Evolutionary Biology

Variations and predictability of epistasis on an intragenic fitness landscape

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eLife Assessment

This paper addresses the significant question of quantifying epistasis patterns, which affect the predictability of evolution, by reanalyzing a recently published combinatorial deep mutational scan experiment. The findings are that epistasis is fluid, i.e. strongly background dependent, but that fitness effects of mutations are predictable based on the wild-type phenotype. However, these potentially interesting claims are **inadequately** supported by the analysis, because measurement noise is not accounted for, arbitrary cutoffs are used, and global nonlinearities are not sufficiently considered. If the results continue to hold after these major improvements in the analysis, they should be of interest to all biologists working in the field of fitness landscapes.

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Abstract

How epistasis hinders or facilitates movement on fitness landscapes has been a longstanding question of interest. Several high throughput experiments have demonstrated that despite its idiosyncrasy, epistatic effects exhibit global statistical patterns. Recently, Papkou et. al. constructed a fitness landscape for a 9-base region in the *folA* gene, which encodes for dihydrofolate reductase (DHFR), in *E. coli*, and demonstrated that despite being highly rugged, the landscape is highly navigable. In this work, using the *folA* landscape, we ask two questions: (1) How does the nature of epistatic interactions change as a function of the genomic background? (2) How predictable is epistasis within a gene? Our results show that epistasis is “fluid” - the nature of epistasis exhibited by a pair of mutations is strongly contingent on the genetic background. Mutations exhibit one of two binary “states”: a small fraction of mutations exhibit extremely strong patterns of global epistasis, while most do not. Despite these observations, we observe that the distribution of fitness effects (DFE) of a genotype is highly predictable based on its fitness. These results offer a new perspective on how epistasis operates within a gene, and how it can be predicted.

Significance Statement.

How a mutation changes organismal fitness is dependent on the genome in which it occurs. This phenomenon is known as epistasis and makes evolution unpredictable. Recent efforts to understand epistasis have led to the identification of statistical patterns in its manifestations. To study how epistasis operates in protein evolution, we analyze a recently reported landscape which quantifies fitness of ~260000 sequences of an *E. coli* gene. We show two previously unknown properties of epistasis: "fluid" (epistasis between mutations is controlled via other epistatic interactions) and "binary" (only a few mutations exhibit statistical patterns; most do not). This work sheds new light on how epistasis manifests in gene sequences. Our results have important consequences for protein & organismal evolution.

Introduction

Mutations decide the fitness of an organism in an environment-dependent fashion. But, the effect of mutations also depends on the genetic background they are occurring in[1]. This phenomenon is referred to as epistasis. Hence, epistasis influences adaptation[2,3]. However, it is largely unpredictable, although a few statistical patterns based on macroscopic traits have been reported in the last few years[4–10]. One way to study genotype-phenotype relationship and patterns of epistasis is by using fitness landscapes[11–13].

Fitness landscape is a multidimensional surface, in which one dimension represents a fitness-related phenotype, and the others genotype. Hence, it serves as a genotype-phenotype map, whose shape, in a given environment, is a consequence of genetic interactions or epistasis[14–17]. If the landscape has many peaks, its structure is called rugged[18–20]. Populations navigating on a rugged landscape are likely to be trapped in local peaks, whose precise identity is dictated by chance and population's starting point on the landscape[21–23]. Alternatively, on smooth landscapes, populations starting from different points on the sequence space all converge to the same global peak[24–27]. Thus, the structure of the landscape also dictates our ability to predict evolution, and this has wide-ranging implications.

Although fitness landscape was conceptualized to explain the relationship between a population's genotype and fitness, it has evolved to explain the relationship between fitness and functional protein-coding sequences[13,28]. This effort to characterize fitness landscapes started by considering a handful of "important" biallelic sites in a protein[27,29–37]. Although such landscapes could explain the pervasiveness of epistasis in protein sequences, they do not allow us to make wide-ranging statistical predictions of the characteristics of the fitness landscape[38]. However, with advancing high-throughput technologies, it has become possible to construct high-dimensional landscapes[39–45].

In this study, we use one such high-dimensional fitness landscape constructed by Papkou and coworkers to understand how epistasis operates in a 9-base pair region of the *folA* gene in *E. coli*[40]. *folA* encodes for dihydrofolate reductase (DHFR) and mutations in the gene are known to confer resistance to the antibiotic trimethoprim[46–50].

Via analysis of the *folA* landscape, we ask three specific questions (Supplement Figure S1): (1) How does the nature of epistasis between two given sites change as a function of the genetic background? (2) Are these changes dependent on the fitness or genotype? (3) Does a given mutation follow already known patterns of global epistasis? If yes, what does it depend on? Our results show that epistasis is "fluid" – i.e., the nature of epistasis two mutations exhibit is a

function of the genetic background. We also show that only a small fraction of mutations follow global epistasis. In fact, mutations can be classified into two groups consisting of ones that show global epistasis and others (comprising of a majority) that do not. We also propose a novel way to estimate and predict distribution of fitness effects (DFE) of a given genotype.

Results

folA fitness landscape in *E. coli*

A fitness landscape of a 9-base pair region of *folA* was generated recently by Papkou and coworkers[40]. The landscape explored a 9-bp region of the gene that has been shown to be important for resistance to the antibiotic trimethoprim[47,51]. All 4^9 variants of *folA* were generated, and grown in media containing the antibiotic. Deep sequencing was used to quantify fitness, and relative fitness of ~99.7% of all variants was obtained. A striking feature of the landscape reported by Papkou and coworkers was that despite being highly rugged with 514 peaks, a majority of the landscape had adaptive access to high fitness peaks. This was because, compared to lower peaks, high fitness peaks had a large basin of attraction.

Our analysis using this dataset shows that with increasing size of the landscape, the number of peaks increase; however, the density of the peaks decreases (Supplement Figure S2 and S3). With this increasing size of the landscape, however, the accessibility of the global peak decreases (Supplement Figure S4). Globally, only small regions of the landscape could be represented as a maximally rugged NK landscape (Supplement Figure S5).

By scanning all mutations on a 9-dimensional landscape, Papkou *et. al.* have created a dataset that allows us to ask specific questions about epistasis and its manifestations on a fitness landscape. The genotypes on the *folA* landscape have been divided into two categories by Papkou *et. al.* into functional (~7% of all points) and non-functional (~93% of all points). This distinction is based on a statistical segregation of the 4^9 points. Since this segregation does not have any functional basis, we call the two groups as “high fitness” and “low fitness”.

Nature of epistasis between two mutations is context dependent or “fluid” nature of epistasis

Epistasis between two mutations, *A* and *B*, can manifest as no, positive, negative, or sign epistasis[52]. While we know several examples of pairs of mutations exhibiting epistasis of each kind[9,52], we do not know if or how often the nature of epistasis exhibited by a pair of mutations changes with the genomic background.

To answer this question, we pick a pair of mutations and compute the fraction of genomes in which these mutations exhibit (a) Positive epistasis (PE), (b) Negative epistasis (NE), (c) Sign epistasis, and (d) No epistasis (No). Sign epistasis was further classified into (i) Reciprocal Sign epistasis (RSE), (ii) Single Sign epistasis (SSE) and (iii) Other Sign epistasis (OSE) based on the number of paths restricted by Darwinian evolution (**Figure 1A**).

For example, the mutation pair (G ◊ A at position 3 and T ◊ C at position 7) exhibits PE in 26.08%; NE in 34.36%; RSE in 0.67%; SSE in 2.31%; OSE in 5.00%; and No Epistasis in 31.57% of all high fitness backgrounds. The corresponding figures for low fitness backgrounds are: PE: 19.41%; NE: 22.61%; RSE: 7.65%; SSE: 5.71%; OSE: 13.16%; and No Epistasis: 30.92%. The exact numbers for all mutations pairs are provided in **Supplement Data-File1**.

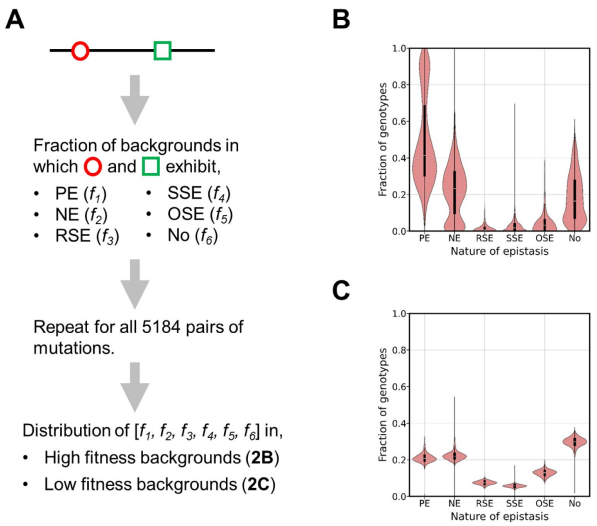


Figure 1.

Nature of epistasis exhibited by two mutations is contingent on the genetic background.

(A) The nature of epistasis exhibited by two mutations (indicated by red circle and green square) in all 4^7 genetic backgrounds was quantified. Numbers f_1 to f_6 indicate the fraction of genomes in which the two mutations exhibit Positive epistasis (PE), Negative epistasis (NE), Reciprocal Sign epistasis (RSE), Single Sign epistasis (SSE), Other Sign epistasis (OSE), and No epistasis (No), and were calculated for this pair of mutations. The process was repeated for every possible pair of mutations. Distribution of the six fractions f_1 to f_6 in **(B)** high and **(C)** low fitness backgrounds is plotted.

We repeat this process for all 5184 ($\binom{9}{2}$ *loci* x $(12)^2$) possible pairs of mutations in the 9-base pair region of *folA*. The frequency distribution of the fraction of all genotypes where a pair exhibits each type of epistasis is shown in **Figure 1B** (for high fitness backgrounds) and **Figure 1C** (for low fitness backgrounds).

Barring a few pair of mutations which exhibit positive epistasis in all/nearly all high fitness backgrounds, nature of epistasis between a pair of mutations is strongly dependent on the genetic background (**Figure 1** and **Supplement Table 1** and **Supplement Data-File2**). In high fitness backgrounds, mutation pairs exhibit positive epistasis most frequently (median 41% of the genotypes), followed by negative epistasis (median 23%) and no epistasis (median 16%), with sign epistasis being relatively rare (**Figure 1B**). In low fitness backgrounds, mutation pairs exhibit no epistasis most frequently (median 30%), followed by negative epistasis (median 22%), positive epistasis (median 21%) and other sign epistasis (median 13%) (**Figure 1C**).

Because of this contingency of the nature of epistasis between two mutations on the genetic background, we propose that epistasis is "fluid".

Functionally important sites cause switch in epistasis more frequently

It has previously been shown that both the secondary structure and location of a residue in a protein dictate the nature of epistasis exhibited by residues[53]. In **Figure 1**, we saw that epistasis changes with genomic background. But, are certain positions more robust to changes in the nature of epistasis than others? In other words, does the location of a mutation in the genetic background dictate the likelihood of epistasis change?

The effect of a locus *X* on changing the nature of epistasis between two mutations was quantified as shown in **Figure 2A**. As shown in **Figure 2B** and **Figure 2C**, upon the introduction of a single mutation at locus *X*, the nature of epistasis switches in more than 50% of cases. The contributions of different loci is different. Positions 4 and 5 on the landscape, which are critical for protein function, are more responsible for changing the nature of epistasis between two mutations, than other sites on the landscape (**Supplement Table 2** and **Supplement Table 3**). This control of nature of epistasis between other sites by functionally important sites is likely an important factor controlling protein evolution.

The switch of the nature of epistasis is most frequent to positive, negative, or no epistasis (**Supplement Figure S6** and **S7**) (also see **Supplement Data-File3**). Switch to sign epistasis is relatively infrequent. Interestingly, in high fitness backgrounds, mutations at functionally important positions (4 and 5, on the landscape) cause a switch to sign-epistasis more frequently, as compared to a mutation at any of the other seven positions. This pattern is not seen in low fitness backgrounds.

The pervasiveness of the change in epistatic interactions is surprising, and makes prediction of evolutionary trajectories harder. However, we also note that change of nature of epistasis due to a mutation is not unique to *folA*. An analysis of a previously published five-point landscape of beta-lactamase gene[29] shows that change in nature of epistasis in common in intramolecular landscapes (Supplement Figure S8). The above results further emphasize that epistasis is "fluid", and that different sites control the switch of epistasis differently.

Synonymous mutations can cause change of nature of epistasis

While historically synonymous mutations were thought to be neutral[54,55], several studies have demonstrated that they can have a wide range of effects on cellular fitness[56–59]. However, their role in changing epistasis between two mutations has not been studied. To study

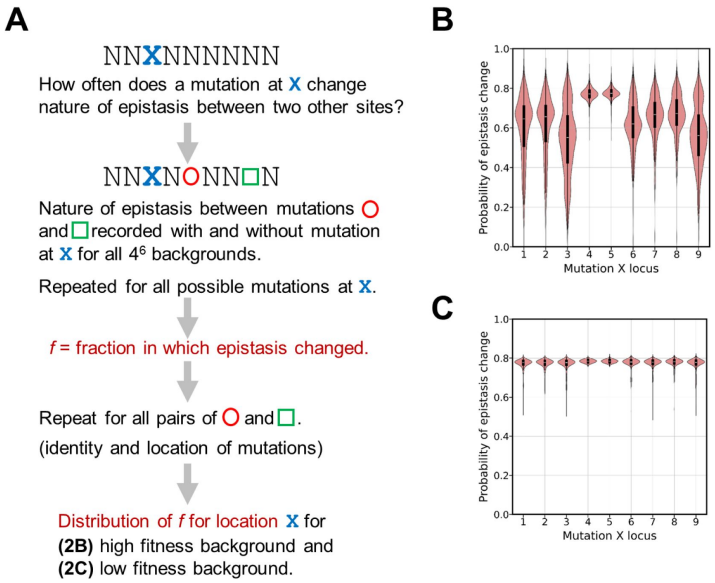


Figure 2.

Different sites on the landscape dictate change in epistasis differently.

(A) To determine the impact of mutation at locus **X** in changing the nature of epistasis between two mutations (indicated by a red circle and a green square), we took the following approach. For a particular pair of mutations, the six sites (indicated by *N*) were fixed, and the nature of epistasis recorded before and after a mutation at **X**. This process was repeated for all 4^6 backgrounds, and the fraction of genomes in which, upon introduction of a mutation at **X**, epistasis between the mutations (red circle and green square) changed, was noted. This gives us ***f***, as indicated in the Figure. This process was repeated for all possible pairs of mutations (red circle and green square); giving a distribution of ***f*** for locus **X**. This distribution of ***f*** is shown in **(B)** for high fitness backgrounds and **(C)** for low fitness backgrounds. Functionally important sites (4 and 5) cause change in epistasis more frequently than others.

this in the *folA* landscape, we note that any two mutations lie in either one or two codons in the *folA* sequence. Depending on the location of these two mutations, there is/are one/two codon(s) that remain unaffected by these mutations. We introduce all possible synonymous mutations in the unaffected codon(s) and ask how frequently does the nature of mutation change? Through this, we seek the likelihood that a synonymous mutation will change the nature of epistasis between two mutations (**Figure 3A**).

In our analysis, we consider T**A**A ↔ T**G**A and TA**A** ↔ TA**G** as synonymous mutations (see discussion). All three codons encode for a stop signal for translation.

Our results shows that synonymous changes can cause change in nature of epistasis frequently (**Figures 3B** and **3C**). In high fitness backgrounds, the likelihood of change of nature of epistasis upon introduction of a synonymous mutation is ~0.45 (**Figure 3B**). In low fitness backgrounds, this number is ~0.75 (**Figure 3C**).

These results clearly demonstrate that change of nature of epistasis can take place via the acquisition of even a synonymous mutation, reaffirming "fluidity" in the nature of epistasis. This property of epistasis makes predicting evolution difficult. However, in recent years, epistasis has been shown to exhibit several statistical patterns, collectively termed as global epistasis[1]. We next check how these patterns hold on the *folA* landscape.

Mutations on the landscape exhibit diminishing returns and increasing costs

Global epistasis suggests that the fitness effect of a mutation is a decreasing function of the background fitness[7,60,61] (**Figure 4A**). In **Figure 4B**, a point represents fitness effect of a single mutation (y-axis) against the fitness of the genotype in which the mutation is introduced (x-axis) on the *folA* landscape. This is done for all mutations in all backgrounds. The line in red indicates the linear fit between the two variables. In both high and low fitness backgrounds, the negative slope of the line indicates presence of global epistasis, although the correlation is not very strong (R^2 = 0.1284 for high fitness backgrounds, & R^2 = 0.1236 for low fitness backgrounds).

Only a small fraction of mutations exhibit global epistasis/ "binary" nature of global epistasis

We next investigate the effect on fitness of each of the 108 (12 possible mutations at each of the 9 sites) mutations on all possible genotypes. **Figure 5** shows that only a few mutations exhibit strong patterns of global epistasis. These mutations are primarily (14 out of 16) at positions (nucleotide 4 and 5) which are functionally most important for *folA*[46]. An overwhelming majority of mutations (77/108) exhibit no correlation (R^2 < 0.2) between their fitness effect and the fitness of the background on which they occur (Supplement Figure S9 and Supplement Table S4).

Therefore, mutations exhibit one of two states depending on whether they follow global epistasis, or not. This indicates the "binary" nature of mutations. In the case of *folA* landscape, only mutations at nucleotide positions critical for function exhibit global epistasis.

A recent work[61] demonstrated that the growth rate at which any mutation switches from being beneficial to deleterious is conserved for all mutations. This growth rate, around which the nature of mutation changes from being beneficial to deleterious was referred to as the pivot growth rate. While the mechanistic origins of pivot growth rate are yet unknown, this phenomenon likely represents a deep fundamental "rule" of how a cell works. We test the existence of the pivot growth rate for each of the 108 mutations in the *folA* landscape. Most (~80%) mutations pivot from

Figure 3.

Introduction of a synonymous mutation changes the nature of epistasis between two interacting mutations.

(A) For a given sequence, two mutations (red circle and green square) could occur in either one or two codons, resulting in two or one unaltered codons (underlined in figure). All possible synonymous mutations were introduced in the unaltered codon(s) and change of nature of epistasis (if any) between the two mutations was noted. This was done for all backgrounds and all pairs of two mutations. Probability that a synonymous mutation at locus *X* leads to a change in nature of epistasis between two mutations in **(B)** high and **(C)** low fitness backgrounds is shown. Mutations resulting in T**A**A ↔ T**G**A and TA**A** — TA**G** are considered as synonymous mutations.

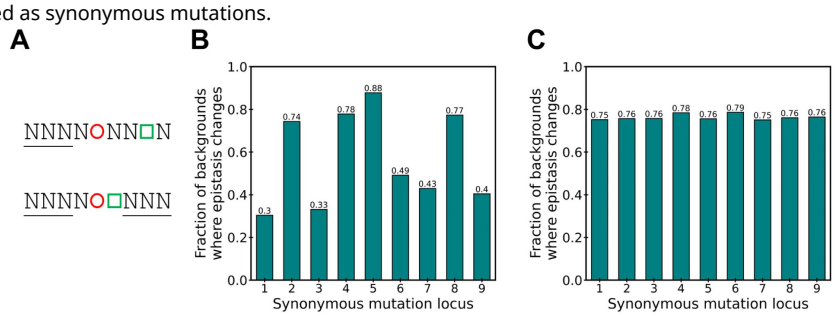
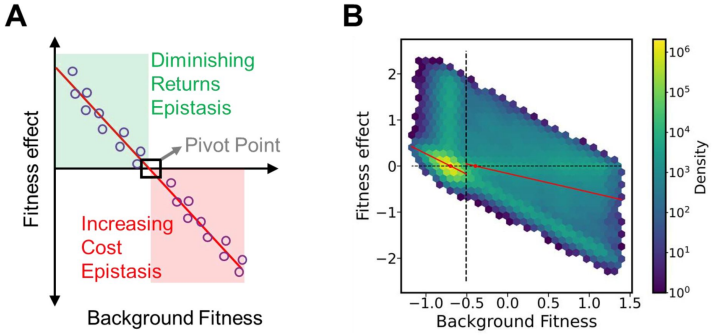


Figure 4.

The *folA* fitness landscape exhibits weak patterns of global epistasis.

(A) Cartoon to illustrate statistical patterns of global epistasis. The beneficial effect of a mutation decreases with an increase in background fitness (Diminishing Returns Epistasis). Beyond a certain background fitness (Pivot Point), the mutation becomes deleterious and its deleterious effects increase as the fitness of the genetic background increases (Increasing Costs Epistasis). **(B)** Mutational effects vs. the background fitness show weak correlation for low fitness (R^2 = 0.1236) (left of dotted line) and high fitness (R^2 = 0.1284) (right of dotted line) backgrounds. The red lines show the best linear fits for the two groups.



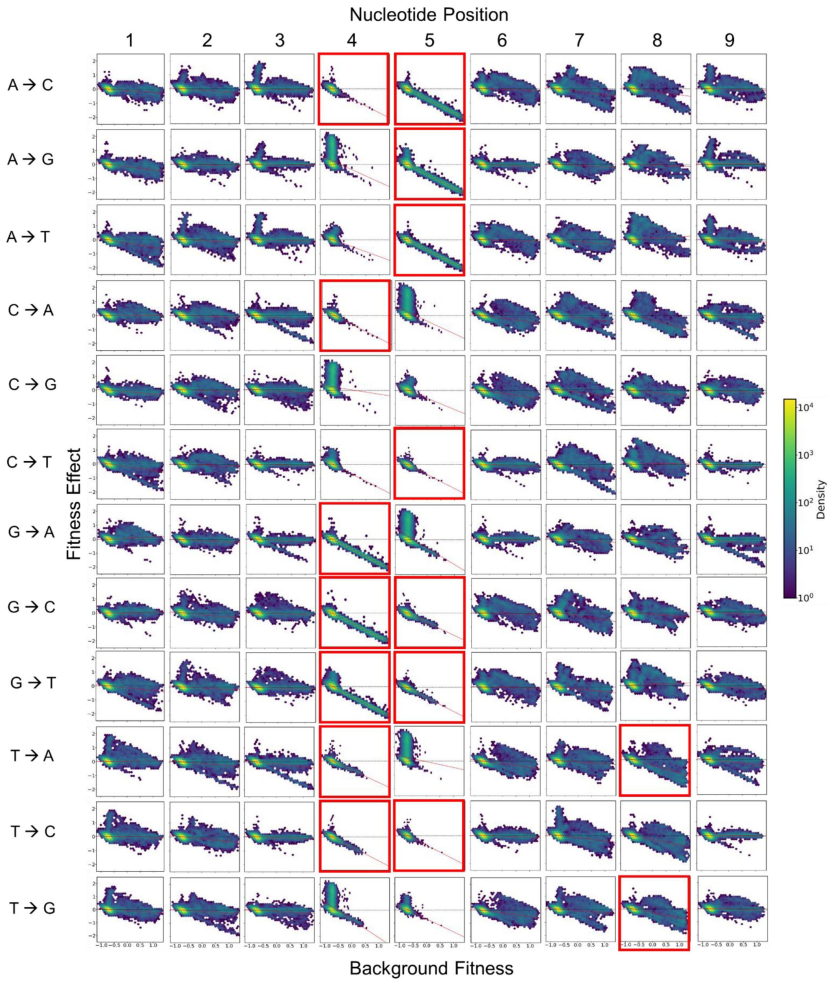


Figure 5.

A small fraction of mutations follow global epistasis.

Only 16 (14 of which are at position 4 and 5) (highlighted in red boxes) out of 108 possible mutations exhibit statistically significant patterns (R^2 > 0.4) of global epistasis. See Supplement Table 1 for statistics for each mutation.

being beneficial to deleterious at a growth rate -0.657 ± 0.0657 (**Figure 6** and **Supplement Table 4**). The presence of a pivot growth rate despite poor statistical correlations between background fitness and the fitness effect of a mutation is a surprising observation.

Predicting DFE from phenotype

The "binary" and "fluid" nature of epistasis discussed in this work make prediction of evolutionary trajectories difficult. As shown in **Figure 5**, only functionally important sites exhibit global epistasis. For most mutations, fitness effects are idiosyncratic. Hence, in the absence of knowledge of the sites which exhibit global epistasis, predictions are likely going to be accurate. From the context of the "fluidity" of epistasis, in the absence of complete knowledge of the genetic background, it is barely possible to comment on nature of epistasis between two mutations. Thus, these two features of epistasis make evolution unpredictable. We now ask if there exists any statistical pattern at all, which would enable the prediction of evolution.

In this context, we define a quantity called "phenotypic DFE", which represents the collective DFE of all genotypes exhibiting near identical fitness (**Figure 7A**). To compute the phenotypic DFE, we binned all genotypes in non-overlapping narrow fitness intervals (see methods). DFE of each genotype was computed by introducing all possible 27 (by introducing all three mutations at each of the 9 positions for a given genotype) mutations. DFE of all genotypes in one interval was averaged to obtain the phenotypic DFE. We next ask how robustly the DFE of a particular genotype of near identical fitness can be predicted solely from the phenotypic DFE.

To test this, we compute the phenotypic DFE from randomly sampled 90% of the genotypes with fitness between f_o and $f_o + \Delta$. The DFE of the remaining 10% of the genotypes in a fitness window was computed separately, and each DFE was compared with the phenotypic DFE. The likelihood of equivalence of each genotype's DFE with the corresponding phenotypic DFE was estimated as the *p*-value of the Mann-Whitney U test. This comparison gives us a distribution of *p*-values, for each background fitness.

The phenotypic DFE of high fitness backgrounds comprised of two peaks. The first peak corresponds to mutations with large deleterious effects, whose magnitude increases with increasing background fitness (**Figures 7B**). The second peak is roughly centered at fitness effect ~ 0. For low fitness backgrounds, the DFE comprised of only one peak, whose mean decreased as the background fitness increased (**Figure 7C**).

Phenotypic DFE is better predicted, than fitness effects of individual mutations, by the background fitness of the genotype. The fraction of genomes for which the phenotypic DFE is statistically significantly different from the actual DFE reduces as the background fitness increases (**Figure 7D**). This effect can also be seen from the distribution of *p*-values of comparisons between phenotypic DFE and actual DFEs, for different background fitness (**Supplement Figure 10**).

Discussion

Genotype-phenotype mapping, especially of proteins, is of great interest in evolutionary biology, cell biology, and genetics[11,46,62–66]. The underlying rules that dictate this mapping are a combination of individual mutation effects, epistatic interactions between the mutations, and between the mutations and the genetic background they occur in[7,29,35,60,61,67–70]. The extent and ubiquity of epistatic interactions is of particular interest, because they are mostly unpredictable and have a direct effect on the shape of the fitness landscape, and consequently, adaptation[34,38,71–76]. Therefore, in order to understand the statistical rules which govern

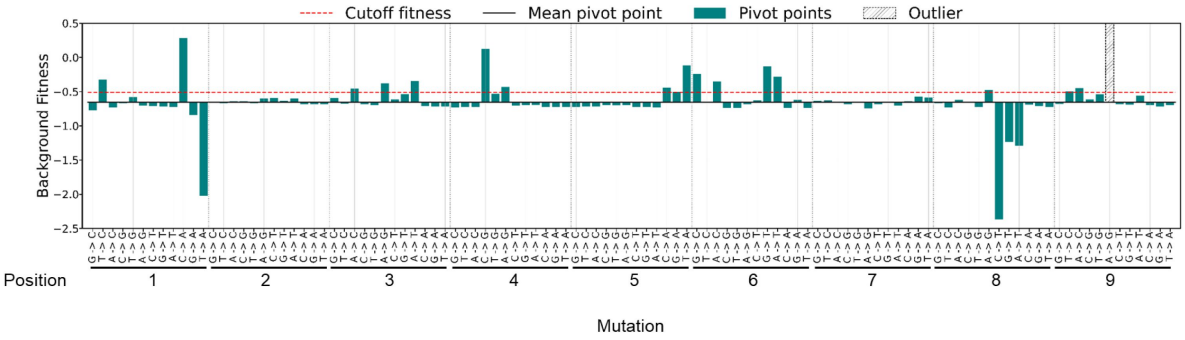


Figure 6.

Most mutations switch from being beneficial to deleterious at the pivot point.

Background fitness (y-axis) at which each of the 108 mutations (x-axis) switches from being beneficial to deleterious. The black solid line shows the average fitness (−0.657) (or pivot) at which a mutation changes from being beneficial to deleterious. Individual bars show the deviation from the mean pivot point for each mutation. More than 80% (86 out of 108) of the mutations exhibit the pivot fitness in the range -0.657 +/-0.0657. The dotted line in red indicates the growth rate used in Papkou et al[40] to differentiate between high and low fitness variants.

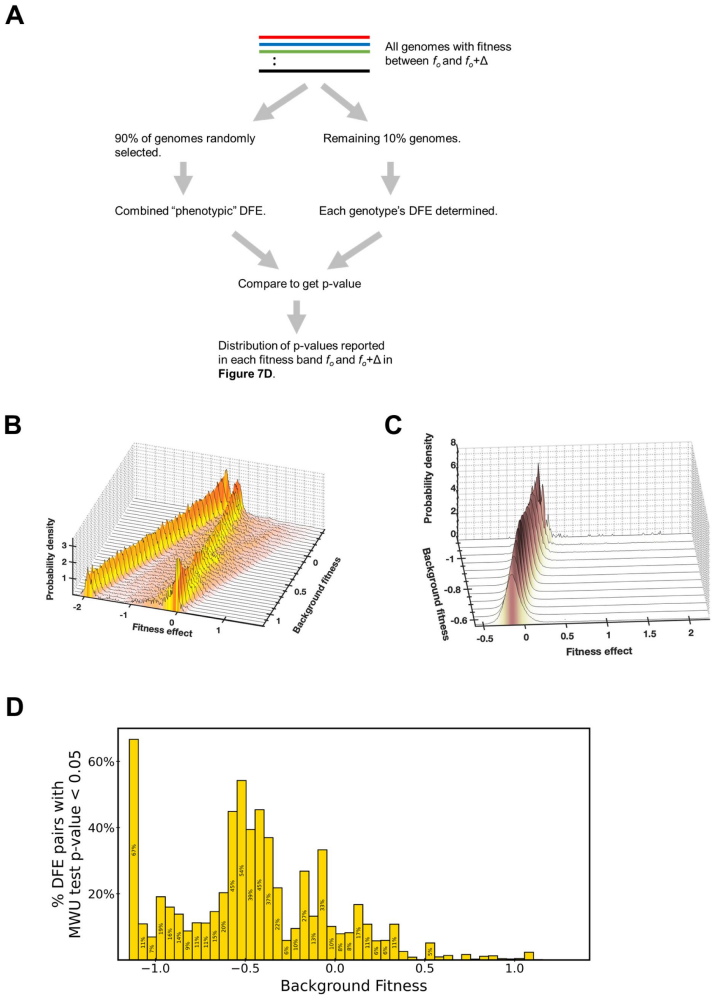


Figure 7.

"Phenotypic DFE" is a predictor of DFE.

(A) Backgrounds with near identical fitness were randomly distributed in two groups comprising 90% and 10% of all backgrounds. The first group was used to define a "phenotypic DFE" (a mean DFE of all genomes in the group). The phenotypic DFE was compared with individual DFEs in the 10% group, and the *p*-value distribution of this comparison was obtained. The process was repeated for genotypes with other fitness. **(B)** Phenotypic DFE of high fitness backgrounds exhibited two peaks. The first peak was at fitness effect ~ 0. The second peak comprised of deleterious mutations, whose magnitude increased with increasing background fitness. **(C)** Phenotypic DFE of low fitness backgrounds exhibited a single peak, whose mean decreased with increasing background fitness. **(D)** Percent of DFEs in a fitness window with p-value < 0.05 when compared with the Phenotypic DFE. As fitness increases, Phenotypic DFE becomes a better predictor of DFE of a genotype.

epistasis on a landscape, we analyzed a recently published *folA* landscape which was constructed by quantifying fitness of more than 260,000 sequences in a 9-base pair region[40]. Mutations in these nine base pairs have been reported to be adaptive[47,49].

Navigability of fitness landscapes decides the likelihood of a population to reach the global fitness maximum. Most fitness landscapes indicate that protein landscapes are rugged, and hence, protein evolution is constrained[47,70,77–79]. But, fitness landscapes are constructed by considering a handful of sites, and there is theoretical evidence to suggest that mutations in other "dimensions" could enable populations to navigate valleys in the landscape[11]. Fisher suggested the same in his correspondence with Wright[80].

A particularly confounding mode of epistasis is sign epistasis, which alters the qualitative nature of a mutational effect, and creates valleys in fitness landscapes. In the *folA* landscape, sign epistasis frequently changes to positive or negative epistasis, indicating "fluidity" in its effects. We also report that synonymous mutations can change the nature of epistasis between two existing mutations. Hence, even if valleys existed, they are not that difficult to navigate. Similar findings have been reported in the past - deleterious mutations making peaks accessible, and neutral mutations being adaptive[81,82]. Additionally, synonymous mutations are known to have fitness effects by changing protein amount (by altering mRNA stability or translation rates)[56,83–87] or structure[88–92]. Our results provide a novel mechanism via which synonymous mutations are relevant, for driving evolutionary change and controlling disease states[93–96].

Interestingly, our analysis shows that the nature of epistasis between two mutations can change by simply changing the stop codon (TAA ↔ TGA or TAA ↔ TAG). This observation holds even when the stop codon is encoded by the first three bases of the 9-bp landscape, and the interacting mutations whose nature of epistasis changes are in the subsequent two codons (which are presumably not even translated). This indicates that the change in the nature of epistasis has likely not do with the protein synthesis but with the mRNA and its effect on cellular fitness.

Premature termination of translation has been known to destabilize the entire transcript[97]. In eukaryotes, elaborate mechanisms are present to deal with potentially toxic effects of truncated proteins[98–101]. However, prokaryotes lack these mechanisms. In fact, a recent study shows that in *E. coli* under stress, despite a premature stop codon in the gene sequence, stop codon read-through rates may be as high as 80%, due to a high probability of a mismatch at a premature stop codon[102]. This is a likely explanation for change of epistasis (or change of fitness) due to alternate premature stop codons in *folA*, when *E. coli* is grown in antibiotic stress.

Neutral mutations can improve evolvability of a sequence, and hence aid the movement on a fitness landscape[103–108]. However, we do not see any evolvability enhancing mutations in the 9-bp *folA* landscape (Supplement Figure S11).

Predicting evolution is a longstanding goal in the field, and global epistasis patterns offered a tool to predict epistatic effects. However, our analyses show that global epistasis often does not hold. Instead, we observe that sequences with similar fitness have similar DFEs, offering some predictability, based on a macroscopic trait, of the adaptive potential of a population[109–111]. The means of these DFEs decreased linearly with an increase in background fitness, despite the mutations on this landscape not following global epistasis patterns (Supplement Figure S12).

We show that the navigability of a landscape can change via mutations in the same protein. Can it also change via mutations elsewhere on the genome? High-dimensional landscapes are necessary to answer this question. Additionally, increasing the dimensionality of the landscape is likely going to provide qualitatively new perspectives of adaptation.

Methods

Calculating Hamming Distance

To calculate the hamming distance between two sequences of equal length, we compare the sequences and count the number of dissimilar loci.

Construction of sequence spaces and landscapes

In order to construct an *n*-base pair sequence space from a parent *p*-base pair sequence space ($p > n$), we choose any $p - n$ loci in the parent sequence space and find all variants in the parent space in which the selected loci are fixed (these loci contain a same sequence). The set of these selected variants are assigned their one hamming distance neighbours to construct a new *n-base* sequence space.

By repeating this process over all $\binom{p}{p-n}$ combinations of selecting the loci to be fixed and the 4^{p-n} permutations of choosing the fixed sequence in each case, we are able to break the parent sequence space into $\left[\binom{p}{p-n} \times 4^{p-n}\right]$ *n*-base pair sequence spaces (Supplement Table 2).

For our study, we use a nine-base pair parent sequence space to generate *n*-base pair sequence spaces ($1 \leq n \leq 9$). Landscapes are generated by mapping fitness values of each sequence in a sequence space corresponding to the ones assigned in the empirical fitness landscape generated by Papkou et al. using a nine base pair gene.

In rare cases where fitness value was not known, we disregarded those variants in all studies.

Finding peaks in a fitness landscape

Number of Peaks in Landscape

To count the number of peaks in fitness landscapes, we find the number of variants that have a higher fitness value than all its one hamming distance neighbours in that landscape.

Peak Probability

To quantify the probability of encountering a fitness peak in any landscape, we find the ratio of number of peaks found to the total number of sequences in that fitness landscape.

Expected number of peaks

In our case (as we are dealing with 4 letter genome), the expected number of peaks in maximally rugged (uncorrelated) NK landscapes is found by $\left(\frac{number\ of\ functional\ variants}{3n+1}\right)$ for an *n*-base pair landscape[20]. The number of peaks predicted by NK *n*+1 landscapes is rounded off to nearest integer.

Fitness effect of mutations

To find the fitness effect of a mutation acting on a genotype, we start with a set of all genotypes in the empirical Papkou et al. fitness landscape which would result in a new sequence formed following the mutation. In all such genotypes, we find the background fitness f_b and the fitness of resulting mutant f_b. The fitness effect of this mutation in this background is determined as the fitness difference between the mutant and the background ($s = f_m - f_b$).

Finding phenotypic DFE

We found the Distribution of Fitness Effects for variants lying in a small slice of fitness value (for our study, we kept the range of this slice to be 0.05). The DFE was constructed by analysing the frequency of fitness effects of all mutations acting on the backgrounds lying in the selected range.

Finding genotypic DFE

For any 9 length genotypic sequence, we found the $9loci \times 3bases = 27$ mutations that may result in a new sequence. We used the frequency of fitness effect of all these mutations to constitute the distribution of fitness effects for any genotype.

Non parametric tests

We found the *p*-value of the two parameter KS test and the Mann– Whitney *U* test using the *python scipy.stats* library.

Linear regression

We found the pivot point fitness of individual mutations via linear regression of background fitness and the selection coefficient of the mutation using the "LinearRegression" model from python sklearn.linear_model library.

Epistasis as function of genetic background

Classifying Epistasis

To quantify the epistasis present in a mutation pair acting on two differing genetic loci, we compute the fitness effect of the individual mutations on a genetic background and the cumulative fitness effect of the two mutations on the same genetic background. If the fitness effect of the individual mutations were s_1 and s_2, while the cumulative effect of the two mutations was s_{12}, then we classify the epistasis into following categories,

1. No Epistasis: If $|s_{12} - (s_1 + s_2)| < 0.05$ i.e. we assume no epistasis was present if the cumulative fitness effect of the two mutations was about the same as the two mutations acting independently on the genetic background.
2. Sign Epistasis: If $s_{12} \times (s_1 + s_2) < 0$ i.e. we classify sign epistasis if the cumulative effect of the two mutations lead to a different sign of fitness effect than the sum of individual fitness effects of the two mutations on the same background. For example, if the combined effect of the mutation pair was beneficial even though the sum of fitness effects of the two mutations on the same background was deleterious, and vice versa.
3. We further classified this epistasis into three categories,
    1. Reciprocal Sign Epistasis: If $s_1 < 0$, $s_2 < 0$ and $s_{12} > 0$ i.e. if the cumulative effect of the two mutations lead the background to a higher fitness, but both shortest paths to this higher fitness point are blocked to darwinian evolution.
    2. Single Sign Epistasis: If exclusively $s_1 < 0$ or $s_2 < 0$ and $s_{12} > 0$ i.e. if exactly one of the two shortest paths leading background to higher fitness are blocked to darwinian evolution.
4. Other Sign Epistasis: All other cases cases classified to sign epistasis.
5. Positive Epistasis: If $s_{12} > s_1 + s_2$ i.e. if the combined fitness effect of the two mutations was more beneficial / less deleterious than the sum of individual effects of the mutation pair on the genetic background.

6. Negative Epistasis: If $s_{12} < s_1 + s_2$ i.e. if the combined fitness effect of the two mutations was less beneficial / more deleterious than the sum of individual effects of the mutation pair on the genetic background.

Epistasis Change with Genetic Background

Having the epistasis dossier generated for all mutation pairs, we compiled all cases of *Positive, Negative, Sign* and *No Epistasis*. For each of these cases, we select the genetic backgrounds and their one mutant neighbours such that their differing mutation locus is unrelated to the loci involved in Epistasis (In our case, we can find $(9-2)loci \times 3bases = 21$ such neighbours for each background).

We then check the nature of epistasis in each of these 21 neighbours on the same mutation pair, and quantify the number of these cases in which nature of epistasis changes.

Finding paths in a sequence space

Set of all variants

We start by listing all the mutations required to convert the starting sequence *A*to the target sequence *T*. If h denotes the minimum number of mutations required to change sequence A to T, then for each step $s \in 1, \cdots, h-1$, we list all variants in the sequence space which are at s hamming distance from the starting variant and $h-s$ hamming distance from the target sequence. The resulting set includes all variants involved at each step for shortest traversal from A to T.

Having the set of all variants at each step in traversal of sequence A to T, we recursively find all $h!$ permutations of paths such that each step only allows one base change while leading the sequence to the target in minimum number of steps.

Effect of neutral mutations on evolvability

To perform this study, we compiled all the $(9loci \times 4bases) = 36mutations$ that are possible among all sequences of *folA* landscape. We then listed all the backgrounds on which these mutations change the genotype, but showcase neutral fitness effect (magnitude of fitness effect < 0.05).

We then allow a second mutation which changes both the background and the mutant genotype. If the selection coefficient of the second mutation on the background is s_1 and on the neutral mutant is s, then the relative increase in evolvability is quantified as: $\left(\frac{s_2 - s_1}{|s_1|}\right)$.

Consider a variant A and a neutral mutation X which results in a variant B ($A \neq B \,|\, s_{AB}| < 0.05$). For any mutation Y taking place on genotypes A and B forming A' and B' such that ($A \neq A' B \neq B'$), we quantify the relative change in evolvability of A from mutation Y due to a neutral mutation X as $\left(\frac{s_{BB'} - s_{AA'}}{|s_{AA'}|}\right)$.

Finding synonymous mutations

We identified the all synonymous codons, i.e. the codons that encode the same amino acid / termination function. For each codon c_i, we then found the list of all synonymous codons which are at 1 Hamming distance from the codon c_i.

Using this data, we were able to identify the set of synonymous mutations for each codon.

Change in mode of epistasis due to synonymous mutation in an extrinsic codon

For any background in DHFR gene, we tested all mutation pairs (*A* and *B*) and identified the type of epistasis exhibited. Since these two mutations can mutate a minimum of one and a maximum of two codons in the 9 base pair gene, at least one codon remains un-mutated. For the mutation pair, we found the un-mutated codon(s), and derived the set of all possible synonymous mutations on the codon(s) of the given background.

We noted the number of instances where introduction of a synonymous mutation (X) at a particular locus (on an un-mutated codon) does or does not change the nature of epistasis for the background and given mutation pair.

We did this analysis separately (all / high fitness / low fitness) backgrounds, their possible mutation pairs (*A* and *B*) and their respective synonymous mutations (X) to identify the overall probability of epistasis change due to synonymous mutation on each locus.

Codes

All codes used in this work and Supplement Data Files are available at: *https://github.com/SainiSupreet/Ecoli-folA-DHFR*. The "readme" file at the repository gives details of how to run codes.

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Additional files

Supplement figures and tables.

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Editors

Reviewer #1 (Public review):

This paper describes a number of patterns of epistasis in a large fitness landscape dataset recently published by Papkou et al. The paper is motivated by an important goal in the field of evolutionary biology to understand the statistical structure of epistasis in protein fitness landscapes, and it capitalizes on the unique opportunities presented by this new dataset to address this problem.

The paper reports some interesting previously unobserved patterns that may have implications for our understanding of fitness landscapes and protein evolution. In particular, Figure 5 is very intriguing. However, I have two major concerns detailed below. First, I found the paper rather descriptive (it makes little attempt to gain deeper insights into the origins of the observed patterns) and unfocused (it reports what appears to be a disjointed collection of various statistics without a clear narrative. Second, I have concerns with the statistical rigor of the work.

(1) I think Figures 5 and 7 are the main, most interesting, and novel results of the paper. However, I don't think that the statement "Only a small fraction of mutations exhibit global epistasis" accurately describes what we see in Figure 5. To me, the most striking feature of this figure is that the effects of most mutations at all sites appear to be a mixture of three patterns. The most interesting pattern noted by the authors is of course the "strong" global epistasis, i.e., when the effect of a mutation is highly negatively correlated with the fitness of the background genotype. The second pattern is a "weak" global epistasis, where the correlation with background fitness is much weaker or non-existent. The third pattern is the vertically spread-out cluster at low-fitness backgrounds, i.e., a mutation has a wide range of mostly positive effects that are clearly not correlated with fitness. What is very interesting to me is that all background genotypes fall into these three groups with respect to almost every mutation, but the proportions of the three groups are different for different mutations. In contrast to the authors' statement, it seems to me that almost all mutations display strong global epistasis in at least a subset of backgrounds. A clear example is C>A mutation at site 3.

1a. I think the authors ought to try to dissect these patterns and investigate them separately rather than lumping them all together and declaring that global epistasis is rare. For example, I would like to know whether those backgrounds in which mutations exhibit strong global epistasis are the same for all mutations or whether they are mutation- or perhaps position-specific. Both answers could be potentially very interesting, either pointing to some specific site-site interactions or, alternatively, suggesting that the statistical patterns are conserved despite variation in the underlying interactions.

1b. Another rather remarkable feature of this plot is that the slopes of the strong global epistasis patterns seem to be very similar across mutations. Is this the case? Is there anything

special about this slope? For example, does this slope simply reflect the fact that a given mutation becomes essentially lethal (i.e., produces the same minimal fitness) in a certain set of background genotypes?

1c. Finally, how consistent are these patterns with some null expectations? Specifically, would one expect the same distribution of global epistasis slopes on an uncorrelated landscape? Are the pivot points unusually clustered relative to an expectation on an uncorrelated landscape?

1d. The shapes of the DFE shown in Figure 7 are also quite interesting, particularly the bimodal nature of the DFE in high-fitness (HF) backgrounds. I think this bimodality must be a reflection of the clustering of mutation-background combinations mentioned above. I think the authors ought to draw this connection explicitly. Do all HF backgrounds have a bimodal DFE? What mutations occupy the "moving" peak?

1e. In several figures, the authors compare the patterns for HF and low-fitness (LF) genotypes. In some cases, there are some stark differences between these two groups, most notably in the shape of the DFE (Figure 7B, C). But there is no discussion about what could underlie these differences. Why are the statistics of epistasis different for HF and LF genotypes? Can the authors at least speculate about possible reasons? Why do HF and LF genotypes have qualitatively different DFEs? I actually don't quite understand why the transition between bimodal DFE in Figure 7B and unimodal DFE in Figure 7C is so abrupt. Is there something biologically special about the threshold that separates LF and HF genotypes? My understanding was that this was just a statistical cutoff. Perhaps the authors can plot the DFEs for all backgrounds on the same plot and just draw a line that separates HF and LF backgrounds so that the reader can better see whether the DFE shape changes gradually or abruptly.

1f. The analysis of the synonymous mutations is also interesting. However I think a few additional analyses are necessary to clarify what is happening here. I would like to know the extent to which synonymous mutations are more often neutral compared to non-synonymous ones. Then, synonymous pairs interact in the same way as non-synonymous pair (i.e., plot Figure 1 for synonymous pairs)? Do synonymous or non-synonymous mutations that are neutral exhibit less epistasis than non-neutral ones? Finally, do non-synonymous mutations alter epistasis among other mutations more often than synonymous mutations do? What about synonymous-neutral versus synonymous-non-neutral. Basically, I'd like to understand the extent to which a mutation that is neutral in a given background is more or less likely to alter epistasis between other mutations than a non-neutral mutation in the same background.

(2) I have two related methodological concerns. First, in several analyses, the authors employ thresholds that appear to be arbitrary. And second, I did not see any account of measurement errors. For example, the authors chose the 0.05 threshold to distinguish between epistasis and no epistasis, but why this particular threshold was chosen is not justified. Another example: is whether the product $s12 \times (s1 + s2)$ is greater or smaller than zero for any given mutation is uncertain due to measurement errors. Presumably, how to classify each pair of mutations should depend on the precision with which the fitness of mutants is measured. These thresholds could well be different across mutants. We know, for example, that low-fitness mutants typically have noisier fitness estimates than high-fitness mutants. I think the authors should use a statistically rigorous procedure to categorize mutations and their epistatic interactions. I think it is very important to address this issue. I got very concerned about it when I saw on LL 383-388 that synonymous stop codon mutations appear to modulate epistasis among other mutations. This seems very strange to me and makes me quite worried that this is a result of noise in LF genotypes.

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Reviewer #2 (Public review):

Significance:

This paper reanalyzes an experimental fitness landscape generated by Papkou et al., who assayed the fitness of all possible combinations of 4 nucleotide states at 9 sites in the E. coli DHFR gene, which confers antibiotic resistance. The 9 nucleotide sites make up 3 amino acid sites in the protein, of which one was shown to be the primary determinant of fitness by Papkou et al. This paper sought to assess whether pairwise epistatic interactions differ among genetic backgrounds at other sites and whether there are major patterns in any such differences. They use a "double mutant cycle" approach to quantify pairwise epistasis, where the epistatic interaction between two mutations is the difference between the measured fitness of the double-mutant and its predicted fitness in the absence of epistasis (which equals the sum of individual effects of each mutation observed in the single mutants relative to the reference genotype). The paper claims that epistasis is "fluid," because pairwise epistatic effects often differs depending on the genetic state at the other site. It also claims that this fluidity is "binary," because pairwise effects depend strongly on the state at nucleotide positions 5 and 6 but weakly on those at other sites. Finally, they compare the distribution of fitness effects (DFE) of single mutations for starting genotypes with similar fitness and find that despite the apparent "fluidity" of interactions this distribution is well-predicted by the fitness of the starting genotype.

The paper addresses an important question for genetics and evolution: how complex and unpredictable are the effects and interactions among mutations in a protein? Epistasis can make the phenotype hard to predict from the genotype and also affect the evolutionary navigability of a genotype landscape. Whether pairwise epistatic interactions depend on genetic background - that is, whether there are important high-order interactions -- is important because interactions of order greater than pairwise would make phenotypes especially idiosyncratic and difficult to predict from the genotype (or by extrapolating from experimentally measured phenotypes of genotypes randomly sampled from the huge space of possible genotypes). Another interesting question is the sparsity of such high-order interactions: if they exist but mostly depend on a small number of identifiable sequence sites in the background, then this would drastically reduce the complexity and idiosyncrasy relative to a landscape on which "fluidity" involves interactions among groups of all sites in the protein. A number of papers in the recent literature have addressed the topics of high-order epistasis and sparsity and have come to conflicting conclusions. This paper contributes to that body of literature with a case study of one published experimental dataset of high quality. The findings are therefore potentially significant if convincingly supported.

Validity:

In my judgment, the major conclusions of this paper are not well supported by the data. There are three major problems with the analysis.

(1) Lack of statistical tests. The authors conclude that pairwise interactions differ among backgrounds, but no statistical analysis is provided to establish that the observed differences are statistically significant, rather than being attributable to error and noise in the assay measurements. It has been established previously that the methods the authors use to estimate high-order interactions can result in inflated inferences of epistasis because of the propagation of measurement noise (see PMID 31527666 and 39261454). Error propagation can be extreme because first-order mutation effects are calculated as the difference between the measured phenotype of a single-mutant variant and the reference genotype; pairwise effects are then calculated as the difference between the measured phenotype of a double mutant and the sum of the differences described above for the single mutants. This paper claims fluidity when this latter difference itself differs when assessed in two different

backgrounds. At each step of these calculations, measurement noise propagates. Because no statistical analysis is provided to evaluate whether these observed differences are greater than expected because of propagated error, the paper has not convincingly established or quantified "fluidity" in epistatic effects.

(2) Arbitrary cutoffs. Many of the analyses involve assigning pairwise interactions into discrete categories, based on the magnitude and direction of the difference between the predicted and observed phenotypes for a pairwise mutant. For example, the authors categorize as a positive pairwise interaction if the apparent deviation of phenotype from prediction is >0.05, negative if the deviation is <-0.05, and no interaction if the deviation is between these cutoffs. Fluidity is diagnosed when the category for a pairwise interaction differs among backgrounds. These cutoffs are essentially arbitrary, and the effects are assigned to categories without assessing statistical significance. For example, an interaction of 0.06 in one background and 0.04 in another would be classified as fluid, but it is very plausible that such a difference would arise due to error alone. The frequency of epistatic interactions in each category as claimed in the paper, as well as the extent of fluidity across backgrounds, could therefore be systematically overestimated or underestimated, affecting the major conclusions of the study.

(3) Global nonlinearities. The analyses do not consider the fact that apparent fluidity could be attributable to the fact that fitness measurements are bounded by a minimum (the fitness of cells carrying proteins in which DHFR is essentially nonfunctional) and a maximum (the fitness of cells in which some biological factor other than DHFR function is limiting for fitness). The data are clearly bounded; the original Papkou et al. paper states that 93% of genotypes are at the low-fitness limit at which deleterious effects no longer influence fitness. Because of this bounding, mutations that are strongly deleterious to DHFR function will therefore have an apparently smaller effect when introduced in combination with other deleterious mutations, leading to apparent epistatic interactions; moreover, these apparent interactions will have different magnitudes if they are introduced into backgrounds that themselves differ in DHFR function/fitness, leading to apparent "fluidity" of these interactions. This is a well-established issue in the literature (see PMIDs 30037990, 28100592, 39261454). It is therefore important to adjust for these global nonlinearities before assessing interactions, but the authors have not done this.

This global nonlinearity could explain much of the fluidity claimed in this paper. It could explain the observation that epistasis does not seem to depend as much on genetic background for low-fitness backgrounds, and the latter is constant (Figure 2B and 2C): these patterns would arise simply because the effects of deleterious mutations are all epistatically masked in backgrounds that are already near the fitness minimum. It would also explain the observations in Figure 7. For background genotypes with relatively high fitness, there are two distinct peaks of fitness effects, which likely correspond to neutral mutations and deleterious mutations that bring fitness to the lower bound of measurement; as the fitness of the background declines, the deleterious mutations have a smaller effect, so the two peaks draw closer to each other, and in the lowest-fitness backgrounds, they collapse into a single unimodal distribution in which all mutations are approximately neutral (with the distribution reflecting only noise).
Global nonlinearity could also explain the apparent "binary" nature of epistasis. Sites 4 and 5 change the second amino acid, and the Papkou paper shows that only 3 amino acid states (C, D, and E) are compatible with function; all others abolish function and yield lower-bound fitness, while mutations at other sites have much weaker effects. The apparent binary nature of epistasis in Figure 5 corresponds to these effects given the nonlinearity of the fitness assay. Most mutations are close to neutral irrespective of the fitness of the background into which they are introduced: these are the "non-epistatic" mutations in the binary scheme. For the mutations at sites 4 and 5 that abolish one of the beneficial mutations, however, these have a strong background-dependence: they are very deleterious when introduced into a high-

fitness background but their impact shrinks as they are introduced into backgrounds with progressively lower fitness. The apparent "binary" nature of global epistasis is likely to be a simple artifact of bounding and the bimodal distribution of functional effects: neutral mutations are insensitive to background, while the magnitude of the fitness effect of deleterious mutations declines with background fitness because they are masked by the lower bound. The authors' statement is that "global epistasis often does not hold." This is not established. A more plausible conclusion is that global epistasis imposed by the phenotype limits affects all mutations, but it does so in a nonlinear fashion.

In conclusion, most of the major claims in the paper could be artifactual. Much of the claimed pairwise epistasis could be caused by measurement noise, the use of arbitrary cutoffs, and the lack of adjustment for global nonlinearity. Much of the fluidity or higher-order epistasis could be attributable to the same issues. And the apparently binary nature of global epistasis is also the expected result of this nonlinearity.

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Reviewer #3 (Public review):

Summary:

The authors have studied a previously published large dataset on the fitness landscape of a 9 base-pair region of the folA gene. The objective of the paper is to understand various aspects of epistasis in this system, which the authors have achieved through detailed and computationally expensive exploration of the landscape. The authors describe epistasis in this system as "fluid", meaning that it depends sensitively on the genetic background, thereby reducing the predictability of evolution at the genetic level. However, the study also finds two robust patterns. The first is the existence of a "pivot point" for a majority of mutations, which is a fixed growth rate at which the effect of mutations switches from beneficial to deleterious (consistent with a previous study on the topic). The second is the observation that the distribution of fitness effects (DFE) of mutations is predicted quite well by the fitness of the genotype, especially for high-fitness genotypes. While the work does not offer a synthesis of the multitude of reported results, the information provided here raises interesting questions for future studies in this field.

Strengths:

A major strength of the study is its detailed and multifaceted approach, which has helped the authors tease out a number of interesting epistatic properties. The study makes a timely contribution by focusing on topical issues like the prevalence of global epistasis, the existence of pivot points, and the dependence of DFE on the background genotype and its fitness. The methodology is presented in a largely transparent manner, which makes it easy to interpret and evaluate the results.

The authors have classified pairwise epistasis into six types and found that the type of epistasis changes depending on background mutations. Switches happen more frequently for mutations at functionally important sites. Interestingly, the authors find that even synonymous mutations in stop codons can alter the epistatic interaction between mutations in other codons. Consistent with these observations of "fluidity", the study reports limited instances of global epistasis (which predicts a simple linear relationship between the size of a mutational effect and the fitness of the genetic background in which it occurs). Overall, the work presents some evidence for the genetic context-dependent nature of epistasis in this system.

Weaknesses:

Despite the wealth of information provided by the study, there are some shortcomings of the paper which must be mentioned.

(1) In the Significance Statement, the authors say that the "fluid" nature of epistasis is a previously unknown property. This is not accurate. What the authors describe as "fluidity" is essentially the prevalence of certain forms of higher-order epistasis (i.e., epistasis beyond pairwise mutational interactions). The existence of higher-order epistasis is a well-known feature of many landscapes. For example, in an early work, (Szendro et. al., J. Stat. Mech., 2013), the presence of a significant degree of higher-order epistasis was reported for a number of empirical fitness landscapes. Likewise, (Weinreich et. al., Curr. Opin. Genet. Dev., 2013) analysed several fitness landscapes and found that higher-order epistatic terms were on average larger than the pairwise term in nearly all cases. They further showed that ignoring higher-order epistasis leads to a significant overestimate of accessible evolutionary paths. The literature on higher-order epistasis has grown substantially since these early works. Any future versions of the present preprint will benefit from a more thorough contextual discussion of the literature on higher-order epistasis.

(2) In the paper, the term 'sign epistasis' is used in a way that is different from its well-established meaning. (Pairwise) sign epistasis, in its standard usage, is said to occur when the effect of a mutation switches from beneficial to deleterious (or vice versa) when a mutation occurs at a different locus. The authors require a stronger condition, namely that the sum of the individual effects of two mutations should have the opposite sign from their joint effect. This is a sufficient condition for sign epistasis, but not a necessary one. The property studied by the authors is important in its own right, but it is not equivalent to sign epistasis.

(3) The authors have looked for global epistasis in all 108 (9x12) mutations, out of which only 16 showed a correlation of $R^2 > 0.4$. 14 out of these 16 mutations were in the functionally important nucleotide positions. Based on this, the authors conclude that global epistasis is rare in this landscape, and further, that mutations in this landscape can be classified into one of two binary states - those that exhibit global epistasis (a small minority) and those that do not (the majority). I suspect, however, that a biologically significant binary classification based on these data may be premature. Unsurprisingly, mutational effects are stronger at the functional sites as seen in Figure 5 and Figure 2, which means that even if global epistasis is present for all mutations, a statistical signal will be more easily detected for the functionally important sites. Indeed, the authors show that the means of DFEs decrease linearly with background fitness, which hints at the possibility that a weak global epistatic effect may be present (though hard to detect) in the individual mutations. Given the high importance of the phenomenon of global epistasis, it pays to be cautious in interpreting these results.

(4) The study reports that synonymous mutations frequently change the nature of epistasis between mutations in other codons. However, it is unclear whether this should be surprising, because, as the authors have already noted, synonymous mutations can have an impact on cellular functions. The reader may wonder if the synonymous mutations that cause changes in epistatic interactions in a certain background also tend to be non-neutral in that background. Unfortunately, the fitness effect of synonymous mutations has not been reported in the paper.

(5) The authors find that DFEs of high-fitness genotypes tend to depend only on fitness and not on genetic composition. This is an intriguing observation, but unfortunately, the authors do not provide any possible explanation or connect it to theoretical literature. I am reminded of work by (Agarwala and Fisher, Theor. Popul. Biol., 2019) as well as (Reddy and Desai, eLife, 2023) where conditions under which the DFE depends only on the fitness have been derived. Any discussion of possible connections to these works could be a useful addition.

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Author response:

Thank you for sharing a detailed review of our manuscript titled, Variations and predictability of epistasis on an intragenic fitness landscape. We have now carefully gone through the reviewers' and the editor's comments and have the following preliminary responses.

(1) **Measurement noise in the *folA* fitness landscape.** All three reviewers and the editors raise the important matter of incorporating measurement noise in the fitness landscape. The paper by Papkou and coworkers makes the fitness measurements of the landscape in six independent repeats. They show that the fitness data is highly correlated in each repeat, and use the weighted mean of the repeats to report their results. They do not study how measurement noise influences their findings. The results by Papkou and coworkers were our starting point, and hence, we built on the landscape properties reported in their study. As a result, we also analyse our results working with the same mean of the six independent measurements.

The main result of the work by Papkou and coworkers is that largest subgraph in the landscape has 514 fitness peaks.

We revisit this result by quantifying how measurement noise changes this number. By doing this, we note the subgraph contains only 127 peaks which are statistically significant. We define a sequence as a peak when its corresponding fitness is greater than all its one-distance neighbours with a p-value < 0.05. This shows that, as pointed out in the reviews, incorporating noise in the landscape results significantly changes how we view the landscape – a facet not included in Papkou et al and the current version of our manuscript.

Not incorporating measurement noise means that the entire landscape has 4055 peaks. When measurement noise is included in the analysis, this number reduces to 137, out of which 136 are high fitness backgrounds (functional).

In the revised version of our manuscript, we will incorporate measurement noise in our analysis. Through this, we will also address the concern regarding the use of an arbitrary cut-off to study "fluid" epistasis. However, we note that arbitrary cut-offs to define DFEs have been recently used (Sane et al., *PNAS*, 2023).

We also note that previous work with large scale landscapes (Wu et al, *eLife*, 2016) also reported a fitness landscape with a single experiment, with no repeats.

(2) **Global nonlinearities and higher-order leading to *fluid* epistasis.** Attempts at building models for higher-order epistasis from empirical data have largely been confined to landscapes of a limited data size. For example, Sailer & Harms, *Genetics*, 2017 propose models for higher-order epistasis from seven empirical data sets, each with less than a 100 data points. Another recent attempt (Park et al, *Nat Comm*, 2024) proposes rule for protein structure-function with 20 fitness landscapes. In this study, only one landscape which used fitness as a phenotype had ~160000 data points (of which only 42% were included for analysis). All other data sets which used fitness as a phenotype contained less than 10000 data points. While these statistical proposals of how higher-order epistasis operates exist, none of them are reliant of large scale, exhaustive network, like the one proposed by Papkou and coworkers.

In the edited manuscript, we will replace our arbitrary cut-off with results of statistical tests carried out based on measurement noise.

Global non-linearities shape evolutionary responses. We would like to emphasize that the goal of this work to study and understand how these global non-linearities result in patterns

on a large fitness landscape by presenting the sum total of these fundamental factors in shaping statistical patterns.

While we understand that we may not have sufficiently explained the effects of global non-linearities on our results, we do not agree with the reviewer's conclusion that our results are artifacts of these non-linearities. We will expand on the role of these nonlinearities on the patterns that we observe (like, fitness being bounded, as pointed out by reviewer 2, or differential impact of a mutation in functional vs. non-functional variants).

We also speculate that changing our arbitrary cut-off (selection coefficient of 0.05) to measurement noise will not alter our results qualitatively.

The question we address in our work is, therefore, how does the nature of epistasis change with genetic background over a large, exhaustive landscape. The nature of epistasis between two mutations is analysed in all 4^7 backgrounds. The causative agents for the change in epistasis will be context-dependent, depending on the precise nature of the two mutations and the background. For instance, a certain background might simply introduce a Stop codon in the sequence. Notwithstanding these precise, local mechanistic explanations, we seek to answer how epistasis changes statistically in a sequence. Investigating statistical patterns which explain switch in nature of epistasis in deep, exhaustive landscapes is a long-term goal of this research.

(3) Last, in our revised manuscript, we will address the reviewers' other minor comments on the various aspects of the manuscript.

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