nature portfolio

Peer Review File

Genetic properties underlying transcriptional variability across different perturbations

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors compare the variability of E coli transcriptional datasets across different protocols, to test the correspondence between the evolutionary acquired and environmentally triggered differences. They indeed find such a correspondence and interpret it in the light of the regulatory network. The study is neat overall and the topic important. I have some comments but I think the work deserves publication in Nature Communications once these comments are addressed.

1. The replicability of microarrays in SI Fig 1 is impressive, however is technical or biological? It would be important to have a sense of the biological replicability. Also, I would like to see the same for the external datasets ENV and EVO, if replicates are available. This would help to assess the significance of the distributions of Fig 1 and the correlations of Fig 2a, as the actual correlation between expression variability may in reality be higher between datasets, it is just that experimental noise masks it.

2. It would be useful to explain more the criterion for enrichment in the main text. For instance, nothing is low-DM-enriched in EVO, how should this be understood?

3. Lines 126-129 page 6 are too vague.

4. Fig. 5a: I am confused about blue-green and light-green

5. page 8 line 196 : “significantly enriched” can you specify the null hypothesis? It should be keeping the number of edge per node and just shuffle them, otherwise there is no particular claim here as the TRcom tend to have many targets.

6. Fig. 6a: what are the colors for in Env?

7. Fig. 6b, 7: I must stay that I start to be lost here because these analyses are very indirect: they consist of correlations between genes contributions to axes of different PCAs done on the transcriptional variability. On top of that, the correlations are not strong. It is fine to point to hypotheses to be tested more directly, but if the other find a more direct way to present their claim, I would find it more credible.

Reviewer #2 (Remarks to the Author):

This is a very interesting paper that attempts to establish the degree of concordance of gene expression variability in response to genetic and environmental perturbations in E. coli. To my

knowledge, this is the first exhaustive experimental test of recent theory on this topic and the study is therefore a much welcome addition to the literature. The use of existing data sets poses some limitations and make it somewhat difficult to assess in places how confident one should be in the results (see specific comments below). Nevertheless, the study is very valuable and likely to inspire further exploration of a topic fundamental to understanding the role of generative processes in evolution.

Specific comments (in order of appearance)

Line 28-30. Here and elsewhere, the authors interpret their results on the basis of the theoretical work done by Watson and colleagues (e.g., Kouvaris et al. 2017. PLoS Comp Biol; Brun Usan et al. 2021. BMC Ecol Evol). That is, they consider the similarity of responses to different perturbations to reflect the evolutionary (and selective) history of GRNs. However, the idea that GRNs 'learn' is not actually tested in this paper, and the conclusion therefore does not follow from the results. In fact, as Brun-Usan et al. (2021, BMC Ecol Evol, not cited by the authors) have shown, the 'alignment' between transcriptional responses to genetic and environmental perturbations is a property of GRNs themselves, without the need for 'structuring in past environments'. The authors should therefore moderate their conclusion. This criticism also affects some of the supplementary discussion, see below.

Line 43-52 and elsewhere. It seems that two rather important studies are missing from the discussion of the similarity of developmental bias in response to different perturbations: Brun-Usan et al. 2021 BMC Ecol Evol and Milocco & Uller 2024. PNAS (perhaps also see Brun-Usan et al. 2023. Bioessays). the results of these studies strengthen the authors theoretical standpoints and provide opportunities for more nuanced explanations of the results (see above and below).

Line 47. It is not entirely clear to me that the work cited is appropriate here. Would this statement not be accompanied by empirical studies that directly compare variation in response to genetic and environmental perturbations (e.g., quantitative genetic studies)?

Lines 45-46 & 49-51. These two sentences seem to say pretty much the same, so there is arguably some scope for streamlining this paragraph (see also comments above).

Lines 57-59. The wording here ('concordance') seems to imply that gene expression responses to genetic and environmental perturbation are biased in similar ways (in line with the theoretical work). However, at least some of the cited literature is a little different in scope. For example, reference 21 shows that genes with greater environmental sensitivity also are sensitive to genetic perturbations (and noise). But that is a statement about gene identity, not concordance of

expression patterns. Perhaps this is not so important, but it may be worth rewriting to make the sentence convey more information about what is known about this topic to date.

Line 69. I am not sure what 'structure of transcriptional regulation' means.

Lines 72-74. As explained above, this study does not really show the effect of past environments on GRNs and their ability to 'generalize'. Better to avoid this conclusion. See also criticism of supplementary discussion below.

Line 79. Please acknowledge the limitations of drawing far-reaching conclusions about transcriptional variability on the basis of a single strain. Please also acknowledge the limitations of contrasting data sets that differ in important respects (particularly important for interpreting how the results are related to past evolution and selection).

Line 111. Please explain this better: 'canalization in… functional categories' is difficult to understand.

Lines 137-145. Here and elsewhere (e.g., line 148), it is not clear that the results really show greater overlap than expected based on chance. It would be helpful if the authors spelled out their results in a way that makes it clear what it means that regulators are 'enriched' (e.g., phrase this to make it explicit what the null assumption of overlap between data sets would be). Alternatively, the authors may wish to include a clear statement at the outset that explains how they tested this statistically.

Line 216. A more informative study here is probably Brun-Usan et al. 2021 BMC Ecol Evol. Also see the recent Milocco & Uller 2024 PNAS (which probably came out after the authors submitted). Same comment applied for line 261.

Lines 267-273. The rationale for these additional data sets is not entirely clear. Please provide some additional information, and also explain why these data sets are 'supplementary' rather than part of the main analyses.

Lines 289-292. This conclusion is not entirely clear. It may need some expansion or connection to the theoretical studies to make sense.

Supplementary Discussions. The authors have done an impressive amount of work that is only presented and discussed in the supplementary material. While I find this work very interesting, it is difficult to evaluate and I am not fully convinced by all the conclusions. Perhaps some of this material should be lifted out of this paper and form the basis for a separate study? For example, the material in Supplementary Discussion 3 is intriguing, but it is nearly impossible (at least to this reader) to evaluate to what extent the conclusions follow from the data.

In general, my main concern with these supplements is that I am not convinced that the authors can successfully connect their results to past adaptive evolution. The data required to do so would arguably be very different than the data the authors have access to in this work. This criticism affects Supplementary Discussion 1 insofar that the speculations about adaptive significance of variability in cell motility are just speculations. It may concern many readers that the authors imply there has been 'selection for evolvability' (I am not sure the authors really imply selection 'for', but rather 'selection of', as in the theoretical models they cite). Overall, it is not clear that Supplementary Discussion 1 adds much to the study, and perhaps the paper would be better without it?

Similar arguments could be made for the other supplementary discussions. The conclusions that the authors draw from the results presented in these supplements (which, again, are difficult to evaluate for the reader) are perhaps too far-reaching. One suggestion is to keep the analyses and results as supplementary information but to avoid drawing conclusions about past selection and adaptation from these data. Again, the data is arguably not very good for this (e.g., comparing a single strain for environmental perturbations with an artificial mutator strain would arguably not be the choice if one wanted to the test the idea that GRNs evolve to 'generalize' as in the model of Kouvaris et al). Thus, while e.g. past selection for plasticity may explain the shared variability in GRNs (and the mechanistic basis for this), the experiments and data needed to actually demonstrate this to be the case are quite demanding.

In summary, the work presented here convincingly demonstrates alignment in gene expression between genetic and environmental perturbations, and reveals features of GRNs that may explain this relationship mechanistically. Those are very important advances. It is less convincing to use these data to argue for a link between past evolution and those features of GRNs. The study may therefore be better if some of the follow-on analyses and inferences in the supplementary discussions are revised.

General comments and notes to the reviewers

We would like to thank the reviewers for their constructive suggestions and comments about our manuscript. By addressing all the comments from the reviewers, we believe that the overall quality of the manuscript has improved significantly.

In addition to the changes made to address the reviewers' comments and some minor stylistic changes, we corrected two mistakes that we noticed while replying to the reviewers' comments:

Revision (Materials and Methods / L499–500):
For GSEA of transcriptional regulators, we also filtered out the transcriptional regulators that regulated fewer than five ~~target genes~~ or more than 1000 genes.

Revision (Results / L148–149):
We focused on the transcription factors and sigma factors that regulate more than four and less than 1001 target genes.

Both sentences describe the condition of GSEA using transcriptional regulators. Accordingly, these two sentences originally need to indicate the same condition. On the first mistakes, the words "more than" were missing in the previous version. The second mistake was related to the first mistake, where the important words "less than 1001" were missing in the previous version. We sincerely apologize for not noticing these mistakes before the initial submission.

In addition to the responses to the reviewers' comments, we have uploaded a marked-up version of the revised manuscript with "_diff.pdf" in the filename.

Point-by-point responses

Responses to Reviewer 1:

General Comment 1

The authors compare the variability of E coli transcriptional datasets across different protocols, to test the correspondence between the evolutionary acquired and environmentally triggered differences. They indeed find such a correspondence and interpret it in the light of the regulatory network. The study is neat overall and the topic important. I have some comments but I think the work deserves publication in Nature Communications once these comments are addressed.

Response: We would like to thank the reviewer for highly evaluating our manuscript. The time and effort invested in reviewing our work have significantly contributed to its improvement. We have addressed all the comments as detailed below.

Comment 1.1

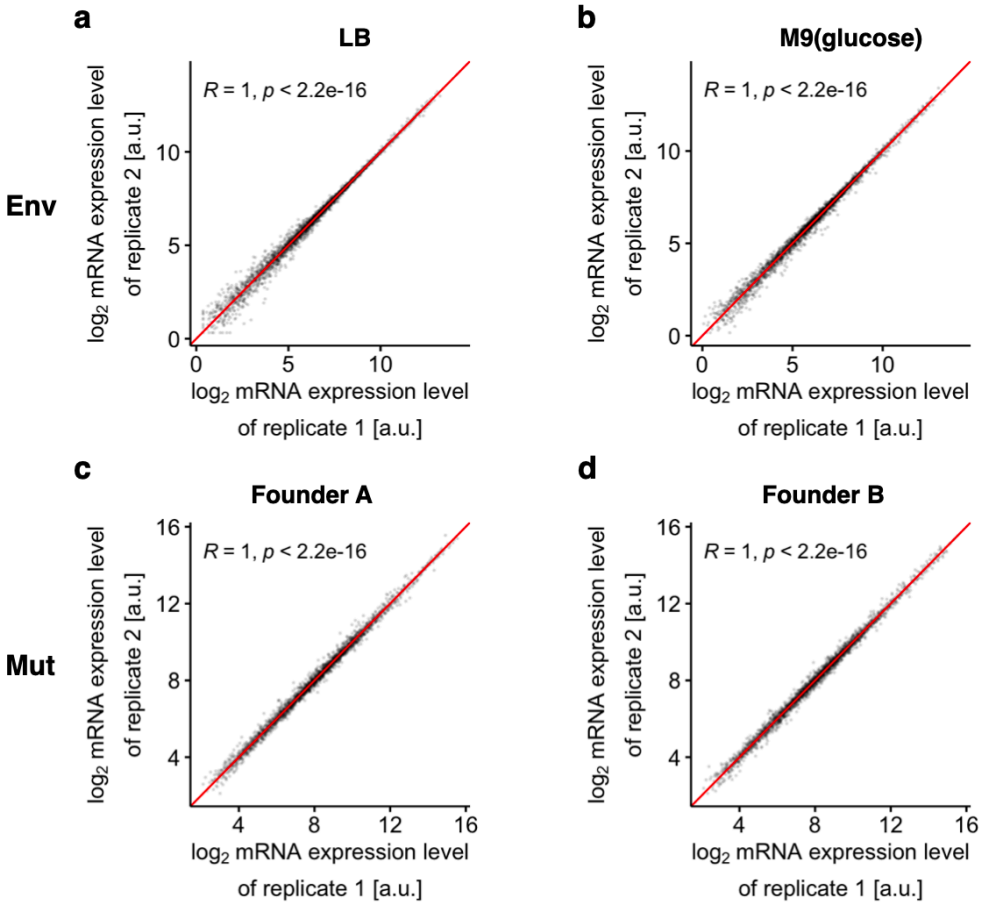
The replicability of microarrays in SI Fig 1 is impressive, however is technical or biological? It would be important to have a sense of the biological replicability. Also, I would like to see the same for the external datasets ENV and EVO, if replicates are available. This would help to assess the significance of the distributions of Fig 1 and the correlations of Fig 2a, as the actual correlation between expression variability may in reality be higher between datasets, it is just that experimental noise masks it.

Response:

Thank you for your positive evaluation of our data quality. **Supplementary Fig. 1** represents biological replicates, not technical ones. In response to the reviewer's comment, we have added figures showing high correlation between biological replicates in the Env dataset (**Supplementary Fig. 1a,b, newly added**). This confirms the high reproducibility of this crucial dataset, supporting our findings. Unfortunately, the individual processed transcriptome profile of each replicate in the Evo dataset were not available; only averaged data between biological triplicates were provided by the original paper. Although we attempted to analyze the corresponding raw fastq files, the necessary information for processing the data was not clearly described in the original paper. While the reproducibility of the Evo dataset was remains unclear, the RNA sequencing used in the Evo dataset differs significantly from the DNA microarray technology used in the Mut. Therefore, the technical noise affecting these two datasets is considered independent. Additionally, PCA of the Evo dataset showed that closely related strains (grouped by phylogroups) tended to have more similar expression in the top three PCs (**Supplementary Fig. 7**), with the exception of one phylogroup (A). These results suggest that most transcriptional variation in the Evo datasets reflects genetic differences rather than noises. Furthermore,

the strong correlation between DM_{evo} and DM_{env} at the functional category level supports the notion that DM_{evo} at the gene level is not dominated by random noise (**Fig. 2b**). Finally, the transcriptome profile of the MG1655 strain cultured under LB in the Evo dataset strongly and positively correlated with that in the Env dataset (Spearman's R=0.85, $p<10^{-15}$), even though the growth phases or detailed genetic backgrounds may not have been identical between the two datasets. Overall, the high reproducibility of the two most important datasets (Env and Mut) was directly confirmed by the high positive correlations between biological replicates. The reproducibility of the Evo dataset is less clear, but the majority of the variance in this dataset appears biologically meaningful.

Revision (Supplementary Fig. 1a,b, added):



Supplementary Fig. 1: Reproducibility of ~~DNA microarray~~transcriptome analysis in Env and Mut.

a,b, Log_2-transformed and quantile-normalized mRNA expression levels of 2622 genes are shown for two biological replicates (replicates 1 and 2) cultured under a complex rich medium (LB, **a**) and a synthetic minimal medium (M9, **b**) in the Env dataset. **c**,**d**, Log_2-transformed and quantile-normalized mRNA expression levels of 2622 genes are shown for two biological replicates (replicates 1 and 2) of founders A (**a**) and B (**b**) in the MA experiment. Each dot represents a different gene. The insets represent Spearman's R and p-values. The red lines represent x= y.

Comment 1.2

It would be useful to explain more the criterion for enrichment in the main text. For instance, nothing is low-DM-enriched in EVO, how should this be understood?

Response:

We apologize for the unclear description of enrichment criteria. Following your suggestion, we have explicitly added the criterion to the corresponding sentences. We also apologize for the unclear Venn diagram shown in **Fig. 2C** (right). The Evo and Mut sets overlapped completely, which led to the misleading impression that nothing was enriched at lower DM values in Evo. In fact, seven GOs were enriched at lower DM values in the Evo dataset. We have revised the figure to include explicit labels. In response to a similar comment from **Reviewer 2** (**Comment 2.11**), we have also added detailed explanations for the Venn diagram with a statistical test.

Revision (L115–132, modified):

To further investigate functional associations in DM values, we ~~Using~~ performed gene set enrichment analysis (GSEA[33]) with GO terms for both higher and lower DM values for each dataset independently. ~~,~~We found several GO terms significantly enriched in higher or lower DM values (BH-adjusted p-value (q)<0.05, GSEA). For instance, we identified genes related to 13 GOs that were significantly overrepresented at higher DM values compared with a uniform distribution. ~~we~~ We identified several common GO terms across all datasets (**Fig. 2c**, **Supplementary Fig. 2**). For higher DM values, more than three GOs were found at every intersection between pairs of datasets. This overlap was significantly more than expected (p<0.0001) based on random drawing from a pool of 81 GOs without replacement. Notably, genes related to flagellum-dependent cell motility and amino acid catabolic processes were consistently enriched with higher DM values in all three datasets (q<0.05, GSEA). Tuning the activity of these functional categories in response to environmental changes is crucial for cells to cope with nutritionally fluctuating environments[34, 35]~~, suggesting the importance of higher transcriptional variability of these genes in the complex habitats[36, 37] of *E. coli* (**Supplementary Discussion 1**)~~. In contrast, genes related to fundamental biological processes related to cell-wall construction and maintenance were consistently enriched at lower DM values for all datasets (q<0.05, GSEA, **Fig. 2c right**), indicating that the expression levels of genes within these categories were relatively robust to perturbations. The number of these common GOs (7) in lower DM values was significantly larger (p<0.0001) than expected (0.06) based on random drawing from a pool of GOs without replacement.

Revision (L149–169, modified):

For the Env dataset, we identified 30 transcriptional regulators enriched in higher DM_{env}, such as FlhDC and Fur, and termed them TR_{env} (q<0.05, GSEA, **Fig. 3a**). This enrichment means that the target

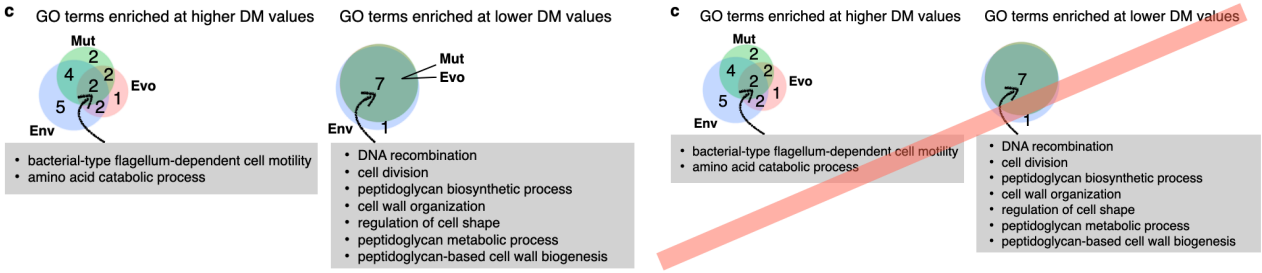
genes of these regulators were overrepresented at higher DM_{env} compared with a uniform distribution. In contrast, we identified only four transcriptional regulators enriched in the lower DM_{env} ($q<0.05$, GSEA). Similar analyses were performed for DM_{evo} and DM_{mut}, independently (**Supplementary Fig. 5**). Consequently, we identified 29 (TR_{evo}) and three transcriptional regulators enriched in higher and lower DM_{evo}, respectively. Similarly, 28 (TR_{mut}) and two transcriptional regulators were enriched in the higher and lower DM_{mut}, respectively ($q<0.05$, GSEA). Thus, fewer transcriptional regulators were ~~enriched~~ overrepresented at lower DM values, suggesting that regulation by transcriptional regulators tends to increase the transcriptional variability of target genes, with some regulatory motifs, such as negative feedback, potentially repressing fluctuations in gene expression, as a minor exception[40].

Next, we explored the similarity between TR_{env}, TR_{evo}, and TR_{mut}, which were identified in each dataset independently. We found that 13 transcriptional regulators, referred to as TR_{com}, were common among the three datasets, corresponding to more than one-third of the transcriptional regulators enriched in each dataset (**Fig. 3b**). The number of TR_{com}s (13) was significantly larger ($p<0.0001$) than the expected number (1.7) for the intersection of the three sets, constructed by random drawing from a pool of 118 TRs without replacement. These results suggest that the target genes regulated by TR_{com} exhibited higher transcriptional variability in response to both environmental and genetic perturbations, indicating that TR_{com}s function as a common mechanism to bias transcriptional variability against various perturbations.

Revision (L263–270, added):

We conducted a similar GSEA for the other two datasets (**Supplementary Fig. ~~9~~10**), identifying 31 regulations common across all three datasets (**Fig. 6e**), including 11 regulations by TR_{com}s, which is consistent with the notion that TR_{com}s are global regulators collectively controlling numerous targets. The number of common regulations (31) was significantly larger ($p<0.0001$) than the expected number (0.2) for the intersection of the three sets constructed by random drawing from a pool of 267 regulations without replacement. Overall, these findings support the idea that the similarity in the direction of transcriptional changes in response to different types of perturbations arises from shared transcriptional regulation.

Revision (Fig. 2c, labeled): left: Revised; right: Previous



Revision (Legend of Fig. 2c, added): **c**, Venn diagram of GO terms enriched in higher (left) and lower (right) DM values in each dataset (GSEA, BH-adjusted p-values ($q<0.05$, **Supplementary Fig. 2**).

The Evo and Mut sets completely overlap in the right Venn diagram. The numbers indicated in the diagram show the number of GO terms enriched in the corresponding set.

Comment 1.3

Lines 126-129 page 6 are too vague.

Response:

We apologize for the vague explanations regarding the results using the KEGG pathways. Using the KEGG pathways as an alternative to GO terms, we confirmed positive correlations in DM values at the pathway level between the three main datasets (Spearman's $R=0.5–0.67$, $p<0.05$). Additionally, we performed GSEA using the KEGG pathways and found that genes related to chemotaxis and peptidoglycan biosynthesis were overrepresented at higher and lower DM values, respectively, across all datasets ($q<0.05$, GSEA). These KEGG pathways are closely related to flagellum-dependent cell motility and cell wall construction in GO terms, respectively. We have added these detailed explanations to the corresponding sentences.

Revision (L132–141, added):

We obtained relevant results using the functional categories defined by the KEGG pathways (**Supplementary Fig. 3**,**4**, **Supplementary Table 6**). There were positive correlations in DM values at the pathway level between the three datasets (Spearman's $R=0.5–0.67$, $p<0.05$). Additionally, genes related to chemotaxes and peptidoglycan biosynthesis were overrepresented at higher and lower DM values, respectively, for all datasets ($q<0.05$, GSEA). These KEGG pathways were closely related to flagellum-dependent cell motility and cell-wall construction in GO terms, respectively. These results indicate frequent associations between canalization/decanalization in transcription level of genes and their functions.~~These results suggest~~, suggesting a certain common mechanism underlying the heterogeneous transcriptional variability associated with functional categories.

Comment 1.4

Fig. 5a: I am confused about blue-green and light-green

Response:

Thank you for pointing out the errors in the legend of **Fig. 5a**. This has been corrected.

Revision (Legend of Fig. 5a, fixed):

a, Yang's bow-tie structure[41] is delineated into seven sectors, with the main sectors highlighted: IN (yellow), OUT (~~light~~ blue green), and the strongly connected component (SCC, ~~blue~~light-green).

Comment 1.5

page 8 line 196 : “significantly enriched” can you specify the null hypothesis? It should be keeping the number of edge per node and just shuffle them, otherwise there is no particular claim here as the TRcom tend to have many targets.

Response: Thank you for the comment. We agree that most TR_{com}s belong to the SCC sector, likely because TR_{com}s tend to have many targets, as you suggested. Following your comment, we have revised the corresponding sentences by removing the enrichment analysis.

Revision (L211–214, modified):

The numbers of nodes in these primary sectors in *E. coli* TRN were seven TRs, 62 TRs and 2715 genes including the TRs for IN, SCC, and OUT, respectively (**Fig. 5e**). ~~Interestingly,~~As expected from their many target genes, TR_{com}s (12 of 13 TR_{com}s) and TR_{high}s (26 of 48 TR_{high}s in Env, Evo, and Mut) ~~were~~ belonged to~~significantly enriched in~~ SCC (~~Hypergeometric test, q <0.05,~~ **Fig. 5d**).

Comment 1.6

Fig. 6a: what are the colors for in Env?

Response: We apologize for the unclear explanation about this figure. Each color represents a different environmental condition in the Env dataset. This explanation has been added to the legend.

Revision (Legend of Fig. 6a, added):

a, First and second principal components (PC) of Env, Evo and Mut arranged from left to right. Points are colored by environmental condition in Env.

Comment 1.7

Fig. 6b, 7: I must stay that I start to be lost here because these analyses are very indirect: they consist of correlations between genes contributions to axes of different PCAs done on the transcriptional variability. On top of that, the correlations are not strong. It is fine to point to hypotheses to be tested more directly, but if the other find a more direct way to present their claim, I would find it more credible.

Response 1/4:

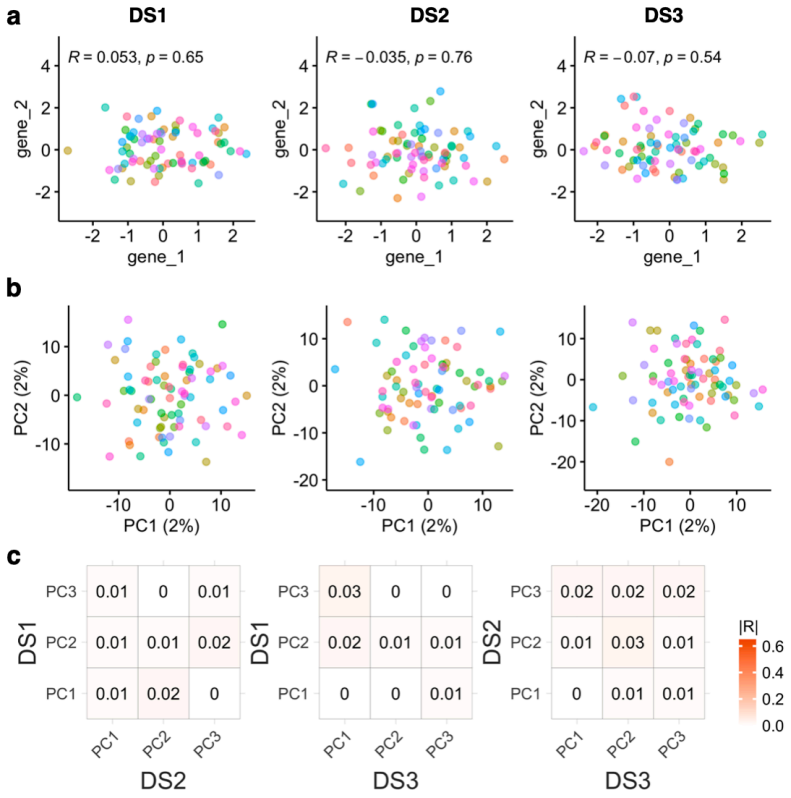
We apologize for not explaining how these relatively weak correlations are meaningful compared to a null hypothesis. The expected magnitude of correlations in PCA strongly depends on the number of

genes in the transcriptome data. To address this point, we computationally generated three datasets, termed DS1–3, to serve as a reference group. Each dataset consists of 2,622 genes and 76 conditions, matching the sizes of the Env dataset. Expression levels were randomly generated for each dataset, following a normal distribution with a mean of 0 and a standard deviation of 1. This ensured the independence of expression levels between genes within and between the three datasets (**Supplementary Fig. 9a, newly added**). We performed PCA independently for each dataset. As expected, at most a few percent of variance was explained by PC1, indicating the absence of significant bias in directionality in these independent datasets (**Supplementary Fig. 9b**). Based on these PCAs, we performed a correlation analysis for the loadings of the top three PCs among the three datasets. We confirmed that the absolute correlation coefficients were very small (~0.03) for all pairs (**Supplementary Fig. 9c**). These results indicate that the correlations in the top three PCs between the three main datasets (Env, Evo, and Mut) were much higher (~0.45) than random expectations. In response to the reviewer's comment, we have added these explanations to the main text.

Revision (L244–247, added):

Similarly, PC1 in the Evo dataset showed a relatively strong correlations with PC1–3 in the Env and Mut datasets. These correlations in loadings were much stronger than expected (~0.03 in Spearman's R) based on independent datasets comprising no correlations in expression levels between genes (**Supplementary Fig. 9c**).

Revision (Supplementary Fig. 9a–c, added):



Supplementary Fig. 9: PCA of the reference datasets comprising computationally generated expression levels.

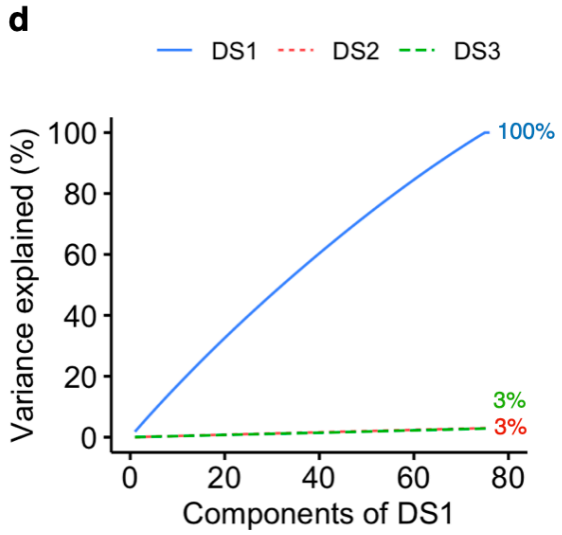
Three datasets, termed DS1–3, were computationally generated to serve as a reference group. Each dataset consists of 2,622 genes and 76 conditions, matching the size of the Env dataset. Expression levels were randomly generated for each dataset, following a normal distribution with a mean of 0 and a standard deviation of 1. This ensured the independence of expression levels between genes within and between datasets. **a**, There was no correlation in expression levels between two representative genes (gene_1 and gene_2) for each dataset (DS1 to DS3 from left to right), as designed. Spearman's R and p-values are shown in the insets. **b**, Pairwise score plots of PC1 and PC2 are shown for each dataset. PCA was performed independently for each dataset. Each color represents a different condition. **c**, A correlation analysis was conducted for the loadings of the top three PCs among the three datasets. The color scale and numbers in the tile represent the absolute values of Spearman's R. **d**, The cumulative variance explained by the PCs defined by the DS1 dataset. Overall, the DS1 dataset explained only 3% of the variance in the DS2 and DS3 datasets, respectively.

Response 2/4:

The purpose of this analysis is to test a theoretical prediction that major PCs are similar between different types of perturbations (Draghi & Whitlock, 2012; Furusawa & Kaneko, 2018). As the reviewer pointed out, the correlation analysis of the loadings might be an indirect method for showing the similarity between the PCs. To validate the similarity of the PCs between the datasets more formally and properly (J. Edward Jackson, A User's Guide to Principal Components, Wiley-Interscience, 2003), we assessed the extent to which the principal components in the Env dataset explained the transcriptional variance in the other two datasets (**Fig. 6c**). We observed that the PCs in the Env dataset explained up to 37% and 39% of the total variance in the Evo and Mut datasets, respectively (**Fig. 6c**). Moreover, the cumulative variance plot displayed a convex upward curve for both the Evo and Mut datasets, as well as Env, supporting the similarity between the top PCs. However, considering the reviewer's comment, this analysis might still be insufficient to demonstrate how these percentages (37%–39%) or the convex upward curves are higher or more extreme than random expectations. To address this issue, we performed a similar analysis for the DS1–3 datasets. This time, we assessed the extent to which the principal components in the DS1 dataset explained the transcriptional variance in the other two datasets (DS2, DS3) (**Supplementary Fig. 9d, newly added**). We confirmed that the cumulative variance for DS1 showed a linear-like curve (blue line), consistent with the top PCs not sufficiently explaining the variance (**Supplementary Fig. 9b**). We also confirmed that the PCs of DS1 explained only a small percentage of the variance in the DS2 and DS3 datasets, respectively, clearly indicating the dissimilarity of the PCs between these random datasets. Compared with this reference group, the percentages of 37%–39% and the convex upward curves in the main datasets were indeed extreme. Based on these results, we believe the claim that the top PCs are similar between the Env,

Evo, and Mut datasets is credible. We have added these additional analyses and explanations to the main text.

Revision (Supplementary Fig. 9d, added):



d, The cumulative variance explained by the PCs defined by the DS1 dataset. Overall, the DS1 dataset explained only 3% of the variance in the DS2 and DS3 datasets, respectively.

Revision (L247–256, added):

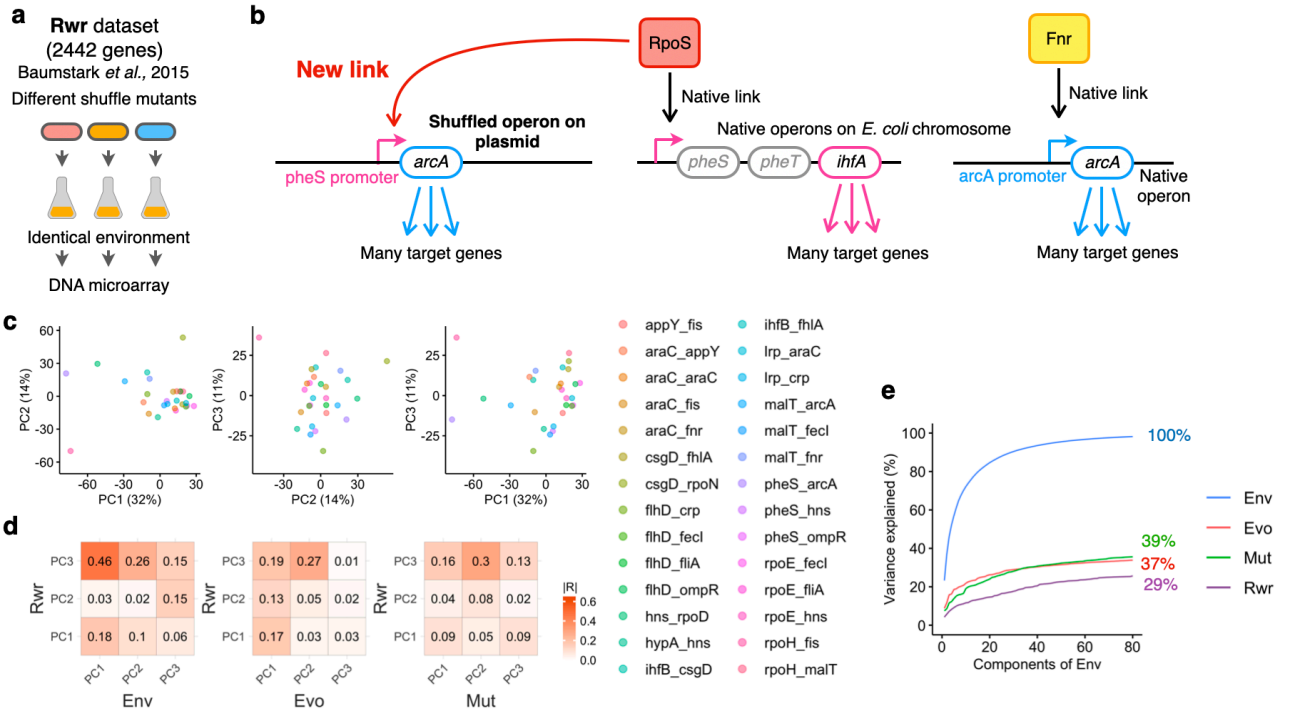
To validate the high similarity of the PCs between the three datasets more formally, we ~~In addition, we~~ assessed the extent to which the principal components in the Env dataset explained the transcriptional variance in the other two datasets. Overall, all principal components in the Env dataset explained up to 37% and 39% of the total variance in the Evo and Mut datasets, respectively (**Fig. 6c**). These fractions were much larger than random expectations (~3%, **Supplementary Fig. 9d**). Moreover, the cumulative variance plot displayed a convex upward curve for both the Evo and Mut datasets, as well as Env, which was distinct from a linear-like curve in random expectations (blue line in **Supplementary Fig. 9d**). These findings support the notion that the major directions of transcriptional variation in response to environmental perturbations are considerably similar to those observed in response to genetic perturbations, despite a large number of genes.

Response 3/4:

We agree that experimental evidence is crucial to support the hypothesis that the similarity of the top PCs is driven by global regulators. While a thorough empirical verification is challenging, some external experimental evidence, as suggested by the reviewer, may provide support. One possible approach is to test whether perturbing the regulatory networks of global regulators affects the directionality of transcriptional variation in the three main datasets. To this end, we analyzed

transcriptome profiles of E. coli mutants with rewired transcriptional regulations (Isalan *et al.*, *Nature*, 2008) that disrupt native transcriptional networks among major global regulators (**Supplementary Fig. 11, newly added**). Each rewired strain contains a unique shuffled synthetic operon comprising a plasmid copy of the native promoter region of one global regulator ('A') and a plasmid copy of a gene encoding another global regulator ('B'). This setup creates new regulatory "links" between randomly selected pairs of global regulators, perturbing the native transcriptional regulations. The transcriptome profiles of these rewired strains were originally obtained by Baumstark *et al*. (*Nat. Commun.*, 2015) under identical environmental conditions. To ensure comparability with the Env dataset, which exhibits high reproducibility, we excluded transcriptional profiles that showed relatively low quality (defined as less than 0.95 in mean Spearman's R between biological triplicates). The remaining profiles were then averaged across the triplicates for each rewired strain. We used a computationally generated random dataset, following a multivariate normal distribution, to confirm that this filtration and averaging process yielded high reliability, comparable to the Env dataset. This process resulted in transcriptional profiles of 28 rewired strains (**Supplementary Fig. 11c**). Using this dataset, termed the Rwr dataset, we performed PCA and investigated how the directionality in transcriptional variance differed from the common directionality shared by the three main datasets (**Supplementary Fig. 11c,d**). We found that the top two PCs in the Rwr dataset showed lower correlations (~0.18 at most) with those in the three main datasets, in contrast to the higher correlations observed between the three main datasets (**Fig. 6b**). Relatively higher correlations were found between the third PC in the Rwr dataset and the top two PCs in the three main datasets. Consistently, the PCs defined by the Env dataset explained at most 29% of the variance in the Rwr dataset, which was lower than that observed in the Mut and Evo datasets (**Supplementary Fig. 11e**). Notably, these percentages were even smaller when the low-reproducibility profiles were not excluded. These results suggest that the directionality in transcriptional variation among rewired strains is less similar to the common directionality shared in the three main datasets. These findings underscore the importance of regulatory networks between global regulators in shaping major directions of transcriptional variability, thus supporting the relevance of our findings. In response to the reviewer's comment, we have now included this analysis in the main text.

Revision (Supplementary Fig. 11, added):



Supplementary Fig. 11: PCA of the Rwr dataset.

a, A schematic representation of the Rwr dataset. Baumstark *et al*[1] cultured different rewired strains of *E. coli*, originally constructed by Isalan *et al*[2], under identical environmental conditions. Transcriptome profiles were obtained using a DNA microarray. A total of 2,442 genes present in the three main datasets were extracted. Transcriptome profiles were $\log_2$-transformed and quantile-normalized. Transcriptome profiles that showed relatively low quality (mean Spearman's R<0.95 between biological triplicates) were removed. The transcriptional profiles were then averaged between the triplicates for each rewired strain. This filtration resulted in transcriptome profiles of 28 rewired strains, termed the Rwr dataset. **b**, A schematic representation of the transcriptional regulation in a representative rewired strain (the pheS_arcA strain). The native promoter region (the pheS promoter) of a global dual transcriptional regulator, *ihfA*, coding for a subunit of IHF, was copied and pasted into a plasmid. The *arcA* gene coding for a global dual regulator ArcA was copied and transcriptionally fused to the plasmid copy of the *pheS* promoter. The native chromosomal copies of these genes and promoters were intact. Consequently, the rewired strain obtained a new regulatory link (red arrow) between two global regulators, RpoS and ArcA, while the native regulatory links (RpoS to IHF or Fnr to ArcA) were retained. The names of the rewired strains consist of the names of the copied promoters (pheS) followed by the names of the copied ORFs (arcA). **c**, Pairwise score plots of PC1–3 are shown. **d**, A correlation analysis for the loadings of the top three PCs among the datasets. PCA was performed independently for each dataset using the common 2,442 genes shared by the four datasets. The color scale and numbers in the tile represent the absolute values of Spearman's R. **e**, The cumulative variance explained by the PCs defined by the Env dataset. Overall, the Env dataset explained only 29% of the variance in the Rwr dataset (purple).

Revision (L268–291, added):

Overall, these findings support the idea that the similarity in the direction of transcriptional changes in response to different types of perturbations arises from shared transcriptional regulation. To further confirm the relevance of these findings, we analyzed transcriptome profiles of *E. coli* mutants harboring rewired transcriptional regulations[44] that perturb native transcriptional regulations between major global regulators (**Supplementary Fig. 11**). Each of the rewired strains has a unique shuffled synthetic operon comprising a plasmid copy of the native promoter region of a global regulator ('A') and a plasmid copy of a gene coding for another global regulator ('B'). Accordingly, promoter-mediated transcriptional regulations controlling expression of 'A' are newly connected to 'B' without mutating native chromosomal regulations. Consequently, these rewired strains have new regulatory "links" between randomly selected pairs of global regulators, perturbing native transcriptional regulations between global regulators. The transcriptome profiles of the rewired strains were originally obtained by Baumstark *et al*[45] under identical environmental conditions. Using this dataset, termed the Rwr dataset, we performed PCA and explored how the directionality in transcriptional variance differed from the common directionality shared by the three main datasets (**Supplementary Fig. 11c,d**). We confirmed that the top two PCs in Rwr showed lower correlations (~0.18 at most) with those in the three main datasets, which was very different from the higher correlations between the three main datasets (**Fig. 6b**). Relatively higher correlations were found between the third PC in Rwr and the top two PCs in the three main datasets. Consistently, the PCs defined by the Env dataset explained at most 29% of the variance in the Rwr dataset, which was lower than those of the Mut and Evo datasets (**Supplementary Fig. 11e**). These results indicated that the directionality in transcriptional variation between rewired strains was less similar to the common directionality shared in the other datasets. These results highlighted the importance of regulatory networks between global regulators in major directions in transcriptional variability, supporting the relevance of our findings.

Revision (Methods / L520–532, added):

Data analysis of the Rwr dataset

The Rwr dataset was derived from transcriptome profiles reported by Baumstark *et al* [45]. In the Rwr dataset, *E. coli* TOP10 (K12) strains, which harbor synthetic shuffled operons [44] (referred to as rewired strains), were cultured under identical environmental conditions. Transcriptome profiles were obtained using the DNA microarray technique (**Supplementary Fig. 11a,b**). A total of 2442 genes, shared across the three main datasets, were extracted. The expression profiles were $\log_2$-transformed and quantile-normalized. To facilitate comparison with the Env dataset, which exhibits high reproducibility, transcriptional profiles that showed relatively low quality (less than 0.95 in mean Spearman's R between biological triplicates) were excluded from the Rwr dataset. The transcriptional profiles were subsequently averaged between the triplicates for each rewired strain. We used a computationally generated random dataset, following a multivariate normal distribution, to confirm that this filtration and averaging process yielded high reliability, comparable to the Env dataset. This filtration resulted

in transcriptional profiles of 28 rewired strains (**Supplementary Fig. 11c**).

Response 4/4:

We agree that additional and more direct empirical validations could further strengthen our findings. We believe it is important to explicitly note this limitation. In response to **Reviewer 2**'s comments (**Comment 2.9**), we have described the limitations of our study more clearly in the Discussion section. Please refer to our response to **Comment 2.9**.

Responses to Reviewer 2:

Comment 2.1

This is a very interesting paper that attempts to establish the degree of concordance of gene expression variability in response to genetic and environmental perturbations in E. coli. To my knowledge, this is the first exhaustive experimental test of recent theory on this topic and the study is therefore a much welcome addition to the literature. The use of existing data sets poses some limitations and make it somewhat difficult to assess in places how confident one should be in the results (see specific comments below). Nevertheless, the study is very valuable and likely to inspire further exploration of a topic fundamental to understanding the role of generative processes in evolution.

Response:

Thank you very much for your high evaluation of our manuscript. Your comments have greatly contributed to improving our manuscript. We fully agree that it is difficult to draw conclusions about past evolution from our research methods, as you pointed out. We have followed all of your suggestions and removed the corresponding sentences from the manuscript. We have addressed all of your comments as detailed below.

Comment 2.2

Line 28-30. Here and elsewhere, the authors interpret their results on the basis of the theoretical work done by Watson and colleagues (e.g., Kouvaris et al. 2017. PLoS Comp Biol; Brun Usan et al. 2021. BMC Ecol Evol). That is, they consider the similarity of responses to different perturbations to reflect the evolutionary (and selective) history of GRNs. However, the idea that GRNs 'learn' is not actually tested in this paper, and the conclusion therefore does not follow from the results. In fact, as Brun-Usan et al. (2021, BMC Ecol Evol, not cited by the authors) have shown, the 'alignment' between transcriptional responses to genetic and environmental perturbations is a property of GRNs themselves, without the need for 'structuring in past environments'. The authors should therefore moderate their conclusion. This criticism also affects some of the supplementary discussion, see below.

Response:

Thank you for pointing out our weak speculations. We agree that it is difficult to draw conclusions about past evolution from our research methods. Following your suggestion, we have now removed the corresponding sentences.

Revision (L27–28, removed):

Our findings highlight the role of gene regulatory networks in shaping the shared phenotypic variability across different perturbations. ~~This study provides insights into how gene regulatory~~

~~networks have been structured in past environments and generalized to new ones.~~

Comment 2.3

Line 43-52 and elsewhere. It seems that two rather important studies are missing from the discussion of the similarity of developmental bias in response to different perturbations: Brun-Usan et al. 2021 BMC Ecol Evol and Milocco & Uller 2024. PNAS (perhaps also see Brun-Usan et al. 2023. Bioessays). the results of these studies strengthen the authors theoretical standpoints and provide opportunities for more nuanced explanations of the results (see above and below).

Response:

Thank you for informing us about these appropriate references. We have now cited these references (Brun-Usan et al. 2021 BMC Ecol Evol as Ref. 12; Milocco & Uller 2024. PNAS as Ref. 13; Brun-Usan et al. 2023. Bioessays as Ref. 14) where appropriate.

Revision (L41–43, added):

Seemingly different types of phenotypic variability may actually be driven by a common mechanism. Theoretical studies have demonstrated that the bias in phenotypic variability is shared by different perturbations[9, 10, 11, 12, 13, 14].

Comment 2.4

Line 47. It is not entirely clear to me that the work cited is appropriate here. Would this statement not be accompanied by empirical studies that directly compare variation in response to genetic and environmental perturbations (e.g., quantitative genetic studies)?

Response:

We apologize for the unclear reference. As you suggested, the corresponding statement should be accompanied by quantitative genetic studies with empirical studies on transcriptional phenotypes. In fact, Rifkin et al. 2005, *Nature* (Ref. 17), performed the corresponding experiments in flies. Following your comment, we have also referred to Landry et al. 2007, *Science* (Ref. 18), which performed similar experiments in yeast.

Revision (L43–45, modified):

Notably, ~~there is~~several quantitative genetic studies identified similarity in the magnitude of variance, where traits sensitive to environmental perturbations tend to be sensitive to genetic perturbations[15, 16, 17, 18].

Comment 2.5

Lines 45-46 & 49-51. These two sentences seem to say pretty much the same, so there is arguably some scope for streamlining this paragraph (see also comments above).

Response:

Thank you for pointing out the redundancy. We have removed the corresponding sentences.

Revision (L41–45, removed):

Seemingly different types of phenotypic variability may actually be driven by a common mechanism. Theoretical studies have demonstrated that the bias in phenotypic variability is shared by different perturbations[9, 10, 11, 12, 13, 14]. ~~Consistently, environmentally induced phenotypic variations often mirror genetically induced phenotypic variations.~~ Notably, ~~there is~~several quantitative genetic studies identified similarity in the magnitude of variance, where traits sensitive to environmental perturbations tend to be sensitive to genetic perturbations[15, 16, 17, 18].

Comment 2.6

Lines 57-59. The wording here ('concordance') seems to imply that gene expression responses to genetic and environmental perturbation are biased in similar ways (in line with the theoretical work). However, at least some of the cited literature is a little different in scope. For example, reference 21 shows that genes with greater environmental sensitivity also are sensitive to genetic perturbations (and noise). But that is a statement about gene identity, not concordance of expression patterns. Perhaps this is not so important, but it may be worth rewriting to make the sentence convey more information about what is known about this topic to date.

Response:

We apologize for the unclear wording. As you pointed out, we described two different concepts using the same word "concordance," which caused confusion in the paragraph. To improve clarity, we have revised the corresponding sentences.

Revision (L55–59, modified):

Despite a growing number of studies demonstrating empirical evidence supporting the similarity both in susceptibility and in directionality of variations in gene expression levels~~concordance~~ between environmental and genetic perturbations~~ly induced and genetically induced phenotypic variations in gene expression levels~~[17, 18, 24], it remains largely unknown how the real regulatory networks account for the observed bias in variability among genes[8].

Comment 2.7

Line 69. I am not sure what ‘structure of transcriptional regulation’ means.

Response:

We apologize for the unclear term. This has been revised to improve clarity.

Revision (L67–68, modified):

Most importantly, we found that the ~~structure of~~ transcriptional regulatorsion accounts for these common biases.

Comment 2.8

Lines 72-74. As explained above, this study does not really show the effect of past environments on GRNs and their ability to ‘generalize’. Better to avoid this conclusion. See also criticism of supplementary discussion below.

Response:

Thank you for pointing out the speculative conclusion. Following your suggestion, we have removed the corresponding sentences.

Revision (L70–71, removed):

Our results suggest that these biases are not random but are functionally significant in coping with fluctuating environments. ~~This study sheds light on how gene regulatory networks have been structured in past environments and generalized to new environments.~~

Comment 2.9

Line 79. Please acknowledge the limitations of drawing far-reaching conclusions about transcriptional variability on the basis of a single strain. Please also acknowledge the limitations of contrasting data sets that differ in important respects (particularly important for interpreting how the results are related to past evolution and selection).

Response:

Thank you for pointing out the important limitations of our study. Following your comment, we have added explanations about the limitation of focusing on a single strain in the **Discussion** section. We also agree that it is difficult to draw conclusions about past evolution from our research methods. Following your suggestion, we have removed the corresponding sentences.

Revision (L364–373, added):

A major limitation of our study is that it focused solely on the transcriptome regulatory networks of a single strain of *E. coli* (MG1655 including its closely-related strains). Currently, most of the reliable knowledge about transcriptional regulatory networks in *E. coli* is heavily restricted to this model strain. Ideally, gathering comprehensive information on the networks of different strains of *E. coli* or other bacterial species with different configurations of transcriptional regulatory networks would enable us to explore how transcriptional variability depends on their transcriptional regulatory networks. This future exploration would also require many transcriptomic profiles under different environmental conditions for each strain to capture the transcriptional variability unique to each. This extension would further underscore the impact of transcriptional regulatory networks on the magnitude and directionality of transcriptional variability.

Comment 2.10

Line 111. Please explain this better: 'canalization in… functional categories' is difficult to understand.

Response:

We apologize for the vague sentence. We have revised the corresponding text by adding examples and combining closely related paragraphs.

Revision (L106–115, modified):

This phenomenon of canalization/decanalization could occur not only at the gene level but also at more complex phenotypes subject to natural selection. ~~For example~~C~~, c~~analization/decanalization in transcriptional changes has been observed in some~~the~~ functional categories of genes in yeast[18, 24]. For instance, genes involved in stress or amino acid metabolism tend to have higher transcriptional variability [24], while genes related to cell growth, maintenance, and cell cycles do not exhibit higher transcriptional variability against genetic perturbations [18]. To explore ~~this possibility~~whether functional associations in DM values were present in bacteria, we utilized a refined subset of Gene Ontology (GO) terms as functional categories (**Methods**) **(Supplementary Table 5**). ~~, u~~Using the mean DM values of genes within each GO term as a measure of transcriptional variability~~. Consequently~~, we observed positive correlations between the datasets even at the functional category level (**Fig. 2b**). ~~These results indicate frequent associations between canalization/decanalization of genes and their functions.~~

Comment 2.11

Lines 137-145. Here and elsewhere (e.g., line 148), it is not clear that the results really show greater

overlap than expected based on chance. It would be helpful if the authors spelled out their results in a way that makes it clear what it means that regulators are 'enriched' (e.g., phrase this to make it explicit what the null assumption of overlap between data sets would be). Alternatively, the authors may wish to include a clear statement at the outset that explains how they tested this statistically.

Response:

We apologize for the unclear explanation of the term "enrichment." **Reviewer 1** also raised a similar concern (**Comment 1.2**). In the original version of our manuscript, we only performed GSEA to identify transcriptional regulators that were overrepresented at higher/lower DM values (q-values < 0.05, GSEA). We had not performed any additional statistical tests to determine how significantly these enriched regulators overlapped between the different datasets. To address this important question, we have now performed the corresponding statistical test. First, we computationally constructed three sets by randomly drawing regulators from a pool of 118 transcriptional regulators, where the number of regulators per set was the same as the number in the real sets (TR_{high} and TR_{low} for each of the three main datasets). We subsequently constructed the Venn diagram for the drawn sets and counted the number of regulators overlapping across the three sets. This procedure was repeated 10,000 times to construct a probability distribution. Using this distribution, we estimated the mean number of overlapping regulators based on random expectation and calculated p-values. We found that the number of TR_{com}s (13) was significantly larger ($p < 0.0001$) than the expected number (1.7) for the intersection of the three sets. A similar analysis was performed for **Figs. 2c**, **3b**, and **6e**. Following the reviewers' comments, we have explicitly added the corresponding statistical criteria for each enrichment analysis as detailed in our responses to **Comment 1.2**.

Comment 2.12

Line 216. A more informative study here is probably Brun-Usan et al. 2021 BMC Ecol Evol. Also see the recent Milocco & Uller 2024 PNAS (which probably came out after the authors submitted). Same comment applied for line 261.

Response:

Thank you very much for informing these appropriate references. We agree that facilitation of genetic evolvability by selection for phenotypic plasticity (Brun-Usan et al. 2021 BMC Ecol Evol) is highly relevant for line 304 (line 261 in previous version). We have now cited these studies (Brun-Usan et al. 2021 BMC Ecol Evol as Ref. 12; Milocco & Uller 2024. PNAS as Ref. 13) accordingly. In line 304, we have also revised the sentence by explicitly adding the words "or be facilitated by".

Revision (L232–234, modified):

Next, we explored the major directions of correlated transcriptional variation among thousands of

genes. Theoretically, genetically caused phenotypic variance is expected to be the greatest along the major axis or the principal component of environmentally caused phenotypic variance[10, 12, 13].

Revision (L305–307, modified):

Heterogeneous phenotypic variability is not a random occurrence devoid of biological significance (**Fig. 2c** and **6f,g**), but rather it may result from or be facilitated by the adaptive generalization of past environments[9, 10, 11, 12, 13] ~~(**Supplementary Discussion 1**)~~.

Comment 2.13

Lines 267-273. The rationale for these additional data sets is not entirely clear. Please provide some additional information, and also explain why these data sets are 'supplementary' rather than part of the main analyses.

Response:

We apologize for the lack of clarity regarding the purpose of using these additional datasets. In our main analyses, we identified similarities in transcriptional variability between environmental and genetic perturbations. The additional datasets were employed to explore the broader applicability of the observed variability. These datasets, however, include transcriptome or proteome profiles obtained under more specific conditions compared to the three main datasets (Env, Evo, and Mut). For example, the Strs and Strs_he datasets consist of transcriptome profiles of adaptive mutants isolated through strong directed evolution, which differs from the Mut dataset that comprises transcriptome profiles of mutants isolated via the MA experiment. Consequently, selection in these two datasets can, to some extent, mask or bias the raw variability. Similarly, the Strs_hp dataset contains transcriptome profiles of *E. coli* cultured under different stressful chemical conditions, which contrasts with the Env dataset that examines different nutrients, culture conditions (e.g., temperature, pH), and a broad spectrum of chemical compounds, including antibiotics. The Noise_ch dataset is unique and distinct from the other datasets, as it provides information about cell-to-cell variability in protein expression levels for each gene but lacks information about covariance between genes, which is needed for PCA. Therefore, we treated these datasets as supplementary rather than primary datasets. Despite these limitations, these additional datasets still provide valuable transcriptome/proteome profiles under several perturbations not covered by the three main datasets, with the exception of Prtn. The Prtn dataset, which includes several environmental conditions similar to those in the Env dataset, was used to test whether the bias in transcriptional variability observed at the transcriptome level is retained at the protein level. In response to the reviewer's suggestion, we have added explanations on how these additional datasets were utilized to draw conclusions. We also note that most of the original Supplementary Discussions have been removed in the revised version. Detailed information on processing each of the additional datasets has been retained and is now labeled as **Supplementary Note**, as detailed in our response to

Comment 2.17.

Revision (L309–324, modified):

These findings imply a similarity in variability in gene expression levels across a broader range of perturbations than previously examined. For instance, genes with higher/lower DM values in Env might also have higher/lower transcriptional variability in response to other environmental perturbations or other nongenetic perturbations (molecular noises) untested in the Env dataset. Similarly, genes with higher/lower DM values in response to random mutations accumulated through genetic drift (Mut) might exhibit higher/lower DM values in response to beneficial mutations accumulated by strong selection in adaptive evolution. –To address ~~this~~these possibilities and validate a broader applicability of the findings from the main datasets, we analyzed five additional datasets for *E. coli*, termed Strs_hp, Strs_he, Strs, Prtn, and Noise_ch (**Fig. 7a**, **Supplementary Figs. 1~~5~~3–2~~2~~1**, **Supplementary ~~Discussions 2~~Note**). These datasets include– transcriptome or proteome profiles obtained under relatively narrower or more specific conditions than the three main datasets (Env, Evo, and Mut), but –under ~~various~~ several perturbations absent in the three main datasets ~~(Env, Evo, and Mut)~~, except for Prtn. The Prtn dataset, which contains several environmental conditions similar to those used in Env, was used to test whether the bias in transcriptional variability was retained at the protein level. The phenotypic variabilities based on these datasets, either at the transcriptional or protein level, were termed DM_{strs_hp}, DM_{strs_he}, DM_{strs}, and DM_{noise_ch} as detailed in **Supplementary Note**.

Revision (L336–347, added):

Thus, transcriptional variability tended to be retained at the protein level, as shown in the Prtn dataset, suggesting the impact of transcriptional regulation on the variability in phenotypes governed by proteins. The similarity in transcriptional variability with Strs_hp supported the impact of the transcriptional variability identified in the three main datasets on broad environmental conditions. Importantly, the similarity with Strs and Strs_he highlighted the impact of transcriptional variability under relaxed selection (Env or Mut) on the evolutionary diversification in transcriptional phenotypes accompanied by adaptive evolution. Interestingly, the transcriptional variability in response to mutations (Mut) was also coupled with stochastic protein noise (Noise_ch), which was previously reported in yeast [18]. Taken together, t~~T~~hese results indicated similar trends in gene-to-gene differences in both the magnitude and major directions of expressional variations across various perturbations, suggesting global canalization/decanalization across different perturbations[12, 13, 14, 21, 47].

Comment 2.14

Lines 289-292. This conclusion is not entirely clear. It may need some expansion or connection to the theoretical studies to make sense.

Response:

We apologize for the unclear conclusion. Following the reviewer's suggestion described below (**Comment 2.15**), Supplementary Discussion 3 has been removed. Consequently, the corresponding sentence in the main text has also been removed.

Revision (L344–347, removed):

Taken together, t~~T~~hese results indicated similar trends in gene-to-gene differences in both the magnitude and major directions of expressional variations across various perturbations, suggesting global canalization/decanalization across different perturbations[12, 13, 14, 21, 47]. ~~These results also imply that the heterogeneous transcriptional variability can both facilitate and constrain the rates and directions of de novo phenotypic evolution not only through neutral processes, as shown in the MA experiment, but also through complex processes, including adaptive processes as demonstrated in the Strs dataset (**Supplementary Discussions 2,3**).~~

Comment 2.15

Supplementary Discussions. The authors have done an impressive amount of work that is only presented and discussed in the supplementary material. While I find this work very interesting, it is difficult to evaluate and I am not fully convinced by all the conclusions. Perhaps some of this material should be lifted out of this paper and form the basis for a separate study? For example, the material in Supplementary Discussion 3 is intriguing, but it is nearly impossible (at least to this reader) to evaluate to what extent the conclusions follow from the data.

Response:

We apologize for the unclear descriptions in the Supplementary Discussions. Following the reviewer's suggestion, Supplementary Discussion 3 and the related speculations have been removed from the manuscript. Additionally, as per the comments below (**Comments 2.16 and 2.17**), we have removed Supplementary Discussion 1, as detailed in our response to **Comment 2.16**. For Supplementary Discussion 2, we have moved this section to the newly labeled **Supplementary Note** and removed conclusive sentences from this section, as detailed in our response to **Comment 2.17**.

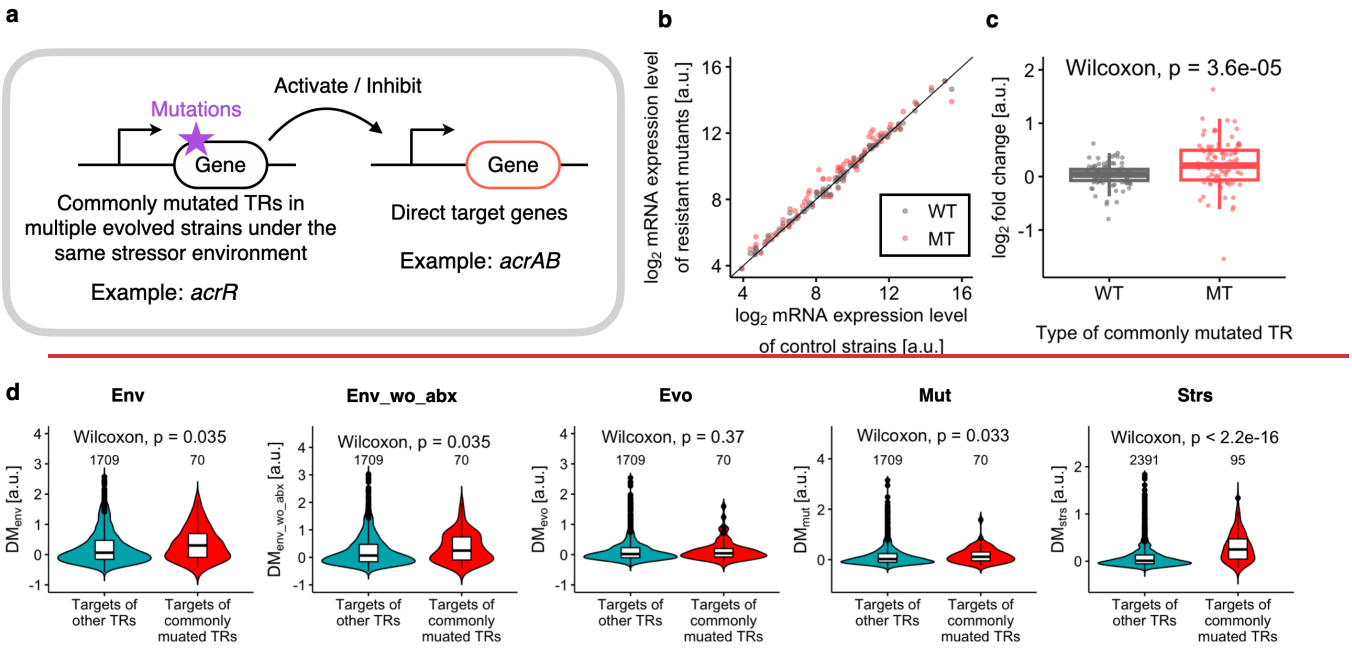
Revision (Supplementary Discussion 3, removed):

~~**Supplementary Discussion 3. Facilitation of adaptive evolution by heterogeneous transcriptional variability**~~

~~In principle, heterogeneous phenotypic variability can either facilitate or constrain evolution depending on the selection acting on traits[31]. In the former case, if the selection in *de novo* evolution is similar to past natural selection, the generalized responses can facilitate *de novo* evolution by~~

~~generating useful variants favored by past natural selection more frequently than other variants, which is known as facilitated variation theory[32]. This can be achieved by increasing the transcriptional variability of useful genes in response to mutations. To test this possibility, we focused on the frequently mutated TRs in adaptive laboratory evolution toward stressors and explored the transcriptional variability of their direct target genes of TRs. Maeda *et al.*[3] identified that several TRs, such as AcrR, were commonly mutated in multiple evolved lines under identical environmental conditions (**Supplementary Fig. 21a**). Most of these frequently identified TRs (six TRs), called commonly mutated TRs, directly regulate the transcription of useful genes for resistance to stressors, such as genes encoding the multidrug efflux pump AcrAB. Accordingly, the upregulation of the expression levels of these target genes is relevant to confer resistance to some stressors. We confirmed that the expression levels of these target genes were higher in mutants carrying mutations in the commonly mutated TRs (36 mutants) than in those without mutations in the commonly mutated TRs (148 mutants), as shown in **Supplementary Fig. 21b,c**. We found that the DM_{strs} of the target genes of the commonly mutated TRs were higher than those of the target genes of the other TRs (Wilcoxon test, $p<0.05$, **Supplementary Fig. 21d**), indicating that the former highly contributed to transcriptional diversification derived from the mutations accumulated during adaptive evolution. Interestingly, the target genes of the commonly mutated TRs also showed higher DM_{env} and DM_{mut} values than those of other TRs. There was no overlap between the culture conditions used in Env and Mut and those used in Strs for the evolution experiments. This trend retained even if we removed the transcriptome profiles obtained in the culture conditions containing any antibiotics from Env. The insignificant difference in DM_{evo} between the two groups might reflect violations due to complex natural selection. These results suggest that the target genes of commonly mutated regulators not only have a useful function in resistance to stressors but also have higher transcriptional variability against different perturbations, including mutations, thereby conferring a greater impact on the increase in resistance level. These results may explain the frequent emergence of mutants resistant to multiple antibiotics at higher resistance levels owing to only a few mutations.~~

Revision (Supplementary Fig. 21 (previous version), removed):



Supplementary Fig. 21: Transcriptional variabilities of the target genes of commonly mutated transcriptional regulators in the Strs dataset.

a, Schematic representation illustrating the relationship between commonly mutated regulators and their target genes. Evolution experiments conducted by Maeda *et al.*[3] identified several TRs that were commonly mutated in multiple evolved lines. Commonly mutated TRs are transcriptional regulators in which mutations are identified in multiple replicative lines under the same environmental conditions. Most of these mutations were confirmed to confer higher resistance to the corresponding stressors than the control strains. Consequently, these mutations were likely fixed in the population through selection for changes in the expression levels of the regulatory target genes. Notably, the target genes of the most commonly mutated TRs included those encoding multidrug efflux pumps. **b**,**c** Expression levels of the union set of direct target genes (95 genes) of commonly mutated TRs (six TRs). The stressor-resistant mutants were divided into two groups: resistant mutants carrying mutations in commonly mutated TRs (36 mutants), denoted as MT, and resistant mutants without mutations in the commonly mutated TRs (148 mutants), denoted as WT. The control strains represent strains that propagated under stress-free conditions (four strains). **b**, Expression levels of WT and MT compared to those of the control strains. Each dot represents the mean expression level of a target gene among the mutants in each mutant group. The black solid line represents y=x. **c**, Box plot of log2-fold changes in the expression levels of the target genes from resistant mutants to control strains, which corresponds to the vertical distance of the expression level to the solid line in panel **b**. **d**, Violine plots of the DM values of different datasets (Env, Env_wo_abx, Evo, Mut, and Strs, from left to right). The Env_wo_abx dataset was constructed by removing the transcriptome profiles obtained in the presence of antibiotics from the Env dataset. The regulatory target genes of the commonly mutated TRs and those of other TRs are colored differently. The numbers of genes in each category are indicated. Lower and upper edges of boxes represent first (q1) and third (q3) quartiles, respectively. The horizontal lines in the boxes represent the

median (m). The whiskers from the boxes extend to the most extreme observed values inside inner fences, m±1.5(q3–q1).

Comment 2.16

In general, my main concern with these supplements is that I am not convinced that the authors can successfully connect their results to past adaptive evolution. The data required to do so would arguably be very different than the data the authors have access to in this work. This criticism affects Supplementary Discussion 1 insofar that the speculations about adaptive significance of variability in cell motility are just speculations. It may concern many readers that the authors imply there has been 'selection for evolvability' (I am not sure the authors really imply selection 'for', but rather 'selection of', as in the theoretical models they cite). Overall, it is not clear that Supplementary Discussion 1 adds much to the study, and perhaps the paper would be better without it?

Response:

We agree that it is difficult to draw any conclusions about past evolution from our research methods, as the reviewer pointed out. Following the reviewer's comment, Supplementary Discussion 1 has been removed.

Revision (Supplementary Discussion 1, removed):

Supplementary Discussion 1. Transcriptional variability coupled with multiple functions in bacterial lifestyle

In the main text, we focused on the regulatory mechanisms underlying transcriptional variability in response to different perturbations. Here, we discuss the relevance of the functional categories accompanied by bias in transcriptional variability in more detail.

According to previous studies, the frequently observed bias in phenotypic variability is not a random occurrence devoid of biological significance, but rather it has evolved to enhance fitness in past environments[5, 6, 7]. If this is indeed the case, transcriptional variability is anticipated to be linked to critical functions relevant to bacterial fitness. As shown in **Fig. 2c**, flagellum-dependent cell motility and amino acid catabolic processes were consistently enriched with higher DM values in the three main datasets (Env, Evo, and Mut). The flagellum plays a crucial role in the colonization of natural *E. coli* habitats such as the mammalian intestine[8], while it requires substantial energy investment in ATP currency for its construction and operation[9]. Therefore, regulating the allocation of cellular resources for motility in response to varying growth conditions is imperative. Indeed, recent research has shown that *E. coli* modulates the expression levels of motility genes to maintain efficient motility while minimizing growth reduction under diverse nutrient conditions[10]. Accordingly, the high transcriptional variability of motility genes in response to different environmental perturbations appears to be useful for *E. coli*. to achieve optimal transcriptional states on demand. Consistent with the need for regulation

in response to environmental changes, motility genes are governed by numerous transcriptional regulators (e.g., FlhDC, FliA, IHF, Crp, and H-NS) and two-component signaling systems[11, 12]. These transcriptional regulators belonged to TR_{com}s and were enriched in higher DM values in all three datasets. Similarly, amino acid catabolic processes are finely tuned by *E. coli* to survive and thrive in fluctuating environments[13] including the transitions from animal intestines to open environments[8, 14]. The genes associated with these processes are under the control of global transcriptional regulators, such as Crp, Lrp, and Nac[15, 16, 17], which also belong to TR_{com}s. Thus, the tunability of both motility and amino acid catabolism in response to environmental conditions is directly related to cellular fitness in natural habitats, supporting the idea that higher transcriptional variability might have evolved to enhance cellular fitness under frequent environmental perturbations in the life cycle of *E. coli*. In contrast, biological processes related to cell-wall construction and maintenance were consistently enriched at lower DM values, indicating that the expression levels of genes within these categories were relatively robust to perturbations (**Fig. 2c**). These processes are fundamental for the maintenance of the cellular body. Therefore, low variability in these genes ensures the stability of cellular life, aligning with the concept of Waddington's canalization[18].

We also detected the functional categories that were enriched in the major directions of transcriptional variations in response to different perturbations. As shown in **Fig. 6f**, **g**, the enrichment of growth-related functional categories in PC1 in Env suggests coupling with changes in growth rate. Notably, we found a positive correlation between PC1 in Env and the growth rates at which the transcriptome profiles were obtained (Spearman's R=0.55, **Supplementary Fig. 6b**), as well as a negative correlation between PC2 in Env and the growth rates (Spearman's R= −0.53). These findings align with the previous observation that the promoter activities of many growth-associated genes (ribosomes and translation) coordinate with growth rate[19]. We note that the PC3 in the Env dataset weakly but positively correlated with the growth rate (Spearman's R= −0.32, p=0.013), even though the PC3 exhibited an enrichment of some GO terms of the growth family in the negative direction. This exception suggests that not all functional categories in the growth family have a robust positive correlation with growth rate. Contrary to the growth-coupled directionality of the transcriptome profiles in the Env dataset, none of the major directions (PC1–3) in the Mut dataset showed significant correlation with growth rates (**Supplementary Fig. 8b**). This result suggests that many mutations can decrease growth fitness, independent of the transcriptome profiles induced by the mutations, even though mutational effects are shared by environmental perturbations. These results imply that the major directionality of transcriptional variations may result from adaptation to environmental perturbations (acquisition of adaptive plasticity) rather than mutations. That is, most of the observed bias in transcriptional variability against genetic perturbations may be a byproduct of adaptive evolution in response to environmental perturbations. We noted that the growth rate of the natural isolates was not available in the Evo dataset because of a lack of growth rate[20].

Overall, the magnitude and major directions of transcriptional variation were closely associated with

~~functional categories relevant to growth and survival in bacterial lifestyles across diverse environments. This supports the notion that non-uniform variability was acquired through adaptive evolution to enhance fitness in the frequently experienced environments.~~

Comment 2.17

Similar arguments could be made for the other supplementary discussions. The conclusions that the authors draw from the results presented in these supplements (which, again, are difficult to evaluate for the reader) are perhaps too far-reaching. One suggestion is to keep the analyses and results as supplementary information but to avoid drawing conclusions about past selection and adaptation from these data. Again, the data is arguably not very good for this (e.g., comparing a single strain for environmental perturbations with an artificial mutator strain would arguably not be the choice if one wanted to the test the idea that GRNs evolve to 'generalize' as in the model of Kouvaris et al). Thus, while e.g. past selection for plasticity may explain the shared variability in GRNs (and the mechanistic basis for this), the experiments and data needed to actually demonstrate this to be the case are quite demanding.

Response:

Thank you again for pointing out our speculative discussions. We agree that it is difficult to draw any conclusions about past evolution from our research methods, as the reviewer mentioned. Following the reviewer's suggestions above (**Comments 2.15** and **2.16**), we have removed Supplementary Discussions 1 and 3, as detailed in our responses to **Comments 2.15** and **2.16**. Additionally, based on the reviewer's suggestion provided in this comment, we have removed the conclusive sentences from Supplementary Discussion 2. The revised section has now been renamed as **Supplementary Note**.

Revision (Supplementary Note, relabeled and modified):

Supplementary ~~Discussion 2~~Note~~.~~ ~~:~~ Detailed descriptions of the five additional datasets~~Transcriptional variability reflects generalized responses against different types of perturbations~~

~~In the main text, we summarize the five additional datasets: Strs_hp, Strs_he, Strs, Prtn, and Noise_ch. In this section, we discuss the implications of each dataset in detail.~~

~~2.~~1. The Prtn dataset~~Transcriptional variability explains protein level variability~~

In the main text, w~~W~~e investigated whether transcriptional variability can extend~~ed~~ to the protein level. For this purpose, we analyzed the proteomic variability of *E. coli* in response to various environmental conditions[3] **(Supplementary Fig. 15)**. This dataset, referred to as the Prtn dataset, contains several environmental conditions similar to those used in the Env dataset, which was suitable for our purpose. Using this dataset, we ~~allowed us to~~ calculated the distance of the standard deviation to a smoothed running median of the standard deviations of the $\log_2$-transformed protein abundance per cell across diverse environmental conditions. The resulting distance, DM_{prtn}, serves as a measure of proteomic

variability in response to different environmental conditions (**Supplementary Fig. 15c,d**). Comparing DM values across different datasets, we observed that genes exhibiting higher DM values at the mRNA level tended to have higher DM_{prtn} (Spearman's R = 0.27–0.43, **Supplementary Fig. 15e**)~~, indicating a likely persistence of transcriptional variability at the protein level (**Fig. 7b**)~~. We also investigated whether the major axes of transcriptional variation were retained at the protein level. To this end, we ~~Employing~~ performed PCA to identify the top three principal components (PC1–3) in the Prtn dataset (**Supplementary Fig. 16a**). The results of correlation analysis for the top three PCs between the Prtn and the three main datasets were provided in the main text (**Fig. 7c**). ~~, w~~We observed that PC1 of the Prtn dataset was correlated with PC1 or PC2 in the Env, Evo, and Mut datasets~~., supporting the similarity in the major directions between these datasets (**Fig. 7c**). Thus, the magnitude and directions of transcriptional variations appear to persist at the protein level. These findings suggest that transcriptional variability influences variability in phenotypes that are governed by protein enzymes.~~ We also confirmed that PC1 in the Prtn dataset correlated with the growth rates at which the proteome profiles were obtained (Spearman's R=–0.96, **Supplementary Fig. ~~14~~16b**), which was consistent with the results from the Env dataset (**Supplementary Fig. 6b**). Over all, the Prtn dataset provided protein-level variability in response to different environmental perturbations~~, supporting the coupling of the major direction of environmentally induced phenotypic variation with growth fitness~~.

2.~~2. Cross-validation to assess generality of transcriptional~~ The Strs_hp dataset~~variability to an independent transcriptome dataset under different environmental perturbations~~

In the main text, ~~Heterogeneous phenotypic variability may result from the generalization of past environments[5, 6]. w~~We investigated whether the magnitude and major directions of transcriptional variation in the ~~Env~~ three main dataset correlated with those in different ~~scenarios~~environmental perturbations. ~~First~~For this purpose, we ~~explored the~~analyzed transcriptional ~~variability~~ profiles in response to environmental conditions that were not ~~covered~~ examined by the Env dataset. ~~To test this hypothesis, w~~We used a compendium of transcriptional profiles obtained from a single strain of *E. coli*, MDS42, cultured under different stressors[4]. We removed the transcriptional profiles obtained under the same stressors as those in the Env dataset. The obtained dataset was called Strs_hp (**Supplementary Fig. 17**). Following the same procedure as for DM_{env}, we calculated DM_{strs_hp} as a measure of transcriptional variability in response to different environmental perturbations caused by stressors (**Supplementary Fig. 17e,f**). We observed strong positive correlations between DM_{strs_hp} and DM_{env} (Spearman's R=0.62, **Fig. 7b**, **Supplementary Fig. ~~16a~~18a**) In addition, DM_{strs_hp} positively correlated with DM_{evo} and DM_{mut} (Spearman's R=0.36, 0.43, **Fig. 7b**). ~~These results further support the presence of generalized transcriptional responses against various perturbations. Next, w~~We also performed PCA to identify the major axes of transcriptional variation in Strs_hp (**Supplementary Fig. ~~17a~~19a**). The results of correlation analysis for the top three PCs between different datasets were provided in the main text (**Fig. 7c**). Compared to PC1–3 in the other datasets, we found that PC1 in the Strs_hp dataset correlated most strongly with PC1 in the Env dataset ~~(**Fig. 7e**)~~. Thus, the Strs_hp dataset provided transcriptional variability in response to different environmental changes that were

absent in the Env dataset~~, indicating that the magnitude and major direction of the transcriptional differences in the Env dataset were likely to reflect the generalized responses to different environmental changes~~.

~~2.~~3. The Strs_he and Strs dataset~~Transcriptional variability can influence transcriptional diversity accompanied by adaptive evolution~~

In the main text, w~~W~~e also investigated whether the magnitude and major directions in the ~~Env~~ three main datasets were shared by transcriptional diversification accompanying adaptive evolution. Unlike neutral evolution driven by genetic drift, mutations accumulated in adaptive evolution constitute only a fraction of the mutations that are likely to be advantageous to an organism's fitness, depending on selective conditions. Accordingly, these biased mutations can affect the magnitude and direction of transcriptional diversification during adaptive evolution, which may violate the ~~applicability of generalized responses~~variation derived from the transcriptional variability identified in the main three datasets. To test this, we analyzed two datasets, Strs_he and Strs, comprising the transcriptome profiles of different mutants of *E. coli* isolated during adaptive laboratory evolution toward resistance to different stressors. The Strs_he dataset consisted of the transcriptome profiles of resistant mutants isolated from eight different stressful conditions[4] **(Supplementary Fig. 17)**. The Strs dataset consisted of resistant mutants under 47 stress conditions[5] **(Supplementary Fig. 20)**. The culture conditions under which the transcriptome profiles were obtained were stressor-free and identical between the mutants and the two datasets. Importantly, the transcriptional profiles derived from the mutants obtained under the same stress conditions as those in the Env dataset were removed. Following the same procedure as that for DM_{mut}, we calculated DM_{strs_he} and DM_{strs} and used them as measures of transcriptional diversification through adaptive evolution. We found DM_{env} strongly and positively correlated with DM_{strs_he} and DM_{strs} (Spearman's R=0.56, 0.68; **Fig. 7b**, **Supplementary Figs. ~~16b~~18b, ~~18f~~20f**). Additionally, DM_{strs_he} and DM_{strs} were moderately positively correlated with DM_{evo} and DM_{mut}. ~~These results support the idea that the generalized responses affected not only transcriptional diversification through neutral evolution (DM_{mut}), but also through adaptive evolution with strong positive selection. Next, w~~We also performed PCA to identify the major axes of transcriptional variation in the two datasets (**Supplementary Figs. ~~17b~~19b**, **~~19~~21**). Compared with PC1–3 in the other datasets, we found that PC1s in Strs_he and Strs correlated the most with PC1 in the Env dataset as explained in the main text (Spearman's R=0.48, 0.61; **Fig. 7c**). Overall, these two datasets provided transcriptional variation caused by genomic mutations accumulated through adaptive evolution~~These results indicate that the magnitude and major direction of transcriptional variation in the Env dataset tend to be preserved across broad evolutionary contexts, including de novo adaptive evolution. This supports the notion that the transcriptional variability in the Env dataset is similar to the transcriptional diversification generated through complex evolution in nature (the Evo dataset)~~.

~~2.~~4. The Noise_ch dataset~~Transcriptional variability couples with canalization/decanalization in molecular noise~~

Several studies have suggested that evolution tends to produce global canalization that buffers phenotypes against genetic and environmental perturbations[7]. Additionally, global canalization can buffer phenotypes against perturbations caused by stochastic protein noise[8, 9]. In the main text, ~~We~~ we investigated this in *E. coli* using a dataset, termed the Noise_ch dataset, comprising cell-to-cell variations in the protein expression levels of genes encoded by endogenous promoters and natural chromosomal positions[6] **(Supplementary Fig. 22)**. To compensate for the mean dependency of protein noise caused by intrinsic noise at lower expression levels[6], we followed a conventional method[10, 11] and calculated DM values as the vertical distance to a smoothed running median of $\log_{10}$-transformed protein noise, representing cell-to-cell variance divided by the square of the mean protein copy number per cell. We termed this relative protein noise DM_{noise_ch} a measure of variability against stochastic perturbations under identical environmental and isogenic conditions (**Supplementary Fig. 2~~20~~c,d**). We found positive correlations (0.21–0.32, Spearman's R) between DM_{noise_ch} and the other DM values in Env, Evo and Mut for highly expressed proteins (**Fig. 7b**, **Supplementary Fig. ~~20e~~22e**). In contrast, we observed only minimal or no significant positive correlations for lowly expressed proteins (**Supplementary Fig. ~~20f~~22f**), which is consistent with previous observations[12]. ~~It is well-known that protein noise is mainly composed of two sources: intrinsic noise dominating lowly expressed proteins and extrinsic noises dominating highly expressed proteins. Intrinsic noise is derived from the stochastic nature of biochemical process[26, 27], while extrinsic noise contains noise propagated from upstream transcriptional regulations[28]. Considering that transcriptional variabilities reflect transcriptional regulation, the positive correlations in highly expressed genes might suggest that a certain common mechanism of transcriptional regulation, such as sensitivity of downstream promoter activity to changes in the activity of upstream regulator[29], influence both variability at the protein expression level against perturbations from stochastic noise and transcriptional variability against environmental and genetic perturbations. This may be an alternative to the network structure discussed in the main text. Thus, our results support the existence of a common mechanism underlying this perturbation.~~

~~Our results support the positive correlation between protein noise and transcriptional plasticity in *E. coli*, as demonstrated by Singh[25].~~ In contrast to a previous study, we strictly distinguished transcriptional variability in response to environmental perturbations from that in response to genetic perturbations. In addition, the protein noise we utilized was independent of plasmid copy number variation and potential confounding effects arising from gene expression from the plasmids. Despite these stringent considerations relative to the previous study, our results consistently revealed robust positive correlations between protein noise and transcriptional plasticity.

Furthermore, our findings align with previous observations in yeast, where relative cell-to-cell variations in the protein expression levels of genes expressed from endogenous promoters and natural chromosomal positions correlated with transcriptional variability in response to genetic and environmental perturbations[11, 13]. Thus, the Noise_ch dataset provided protein-level variability in response to stochastic molecular noises.~~These findings suggest that global canalization may be~~

~~prevalent across different organisms.~~

Comment 2.18

In summary, the work presented here convincingly demonstrates alignment in gene expression between genetic and environmental perturbations, and reveals features of GRNs that may explain this relationship mechanistically. Those are very important advances. It is less convincing to use these data to argue for a link between past evolution and those features of GRNs. The study may therefore be better if some of the follow-on analyses and inferences in the supplementary discussions are revised.

Response:

Thank you very much for your high evaluation of our manuscript. We have addressed all of the comments as detailed above. Following the reviewer's suggestions, we have removed the sentences drawing conclusions about past evolution from the main text as well as from the supplementary discussions. We believe that the revised manuscript is now more balanced.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my requests, I am fine with the manuscript being published.

Reviewer #3 (Remarks to the Author):

I enjoyed reading the study by Tsuru et al, it asks an interesting and clearly posed question (asking whether variability in gene expression due to environmental and genetic perturbations would be correlated), and provides evidence for prior theoretical hypotheses about the connection of either to the structure of transcriptional regulation.

Having been added as a "substitute" reviewer only at the second stage, I benefited from reading the thoughtful comments of my colleagues from previous rounds. I believe they were addressed satisfactorily by the authors, and that the manuscript benefited from these changes. In this respect, there are only a couple residual minor issues that I spotted; I mention them at the very end. Given the substantial edits the manuscript underwent already, and seeing as it benefited the quality, my inclination is not to subject the authors to minor quibbles.

However, I do have one larger concern. The authors' interpretation is that high DM is evidence of high sensitivity to perturbations (genetic or environmental). But this could also be caused by any number of confounding factors. I don't see how this claim can possibly be made without a suitable negative control, and as far as I could tell, the authors don't have one. What am I missing?

Some genes show high variability. Others show low variability. The authors acknowledge that some of this could be coming from "uninteresting" sources, e.g. correlation with something else -- like the mean. Specifically for that ONE potential confounder, they correct for this effect by transforming from raw deviations to their "DM" metric, "the median deviation of std from the spline-averaged value of similar mean expression".

But one could come up with all sorts of other quantities to put on the x axis, which could also conceivably have an common effect on expression noise. For example, let's take a gene whose expression has a strong temporal pattern. Such a gene would presumably show an abnormally large variability in each of the assays, and would enhance the correlation (such as reported in Fig. 2a). However, this would have nothing to do with the claimed interpretation.

Thus, the authors' solution (acknowledging possible effect of mean and thus looking at DM instead of raw std) is in my mind insufficient - the issue is broader, and the solution must be broader as well.

The GO analysis provides circumstantial evidence of varying strength, and my intuition is that the effects reported are likely real, which is why I read it with interest. But the fact remains that without a negative control, the authors' interpretation is speculative. A reader who spots this potential issue at Fig. 1 + Fig. 2A would stop assimilating any other information until they see this concern addressed. Thus, for the manuscript to stand, this issue either needs to be resolved (a negative control added), or addressed directly (in lieu of discussion of possible effects of specifically the mean, as if it were the only possible concern to worry about).

So, I believe one of the following should happen.

• Either the authors can add a suitable negative control. This is definitely the preferred option, and would eliminate all concerns, making this a strong manuscript. Are there any results on e.g. sampling same cells multiple times in same conditions? It seems to me like the variation observed in such an experiment is what one would want to normalize by...

• Alternatively, if adding a negative control is logistically impossible, there needs to be a very explicit paragraph VERY early on (in the discussion of Fig. 1) which tackles this head-on -- acknowledges that the study is effectively performed without a negative control, but the authors believe this is ok because of specific arguments X, Y and Z. Or because they offer an alternative path to validation, etc. In my mind, the strength of the work would rather depend on the strength of this argument...

• Or perhaps I'm missing something? If so, the text would benefit from a clarification for readers who might be similarly confused.

And as promised, the two minor residual issues left over from addressing previous comments:

1. The edit from "Most importantly, we found that the structure of transcriptional regulation accounts for these common biases." into "Most importantly, we found that transcriptional regulators accounts for these common biases."

-->

I'm not sure this addressed the reviewer's question. To the extent that the first phrasing was vague, the rephrasing didn't much help. What does it mean that a transcriptional regulator accounts for bias? Could this statement be made sharper and ideally quantitative? (E.g., a specified fraction of variance in XX is explained by YY)? For a summary statement summarizing what the paper showed, this is a little too "fluffy". And at the same time, the categorical-sounding "accounts for" sounds stronger than what was shown.

1. Lines 106-111 - these lines were added in revisoin, and the logic of how it fits into the paragraph is very unclear. "For instance, genes involved in stress or amino acid metabolism tend to have higher transcriptional variability, while genes related to cell growth, maintenance, and cell cycles do not exhibit higher transcriptional variability against genetic perturbations". This is confusing to me because "not exhibiting higher variability" appears to be cited as being good evidence of something, or an example of something, but this sounds like a null result? Or was it shown that they exhibit unexpectedly low variability?

Reviewer #3 (Remarks on code availability):

Did not review the code.

Point-by-point responses

Responses to Reviewer 1:

Comment 1.1

The authors have addressed my requests, I am fine with the manuscript being published.

Response:

We would like to thank the reviewer for being satisfied with our revised manuscript. The time and effort invested in reviewing our work have significantly contributed to its improvement.

Responses to Reviewer 3:

General comments:

Comment 3.1

I enjoyed reading the study by Tsuru et al, it asks an interesting and clearly posed question (asking whether variability in gene expression due to environmental and genetic perturbations would be correlated), and provides evidence for prior theoretical hypotheses about the connection of either to the structure of transcriptional regulation.

Response:

We would like to thank the reviewer for highly evaluating our manuscript. The time and effort invested in reviewing our work have significantly contributed to its improvement. We have addressed all the comments as detailed below.

Comment 3.2

Having been added as a "substitute" reviewer only at the second stage, I benefited from reading the thoughtful comments of my colleagues from previous rounds. I believe they were addressed satisfactorily by the authors, and that the manuscript benefited from these changes. In this respect, there are only a couple residual minor issues that I spotted; I mention them at the very end. Given the substantial edits the manuscript underwent

already, and seeing as it benefited the quality, my inclination is not to subject the authors to minor quibbles.

Response:
Thank you very much for reviewing our responses to the reviewers in the previous round. We really appreciate your high evaluation of our previous revisions.

Major comments:

Comment 3.3

However, I do have one larger concern. The authors' interpretation is that high DM is evidence of high sensitivity to perturbations (genetic or environmental). But this could also be caused by any number of confounding factors. I don't see how this claim can possibly be made without a suitable negative control, and as far as I could tell, the authors don't have one. What am I missing?
Some genes show high variability. Others show low variability. The authors acknowledge that some of this could be coming from "uninteresting" sources, e.g. correlation with something else -- like the mean. Specifically for that ONE potential confounder, they correct for this effect by transforming from raw deviations to their "DM" metric, "the median deviation of std from the spline-averaged value of similar mean expression".
But one could come up with all sorts of other quantities to put on the x axis, which could also conceivably have an common effect on expression noise. For example, let's take a gene whose expression has a strong temporal pattern. Such a gene would presumably show an abnormally large variability in each of the assays, and would enhance the correlation (such as reported in Fig. 2a). However, this would have nothing to do with the claimed interpretation.
Thus, the authors' solution (acknowledging possible effect of mean and thus looking at DM instead of raw std) is in my mind insufficient - the issue is broader, and the solution must be broader as well.
The GO analysis provides circumstantial evidence of varying strength, and my intuition is that the effects reported are likely real, which is why I read it with interest. But the fact remains that without a negative control, the authors' interpretation is speculative. A reader who spots this potential issue at Fig. 1 + Fig. 2A would stop assimilating any other information until they see this concern addressed. Thus, for the manuscript to stand, this issue either needs to be resolved (a negative control added), or addressed directly (in lieu of discussion of possible effects of specifically the mean, as if it were the only possible

concern to worry about).
So, I believe one of the following should happen.
• Either the authors can add a suitable negative control. This is definitely the preferred option, and would eliminate all concerns, making this a strong manuscript. Are there any results on e.g. sampling same cells multiple times in same conditions? It seems to me like the variation observed in such an experiment is what one would want to normalize by...
• Alternatively, if adding a negative control is logistically impossible, there needs to be a very explicit paragraph VERY early on (in the discussion of Fig. 1) which tackles this head-on -- acknowledges that the study is effectively performed without a negative control, but the authors believe this is ok because of specific arguments X, Y and Z. Or because they offer an alternative path to validation, etc. In my mind, the strength of the work would rather depend on the strength of this argument…
• Or perhaps I'm missing something? If so, the text would benefit from a clarification for readers who might be similarly confused.

Response:
Thank you very much for highlighting these important points. To address the reviewer's concerns, we conducted the following analyses.

As the reviewer noted, some *E. coli* genes exhibit fluctuations in expression that follow specific temporal patterns. Unlike animals and plants, *E. coli* lacks developmental stages, and its temporal fluctuations in gene expression are associated with the cell cycle. Recently, Pountain et al. (2024, *Nature*) performed single-cell transcriptomics for *E. coli* and identified cell-cycle-associated temporal fluctuations in gene expression levels, primarily driven by replication-dependent changes in gene dosage. In the transcriptomic analysis used in our current study, however, the bacterial cultures were unsynchronized, containing cells at various stages of the cell cycle (Pountain et al. *Nature* 2024). Therefore, the expression levels represent the population averages of over 10^6 cells for each transcriptional profile. As a result, the temporal fluctuations are expected to be averaged out, making it unlikely that they would confound the DM values. To verify this, we examined the relationship between the known temporal variability in mRNA expression levels, as reported in the previous study, and the DM values. Pountain et al. measured cell cycle-dependent changes in expression levels and quantified the amplitude of these fluctuations as the fold change from trough to peak in mRNA levels throughout the cell cycle. Larger amplitudes represent higher temporal variability in mRNA expression without environmental or genetic perturbations. We confirmed that there was no positive correlation between the DM values and the amplitude of cell cycle expression

(**Supplementary Fig. 2a, newly added**). This result is consistent with the expectation that our transcriptomic profiles reflect population averages from unsynchronized *E. coli* populations. Thus, the DM values do not primarily reflect cell-cycle-associated temporal fluctuations.

In principle, replication-dependent changes in gene dosage could still interfere with the DM values in another way, even for unsynchronized cultures. At higher growth rates, overlapping cycles of replication occur, leading to higher copy numbers of genes located near the origin of replication (*oriC*). At slower growth rates, replication overlap does not occur, making gene dosage near *oriC* more sensitive to growth rate changes. This could potentially enhance transcriptional variability of genes located near *oriC* across different environmental or genetic conditions. To test whether this effect influences the DM values, we explored the relationship between DM values and the genomic distance from *oriC* (**Supplementary Fig. 2b, newly added**). We found no significant negative correlation, suggesting that replication-dependent gene dosage effects did not significantly confound the DM values.

In addition to using unsynchronized cultures, we were mindful of the growth phase when performing transcriptomics for the Mut and Evo datasets. A previous study (Caglar et al., 2017, *Scientific Reports*) confirmed that transcriptome profiles obtained at different time points during the exponential growth phase are highly similar compared to those obtained under different environmental conditions or growth phases. Therefore, transcriptomics of *E. coli* is generally conducted during the exponential growth phases to minimize the effects of temporal fluctuations in gene expression levels. Following this conventional method, we performed transcriptomics on exponentially growing cells in the same environmental conditions for the Mut and Evo datasets. In contrast, the Env dataset inherently included different growth conditions, and sampling times varied with environmental conditions, meaning some samples were taken outside of the exponential growth phase. To assess the impact of these samples on DM_{env}, we constructed a refined dataset based on information from the original papers in the PRECISE database, termed Envexp, which included only transcriptome profiles from the exponential growth phase. Using this dataset (78 profiles, 37 unique environmental conditions), we recalculated DM values, termed DM_{envexp}, and confirmed a high correlation between DM_{env} and DM_{envexp} (**Supplementary Fig. 3, newly added**). We also found that DM_{envexp} positively correlated with DM_{evo} and DM_{mut}, with correlation levels comparable to those seen with DM_{env} (Fig. 2a). These results indicate that samples taken outside of the exponential growth phase did not significantly interfere with DM_{env}.

Overall, our findings suggest that DM values primarily reflect transcriptional

changes in response to environmental or genetic perturbations, rather than temporal fluctuations unrelated to these perturbations. Unfortunately, both RNA sequencing and microarray techniques used in this study are invasive methods of measuring gene expression, making it impossible to sample the same cells multiple times under the same conditions. In line with the reviewer's second advice, we have incorporated these analyses and explanations regarding the interpretation of DM values into the revised manuscript.

Revision (L 106–153, modified):

The DM values of each gene in the dataset was designated as DM_{env}, DM_{evo}, DM_{mut} for the Env, Evo, and Mut datasets, respectively (**Fig. 1f**, **Supplementary Table 4**).

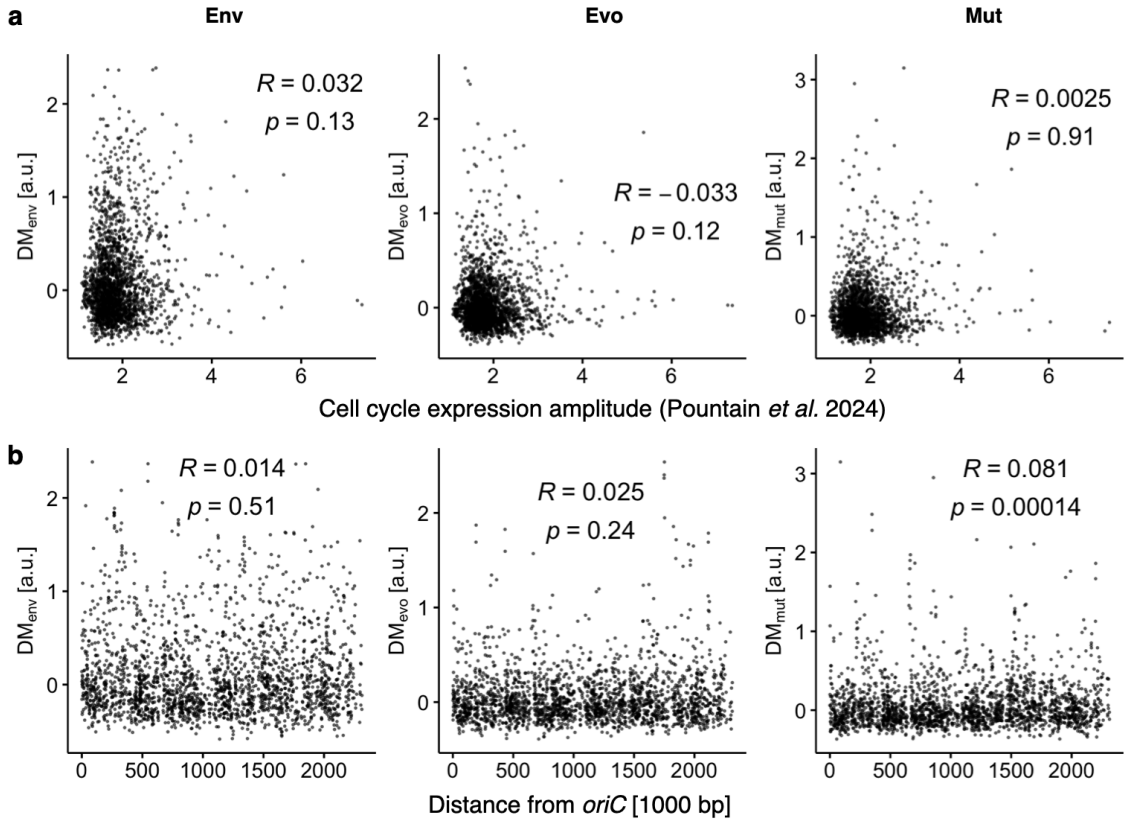
Since each transcriptome profile for all datasets was obtained from millions of cells in unsynchronized cultures, gene expression levels are expected to be independent of the well-known temporal fluctuations in expression associated with the cell cycle. Unlike animals and plants, *E. coli* lacks developmental stages, and its temporal fluctuations in gene expression are primarily linked to replication-dependent changes in gene dosage [33]. To verify that cell-cycle fluctuations did not confound our results, we examined the relationship between known temporal variability in mRNA expression and DM values. Pountain et al. performed single-cell RNA sequencing on the *E. coli* K12 MG1655 strain and quantified cell-cycle-dependent changes in gene expression. The amplitude of these fluctuations was measured as the fold change from trough to peak in mRNA levels within a cell cycle. Larger amplitudes represent higher temporal variability in mRNA expression without environmental or genetic perturbations. We confirmed that there was no positive correlation between DM values and the amplitude of cell cycle expression (**Supplementary Fig. 2a**). This is consistent with the expectation that our transcriptomic profiles reflect the population averages of unsynchronized *E. coli* cultures, and thus DM values did not reflect cell-cycle-associated temporal fluctuations.

In principle, replication-dependent changes in gene dosage could still influence DM values, even in unsynchronized cultures. At higher growth rates, overlapping cycles of replication occur, leading to higher copy numbers of genes located near the origin of replication (*oriC*). In slower growth conditions, this overlap does not occur, making gene dosage near *oriC* more sensitive to growth rate changes. This could potentially increase the transcriptional variability of genes near *oriC* across different environmental or genetic conditions. To test this, we explored the relationship between DM values and the genomic distance from *oriC* (**Supplementary Fig. 2b**). We found no significant negative

correlation, suggesting that replication-dependent gene dosage effects did not significantly confound the DM values.

In addition to using unsynchronized cultures, we also accounted for the growth phase of the cultures in the Mut and Evo datasets. A previous study confirmed that transcriptome profiles obtained at different time points during the exponential growth phase under identical conditions are highly similar, especially when compared to profiles obtained under different environmental conditions or during other growth phases [34]. To minimize the risk of unknown temporal fluctuations in expression levels in the absence of environmental changes, we performed transcriptomics on populations of exponentially growing cells under identical conditions for both the Mut and Evo datasets. Ideally, sampling the same cells multiple times under the same conditions could further validate these assumptions. However, both RNA sequencing and the microarray techniques used in this study are invasive methods for measuring gene expression, making this approach impractical. Unlike the other datasets, the Env dataset involved different growth conditions, and sampling times varied depending on the environment, meaning that some samples were taken outside the exponential growth phase. To assess the impact of these profiles on DM_{env}, we extracted the transcriptome profiles from the exponential growth phase within the Env dataset to construct a refined dataset, termed Envexp, based on information from the original papers in the PRECISE database. Recalculating DM values (DM_{envexp}) from this dataset (78 profiles, 37 unique environmental conditions, **Supplementary table 1**), we observed a high correlation between DM_{env} and DM_{envexp} (**Supplementary Fig. 3**). Additionally, we confirmed that DM_{envexp} positively correlated with DM_{evo} and DM_{mut}, with correlation levels nearly equivalent to those of DM_{env} (**Fig. 2a**). These results indicate that samples taken outside the exponential growth phase did not significantly affect DM_{env}. Thus, we conclude that DM_{env} primarily reflects transcriptional changes in response to environmental perturbations, rather than temporal changes independent of environmental conditions. Overall, DM values are reasonably assumed to reflect transcriptional changes in response to environmental or genetic perturbations, rather than temporal fluctuations without such perturbations.

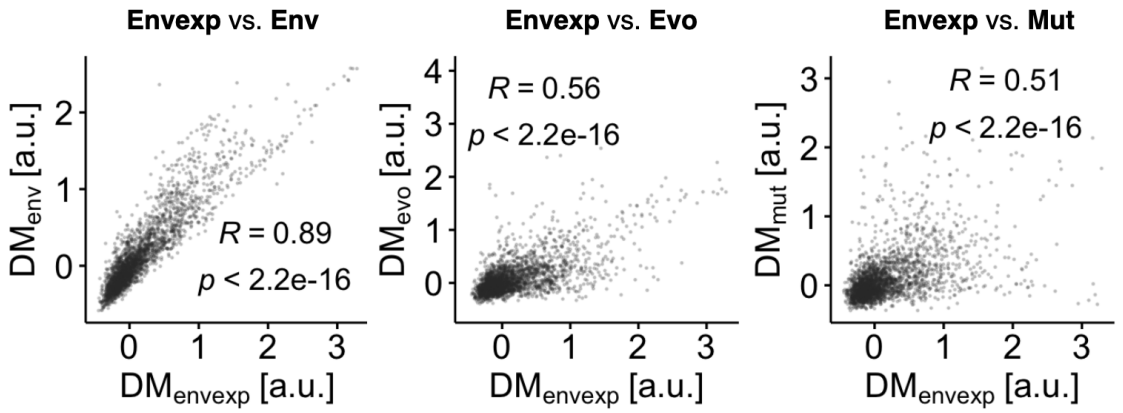
Revision (Supplementary Fig. 2, newly added):



Supplementary Fig. 2: Relationship between DM values and cell-cycle-associated transcriptional changes.

a, The relationship between DM values and cell cycle expression amplitude. The cell cycle expression amplitude was obtained from Pountain *et al* and is defined as the fold change from trough expression to peak expression in the temporal dynamics of mRNA expression levels within the cell cycle. **b**, The relationship between DM values and the shortest distance from *oriC* on the chromosome of *E. coli* K12 MG1655. The insets represent Spearman's R and p-values.

Revision (Supplementary Fig. 3, newly added):



Supplementary Fig. 3: Relationship between DM values based on exponential growth phase samples.

Transcriptome profiles obtained during the exponential growth phase were extracted from the original Env dataset to construct the Envexp dataset based on information from the original papers in the PRECISE database. The DM values of genes were calculated from this filtered dataset (78 profiles, 37 unique environmental conditions) and termed DM_{envexp}. The relationship between DM_{env}, DM_{evo}, DM_{mut} and DM_{envexp} is shown. The insets represent Spearman's R and p-values. DM_{evo} and DM_{mut} were originally derived from exponential growth phase samples.

Minor comments:

Comment 3.4

And as promised, the two minor residual issues left over from addressing previous comments:

1. The edit from "Most importantly, we found that the structure of transcriptional regulation accounts for these common biases." into "Most importantly, we found that transcriptional regulators accounts for these common biases."

I'm not sure this addressed the reviewer's question. To the extent that the first phrasing was vague, the rephrasing didn't much help. What does it mean that a transcriptional regulator accounts for bias? Could this statement be made sharper and ideally quantitative? (E.g., a specified fraction of variance in XX is explained by YY)? For a summary statement summarizing what the paper showed, this is a little too "fluffy". And at the same time, the categorical-sounding "accounts for" sounds stronger than what was shown.

Response:

Thank you very much for pointing out our unclear explanations. To improve the logical flow with the preceding and following sentences, the corresponding sentence have been revised by adding more clearer explanations about our findings.

Revision (L 65–74, added):

In addition, the major directions, or the top principal components, of correlated variation among thousands of genes were shared by different perturbations, demonstrating a common bias in the directionality of transcriptional changes. We identified 13 global transcriptional regulators that underlie this shared transcriptional variability across various perturbations. Genes regulated by these key regulators tended to exhibit greater transcriptional variability compared to those regulated by other transcriptional regulators. Moreover, these global regulators orchestrated coordinated transcriptional changes in their target genes, contributing to the predominant directionality of transcriptomic shifts observed across different perturbations.~~Most importantly, we found that the transcriptional regulators account for these common biases.~~ These empirical findings support the relevance of the gene regulatory networks in explaining the bias in phenotypic variability shared across different perturbations.

Comment 3.5

1. Lines 106-111 - these lines were added in revisoin, and the logic of how it fits into the paragraph is very unclear. "For instance, genes involved in stress or amino acid metabolism tend to have higher transcriptional variability, while genes related to cell growth, maintenance, and cell cycles do not exhibit higher transcriptional variability against genetic perturbations". This is confusing to me because "not exhibiting higher variability" appears to be cited as being good evidence of something, or an example of something, but this sounds like a null result? Or was it shown that they exhibit unexpectedly low variability?

Response:

We are sorry for our confusing expression about the previous study (Landry and Hartl, *Science*, 2007). The study actually performed an overrepresentation test to show that genes related to cell growth, maintenance, and the cell cycle tend to exhibit lower transcriptional variability in response to genetic perturbations. The corresponding sentence has now been revised.

Revision (L 160–163, modified):

For instance, genes involved in stress or amino acid metabolism tend to have higher transcriptional variability [24], while genes related to cell growth, maintenance, and cell cycles tend to have lower transcriptional variability against genetic perturbations, compared to random expectations~~do not exhibit higher transcriptional variability against genetic perturbations~~ [18].

REVIEWERS' COMMENTS

Reviewer #3 (Remarks to the Author):

Thank you for addressing my comments. Hopefully this extended discussion of my initial concern will help the readers.