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Pleiotropic effects of *trans*-regulatory mutations on fitness and gene expression

Pétra Vande Zande[1], **Mark S. Hill**[2], **Patricia J. Wittkopp**[1,2,*]

[1]Department of Molecular, Cellular, and Developmental Biology, University of Michigan; Ann Arbor, MI, USA.

[2]Department of Ecology and Evolutionary Biology, University of Michigan; Ann Arbor, MI, USA.

Abstract

Variation in gene expression arises from *cis*- and *trans*-regulatory mutations, which contribute differentially to expression divergence. Here, we compare the impacts on gene expression and fitness for *cis*- and *trans*-regulatory mutations in *Saccharomyces cerevisiae*, with a focus on the *TDH3* gene. We use the effects of *cis*-regulatory mutations to infer effects of *trans*-regulatory mutations attributable to impacts beyond the focal gene, revealing a distribution of pleiotropic effects. *Cis*- and *trans*-regulatory mutations had different effects on gene expression, with pleiotropic effects of *trans*-regulatory mutants impacting expression of genes both in parallel to and downstream of the focal gene. The more widespread and deleterious effects of *trans*-regulatory mutations we observed are consistent with their decreasing relative contribution to expression differences over evolutionary time.

One-Sentence Summary:

This study quantifies the pleiotropic impacts of *trans*-regulatory mutations on fitness and gene expression relative to a focal gene.

Heritable variation in gene expression is widespread within and between species and often contributes to phenotypic diversity (1). This variation arises from mutations that alter activity of the regulatory networks that control gene expression. Each regulatory mutation can act in *cis* or in *trans* with respect to a specific gene. *cis*-regulatory mutations tend to be located close to the focal gene and often impact non-coding sequences that regulate

*Corresponding author wittkopp@umich.edu.

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Data and materials availability: RNA-seq data is available at GEO accession GSE175398. Code used for data analysis, as well as supporting data files, are available from GitHub at https://github.com/pvz22/Trans-reg_pleiotropy with the version of record at time of publication available at Zenodo under DOI 10.5281/zenodo.6567260 (27). All other data is included with the manuscript or supplementary material. Mutant strains of *S. cerevisiae* available upon request.

the focal gene's expression, such as promoters or enhancers. *trans*-regulatory mutations can be located anywhere in the genome and can impact either coding or non-coding sequences of genes that influence the focal gene's expression through activity of a diffusible molecule such as a protein or RNA. Prior work has shown that *trans*-regulatory variants appear to be the primary source of mRNA expression differences within a species but the relative contribution of *cis*-regulatory variants often increases with evolutionary time (2-6). Understanding how and why these classes of regulatory mutations contribute differently to variation in gene expression is important for understanding how gene expression evolves.

Differences in the way *cis*- and *trans*-regulatory mutations affect gene expression might contribute to a preferential fixation of *cis*-regulatory variants relative to *trans* (6-9). A *cis*-regulatory mutation alters expression of a focal gene, which can in turn have effects on expression of downstream genes (orange box in Figure 1). By contrast, a *trans*-regulatory mutation affecting expression of the same focal gene might have effects comparable to the *cis*-regulatory mutation plus independent effects on expression of other genes, each with its own downstream consequences (blue box in Figure 1). *Trans*-regulatory mutations are thus predicted to have more wide-spread effects on gene expression than mutations altering expression of the focal gene in *cis.* Consequently, *trans*-regulatory mutations might be fixed less often than *cis*-regulatory mutations because mutations that are more pleiotropic (i.e., affect more traits) are predicted to be more deleterious (10). This hypothesis has been difficult to test directly, however, because it is notoriously difficult to disentangle the effects of a pleiotropic mutation on one trait from its effects on others (11-13).

Here, we examine the pleiotropic effects of *trans*-regulatory mutations by using *cis*-regulatory mutations to separate the effects of a *trans*-regulatory mutation caused by its impact on a focal gene from its effects caused by impacts on other genes. We separate these mutational effects for fitness and gene expression by measuring relative growth rate and expression profiles (using RNA-seq) for strains of *S. cerevisiae* carrying either a *cis*- or *trans*-regulatory mutation affecting expression of the focal gene. We focus on mutations affecting expression of the *TDH3* gene in the baker's yeast *Saccharomyces cerevisiae,* which encodes a glyceraldehyde-3-phosphate dehydrogenase (GAPDH), because prior studies have systematically identified and isolated individual *cis*- and *trans*-regulatory mutations that affect its expression (14-16). To the best of our knowledge, comparable data are not available for other genes in *S. cerevisiae* or other eukaryotic species.

We analyzed the effects of 5 *cis*-regulatory mutants on fitness and gene expression that caused expression of *TDH3* to vary from 0% to ~135% of wild-type levels (Data S1). These mutants were selected to cover the range of effects observed previously for 348 mutant alleles of the 678 bp *TDH3* promoter (14, 17, 18). The five *cis*-regulatory mutants were genetically identical except that the 0% *TDH3* expression strain carried a deletion of the *TDH3* gene, the 20%, 50%, and 85% *TDH3* expression strains each carried a single point mutation in the *TDH3* promoter, and the 135% *TDH3* expression strain carried a duplication of the *TDH3* allele harboring a point mutation with a copy of URA3 separating the two copies of *TDH3* (Fig. S1). The impact of each of these mutations on gene expression was determined by comparing expression in these strains to expression in the progenitor strain lacking any mutations in *TDH3*, either without (for the 0, 20, 50, and 85% strains) or

with (for the 135% strain) the extra *URA3* gene (Fig. S1, see Methods). In parallel, we analyzed the effects of 35 *trans*-regulatory mutant strains carrying mutations that caused *TDH3* expression to vary from ~6% to ~130% of wild-type levels (Data S1). 26 of these 35 strain carried single mutations with a range of effects similar to those captured by the 5 *cis*-regulatory mutants as well as those observed for >1400 *trans*-regulatory mutations introduced randomly by ethyl methanesulfonate (EMS) (15). The remaining 9 strains each carried 1 to 6 mutations in either *RAP1* and *GCR1*, which directly regulate *TDH3* (16) (Fig. S1, Data S1).

To separate the fitness effects of *trans*-regulatory mutations attributable to changes in *TDH3* expression from the fitness effects attributable to the pleiotropic impacts of these mutations on other genes, we first defined the relationship between *TDH3* expression and fitness using only the *cis*-regulatory mutants. Relative fitness was estimated for each mutant based on measures of growth rate under the same conditions used to grow cells for expression profiling (see Methods). To predict the fitness effects of any change in *TDH3* expression between 0 and 135% of wild-type expression, we fit a local polynomial regression (LOESS) curve to these data. The continuous relationship between fitness and *TDH3* expression level observed (Fig. 2A) is consistent with prior work using competitive growth to estimate fitness of 43 *cis*-regulatory mutations in *TDH3* (17, 18).

Using these inferred effects of changes in *TDH3* expression on fitness, we estimated the pleiotropic fitness effects of each *trans*-regulatory mutant by comparing its measured fitness to the fitness predicted for a *cis*-regulatory mutant with the same change in *TDH3* expression (Fig. 2B). Excluding 2 flocculant *trans*-regulatory mutants for unreliable estimates of growth rate, 52% (17/33) of mutants had significant deleterious pleiotropic effects based on the LOESS regression curve falling above their 99% confidence intervals for fitness. Only 1 mutant had a significant beneficial pleiotropic effect; this mutant had a mutation in *IRA2*, which has also been found to harbor beneficial mutations in other studies (19-21). The remaining 15 *trans*-regulatory mutants (45%) showed fitness effects comparable to *cis*-regulatory mutants with similar impacts on *TDH3*. Overall, this empirical distribution of pleiotropic fitness effects was skewed toward deleterious effects (Fig. 2C). Mutations with the largest deleterious fitness effects tended to also cause the largest decreases in *TDH3* expression, although some mutations with large deleterious effects had little impact on *TDH3* expression (Fig. 2C, insert). An additional 1106 gene deletions that affected *TDH3* expression in *trans* showed a similar distribution of pleiotropic effects (Fig. S2, (22, 23))

To test the idea that *trans*-regulatory mutations are more likely to be deleterious because they tend to be more pleiotropic, we compared the number of genes considered significantly differentially expressed in the *cis*- and *trans*-regulatory mutants at a false discovery rate (FDR) of 10%. Overall, we found no statistically significant difference in the median number of differentially expressed genes between the 5 *cis*- and 35 *trans*-regulatory mutants (Fig. 2D, permutation test p-value: 0.14, Fig. S3A) but observed significantly more variable effects for the *trans*-regulatory mutants (permutation test p-value = 0.01, Fig. S3B). Although the small number of *cis*-regulatory mutants included limited the power of this test, the absence of a larger median effect of *trans*-regulatory mutants might be due to differences

in the severity of mutational effects between the sets of *cis*- and *trans*-regulatory mutants examined. To test this possibility, we examined the effects of *cis*- and *trans*-regulatory mutants on the number of differentially expressed genes while taking their impact on *TDH3* expression into account. We found that most *trans*-regulatory mutants showed more widespread effects on gene expression than *cis*-regulatory mutants with the same effect on *TDH3* expression (Fig. 2E).

As shown in Figure 1, *trans*-regulatory mutants are assumed to have effects on expression of genes downstream of the focal gene similar to *cis*-regulatory mutations but to also have additional pleiotropic effects on expression of other genes in parallel. To test this assumption, we sought to separately analyze the effects of *trans*-regulatory mutants on expression of genes downstream of and in parallel to *TDH3*. To identify the set of genes downstream of *TDH3*, we identified genes whose expression was significantly altered in the TDH3 null mutant. We found 140 such genes (10% FDR, p-value=0.002, Data S3), excluding *TDH3* itself. 49 (35%) of these genes were under-expressed in the null mutant relative to the wild-type strain, and 91 (65%) were over-expressed (Data S3). This gene set was significantly enriched for genes encoding proteins involved in glycolytic processes (Fig. S4), suggesting that many expression changes observed in the *TDH3* null mutant might be due to a homeostatic response of the cells to maintain metabolism in the absence of the TDH3p enzymatic activity involved in glycolysis and gluconeogenesis (24). These downstream genes were also enriched for genes associated with the gene ontology terms DNA biosynthesis, integration, and transposition (Fig. S4), which might result from non-metabolic functions of TDH3p in processes such as transcriptional silencing and rDNA recombination (25).

The median absolute $\log_2$ fold expression changes observed for this set of 140 genes downstream of *TDH3* decreased monotonically as *TDH3* expression approached wild type and was smallest in the *cis*-regulatory mutant overexpressing *TDH3* (Fig. 3A). 122 (87%) of these 140 genes showed a significant linear relationship with *TDH3* expression in the 5 *cis*-regulatory mutants (10% FDR, p-value = 0.09, Fig. S5A), with 44 positively correlated and 78 negatively correlated (Fig. 3B, Fig. S5B). For example, the *GPD2* gene, which encodes an enzyme two steps away from *TDH3* in the metabolic network, increased linearly when *TDH3* expression was decreased by *cis*-regulatory mutations (Fig. 3C).

A heatmap showing expression with hierarchical clustering for these 140 genes downstream of *TDH3* in the 40 *cis*- and *trans*-regulatory mutants also visually showed the correlation with *TDH3* expression in the *cis*-regulatory mutants (Fig. 3D, orange cluster). The *cis*-regulatory mutant overexpressing *TDH3* (135%TDH3) had opposing effects on expression of these genes that caused it to cluster separately, with expression most similar to two *trans*-regulatory mutants (bearing mutations in *NAR1* and *IRA2*) that also caused overexpression of *TDH3* (Fig. 3D, green cluster). The other 33 *trans*-regulatory mutants had more distinct patterns of expression for these genes (Fig. 3D), with some mutants (e.g., RAP1484 and IRA2) showing significant changes in expression for many genes despite minimal impacts on *TDH3* expression (Fig. 3E). These observations suggest that the pleiotropic effects of *trans*-regulatory mutants include impacts on expression of genes downstream of *TDH3* that are not explained by their impact on *TDH3* (Fig. 3F).

To further explore this possibility, we used the linear models fitted to the *cis*-regulatory mutant data to predict the change in expression expected for each downstream gene due to the impact of the *trans*-regulatory mutant on *TDH3* expression alone. Deviations from these expectations indicate pleiotropic effects of *trans*-regulatory mutants on the expression of genes downstream of *TDH3*. For example, 6 of the 35 *trans*-regulatory mutants showed evidence of a pleiotropic effect on expression of *GPD2*, as indicated by a change in *GPD2* expression further than one standard error outside of the 95% prediction interval for the expression change expected based on *cis*-regulatory mutants (Fig. 3G). Such pleiotropic effects were observed for at least one *trans*-regulatory mutant for 111 of the 122 genes downstream of *TDH3*, with the magnitude of the pleiotropic effects (measured as residuals from the gene-specific regression models based on the *cis*-regulatory mutants) varying among *trans*-regulatory mutants and genes (Fig. 3H).

These pleiotropic effects on expression of genes downstream of *TDH3* are in addition to the pleiotropic effects of *trans*-regulatory mutants in parallel to *TDH3*. A heatmap of expression with hierarchical clustering for the 5806 genes not classified as downstream of *TDH3* shows these other effects (Fig. 4A). 14 of the *trans*-regulatory mutants showed minimal effects on expression of these genes (purple cluster in Fig. 4A). The four hypomorphic *cis*-regulatory mutants clustered together (orange cluster in Fig. 4A) and showed additional effects that appeared to scale with *TDH3* expression level (consistent with Fig. S5), suggesting that there might be more genes downstream of *TDH3* than were called differentially expressed in the *TDH3* null mutant with the statistical thresholds used. The *trans*-regulatory mutants with larger effects tended to cluster according to related phenotype or function, such as the two flocculant strains (bearing mutations in *CYC8* and *SSN2)*, genes involved in adenine biosynthesis (*ADE4, ADE5,* and *ADE6),* and large-impact mutations in *GCR1*. Finally, the two *RAP1* mutants with the largest impacts on *TDH3* expression (RAP154 and RAP1238) showed the most different expression patterns from the other mutants and each other.

Comparing the impacts of *trans*-regulatory mutations on gene expression downstream of and in parallel to *TDH3* showed that mutants affecting expression of many genes in parallel to *TDH3* tended to also affect expression of many genes downstream of *TDH3* (Fig. 4B). This observation indicates that some *trans*-regulatory mutants had larger or smaller overall effects on the transcriptome. However, the impact of a mutation on *TDH3* expression was not a strong predictor for this overall effect: some mutants with minimal effects on *TDH3* expression altered expression of thousands of other genes, whereas other mutants changed *TDH3* expression up to 30% but altered expression of less than 100 other genes (Fig. 2E). The overall number of genes whose expression was affected by a *trans*-regulatory mutation was a strong predictor of the relative fitness of the mutant (Fig. 4C, consistent with (26)), although two *trans*-regulatory mutants (*IRA2* and *NAM7*) were notable exceptions (Fig. 4C). Directly comparing the pleiotropic effects of *trans*-regulatory mutations inferred for fitness and expression of genes in parallel to *TDH3* showed a very similar relationship (Fig. 4D) because the *cis*-regulatory mutants had much smaller effects on both fitness and expression of other genes than *trans*-regulatory mutants with similar effects on *TDH3* expression (Fig. 2B,E).

In summary, we developed a framework to decompose the effects of *trans*-regulatory mutations attributable to their impact on expression of a focal gene from their impact on other genes in the genome. These data support the widely held but rarely tested assumptions that *trans*-regulatory mutations tend to be more pleiotropic and more deleterious than *cis*-regulatory mutations, but also provide a more nuanced view of these differences at the level of specific mutations and show that the pleiotropic effects of *trans*-regulatory mutations might often affect expression of genes both in parallel to and downstream of the focal gene. Studies such as this one that empirically determine and compare the properties of different types of mutations affecting gene expression are critical for understanding how regulatory systems evolve.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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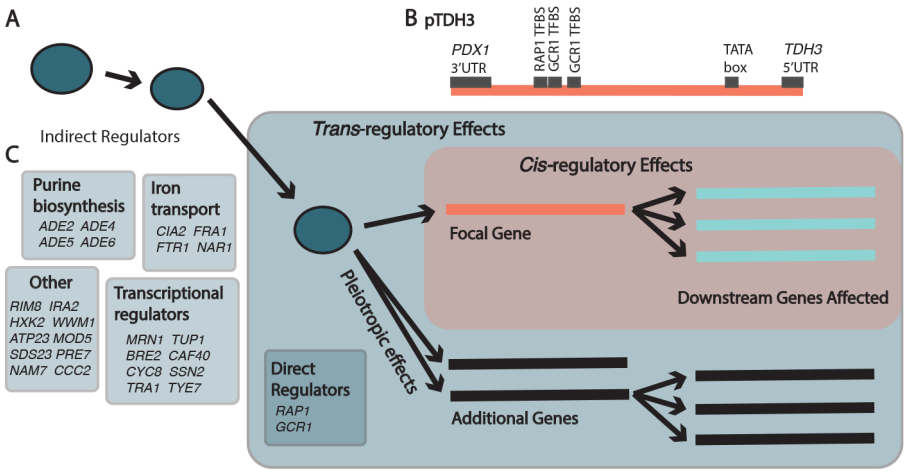

Fig. 1. *cis*- and *trans*-regulatory mutations have different effects on gene expression.

(**A**) *Trans*-regulatory mutations (blue), in either indirect or direct regulators, influence expression of a focal gene (orange), which in turn influences expression of downstream genes (light blue) either directly or indirectly, and can also impact additional genes (black). (**B**) Schematic shows the 678 bp *cis*-regulatory sequence (promoter) for the *S. cerevisiae TDH3* gene (*pTDH3*) used as a focal gene for this work (see Fig S1, Data S1). (**C**) Previously identified (16) *trans*-regulators of *TDH3* expression harboring mutations tested in this work (see Fig S1 for detail).

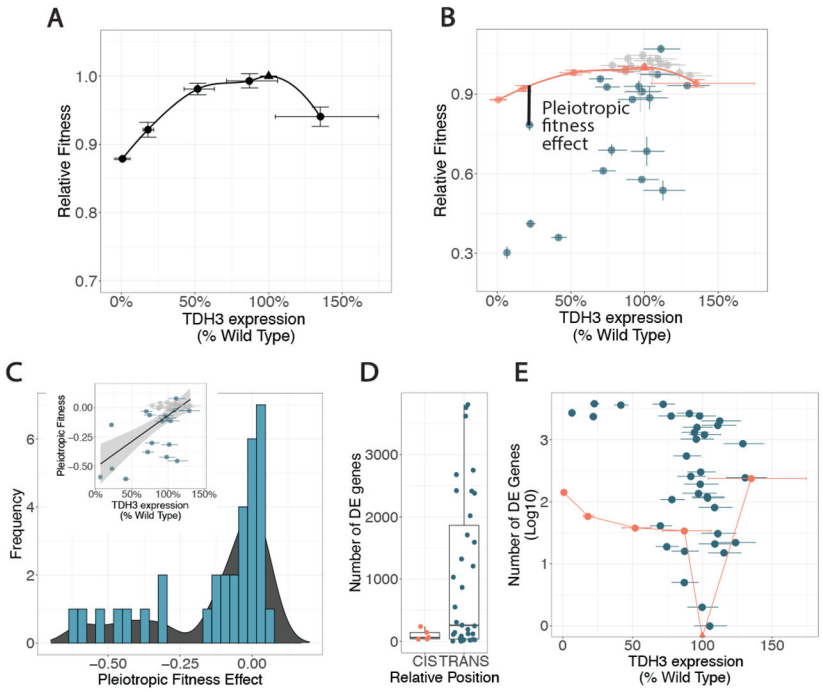

Fig. 2. Pleiotropic effects of *trans*-regulatory mutations on fitness and gene expression.

(**A**) Relative fitness is shown for *cis*-regulatory mutants and a wild-type strain (triangle) with different levels of *TDH3* expression. Line fitted with a local polynomial regression (LOESS). (**B**) Relative fitness and *TDH3* expression of *trans*-regulatory mutants is shown, with the *cis*-regulatory mutants and fitted LOESS curve from (A) in orange. Pleiotropic fitness effect of one *trans*-regulatory mutant indicated with solid black line. Mutants with significant pleiotropic fitness effects are blue, not significant are grey. (**C**) Histogram summarizes pleiotropic fitness effects of all *trans*-regulatory mutants. A smoothed density distribution derived from this histogram is underlaid in black. Inset shows the same pleiotropic fitness effects plotted vs the effect on *TDH3* expression including a robust linear regression (black line). Grey shaded area is the 95% confidence interval. (**D**) Number of significantly differentially expressed (DE) genes at a 10% FDR is shown for *cis*-regulatory mutants (orange) and *trans*-regulatory mutants (blue). Boxplots show median and quartile values. (**E**) The $\log_{10}$ number of significantly differentially expressed genes at a 10% FDR is shown for each mutant, plotted according to the mutant's impact on *TDH3* expression. *cis*-regulatory mutants (circles) and wild type strain (triangle) are connected by straight line segments. In all panels, error bars represent one standard error of the mean.

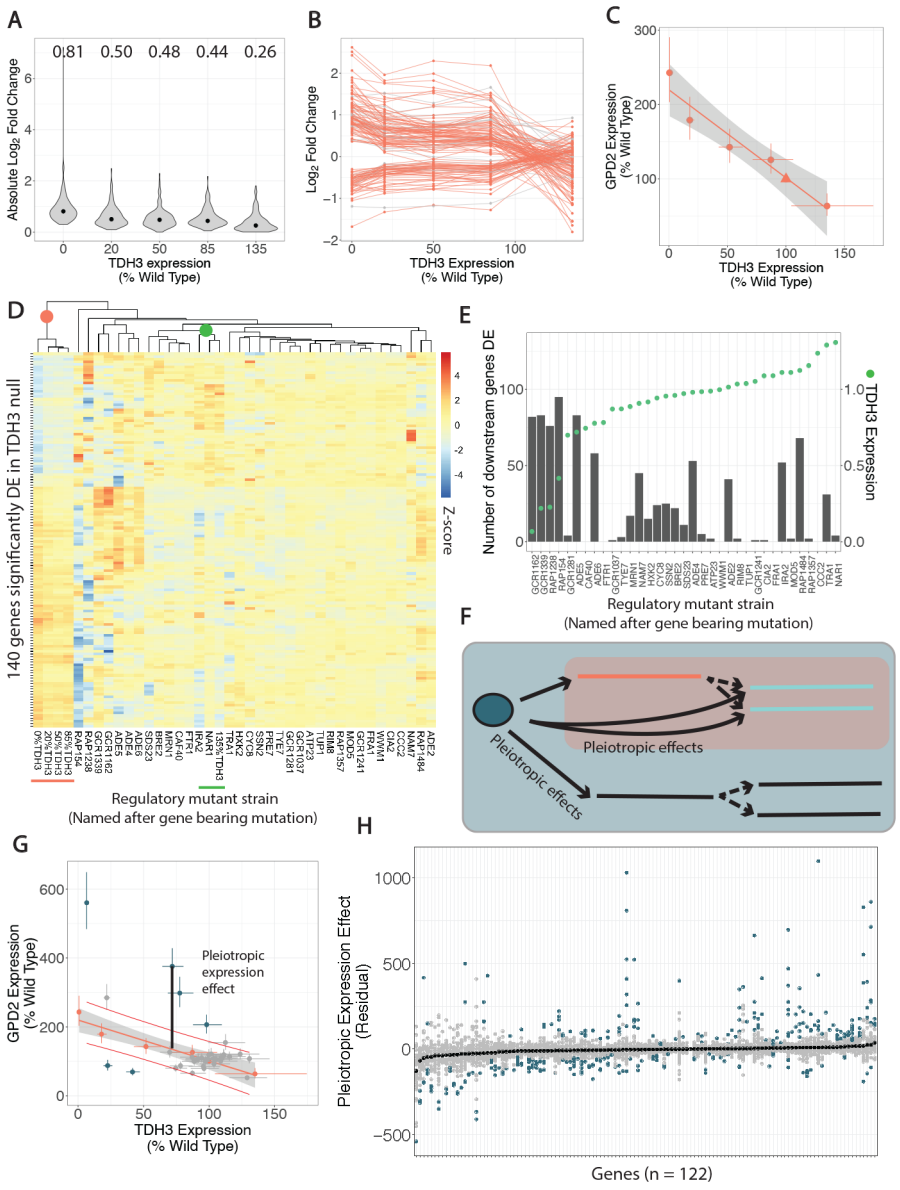

Fig. 3. *cis*- and *trans*-regulatory mutants have distinct effects on expression of genes downstream of *TDH3*.

(**A**) Violin plots show absolute $\log_2$ fold changes in the *cis*-regulatory mutants for the 140 genes identified as downstream of *TDH3* because they were significantly differentially expressed (DE) in the *TDH3* null mutant. Median absolute $\log_2$ fold changes are shown above each plot and indicated with black dots. (**B**) Log_2 fold changes in *cis*-regulatory mutants are shown for the same 140 genes downstream of *TDH3* connected by line segments. Genes whose expression was not significantly linearly correlated with *TDH3* expression are shown in grey. (**C**) Expression of *TDH3* and *GPD2* is shown for the *cis*-regulatory mutants (circles) and the wild type strain (triangle). The best fit linear regression line and 95% confidence interval (grey shaded area) are shown. (**D**) A heatmap of the 140 genes downstream of *TDH3* shows $\log_2$ fold changes in all *cis*- and *trans*-regulatory mutants in which genes are rows and mutants, named after the gene bearing the mutation in that

mutant, are columns. Color intensity is scaled by row (by gene) and represents z-scores. Mutants are hierarchically clustered as shown by the dendrogram. (**E**) The number of downstream genes that are also significantly differentially expressed in each *trans*-regulatory mutant is shown as a column relating to the left y-axis. The expression level of *TDH3* in that mutant is shown as a green point relating to the right y-axis. (**F**) Schematic shows that *trans*-regulatory mutants can have pleiotropic effects on genes downstream of *TDH3* not mediated by their impact on *TDH3* as well as pleiotropic effects on genes in parallel to *TDH3*. (**G**) Expression of *GPD2* and *TDH3* is shown for the *trans*-regulatory mutants, with the expression and linear regression from *cis*-regulatory mutants from (C) included in orange. Red lines delineate 95% prediction intervals for the *cis*-regulatory mutant relationship between *TDH3* and *GPD2*. Effects of *trans*-regulatory mutants on *GPD2* that are not explained by their impact on *TDH3* (pleiotropic expression effects, example illustrated by black line) are colored blue. (**H**) For each of the 122 genes downstream of *TDH3* with a significant linear relationship to *TDH3* expression in the *cis*-regulatory mutants (x-axis), the pleiotropic expression effect (y-axis), as illustrated in (G), is shown. For each gene, each point represents a different *trans*-regulatory mutant. Genes are ordered on the x-axis by median residual (black points). Blue points indicate mutants with significant pleiotropic expression effects, while grey points indicate non-significant pleiotropic expression effects.

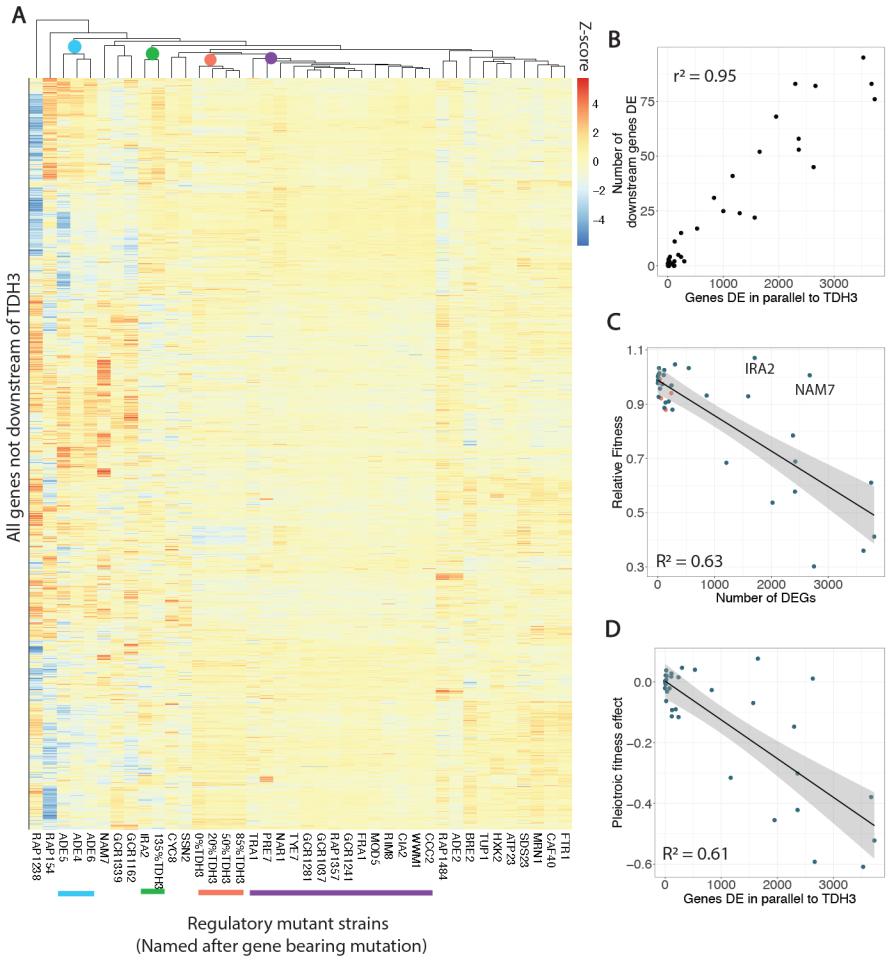

Fig. 4. Larger pleiotropic expression effects of *trans*-regulatory mutants correlate with negative fitness effects.

(**A**) A heatmap shows $\log_2$ fold changes for all genes not downstream of *TDH3* as estimated by DESeq2, in which rows are genes and columns are mutants. Color intensity is scaled by row (by gene) and corresponds to z-scores. Hierarchical clustering of mutants is shown by the dendrogram above. Three of the four *trans*-regulatory mutants bearing mutations in the adenine biosynthesis pathway cluster together and are marked by a blue dot and line. The *cis*-regulatory mutant overexpressing *TDH3* clusters with *IRA2* (green), while the *cis*-regulatory mutants with reduced *TDH3* expression cluster together (orange). A large cluster of *trans*-regulatory mutants with small effects across the genome are marked by a purple dot and line. (**B**) For all *trans*-regulatory mutants, the number of downstream genes differentially expressed is positively correlated with the number of genes differentially expressed in parallel to *TDH3* in that mutant. (**C**) For both *cis*- (orange) and *trans*- (blue) regulatory mutants, the total number of differentially expressed genes is a strong predictor of relative fitness. A linear model is shown with grey shading representing 95% confidence intervals and two outliers, *IRA2* and *NAM7* are indicated. (**D**) For all *trans*-regulatory mutants with fitness data (*i.e.,* excluding the two flocculant strains), the pleiotropic fitness effect, as defined in Fig. 2B, is plotted vs the number of genes differentially expressed in

parallel to *TDH3* in that *trans*-regulatory mutant. A linear regression is shown in black with 95% confidence intervals in grey.