Environment by environment interactions (ExE) differ across genetic backgrounds (ExExG)

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Abstract

While the terms "gene-by-gene interaction" (GxG) and "gene-by-environment interaction" (GxE) are commonplace within the field of quantitative and evolutionary genetics, "environment-by-environment interaction" (ExE) is a term used less often. However, in this study, we find that environment-by-environment interactions are common and differ for different genotypes (ExExG). To reach this conclusion, we analyzed a large dataset of roughly 1,000 mutant yeast strains with varying degrees of resistance to different antifungal drugs. Many researchers endeavor to predict combinations of drugs that are more lethal than either single drug. But we show that the effectiveness of a drug combination, relative to the effectiveness of single drugs, often varies across different drug resistant mutants. Even mutants that differ by only a single nucleotide change can have dramatically different drug x drug (ExE) interactions. Studying how ExE interactions change across genotypes (ExExG) is not only important when modeling the evolution of pathogenic microbes. High throughput screens of GxG and GxE have taught us about the basic cell biology and gene regulatory networks underlying genetic interactions. ExExG has been omitted but stands to impart similar lessons about the architecture of living systems. In this study, we call attention to ExExG, measure its prevalence, introduce a new framework that in some instances better predicts its direction and magnitude, and make the case for further study of this type of genetic interaction.

Introduction

Over 100 years ago, William Bateson (Bateson, 1902) and R. A. Fisher (Fisher, 1919) defined the term "epistasis" to describe genetic interactions. Broadly speaking, genetic interactions are cases where the combined effect of two genetic changes is not simply their sum (Ogbunugafor et al., 2016; Phillips, 1998). Or, as one colloquial definition frames it, epistasis is the "surprise at the phenotype when mutations are combined, given the constituent mutations' individual effects" (Weinreich et al., 2013). These sorts of interactions have been of interest, in both classical and modern settings, because they undermine one of the major goals of modern biology: predicting the phenotypic effects of mutations (Brandon Ogbunugafor & Scarpino, 2022; Eguchi et al., 2019; Sackton & Hartl, 2016; Zhou et al., 2022). They have sparked debate about the extent to which interactions impede such prediction efforts (Ehrenreich, 2017; Huang & Mackay, 2016) and what types of interactions, e.g. gene x gene (GxG) or gene x environment (GxE), are important (Ogbunugafor, 2022). Genetic interactions have become of increasing interest to other disciplines as well (Domingo et al., 2019). For example, genetic interactions have suggested which genes participate in the same regulatory modules (Boone et al., 2007; Segal, Shapira, et al., 2003), predicted which evolutionary trajectories are most likely (Brown et al., 2010; Ogbunugafor et al., 2016), and revealed global constraints on protein evolution (Weinreich et al., 2006) and adaptive evolution (Kryazhimskiy et al., 2014). Given their broad utility to biologists, many useful mathematical frameworks exist for quantifying GxG (Mani et al., 2008), GxE (Agrawal & Whitlock, 2010) and GxGxE (Diaz-Colunga et al., 2023; Ogbunugafor, 2022; Ogbunugafor et al., 2016). Further, many experimental frameworks have comprehensively surveyed GxG or GxGxG (Brown et al., 2010; Kuzmin et al., 2018; New & Lehner, 2019; Taylor & Ehrenreich, 2014; Weinreich et al., 2006), GxE (J. Z. Chen et al., 2022; S.-A. A. Chen et al., 2023; Lindsey et al., 2013), or GxGxE (Ardell et al., 2023; Costanzo et al., 2021; Flynn et al., 2013; Ghenu et al., 2023; Lindsey et al., 2013). But one type of interaction has remained almost completely ignored by quantitative geneticists: ExE (**Figure 1A**).

Here, we define ExE (i.e. environment-by-environment interactions) as when the combined effect of two environments on phenotype is unexpected given their individual effects (**Figure 1B**). For example, if a microbe grows slowly in a high salt environment and equally slowly in a high temperature environment, but does not grow even slower in a high salt plus high temperature environment, this would be unexpected under an additive model and herein termed "ExE". Perhaps the reason for the near omission of the term "ExE" in the quantitative genetics literature is obvious: there is no genetic component (no "G"), so those who map the effects of genetic changes onto phenotype have less interest in quantifying ExE. Whatever the reason that ExE interactions are not a current research focus, there are several reasons it may be worthwhile to turn attention towards ExE. For one, understanding why environments have non-additive effects on phenotype stands to expand knowledge about regulatory network architecture (Yeh et al., 2006, 2009), as have GxG and GxE models (Boone et al., 2007; Segal, Wang, et al., 2003). Further, if ExE often varies across genetic backgrounds, in other words, if ExExG is common, then quantitative and evolutionary geneticists can incorporate ExExG interactions into models that predict the phenotypic effects of mutation. Note, ExExG is not the same phenomenon as GxGxE (**Figure 1C–D**).

To date, only a handful of papers mention ExExG (Sardi et al., 2018), but most are specifically focused on either multidimensional plasticity (Morel-Journel et al., 2020; Verspagen et al., 2020; Westneat et al., 2019), or environments that are experienced subsequently rather than simultaneously (Avinun et al., 2018; Starr et al., 2021; Sun & Cao, 2024).

A key reason to study ExE pertains to drug resistance (Foucquier & Guedj, 2015; Madani Tonekaboni et al., 2018; Russ & Kishony, 2018), though it is termed "drug interaction" (Gjini & Wood, 2021; Yeh et al., 2009) or occasionally "drug epistasis" (Michel et al., 2008) rather than "ExE". There is practical interest in finding pairs of drugs that interact 'synergistically', i.e., the combination of both drugs is deadlier than one would predict based on either single drug (**Figure 1B**) (Cacace et al., 2023; Liu et al., 2021; Meyer et al., 2020; Mikhail et al., 2019; Roell et al., 2017). But just as genotype-phenotype mapping studies focus less on environment interactions, drug synergy studies focus less on genetic interactions. For example, several studies suggest that if one understands the cell biology underlying drug interactions, one can predict synergy (Feala et al., 2010; Pan et al., 2023; Yang et al., 2020), but this ignores that mutations may change the underlying drug interactions (Schmidlin et al., 2024). Other studies describe the biggest challenge in detecting synergy as there being more possible drug combinations than one can study (Madani Tonekaboni et al., 2018; Menden et al., 2019; Pan et al., 2023), but this ignores that studying every drug combination in every genetic background would be orders of magnitude more difficult. Despite the combinatorics challenge, heroic efforts have been made to measure massive numbers of drug interactions (Menden et al., 2019), including higher-order interactions (Lozano-Huntelman et al., 2021), which have fueled sophisticated multidrug treatment strategies and evolutionary models (Baym et al., 2016). But these treatments and models could fail if mutations change the drug interactions on which they are based. Thus, systematic study of the extent to which drug interactions change across genetic backgrounds (ExExG) is needed.

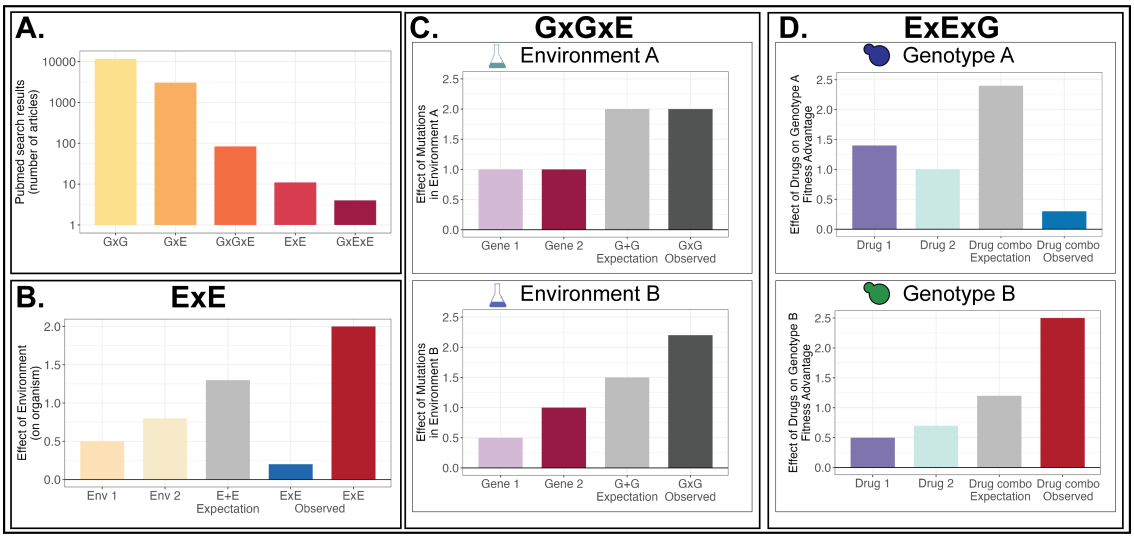

Figure 1: Comparative visualizations of ExE, GxGxE and ExExG interactions.(A) ExE interactions are understudied. Search results retrieved from Pubmed on May 3, 2024 demonstrate that publications describing ExE interactions, including GxExE, show substantial disparities when compared to simpler interactions like GxG

and GxE which have significantly greater representation. Complete search term results are located in table S1. **(B)** A cartoon to define ExE. Environments 1 and 2 have unique effects on organism fitness (light orange and light yellow). When exposed to both environments simultaneously it is possible that the effect on fitness is additive (E+E, indicated by gray), synergistic (fitness is lower than expected, indicated by blue) or antagonistic (fitness is higher than expected, indicated by red). **(C)** A cartoon to define GxGxE. GxGxE interactions investigate the effect of the same mutations (light and dark pink) in two or more environments. In this cartoon, the effects of gene 1 and gene 2 are additive (G+G = GxG) in environment A, but produce unexpected interactions in environment B (G+G < GxG). **(D)** A cartoon to define ExExG. In this manuscript, fitness advantages are used to delve into ExExG interactions. Fitness is always measured relative to a reference strain, which is the ancestor of all the drug resistant mutants we study. Different drug resistant genotypes are exposed to the same single drugs (Drug 1, purple and Drug 2, teal) and their combination (Drug combo, gray). For illustration, genotype A is resistant to drug 1 and 2 but when these drugs are combined, it exhibits synergy (drug combo observed, blue). In opposition, genotype B is moderately resistant to drug 1 and 2 but when exposed to the combination, the fitness is greater than expected (drug combo observed, red).

This type of systematic study has recently become possible due to evolution experiments that utilize DNA barcodes (Levy et al., 2015; Schmidlin et al., 2024) to create thousands of adaptive microbial strains that each possess only a small number of genetic differences and are highly tractable, meaning their fitness relative to a common ancestor can be measured in many conditions using pooled barcoded competitions. Here, we take a large collection of roughly 1,000 antifungal drug-resistant yeast mutants evolved using this method and ask how often fitness in multidrug environments is predicted by fitness in single drug environments (**Figure 1D**). We find substantial ExE (i.e., multidrug fitness is not easily predicted by single drug fitness). We also find substantial ExExG (i.e. the magnitude and direction of ExE are different across different mutants). We demonstrate that single point mutations often alter ExE and that even similar adaptive mutants that emerge from the same evolution experiment can have different ExE. Given the prevalence of ExExG in our data, we next explored some new ways to study ExE and ExExG. We applied a GxG model to better predict ExE for some mutants. We also observed that diverse mutants cluster into groups with characteristic ExE, implying the ExE of some mutants can be used to predict ExE of others. In general, our findings call for greater study of ExExG across disciplines, including among scientists interested in modeling the evolution of drug resistance, scientists interested in how gene expression responds to environmental change, and scientists interested in predicting phenotype from genotype.

Results and Discussion

Environment by environment (ExE) interactions vary across drug pairs

In order to study environment-by-environment interactions, we compared data from pooled fitness competitions conducted in 4 environments each containing a single drug to data from 4 environments representing all pairwise combinations of these drugs (Schmidlin et al., 2024) **(Figure 2A)**. We asked if multidrug fitness of 1000 drug-resistant mutants was easily predicted by fitness in each single drug environment. We used four different models **(Figure 2B)** to predict fitness in the drug combination environments, including the simple additive model depicted in **figure 1B** and other common models (Bliss, 1939; Foucquier & Guedj, 2015; Madani Tonekaboni et al., 2018; Meyer et al., 2020; Yeh et al., 2009). None of the models we tried accurately predicts fitness in all four drug combinations. For example, fitness in the combined low rad + low flu environment (LRLF) is often predicted by taking the higher fitness of the low rad and low flu single drug environments (**figure 2B**; leftmost panel; median falls on the zero line when using the highest single agent "HSA" model). But this same model tends to overpredict fitness in the high rad + low flu environment and underpredict fitness in the low flu + high rad environment (**Figure 2B**; middle panels; medians of HSA model fall farther from the zero line). Overall, there appears to be a good deal of ExE interaction. In other words, there are many cases where fitness in multidrug environments is not predicted by fitness in single drug environments.

Like previous studies, we noticed that the direction of ExE interaction is sometimes specific to a multidrug environment (Lozano-Huntelman et al., 2021; Yeh et al., 2006). For example, most of the models we tried tend to overpredict fitness in the high rad + low flu environment (HRLF). In other words, this combination of drugs is "synergistic", meaning it hinders fitness more than expected based on the fitness effects of both single drugs (**Figure 2B**; third panel, more points are blue and most boxplot medians fall below the zero line). The opposite tendency, "antagonism", appears more common in the low rad + high flu environment (LRHF). Fitness in this drug combination is often greater than expected based on fitness in the relevant single drug conditions (**Figure 2B**; second panel, more points are red and more boxplot medians fall above the center line). These trends are important because identifying synergistic drug combinations (those that are more detrimental than expected) could be helpful in treating viral (Jin et al., 2021), bacterial (Roemer & Boone, 2013), and fungal infections (Kane & Carter, 2022), and cancers (Menden et al., 2019). Identifying drug pairs that interact antagonistically could be helpful as well by suggesting functional relationships between drug targets and strategies for restraining the evolution of drug resistance (Lozano-Huntelman et al., 2021; Yeh et al., 2006, 2009).

But to what extent is synergy or antagonism a property of a drug pair? Even for drug pairs in which most of the mutants we study have lower fitness than expected, there are a few mutants that have unexpectedly high fitness (**Figure 2B**; there are always a number of red points even when most points are blue). So we next asked to what extent ExE varies across drug pairs versus across different mutants.

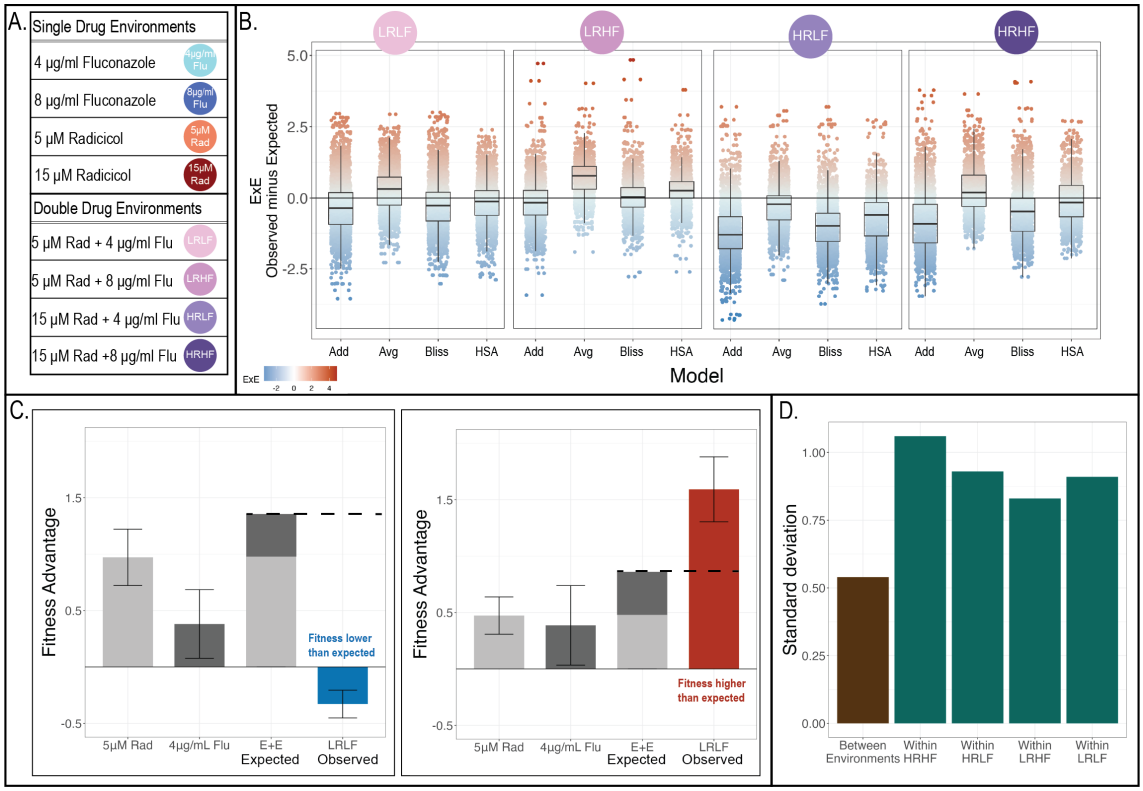

Figure 2: ExE interactions vary across drug pairs and across mutants. (A) We predict fitness in four double drug environments from fitness in the relevant single drug environments. **(B)** Environment-by-environment interactions are revealed when fitness in a double drug environment deviates from the expectation generated by the relevant single drug environments. Four different models (horizontal axis) are used to calculate expected fitness for each of roughly 1000 mutants per drug pair (LRLF: n=1688; LRHF: n=850; HRLF: n=1318; HRHF: n=1023). Points representing each mutant are colored blue when a mutant's fitness is worse than expected (synergy), and red when fitness is higher than expected (antagonism). Boxplots summarize the distribution across all mutants, displaying the median (center line), interquartile range (IQR) (upper and lower hinges), and highest value within 1.5 × IQR (whiskers). **(C)** Some mutants have different ExE interactions than others. The left panel displays the fitness of a yeast strain with a mutation in the HDA1 gene. It has lower fitness in the LRLF double drug environment than expected based on the simple additive model depicted in figure 1. The right hand panel shows a different yeast strain that has higher fitness than expected in the same environment. Error bars represent the range of fitness measured across two replicate experiments. Fitness is always measured relative to a reference strains, which is the shared ancestor of all mutant strains. **(D)** ExE interactions vary more across mutants than they do across drug pairs. The vertical axis displays the standard deviation across all four environments (brown) or across all roughly 1,000 mutants (green) when ExE is predicted using an additive model.

ExE interactions vary more across mutants than they do across drug pairs

The drug resistant mutants we study were created in previous work by evolving a barcoded ancestral yeast strain in 12 different environments, including the 8 in **figure 2A** (Schmidlin et al., 2024). Each mutant yeast strain differs from their shared ances-

tor by, on average, a single point mutation (Levy et al., 2015; Schmidlin et al., 2024). Yet, despite this similarity at the genetic level, there is variation in ExE (**figure 2B**; see spread of points along vertical axis). To point to an example, one of these evolved yeast strains has a single point mutation in the HDA1 gene. It has unexpectedly low fitness in the LRLF environment given its fitness advantage in the relevant single drug environments (low rad: 5uML Rad and low flu: 4ug/mL Flu) (**Figure 2C**; left panel; error bars reflect range across 2 replicates). However, another (unsequenced) one of these evolved mutants has unexpectedly high fitness in this environment (**Figure 2C**; right panel; error bars reflect range across 2 replicates). The fitness of all mutants is measured relative to a reference strain, which is their shared ancestor (Schmidlin et al., 2024).

While our previous work focused on 774 mutants with high quality fitness measurements in all 12 environments, here we are able to expand that collection. We do so by allowing each drug pair to have a unique dataset consisting of all mutant strains for which fitness was robustly measured in the relevant double and single drug conditions, plus a control condition with no drugs (LRLF: n=1688; LRHF: n=850; HRLF: n=1318; HRHF: n=1023). These datasets include 810 overlapping mutants for each of which we calculated ExE in all four drug pairs.

Overall, we found that ExE interactions vary at least as much across genotypes as they do across drug pairs. When using a simple additive model, the median amount of ExE varies across environments from -1.35 in HRLF to -0.3 in LRHF, with a standard deviation across all 4 drug pairs of 0.52 (**Figure 2D**; leftmost bar). This standard deviation is smaller than the standard deviation across mutants within each environment, which ranges from 0.8 to 1.05 (**Figure 2D**). In sum, these results suggest that ExExG is prevalent. Our follow-up analyses provide additional evidence that ExExG indeed reflects how ExE varies across different genes and strains.

Mutations in different genes have different ExE interactions

Of the 810 drug resistant yeast strains present across all environments we survey, 62 have been previously sequenced at high enough coverage to identify the single nucleotide mutations that likely underlie drug resistance (Schmidlin et al., 2024). A few genes appear to be common targets of adaptive mutation such that we can ask whether mutants in the same gene tend to have similar ExE interactions. For example, 35/62 sequenced drug-resistant strains have different mutations to either the PDR1 or PDR3 paralogs. Other genes, such as SUR1, GBP2 and IRA1, were also found to be mutated in multiple different strains, though far less frequently than PDR1/3. Mutations to the same gene tend to have similar effects on fitness (**Figure 3**; error bars reflect standard deviation across all strains with mutations to a given gene).

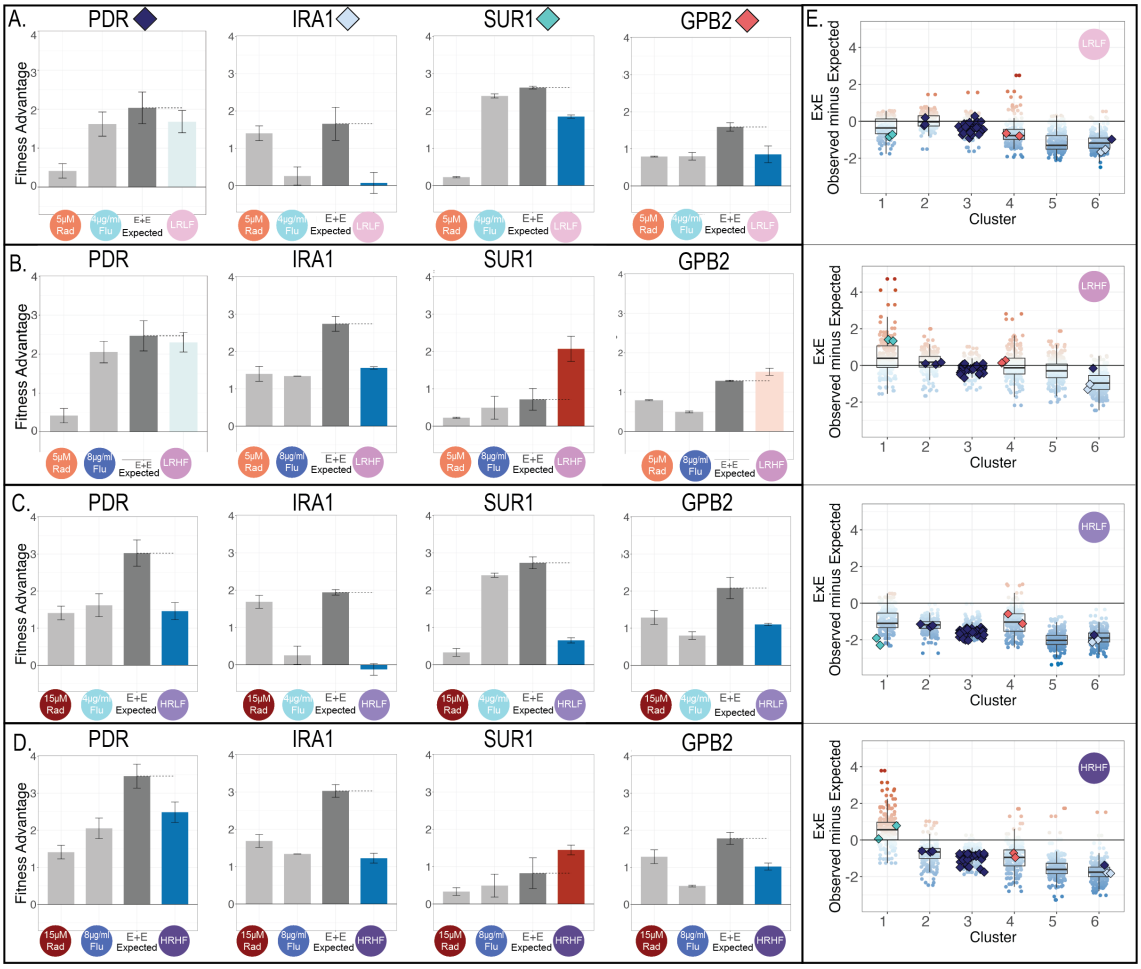

Figure 3: A few mutations can change a drug pair from having a synergistic to an antagonistic effect. Fitness advantages of strains with mutations in either PDR1/3 (n=35), IRA1 (n=3), SUR1 (n=2), GPB2 (n=2), relative to unmutated reference strains. Light gray bars represent average fitness in single drug environments, dark gray bars represent fitness predictions in double drug environments made using an additive model, and colored bars represent average fitness in double drug environments (colored blue when fitness is lower than prediction and red when fitness exceeds the prediction. Colors lighten when within 0.5 of the expected value. The type and magnitude of ExE interaction present appears to be similar across mutations to the same gene, but different across mutations to different genes. Each row corresponds to one of the double drug environments we study, including **(A)** LRLF, **(B)** LRHF, **(C)** HRLF, **(D)** HRHF. **(E)** ExE profiles for 774 mutants in each studied drug combination broken down by cluster assigned in (Schmidlin et al., 2024). Mutants are colored by their type of ExE interaction. Here, mutants that experience synergistic interactions are noted with a blue point while antagonistic interactions are noted with a red point. Colors lighten as both sides approach the expected E+E (0). Sequenced mutants from A-D are shown by colored diamonds. Within each cluster, ExE interactions of each mutant are more similar to each other and to the cluster's sequenced mutants. Boxplots summarize the distribution across all mutants, displaying the median (center line), interquartile range (IQR) (upper and lower hinges), and highest value within 1.5 $\times$ IQR (whiskers).Comparative visualizations of ExE, GxGxE and ExExG interactions.

Overall, we find that mutations to the same gene tend to have similar ExE inter-

actions (**Figure 3**). For example, the 35 PDR1/3 mutants tend to have lower fitness than expected by an additive model in the LRHF environment (**Figure 3A**; left), but not to the same degree as do IRA1 mutants, some of which actually have a slight disadvantage in that double drug environment despite being adaptive in both single drug conditions (**Figure 3A**; middle). And in a different double drug environment, the fitness of all evolved yeast strains with mutations to either PDR1 or PDR 3 is fairly well predicted by an additive model (**Figure 3B**; left). But an additive model dramatically underestimates the fitness of mutations to the SUR1 gene in the same environment (**Figure 3B**; right). Across all four double drug environments and all 4 common targets of adaptation we sequenced, the type and magnitude of ExE interactions depends on which gene is mutated (**Figure 3**).

Our observation that ExE varies across mutants does not necessarily arise because we collected adaptive mutants across 12 different selective pressures (Schmidlin et al., 2024). Mutants that emerge in response to the same selection pressure can have different ExE. For example, IRA1 and GPB2 are both negative regulators of glucose signaling, and both are common targets of adaptation in response to glucose limitation (Kinsler et al., 2020; Schmidlin et al., 2024; Venkataram et al., 2016). Here, we show that these genes demonstrate different ExE interactions. IRA1 mutants perform worse than expected in LRHF, while GPB2 mutants perform better than expected given their meager fitness advantages in the relevant single drug conditions (**Figure 3B**).

In terms of synergy vs antagonism, our results suggest that a small number of mutations can change a drug combination from having a synergistic to an antagonistic effect. For example, **figure 2C** shows a case where LRLF acts synergistically on a yeast strain harboring a single nucleotide mutation to the HDA1 gene, but acts antagonistically on a different evolved yeast mutant. Similarly, **figure 3** shows cases where a drug pair changes from having a synergistic to an antagonistic effect across different mutants. The extreme sensitivity of synergy to the effect of single mutations has important implications for the development of multidrug strategies that rely on drugs having synergistic or antagonistic effects.

Hundreds of mutants fall into a few groups with characteristic ExE interactions

The above observations highlight the prevalence of ExExG. They beg questions about to what extent there are trends that can help us predict ExE of some mutants from other mutants. These observations also beg questions about the underlying cellular mechanisms that cause ExE interactions to change from one mutant to the next. Both types of questions are related because mutations that affect drug resistance through similar cellular mechanisms may have similar ExE, such that understanding the mechanisms underlying ExE may help predict its direction and magnitude.

We previously showed that many (774) of the yeast strains we study cluster into a small number of groups (6) that each may affect fitness via distinct cellular mechanisms (Schmidlin et al., 2024). Here, we find that mutants from the same cluster tend to have more similar ExE (**Figure 3E**). For example, the two yeast strains with

mutations to SUR1 (**Figure 3**) clustered together with 107 other strains that have fitness advantages in low (but not high) concentrations of fluconazole (Schmidlin et al., 2024). On average, ExE interactions across these 109 yeast strains are predicted by the behavior of the SUR1 strains in **figure 3**; they tend to behave synergistically in drug combinations containing low flu (**Figure 3E**; cluster 1 in LRLF & HRLF), and antagonistically in combinations containing high flu (**Figure 3E**; cluster 1 in LRHF & HRHF). Similarly, 31 of the 35 yeast strains with mutations to either PDR1 or PDR3 clustered together with 127 other yeast strains that have fitness advantages in all single and double drug environments (Schmidlin et al., 2024). On average, ExE interactions across these strains are predicted by the behavior of the PDR strains in **figure 3**; they are synergistic across all drug pairs (**Figure 3E**; cluster 2). This synergism (i.e., mutants are less fit than predicted by an additive model) seems consistent with the mechanism underlying drug resistance in PDR strains. PDR1 and PDR3 regulate a pump that eliminates drugs from cells (Fardeau et al., 2007; Osset-Trénor et al., 2023). Perhaps the rate at which this pump removes drug from cells does not increase linearly as more drug is added, therefore an additive model overestimates fitness in double drug environments.

Considering ExExG suggests a nuanced model for predicting ExE

Our clustering analysis suggests that ExE interactions for some mutants might be predicted from others. This is one way that embracing the dependence of ExE on G can help make ExE predictions. Modeling ExE in the same way that genetic interactions are modeled is another potential way to improve ExE predictions. For example, we found it surprising when some mutants that resisted two single drugs lost their fitness advantage when those single drugs were combined (**Figure 4A**; left). However, this loss of fitness is sometimes predictable when we use GxG models to study ExE (**Figure 4**; left side). The key is that GxG models incorporate information from a wildtype individual (Figure 4B). We can mimic this framework to model ExE by incorporating information from an environment lacking drugs. This lets us model the "effect" of each single drug similarly to how models of epistasis model the "effect" of each single mutation (Domingo et al., 2019) (**Figure 4B** – C). Once this effect is measured, it creates an expectation for how addition of this drug will modify fitness (**Figure 4C**; purple diamond). We call our model the "Drug Effect" (DE) model because, like the GxG framework upon which it is based, it assumes that a perturbation (e.g., an environmental change) has a static effect on a given mutant's fitness.

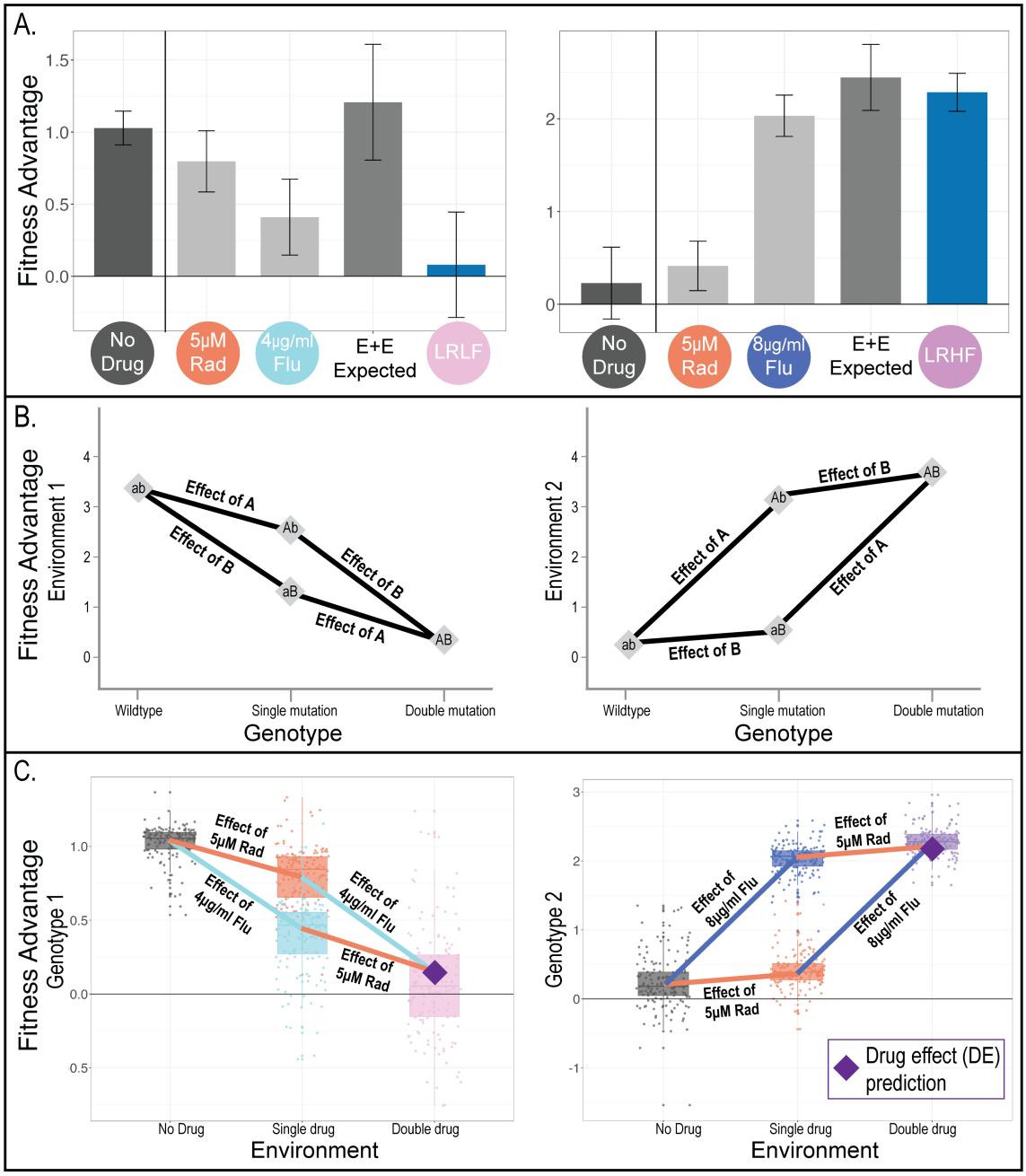

Figure 4: Classical GxG framework inspired a new "drug effect" (DE) model that accurately predicts the behavior of some drug resistant mutants in double drug environments. (A) An additive model makes poor fitness predictions for the 145 mutants in the left panel, but not for the 158 mutants in the right panel. Another key difference is that the mutants in the left panel have fitness advantages over the reference strain in the no drug environment, while the mutants in the right panel do not. The mutants in each panel clustered together in previous work based on their fitness in 12 environments (Schmidlin et al., 2024). Dark gray bars represent average fitness in no drug, light gray bars represent average fitness in single drug environments, medium gray bars represent fitness predictions in double drug environments made using an additive model, and colored bars represent average fitness in double drug environments. Error bars represent standard deviation.**(B)** Classic GxG predictive models are different from the classic ExE predictive models in panel A. They

add together the "effect" of each single mutation rather than the fitness of each single mutant to predict the fitness of the double mutant (Domingo et al., 2019). The left panel provides an example where the wildtype (ab) has a fitness advantage in environment 1. Gaining mutation A or B results in decreased fitness. Subtracting the effect of both A and B allows for the correct prediction of the double mutant's (AB) fitness in environment 1. The right panel presents a second environment where the wildtype fitness is improved by mutations A and B. Here adding the effect of both A and B results in accurate prediction of the double mutant's fitness.**(C)** Repurposing the GxG model in panel B to predict ExE results in accurate predictions. Boxplots summarize the distribution across all mutants, displaying the median (center line), interquartile range (IQR) (upper and lower hinges), and highest value within 1.5 $\times$ IQR (whiskers). No drug is shown in dark gray, single drugs in blue/orange and double drugs in pink/purple. The effect of each drug is represented by a colored line matching that of the single drug. The average prediction of the DE model for both groups of mutants is shown by a purple diamond.

To better illustrate the DE model, consider that the decisive difference between the mutants in **figure 4A** left and right is their fitness in conditions lacking any drug. The mutants on the left have a fitness advantage in conditions lacking drug (**Figure 4A**; no drug). While the mutants on the left also have a fitness advantage in each single drug, the "effect" of each single drug on fitness is actually negative. These drugs reduce the fitness advantage. The DE model thus correctly predicts that the effect of combining both drugs will be a further reduction in fitness (**Figure 4C**; left) while an additive model fails to make an accurate prediction (**Figure 4A**; left). But the mutants on the right have no advantage in the no drug environment, and the "effect" of adding each single drug is actually to improve their relative fitness (**Figure 4A**; right). Here, the DE model performs similarly to a classic additive model in predicting fitness in the multidrug environment (**Figure 4**; right). An important caveat is that, although the DE framework makes reasonable fitness predictions for these two drug pairs, it fails in many other environments and for many other genotypes, again highlighting the prevalence of ExExG.

Methods

Data acquired from experimental evolutions and fitness competitions

All data presented in this work was collected as previously described in (Schmidlin et al., 2024). Briefly, 300,000 barcoded yeast lineages were evolved for 7 weeks in 10 drug conditions and 2 controls. From these evolutions, 21,000 (2k from each evolution) colonies were selected for a fitness remeasurement experiment. Barcode sequencing was performed every 48 hours and log-linear changes in barcode frequencies over 4 time points were used to infer fitness. From this subset, a final collection of 774 lineages, characterized by greater than 500 barcode reads from each of the 12 environments, were analyzed from this previous study. However, there are additional lineages that have greater than 500 barcode reads/condition if you require fewer conditions. Since we were interested in ExE interactions, we created four improved datasets that con-

tained lineages present in the no drug control, both single drugs that made up the combination and the double drug combination. Datasets were improved as follows: LRLF: n=1688; LRHF: n=850; HRLF: n=1318; HRHF: n=1023.

Definitions for drug interaction models

Several models were used to quantify drug interactions and are defined as follows:
1. Additive Model (E+E): The fitness of each lineage in the defined drug combination is determined by the sum of the relative fitness values in drug environment 1 and drug environment 2. For our work here, this constitutes the expected model.

2. Bliss Independence Model (Bliss): Prior to calculation, each fitness value was converted to a percentage based on the maximum observed fitness value in the respective drug combination (DC). The formula is as follows: (Fitness in drug environment 1 + fitness in drug environment 2 - (Fitness in drug environment 1* fitness in drug environment 2))*maxDC.

3. Highest Single Agent Model (HSA): This model reports the maximum fitness value among the single drugs present in the combination.

4. Average Model (Avg): The model fitness in the drug combination is represented as an average between the two single drugs.

5. Drug Effect Model (DE): This model first finds the fitness value for a single drug, then from this value subtracts the fitness of the lineage in no drug from the fitness of the lineage in the second single drug. The result is the prediction for the drug combination.
All code is available on OSF under the project: Environment by environment interactions (ExE) differ across genetic backgrounds (ExExG).

Quantifying ExE for 774 lineages in four drug combinations
In order to quantify the amount of ExE captured in our dataset, we first estimated the fitness of each lineage in the four drug combination environments using log linear slope as previously described (Schmidlin et al., 2024). Five predictions, one for each model above, were made for each lineage in the dataset. Once predictions were calculated, they were subtracted from the known fitness. Differences that did not equal 0 (truth minus prediction) were considered to have environment by environment interactions and are reported as ExE.

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SUPPLEMENT

Table 1 | Pubmed search terms and results for Figure 1A

Search term on Pubmed (3May23)	Number of articles*	Earliest mention
For GxG	**Total: 11,565**	**1951**
"epistasis"	10,531	
"gene-by-gene"	675	
'GxG"	264	
"genotype-by-genotype"	7	
For GxE	**Total: 3048**	**1977**
"genotype-by-environment"	1355	
"GxE"	911	
"gene-by-environment"	782	
For ExE	**Total: 11**	**2012**
"ExE"	874**	
"environment-by-environment"	11	
For GxGxE	**Total: 4**	**2018**
"gene-by-environment-by-environment"	2	
"genotype-by-environment-by-environment"	1	
"GxGxE"	1	

* Search results do not take into account articles that are present in multiple search terms. ** "ExE" resulted in 874 articles, however, the vast majority of these correspond to terms not related to environment-by-environment interactions such as endurance exercise and exe genes.