Gene expression profiling of the developing *Drosophila* CNS midline cells

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Abstract

The *Drosophila* CNS midline cells constitute a specialized set of interneurons, motorneurons, and glia. The utility of the CNS midline cells as a neurogenomic system to study CNS development derives from the ability to easily identify CNS midline-expressed genes. For this study, we used a variety of sources to identify 281 putative midline-expressed genes, including enhancer trap lines, microarray data, published accounts, and the Berkeley *Drosophila* Genome Project (BDGP) gene expression data. For each gene, we analyzed expression at all stages of embryonic CNS development and categorized expression patterns with regard to specific midline cell types. Of the 281 candidates, we identified 224 midline-expressed genes, which include transcription factors, signaling proteins, and transposable elements. We find that 58 genes are expressed in mesectodermal precursor cells, 138 in midline primordium cells, and 143 in mature midline cells—50 in midline glia, 106 in midline neurons. Additionally, we identified 27 genes expressed in glial and mesodermal cells associated with the midline cells. This work provides the basis for future research that will generate a complete cellular and molecular map of CNS midline development, thus allowing for detailed genetic and molecular studies of neuronal and glial development and function.

Keywords

CNS; Development; *Drosophila*; Gene expression; Mesectoderm; Midline; Midline glia; Neurogenesis

Introduction

One major goal of developmental neuroscience is to understand the molecular mechanisms that generate the neurons and glia that populate the central nervous system (CNS). This includes identifying the entire complement of RNAs and proteins present in each developing and mature cell type. Recent technical achievements along with other bioinformatic, biochemical, and genetic techniques now allow comprehensive, genome-wide views regarding how genes are regulated, interact, and function to generate diverse CNS cell types.

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The *Drosophila* CNS midline cells consist of a small number of neurons and glia (Bossing and Technau, 1994) and constitute an excellent system for neurogenomic analysis of CNS development. These cells resemble the vertebrate floorplate, which lies along the ventral midline of the spinal cord, as both tissues are important sources of developmental signals (Dickson, 2002; Ruiz i Altaba et al., 2003). Several aspects of CNS midline cell development have been well-characterized, including initial specification of the midline cells by the Single-minded (Sim) and Tango (Tgo) bHLH-PAS proteins (Crews, 2003), midline glial apoptosis (Bergmann et al., 2002), and the roles of midline cell-derived signaling in controlling axon guidance (Araujo and Tear, 2003) and ventral ectoderm formation (Kim and Crews, 1993; Schweitzer and Shilo, 1997). However, relatively little is known regarding how midline neurons and glia are generated.

The *Drosophila* CNS midline cells arise in the blastoderm embryo as two cellular stripes, referred to as mesectoderm anlage *in statu nascendi* (ISN) (Fig. 1A) (midline stages are those described by Tomancak et al., 2002). At the end of the mesectoderm ISN stage (stages 5–6), gastrulation brings the mesectodermal stripes together at the ventral midline. In the subsequent mesectoderm anlage stage (stages 7–8), the midline precursors undergo a synchronous cell division to give rise to 16 midline progenitor cells per segment (Fig. 1B). During embryonic stages 9–12, these 16 midline cells undergo cell shape changes, cell division, and differentiation to form the midline primordium (Fig. 1C). In the mature midline stage (stages 13–17), CNS midline cells undergo cell movement, apoptosis, and axonogenesis to form ~3 mature midline glia and 15–18 midline neurons.

At the beginning of the mature midline stage, the midline glia consist of two distinct groups, anterior and posterior (Fig. 1D). The anterior midline glia, a group of ~7–8 cells, are reduced by apoptosis to ~3 cells, which become the mature midline glia (Dong and Jacobs, 1997) (Fig. 1E). The mature midline glia ensheathe the axon commissures, and their survival is dependent on axon-directed epidermal growth factor receptor (Egfr) signaling (Bergmann et al., 2002;Jacobs, 2000). The posterior midline glia are a group of ~6 cells that are spatially distinct from the anterior midline glia and, as yet, have no defined function. By stage 16, all posterior midline glia have undergone apoptosis and do not contribute to the population of mature midline glia (Dong and Jacobs, 1997).

Midline neurons (Fig. 1E) form via two distinct mechanisms, the single division of individual midline precursors (MPs) (Bate and Grunewald, 1981) and the stem cell division of the median neuroblast (MNB) (Goodman and Spitzer, 1979). In each segment, five MPs each divide once to give rise to characteristic neuronal progeny. The two MP1 interneurons are identical, while the two MP3 interneurons (H cell and H cell-sib) show different axonal trajectories and likely have different functional roles (Bossing and Technau, 1994;Schmid et al., 1999). The *Drosophila* ventral unpaired median (VUM) neurons consist of three interneurons and three motorneurons (Bossing and Technau, 1994;Landgraf et al., 1997;Siegler and Jia, 1999;Sink and Whitington, 1991). Based on their development, gene expression, and axonal trajectories (Goodman, 1982;Jia and Siegler, 2002), the *Drosophila* VUM neurons are likely homologous to the grasshopper MP4–6 neurons. Midline neurons are also generated by the single median neuroblast in each segment that divides in an asymmetric stem cell mode giving rise to five to eight neurons (Bossing and Technau, 1994). The *Drosophila* MNB progeny consist predominantly of interneurons, but some cells are likely to be motorneurons (Bossing and Technau, 1994).

There are also three accessory cell types that lie along the midline but are not derived from mesectodermal cells (Fig. 1F). These include (1) dorsal median (DM) cells that are mesodermal and act as guidance cues for a subset of motor axon nerves (Luer et al., 1997;Zhou et al., 1997); (2) channel glia, which line the midline channel that spans the dorsal–ventral axis of

the CNS (Ito et al., 1995); and (3) medial-most cell body glia (MM-CBG) that are closely associated with and may support the VUM neurons (Ito et al., 1995).

Despite advances in our understanding of midline cell architecture, a number of aspects of midline cell development are poorly understood. These include (1) how midline neurons and glia acquire their individual cell fates; (2) how midline progenitor cells assume either a neuroblast or midline precursor mode of cell division; (3) how different MPs generate either identical or different pairs of neurons; (4) how the functional properties of midline neurons and glia are generated; and (5) how transcription factors and signaling proteins work combinatorially to control gene expression throughout midline development. One way to begin addressing these issues is to define the developmental gene expression profile of each midline cell type. To this end, we identified 224 genes expressed in midline cells and determined their spatiotemporal patterns of expression, thus establishing a database of genes expressed at all stages in all midline and midline accessory cells. This is an important step towards a comprehensive molecular and genetic analysis of CNS midline cell development and function.

Materials and methods

Drosophila strains

P[AA41-lacZ; ry$^+$] ry, an enhancer trap line which expresses lacZ in midline glia (Crews et al., 1992), was used as the standard lab stock for in situ hybridization. The 123 enhancer trap strains with midline *lacZ* expression were acquired from multiple sources including our lab, Corey Goodman, W. Janning, and the Bloomington *Drosophila* Stock Center. Additional lines used for cell identification were obtained from Gerd Technau, Andreas Prokop, and Corey Goodman. These include *C544-Gal4* (MP1s), *MzVUM-Gal4* (motorneuronal VUMs), *Mz840-Gal4* (MM-CBG), *Mz820-Gal4* (channel glia), and *slit-Gal4* (midline glia). *UAS* constructs used include *UAS-tau-lacZ* (Hidalgo et al., 1995), and *UAS-GFP-lacZ.nls* (Y. Hiromi and S. West, unpublished). Microarray misexpression experiments employed *daughterless-Gal4* (*da-Gal4*) (Giebel et al., 1997), *scabrous-Gal4* (*sca-Gal4*) (Hinz et al., 1994), and *UAS-sim-GFP* (Estes et al., 2001).

Inverse PCR and sequencing of enhancer trap line genomic DNA

Genomic sequence flanking P-element insertions was determined using inverse PCR (Liao et al., 2000) and DNA sequencing on genomic DNA isolated from fly strains carrying enhancer trap insertions. One hundred twenty-three lines were sequenced. Fifteen lines had insertions at the same position as another insertion. These may represent either (1) insertions that were not independent; (2) contamination of fly stocks; (3) mislabeling; or (4) independent insertions that landed in the same location. Of the 108 independent insertions, 32 were 5′ to reported exon 1, 36 were in introns, 23 were in the 5′ untranslated region of the transcription unit, 1 was in the coding sequence, 1 was in the 3′ untranslated region, 4 were found 3′ to the transcription unit, and 11 were within or near transposable elements. Of the transposable element insertions, 10 were within the body of the element and 1 was 5′ to the element.

Microarray analysis

Embryo collection and strains—*sim* overexpression embryos were the progeny of *UAS-sim-GFP* flies (Estes et al., 2001) mated to either *sca-Gal4* or *da-Gal4* flies. Two control embryo populations were used: (1) *yw^{67}* and (2) *UAS-sim-GFP* (with no *Gal4* driver present). Embryos were collected for 3 h on grape juice/agar plates and then aged for 5 h at room temperature. After collection, the embryos were dechorionated with 50% bleach, collected in PBT (1× PBS, 0.1% Triton-X-100), and kept frozen until RNA was prepared.

RNA preparation and probe generation—Total RNA was prepared from embryos using the Qiagen RNAeasy kit. Three independent RNA preparations were used in hybridization experiments for each condition. The RNA was used to generate labeled cRNA probes for microarrays. This was done by first using the RNA as a template to produce cDNA with Superscript II reverse transcriptase and a T7-(T)$_{24}$ primer. Second strand cDNA was synthesized with *E. coli* DNA ligase, *E. coli* DNA polymerase, and RNase H. After second strand synthesis, the samples were purified using RNase A, proteinase K, and phenol extraction, followed by ethanol precipitation. Biotinylated antisense cRNA was produced with an Enzo Diagnostics kit. Unincorporated NTPs were removed with an RNeasy spin column, and then the samples were ethanol precipitated, quantified, and fragmented.

High-density oligonucleotide array hybridization and data analysis—After prehybridization at 45°C for 15 min, the labeled, fragmented cRNA was hybridized to *Drosophila* Affymetrix microarray chips for 16 h at 45°C at the UNC Functional Genomics Core Facility. After hybridization, the arrays were washed in an Affymetrix Fluidics station and stained with streptavidin-R phycoerythrin conjugate (Molecular Probes). After hybridization and washing, the expression signals of each gene were obtained by scanning the microarray chips. Values from the chips were analyzed using Affymetrix software that generated normalized average difference values for each gene. To determine the genes that are up-regulated in embryos overexpressing *sim*, we entered the data obtained from the Affymetrix software into GeneSpring (Silicon Genetics). The *sca-Gal4* × *UAS-sim-GFP* embryos were compared to the *yw*[67] embryos, while the *da-Gal4* × *UAS-sim-GFP* embryos were compared to *UAS-sim-GFP* embryos. The data was normalized to median values for each gene and a *t-test* was performed on a gene-by-gene basis. When compared to wild-type controls, genes that had significantly increased by ≥1.2× ($P < 0.05$) in one of the three experimental conditions were designated as "increased". Genes that showed ≥1.2× difference ($P < 0.05$) in expression levels between the two control conditions (*yw*[67] and *UAS-sim-GFP*) were eliminated from the lists of increased genes.

Sources of cloned DNA

cDNA clones from the BDGP collection (v1.0 and v2.0; Open Biosystems) were used to prepare in situ hybridization probes for most genes. Two genes, *CG12648* and *scute*, were not represented in the BDGP collection and were PCR-amplified directly from genomic DNA using gene-specific primers. The PCR products were cloned into pGEM-T easy (Promega) to facilitate in vitro transcription.

Embryo collection and fixation

Drosophila embryos were collected on grape juice agar plates. Timed collections were performed corresponding to three different staged groupings (s4–10, s11–12, and s13–17). Embryos were dechorionated by immersing in 50% bleach for 5 min. Dechorionated embryos were fixed at the interface of heptane and 4% formaldehyde/1× PBS for 30 min, suspended in 50% methanol/50% heptane, and vigorously shaken to remove vitelline membranes. Embryos were then methanol dehydrated by rinsing 5× in 100% methanol and stored at −20°C. Prior to in situ hybridization or immunostaining, the three staged groups of embryos were combined in equal proportions to achieve equal representation of all embryonic stages.

In situ hybridization

Most steps of the in situ hybridization procedure were carried out using modifications of protocols from the BDGP Methods website (http://www.fruitfly.org/about/methods/index.html) (Tomancak et al., 2002). DNA templates for digoxigenin (dig)-labeled RNA probe preparation were PCR-amplified from individual clones such that the amplified insert was

flanked by an RNA polymerase initiation site allowing in vitro antisense probe transcription. PCR amplification of inserts cloned into the pBluescript SK$^-$ and pFlc-1 vectors was carried out using the SK-30 (5′ GGG-TAA-CGC-CAG-GGT-TTT-CC 3′) and SK-Met (5′ ATG-ACC-ATG-ATT-ACG-CCA-AGC 3′) primers. To amplify inserts in the pOT2a and pOTB7 vectors, primers OT2Afor (5′ GAA-CGC-GGC-TAC-AAT-TAA-TAC-A 3′) and OT2Arev (5′ GCC-GAT-TCA-TTA-ATG-CAG-GT 3′) were used. To amplify inserts in the pGEM-T easy vector, a gene-specific primer and the M13/pUCfor (5′ CCC-AGT-CAC-GAC-GTT-GTA-AAA-CG 3′) primer were used. DNA templates were amplified from single bacterial colonies streaked from the *Drosophila* cDNA collections or from diluted plasmid minipreps. PCR was performed using the following conditions: 1× PCR buffer II (Applied Biosystems), 200 µM dNTP, 1.5 mM MgCl$_2$, 0.3 µM each primer, 0.675 units Amplitaq DNA polymerase (Applied Biosystems), and 0.015 units cloned Pfu polymerase (Stratagene). PCR cycling was preceded by a 5-min incubation at 95°C to lyse bacterial cells and followed by a 10-min incubation at 72°C. PCR cycling consisted of 35 cycles of 1 min at 95°C, 1 min at 50°C, and a 72°C extension starting at 5 min and increasing by 6 s each cycle. DNA templates were sized on a 1% agarose gel and precipitated with sodium acetate prior to dig-RNA probe preparation. Using these protocols, we successfully amplified 320 of the total 331 cDNAs.

Synthesis of dig-UTP RNA probes was performed in 96-well plates. To generate antisense RNA probes for each cloned cDNA, 10 µl of PCR product was ethanol precipitated, resuspended in DEPC-treated H$_2$O, and in vitro transcribed using SP6 (New England Biolabs), T3 (Promega), or T7 (New England Biolabs) polymerases. All reactions were performed at 37°C for 3 h and contained the following: 1× transcription buffer, 10 mM DTT, 1× nucleotide labeling mix (Roche) containing dig-UTP, 20 units RNasin (Promega), and 8 units of RNA polymerase in a 10 µl final volume. RNA probes were DNase-treated with 1 µl of RQ1 DNase (Promega) for 30 min at 37°C. RNA probes were checked for size and concentration by gel electrophoresis on a 1.2% agarose gel. All probes were fragmented by adding an equal volume of Na$_2$CO$_3$ (pH 10.2) and incubated at 60°C for 15 min, followed by ethanol precipitation and resuspension in 150 µl of probe resuspension buffer (50% formamide, 5 mM Tris–HCl pH 7.5, 0.5 mM EDTA and 0.01% Tween-20). Transcription reactions that yielded significantly less RNA than expected were resuspended proportionally.

Hybridization was carried out in 96-well plates using an existing protocol (Tomancak et al., 2002) with the following modifications. Approximately 25 Al of embryos (encompassing stages 4–17) were hybridized with each RNA probe. Probes were diluted 1:100 in hybridization buffer (50% glycerol, 4× SSC, 5% dextran sulfate, 0.01% Tween-20) and hybridization was performed in a total volume of 100 µl at 55°C for ≥18 h. Anti-dig-alkaline phosphatase (AP) antibody (Roche) was used at 1:2000 in a total volume of 100 µl/well, and AP color reagent [175 µg/ml BCIP, 225 µg/ml NBT, 50 mM MgCl$_2$, 100 mM NaCl, 100 mM Tris (pH 9.5), 0.01% Tween-20] was used to detect hybridization signal. The color reaction was stopped by washing embryos 5× in PTX (1× PBS, 0.1% Triton X-100).

Fluorescent development of hybridized embryos was performed using the protocol of D. Leaman and U. Gaul (personal communication). Hybridized embryos were blocked in 0.5% blocking buffer (Perkin Elmer) and incubated in anti-dig-POD antibody (Roche) diluted 1:50 in 0.5% blocking buffer for 1 h. Following three 10-min PT (1× PBS, 0.1% Tween-20) washes, embryos were incubated for 2 h in TSA-Cy3 or TSA-Cy5 diluted in amplification diluent (Perkin Elmer). TSA reactions were stopped by three 5-min PT washes. Fluorescent RNA probe development was followed by immunostaining (see below).

Stained embryos were exchanged into 70% glycerol/ 1× PBS and imaged with a Zeiss Axiophot microscope with Nomarski optics, Sony 3XDD CCD video camera, and Zeiss Axiovision software. For immunofluorescent analysis, embryos were mounted in Aqua Poly/Mount

(Polysciences, Inc.). Confocal images were obtained using a Zeiss LSM 510 laser scanning system equipped with individual Argon and HeNe lasers for excitation at 488, 543, and 633 nm.

Immunostaining

Fixed embryos were rehydrated for 5 min in a solution of 1:1 methanol/PTX, washed 30 min in PBT (1× PBS, 0.1% BSA, 0.1% Triton X-100), and blocked 30 min in PBT-5% Normal Goat Serum (NGS). Embryos were incubated in 1° antibody diluted in PBT-NGS overnight at 4°C, washed 3× for 5 min and 4× for 30 min in PBT, and blocked for 30 min in PBT-NGS. Embryos were incubated in 2° antibody diluted in PBT-NGS for 2 h at room temperature, washed 3× for 5 min and 4× for 30 min in PBT, and visualized for fluorescence or stained with HRP. For HRP staining, embryos were incubated in diaminobenzidine (DAB) solution (1× PBS, 0.05% Tween-20, 0.3 mg/ml DAB) for 2 min and mixed with H_2O_2 to a final concentration of 0.03%. The 1° antibodies were used at the following dilutions: mouse anti-Engrailed (En) 4D9 (1:25) (Patel et al., 1989), rabbit anti-β-galactosidase (1:1000, Cappel), and mouse anti-Tau (1:500, Sigma). For 2° antibodies, we used α-rabbit-HRP (Sigma), α-mouse-Alexa 488, and α-rabbit-Alexa 647 (Molecular Probes) at a 1:200 dilution.

Results

Identification of midline–expressed genes

We assembled a database containing genes either known or potentially expressed in CNS midline cells. There were four sources of midline-expressed genes: (1) *Drosophila* enhancer trap lines; (2) microarray data for genes overexpressed in embryos that possess excess midline cells; (3) genes analyzed by in situ hybridization by the BDGP and other labs; and (4) genes identified via literature searches, annotated either by FlyBase or our-selves. While a few genes with broad expression in the CNS were included in the database, genes with ubiquitous expression or those without conspicuous midline expression were not considered.

Enhancer trap lines

There have been several P-element enhancer trap screens that identified midline-expressed genes (Crews et al., 1992; Kasai et al., 1992; Klämbt et al., 1991; Matthews and Crews, 1999; Peter et al., 2002). We accumulated 123 enhancer trap lines with detectable expression in the embryonic CNS midline cells. Several genes corresponding to P-element insertions from these screens have been previously identified and characterized (e.g., Hu et al., 1995; Klämbt, 1993; Matthews and Crews, 1999), but most have not. We used inverse PCR, DNA sequencing, and the *Drosophila* genome sequence to identify the location of each P-element insertion and the corresponding gene.

Sequencing of the 123 enhancer trap lines revealed 108 unique insertions corresponding to 76 distinct genes. The most prominent hot spots in this collection were *pum* (six insertions), *CG32423* (five insertions), *esg* (five insertions), *lola* (five insertions), *TE-opus* (four insertions), *dve* (four insertions), and *sog* (four insertions). Eleven insertions identified 7 different transposable element families. While there were previous reports that transposable elements were expressed in midline cells (Ding and Lipshitz, 1994; Lammel and Klambt, 2001; Tomancak et al., 2002), these results indicate that this is a widespread phenomenon. Of the 76 enhancer trap genes, 46 had available cDNA clones. We find that 39 of these are expressed in the CNS midline cells (Table 1), while the remaining 7 are not.

Microarray analysis

When *sim* is misexpressed in the neuroectoderm, a number of genes expressed in midline cells are ectopically expressed throughout the lateral CNS (Estes et al., 2001; Nambu et al., 1991). We used this paradigm to identify additional genes expressed in the CNS midline cells by comparing RNA populations between wild-type and *sim* misexpression embryos using microarrays. We used the *Gal4/UAS* system to misexpress a functional Sim-GFP fusion protein throughout the neuroectoderm and CNS using *sca-Gal4* and *da-Gal4*. Control and experimental RNA from these misexpression embryos were labeled and hybridized to Affymetrix *Drosophila* GeneChips. We made RNA comparisons during the midline primordium stage, a time when midline progenitors are developing into midline neurons and glia.

Of the 13,601 genes arrayed on the chip, the *da-Gal4* × *UAS-sim-GFP* embryos had 380 genes up-regulated with 77 up-regulated ≥2× (data available upon request). *sca-Gal4* × *UAS-sim-GFP* embryos had 419 genes up-regulated with 112 increased ≥2×. Examination of the up-regulated genes revealed several points. (1) The positive control genes, *sim* and *wrapper*, which are relatively specific for midline cells (Crews et al., 1988; Noordermeer et al., 1998), were increased in both paradigms. *wrapper* was increased 3× ($P < 0.03$) in *da-Gal4* × *UAS-sim-GFP* and 3× ($P < 0.02$) in *sca-Gal4* × *UAS-sim-GFP* embryos. *sim* was increased 15× ($P < 0.03$) in *da-Gal4* × *UAS-sim-GFP* and 6× ($P < 0.01$) in *sca-Gal4* × *UAS-sim-GFP* embryos. However, in these experiments, we were unable to distinguish the relative contributions of *sim* misexpression and *sim* autoactivation. (2) Few genes [25] were up-regulated in both paradigms. Furthermore, no gene showed expression ≥2× in both paradigms, with the exception of *sim* and *wrapper*. (3) While a number of genes known to be expressed in the CNS midline cells were up-regulated in one or more paradigms [17], most of the midline-expressed genes studied by in situ hybridization in this report (see below) were not. (4) Many genes up-regulated in one or more of the paradigms do not show midline expression according to published reports.

We tested 21 genes that were up-regulated in *sca-Gal4* × *UAS-sim-GFP* embryos compared to wild-type by in situ hybridization (Table 1). Six genes showed midline expression, 14 had expression elsewhere (gut expression was common), and 1 did not show any embryonic expression. The expression of the six genes showing midline expression was not specific to midline cells and midline expression was weak or transient. While the microarray experiments allowed identification of novel midline-expressed genes, the approach had many false positives and failed to identify many midline-expressed genes. This may be due, in part, to the restriction of our analysis to mid-embryogenesis (stages 11–12), which will not identify genes expressed later in midline development. Moreover, most midline-expressed genes are expressed in many non-midline cell types during mid-embryogenesis. Since mid-line cells constitute only ~2% of all embryonic cells, the use of whole embryos to isolate RNA likely increases background expression, and thus decreases signal to noise ratios for broadly expressed genes.

Midline genes identified from Drosophila databases

There were 89 genes annotated in FlyBase as of February 2004 that have midline expression based on published accounts. The BDGP gene expression site (Tomancak et al., 2002) has identified 132 genes that are expressed in CNS midline cells. All categories that denote expression in CNS midline cells were included for analysis. Another database, listed on FlyBase (Consortium, 2003), contains ESTs derived from embryonic membrane-bound polysomal RNA (Kopczynski et al., 1998) that includes 22 genes expressed in CNS midline cells. Additional midline-expressed genes were identified by inspection of various published articles (e.g., Brody et al., 2002; Freeman et al., 2003). There is overlap between genes contained in these data sets, and including all sources, we identified 387 potential midline-expressed genes. Although these genes are reported to be expressed in midline cells, our

analysis extends these findings by carefully mapping the spatiotemporal expression profile of each gene with respect to each midline cell type.

In situ hybridization experimental results

Of the 387 potential midline-expressed genes in our database, cDNA clones were available for 331. For each of these 331 cDNAs, dig-labeled RNA probes were generated and hybridized to staged *Drosophila* embryos using a 96-well plate procedure. In this study, we define CNS midline cells as those cells that lie along the CNS midline including cells derived from *sim*-positive mesectodermal cells (MP1, MP3, VUM, MNB, and midline glial lineages), as well as the mesodermal DM cells, and MM-CBG and channel glia that are both derived from the lateral CNS and migrate to the midline. The only exceptions are the apoptotic cells that accumulate at the midline between the CNS and epidermis in late stage embryos. These cells were ignored for the purposes of this analysis. Of the 331 genes assayed, 282 showed detectable hybridization with 224 expressed in midline cells.

Expression profiles of CNS midline-expressed genes

To create a database of midline gene expression profiles, we analyzed embryos at each developmental stage pertinent to embryonic CNS development (stages 5–17) (Campos-Ortega and Hartenstein, 1997). These stages include (1) mesectoderm ISN (stages 5–6); (2) mesectoderm anlage (stages 7–8); (3) midline primordium (stages 9–12); and (4) mature midline cells (stages 13–17) (Fig. 1). The annotation of these developmental time periods corresponds to the terminology proposed by the BDGP gene expression group (Tomancak et al., 2002). It is common for genes to be expressed in multiple stages and in multiple midline and lateral CNS cell types. Using alkaline phosphatase (AP) staining we can identify gene expression in subsets of midline cells. The anterior and posterior midline glia occupy opposite ends of the midline segment while midline neurons can be recognized as medial neurons (MP1 and MNB progeny) or ventral neurons (MP3 and VUM) based on their position in the segment. Midline accessory cells also occupy characteristic positions with respect to the mature midline cells. Definitive identification of expression in individual cell types requires confocal analysis (see below), and will, largely, be the subject of subsequent work. Table 1 summarizes the expression profile of each gene during each time period and in each mature cell type.

Mesectoderm ISN expression (stages 5–6)

The mesectoderm ISN occurs in blastoderm and gastrula stage embryos (stage 5–6) when mesectodermal cells cannot be recognized by morphology but only by gene expression (Fig. 1A and Fig. 2). We find 37 genes expressed in the mesectoderm ISN. These include transcription factors (14) and proteins implicated in cell signaling (12), consistent with early roles for these genes in dictating midline cell fates. We have subdivided these into two categories: genes expressed in all mesectoderm ISN cells (18) and genes expressed in a subset of mesectoderm ISN cells (19). Representatives of each of these classes of mesectoderm ISN-expressed genes are shown in Fig. 2 while a comprehensive list is found in Table 1. Not included are gap genes and others with either widespread or narrowly restricted expression patterns that are unlikely to play a role in the development of mesectoderm ISN cells or their progeny. Of the 18 genes expressed in all mesectoderm ISN cells, some are expressed exclusively in the mesectoderm, such as *sim*, *HLHmβ*, *Sema-1b*, and *CG9598* (Figs. 2A–D), whereas others including *brk* and *Tom* are expressed in the mesectoderm and neuroectoderm (Figs. 2E–F). There is a significant body of work describing the regulation of mesectoderm ISN gene expression by the embryonic dorsal–ventral (D/V) patterning cascade and the *Notch* signaling pathway (Kasai et al., 1998;Morel and Schweisguth, 2000;Stathopoulos and Levine, 2002), thus providing many tools to understand the regulation of these mesectoderm ISN-expressed genes.

Genes that are expressed in subsets of mesectoderm ISN cells are expressed as either repeating stripes that bisect the mesectoderm ISN or small numbers of mesectoderm ISN cells. These genes include the well-known segmentation genes, such as *dpn* (Fig. 2G), *pxb* (Fig. 2H), *wg*, *en*, *gsb-n*, and *slp2* as well as additional genes such as *Sema-5c*, *Mes2*, and *Ect3*. Although their functions in midline cells are unclear, many of these genes are likely involved in defining different classes of midline cell types (Hummel et al., 1999), similar to their roles in intrasegmental patterning of the epidermis and lateral CNS (Bhat, 1999).

Mesectoderm anlage expression (stages 7–8)

The mesectoderm anlage stage occurs after gastrulation (stages 7–8) when the two populations of mesectoderm ISN cells meet at the ventral midline and undergo a synchronous cell division producing 16 midline cells per segment (Fig. 1B) (Foe, 1989). There are 22 genes expressed in all mesectoderm anlage cells, including *sim*, *Wnt8*, and *edl* (Figs. 2I–K), and 19 expressed in subsets, including *Mes2*, *wg*, *slp2*, *esg*, and *Ect3* (Figs. 2L–P). More than half of these genes (22/41) are also expressed in the mesectoderm ISN in similar patterns. Only 10 genes expressed in all mesectoderm anlage cells and six in subsets of cells initiate transcription during this stage. Consistent with roles in regulating the division and fate of midline cells, this newly expressed group includes genes involved in cell division (*CycE, stg*), transcriptional control (*esg, Hgtx*), and cell signaling (*btl, edl, tkv, tsl*).

Midline primordium expression (stages 9–12)

During the midline primordium stage, midline precursor cells divide, change shape, migrate, and differentiate. There are 138 genes expressed in the midline primordium (Table 1; Fig. 3). All but six of the genes expressed in the mesectoderm anlage continue expression in the midline primordium. However, eight genes, including *rho* and *vvl*, refine their expression from all mesectoderm anlage cells to a subset of midline primordium cells. In contrast, four genes, including *CenB1A* and *Stat92E*, expand their expression from subsets of mesectoderm anlage to all midline primordium cells. Of note, 67% (92/138) of the midline primordium-expressed genes initiate their expression during this stage. Several genes (37) are expressed in all midline primordium cells (Figs. 3A–D) including *cdi*, *CenB1A*, *Ect3*, *mfas*, *rho*, *sog*, *Tl*, and *Wnt8*. These genes mimic the expression pattern of sim and are likely direct transcriptional targets of SimDTgo heterodimers (Ohshiro and Saigo, 1997;Sonnenfeld et al., 1997;Wharton et al., 1994;Zelzer and Shilo, 2000). Most genes at this stage are expressed in cellular subsets (101) (Figs. 3E–X). These genes label a range of subsets including large and small groups of cells (Figs. 3E–L) as well as single cells (Figs. 3M–P) and are noteworthy since they reveal the subdivision of midline progenitors into distinct cell types. It is likely that these genes are regulated by SimDTgo in combination with other transcription factors. Of note, a large number of transcription factors are expressed in subsets of midline primordium cells (see below). Many of the midline primordium-expressed genes are also expressed in mature midline neurons (41; Figs. 3Q–R) and glia (35; Figs. 3S–T) and will be useful in determining developmental relationships between midline primordium and mature midline cells.

The 138 genes expressed in midline primordium cells include characterized (Figs. 3E–R, T) and uncharacterized (Figs. 3S, U–X) genes. Transcription factors compose the largest class. There are 40 putative transcription factors, including 15 zinc finger proteins, 9 homeobox proteins, and 7 bHLH proteins. Interestingly, zinc finger genes such as cas and *Kr* (Figs. 3E, L) play prominent roles in generating ganglion mother cell (GMC) diversity in the lateral CNS (Isshiki et al., 2001; Kambadur et al., 1998) and may play a similar role in generating the diversity of MNB progeny. Another set of related zinc finger proteins that function together in development of the lateral CNS neurons are *esg*, *sna*, and *wor* (Fig. 3Q) (Ashraf and Ip, 2001; Ashraf et al., 1999; Cai et al., 2001). All three are expressed in the midline primordium and have different expression profiles that likely overlap in some midline cells. An additional

set of genes important in lateral CNS development and expressed in the midline primordium are the proneural genes *sc* and *l(1)sc* (Figs. 3I, N). Similar to their role in lateral CNS neuroblast formation (Jimenez and Campos-Ortega, 1990; Skeath and Doe, 1996), these genes may be important for the formation, differentiation, and division of the MNB and its progeny.

Another 41 genes expressed in the midline primordium are membrane or intracellular signaling proteins or membrane receptors involved in cell adhesion. These genes include *argos, btl, glec* (Fig. 3K), *robo* (Fig. 3F), *Tl, tsl*, and *wrapper*, some of which have been shown to be important for midline cell migration, apoptosis, midline-controlled axonogenesis, and glial-neuron interactions (Jacobs, 2000).

Mature midline cells (stages 13–16): midline glial expression

The midline glia are specified as two separate populations of cells, anterior and posterior, that are reduced by apoptosis to generate the ~3 mature midline glia in each segment. We identified 50 genes expressed in midline glia (Table 1; Fig. 4). Most (22) are expressed in both anterior and posterior midline glia (Figs. 4A–D), 11 are expressed prominently in anterior midline glia (Figs. 4E–H), and 4 are expressed prominently in posterior midline glia (Figs. 4 I–L). An additional 13 genes are expressed in midline glia, but it is unclear whether they are expressed in all or a subset of midline glia. Many of the genes expressed in midline glia are also expressed in the midline primordium (35/50), potentially providing markers for glial precursors in the primordium stage. Several genes (10) are expressed in both midline glia and other glial cells (Figs. 4M–N), indicating that midline and lateral glia can share glial-expressed genes. Additionally, 13 genes are expressed in both midline glia and midline neurons (Figs. 4O–P). Genes expressed in midline glial cells are varied in nature, including transcription factors, signaling proteins for the *spitz, netrin*, and *slit pathways*, intracellular signaling proteins, and genes whose functions have not been tested experimentally (Figs. 4A–B, E–H, K–N, P). There are also genes, including *CG8291*, *CG31116*, *CG31634*, and *Mdr65*, that resemble ion channels and transport proteins that may be involved in glial function.

Mature midline cells (stages 13–16): midline neuronal expression

Midline neurons consist of a number of different interneurons and motorneurons from distinct midline lineages. We have identified 106 genes expressed in mature midline neurons: 52 in subsets of midline neurons, 29 in all or most midline neurons, and 25 whose expression is too complex or obscured to make an accurate assignment (Table 1; Fig. 5). Approximately half of these genes (51) initiate expression during the midline primordium stage, suggesting that many neuronal cell fates are likely determined prior to the mature midline stages (Table 1; compare Fig. 4Q to Fig. 5C and Fig. 4R to Fig. 5J). Genes expressed in the mature midline neurons can be categorized by their appearance in the medial or ventral regions of the CNS (Fig. 1E). The medial group includes the two MP1 neurons and the 5–8 MNB progeny, while the ventral group includes the MP3 and VUM neurons. Of the 52 genes identified in subsets of midline neurons, 16 are expressed in medial neurons (Fig. 5A–D, L, O–P) and 36 in ventral neurons (Fig. 5E–H, I–K, M–N). Among genes expressed in midline neurons, notable are genes associated with neural function. These include neurotransmitter receptors (*Glu-RI*, *5-HT1a*, *5-HT7, Nmdar1, NPFR1, NPFR76F*) and proteins involved in neurotransmitter synthesis (*Gad1*, *ple*). In addition, this class contains many functionally uncharacterized genes (Fig. 5M–P). With their well-defined complement of gene expression, midline neurons will be a useful system for studying the molecular genetics of neural function.

Mature midline (stages 13–16): midline accessory cell expression

A number of cells that lie along the CNS midline are derived from the lateral CNS or mesoderm. These include the MM-CBG, channel glia, and DM cells. Some of the genes that are expressed in the MM-CBG and channel glia were previously described in a genome-wide search for glial-

expressed genes (Freeman et al., 2003). While a systematic analysis of accessory cell gene expression was not a major goal of this screen, a number of genes described from various sources as "midline" are expressed in accessory cells, and for this reason they have been included.

The MM-CBG consist of two to four glial cells per segment (two in abdominal and four in thoracic segments) that lie adjacent to the VUM neurons (Fig. 1F) (Ito et al., 1995). Their function is unknown; however, due to their close proximity, MM-CBG may act as support cells for the VUM neurons. These cells originate from lateral CNS neuroblast 6–4 and then migrate to their final position adjacent to the midline (Higashijima et al., 1996;Schmidt et al., 1997). MM-CBG can be recognized based on their cell morphology, location, and co-localization with *Mz840–Gal4*, an enhancer trap line expressed in MM-CBG (Fig. 6I–L) (Ito et al., 1995). We identified 12 genes expressed in MM-CBG (Table 1; Fig. 6A, B, I–L). Two genes, *CG1124* (Fig. 6A, I–L) and nrv2, were also expressed in midline glia. The genes expressed in MM-CBG are characteristic of glia, and include five that encode transporters (*CG6070*, *CG10960*, *Eaat1*, *nrv1*, *nrv2*). Studying the MM-CBG and VUM neurons will potentially provide insight into glial–neuronal interactions.

The channel glia consist of six cells, two located dorsally, two medially, and two ventrally, that line the channel that lies along the midline between the posterior commissure and anterior commissure of the next ganglion (Fig. 1F) (Ito et al., 1995). Like MM-CBG, the channel glia are derived from non-mesectodermal cells, including lateral CNS neuroblast 7–4, and migrate towards the midline (Schmidt et al., 1997). Channel glia can be identified based on their glial-like morphology, their location along the midline channel, and expression of previously identified markers, *Mz820–Gal4* (Fig. 6O) and *en* (Fig. 6M) (Ito et al., 1995). In our AP screen, six genes were found to be expressed in channel glia (Table 1;Fig. 6C, D, M–P). In addition to *repo*, a transcription factor shown to be expressed in many glia including channel glia (Ito et al., 1995), the functions of other channel glia-expressed genes are diverse and include a G protein-coupled receptor (*CG4322*), an esterase (*Gli*), a metallopeptidase (*CG6225*), and a transporter (*CG3168*).

The two mature DM cells in each segment are mesodermal derivatives that lie above the CNS (Fig. 1F). DM cells are associated with the midline channel and, thus, reside between the posterior commissure and the anterior commissure of the next ganglion. The DM cells extend a process to the body wall muscle attachment sites. CNS motorneurons and neurosecretory cells that form the transverse nerve use these processes to navigate toward their muscle synaptic targets (Chiang et al., 1994). The formation of mature DM cells is dependent on an unknown signal from the CNS midline cells (Luer et al., 1997;Zhou et al., 1997). The paired DM cells are easily identified based on their number, position atop the CNS, and characteristic morphology. These cells are noted for their expression of the *buttonless gene* (Chiang et al., 1994) and, like channel glia, numerous genes encoding basement membrane components (Fessler and Fessler, 1989). In our AP screen, 10 genes were expressed in the DM cells (Table 1; Fig. 6E–F), including the TE 412 transposable element (Fig. 6G). The genes expressed in DM cells are varied, including two transcription factors (*zfh1* and *CG12648*), a cell adhesion protein (*fas1*), a protease (*Mmp1*), a basement membrane component (*prc*), a G protein-coupled receptor (*CG4322*), and a transporter (*CG4726*).

Transposable elements

There are 96 families of transposable elements in *Drosophila*, constituting 1596 individual elements (Kaminker et al., 2002). Analysis of midline-expressed enhancer trap lines revealed 11 independent insertions into seven families of transposable elements. We believe the enhancer traps are recapitulating the expression of transposable elements and not nearby genes because 10/11 lines show P-element insertion into the body of the transposable element within

the putative coding sequence. Other sources implicated midline expression of four additional transposable element families. Of these 11 total transposable element families, we analyzed seven by in situ hybridization. Six showed prominent midline expression (Table 1; Fig. 6G–H), and one did not. Since probes were used for each transposable element that would cross-hybridize with many or all members of the family, midline expression is likely to be from multiple members of the family, not just the element containing the enhancer trap insertion. Midline expression of transposable elements is diverse: *TE 412* is expressed in DM cells (Fig. 6G), *TE Beagle* in midline neurons and glia (Lammel and Klambt, 2001) and *TE copia* (Fig. 6H), *TE 1371*, *TE Doc*, and *TE Rt1a* in midline neurons. *TE 412* and *TE Doc* are also expressed in the midline primordium. While the significance of transposable element midline expression is unknown, it appears to be relatively widespread and may serve a function in the evolution of transcriptional regulation (Bennetzen, 2000).

High-resolution identification of midline gene expression

One of the long-term goals that will assist genetic analysis of midline cell development and function is to determine the expression of each midline-expressed gene at the single cell level. This currently involves the use of cellular markers that unambiguously identify specific midline cell types, multi-label fluorescent staining, and confocal microscopy. Previous studies have identified midline cell types based on the position of cell bodies and, in the case of neurons, axonal morphology (Bossing and Technau, 1994; Schmid et al., 1999). Here we demonstrate how in situ hybridization can be systematically employed with appropriate midline cell markers to localize midline gene expression to specific mature midline cell types. Transgenic fly strains that drive expression of *Gal4* in subsets of midline neurons and glia are particularly useful since they can be combined with *UAS* reporter genes that allow visualization of nuclei, cytoplasm, or axons. The *Gal4* strains include *MzVUM-Gal4* (VUM motorneurons), *C544-Gal4* (MP1 neurons), and *slit-Gal4* (anterior and posterior midline glia) (Landgraf et al., 2003; Scholz et al., 1997). When crossed to *UAS* reporters and double-labeled, we can correlate the neuronal or glial cell bodies directly with midline gene expression. In these experiments, we also use En antibody staining to mark the VUM interneurons and several progeny of the MNB (Jia and Siegler, 2002).

MzVUM-Gal4 × UAS-tau-lacZ stains the soma of the three motorneuronal VUM neurons, which are also En-negative (Siegler and Jia, 1999), as confirmed by visualization of the characteristic motorneuronal axonal trajectories (Fig. 7A) (Bossing and Technau, 1994; Landgraf et al., 1997; Sink and Whitington, 1991). In our AP staining experiments, we identified *Snf4Ay* as expressed in ventral neurons (Fig. 5I). When *MzVUM-Gal4 × UAS-GFP-lacZ.nls* embryos are immunostained for En and β-gal, and hybridized to *Snf4Ay*, we observed overlap between *Snf4Ay* and *lacZ* expression, but not En (Fig. 7B–E). This demonstrates that *Snf4Ay* is expressed in the three VUM motorneurons and not the VUM interneurons. Additional *Snf4Ay* expression anterior to the motorneuronal VUM staining may be localized to one or both of the MP3 progeny.

C544-Gal4 × UAS-tau labels two MP1 neurons, as identified by their unique interneuronal projections (Fig. 7F). In *C544-Gal4 × UAS-GFP-lacZ.nls* embryos, we observe colocalization of the MP1 β-gal stain with the expression of *odd* (Fig. 7G–J), a gene we determined by AP staining to be expressed in medial neurons (Fig. 5L). This result proves the expression of *odd* in MP1 neurons, as previously proposed (Spana et al., 1995).

Both anterior and posterior midline glia can be visualized using a *Gal4* transgene driven by the *slit* promoter (Fig. 7K) (Wharton and Crews, 1993). In our AP expression screen, we classified *CG7663* as expressed prominently in posterior midline glia (Fig. 4K–L). Using *slit-gal4* driving the expression of *UAS-GFP-lacZ.nls*, we confirmed *CG7663* expression co-localized with *slit-Gal4* in the posterior glia (Fig. 7L–O). Interestingly, the *CG7663* expression

also co-localizes with En, indicating that at least a subset of posterior glia express both En and *slit* (Fig. 7L–O). These results indicate that the use of appropriate makers and imaging techniques can unambiguously identify distinct midline cell types. Extending this type of analysis to all midline-expressed genes will allow for the creation of a comprehensive molecular map of midline cell development.

Discussion

To begin a detailed analysis of the development and function of the *Drosophila* CNS midline cells, we assembled a database containing genes likely to be expressed in midline cells. In this report, we cataloged the expression patterns of 282 genes at each stage of CNS development and demonstrated midline cell expression for 224 (80%). These include (1) previously identified as well as novel genes; (2) genes expressed in all or most midline cells; (3) genes expressed in specific midline cell types, including subsets of midline progenitors; and (4) genes expressed in midline accessory cells. These data together with the genetic and genomic resources available in *Drosophila* provide a strong foundation for the future study of CNS midline cells.

A database of midline-expressed genes

The overall goal of this project is to study the development and function of the CNS midline cells as a model for understanding the molecular and genetic control of neural and glial cell development and function. There are a number of attractive reasons for studying CNS midline cell development: (1) midline cells occupy a discrete location within the embryo and are made up of diverse cell types, including interneurons, motorneurons, and glia; (2) midline cells have many developmental roles; (3) genes expressed in midline cells are particularly easy to identify based on their unique stripe of expression along the A/P axis of the embryo; and (4) there are a number of existing genes known to control aspects of their development and function.

To harness the full utility of this neurodevelopmental model system, we have begun to establish a comprehensive molecular map of CNS midline cell development. At the level of resolution of AP in situ hybridization, we were able to subdivide gene expression in mature midline cells into two neuronal categories (ventral and medial), two glial categories (anterior and posterior), and three accessory cell types. We have also demonstrated, using the *Gal4/UAS* system, in combination with fluorescent in situ hybridization and confocal analysis, that we can determine the extent of midline gene expression at the single cell level. These technologies together with the relatively simple organization of the CNS midline cells will allow the expression of each gene in the midline database to be assigned to specific midline cell types at each stage of development. Once assembled, this molecular map of gene expression will allow large-scale neurogenetic analysis in which scores of genes can be examined simultaneously in mutant or RNAi embryos that show defects in midline cell development, gene expression, or function. Since co-expressed genes have the potential to biochemically and genetically interact, the molecular map will likely be useful in predicting gene regulatory and protein–protein interactions that can be tested directly or cross-referenced with existing *Drosophila* protein and genetic interaction databases (Breitkreutz et al., 2003; Giot et al., 2003).

Using recent data that estimate the total number of Drosophila genes at ~16,000 (Hild et al., 2003) and the percentage of midline expressed genes compared to total examined (3%) from the BDGP expression database (Tomancak et al., 2002), we estimate that, in total, ~480 genes will be expressed in the CNS midline cells. These estimates indicate that we have already identified the expression patterns of roughly half of all genes expressed in midline cells. Thus, the continued analysis of gene expression patterns during *Drosophila* embryogenesis, both by individual labs and large-scale genome projects, will likely identify all CNS midline-expressed genes in the near future. With the completion of a comprehensive molecular map, the

Drosophila CNS midline cells will be an excellent system for neurogenomic research given the large number of genes identified, the detailed view of their expression, and the advantages of *Drosophila* as an organism for molecular genetic research.

Acknowledgments

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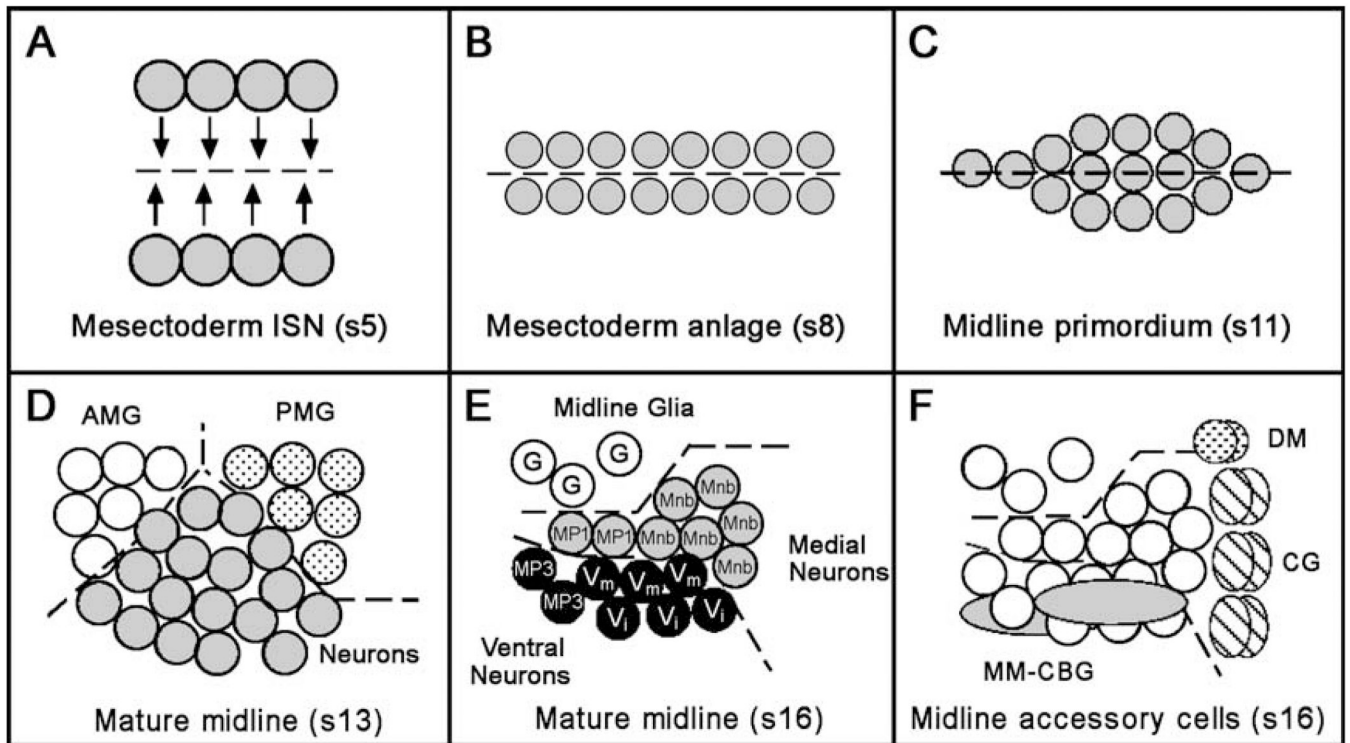
Fig. 1.
Schematic summary of CNS midline cell development. In all panels, a single segment is shown with anterior to the left. Embryonic stages are indicated by "s#". (A) Mesectoderm ISN stage, ventral view. Two stripes of mesectodermal cells reside on either side of the mesoderm in the blastoderm embryo (stage 5). Dotted line indicates ventral midline of embryo. There are four cells/segment on each side. Arrows represent how the mesectodermal cells move together at the ventral midline during gastrulation (stage 6) as the mesoderm invaginates. (B) Mesectoderm anlage stage, ventral view. During the mesectoderm anlage stage (stages 7–8), the mesectodermal cells meet at the midline and then undergo a synchronous cell division, resulting in 16 cells per segment. (C) Midline primordium stage, ventral view. During the midline primordium stage, midline cells rearrange from a two-cell wide planar array into a cell cluster. Midline cells within these clusters differ slightly in their dorsal/ventral positions. (D) Mature CNS midline cells, stage 13. Sagittal view, dorsal up. At stage 13, two populations of midline glial cells become evident. The anterior midline glia (AMG; open circles) are reduced by apoptosis but ultimately will ensheathe the commissures while all posterior midline glia (PMG; dotted circles) will undergo apoptosis. Midline neurons (shaded circles) occupy the space between and below the midline glia. Dotted lines separate the different cell groups. (E) Mature CNS midline cells, stage 16. Sagittal view, dorsal is up. The PMG have undergone apoptosis and are absent, whereas the AMG give rise to ~3 mature glia (G, open circles). Midline neurons have migrated to their final positions within the ganglion. Medial neurons include MP1 neurons (MP1, shaded circles) and the progeny of the MNB (Mnb, shaded circles). Ventral neurons include VUM motorneurons (Vm, black circles), VUM interneurons (Vi, black circles), and MP3 neurons (MP3, black circles). (F) Midline accessory cells shown in relation to midline neurons and glia (open circles). Two DM cells (dotted circles) lie atop the CNS near the midline channel, which is lined by six-channel glia (CG; hatched ovals). The two MM-CBG in each segment (shaded ovals) are closely associated with the ventral neurons.
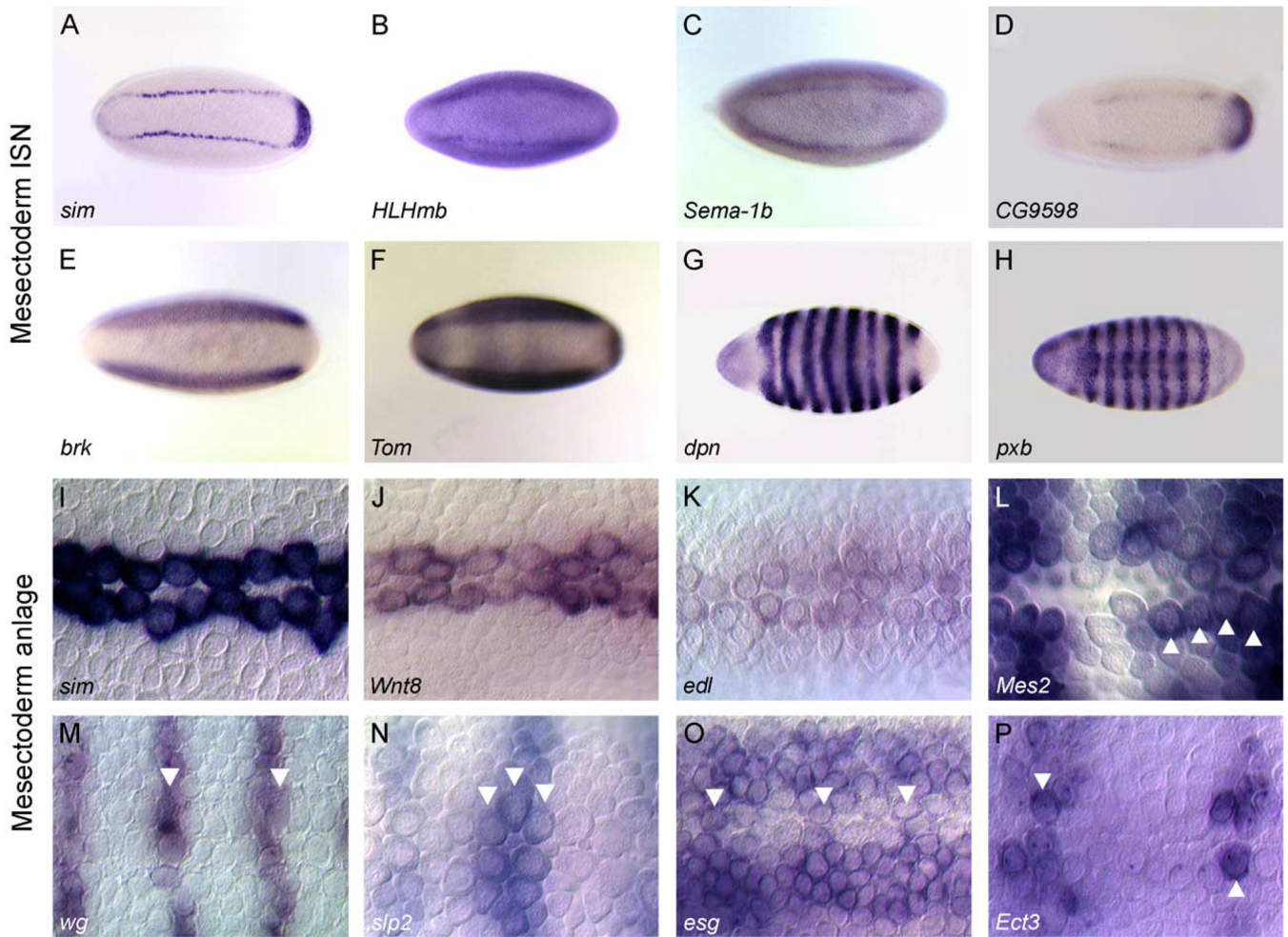
Fig. 2.
Gene expression during the mesectoderm ISN and mesectoderm anlage stages. Whole-mount embryos hybridized in situ showing the expression patterns of genes expressed in: (A–H) mesectoderm ISN, and (I–P) mesectoderm anlage. All views are ventral with anterior to the left. Gene names are shown at the bottom left. Representative genes are shown for three classes of mesectoderm ISN expression: (A–D) genes with relatively specific expression in all mesectoderm ISN cells, (E–F) genes expressed in all mesectoderm ISN cells and showing additional expression in other neuroectodermal cells, and (G–H) genes with pair-rule patterns expressed in subsets of mesectoderm ISN cells. (I–P) Shown are genes expressed in: (I–K) all mesectodermal anlage cells, and (L–P) subsets of mesectodermal anlage cells. Arrowheads indicate stained mesectodermal cells. Note the number of mesectoderm anlage cells labeled per hemisegment varies for different genes. Magnification is 12.5× for A–H and 80× for I–P.
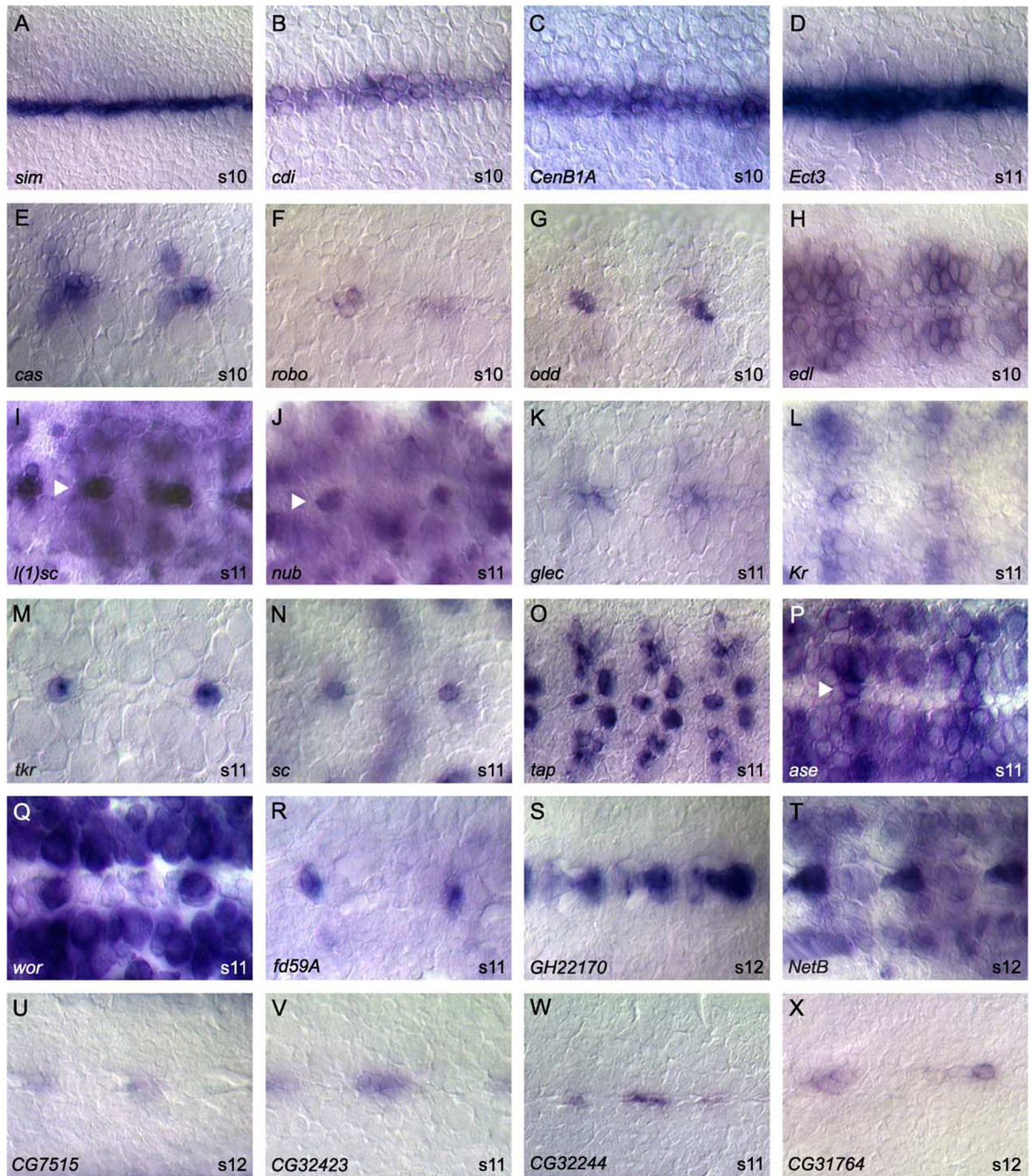
Fig. 3.
Midline primordium gene expression. Whole-mount stages 9–12 embryos hybridized to RNA probes. All views are ventral with anterior to the left. Arrowheads denote midline cells in embryos with high surrounding expression. Representatives of two major classes of gene expression are shown: (A–D) expression in all midline cells, and (E–X) expression in subsets of midline cells. Genes expressed in subsets can be further divided into those expressed in: (E–L) multiple primordium cells, and (M–P) single primordium cells. (Q–T) A subset of genes expressed in the primordium stages maintains expression in (Q–R) mature midline neurons or (S–T) midline glia. Compare (S) *GH22170* in the primordium to *GH22170* in the mature midline glia (Figs. 4A–B), and (Q) *wor* in the primordium to *wor* in midline neurons (Fig. 5C).

(U–X) Shown are genes of currently uncharacterized function expressed in subsets of midline primordium cells. Magnification is 80× for all except Q (100×).
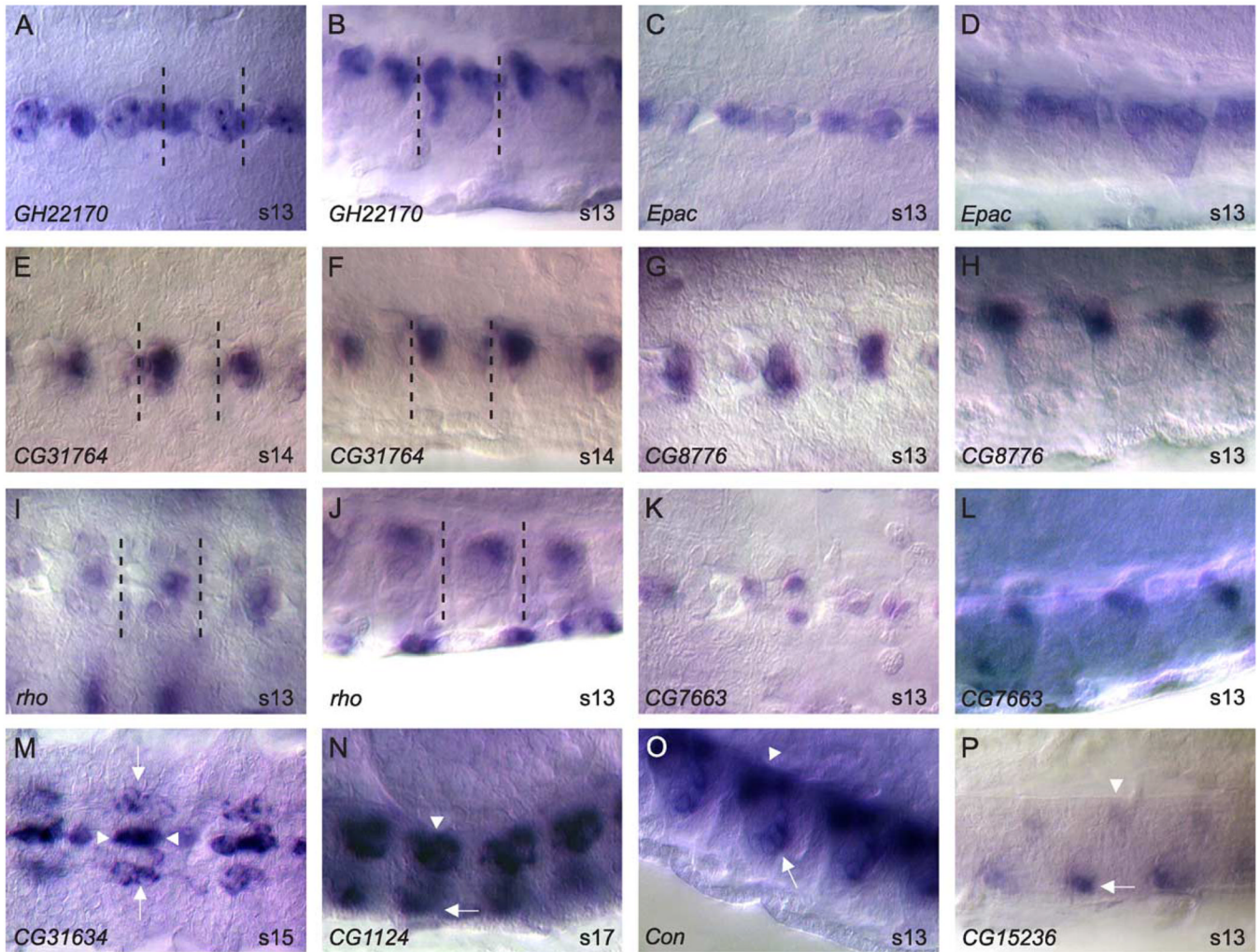
Fig. 4.
Midline glial gene expression. Whole-mount stages 13–17 embryos hybridized to RNA probes.
(A–L) Each gene is shown twice with a ventral view on the left and sagittal view on the right.
Anterior is left and dorsal top for sagittal views. Dotted lines enclose a single ganglion. (A-D)
Two genes expressed in both anterior and posterior midline glia. (E–H) Two genes expressed
prominently in anterior midline glia. (I–L) Two genes expressed prominently in posterior
midline glia. (M–P) Ventral (M) or sagittal (N–P) views of embryos hybridized with probes
to genes expressed in midline glia and other cell types. (M) *CG31634* is expressed in both
midline glia (arrowhead) and longitudinal glia (arrow). (N) *CG1 124* is expressed in both
midline glia (arrowhead) and MM-CBG (arrow). (O–P) Examples of genes expressed in both
midline glia (arrowhead) and midline neurons (arrow). Magnification is 80x.
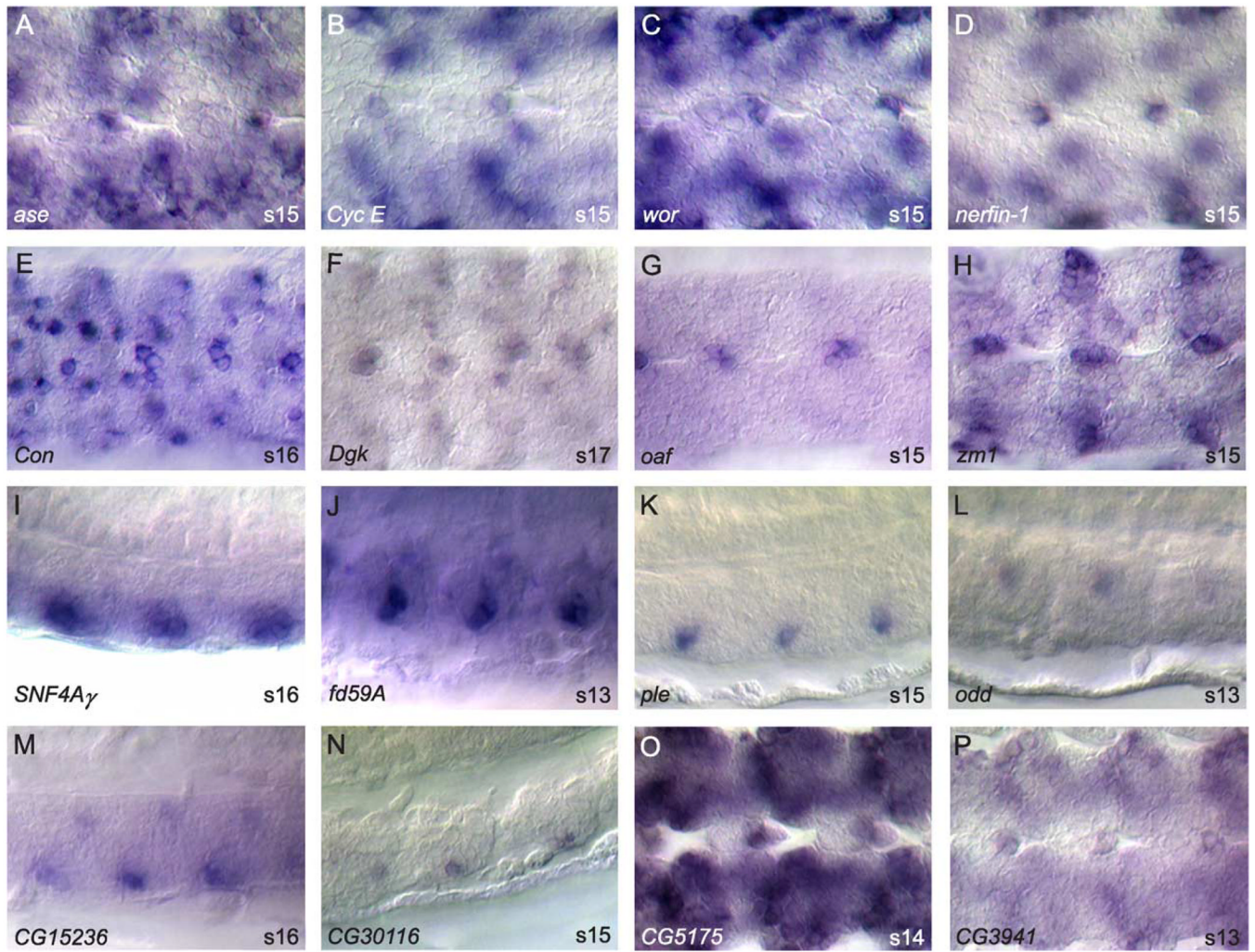
Fig. 5.
Midline neuronal expression. Whole-mount stages 13–17 embryos hybridized to RNA probes. Anterior is to the left and dorsal top for sagittal views. (A–H) Ventral views of genes expressed in: (A–D) medial neurons, and (E–H) ventral neurons. (I–L) Sagittal views showing the dorsal/ventral position of genes expressed in (I–K) ventral neurons and (L) medial neurons. (M–P) Examples genes of uncharacterized function expressed in (M–N) ventral neurons (sagittal views), and (O–P) medial neurons (ventral views). Magnification is 80x.
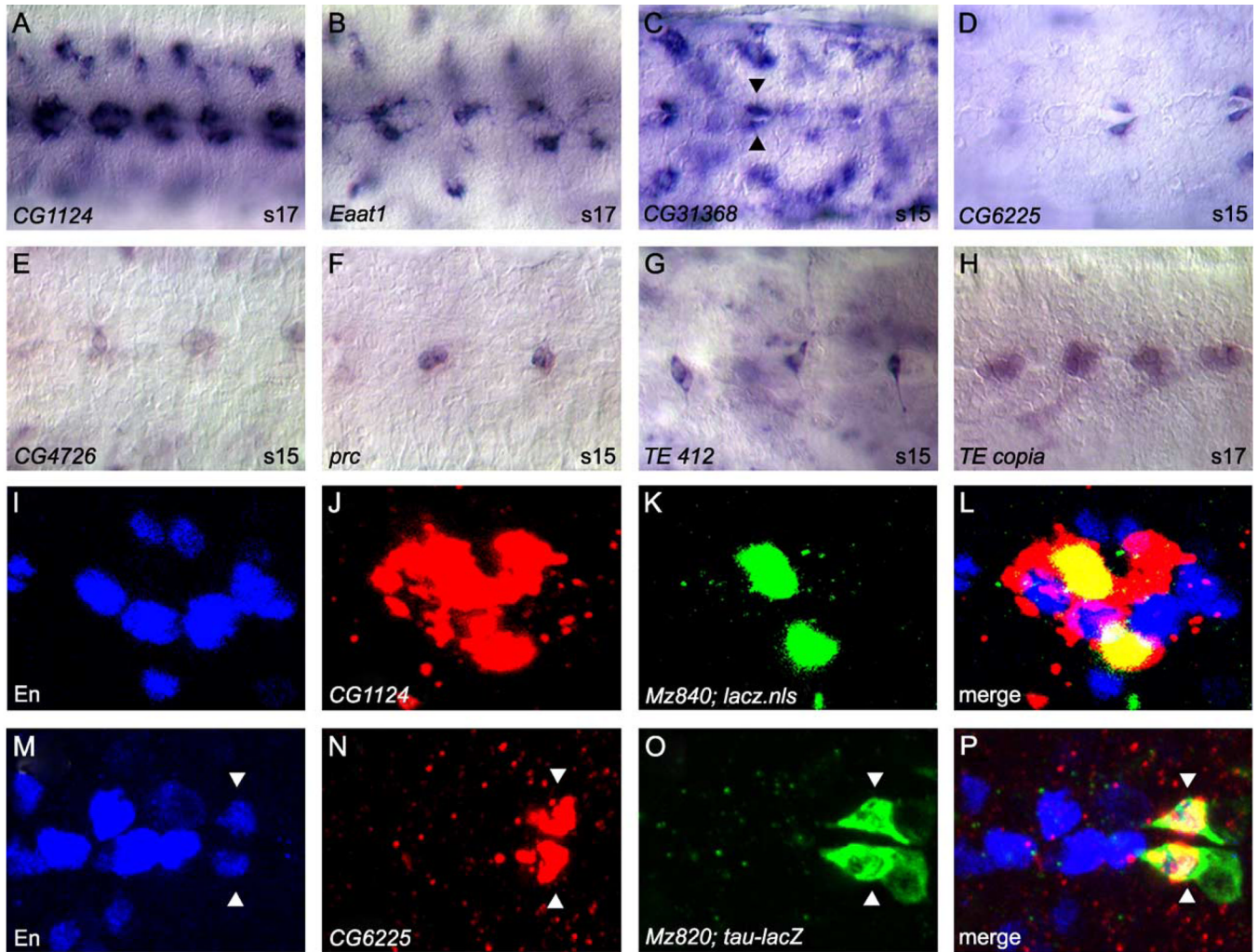
Fig. 6.
Midline accessory cell gene expression and midline-expressed transposable elements. (A–H) Whole-mount stage 15 or 17 embryos were hybridized to RNA probes and visualized by AP/DIC imaging. All views are ventral with anterior to the left. Magnification is 80x. (A–G) Examples of genes expressed in subsets of midline accessory cells, including (A–B) MM-CBG, (C–D) channel glia (arrowheads), and (E–G) DM cells. (G–H) Transposable element expression in midline and accessory cells, including (G) DM cells and (H) ventral neurons. (I–P) Confocal images of multi-label fluorescent in situ hybridization. An individual ganglion is shown for each panel. All views are ventral with anterior to the left. (I–L) Stage 16 *Mz840-Gal4 × UAS-GFP-lacZ.nls* embryo co-labeled with: (I) anti-En, (J) *CG1124* RNA probe, and (K) anti-β-gal. (L, merge image) *CG1124* expression (red) colocalizes with *Mz840-* driven nuclear β-gal (green) expression in MM-CBG. MM-CBGs are positioned just lateral to En-positive VUM cells. The in situ fluorescence stains the cytoplasm and is usually broader than the nuclear staining observed with immunostaining of *GFP-lacZ.nls* or nuclear proteins. (M–P) Stage 15 *Mz820-Gal4 × UAS-tau-lacZ* embryo co-labeled with: (M) anti-En, (N) *CG6225* RNA probe, and (O) anti-β-gal. (P, merge image) *CG6225* expression (red) co-localizes with *tau-lacZ* fusion protein (green) expressed in channel glia (arrowheads) via the *Mz820-Gal4* enhancer trap line. CG6225-positive channel glia also express *en*.
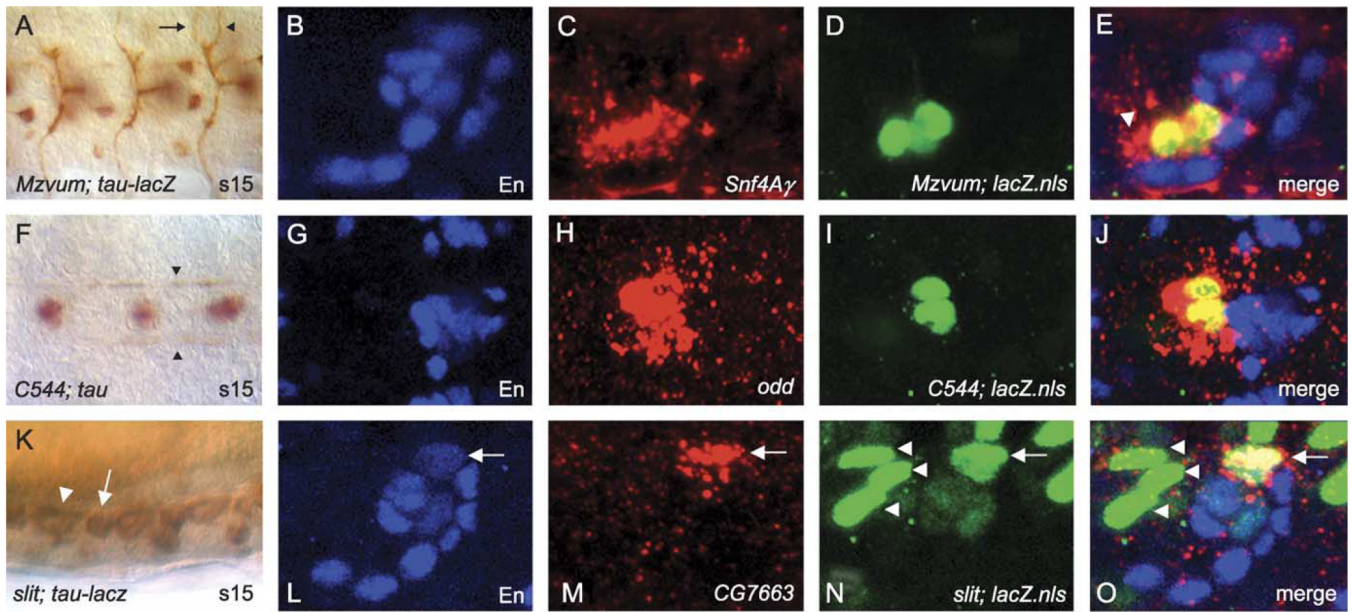
Fig. 7.
Cellular correspondence of midline gene expression. (A, F, K) DIC images of *Gal4×UAS-tau* or *UAS-tau-lacZ* embryos immunostained with anti-Tau or anti-β-gal. (B–E, G–J, L–O) Confocal images showing midline cell co-localization of RNA probes with defined midline cell markers. Anterior is to the left in all cases, and for sagittal views, dorsal is up. (A) Ventral view of stage 15 *MzVUM-Gal4×UAS-tau-lacZ* embryo showing the VUM motorneuron axonal trajectories that project out the intersegmental (arrow) and segmental (arrowhead) nerve roots. The VUM motorneuron cell bodies are out of the plane of focus. (B–E) Sagittal view of an individual ganglion from a *MzVUM-Gal4×UAS-GFP-lacZ.nls* embryo stained for (B) anti-En (blue), (C) *Snf4Aγ* RNA (red), (D) anti-β-gal (green), and (E) merge image showing that *Snf4Aγ* RNA co-localizes with β-gal-stained VUM motorneurons. Additional expression is detected just anterior to VUM motorneurons (arrowhead) and may represent staining in MP3 neurons. (F) Ventral view of a stage 15 *C544-Gal4×UAS-tau* embryo stained with anti-Tau showing the MP1 axonal trajectories along the longitudinal connectives (arrowheads). (G–J) Ventral view of an individual ganglion from a stage 16 *C544-Gal4×UAS-GFP-lacZ.nls* embryo stained for (G) anti-En (blue), (H) *odd* RNA (red), (I) anti-β-gal (green), and (J) merge image showing that *odd* RNA co-localizes with β-gal-stained MP1 neurons. (K) Sagittal view of stage 15 *slit-gal4×UAS-tau-lacZ* embryo showing anterior (arrowhead) and posterior (arrow) glia within a single ganglion. (L–O) Sagittal view of a single ganglion from a *slit-Gal4×UAS-GFP-lacZ.nls* embryo stained for (L) anti-En (blue), (M) *CG7663* RNA (red), (N) anti-β-gal (green), and (O) merge image showing that *CG7663* RNA co-localizes with β-gal and En-stained posterior glia (arrow). Posterior glia (arrow) can be easily distinguished from the three anterior glia (arrowheads) based on their relative positions along the A–P axis.

Table 1

Genes expressed in midline cell types at each stage of development

Name	Function	Midline expression								ID
		I	A	P	MG	MN	DM	MM	CG	
18w	Transmembrane receptor	—	All	Sub	—	—	—	—	—	—
5-HT1A	Serotonin receptor	—	—	—	—	All	—	—	—	—
5-HT7	Serotonin receptor	—	—	—	—	Ven	—	—	—	—
α-Cat	Cytoskeletal protein binding	—	—	Sub	Unc	Unc	—	—	—	—
Acf1	Chromatin accessibility complex	—	—	Sub	—	—	—	—	—	—
aly	Spermatogenesis	—	—	—	—	Ven	—	—	—	—
apt	Myb domain transcription factor	—	—	All	All	—	—	—	—	ET
argos	EGF receptor antagonist	—	—	Sub	AMG	—	—	—	—	ET
ase	bHLH transcription factor	—	—	Sub	—	All	—	—	—	—
asp	Microtubule binding	—	—	Sub	—	—	—	—	—	MA
β-Spec	Cytoskeletal protein binding	—	—	—	—	Ven	—	—	—	—
bib	Connexon channel	—	—	Sub	—	—	—	—	—	—
bmb	Unknown	—	All	Sub	—	—	—	X	—	—
brk	Transcription factor	All	All	All	—	Unc	—	—	—	—
btl	Receptor tyrosine kinase	—	Sub	Sub	—	—	—	—	—	ET
CaBP1	Protein disulfide isomerase	—	—	Sub	—	Ven	—	—	—	—
cact	Transcription factor binding	—	—	Sub	—	All	—	—	—	ET
Cad74A	Cadherin domain	—	—	Sub	—	—	—	—	—	—
Caki	Guanylate kinase	—	—	All	—	All	—	—	—	—
cas	Zinc finger transcription factor	—	—	Sub	—	—	—	—	—	—
cdi	Receptor serine/threonine kinase	—	—	All	All	—	—	—	—	ET
cenB1A	ARF GTPase activator	Sub	Sub	All	—	—	—	—	—	—
CG1124	Unknown	—	—	—	AMG	—	—	X	—	—
CG2165	Calcium-transporting ATPase	—	—	Sub	—	—	—	—	—	—
CG2310	Unknown	—	—	—	—	—	—	—	X	—
CG3097	Carboxypeptidase A	—	—	Sub	—	—	—	—	—	—
CG3168	Carbohydrate transporter	—	—	—	—	—	—	—	X	—
CG3171	G-protein coupled receptor	—	—	—	Unc	—	—	—	—	ET

Name	Function	Midline expression								ID
		I	A	P	MG	MN	DM	MM	CG	
CG3409	Monocarboxylic acid transporter	–	–	All	–	All	–	–	–	–
CG3941	Zinc finger transcription factor	–	–	Sub	–	Med	–	–	–	–
CG4133	Zinc finger domain	–	–	Sub	–	All	–	–	–	–
CG4322	G-protein coupled receptor	–	–	–	–	–	X	–	X	–
CG4455	Unknown	–	–	All	–	All	–	–	–	ET
CG4532	Actin binding	–	–	Sub	–	All	–	–	–	–
CG4726	Inorganic phosphate transporter	–	–	–	–	–	X	–	–	–
CG5175	Unknown	–	–	–	–	Med	–	–	–	–
CG5358	Arginine *N*-methyltransferase	–	–	Sub	–	–	–	–	–	MA
CG5629	Unknown	–	–	Sub	–	Ven	–	–	–	–
CG5746	Unknown	–	–	–	–	All	–	–	–	–
CG5854	NAD(P)-binding domain	–	–	Sub	–	Med	–	–	–	–
CG6070	Amino acid transporter	–	–	–	–	–	–	X	–	–
CG6201	Hydrolase	–	–	–	–	All	–	–	–	–
CG6218	Carbohydrate kinase	–	–	–	–	–	–	X	–	MA
CG6225	Metallopeptidase	–	–	–	–	–	–	X	–	–
CG6783	Fatty acid binding	–	–	–	–	–	–	X	–	–
CG7224	Unknown	–	–	All	–	All	–	–	–	–
CG7271	Unknown	–	–	Sub	AMG	–	–	–	–	–
CG7515	Guanyl-nucleotide exchange factor	–	–	Sub	AMG	–	–	–	–	MA
CG7663	Unknown	–	–	Sub	PMG	–	–	–	–	–
CG8026	Mitochondrial carrier	–	–	Sub	–	–	–	–	–	ET
CG8291	Serotonin transporter	–	All	All	All	–	–	–	–	ET
CG8709	Unknown	–	Sub	Sub	–	All	–	–	–	–
CG8776	Carbon-monoxide oxygenase	–	–	Sub	AMG	–	–	–	–	ET, MA
CG8965	RA domain	–	–	Sub	AMG	–	–	–	–	–
CG9598	PDZ domain	All	All	All	–	–	–	–	–	–
CG9634	Metalloendopeptidase	Sub	Sub	All	Unc	Med	–	–	–	–
CG10960	Sugar transporter	–	–	–	–	–	–	X	–	–
CG11248	Microtubule binding	–	–	All	–	Unc	–	–	–	MA

Name	Function	Midline expression								ID
		I	A	P	MG	MN	DM	MM	CG	
CG11798	Zinc finger domain	–	–	–	–	All	–	–	–	–
CG11880	Transporter	–	–	–	–	–	X	–	–	MA
CG11902	Zinc finger transcription factor	–	–	–	–	–	–	X	–	–
CG12648	bHLH transcription factor	–	–	–	–	–	X	–	–	–
CG12910	C2 calcium/lipid-binding domain	–	–	–	Unc	–	–	–	–	–
CG13333	Unknown	–	–	All	Unc	Unc	–	–	–	–
CG13532	Immunoglobulin domain	–	–	–	–	Ven	–	–	–	–
CG14059	Unknown	–	–	–	–	Ven	–	–	–	–
CG14883	Unknown	–	–	–	–	All	–	–	–	–
CG15236	Unknown	–	–	–	PMG	Ven	–	–	–	–
CG16876	bHLH domain	–	–	–	–	–	–	X	–	–
CG30116	Apoptotic protease activator	–	–	–	–	Ven	–	–	–	–
CG30342	Unknown	–	–	–	–	All	–	–	–	MA
CG31116	Voltage gated chloride channel	–	–	Sub	All	–	–	–	–	–
CG31145	Nucleotide hydrolase domain	–	–	Sub	All	Ven	–	–	–	ET
CG31235	Oxidoreductase	–	–	Sub	–	–	–	–	–	–
CG31634	Organic anion transporter	–	–	–	All	–	–	–	–	–
CG31640	Receptor tyrosine kinase	–	–	–	–	Ven	–	–	–	–
CG31757	Phosphodiesterase	–	–	–	–	Ven	–	–	–	–
CG31764	Unknown	–	–	–	AMG	–	–	–	–	–
CG32030	Actin binding	–	–	Sub	All	–	–	–	–	ET
CG32046	Unknown	–	–	Sub	–	Unc	–	–	–	–
CG32244	Unknown	–	–	Sub	All	–	–	–	–	–
CG32423	RNA–binding	–	–	Sub	All	Unc	–	–	–	ET
CG32594	Unknown	–	–	All	–	–	–	–	–	–
ci	Zinc finger transcription factor	–	–	All	–	All	–	–	–	–
Cnx99A	Chaperone	–	Sub	–	–	–	–	–	–	–
comm	Protein binding	–	–	Sub	Unc	–	–	–	–	–
comt	ATPase	–	–	Sub	–	All	–	–	–	–
Con	Structural molecule	–	–	Sub	Unc	Ven	–	–	–	ET

Name	Function	Midline expression								ID
		I	A	P	MG	MN	DM	MM	CG	
corn	Microtubule binding	–	–	Sub	Unc	–	–	–	–	ET
cyc	bHLH PAS transcription factor	–	–	–	–	Med	–	–	–	–
Cyc E	Cyclin-dependent protein kinase	Sub	Sub	–	–	Med	–	–	–	–
D	HMG transcription factor	All	All	Sub	–	–	–	–	–	–
danr	Homeobox containing	–	Sub	All	–	All	–	–	–	–
Dg	Cytoskeletal anchoring	–	–	All	–	–	–	–	–	–
Dgk	Diacylglycerol kinase	–	–	–	Unc	Ven	–	–	–	–
Dip3	Unknown	–	–	Sub	–	All	–	–	–	MA
Dl	Notch binding receptor	–	–	Sub	–	–	–	–	–	–
dpld	Zinc finger transcription factor	–	–	–	–	–	–	X	–	–
dpn	bHLH transcription factor	Sub	–	Sub	–	–	–	–	–	–
dve	Homeobox transcription factor	–	–	All	AMG	Med	–	–	–	ET
E2F	E2F transcription factor	–	–	Sub	–	–	–	–	–	–
Eaat1	Amino acid transporter	–	–	–	–	–	–	X	–	–
Eaat2	Amino acid transporter	–	–	–	–	Ven	–	–	–	–
Ect3	Beta-galactosidase	Sub	Sub	All	All	–	–	–	–	–
edl	MAPK signaling component	–	All	Sub	–	–	–	–	–	–
Egfr	Receptor tyrosine kinase	Sub	–	–	All	–	–	–	–	–
emc	HLH transcriptional corepressor	–	–	–	–	Unc	–	–	–	–
en	Homeobox transcription factor	Sub	Sub	Sub	–	All	–	–	–	ET
Epac	Guanyl-nucleotide exchange factor	–	–	Sub	All	–	–	–	–	–
Ephrin	Ephrin receptor binding	–	–	–	–	Ven	–	–	–	–
esg	Zinc finger transcription factor	–	Sub	Sub	–	–	–	–	–	ET
Fas1	Fas domain	–	–	Sub	–	Unc	X	–	–	–
fd59A	Forkhead transcription factor	–	Sub	Sub	–	Ven	–	–	–	–
FK506-bp2	Peptidyl-prolyl cis–trans isomerase	–	–	Sub	–	–	–	–	–	ET
futsch	Microtubule binding	–	–	–	–	Ven	–	–	–	–
Gad1	Glutamate decarboxylase	–	–	–	–	Unc	–	–	–	–
Gdi	GDP–dissociation inhibitor	–	–	Sub	–	Unc	–	–	–	–
GH22170	Unknown	–	–	Sub	All	–	–	–	–	ET

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Name	Function	Midline expression								ID
		I	A	P	MG	MN	DM	MM	CG	
glec	Carbohydrate binding	–	All	Sub	All	–	–	–	–	ET
Gli	Serine esterase	–	–	–	–	–	–	–	X	–
Glu-Rl	Glutamate–gated ion channel	–	–	–	–	Ven	–	–	–	–
gsb-n	Homeobox transcription factor	Sub	–	Sub	–	–	–	–	–	–
gukh	Unknown	All	All	Sub	–	Ven	–	–	–	–
HGTX	Homeobox transcription factor	–	All	All	–	Med	–	–	–	–
HLHmβ	bHLH transcription factor	All	All	All	–	–	–	–	–	–
Hrb87F	RNA binding	–	–	–	–	Unc	–	–	–	–
jing	Zinc finger transcription factor	–	–	–	All	–	–	–	–	–
kek1	Receptor serine/threonine kinase	–	–	Sub	–	All	–	–	–	ET
klar	ATPase	All	–	Sub	–	Unc	–	–	–	ET
klg	Immunoglobulin domain	–	–	–	–	Ven	–	–	–	ET
Klp61F	Microtubule motor protein	–	–	Sub	–	–	–	–	–	–
knrl	Zinc finger transcription factor	–	–	Sub	–	–	–	–	–	–
Kr	Zinc finger transcription factor	–	–	Sub	–	All	–	–	–	–
Kr-h1	Zinc finger transcription factor	–	–	Sub	–	Unc	–	–	–	–
l(1)G0003	C2 calcium/lipid-binding domain	–	–	Sub	All	–	–	–	–	ET
l(1)sc	bHLH transcription factor	–	–	Sub	–	–	–	–	–	–
l(2)02045	Regulator of G-protein signaling	–	–	–	AMG	–	–	–	–	–
lbm	Receptor signaling protein	–	–	–	–	Unc	–	–	–	–
lcs	Unknown	–	–	Sub	–	–	–	–	–	–
lin-28	Zinc finger domain	–	–	Sub	–	–	–	–	–	–
mab-2	Unknown	–	–	–	–	Ven	–	–	–	–
mam	Unknown	–	–	–	–	Unc	–	–	–	–
mas	Serine-type endopeptidase	–	–	Sub	–	–	–	–	–	–
Mcm5	DNA helicase	–	–	Sub	–	–	–	–	–	–
Mdh	Malate dehydrogenase	–	–	Sub	–	All	–	–	–	ET
Mdr65	Multidrug transporter	–	–	–	Unc	–	–	–	–	–
Mes2	Unknown	Sub	Sub	Sub	–	–	X	–	–	MA
mfas	Fas domain	–	–	All	Unc	–	–	–	–	–

Name	Function	Midline expression								ID
		I	A	P	MG	MN	DM	MM	CG	
Mmp1	Metalloendopeptidase	–	–	Sub	–	–	X	–	–	–
nerfin–1	Zinc finger transcription factor	–	–	Sub	–	Med	–	–	–	–
NetA	Secreted protein	–	All	Sub	All	–	–	–	–	–
NetB	Secreted protein	–	–	Sub	All	–	–	–	–	–
neur	Ubiquitin-protein ligase	All	–	Sub	–	–	–	–	–	ET
Nmdar1	Ligand-gated ion channel	–	–	–	–	Unc	–	–	–	–
NPFR1	Neuropeptide receptor	–	–	–	–	Ven	–	–	–	–
NPFR76F	Neuropeptide receptor	–	–	–	–	Unc	–	–	–	–
Nrg	Immunoglobulin domain	–	–	–	–	All	–	–	–	–
nrv1	Na/K-exchanging ATPase	–	–	–	–	–	X	–	–	–
nrv2	Na/K-exchanging ATPase	–	–	–	–	–	X	–	–	–
nub	Homeobox transcription factor	–	–	Sub	–	Unc	–	–	–	–
nvy	TAF domain transcription factor	–	–	Sub	–	All	–	–	–	–
oaf	Unknown	–	–	–	–	Ven	–	–	–	–
odd	Zinc finger transcription factor	Sub	–	Sub	–	Med	–	–	–	–
Orct	Organic cation transporter	–	–	All	–	–	–	–	–	MA
pdm2	POU Homeobox transcription factor	–	Sub	–	–	Med	–	–	–	–
Ptslre	Receptor serine/threonine kinase	–	–	–	–	Unc	–	–	–	–
ple	Tyrosine 3-monooxygenase	–	–	–	–	Ven	–	–	–	–
prc	Matrix component	–	–	–	–	–	X	–	–	–
pros	Transcription factor	–	–	Sub	–	–	–	–	–	–
Ptp99A	Receptor tyrosine phosphatase	–	–	–	–	Unc	–	–	–	–
Pvf3	VEGF receptor binding	–	–	Sub	–	Unc	–	–	–	–
pxb	Unknown	Sub	Sub	Sub	–	Unc	–	–	–	–
ran	Small monomeric GTPase	–	–	Sub	–	–	–	–	–	–
ref(2)P	Transcription factor	–	–	–	–	Med	–	–	–	–
repo	Homeobox transcription factor	–	–	–	–	–	–	–	X	–
rhea	Actin binding	–	–	–	–	Ven	–	–	–	–
rho	Serine-type peptidase	All	All	All	PMG	Ven	–	–	–	ET
robo	Transmembrane receptor	–	–	Sub	–	–	–	–	–	–

Name	Function	Midline expression								ID
		I	A	P	MG	MN	DM	MM	CG	
rst	Immunoglobulin domain	–	–	All	–	–	–	–	–	–
S	EGF ligand processing	–	All	All	–	Ven	–	–	–	–
sc	bHLH transcription factor	All	–	Sub	–	–	–	–	–	–
sca	Receptor binding	All	–	Sub	–	–	–	–	–	–
scb	Laminin receptor	–	–	–	PMG	–	–	–	–	–
Sema-1b	Receptor	All	All	All	–	–	–	–	–	–
Sema-5c	Receptor	Sub	Sub	Sub	–	–	–	–	–	ET
sim	bHLH PAS transcription factor	All	All	All	All	–	–	–	–	–
slp2	Forkhead transcription factor	Sub	Sub	Sub	–	–	–	–	–	–
sna	Zinc finger transcription factor	–	–	All	All	–	–	–	–	–
SNF4Ag	Receptor serine/threonine kinase	–	–	Sub	–	Ven	–	–	–	–
sno	Unknown	All	–	–	–	–	–	–	–	–
sob	Zinc finger transcription factor	Sub	–	–	–	–	–	–	–	–
sog	Torso signaling	All	All	All	–	–	–	–	–	ET
Stat92E	SH2 transcription factor	Sub	Sub	All	–	–	–	–	–	ET
stg	Protein tyrosine phosphatase	–	All	–	Unc	Ven	–	–	–	–
Sulf1	N-acetylglucosamine-6-sulfatase	–	–	Sub	–	Med	–	–	–	–
SytIV	Phospholipid binding	–	–	–	–	All	–	–	–	–
tap	bHLH transcription factor	–	–	Sub	–	Med	–	–	–	–
TE 1731	Transposable element	–	–	–	–	Unc	–	–	–	–
TE 412	Transposable element	–	–	–	–	–	X	–	–	–
TE Beagle	Transposable element	–	–	–	AMG	Unc	–	–	–	ET
TE copia	Transposable element	–	–	–	–	Ven	–	–	–	–
TE Doc	Transposable element	–	–	All	–	Ven	–	–	–	ET
TE R1a	Transposable element	–	All	–	–	All	–	–	–	ET
th	Ubiquitin–protein ligase	Sub	–	Sub	–	All	–	–	–	ET
timeout	Myb DNA binding domain	–	–	–	–	Ven	–	–	–	–
tkr	BTB/POZ domain	–	–	Sub	–	All	–	–	–	ET
tkv	Type I TGF-β receptor	–	All	–	AMG	–	–	–	–	–
Tl	Cytokine binding	–	–	All	–	Ven	–	–	–	ET

Name	Function	Midline expression								ID
		I	A	P	MG	MN	DM	MM	CG	
tok	Procollagen C-endopeptidase	–	–	All	–	All	–	–	–	–
Tom	Unknown	All	–	Sub	–	–	–	–	–	–
Trafl	Receptor binding	Sub	–	Sub	–	Med	–	–	–	–
trbl	Receptor serine/threonine kinase	Sub	–	Sub	–	–	–	–	–	ET
trf2	General transcription factor	–	–	All	Unc	Unc	–	–	–	ET
tsl	Torso binding	–	Sub	Sub	All	–	–	–	–	–
Tsp42Ee	Unknown	–	–	–	–	Ven	–	–	–	–
twi	bHLH transcription factor	All	–	–	–	–	–	–	–	–
Ubx	Homeobox transcription factor	–	–	All	–	–	–	–	–	–
vvl	POU Homeobox transcription factor	–	–	All	–	–	–	–	–	–
wg	Frizzled-2 binding	Sub	Sub	Sub	–	–	–	–	–	–
Wnt8	Wnt family	All	All	All	–	–	–	–	–	–
wor	Zinc finger transcription factor	–	–	Sub	–	Med	–	–	–	–
wrapper	Immunoglobulin domain	–	–	Sub	All	–	–	–	–	–
zfh1	Zinc finger transcription factor	All	All	Sub	–	Ven	X	–	–	–
zfh2	Zinc finger transcription factor	–	–	Sub	–	All	–	–	–	–

I—mesectoderm ISN; A—mesectoderm anlage; P—midline primordium: All—all midline cells; Sub—subset of midline cells. MG—midline glia: AMG— anterior midline glia, PMG—posterior midline glia, All—all midline glia, Unc—uncertain midline glial localization. MN—midline neurons: Ven—ventral midline neurons, Med—medial midline neurons, All—all midline neurons, Unc—uncertain midline neuronal localization. DM—dorsal median cell, MM— MM-CBG; CG—channel glia: X—expression. ID, identification: ET—enhancer trap, MA—microarray. Functional assignments provided by Flybase.