Dual role for *Drosophila lethal of scute* in CNS midline precursor formation and dopaminergic neuron and motoneuron cell fate

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**SUMMARY**

Dopaminergic neurons play important behavioral roles in locomotion, reward and aggression. The *Drosophila* H-cell is a dopaminergic neuron that resides at the midline of the ventral nerve cord. Both the H-cell and the glutamatergic H-cell sib are the asymmetric progeny of the MP3 midline precursor cell. H-cell sib cell fate is dependent on Notch signaling, whereas H-cell fate is Notch independent. Genetic analysis of genes that could potentially regulate H-cell fate revealed that the *lethal of scute* ([l(1)sc], *tailup* and *SoxNeuro*) transcription factor genes act together to control H-cell gene expression. The *l(1)sc* bHLH gene is required for all H-cell-specific gene transcription, whereas *tailup* acts in parallel to *l(1)sc* and controls genes involved in dopamine metabolism. *SoxNeuro* functions downstream of *l(1)sc* and controls expression of a peptide neurotransmitter receptor gene. The role of *l(1)sc* may be more widespread, as a *l(1)sc* mutant shows reductions in gene expression in non-midline dopaminergic neurons. In addition, *l(1)sc* mutant embryos possess defects in the expression of MP4-6 midline precursor and the median neuroblast stem cell, revealing a proneural role for *l(1)sc* in midline cells. The Notch-dependent progeny of MP4-6 are the mVUM motoneurons, and these cells also require *l(1)sc* for mVUM-specific gene expression. Thus, *l(1)sc* plays an important regulatory role in both neurogenesis and specifying dopaminergic neuron and motoneuron identities.

**KEY WORDS:** bHLH, Dopamine, *Drosophila*, *Lethal of scute*, Neural cell fate, *Tailup*

**INTRODUCTION**

Complex behaviors require the coordinated action of diverse ensembles of neurons. Each neuron contains a distinct combination of neural function genes, which include genes encoding neurotransmitter biosynthetic enzymes, neuropeptides, vesicular transporters, membrane transporters, neurotransmitter receptors and axon guidance proteins. One of the key goals in developmental neuroscience is to understand how neural gene expression is regulated, and how this defines each neuron and its precursors during development. Not only will this help explain how neuronal diversity is generated, but provide insights into the origins of human nervous system disease and the development of effective therapies.

Efforts to reach these goals have been systematically applied towards studying the development of dopaminergic neurons because of their prominent neurobiological roles in reward, emotion and locomotory pathways, and their importance in neurodegenerative diseases. Parkinson’s disease is characterized by the loss of midbrain dopaminergic neurons, and a major goal of neural stem cell research is to generate dopaminergic neurons in vitro for cell replacement therapies to counteract Parkinson’s disease. Studies in vertebrates have identified multiple regulatory proteins that are required for controlling gene expression and development of dopaminergic neurons (Smidt and Burbach, 2007). Recent work further showed that an Ets-family transcription factor is evolutionarily conserved between *C. elegans* (AST-1) and mammals (ETV1), and controls dopaminergic neuron gene expression in both organisms (Flames and Hobert, 2009).

In insects, dopaminergic neurons are found in both the nerve cord and brain (Monastirioti, 1999). One of the best-characterized insect dopaminergic neurons is the H-cell (named for its ‘H’-like axonal projections), which is present in the CNS midline cells of the nerve cord. The H-cell was first described in grasshopper as one of the two progeny of the Midline Precursor 3 (MP3) cell (Goodman et al., 1981), and shown in the moth *Manduca sexta* to be dopaminergic (Mesce et al., 2001). The H-cell midline interneuron is also present in *Drosophila* (Bosson and Technau, 1994; Budnik and White, 1988; Schmid et al., 1999; Wheeler et al., 2006), and similar to other dopaminergic neurons expresses a set of genes encoding dopamine biosynthetic enzymes, including *pale* (*ple*; which encodes tyrosine hydroxylase) and *dopa decarboxylase* (*Ddc*). The H-cell also expresses a vesicular monoamine transporter (*Vmatt*), dopamine membrane transporter (*DAT*) and neurotransmitter receptors that receive input for serotonin (*5-HT1A*), glutamate (*Glu-R1*) and neuropeptide F (*NPFFR1*) (Wheeler et al., 2006). This characteristic pattern of gene expression and its ‘H’ axonal projection, to a large degree, constitute the unique character of the H-cell.

Recent work has provided insight into the origins of midline neurons and glia (Fig. 1). Around the time of gastrulation, the *single-minded* midline master regulatory gene activates the midline developmental program (Crews, 1998), and soon after 3 MP equivalence groups (MP1, MP3, MP4) of five or six cells/each form (Wheeler et al., 2008). Notch signaling selects one cell from the MP1 group to become an MP1 and the others become midline glia (MG). The same occurs for the MP3 group, with one cell becoming...
an MP3 and the others MG. Development of the MP4 group is more complex, with sequential Notch-dependent formation of MP4 followed by MP5, MP6 and the median neuroblast (MNB). Each MP undergoes a single division that leads to two neurons. For MP3-6, this involves binary cell fate decisions: MP3 gives rise to the dopaminergic H-cell and glutamatergic H-cell sib interneurons, and MP4-6 each gives rise to a GABAergic iVUM interneuron and glutamatergic/octopaminergic mVUM motoneuron pair. The differences in MP3-6 neuron cell fate are due to the asymmetric localization of the Numb protein, which is high in H-cell and mVUMs, but low in H-cell sib and iVUMs, and differential Sanpodo localization (Wheeler et al., 2008). Although Notch signaling directs H-cell sib and iVUMs to their fates, it is blocked in H-cell and mVUMs due to the presence of Numb (Lundell et al., 2003; Wheeler et al., 2008). Thus, H-cell sib and iVUM cell fate and gene expression are dependent on Notch signaling, and a different regulatory program governs H-cell and mVUM fates.

In this paper, we ask the question: what regulatory proteins govern Notch-independent H-cell and mVUM fate and gene expression? We also address how the two types of midline precursors, MPs and MNB, form, proneural genes of the bHLH protein (Alonso and Cabrera, 1988), Tailup (Tup; Islet) Lim-homeodomain protein (Thor and Thomas, 1997) and the Sox family protein SoxNeuro (SoxN) (Bauescher et al., 2002; Cremazy et al., 2000; Overton et al., 2002), work together to control overlapping aspects of H-cell gene expression. In addition, l(1)sc regulates mVUM motoneuron gene expression. All three proneural members of the Drosophila achaete-scute complex (AS-C) (l(1)sc and sc) are expressed in MPs in distinct patterns, and l(1)sc is required for the formation of MP4-6 and the MNB. Thus, l(1)sc controls both midline precursor formation and, in combination with SoxN and tup, controls H-cell-specific gene expression and cell fate. Both the l(1)sc and tup genes may also function together more broadly and control non-midline dopaminergic neuron gene expression.

MATERIALS AND METHODS
Drosophila strains
Drosophila strains used included: scM6 (Gomez-Skarmeta et al., 1995), Df(l)ase-1 (Gonzalez et al., 1989), Df(l)sc10-1 (Villares and Cabrera, 1987), Df(l)sc-B57 (Jimenez and Campos-Ortega, 1990), SoxN(scM6)2 (Buescher et al., 2002), tup31 (Thor and Thomas, 1997) and tup1 (Thor and Thomas, 1997). Ga4 and UAS lines employed were: sim-Gal4 (Xiao et al., 1996), UAS-tau-GFP (Wheeler et al., 2006), UAS-l(1)sc (Parras et al., 1996), UAS-sc (Parras et al., 1996) and UAS-elav (Koushika et al., 1996).

L(1)sc and Sc antisera
Polyclonal antibodies against full-length L(1)sc and Sc proteins were generated by injecting both guinea pigs and rats (Pocono Rabbit Farm) with N-terminal 6His-L(1)sc and 6His-Sc fusion proteins generated in E. coli. The specificity of both antibodies was confirmed by the following observations: (1) the lateral CNS staining was identical to published accounts for L(1)sc and Sc (Martin-Bermudo et al., 1991; Skeath et al., 1992); and (2) immunoreactivity in neural precursors was absent in the corresponding mutant strains [Df(l)sc-B57 for L(1)sc and Sc, and Df(l)sc10-1 for Sc].

In situ hybridization, immunostaining, microscopy, and statistical analysis
Embryo collection, in situ hybridization, immunostaining and confocal imaging were performed as previously described (Kearney et al., 2004; Wheeler et al., 2006; Wheeler et al., 2008). Digoxigenin-labeled or biotin-labeled antisense RNA probes for 5-HT1A, BarH1, CG13565, CG15236, CG16778, DAT, dgk, Glu-R1, NPER1, nubbins, ple, pmd2, Tbh, wor, wrapper and VGlut for in situ hybridization were generated from cDNA clones from the Drosophila Gene Collection (Open Biosystems). The clone used to make the tup probe was derived from PCR of Drosophila genomic DNA, and the Vmat probe was derived by PCR of Drosophila adult head cDNA. Primary antibodies used were: rabbit anti-Cas (Kambadur et al., 1998), guinea pig anti-Eve (Asian Distribution Center for Segmentation Antibodies), mouse and rat anti-Elav (Developmental Studies Hybridoma Bank), rabbit anti-GFP (Abcam), guinea pig anti-Lim3 (Brohier and Skeath, 2002), rabbit anti-SoxN (John Nambu), mouse anti-Tau (Sigma), rat anti-Tup (Brohier and Skeath, 2002), and guinea pig anti-Zfh1 (Fortini et al., 1991). Alexa Fluor-conjugated secondary antibodies were used (Molecular Probes), and the Tyramide Signal Amplification System (Perkin Elmer) was employed for some experiments. Abdominal segments 1-8 were analyzed for each experiment. Statistical analysis was performed using Fisher’s Exact Test. Percentages and P values for each experiment are listed in Tables 1, 2 and Tables S1, S2 in the supplementary material.

RESULTS
Expression of H-cell neural function and transcription factor genes
MP3 divides at early stage 11 (~5 hours post-fertilization) to give rise to H-cell and H-cell sib. Expression of H-cell neural function genes that are involved in dopamine biosynthesis,
Fig. 2. H-cell gene expression. Sagittal views of single segments of sim-Gal4 UAS-tau-GFP embryos at stage 14. Embryos were stained for GFP or Tau (blue, midline cell cytoplasm is GFP+ and cell nuclei are GFP–) and additional antibody and in situ hybridization probes. Italicized gene names represent RNA and unitalicized capitalized names represent protein. (A–G) In all panels, ple expression (green) indicates the H-cell. (A–D) The neural function genes (A) DAT, (B) NPFR1, (C) Vmat and (D) S-HT1A (all magenta) colocalize with ple in the H-cell. (C) The Vmat gene is expressed in the H-cell (arrowhead) and in three mVUMs (arrows). (D) The S-HT1A gene is expressed in the H-cell (white arrowhead), H-cell sib (yellow arrowhead) and two MP1 neurons (arrows). (E–G) The transcription factor genes (E) SoxN, (F) BarH1 and (G) Tup (all magenta) colocalize with ple in the H-cell.

Dopaminergic neuron development begins at late stage 13 (~10 hours post-fertilization). Fig. 2A–D shows the expression of the ple, DAT, NPFR1, Vmat and S-HT1A neural function genes in the H-cell. These genes were previously mapped to the H-cell by in situ hybridization (Wheeler et al., 2006). Previous expression analysis identified 36 transcription factor genes known to be expressed in midline cells (Wheeler et al., 2006; Wheeler et al., 2008), and identified SoxN as being expressed in the H-cell (Wheeler et al., 2006) (Fig. 2E). In addition, both the BarH1 (Reig et al., 2007) and tup (Thor and Thomas, 1997) transcription factor genes had previously been shown to be expressed in the H-cell (Fig. 2F,G; Fig. 3A–D). In this paper, we further examine the expression of each gene during H-cell development, and also demonstrate that two members of the AS-C gene family, l(1)sc and sc, are expressed in the H-cell (Fig. 3E–J; see Fig. S1A–E in the supplementary material). The genes and proteins analyzed were assigned to specific midline cell types by the use of co-staining with midline cell type-specific markers in sim-Gal4 UAS-tau-GFP embryos [data not shown; see Wheeler et al. (Wheeler et al., 2008) for details]. The BarH1, SoxN, and tup genes are each expressed at stage 11 and remain on through the end of embryonic development. BarH1 is not expressed in MP3, but is present in the H-cell beginning at late stage 11 (Fig. 2F, Fig. 3I), and is absent in the H-cell sib. SoxN is expressed in both the H-cell and H-cell sib until the end of stage 13, and then becomes localized to only the H-cell (Fig. 2E). tup is absent in MP3, initially present in both the H-cell and H-cell sib, but by late stage 11 is preferentially localized to only the H-cell (Fig. 2G and Fig. 3A–D). Expression is summarized in Fig. 4. Each of these genes is expressed before the appearance of H-cell neural function gene expression, and could potentially regulate their expression either directly or indirectly.

**AS-C genes are expressed in midline precursors and their neuronal progeny**

The AS-C consists of three proneural basic-helix-loop-helix (bHLH) genes, ac, l(1)sc and sc (Campuzano and Modolell, 1992), and the asense (ase) bHLH neural precursor gene (Brand et al., 1993; Dominguez and Campuzano, 1993; Gonzalez et al., 1989). The proneural bHLH genes play important roles in CNS and sensory cell development (Bertrand et al., 2002). Previous work has shown that l(1)sc was expressed in midline cells (Bossing and Brand, 2006; Kearney et al., 2004) and we earlier described the expression of ase in MPs and MNB (Wheeler et al., 2006). However, detailed expression analysis of the ac, l(1)sc and sc genes had not been carried out. Consequently, we examined their expression by immunostaining with antibodies we generated against the L(1)sc and Sc proteins, as well as an existing Ac monoclonal antibody.

**L(1)sc**

L(1)sc is present in all midline neuronal precursors, including MP1, MP3, MP4, MP5 and MP6 at stages 10-11 and MNB at stages 10-12 (Fig. 3E-H,J). L(1)sc remains present in the newly divided neurons of MP3 (the H-cell, H-cell sib), MP5 (mVUM5, iVUM5) (Fig. 3F,G) and MP6 (mVUM6, iVUM6) (not shown). Although both the H-cell and H-cell sib initially possess high levels of L(1)sc protein (Fig. 3F), the amount is greatly reduced in the H-cell sib as stage 11 progresses, whereas levels remain high in the H-cell (Fig. 3G-I). Similar L(1)sc dynamics are observed for the VUMs; L(1)sc is initially present in both mVUM5 and iVUM5 (Fig. 3F), but levels later become higher in mVUM5 with respect to iVUM5 (Fig. 3G) (the same dynamics apply to mVUM6 and iVUM6; not shown). By contrast, L(1)sc is present in MP1 and MP4 (Fig. 3E,F), but is undetectable in their progeny (Fig. 3F-H). By the end of stage 11, L(1)sc is no longer detectable in midline neurons (Fig. 3J), including H-cell and mVUMs. L(1)sc is present in PMG from stages 10-12 (Fig. 3E-H,J) and occasionally appears weakly in 2 AMG (data not shown).

**Sc**

Sc is present in the MP1, MP3, MP4, MP5, MP6 and MNB precursors (see Fig. S1A-D in the supplementary material). Unlike L(1)sc, the Sc protein is absent in the newly divided MP and MNB neurons. Sc appears in the H-cell beginning at stage 14 (see Fig. S1E in the supplementary material) and remains on throughout embryonic development. Sc is not present in any other midline neurons. Sc is present in PMG from stages 10-11, but absent in AMG (see Fig. S1A-C in the supplementary material).
Fig. 3. The *tup* and *l(1)sc* genes are expressed in midline precursors and their neuronal progeny. Sagittal views of single segments of *sim-Gal4 UAS-tau-GFP* embryos stained with (A-D) anti-*Tup* (magenta), (E-J) anti-*L(1)sc* (magenta), (A-H) *pdm2*/*nubbin* (blue), (I) *BarH1* (blue) and (J) *wor* (blue). Embryonic stages are indicated on the left: 10, early 11 (E11), mid-11 (M11), late 11 (L11) and 12. Embryos are progressively older moving from top to bottom. Each segment shows the magenta and blue channels separately, along with a merge image. Arrowheads indicate MPs and arrows indicate neurons. In all panels the following designations apply: MP1 (green arrowhead), MP3 (yellow arrowhead), MP4 (white arrowhead), MP5 (blue arrowhead), MP6 (red arrowhead), MNB (purple arrowhead), H-cell (H), H-cell sib (Hs), mVUM4 (m4), iVUM4 (i4), mVUM5 (m5) and iVUM5 (i5). (A-I) Combined *pdm2* and *nubbin* expression (*pdm*; blue) marks MP1, MP3, MP4 and their neuronal progeny, but is absent in MP5 and MP6 and their progeny, in MNB and in MG. (A-A*) At early stage 11 immediately after MP3 division, *Tup* is absent from the H-cell (white arrows) and H-cell sib (yellow arrows). (B-B*) Later during stage 11, *Tup* is present in both the H-cell and H-cell sib. (C-C*) By the end of stage 11, *Tup* is present in the H-cell, but is absent from the H-cell sib. (D-D*) Higher magnification view of C. (E-E*) At stage 10, *L(1)sc* is present in *pdm*+ MP3 (yellow arrowheads), *pdm*+ MP4 (white arrowheads), *pdm*+ MP5 (blue arrowheads), and MP6, MNB and PMG (*; these cell types cannot be distinguished at this time). *L(1)sc* is absent in MP1 (green arrowheads). (F-F*) At early stage 11, *L(1)sc* is present at low levels in MP1 (green arrowheads) and at high levels in the newly divided MP3 neurons (H-cell and H-cell sib; yellow arrows). It is absent in the MP4 neurons (white arrows) and present in both MP5 neurons (mVUM5 and iVUM5; blue arrows) and MP6 (red arrowheads). Levels are high in the PMG and MNB (*). (G-G*) *L(1)sc* levels are higher in the H-cell (yellow arrows) than in the H-cell sib (yellow arrows), absent in VUM4s (white arrows) and present in mVUM5s (*; blue arrows), but not iVUM5s (blue arrows). (H-H*) *L(1)sc* is present in the H-cell (white asterisks), but levels are greatly reduced in the H-cell sib, absent in VUMs, and present in MNB (purple arrowhead) and PMG (blue asterisks). (I-I*) High-magnification view of a late stage 11 embryo stained for *L(1)sc* and *BarH1*. *BarH1* expression is restricted to the H-cell, and this image demonstrates the higher levels of *L(1)sc* in the *BarH1*+ H-cell (yellow arrows) compared with the H-cell sib. (J-J*) Expression of *L(1)sc* overlaps with *wor*, which is expressed in the MNB (white asterisks, purple arrowheads) and is also present in PMG (blue asterisks).
**Ac**

Ac is present in MP1 and transiently in MP5, MP6 and MNB at stages 10-11 (see Fig. S1F-H in the supplementary material). Ac prominently remains on in the MP1 neurons after division throughout embryogenesis (see Fig. S1H-J in the supplementary material). By contrast, Ac is absent in all other midline neurons and MG.

In summary, the three AS-C proneural genes are all expressed in the midline cells in a dynamic manner (see Fig. 4 for a schematic summary). Although they partially overlap in expression, each gene has a unique pattern of midline expression. In the case of ac and sc, this contrasts with their expression in embryonic lateral neuroblast and sensory organ precursors, where their expression closely overlaps, owing to use of a shared cis-regulatory region (Skeath and Carroll, 1991; Skeath et al., 1992). Regarding H-cell development, l(1)sc and sc, but not ac, are expressed in MP3 and could be involved in MP3 formation. Most significantly, l(1)sc is expressed early in H-cell and H-cell sib development, and could influence cell fate in either cell type, although it remains on longer in H-cell. The sc gene is also expressed specifically in H-cell, but relatively late in development. Similarly, expression of l(1)sc, ac and sc in MP1, MP4, MP5, MP6 and MNB could influence MP formation and division, and l(1)sc could control mVUM cell-type-specific expression, as it is preferentially expressed in mVUMs compared with iVUMs.

**l(1)sc is required for H-cell gene expression**

Based on the expression of l(1)sc and sc (but not ac) in the H-cell and their potential roles in H-cell development, we analyzed mutants of the AS-C to assess their role in H-cell development and gene expression. The mutants analyzed included: (1) Df(1)sc-B57 [mutant for ac, l(1)sc, sc and ase], (2) Df(1)sc19 [mutant for ac, l(1)sc and sc], (3) sc<sup>66</sup> (mutant for sc), (4) Df(1)sc10-I (mutant for ac and sc) and (5) Df(1)ase-I (mutant for ase). In Df(1)sc-B57 mutant embryos, there was an absence of expression (ranging from 43-76% of segments analyzed) for the H-cell neural function genes DAT, NPFR1, ple and Vmat (Fig. 5A-D,H-K; Table 1). In wild-type embryos, Vmat was expressed in the pdm<sup>+</sup> H-cell and the three pdm<sup>+</sup> mVUMs (Fig. 5D). In Df(1)sc-B57 embryos (Fig. 5K), Vmat was absent in the H-cell in 43% of segments, absent from all mVUMs in 39% of segments, present in only one mVUM in 43% of segments and present in two mVUMs in 17% of segments (Tables 1, 2). This result indicated that AS-C genes are required for H-cell and mVUM gene expression. BarH1, SoxN and tup transcription factor gene expression was assayed in Df(1)sc-B57 stage 14-16 mutant embryos, when expression of these genes is restricted to only the H-cell in wild type (Fig. 5E-G,L-O; Table 1). BarH1 expression was absent in 63% of segments, and SoxN was absent in 47% of segments. By contrast, Tup protein was present in Df(1)sc-B57 embryos in one cell in 71% of segments, two cells in 27% of segments and was absent in 2% of segments. Both Tup cells are also pdm<sup>+</sup>, so the identity of the two Tup<sup>+</sup> cells are likely to be the H-cell and H-cell sib, but this is unproven as all H-cell markers were reduced or absent in Df(1)sc-B57, and we did not detect colocalization between Tup and the CG13565 H-cell sib marker when analyzed together (0/7 segments). In wild-type embryos, CG13565 is present in H-cell sib in 54% of segments (Table 1). In Df(1)sc-B57 mutant embryos, CG13565 was present in 46% of segments, only slightly lower than wild type (Table 1). Thus, the H-cell sib was not transformed into an H-cell in most embryos. However, it remains possible that H-cell sib neurons lose CG13565 expression when coupled with ectopic tup in Df(1)sc-B57 mutant embryos.

The 5-HT1A, pdm2 and nub genes are expressed in both H-cell and H-cell sib (Wheeler et al., 2006), and their expression was unaffected in Df(1)sc-B57 mutants (Fig. 5H,M,P,R). Lim3 is expressed in MP1 neurons, and its expression was also unaffected in Df(1)sc-B57 mutant embryos (Fig. 5Q,S). These data suggest that genes with dual expression in both H-cell and H-cell sib and genes expressed in MP1 neurons are not regulated by the AS-C.

The Df(1)sc-B57 mutant strain provided strong evidence for defects in H-cell gene expression, but is mutant for four regulatory genes [ac, ase, l(1)sc, sc]. Similar defects in DAT and ple expression were observed in another mutant strain, Df(1)sc19 [mutant for ac, l(1)sc and sc] (data not shown). The analysis of additional mutations in the AS-C locus suggested that the l(1)sc gene is the principal AS-C gene required for H-cell gene expression, as H-cell gene expression was largely unaltered in Df(1)sc10-I (ac<sup>+</sup> sc<sup>+</sup>, sc<sup>66</sup> (sc<sup>+</sup>) and Df(1)ase-I (ase<sup>+</sup>) (see Fig. S2A,B,F,E and Table S1 in the supplementary material). In sc<sup>66</sup> (sc<sup>+</sup>), BarH1, DAT, NPFR1, ple, SoxN and tup expression was unaffected (see Fig. S2I,K,M-P and Table S1 in the supplementary material). This indicates that sc may play a minor role in regulating H-cell gene
expression, but, more generally, demonstrates that ac, sc and ase do not significantly contribute to the Df(1)sc-B57 defects by themselves. Without a l(1)sc single-gene mutant available, we attempted transgenic l(1)sc RNAi experiments, but these failed to significantly reduce l(1)sc levels or H-cell gene expression. However, rescue experiments employing sim-Gal4 UAS-l(1)sc successfully rescued the Df(1)sc-B57 phenotype. In these experiments, ple^H-cells were present in 92% of rescue segments, compared with only 24% ple^-segments that were present in Df(1)sc-B57 (see Table S2 in the supplementary material). These data indicate that l(1)sc is a key regulator of H-cell gene expression. This is consistent with the prominent H-cell-specific localization of the L(1)sc protein.

**Misexpression of l(1)sc activates H-cell gene expression**

To gain further insight into l(1)sc and sc function, we individually misexpressed both genes in all midline cells in sim-Gal4 UAS-l(1)sc and sim-Gal4 UAS-sc embryos, and assayed stage 14-16 embryos for alterations in H-cell gene expression. In sim-Gal4 UAS-l(1)sc embryos, L(1)sc protein was present in all midline cells through mid-stage 11, but was absent in midline neurons by late stage 11, while persisting in MG past stage 16. When l(1)sc was misexpressed, ple expression was observed in an extra cell in addition to the H-cell in 18% of segments analyzed (Fig. 6A,B; Table 1). The additional ple^cell was an H-cell sib based on co-expression of ple with CG13565, an H-cell sib-specific marker (Fig. 6B). As CG13565 was still expressed in the ple^-cell, this indicated that H-cell sib was not completely transformed into an H-cell in all segments, but rather that l(1)sc was able to activate H-cell-specific gene expression in H-cell sib. Ectopic ple expression was not observed in any other midline cell type besides H-cell sib, suggesting that only H-cell sib has the requisite transcriptional co-activators, chromatin structure or other factors required for L(1)sc to activate H-cell-specific transcription. l(1)sc misexpression also induced the expression of additional H-cell specific genes, including BarH1, DAT, NPFR1, SoxN, tup and Vmat in an extra cell in 14-24% segments analyzed (Fig. 6C-H; Table 1). In all cases, the two cells were an H-cell and H-cell sib. The Vmat gene, which is expressed in both the H-cell and mVUMs in wild-type embryos, showed an increase in l(1)sc misexpression embryos.
from four cells in wild type to five to eight cells (Fig. 6H). One of the additional cells was an H-cell sib, based on co-expression with ple; the others were presumably iVUMs that ectopically expressed Vmat. This demonstrated that l(1)sc can ectopically activate both H-cell and mVUM gene expression. By contrast, misexpression of sc did not result in expanded ple or tup expression (Fig. 6I), even though Sc protein was present in all midline cells until mid-stage 11, similar to l(1)sc protein dynamics in l(1)sc misexpression experiments. These results indicated that l(1)sc has the ability to activate most, if not all, genes specifically expressed in H-cell, whereas sc, despite its close sequence homology to l(1)sc, was not able to induce ectopic H-cell specific gene expression. Use of elav-Gal4 to express l(1)sc in post-mitotic neurons failed to elicit ectopic H-cell gene expression, suggesting that the presence of high levels of l(1)sc in MP3 or its newly-born progeny is required to ectopically activate H-cell-specific gene expression.

**SoxN and tup regulate aspects of H-cell gene expression**

Two other transcription factors that regulate H-cell gene expression are SoxN and Tup. The tup gene, like l(1)sc, is expressed in H-cell and H-cell sib, and then localizes to only the H-cell (Fig. 3A-D). Previous work has demonstrated that tup was required for ple and ddc expression (Thor and Thomas, 1997). Genetic analyses of tup+ and tupp+ mutant embryos with additional neural function genes indicated that tup function is required for ple and DAT expression.

**Table 1. Summary of H-cell, H-cell sib and dorsal lateral dopaminergic neuron genetic data**

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Cell type refers to the wild-type cell in which each gene is expressed. DL DA neuron refers to dorsal lateral dopaminergic neuron. Values represent the number of segments in which no, one or two cells expressed the gene. Percentages are indicated in parentheses. In Df(1)sc-857, there was a single appearance of two Vmat+ pdm+ cells, indicating that the two cells were probably the H-cell and H-cell sib, and not VUM neurons. P<0.0001 for all Df(1)sc-857 and rescue experiments, except tup (P=0.4943) and CG13565 (P=0.7622). P<0.0163 for the sim-Gal4 UAS-l(1)sc experiments, except for Vmat (P=0.0556).
Fig. 7. SoxN and tup control different aspects of H-cell gene expression. Confocal images of stages 14-16 (A-D) wild-type, (E-G) tup
embryos, and (H) SoxN(5417S) embryos. (A-D) Wild-type embryos show H-cell expression of ple, NPFR1, DAT, Vmat and tup. (E-G) tup
mutant embryos showed an absence of (E) ple and (F) DAT expression, but (E-G) NPFR1 and (G) Vmat expression was unaffected. In tup
mutants, NPFR1 expression indicates the presence of the H-cell. (H) SoxN mutant embryos lacked NPFR1 expression, but tup expression was unaffected.

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in 82% of segments, compared with its presence in three neurons in wild type (Fig. 8K,O,S). \(CG16778\) was absent in 24% of segments and present in only one cell in 12% of segments (Fig. 8K,O,S), instead of two cells observed in wild type. Expression of \(cas\) was absent in 50% of segments, and reduced to only two cells in another 50% of segments (instead of four cells in wild type) (Fig. 8M,Q,U). These observations demonstrated that \(l(1)sc\) is required for the generation of MP6 and VUM6 neurons, and either MP4/VUM4s or MP5/VUM5s or both [we cannot distinguish these cell types in \(Df(1)sc-B57\)].

Analysis of mVUM and iVUM gene expression in \(Df(1)sc-B57\) mutant embryos indicated that the two surviving VUMs (on average) were likely to be an iVUM/mVUM pair in which mVUM-specific gene expression was reduced. Not only are the
levels of the mVUM marker Tbh greatly reduced in Df(1)sc-B57 (Fig. 8O,S), but the expression of Vmat, zfh1 and dgk, which is present in mVUMs but not in iVUMs (Wheeler et al., 2006; Wheeler et al., 2008), was reduced in Df(1)sc-B57 (Fig. 5D,K; Fig. 8PT; see Fig. S4A,C in the supplementary material). By contrast, iVUM-expressed genes, including en and CG15236 (Wheeler et al., 2006), were not affected (Fig. 8N,R,V; see Fig. S4B,D in the supplementary material). Expression of Tbh was unaffected in Df(1)sc10-1 or Df(1)ase-1 mutant embryos, suggesting that the defects in mVUM gene expression seen in Df(1)sc-B57 mutant embryos were dependent on l(1)sc function (see Fig. S2C,G; Table S1 in the supplementary material).

The l(1)sc gene plays an important proneural role in the formation of neuroblasts in the Drosophila embryonic CNS (Jimenez and Campos-Ortega, 1990; Younossi-Hartenstein et al., 1997). As it is also prominently expressed in the MNB, we assayed Df(1)sc-B57 embryos for defects in MNB formation by assaying wor expression, a gene required for NB formation (Ashraf et al., 1999; Ashraf and Ip, 2001; Cai et al., 2001). Analysis of Df(1)sc-B57 mutant embryos indicated that wor expression was absent from the MNB in 93% of segments (Fig. 8E,J; Table 2), consistent with the observation by Jiménez and Campos-Ortega (Jiménez and Campos-Ortega, 1999; Ashraf and Ip, 2001). Analysis of Df(1)sc-B57 embryo expression in the midline confirmed this observation (Fig. S5F,G). NB4-2 gives rise to GMC4-2a and its neuronal progeny, which are present in Df(1)sc-B57 mutant embryos, indicating that the l(1)sc gene function is dependent on L(1)sc localization in the MNB.

The results suggest that AS-C function, potentially l(1)sc, is required for gene expression in the dorsal lateral dopaminergic neurons, although whether it mechanistically functions similar to its role in the H-cell will depend on more detailed studies of the dorsal lateral dopaminergic neuronal cell lineage, L(1)sc localization and genetic analyses.

### DISCUSSION

**l(1)sc selectively controls midline precursor formation**

The formation of midline neural precursors (five MPs and the MNB) is a dynamic, yet stereotyped process. The MPs undergo cellular changes in which their nuclei delaminate from an apical position within the ectoderm and move to the basal (internal) surface. There they divide after orienting their spindles. The precursors arise in a distinct order: MP4 → MP3 → MP5 → MP4 → MP6 → MNB (Wheeler et al., 2008). We demonstrated that the l(1)sc gene is required for the formation of the MP4-6 and MNB precursors and their neuronal progeny (Fig. 10A). As we cannot definitively distinguish MP4 from MP5 in Df(1)sc-B57, there is some uncertainty whether both cell types are regulated by l(1)sc. However, as most segments only possess two VUMs, and those are VUM6s in over 60% of segments, it is likely that both MP4 and MP5 are commonly affected in Df(1)sc-B57, in addition to MP6. The ac and sc genes are both expressed in MPs and MNB, yet do not appear to play a significant role in MP and MNB formation. Although l(1)sc is the major proneural gene that controls formation of embryonic neuroblasts (Jimenez and Campos-Ortega, 1990; Younossi-Hartenstein et al., 1997), relatively little is known about how it functions and the identity of the genes it regulates.
of relevant target genes. In one study, it was shown that morphological changes that accompany neuroblast formation were dependent on $l(1)sc$ function (Stollewerk, 2000). This is likely to be the case for $l(1)sc$ and MP4-6 and MNB development, as MP4-6 and MNB delamination or division was commonly absent in $Df(1)sc-B57$. One key question is what activates or maintains $l(1)sc$ expression in MP3-6 and MNB? Signaling by hedgehog ($hh$) is likely to be important, as no midline $l(1)sc$ expression is present in $hh$ mutant embryos (Bossing and Brand, 2006).

Although all MPs and MNB express $l(1)sc$, only MP4-6 and MNB were affected in mutants – formation of MP1 and MP3 were unaffected. These differences are unlikely to be solely due to different levels of L(1)sc protein or to a combination of Ac, L(1)sc

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**Fig. 9. Reduction of DAT and ple expression in $Df(1)sc-B57$ dorsal lateral dopaminergic neurons.** Horizontal views of two segments of stage 16 (A-B") wild-type and (C-D") $Df(1)sc-B57$ embryos. All embryos contained sim-Gal4 UAS-tau-GFP and were stained for GFP (blue). (A-B") In wild-type embryos, (A-A") DAT and (B-B") ple (both magenta) expression was present in the dorsal lateral dopaminergic neuron (arrows) in each of the four hemisegments shown and colocalized with Tup (green). (C-D") In $Df(1)sc-B57$ embryos, (C-C") DAT and (D-D") ple expression was present in the dorsal lateral dopaminergic neuron (arrows) in only one hemisegment, and was absent in the other three hemisegments.

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**Fig. 10. Summary of midline precursor formation and neuron-specific gene expression.** The genetic results presented in this paper. (A) From a group of three neural precursor equivalence groups (MP1, MP3 and MP4), Notch signaling partitions midline precursors at stage 10 into MPs, MNB, AMG and PMG. As shown here, $l(1)sc$ acts as a proneural gene to promote the formation of MP4-6 and MNB, but not MP1, MP3, AMG or PMG. (B) The H-cell, H-cell sib, mVUMs and iVUMs are represented along with their regulatory pathways and target genes. The $tup$ and $l(1)sc$ genes play important roles in H-cell-specific gene expression, and $l(1)sc$ plays an important role in mVUM-specific gene expression. H-cell sib and iVUM-specific gene expression is largely controlled by Notch signaling. Gene expression in common to H-cell and H-cell sib or to mVUMs and iVUMs is controlled by separate pathways.
and Sc. L(1)sc protein levels are relatively constant among all five MPs and MNB, both L(1)sc and Sc are present in all MPs and MNB, and Ac, L(1)sc and Sc are present in MP1 as well as MP5,6 and MNB, yet no defects in MP1 and MP3 delamination or cell division were observed. Instead, the ability of l(1)sc to direct development of some MPs and not others may reflect the different cell states (and distinct co-factors) of the precursor populations from which each MP arises (Powell and Jarman, 2008). Similarly, l(1)sc controls expression of different genes in the H-cell compared with mVUMs, probably based on their different origins (MP3 versus MP4-6). Variability in the genetic control of midline MP formation extends to the non-midline MP2 cells. The MP2s require both ac and sc for MP formation and differentiation, whereas l(1)sc does not play a role (Skeath and Doe, 1996). Thus, MP2 and midline MPs (MP4-6) each require AS-C gene activity for proneural and differentiation functions, but use different AS-C family members.

Multiple regulatory pathways control H-cell gene expression

At least two distinct genetic programs control H-cell gene expression: (1) H-cell-specific gene expression is controlled by l(1)sc, tup and SoxN, and (2) unknown factors control gene expression that is present in both the H-cell and H-cell sib (Fig. 10B). All H-cell-specific gene expression requires l(1)sc function. tup acts in parallel to control important aspects of H-cell gene expression, including the DAT, ddc and ple genes. SoxN acts downstream of l(1)sc to control NPFRI expression. H-cell neural function gene expression begins at stage 13, well after l(1)sc expression is absent, indicating that l(1)sc is unlikely to directly regulate these genes. However, Tup is present after stage 13 and could directly regulate DAT, ddc and ple; SoxN is also present and could directly regulate NPFRI. The l(1)sc gene regulates mVUM gene expression in a manner similar to its control of H-cell expression (Fig. 10B), but does so independently of tup, which is not expressed in mVUMs. We note that L(1)sc protein is present at higher levels in H-cell than mVUMs (Fig. 3F-H), although the significance of this is unclear. Expression of genes common to both H-cell and H-cell sib cells, including 5-HT1A, Glu-RII and tup, were not affected in l(1)sc or Notch pathway mutants (Wheeler et al., 2008), indicating a second distinct regulatory pathway. This was also observed for genes expressed in common between mVUMs and iVUMs.

l(1)sc and tup regulation of H-cell and H-cell sib gene expression

The relationship between l(1)sc and tup in controlling H-cell-specific gene expression is complex. Both genes are initially expressed in the H-cell and H-cell sib after MP3 division, but expression of both is soon restricted to the H-cell. Misexpression of l(1)sc resulted in the ectopic expression of tup in the H-cell sib, similar to other H-cell-specific genes. However, in l(1)sc mutants, tup expression was not absent in the H-cell, but instead tup expression remained present in the H-cell and sometimes in two cells: one was the H-cell and the other was (probably) the H-cell sib. In addition, l(1)sc expression was not affected in tup mutants. These results indicated that: (1) l(1)sc and tup act in parallel in the H-cell to regulate dopaminergic pathway gene transcription; and (2) l(1)sc downregulates tup in the H-cell sib, indicating a role for l(1)sc in H-cell sib development. The best marker for the H-cell sib is CG13565, although it is expressed in wild type in only 54% of segments. In Df(l)sc-B57 mutant embryos, CG13565 was expressed in 46% of segments, similar to wild type. However, given its variability of gene expression in Df(l)sc-B57 mutants (Table 1) and the normal variability of CG13565 expression, it remains possible that l(1)sc (and tup) may play roles in H-cell sib development. Additional experiments are necessary to determine how l(1)sc and tup function together to control H-cell-specific gene expression.

How common is l(1)sc control of neuron-specific gene expression?

Within midline cells, l(1)sc plays important roles in controlling H-cell and mVUM gene expression, while playing relatively insignificant roles in MP1, H-cell sib and iVUM neuronal gene expression. We have begun to address whether non-midline neuronal gene regulation is regulated by l(1)sc. Significantly, Df(l)sc-B57 mutant embryos show a strong reduction in DAT and ple expression in the non-midline dorsal lateral dopaminergic neurons. Thor and Thomas (Thor and Thomas, 1997) showed that ple expression in these cells was also reduced in tup mutant embryos. Although more detailed cellular and genetic studies are required to bolster these observations, these data raise the possibility that both l(1)sc and tup may regulate gene expression in both midline and non-midline dopaminergic neurons. More generally, l(1)sc control of neuron-specific gene expression is likely to be uncommon. This is based on the observation that in the developing CNS, there is little L(1)sc protein colocalizing with newly divided Elav’ neurons or GMCs.

Evolutionary aspects of Drosophila dopaminergic neuron regulation

Because of the key neurobiological and medical importance of dopaminergic neurons, there has been intensive analysis of the regulatory factors that control their development in vertebrates and C. elegans. Are the regulatory programs involved in dopaminergic neuron differentiation conserved between insects, worms, and mammals? The two key regulatory proteins that control Drosophila H-cell dopaminergic differentiation are l(1)sc and tup. In vertebrates the bHLH genes mouse achaete-scute homolog [Mash1; homolog of l(1)sc] and neurogenin 2 (Ngn2) play roles in midbrain dopaminergic neuron development, although the role of Mash1 is secondary to Ngn2, which has a key function in dopaminergic differentiation (Kele et al., 2006). However, Mash1 (as well as Ngn2) can initiate neurogenic programs of other neuronal cell types. This was emphasized demonstrated in recent work in which forced expression of Mash1 and two other transcription factor genes converted murine fibroblast cells to neurons (Vierbuchen et al., 2010). The mammalian orthologs of Drosophila tup, Isl1 and Isl2, play important roles in motoneuron differentiation (Tsuchida et al., 1994), but have not been reported to influence dopaminergic neuron development and gene expression. Recently, C. elegans and vertebrate ETS family transcription factor genes were shown to directly regulate dopamine pathway gene expression (Flames and Hobert, 2009). It will be important to identify the transcription factors in Drosophila that directly regulate dopaminergic neuron function genes and connect them to the regulatory genes we identified in this paper.

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Dopaminergic neuron development

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.orglookup/suppl/doi:10.1242/dev.056507/-/DC1

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Values represent the number of segments in which none, one, two or three cells expressed the gene. Percentages are indicated in parentheses. $P>0.1737$. 

Table S1. Summary of $sc^{en}$, $D(f)(1)sc10^{-1}$ and $D(f)(1)ase^{-1}$ mutant genetic data.
Table S2. *Df*(1)sc-*B57* rescue data

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Rescue of *Df*(1)sc-*B57* mutants by sim-Gal4 UAS-l(1)sc. Values represent the number of segments, and percentages are indicated in parentheses. *P*=0.0442 for H-cell (ple expression) wild type/rescue; *P*=0.0001 for *Df*(1)sc-*B57/rescue; *P*=1.000 for MNB (wor expression) wild type/rescue; *P*=0.0001 for *Df*(1)sc-*B57/rescue.