nature genetics

Genetic reconstruction of a functional transcriptional regulatory network

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Although global analyses of transcription factor binding provide one view of potential transcriptional regulatory networks[1,2], regulation also occurs at levels distinct from transcription factor binding[3,4]. Here, we use a genetic approach to identify targets of transcription factors in yeast and reconstruct a functional regulatory network. First, we profiled transcriptional responses in *S. cerevisiae* strains with individual deletions of 263 transcription factors. Then we used directed-weighted graph modeling and regulatory epistasis analysis to identify indirect regulatory relationships between these transcription factors, and from this we reconstructed a functional transcriptional regulatory network. The enrichment of promoter motifs and Gene Ontology annotations provide insight into the biological functions of the transcription factors.

We grew each of 263 transcription factor knockout strains (obtained from Open Biosystems) as replicates (**Supplementary Table 1** online) and compared mRNA expression of each of these strains with a wild-type strain using microarrays. We used a weighted error model[5] that integrated replicate data to assign *P* values and identify target genes that were significantly differentially expressed (**Supplementary Methods** online). Expression data confirmed that a given transcription factor gene was deleted in a given knockout strain (**Fig. 1a,b**). Furthermore, PCR analysis showed that 18 out of 21 strains with the greatest likelihood of errors had the correct transcription factor gene deleted (data not shown). The three remaining strains had few targets (0, 3 and 19, respectively) and did not affect the data significantly.

We defined our unrefined transcription factor target network as the cumulative set of significantly differentially expressed genes in each deletion strain. Although there was statistically significant overlap between transcription factor targets identified in our unrefined network and targets identified by chromatin immunoprecipitation (ChIP)-chip[1], this overlap was low (**Supplementary Table 2** online). However, when we compared our data for *RAP1* targets with a different high-quality Rap1 ChIP-chip data set[6], the overlap improved. Of the 354 Rap1 targets in the high-quality ChIP-chip data set and the 537 targets we defined, 144 were shared, compared with only 71 targets shared between our data set and the large-scale ChIP-chip data set[1]. We observed similar trends with high-quality ChIP-chip data sets for Hsf1 (ref. 7) and Swi4 (ref. 8), suggesting that data quality may be one reason for the low overlap.

Another possible explanation for the low overlap is that only a subset of bound transcription factors may affect a target gene's expression, depending on the positioning and orientation of binding sites and the presence of other proteins. There are several additional possible explanations for the small overlap between affected genes and binding targets. First, a transcription factor can regulate secondary targets via regulatory cascades. Therefore, the unrefined knockout targets are likely to include both direct and indirect targets. Second, different transcription factors occupying a promoter could compensate for each other's loss, masking the deletion effect. Finally, a transcription factor could bind a promoter under normal growth conditions but function only under specific environmental conditions, suggesting that regulation occurs not by

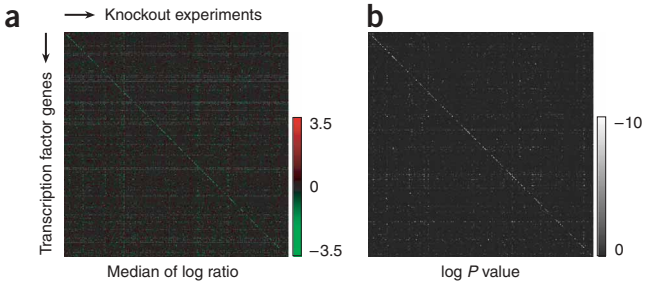

Figure 1 Validation of the transcription factor deletion strains and expression profiling data. (**a**) Expression of 263 transcription factor genes in the corresponding knockout strains, relative to wild-type strains. Expression is represented by the arithmetic mean of log-ratios from biological replicate experiments. Columns and rows represent experiments and genes, respectively, and are sorted in the same order. The green diagonal confirms the apparent downregulation of the transcription factor genes in the corresponding deletion strain. (**b**) *P* values of the expression changes in the same order as in **a** (calculated using an error model) show that the loss of expression of a transcription factor gene in its deletion strain is statistically significant.

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Received 26 December 2006; accepted 1 March 2007; published online 8 April 2007; doi:10.1038/ng2012

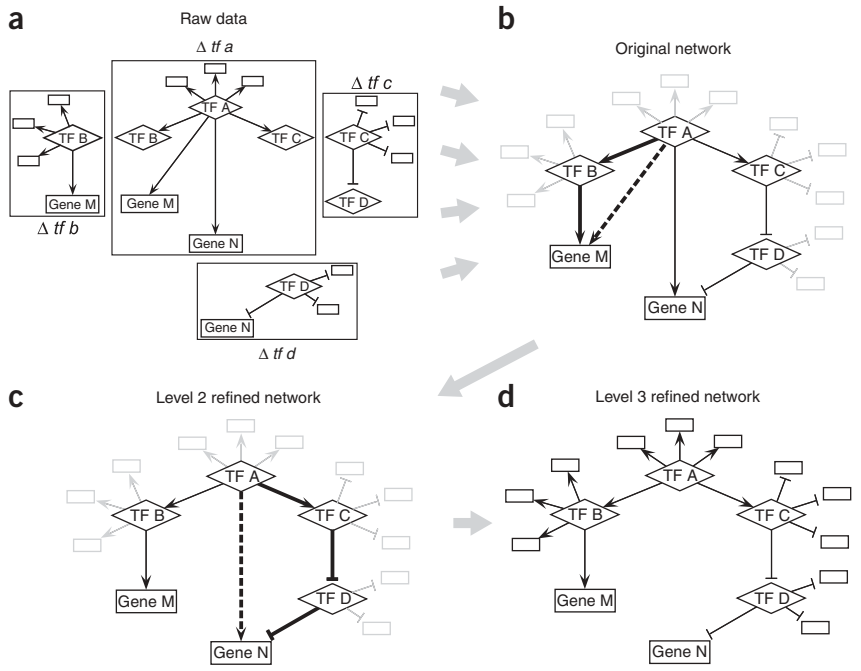

Figure 2 Regulatory epistasis analysis and functional network refinement. Transcription factors (diamonds) and their regulated genes (rectangles) are vertices, and regulatory relationships are edges. The sign of the edge is indicated by an arrow for activation and a line ending in a 'T' for repression. (**a**) The primary data consisted of the target set for each of the 268 transcription factors, defined using the error model. (**b**) Primary data were integrated and rendered as the original unrefined network, including secondary and further levels of regulation. (**c**) First level of network refinement. In the unrefined network, if transcription factor A activated transcription factor B and gene M, but transcription factor B itself activated gene M, and if the confidence of transcription factor A regulating gene M was lower than for transcription factor B regulating gene M, then the regulation of gene M by transcription factor A was presumed to be indirect and was therefore erased. The latter constraint ensured that valid multi-edge circuits such as feed-forward loops (in which M could be regulated combinatorially by transcription factor A and transcription factor B) were not invariably removed. (**d**) An additional refinement step similar to **c**, except that the indirect edge that was removed bridged a three-step direct interaction series at the preceding level, resulting in a level 3 refined network. Logical consistency for regulatory edges was maintained at all times. The level 4 refined network (not shown) was the final refined network.

the mere binding of a transcription factor to a promoter but rather at a separate step.

We addressed each of these possibilities in turn. We traced all secondary and tertiary targets along binding regulatory chains for each transcription factor[1,2] and then compared this extended binding target set to our functional targets. Although the overlap improved (**Supplementary Table 2**), the overall *P* value did not, probably because such extended binding chains include increasing numbers of false positive targets.

Next, we reconstructed a refined transcriptional regulatory network by removing from the unrefined network indirect regulatory interactions inferred from a network model. Several strategies of analyzing expression profiles by epistasis and network refinement have been described[9–11]. We designed a regulatory epistasis approach to identify indirect regulation and to reconstruct a refined transcription factor target network. We modeled the data as a directed-weighted graph and integrated subnetworks to form the initial unrefined network (**Fig. 2a**,**b**). In our network model, transcription factors and their regulated genes are nodes, and regulatory relationships are edges. We identified putative indirect regulatory edges and erased them in the network (**Fig. 2c**,**d**). We iterated this process until level 4, beyond which we could not remove any indirect regulatory edges, to yield our final refined network (**Supplementary Methods**). In this refined network, 45% of all genes were significantly regulated by at least one transcription factor; 138 transcription factors had more activated targets than repressed targets, whereas 114 transcription factors had more repressed targets than activated targets. Overall, 9,659 targets were activated, and 4,595 were repressed in the refined network.

The refined transcriptional regulatory network showed several expected properties in terms of the relationship of transcription factor binding to expression of a target gene. When the transcription factor bound to a promoter was deleted, the expression of the downstream gene was much more likely to be affected than the background (**Fig. 3**). Expression from promoters that were detectably occupied by a single transcription factor were even more likely to be affected by deletion of that potentially major or sole regulator (**Fig. 3**). Conversely, promoters whose downstream genes were significantly regulated in our refined functional network were more likely to be occupied by the appropriate transcription factor (**Supplementary Fig. 1** online). Thus, there was significant overlap between binding targets defined by ChIP-chip and functional targets defined by deletion analysis and network refinement.

We next focused on identifying condition-specific targets for a subset of 23 transcription factors that potentially regulated transcription during heat shock by repeating our profiling experiments during heat shock (**Supplementary Table 1**). For the essential transcription factor *HSF1*, we used a conditional shut-off strain[12]. We identified 695 targets for these transcription factors during heat shock. Regulated targets of Hsf1 under heat shock agreed well with previous high-quality binding data[7]; 65 of its 223 functional target promoters were occupied by Hsf1 under heat shock, compared with zero overlap in a large-scale data set where Hsf1 binding was also analyzed after heat shock[2].

To begin to characterize our refined functional network, we carried out upstream motif analysis and Gene Ontology (GO) analysis. For each transcription factor, we analyzed activated targets separately from repressed targets. Such analysis of activated and repressed targets separately is not possible with targets defined solely by transcription factor binding. We first quantified the enrichment of previously known transcription factor motifs[2,13–15] in the corresponding target promoters (**Supplementary Methods**). Of the 102 transcription factors with known promoter motifs, 40 showed significant enrichment of the motif in target promoters (false discovery rate (FDR) = 2.6%) (**Fig. 4a** and **Supplementary Table 3** online). Next, we used AlignACE[16], MEME[17] and MDscan[18] for *de novo* promoter motif discovery (**Supplementary Methods**). For 106 transcription factors, of which 61 had no previously defined binding motifs, we identified new high-confidence binding motifs (**Fig. 4b** and **Supplementary Table 3**).

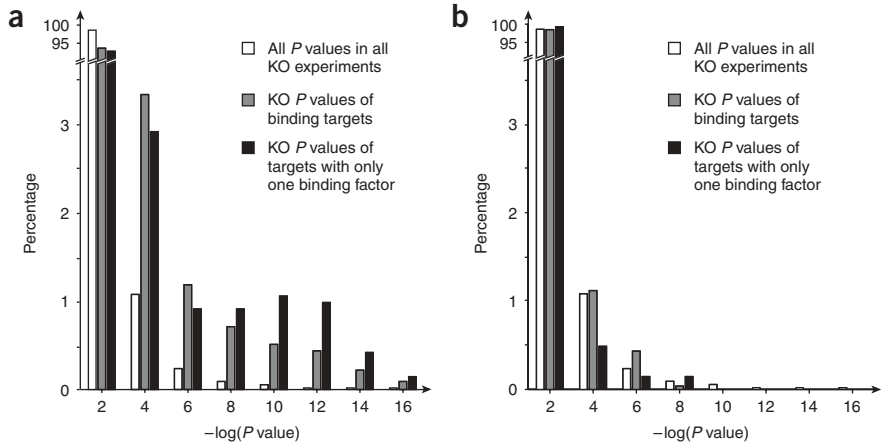

Figure 3 Overlap between transcription factor–regulated targets and binding targets. The plots show histograms of *P* values for the change in expression of genes, expressed as the negative log. (**a**) Distribution of *P* values in our final refined network. Open bars represent *P* values for expression changes in all genes; gray bars represent genes whose promoters were occupied by the corresponding transcription factor; black bars represent genes whose promoters were occupied by only the corresponding transcription factor. (**b**) Control histogram similar to **a** but using a randomly permuted knockout target data set.

We developed GO analysis software that cumulatively evaluated enrichment of functional annotations among targets in the refined network at increasingly higher levels of the GO hierarchy. This enabled us to detect enrichment among transcription factor targets of 'composite' parent annotations in the GO hierarchy even if their 'raw' child annotations, taken individually, were not significantly enriched (**Supplementary Methods**). At a stringent significance threshold ($P = 4 \times 10^{-5}$), there were 1,114 enriched annotations (213 distinct) covering 156 transcription factors (**Supplementary Table 4** online). Approximately 35% of the enriched annotations were consistent with known transcription factor functions (**Table 1**). At a relaxed threshold ($P = 0.001$), there were 2,228 enriched annotations (433 were distinct) covering 236 transcription factors. GO annotation enrichment among targets that were concordantly either activated or repressed provided strong implications about the biological role of the transcription factor in the cell. For example, we observed the enrichment of five GO categories related to glucose metabolism among the activated targets of *GCR2*, clearly indicating that this regulator serves as an activator of this process. Among 131 transcription factors with marginal or no target overlap between the large-scale binding data and our deletion data, 33 showed a functional overlap, in that the same promoter motifs and/or GO annotations were enriched among targets in both data sets. We found dozens of examples of GO annotation pairs that were co-enriched by multiple transcription factors, suggesting connections between the corresponding biological themes. For example, 16 transcription factors coregulate polyamine and spermine transport as well as response to stress, and 13 transcription factors regulate cell wall genes as well as RNA binding proteins, suggesting coordination between these processes in the cell (**Supplementary Table 4**).

We tested five transcription factors that affected GO annotations not previously associated with them. Of these, *rtg3*Δ and *aft1*Δ (in which glutamate biosynthesis genes and chaperones, respectively, were significant targets) had growth defects under appropriate conditions (**Fig. 4c**). Although *RTG3* has been associated previously with glutamate biosynthesis, this is not reflected in its SGD annotation[19,20]. *AFT1* has a known role in iron transport but had only been predicted by computational analysis to affect the stress response[21]. Expression profiling of *rtg3*Δ and *aft1*Δ grown in minimal medium or heat shock, respectively, confirmed that *RTG3* regulates genes involved in glutamate biosynthesis, and *AFT1* regulates stress response genes.

Several aspects of our functional transcription factor regulatory network are noteworthy. First, few indirect regulatory edges (1.2%) were removed by our epistasis analysis. We extended our epistasis analysis to exclude nonspecific effects on transcription in the deletion strains, as well as post-transcriptional effects mediated by RNA binding proteins (RBPs). If a transcription factor deletion were to affect genes nonspecifically, this indirect effect on transcription ultimately would still have to be mediated by some other transcription factors. If such indirect effects were prevalent, we would expect to see instances where the targets of a transcription factor largely overlapped with the targets of another transcription factor, even though the latter transcription factor was not a target of the former. Alternatively, if a transcription factor deletion affected genes at a post-transcriptional

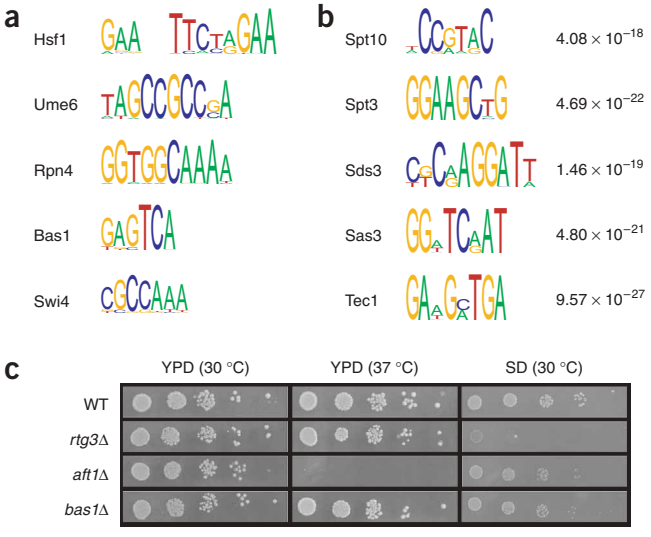

Figure 4 Enrichment of sequence motifs and biological impact of transcription factor deletions. (**a**) Examples of known binding motifs for transcription factors recovered in the promoters of targets in the refined network. (**b**) Newly described sequence motifs not previously known to be associated with the corresponding transcription factor. Numbers at right represent significance of motif enrichment in target promoters relative to background. (**c**) Growth defects of transcription factor knockout strains newly predicted by GO overrepresentation analysis of the refined network. YPD is rich medium, and SD is synthetic minimal medium with dextrose, uracil, histidine, methionine and leucine. Aft1 was predicted to activate chaperone genes, and the *aft1*Δ strain is defective for growth at 37 °C. Rtg3 was predicted to activate glutamate biosynthesis, and the *rtg3*Δ strain is defective for growth on minimal medium. Of the five strains we tested, *bas1*Δ, *ric1*Δ and *pho2*Δ showed no corresponding growth defect; only *bas1*Δ is shown here.

Table 1 Examples of significantly enriched Gene Ontology (GO) annotations in the refined network

Transcription factor	Functional description[a]	Enriched GO annotation	*P* value
TEC1	Transcriptional regulator of Ty1 expression	Ty element transposition	4.15×10^{-26}
GCR2	Transcriptional activator of genes involved in glycolysis	Glucose metabolism	3.18×10^{-31}
TYE7	May function as a transcriptional activator in Ty1-mediated gene expression	Ty element transposition	2.19×10^{-23}
GAL80	Transcriptional repressor of GAL genes in the absence of galactose	Galactose metabolism	1.63×10^{-11}
ARG80	Involved in regulation of arginine-responsive genes	Arginine biosynthesis	1.53×10^{-10}
MSN2	Transcriptional activator, activated in stress conditions	Cellular response to nitrogen starvation	1.02×10^{-10}
ARG81	Involved in the regulation of arginine-responsive genes	Arginine biosynthesis	6.37×10^{-9}
GCR1	Transcriptional activator of genes involved in glycolysis	Glycolysis	3.38×10^{-9}
CST6	Activates transcription of genes for use of non-optimal carbon sources	Hexose metabolism	4.15×10^{-9}
ACE2	Localizes to daughter cell nuclei after cytokinesis and delays G1 progression in daughters	Cytokinesis, completion of separation	8.76×10^{-10}
BAS1	Myb-related transcription factor involved in regulating basal and induced expression of genes of the purine and histidine biosynthesis pathways	Purine nucleotide biosynthesis	8.38×10^{-11}
OPI1	Functions in negative regulation of phospholipid biosynthetic genes	Phosphatidylcholine biosynthesis	1.12×10^{-8}

[a]Transcription factor functional descriptions are from the *Saccharomyces* Genome Database.

step, this effect could be presumed to be mediated by RBPs. If so, the targets of the RBP should largely overlap with the transcription factor deletion targets. To check for the former possibility, we compared all the transcription factor deletion target sets against each other. To check for the latter possibility, we compared published genome-wide RBP targets (**Supplementary Table 5** online) with targets in our functional network. We did not observe any indication in either case that indirect transcriptional or post-transcriptional regulation was responsible for the effects of a transcription factor deletion (**Supplementary Methods**). This suggests that during normal growth, regulation by a transcription factor is not propagated appreciably via extended cascades involving other transcription factors or by indirect regulatory steps. Thus, although transcription factor regulatory cascades are important during progressive events like the cell cycle[22], they seem to be less significant during steady state growth. This finding is consistent with studies suggesting that temporal and developmental transcription networks differ with regard to the length of cascades[23].

Second, the discrepancy between binding targets and regulated targets was not entirely accounted for by condition-specific effects of transcription factors. Genes expressed at high levels in rich medium and detectably occupied by only one transcription factor might be expected to be strongly responsive to the deletion of that transcription factor. However, this subset of genes was no more likely to be affected by deletion of the major occupying transcription factor (**Supplementary Fig. 1**). It is possible that there is unaccounted redundancy owing to the binding of unrecognized transcription factors[24] that is not reflected in available ChIP-chip data sets. Third, many promoters occupied by transcription factors under normal growth conditions are not actively regulated, pointing to extensive control of transcription at a step distinct from transcription factor binding. To investigate further, we used *HSF1* as an example and profiled expression changes after overexpressing *HSF1* (ref. 25). Overexpression, and thus increased binding of Hsf1, activated most of its downstream targets. However, 28% of the targets that were clearly occupied by Hsf1 and activated by temperature shift were not activated by increasing the binding of Hsf1 caused by its overexpression (**Supplementary Table 6** online). Thus, this subset of Hsf1 binding targets shows that a step distinct from DNA binding is needed for induction by a temperature shift. To estimate the minimal set of transcription factor targets that could involve such complex control beyond transcription factor binding, we considered the subset of genes that were occupied by only one transcription factor in the large-scale ChIP-chip data sets. We calculated the fraction of these genes that were not regulated in our network of affected genes, which comprises the set of genes likely to be regulated at a post-DNA binding step. We found 101 transcription factors where three or more targets that were occupied by the transcription factor were not regulated by its deletion (**Supplementary Fig. 1**).

Our data set provides, for the first time, a distinction between activated and repressed transcription factor targets on a genome-wide scale and thus enables more accurate modeling of global transcription in terms of the functional effect of a transcription factor upon a target, as well as its binding. The approach described here is can be extended for systematic analysis of gene regulation under a range of different growth conditions in yeast and other organisms.

METHODS

Strains. Yeast knockout strains were obtained from Open Biosystems and were derived from a BY4741 (*MATa his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0) parent[26]. The $TetO_7$-promoter regulated mutant strains were also obtained from Open Biosystems and were based on a BY4741 derivative (*URA3*::*CMV-tTA MATa his3*Δ1 *leu2*Δ0 *met15*Δ0)[12]. **Supplementary Table 1** shows a complete list of the strains used in this study.

Growth conditions. For each of the 263 nonessential transcription factors, knockout strains were grown in YPD medium to mid-log phase and then harvested. For six essential transcription factors, $TetO_7$-promoter regulated mutant strains[12] were grown in YPD medium in the presence or absence of 10 μg/ml doxycycline (Sigma) for 14–16 h and then harvested. We could not identify significant targets for one of these 269 transcription factors. For 22 of these transcription factors implicated in the heat shock response, deletion strains were grown in YPD medium to mid-log phase and then were subjected to a 15-min heat shock at 39 °C before harvest. For $TetO_7$-*hsf1*, the strain was grown similarly and harvested before or after a 15-min heat shock at 39 °C. Each experiment was independently carried out at least twice. A wild-type strain was cultured together with each batch of mutants as a growth control. **Supplementary Table 1** contains additional information on the growth rate of all the strains used.

Expression profiling. Total RNA samples from the harvested cells were extracted as described[27]. Yeast whole-genome microarrays were manufactured

as described[7]. cDNA generation, CyDye incorporation, array hybridization and scanning were also carried out as described[7,27]. cDNA samples from wild-type controls were generally labeled with Cy3, and samples from knockout strains were generally labeled with Cy5.

Data analysis. Microarray quantification was done using GenePix 5.x or 6.x software (Molecular Devices), and data were uploaded to the Longhorn Array Database[28]. Because we used a common reference RNA design for all microarray hybridizations, we calculated the true relative expression level of each gene in the mutant relative to the wild-type, normalizing for growth and reference batch effects[29]. To identify significantly regulated target genes, we adapted an error model[5], which generates a normalized, confidence-weighted measure of each ratio called the *X* score (**Supplementary Methods**). We used normalized microarray data to generate *X* scores in MATLAB and performed weighted duplicate averaging with custom Java software. A *P* value for every gene in the resulting data set was calculated in MATLAB based on the averaged *X* scores. We used a *P* value threshold of 0.001 to define the target set for every transcription factor. For further details on the directed weighted graph approach, sequence motif analysis and GO analysis, see **Supplementary Methods**.

Accession codes. Expression data from this study have been deposited in the Gene Expression Omnibus (GEO) database under the series accession number GSE4654.

URLs. GEO is found at http://www.ncbi.nlm.nih.gov/geo/. Primary data are also available at http://www.iyerlab.org/tfnetwork.

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Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank members of the Iyer lab for help with microarray printing; T. Hughes, B. Ren, H. Dai and M. Robinson for advice on the error model; E. Marcotte for discussions and suggestions and J. Gu for statistical advice. This work was supported by grants from the US National Institutes of Health (NIH) and the US National Science Foundation (V.R.I.), by a training grant from the US National Institute on Alcohol Abuse and Alcoholism and by a University of Texas Continuing Fellowship (P.J.K.).

AUTHOR CONTRIBUTIONS

Z.H., P.J.K. and V.R.I. designed the study, Z.H. did the microarray experiments, P.J.K. wrote the software, P.J.K. and Z.H. did the analysis and wrote the supplementary information and Z.H. and V.R.I. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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