Gene Function Prediction Based on Developmental Transcriptomes of the Two Sexes in *C. elegans*

**Graphical Abstract**

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**In Brief**
Taking advantage of a unique developmental feature of *C. elegans* males, Kim et al. compare transcriptomes of the two sexes to identify male-specific and nervous-system-enriched genes. The dataset and mining methods used provide a rich resource for future gene discovery.

**Highlights**
- Transcriptomes of *C. elegans* males and hermaphrodites are compared
- Comparison reveals 1,751 genes expressed at a higher level in males
- The male-upregulated set of genes is enriched for previously unannotated genes
- Functional gene clusters defined by gene co-expression analysis are validated

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Gene Function Prediction Based on Developmental Transcriptomes of the Two Sexes in C. elegans

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SUMMARY

We compare whole-animal RNA-seq transcriptomes for C. elegans males and hermaphrodites from the late L3 larval stage to young adulthood. During this interval, male sexual structures develop, including extensive neurogenesis and synaptogenesis that nearly doubles the size of the nervous system. Previous genome-wide expression studies in C. elegans have usually focused on only one sex—the hermaphrodite—and there are a relatively large number of genes that remain without meaningful annotation. In the present study, differential expression analysis of the RNA-seq data revealed 1,751 genes expressed at a higher level in the male. By differential expression and co-expression analyses, we identified transcription factors required for differentiation of male genital structures, semen proteins, and candidates for components of synapse function. Comparison with other prediction tools suggests that our dataset can expand gene predictions. The results validate the dataset as a rich resource for future gene discovery in C. elegans.

INTRODUCTION

Genome-wide transcriptional expression profiles have greatly enhanced our understanding of how genes are regulated and what their roles are in biological systems. One promising strategy to reveal potential functions of unknown genes is a gene co-expression approach, where functions of unknown genes can be inferred from the similarity of their expression profiles to those of genes with known functions. This approach has been applied in global expression analysis of Caenorhabditis elegans (Kim et al., 2001) and developed as a computational tool for gene discovery with a large compendium of gene expression profiles (SPELL: http://spell.caltech.edu:3000; Hibbs et al., 2007).

Despite the considerable progress in functional annotation for C. elegans genes through global expression and mutational studies as well as many studies focused on single genes, a large fraction of predicted genes still remains without functional assignment. The functions of protein-coding genes can be inferred from similarity of protein structure or amino acid sequence to genes of known function or from mutant phenotype. When this information is available for a gene in C. elegans, a functional gene name is assigned following standard nomenclatural rules. Of the approximately 20,000 predicted protein-coding genes in the C. elegans genome, 9,761 (48%) have such assigned gene names. The remaining 10,601 genes are named by their position in the genomic sequence (WormBase [WS250]: http://www.wormbase.org). For these genes, little is known about their biological function. One possible explanation for unassigned genes is that males and their behaviors have been largely excluded from prior studies. Thus, genes that act or are expressed predominantly or exclusively in the male may have been overlooked.

Sexually dimorphic gene expression shapes phenotypic differences between the two sexes during development (Ellegren and Parsch, 2007). Temporal gene expression profiles of the two sexes may allow us to predict functional roles of individual genes by providing the timing of action for the gene products during sexual maturation. Up to now, most temporal gene expression studies in C. elegans focused on only one sex—the hermaphrodite—whereas some included the male of only one stage, either L4 or adult (Gerstein et al., 2010; Hillier et al., 2009; Jiang et al., 2001; Reinke et al., 2000, 2004; Snoek et al., 2014; Spencer et al., 2011). To our knowledge, only one study, in which microarrays were applied to different larval stages (L2–L4) of both hermaphrodite and a genetically masculinized worm population, has examined temporal gene expression of both sexes, identifying over 300 sex-enriched genes (Thoemke et al., 2005).

When compared to the self-fertilizing hermaphrodite, the C. elegans male has a distinct body morphology, a different gonad system, 40 male-specific muscles, and 85 additional neurons that contribute to the copulatory structures and behavior (Emmons, 2014). The morphological differentiation of the male, including the addition of the male-specific cells and the formation of over 8,000 synapses, arises mostly at the third larval stage (L3) and later (Jarrell et al., 2012; Sulston et al., 1980). By contrast, hermaphrodite differentiation is largely complete by this time and primarily involves initiation of gametogenesis. This developmental distinction provides an opportunity to identify genes that are specifically upregulated during the neurogenic and morphogenetic episode associated with male sexual maturation as well as genes that function specifically in adult male reproduction.

Here, we report an expression analysis of the developmental transcriptome ranging from late L3 to adult in the two sexes of
C. elegans using whole-animal RNA sequencing (RNA-seq). As predicted, among 21,143 genes with detectable expression, a large number are upregulated in the male during this interval. We employed differential expression, unbiased gene correlation analysis, and guilt-by-association approaches to identify transcription factors regulating male morphogenesis, semen components, synaptic components, and putative components for cilia function. From the results, functions for a large number of genes may be inferred, making this dataset a rich source of future gene discovery.

RESULTS

RNA-Seq Transcriptomes of the Two Sexes during Sexual Maturation
To analyze gene expression during sexual maturation in C. elegans, we performed RNA-seq for five samples for each sex ranging at 6-hr intervals from late L3 to young adult stages (Figures 1A and 1B). Approximately 140 million sequencing reads from the ten samples (8–28 million reads per sample) were mapped to the C. elegans genome (WS190). We considered a gene as expressed if there were a total of five or more reads summing across the ten samples. We obtained expression data for a total of 21,143 genes, including 17,967 protein-coding genes covering 88% of ~20,000 predicted protein-coding genes annotated in C. elegans (WormBase [WS250]: http://www.wormbase.org). The expression data are made available in the Supplemental Information (Table S1).

Principal-component analysis revealed a robust difference between the sets of genes expressed by the two sexes during sexual maturation. During the late L3 stage, when sexual differentiation is not yet prominent, overall gene expression profiles of the two sexes are similar, but they diverge as worms develop into the adult stage (Figure 1B).
As an initial assessment of the gene sets, we analyzed the data for genes with different overall levels of expression in the two sexes. By comparing two sets of pooled data from the five samples for each sex, we identified 1,751 genes (1,645 protein coding) expressed at a higher level in the male, consistent with the burst of differentiation in the male, whereas 68 genes (66 protein coding) were expressed at a higher level in the hermaphrodite (false discovery rate < 1%; >4-fold change; Figure 1C; Table S2). These are minimum estimates because variation across the time points reduces the statistical power of the comparison. As expected, the male-enriched set included many genes for known sperm proteins (e.g., major sperm protein gene family) and several transcription factors known to be critical for male development (e.g., *egl-5*, *mab-23*, and *dmd-3*; Emmons, 2014), whereas the hermaphrodite-enriched set included genes that are known to be expressed specifically in the hermaphrodite or in the hermaphrodite-specific cells (e.g., *vit-2* and *cht-3*; Yi and Zarkower, 1999; Mounsey et al., 2002). Gene ontology (GO) term analysis showed that the male-enriched set was overrepresented with the GO terms related to protein kinases and phosphatases, which is a characteristic of sperm-enriched genes (Reinke et al., 2000; Table S3).

### Transcription Factors for Male Differentiation

In order to assess the possibility for gene discovery based on the differential expression levels, we examined transcription factor genes in more detail. As the male-specific transcription factors noted above were present in the male-enriched set, we sought to identify additional transcription factors expressed at a higher level in the male. By combining the male-enriched set with a list of putative transcription factor genes in the *C. elegans* genome (Reinke et al., 2013), we identified 17 male-enriched transcription factors (Figure 2A). In addition to the four genes mentioned above (the three doublesex/MAB-3 [DM] domain transcription factor genes *mab-3* [Raymond et al., 1998], *mab-23* [Lints and Emmons, 2002], and *dmd-3* [Mason et al., 2008] and the homeobox gene *egl-5* [Ferreira et al., 1999]), the list included an additional transcription factor known to be involved in male development, the COUP (chicken ovalbumin upstream promoter) transcription factor gene *unc-55* (Shan and Walthall, 2008).

To test whether the members of the remaining set are also required for male development, we examined four available mutant males. There were no obvious defects in gross morphology or morphology of the gonad in the mutant males (data not shown). However, two out of four mutants tested showed slightly abnormal ray formation in the male tail. The wild-type male has nine bilateral pairs of rays in its tail; however, in mutant males for two genes *ceh-48* and *T20H4.2*, missing or fusion of the ray structure was frequently observed (Figure 2B), indicating these genes encode putative transcription factors required for male tail development. To further observe the structure of the nervous system in the four mutants, we used a *pkd-2p::gfp* reporter to visualize some male-specific neurons, including RnBs, CEMs, and HOB (Jia and Emmons, 2006). We did not find any observable defects in cell number, cell body location, or process placement of these neurons, except for occasional misplacement of RnB dendrites corresponding to missing or fusion of the rays described above (data not shown).
We also examined expression of all 11 members of the DM domain gene family. DM domain transcription factors are evolutionarily conserved regulators of sexual development in many metazoans (Matson and Zarkower, 2012). Hierarchical clustering analysis divided these genes into two groups: higher expression in male (>2-fold change; dmd-3, dmd-4, dmd-6, dmd-10, dmd-11, mab-3, and mab-23) and similar or lower expression in male (<1.3-fold change; dmd-5, dmd-7, dmd-8, and dmd-9; Figure S2). Thus, it is possible that the four DM domain genes dmd-4, dmd-6, dmd-10, and dmd-11 may also control male development along with mab-3, mab-23, and dmd-3.

Unbiased Gene Correlation Analysis Partitioned Genes into Multiple Modules for Diverse Functions

We next treated each time point as a separate data point and determined the co-expression relationships between genes by performing weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008). This method partitions a gene set into modules defined as branches of the co-expressed gene cluster tree selected by a tree-cutting method. By this analysis, the set of 21,143 genes was partitioned into 27 modules with sizes ranging from 105 to 5,747 genes (Figure 3A; Table S1). Six major modules, each containing more than 1,000 genes, were readily associated by enrichment of...
GO terms with nervous system function (Mod1 and Mod2), semen/sperm development (Mod3), cuticle/hypodermis formation (Mod4), and germline/oocyte development (Mod5 and Mod6; Table 1; Figure 3A). However, each module was also enriched with more than one functional category: Mod1 also contained genes for muscle development, whereas Mod3 contained a set of genes related to neuropeptide signaling. Most of the male- or hermaphrodite-enriched genes described above belonged, respectively, to Mod3 or Mod6 (Table 1), suggesting that most of these sex-enriched genes function in various aspects of gamete development and reproduction.

The temporal gene expression patterns of the genes in the six major modules were well correlated with the presumptive functions of genes in these modules (Figure 3B). Genes in Mod1 and Mod2 showed relatively higher expression in the male compared to the hermaphrodite during late developmental stages from L4 to adult, as expected given the addition of many male-specific neurons and muscles at these stages (Sulston et al., 1980). Genes in Mod3 or Mod5 and Mod6 (Table 1), suggesting that most of these sex-enriched genes function in various aspects of gamete development and reproduction.

Table 1. Representative Gene Ontology Terms, Level of Expression, and Number of Sex-Enriched Genes in WGCNA Modules

| Module | No. of Genes | GO Term (Fold Enrichment) | Median Expression Level (Interquartile Range) | No. of Sex-Enriched Genes |
|--------|--------------|---------------------------|-----------------------------------------------|----------------------------|
|        |              |                           | Male (Interquartile Range) | Herm. (Interquartile Range) | Male | Herm. |
| Mod1   | 2,462        | Synaptic transmission (3.5) | 600 (199–1,737) | 316 (102–1,022) | 26 |
|        |              | Ion transport (2.4)         |  |  |  |
|        |              | Muscle development (3.6)    |  |  |  |
| Mod2   | 1,166        | GPCR signaling (3.1)        | 126 (59–315) | 31 (14–89) | 96 |
|        |              | Cilium (7.5)                |  |  |  |
|        |              | Synapse (2.5)               |  |  |  |
| Mod3   | 3,467        | Sperm development (8.9)     | 864 (185–2,675) | 109 (12–392) | 1,625 |
|        |              | Phosphorylation (2.8)       |  |  |  |
|        |              | Neuropeptide signaling (2.6) |  |  |  |
| Mod4   | 1,421        | Cuticle (7.4)               | 578 (143–1,933) | 492 (134–1,714) |  |
|        |              | Molting cycle (2.8)         |  |  |  |
|        |              | Cell adhesion (3.5)         |  |  |  |
| Mod5   | 1,016        | Ribosome (15.8)             | 1,354 (250–5,336) | 2,289 (520–8,737) | 8 |
|        |              | Growth (2.6)                |  |  |  |
|        |              | Mitochondrion (4.4)         |  |  |  |
| Mod6   | 5,747        | Cell cycle (2.4)            | 993 (76–2,676) | 2,028 (280–5,033) | 59 |
|        |              | RNA processing (2.2)        |  |  |  |
|        |              | Transcription (2.6)         |  |  |  |
| Others | 5,864        |                            | 23 (8–337) | 16 (3–310) | 4 |

\*Representative GO terms that are significant at p < 0.05 using both DAVID and PANTHER are shown. Fold enrichment scores from DAVID are shown in parentheses.
\*\*Expression level is indicated as a median of normalized count reads.
\*\*\*Sex-enriched genes are differentially expressed genes in males (n = 1,751) and hermaphrodites (n = 68) shown in Figure 1C.

One large set of predicted genes about which little is known consists of over 280 genes encoding C-type lectin-like domains (clec genes). Some clec genes have been shown to be upregulated in the male (Thoemke et al., 2005; Miersch and Döring, 2012), others upon infection with pathogens (O’Rourke et al., 2006), whereas one is required for nervous system patterning (Kulkarni et al., 2008). As a validation of module assignment, we analyzed the tissue-specific expression patterns of several clec genes. Two clec genes selected from Mod1 (clec-64 and clec-199) were expressed in subsets of head neurons in both sexes (Figure 3D). Although genes in Mod1 were expected to have additional expression in the male compared to the hermaphrodite, we did not observe robust expression of the two clec genes in the male tail except for occasional faint expression, possibly resulting from incomplete promoter sequences we used. Two clec genes selected from Mod3 (clec-207 and clec-219) were expressed in the male-specific gonadal structure called vas deferens (Figure 3E). These results are consistent with module assignment and indicate that the function of unknown genes can be suggested from the modules to which they are assigned. As each module contains many uncharacterized genes, these data should be a rich source for gene discovery.
Putative Semen Protein Genes Identified by a “Guilt-by-Association” Approach

To further identify subsets of genes functioning closely together in a specific biological process, we adopted a correlation-based guilt-by-association approach. In this approach, genes are sought with expression patterns highly correlated with a set of known genes in a pathway or process (probes; see Experimental Procedures). Using this approach, we sought to identify components of semen. We expected that a set of genes in Mod3 might produce semen components. Seminal fluid proteins are important for reproductive success in many organisms. Although studied extensively in insects (reviewed in Avila et al., 2011), less is known about semen components in C. elegans, with there being just two studied examples, TRY-5 and PLG-1 (Palopoli et al., 2008; Smith and Stanfield, 2011). In our dataset, try-5 is placed in Mod3, whereas plg-1 is not expressed because this gene was disrupted by a retrotransposon insertion in the laboratory strain (Palopoli et al., 2008).

We used four genes (clec-161, clec-197, F58A4.1, and T26A8.3) as probes, because they were highly co-regulated in the RNA-seq data (Pearson correlation coefficient > 0.9720) and known to be expressed in the same region of the male gonad, the vas deferens, the presumptive site of semen generation and storage (Thoemke et al., 2005). We found 54 genes in Mod3 co-expressed with these four probe genes (correlation coefficient > 0.9720; Figure 4A). This gene list included the two clec genes we randomly selected from Mod3 (clec-207 and clec-219) and showed above are expressed in the vas deferens (Figures 3D and 3E). None of the 54 genes have previously been functionally characterized, with only six of them having human orthologs; 44 do not have assigned gene names (Table S4).

We further tested the three most highly expressed genes among the 54 co-expressed genes to determine whether their protein products were indeed components of semen. Using promoter::GFP fusion transgenes, we found that all three are expressed in the male gonad and in vas deferens for F59B2.12 protein. Time course images showing transfer of INS-31 from male to hermaphrodite during mating. To facilitate observation of mating behavior, slower-moving unc-119 mutant hermaphrodites were used. INS-31::sfGFP (green) is visible within the seminal vesicle of a male before spicule insertion (a) and moves into the lumen of vas deferens after insertion until ejaculation (b). During ejaculation, INS-31::sfGFP is transferred into the vulva region of the hermaphrodite (c) and remains diffused in the uterus after mating (d).

We examined protein localization for INS-31 and F59B2.12 using translational reporter lines. We found that their protein products are localized to the vesicular structures in the seminal vesicle region for INS-31 and in vas deferens for F59B2.12 protein. Expression of these genes was not previously known, except ins-31, which was shown to be expressed in the male gonad (Thoemke et al., 2005). There was no detectable expression in any stages of hermaphrodites (data not shown). We further tested the three most highly expressed genes among the 54 co-expressed genes to determine whether their protein products were indeed components of semen. Using promoter::GFP fusion transgenes, we found that all three are expressed in the male gonad as expected: ins-31 in the seminal vesicle; F59B2.12 in the vas deferens; and B0207.5 in the valve region of the vas deferens (Figures 4B and 4C). Expression of these genes was not previously known, except ins-31, which was shown to be expressed in the male gonad (Thoemke et al., 2005). There was no detectable expression in any stages of hermaphrodites (data not shown). We examined protein localization for ins-31 and F59B2.12 (we did not include B0207.5 because it is expressed in the valve cells that are not well characterized anatomically) using translational reporter lines. We found that their protein products are localized to the vesicular structures in the seminal vesicle and vas deferens, respectively (Figure 4D). INS-31 protein was also found in coelomocytes in...
males (data not shown). Both INS-31::sfGFP and F59B2.12::sfGFP (superfolder GFP) fusion proteins were transferred from males to hermaphrodites during mating (Figures 4E and S3). Thus, these proteins are components of semen.

To compare our candidate gene list with those obtained using other gene-prediction tools, we utilized a co-expression-based SPELL (Hibbs et al., 2007) and a proteome-scale gene network WormNet (Cho et al., 2014). For direct comparison, we identified the same number of candidate genes in the SPELL database that are most highly correlated with our four semen probe genes (top 54 genes). However, we could obtain only 31 genes from WormNet with the same probe genes, even though WormNet usually gave a list of top 200 genes when we used well-studied genes as probes; this is possibly due to the lack of functional information for the probe genes, as these genes have exclusive expression in males and thus have been understudied previously. None of these candidate genes have previously been related to semen function. Module assignment revealed that all but one gene (98%) yielded by SPELL belong to the highly male-enriched Mod3, whereas only three genes (10%) in WormNet belong to Mod3 (Figure 4F). Our candidate gene list shared 18 genes with the SPELL gene list, but not with the WormNet gene list (Figure 4F; Table S4). The shared gene list contained two semen protein genes (ins-31 and F59B2.12) shown above, suggesting that the number of candidate genes for semen proteins could be reduced by utilizing co-expression data like SPELL. In addition, there exists a considerable number of semen candidate genes exclusively predicted by our approach. This result shows that our dataset can generate a set of candidate genes for semen components.

**Candidate Genes for Synapse Formation and Function**

Using the same guilt-by-association approach, we sought to identify synaptic proteins. In view of the burst of synaptogenesis during maturation of the male, transcripts of genes involved in synapse formation or function are expected to be elevated in the male-enriched modules (Mod1–Mod3). Previously, a large-scale RNAi screen in C. elegans identified over 100 genes required for synapse function based on mutant response to the acetylcholinesterase inhibitor aldicarb (Sieburth et al., 2005); however, there are likely to be many additional synaptic components that remain to be discovered, as proteomic studies have revealed several hundred factors residing in synapses (Bayés and Grant, 2009).

To obtain a probe gene set, we first searched conserved genes for synapse function from the literature (Jin, 2005; Jin and Garner, 2008; Richmond, 2005; Sudhof and Rizo, 2011) and selected five genes, including cpx-1 (complexin), rab-3 (rab3), ric-4 (SNAP-25), unc-13 (Munc13), and unc-104 (KIF1A/kinesin-3), because they were highly co-regulated in the RNAi screen in C. elegans identified over 100 genes required for synapse function based on mutant response to the acetylcholinesterase inhibitor aldicarb (Sieburth et al., 2005); however, there are likely to be many additional synaptic components that remain to be discovered, as proteomic studies have revealed several hundred factors residing in synapses (Bayés and Grant, 2009).

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Of the 47 genes not previously associated with synapses, we further focused on 29 genes that have human orthologs (Table S5), because synaptic components in general are likely to be conserved across diverse taxa. The expression patterns of 18 of these genes were previously annotated in WormBase. For ten (out of 11) of the remaining genes, we examined their expression using promoter::GFP fusion transgenes (Figure S4). All 28 examined genes were expressed in neurons and/or muscles (Figure S5), indicating that these genes act in most relevant tissues for synapse function. In all cases we tested, outside of the male-specific nervous system in the tail, expression was similar in both sexes; hence, these genes are expected to have similar functions in both sexes.

To address further whether these genes are required for synaptic transmission, we tested response of available mutants to aldicarb. As aldicarb perturbs the breakdown of acetylcholine at neuromuscular synapses, aldicarb-resistant or hypersensitive mutants have a decrease or increase in acetylcholine signaling, respectively (Mahoney et al., 2006). Among the 29 genes with human orthologs, mutations were available for 12 genes from the Caenorhabditis Genetics Center, and we used these mutants for an aldicarb assay. We found two resistant mutants, ckr-1 and W10C8.5, and one hypersensitive mutant, R13A5.9 (Figure 5D), suggesting that these gene products function at least in cholinergic synapses. ckr-1 and W10C8.5 encode a cholecystokinin receptor homolog and glutamine synthetase/guanido kinase domain protein, respectively; R13A5.9 encodes a major facilitator superfamily domain protein. None of these genes were previously implicated in synapse function or found in the previous large-scale RNAi screen using aldicarb (Sieburth et al., 2005). Taken altogether, these results suggest that this set of genes highly correlated in their expression patterns with known synaptic genes may identify previously unrecognized components of synapses or proteins required for synapse formation or function.

To compare our candidate gene list with those obtained using other gene-prediction tools, we obtained candidate gene lists using SPELL and WormNet. For each dataset, we identified the 63 most highly correlated genes with the five synaptic probe genes. All analyses performed well based on their ability to identify previously known genes for synapse function: among the 63 genes, 17% (this study), 27% (SPELL), and 19% (WormNet; Figure 5E). These rates were further increased in shared gene lists between the datasets. For example, we found 14 overlapping genes between our candidate list and the SPELL list, of which six (nx-1, rbf-1, ida-1, unc-14, mpz-1, and gar-2) have known synaptic function and two (snt-4 and DH11.5; also shared by the WormNet list) are synaptotagmin homologs involved in synaptic vesicle trafficking (total 57%; Figure 5E; Table S5). The remaining six genes include R13A5.9 for which mutant showed a hypersensitive response to aldicarb in our analysis (Figure 5D). However, two genes for the resistant mutants in the aldicarb assay (ckr-1 and W10C8.5) were predicted by our approach.
These results show that our dataset can expand a candidate gene list for synapse function.

**Prediction of Putative Ciliary Genes**

As a final example, we examined the possibility of identifying components of cilia, which are conserved macromolecular structures. In addition to the 60 ciliated neurons present in both sexes, males generate 52 additional ciliated neurons during late-larval maturation (Sulston et al., 1980; White et al., 1986). Therefore, it is expected that transcripts of many ciliary genes will be enriched in the male when compared to the hermaphrodite during this period, and components of cilia may be found by a co-expression approach. For example, three known ciliary genes, *bbs-1*, *che-13*, and *osm-5*, are expressed in most of the ciliated sensory neurons (Efimenko et al., 2005) and are highly co-regulated within Mod1 (correlation coefficient > 0.9226). Forty-six genes are equally co-expressed with these three genes, all of which belonged to Mod1 (Table S6). Only three of these genes (*bbs-9, dyf-2*, and *tub-1*) have previously been implicated in ciliary functions (Blaque et al., 2005; Efimenko et al., 2006; Mukhopadhyay et al., 2005). Of the 21 out of the 46 genes with known expression patterns in WormBase, 16 genes (76%) have expression in some of the ciliated sensory neurons. It remains to be determined whether some of the remaining 43 co-expressed genes also function in cilia.

**DISCUSSION**

In *C. elegans*, most male-specific structures, together with the addition of a large number of synapses, arise during a short interval of late larval development (Emmons, 2014; Sulston et al., 1980). This brief episode of neurogenesis and morphogenesis...
is expected to be accompanied by relatively rapid changes in overall gene expression (see Figure 1B). This unique developmental feature makes possible the generation of temporal gene expression profiles with great resolution, which provide a template for functional prediction not only of male-specific genes but also genes with functions in both sexes that are upregulated specifically in the male during this period. In this study, we used temporal gene expression profiles to predict gene functions that are sex specific (e.g., male transcription factors and semen components) as well as potentially shared between both sexes (e.g., factors for synapse function and components of cilia). The validation experiments for some candidate genes support our predictions of genetic functions.

Prediction of gene functions through co-expression approaches is based on the similarity of gene expression profiles across multiple samples or conditions. It is thought that adding more datasets obtained from different conditions increases the predictive power of the method (D’haeseleer et al., 2000). In our analyses, we used ten samples comprising five different time points for each sex at 6-hr intervals from late L3 to young adult stages. Recently, it has been reported that, during the transition from larval to adult stages, the hermaphrodite shows rapid gene expression shift that was not previously recognized when sampled at hourly intervals (Snoek et al., 2014). Thus, we expect that the predictive power of co-expression analysis can be increased by sampling more-temporal data points during the interval we examined here.

The use of gene transcriptional co-expression analysis to predict gene functions rests on the assumption that subsets of genes that encode proteins primarily or exclusively functioning together in a pathway or molecular complex will be coordinately transcribed. In addition to the sets of genes we identified—transcription factors for male development, semen proteins, and putative synaptic components—many other pathways can be explored with our data. These could include genes for muscle development (Mod1), cuticle and molting (Mod4), ribosome-associated proteins (Mod5), and oocyte development (Mod6). Other pathways and cell structures that involve conserved protein complexes include RNA processing, chromatin, and transcription.

Although our guilt-by-association approach identified several candidate genes with functions possibly related to the functions of the probe gene set, the limited sample size makes it difficult to assess the overall success of the method. We expect that this is the case especially for the synaptic and ciliary candidates, which do not show robust sex-biased expression. We found that three out of 12 mutants were defective in synaptic transmission, but we do not know what the background frequency would be among all genes in the nervous-system-related modules. For ciliary function, we predicted 46 candidate genes, only three of which are known cilia genes and one of which (unc-104) may function in cilia (Ou et al., 2007). One way to increase the power of prediction is to utilize existing databases in conjunction with ours, such as SPELL (Hibbs et al., 2007) or WormNet (Cho et al., 2014), as described in Figures 4F and 5E.

We detected significant transcription for 88% of the approximately 20,000 predicted protein-coding genes in the C. elegans genome. Of these genes, 8,273 (46%) have assigned functional gene names based on structural or phenotypic information. Interestingly, in the male-enriched set of 1,645 protein-coding genes, only 365 have functional gene names (22%); the remaining 1,280 (78%) have only sequence-based names and usually no listed mutant phenotype. Our modularity analysis partitions the 21,143 protein- and non-protein-coding genes in a highly informative manner, and genes without functional gene names are distributed throughout all the modules. For many of these genes, functions may be inferred from the functions of their nearest neighbors in the co-expression tree.

**EXPERIMENTAL PROCEDURES**

**Synchronized Worm Preparation**

To generate worm populations containing large numbers of males, *him-5(e1490)* V worms were grown at 20°C on standard nematode growth media (NGM) plates with OP50 E. coli as a food source. To obtain pure preparations of males and hermaphrodites of a specific age, individual animals were selected by hand from staged, synchronized populations, obtained as follows. Gravid adult hermaphrodites were allowed to lay eggs overnight. The following day, hermaphrodites and any larval worms were gently washed off the agar, leaving eggs adhering to the surface. Newly hatched L1 worms were then harvested at 1-hr intervals by gently washing them off the plate with M9 buffer. The resulting worms were grown at 20°C to a desired time point, and then males or hermaphrodites were individually selected over an interval of 1 hr (up to 600 worms per time point). For NGM agar, M9, and general worm methods, consult Brenner (1974).

To cover a certain developmental stage, worms from three time points were pooled into one sample, resulting in five samples for each sex:

- samples M1 and H1: 30, 32, and 34 hr post-hatching (correspond to late L3);
- samples M2 and H2: 36, 38, and 40 hr post-hatching (correspond to early L4);
- samples M3 and H3: 42, 44, and 46 hr post-hatching (correspond to late L4);
- samples M4 and H4: 48, 50, and 52 hr post-hatching (correspond to young adult); and
- samples M5 and H5: 54, 56, and 58 hr post-hatching (correspond to young adult).

Each sample was washed three times in M9 buffer and stored at −80°C.

**RNA Isolation, RNA Sequencing, and Data Processing**

Total RNA was extracted using TRIzol (Invitrogen); DNA was removed with TURBO DNA-free (Life Technologies). Concentration and quality of RNA was determined using the Agilent 2100 Bioanalyzer. For each sample, a total of 1 μg of RNA was submitted to the Genomics Core Facility of the Albert Einstein College of Medicine for library preparation and RNA sequencing. Library preparation followed the protocol described at http://wasp.einstein.yu.edu/index.php/Main_Page, and sequences were determined for 100-bp single-end reads using the Illumina HiSeq2500. Sequence reads were aligned to the C. elegans reference genome (WS190) using GSNAP (version 2012-07-12; Wu and Nacu, 2010). Assignment of reads to genes was performed with htseq-count in HTSeq (version 0.5.3p3; Anders et al., 2015). Both of these steps were performed by the Genomics Core Facility. The resulting read count data were normalized using R/Bioconductor package DESeq (Anders and Huber, 2010). After genes with fewer than five reads total across the ten samples were removed, significant expression data were obtained for 21,143 genes. The normalized read counts were utilized for principal-component and differential expression analyses using DESeq or further log-transformed as log2(1 + x) for co-expression analysis.

**Weighted Gene Co-expression Network Analysis**

Weighted gene co-expression network analysis was performed using R package WGCNA (Langfelder and Horvath, 2008). The log-transformed data were used to generate a matrix of the Pearson correlations between all pairs of
genes across the samples. A weighted correlation network was created by using the correlation coefficients with the power $|\beta| = 6$ if a coefficient is more than zero and otherwise the value is zero. The value $|\beta| = 6$ was chosen as a saturation level for a soft threshold of the correlation matrix, based on the approximation of scale-free topology (Zhang and Horvath, 2005). To minimize effects of noise and spurious connections, the correlation matrix was transformed to the topological overlap matrix using a “TOMsimilarity” function implemented in the WGCNA package (Langfelder and Horvath, 2008). The topological overlap matrix was then used to group highly co-expressed genes by performing average linkage hierarchical clustering. The Dynamic Hybrid Tree Cut algorithm was used to cut the hierarchical clustering tree (Langfelder et al., 2008), and modules were defined as the branches resulting from this tree cutting. The module eigengene, the first principal component of the module, was used to further merge highly correlated modules (correlation coefficient > 0.8).

**Guilt-by-Association Approach**

The guilt-by-association approach was used to identify genes co-expressed with known genes (probes) for a specific function or expression pattern. To obtain a manageable number of probe genes, we selected probes fulfilling two criteria: (1) known from other work to have a common expression pattern and/or function and (2) highly co-regulated in our data (correlation coefficient > 0.9). A list of the genes considered to be co-expressed with the probe genes was obtained by finding all genes having correlation coefficients with each of the probe genes that were greater than that of the least-similar pair of probe genes.

**Gene Ontology Analysis**

Functional annotation for the expressed gene list was performed with two annotation tools: the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 and protein analysis through evolutionary relationships (PANTHER) version 10.0. EASE Score, a modified Fisher Exact p value, was used to calculate enrichment p value in DAVID (Huang et al., 2009). Statistical overrepresentation test was performed to obtain enriched GO terms in PANTHER (Mi et al., 2013). To compare the two annotation tools in generating enriched GO terms, we used “GOTERM_BP_FAT” for DAVID and “GO-Slim Biological Process” for PANTHER as annotation datasets in Table S3. Representative GO terms in Table 1 are significant at p < 0.05 in both DAVID and PANTHER.

**Comparison of Gene Prediction Using Existing Datasets**

Two gene-prediction tools, SPELL (version 2.0.3) and WormNet (version 3) that allow multiple query genes, were used to compare candidate gene lists (Hibbs et al., 2007; Cho et al., 2014). For direct comparison, we obtained the same number of candidate genes that show high scores with the probe (query) genes used in this study. Previously known genes for semen proteins or synapase function were searched in WormBase (http://www.wormbase.org).

**C. elegans Strains**

N2 Bristol or CB4088 (*him-5*(*e1490*)) was used as the wild-type reference strain. The following mutant alleles were used in this study: LGI: ckr-1(ok2502); ant-1(ok285); tom-1(ok283); T04D3.3(ok2924); W10C8.5(ok2723); and Y18D10A.6(ok549), LGII: C52E12.6(ok7324) and F57F10.1(ok368), LGIII: gmp-3(ok3283); pros-1(ok903); unc-119(ed3); R13A5.9(ok3733); and T20H4.2(ok2547), LGIV: C17H12.9(ok1395) and Y73B8B.19(ok1188), and LGV: ric-4(m1088); sul-2(kg187); lin-1(ok1088); and T11A8.6(ok1866). To examine defects in male tail ray morphology, four mutants, for ceh-45, lst-5, gmb-3, and T20H4.2, were crossed four times into *him-5*(*e1490*) background. These mutants were further crossed with *bxEx17 (pkd-2p::gfp; Jia and Emmons, 2006)* to visualize male-specific neurons, including RnBs, CEMs, and HOB.

**Transgenic Strains**

All transgenes were generated using a PCR fusion method (Hobert, 2002). For a transcripational reporter, a genomic fragment containing the upstream sequence of a gene (~1,000–2,000 bp) was PCR amplified from N2 worms and then fused to GFP or sfGFP. For translational reporters of *ins-31* and *FS9B2.12*, a genomic fragment containing upstream sequence and coding region was PCR amplified and fused to GFP or sfGFP at its C terminus. The resulting PCR products were injected into *him-5*(*e1490*) worms at 50–100 ng/µL with co-injection marker pRF4 (rol-6(su1006)) or ttx-3p::gfp to generate the following strains: bx*Ex*171 (*C295F3.3p::gfp; amplified region [bp]: −1,102−4 bp; bx*Ex*172 (amps-3p::gfp: −2,259−6 bp; bx*Ex*173 (amp-1p::gfp: −2,058−6 bp; bx*Ex*174 (apw-2p::gfp: −2,021−4 bp; bx*Ex*175 (DH11.5p::gfp: −2,006−1 bp; bx*Ex*176 (F27C1.11p::gfp: −893−1 bp; bx*Ex*177 (M117.1p::gfp: −2,096−1 bp; bx*Ex*178 (T1946.4p::gfp: −999−1 bp; bx*Ex*179 (W10C8.5p::gfp: −2,065−1 bp; bx*Ex*180 (kaa-2p::gfp: −2,049−1 bp; bx*Ex*189 (clec-64p::gfp: −873−1 bp; bx*Ex*190 (clec-207p::gfp: −1,026−4 bp; bx*Ex*191 (clec-199p::gfp: −854−16 bp; bx*Ex*192 (clec-219p::gfp: −2,807−9 bp; bx*Ex*193 (B0207.5p::gfp: −874−1,265 bp; bx*Ex*198 (ins-31p::sfgfp: −2,035−1 bp; bx*Ex*199 (FS9B2.12p::gfp: −993−1 bp; bx*Ex*200 (ins-31p::sfgfp: −2,035−750 bp; and bx*Ex*197 (FS9B2.12p::sfgfp: −993−3,247 bp).

**Microscopy**

Worms were mounted on 5% agar pads on glass slides using 10 mM sodium azide (Shaham, 2005). Worms were observed with Nomarski or fluorescence microscopy (Zeiss Axio Imager.A1 or Z2), and images were acquired using a camera (AxioCam; Zeiss) and processed using AxioVision (Zeiss). Figures were prepared using ImageJ software.

**Aldicarb-Sensitivity Assay**

Aldicarb (1 mM; Sigma-Aldrich) assays were performed using young adult hermaphrodites at least three times as described (Mahoney et al., 2006).

**ACCESSION NUMBERS**

The accession number for the sequence data reported in this paper is NCBI: GSE83216.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.051.

**AUTHOR CONTRIBUTIONS**

B.K. and S.W.E designed the experiments. B.K. and S.W.E analyzed the data, and wrote the paper. B.K. and B.S. conducted the experiments.

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**REFERENCES**

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biol. 11, R106.

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169.

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D., and Wolfner, M.F. (2011). Insect seminal fluid proteins: identification and function. Annu. Rev. Entomol. 56, 21–40.

Bayés, A., and Grant, S.G. (2009). Neuroproteomics: understanding the molecular organization and complexity of the brain. Nat. Rev. Neurosci. 10, 635–646.
Blacque, O.E., Perens, E.A., Boroevich, K.A., Inglis, P.N., Li, C., Warner, A., Khattra, J., Holt, R.A., Ou, G., Mah, A.K., et al. (2005). Functional genomics of the cilium, a sensory organelle. Curr. Biol. 15, 935–941.
Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics 77, 71–94.
Cho, A., Shin, J., Hwang, S., Kim, C., Shim, H., Kim, H., Kim, H., and Lee, I. (2014). WormNet v3: a network-assisted hypothesis-generating server for Caenorhabditis elegans. Nucleic Acids Res. 42, W78–W82.
D’haeseleer, P., Liang, S., and Somogyi, R. (2000). Genetic network inference: from co-expression clustering to reverse engineering. Bioinformatics 16, 707–726.
Elfenbenko, E., Bubk, K., Mak, H.Y., Holzman, T., Leroux, M.R., Ruvkun, G., Thomas, J.H., and Swoboda, P. (2005). Analysis of xbx genes in C. elegans. Development 132, 1923–1934.
Elfenbenko, E., Blacque, O.E., Ou, G., Haycraft, C.J., Yoder, B.K., Scholey, J.M., Leroux, M.R., and Swoboda, P. (2006). Caenorhabditis elegans DFF-2, an orthologue of human WDR19, is a component of the infratragal transport machinery in sensory cilia. Mol. Biol. Cell 17, 4801–4811.
Ellegren, H., and Parsch, J. (2007). The evolution of sex-biased genes and sex-biased gene expression. Nat. Rev. Genet. 8, 689–698.
Emmons, S.W. (2014). The development of sexual dimorphism: studies of the Caenorhabditis elegans male. Wiley Interdiscip. Rev. Dev. Biol. 3, 239–262.
Ferreira, H.B., Zhang, Y., Zhao, C., and Emmons, S.W. (1999). Patternning of Caenorhabditis elegans posterior structures by the Abdominal-B homolog, egl-5. Dev. Biol. 207, 215–228.
Gerstein, M.B., Li, Z.J., Van Nostrand, E.L., Cheng, C., Arshinoff, B.I., Liu, T., Yip, K.Y., Robilotto, R., Rechtsteiner, A., Ikegami, K., et al.; modENCODE Consortium (2010). Integrative analysis of the Caenorhabditis elegans genome by the modENCODE project. Science 330, 1775–1787.
Hibbs, M.A., Hess, D.C., Myers, C.L., Huttenhower, C., Li, K., and Troyanskaya, O.G. (2007). Exploring the functional landscape of gene expression: directed search of large microarray compendia. Bioinformatics 23, 2692–2699.
Hiller, L.W., Reinke, V., Green, P., Hirst, M., Marra, M.A., and Waterston, R.H. (2009). Massively parallel sequencing of the polyadenylated transcriptome of C. elegans. Genome Res. 19, 657–666.
Hobert, O. (2002). PCR fusion-based approach to create reporter gene constructs for expression analysis in transgenic C. elegans. Biotechniques 32, 728–730.
Huang, W., Sherman, B.T., and Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57.
Jarrell, T.A., Wang, Y., Bloniarz, A.E., Brittin, C.A., Xu, M., Thomas, J.N., Albertson, D.G., Hall, D.H., and Emmons, S.W. (2012). The connectome of a male Caenorhabditis elegans. PLoS ONE 7, e44748.
Jin, Y. (2005). Synaptogenesis (WormBook). http://dx.doi.org/10.1895/wormbook.1.44.1.
Jin, Y., and Garner, C.C. (2008). Molecular mechanisms of presynaptic differentiation. Annu. Rev. Cell Dev. Biol. 24, 237–262.
Johnstone, I.L. (2000). Cuticle collagen genes. Expression in Caenorhabditis elegans. Trends Genet. 16, 21–27.
Kim, S.K., Lund, J., Kiraly, M., Duke, K., Jiang, K., Stuart, J.M., Eizinger, A., Wylie, B.N., and Davidson, G.S. (2001). A gene expression map for Caenorhabditis elegans. Science 293, 2087–2092.
Kulkarni, G., Li, H., and Wadsworth, W.G. (2008). CLEC-38, a transmembrane protein with C-type lectin-like domains, negatively regulates UNC-40-mediated axon outgrowth and promotes presynaptic development in Caenorhabditis elegans. J. Neurosci. 28, 4541–4550.
Langfelder, P., and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9, 559.
Langfelder, P., Zhang, B., and Horvath, S. (2008). Defining clusters from a hierarchical cluster tree: the Dynamic Tree Cut package for R. Bioinformatics 24, 719–720.
Lints, R., and Emmons, S.W. (2002). Regulation of sex-specific differentiation and mating behavior in C. elegans by a new member of the DM domain transcription factor family. Genes Dev. 16, 2390–2402.
Mahoney, T.R., Luo, S., and Nonet, M.L. (2006). Analysis of synaptic transmission in Caenorhabditis elegans using an aldicarb-sensitivity assay. Nat. Protoc. 1, 1772–1777.
Mason, D.A., Rabinowitz, J.S., and Portman, D.S. (2008). dmd-3, a doublesex-related gene regulated by tra-1, governs sex-specific morphogenesis in C. elegans. Development 135, 2373–2382.
Matson, C.K., and Zarkower, D. (2012). Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. Nat. Rev. Genet. 13, 163–174.
Miersch, C., and Döring, F. (2012). Sex differences in carbohydrate metabolism are linked to gene expression in C. elegans. PLoS ONE 7, e44748.
Mounsey, A., Bauer, P., and Hope, I.A. (2002). Evidence suggesting that a fifth of annotated Caenorhabditis elegans genes may be pseudogenes. Genome Res. 12, 770–775.
Mukhopadhyay, A., Deplancke, B., Walhout, A.J., and Tissenbaum, H.A. (2005). C. elegans tubby regulates life span and fat storage by two independent mechanisms. Cell Metab. 2, 35–42.
O’Rourke, D., Baban, D., Demidova, M., Mott, R., and Hodgkin, J. (2006). Genomic clusters, putative pathogen recognition molecules, and antimicrobial genes are induced by infection of C. elegans with M. nematophilum. Genome Res. 16, 1005–1016.
Ou, G., Koga, M., Blacque, O.E., Murayama, T., Ohshima, Y., Schafer, J.C., Li, C., Yoder, B.K., Leroux, M.R., and Schoele, J.M. (2007). Sensory cilio genesis in Caenorhabditis elegans: assignment of IFT components into distinct modules based on transport and phenotypic profiles. Mol. Biol. Cell 18, 1554–1569.
Page, A.P., and Johnstone, I.L. (2007). The cuticle. WormBook. 1–15. http://dx.doi.org/10.1895/wormbook.1.138.1.
Palopoli, M.F., Rockman, M.V., TinMaung, A., Ramsay, C., Curwen, S., Aduna, A., Laurita, J., and Kruglyak, L. (2008). Molecular basis of the copulatory plug polymorphism in Caenorhabditis elegans. Nature 454, 1019–1022.
Raymond, C.S., Shamu, C.E., Shen, M.M., Seflert, K.J., Hirsch, B., Hodgkin, J., and Zarkower, D. (1998). Evidence for evolutionary conservation of sex-determining genes. Nature 391, 691–695.
Reinke, V., Smith, H.E., Nance, J., Wang, J., Van Doren, C., Begley, R., Jones, S.J., Davis, E.B., Scherer, S., Ward, S., and Kim, S.K. (2000). A global profile of germline gene expression in C. elegans. Mol. Cell 6, 605–616.
Reinke, V., Gil, I.S., Ward, S., and Kazmer, K. (2004). Genome-wide germ-line-enriched and sex-biased expression profiles in Caenorhabditis elegans. Development 131, 311–323.
Reinke, V., Krause, M., and Okkema, P. (2013). Transcriptional regulation of gene expression in C. elegans. WormBook. 1–34. http://dx.doi.org/10.1895/wormbook.1.45.2.
Richmond, J. (2005). Synaptic function. WormBook. 1–14. http://dx.doi.org/10.1895/wormbook.1.68.1.
Shaham, S. (2005). Methods in Cell Biology. WormBook. http://dx.doi.org/10.1895/wormbook.1.49.1.
Shan, G., and Wathall, W.W. (2008). Copulation in C. elegans males requires a nuclear hormone receptor. Dev. Biol. 322, 11–20.
Sieburth, D., Ch’ng, Q., Dybbs, M., Tavazoie, M., Kennedy, S., Wang, D., Dupuy, D., Rual, J.F., Hill, D.E., Vidal, M., et al. (2005). Systematic analysis of genes required for synapse structure and function. Nature 436, 510–517.
Smith, J.R., and Stanfield, G.M. (2011). TRY-5 is a sperm-activating protease in Caenorhabditis elegans seminal fluid. PLoS Genet. 7, e1002375.

Snoek, L.B., Sterken, M.G., Volkers, R.J., Klatter, M., Bosman, K.J., Bevers, R.P., Riksen, J.A., Smart, G., Cossins, A.R., and Kammenga, J.E. (2014). A rapid and massive gene expression shift marking adolescent transition in C. elegans. Sci. Rep. 4, 3912.

Spencer, W.C., Zeller, G., Watson, J.D., Henz, S.R., Watkins, K.L., McWhirter, R.D., Petersen, S., Sreedharan, V.T., Widmer, C., Jo, J., et al. (2011). A spatial and temporal map of C. elegans gene expression. Genome Res. 21, 325–341.

Sudhof, T.C., and Rizo, J. (2011). Synaptic vesicle exocytosis. Cold Spring Harb. Perspect. Biol. 3, a005637.

Sulston, J.E., Albertson, D.G., and Thomson, J.N. (1980). The Caenorhabditis elegans male: postembryonic development of nongonadal structures. Dev. Biol. 78, 542–576.

Thoemke, K., Yi, W., Ross, J.M., Kim, S., Reinke, V., and Zarkower, D. (2005). Genome-wide analysis of sex-enriched gene expression during C. elegans larval development. Dev. Biol. 284, 500–508.

White, J.G., Southgate, E., Thomson, J.N., and Brenner, S. (1986). The structure of the nervous system of the nematode Caenorhabditis elegans. Philos. Trans. R. Soc. Lond. B Biol. Sci. 314, 1–340.

Wu, T.D., and Nacu, S. (2010). Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics 26, 873–881.

Yi, W., and Zarkower, D. (1999). Similarity of DNA binding and transcriptional regulation by Caenorhabditis elegans MAB-3 and Drosophila melanogaster DSX suggests conservation of sex determining mechanisms. Development 126, 873–881.

Zhang, B., and Horvath, S. (2005). A general framework for weighted gene co-expression network analysis. Stat. Appl. Genet. Mol. Biol. 4, e17.