Cell-Specific mRNA Profiling of the Caenorhabditis elegans Somatic Gonadal Precursor Cells Identifies Suites of Sex-Biased and Gonad-Enriched Transcripts

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ABSTRACT The Caenorhabditis elegans somatic gonad differs greatly between the two sexes in its pattern of cell divisions, migration, and differentiation. Despite decades of study, the genetic pathways directing early gonadal development and establishing sexual dimorphism in the gonad remain largely unknown. To help define the genetic networks that regulate gonadal development, we employed cell-specific RNA-seq. We identified transcripts present in the somatic gonadal precursor cells and their daughter cells of each sex at the onset of sexual differentiation. We identified several hundred gonad-enriched transcripts, including the majority of known regulators of early gonadal development, and transgenic reporter analysis confirmed the effectiveness of this approach. Before the division of the somatic gonad precursors, few sex-biased gonadal transcripts were detectable; less than 6 hr later, after their division, we identified more than 250 sex-biased transcripts, of which about a third were enriched in the somatic gonad compared to the whole animal. This indicates that a robust sex-biased developmental program, some of it gonad-specific, initiates in the somatic gonadal precursor cells around the time of their first division. About 10% of male-biased transcripts had orthologs with male-biased expression in the early mouse gonad, suggesting possible conservation of gonad sex differentiation. Cell-specific analysis also identified approximately 70 previously unannotated mRNA isoforms that are enriched in the somatic gonad. Our data illustrate the power of cell-specific transcriptome analysis and suggest that early sex differentiation in the gonad is controlled by a relatively small suite of differentially expressed genes, even after dimorphism has become apparent.

KEYWORDS sex determination gonad RNA-seq Z1/Z4 C. elegans genetics of sex
gesting that TRA-1 may be regulating only a small subset of genes

Hodkin 1992). Surprisingly, very few transcripts enriched in the so-

transcripts enriched in the gonad compared to the whole animal, in-

The majority of the dissociated cell suspension was sorted by FACS to isolate the gonadal cells. An aliquot of the dissociated cells was removed prior to FACS to be used as a whole animal control. (E) RNA was isolated and RNA-seq performed (see Materials and Methods).

(Spencer et al. 2011, 2014). Here we use RNA-seq of purified cells to
define the transcriptome of the somatic gonad primordium in each sex
in order to delineate components of the distinct genetic networks that
regulate organ-specific and sex-specific gonadal development. We ex-

Figure 1 Schematic of cell-specific RNA-sequencing. (A) Embryos harvested using strains that give rise to pure populations of either sex. (B) Animals were hatched in the absence of food to synchronize growth. After hatching, larvae were fed to allow development to proceed. (C) Animals were harvested at the appropriate developmental time point after disrupting the cuticle and dissociating and filtering the single cells. (D) The majority of the dissociated cell suspension was sorted by FACS to isolate the gonadal cells. An aliquot of the dissociated cells was removed prior to FACS to be used as a whole animal control. (E) RNA was isolated and RNA-seq performed (see Materials and Methods).

MATERIALS AND METHODS

Strains

Z1/Z4 and their daughter cells were isolated for transcriptional profiling from DZ685 [sol-1(y9) X; rdIs4 (ehn-3a(promoter::Venus)]) in hermaphrodites. The rdIs4 expresses the Venus fluorescent protein exclusively in Z1/Z4 and their descendants. To collect large populations of phenotypically male animals DZ683 {tra-2(ar221) II; xol-1(y9) X; rdIs4 [ehn-3a(promoter::Venus)]} was used. This strain has a temperature-sensitive mutation in tra-2, a component of the global sex determination pathway, tra-2(ar221) xol-1(y9) animals reared at permissive temperature develop as hermaphrodites, and those reared at restrictive temperature develop as well masculinized XX pseudomales (Hodkin 2002), which will be referred to as males. The xol-1(y9) mutation kills spontaneous XO males by disrupting sex chromo-
some dosage compensation and also enhances masculinization by tra-2(ar221). Dissociated cells from DZ685 and DZ683 that were not sorted by fluorescence-activated cell sorting (FACS) were used to collect whole animal populations of cells for the later time point, and both time points, respectively. xol-1(y9)X, a strain lacking rdIs4, was used for the hermaphrodite whole animal sample for the earlier time point.

Nematode culture

To produce large populations of animals for isolation of cells from the
gonadal primordium, cultures of synchronized animals were reared by
the following method: animals were first cultured on standard 60 mm
nematode growth medium (NGM) plates (Stiernagle 2006) until food
was exhausted. Each starved 60-mm NGM plate was used to inoculate
two 150-mm bactopeptone-rich 8P plates with four times the usual
amount of cholesterol supplementation (Bianchi and Driscoll 2006). Strains were grown at 15° until plates were full of gravid adults (about
and transferred to a chilled 15 ml conical tube. Live larvae were then collected by sucrose flotation in 9 ml of ice cold 70% sucrose mixed with 6 ml of ice-cold M9 and centrifuged immediately at 4°C for 5 min at 2000 rpm. Up to 10 ml of solution containing larva was removed from the tube with polished blunt Pasteur pipet. Collected larvae were diluted to 50 ml with M9 in a 50-ml conical tube and washed twice for 2 min at 2000 rpm to remove sucrose. Approximately 1.2 ml of packed larvae were pelleted by centrifugation at 7000 rpm for 1 min in 1.5 ml microfuge tubes, washed with sterile water and pelleted again, with a maximum volume of 200 μl of packed worms per tube.

Cell isolation from the collected larvae largely followed Zhang et al. (2011) with modifications to allow isolation of larger numbers of cells, which included adjusting volumes, incubations, amount of mechanical force by pipetting, and filtering of debris after dissociation. With these modifications, we were able to dissociate a five to ten times larger population of healthy dissociated cells than before. We also fed larvae a normal bacterial diet rather than the axenic medium used in previous isolations.

To disrupt the cuticle, each tube of washed and pelleted animals was incubated for 2 min in 500 μl of freshly thawed sterile SDS-DTT solution (Zhang et al., 2011), and then 1 ml of egg buffer (Strange et al. 2007) was added and tubes were spun immediately for 1 min at 7000 rpm. Supernatant was removed and pelleted animals were washed five times with 1 ml egg buffer. Pelleted SDS-DTT-treated worms were then suspended in 500 μl of 15 mg/ml pronase (P8811; Sigma-Aldrich, St. Louis, MO) in egg buffer and incubated 7 min at room temperature and then pipetted with a standard 1-ml plastic pipet for 15 min continuously in pronase solution to dissociate cells by mechanical force. Animals were incubated in pronase for an additional 3–15 min after pipetting. One milliliter of L-15 medium supplemented with 10% fetal bovine serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin (Strange et al. 2007) was added to each tube to quench pronase digestion, and cells were pelleted by centrifugation at 10,000 rpm for 5 min at 4°C. Pellets were washed twice with L-15/FBS and resuspended in 1 ml L-15/FBS. A small sample of cell suspension was examined on a microscope slide to verify the presence of green fluorescent protein (GFP)-positive single cells and the overall effectiveness of the cell dissociation. Resuspended cells were filtered with a 5 μm filter (EMD Millipore) to separate individual cells from carcasses and debris, and filtered cells were pelleted by centrifugation at 10,000 rpm for 5 min at 4°C. Cells were resuspended in egg buffer with 5 μg/ml of propidium iodide to identify and remove dead cells during cell sorting and then sorted by FACS at the University of Minnesota Flow Cytometry Resource on an AriaII cell sorter with a 70 μm diameter nozzle. To exclude auto–fluorescent cells during FACS, strains harboring rdls4 were compared to zol-1(y59)X, a strain lacking GFP.

**RNA extraction and sequencing**

Sorted cells were collected directly into TRIzol (Ambion, Carlsbad, CA) for RNA extraction. RNA was purified using RNeasy MiniElute spin column kits (Qiagen, Valencia, CA). RNA was depleted by Ribo-Zero (Epitect, Madison, WI). Initial experiments used Ovation RNA-Seq System V2 (NuGEN, San Carlos, CA) to amplify cDNA, but subsequent experiments proceeded directly to TruSeq (Illumina, San Diego, CA) from the rRNA-depleted sample but omitting the poly-A selection of mRNA. Next generation sequencing was performed at the University of Minnesota Genomics Center using single-end 50 bp reads. Sequences were mapped to the C. elegans genome (USCS ce10, Wormbase

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**Figure 2** Green fluorescent protein (GFP) expression in smotic gonadal precursor cells. Differential interference contrast (DIC) and fluorescence micrographs of (A) gonad of a hermaphrodite larva at 9.5 hr postfeeding. (B) Gonad of a male larva at 9.5 hr postfeeding. Only Z1 is visible in the male, because Z4 lies outside focal plane. Prior to the division of Z1/Z4 the gonad appears identical between the sexes. (C) Gonad of a hermaphrodite larva at 15 hr postfeeding after the division of Z1/Z4. Distal daughters are marked by filled arrowheads and proximal daughters are marked by open arrowheads. (D) Gonad of a male larva at 15 hr postfeeding after Z1/Z4 daughters have migrated and gonads become sexually dimorphic. Note that distal daughters of Z1/Z4 are smaller. (E) Cell suspension after dissociation. To obtain a large population of healthy dissociated cells, dissociation was stopped while a portion of whole animals were still undissociated. (F) A population of single cells generated by filtering dissociated sample. Scale bar is 5 μm. (G) Density plot shows clear separation by FACS of GFP positive and Pl negative Z1/Z4 daughters, which are outlined by the green box.

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5 days, shifted to room temperature for about 90 min and then animal were washed from the plates with M9 (Stiernagle 2006). To obtain sufficient material for transcriptional profiling, approximately 20–40 million embryos of each sex were collected for each sample replicate. Eggs were isolated by bleaching, washed repeatedly with M9 to remove any trace of bleach, (Stiernagle 2006) and allowed to hatch in ∼300 ml M9 overnight while shaking at 250 rpm at the restrictive temperature of 23.5°C. Since RNA was isolated directly from FAC-sorted dissociated cells without cell culture, hatched larvae could be fed a normal bacterial diet instead of an axenic medium. Hatched larvae were plated on NA22-seeded 150-mm 8P plates in small droplets with approximately 1–4 million larva per plate and incubated at 23.5°C for 9.5 or 15 hr prior to isolation of Z1/Z4 or their daughters.

**L1 cell isolation**

Mid- and late-L1 animals fed for either 9.5 or 15 hr were recovered from plates with ice-cold M9, washed three to four times with ice-cold M9,
Table 1 Correlation of replicates

| Replicates Compared                  | Spearman’s Correlation, 9.5-hr Time Point | Spearman’s Correlation, 15-hr Time Point |
|--------------------------------------|--------------------------------------------|-----------------------------------------|
| Hermaphrodite gonadal cells          | 0.936, 0.939, and 0.966                    | 0.962                                   |
| Male gonadal cells                   | 0.920, 0.930, and 0.935                    | 0.929                                   |
| Hermaphrodite whole animal           | 0.946, 0.909, and 0.912                    | 0.963                                   |
| Male whole animal                    | 0.896, 0.913, and 0.946                    | 0.968                                   |
| Hermaphrodite gonadal cells,         |                                            |                                         |
| using NuGEN                          |                                            |                                         |

WS220) using Illumina-supplied annotations. Reads were mapped using TopHat v1.4.1 and differential expression was analyzed using Cuffdiff v2.1.1 (Trapnell et al. 2012).

Gene ontology
To determine transcripts that were overrepresented in the gonad-enriched datasets, the PANTHER Overrepresentation Test (release 20150430) was used (Mi et al. 2013). The annotation version and release date were GO Ontology database released 2015-07-07. To determine transcripts that had sex-biased function, DAVID Bioinformatics Resources 6.7 was used (Huang da et al. 2009).

Expression analysis and microscopy
Promoter regions containing between 400 bp and 3.6 kb of DNA from upstream of the start codon (Supporting Information, Table S1) were cloned into the GFP expression vector pPD95.75. Reporter constructs (20 ng/µl) were injected into the distal gonads of adult him-8(e1489) hermaphrodites and stable transgenic lines were assayed for GFP expression. Differential interference contrast (DIC) and fluorescent images were acquired on a Zeiss motorized Axioplan 2 microscope using an AxioCam MRm camera and AxioVision software (Zeiss, Jena, Germany).

Motif analyses
DREME was used to identify over-represented motifs and then Tomtom was used to compare each DREME-generated motif against a database of known motifs. FIMO was then used to identify which promoter regions of transcripts contained the DREME-generated motif. Allinformatics tools can be found at: http://meme-suite.org.

RNAi analyses
Bacterial clones (Kamath and Ahringer 2003) obtained from SourceBioSciences were grown overnight in Luria-Bertani (LB) medium and seeded onto NGM medium containing 0.02% lactose to induce dsRNA expression. Larval L4 hermaphrodite animals were placed on RNAi plates and incubated at 22°. On the following day, adult animals were moved to a second RNAi plate and rearing continued at 22°. Male young adult progeny were scored for phenotypic defects.

Hyper-geometric calculations
To determine whether there is significant overlap between two datasets the following website was used: http://nemates.org/MA/progs/overlap_stats.html. A total of 19,862 genes per genome was used for all calculations. This was the total number of genes mapped in WS220 available in iGenomes for TopHat v1.4.1, which was used in all bioinformatics analyses. Comparisons were made to determine significant overlap between sex-biased transcripts and genes near TRA-1 binding sites (P < 0.05) as well as to determine whether sex-biased or gonad-enriched transcripts were significantly over-represented on a chromosome. (P values of < 0.01 and 0.001 are indicated by * and **, respectively).

Data availability
Strains are available upon request. Illumina sequence data for RNA-seq have been deposited at NCBI with accession number GSE71772.

RESULTS
Sexually dimorphic mRNA expression in the somatic gonadal primordium
Single-sex larval populations were harvested and cells dissociated at two time points during the first larval stage (L1) using a method we optimized as described in Materials and Methods (Figure 1). Because pure populations of XO larval males cannot be obtained, we generated pure populations of larval XX phenotypic males using a strain containing a conditional mutation in the sex determination gene tra-2. At the restrictive temperature this strain gives rise to XX pseudomale animals that are anatomically male and can sire progeny. We dissociated synchronized larvae and isolated either the somatic gonadal precursor cells (SGPs) Z1 and Z4 in mid-L1 or their daughters (Z1a/p and Z4a/p) toward the end of L1, after the Z1/Z4 divisions occurred in the vast majority of animals and when the gonad is overtly sexually dimorphic (Figure 2, C and D). In males, the first SGP division has a more pronounced asymmetry, giving rise to markedly smaller distal daughter cells (Z1a and Z4p). These distal daughters migrate posteriorly in the male, while the proximal daughter cells migrate anteriorly. In contrast, in the hermaphrodite there is no gonadal cell migration at this time. To enable isolation of the SGPs or their daughters by FACS, both strains also harbored an integrated array containing an ehn-3a[promoter]:Venus fusion, which is expressed exclusively in the Z1/Z4 lineage (Figure 2, A–D). These strains also contained a mutation in the sol-1 gene, which kills spontaneous XO males and also enhances the phenotype of tra-2 (Hodgkin 2002). We isolated 100–300 ng of RNA from 300,000–600,000 FACS-sorted gonadal cells per replicate (~0.4 pg total RNA/cell).

Optimization of RNA isolation
In initial experiments we isolated approximately 25 ng of total RNA from each sample. Because of the very small amount of RNA isolated, we first used Ovation RNA-Seq System V2 (NuGEN) to synthesize and amplify cDNA prior to RNA-seq. We found that the correlation of replicates was subpar using this method (Table 1). After optimizing our dissociation protocol (see Materials and Methods), we were able to harvest 100–300 ng of total RNA. We therefore tested whether RNA-seq using TrueSeq (Illumina), which omits linear RNA amplification, might allow a better correlation of replicates. Indeed, TrueSeq yielded high correlations of replicates even with a limited quantity of RNA (Table 1), and was therefore used for all experiments presented here.
Samples were harvested in triplicate for the earlier time (profiling Z1/Z4) and in duplicate for the later time (profiling Z1/Z4 daughters). Spearman’s correlations of pairwise comparisons between the 20 samples confirmed a high correlation between replicates within the same sample (Figure 3A). At the earlier time there are few morphological differences between the sexes: males have a ventral coelomocyte located more posteriorly, they have four male-specific cephalic male (CEM) sensory neurons and they lack two hermaphrodite-specific neurons (HSNs) that undergo sex-specific programmed cell death during embryogenesis. Also the posterior B and Y blast cells are present in both sexes but have larger nuclei in males (Emmons 2005; Sulston and Horvitz 1977). Consistent with this limited anatomical dimorphism, fewer than 50 mRNAs exhibited sex-biased expression in whole larvae at the earlier time (Table 2). Our cutoff for enrichment was log2(twofold) with an uncorrected P value of < 0.025 and a false discovery rate (FDR) value < 0.05. In addition, while genetic experiments indicate that gonadal sex is determined prior to this time (Klass et al. 1976; Nelson et al. 1978), fewer than 30 transcripts showed sex-biased expression in SGPs at the earlier time point. At the later time we identified 269 sex-biased transcripts in the SGP daughters but only 27 in cells from whole larvae. This difference illustrates that sex-specific profiling of whole animals masks sex-biased expression limited to small numbers of cells.

Principal component analysis (PCA) can indicate the degree of similarity between samples based on the degree of covariance. We performed a PCA of gene expression in the 20 samples based on the read depth for each gene [reads per kilobase of transcript per million mapped reads (RPKM)] and found that they grouped in four specific clusters. Developmental times and tissues were separable but samples from different sexes grouped together (Figure 3B). Sex differences likely were not separable by PCA because there were very few gene expression differences between the sexes at each time point for either whole body or gonadal cells (0.13%–1.4% of mRNAs had significant sex-biased expression). As a consequence, the high correlation of replicates (Figure S1) was critical for reliably identifying the rare sex-biased transcripts.

**Validation of SGP transcriptomes**

To validate the gonadal transcriptome data (Table S2), we examined the representation of genes previously shown to be expressed in the SGPs. Among the mRNAs that were enriched in Z1/Z4 and their daughters relative to the entire larva, or expressed in Z1/Z4 and their daughters, to the best of our knowledge we identified all transcripts with previously described SGP expression. The majority of these transcripts were identified as significantly gonad-enriched in our analysis: fkh-6 (Chang et al. 2004), tra-1 (Mathies et al. 2004), gem-4 (Church and Lambie 2003), ceh-22 (Lam et al. 2006), cyd-1 (Tilmann and Kimble 2005), sys-1 (Miskowski et al. 2001), dsh-2 (Chang et al. 2005), and lin-26 (den Boer et al. 1998). Several other known SGP-expressed genes, [gon-14 (Walker et al. 2004), gon-2 (Sun and Lambie 1997), gon-4 (Friedman et al. 2000), and pop-1 (Siegfried et al. 2004)], also were identified as expressed in SGPs but did not meet our cutoff of log2(twofold) for enrichment in gonadal primordial cells due to their expression in other tissues. Based on our detection of previously described SGP-expressed genes, it appears that our data set identifies a majority of this gene class.

To further assess the SGP transcriptome data, we analyzed expression from transcriptional reporter transgenes containing from 400 bp to 3.6 kb of upstream sequences from each of ten genes showing SGP-enriched mRNA expression fused to GFP at the transcriptional start site. Seven of the ten reporters were expressed in the somatic gonadal precursor cells (sde-1, R11A5.3, F52D2.7, ckb-3, K03B8.8, ceh-8, pes-1); the others (pho-10, xtr-2, T13F3.8) may have lacked essential regulatory elements, such as intronic enhancers, or might have been false positives. Among the reporters expressed in the gonad, some were expressed only...
phenotypic analyses of male-biased, gonad-enriched transcripts

Of the mRNAs enriched in the gonad, 5.4% (earlier time) and 11.5% (later time) of the genes were previously reported to have a sterile phenotype based on either mutant alleles or RNAi. This relatively small proportion of gonadally-enriched mRNAs required for fertility may indicate significant genetic redundancy. In an attempt to uncover redundant requirements in gonadal function for the sex-biased and gonad-enriched transcripts expressed in the somatic gonad, we therefore performed RNAi of 44 male-biased, gonad-enriched mRNAs in four sensitized strains (1) tra-1(e1834); fkh-6(e216), (2) him-8(e1489); egl-5(m868), him-8(e1489), and (4) hhl-3(ot354), him-8(e1489) carrying mutations causing mild to more severe male gonadal defects. Three of the four genes are known to display partial redundancy with other gonadal regulators in males (Kalisi et al. 2010; J. Kimble, personal communication).

Identification of novel isoforms

Transcriptome analysis identified previously unannotated mRNA isoforms from 66 genes with gonad-enriched expression (20 unannotated isoforms were enriched at the earlier time, 14 at the later time, and 32 were enriched at both times, as indicated in Table S2 and Table S3). These unannotated isoforms used undescribed alternative transcriptional start sites or exons. (Figure 5A) or omitted specific exons that had previously appeared constitutive. Six mRNAs that were sex-biased in the SGP-daughters but not gonad-enriched were also expressed as unannotated isoforms. In contrast to a recent report of more than 300 sex-biased isoforms in the adult germline (Ortiz et al. 2014), we did not detect sex-biased mRNA isoforms in the somatic gonad; however, two genes (glna-1 and fbxc-40), one of which is shown in Figure 5B expressed two distinct unannotated mRNA isoforms, one at the earlier time in Z1/Z4 and the other at the later time in Z1/Z4 daughters. It will be of interest to test whether these gonad-enriched isoforms have specialized functions.

Correlations of transcripts with similar temporal, gonadal, or sex-biased expression

We next examined the pattern of transcript enrichment in the two sexes at each time. At the earlier time, gonadal transcriptomes were very similar in the two sexes, but they diverged at the later time (Table S2). Only 40% of mRNAs that were enriched in the gonad at the earlier time in either sex remained significantly enriched at the later time and many new mRNAs became gonad-enriched (Figure 6A, top portion of heatmap; Table S2), suggesting that the network of genes expressed during gonadal development is dynamic during this period. Of 269 mRNAs with sex-biased expression in the Z1/Z4 daughters, 29% also were enriched in the gonad and therefore may act primarily in that tissue (Table S3); 33% and 26% of mRNAs that were hermaphrodite-biased or male-biased, respectively, were enriched in the gonad, suggesting similar levels of sexual specialization in the gonads of each sex (Figure 6B).

Candidate regulators of the gonadal transcriptome

We hypothesized that transcripts showing similar enrichment profiles might be regulated by the same cis-regulatory elements and trans-acting factors. Due to the generally compact nature of C. elegans promoter regions (Blazie et al. 2015), it is feasible to search for regulatory elements within undefined promoter regions. We defined the promoter region as 1000 bp upstream of the transcriptional start site and used DREME (Bailey 2011) to identify motifs overrepresented in the promoter regions of genes biased for expression in one sex and underrepresented in the promoter regions of genes biased for expression in the other sex. The promoter regions of hermaphrodite-biased genes expressed in Z1/Z4 daughter cells were enriched for the motif ACTTKAAC, present in 18% of 120 hermaphrodite-biased genes (Table S3), while this motif appeared in less than 1% of the promoter regions of male-biased genes at the same time point. We then used FIMO (Grant et al. 2011) to identify the promoter regions of hermaphrodite-enriched genes that contained the ACTTKAAC motif (indicated in Table S3). Further analysis using Tomtom (Gupta et al. 2007) to compare the hermaphrodite-enriched motif ACTTKAAC against a database of known motifs identified HLH-2 as a potential trans-acting factor binding this motif. We found hhl-2 mRNA to be expressed in the somatic gonad in both sexes. However, HLH-2 has been shown to act
as a DNA-binding heterodimer (Portman and Emmons 2000; Thellmann et al. 2003), indicating that HLH-2 could be acting through the hermaphrodite-enriched motif, presumably in concert with other, sex-limited, factors. Of the seven genes with which HLH-2 is known to interact (ces-1, egl-43, hlh-3, hlh-14, log-2, lin-12, lin-32) only one gene, hlh-3, has sex-limited expression in the somatic gonadal precursor cells, with higher expression in male cells. This could indicate that an HLH-3/HLH-2 heterodimer acts in males to repress hermaphrodite-biased transcripts or alternatively HLH-2 may have another partner, yet to be identified, in the gonad.

We did not identify an enriched motif in male-biased transcripts but previous work has identified two transcriptional regulators, TRA-1 and FKH-6, which are crucial for male-specific gonadal development. Some of the male-biased transcripts identified here are likely to be regulated in the somatic gonad by one or both of these transcription factors. TRA-1 (Zarkower and Hodgkin 1992) is a GLI homolog that regulates sex determination by repressing genes involved in male differentiation (Chen and Ellis 2000; Conradt and Horvitz 1999; Yi et al. 2000). TRA-1 is required only in hermaphrodites in most tissues, but in the somatic gonad TRA-1 not only promotes hermaphrodite development in XX animals but also acts in both sexes to regulate SGP position and polarity and promote proliferation of gonadal cells (Mathies et al. 2004; Schedl et al. 1989). To identify potential mediators of TRA-1-dependent gonadal development, we asked whether any of the sex-biased gonadal transcripts identified here also are likely direct targets of TRA-1. A recent study identified 184 in vivo TRA-1 binding sites in XX larvae (Berkseth et al. 2013). Four of the 149 male-biased gonadal transcripts (ztf-22, ccc-1, col-176, and C26B9.6) were from genes near TRA-1 binding sites, which was a statistically significant hyper-geometric probability of overlap between these two data sets. Additional mRNAs that were enriched in the gonad and near TRA-1 binding sites are noted in Table S2. Future experiments can test whether these genes are downstream effectors of TRA-1 in the SGPs. Because the previous TRA-1 ChIP-seq was performed on L2 and later larvae there may be additional TRA-1 SGP targets during L1 that were not identified. FKH-6 also is essential for proper differentiation of somatic cells in the male gonad and functions in parallel with TRA-1 to promote cell proliferation in the male somatic gonad (Chang et al. 2004). We investigated the occurrence of FKH-6 binding sites in genes with gonad-enriched expression, but the low sequence complexity of the FKH-6 binding site (Kalis et al. 2010) precluded useful enrichment analysis. Determining the direct targets of gonadal transcription factors may provide valuable entry points to identify the regulatory networks controlling sex-specific gonadal gene expression during this period.

Previous studies found that genes with female-biased expression in the adult soma are enriched on the X chromosome in adult animals, while no bias was observed in adult males or larval animals of either sex (Reinke et al. 2004; Thoeime et al. 2005). We therefore examined chromosomal locations of the sex-biased and gonad-enriched transcripts identified in this study. We found that gonad-enriched genes from both sexes and both time points were significantly enriched for expression on the X chromosome. Additionally transcripts that were male-biased in Z1/Z4 daughters were also significantly enriched on the X chromosome (Figure 7).

**Enrichment of functional gene classes**

Gene ontology analysis of mRNAs enriched in Z1/Z4 and their daughters revealed the anticipated enrichment for GO terms related to reproductive processes (2.18- and 3.04-fold enrichment compared to whole animal for Z1/Z4 and their daughters, respectively; for mapped genes, see Materials and Methods), gonad development (2.33- and 3.81-fold enrichment) and sex differentiation (2.31- and greater than 5-fold enrichment). We also found more specialized functions, some of which were enriched in only one sex. In particular, the male-biased gonadal transcriptome was enriched for expression of genes involved in cell migration, consistent with the male-specific cell movements that occur during this period (Table S3). Additionally, the male-biased gonadal transcriptome included a number of kinases, Patched-related genes and
homeobox genes, which may provide insights into mechanisms of male-specific gonadal development. Finally, the hermaphrodite-biased gonadal transcriptome was enriched for expression of endopeptidase inhibitors while the male-biased transcriptome was enriched for expression of peptidases, suggesting a possible post-translational mechanism for gonadal sex differentiation.

Sex-biased gonadal expression shared in worm and mouse

Gene expression at the onset of gonad sexual differentiation also has been studied in the mouse (Jameson et al. 2012). To ask whether related genes may function in gonadal differentiation in nematodes and mammals, we compared sex-biased gonadal transcripts from the two species. Approximately 10% of the C. elegans male-biased transcripts identified in this study have mammalian orthologs with male-biased expression in somatic supporting cells of the early fetal gonad of the mouse, while 3% of the C. elegans hermaphrodite-biased transcripts have mouse orthologs with female-biased expression in supporting cells (Table S3).

DISCUSSION

We have described an optimized method for single cell transcriptional profiling of C. elegans larvae that we have used to define the transcriptomes of the SGPs and their daughters in each sex, identifying many mRNAs whose expression is enriched in the early SGP lineage in one or both sexes. Transcriptome analysis identified all of the genes previously known to be specifically expressed in Z1/Z4 and their daughters as well as all of the genes previously known to be essential for early larval gonadal development, suggesting that the transcriptomes we generated include the majority of transcripts strongly enriched in the early larval somatic gonad. We also identified a number of previously unannotated mRNA isoforms. The times analyzed include the onset of sex-specific gonadal development and comparison of Z1/Z4 transcriptomes with those of their daughters less than 6 hr later revealed the emergence of a suite of sex-biased transcripts that are likely to mediate differential gonadal development in the two sexes. The sex-biased expression of homologous genes in somatic supporting cells of the gonad in both C. elegans and mice, particularly in males, may indicate evolutionary conservation of part of the somatic gonad sexual differentiation program.

Profiling isolated cells identified ten times more sex-biased transcripts than whole animal analysis during the same period. Due to this higher sensitivity, cell type specific transcriptome analysis is likely to have broad utility in elucidating developmental programs in other tissues and organs. In principle, cell type specific enrichment and sex bias can be determined for any larval cell type where
appropriate sorting markers are available. Attractive targets for such analysis would be larval blast cells with highly dimorphic development, such as the M, T, or B cells (Sulston and Horvitz 1977). Cell type specific transcriptome analysis can also allow much more sensitive analysis of the effects of mutations or environmental factors in cell types of particular interest. For example, mutational effects have previously been studied by transcriptome comparisons in the VA neural cells (Von Stetina et al. 2007).

We performed extensive RNAi and mutant analysis (data not shown) of the gonad-enriched genes, focusing on those with male-biased expression. Strikingly, RNAi of male-biased mRNAs revealed almost no new phenotypes even when RNAi was performed on sensitized backgrounds. In light of the strong Z1/Z4 lineage enrichment of the transcripts tested, this result suggests a high level of functional redundancy in the early somatic gonad and/or minor roles in gonadal development for many gonad-enriched mRNAs. Given the direct role of the gonad in reproduction, a genetic buffering system to prevent severe effects from the loss of many gonadally-expressed genes would perhaps be expected. Future studies may need to employ network-based approaches to elucidate the functional roles of the gonad-enriched transcripts we have identified. Possible approaches include focusing on functional classes (e.g., Table S3) or gonadal gene regulatory networks. As an entry point to determine potential genetic networks, transcripts with similar expression profiles may be useful. Transcripts that have similar patterns for gonadal enrichment and sex-biased expression could be regulated by common factors and therefore contain similar regulatory elements. One example of transcripts that could be regulated in the same manner is the group of four at the bottom of the heat map in Figure 6B. These transcripts are male-biased, yet they are gonad-enriched compared to the whole animal in both males and hermaphrodites and could be regulated by a similar mechanism. Identifying the cis-regulatory elements and trans-acting regulators, which should be expressed in the gonad, could better define the shape of the gonadal genetic networks. The enrichment of a putative HLH-2 binding site in promoters of hermaphrodite-biased genes may provide a first step. Analyzing the direct targets of key transcriptional regulators including HLH-2, TRA-1, and FKH-6 may provide insight into how the gonadal sex differences are established.

We identified more than 70 genes that have isoforms that were not previously annotated. In many cases the annotated isoform is expressed in the whole animal while the gonadal cells express a novel isoform (Figure 5A). It will be important to determine whether these novel isoforms provide gene functions specific to gonadogenesis. While the C. elegans genome has been well studied it is likely that transcriptome
analysis in other cell types also will identify additional novel mRNA isoforms with restricted expression domains.

Conclusion
To our knowledge these data represent the only sex-specific transcriptome analysis of a C. elegans somatic tissue. We identified several hundred gonad-enriched transcripts, including a number of novel isoforms, as well as a small suite of sex-biased transcripts. The transcriptomes we report provide insights into the processes that drive early gonadal development and should provide a useful resource to further define how development of the bipotential C. elegans SGPs is coordinated to form two distinct organs with very different organizations and functions.

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Figure 7  Gonad-enriched transcripts were significantly enriched on the X chromosome. Chromosomal distribution of gonad-enriched transcripts in either Z1/Z4 or Z1/Z4-daughters (for either the male or hermaphrodite) and sex-biased transcripts. The representation factor is the ratio of observed to expected transcripts for each chromosome. A hyper-geometric probability test was used to determine any statistically significant deviation of observed from expected. P values of < 0.01 and 0.001 are indicated by " and **, respectively.
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