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Transcriptional Networks for Alcohol Sensitivity in *Drosophila melanogaster*

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ABSTRACT

Understanding the genetic architecture of polygenic traits requires investigating how complex networks of interacting molecules mediate the effect of genetic variation on organismal phenotypes. We used a combination of *P*-element mutagenesis and analysis of natural variation in gene expression to predict transcriptional networks that underlie alcohol sensitivity in *Drosophila melanogaster*. We identified 139 unique *P*-element mutations (124 in genes) that affect sensitivity or resistance to alcohol exposure. Further analyses of nine of the lines showed that the *P*-elements affected expression levels of the tagged genes, and *P*-element excision resulted in phenotypic reversion. The majority of the mutations were in computationally predicted genes or genes with unexpected effects on alcohol phenotypes. Therefore we sought to understand the biological relationships among 21 of these genes by leveraging genetic correlations among genetically variable transcripts in wild-derived inbred lines to predict coregulated transcriptional networks. A total of 32 "hub" genes were common to two or more networks associated with the focal genes. We used RNAi-mediated inhibition of expression of focal genes and of hub genes connected to them in the network to confirm their effects on alcohol-related phenotypes. We then expanded the computational networks using the hub genes as foci and again validated network predictions. Iteration of this approach allows a stepwise expansion of the network with simultaneous functional validation. Although coregulated transcriptional networks do not provide information about causal relationships among their constituent transcripts, they provide a framework for subsequent functional studies on the genetic basis of alcohol sensitivity.

KNOWLEDGE of the genetic basis of phenotypic variation is critical for predicting disease risk and individual therapeutic treatments in human populations, understanding processes maintaining genetic variation in natural populations, predicting adaptive evolution, and selective improvement of agriculturally important species. Most phenotypic variation is genetically complex, attributable to multiple segregating quantitative trait loci (QTL) with small, environmentally sensitive, and often context-dependent allelic effects (Falconer and Mackay 1996; Lynch and Walsh 1998). Many recent large genome-wide studies mapping QTL affecting diseases and complex traits in human populations have identified novel loci and led to new biological insights about the biology of disease, but taken together these variants only explain a small fraction of the total segregating variation (Altshuler *et al.* 2008; Donnelly 2008; Frazer *et al.* 2009; Manolio 2010). Genetic dissection of complex traits in model genetic organisms, in which mutagenesis as well as analysis of segregating polymorphisms can be applied, also reveal that many novel, pleiotropic alleles affect most traits; these alleles often have sex-, environment- and genetic background-specific effects (Falconer and Mackay 1996; Flint and Mackay 2009).

The current challenge facing the genetic analysis of complex traits is to understand how allelic variants at hundreds of loci act together to affect organismal phenotypes. The key to solving this challenge is the realization that new mutations and segregating variants do not affect organismal phenotypes directly, but do so via complex interacting networks of transcriptional, protein, metabolic, and other molecular endophenotypes (Anholt *et al.* 2003; Sieberts and Schadt 2007; Chen *et al.* 2008; Emilsson *et al.* 2008; Keller *et al.* 2008; Ayroles *et al.* 2009; Cookson *et al.* 2009; Dobrin *et al.* 2009; Edwards *et al.* 2009a; Harbison *et al.* 2009; Morozova *et al.* 2009; Schadt 2009; Schadt *et al.* 2009; Winrow *et al.* 2009). If we know the network elements (genes) and connections (*cis*- and *trans*-regulation) associated with any complex phenotype, we can begin to make predictions about the effects of genetic perturbations on the organismal trait and whole network responses to such perturbations.

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The wealth of genetic and genomic resources, diversity of complex phenotypes, and ability to rear large numbers of genetically identical individuals under controlled environmental conditions makes *Drosophila melanogaster* an attractive model organism for elucidating general principles of systems genetics of complex traits. Two complementary approaches have been used to begin to define coregulated transcriptional networks associated with complex traits. The first involves transcriptional profiling of mutations affecting the trait that have been generated in a common isogenic background; genes encoding coregulated transcripts are themselves candidate genes affecting the trait (Anholt *et al.* 2003; Dubnau *et al.* 2003; Keegan *et al.* 2007; Bauer *et al.* 2008; Rollmann *et al.* 2008; Seugnet *et al.* 2009; Magwire *et al.* 2010; Slack *et al.* 2010). However, the expense of whole-genome expression analysis limits the number of mutations studied to only a few of the large number of mutations affecting each trait, and the pleiotropic effects of each mutation on multiple traits confounds our ability to infer which of many coregulated transcripts are associated with the focal trait. The second approach utilizes populations of lines suitable for linkage or association mapping of complex traits in which genome-wide expression and organismal phenotypes have been jointly assessed (Passador-Gurgel *et al.* 2007; Ayroles *et al.* 2009; Harbison *et al.* 2009; Morozova *et al.* 2009). Quantitative trait transcripts associated with the trait can then be grouped into correlated transcriptional networks associated with the trait. The limitation of this approach is that only coregulated transcripts associated with allelic variation in the study population can be assessed.

Here, we combine mutagenesis with transcriptional variation in wild-derived Drosophila lines to derive computationally predicted networks that affect alcohol sensitivity. We chose alcohol sensitivity as an example of a complex trait because alcohol is a well-defined, ecologically relevant chemical cue for localization of decomposing fruit on which flies feed, and understanding genetic networks associated with alcohol sensitivity in flies is likely to show evolutionary conservation with relevance to human alcohol consumption and addiction (Park *et al.* 2000; Rothenfluh and Heberlein 2002; Guarnieri and Heberlein 2003; Mackay and Anholt 2006; Li *et al.* 2008; Morozova *et al.* 2009). Furthermore, alcohol knockdown time can be readily and accurately measured in an "inebriometer" (Weber 1988). When exposed to alcohol, flies initially become hyperactive, but soon lose postural control and fall through the inebriometer at a rate that is correlated with their sensitivity. Following recovery, a second exposure reveals the development of tolerance reflected in a longer knockdown time (Moore *et al.* 1998; Scholz *et al.* 2000; Singh and Heberlein 2000; Cowmeadow *et al.* 2005, 2006; Scholz *et al.* 2005; Wen *et al.* 2005; Morozova *et al.* 2006, 2009; Kong *et al.* 2009).

We screened a collection of 653 co-isogenic *P[GT1]*-element insertional mutations (Bellen *et al.* 2004) and identified 162 mutations that affect alcohol sensitivity. We then constructed networks of genetically correlated transcripts centered on a subset of these loci as focal genes, using whole-genome expression data from 40 inbred wild-derived lines (Ayroles *et al.* 2009). We validated hub genes in these networks by RNAi-mediated suppression of expression of target genes and identified interconnected genes that showed altered regulation of gene expression. We then assessed the effects of those genes on alcohol sensitivity and constructed new networks around the new focal genes. This iterative approach enables the gradual buildup of genetic networks for alcohol sensitivity and resistance with simultaneous functional validation. This approach is, in principle, applicable to any complex trait that can be analyzed with mutations and for which genetically correlated transcriptional networks can be constructed.

MATERIALS AND METHODS

Drosophila stocks: Homozygous lines containing *P[GT1]* elements in or near candidate genes in a co-isogenic *Canton-S (A–F)* background were generated as part of the Berkeley Drosophila Gene Disruption Project (Bellen *et al.* 2004). Revertant alleles were generated using crossing schemes that preserved the co-isogenic background of the revertant lines. For example, we constructed chromosome *2* revertant lines by crossing w^{1118}; *P; iso3* females to w^{1118}; *CyO/Sp*; *SbΔ2–3/TM6,Tb* males. We mated male offspring of genotype w^{1118}; *CyO/P*; *SbΔ2–3/iso3* to w^{1118}; *CyO/Sp*; *iso3* females, and crossed single male offspring of genotype w^{1118}; *CyO/*P^{-}; *iso3* in which the *P* element had excised to w^{1118}; *CyO/Sp*; *iso3* females. In the following generation, we mated males and females of genotype w^{1118}; *CyO/*P^{-}; *iso3 inter se* to make a homozygous revertant stock of genotype w^{1118}; P^{-}; *iso3*. w^{1118} and *iso3* are the two isogenic chromosomes of the appropriate *Canton-S* strain; *P* refers to the chromosome with the *P*-element insertion; and P^{-} indicates a *P*-element excision allele. Similar crosses were used to construct *X* and chromosome *3* revertant alleles (Rollmann *et al.* 2006). We verified excision of the *P[GT1]* element by PCR, using primers flanking the insertion site, followed by sequencing of the amplification products.

Seven RNAi transgenic fly lines (51977^{fng}, 100739^{Thor}, 104005^{Cyp1}, 104016^{Men}, 106641^{Pdk}, 106875^{H15}, and $107177^{Mlc\text{-}c}$) and the progenitor control line (60000) were obtained from the Vienna Drosophila RNAi Center (VDRC) from the φC31 RNAi library stocks (Dietzl *et al.* 2007). An ubiquitous *tub-Gal4*^{75138} driver line was obtained from the Bloomington Drosophila Stock Center. This line was supplemented with a *UAS-GAL4* enhancer. We crossed males from each of the transgenic *UAS-RNAi* lines to virgin females from the *tub-Gal4* driver line to suppress the expression of the gene of interest in hybrid F_1 offspring.

Fly stocks were reared under standard culture conditions on cornmeal–molasses–agar medium at 25°, 60–75% relative humidity, and a 12 hr light/dark cycle. Flies were not exposed to CO_2 anesthesia for at least 24 hr prior to assays for alcohol sensitivity.

Quantitative assay for alcohol sensitivity and tolerance: We assessed ethanol sensitivity for males only from 653 lines with single *P[GT1]* transposon insertions and their co-isogenic

control line with two replicate measurements ($N = 70$ per replicate) per line. The replicates for each line were assessed on different days together with the appropriate co-isogenic control. We placed the flies in an inebriometer, a glass column with slanted mesh partitions (Weber 1988) preequilibrated with ethanol vapor, and collected them as they fell through the column at 1-min intervals. The mean elution time (MET) is a measure of alcohol sensitivity. Following the initial screen, we retested males from 210 *P[GT1]*-element insertion lines with significantly different mean elution times from their controls and females from a subset of these lines (four to five replicates per line, $N = 60$–70 per replicate). We determined alcohol sensitivity of revertant alleles simultaneously with the *P*-element mutant lines and the *Canton-S* control (five replicates per line, $N = 70$ per replicate).

We assessed both ethanol sensitivity and induction of tolerance for *tub-Gal4;UAS-RNAi* and progenitor F_1 offspring by recording elution times upon initial exposure to ethanol (E1) and a second exposure (E2) of the same flies 2 hr later (five replicates per line per sex, $N = 70$ per replicate). The scaled difference of METs between the second and first exposures is a measure of the induction of tolerance, *i.e.*, $(E2 - E1)/(0.5(E1 + E2))$ (Morozova *et al.* 2009).

Quantitative genetic and statistical analyses: We used a one-way ANOVA model with *post hoc* Dunnett's test ($Y = \mu + L + \varepsilon$) to assess differences in ethanol sensitivity between replicate measurements of each individual *P[GT1]*-element insertion line and its co-isogenic control at $P < 0.05$, where μ is the overall mean, L is the random main effect of line (*P*-element insertion *vs.* control lines), and ε is the environmental variance between replicates. The measurements for replicates were randomized over time along with appropriate contemporaneous *P*-element free *Canton-S* controls. We did not find significant differences between control replicates done on different days. We estimated the total mutational variance among lines as σ_L^2 (the among-line variance component). The total phenotypic variance is $\sigma_P^2 = \sigma_L^2 + \sigma_E^2$, where σ_E^2 is the environmental variance component. We estimated broad sense mutational heritabilities as $H^2 = \sigma_L^2/\sigma_P^2$. We quantified the standardized mutational effects of the *P*-element mutations as a/σ_P, where a is one-half of the difference between the homozygous mutant and control line, and σ_P is the phenotypic standard deviation of the control (Falconer and Mackay 1996).

Functional gene annotations are based on FlyBase (Drysdale and Crosby 2005; Tweedie *et al.* 2009) and Affymetrix (Netaffx Analysis Center, http://www.affymetrix.com) compilations. Gene ontology enrichment analysis used the DAVID program (Huang da *et al.* 2009).

To identify networks of genetically correlated transcripts, we computed the sex-centered correlation r_{ij} between all pairs of significant transcripts i and j among 40 wild-derived inbred lines (Ayroles *et al.* 2009). To enable visualization and to retain the most highly correlated transcripts, we used the absolute correlations $|r_{ij}|$ to construct transcriptional networks around focal genes at an arbitrary threshold level determined for each gene individually.

Quantitative RT–PCR: We quantified mRNA levels by quantitative RT–PCR with the SYBR Green detection method according to the protocol from Applied Biosystems (Foster City, CA), using the ABI PRISM 7900 sequence detection system (Applied Biosystems). We used the *glyceraldehyde-3-phosphate dehydrogenase* gene as an internal standard. Three independent replicates of total RNA were isolated from *P[GT1]*-element insertion lines or *tub-GAL4;UAS-RNAi* and progenitor line F_1 offspring using the Trizol reagent (GIBCO-BRL, Gaithersburg, MD), and cDNA was generated from 100 ng of total RNA by reverse transcription using the high capacity cDNA reverse transcription kit (Applied Biosystems). Primer Express software (Applied Biosystems) was used to design transcript-specific primers to amplify 100- to 150-bp regions of genes of interest. Primers were designed to encompass common regions of alternative transcripts. Negative controls without reverse transcriptase were used for all genes to exclude potential genomic DNA contamination. Samples in each run were normalized relative to a control sample, using $2^{-\Delta\Delta Ct}$ values (Abi 2001). Statistical significance for differences in gene expression levels was determined by two-tailed Student's *t*-tests on ΔCt values.

To examine developmental stage-specific gene expression levels in *P[GT1]*-element insertion lines and revertant alleles, we analyzed relative levels of expression as described above after extraction of triplicate RNA samples from embryos between 5 and 8 hr after oviposition, third instar larvae, pupae, adult heads and bodies, and whole adult flies.

RESULTS

To identify transcriptional networks for increased sensitivity or resistance to ethanol exposure we first used mutational analysis to identify genes that directly impact alcohol sensitivity in an isogenic background. We then capitalized on natural variation in wild-derived inbred lines to integrate these genes into coregulated networks. Critical hub genes in these networks were then verified by quantitative RT–PCR.

Identification of co-isogenic *P[GT1]*-transposon insertion lines with altered alcohol sensitivities: We measured the METs of 653 lines with single *P[GT1]*-transposon insertional mutations in common *Canton-S (A–F)* genetic backgrounds (Lukacsovich *et al.* 2001; Bellen *et al.* 2004). We found significant variation in alcohol sensitivity among the *P[GT1]*-insertion lines (Figure 1 and supporting information, Table S1). The average deviation of METs from the control for all 653 lines was 0.5 ± 0.2 min, with a range from -3.7 min to 7.7 min. The average mutational effect (Falconer and Mackay 1996) was 0.45 min, with a range of -0.7 to 1.7 min. The broad sense mutational heritability for alcohol sensitivity was $H_M^2 = 0.17$. The range of variation in alcohol sensitivity among the *P*-element insertion lines is comparable to that previously observed among 40 wild-derived inbred lines after a single ethanol exposure with broad sense heritability of 0.244 (Morozova *et al.* 2009).

We identified 210 *P[GT1]* lines with significant deviations from the controls from the initial screen, and retested these lines with five additional replicates per assay. The second screen confirmed that 162 mutant lines were significantly different from the control (77% of the lines detected in the initial screen and 28.7% of all 653 lines tested), with 27 sensitive and 135 resistant lines (Table S2). These 162 lines included 139 unique *P[GT1]*-insertion sites with 124 insertions in or near known genes (Table S2). Seventy-two lines are significantly different from the control even after Bonferroni correction for multiple testing ($P < 0.0003$). Only a few sensitive

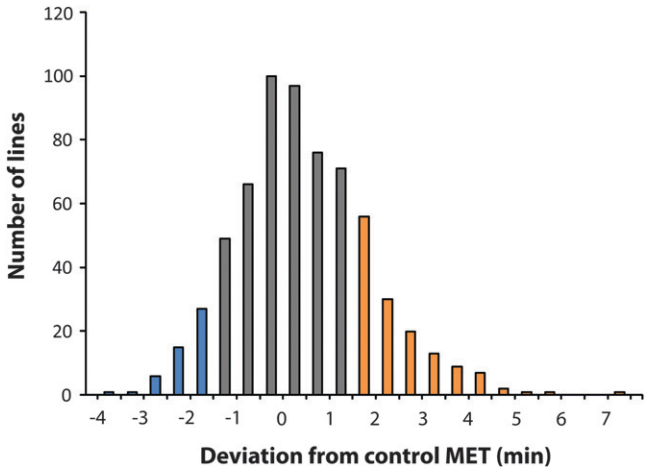

Figure 1.—Distribution of mean elution times (MET) of 653 *P[GT1]*-insertion lines. Data are standardized by calculating the deviation of the MET for each line from its contemporaneously tested control line. The MET (±SE) was 7.3 ± 0.03 min for males for the *Canton-S (A)* control line; 6.0 ± 0.01 min for the *Canton-S (B)* control line; 5.9 ± 0.07 min for the *Canton-S (C)* control line; 7.1 ± 0.11 min for the *Canton-S (D)* control line; 4.0 ± 0.02 min for the *Canton-S (E)* control line and 4.96 ± 0.011 min for the *Canton-S (F)* control line.

lines were confirmed after retesting, because the inebriometer assay is biased toward detecting alcohol resistance rather than hypersensitivity—the time it takes for flies to pass through the inebriometer sets the low threshold for the assay. In addition, *Canton-S (E)* flies had an elution time of 3.9 ± 0.1 min, precluding the detection of hypersensitivity in this genetic background.

The insertion site of the *P[GT1]*-element could be determined unambiguously in all but 5 of the 162 lines. A total of 10 insertions were in regions with no annotated gene within 2 kb of the *P[GT1]*-element insertion site. Several genes were tagged by multiple *P[GT1]*-element insertions (*e.g.*, *Beadex*, *capricious*, *Calreticulin*, *High mobility group protein D*, *Malic enzyme*, *Mnt*, *Thor*, *wing blister*, Table S2). In addition, 51 out of 162 lines with altered alcohol sensitivity encode predicted transcripts with unknown functions.

Gene ontology analysis and correlated phenotypes: Gene ontology (GO) analysis reveals diverse categories of genes that confer increased sensitivity or resistance to ethanol (Figure 2). Two GO categories are prominently associated with increased sensitivity to ethanol: olfactory behavior and nervous system development. The group of lines with increased resistance to ethanol exposure also showed enrichment for the "nervous system development" category, but in addition was enriched for genes associated with imaginal disc, organ, tissue and sensory organ development, as well as cell motility and neurogenesis. Most of the mutations that affect alcohol sensitivity have pleiotropic effects on other complex traits, such as startle-induced locomotion (Yamamoto *et al.* 2008), aggression (Edwards *et al.* 2009b), sleep (Harbison and Sehgal 2008), and starvation stress resistance (Harbison *et al.* 2005) (Table S3). We found significant positive correlations with aggression ($N = 43$, $r = 0.35$, $P = 0.012$) (Edwards *et al.* 2009b), lifespan ($N = 142$, $r = 0.32$, $P < 0.0001$) (Magwire *et al.* 2010), and 24 hr sleep ($N = 22$, $r = 0.52$, $P = 0.008$; Figure S1) (Harbison and Sehgal 2008).

To assess the correlation for alcohol sensitivity between the sexes, we examined differences in responses to a single ethanol exposure between males and females from 11 sensitive and 16 resistant *P[GT1]*-element insertion lines. We did not observe significant sex differences ($r = 0.98$, $P < 0.0001$; Figure S2). This lack of sexual dimorphism agrees with our previous study on alcohol sensitivity in 40 inbred wild-derived lines (Morozova *et al.* 2009), but contrasts with other traits examined in these lines, including sleep (Harbison and Sehgal 2008), startle behavior (Yamamoto *et al.* 2008), and olfactory avoidance behavior (Sambandan *et al.* 2006), where significant differences in mutational effects between the sexes have been reported.

Analysis of *P[GT1]*-element revertant alleles: To demonstrate that the effects on alcohol sensitivity were due to the *P[GT1]* insertions rather than unrelated mutations in the genome, we excised the *P[GT1]* elements in a subset of the mutants. We chose two sensitive (*Sip1* and *psq*) and five resistant (*Fs*, *CG14430*, *CG14591/SCAP*, *Men*, and *Osi9*) *P[GT1]*-element mutations and created revertant alleles for these lines. In addition, we used a previously generated revertant for BG01214 [*sugarless* (*sgl*)] (Edwards *et al.* 2009b) and BG01272 (*Tre1/Gr5a*) (Figure S3; Rollmann *et al.* 2006). We sequenced the revertant alleles and identified at least one precise revertant for each line except *CG14430* and *Tre1/Gr5a*, where excision was imprecise.

We showed that the MET of the excision lines reverted to the control phenotype, including the imprecise excisions of *CG14430* and *Tre1/Gr5a* (Figure 3A). The *Osi9* revertant allele showed a MET significantly lower than both the mutant and control lines, possibly due to a mutation created during mobilization of the *P[GT1]* element. We characterized gene expression of the mutations and revertant alleles using qRT–PCR analysis at four developmental stages: embryos, larvae, pupae, and adults, as well as adult heads and bodies, separately (Figure 3B). All mutant lines showed changes in levels of expression in one or more developmental stages. Expression of *psq* is higher in the *P[GT1]*-element insertion line at all developmental stages, except embryos, as observed previously (Sambandan *et al.* 2006). Transcript levels in *Sip1* and *Fs* mutants were lower in pupae but increased in adults. *Men* and *sgl* mutants were associated with decreased transcript levels throughout development. *Osi9* and *Tre1* mutants had reduced levels of gene expression in embryos but increased levels at all other stages except pupae. Transcript levels in the *SCAP* mutant were increased in adults, but decreased in larvae. Finally, the *CG14430* mutant had reduced levels

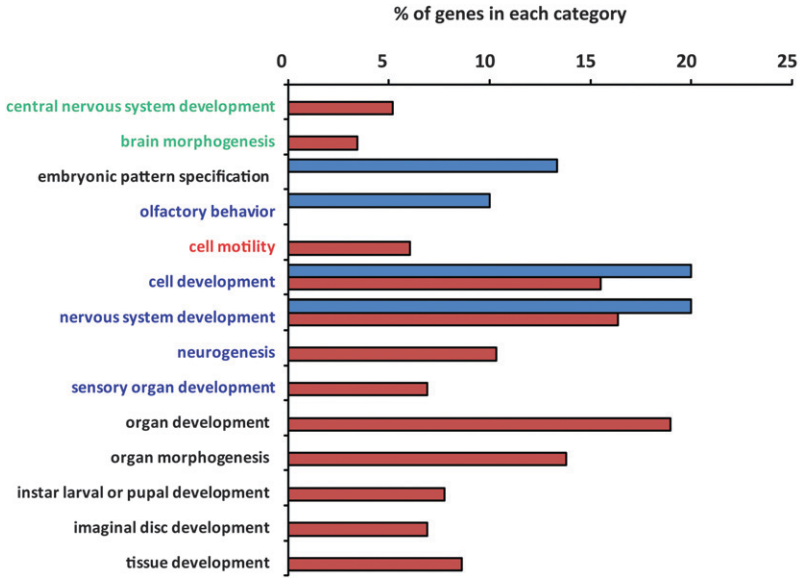

Figure 2.—Biological function gene ontology categories of candidate genes with mutations associated with ethanol sensitivity. The bars indicate the percentage of genes in each overrepresented category for mutations increasing (blue bars) and decreasing (red bars) alcohol sensitivity compared to controls at $P < 0.05$. GO categories that contain overlapping genes are indicated with the same font color.

of gene expression in embryos and pupae, and increased levels in adult bodies. These results are consistent with the notion that insertions of transposons in regulatory regions of genes can alter their temporal expression during development, as reported previously (Sambandan *et al.* 2006).

To characterize revertant alleles further, we estimated transcript levels of the candidate genes at those developmental stages at which *P[GT1]*-element insertions significantly affected expression levels (Figure 3C). Transcript levels of revertant alleles were not different from controls for all candidate genes with the exception of *Men* and *CG14430*, where transcript levels were significantly different from both mutant and control. This cannot be readily explained, as the *P[GT1]*-element excision for *Men* was precise. For *CG14430*, partial restoration of transcript level can be explained by imprecise excision of the *P* element. Alcohol sensitivity for both of these revertant alleles, however, was not distinguishable from the control (Figure 3A).

Systems genetics analysis: We took advantage of a previous study, which quantified genetic variation for 10,096 transcripts among 40 wild-derived inbred lines (Ayroles *et al.* 2009). These data have been used previously to regress phenotypic values of traits of interest on transcript abundance to identify candidate genes affecting these phenotypes, including alcohol sensitivity and tolerance (Morozova *et al.* 2009), aggression (Edwards *et al.* 2009a), and sleep (Harbison *et al.* 2009). Here, we assessed the extent to which the 10,096 transcripts were genetically correlated with genes affecting alcohol sensitivity identified in this study. This approach is limited by two assumptions, which require verification. First, we are assuming that genome-wide effects of mutational variation on the transcriptome will be the same as segregating variation. Second, we assume that genetic correlation is the result of coregulation of gene expression. If these assumptions are correct (as we will argue below), then we are justified in utilizing previously characterized transcriptional genetic networks from a natural population to infer coregulated networks in the mutant backgrounds.

We built transcriptional genetic correlation networks using 9 genes with mutations associated with increased alcohol sensitivity and 12 genes with mutations associated with alcohol resistance as focal genes and the transcriptional profiling data from the 40 wild-derived inbred lines (Ayroles *et al.* 2009). This analysis reveals highly interconnected transcriptional networks for each focal gene (Figure 4). Thirty-two genes occur in two or more networks. Thirteen of these 32 genes encode predicted transcripts of unknown functions. Correlated transcripts interconnect not only within alcohol-related phenotypic groups (*i.e.*, networks around focal genes associated with enhanced sensitivity or resistance to alcohol), but also between phenotypic groups, indicating the complex genetic architecture that underlies alcohol sensitivity.

Gene ontology analyses for each predicted coregulated transcriptional network separately reveals that the major biological function categories that overlap between sensitive and resistant gene networks include regulation of primary metabolic process, nervous system and organ development, oogenesis, cell cycle phase, and positive and negative regulation of cellular process (Figure 5). At the same time transcripts affecting transcription, embryonic axis specification, and immune system development are enriched in transcriptional networks associated with increased sensitivity to alcohol. Transcripts affecting RNA processing are only associated with the *CG10778* network. Synaptic transmission, response to light, and oxidative phosphoryla-

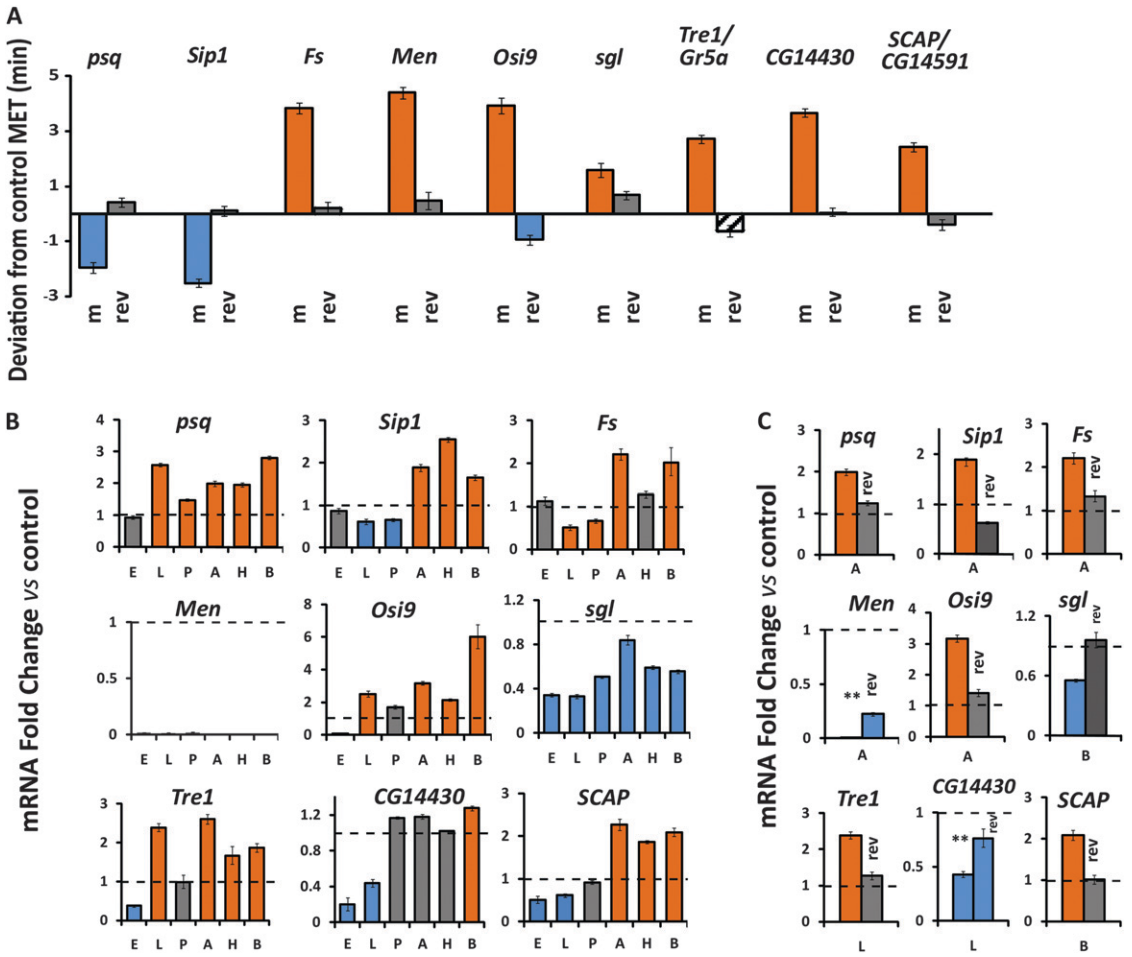

Figure 3.—Phenotypic reversion of alcohol sensitivity by *P*-element excisions. (A) METs of *P[GT1]*-element insertion lines and revertant alleles. METs are shown as deviations from the *Canton-S (B)* control line ± SE. Blue bars indicate sensitive lines with MET lower than the control ($P < 0.05$); orange bars indicate resistant lines with MET higher than the control ($P < 0.05$); gray bars indicate no significant difference in MET; the hatched bars indicate an imprecise revertant allele. m, mutant; rev, revertant line. (B) Quantitative RT–PCR analysis of candidate genes affecting alcohol sensitivity at different developmental stages. Levels of mRNA for *P[GT1]*-element insertion line are indicated relative to the level in the *Canton-S (B)* control (dotted line). Blue bars indicate expression levels that are lower than the control; orange bars indicate expression levels that are higher than the control ($P < 0.05$). Gray bars indicate no significant difference in mRNA expression levels. mRNA expression was assessed at four developmental stages: embryos aged 10–12 h after egg laying (E), third instar larvae (L), pupae (P), adults (A), and adult heads (H) and bodies (B). Standard errors were obtained using Ct values normalized to an internal control (*Gpdh*). (C) Quantitative RT–PCR analysis of revertant alleles (rev) of candidate genes at selected developmental stages at which *P[GT1]*-element insertion affects expression (A, adults; B, bodies; L, third instar larvae). $^{**}P \leq 0.001$ indicates a significant difference between mutant and revertant alleles.

tion appear as unique gene ontology categories for transcriptional networks associated with increased resistance to alcohol. Genes annotated as being associated with translation are only apparent in the *CG14430* network, where fatty acid and oxoacid metabolic processes are significantly interconnected in the transcriptional network around the *Men* focal gene. Finally, gene products implicated in signal transduction are enriched in the *Tre1* module.

Previously, we identified 1,133 transcripts associated with one or more alcohol phenotypes by regressing phenotypic values on transcript abundance among the 40 wild-derived inbred lines (Morozova *et al.* 2009) as well as 2,615 transcripts associated with correlated transcriptional responses to divergent artificial selection for alcohol sensitivity and resistance (Morozova *et al.* 2007). Here, we combined identification of candidate genes by *P[GT1]*-element insertional mutagenesis with whole-genome transcriptional data to build networks of highly interconnected genes on the basis of genetic correlations among transcripts in a natural population. We predicted to find more overlap between our previously documented transcriptional networks and the networks shown in Figure 4 than expected by chance. Indeed, we found 78 transcripts and 95 transcripts that were in common ($\chi_1^2 = 23$, $P < 0.0001$ and $\chi_1^2 = 26$, $P < 0.0001$) between the present study and Morozova *et al.* (2009) and Morozova *et al.* (2007),

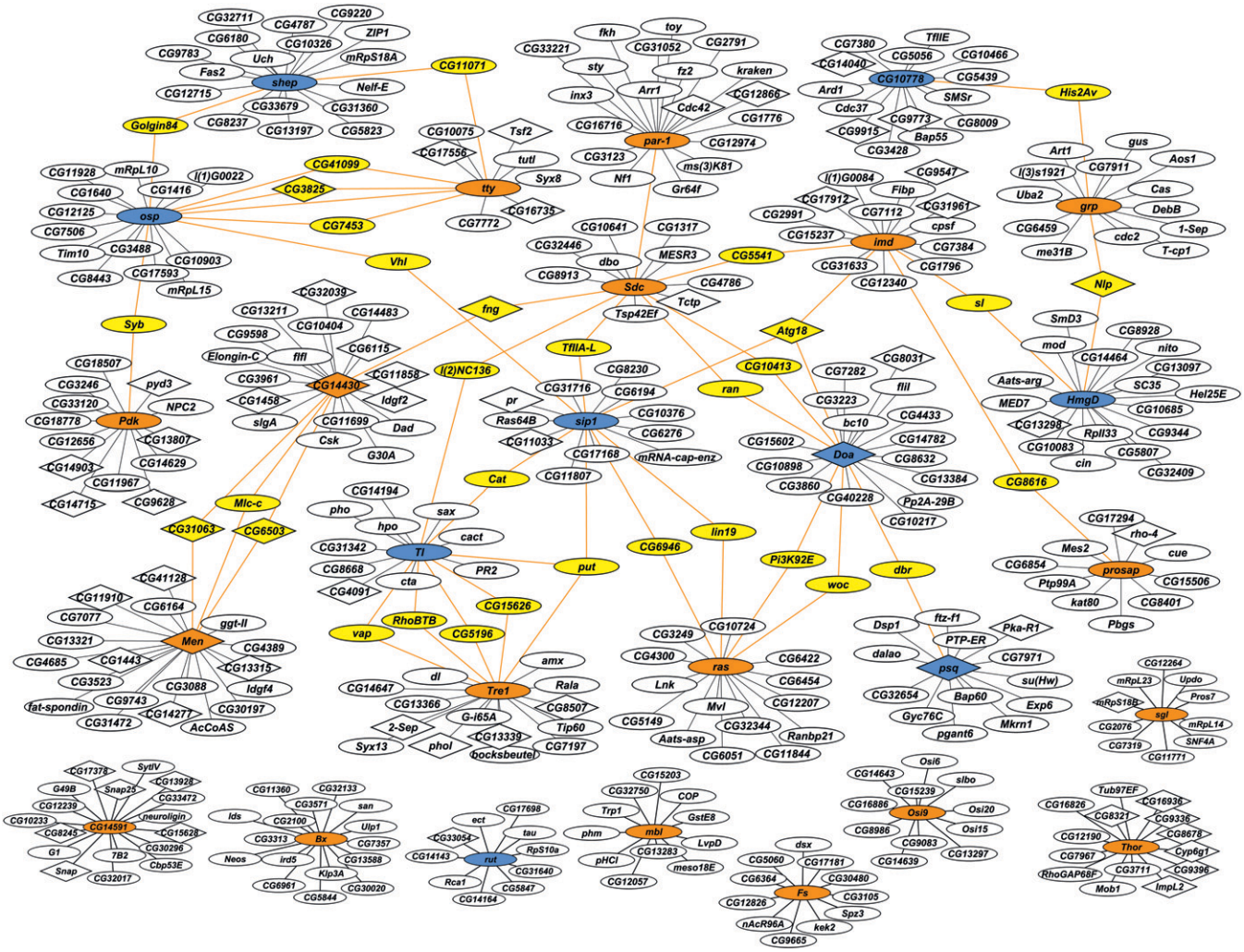

FIGURE 4.—Highly connected networks centered on focal candidate genes affecting alcohol sensitivity. Focal genes for which *P[GT1]*-element insertions are associated with increased alcohol sensitivity are shown on a blue background, and focal genes for which mutations are associated with increased alcohol resistance are shown on an orange background. For visualization, only the most strongly correlated transcripts are depicted for each focal gene. Absolute transcriptional genetic correlations for all genes in networks around focal genes are ≥0.8 for *grp*; ≥0.75 for *Tl*; ≥0.7 for *ras*, *Tre1*, *CG10778*, and *Sip1*; ≥0.6 for *Bx*, *osp*, *Sdc*, and *shep*; ≥0.55 for *Doa*, *HmgD*, and *psq*; ≥0.5 for *CG14430*, *CG14591*, *Thor*, *sgl*, *tty*, *Osi9*, and *Pdk*; ≥0.45 for *Men*, *Prosap*, and *par-1*; ≥0.4 for *imd*, *Fs*, *mbl*, and *rut*. Genes shared by more than two different networks are indicated on a yellow background and connected by orange lines. Diamond shapes indicate transcripts previously associated with repeated ethanol exposures (MOROZOVA *et al.* 2009).

respectively, including 11 focal candidate genes (*Bx*, *CG14430*, *CG14591*, *Doa*, *Men*, *Pdk*, *Prosap*, *psq*, *Sdc*, *Sip1*, and *Tl*; Figure 4). Fifty-five of these transcripts encode gene products of unknown functions (Table S4). Imaginal disc, nervous system, organ development, and olfactory behavior were enriched among the major biological function gene ontology categories for the common transcripts.

The complex interconnectivity of the networks can be broken down into subsets of focal genes and associated networks that contribute to resistance to alcohol (Figure 6A). This reveals an underlying network structure of hub genes associated with alcohol resistance, including *Cyp1*, *fng*, *H15*, and *Mlc-c*.

Validation of candidate genes and interconnected networks: We used RNAi lines from the Vienna Drosophila Research Center collection (DIETZL *et al.* 2007) to validate candidate genes found by the *P[GT1]*-element screen and to test our computationally predicted networks. We focused on a subset of candidate genes in which *P[GT1]*-element insertions lead to increased alcohol resistance, including *Men*, *Pdk*, and *Thor* (Figure 6A).

To validate the connectivity of the predicted networks, we chose four genes, *Cyclophilin 1* (*Cyp1*), *fringe* (*fng*), *H15*, and *Myosin light chain cytoplasmic* (*Mlc-c*) that were common for two or more predicted networks (Figure 6A). We crossed lines containing *UAS-RNAi*

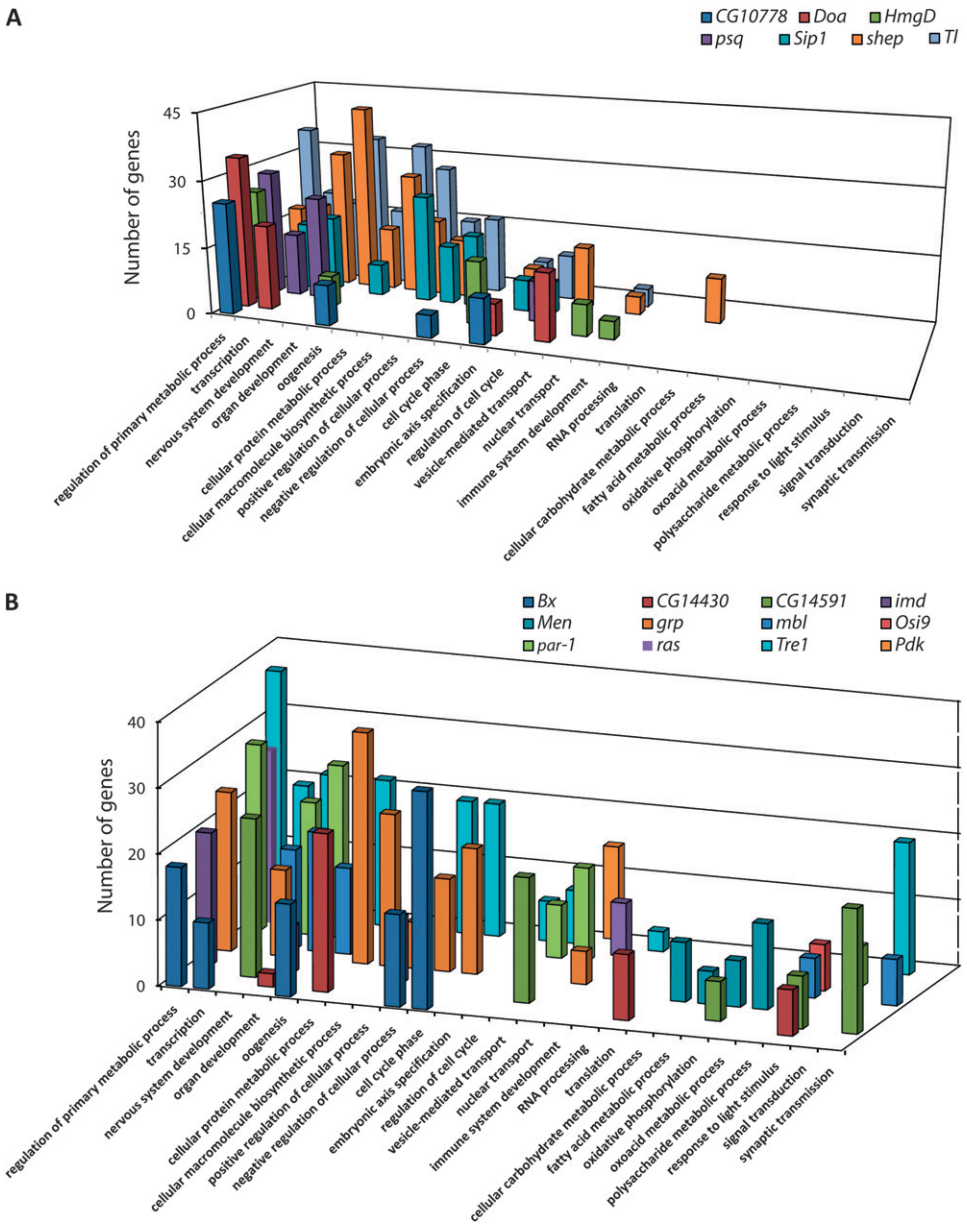

FIGURE 5.—Overrepresentation of gene ontology biological function categories for gene networks centered on focal candidate genes associated with increased ethanol sensitivity (A) and increased ethanol resistance (B). Focal genes from Figure 4 are color coded and the numbers of genes overrepresented in each network associated with these genes are represented by bars in corresponding colors ($P < 0.05$).

constructs for each target gene and the progenitor control to a ubiquitous *tubulin-Gal4*; driver line to suppress the expression of the target genes. F_1 *tub-GAL4;UAS-RNAi* hybrids were used for both molecular and behavioral experiments. The efficiency of RNAi-mediated suppression of individual target genes in *tub-GAL4;UAS-RNAi* F1s was assessed by qRT–PCR measured on whole flies (Figure 6B). Transcript levels of the target genes were suppressed in all cases compared to the progenitor control line and varied from 20% (*H15* females) to 90% (*Cyp1* males) reduction in gene expression. Next, we measured METs after an initial exposure to ethanol (E1) and after a second exposure 2 hr later (E2). As illustrated in Figure 6C, RNAi-mediated suppression of *fng*, *Mlc-c*, and *Pdk* resulted in greater sensitivity to a single ethanol exposure than the control, while RNAi-mediated suppression of *Cyp1*, *H15*, *Men*, and *Thor* resulted in enhanced resistance. A second exposure showed development of tolerance through a shift in MET, except for *Mlc-c* and *Pdk* males. The MET averaged over all RNAi hybrids was greater after the second (11.1 min) than the first exposure (7.8 min), again indicating the development of tolerance. We also quantified tolerance (T) as the shift in MET between the first and second exposures, scaled by their average values ($T = (E2 - E1)/0.5(E1 + E2)$). Overall, we did not find significant changes in tolerance with the exception of *fng*, which showed a significant increase in tolerance in both sexes (Figure 6C).

In summary, using *UAS-RNAi* lines we independently confirmed that the three candidate genes tested (*Men*, *Pdk*, and *Thor*) contribute to alcohol sensitivity. In addition we showed that genes in the same transcriptional genetic network as *Men*, *Pdk*, and *Thor* (*Cyp1*, *fng*, *H15*, and *Mlc-c*) also affect alcohol sensitivity.

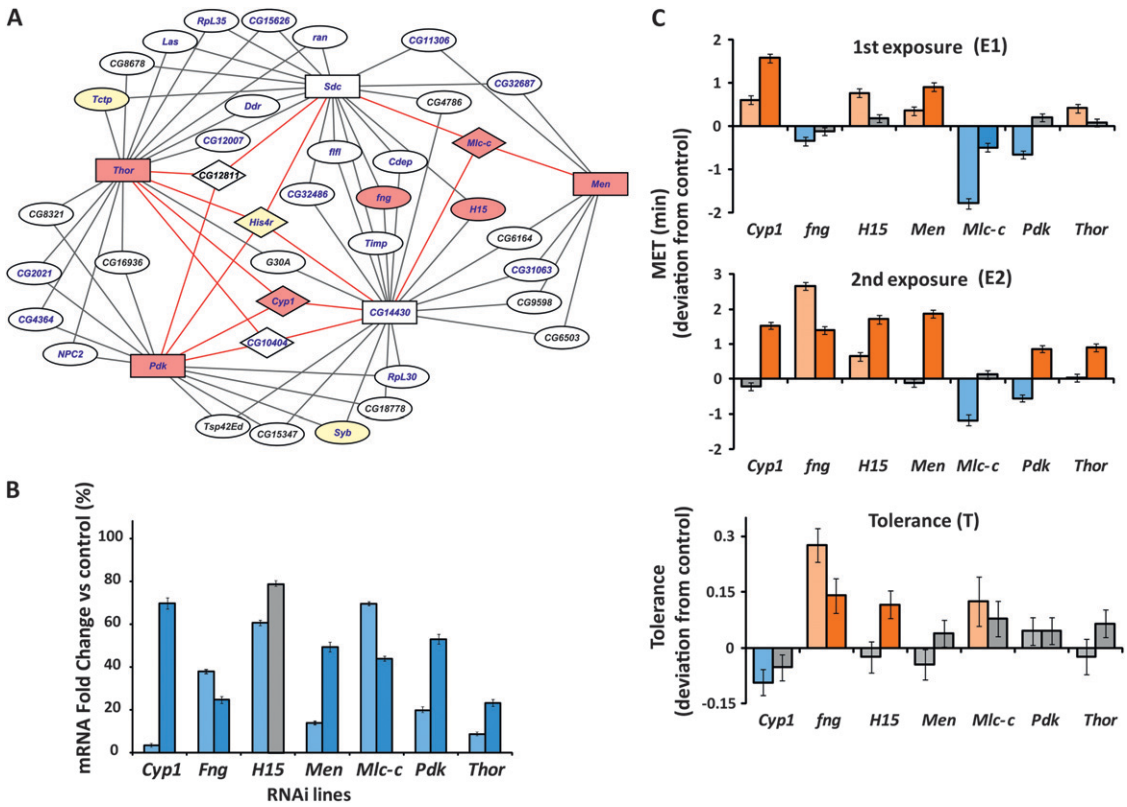

FIGURE 6.—Validation of computationally predicted genetic networks. (A) Highly interconnected genetic network associated with alcohol resistance. Rectangles indicate the most highly connected focal genes from Figure 4 used to build the network. RNAi targeted genes used to validate the connectivity are shown on a pink background. All genes depicted were shared by at least two networks and have absolute transcriptional genetic correlations $|r| \geq 0.45$ for *Men*; ≥ 0.50 for *CG14430*, *Pdk*, and *Thor*; and ≥ 0.6 for *Sdc*. Additional genes that were selected for further analysis are indicated on a yellow background. Drosophila genes with annotated human orthologs are indicated in blue font. (B) Relative fold changes in mRNA for RNAi targeted genes. Levels of mRNA for each gene are depicted relative to the level of the control (equals 100% of the mRNA expression level). Blue bars indicate expression levels that are lower than the control with lighter bars indicating males and darker bars, females. Gray bars indicate no significant difference in mRNA expression levels. Standard errors were obtained using Ct values normalized to an internal control (*Gpdh*). (C) Variation in MET among *tub-GAL4;UAS-RNAi* lines after a first (E1) and a second (E2) exposure to ethanol and as a results of tolerance ($T = (E2 - E1)/0.5(E1 + E2)$). MET is shown as the deviation from the control line ± SE, with males in lighter shades and females in darker shades for each gene. Blue bars indicate sensitive lines with MET lower than the control ($P < 0.05$); orange bars indicate resistant lines with MET higher than the control ($P < 0.05$); gray bars indicate no significant difference in MET.

Predictive value of transcriptional connectivity: Previous studies showed that networks of correlated transcripts can be used to identify new candidate genes affecting a phenotype (EDWARDS *et al.* 2009a; HARBISON *et al.* 2009; MOROZOVA *et al.* 2009). We tested whether we could use an iterative process to expand our networks by constructing new networks of correlated transcripts. We used *Cyp1*, *fng*, *H15*, and *Mlc-c* as focal genes together with transcriptional profiling data from the 40 wild-derived inbred lines (AYROLES *et al.* 2009) to assess the genome-wide connectivity of these four transcripts. This resulted in a highly connected ensemble of 85 genes, with 65, 17, and 3 genes shared by two, three, and four modules around the focal genes, respectively (Figure 7A). The strength of absolute correlation of the transcripts for these networks was surprisingly high: ≥0.65 for *H15* and *fng*, and ≥0.75 for *Mlc-c* and *Cyp1*. Biological function GO categories of the 85 transcripts showed enrichment for protein metabolic processes, microtubule cytoskeleton organization, vesicle-mediated transport, and translation. Thirty nine of the 85 transcripts from computationally predicted networks were common between differentially expressed transcripts from at least one of the previous studies (MOROZOVA *et al.* 2006, 2007, 2009) (Table S5). It should be noted that 82% of these Drosophila transcripts have human orthologs.

Since expression levels of the focal genes in corresponding *tub-GAL4;UAS-RNAi* lines were suppressed (Figure 6B), we predicted that transcripts genetically correlated with the focal genes would also show altered transcript levels in the *Cyp1*, *fng*, *H15*, and *Mlc-c* RNAi backgrounds. Indeed, expression of the most highly interconnected transcripts, such as *Histone H4 replacement* (*His4r*), *Tctp*, and *Synaptobrevin* (*Syb*) were altered in the RNAi-hybrid lines in both sexes (Figure 7B). Since

these transcripts were among the correlated transcripts in networks containing *Pdk* and *Thor*, we tested their expression levels in *tub-GAL4;UAS-RNAi* hybrids with reduced expression of *Pdk* and *Thor*. Here, we also found reduced expression levels of the *His4r*, *Tctp*, and *Syb* transcripts (Figure 7B).

DISCUSSION

We have applied a combination of mutational and computational approaches using *P*-element insertional mutagenesis while capitalizing on naturally occurring variation in genome-wide transcript abundance levels to predict transcriptional networks that determine alcohol sensitivity in *D. melanogaster*. This approach enables a systematic walk through the transcriptome to identify interconnected hub genes that can serve as landmarks for iterative expansion of the network. The hub genes also serve as cornerstones for subsequent comparative genomic approaches in which orthologous networks for alcohol-related phenotypes in other species, including humans, can be superimposed on the transcriptional network for alcohol sensitivity in Drosophila. The feasibility of such a translational approach has been demonstrated previously by implicating cytoplasmic malic enzyme 1 in human drinking behavior on the basis of the identification of *Men* as a gene associated with alcohol sensitivity in Drosophila (Morozova *et al.* 2009).

***P[GT1]*-element mutations affecting alcohol sensitivity:** Approximately 29% of the *P*-element mutations tested for alcohol sensitivity and resistance differ significantly from the co-isogenic *P*-element free controls. This percentage demonstrates a large mutational target for alcohol sensitivity and indicates that a large fraction of the genome contributes to alcohol sensitivity, implicating extensive pleiotropy (Table S3). Indeed, most of the mutations that affect alcohol sensitivity have pleiotropic effects on other complex traits, including aggression (Edwards *et al.* 2009b), life span (Magwire *et al.* 2010), and sleep (Harbison and Sehgal 2008) (Table S3).

The large mutational target appears to be a characteristic of quantitative traits. For example, ~37% of *P*-element insertion lines tested for startle-induced locomotion differed significantly from the *P*-element–free, isogenic *Canton-S* control line (Yamamoto *et al.* 2008). About 6% of *P*-element insertion lines had aberrant olfactory avoidance behavior (Sambandan *et al.* 2006). A total of 35% of the *P*-element insertion lines tested for aggression exhibited levels of aggression that differed significantly from the control (Edwards *et al.* 2009b). Fifteen percent of *P*-element insertions with known effects on physiology, development, and behavior affect 24-hr sleep time (Harbison and Sehgal 2008). The numbers of abdominal and sternopleural bristles were affected by 38.3% of co-isogenic *P*-element insertions (Norga *et al.* 2003).

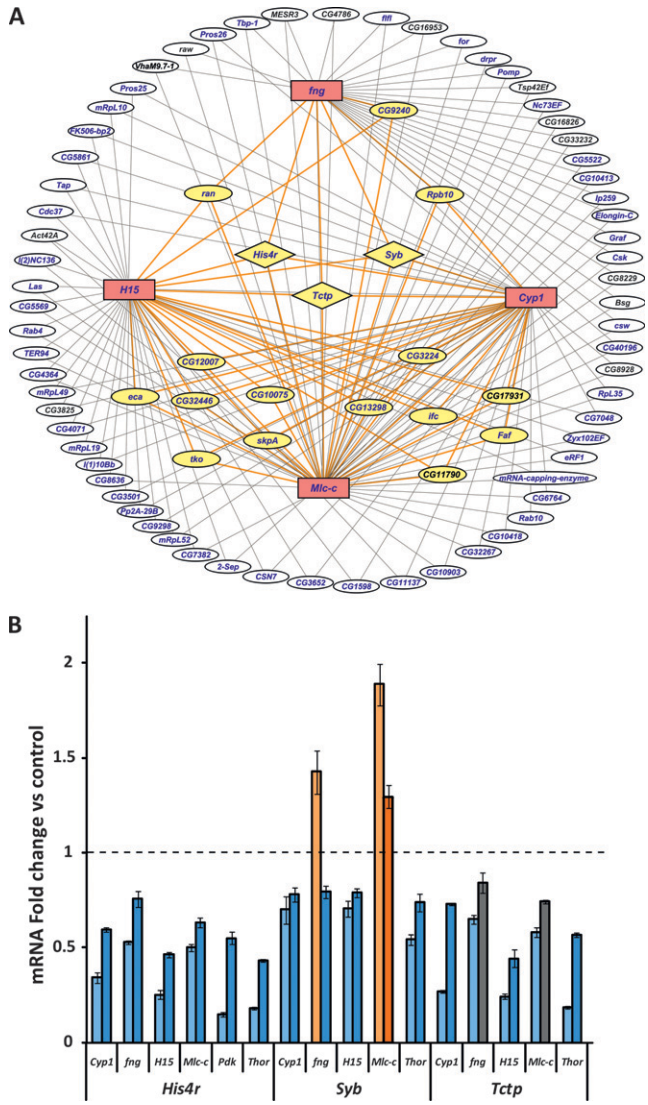

Figure 7.—An expanded network for alcohol sensitivity derived from highly interconnected genes. (A) Hierarchical network of interconnected genes based on connectivity with the focal genes from Figure 6A (red squares). Only genes connected to more than two of the focal genes from Figure 6A are indicated. Absolute transcriptional genetic correlations were ≥0.65 for transcripts clustered around *H15* and *fng*, and ≥0.75, for transcripts clustered around *Mlc-c* and *Cyp1*. Genes connected to two focal genes are shown at the periphery of the circle in white ovals and connected by gray lines. Genes interconnected by three and more networks are indicated on a yellow background and connected by orange lines. Diamond shapes indicate genes connected to all four focal genes. Drosophila genes with annotated human orthologs are indicated in blue. (B) Relative fold changes in mRNA levels for transcripts covariant with *His4r*, *Syb*, and *Tctp* in lines in which expression of *His4r*, *Syb*, and *Tctp* is suppressed by RNAi. Levels of mRNA for each gene are depicted relative to the level in the control (dashed black line). Blue bars indicate expression levels that are lower than the control. Orange bars indicate expression levels that are higher than the control. Gray bars indicate no significant difference in mRNA expression levels. Data for males are in lighter shades; data for females are in darker shades. Standard errors were obtained using Ct values normalized to an internal control (*Gpdh*).

We found a significant positive correlation between alcohol sensitivity and the number of aggressive encounters under conditions of competition for a limited food source (Figure S1). Thus, mutations associated with ethanol resistance are also associated with increased aggressive behavior. We also found a positive correlation with 24-h sleep and lifespan (Figure S1). Thus, mutations associated with resistance to ethanol exposure sleep longer and live longer.

Several of the genes implicated in alcohol sensitivity in Drosophila in this study have been implicated previously, either as genes that are differentially expressed after repeated exposures to ethanol in a *Canton-S (B)* background (Morozova *et al.* 2006), genes associated with correlated transcriptional responses to divergent artificial selection for alcohol sensitivity and resistance (Morozova *et al.* 2007), or genes computationally implicated in transcriptional networks for alcohol sensitivity and tolerance in 40 inbred wild-derived lines (Morozova *et al.* 2009) (Table S6). Genes that were consistently identified in all studies include *CG12505*, *lola*, *Men*, and *Pdk*. *CG12505* is also involved in starvation resistance (Mattaliano *et al.* 2007). *lola* encodes a transcription factor required for axon growth and guidance in Drosophila (Giniger *et al.* 1994), and plays a role in aggression (Edwards *et al.* 2009b) and startle behavior (Yamamoto *et al.* 2008). *Men* and *Pdk* encode malic enzyme and pyruvate dehydrogenase kinase, respectively, and function in energy metabolism (Morozova *et al.* 2006).

Systems genetics analysis: *P*-element insertional mutations can cause widespread alterations in transcript levels throughout the genome (Anholt *et al.* 2003; Harbison *et al.* 2005; Rollmann *et al.* 2006). It is reasonable to hypothesize that genes that are coregulated in mutant backgrounds are themselves candidate genes affecting the trait. Here, we used a combination of *P*-element insertional mutagenesis and analysis of natural variation to derive computational networks that underlie alcohol sensitivity in *D. melanogaster*. An iterative approach, which uses RNAi-mediated inhibition of expression of focal genes to expand computational networks, while at the same time confirming effects of hub genes on the phenotype, presents a paradigm for stepwise expansion of genetic networks associated with a specific phenotype. Gene ontology analysis of networks built around hub genes associated with high or low sensitivity to alcohol exposure that contain genes associated with specific biological processes (*e.g.*, RNA processing, synaptic transmission, signal transduction), whereas other networks are composed of more diverse covariant transcripts. Despite this complexity, *P*-element mutagenesis allows the identification of gene ensembles associated with high sensitivity or resistance to alcohol exposure. The boundaries between these networks are, however, fluid, as is evident from our observation that *P*-element insertions in genes that affect alcohol sensitivity can either be recruited in networks associated with low or high sensitivity depending on the precise insertion site and consequently the precise effect of the *P* element on transcription.

We used 9 *P*-element–tagged alleles associated with increased alcohol sensitivity, and 12 alleles associated with alcohol resistance to build correlated transcriptional networks associated with each hub gene (Figure 4). We can validate the predicted transcriptional networks by measuring transcript levels of genes connected to a *P*-element–tagged focal gene. For example, a previous study showed that expression levels of *CG31063*, *CG6503*, *CG1443*, *CG14277*, *CG12262*, *CG32032*, *CG11200*, *CG4786*, *CG5399*, and *Obp18a* were altered in the mutant $Men^{BG02365}$ *P*-element insertion background (Morozova *et al.* 2009). It should be noted that *CG31063*, *CG5399*, *CG6503*, *CG14277*, and *CG32032* have previously unknown biological functions; thus, their connectivity within a network associated with a phenotype, *i.e.*, alcohol sensitivity, provides a functional annotation for these genes.

The complexity of interconnections between clusters and the number of hub genes could be simplified by considering only a subset of genes with *P*-element–tagged alleles, including *CG14430*, *Men*, *Pdk*, *Sdc*, and *Thor*, as focal genes. All five mutant lines have increased ethanol resistance relative to the control. *CG14430*, *Men*, and *Pdk* have been associated previously with changes in transcript abundance after a second exposure to ethanol (Morozova *et al.* 2009); *Men* and *Pdk* also showed differential expression after artificial selection for alcohol sensitivity (Morozova *et al.* 2007) and after repeated exposures to ethanol in the *Canton-S (B)* genetic background (Morozova *et al.* 2006). Repeated exposures to ethanol also resulted in changes in *Thor* transcript abundance (Morozova *et al.* 2006). We built transcriptional networks around these genes (Figure 6) and showed that RNAi targeted against *Men*, *Pdk*, and *Thor*, *Mlc-c*, *H15*, *Cyp1*, and *fng* decreases their expression. We confirmed not only that expression of these genes was down-regulated, but also that alcohol sensitivity in RNAi lines targeting these genes was altered upon repeated exposure to ethanol (Figure 6). We then chose *Mlc-c*, *H15*, *Cyp1*, and *fng* for further validation, as these genes emerged as hub genes.

Next, we constructed new predicted networks of genes using *Cyp1*, *fng*, *H15*, and *Mlc-c* as focal genes. Unexpectedly, we found an even more highly connected network of 85 genes shared by two and more gene clusters (Figure 7). We chose *His4r*, *Syb*, and *Tctp* for validation because they showed the highest interconnectivity level and were correlated with one of the focal genes in Figures 4 and 6. In addition, *His4r* and *Tctp* showed differential expression after a second ethanol exposure in 40 wild-derived lines (Morozova *et al.* 2009) (Table S5), and *Syb* was differentially expressed

among selection lines for alcohol sensitivity (Morozova *et al.* 2007) and after repeated ethanol exposures in a *Canton-S (B)* line (Morozova *et al.* 2006). An alcohol-related phenotype has also been reported for the murine ortholog of *Syb* (*Vamp2*) (Mulligan *et al.* 2006). Using RT–PCR analysis we confirmed that expression levels of *His4r*, *Syb*, and *Tctp* were altered in all of the RNAi lines targeting *Cyp1*, *H15*, *fng*, *Mlc-c*, and *Pdk* in both sexes.

The error rate underlying the predicted networks is unknown. Thus, it is possible that some of the transcripts interconnected with focal genes may not be associated with alcohol sensitivity. Determination of the error rate in building iterative networks can only be done empirically. On the basis of Morozova *et al.* (2009), the error rate for genes coregulated with *Malic enzyme* and associated with a second exposure to ethanol, estimated by qRT–PCR, was 25% at $r > 0.3$. Similarly, the error rate for genes coregulated with *pipsqueak* and associated with an initial exposure to ethanol, estimated by qRT–PCR, was 26% at $r > 0.3$. Harbison *et al.* (2009) calculated an error rate of 19% at $r > 0.7$ for covariant transcriptional networks associated with sleep phenotypes. In building these networks, the number of interconnected hub genes can be increased or decreased by adjusting the criterion for strength of correlation. However, reduction of the threshold for correlation will introduce more false-positive associations.

To reduce the false-positive rate, we used two procedures. First, we focused only on genes with the strongest correlations with focal genes known to affect alcohol sensitivity. Second, we performed validation of several interconnected genes both by qRT–PCR and, wherever possible, by assessing the effect on the phenotype. These safeguards protect the iterative networking approach we describe from accumulating incremental errors. The observation that 39 of the 85 transcripts from computationally predicted networks were common between differentially expressed transcripts from at least one or more previous studies (Morozova *et al.* 2006, 2007, 2009) (Table S5) provides further validation for the networks despite the lack of a precise false-positive error estimate.

Previous studies have shown associations between polymorphisms in *Adh* and *Aldh* with ethanol sensitivity in Drosophila (Fry and Saweikis 2006; Fry *et al.* 2008). Unfortunately, our collection of *P*-element insertion lines did not contain an insertion affecting *Adh* or *Aldh*. Furthermore, the strength of correlations of *Adh* or *Aldh* transcripts with any of our focal genes did not reach our minimal arbitrary threshold for significance ($r > 0.45$).

Our results demonstrate that sensitivity to alcohol exposure is determined by the composition and dynamics of its genome-wide transcriptional underpinnings. Although coregulated transcriptional networks do not provide information about causal relationships among their constituent transcripts, they provide a useful context to guide subsequent functional studies. Our studies also emphasize the importance of assessing the genetic context when evaluating the effects of single genes on alcohol sensitivity and to note that selection for low or high sensitivity acts on genetic networks rather than on single alleles.

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GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.110.125229/DC1

Transcriptional Networks for Alcohol Sensitivity in *Drosophila melanogaster*

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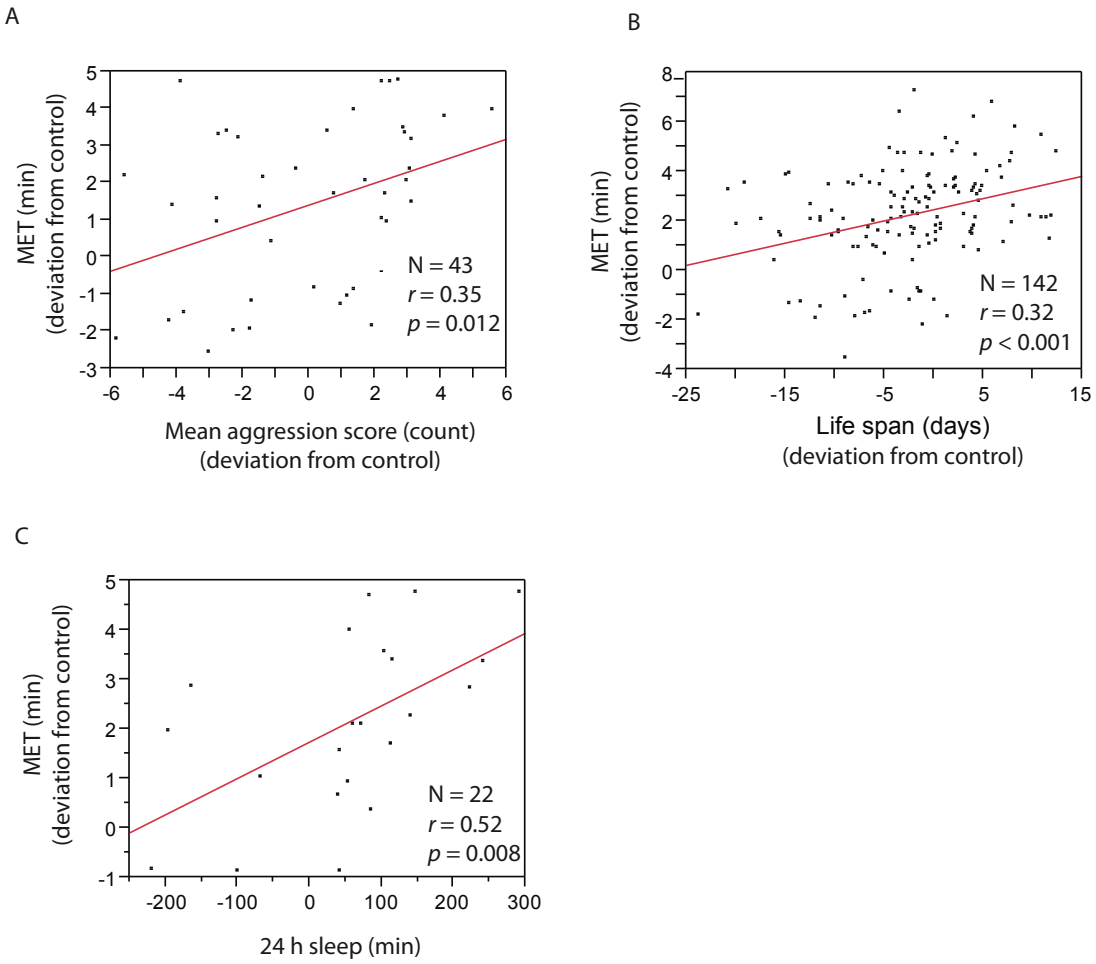

FIGURE S1.—Phenotypic correlations between alcohol sensitivity in males and (A) aggression , (B) lifespan and (C) sleep in *P[GT1]*-element insertion lines.

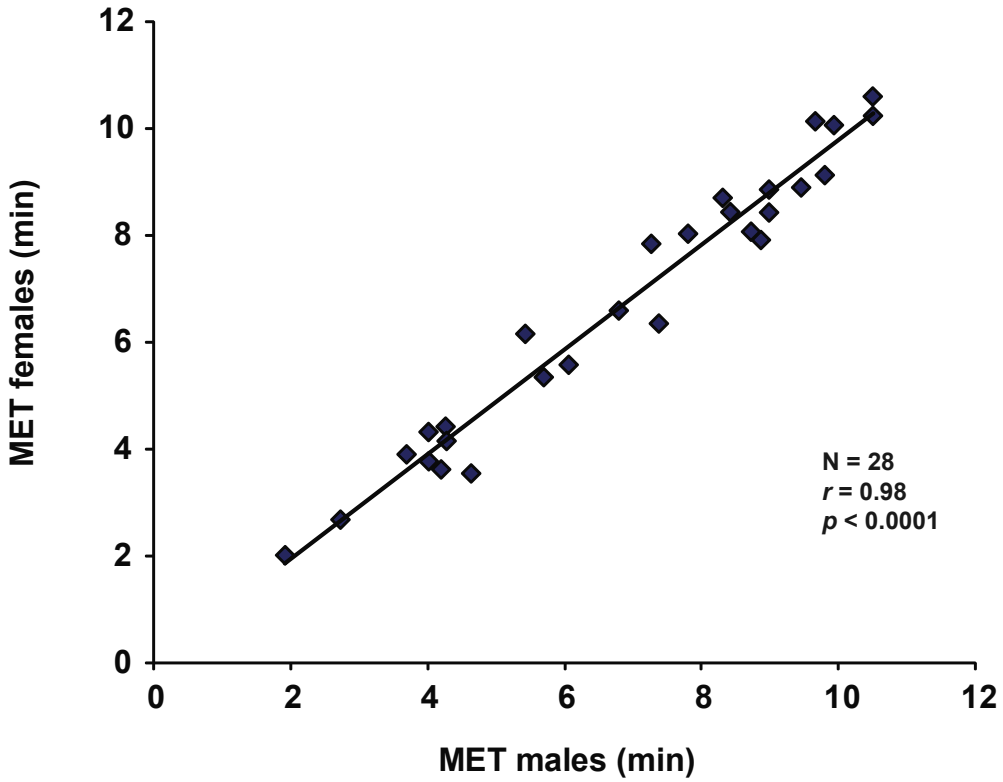

FIGURE S2.—Phenotypic correlation between METs in males and females in *P[GT1]*-element insertion lines.

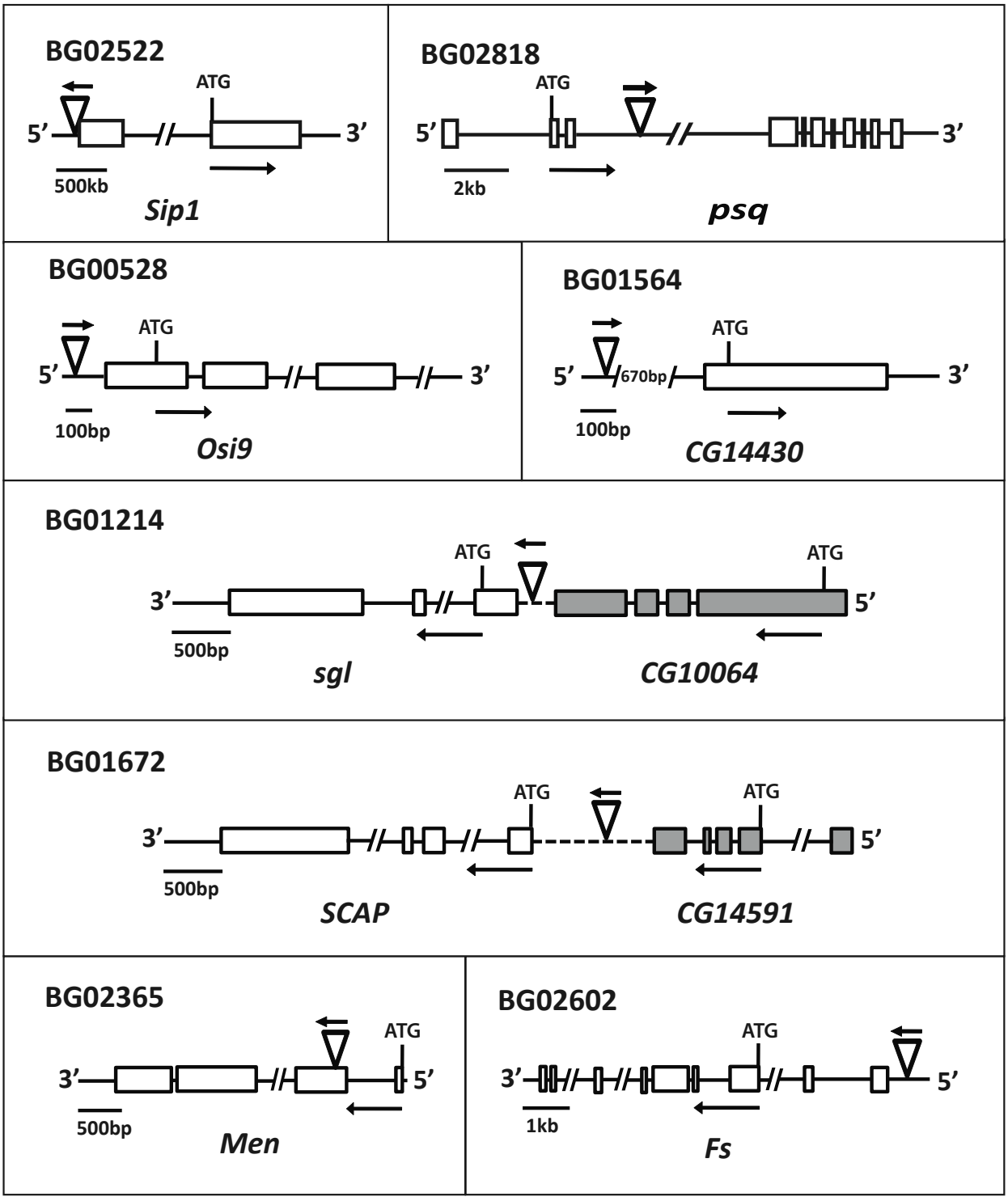

FIGURE S3.—Diagram of *P[GT1]*-insertion sites in candidate genes. Inverted open triangles indicate the *P[GT1]*-element insertion sites. BG line designations are indicated in the upper left corner of each panel. Open boxes indicate exons. Orientations of the candidate gene and the *P[GT1]*-element are indicated by the long arrows below each diagram and the small arrows above the inverted triangles, respectively. ATG indicates the position of the translation initiation site of the coding sequence.

TABLE S1

Analyses of variance of mean elution time after a single exposure to ethanol of 653 *P[GT1]* insertion lines in males.

Source	d.f.	SS	F	*P*	σ^2
Line (*L*)	652	241647	39.6	<0.0001	1.85
Error	126906	1429311			9.36

d.f., degree of freedom; SS, sum of squares; F, F-statistics; σ^2, variance component

TABLES S2 - S6

Tables S2 – S6 are available for download as Excel files athttp://www.genetics.org/cgi/content/full/genetics.110.125229/DC1.

Table S2: *P[GT1]* insertion lines contributing to alcohol sensitive/resistant phenotypes.

Table S3: Pleiotropic effects of *P[GT1]*-element insertions on multiple phenotypes.

Table S4: Overlap between GO categories of transcripts clustered around focal candidate genes in Figure 4 and transcripts previously associated with ethanol exposures.

Table S5: Transcripts clustered around focal candidate genes in Figure 7, that have been previously associated with ethanol exposures.

Table S6: Genes implicated in alcohol sensitivity in Drosophila in this study and previously published studies.