

A survey of ovary-, testis-, and soma-biased gene expression in *Drosophila melanogaster* adults

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Abstract

Background: Sexual dimorphism results in the formation of two types of individuals with specialized reproductive roles and is most evident in the germ cells and gonads.

Results: We have undertaken a global analysis of transcription between the sexes using a 31,464 element FlyGEM microarray to determine what fraction of the genome shows sex-biased expression, what tissues express these genes, the predicted functions of these genes, and where these genes map onto the genome. Females and males (both with and without gonads), dissected testis and ovary, females and males with genetically ablated germlines, and sex-transformed flies were sampled.

Conclusions: Using any of a number of criteria, we find extensive sex-biased expression in adults. The majority of cases of sex differential gene expression are attributable to the germ cells. There is also a large class of genes with soma-biased expression. There is little germline-biased expression indicating that nearly all genes with germline expression also show sex-bias. Monte Carlo simulations show that some genes with sex-biased expression are non-randomly distributed in the genome.

Background

Sexual dimorphism varies enormously from the morphologically indistinct yeast mating types, to extreme differences that can lead to the misclassification of males and females as distinct species. While the *Drosophila* sexes show relatively modest sexually dimorphic somatic body plans there is striking sexual dimorphism in the germline [1]. Indeed, it is difficult to imagine cell types that differ more than eggs and

sperm. During the last century a handful of genes involved in the regulation of somatic (for example, *Sex-lethal* [2], *transformer* [3], *transformer-2* [4], *doublesex* [5], *intersex* [6]) and germline sexual identity (for example, *ovo* [7], *sans fille* [8], *ovarian tumor* [9], and *stand still* [10]) have been identified, but we know very little about the effector genes that actually result in a sexually dimorphic state. Major terminal genes in the current models of somatic and germline sex

determination are a transcription factor (*doublesex*) and an RNA binding protein (*Sex-lethal*) [11,12]. These molecules must orchestrate a cascade of effector functions that result in sexually dimorphic gametes and the somatic support functions required for their union at fertilization.

Whilst we know relatively little about how germline sexual identity is determined in *Drosophila*, the downstream process of oogenesis has been well studied. Egg production occurs in the ovary [13]; each ovary consists of a cluster of 16-20 ovarioles where the assembly line-like production of germline and somatic cell units (egg chambers) progresses along the length of the ovariole. The germline component of each egg chamber contains the differentiated products of a single germline stem cell division. The differentiating stem cell daughter undergoes four rounds of incomplete cytokinesis to produce an interconnected 16-cell cyst surrounded by a somatic follicular epithelium.

One of these 16 germline cells becomes the oocyte and the remaining 15 cells develop into supporting nurse cells. The 16-germline cells within cysts are connected by an intercellular network facilitating the active transport of macromolecules from the nurse cells into the growing oocyte. These components include basic cellular machinery such as ribosomes, and a vast assortment of proteins and RNA species that support early embryonic development. For example, the dorsal/ventral and anterior/posterior axes of the future embryo are laid down during oogenesis [14]. The somatic follicular epithelium surrounding each egg chamber is an important source of structural proteins, such as yolk and egg shell proteins, that become incorporated into the oocyte, as well as patterning information. Most of the yolk proteins are produced distantly in non-gonadal fat body tissue [15]. Thus, while much of the egg is constructed by the cells of the egg chamber, there is significant contribution from distant organs. Finally, additional somatic functions in the female reproductive tract and female mating behaviors are required for productive gamete function [16]. A global analysis of gene expression in adult females therefore captures the genes required for all of the stages of oogenesis from stem cell to early embryo.

Analogously, the *Drosophila* testis contains developing gametes, from the stem cells at the apical tip of the testis to fully functional sperm [17]. As in the ovary, the germline stem cell division in the testis produces cysts of 16 primary spermatocytes. However, in males all these primary spermatocytes undergo meiosis resulting in a cyst of 64 spermatids. Each spermatid then follows an elaborate differentiation program of cytoskeletal and nuclear rearrangements to form a mature sperm cell. These changes are quite remarkable. For example, sperm chromatin is nearly crystalline and the nucleus changes from a round structure to a highly elongated and slightly hooked shape. Cytoskeletal rearrangement is equally dramatic. The round spermatid forms a flagellar axoneme

that is nearly half the length of the adult. Mitochondrial differentiation in the axoneme is also striking. Individual mitochondria fuse into two large and interleaved structures extending along the length of the flagellum. Thus, while the structure of a sperm cell might suggest a simplistic developmental program for spermatogenesis (DNA and a motor), shedding the features that characterize virtually all other cells in the body (such as packing DNA into nucleosomes) is an enormous reengineering feat. As is the case in females, the male reproductive tract and male behavior are required for fertility [18]. Therefore a global analysis of gene expression in adult males captures the genes required for all of the stages of spermatogenesis from stem cell to the fertilized egg.

Not surprisingly, both genetic and classic molecular studies indicate that spermatogenesis and oogenesis are complex events requiring extensive and often sex-specifically deployed information [13,17]. More recently, global gene expression studies using printed cDNAs, expressed sequence tags (ESTs), and full transcriptome microarrays have revealed extensive overall sex differential expression [19-24], with gene expression in the germline and gonads being particularly striking [19,20,24]. Here we report gene expression profiles as a function of sexual dimorphism and sex determination in *Drosophila* as analyzed using a platform including 93% of predicted genes from version 1.0 of the *Drosophila* genome [25] and 75% of release 3.1. This article includes the dataset from Parisi *et al.* [24] on gene expression in adults, augmented with additional microarray experiments to further track the source of sexually dimorphic expression.

There are many stories embedded in the expression data reported here. We touch on only a few to illustrate the value of the dataset. The most significant aspect of the survey we report here is the creation of a dataset that can be mined by other researchers interested in gametogenesis and sexual dimorphism. To that end, genes showing differential expression have been organized into easy to browse tables that include internet links to FlyBase [26,27], the compendium of genome information for *Drosophila*. We have also deposited all the data at the Gene Expression Omnibus [28,29] so that those interested in large-scale reanalysis can easily download the entire dataset.

Results

Experimental design

We used a series of experiments to delimit germline and somatic sex-biased expression. By comparing flies with and without a germline we can identify genes with germline-dependent expression bias [21,23]. While most genes showing germline-dependent expression are expressed within the germline cells, this class also includes somatically-expressed genes induced or de-repressed by the presence of germ cells. Progeny of homozygous *tudor¹* mothers have no germline and are useful in differentiating somatic and germline-biased

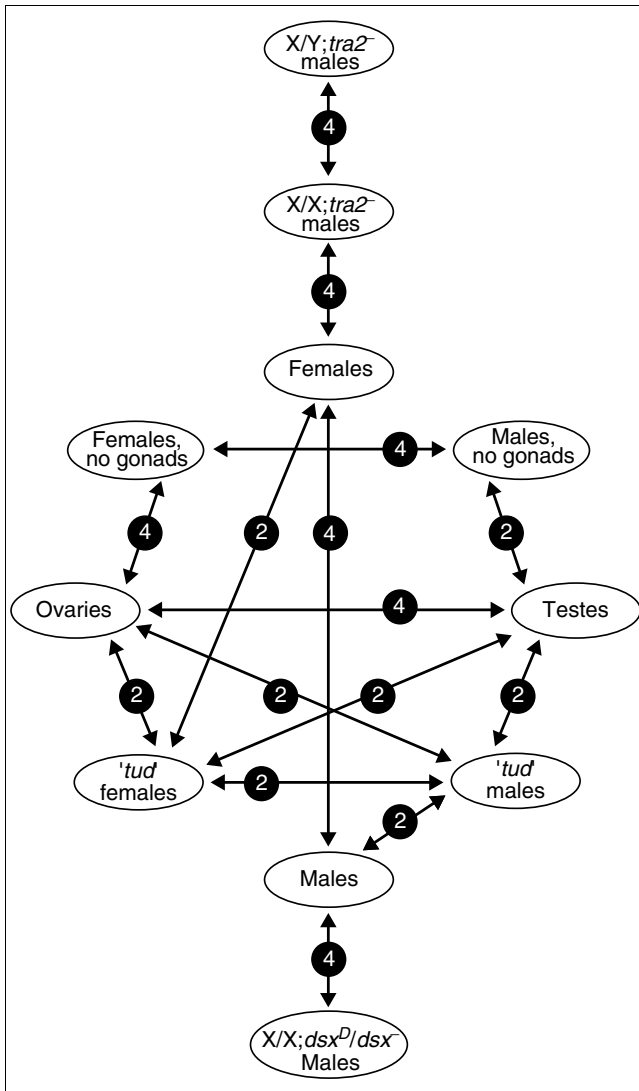


Figure 1
Microarray experimental design. Sex biased gene expression data was derived from 44 microarray hybridizations testing 15 conditions. All experimental conditions included at least one biological replicate and most also include dye-flip hybridizations for additional replicates. Abbreviated genotypes of the samples are shown in light ellipses with the total number of replicated hybridizations in dark circles between the two samples. Full genotypes of the flies used are as follows: X/Y;tra2⁻ males are w^{67c}/BsY;tra2^B/Df(2R)trix, X/X;tra2⁻ sex transformed males are w^{67c}/+;tra2^B/Df(2R)trix, X/X;dsx^D/dsx⁻ males are (+/+; dsx^{M+R45}/dsx^{swe}. 'tud' males and females are the progeny of homozygous tud¹ bw¹ sp¹ females mated to tud¹ bw¹ sp¹/CyO males and themselves are genotypically tud¹ bw¹ sp¹ homozygotes. Females and males are whole adult y¹ w^{67c} flies. Ovary, testis and no gonad samples are also derived from y¹ w^{67c} flies.

expression [30]. Another measure of germline contribution was obtained by dissection. The gonad samples provide a measure of gene expression in the germline and somatic components of the gonad. Whilst the gonads clearly contain both tissues, the vast majority of the mRNA is derived from the germline cells. Indeed, we were unable to collect sufficient

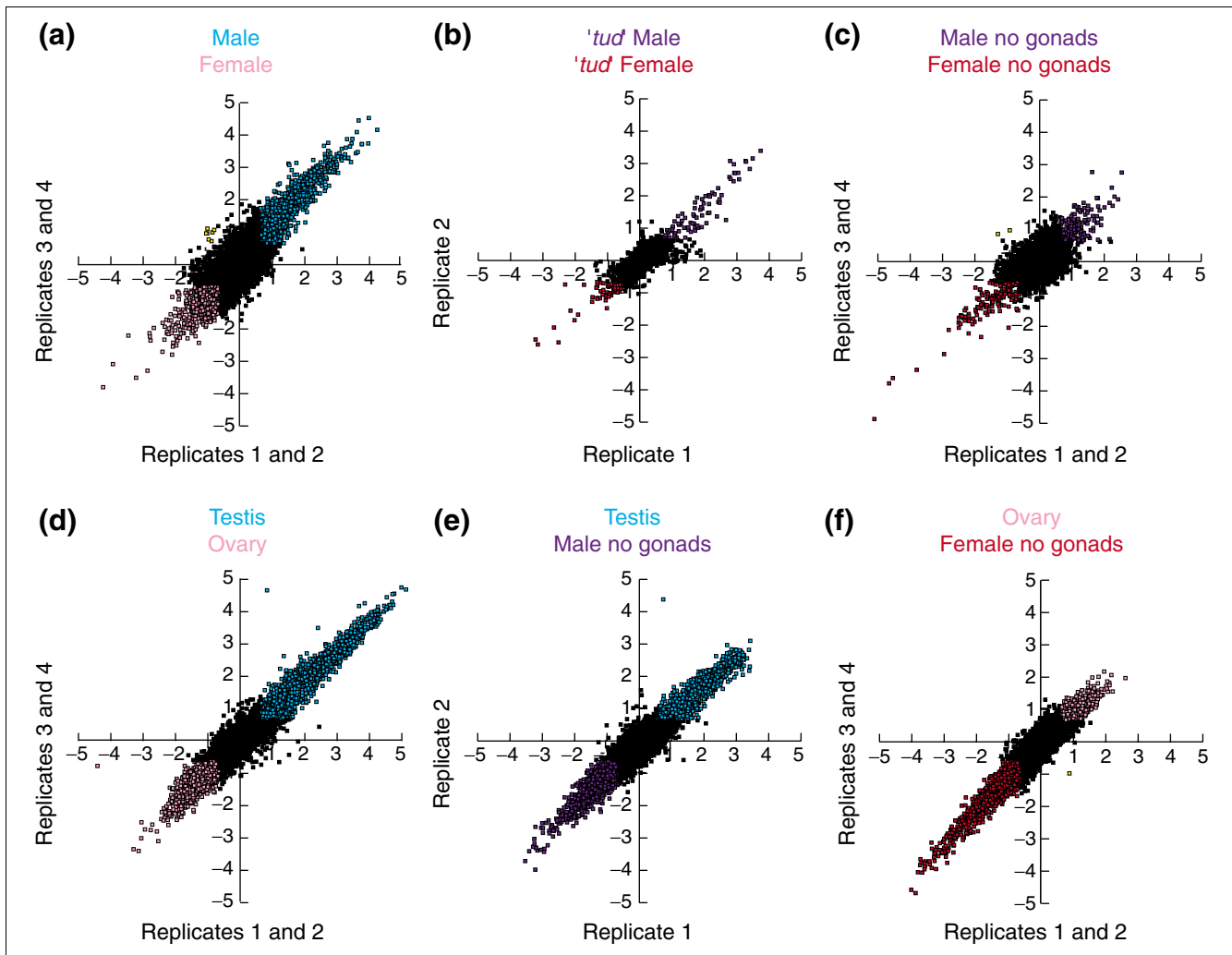
mRNA (600 ng of polyA+) from dissected germline-less gonads to perform a single microarray experiment. Finally, wildtype *Drosophila* males are XY and females are XX. We reversed the sex of XX flies from female to male using mutations in *tra2* and *dsx* [31]. These sex transformed flies have rudimentary testes with very few germline cells [32] and are thus phenotypically similar, although less extreme, than the male progeny of *tudor*¹ mothers [1]. By performing replicate microarray experiments on these diverse samples we obtain a robust picture of sexually dimorphic gene expression in the germline and soma.

Pairs of mRNA samples (Figure 1) labeled using Cy3 or Cy5 labeled nonomers were applied to Incyte Fly Gene Expression Microarrays (FlyGEM) and the Cy3 and Cy5 channel intensity values were extracted for study as previously reported [33]. The microarray was designed against release 1 of the *Drosophila* genome and has been updated to release 3.1. There are many changes in the annotation and future changes are also likely. As a result some of the microarray elements no longer correspond to officially recognized genes (although some detect transcripts) and some transcripts are now recognized by multiple element species. Therefore we use the expression from the 14,611 DNA element species pairs corresponding to *Drosophila* transcripts (there are at least two of each printed on the array plus many control elements for a total of 31,464) as a metric for 'genes'. Using element species rather than gene names assures that the dataset can be updated as gene models change in the ongoing annotation effort.

In order to characterize a gene as showing biased expression in a particular tissue or sex, one is forced to use arbitrary statistical or magnitude cut-offs. In our replicated heterotypic hybridizations we used both a conservative measure to capture the highest confidence data and noisier, but more inclusive, clustering methods. We express sex-biased and tissue-biased gene expression in terms of percent of the genome for didactic purposes only, as these numbers depend greatly on the arbitrary cut-off. For gene lists, see the extensive set of tables and links to FlyBase in the Additional data files.

Expression analyzed by ratio

We have used conservative calls on competitive pairwise experiments at two-fold. An extensive set of homotypic hybridization experiments (mean r² of 0.754, and a standard deviation of 0.097) has shown that the 99.5% tolerance interval for measurements on the FlyGEMs is <1.5-fold, and unlike the case with some array platforms the particular element sequence does not contribute significantly to variance [33]. Therefore, this global statistic can be applied to individual elements. Outliers are very effectively dealt with by replicates (Figure 2). In heterotypic hybridizations reported here, only 0.01% of the elements (representing 0-6 in each comparison) showed two-fold differences that fail to correlate with the experimental variables in question. Briefly, replication

**Figure 2**

Sex-biased differential expression in *Drosophila*. Scatter plots show global expression in *Drosophila* testing different sex and tissue conditions. Data are pairwise comparisons of natural log (Ln) Cy3/Cy5 signal ratios averaged from dye flipped and biological replicate experiments. Black color indicates expression ratios that fall within a two-fold cutoff. Microarray element species greater than two-fold are color coded as indicated on each scatter plot. Yellow points indicate 2-fold differences that fail to correspond to the expression variables analyzed. The number of element species included in each pairwise comparison that met stringency conditions are indicated in parentheses. (a) $y^l w^{67c}$ males versus $y^l w^{67c}$ females ($n = 10,688$); (b) male versus female progeny of homozygous $tud^1 bw^1 sp^1$ females ($n = 12,836$); (c) $y^l w^{67c}$ males, no gonads versus $y^l w^{67c}$ female, no gonads ($n = 9,778$); (d) $y^l w^{67c}$ testis versus $y^l w^{67c}$ ovary ($n = 11,338$); (e) $y^l w^{67c}$ testis versus $y^l w^{67c} y^l w^{67c}$ male, no gonads ($n = 12,461$); (f) $y^l w^{67c}$ ovary versus $y^l w^{67c}$ female, no gonads ($n = 11,223$).

effectively eliminates false positives for tissue-biased expression in theory and practice.

In comparisons of whole adult females and males, 17% of the element species reporting in all experiments showed a greater than two-fold sex-biased hybridization intensity (Figure 2a). Female-biased hybridization intensity was less extensive (6%) than male-biased hybridization intensity (11%). A series of experiments suggest that most sex-biased hybridization intensity is due to the germline (Figure 2; see also tables in the Additional data). We found that 1.5% of array element species show sex-biased hybridization in experiments using probes

derived from flies with no germ cells (Figure 2b). This distribution was asymmetric, with greater numbers of element species showing high hybridization to male samples (0.9%) than to female samples (0.4%). Similarly, when male or female carcasses were compared, 3% of the element species showed sex-biased hybridization (Figure 2c). The greater degree of sex-biased hybridization observed in the carcass samples may reflect strain differences, which can be substantial [21,23], or germline-dependent expression in the soma. We are currently scrutinizing these possibilities with additional array experiments using flies of identical genotypes, with and without germlines. Briefly, these data indicate that relatively little

of the sex-biased expression in whole flies is attributable to the soma.

While the contribution of the germline to sexually dimorphic gene expression can be approximated by examining the fraction of the sex-biased expression in whole wildtype flies minus the sex-biased expression in the soma, a more direct measurement is obtained by hybridizing mRNA from dissected gonads. As we show below, this substantially concentrates the mRNAs from germline cells and increases the depth of the observed expression profile. Ovary and testis samples exhibit the highest degree of sex-biased hybridization in our experiments (Figure 2d). A total of 29% of element species exhibit ovary-biased or testis-biased hybridization. This is in contrast to 14-16% of element species deduced to show ovary or testis-biased hybridization by subtracting the somatic sex-biased genes from the list of element species exhibiting sex-biased hybridization in whole adults. The expression profile is distinctly asymmetric with nearly twice as many element species showing greater than two-fold hybridization to testis samples (18%) versus ovary samples (11%). Additionally, there were far more element species showing a large magnitude of testis-biased hybridization. For example, 2% of element species showed >20-fold hybridization intensity in the testis channel, while <<1% of element species showed a similar magnitude in the ovary channel ($n = 4$). These data indicate that the cells of the testis deploy a larger battery of specific genes than those of the ovary. Additionally, these data confirm that the most sexually dimorphic tissues, the gonads, show the most distinct gene expression profiles.

A priori, one might expect that the soma would be quite different from the germline, but to what extent is this due to the expression of germline-specific or soma-specific gene batteries? To address this question, we have also examined germline versus somatic expression within a given sex. As expected we observed a high degree of testis-biased hybridization in competitive hybridizations with samples from either males with no or few germline cells (males from *tudor*² mothers, and females transformed somatically into males; not shown) or from males with gonads removed (Figure 2e). A total of 14% of element species hybridize preferentially to labeled testis samples, supporting the idea that the gene batteries deployed in a testis-biased manner are extensive. Additionally, these experiments reveal a high degree of soma-biased hybridization - 10% of element species are preferentially hybridized to samples from males with the gonads removed (Figure 2e).

Somewhat surprisingly, comparing females with or without germlines did not show the same pattern of soma versus germline hybridization that was evident in the males. Ovary-biased hybridization was modest (4%) in experiments measuring hybridization of samples from ovary versus samples from females with the ovaries removed (Figure 2f). In contrast, soma-biased hybridization was extensive (12%) as was the case when examining male soma and germline (Figure

2e). Thus, unlike what is seen in males, the asymmetry in these ratio plots favors the soma. This suggests that most of the genes expressed in the female soma are also expressed in the ovary and that many genes are expressed specifically in the female soma. The soma is distinguished from the germline by a high degree of soma-biased gene expression.

Expression analyzed by intensity

An overview of all sex-biased gene expression is provided by examining a self-organized heat map of the cross-normalized intensities from each of the hybridizations (Figure 3). This also provides an independent method for building gene lists. As expected from the pairwise comparisons, the prominent clusters in this map highlight groups of element species exhibiting testis-biased (12%), ovary-biased (9%), and soma-biased (11%) hybridization. The clusters of element species showing testis-biased hybridization are most dramatic, both in terms of the number showing sex-bias and in terms of the degree of hybridization bias. This global overview shows very little overt sex-biased expression in the soma, and very little germline-biased expression. In agreement with the scatter-plot analysis, the heat map indicates that the main batteries of differentially expressed genes distinguish the soma from the germline, and the female germline from the male germline. Again, the lack of an overt class of genes showing germline-biased expression indicates that the male and female germlines have very little in common, other than the absence of expression from a large class of genes that appear to have soma-biased function.

Cross-validation of expression by Northern blotting

It is standard practice to confirm the expression of genes of interest based on microarray data by RT-PCR or Northern blotting, although this is not practical when thousands of genes show biased expression. Additionally, this confirmation is usually restricted to genes showing differential expression, and therefore does not allow for a good estimate of sex-biased gene expression missed in the FlyGEM experiments. We selected 75 element species from the FlyGEM experiments, across the range of observed intensities and sex-biases, for Northern blotting experiments on adult males and females with and without germlines (Figure 4a). Signal intensities from each lane on the Northern blots were measured for each experiment and then used to calculate differential expression ratios among the four sample lanes in a fashion analogous to pairwise microarray comparisons (absolute intensities correlated very poorly, indicating that the techniques have different signal and noise characteristics even though the same amplicons were used). Plotting the ratios from Northern blots versus microarrays demonstrates consistent relative measurement of RNA between the two experimental systems (Figure 4b,c,d,e). The majority of the data points fall along the diagonal for wildtype males and females. In the other comparisons, both the microarray and Northern ratios tend to cluster near the origin (a ratio of 1 is 0 in log space) due to lower sex-biased expression in the germlineless flies of

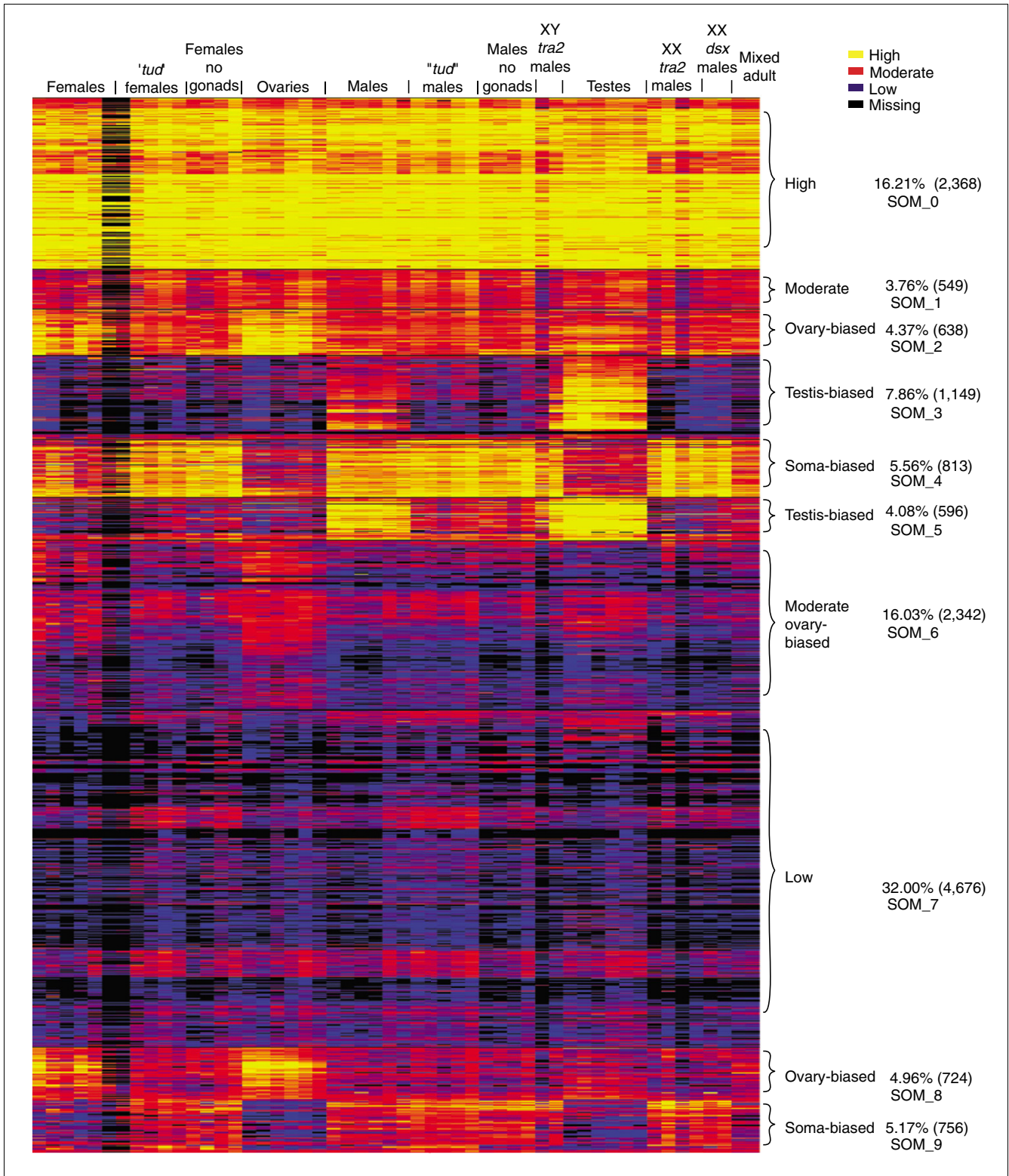


Figure 3 (see legend on next page)

Figure 3 (see previous page)

Heat diagram of intensities. Self organizing maps (SOMs) were used to generate an image of clustered intensity data from 26 pairwise experiments. The individual channels from these experiments are parsed out and arranged by tissue type as indicated in the top row text. The normalized intensities are indicated as high expression (yellow), moderate (red), low (blue) and missing value (below background; black). The diagram represents averaged data from the duplicated elements within each microarray. Brackets show ten SOM clusters with the tissue type and percentage of the total number of microarray elements in the right text column.

tudor¹ mothers. Thus, ratios determined by Northern blotting and FlyGEM compare favorably.

Cross-validation of expression with the developmental time course

Comparing array results across platforms can cross-validate a larger fraction of the genome. Confidence is heightened for element species showing biased expression on two platforms [34]. A *Drosophila* developmental profile including males and females with and without a germline has also been performed on a cDNA array platform (GEO accession GPL218) [20]. This array is less complete than the FlyGEM and is biased against testis gene expression as no clones from testis cDNA libraries were included in the platform. Thus, overall estimates of sex-biased gene expression are expected to vary between the platforms. However, FlyGEM element species designed against the genes represented by cDNA inserts should correlate if both platforms are performing as expected. We extracted data from 2,536 GPL218 elements also present on the FlyGEM platform for comparative analysis.

The correlation between male/female hybridization ratios was quite good (Figure 5a,b). The trend lines clearly demonstrate that elements showing sex-biased expression on one platform show similar sex-biased expression on the other if one applies a greater than two-fold cut-off to these ratios. The only clearly artifactual result from this comparison is the presence of a substantial number of elements reporting sex-bias only in Arbeitman *et al.* [20]. This may be due to cross-hybridization as a result of low sequence complexity and repeats in the cDNA inserts - a common problem with cDNA arrays [35]. Nevertheless, this comparison clearly cross-validates the expression profiles for a substantial set of genes. Indeed, the r^2 for male/female ratios across platforms is 0.447, which is similar to the correlation observed between replicate hybridizations within the time course dataset ($r^2 = 0.334 \pm 0.114$).

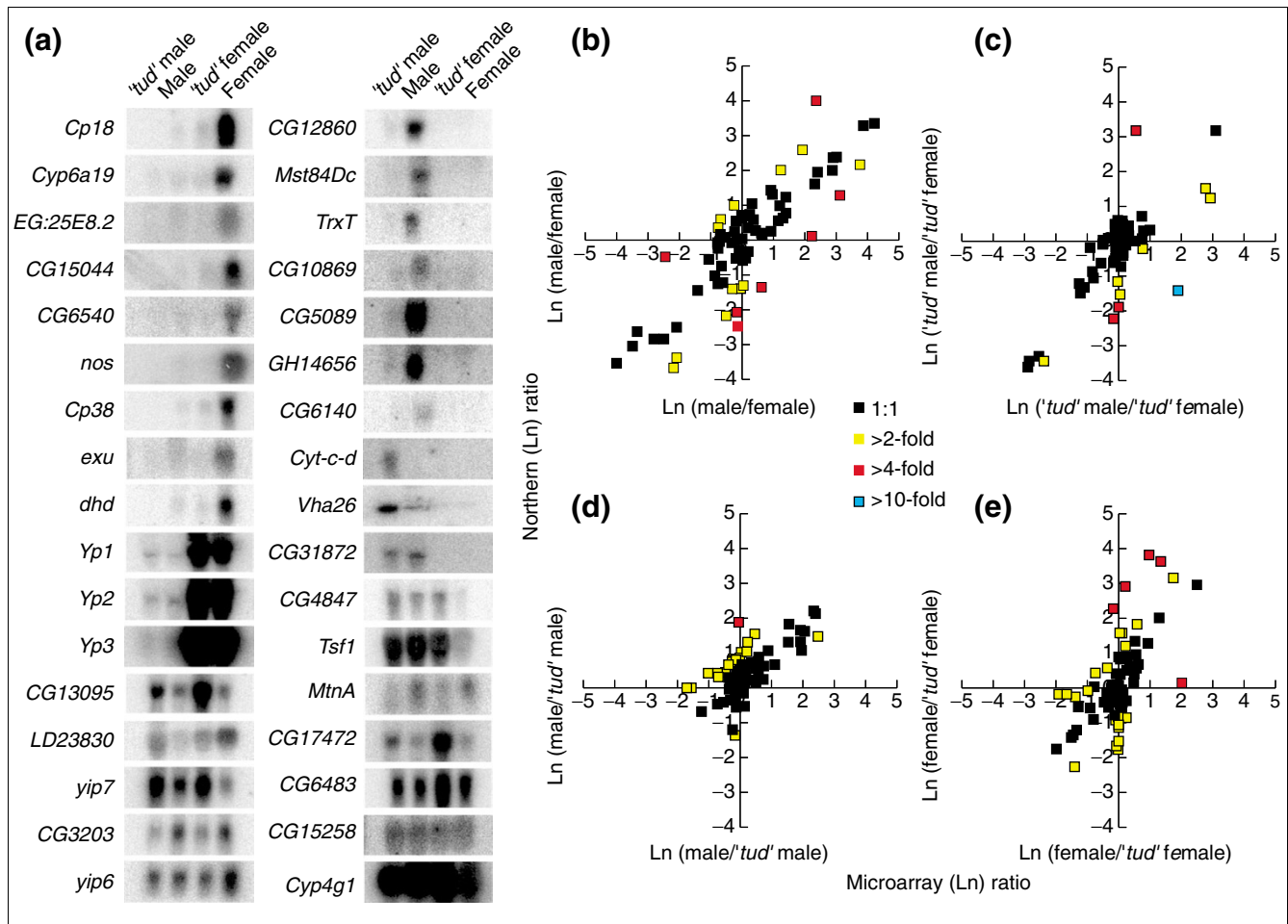
We then tested whether expression ratios from our Northern analysis correlated to ratios derived from Arbeitman *et al.* [20]. We have Northern data in 16/2,536 elements common to both platforms. The male versus female and 'tud' male versus 'tud' female ratios were plotted (Figure 5c,d). The Arbeitman *et al.* [20] data to Northern (Figure 5c,d) and FlyGEM to Northern (Figure 4b,c) correlation was similar.

The Arbeitman *et al.* [20] data has been recently re-evaluated in a more statistically rigorous manner to extract the high

confidence data from the moderately reproducible primary data [36]. The specific aim of this re-evaluation was to identify genes with sex-specific expression patterns. If the set of cDNA amplicons that most reliably hybridize to female or male samples can also be used to categorize female and male samples of differing genotype from another lab, then confidence in that list of genes is augmented. We therefore took a list of gene identifiers from this more recent study, extracted our corresponding data, and generated a cluster map of samples (Figure 5e,f). This small subset of elements was sufficient for the unsupervised classification of whole females and males, despite disparate genetic backgrounds. Tissues were also appropriately juxtaposed on the sample tree. Collectively, the scatterplot and heat map comparisons indicate that FlyGEM and Arbeitman *et al.* [36] data are in broad agreement and suggests that a full meta-analysis of *Drosophila* expression data from multiple platforms will be fruitful.

Well-characterized genes are correctly scored

Genes known to have sex-biased expression or function should be present in the appropriate ovary-, testis-, or soma-biased lists. We have mapped the FlyGEM element species to the current release of the *Drosophila* genome and generated a large set of lists with links to Flybase gene report pages (see Additional data). Following these links leads to more links (for example, literature, additional array data, yeast two-hybrid maps) and following these trails lends further confidence in the array data. For example, on lists of element species showing ovary-bias (Table 21 in Additional data; see also Tables 5, 15, 17, 30, 24, 36, and 38), we find six that represent chorion proteins (*Cp15*, *Cp16*, *Cp18*, *Cp19*, *Cp36*, and *Cp38*). Chorion proteins are components of the egg shell, and are known to be highly over-expressed in the ovary [37,38]. Similarly, structural components of sperm such as *beta-tubulin85D* [39], *tektin-A* [40] and *don juan* [41] are found on testis-biased lists (Additional data, Table 22; see also Tables 6, 7, 9, 31, 33, and 39). A number of other genes showing biased hybridization are known to be required for female or male fertility. These include *alpha-tubulin67C*, which is represented by over 20 female sterile alleles [42] and *dead-head*, which is represented by four female sterile alleles and a single lethal allele [43]. Genes represented by male sterile alleles (for example, *boule* [34] and *fuzzy onions* [44]) are found on testis-biased lists. The *boule* gene is also required in mammalian spermatogenesis [34], highlighting the potential utility of this dataset for those interested in spermatogenesis in mammals. Genes known to encode products required maternally for germline formation in the resulting embryo

**Figure 4**

Northern and microarray comparison. A series of 75 Northern blots with total RNA from germline-less ('tud') male, wildtype (*y^l w^{67c}*) male, germline-less ('tud') female and wildtype (*y^l w^{67c}*) female whole flies were probed with the PCR products identical to those printed as microarray elements. (a) 34 Northern blot images show a range of expression patterns among the input RNA samples. Phosphorimaging of the radioactive signal from Northern blots gave expression ratios between the RNA sample types for each Northern probe. (b-e) Corresponding (Ln) transformed Northern and microarray element ratios averaged from multiple experiments are shown as four scatterplots comparing expression between (b) *y^l w^{67c}* males versus *y^l w^{67c}* females; (c) 'tud' males versus 'tud' females; (d) *y^l w^{67c}* males versus 'tud' males and (e) *y^l w^{67c}* females versus 'tud' females. The y and x axes are expression ratios derived from Northern blot and microarray ratios respectively. Over two-, over four- and over tenfold differences in ratio values between the Northern and microarray experiments are indicated in yellow, red and blue.

are represented on lists of genes showing ovary-biased hybridization (for example, *oskar* [45], *mago nashi* [46], *vasa* [45], *smaug* [47], and *cyclinB* [48]) as are genes required for germline cell division within the ovary (for example, *piwi* and *fs(1)Ya* [49]). Even among the genes with little functional information, there is evidence to cross validate some of them. For example, *BEST:GH14656* is represented by 13 testis ESTs from the NIH collection and just two ESTs from the BDGP head library [19,50].

In addition, there are a number of genes showing soma-biased expression in our experiments (Additional data, Table 23; see also Tables 9, 18, 37, and 40). Examples include *actin88F* [51], *flightin* [52], *Tropomyosin1*, *Tropomyosin2*, and *upheld* [53] that are known to be required for flight mus-

cle function. Similarly, genes involved in phototransduction (*arrestin-2* [54], *ninaC* [55] and five genes encoding rhodopsins [56]) exhibit soma-biased expression, as do genes expressed in the fat body which are immune response genes (*Drosomyacin* [57]), or genes that may be required for energy storage (*fat body protein 1* [58] and *lipid storage droplet protein 1* [59]). Thus, many of the class of genes showing soma-biased expression are genes used for well-defined soma-specific functions.

Briefly, many of the genes expected to show biased expression do. Additionally, given the importance of these known genes, it seems likely that there will be interesting functions among the unstudied genes showing biased expression in our experiments.

Systematic analysis of gene function

Browsing offers some degree of confidence, but is not amenable to the analysis of large datasets. The Gene Ontology (GO) consortium [60] has provided a controlled vocabulary to describe gene function allowing us to determine rigorously which functions are over-represented in a particular expression profile. We queried our element species lists with the GO term analysis tool 'Database for Annotation, Visualization and Integrated Discovery' (DAVID) and 'Expression Analysis Systematic Explorer' (EASE) [61].

The results of GO term analysis are reassuring. Genes with oogenic functions [13] are highly over-represented among element species with ovary-biased expression (Figure 6a). The most highly over-represented categories are associated with metabolism ($P < 1 \times 10^{-20}$) and are probably associated with biosynthesis required for the manufacture of eggs. Similarly, ribosomes are highly over-represented cellular components ($P < 2 \times 10^{-42}$) as are ribosomal proteins in the protein GO category ($P < 8 \times 10^{-52}$). Genes encoding translational initiation complexes are also highly over-represented ($P < 2 \times 10^{-22}$). This is consistent with the high level of protein production during egg development and the high ribosomal content maternally loaded into the egg. Indeed, mutations in many ribosomal protein genes result in female sterility due to defective egg formation [62]. Also prominently represented are genes involved in DNA metabolism and cell cycle regulation ($P < 1 \times 10^{-12}$, $P < 1 \times 10^{-7}$). These functions may be required for the dramatic endoreplication of nurse cells during oogenesis. Similarly, the over-representation of transcripts from genes required for DNA replication ($P < 1 \times 10^{-9}$), chromatin architecture ($P < 4 \times 10^{-5}$), nuclear organization ($P < 1 \times 10^{-4}$), and DNA packaging ($P < 5 \times 10^{-5}$), may be required for nurse cell polyploidization [13] or for the rapidly dividing embryonic cells which rely on maternally deposited gene products. Finally, molecular genetic analysis of oogenesis has revealed that a substantial number of these gene products include RNA binding proteins, and this class is also over-represented in ovaries in our microarray data ($P < 2 \times 10^{-6}$).

The representation of GO terms in the testis-biased element species lists was similarly examined (Figure 6b). Again, the most significantly over-represented GO terms are consistent with what we know about spermatogenesis [17]. Genes encoding microtubule ($P < 2 \times 10^{-9}$) or movement ($P < 3 \times 10^{-8}$) function are among the element species with testis-biased expression. These are required for the extensive cytoskeletal reorganization during spermiogenesis [17]. Genes encoding transport proteins ($P < 2 \times 10^{-6}$), including those with dynein-associated ATPase activity ($P < 1 \times 10^{-9}$) are over-represented. Again, this is consistent with the motile nature of sperm. Chaperones ($P < 3 \times 10^{-4}$) are over-represented, which is in agreement with the known requirement for heat shock proteins during spermatogenesis [63]. In testis, cellular component analysis shows high over-representation of gene products associated with mitochondria ($P < 9 \times 10^{-14}$),

membranes ($P < 1 \times 10^{-4}$) and the cytoskeleton ($P < 3 \times 10^{-7}$) which is almost certainly due to requirements for sperm axoneme structure.

Non-gonadal female somatic tissues show sex-biased expression of genes related to reproductive processes (Figure 6c). Vitellogenesis ($P < 2 \times 10^{-5}$) is required for egg development, as the vitellogenin or yolk proteins synthesized in the female fat body are the major component of a mature egg. The second category of proteolytic processing ($P < 8 \times 10^{-4}$) might reflect the process required for generating yolk proteins. Ribosomal protein encoding genes are also over-represented ($P < 1 \times 10^{-6}$) in the non-gonadal female soma transcription profile. Similarly, element species encoding lysosome components ($P < 2 \times 10^{-5}$) are over-represented. These functions probably reflect the dual anabolic and catabolic metabolism required to support egg development.

The male reproductive tract includes several additional somatic structures in addition to the testis. For example, the seminal fluid components of the ejaculate are produced in the accessory glands. These glandular secretions mediate sperm competition, reduce post copulatory mating by recipient females, and increase egg laying rates [18]. These element species with 'extracellular' function are over-represented in the male expression profile ($P < 4 \times 10^{-5}$) (Figure 6d).

Gene expression neighborhoods

A whole genome transcriptional profile enables us to investigate the genomic organization of tissue-specific gene batteries. Previous experiments have shown that there are significantly fewer genes with male-biased expression on the X chromosome [23,24]. The fourth chromosome is enriched in genes with soma-biased expression (not shown). On a more local level, *Drosophila* genes with tissue-, temporally-biased expression patterns have been shown to be concentrated in neighborhoods of contiguous genes [64]. We asked if genes with sex-biased expression show a similar sub-chromosomal level organization.

We first examined a heat map of cross-normalized intensities by position along the chromosome arms (not shown). While there were patterns of alternating bands of genes showing greater or lesser intensity along the chromosomes, similar but not as obvious as those reported for embryo and adult [64], these patterns were also seen in randomized datasets, albeit at a lower frequency. Additionally, these patterns were less striking than those seen when plotting normalized intensity versus process parameters such as position in the microarray. (The FlyGEM platform we used is printed randomly with respect to genome position, so these process artifacts do not affect our previously reported reduction in the density of genes with male-biased expression on the X chromosome [24], nor the neighborhoods reported below.)

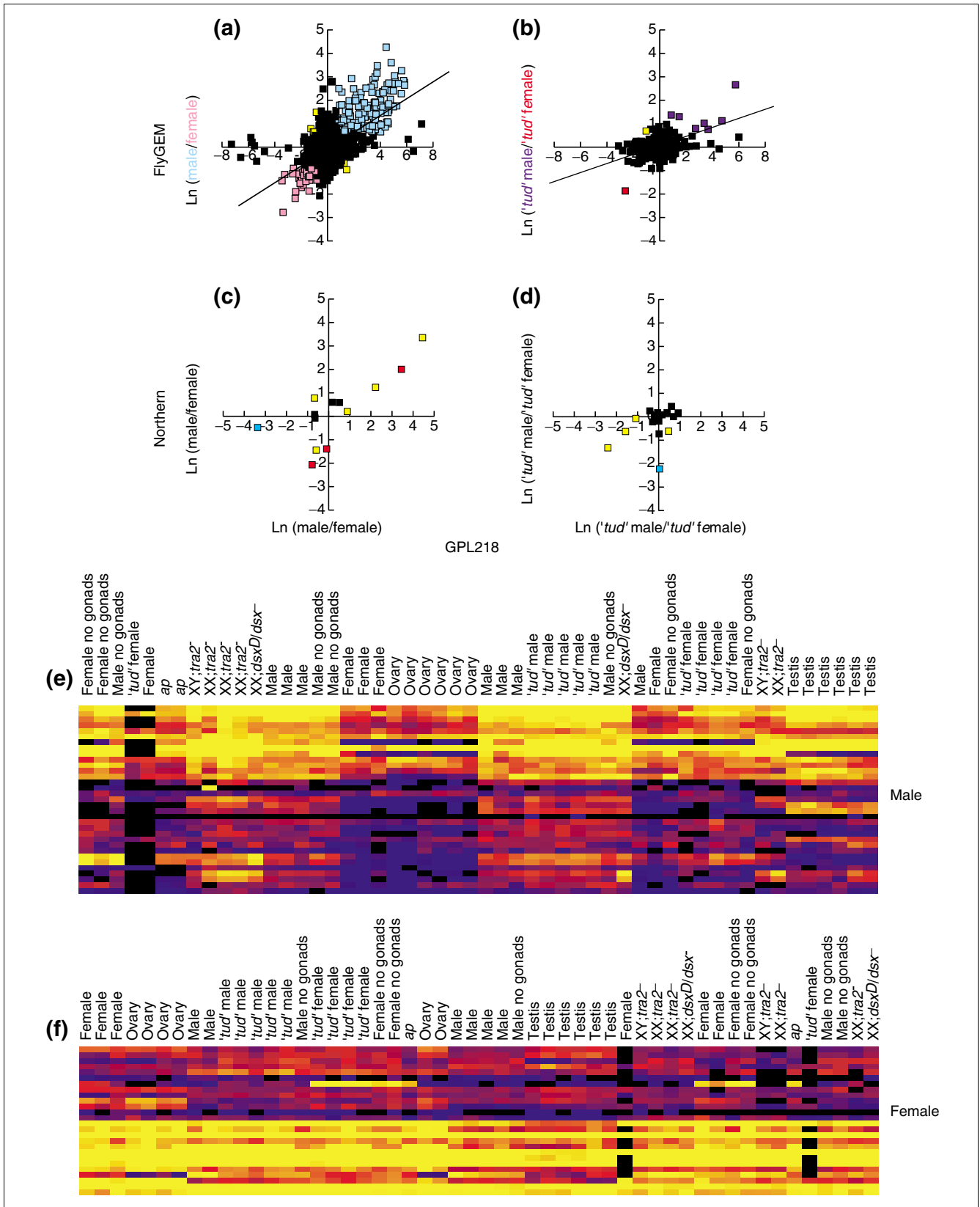


Figure 5 (see legend on next page)

Figure 5 (see previous page)

Meta-analysis of Arbeitman et al. [20,36] data. **(a,b)** Scatterplots of averaged (Ln) ratios for (a) male versus female and (b) 'tud' male versus 'tud' female comparing data from experiments performed with the FlyGEM and GPL218 platforms. Two-fold sex-biased expression in both datasets are indicated in blue (male-biased) and pink (female-biased). Those showing two-fold male-biased expression in one data set, but two-fold female-biased expression in the other are shown in yellow. **(c,d)** Comparison of Northern expression ratios for 16 probes versus averaged (Ln) ratios from Arbeitman et al. are shown in scatterplots for (c) male versus female and (d) 'tud' male versus 'tud' female. The 16 probes represent CG13263, CG8994, CG3972, CG10701, CG1088, CG7961, G10961, CG6206, CG4586, CG13095, CG5089, CG4847, CG6483, CG8549, CG7660, CG6457. Two-, four- and 10-fold deviations from 1:1 ratio are indicated by yellow, red and blue color (see Figure 4). **(e,f)** Clustering of FlyGEM normalized intensity data was performed for sex-biased somatic genes from Table 2 of Arbeitman et al. [36]. (e) 33 male and (f) 26 female genes are shown as intensity heat clustergrams. The normalized intensities are indicated as high expression (yellow), moderate (red), low (blue) and below background (black). (Intensities are represented by color as in Figure 4). Columns show 52 channels parsed from 26 pairwise FlyGEM microarray experiments. Clustering shows soma-bias corroborating the Table 2 lists. The array element species represented in rows from top are listed as follows: (e) CG12268, CG3359, CG3359, CG3359, CG5740, CG7050, CG7157, CG7178, CG7178, CG7748, CG8110, CG8552, CG9456, CG12558, CG14024, CG15097, CG16820, CG17843, CG18284, CG3359, CG5411, CG5455, CG6518, CG6716, CG6844, CG7178, CG7178, CG7342, CG8708, CG8708, CG8909, CG9519; (f) CG10281, CG10566, CG1090, CG11248, CG12269, CG1646, CG17012, CG7129, CG7702, CG7899, CG8327, CG8370, CG9547, CG10944, CG12740, CG14792, CG18525, CG3195, CG3751, CG4087, CG5821, CG7777, CG8453, CG8705, CG9696, CG9699.

Given that the concept of gene expression neighborhoods is relatively new, we were interested in identifying the most significant examples for detailed future study. To do so, we examined the distribution of element species with both highly ovary-, testis-, or soma-biased expression and high intensity to avoid low intensity noise. This resulted in 791 element species with testis-biased hybridization, 129 element species with ovary-biased hybridization, and 287 element species with soma-biased hybridization, which we mapped onto the genome (Figure 7). We also generated randomized datasets to determine if the distribution pattern of ovary-biased, testis-biased, or soma-biased expression is non-random. To do this, 100,000 randomizations were performed for each gene position over windows (consecutive genes along the chromosome) ranging between five to 200 genes. We used sliding windows of various sizes to determine how often an equal or greater number of elements reporting highly biased expression occurred in a random set versus the real data. A total of 20 gene expression neighborhoods were identified with a P -value $< 10^{-3}$, 15 at $P < 10^{-4}$ and seven at the lowest possible P -value of $< 10^{-5}$ (0/100,000). The most clear-cut examples of gene expression neighborhoods include approximately 10 genes within a chromosomal region spanning between 20 to 40 genes (Figure 8). This is consistent with the report of 10- to 20-gene expression neighborhoods shown in microarray analysis of *Drosophila* embryonic versus adult transcription [64].

There are several possible reasons for co-expression of neighboring genes. One possibility is that they are members of gene families or are part of the same transcription unit. Indeed, a recent article suggests that operons and duplicate genes account for many of the gene expression neighborhoods in *Caenorhabditis elegans* [65]. While there is no evidence for extensive operon structure in *Drosophila*, genome annotation errors might potentially introduce artificial evidence for gene expression neighborhoods if genes thought to be distinct are in fact derived from a common transcript. Alternatively, gene expression neighborhoods might reflect the

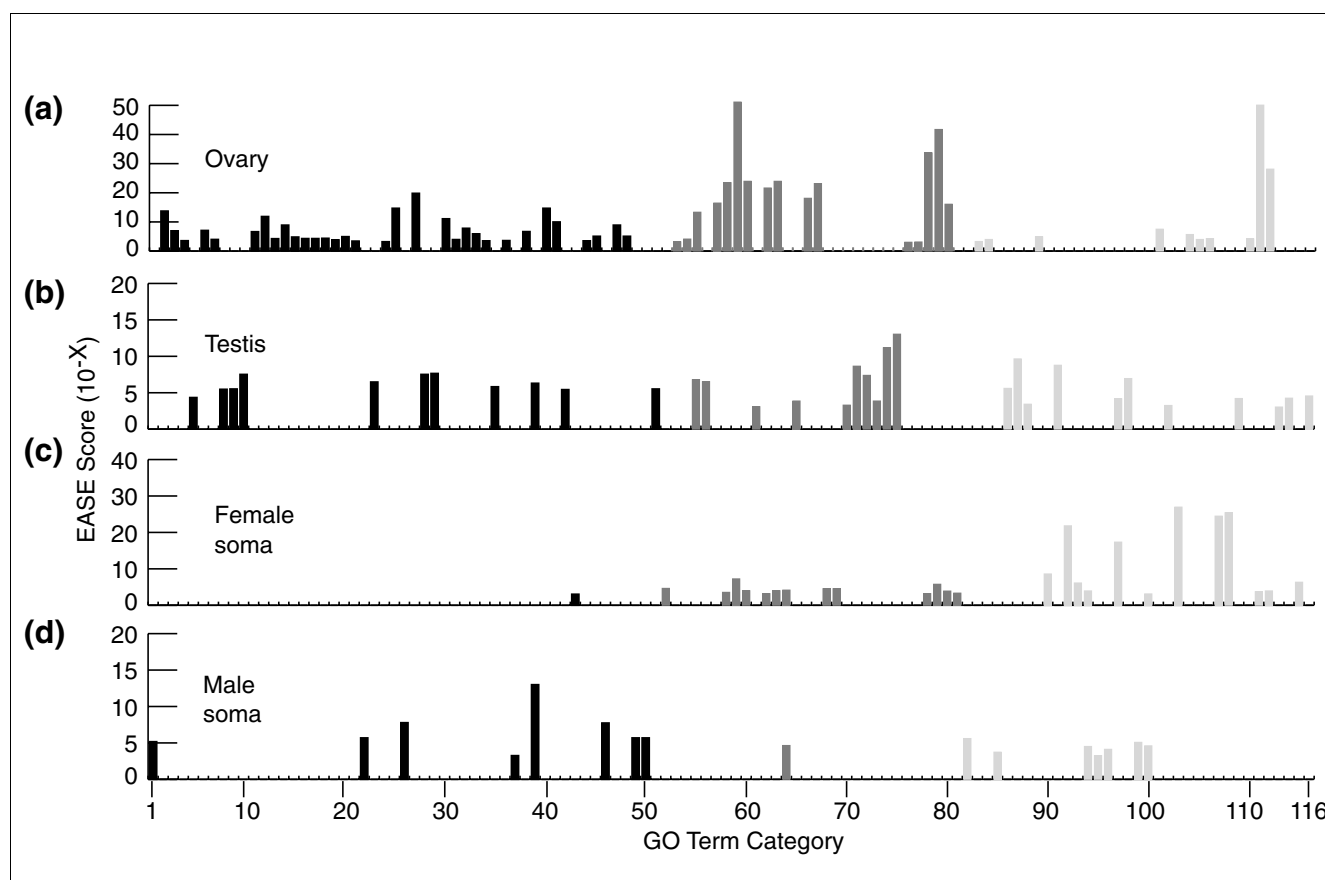
local transcriptional environment via a neighborhood-wide control structure [66,67]. In order to distinguish between these models, to a first approximation, we examined the seven $P < 10^{-5}$ neighborhoods more closely.

All of the most significant gene expression neighborhoods contain genes expressed from either strand, indicating that neither polycistronic messages nor annotation errors are causal (Figure 8). Additionally, there are no overt gene families or groups of gene ontology terms associated with six of the neighborhoods, suggesting that they are unlikely to be derived by gene duplication, although one is a neighborhood of paralogous genes (Figure 8e). This latter soma-biased gene expression neighborhood consists of a group of distinct genes encoding trypsin-like proteases [68]. Such gene arrangements usually represent gene duplications from a common ancestor and are often co-expressed. The unusually high number of *Drosophila* genes encoding trypsins has been previously noted [69], it appears that repeated gene duplication may be the cause.

Discussion

A large fraction of the *Drosophila* genome shows sex-biased expression

We have carefully cataloged sex-biased expression in *Drosophila* adults using 88 samples of wild-type flies and tissues, germline-ablated flies, and flies with sex transformed genotypes with 14,611 pairs of elements representing exons from release 1 of the *Drosophila* genome. We have also carefully examined platform performance [33] and have extensively analyzed the dataset for internal consistency as well as cross-validation with other array datasets and Northern blotting. The lists of genes in the survey of sex-biased expression will be a valuable resource for ultimately understanding how sexual dimorphism is genetically encoded and executed. Additionally, this dataset has already proven valuable in the study of rapidly evolving genes with male-biased expression and in understanding X chromosome evolution [23]. Sex-biased

**Figure 6**

An analysis of Gene Ontology term categories. The results show categories over-represented in (a) ovary, (b) testis, (c) female soma and (d) male soma queried by the three GO ontologies of biological process (black bars), cellular component (dark gray bars) and molecular function (light gray bars). Histograms represent significant over-representation ($P < 0.001$) of element species for 116 GO categories. The y axis scale shows P -values of the modified F-statistic (EASE score). The GO terms for each column are: 1, behavior; 2, biosynthesis; 3, cell cycle; 4, cell growth and/or maintenance; 5, cell organization and biogenesis; 6, cell proliferation; 7, chromosome organization and biogenesis (sensu Eukarya); 8, cytoplasm organization and biogenesis; 9, cytoskeleton organization and biogenesis; 10, cytoskeleton-dependent intracellular transport; 11, DNA dependent DNA replication; 12, DNA metabolism; 13, DNA packaging; 14, DNA replication; 15, DNA replication and chromosome cycle; 16, eggshell formation; 17, eggshell formation (sensu Insecta); 18, establishment and/or maintenance of chromatin architecture; 19, G2/M transition of mitotic cell cycle; 20, gametogenesis; 21, insect chorion formation; 22, insemination; 23, intracellular transport; 24, M phase of mitotic cell cycle; 25, macromolecule biosynthesis; 26, mating behavior; 27, metabolism; 28, microtubule-based movement; 29, microtubule-based process; 30, mitotic cell cycle; 31, nuclear organization and biogenesis; 32, nucleobase, nucleoside, nucleotide and nucleic acid metabolism; 33, oogenesis; 34, oogenesis (sensu Insecta); 35, organelle organization and biogenesis; 36, ovarian follicle cell development (sensu Insecta); 37, oviposition; 38, physiological processes; 39, post-mating behavior; 40, protein biosynthesis; 41, protein metabolism; 42, protein modification; 43, proteolysis and peptidolysis; 44, regulation of cell cycle; 45, reproduction; 46, reproductive behavior; 47, S phase of mitotic cell cycle; 48, sexual reproduction; 49, sperm competition; 50, sperm displacement; 51, transport; 52, vitellogenesis; 53, cell; 54, chaperonin-containing T-complex; 55, cytoplasm; 56, cytoskeleton; 57, cytosol; 58, cytosolic large ribosomal subunit (sensu Eukarya); 59, cytosolic ribosome (sensu Eukarya); 60, cytosolic small ribosomal subunit (sensu Eukarya); 61, dynein complex; 62, eukaryotic 43S pre-initiation complex; 63, eukaryotic 48S initiation complex; 64, extracellular; 65, inner membrane; 66, intracellular; 67, large ribosomal subunit; 68, lysosome; 69, lytic vacuole; 70, membrane; 71, microtubule associated complex; 72, microtubule cytoskeleton; 73, mitochondrial inner membrane; 74, mitochondrial membrane; 75, mitochondrion; 76, pre-replicative complex; 77, replication fork; 78, ribonucleoprotein complex; 79, ribosome; 80, small ribosomal subunit; 81, alpha-mannosidase activity; 82, aminopeptidase activity; 83, ATP dependent helicase activity; 84, ATP dependent RNA helicase activity; 85, carbohydrate binding activity; 86, carrier activity; 87, catalytic activity; 88, chaperone activity; 89, chromatin binding; 90, chymotrypsin activity; 91, dynein ATPase activity; 92, endopeptidase activity; 93, enzyme activity; 94, exopeptidase activity; 95, galactose binding activity; 96, hormone activity; 97, hydrolase activity; 98, hydrolase activity, acting on acid anhydrides, involved in cellular and subcellular movement; 99, leucyl aminopeptidase activity; 100, metalloexopeptidase activity; 101, nucleic acid binding; 102, oxidoreductase activity; 103, peptidase activity; 104, RNA binding; 105, RNA dependent ATPase activity; 106, RNA helicase activity; 107, serine-type endopeptidase activity; 108, serine-type peptidase activity; 109, small protein conjugating enzyme activity; 110, structural constituent of chorion (sensu Insecta); 111, structural constituent of ribosome; 112, structural molecule activity; 113, thiolester hydrolase activity; 114, transporter activity; 115, trypsin activity; 116, ubiquitin conjugating enzyme activity.

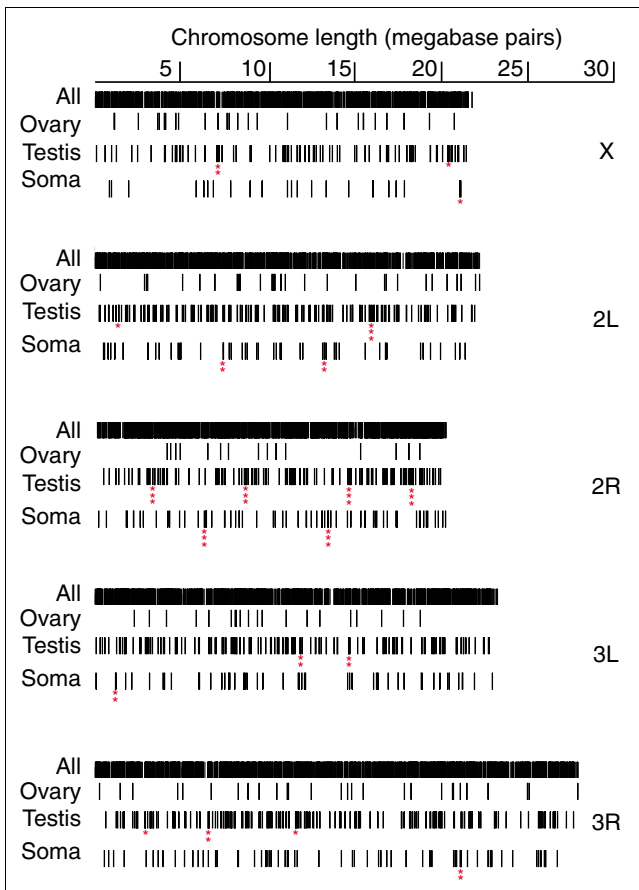


Figure 7
 Distribution of genes with high ovary-, testis-, and soma-biased expression in the genome. Gene positions are shown on the chromosome arms to scale with the positions of all genes on the five major chromosome arms (fourth and Y chromosomes not shown) presented as single tick marks for each in the upper column (All). The positions of genes represented by element species highly expressed in gonads and somatic tissues are presented in adjacent rows. The element species defined as highly expressed in the three tissue categories were up in multiple pairwise comparisons; ovary-biased (from microarrays versus both testis and female carcass), testis-biased (versus both ovary and male carcass) and somatic-biased (common among male carcass versus testis, and female carcass versus ovary). Asterisks indicate gene neighborhoods identified by Monte Carlo simulations with highly significant *P* values (**P* < 10⁻³, ***P* < 10⁻⁴, ****P* < 10⁻⁵).

expression may also be a convenient tool for understanding functional consequences and mechanistic underpinnings of gene expression neighborhoods.

In broad terms, we identify three large batteries of genes with ovary-biased, testis-biased and soma-biased expression. The germline and soma of *Drosophila* are the first lineages to separate in the developing embryo, so we tend to think of germline versus somatic tissue as being perhaps the most significant dichotomy. In addition to early divergence in the embryo, the germlines of females and males are functionally similar - they undergo meiosis and produce gametes. One

might therefore expect that female and male germ cells would express many genes that are silent in somatic cells. There is a small core of genes showing germline-biased expression (~3%); our data, however, suggest that most of the genes showing germline-dependent expression also show sex-biased expression. Essentially, adult ovary and testis are as different from each other as they are from somatic cells. A major difference between the germline and soma is the absence of gene expression from loci encoding terminal differentiation proteins (for specific adult somatic structures such as eyes and muscles) in the germline.

The lack of a large battery of genes showing germline-bias could also be due to expression of sperm or egg differentiation products that overwhelm the signal from genes expressed in the stem cells, which show no overt sexual dimorphism. Early undifferentiated germ cells from males and females would be expected to have more similar gene expression profiles. However, preliminary global analysis of transcripts in undifferentiated germ cells are also revealing a modest set of genes with germline-biased expression. The germline transcription profile is defined by the transcripts from genes encoding somatic terminal differentiation products and by sex. Germline development is under the control of sex determination signals from the time the embryonic genome is activated [70]. Perhaps it is better to think of the male and female germlines as quite distinct entities, rather than lumping them together as 'germline'.

Sex-biased expression in ovaries and testis were by far the most dramatic in terms of both numbers of genes and the degree of sex-bias. While most of this sex-biased expression is clearly explained by expression within the germline, there also appears to be striking germline-dependent expression outside of the gonads. Flies with and without a germline are often used to distinguish between genes that are expressed in the germline from those that are expressed in the soma [30]. Technically, a gene expressed in wildtype adults but not in adults missing a germline is said to be germline-dependent, not germline expressed. In comparing flies without a germline to those whose gonads had been removed, we found that there was greater sex-biased expression in the latter. These data suggest that the germline acts at a distance to condition the soma. This does not appear to be due to strain differences, as preliminary analysis of gonadectomized flies of identical zygotic genotypes but which had or were missing germ cells, shows substantial gene expression differences. Thus, it appears that much of the sex-biased expression in the *Drosophila* soma is due to conditioning by the germline, rather than the somatic sex determination hierarchy.

Data mining

A catalog of gene expression data is of limited value if it cannot be easily accessed. In addition to depositing all the array data at GEO, we have run a number of queries and provided the resulting gene lists in the Additional data files. Any of the

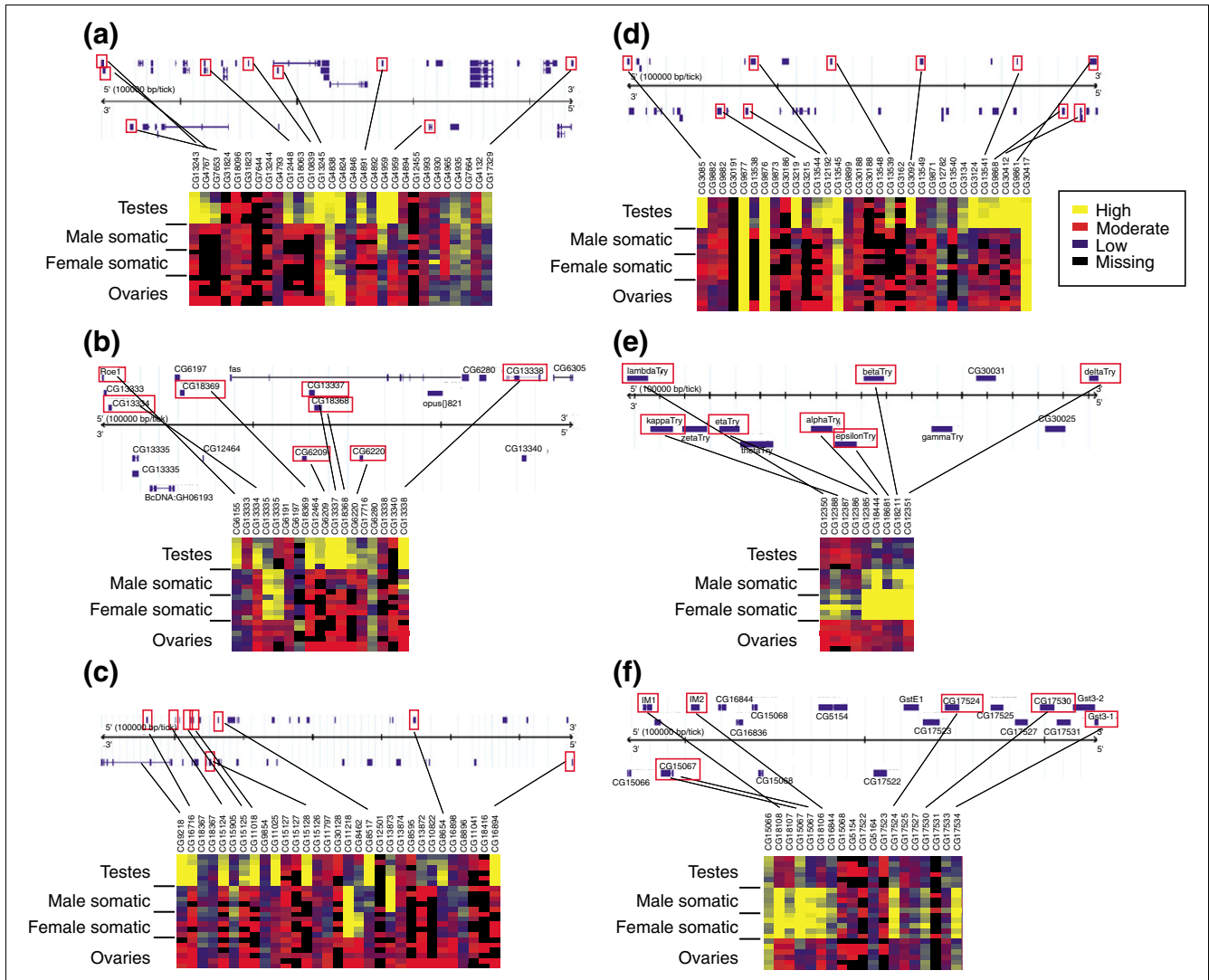


Figure 8

Gene neighborhoods. Monte Carlo simulations (see Materials and methods) were used to identify statistically significant clusters of testis- and soma-biased genes using a series of window sizes ranging from five to 200 consecutive genes. Examples are shown for six gene neighborhoods that were found zero times in 100,000 replicated randomization tests. GADFLY [26] annotation images of the chromosomal regions for each neighborhood indicating high expression (yellow), moderate (red), low (blue) and below background (black). (a) A testis-biased cluster at cytological position 2L:35D4-F435, (b) a testis-biased cluster at 2R:50B1-50C6, (c) a testis-biased cluster at 2R:56E1-F9, (d) a testis-biased cluster at 2R: 59C3-D6, (e) a soma-biased cluster at 2R:47E1-F5 and (f) a soma-biased cluster at 2R:55C6-C8.

lists of element species in the Additional data associated with this manuscript may be browsed in order to confirm the performance of the platform with a gene of interest, or for finding a novel expression pattern for a known gene. We have highlighted just a few of the stories that are embedded in the array dataset reported here. To list just a few examples, the preliminary data reported here suggest that germ cells are dosage compensated [24]. The dot fourth chromosome is especially active in the soma. Odorant-binding protein-encoding genes with highly sex-biased expression might be quite interesting candidates in the pheromone response.

It is our hope that this and other datasets generated on this platform will be useful to those who are interested in data mining for candidate genes. Additionally, there was reasonably good agreement between the array datasets here and those of Arbeitman *et al.* [20,36], which suggests that *Drosophila* array data from different laboratories will be directly comparable. This further suggests that array data will be broadly useful to the wider *Drosophila* community. However, it is also clear that there will be artifacts in array datasets, and indeed in the underlying genome annotations. Strain differences and the presence of genetic markers are also likely

to confound results; array data should be viewed with this firmly in mind.

Materials and methods

Animals and treatments

We used standard *Drosophila* techniques throughout. Flies were grown at $25 \pm 0.5^\circ\text{C}$ on GIF or PB media (KD Scientific, Columbia, MD, USA) and aged 5-7 days prior to use. Most alleles and transgenes have been previously described and can be found, with references, at FlyBase [26]. Flies wildtype for sex were of the genotype $y^1 w^{67c}$. To study the contribution of the body as a whole and the gonads, flies were dissected and snap frozen as described [19]. To study the germline-dependent gene expression profiles, samples were prepared from the progeny of homozygous $tud^1 bw^1 sp^1$ females. Flies were dissected to confirm germline loss ($tudor^1$ is not fully penetrant for germlinelessness, only individuals with atrophic gonads were retained) as above. This sham dissection also facilitates comparisons to gonadectomized samples. The effects of somatic sex determination mutants on sex-biased gene expression included examining sex-transformed flies using *doublesex* (+/+; dsx^{M+R45}/dsx^{swe}) and *transformer 2* ($w^{67c}/+; tra2^B/Df(2R)trix$) mutants. Sibling $w^{67c}/B^sY; tra2^B/Df(2R)trix$ were used in a direct comparison for one group of experiments.

Microarray hybridization

An extensive platform description is available [71] and at the Gene Expression Omnibus website [29] under accession number GPL20. RNA isolation, labeling, and hybridization have been previously described [71].

Microarray intensity quantification

Hybridized slides were read on a GenePix 4000 A to generate TIFF images which were imported into GemTools (Incyte Genomics, Palo Alto, CA, USA) for spot finding. Data was normalized and corrected in Array Qualifier (Novation Biosciences, Palo Alto, CA, USA). Intensities <2.5-fold above local background were discarded. Raw intensity data and corrected data from Array Qualifier were loaded into an Access database and analyzed in Excel (Microsoft, Redmond, WA, USA). The GEO data accessions for these hybridizations are: GSM2456, GSM2458, GSM2460, GSM2462, GSM2464, GSM2466, GSM16542, GSM16554, GSM16556, GSM16569, GSM16570, GSM16572, GSM16574, GSM16576, GSM16578, GSM16580, GSM2457, GSM2459, GSM2461, GSM2463, GSM2465, GSM2467, GSM16650, GSM16555, GSM16571, GSM16573, GSM16575, GSM16577, GSM16579, GSM16581, GSM16582, GSM16584, GSM16586, GSM16588, GSM16593, GSM16613, GSM16614, GSM16615, GSM16619, GSM16583, GSM16585, GSM16587, GSM16589, GSM16609.

Analysis of ratios

For analysis of ratios, the Cy3: Cy5 signal for each element was averaged over biological replicate experiments and dye flips

and then averaged from duplicate elements. Elements failing to return a ratio value from duplicates were excluded. Boolean queries were run to generate gene lists of particular interest (for example, high in ovary and female, and low in all others). For analysis by intensity, array channels were cross-normalized in Array Qualifier.

For array to array comparisons, the GPL218 platform table and 40 sample datasets representing 20 biologically replicated adult stages were downloaded from the GEO website. For comparison to the GPL20 data, Log₂ VALUE data (ratio of experiment to reference sample) from eight sample sets representing four biological replicates for five day adult Canton S or *tud* males and females (GEO accessions GSM3551, GSM3552, GSM3518, GSM3519, GSM3531, GSM3532, GSM3537, GSM3538) were converted to Ln values. The ratios from each biological replicate pair were averaged and these experiment/reference sample ratios were then rearranged into male/female and *tud* male/*tud* female ratios for parallel platform comparison. A total of 2,536 overlapping elements between GPL20 and GPL218 were selected based on Flybase IDs and plotted against the GPL20 counterpart (sample accession: GSM2456, GSM2458, GSM2457, GSM2459, GSM16582, GSM16583) or Northern ratios.

Clustering

To analyze sex-biased gene expression as normalized intensities among multiple microarray experiments, heat diagrams were generated by the Cluster 3.0 for Mac OSX program [72] formatted for OSX by Michiel deHoon [73]. A normalized set of signal intensity data was prepared for Cluster by averaging duplicate spots (the two duplicate features within an array) from 26 cross normalized microarray experiments. The sample columns were fixed, while genes were clustered. A self organizing map (SOM) was made using default parameters (10 clusters). The resulting SOM file was then used to perform k-means clustering. The output file was visualized with JavaTreeview (adapted by Alok Saldanha at Stanford University) [74].

A similar heat diagram was processed for analyzing the gene neighborhoods using this normalized dataset. A Treeview-generated image of the normalized intensities was made using an input data file that sorted the data for each gene by position along the chromosome. Data was sorted first by chromosome arm and then by distance along the chromosome using the first nucleotide of the microarray element amplicon as the positional reference relative to the annotation.

To produce the array to array comparison heat map, normalized intensity data from the FlyGEM platform was extracted for gene identifiers from Arbeitman *et al.*, Table 2 [36]. Heat maps were performed by k-means clustering of 26 female and 33 male soma-biased element species. Both genes and samples were clustered.

GO term analysis

Analysis of gene function was done using the vocabulary of the Gene Ontology Consortium [75] and applying it to the pairwise expression ratio gene lists processed through the bioinformatics tools DAVID and EASE [76] (search performed November, 2003). The LocusLink identifiers for all microarray features showing greater than two-fold differential expression ratios were loaded into the DAVID website and search parameters set to include all available categories from the three GO ontologies (biological process, cellular component and molecular function). A statistical measure of the representation of all GO categories was done with the EASE bioinformatics tool. The statistical probability for representation of genes within a given category relative to the total number of genes with GO function terms (EASE score) was calculated for each category using the *Drosophila*-specific database as a background filter. *Drosophila* features with GO term categories with EASE scores of $P < 0.001$ were reported.

Monte Carlo

To test if genes with sex-biased expression are non-randomly distributed into neighborhoods, we performed 100,000 simulations per window. The input data are stringently selected lists of genes that are differentially expressed in ovaries (ovary versus testis or females, no gonads), testis (testis versus ovary or males, no gonads) and somatic tissue (female and male, no gonads versus their respective gonads). The microarray data elements include those shown to be greater than three standard deviations above the mean local background in one channel while the signal from the other channel was within background levels. These lists are binary. The chromosomal locations for all genes were mapped on the genomic scaffolds from version 3.1 of the *Drosophila* genome annotation using the first nucleotide position from the sequence of each microarray element. Redundant elements were then removed based on their primer sequences to minimize spurious clustering.

To control for non-random distribution of gene models on chromosomes, we made use of gene order rather than exact chromosomal position. This is addressed using a nonparametric permutation test approach [77]. Because there is a multiple testing issue [78], a large number of dependent tests (since the windows are allowed to overlap) are performed, one for every gene window, so the measures of significance have to be calculated to account for the number of tests performed.

We determined a significance level to associate with the test of clustering in a given window as follows. In the given window (w) we determine the number (n) of sex-biased genes. A series of 100,000 Monte Carlo iterations are carried out in which the sex-biased genes and the non-differentially expressed genes are shuffled among the gene positions and the relative frequency of at least n sex-biased genes occurring in some window of the same size as w on the chromosome is

determined. Thus, our empirical P -value can be interpreted as the probability of such a degree of clustering occurring in at least some window of the size by chance alone.

Northern versus microarray

A subset of 75 element species were chosen as probes for Northern blotting on Hybond-N+ membranes (Amersham, Piscataway, NJ, USA) essentially as described in [79]. Probes were selected to cover the full range of absolute intensities and male/female differential expression revealed in microarray experiments. Blotted mRNAs were from flies wildtype with respect to sex and germline ($y^1 w^{67c}$) or from flies with no germline (progeny of homozygous *tudor¹* mothers). These same genotypes were used for labeling reactions in microarray experiments. Amplicon probes were made using the same primer pairs used in microarray construction and were labeled using Redi-prime II (Amersham). Northern blots were hybridized at 42° in UltraHyb (Ambion, Houston, TX, USA) in 15 ml conical tubes in a bacterial shaker. Blots were imaged on a Storm 860 phosphorimager and quantified using ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA). A total of 73 Northern blots were successful (passing visual inspection and showing bands above background, see Figure 4a). While absolute intensities between microarrays and Northern blots showed poor correlation (not shown), which is perhaps expected for different assays with different efficiencies, the relative measures showed very good correlation.

Additional data files

The following additional data files are available: a zip file containing 42 Excel tables (Additional data file 1) and an rtf file detailing the contents of the tables (Additional data file 2).

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