Model-based transcriptome engineering promotes a fermentative transcriptional state in yeast

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The ability to rationally manipulate the transcriptional states of cells would be of great use in medicine and bioengineering. We have developed an algorithm, NetSurgeon, which uses genomewide gene-regulatory networks to identify interventions that force a cell toward a desired expression state. We first validated NetSurgeon extensively on existing datasets. Next, we used NetSurgeon to select transcription factor deletions aimed at improving ethanol production in Saccharomyces cerevisiae cultures that are catabolizing xylose. We reasoned that interventions that move the transcriptional state of cells using xylose toward that of cells producing large amounts of ethanol from glucose might improve xylose fermentation. Some of the interventions selected by NetSurgeon successfully promoted a fermentative transcriptional state in the absence of glucose, resulting in strains with a 2.7-fold increase in xylose import rates, a 4-fold improvement in xylose integration into central carbon metabolism, or a 1.3-fold increase in ethanol production rate. We conclude by presenting an integrated model of transcriptional regulation and metabolic flux that will enable future efforts aimed at improving xylose fermentation to prioritize functional regulators of central carbon metabolism.

Significance

The ability to engineer specific behaviors into cells would have a significant impact on biomedicine and biotechnology, including applications to regenerative medicine and biofuels production. One way to coax cells to behave in a desired way is to globally modify their gene expression state, making it more like the state of cells with the desired behavior. This paper introduces a broadly applicable algorithm for transcriptome engineering—designing transcription factor deletions or overexpressions to move cells to a gene expression state that is associated with a desired phenotype. This paper also presents an approach to benchmarking and validating such algorithms. The availability of systematic, objective benchmarks for a computational task often stimulates increased effort and rapid progress on that task.


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that are differentially expressed (DE) between the source and target cells (not those that are activated by posttranscriptional mechanisms). Finally, these systems have been tested on only a handful of conversion tasks, perhaps because of the technical challenges of mammalian cell culture, transdifferentiation, and cell type determination.

In this paper, we introduce and systematically evaluate NetSurgeon, a broadly applicable algorithm for recommending TF deletions or overexpressions to move cells from an initial (current) expression state to any desired state associated with any desired phenotype. The only inputs it requires are one expression profile of the initial state, one expression profile of cells in the target state, and an approximate transcriptional regulatory map indicating the direct targets of each TF. The network map can be derived from gene expression data by NetProphet (14) or other network inference algorithms, from TF binding motifs determined in vitro, or from in vivo binding data derived by methods such as chromatin immunoprecipitation sequencing (ChIP-seq) or Calling Cards (15, 16). Unlike the CellNet recommendation system (8), NetSurgeon considers all genes that are DE between the initial and target states, not only those that increase, it considers interventions on all TFs whether or not they are DE, and it has no bias toward TFs with large numbers of targets. Instead, it prefers interventions on TFs most of whose targets are predicted to move in the desired direction upon deletion or overexpression of the TF. Because it considers the predicted direction of change, NetSurgeon has no bias toward activators over repressors or overexpression over knockdown. CellNet, by contrast, considers only interventions in which activators are overexpressed. We also introduce a systematic, genome-scale evaluation procedure for general transcriptome engineering algorithms. Saccharomyces cerevisiae is ideal for evaluating transcriptome engineering because of the ease of experimental testing in yeast and the wealth of data available for TF network mapping and algorithmic validation (17–20).

After evaluation on existing yeast data, we apply NetSurgeon to the goal of improving xylose fermentation in S. cerevisiae. Xylose is a five-carbon sugar that is found, together with glucose and other sugars, in cellulosic biomass. Although S. cerevisiae is the most commonly used organism for industrial ethanol production, its inefficient fermentation of pentoses has hampered the production of ethanol from lignocellulosic biomass (21). Recombinant yeast strains expressing transgenes that facilitate integration of xylose into central carbon metabolism have been constructed (22, 23). However, when they are grown in mixed glucose/xylose cultures, which would be encountered in lysates of cellulosic biomass, they rapidly ferment all available glucose and then shift into a respiratory metabolic state in which little if any ethanol is produced (23). This led us to hypothesize that engineering yeast cells to have a gene expression profile in xylose similar to the profile of wild-type (WT) cells in glucose would increase ethanol yield.

Results

Algorithmic Approach to Transcriptome Engineering. Our approach consists of three steps. First, we build a genome-wide map of the network of direct, functional regulation (Fig. 1A). Each TF–target relationship in this map is labeled as either activating or
repressing. Second, we define starting and goal transcriptional states and apply NetSurgeon, which searches through all possible interventions (TF deletions or overexpressions) to identify those that are likely to move the transcriptional state toward the goal state (Fig. 1B). Finally, we construct strains containing the predicted best interventions, profile their transcriptomes, and quantitatively assay the phenotypes of interest—in this study, sugar consumption and the production of biomass and major metabolic products (Fig. 1C).

NetSurgeon assigns a score to each possible intervention, representing its confidence that the intervention will yield a substantial shift toward the goal state. The score is loosely based on the cumulative hypergeometric distribution, where the universe is the set of all genes. The “positives” are the genes that are significantly differently expressed between the initial state and the goal state, optionally intersected with a set of genes involved in a process of interest (Fig 1D). In the hypergeometric enrichment calculation, the “draws” are the target genes of the TF on which the intervention acts, as specified by the input network map. The successes are the positives drawn—that is, the targets of the TF that are also DE between initial and goal states. To count as a success, a gene must not only be a target of the TF, it must also be predicted to move in the direction of the goal state as a result of the intervention (Fig. 1D). Deletion of a TF is predicted to increase the expression of targets it represses and decrease expression of targets it activates. Overexpression of a TF is predicted to have the opposite effects.

The NetSurgeon score is not used for hypothesis testing—that is, rejecting a null hypothesis. It is simply a convenient formula for calculating a score that increases when either of two numbers (the number of targets or the fraction of targets that are DE between the initial and goal states) increases while the other is held constant. The NetSurgeon score differs from the hypergeometric probability of obtaining at least $Sk$ successes when drawing a sample of size $qi$ where $Sk$ is the set of indices of genes whose $q$ value is below the threshold. The sum of $W_i$ over all DE genes is 1. The number of successes for intervention $k$, $Sk$, is the total number of DE genes times the sum of the weights of the DE genes that are targets of the TF:

$$S_k = ||D|| \sum_{i \in T_k} W_i,$$

where $T_k$ is the index set of targets that are predicted to move toward the goal state as a result of the intervention. The NetSurgeon score for intervention $k$ given a fixed network is then the hypergeometric probability of obtaining at least $Sk$ positives when drawing a sample of size $T$ from a universe of all genes containing $||D||$ positives, where $T$ is the total number of targets of the perturbed TF.

For a given network map in which each gene is categorized as repressed, activated, or unregulated by each TF, the scores for interventions are calculated as above. However, network-mapping algorithms typically return a list of possible TF–target relations ranked from most confident to least confident; setting a confidence threshold is left to the user. NetSurgeon therefore makes the score calculation described above using various thresholds and takes the maximum score over all thresholds considered. It considers a threshold that includes only the top 500 interactions, the top 1,000, the top 1,500, and so on, down to the top 40,000.

**Building a TF Network Map.** To test this approach, we first built an integrated gene-regulatory network map by combining separate functional and physical maps. The functional map was built by using NetProphet, an expression-based mapping algorithm that combines a differential expression analysis and a regression analysis (14). Each possible TF–target pair is assigned a differential expression score that is equal to the log odds that the putative target is DE when the TF is perturbed, given the available replicate expression profiles. Each pair is also assigned a regression score based on a regularized regression of the expression level of the target gene (the dependent variable) against the expression levels of all TFs (the predictor variables). The regression score for a given TF–target pair is simply the regression coefficient assigned to the TF’s expression level as a predictor of the target’s expression level. The differential expression and regression scores are combined to provide a final score representing NetProphet’s confidence that the TF binds and regulates the target (see ref. 14 for details). For this paper, we ran NetProphet with 320 potential regulators including proteins with DNA-binding domains, chromatin factors, and other members of DNA-binding protein complexes that regulate gene expression. The input data were gene expression profiles of 269 regulator deletion strains grown in rich medium with glucose (19). For the 51 potential regulators for which there was no expression-profiled deletion, strain 0 was used as the differential expression component (the number of targets or the fraction of targets that are DE expressed to produce the goal state, or even that the goal state was achieved, before the intervention acts, as specified by the input network map. The NetSurgeon score differs from the hypergeometric probability of obtaining at least $Sk$ successes when drawing a sample of size $qi$ where $Sk$ is the set of indices of genes whose $q$ value is below the threshold. The sum of $W_i$ over all DE genes is 1. The number of successes for intervention $k$, $Sk$, is the total number of DE genes times the sum of the weights of the DE genes that are targets of the TF:

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For a given network map in which each gene is categorized as repressed, activated, or unregulated by each TF, the scores for
The 320 included 75 “distractor” regulators that were present in the NetProphet+PWM network but did not correspond to goal states. We plotted the cumulative percentage of goal states for which NetSurgeon ranked the best intervention (the one that actually produced the goal state profile) at or above each rank (Fig. 2B, green). We compared NetSurgeon performance to random ranking of TF deletions (Fig. 2B, dotted black) and found that NetSurgeon assigns higher ranks to the correct interventions (Mann–Whitney U test, $P < 10^{-23}$). NetSurgeon ranks the correct intervention within the top five, a reasonable number to test experimentally, for 91 goal states, which is 24 times better than chance ($P < 10^{-96}$). We also ran NetSurgeon on 100 random networks with the same topology as the NetProphet+PWM network (Fig. 2B, gray) and observed performance at random chance levels, indicating that an accurate network is critical for NetSurgeon.

We then repeated the analyses described above using goal states defined by cells cultured in conditions even further from those used to construct the input network map. These goal states consisted of 63 expression profiles obtained from regulator overexpression strains grown in SC+Gal (24) (defined medium with galactose), whereas the NetProphet+PWM network was built using expression profiles from regulator deletion strains growing in YP+Glc (rich carbohydrate medium).
medium with glucose). As expected, performance on this more challenging task was not as good as when the goals states were from regulator deletion strains grown on SC+Glc. Nonetheless, NetSurgeon assigned higher ranks to the correct interventions compared with randomly assigned ranks (Mann–Whitney U test, \( P < 10^{-4} \)) and assigned the best intervention a top five rank for 8 of the 63 goal states (15%), an eightfold improvement over chance (\( P < 10^{-7} \)).

To assess the practicality of NetSurgeon-guided transcriptome engineering, we ran NetSurgeon on the NetProphet+PWM network and the SC+Glc goal set (20) and computed the median number of top-ranked interventions that would need to be tested to identify the single most useful intervention. NetSurgeon can identify the single most useful intervention (the one that produced the goal state) in a median of 22 predictions, whereas random guessing would take 161 tries. An intervention was deemed useful if it reduced the distance between the WT cells and the goal by at least 10% (Fig. 2D). NetSurgeon can identify at least one useful intervention in a median of 13 predictions, whereas random guessing would take 134 tries.

**NetSurgeon Accuracy Depends on the Accuracy of the TF Network Map.** To evaluate the effect of network accuracy on NetSurgeon performance, we applied NetSurgeon to network maps inferred from the same expression dataset as before but using different algorithms for TF network mapping: CLR (25), LASSO regression (26), NetProphet (27), and NetProphet integrated with PWM scores. We first evaluated the structural accuracy of the five maps by their level of support from ChIP data. The results show that NetProphet is more accurate than a simple LASSO regression approach, which is more accurate than CLR, consistent with our previously published comparison of these algorithms (14) (see ref. 27 for a recent review of TF network mapping algorithms.) As expected, scoring potential TF-target relations by the correlation of the TF’s expression level with target expression level performed worse than CLR, which postprocesses correlation coefficients. Also as expected, supplementing NetProphet scores with scores reflecting the TF’s potential for binding the sequences in the promoter region of each gene improved accuracy above that of NetProphet alone. We then plotted the accuracy of NetSurgeon when using each of these five input maps on our two goal sets—the TF-deletion strains in SC+Glc and TF-overexpression strains in SC+Galactose—against the accuracy of the input map (Fig. 2E). This shows a clear pattern of improved NetSurgeon performance with more accurate network maps.

**Application of Transcriptome Engineering to Ethanol Production.** We next applied NetSurgeon to the industrially relevant problem of ethanol production in a mixed glucose–xylose coculture. Principal-components analysis of RNA-seq data from *S. cerevisiae* cells grown with various carbon sources, including xylose, indicated that cells growing on xylose were in a transcriptional state with some characteristics of cells grown in 5% (wt/vol) glucose, a fermentative state, and some characteristics of cells grown in 1.3% (wt/vol) ethanol, a respiratory state (Fig. 3A). As *S. cerevisiae* cells do not natively consume xylose, we hypothesized that they were unable to recognize xylose as a fermentable carbon source and therefore entered into a transcriptional state that was nonoptimal for fermentative metabolism. We therefore sought to identify interventions that would shift the cells growing in xylose from the transcriptional state of WT cells growing in xylose (initial state) to the transcriptional state of WT cells growing on glucose (goal state). To apply NetSurgeon to this problem, we generated a network map using all available expression profiles and DNA binding motifs (Full-NetProphet+PWM) and attempted to optimize the expression of 445 genes involved in carbohydrate metabolism. Specifically, we calculated the NetSurgeon score using a subset of the genes that are DE between the initial and goal states—the 445 genes that are annotated in GO or KEGG with the terms Glycolysis, Pentose Phosphate, TCA Cycle, Oxidative Phosphorylation, Carbohydrate Metabolism, Hexose Metabolism, or Pentose Metabolism. Using this network map, NetSurgeon produced a ranked ordered list of regulators whose deletion was predicted to force the system toward the goal state. We tested the top eight interventions, deletion of *CAT8* (catalytic repression), *HAP4* (heme activator protein), *MSN2* (multicopy suppressor of SNF1 mutation), *MSN4* (multicopy suppressor of SNF1 mutation), *GIS1* (Glc1-2 suppressor), *AFT2* (activator of Fe [iron] transcription), or *USV1* (up in starvation), in the H2217-7 yeast strain (28). As a limited comparison of our algorithmically selected deletions with expert intuition, we also deleted the master regulator *SNF1*, the yeast ortholog of AMP kinase, which inactivates TFS responsible for glucose repression (29).

The selected interventions looked promising on the basis of existing literature. Cat8 and Hap4 are respiratory factors active in the general cellular response to xylose, and deletion of *HAP4* was recently shown to improve cellobiose consumption rates (28, 30). MSN2 and MSN4, encoding stress-associated factors, were observed to be highly up-regulated in xylose and their transcriptional targets misregulated (31). *ADR1* is an activator of *ADH2*, a glucose-repressed gene that encodes an alcohol dehydrogenase that catalyzes a key step in ethanol consumption (32, 33). Usv1, Gis1, and Aft2 have been shown to have clear roles in the yeast transcriptional response to nonfermentable carbon sources and general stress response (34–36).

Aerobic batch fermentations were used to assess the outcome of our interventions at both the transcriptional and the metabolic levels. Cells were inoculated into synthetic complete medium (SC) supplemented with 2% (wt/vol) glucose and 5% (wt/vol) xylose at OD600 = 1.0 ± 0.2 and grown for 48 h. Samples were taken for RNA-seq at 4 and 24 h, representative of the glucose–xylose and xylose phases. Samples were also taken for analyzing output metabolites by HPLC at time points throughout the 48-h fermentation. The results showed a glucose–xylose phase, during which both sugars were consumed and ethanol was produced, followed by a xylose phase during which glucose was largely depleted and ethanol was consumed along with xylose (Fig. 3B).

**Transcriptome Engineering Promotes a Fermentative State.** Analysis of our RNA-seq data revealed that 2,887 genes are DE between the 4-h time point, when glucose is abundant, and the 24-h time point, when glucose has been depleted, by at least twofold in WT cells (42% of all genes; Fig. 4A, WT). Six of the eight NetSurgeon-selected interventions lowered the number of DE genes. The best intervention, *cat8Δ*, reduced the number of DE genes by 22%, performing much better than the choice of our human expert, *snf1Δ*. To examine this from another perspective, we calculated the Euclidean distance between the goal state (the fermentative state defined by WT expression profile in the glucose–xylose phase) and the
state of the engineered strain in the xylose phase (Fig. 4B). Six of eight NetSurgeon interventions lowered the Euclidean distance between the two phases, with an average reduction of 21%. The cat8Δ intervention reduced the genome-wide expression distance by 28%. Among the 445 carbon metabolism genes that NetSurgeon targeted for optimization, cat8Δ reduced the Euclidean distance by 36% (Fig. 4C).

We also evaluated the ability of each TF deletion to promote a fermentative state in 11 pathways of central carbon metabolism (Fig. 4D). Seven of the eight NetSurgeon-selected interventions reduced the Euclidean distance between the initial (T24, respiratory) and goal (T4, fermentative) state expression levels of the genes in at least one of the central carbon metabolism pathways evaluated. Six of these interventions improved the expression of glycolytic genes, and five of them also improved the expression of TCA cycle genes. Deletion of CAT8 promoted a fermentative state in many metabolic pathways essential for xylose fermentation, including genes involved in glucose utilization, the pentose phosphate pathway, glycolysis, the TCA cycle, and acetate/glycerol production. It moved all of the TCA cycle genes toward their expression levels in the goal state (Fig. 4E), although a few genes moved only a small fraction of the way toward their goal state levels (e.g., CIT3, IDP2) and several overshot their goals by a small margin. Considering all genes encoding enzymes in the TCA cycle, deletion of CAT8 reduced the Euclidean distance to the goal state by 60%. These observations highlight the power of transcriptome-level interventions to modulate the expression of many more genes than is feasible by traditional, one-gene-at-a-time genetic engineering.

Identification of Transcriptional States Associated with Improved Fermentation. To assess changes in metabolic behavior following our transcriptional interventions, we profiled metabolic intake and

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**Fig. 4.** NetSurgeon interventions promote a fermentative transcriptional state. (A) Number of twofold or greater DE genes between the WT strain in the glucose-plus-xylose (fermentative) phase and each deletion mutant in the xylose-only (respiratory) phase. (B) Euclidean distance between the full expression profile of WT cells in the fermentative phase and the expression profiles of deletion mutants in the respiratory phase (lower is better). (C) Same as B, restricted to 445 genes encoding metabolically active proteins. (D) Euclidean distance between the expression profiles of selected metabolic pathways in WT cells in the fermentative phase and their profiles in deletion mutants in the respiratory phase (greener is better). (E) The expression of TCA cycle genes in WT cells in the fermentative phase (goal state, red), WT cells in the respiratory phase (initial state, green), and the cat8Δ deletion mutant in the respiratory phase (blue), on a log scale. Arrowheads indicate the direction the expression of each gene in cat8Δ (blue) would have to move to get closer to the goal (green). For most TCA cycle genes that show a substantial difference between the initial and goal states, the cat8 deletion moves their expression substantially toward the goal state.

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output via HPLC. We calculated the carbon import rate per unit biomass for each strain and displayed the percentage of input carbon that is directed to each of the major carbon fates in each growth phase (Fig. 5 A and B). Carbon import rates declined in the xylose

Fig. 5. Transcriptome engineering alters carbon uptake rates and commitment ratios but does not prevent the transition to respiratory metabolism. (A) The absolute rate of carbon uptake (Top) and the distribution of carbon fates (Bottom) during the fermentative (glucose-plus-xylose) phase. (B) Same as A for the respiratory (xylose) phase. Red in Top panel indicates net ethanol consumption. (C) Specific rate of glucose consumption (fermentative phase); asterisks indicate a difference from WT with \( P < 0.05 \) by Benjamini–Hochberg-corrected t test. (D) Same as C for xylose consumption. (E) Same as C for acetic acid production. (F) Same as C for ethanol production.
phase by 86%, on average, and the cells significantly shifted their carbon commitment from fermentative to respiratory processes in the xylose phase. The proportion of input carbon released as CO$_2$ increased from a mean of 24% in the glucose–xylose phase to 89% in the xylose-only phase.

Although the interventions we tested did not prevent the transition from fermentative to respiratory metabolism, they did affect carbon fate significantly. Fractional carbon commitment to every measured metabolite was altered by at least one of the TF deletions. The xyitol fraction was significantly increased by interventions affecting TFs associated with respiratory processes, a potential side effect of the respiratory factors regulating the promoters of the XYL1, XYL2, and XKS1 transgenes, which encode enzymes required for integrating xylose into central carbon metabolism. Interestingly, all significant changes in the fraction of input carbon allocated to ethanol or biomass were reductions. The deletions of SNF1, HAP4, USV1, GIS1, MSN4, and AFT1 significantly reduced the fraction of carbon committed to ethanol (average, 26%). Deletions of CAT8 and HAP4 reduced carbon commitment to biomass by 33% and 38%, respectively, in the glucose–xylose phase.

We also analyzed the specific rate (rate per unit biomass) of production of each metabolite by each strain (Fig. 5 C–F). This differs from the previous analysis, which focused on the fraction of carbon input allocated to each carbon fate. Although none of the interventions increased the fraction of input carbon allocated to ethanol, some interventions increased the specific rate of carbon input and hence the specific rate of ethanol production in the glucose–xylose phase. Interventions on respiratory regulators (Cat8, Hap4, and Adr1) improved the specific rate of glucose consumption between 11% and 40% (Fig. 5C). The hap4Δ and msn4Δ mutants improved the specific rate of xylose consumption by 170% and 120%, respectively (Fig. 5D). Acetic acid, a fermentation by-product that has been demonstrated to inhibit glycolysis (37), was produced at lower specific rates in the hap4 and cat8 mutants (53% and 83%; Fig. 5E). Importantly, the specific rate of ethanol production was significantly increased by 22% and 31% in the cat8 and hap4 mutants (Fig. 5F). These strains directed a smaller fraction of the carbon they consumed toward biomass than WT and their peak ethanol levels were significantly higher than those of WT (by 14% and 19%, respectively) and occurred at approximately the same time as WT (24 h). Among the stress-associated factors, we found that the deletion of USV1, MSN2, MSN4, and AFT2 significantly reduced the specific rate of ethanol production (22% on average; Fig. 5F). Taken together, these data demonstrate that transcriptional engineering can generate significant changes in metabolic behavior.

**An Integrated Model of Transcriptional Regulation and Metabolic Flux Identifies Direct Regulators of Metabolic Flux.** In the previous sections, we presented both gene expression data and metabolic data on WT yeast and nine TF deletion mutants at multiple time points. We now integrate these datasets to produce a qualitative model linking transcription factors to metabolic fluxes. The purpose of the model is to summarize and integrate the findings reported in this paper and to suggest directions for future work.

As described above, major sources of carbon input and output were measured by HPLC (for soluble metabolites) and optical density (for biomass). These inputs and outputs are shown around the outside of Fig. 6. We then generated a system of linear equations encoding stoichiometric constraints on carbon fluxes across nine metabolic pathways: glucose import, xylose import, the pentose phosphate pathway, glycolysis, the TCA cycle, acetate production, ethanol production, biomass, and residual (CO$_2$).

![Fig. 6](image_url) An integrated map of transcriptional regulation in central carbon metabolism. Metabolites around the outside are measured, except for the residual, which is carbon consumed minus carbon allocated to all measured carbon fates. Mauve rectangles: selected pathways of central carbon metabolism, including the xylose utilization pathway whose components are encoded by transgenes. Black arrows: connections along which carbon can flow from one pathway to another. Green circles: transcriptional regulators. Blue arrows: inferred transcriptional activation. Red T-head lines: inferred transcriptional repression. Solid lines indicate that the TF directly regulates one or more genes encoding enzymes involved in the pathway, according to the Full-NetProphet+PWM network.

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**Table:**

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gycerol production and consumption, acetate production/consumption, ethanol production and consumption, and biomass production (Fig. 6, rectangles and black arrows). These equations, together with our measurements and an approximate partitioning of biomass into components emanating from each pathway, enabled us to calculate fluxes through the pathways. Together, our measurements and the assumption that most of the unaccounted-for carbon was released as CO_2 fully constrained the internal fluxes. Unlike many flux balance analyses (e.g., refs. 38 and 39), our fully constrained model did not require the assumption that fluxes are distributed in a way that maximizes biomass production.

To link transcriptional states with metabolic flux states, we computed the Pearson correlation coefficient between the expression of each TF in the yeast genome and the carbon flux through each pathway. The correlation was calculated across all strains and time points described above. We also generated a null distribution of correlation coefficients by correlating randomly permuted gene expression patterns with the metabolic fluxes. We then identified TFs whose expression is significantly correlated with metabolic flux (false-discovery rate ≤ 0.01) and generated edges (Fig. 6, red and blue lines) linking correlated TFs (Fig. 6, green circles) with each metabolic pathway. Interactions between TFs and pathways were hypothesized to be direct (solid lines) if our TF network map supported the TF as a direct regulator of one or more genes encoding proteins in the pathway. This analysis of our data suggested three transcriptional regulators that may be deeply interconnected with biochemical pathways important for xylose metabololism and fermentation.

\( CAT8 \) expression was significantly correlated with carbon flux through the xylose utilization, the pentose phosphate, and the TCA cycle. Msn4 was predicted to directly regulate genes involved in xylose utilization and the pentose phosphate pathway, and flux through these pathways was negatively correlated with \( MSN4 \) expression. Pdr3 was suggested as a regulator of glycolytic genes, and flux through glycolysis was positively correlated with \( PDR3 \) expression. This integrated model suggests important transcriptional regulators that may control both transcriptional and metabolic state and therefore represent high-value targets for future transcriptomic engineering of ethanol productions.

**Discussion**

In this paper, we introduced NetSurgeon, an algorithm that uses a map of direct transcriptional regulation to select TFs whose deletion or overexpression will move a cell’s transcriptional state toward a specified goal. The availability of comprehensive TF deletion and overexpression collections in S. cerevisiae enabled us to systematically assess NetSurgeon’s accuracy on a genome-wide scale. Given the expression profile of a cell in which a single TF has been deleted, NetSurgeon can identify that TF in a median of 22 predictions, whereas random guessing would take 161 tries. We observed that network maps built with expression data from one environmental condition can be successfully used to identify interventions for achieving goal states defined by profiles in different environmental conditions. We also demonstrated that TF network maps enriched for direct regulatory relationships are important for this task—network maps generated by Net Prophet together with PWM models led to selections that were substantially better than those made by using maps based on expression correlation or CLR (25).

We applied NetSurgeon to optimizing yeast for ethanol production from glucose–xylose coculture. NetSurgeon selected 26 genes, making optimization of this pathway’s expression level a difficult task for one-gene-at-a-time engineering. Deletion of \( CAT8 \) moved all 26 genes of the TCA cycle toward the fermentative state, showing that the manipulation of transactors that have evolved to regulate a naturally occurring transcriptional state can greatly facilitate transcriptome engineering.

Transcriptome engineering is a relatively new endeavor with a bright future. We have demonstrated that transcriptional network maps enriched for direct regulatory interactions are highly valuable for identifying the key regulators that mediate a state transition and prioritizing them for genetic intervention. By exploiting collections of expression profiles from TF deletion or overexpression strains, we have established clear benchmarks by...
which transcriptome engineering algorithms can be compared with one another, which may accelerate progress (41). We have also demonstrated that transcriptome engineering can be used to move cellular behavior—in this case, metabolic behavior—toward a desired goal. Our dataset of 8,055 metabolic measurements with 73 matched RNA-seq profiles across 14 genotypes will enable future efforts to maximize ethanol production by manipulating key transcriptional regulators. The software that further progress in transcriptome engineering will lead to improvements in bioproduction, personalized medicine, and stem cell engineering.

Materials and Methods

A software package implementing NetSurgeon is available from the Brent Laboratory web page: mblab.wustl.edu/software.html. The NetSurgeon scoring algorithm (described at the beginning of Results) can be expressed in the following formula for the score of intervention k:

\[ S_k = \frac{\log q_{\text{true}} - \log q_{\text{false}}}{\log q_{\text{true}} - \log q_{\text{false}}} \]

where \( h \) is the hypergeometric probability function, \( S_k (\text{the positives in the sample}) \) is defined below, G (the population) is the index set of all genes in the genome, \( T_i (\text{the sample}) \) is the index set of targets of the perturbed regulator among the \( I \) most confidently predicted regulatory interactions in the network that are predicted to move toward the goal state as a result of the intervention, and D (the positives) is the set of indices of genes that are DE between the initial and goal state (i.e., those whose q value is below the user-determined threshold).

The number of positives in the sample for intervention k is defined to be the following:

\[ q_i = \min\{\text{DE} \mid a \} \]

where q is the false-discovery rate for gene i and all those q values < q are being DE between the initial and goal states.

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Supporting Information

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**SI Materials and Methods**

*Saccharomyces cerevisiae* Fermentations. All *S. cerevisiae* strains were grown aerobically in 60 mL of synthetic complete medium (SC) at 30 °C in 250-mL baffled Erlenmeyer flasks shaken at 225 rpm. Cultures for identification of differential expression associated with carbon sources were grown in triplicate in 50 g/L glucose, 50 g/L xylose, or 1.3 g/L ethanol for 8 h before sampling for RNA-seq. Cultures of cells for evaluating the impact of transcriptional interventions were performed in triplicate and initiated by inoculating overnight cultures into 60 mL of SC supplemented with 20 g/L glucose and 50 g/L xylose resulting in a density of OD600 = 1.0 ± 0.2. Samples taken for RNA-seq were aliquoted from the primary culture, spun down at 3,000 × g, and frozen in liquid nitrogen. The samples for HPLC were supernatants collected after centrifugation of cell samples at 12,000 × g for 3 min before snap freezing for storage in a dry ice/ethanol bath. All samples were stored at −80 °C. At least two independent experiments of three biological replicates were performed for each genotype evaluated by HPLC.

**HPLC.** The concentration of input and output soluble metabolites was analyzed by using HPLC. Supernatant solutions were stored at −80 °C and filtered through 0.22-μm syringe filter before HPLC analysis. Metabolites were eluted from an Aminex HPX-87H column maintained at 65 °C and peaks detected by a Waters 2414 refractive index detector. Identified peaks were quantified through integration and interpolated against serial dilutions of standards for glucose, xylose, xylool, glycerol, acetic acid, and ethanol. Analysis of HPLC data was performed on each biological replicate, with metabolic input/output relationships quantified across each fermentation and pooled into a single distribution based on genotype. Turbidity measurements were converted into units of grams biomass per liter based on the turbidity to biomass conversion factor published (23). To evaluate internal carbon flux, a system of linear stoichiometric constraints was developed for certain branch points in key pathways of *S. cerevisiae* central carbon metabolism (SI Materials and Methods). This system of equations was fit to experimentally measured parameters of carbon import and export for each genotype across the glucose/xylose and xylose-only phases of each fermentation.

RNA Sequencing. Total mRNA was isolated using the yeast RiboPure kit (Life Technologies). Libraries for RNA-seq were prepared as in ref. 42 except that the selection of poly(A) RNA from the total RNA isolated was accomplished using an mRNA Catcher Plus kit (Life Technologies) and the resulting poly(A) RNA was subsequently sheared by incubating in TURBO DNA-free buffer at 75 °C for 10 min and purified with a QIAquick PCR Purification kit (Qiagen).

**Data Analysis Methods.**

**HPLC raw data output/structuring.** Analyte detection, peak identification, and peak integration were all performed using the Empower 3 software suite. Following initial labeling by Empower 3, individual peaks were manually confirmed and computationally identified peak boundaries adjusted to minimize error. The area under each labeled peak was calculated as a representation of analyte concentration. Due to the lack of batch data export or API access within Empower 3, tabular data for each HPLC run were manually exported from Empower 3 into a flat text file. Following raw data output, each peak was manually labeled with the molecule responsible for peak generation. Manual labeling was performed through comparison of peak retention time and area against authentic standards for each analyte. This process generated an annotated, comma-separated file suitable for downstream analysis.

**Batch effect normalization.** HPLC and refractometry error were controlled for on a per-replicate basis. Data for each replicate were normalized for day effect as follows. All analyte concentrations were divided by the ratio of the HPLC reading for glucose at time 0 (expected to be 20 g/L because that is how the medium was prepared) and an authentic standard of 20 g/L glucose. This reduced the median coefficient of variation among biological replicates by 41%.

**Biomass/HPLC data integration, replicate-based analysis.** Measurements of metabolite production from HPLC analysis and turbidity-based biomass measurements collected from spectrophotometry were integrated into a single data structure based on experimental date, strain genotype, and replicate number. All downstream analyses, including stoichiometric flux balance analysis, were performed on a per-replicate basis.

**Evaporative normalization.** The presence of a significant evaporative effect on metabolite concentration was observed across the 48-h fermentations. Pilot experiments using standard fermentation media (synthetic complete medium, minus histidine, 20 g/L glucose, 50 g/L xylose) but lacking cells, indicated that all metabolites increased in concentration by ∼20% across a 48-h mock fermentation. To control for this, a quadratic polynomial was fitted to 10 mock fermentations sampled for HPLC analysis across 120 h. For each replicate, across all time points, the predicted evaporative effect was calculated and removed from HPLC and biomass measurements.

**Analyte interpolation.** Analyte measurements were interpolated against serial dilutions of purified standards obtained from Sigma-Aldrich. Standards were diluted across physiologically relevant concentration ranges and then assayed by HPLC. A linear equation was fit to each standard and used to quantify each analyte.

**Outlier identification.** Treatment of outliers was performed according to the National Institute of Standards and Technology (NIST) Engineering Statistics Handbook 1.3.5.17 (NIST/SEMATECH, 2012). The generalized extreme Studentized deviate test was used for formal outlier identification, with input parameters of α = 0.05 and 2 for the upper bound of total outliers allowed within a distribution.

**Calculation of analyte rates of change, specific rates of change, and carbon commitment ratios.** To examine the effect of transcriptional interventions on cellular metabolism, the analytical process required the identification of biological steady states across which analyte rates of input and output could be calculated and transformed into steady-state models of internal carbon flux. Analysis of ethanol production and consumption indicated that there were three steady states present across the 48-h fermentation. The period from 3 to 12 h was defined as a fermentative metabolic state associated with high external glucose concentrations. A second fermentative phase, associated with low external glucose concentrations, was identified across the 12- to 20-h period. All assayed genotypes transitioned into a respiratory metabolic state during the 24- to 48-h window of the fermentation—this period was therefore used to represent the metabolic state associated with xylose catabolism. A lag phase during the diauxic shift from glucose to xylose and ethanol metabolism was observed from 20 to 24 h, and this time period was excluded from downstream analyses. Analysis of metabolic rates of change, carbon commitment calculations, and stoichiometric flux balance analysis focused on the 3- to 12- and 24- to 48-h fermentative periods for which RNA-seq data were available.

**RNA-seq.** Two to three biological replicates of each fermentation strain genotype, growth condition, and time point combination were profiled for all RNA-seq samples, the mean and median
sequencing depth was 7.2 and 6.9 million reads, respectively. The interquartile range of sequencing depth was 4.8–9.1 million reads. Sequenced reads were aligned to the S. cerevisiae S288C reference genome release R63 using TopHat, version 2.0.4 (43), and Bowtie, version 0.12.8 (44). Reads that aligned uniquely to the reference sequence were considered for gene expression quantification with Cufflinks, version 2.0.2 (45). Gene expression was quantitated against known gene boundaries from the gene annotations provided by the SGD S288C reference genome release R63. Gene expression was normalized using the Cufflinks upper-quartile normalization option. After gene expression quantification, samples not passing the following quality control filters were removed from consideration. Each sample was required to be sequenced at a depth of at least 750,000 reads. The expression of the deleted gene in mutant expression profiles was required to be less than 10% of its expression in WT. The mean and median expression of the deleted genes were 1.3% and 0% of the WT levels, respectively. The low levels of expression that remained for some samples were likely the result of errors in the sequencing process itself, either due to contamination between samples or a low level of error in matching barcodes for multiplexed samples to reads. In addition, within each replicate set containing three biological replicates, outlying gene expression profiles were detected and removed if the outlier caused the median of all genes’ coefficients of variation (the SD divided by the mean) to be greater than 0.5 for the replicate set. In total, we selected the PWM binding potential scores for the pair of replicate gene expression profiles to be less than 0.8. Differential expression analysis comparing initial and goal state expression profiles was performed by using LIIMA (46) with the voom transformation applied to read counts (47). The number of reads mapping to each gene was determined by using the htseq-count tool in version 0.5.3 of the HTSeq Python package (48). DE genes responding to the treatment factor were selected based on a q value (false-discovery rate) cutoff of less than or equal to 0.05.

**Gene-Regulatory Network Construction.** We took an integrative approach to constructing a gene-regulatory network model by building separate network models from functional and physical data and then combining them. An integrative approach to the network-building process was chosen because integration often leads to more accurate and complete inferred network models (49). The functional gene-regulatory network model is built using NetProphet (14), a method for inferring direct, functional regulation from gene expression data. NetProphet works by combining differential expression analysis with a coexpression analysis. The LASSO regression coexpression component of NetProphet was run on four S. cerevisiae expression profile datasets, three of which are previously published large-scale datasets (19, 24, 50), and the last of the four was a RNA-seq dataset of S. cerevisiae cells grown with various single carbon sources. The four resulting network models were combined into a final coexpression regulatory network model by computing the geometric mean of scores assigned to each TF–target pair. A network model based on differential expression analysis was constructed by using the target differential expression information for all regulators deleted in the Hu et al. (19) dataset supplemented with the target differential expression information for overexpressed regulators in the Chua et al. (24) dataset that were not deleted in the Hu et al. dataset. The NetProphet weighted averaging scheme was used to combine the final coexpression and differential expression networks, producing the fully integrated functional regulatory network. This fully integrated functional regulatory network was normalized to a [0, 1] range by dividing each interaction score by the maximum of all interaction scores.

The physical network model was built using TF binding potential estimates generated by scanning a collection of position weight matrix (PWM) models over all yeast promoters. PWMs for 196 S. cerevisiae TFs were extracted from the SceTF database (17). To estimate the binding potential of each TF with a PWM on each gene’s promoter, FIMO (51) was used to scan the TF’s PWM over the promoter of each S. cerevisiae gene using the default FIMO background model. Promoters were defined as extending from 800 bases upstream of the transcription start site (excluding sequence from neighboring ORFs) to 200 bases downstream from the transcription start site. Binding sites that were identified by FIMO at a value of $P < 0.005$ were considered in subsequent analyses. Two models of binding were considered. For each TF, the strong site model ranks promoters containing one or more significant binding sites according to the negative log $P$ value of the most significant site. The weak site model ranks promoters containing one or more significant binding sites by the sum of the negative log $P$ values for all significant sites in a promoter. The final PWM binding potential scores for each TF were normalized into a [0, 1] interaction score range by dividing each binding potential score of a TF by the maximum of all binding potential scores for the TF.

We created our overall gene-regulatory network model by assigning a score to each TF–target gene pair that was equal to the geometric mean of the scores assigned to the pair in the functional and physical networks. This scoring function enforced that high scoring interactions are likely to be direct and functional due to their support by both binding and expression evidence.

**NetSurgeon in Silico Validation.** We assessed the ability of NetSurgeon to select regulator interventions (deletions and overexpressions) for moving transcriptional state toward a goal state. To accomplish this, we evaluated NetSurgeon’s ability to identify the correct regulator intervention for goal states defined by postintervention expression profiles from two S. cerevisiae datasets. The first contains 245 regulator deletion expression profiles from cells growing on synthetic complete medium with glucose (SC+Gal) (20). The second contains 63 regulator overexpression expression profiles from cells grown in synthetic complete medium with galactose (SC+Glc) (24). In each case, the initial state was the WT expression profile from the same dataset. For each expression profiling dataset, we transformed NetSurgeon’s scores for interventions aimed at achieving each goal state into ranks. We then compared NetSurgeon’s accuracy to randomly ranking regulator interventions and to intervention ranks produced by running NetSurgeon on 100 random networks with the same topology as the NetProphet+PWM network. In addition, we also evaluated the number of NetSurgeon selected interventions required to recover all useful interventions, the most useful intervention, and any useful intervention. The most useful intervention was defined as the intervention that decreased the Euclidean distance between the intervention expression state and the goal state by the most. This was, by definition, the intervention that produced the goal state expression profile.

To investigate the relationship between NetSurgeon accuracy and transcriptional network accuracy, we first inferred transcriptional networks using five different inference algorithms: expression correlation, CLR (25), LASSO regression (26), NetProphet alone (14), and NetProphet integrated with PWM scores. Each algorithm was used to infer a transcriptional network from a gene expression dataset consisting of 269 regulator deletions strains grown inYPD (19). The structural accuracy of each resulting network was assessed by comparing the ranked network interaction predictions to the network structures defined by a collection ChIP-seq and ChIP-chip experiments. The ChIP data were compiled as previously described (14). In total, the ChIP data provides 29,260 experimentally validated links between 187 regulators and 5,566 genes, and the ChIP-based network is assigned binary scores based on the presence of ChIP-validated interactions. The area under the precision recall curve at the 5% recall point was used to assess the accuracy of each inferred network compared with the ChIP-defined true network.
These inferred networks were also used to make NetSurgeon intervention selections on both goal state expression datasets. NetSurgeon’s accuracy was assessed by the area under the line representing the cumulative percentage of goal states for which NetSurgeon ranked the best intervention (the one that actually produced the goal state profile) at or above each rank. The R glm function was used to investigate the relationship between network accuracy and NetSurgeon accuracy.

**RNA-seq Batch Effect Removal.** Before using the RNA-seq data for downstream analysis, we identified technical noise in the expression profiling data that varied with the experimental batches. These batch effects were removed from RNA-seq read counts using the RUVg function in the RUVSeq R package (52). RUVg identifies and removes unwanted variation related to technical effects through factor analysis on a set of negative control genes that were not expected to respond to the biological treatments. We defined a set of 192 negative control genes by identifying genes that are not predicted to be regulated by any of the deleted regulators in the 100,000 highest scoring interactions according to the NetProphet PWM network scores. Genes were then eliminated from this list if their expression changed significantly (log twofold change < 1.3 and adjusted P value > 0.1) between any pair of our experiment profiles (and hence the power to detect statistically significant differences) was not consistent among all intervention strains.

Intervention Strain Fermentative Expression State Convergence. For each intervention strain we constructed and profiled, we measured the distance between the expression profile of the WT strain at 4 h (fermentative phase with glucose plus xylose) and the intervention strain at 24 h (xylose only). The goal, of course, was to find interventions that reduced this distance. We used two distance metrics. Our first metric quantified the number of twofold DE genes as the distance between two expression profiles. These genes were identified by applying LIMMA (46), with a design matrix considering factors for treatment and batch effects, on voom-corrected raw read counts as described above. We choose to use a twofold-change cutoff, rather than a statistical significance cutoff, because the number of replicate expression profiles (and hence the power to detect statistically significant differences) was not consistent among all intervention strains. Our second metric was Euclidean distance, calculated by using the R function dist on batch-corrected log₂ cpm profiles.

**Stoichiometric Flux Balance Analysis.** Internal carbon fluxes were described by fitting a system of equations designed to describe the central carbon metabolism of *S. cerevisiae*. The model was then fitted to measured cellular inputs and outputs using specific rates of analytic change in units of grams carbon per grams cell per hour. The flux balance analysis equations are as follows:

\[ V_6 = \text{rate}_{\text{glucose}}, \]
\[ V_7 = (V_9 + V_{31}) \times P_{\text{glucose}} + (V_9 + V_{31}) \times P_{\text{galactose}} + (V_9 + V_{31}) \times P_{\text{acetic acid}} + (V_9 + V_{31}) \times P_{\text{ethanol}}, \]
\[ V_8 = \text{rate}_{\text{biomass}} \times \text{percent}_{\text{carbohydrate}} \times gC12_{\text{carbohydrate}}, \]
\[ V_9 = \text{rate}_{\text{biomass}} \times \text{percent}_{\text{nucleic acid}} \times gC12_{\text{nucleic acid}}, \]
\[ V_{10} = V_5 + V_{32} - \left( V_7 \times P_{\text{glucose}} \right) - \left( V_8 \times P_{\text{glucose}} \right) - \left( V_8 \times P_{\text{galactose}} \right), \]
\[ V_{11} = \left\{ \frac{\text{rate}_{\text{glycerol}}} {\text{rate}_{\text{glycerol}}} \geq 0 \right\}, \]
\[ V_{12} = V_{14} + (V_8 \times P_{\text{glycerol}}) + (V_9 + V_{31}) \times P_{\text{glycerol}} - (V_11 \times P_{\text{ethanol}}), \]
\[ V_{13} = V_{10} + V_5 + (V_30 \times (V_8 \times P_{\text{glycerol}})) - (V_9 + V_{31}) \times P_{\text{glycerol}} - (V_11 \times P_{\text{xylose}}) - (V_11 \times P_{\text{glucose}}) - (V_11 \times P_{\text{galactose}}). \]

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\[ V_{28} = (V_8 + V_9) \times P_{ethanol} + (V_8 + V_9) \times P_{acetic acid} + (V_{11} \times P_{ethanol}) + (V_{11} \times P_{acetic acid}) \]

\[ V_{29} = V_{28} \times V_{29C12ratio} \]

\[ V_{30} = \begin{cases} \text{rate}_{\text{glycerol}} \geq 0 : 0 \\ \text{rate}_{\text{glycerol}} < 0 : |\text{rate}_{\text{glycerol}}| \end{cases} \]

\[ V_{31} = V_9 \times V_{31C12ratio} \]

\[ V_{32} = |\text{rate}_{\text{galactose}}| \]

\[ V_{33} = |\text{rate}_{\text{acetic acid}}| \]

**Integration of Transcriptional Network State with Carbon Fluxes.** To link the transcriptional regulatory network with pathway carbon fluxes, we identified significant correlations between transcription factor expression (TF) levels and calculated metabolic fluxes. For each flux, the Pearson correlation coefficient (PCC) was calculated between the batch-corrected log2 cpm profile of each annotated TF-encoding gene and flux through each pathway. A null distribution of PCCs was generated by computing 10,000 PCCs between the carbon fluxes and randomized gene expression profiles. The random expression profiles were generated by sampling from all genes a log2 cpm expression value once per strain. This null distribution of PCCs was used to calculate the significance of each calculated PCC within the experimental set. In Fig. 6, PCCs with a false-discovery rate of ≤1% between transcription factors and pathway carbon fluxes were plotted.