curr. Opin. Struct. Biol.* **26**, 104–112 (2014).

32. Schymkowitz, J. *et al.* The FoldX web server: an online force field. *Nucleic Acids Res.*

33, W382–8 (2005).

33. Stolworthy, T. S. *et al.* Yeast cytosine deaminase mutants with increased thermostability

impart sensitivity to 5-fluorocytosine. *J. Mol. Biol.* **377**, 854–869 (2008).

34. Mayrose, I., Graur, D., Ben-Tal, N. & Pupko, T. Comparison of site-specific rate-

inference methods for protein sequences: empirical Bayesian methods are superior.

Mol. Biol. Evol. **21**, 1781–1791 (2004).

35. Tarassov, K. An in vivo map of the yeast protein interactome. *Science* **320**, (2008).

36. Freschi, L., Torres-Quiroz, F., Dubé, A. K. & Landry, C. R. qPCA: a scalable assay to

measure the perturbation of protein--protein interactions in living cells. *Mol. Biosyst.* **9**,

36–43 (2013).

37. Chang, A. *et al.* BRENDA, the ELIXIR core data resource in 2021: new developments

and updates. *Nucleic Acids Res.* **49**, D498–D508 (2021).

38. Jumper, J. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* **596**,

583–589 (2021).

39. Mirdita, M. *et al.* ColabFold - Making protein folding accessible to all. *bioRxiv*

2021.08.15.456425 (2022) doi:10.1101/2021.08.15.456425.

40. Pokusaeva, V. O. *et al.* An experimental assay of the interactions of amino acids from

orthologous sequences shaping a complex fitness landscape. *PLoS Genet.* **15**,

e1008079 (2019).

41. Goodwin, M. L. & Drew, R. H. Antifungal serum concentration monitoring: an update. *J.*

Antimicrob. Chemother. **61**, 17–25 (2008).

42. Oliver, J. D. *et al.* F901318 represents a novel class of antifungal drug that inhibits

dihydroorotate dehydrogenase. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 12809–12814

(2016).

43. Hoenigl, M. *et al.* The Antifungal Pipeline: Fosmanogepix, Ibrexafungerp, Olorofim,

Opelconazole, and Rezafungin. *Drugs* (2021) doi:10.1007/s40265-021-01611-0.

44. Verweij, P. E., Te Dorsthorst, D. T. A., Janssen, W. H. P., Meis, J. F. G. M. & Mouton, J.

W. In vitro activities at pH 5.0 and pH 7.0 and in vivo efficacy of flucytosine against

Aspergillus fumigatus. *Antimicrob. Agents Chemother.* **52**, 4483–4485 (2008).

45. Gsaller, F. *et al.* Mechanistic Basis of pH-Dependent 5-Flucytosine Resistance in

Aspergillus fumigatus. *Antimicrob. Agents Chemother.* **62**, (2018).

46. Garland, T., Jr. Trade-offs. *Curr. Biol.* **24**, R60–R61 (2014).

47. Chang, Y. C. *et al.* Moderate levels of 5-fluorocytosine cause the emergence of high

frequency resistance in cryptococci. *Nat. Commun.* **12**, 3418 (2021).

48. Billmyre, R. B., Applen Clancey, S., Li, L. X., Doering, T. L. & Heitman, J. 5-

fluorocytosine resistance is associated with hypermutation and alterations in capsule

biosynthesis in Cryptococcus. *Nat. Commun.* **11**, 127 (2020).

49. Stiffler, M. A., Hekstra, D. R. & Ranganathan, R. Evolvability as a function of purifying

selection in TEM-1 β-lactamase. *Cell* **160**, 882–892 (2015).

50. Esposito, D. *et al.* MaveDB: an open-source platform to distribute and interpret data

from multiplexed assays of variant effect. *Genome Biol.* **20**, 223 (2019).

51. McManus, B. A., Moran, G. P., Higgins, J. A., Sullivan, D. J. & Coleman, D. C. A

Ser29Leu substitution in the cytosine deaminase Fca1p is responsible for clade-specific

flucytosine resistance in Candida dubliniensis. *Antimicrob. Agents Chemother.* **53**,

4678–4685 (2009).

52. Wang, Z. & Zhang, J. Abundant indispensable redundancies in cellular metabolic

networks. *Genome Biol. Evol.* **1**, 23–33 (2009).

53. Brachmann, C. B. *et al.* Designer deletion strains derived from Saccharomyces

cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene

disruption and other applications. *Yeast* **14**, 115–132 (1998).

54. Gietz, R. D. & Schiestl, R. H. High-efficiency yeast transformation using the LiAc/SS

carrier DNA/PEG method. *Nat. Protoc.* **2**, (2007).

55. Janke, C. *et al.* A versatile toolbox for PCR-based tagging of yeast genes: new

fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **21**, 947–

962 (2004).

56. Goldstein, A. L. & McCusker, J. H. Three new dominant drug resistance cassettes for

gene disruption in Saccharomyces cerevisiae. *Yeast* **15**, 1541–1553 (1999).

57. DeLuna, A., Springer, M., Kirschner, M. W. & Kishony, R. Need-based up-regulation of

protein levels in response to deletion of their duplicate genes. *PLoS Biol.* **8**, e1000347

(2010).

58. Casadaban, M. J. & Cohen, S. N. Analysis of gene control signals by DNA fusion and

cloning in Escherichia coli. *J. Mol. Biol.* **138**, 179–207 (1980).

59. Yachie, N. *et al.* Pooled-matrix protein interaction screens using Barcode Fusion

Genetics. *Mol. Syst. Biol.* **12**, 863 (2016).

60. Andrews, S. *FastQC: A quality control analysis tool for high throughput sequencing*

data. (Github).

61. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina

sequence data. *Bioinformatics* **30**, 2114–2120 (2014).

62. Hunter, J. D. Matplotlib: A 2D Graphics Environment. *Computing in Science Engineering*

9, 90–95 (2007).

63. Harris, C. R. *et al.* Array programming with NumPy. *Nature* **585**, 357–362 (2020).

64. Reback, J. *et al. pandas-dev/pandas: Pandas 1.3.4.* (2021).

doi:10.5281/zenodo.5574486.

65. Waskom, M. seaborn: statistical data visualization. *J. Open Source Softw.* **6**, 3021

(2021).

66. Virtanen, P. *et al.* SciPy 1.0: fundamental algorithms for scientific computing in Python.

Nat. Methods **17**, 261–272 (2020).

67. Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G. & Neufeld, J. D.

PANDAseq: paired-end assembler for illumina sequences. BMC

Bioinformaticshttps://doi.org/10.1186/1471-2105-13-31 (2012).

68. Rognes, T., Flouri, T., Nichols, B., Quince, C. & Mahé, F. VSEARCH: a versatile open

source tool for metagenomics. *PeerJ* **4**, e2584 (2016).

69. Rice, P., Longden, L. & Bleasby, A. EMBOSS: the European Molecular Biology Open

Software Suite. Trends Genet.https://doi.org/10.1016/S0168-9525(00)02024-2 (2000).

70. Ryan, O. W., Poddar, S. & Cate, J. H. D. Crispr–cas9 genome engineering in

Saccharomyces cerevisiae cells. *Cold Spring Harb. Protoc.* **2016**, (2016).

71. Amberg, D. C., Burke, D. J., Burke, D. & Strathern, J. N. *Methods in Yeast Genetics: A*

Cold Spring Harbor Laboratory Course Manual. (CSHL Press, 2005).

72. Marchant, A. *et al.* The role of structural pleiotropy and regulatory evolution in the

retention of heteromers of paralogs. *Elife* **8**, (2019).

73. Usmanova, D. R. *et al.* Self-consistency test reveals systematic bias in programs for

prediction change of stability upon mutation. *Bioinformatics* **34**, 3653–3658 (2018).

74. Howe, K. L. *et al.* Ensembl 2021. *Nucleic Acids Res.* **49**, D884–D891 (2021).

75. Chorostecki, U., Molina, M., Pryszcz, L. P. & Gabaldón, T. MetaPhOrs 2.0: integrative,

phylogeny-based inference of orthology and paralogy across the tree of life. *Nucleic*

Acids Res. **48**, W553–W557 (2020).

76. Byrne, K. P. & Wolfe, K. H. The Yeast Gene Order Browser: combining curated

homology and syntenic context reveals gene fate in polyploid species. *Genome Res.* **15**,

1456–1461 (2005).

77. Edgar, R. C. MUSCLE: a multiple sequence alignment method with reduced time and

space complexity. *BMC Bioinformatics* **5**, 113 (2004).

78. Lõoke, M., Kristjuhan, K. & Kristjuhan, A. Extraction of genomic DNA from yeasts for

PCR-based applications. *Biotechniques* **50**, 325–328 (2011).

79. Schlecht, U., Miranda, M., Suresh, S., Davis, R. W. & St Onge, R. P. Multiplex assay for

condition-dependent changes in protein-protein interactions. *Proc. Natl. Acad. Sci. U. S.*

A. **109**, 9213–9218 (2012).

80. Diss, G. & Lehner, B. The genetic landscape of a physical interaction. *Elife* **7**, (2018).

81. Pettersen, E. F. *et al.* UCSF ChimeraX: Structure visualization for researchers,

educators, and developers. *Protein Sci.* **30**, 70–82 (2021).

82. Letunic, I. & Bork, P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic

tree display and annotation. *Nucleic Acids Res.* **49**, W293–W296 (2021).

1288

 # Figure Legends

Figure 1: Fcy1 as a model for resistance-function trade-offs. A) Structure of the Fcy1 homodimer (pdb: 1p6o[15]) and two reactions it catalyzes with opposed effects on fitness. To explore *FCY1*'s functional landscape, each codon was mutated one by one to all other possibilities to generate a comprehensive pool of mutants. The mutants were split into three partly overlapping fragments, and studied in three pools: pool 1 covers from position 2-67, pool 2 from 49-110 and pool 3 from 93 to 158. The resulting libraries were then integrated at the native *S. cerevisiae* locus using CRISPR-Cas9 genome editing, therefore preserving its endogenous transcriptional regulation. **B)** Workflow for the pooled mutants growth assays. The FCY1 mutant libraries were expanded by a round of culture in non-selective media before being split and transferred into 5-FC and cytosine media where mutants competed for around ~20 generations. Variant abundance before and after competition was measured by high-throughput sequencing. **C)** Trade-off of different intensities between resistance to 5-FC and growth using cytosine. In a weak trade-off scenario (light grey), resistance mutations that preserve the canonical enzyme function exist. Resistance can coexist with cytosine use. A strong trade-off (dark) instead implies that mutations that lead to resistance necessarily lead to loss the of cytosine deamination activity.

Figure 2: The functional landscape of Fcy1 identifies all resistance mutations. A) Fcy1
functional landscape in 5-FC media and **B)** in cytosine media. Red dots represent the wild-
type amino acid at each position. The score represents the DMS mutant $\log_2$ fold-change,
scaled by the median of scores of synonymous and nonsense mutations (see methods). The
leftmost column on both heatmaps represents the scores for stop-codons (*). A positive
DMS score indicates faster growth. **C)** Position-wise median 5-FC and **D)** cytosine DMS
scores of missense mutants, mapped onto the Fcy1 homodimer (PDB: 1p6o[15]). The color
scaling across the four panels is based on the 2th and 98th percentile of the 5-FC scores
distribution.
1317

**Figure 3: Fcy1 amino acid substitutions can lead to 5-FC resistance through multiple
mechanisms. A)** 5-FC and cytosine DMS scores of mutations at the substrate binding and
catalytic residues (substrate), zinc cofactor binding residues (Zinc), residues involved in
binding stabilization through Van der Waals interactions (V.d.Waals) and residues involved
in guiding the exit of the product (exit). The score for the silent and nonsense variants for
each position are shown in green and red respectively. **B)** 5-FC and cytosine DMS scores of
missense variants as a function of their presence in orthologous sequences (n=1,078
observed, n=1,890 unobserved). The difference between medians was tested using Mood's
median test: for 5-FC scores, $p=8.12 \times 10^{-134}$, and for cytosine, $p=2.68 \times 10^{-69}$. **C)** 5-FC and
cytosine DMS score distributions for residues as a function of their distance to the dimer
interface. n=531 variants at the interface, n=722 variants near the interface, and n=1,715
distant variants. **D)** Evolutionary rates of residues at, near or distant to the dimer interface.
E) The stability of the Fcy1-Fcy1 complex measured by DHFR-PCA for varinats clustering
with silent mutations is higher than for those clustering with nonsense mutations (p=0.006,
Mood's median test, n=10 silent-like mutants, n=18 nonsense-like mutants). In DHFR-PCA
assay, growth in methotrexate (MTX) (y-axis) is coupled with the amount of complex formed.
F) Two dimensional representation of 5-FC and cytosine DMS scores for all variants. Silent
mutants are shown in green, nonsense in red.
1336

Figure 4: Fcy1 mutants are constrained to a small region of phenotypic space. A) Growth rates in 5-FC and cytosine of 87 validation mutants in the pooled competition conditions (SC + 12μM 5-FC or SC -Ura + 84 μM cytosine. The variants were assigned to different clusters based on DMS scores. Green variants clustered with silent mutations and exhibit a phenotype close to the wild-type. Red variants cluster with nonsense mutations, implying complete loss of function. Blue mutants had both low 5-FC DMS and cytosine DMS scores. Variants falling outside these clusters are shown in grey. The grey area represents the 95% confidence interval around the log-log linear regression slope fit. The inset shows the values on a linear scale. Variants that had 5-FC and DMS scores suggesting they might be mutants that escape the trade-off are shown as squares. **B)** Dose-response curve of wild-type *FCY1* to 5-FC and **C)** to cytosine. The Hill equation parameters were estimated using a non-linear least-square fit on relative growth rates, and the grey area represents the uncertainty around the dose-response curves based on the 95% confidence interval of the measured IC_{50} and hill coefficients. The inset shows the data on a linear scale.

Figure 5: Dose-response parameters predict the properties of Fcy1 mutants. A)
Relationship between growth in 5-FC and cytosine when concentrations drop beneath the
IC_{50} based on the dose-response curves. Growth in cytosine media is abolished so quickly
by lower flux that the same change has little effect on 5-FC resistance. **B)** Effect of reduced
Fcy1 flux on growth inhibition by 5-FC and **C)** growth in cytosine media based on the dose-
response curve parameters. The grey lines show the predicted dose-response curves if Fcy1
metabolic flux is reduced. The blue line represents the phenotype of a Fcy1 variant which
reduces pathway flux by 50% in validation conditions (12 µM 5-FC or 84 µM cytosine). **D)**
Response of validation mutants to higher concentrations of 5-FC and cytosine. The mutants
were ordered based on their position along the trade-off front, starting from the top left in
Figure 4A (no 5-FC resistance, full growth in cytosine). The colored dots above represent the
cluster to which the mutants were assigned based on the validation data. Variants with
mutations predicted to affect stability significantly are annotated with triangles: mutations
annotated with grey triangles have a ΔΔG of 1-5 Kcal/mol and those with black triangles a
ΔΔG >5 Kcal/mol.

Figure 6: The resistance-function trade-off is conserved in the yeast *FCY1* ortholog of
the pathogen *C. neoformans.* A) Structural alignment of cnFcy1 (in orange) as predicted
by AlphaFold and scFcy1 (in grey, pdb: 1p6o). **B)** 5-FC resistance spot dilution assay
phenotypes (see Figure S18 for images) for matched variant pairs, where S: sensitive, r: low
growth and R: full resistance. Variant pairs are colored by the position of the *scFCY1* variant
in the trade-off front. **C)** Resistance-function trade-offs of *scFCY1* and *cnFCY1* variants in
the same conditions as the pooled DMS assays (SC + 12μM 5-FC or SC -Ura + 84 μM
cytosine). The parental strain (BY4742, positive control) and the deletion strain (*fcy1Δ*,
negative control) are shown as triangles. sc*FCY1* variants are shown in grey and those of
cnFcy1 in orange, with the wild-type codon optimized sequence shown as a square.