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Natural Variation, Functional Pleiotropy and Transcriptional Contexts of *Odorant Binding Protein* Genes in *Drosophila melanogaster*

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ABSTRACT

How functional diversification affects the organization of the transcriptome is a central question in systems genetics. To explore this issue, we sequenced all six *Odorant binding protein* (*Obp*) genes located on the *X* chromosome, four of which occur as a cluster, in 219 inbred wild-derived lines of *Drosophila melanogaster* and tested for associations between genetic and phenotypic variation at the organismal and transcriptional level. We observed polymorphisms in *Obp8a*, *Obp19a*, *Obp19b*, and *Obp19c* associated with variation in olfactory responses and polymorphisms in *Obp19d* associated with variation in life span. We inferred the transcriptional context, or "niche," of each gene by identifying expression polymorphisms where genetic variation in these *Obp* genes was associated with variation in expression of transcripts genetically correlated to each *Obp* gene. All six *Obp* genes occupied a distinct transcriptional niche. Gene ontology enrichment analysis revealed associations of different *Obp* transcriptional niches with olfactory behavior, synaptic transmission, detection of signals regulating tissue development and apoptosis, postmating behavior and oviposition, and nutrient sensing. Our results show that diversification of the *Obp* family has organized distinct transcriptional niches that reflect their acquisition of additional functions.

THE manifestation of complex traits depends on the coordinated expression of ensembles of genes (Falconer and Mackay 1996; Lynch and Walsh 1998; Stranger *et al.* 2007; Chen *et al.* 2008; Zhu *et al.* 2008; Ayroles *et al.* 2009; Mackay *et al.* 2009; Schadt 2009). Such ensembles are dynamic and sensitive to the environment and, therefore, best studied in an organism like *Drosophila melanogaster*, in which both the genetic background and the environment can be controlled precisely. Previous studies on isogenic *D. melanogaster* carrying single *P*-element insertions showed that epistatic interactions at the level of the behavioral phenotype are mirrored at the level of the transcriptome and that the introduction of a new mutation affects the expression of wide-ranging ensembles of genes (Anholt *et al.* 2003). Whole-genome transcript analysis of 40 wild-derived inbred lines showed that the transcriptome is genetically highly intercorrelated (Ayroles *et al.* 2009). Further, modules of genetically correlated transcripts form transcriptional networks associated with a variety of complex traits (Ayroles *et al.* 2009; Harbison *et al.* 2009; Morozova *et al.* 2009).

The high interconnectivity of the transcriptome raises the question of how new genes that arise as a consequence of gene duplication acquire a new transcriptional niche and how their functional diversification is accommodated in the organization of the transcriptome. We approached this question using the multigene family of *Odorant binding protein* (*Obp*) genes as a model. Odorant binding proteins are thought to facilitate the transfer of apolar odorants in the aqueous antennal perilymph to membrane-associated odorant receptors and have been implicated as essential intermediaries in pheromone detection (Xu *et al.* 2005; Ha and Smith 2006) and insect–host plant interactions (Matsuo *et al.* 2007). However, members of the *Obp* family are also expressed in the fat body (Fujii and Amrein 2002) and the male accessory gland (Ayroles *et al.* 2009). Furthermore, altered regulation of expression of *Obp* genes has been observed following mating (McGraw *et al.* 2004; Zhou *et al.* 2009); following exposure to starvation stress (Harbison *et al.* 2006); during development of alcohol tolerance (Morozova *et al.* 2006); as a

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correlated response to artificial selection for divergent levels of copulation latency (Mackay *et al.* 2005), aggression (Edwards *et al.* 2006), and sensitivity to alcohol (Morozova *et al.* 2007); between young and old flies (Zhou *et al.* 2009); and as a consequence of pleiotropic effects arising from single *P*-element induced mutations that affect olfactory behavior (Anholt *et al.* 2003). These observations indicate that the functions of odorant binding proteins may not be limited to odorant binding *per se*, but that expansion of the *Obp* family has resulted in functional diversification.

The genome of *D. melanogaster* contains 51 *Obp* genes, many of which occur as clusters (Hekmat-Scafe *et al.* 2002). Previously, we examined *Obp* clusters on the second and third chromosomes (Wang *et al.* 2007, 2010). Since *X*-linked loci can be under different selection pressures in males and females (males are hemizygous, exposing deleterious alleles to selection every generation) and the effective population size of *X*-linked genes is three-fourths that of autosomes, we were especially interested in analyzing *Obp* genes on the *X* chromosome. Thus, we chose all six *Obp* genes on the *X* chromosome for our experiments. Two of those genes are at separate loci (*Obp8a* and *Obp18a*), while the other four form a cluster without intervening genes (*Obp19a–d*). We sequenced these genes in 219 inbred wild-derived lines from the Raleigh population and asked to what extent polymorphisms in these genes are associated with variation in olfactory behavior in response to four different odorants and variation in life span, as previous studies demonstrated a link between olfaction and life span both in flies (Libert *et al.* 2007) and in *Caenorhabditis elegans* (Alcedo and Kenyon 2004). We detected significant associations with polymorphisms in *Obp8a*, *Obp19a*, *Obp19b*, and *Obp19c* and variation in olfactory responses, as well as significant associations between polymorphisms in *Obp19d* and variation in life span. We used reverse-transcriptase PCR followed by real-time PCR to show that some of these polymorphisms result in altered levels of transcript abundance. We also determined the transcriptional context, or "niche," for each *Obp* gene by identifying expression polymorphisms where genetic variation in these *Obp* genes was associated with variation in expression of transcripts genetically correlated to each *Obp* gene. We then examined these transcriptional contexts to assess if these niches also supported functional specialization for each of the *Obp* genes examined. We found that the majority of transcripts within each niche were unique to that transcriptional context and enriched for different gene ontology categories such as olfactory behavior, synaptic transmission, detection of signals regulating tissue development and apoptosis, postmating behavior and oviposition, and nutrient sensing. Both our genotype–phenotype associations and these transcriptional niches indicate that the *Obp* family has undergone divergent functional specializations.

MATERIALS AND METHODS

***D. melanogaster* lines:** We used 219 inbred lines generated from single inseminated wild-caught females from a Raleigh, North Carolina population by 20 generations of full-sib mating. Flies were reared on cornmeal–molasses–agar medium at 25°, 60–75% relative humidity, and a 12-hr light–dark cycle.

Sequencing: Genomic DNA from pools of 30 flies (15 males and 15 females) from each of the 219 lines was extracted using the Puregene DNA extraction kit (Gentra, Minneapolis). PCR primers were designed to amplify overlapping segments corresponding to coding regions and 5′- and 3′-untranslated regions of the six *Obp* genes (*Obp8a*, *Obp18a*, and the *Obp19a–d* cluster). Sequences from the different inbred lines generated by PCR were aligned using the BioEdit 7.0.9 software (Hall 1999). GenBank accession numbers for the sequences are GQ919302–GQ920615.

Quantitative RT–PCR: Messenger RNA levels were quantified using reverse-transcriptase PCR followed by real-time PCR using the SYBR Green detection method [Maxima SYBR Green/Rox qPCR Master Mix (2×); Fermentas Life Sciences, Burlington, Ontario, Canada] and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). For all samples, the housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) was used as an internal control. To investigate the SNP of interest we sampled 10 individuals of each sex from five lines in each homozygous genotypic class. Total RNA was isolated from three independent biological replicates per line using the Trizol reagent (GIBCO-BRL, Gaithersburg, MD). Subsequently, cDNA was generated from 50 ng of total RNA by reverse-transcription PCR using the High Capacity cDNA reverse transcription kit (Applied Biosystems). Primer3Plus (Untergasser *et al.* 2007) was used to design real-time PCR primers. We used a mixed linear model, $Y = \mu + G + S + S \times G + L(G) + S \times L(G) + B(L(G) \times G \times S) + \varepsilon$, where Y denotes the average normalized C_T value, G is genotype (fixed), S is sex (fixed), $L(G)$ is line within genotype (random), and B is the biological replicate nested within line by genotype by sex (random) and the error variance (ε), to determine whether there was a significant difference between the two genotypic classes. This model was run in SAS using PROC MIXED (Sas Institute 1999).

Phenotypes: Olfactory behavior was quantified using the well-established "dipstick" assay (Anholt *et al.* 1996). Briefly, five flies of the same sex are placed in a culture vial and exposed to a cotton wool swab dipped in odorant solution inserted to a standard depth near the top of the vial. The vial is placed horizontally and after a 15-sec acclimatization period the number of flies in a marked compartment remote from the odor source is counted 10 times at 5-sec intervals. The average of these measurements is recorded as a response score and the assay is repeated multiple times on individuals of the same genotype. A theoretical score >2.5 indicates repulsion, whereas scores <2.5 indicate attraction to the odorant. Pilot experiments on 5 of the 219 lines established that a concentration of 3.5% (v/v) provided optimal resolution for evaluating variation in olfactory behavior for acetophenone and hexanol and 0.3% (v/v) for hexanal in these lines. We measured olfactory behavior of 4- to 8-day-old flies from 219 wild-derived inbred lines in single-sex groups of five flies per replicate and 10 replicates per sex. Previously, responses to benzaldehyde were measured for 139 of these lines (Wang *et al.* 2007) and we incorporated these data in our study (the same phenotypic values for benzaldehyde were used by Wang *et al.* 2010 and Rollmann *et al.* 2010). All assays were conducted between 1:00 and 4:00 pm at 25° and 70% humidity. The experimental design was randomized such that measure-

ments on individual lines were collected over several days to average environmental variation. Longevity was assayed as previously described (Vieira *et al.* 2000). Briefly, 25 virgin males and females per line were collected in a 24-hr period and 2-day-old flies were housed in five replicate vials with five same-sex individuals per vial. Flies were transferred to fresh medium every 2 days and the number of live flies was recorded until all were dead.

Quantitative genetic analyses: We partitioned phenotypic variance between sexes (S, fixed), lines (L, random), the $S \times L$ interaction (random), and the error variance (ε) for each trait assayed using ANOVA. We estimated broad-sense heritabilities (H^2) as $H^2 = (\sigma_L^2 + \sigma_{SL}^2)/(\sigma_L^2 + \sigma_{SL}^2 + \sigma_E^2)$, where σ_L^2, σ_{SL}^2, and σ_E^2 are the among-line, sex × line, and within-line variance components, respectively.

Association analysis: General linear models [$Y = \mu + M + S + S \times M + \varepsilon$, where Y denotes the phenotypic values, M the polymorphism (fixed), and S the sex (fixed)] were used to identify polymorphisms associated with variation in organismal phenotypes. For each phenotype all tested polymorphisms were permuted 1000 times and subsequently the same model was run for each polymorphism–phenotype pair in the permuted data set. We set the empirically derived significance threshold value equal to the 95th percentile of the polymorphism–phenotype ranked P-values (Churchill and Doerge 1994).

Transcriptional networks: Whole-genome transcript abundance profiles were obtained previously from duplicate whole-body RNA samples of males and females separately from 40 of the wild-derived inbred lines, using Affymetrix Drosophila Genome 2.0 expression microarrays (Table S1 in Ayroles *et al.* 2009). We defined a transcriptional network as all transcripts that significantly associated (a nominal P-value <0.001) with a marker in the gene of interest (*Obp8a*, *Obp18a*, *Obp19a*, *Obp19b*, *Obp19c*, and *Obp19d*) and were genetically correlated ($|r_{Gij}| = 0.4$) with the corresponding transcript of interest. We used a regression model [$Y = \mu + S + M + S \times M + \varepsilon$, where Y denotes the transcript abundance, S the sex (fixed), and M the marker covariate (fixed)] to identify transcripts significantly associated with genotypic variation. Singletons and markers in high linkage disequilibrium (LD) with another marker ($r^2 > 0.8$), as measured in the 40 lines, were discarded from the analysis as well as the association analysis described above to reduce the penalty for multiple testing using the Bonferroni criterion. Markers dropped from high linkage disequilibrium pairs were chosen randomly. We estimated the genetic correlation r_{Gij} between the transcripts of interest within the whole-genome transcript profile. Genetic correlations were estimated as $r_G = \mathrm{cov}_{ij}/\sigma_i\sigma_j$, where cov_{ij} is the covariance of the measure of expression (median $\log_2$ signal intensity of perfect-match probes) between the two transcripts (males and females), and σ_i and σ_j are the square roots of the genetic variance components for the two transcripts (males and females). To annotate which transcripts were significantly associated with a phenotype we used the same regression models as those previously described as well as association analyses previously conducted for longevity, starvation resistance, and copulation latency (Ayroles *et al.* 2009).

RESULTS

***Obp* genes harbor polymorphisms associated with olfactory behavior and life span:** We evaluated the extent of phenotypic variation for responses to two aromatic odorants, benzaldehyde and acetophenone; two aliphatic odorants, hexanol and hexanal; and life span among the population of inbred wild-derived lines, for males and females (Figure 1; supporting information, Table S1). As expected from previous studies on this population (Wang *et al.* 2007, 2010; Ayroles *et al.* 2009; Harbison *et al.* 2009; Morozova *et al.* 2009), there is extensive variation for all traits as well as significant differences between the sexes (Figure 1, Table S2). Broad sense heritability (H^2) estimates ranged from 0.28 to 0.43 for olfactory responses, and for life span $H^2 = 0.43$. Thus, there is a significant genetic contribution to the observed phenotypic variance, as required for association analyses.

We identified 122 polymorphisms among these six *Obp* genes, with the smallest number of polymorphisms (8) in *Obp18a* and the largest number of polymorphisms (37) in *Obp19b*. LD analysis among these six genes shows only small pockets of LD, which indicates extensive historical recombination (Figure S1). Only one polymorphic marker of pairs that were in high LD ($r^2 = 0.8$) was retained for association studies and singletons were discarded, leaving 87 polymorphisms for association analyses. We used a conservative Bonferroni correction for multiple testing [$\log(1/P) = 3.24$] as well as permutation analyses to establish thresholds for significance.

Polymorphisms associated with variation in olfactory responses were observed in *Obp8a*, *Obp19a*, *Obp19b*, and *Obp19c* (Figure 2). We observed significant associations for variation in response to benzaldehyde and C277T, a synonymous substitution in the second exon of *Obp19a*, and three polymorphisms in *Obp19b* (G95A, a synonymous substitution in the first exon; T1034G in the 3′ untranslated region; and I195D in intron 1). The C277T polymorphism in *Obp19a* exceeds the permutation threshold, but not the Bonferroni-corrected significance threshold, for association with variation in response to acetophenone (Figure 2, Table S3). *Obp8a* contains a variable-size in-frame insertion/deletion (I281D) in exon 2, which deletes one, two, three, or four consecutive alanines [when two or three alanines are deleted, the first alanine of this insertion/deletion (indel) is substituted for a valine] and is associated with variation in response to hexanol (Figure 2, Table S3). T1100G in the 3′-untranslated region of *Obp19b* is associated with variation in response to hexanol (Figure 2, Table S3) and T338G in the first intron of *Obp19c* is associated with variation in response to hexanal. Thus, variation in responses to different, even structurally similar, odorants are associated with different SNPs in different *Obp* genes.

We detected two polymorphisms that were strongly associated with life span in *Obp19d*, T454G in the 5′-untranslated region and C697T, a synonymous substitution in the fourth exon (Figure 2, Table S3). We did not find significant associations with any of the traits and polymorphic markers in *Obp18a* (Figure 2).

Effects of polymorphisms in *Obp* genes on transcript abundance: Although it is formally possible that a noncoding polymorphism that affects the phenotype, such as

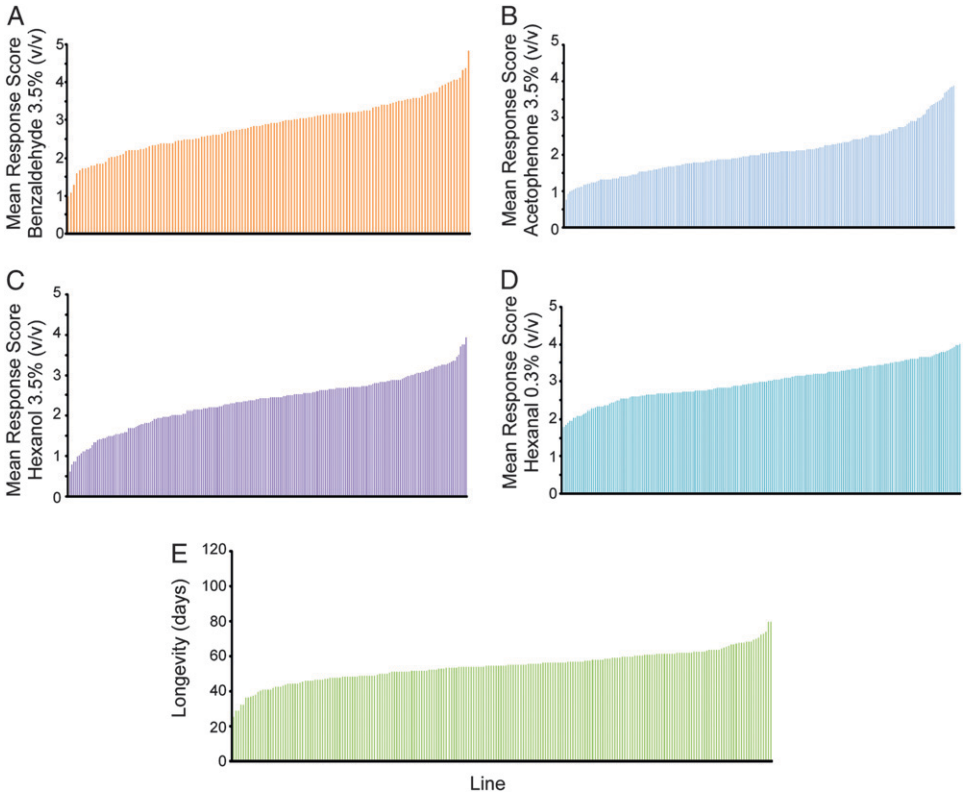

FIGURE 1.—Phenotypic variation in mean olfactory responses to benzaldehyde (A), acetophenone (B), hexanol (C), hexanal (D), and longevity (E) among 219 inbred wild-derived lines of *D. melanogaster*. Mean phenotypic values for each line and sex are given in Table S1. Genetic correlations among the traits are listed in Table S5.

the T1100G polymorphism in *Obp19b*, is in linkage disequilibrium with an unobserved polymorphism in a nearby gene region that causally affects expression, this is unlikely as linkage disequilibrium decays rapidly in Drosophila; thus, the unobserved causal site would have to be in very close proximity. To address this issue and to understand how the *Obp* polymorphisms identified above affect trait variation we investigated transcript abundance in lines varying for a subset of these markers. Despite the likelihood that variations in transcript abundance arising from a single polymorphic site that affect phenotypic variation might be too subtle to be detectable by RT–PCR, we were able to show that two polymorphisms, the in-frame indel I281D in *Obp8a* and the T1100G polymorphism in *Obp19b*, both associated with variation in response to hexanol, modify levels of transcript abundance either directly or indirectly. To resolve these effects, we selected for *Obp8a* five lines that were homozygous for the insertion and showed elevated responses to hexanol (response scores were 3.03 ± 0.46 for males and 2.77 ± 0.09 for females; values are pooled averages ± SEM) and five lines that were homozygous for the deletion and showed reduced responses to hexanol (response scores were 1.19 ± 0.22 for males and 0.98 ± 0.17 for females). RT–PCR showed that transcript levels in males were significantly lower in lines with the deletion than in those with the insertion, whereas the deletion did not affect transcript levels in females (Figure 3A). It should be noted, however, that structural variation at the protein level is also likely to contribute to phenotypic variation in olfactory responsiveness in the case of *Obp8a* as the in-frame indel determines the absence or presence of consecutive alanines.

We performed the same analysis on the T1100G SNP in *Obp19b* and selected five lines homozygous for the T allele and five homozygous for the G allele. Corresponding olfactory response scores for hexanol were 3.25 ± 0.08 for males, 3.11 ± 0.06 for females, and 0.88 ± 0.15 for males, 0.90 ± 0.11 for females, respectively. We found that transcript levels of *Obp19b* were significantly higher in lines homozygous for the T allele than for the G allele in both males and females (Figure 3B).

***Obp* genes occupy distinct network niches in the transcriptome:** We asked whether expression levels of these *Obp* genes are closely correlated within a single transcriptional context or whether they occupy distinct niches within the transcriptome. To address this question, we computed genetic correlations (r_G) between variation in transcript abundance of each *Obp* gene and all other transcripts that were genetically variable among a subset of 40 of the inbred wild-derived lines (AYROLES *et al.* 2009). We assume this subset of 40 lines to be a representative sample of the population, because these 40 lines span the phenotypic range observed for the larger population and because of the statistical significance of the confidence levels of connectivity between transcripts and focal *Obp* transcripts. Transcripts genetically correlated with the focal *Obp* gene at an absolute value of $|r_G| > 0.4$ and that were significantly associated with a polymorphism within the focal *Obp* gene (nom-

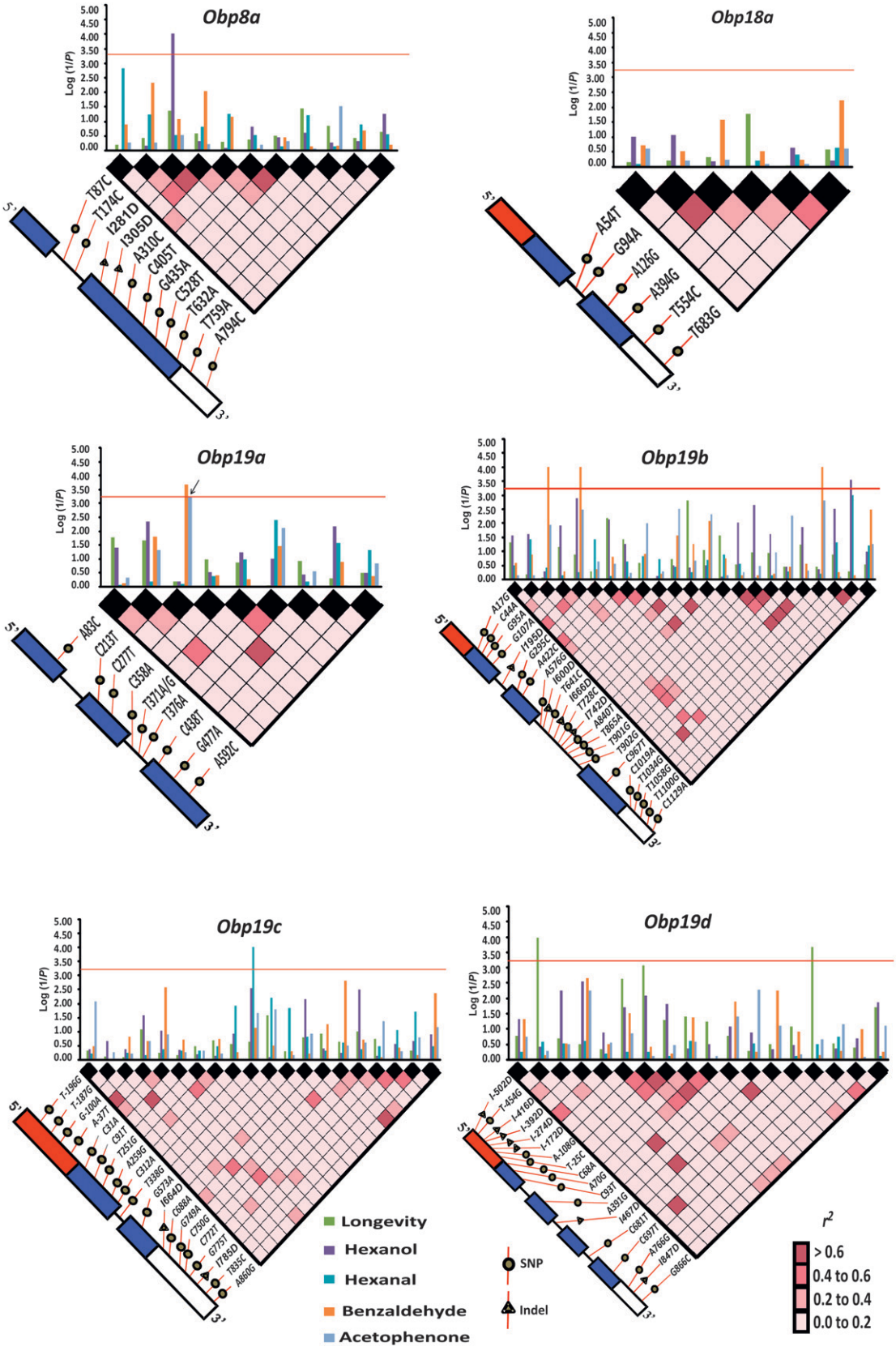

FIGURE 2.—Associations between polymorphisms in *Obp8a*, *Obp18a*, *Obp19a*, *Obp19b*, *Obp19c*, and *Obp19d* and variation in olfactory responses and longevity. The red horizontal line represents the significance threshold determined by Bonferroni correction for the number of markers tested. The arrow indicates an association with a marker in *Obp19a* and variation in response to acetophenone that does not pass the Bonferroni correction but does pass the significance threshold determined by permutation.

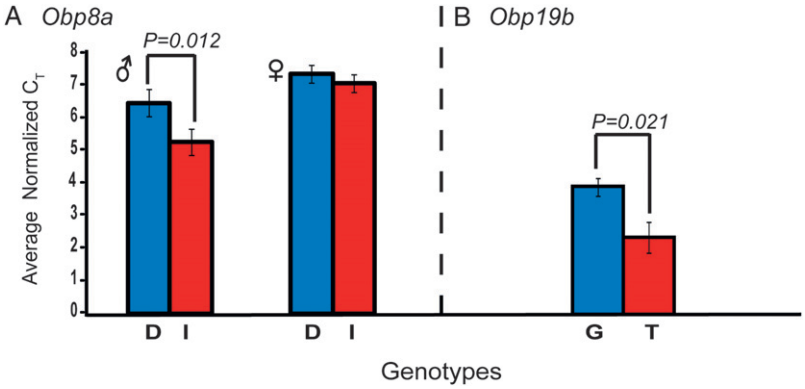

FIGURE 3.—Transcript abundance comparisons between genotypic classes of *Obp8a* and *Obp19b*. Transcript levels were measured by RT–PCR of five lines with high and five lines with low response scores to hexanol. Data are standardized to *Gapdh* and presented as averaged normalized C_T values. Note that averaged normalized C_T values are inversely proportional to transcript abundance. (A) Transcript levels of *Obp8a* in lines homozygous for the deletion (D) or insertion (I) polymorphisms at I281D, measured for males and females separately. (B) Transcript levels of *Obp19b* in lines homozygous for alternative alleles (G and T) at T1100G, measured for sexes pooled. Red bars indicate higher olfactory response values, and blue bars indicate lower olfactory response values.

inal $P < 0.001$) were used to construct covariant transcriptional modules centered around each *Obp* transcript (Figure 4). This analysis revealed that the six *Obp* genes occupy distinct transcriptional niches. *Obp*-centered transcriptional niches varied greatly in size from 8 transcripts connected with *Obp8a* to 173 transcripts connected with *Obp19b*. Whereas the transcriptional niches of the individual *Obp*s are well delineated, some shared transcripts interconnect them, reflecting the overall large-scale genetically correlated transcriptome (AYROLES *et al.* 2009) (Figure S2).

To assess pleiotropy of genetically correlated transcriptional niches centered around each *Obp* transcript in our wild-derived *D. melanogaster* population, we regressed phenotypic values of olfactory responses to benzaldehyde, acetophenone, hexanol and hexanal, life span, resistance to starvation stress (AYROLES *et al.* 2009), and copulation latency (AYROLES *et al.* 2009) on transcript abundance levels and identified transcripts significantly associated with each trait (nominal $P < 0.01$; Figure 4, Table S4).

We next asked to what extent those polymorphisms in *Obp* genes associated with abundance of genetically correlated transcripts were also associated with organismal phenotypes. We found that the C277T substitution in *Obp19a*, which was associated with response to benzaldehyde and acetophone, was associated with variation in abundance of 30 different transcripts, including 5 transcripts associated with the same two organismal phenotypes. Three polymorphisms in *Obp19b*, G95A, I195D (both associated with response to benzaldehyde), and T1100G (associated with response to hexanol), were associated with variation in the abundance of 12, 14, and 16 transcripts, respectively. Associations of polymorphisms in *Obp* genes both with variation in organismal phenotype and with variation in abundance of genetically correlated transcripts may reflect functional connectivity of these transcripts with relation to the phenotype (in this case response to benzaldehyde or acetophenone) or pleiotropy due to ripple effects in the transcriptome that emanate from the introduction of mutations in the genome (ANHOLT *et al.* 2003).

Transcriptional niches support functional diversification: We explored an alternative avenue to gain insights into the functional significance of the six *Obp*-centered transcriptional niches by determining whether certain gene ontology (GO) categories would be overrepresented among transcripts of each niche, using the DAVID analysis algorithm (DENNIS *et al.* 2003) (Figure 5A). *Obp8a* did not show detectable enrichment for GO categories, due to the small size of the niche. The niche of *Obp18a* was enriched for transcripts associated with learning and olfactory behavior. The *Obp19a* niche was enriched for transcripts associated with synaptic transmission; the *Obp19b* niche was enriched for GO categories collectively dedicated to detection of external signals that activate signal transduction processes that regulate tissue development and apoptosis; transcripts connected with *Obp19c* are enriched for GO categories of postmating behavior and oviposition; and the network centered around *Obp19d*, which contains polymorphic markers associated with variation in life span, is enriched for transcripts involved with nutrient sensing and synaptic transmission (Figure 5A). Thus, members of the *Obp19a–d* cluster interact with transcripts in discrete transcriptional niches that support their functional diversification.

We also examined the expression levels of the six *Obp* genes in the FlyAtlas database (CHINTAPALLI *et al.* 2007) to assess whether these data supported the functional diversification that was suggested by the GO analysis. The highest levels of expression of *Obp8a* were found in the male accessory gland, and expression of *Obp18a* was in the head and hindgut (Figure 5B). *Obp19a*,

The diagrams below the graphs indicate the degree of linkage disequilibrium between markers, as measured by r^2. The structures of the genes and positions of the polymorphic markers are indicated schematically with red boxes representing 5′-untranslated regions, blue boxes showing exons, and white boxes showing 3′-untranslated regions. For each marker, a circle indicates a single-nucleotide polymorphism (SNP) and a triangle indicates an indel.

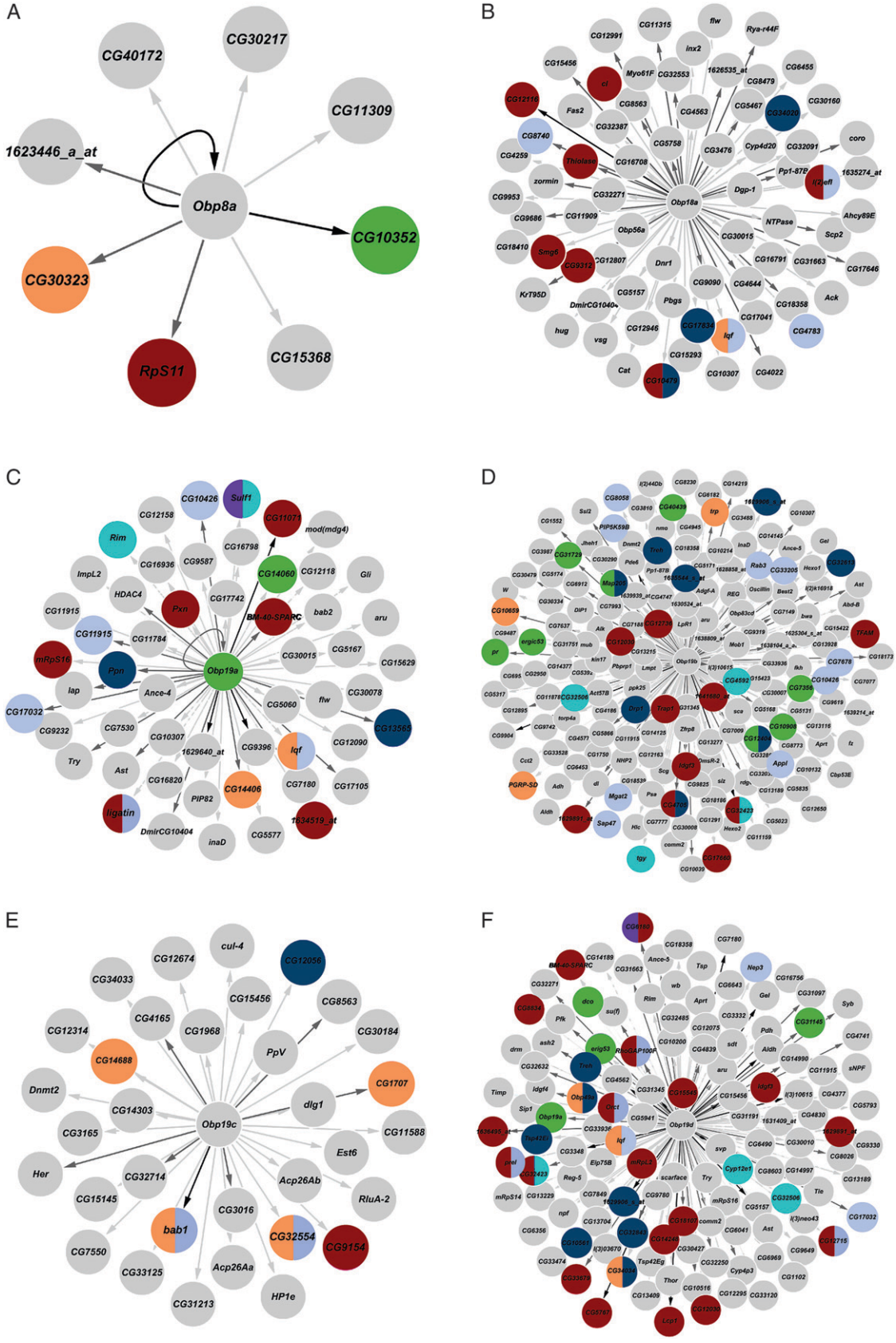

FIGURE 4.—Transcriptional niches of six *Obp* genes. The diagrams depict transcriptional niches of genetically correlated transcripts associated with polymorphisms after accounting for sexual dimorphism in *Obp8a* (A), *Obp18a* (B), *Obp19a* (C), *Obp19b* (D), *Obp19c* (E), and *Obp19d* (F). Black arrows indicate a marker–transcript association of $P \leq 0.00001$, dark gray arrows indicate $P \leq 0.0001$, and light gray arrows indicate $P \leq 0.001$. Transcripts that are significantly associated with a phenotype after regression

Obp19b, and *Obp19d* are expressed in the head, but at different levels, with *Obp19b* showing low levels of expression and *Obp19d* high levels in head and brain (Figure 5B). Interestingly, *Obp19c* expression is high in ovaries (Figure 5B), consistent with the enrichment in its associated transcriptional network of GO categories of oviposition and postmating behavior (Figure 5A). It should be noted that transcripts associated with organismal phenotypes that covary in transcriptional profiles obtained from whole flies do not necessarily have to be expressed in the same cells or tissues. It is tempting to speculate that odorant binding proteins in the accessory gland and ovaries may serve as carriers for hormones that modulate female postmating behavior and physiology.

Next, we used the FlyAtlas annotations to characterize the overall expression patterns of transcripts within each niche (Figure 5C). Whereas expression of transcripts is distributed among all tissues documented in FlyAtlas, enrichment of expression is observed in the accessory gland for *Obp8a* and *Obp19c* (Figure 5C). The enriched expression in the accessory gland for *Obp8a*-associated transcripts, although consistent with expression of *Obp8a* itself in the accessory gland (Figure 5B), must be interpreted with caution given the small size of this niche (Figure 4). Enrichment of covariant transcripts in the male accessory gland in the niche associated with *Obp19c* is intriguing given the high expression of *Obp19c* in the female ovaries (Figure 5B).

DISCUSSION

Previous analysis of *Obp* genes across 12 sequenced Drosophila genomes showed that purifying selection and tandem gene duplications have driven the evolution of this gene family, while preserving the arrangement of *Obp* genes as clusters (Vieira *et al.* 2007). It has been postulated that different functional constraints might indicate functional specialization of odorant binding proteins (Vieira *et al.* 2007; Sánchez-Gracia and Rozas 2008). Here, we focused on all six *Obp* genes located on the *X* chromosome and inferred the transcriptional contexts of each by identifying expression polymorphisms where genetic variation in these *Obp* genes was associated with variation in expression of transcripts genetically correlated to each *Obp* gene. It should be noted that polymorphisms that are located upstream or downstream from the sequenced regions that might affect variation in gene expression and, hence, contribute to phenotypic variation, are not detected in our study. Our analyses, however, show that these *Obp* genes occupy distinct transcriptional niches and that their diversification may facilitate functional pleiotropy.

We detected associations between sequence variation in these *Obp* genes and responses to four odorants and, for *Obp19d*, longevity. The four odorants chosen in this study occur in fruits from host plants on which flies from the Raleigh population feed (*e.g.*, apples and peaches). Thus, these odorants may represent ecologically relevant cues for survival in the natural environment. It is of interest to note that the *Obp19b* and *Obp19d* transcriptional niches contain a large number of transcripts correlated with life-history traits, including resistance to starvation stress, copulation latency, and life span (Ayroles *et al.* 2009). Previous studies demonstrated a link between olfaction and life span both in flies (Libert *et al.* 2007) and in *C. elegans* (Alcedo and Kenyon 2004). Caloric restriction is proportionately related to life span (Piper and Partridge 2007), and there is an inverse relationship between mated state and longevity, at least in Drosophila females (Wolfner 2002).

Only one of the associated polymorphisms (I281D in *Obp8a*) alters the amino acid sequence of the encoded protein. Therefore, it is likely that the majority of associated polymorphisms modulate expression levels; influence alternative splicing; or affect mRNA structure, stability, or translation efficiency (Nackley *et al.* 2006; Wang *et al.* 2007). It is not surprising that a large number of our significant polymorphisms are located in noncoding regions, considering the large number of regulatory polymorphisms implicated in human disease and complex traits in other model organisms (Knight 2005; Flint and Mackay 2009). Using reverse-transcriptase PCR followed by real-time PCR we were able to illustrate how one of these noncoding polymorphisms might give rise to phenotypic variation, in this case response to hexanol, by modulating transcript levels (Figure 3).

We did not detect associations between polymorphisms in *Obp18a* and the phenotypes examined. It is possible that *Obp18a* does not contribute to variation in olfactory responses to any of the four odorants or life span in this population. However, we cannot exclude the possibility that a larger sample size might reveal associations of smaller effect.

To further understand how *Obp* genes contribute to trait variation, we identified distinct transcriptional niches (Figure 4, Figure S2) with respect to each of the six *Obp* genes. Whereas the niches contain transcripts with expression levels that are significantly associated with variation in olfactory responses, *Odorant receptor* (*Or*) and *Gustatory receptor* (*Gr*) genes are notably absent. The Affymetrix expression microarrays used for these studies contained probe sets for only 10 *Or* genes and 11 *Gr* genes, but 8 of the *Or* genes and 4 of the *Gr* genes did

analysis ($P < 0.01$) are in colors other than light gray (dark blue, starvation resistance; maroon, copulation latency; green, longevity; purple, response to hexanol; turquoise, response to hexanal; orange, response to benzaldehyde; light blue, response to acetophenone). Transcript profiles and phenotypic values for starvation stress resistance and copulation latency were published previously (Ayroles *et al.* 2009). See also Table S4.

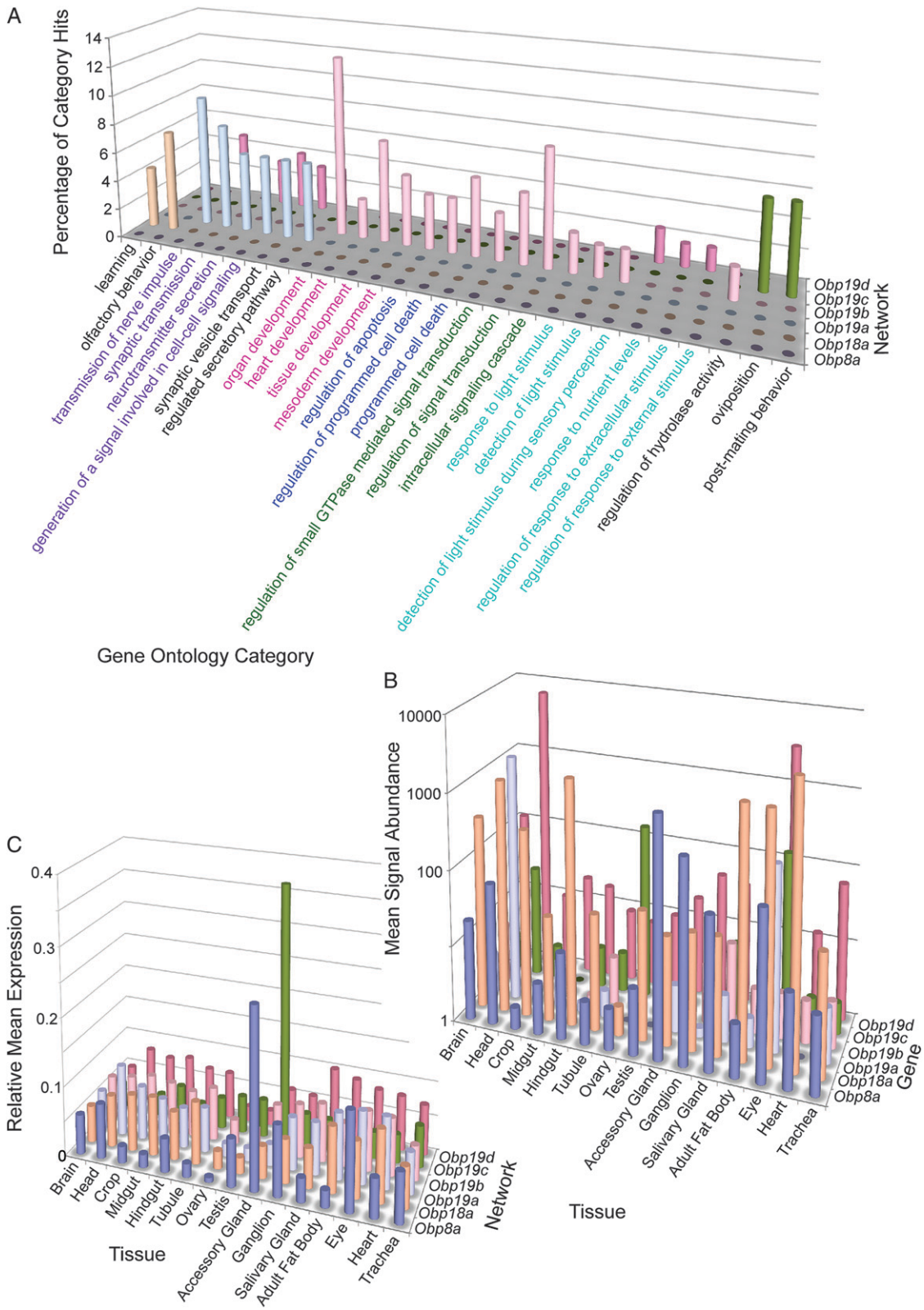

FIGURE 5.—Characterization of transcriptional niches. (A) Gene ontology categories enriched within *Obp*-centered transcriptional niches ($P < 0.05$). Data are based on level 4 of the DAVID analysis (DENNIS *et al.* 2003). GO categories in the same color font are related in the GO hierarchy (black font indicates unrelated categories). (B) Expression of *Obp8a*, *Obp18a*, *Obp19a*, *Obp19b*, *Obp19c*, and *Obp19d* in different fly tissues according to FlyAtlas (CHINTAPALLI *et al.* 2007). (C) Expression of transcripts in niches associated with *Obp8a*, *Obp18a*, *Obp19a*, *Obp19b*, *Obp19c*, and *Obp19d* in different fly tissues according to FlyAtlas (CHINTAPALLI *et al.* 2007).

not show variation in expression in this population under the condition in which the expression profiles were obtained (Ayroles *et al.* 2009). Future experimentation and analysis using expression profiles obtained after exposure to odorants might uncover associations between sequence variation in these 6 *Obp* genes and *Or* and *Gr* transcript abundance that went undetected in our study.

Both associations between sequence variation in the *Obp* genes and our five traits (see *Obp19b* and *Obp19c* in Figure 2) and associations between transcripts within each *Obp* transcriptional niche and a variety of traits (Figure 4) suggest that there is pervasive pleiotropy within the *Obp* gene family as well as within individual *Obp* genes. There was no predictable relationship, when we compared traits that are associated with sequence variation within the *Obp* genes and traits that are associated with transcript variation within the corresponding transcriptional niches. Rather, correlations with transcript levels and various traits were interspersed across all six niches. While we cannot exclude the possibility that causal sequence variation outside the region sequenced could explain this observation, lack of correlation can be accounted for by pervasive pleiotropy. Because of the complex interactions of many transcripts as well as sequence variation within many genes that contribute to trait variation (Flint and Mackay 2009), it is not surprising that traits that are associated with individual *Obp* genes would not necessarily be associated with transcripts in their corresponding transcriptional niches.

In assessing tissue-specific expression of *Obp* genes and correlated transcripts, it should be noted that our transcriptional profiles are based on RNA derived from whole flies. Pooling RNAs across all tissues is not likely to give us false positives, but will give false negatives in an amount that is proportional to how tissue-specific transcripts are and their relative abundance in the tissues in which they are specifically expressed. Furthermore, since we are assessing variation in expression among lines, we expect similar results from analysis of whole flies and dissected tissues when the expression of a given *Obp* gene varies among tissues as well as among lines, if there is no tissue-by-line interaction.

It should be noted that the approaches described here can infer only functions for which genes display natural variation. Complementary analyses, such as targeted gene disruption, could uncover other phenotypes affected by these *Obp* genes, providing further insights in their pleiotropic functions. In addition, the composition of transcriptional networks and strengths of interactions among their members are likely to be sensitive to environmental effects. Expression levels of *Obp* genes are modulated by physiological and social conditions (Zhou *et al.* 2009), and epistatic interactions within ensembles of genes change under different environmental conditions (Sambandan *et al.* 2006). Thus, the *Obp*-centered transcriptional niches described here should be viewed as "one-time snapshots" of dynamic ensembles. Nonetheless, previous studies showed that the baseline transcriptional organization obtained under standard conditions can serve as a reliable indicator for associations with a wide range of *post hoc* measured organismal phenotypes, including behaviors (Ayroles *et al.* 2009). For example, transcriptional modules could be associated with various sleep and activity measures (Harbison *et al.* 2009), as well as sensitivity to alcohol exposure and development of tolerance (Morozova *et al.* 2009), and correlations between transcript levels were validated in each case in a sample of phenotype-associated genes by measuring gene expression in corresponding *P*-element insertion lines (Harbison *et al.* 2009; Morozova *et al.* 2009).

Finally, currently ongoing whole-genome sequencing efforts will result in the availability of complete sequences of the genomes of 192 inbred wild-derived lines. The future availability of these sequences will for the first time enable genome-wide association analyses in Drosophila, including an expanded analysis of the entire *Obp* gene family. Our studies on these six *Obp* genes, however, already underscore the functional pleiotropy of odorant binding proteins, which is likely a characteristic feature of the entire *Obp* family, and demonstrate that distinct transcriptional niches have evolved during evolution of the *Obp* gene family.

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GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.110.123166/DC1

Natural Variation, Functional Pleiotropy and Transcriptional Contexts of *Odorant Binding Protein* Genes in *Drosophila melanogaster*

Gunjan H. Arya, Allison L. Weber, Ping Wang, Michael M. Magwire, Yazmin L. Serrano Negron, Trudy F. C. Mackay and Robert R. H. Anholt

DOI: 10.1534/genetics.110.123166

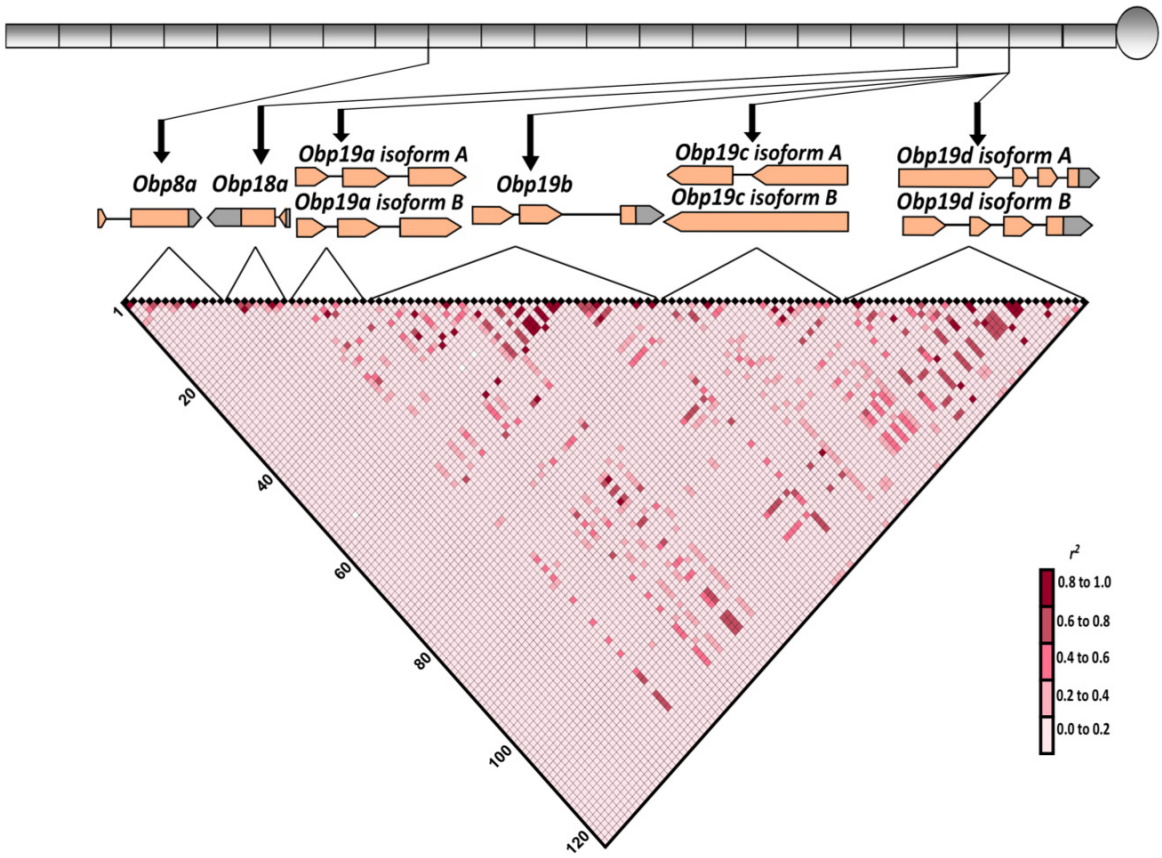

FIGURE S1.—Schematic representation of the locations and corresponding transcripts of six *Obp* genes on the X-chromosome. The linkage disequilibrium diagram below shows pairwise comparisons among polymorphic markers among all six *Obp* genes.

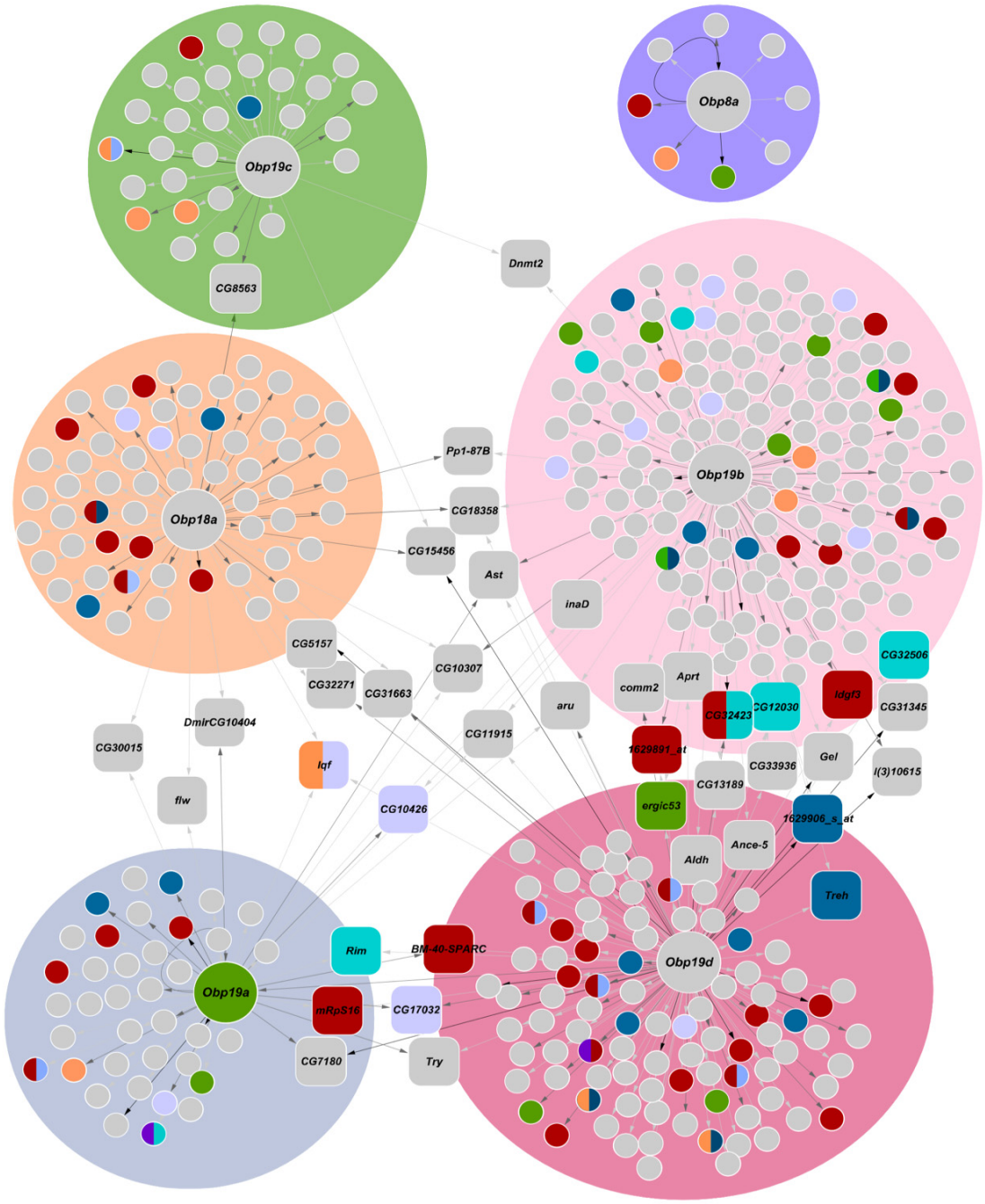

FIGURE S2.—Connectivity among the Obp-centered networks shown in Figure 4. Transcripts associated with more than one *Obp* network are indicated in rectangles. Transcripts that are significantly associated with a phenotype after regression analysis ($P < 0.01$) are in colors other than light gray (dark blue, starvation resistance; maroon, copulation latency; green, longevity; purple, response to hexanol; turquoise, response to hexanal; orange, response to benzaldehyde; light blue, response to acetophenone).

TABLE S1

Line means for responses to four odorants and longevity.

Table S1 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.110.123166/DC1.

TABLE S2

Quantitative genetic parameters of phenotypes for 219 inbred wild-derived *Drosophila melanogaster* lines from the Raleigh, USA population.

Trait	Mean	σ_L^{2a}	σ_{SL}^{2b}	σ_G^{2c}	σ_E^{2d}	σ_P^{2e}	H^{2f}	CV_G^g	CV_E^h
Mean Response Score Benzaldehyde 3.5% (v/v)	2.8553	0.41763****	0.04682****	0.46445	0.61652	1.0810	0.43	23.87	27.50
Mean Response Score Acetophenone 3.5% (v/v)	2.0539	0.35078****	0.01240*	0.36318	0.71434	1.0775	0.34	29.34	41.15
Mean Response Score Hexanol 3.5% (v/v)	2.338	0.36049****	0.01899***	0.37948	0.51305	0.8925	0.42	26.35	30.63
Mean Response Score Hexanal 3.5% (v/v)	2.992	0.20693****	0.01411**	0.22104	0.55914	0.7802	0.28	15.71	24.99
Longevity (days)	54.35	57.740****	30.009****	87.749	115.80	203.55	0.43	17.23	19.80

[a] Among line variance component. [b] Sex by line interaction variance component. [c] Total genetic variance ($\sigma_L^2 + \sigma_{SL}^2$). [d] Variance within replicates or lines. [e] Total phenotypic variance ($\sigma_G^2 + \sigma_E^2$). [f] Broad sense heritability (σ_G^2/σ_P^2). [g] Coefficient of genetic variation ($100\sigma_G$/Mean). [h] Coefficient of environmental variation ($100\sigma_E$/Mean). Asterisks indicate *P*-values from ANOVA F-ratio significance tests. ***** $P < 0.05$; ******, $P < 0.01$; ******* $P < 0.001$; ******** $P < 0.0001$. The fixed sex term was significant for all five traits.

TABLE S3

Polymorphic markers tested for association with the olfactory behavior and longevity and the corresponding log (1/*P*) values. The Bonferroni log (1/*P*) threshold for each trait is given in parentheses after the title in the trait columns. Log (1/*P*) values that meet the Bonferroni correction are indicated by two asterisks and the log (1/*P*) value that meet the threshold calculated using a permutation test, but not the Bonferrroni threshold, is indicated by one asterisk.

Table S3 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.110.123166/DC1.

TABLE S4

***P*-values for associations between polymorphic markers and transcripts within *Obp*-centeredmodules, and associations between these transcripts and various organismal traits.**

Table S4 is available for download as an Excel file at http://www.genetics.org/cgi/content/full/genetics.110.123166/DC1.

TABLE S5

Genetic correlations among phenotypes for 219 wild-derived *Drosophila melanogaster* inbred lines from the Raleigh, USA population.

A.

Trait	Mean Response Score acetophenone 3.5% (v/v)	Mean Response Score hexanol 3.5% (v/v)	Mean Response Score hexanal 3.5% (v/v)	Longevity (days)
Mean Response Score benzaldehyde 3.5% (v/v)	0.507 (0.000)	0.066 (0.440)	0.274 (0.001)	-0.088 (0.303)
	Mean Response Score acetophenone 3.5% (v/v)	-0.024 (0.724)	0.146 (0.031)	0.005 (0.941)
		Mean Response Score hexanol 3.5% (v/v)	0.060 (0.377)	0.003 (0.965)
			Mean Response Score hexanal 3.5% (v/v)	0.063 (0.353)

B.

Trait	Mean Response Score Benzaldehyde 3.5% (v/v)	Mean Response Score Acetophenone 3.5% (v/v)	Mean Response Score Hexanol 3.5% (v/v)	Mean Response Score Hexanal 3.5% (v/v)	Longevity (days)
Mean Response Score Benzaldehyde 3.5% (v/v)		0.472 (0.000)	0.083 (0.331)	0.351 (0.000)	-0.162 (0.057)
Mean Response Score Acetophenone 3.5% (v/v)	0.537 (0.000)		-0.035 (0.606)	0.162 (0.016)	-0.051 (0.453)
Mean Response Score Hexanol 3.5% (v/v)	0.059 (0.490)	-0.014 (0.837)		0.039 (0.566)	0.037 (0.586)
Mean Response Score Hexanal 3.5% (v/v)	0.279 (0.000)	0.133 (0.049)	0.079 (0.244)		0.066 (0.331)
Longevity (days)	-0.018 (0.833)	0.058 (0.393)	-0.032 (0.638)	0.061 (0.369)	

P-values from tests of significant differences from zero are in parentheses. (A) Sexes pooled. (B) Correlations above the diagonal are for females and values below the diagonal are for males.