Close head-to-head juxtaposition of genes favors their coordinate regulation in *Drosophila melanogaster*☆

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Abstract **This report identifies a large number of gene-pairs in *Drosophila melanogaster* that share a common upstream region. 877 gene-pairs (~12% of the genome) are separated by less than 350 bp in a head-to-head orientation. This positional relationship is more highly favored in flies than in other organisms. These gene pairs have a higher correlation of expression than similarly spaced genes that have head-to-tail or tail-to-tail orientations. Thus, the positional arrangement of genes appears to play a significant role in coordinating relative expression patterns and may provide clues for identifying the functions of unknown genes.**

Keywords: Bidirectional promoter; Head-to-head genes; Genome-wide; Sphingolipid metabolism; Intergenic; Co-regulation

1. Introduction

One of the most effective methods for deriving the molecular and cellular functions of genes is to ask which known genes are related to the same function. This is the rationale behind the yeast two-hybrid system and co-immunoprecipitation assays. By identifying physical interactions between proteins, these methods provide a powerful means by which to elucidate novel functions of a gene by relating it to a gene of known function. However, proteins need not physically interact to be functionally related. Such is the case for enzymes involved in the same biosynthetic pathway. The traditional method for finding interactions in such a system is by way of genetic screens, but this can be logistically prohibitive and time-consuming. Another approach has been proposed in which functionally related genes can be identified based on their expression patterns [1,2]. Related genes can be organized into ''syn-expression'' groups to identify smaller pools of candidates. This concept relies on the idea that all of the genes encoding enzymes of a given biochemical pathway, for instance, must be coordinately transcribed for the pathway to function optimally. This report provides evidence that the physical arrangement of genes in *Drosophila melanogaster* may contribute to this coordinated expression.

There are many examples in a number of different species that demonstrate that the physical arrangement of protein-coding genes on chromosomes is not random. Functionally related genes organize within poly-cistronic transcripts in bacteria and *Caenorhabditis elegans* [3,4], cluster within large chromosomal regions in *Drosophila* [5–7] and humans [8], and, in mammalian genomes, may share bi-directional promoters [9–13] or arrange in tandem [14]. All of these organizational methods share the benefit of facilitating the synchronous expression of genes that are coordinately required for a given cellular function by minimizing the energy required for chromatin reorganization and maximizing the local concentration of transcriptional apparati. Interestingly, it has been recently proposed that operons in *C. elegans* may be exploited to extrapolate functional relationships between mammalian proteins [15,16].

In the present study, a genome-wide analysis has shown that there are a large number of gene pairs that are closely juxtaposed in a head-to-head orientation in *D. melanogaster*, and a large percentage of those gene pairs appear to be co-regulated. The relevance of this observation is supported by an analysis of the physical arrangement and expression patterns of genes neighboring those that encode sphingolipid metabolic enzymes. Thus, the identification of closely juxtaposed head-to-head gene pairs in *Drosophila* may be useful in predicting novel functions based on associations to known neighboring genes that are likely to be co-regulated.

2. Materials and methods

2.1. Identification of gene pairs

Gene annotations for each organism were obtained from the following sources: the Berkeley *Drosophila* Genome Project's release 3.1 annotations (http://www.fruitfly.org/sequence/download.html), the *Saccharomyces* Genome Database 11/26/2003 annotations (http://www.yeastgenome.org/DownloadContents.shtml), WormBase gene dump 3/1/2004 (http://www.wormbase.org), and Trinklein et al. [9] (http://www.genome.org). In each case, '−' strand genes were identified whose nearest upstream neighbor is located on the '+' strand. These genes comprised the identifying member of the head-to-head gene pair set. Intergenic distance was determined by subtracting the transcription start site (TSS) of the '−' strand gene from the TSS of its '+' strand cognate. Intergenic distances of head-to-tail pairs were depicted as the difference between the TSS of a gene and the transcription termination (TT) of its nearest upstream neighbor. Tail-to-tail distances were measured from TT to TT.

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2.2. Comparison of D. melanogaster *and* Drosophila pseudoobscura

100 head-to-head, head-to-tail, and tail-to-tail gene-pairs were selected for comparison. In each case, the most coordinately expressed gene-pairs (see below) separated by 250–350 bp were analyzed using the VISTA genome browser. (http://gsd.lbl.gov/vista/index.shtml) *D. melanogaster* gene-pairs that were not adjacent on the same genomic contig in *D. pseudoobscura* were considered disrupted.

2.3. Microarray analysis

Microarray data were obtained from the Berkeley *Drosophila* Genome Project as described [17] (http://www.fruitfly.org/cgi-bin/ex/insitu.pl). Averages of triplicates from 12 developmental time points were obtained from the Affymetrix chip data representing most of the ~14 000 genes in the *Drosophila* genome. Expression correlation between paired genes was determined using Pearson correlation coefficients.

2.4. In situ hybridization

In situ hybridizations were performed essentially as described [18]. Briefly, mixed stage embryos were collected on grape agar plates, dechorionated, devitellinized, and fixed with paraformaldehyde. Embryos were hybridized to digoxygenin-labeled antisense riboprobes, incubated with an alkaline-phosphatase conjugated anti-digoxygenin antibody and developed with NBT/BCIP. Probes used in this study were generated by PCR amplification of the target and subsequent cloning into pCRII-TOPO (Invitrogen cat# K4600-01).

3. Results

3.1. Close juxtaposition of genes occurs at a high frequency in D. melanogaster

A genome-wide survey of intergenic distance comparing head-to-head gene pairs with head-to-tail and tail-to-tail gene pairs in *D. melanogaster* reveals little difference in the mean spacing among the groups (4674, 4575, and 4965 bp, respectively). However, there are notable differences in the distributions of the frequency of the intergenic interval size (Fig. 1). Specifically, there is a higher proportion of gene pairs separated by less than 350 bp when oriented head-to-head compared to head-to-tail controls. 29% of the gene pairs in this orientation fall within this category compared to 23% of the head-to-tail genes ($P < 0.001$). These head-to-head closely juxtaposed genes (CJGs) comprise a large proportion of the protein-coding genes (877 gene pairs, roughly 12% of the genome), considering the small window of intergenic spacing that defines this category. Additionally, there is a notable difference between the distributions of head-to-head and tail-to-tail gene pairs. Although a similarly large percentage of tail-to-tail gene pairs are also separated by <350 bp (30%), the majority of these are *very* closely juxtaposed. More than half of these intergenic spaces are less than 100 bp.

To determine if these relationships were maintained in closely related species, 100 head-to-head, head-to-tail, and tail-to-tail gene-pairs separated by 250–350 bp in *D. melanogaster* were assessed for conservation in *D. pseudoobscura*. Head-to-tail and tail-to-tail gene pairs had modest increases in the number of gene-pairs disrupted by chromosomal rearrangement, 28% ($P < 0.001$) and 21% ($P < 0.05$), respectively, compared to the 14% observed in head-to-head gene-pairs. Therefore, positional arrangement of gene-pairs, that are in a head-to-head orientation, is highly conserved within the genus and more likely to be conserved than alternative arrangements.

Interestingly, head-to-head CJGs seem not to be prevalent in the yeast and *C. elegans* genomes. Yeast genes are highly clustered, presumably due to the small genome size, but lack a bias toward a head-to-head orientation (Fig. 1). *C. elegans* apparently relies less on gene clustering with fewer than 1% of its predicted transcripts having a cognate CJG within 350 bp (Fig. 1). The notable peak in the frequency of head-to-tail genes separated by 200–300 bp may reflect the use of operons in this organism, in which tandemly arrayed open reading frames are transcribed as a poly-cistronic message.

The frequency of head-to-head juxtaposition in *Drosophila* is similar to the recently reported bimodal distribution of intergenic spacing found between head-to-head genes in the human

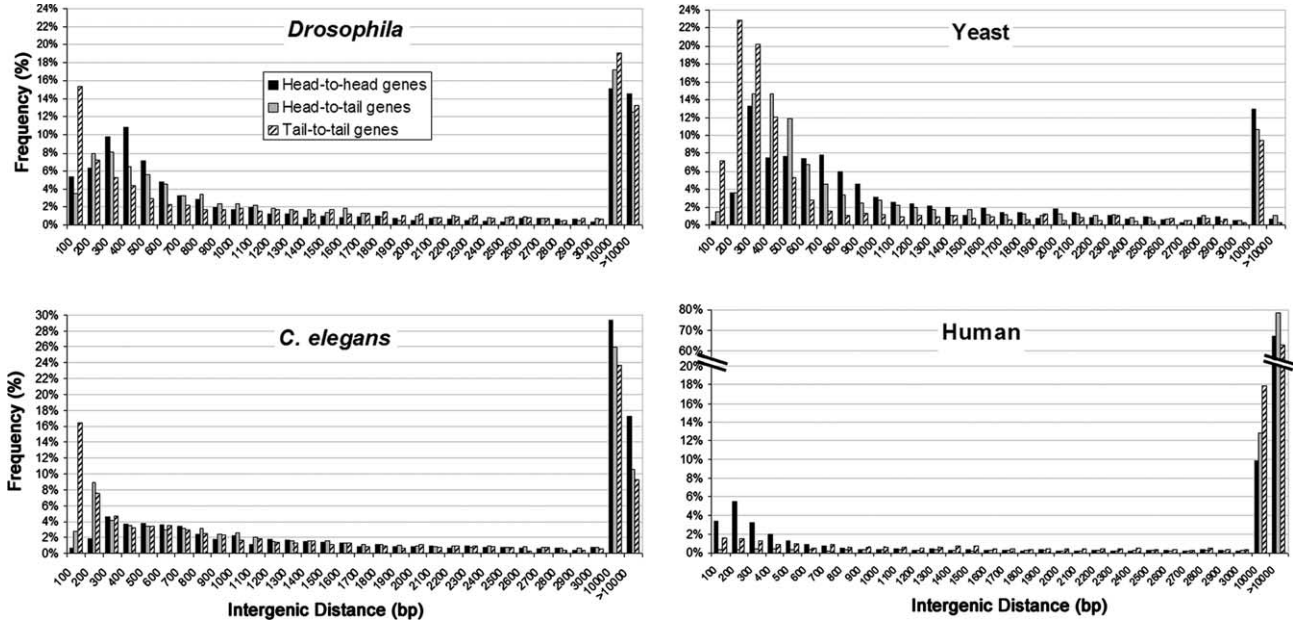

Fig. 1. Frequency of intergenic spacing. Comparison of the distribution of spacing between head-to-head genes (TSS to TSS), head-to-tail genes (TSS to TT), and tail-to-tail genes (TT to TT) among four divergent species. Frequency indicates percent of gene pairs within the indicated intergenic distance interval.

genome [9]. In fact, the selective pressure to maintain close proximity is particularly evident in humans considering that there is no preference for close gene juxtaposition when gene pairs are in the head-to-tail orientation (Fig. 1). The differences between humans and flies may be due to the large expansion of the human genome (~2800 vs. ~140 Mb) compared to the relatively modest increase in the number of protein-coding genes (~25 000 vs. ~14 000).

3.2. Coordinate expression of head-to-head CJGs

The observation that there is such a high occurrence of genes in *Drosophila* sharing a small upstream region suggested that this intergenic spacing has functional significance. Indeed, analysis of microarray data indicates a tendency for head-to-head CJG pairs to be coexpressed. A data set was obtained from Affymetrix chips representing most of the 14 000 genes in the *Drosophila* genome. These chips had been probed with cDNA made from staged embryos at 12 time-points throughout embryogenesis. When dynamic expression levels of gene pairs are compared by Pearson correlation analysis, head-to-head CJGs show higher expression correlations than either head-to-tail or tail-to-tail gene pairs with the same spacing or head-to-head gene pairs whose intergenic spacing is greater than 350 bp

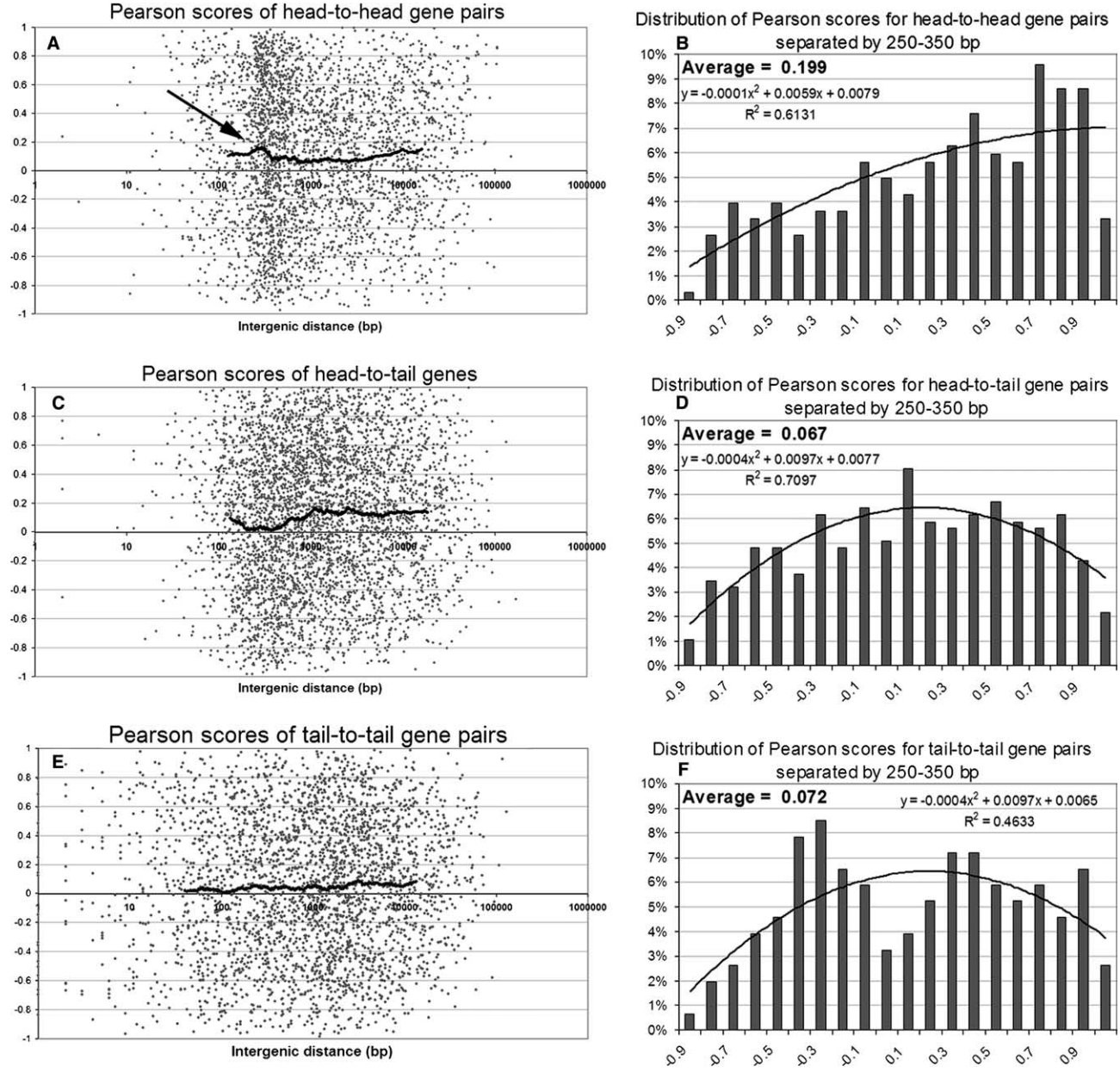

Fig. 2. Head-to-head CJGs show coordinate expression. Expression patterns, as determined by microarray analysis, of head-to-head gene pairs (A), head-to-tail gene pairs (C), and tail-to-tail gene pairs (E) were compared by Pearson analysis and plotted as a function of intergenic distance. The black line represents a moving average with a 500-unit window. There is an apparent clustering of high correlation coefficients in head-to-head pairs between 250 and 350 bp producing a peak in the moving average (arrow). Distribution of Pearson correlation coefficients for gene pairs separated by 250–350 bp in the head-to-head (B), head-to-tail (D), and tail-to-tail (F) orientations.

Table 1
Biological or biochemical functions of selected head-to-head CJG pairs from the 59 bp that had Pearson correlation coefficients >0.85

Distance	Pearson coefficient	"–" strand gene	Known or predicted function	"+" strand gene	Known or predicted function
289	0.938494	CG6840 (*Rpb11*)	RNA polymerase II [24]	CG15141	Zinc-finger protein
251	0.933272	CG7825 (*Rad17*)	DNA repair [25]	CG3509	Histone H1/H5
257	0.931395	CG7581 (*Bub3*)	Mitotic checkpoint [26]	CG1911	Chromosome condensation
321	0.926499	CG11988 (*neur*)	Ubiquitin ligase, mesoderm development, neurogenesis [27]	CG11990	Signal transduction
294	0.923159	CG4260 (*α-Adaptin*)	Vesicle transport [28]	CG4063 (*ebi*)	EGF receptor signaling [29]
77	0.922193	CG16912	Tyrosine-tRNA ligase activity	CG3589	Protein metabolism
101	0.876024	CG6007 (*gatA*)	Glutamyl-tRNA (Gln) amidotransferase activity	CG6009 (*P5cr*)	Pyrroline-5-carboxylate reductase activity [30]
247	0.854102	CG10075	Cytochrome bc1 complex	CG9943 (*Surf1*)	Cytochrome biogenesis [31]
53	0.859492	CG18780 (*TrfP*)	RNA polymerase II [32]	CG7562 (*Trf*)	TFIID complex [33]

Except where noted, predicted functions were determined by curated genome annotation at the Celera Annotation Jamboree, 1999.

Table 2
Embryonic expression of sphingolipid metabolic genes and their juxtaposed gene pair cognate determined by in situ hybridization

Intergenic distance (bp)	Pearson score	Sphingolipid metabolic gene	Embryonic expression	Cognate CJG	Presumed function of cognate	Embryonic expression	Co-expression
52 (353)[g]	0.744	*S1P lyase (Sply)*	Syncytial blastoderm; stage 7–9 PMG/AMG; >stage 11 mid-/hind-gut[a,b,c]	*CG6984*	Enoyl-CoA hydratase	Syncytial blastoderm; stage 7–9 PMG/AMG; >stage 11 mid-/hind-gut[a]	+++
145	N/A	*Sphingosine kinase 2 (Sk2)*	Syncytial blastoderm, stage 7–9 PMG/AMG; stage12–13 mesoderm[a,c,d]	*CG1893*	Phospholipid scramblase	Syncytial blastoderm, stage 7–9 PMG/AMG; stage 12–13 mesoderm[a,e]	+++
182	−0.192	*Sphingomyelinase (CG32052)*	Stage 16 antennae and head sensilla[c]	*Ilp4*	Insulin-like	Stage 5–8 mesoderm; stage 12–16 midgut[a,f]	–
320	0.855	*Sphingomyelinase (CG12034)*	Syncytial blastoderm[c]	*CG14967*	?	Syncytial blastoderm[a]	++++
400	−0.640	*Ceramidase (CDase)*	Stage 10–13 prohemocytes; stage 16 PMG[c]	*CG2224*	Cytokine signaling	CNS[a]	–
549	0.373	*Sphingomyelinase (CG15533)*	Maternal, stage 5–16 ubiquitous[c]	*CG2218*	UBC interacting	Maternal[a]	+
694	−0.108	*Ceramidase (bwa)*	Stage 5 anterior stripe; stage10–11 amnioserosa; optic lobe; stage 13–16 midgut[c]	*CG10728*	WD40	Stage 5 anterior stripe; stage 7–10 ectoderm; stage 13–16 midgut[a,e]	+
1234	N/A	*Ceramide kinase (CG16708)*	Syncytial blastoderm; stage 11–13 hindgut; stage 15–16 midgut lumen[c,e]	*CG31542*	?	Absent[a]	–
N/A	0.578	*Sphingosine kinase 1 (Sk1)*	Stage 6–11 AMG/PMG, mesoderm; stage 15–16 midgut/hindgut[a,c,d]	*CG11727*	?	Stage 12–16 salivary glands, stage 16–17 CNS[a]	–

Each pair has a head-to-head orientation except *Sk1*/*CG11727* (see text).
[a] Current study.
[b] Herr et al. [18].
[c] Renault et al. [19].
[d] Herr et al. [22].
[e] BDGP Gene Expression Report.
[f] Brogiolo et al. [34].
[g] Although the intergenic space between *Sply* and *CG6984* is only 54 bp, there are two TSSs for *Sply*. The second yields an intergenic space of 353 bp.

(Fig. 2A, C, and E). A moving average shows a peak Pearson correlation coefficient at a 300-bp interval. This spacing seemed particularly amenable to co-regulation as illustrated by the distribution of Pearson scores for gene pairs separated by 250–350 bp. Head-to-tail and tail-to-tail gene pairs that are separated by 250–350 bp are fitted by a bell-shaped curve consistent with a random distribution of Pearson correlation coefficients (Fig. 2D and F). Head-to-head CJGs sharing this spacing, however, are markedly "top heavy" (Fig. 2B) in that there are proportionally more gene pairs (30% vs. 18% and 20%) with correlation values >0.6 compared to the head-to-tail and tail-to-tail control groups.

Taken together, head-to-head CJGs (<350 bp) have an average Pearson correlation coefficient of 0.141 in contrast to an average of 0.061 for head-to-tail genes and 0.033 for tail-to-tail genes with the same spacing. There are 59 closely juxtaposed head-to-head gene pairs with very high Pearson correlation scores (>0.85) in the *D. melanogaster* genome. Examples of those gene pairs that are predicted to be involved in the same cellular processes are illustrated in Table 1.

3.3. Analysis of genes closely juxtaposed to sphingolipid metabolic genes

In order to more closely examine the significance of gene juxtaposition in a defined system, we identified the sphingolipid metabolic pathway as a biochemical pathway that would be suitable for this analysis. The genes involved in sphingolipid metabolism are conveniently suited to exemplify the coordinate expression of head-to-head CJGs for two reasons: (1) These genes have been characterized to various extents in previous studies [18–22] and their expression patterns and enzymatic functions are known and (2) 8 of the 11 genes examined have a head-to-head neighbor, providing a gradient of intergenic spacing ranging from 52 to 1234 bp (Table 2).

Whereas, gene pairs that are separated by more than 350 bp share few expression patterns, three of the four gene pairs separated by less than 350 bp are coordinately expressed both spatially and temporally (Table 2, Fig. 3); however, there are some differences in relative expression level. For example, whereas *CG6984* expression is robust throughout the endoderm at stages 7–9, *Sply* expression is present but much lower relative to its expression during other stages.

To control for gene proximity without a shared *cis* region, the expression of *Sphingosine kinase 1* (*Sk1*) was compared to that of *CG11727*. *Sk1* does not have a cognate head-to-head CJG and instead lies within an intron of *CG11727* on the opposite strand. The expression patterns of these genes are divergent and fail to overlap at any point during embryogenesis (Table 2, Fig. 3). Although these genes are not expressed in any of the same tissues, the Pearson correlation coefficient is deceptively high (0.578) due to their coincidental temporal

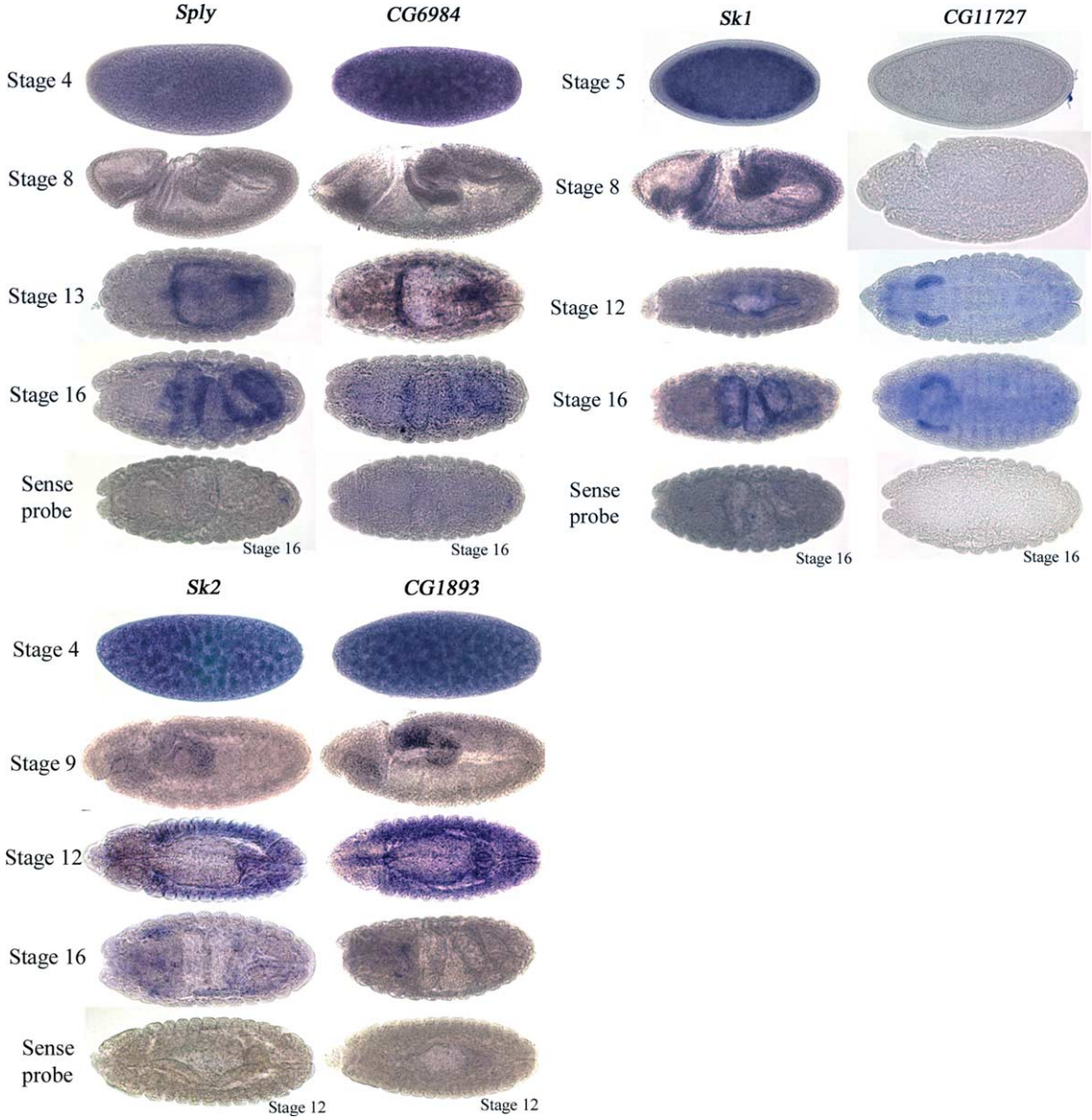

Fig. 3. Embryonic expression of sphingolipid metabolic genes and their CJGs. Expression of *Sply* and *CG6984* coincides throughout development (see Table 2) with the exception that the relative expression of *Sply* at stages 7–9 is noticeably lower. *Sk2* and *CG1893* are highly coexpressed with a particularly notable synchrony of transient expression in the stage-13 mesoderm. (Approximately half of stage-13 embryos exhibit mesodermal staining in both cases.) Expression of *Sk1* does not overlap that of *CG11727* despite their chromosomal proximity. (*Sk1* is within an intron of *CG11727*.) Embryos of relevant stages hybridized to sense-strand control probes lack detectable signal and are shown to demonstrate probe specificity. All embryos are oriented with anterior left. Stage 4–9 embryos are shown with a lateral view. Stages 12–16 are shown with a dorsal view.

coupling. Both are expressed at their highest level during late embryogenesis.

4. Discussion

Although the clustering of related genes into discrete chromosomal domains has been documented in *Drosophila* [5], the unexpected high frequency of CJGs has not been reported previously. The observations presented here suggest a general bias *toward* tight gene clustering in *Drosophila* as exemplified by the high frequency of tail-to-tail genes separated by <100 bp. However, gene pairs in a head-to-head orientation are less frequently observed with intergenic spacing <100 bp, suggesting a competing bias *against* disruptions of the *cis*-regulatory regions (Fig. 1). These disruptions are apparently minimized by an intergenic distance of at least 300 bp. Gene pairs that approach this limit in a head-to-head orientation have the benefit of intact, shared promoter elements in addition to chromosomal proximity. Interestingly, those gene pairs also exhibit the highest level of coordinate expression among all gene pair permutations analyzed in this study.

Head-to-tail and tail-to-tail gene pairs also tend to have positive Pearson correlation scores (Fig. 2C and E), which supports a model that chromosomal proximity alone is sufficient to confer some degree of coordinate regulation. It is notable that there are small peaks in the average correlation scores for head-to-tail gene pairs separated by ~1000 and ~2500 bp (Fig. 2C). This may be due to statistical drift or to another mechanism of transcriptional regulation possibly involving higher-order chromatin architecture.

Previous observations in the human genome have identified the presence of bidirectional promoters [9–13]. These studies have characterized certain features more common to this class of elements (e.g. higher GC content and low occurrence of TATA-boxes; true also in *Drosophila*), but are yet to identify clearly defined sequence motifs that distinguish them from unidirectional promoters. We reason that the localization of the transcriptional machinery to the *cis* region of a gene is sufficient to favor gene activation. Therefore, the activation of one gene in a head-to-head pair will favor the co-activation of its cognate CJG by recruiting regulatory proteins, thus increasing their local concentrations in the shared *cis* region.

Phylogenic differences in the frequency of CJGs demonstrate a marked heterogeneity in chromosomal organization (Fig. 1). This, presumably, is indicative of mechanistic differences in regulating gene expression brought about by variations in genome size and organism complexity.

Perhaps, the most useful application of the CJG model is that it offers the potential to predict unknown gene functions. For example, the action of phospholipid scramblase is necessary and sufficient for the activation of cytosolic sphingomyelinase by increasing its access to the substrate (sphingomyelin) [23]. Although functionally related, phospholipid scramblase and sphingomyelinase do not physically interact and their relationship could not have been discovered by yeast two-hybrid or co-immunoprecipitation assays. Interestingly, a phospholipid scramblase isoform is closely juxtaposed in a head-to-head orientation to another sphingolipid metabolic enzyme (*Sk2*) in *Drosophila*. Given this physical relationship, their developmental coordinate expression (Fig. 3), and their potential biochemical relationship, it seems unlikely that they do not share a common biological function.

This concept may be applied to reveal new relationships between genes that require co-activation for cellular activity. For example, *CG11807* is a homolog of a gene involved in cell-cycle progression. Its cognate CJG, *CG8090*, encodes a G-protein-coupled receptor of unknown function that may signal this proliferation. In addition, unlikely relationships may be discovered. For example, *rpk*, a gonad-specific sodium channel, may somehow function in concert with its CJG, *Dip2* (an endopeptidase) (Supplemental table).

It is tempting to speculate that novel gene functions may have arisen by the chance juxtaposition of genes during random chromosomal rearrangement. Genes that suddenly become co-regulated may elicit new cellular responses, possibly providing a mechanism for the evolution of such processes as signal transduction, biosynthetic pathways, or differentiation of specialized cells types. Although not required for coordinated gene regulation in eukaryotes, close juxtaposition of genes clearly has functional relevance, and understanding this relationship will help unravel the complexity of the organization of transcriptomes.

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