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adrift, a novel *bnl*-induced *Drosophila* gene, required for tracheal pathfinding into the CNS

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SUMMARY

Neurons and glial cells provide guidance cues for migrating neurons. We show here that migrating epithelial cells also contact specific neurons and glia during their pathfinding, and we describe the first gene required in the process. In wild-type *Drosophila* embryos, the ganglionic tracheal branch navigates a remarkably complex path along specific neural and glial substrata, switching substrata five times before reaching its ultimate target in the CNS. In *adrift* mutants, ganglionic branches migrate normally along the intersegmental nerve, but sporadically fail to switch to the segmental nerve and enter the CNS; they wind up meandering along the ventral epidermis instead. *adrift* encodes a novel nuclear protein with an evolutionarily conserved motif. The gene is required in the trachea and is expressed in the leading cells of migrating ganglionic branches where it is induced by the *branchless* FGF pathway. We propose that Adrift regulates expression of tracheal genes required for pathfinding on the segmental nerve, and FGF induction of *adrift* expression in migrating tracheal cells promotes the switch from the intersegmental to the segmental nerve.

Key words: Cell migration, FGF, Epithelial-CNS interaction, *adrift*, *Drosophila*, *branchless*

INTRODUCTION

Developing neurons undergo complex migrations to construct a functional nervous system. An important area of neurobiological research is aimed at identifying and characterizing the areas that guide these migrations. Many such cues are presented by developing neurons and glial cells, and the nervous system provides a highly diverse array of positional cues. This positional information potentially could be used by other migrating cells. In this paper, we demonstrate that migrating tracheal cells in *Drosophila* contact specific neural and glial cells as they navigate to precise targets in the CNS, and we describe a novel tracheal gene called *adrift* that is required for the process.

The *Drosophila* tracheal system is a branched network of epithelial tubes that transports oxygen throughout the body. It develops from 20 ectodermal cell clusters, each containing approximately 80 cells. Each cluster invaginates, forming an epithelia sac, and then primary, secondary and terminal branches sequentially bud from each sac to generate the 20 tracheal hemisegments (Fig. 1A). Initially, six multicellular buds form and grow out along stereotyped paths to generate the six primary branches. One of these, the ganglionic branch (GB), which is the focus of this paper, targets the ventral nerve cord. The leading cell of the GB forms a unicellular tube called a secondary branch, as do the leading cell or cells of other primary branches. These secondary branches grow toward different target tissues and later ramify into the terminal branches that supply the targets with oxygen (Samakovlis et al., 1996a; Manning and Krasnow, 1993).

The critical factor that controls the outgrowth of primary branches is the product of the *branchless* (*bnl*) gene, a homologue of vertebrate FGFs (Sutherland et al., 1996). *bnl* is expressed in discrete clusters of ectodermal and mesodermal cells arrayed around each tracheal sac. The secreted growth factor functions as a chemoattractant that activates the Breathless (Btl) FGF receptor (Klämbt et al., 1992) on nearby tracheal cells and guides their migrations as they grow out and form primary branches. Branchless signalling also induces expression of genes, including *pointed* (Scholz et al., 1993) and *DSRF* (Guillemin et al., 1996), required for secondary and terminal branching in the leading cells of the primary branches, and these cells go on to form secondary and subsequently terminal branches. The signals that guide outgrowth of secondary and terminal branches are unknown.

In this paper, we investigate the cellular and molecular mechanisms that guide outgrowth of secondary ganglionic branches that grow into the ventral nerve cord. We show that these branches navigate a remarkably complex and tortuous path, tracking along five different neural and glial substrata to

reach their ultimate CNS target. We identify mutations in a gene, which we call *adrift*, that affect the first substratum switch causing failure of GBs to enter the CNS and misrouting along the ventral epidermis. Molecular characterization of the *adrift* gene demonstrates that it encodes a novel nuclear protein. Expression of the gene is induced in the lead cell of the migrating ganglionic branch by the *bnl* FGF pathway, whereupon it promotes the switch from one neural substratum to another.

MATERIALS AND METHODS

Drosophila strains

The Pantip-4 P[*lacZ*] enhancer trap line (5405) was generated in the laboratory of M. Scott. Excision alleles of this P transposon were generated as described (Robertson et al., 1988). The 170 excision alleles obtained were balanced over CyO*ftzlacZ* and then screened for lethality and for embryonic tracheal phenotype after staining with mAb2A12 antibody. The screen yielded several homozygous viable revertants and the two *adrift* alleles described here, aft^1 (excision 70) and aft^2 (excision 28). Other P[*lacZ*] enhancer trap markers used were trachealess-*lacZ* (1-eve-1; Perrimon et al., 1991), breathless-*lacZ* (T1; Bier et al., 1989), pointed-*lacZ* (pnt^{7825}/Pantip-1; Samakovlis et al., 1996a), sprouty-*lacZ* ($spry^{9143}$/Pantip-2; Samakovlis et al., 1996a) and repo-*lacZ* ($repo^{2138}$; Xiong et al., 1994). The following null or strong loss-of-function alleles were used: bnl^{P1} (Sutherland et al., 1996), btl^{LG19} (Klämbt et al., 1992), $pnt^{\Delta 88}$ (Scholz et al., 1993) and $pruned^{ex84}$ (Guillemin et al., 1996). Strains used for misexpression studies using the GAL4/UAS system (Brand and Perrimon, 1993) were: UAS-*bnl* B4-2 (Sutherland et al., 1996), *btl*-GAL4 (Shiga et al., 1996) and hsGAL4 (described on Flybase).

Immunostaining and in situ hybridization of embryos

Embryo fixation, staining and light microscopy were as described (Samakovlis et al., 1996b). The tracheal lumen-specific antibodies used were rabbit polyclonal antiserum TL-1 (from J. O'Donnell) diluted 1:2000 and mAb2A12 diluted 1:5. The anti-DSRF monoclonal antibody was mAb2-161 (1:1000) from M. Gilman (Ariad Corporation, Boston, MA). Rabbit polyclonal antibody against β-galactosidase (Cappel) was used at 1:1500. mAb22C10 against peripheral nervous system (Fujita et al., 1982; Grenningloh et al., 1991) was used 1:50. Biotin-, and Cy3-conjugated secondary antibodies (Jackson Laboratories) were used at 1:300 dilution.

To prepare the Adrift antiserum, a DNA fragment from *aft* cDNA 3 that encodes amino acids 371-700 was inserted in frame to the 6xHis coding sequence in pRSETB (Invitrogen). The histidine-tagged Adrift fusion protein was expressed in *E. coli*, purified on Ni-NTA resin (QIAGEN) and used for immunization of rabbits at Agrisera (Umeå). The antiserum was affinity purified on Ni-NTA resin coupled with the histidine-tagged Aft fusion protein and used at 1:5000 final dilution for western blots and immunohistochemistry.

In situ hybridization of whole-mount embryos (Lehmann and Tautz, 1994) was done with random-primed, digoxigenin-labeled *aft* cDNA as probe. Similar results were obtained with the 1.2 kb *Eco*RI *aft* genomic fragment. In situ hybridisation followed by antibody staining was as described (Kopczynski et al., 1996). Embryo staging was according to Campos-Ortega and Hartenstein (1985).

To examine the effects on *adrift* expression of ectopic *branchless* expression, UAS-*bnl*B4-2;Pantip-4/hsGAL4 embryos were collected for approximately 4 hours at 21°C, aged for 7 hours at 21°C, heat-shocked once for 20 minutes, and then aged 9 hours at 21°C. After fixation, embryos were stained with mAb2A12 and anti-β-galactosidase antibodies.

Electron microscopy

Late stage 16 embryos were prepared for EM as described (Tepass and Hartenstein, 1994) except that the fixative was 25% glutaraldehyde, 4% paraformaldehyde and 3% tannic acid in 0.1 M phosphate buffer. Specimens were viewed by a JEOL 100 CX electron microscope. Serial sections of three different abdominal tracheal metameres from two embryos were examined.

Molecular biology

Genomic DNA flanking the Pantip-4 P[*lacZ*] insert was obtained by plasmid rescue in *E.coli* after cleavage of the genomic DNA with *Eco*RI or *Bam*HI. Northern blots of poly(A)$^+$ RNA, screening of genomic and cDNA libraries and sequencing were done according to Sambrook et al. (1989). Restriction mapping of 6 positive clones from a cosmid library (gift from J. Tamkun) resulted in a genomic map of 60 kb around the insertion site of the P-element. The *aft* cDNA 2A, a 1597 nt partial cDNA clone, was isolated from a lambda ZAP embryonic cDNA library (from P. Hurban) using the 1.2 kb *Eco*RI *aft* genomic DNA fragment as probe. Subsequently, cDNA 2A was used to probe a pupal library (from S. Stowers) and identified *aft* cDNA 3, a 2248 nt cDNA clone. *Exo*III deletions of this cDNA were generated and used as templates for dideoxy nucleotide sequencing. cDNA 3 was used to screen an embryonic plasmid cDNA library (Brown and Kafatos, 1988), which yielded a slightly longer *aft* cDNA 10 (2324 nt). This cDNA was sequenced on both strands using primers based on the cDNA 3 sequence. To define intron/exon boundaries, the relevant genomic fragments were subcloned into pBluescript and sequenced. Approximately 10^6 phage or colonies were screened from each of the libraries.

Molecular analysis of the *aft* mutations

Genomic DNA was prepared from aft^1 and aft^2 homozygous flies, the parental strain and wild-type (Canton-S) flies and characterised on genomic Southern blots hybridized with fragments from cosmids surrounding the P-element insertion site and covering the *aft* gene. A higher resolution analysis was conducted by PCR of this genomic DNA using primers specific for the region flanking the P-element insertion site (primer A: 5′-CCTTCGACGGCGATGATCGTC-3′ and primer B: 5′-GCGGAGAACGTAACTCAAGAC-3′ deriving from wild-type cosmid sequences located 405 nt and 933 nt, respectively, upstream from the first nucleotide found in the *aft* cDNA). The PCR products were purified and directly sequenced using the same primers. Sequence comparisons between PCR amplified fragments from the aft^1 mutant and from the corresponding region in wild-type revealed that in aft^1 there is a 23 bp insertion deriving from the P-element in addition to a duplication of 8 bp of genomic sequence. In aft^2 there is a 1.2 kb insert remnant from the P-element.

Transgenes and misexpression studies

To construct the UAS-*aft* transgene, a *Not*I-*Kpn*I fragment from pBluescript-*aft* cDNA 3 plasmid was cloned into the pUAST vector (Brand and Perrimon, 1993). For the hs-*aft* transgenic fly strain, a *Not*I-*Xho*I fragment from the same plasmid was cloned into pPCaSpeR-hs vector (from C. Thummel). Strains carrying the transgenes were obtained by P-element mediated transformation of w^{1118} strain (Spradling, 1986). Two independent strains, with single insertions on the second and third chromosome respectively, were characterized for each of the two constructs.

For the rescue experiment, a *btl*-GAL4 driver strain (Shiga et al., 1996) was used that expresses GAL4 in all tracheal cells from stage 11. Embryos of the cross aft^1/CyO*hblacZ*;UAS-*aft*/+ × aft^1/CyO*hblacZ*;*btl*GAL4/+ were collected at 21°C for 8 hours then transferred to 29°C for 8 hours to maximize GAL4 activity. Embryos were then fixed and stained for Adrift, tracheal lumen and β-galactosidase to score affected GBs in homozygous mutant embryos that express ectopic Adrift.

To analyze the Adrift protein on western blots, wild-type embryos

and embryos carrying the hs-*aft* construct were collected for 16 hours at 21°C, heatshocked for 45 minutes at 37°C and allowed to recover for 1 hour at 18°C. Embryos were dechorionated and homogenized in sample buffer. Electrophoresis and immunoblotting were performed according to Sambrook et al. (1989). The anti-Aft antibody was diluted 1:5000 for immunoblots and hybridizing bands were detected with the Amersham ECL reagent according to the manufacturer.

RESULTS

Pathfinding of the ganglionic tracheal branch in the CNS

Cell-specific tracheal and CNS markers were used to define the movements and contacts of the migrating tracheal ganglionic branches (GB) as they grow into the ventral nerve cord (VNC). There are twenty ganglionic branches, two in each Tr1 tracheal hemisegement and one in each hemisegment from Tr2 to Tr9 (Fig. 1B) (Manning and Krasnow, 1993). The navigation pathway of each GB is complex but highly stereotyped, involving interactions with two different nerves and several glial cell types along the way. The path followed by the 14 abdominal GBs is the same in each segment; it is summarized in Fig. 1J and described in detail below. The path followed by the six thoracic GBs is similar except they turn dorsally and anteriorly as they approach the midline in the VNC (not shown).

The typical GB is an epithelial tube composed of seven cells (Samakovlis et al., 1996a). As the GB buds and grows out, a single cell called GB1 at the end of the branch leads the migrations (Fig. 1A,C); the remaining six GB cells (GB2-GB7) follow in line behind (Samakovlis et al., 1996a). At embryonic stage 13, the GB cells migrate ventrally towards the CNS, in close contact with the intersegmental nerve (ISN) (Fig. 1D and data not shown). The GB cells are attracted towards the CNS by two ventrolateral clusters of epidermal cells expressing the *bnl* FGF (Sutherland et al., 1996). At stage 14, as the tip of the GB approaches the entry point into the VNC, expression of *bnl* ceases. The lead cell (GB1) turns posteriorly, breaks contact with the ISN and changes substratum to associate with the segmental nerve (SN) (Fig. 1D). During stage 15, GB1 migrates along the SN as it traverses laterally and ventrally inside the VNC, GB2 follows GB1 onto the SN. The other GB cells (GB3-GB7) also release contact with the ISN but they do not bind the SN or enter the VNC and they end up suspended in the body cavity (Fig. 1C). A group of CNS glial cells called exit glia (see Goodman and Doe, 1993 for nomenclature) surround the point where the GB cells switch from the ISN to the SN, suggesting that they may play a role in the switching (Fig. 1E).

As the GB1 cell continues medially on the ventral side of the neuropil during stage 16, it comes in close contact with the ventral longitudinal glial cells (Fig. 1F). Just before reaching the midline glia, GB1 turns abruptly and migrates dorsally (Fig. 1G) to reach the dorsal side of the neuropil. It then turns again and extends posteriorly on the dorsal longitudinal glial tracts toward the neighboring hemisegment (Fig. 1G; data not shown).

EM analysis of sagittal sections of the VNC of stage 16 wild-type embryos confirmed the direct association between GB cells and various glial cells along the migration pathway. Inside the VNC, just beyond the point of the ISN to SN switch, a segmental nerve root glial cell (SNG) (Ito et al., 1995) completely engulfs both the GB and the SN (Fig. 1H). In serial sections that allow one to follow the GB towards its tip, contacts between GB1 and longitudinal glia and dorsoventral channel glia are also seen (Fig. 1I; data not shown).

Ganglionic branches are misrouted in *adrift* mutants

The Pantip-4 P(*lacZ, w*$^{+}$) transposon is located at chromosomal position 54F and expresses the β-galactosidase marker in the lead cells of the GB and other growing primary branches (Samakovlis et al., 1996a). The original insertion caused weak, sporadic defects in GB outgrowth (not shown). By introducing a source of transposase, 170 w^{-} transposon excision alleles were obtained. Two homozygous viable alleles (excisions 28 and 70) displayed a similar tracheal phenotype and failed to complement for this function. We refer to the alleles as *adrift*^{1} and *adrift*^{2} because of their tracheal phenotypes described below. Molecular analysis indicates that *aft*^{1} represents the zygotic null condition for the tracheal function of the gene (see below) and was the focus of the phenotypic analysis.

In *aft* mutants, GBs sporadically stalled or were misrouted and failed to follow their stereotyped path into the CNS. In *aft*^{1} mutants, 18% of GBs (*n*=300) missed the entry point into the VNC and continued to migrate along the ventral epidermis (Fig. 2A; see also below). An additional 6% of GBs stalled at or before reaching the VNC. Neither defect was seen in wild-type controls (*n*=400). Double labeling with tracheal and neural markers demonstrated that GBs in homozygous *aft*^{1} embryos grew normally over the ISN during the initial phase of their ventral migration but sporadically failed to make the switch to the SN and associate with the exit glial cells (Fig. 2B,C). Most of the affected branches instead turned posteriorly and continued to migrate along the ventral epidermis, forming a characteristic ‘hook’ structure (Fig. 2A, top arrow).

The defect in GB guidance in *aft*^{1} mutants does not result from grossly aberrant differentiation of the tracheal cells. The cells retained their ability to migrate and expressed all of the appropriate secondary and terminal branch markers tested including pointed^{7825} *lacZ*, sprouty^{9143} *lacZ*, Pantip-4 *lacZ* and DSRF (Fig. 2D,E; data not shown). Furthermore, the structure of the nerves and glial cells normally contacted by the growing GB were unaffected in the mutant (Fig. 2B,C; data not shown). We conclude that *aft* is specifically required to promote recognition and association of the GB1 cell with the SN and glial cells at the first guidepost in its navigation into the CNS.

adrift encodes a nuclear protein with a novel, evolutionarily conserved motif

Genomic DNA fragments flanking the Pantip-4 P-element insertion were recovered by plasmid rescue in *E. coli* and used to probe a wild-type *Drosophila* genomic library. Seven overlapping cosmids were obtained and a genomic map covering 60 kb surrounding the transposon insertion site was constructed (Fig. 3A). Genomic fragments near the insertion site were used to probe northern blots of embryonic mRNA. A 2.3 kb transcript located ~0.7 kb from the insertion site was identified (Fig. 3A,B) and found to be expressed in the trachea in the same pattern as the *lacZ* reporter in the Pantip-4 strain (see below). This transcription unit corresponds to the *aft* gene because its expression was reduced or absent in the *aft* mutants

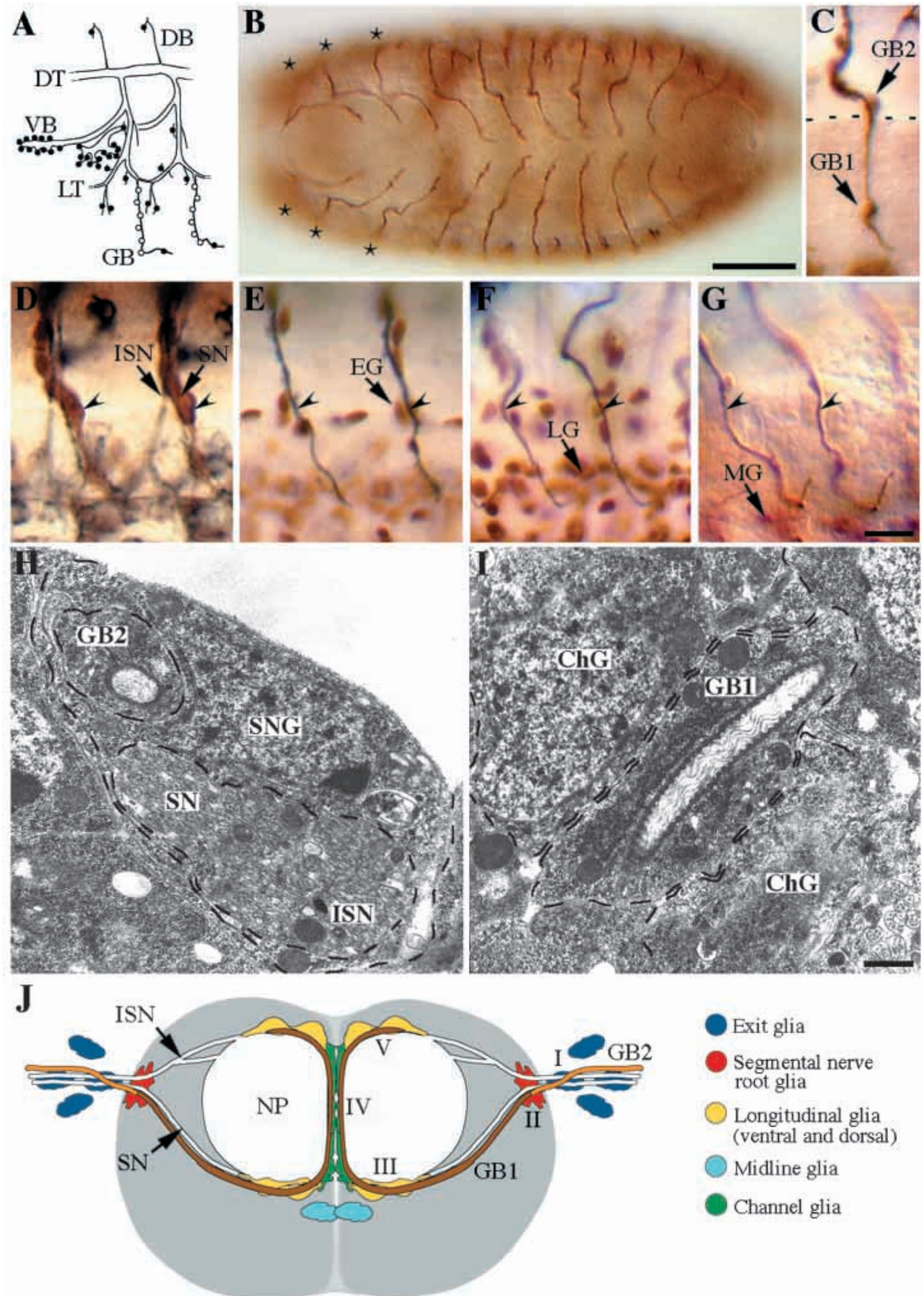


Fig. 1. Pathfinding of the tracheal ganglionic branch into the CNS. (A) Diagram of two abdominal tracheal hemisegments at embryonic stage 15 (lateral view, anterior left). The primary branches are labeled: DB, dorsal branch; DT, dorsal trunk; VB, visceral branch; LT, lateral trunk; GB, ganglionic branch. The nucleus of the GB terminal cell and other terminal cells that lead the outgrowth of primary branches and later form secondary and terminal branches are indicated by the black circles. The remaining 6 cells of the GB are indicated by the open circles. The cells are named (GB1-GB7) according to their position in the branch starting with the terminal cell (GB1, black circle). (B) Ventral view of a wild-type stage 16 embryo stained for mAb2A12 antigen in the tracheal lumen (brown). The plane of focus is on the ventral nerve cord to show the 20 GBs that have migrated into the CNS. The six thoracic GBs are marked by asterisks. (C-G) Stage 15 or 16 embryos carrying one copy of the indicated *lacZ* enhancer trap markers were double stained for a tracheal and a neural or glial marker. Close up of the tips of one (C) or two GBs (marked with concave arrowheads, D-G) are shown as they migrate into and through the CNS (ventral view, anterior left). (C) Stage 16 embryo carrying a *btl-lacZ* reporter (T1) double stained for β-gal to show nuclei of tracheal and CNS midline cells (both brown) and mAb2A12 antigen to show tracheal lumen (also brown). The nucleus of the GB1 cell is in the CNS and the GB2 nucleus lies just outside. The CNS border is outlined by black dots. (D) Stage 15 embryo carrying a *trh-lacZ* reporter (1-eve-1) double stained for β-gal to show tracheal cells (brown) and mAb22C10 antigen to show nerves (blue). GBs veer posteriorly just before entering the CNS, releasing contact with the intersegmental nerve (ISN) and associating with the segmental nerve (SN). (E,F) Embryos carrying a *repo-lacZ* reporter ($repo^{2138}$) double stained for β-gal to show glial cells (brown) and mAb2A12 antigen to show tracheal lumen (blue). On their way into the CNS at stage 15 (E), each GB is in close contact with exit glial cells (EG). Two hours later at stage 16 (F), each GB migrates across the ventral side of the longitudinal glia (LG). (G) Stage 16 embryo stained as in C. When the GB reaches the midline glial cells (MG), it turns and migrates dorsally through the dorsoventral channel. (H,I) Electron micrographs of sagittal sections of stage 16 CNS showing contacts between GB cells and CNS cells. Cell contacts are outlined (broken lines). (H) At the entry point into the CNS the GB2 cell, the segmental nerve root (SN) and the intersegmental nerve root (ISN) are all enveloped by a segmental nerve root glial cell (SNG). (I) As the GB migrates dorsally along the dorsoventral channel, the GB1 cell is in direct contact with channel glial cells (ChG). (J) A simplified transverse projection of the nerve cord (grey) in a stage 16 embryo to illustrate the position of the GB (orange/brown) in the CNS. The steps in GB migration are as follows. (I) The GB switches substratum from the ISN to the SN root just before entering the ventral nerve cord. (II) GB migrates into the CNS along the SN. GB2 (orange) and the SN are both enveloped by a SN root glial cell. (III) GB1 (brown) extends towards the ventral surface of the neuropil (NP) to associate with the ventral longitudinal glial cells; GB2 remain at the CNS periphery. (IV) At the ventral midline, GB turns and migrates dorsally between the two neuropils in close contact with channel glial cells. (V) When GB reaches the dorsal side of the neuropil, it associates with the dorsal longitudinal glial cells and extends laterally and posteriorly. Bar in B, 50 μm; bar in C-G, 10 μm; bar in H and I, 0.7 μm.

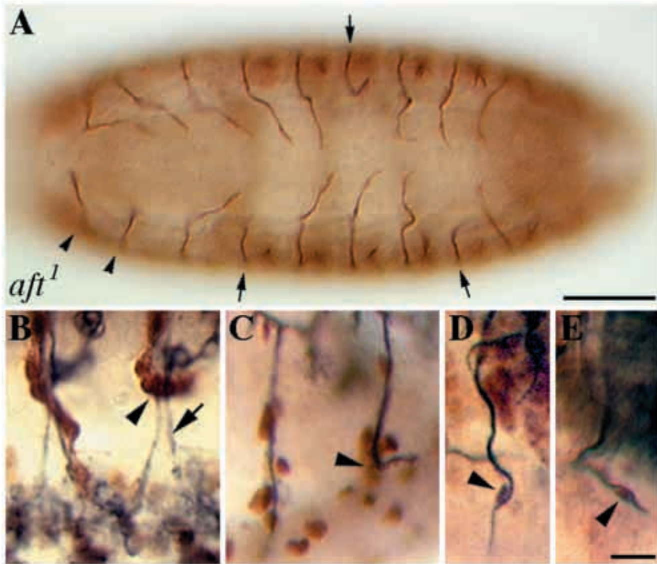


Fig. 2. Ganglionic branches are misrouted in *aft* mutants. (A) Stage 16 *aft*^{1} mutant embryo stained with mAb2A12 to visualize tracheal lumen. GBs sporadically fail to enter the CNS and instead migrate posteriorly forming ‘hooks’ (arrows) or stall near the entry site (arrowheads). The hooks on the bottom curve are out of the focal plane. The most posterior pair of GBs are outside the focal plane. (B) Close-up of two GBs in a stage 15 *aft*^{1} mutant carrying one copy of a *trh-lacZ* reporter (1-eve-1). The embryo was stained for β-gal to show tracheal cells (brown) and mAb22C10 antigen to show nerves (blue). The misrouted GB (arrowhead) released the ISN as in wild type (see Fig. 1D) but failed to attach to the SN (arrow). (C) Close up of two GBs in a stage 15 *aft*^{1} mutant carrying one copy of a *repo-lacZ* reporter (repo^{2138}). The embryo was stained for β-gal to show glial cells (brown) and mAb2A12 antigen to show tracheal lumen (blue). The misrouted branch turns posteriorly at a point close to the exit glial cells (just below focal plane at arrowhead) but is not associated with these cells as are wild-type GBs (see Fig. 1E). (D) Stage 16 *aft*^{1} mutant stained with mAb2A12 (blue) and mAb against the tracheal terminal cell marker DSRF (brown). The GB1 cell (arrowhead) in this misrouted branch expresses the marker normally. (E) Stage 16 *aft*^{1} mutant carrying one copy of a *spry*-lacZ reporter (spry^{9143}) and stained for β-gal (brown) and mAb2A12 (blue). The terminal cell (arrowhead) expresses the *spry* marker normally. All panels show ventral or ventrolateral views (anterior left). Bar in A, 50 μm; bar in B-E, 10 μm.

and the tracheal phenotype of *aft* mutants could be rescued by expression of a representative cDNA (see below).

Three cDNA clones corresponding to the identified transcription unit were obtained by screening embryonic and pupal cDNA libraries. Restriction mapping and sequence analysis of the cDNAs and the corresponding genomic region established the intron-exon structure shown in Fig. 3A. The longest cDNA contains a 700 residue long ORF (Fig. 3C) and an NCBI BLAST search of the combined databases identified several related cDNAs and Expressed Sequence Tags from different species. All show 18-28% identity to a 184 residue region in the N-terminal half of the predicted Adrift protein (residues 135-319; Fig. 3D). We call this the Adrift domain. The only other characterized gene containing an Adrift domain is the yeast YCL54 gene. Temperature-sensitive mutants in YCL54 were isolated in a screen for mutants defective in mating type silencing in yeast (Loo et al., 1995) but the molecular function of YCL54 protein in the process is unknown. There were no other significant similarities between the predicted Adrift ORF and the sequences deposited in the databases.

To examine the subcellular localization of Adrift protein, we raised polyclonal antisera against a recombinant form of the protein consisting of the C-terminal half of Adrift fused to a six histidine epitope tag. On western blots of *Drosophila* embryo extracts, the affinity-purified antiserum recognized a single 72 kDa species, in good agreement with the calculated molecular weight. This species was strongly induced in extracts of heat-shocked embryos carrying a construct including the *aft* cDNA3 under the control of the hsp70 promoter, confirming that it is the Adrift protein (data not shown). Immunolocalization studies in wild-type embryos detected Aft protein in the nuclei of all cells at cellular blastoderm stage (Fig. 4A,B), presumably derived from the abundant maternal *aft* mRNA (see below), and also in nuclei of the gonads, epidermis and brain lobes of older embryos (Fig. 4C,D). Unfortunately, the antisera were not sensitive enough to reproducibly detect the endogenous tracheal expression of the gene. However, when *aft* was overexpressed in the developing tracheal system using the UAS/GAL4 system, Aft antigen was readily detected and was predominantly nuclear (Fig. 4E).

adrift is expressed dynamically and required in migrating ganglionic branches

The pattern of *adrift* transcription during embryonic development was determined by northern blot analysis, whole-mount in situ hybridization and analysis of an *adrift lacZ* reporter (Pantip-4). The gene is maternally expressed and transcripts are evenly distributed during the syncytial blastoderm stage; most of the maternal transcripts are degraded during early embryogenesis (Fig. 3B and data not shown). Zygotic transcription is first detected by in situ hybridization in mid-embryogenesis in the developing tracheal system and later in the developing gonad (Fig. 5B,J). Tracheal expression is highly dynamic as revealed by both in situ hybridization and expression of the *adrift lacZ* reporter. At stage 11, the gene is expressed weakly in all tracheal cells (not shown). As the primary branches bud and grow out during stages 12 and 13, the gene is preferentially expressed in the leading cells of the GB and other growing primary branches (Fig. 5A,B). During stages 14 and 15, expression becomes further restricted to just the GB1 terminal cell (Fig. 5C,I) and other terminal cells (Fig. 5E-H) that lead the migrations toward the CNS and other target tissues.

No tracheal expression was detected in *aft*^{1} mutant embryos (Fig. 5D) and expression was severely reduced in *aft*^{2} mutants (data not shown). Maternal and gonadal expression, however, were detected in the mutants (Fig. 5K and data not shown), indicating that *aft*^{1} and *aft*^{2} selectively affect tracheal expression of the gene. Analysis of the molecular lesions in the alleles identified alterations at the P-element insertion site upstream of the coding region, suggesting that this may be a *cis*-regulatory region important for tracheal expression of the gene (see legend to Fig. 3).

An *aft* transgene expressed specifically in the developing tracheal system was constructed and used to confirm that *aft* function is specifically required in the migrating tracheal cells. A

UAS-*aft* transgene was crossed to a *btl*-GAL4 driver line to drive *aft* expression in all tracheal cells from stage 11 onwards (Shiga et al., 1996). Tracheal expression of the *aft* transgene almost completely rescued the misrouting phenotype of homozygous aft^1 mutant embryos. 24% of GBs are affected in aft^1 mutants whereas only 1% of GBs were affected in aft^1 mutants expressing the *adrift* transgene (Table 1). Thus, *aft* is required in migrating tracheal cells to find their targets in the CNS.

adrift expression is regulated by the Branchless FGF pathway

adrift is expressed in the leading cells of growing tracheal branches, near clusters of *branchless* FGF-expressing cells and in a pattern very similar to that of several known *branchless*-induced genes including *pointed* (Samakovlis et al., 1996a), DSRF/*pruned* (Samakovlis et al., 1996a) and *sprouty* (Hacohen et al., 1998). This suggested that *adrift* expression might also be induced by the *bnl* signalling pathway. We examined expression of the *adrift lacZ* reporter in embryos mutant for four components of the *branchless* pathway: *bnl* (Sutherland et al., 1996), *btl* (Klämbt et al., 1992), *pnt* (Scholz et al., 1993) and *pruned* (Guillemin et al., 1996). Initial expression of the *adrift* reporter in stage 11 tracheal cells was normal in all four mutants (not shown), but subsequent expression in the leading cells of the branches was absent in *bnl*, *btl* and *pnt* mutants (Fig. 6B-D). Expression in *pruned* mutants was unaffected (data not shown). In a complementary experiment in which *bnl* was misexpressed under the control of the hsp70 promoter, expression of the *adrift* reporter expanded to include additional cells in each branch (Fig. 6E). Thus, the Branchless FGF pathway induces *aft* expression in the leading cells of tracheal branches, and this induction requires the *bnl* FGF, the *btl* FGF receptor and the *pointed* ETS domain transcription factor.

DISCUSSION

GB tracheal cells navigate a complex pathway to reach their targets in the CNS

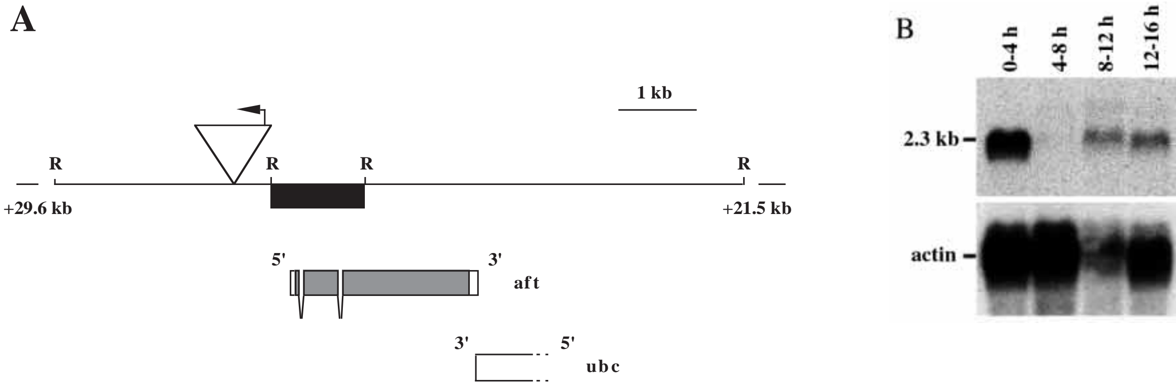
We have shown that the GB1 cell at the end of each migrating GB reaches the CNS at stage 15 and migrates more than 50 μm over the next 5 hours, following a remarkably complex path comparable to the most elaborate paths known in axonal pathfinding in *Drosophila*. We can conceptually divide the GB migration path into five segments based on the direction of migration and the migration substratum and cell contacts (see Fig. 1J). Initially, the GB tracks along the ISN towards the CNS (segment I). At the CNS entry point, the GB switches onto the SN and associates with exit glia nearby. In the CNS, the GB1 cell grows along the SN and segmental nerve root glia (segment II) and then the ventral longitudinal glia (segment III), approaching the midline. At the midline, it turns and extends dorsally along the dorsoventral channel surrounded by the channel glia (segment IV). Once it reaches the dorsal side of the neuropil, the GB1 cell turns back away from the midline, contacting the dorsal longitudinal glia and then continuing migration posteriorly along the longitudinal connectives and surrounding glia towards the next hemisegment (segment V), completing its embryonic migrations. During larval life, GB1 grows again, this time ramifying extensively into fine terminal branches that provide the ~230 neurons in each hemisegment with oxygen (E. Johnson, J. Jarecki, and M. A. K., unpublished data).

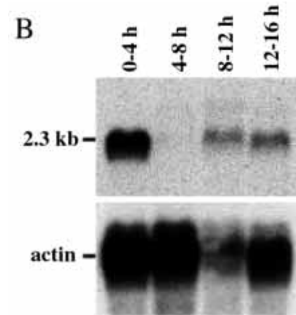
Each segment of this journey is presumably governed by distinct signalling events between the tracheal cell and its immediate environment. The only signal identified so far is the *branchless* FGF, which is expressed in discrete clusters of epidermal cells and attracts the GB toward the CNS during the first segment of the journey. The signals that guide the subsequent migrations into and around the CNS are unknown. Once in the CNS the GB1 cell comes in close contact with various neurons and glia. It thus is exposed to many of the same signals that guide axonal outgrowth and some of these could be used to guide GB migrations in the CNS. Indeed, several cell surface molecules implicated axon guidance, including Fasciclin II and Neuroglian, are also expressed on the surface of growing GB cells (Bieber et al., 1989; Van Vactor et al., 1993).

Fig. 3. Structure of the *aft* locus and gene product. (A) Map of the *aft* locus. The intron-exon structure of the *aft* cDNA and a neighboring, overlapping gene (ubiquitin-conjugating enzyme, ubc) are shown below the genomic map; the shaded areas are coding regions. The position of the P[*lacZ*] insert in Pantip-4 is indicated (large triangle); the arrow shows the direction of *lacZ* transcription. Sequencing of genomic DNA encompassing the transposon in the Pantip-4 strain mapped the insertion site 723 bp upstream of the first nucleotide found in the sequence of the cDNA clone. Molecular defects in the excision mutants were analyzed by genomic DNA blots, using cosmid fragments deriving from the *aft* gene as probes, and by direct sequencing of PCR fragments deriving from genomic DNA of aft^1 and aft^2 flies. Sequence comparisons between PCR amplified fragments from the genomic region around the P-element insertion site in aft^1 and from the corresponding region in wild-type revealed that in aft^1 there is a 23 bp insertion deriving from the P element in addition to a duplication of 8 bp of genomic sequence. In aft^2 there is a 1.2 kb insert remnant from the P element. The black box shows the 1.2 kb genomic *Eco*RI fragment used to identify the *aft* cDNA. R, *Eco*RI. (B) Northern blot of embryonic mRNA from embryos of the indicated ages probed with a radiolabeled 1.2 kb *aft* genomic *Eco*RI fragment (upper panel, 48 hour exposure) and an actin5C probe (Fyrberg et al., 1980) (lower panel, 15 hour exposure) as a loading control. The *aft* probe detects a single 2.3 kb transcript. Zygotic transcript is first detected at 8 and 12 hours; the high level of transcript in 0- to 4-hour-old embryos is apparently of maternal origin. (C) Sequence of *aft* cDNA and the predicted protein (GenBank accession number AF117649). In-frame stop codons following the coding sequence are underlined. The Adrift domain shown in D is highlighted in gray. Arrowheads indicate the intron positions. (D) Sequence similarity between 184 amino acids of the predicted *D. melanogaster* Adrift protein (D.m.) and portions of five other known or predicted proteins. Shaded boxes mark amino acid identities between Adrift and the others identified by FASTA3 searches (Pearson and Lipman, 1988) of the non-redundant amino acid database. Brackets mark sequence blocks conserved between all sequences as identified by a multiple sequence alignment tool (Depiereux et al., 1997). No obvious homology was detected in sequences outside this domain. A.s, African swine fever virus pEP424R (accession number U18466, 28% identity), H.s, a novel human gene (D43949, 25%), E.p, *Epiphyas postvittana* nucleopolyhedrovirus (AF037358, 25%), C. e, *Caenorhabditis elegans* R74.7 (Z36238, 20%), S.c, *Saccharomyces cerevisiae* hypothetical 34.7 kDa protein in ORC-TIP1 intergenic region (Z35930, 18%).

The *adrift* gene is required in the trachea for efficient entry into the CNS

One of the most challenging transitions on the GB migration path occurs at the entry into the CNS. At late stage 14, all seven cells of the branch have migrated ventrally in response to *bnl* signalling, and the lead cell, GB1, has been induced to express secondary and terminal branching genes. The GB1 cell leaves the ISN and associates with the SN. This substratum switch





C

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As   128 AFCNCELPGGFISAINHFNYTMMHYPTFNWVASSLYPSSETDALEDHYGLYQCNPDNWLMQSPLLKKNVDYNDGDVTIASNVKNLALRATQRLTP--IHLYTADGGINVGHD 237
H.s.  45 FADVCAGPGGFSEYVLWRKKWHAKGFGMTLKGPNDFKLEDFYSASSELFEPYYGEGGIDGD------------GDITRPENISAFRNFVLDNTDRKGVHFLMADGGFSVEGQ 144
E.p.  59 ALDLCGGFGEFAAYTMARNPLCRVFGVTLIANAPYKRVVQNHSNFVNVVGPDGT-------------------GDVLDKNVLFDLSVACGNA-----CDLVLADGAVDAAGR 146
C.e.  45 AVDLCAAPGSWSQVLSKRLYEEDQEAKIVAIDLQPMAPIPGVIQLQ---------------------------GDITSVDTANQVIKHFSGEK----SDIVICDGAPDVTGI 125
S.c.  47 VVDLCAAPGSWSQVLSRKLFDESPSSDKEDRKIVSVDLQPMSPIPHVTTLQ----------------------ADITHPKTLARILKLFGNEK----ADFVCSDGAPDVTGL 132

D.m. 243 PDCQEEIVVRLFFAEVLSALRILSSGGNFLVKMFTLFEACSVSLLYTLNCIFEEVHIFKPATSKRGNSEVYVICLNY 319
As   238 YNKQEELNLKLHFGQALTGLLSLSKGGNMILKHYTLNHAFTLSLICVFSHFFEELYITKPTSSRPTNSETYIVGKNR 314
H.s. 145 ENLQEILSKQLLLCQFLMALSIVRTGGHFICKTFDLFTPFSVGLVYLLYCCFERVCLFKPITSRPANSERYVVCKGL 221
E.p. 147 ENEQETINRALILRETQLALICLRVGGNCVLKVFDAFHNETLDVLERFARHFARWRLFKPPSSRPANSERYLVCINK 223
C.e. 126 HSLDEFMQAELILAAFNITSHVLKEGGNFLAKIFRSRNSSLLYAQMKKY--FKKVYLAKPRSSRQSSCEAFVLCLDY 200
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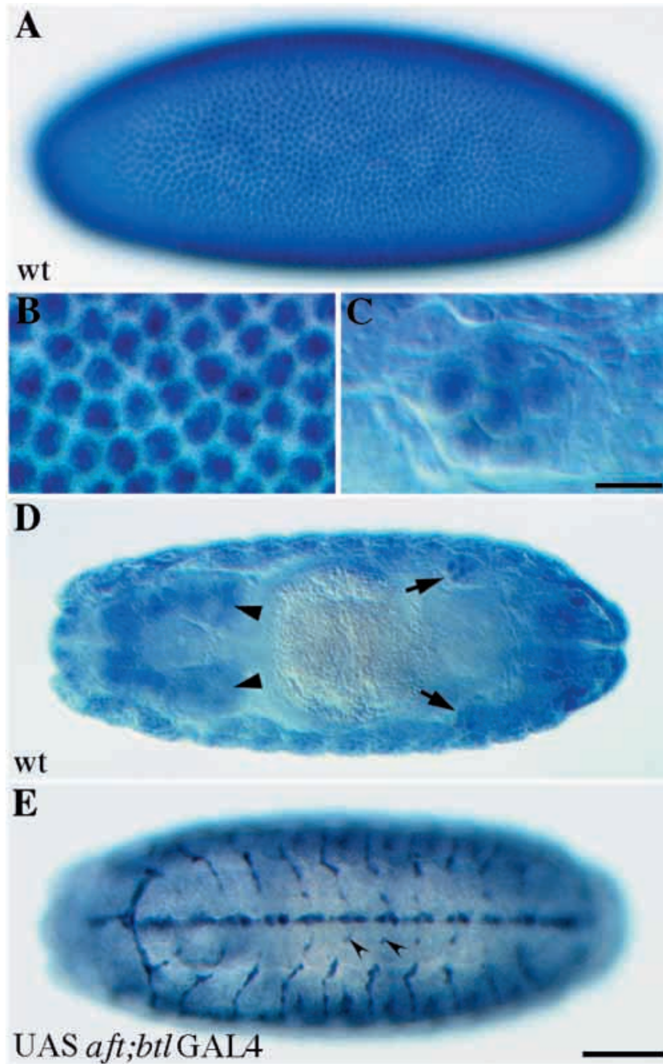

Fig. 4. Immunostaining of Adrift protein in wild-type embryos (A-D) and transgenic embryos overexpressing Aft (E; UAS-*aft/btl*-GAL4). (A) Stage 5 embryo. Aft protein is detected throughout the blastoderm. (B) Close-up of embryo in A. Aft protein is localized to the nucleus. (C,D) Close up (C) and lower magnification view (D) of stage 15 embryo (ventral view) showing Aft expression in nuclei of the gonads (arrows). In D, Aft protein is also seen in the brain lobes (arrowheads) and epidermis. (E) Stage 16 embryo (ventral view) overexpressing Aft protein in all tracheal cells and in midline cells of the CNS. Concave arrowheads indicate nuclear expression in the terminal GB1 cells of ganglionic branches. Bar in A, D and E, 50 µm; bar in B and C, 10 µm.

occurs at the CNS exit junction, which is composed of the two major nerve roots surrounded by a specialized group of exit glial cells. The junction is also a plexus region where motorneurons leaving the CNS switch pathways and selectively fasciculate into five major nerve branches destined for different regions of the musculature (Goodman and Doe, 1993; Van Vactor et al., 1993; Landgraf et al., 1997). Thus, the GB1 cell must not only release its old substratum but also select a new substratum from among several glial cells and neuronal projections. The GB2 cell makes a similar choice when it crosses the same junction ~1 hour later.

Table 1. Rescue of *aft*^{1} mutant by tracheal expression of a UAS-*aft* transgene

Genotype	Affected ganglionic branches* (%)
Oregon^R (wild type)	0 (*n*=400)
aft^1/*aft*^{1}	24 (*n*=300)
aft^1/*aft*^{1};UAS-*aft*/*btl*-GAL4	1 (*n*=300)

*Misrouted or stalled ganglionic branches.

In *aft* mutants, most of the affected GBs migrate into the exit junction, release contact with the ISN, and extend posteriorly towards the SN but fail to attach to it or the surrounding glia. Instead, they continue migrating posteriorly along the ventral epidermis and muscle. This suggests that *adrift* functions in a signalling pathway that attracts the GB onto the SN or fixes it on it once there. The expression pattern of the gene and mutant rescue by an *aft* transgene expressed in migrating tracheal cells demonstrate that *aft* is required in the cells that receive the signal and not in the signalling cells or the SN cells to which the GB cells adhere.

What might be the source of this signal? The most obvious candidates are the SN neurons or surrounding glial cells. Several axons including those of the RP1, RP3, RP4 and RP5 motoneurons switch from ISN to SN as they leave the CNS at the exit junction (Goodman and Doe, 1993). These might express short-range attractive signals or adhesion molecules and provide a bridge for the growth of the GB from the ISN to SN. Other potential sources of the signal are the three or four exit glia cells and the segmental nerve root glia that are intimately associated with the GB at the exit junction. In the absence of mutants that selectively affect these glial subtypes, we have not been able to specifically assess their roles in GB pathfinding. However, preliminary analysis of the *glial cells missing* (*gcm*) mutant (Jones et al., 1995; Vincent et al., 1996) in which many glial cell types fail to differentiate properly, has revealed several GB pathfinding phenotypes including misrouted branches that do not enter the CNS, supporting a role for glial cells in GB pathfinding events.

adrift function and regulation by the Branchless FGF pathway

The localized and dynamic expression of the *bnl* FGF in the epidermis and other tissues guides tracheal cell migrations during primary branching including outgrowth of the GB towards the CNS. In addition to this chemoattractant function, the *bnl* FGF also induces expression of genes required for secondary and terminal branches and thus selects where the next generation of branches will sprout (Lee et al., 1996; Sutherland et al., 1996). Our results identify a third function of *bnl* signalling in tracheal development – induction of a gene involved in pathfinding.

Induction of *adrift* expression at the end of the migrating GB promotes the switch from the ISN to the SN. Although we do not know at the molecular level how Adrift promotes this pathfinding switch, the nuclear localization of Adrift protein and its sequence similarity to a gene implicated in transcriptional silencing in yeast suggest that Adrift may regulate expression of other genes, perhaps one involved in recognition and adhesion to the SN or nearby glia. Adrift and the yeast protein may define a new class of regulatory proteins

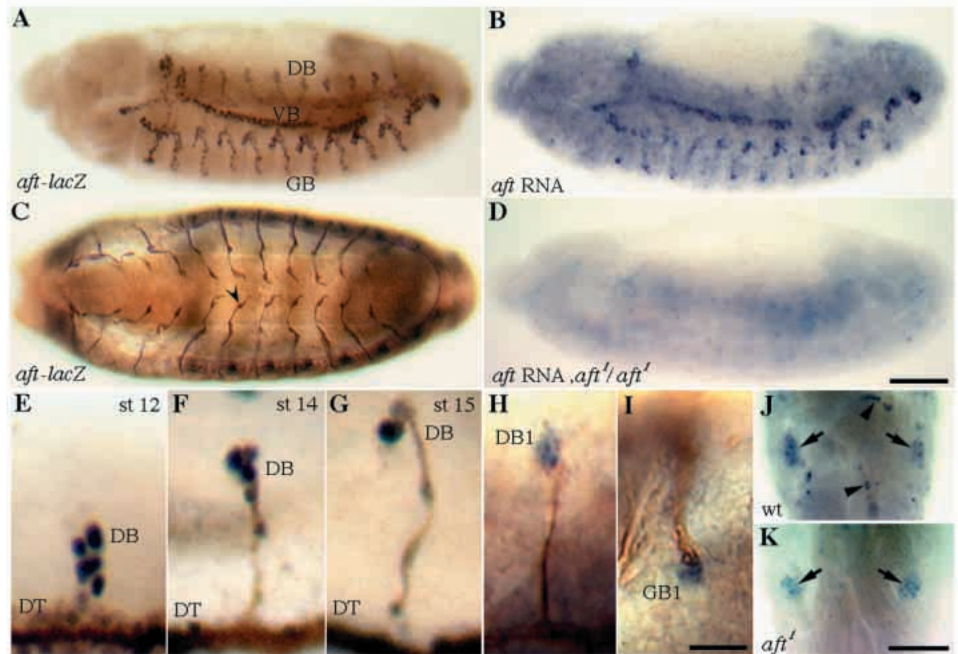

Fig. 5. Embryonic expression pattern of *aft*. *aft* expression was detected by in situ hybridization with an *aft* cDNA or by β-gal antibody staining (blue) of embryos carrying one copy of the *aft-lacZ* marker Pantip-4 (A,C,E-G); some embryos were also stained with mAb2A12 (C,E-G) or TL-1 antisera (H,I) to show tracheal lumen (brown). (A) Stage 12/13 *aft-lacZ* embryo (lateral view, anterior left). The *aft-lacZ* marker is expressed in the ganglionic branch and other growing primary branches including the DBs (DB, dorsal branch; VB, visceral branch; GB, ganglionic branch). (B) Stage 12/13 embryo (lateral view). *aft* RNA is expressed in the leading cells of the GB and other growing primary branches including the DBs. (C) Stage 16 *aft-lacZ* embryo (ventral view, anterior left). Expression of the marker has restricted to the GB1 cells (arrowhead) and other tracheal terminal cells (not shown). (D) Stage 12-13 *aft*^{1} mutant (lateral view). No expression of *aft* RNA is detectable in the trachea. Compare to wild-type embryo in B which was treated identically. (E-G) Close-up views of growing dorsal branch in embryos carrying one copy of the *aft-lacZ* marker. Note the dynamic restriction of marker expression to the leading cells. DB, dorsal branch; DT, dorsal trunk. (E) Stage 12. The marker is expressed in all cells of the budding DB. (F) Stage 14. Expression becomes restricted to the leading cells of the DB. (G) Stage 15. Strong expression is seen only in the DB1 terminal cell. Weak residual β-gal can be detected in the other DB cells. (H) Close up view of growing dorsal branch in wild-type stage 14 embryo showing a similar restriction of *aft* RNA to the DB1 cell. (I) Close-up view of growing ganglionic branch in wild-type stage 14 embryo showing similar restriction of *aft* RNA to the GB1 cell. (J) *aft* RNA is detected in the gonads (arrows) of the stage 15/16 wild-type embryo (ventral view, anterior up). (K) Gonadal expression remains in the *aft*^{1} mutant (arrows). However, tracheal expression seen in the wild-type (arrowheads in J) is missing in the mutant. Bar in A-D, 50 µm; bar in E-I, 10 µm, bar in J and K, 50 µm.

as a similar conserved motif was found in several anonymous predicted proteins in other organisms from *C. elegans* to humans (Fig. 3D).

aft expression is induced by Bnl signalling at the ends of all growing primary branches. However, *aft* mutations affect only the GBs. The lack of effect in other branches could be due to persistence of a low level of maternal *aft* expression that is sufficient for function in these branches. Alternatively, *aft* might be fully redundant or have no function in other branches; even in GB pathfinding, it does not appear absolutely essential as many GBs reach their proper targets in the mutants. Another gene called *unplugged* (*unp*), which encodes a nuclear homeobox protein, is also variably required for GB entry into the CNS (Chiang et al., 1995). The perception and response to cues provided at the CNS entry site may thus require the combined action of *aft* and *unp*. Because *unp* is expressed in

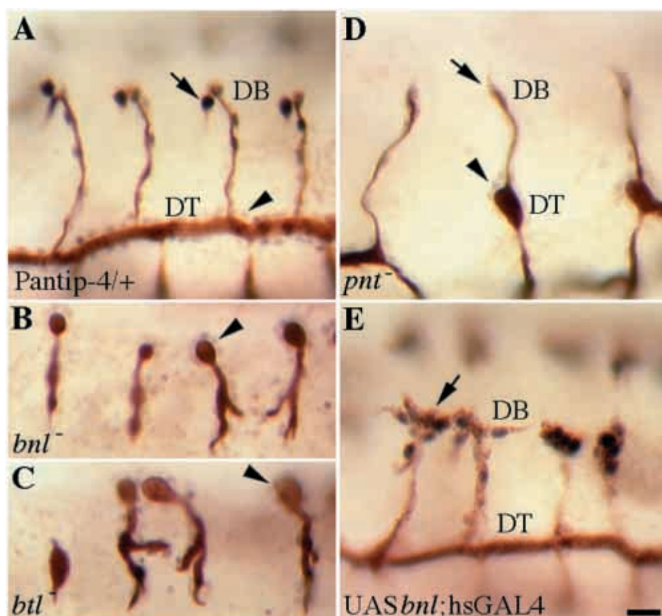

Fig. 6. The Branchless FGF pathway regulates *aft* expression. *aft* expression was monitored in different Branchless pathway mutants using the *aft-lacZ* marker Pantip-4. The stage 15 embryos are double stained for β-gal (blue) to show *aft-lacZ* expression and mAb2A12 antigen to show tracheal lumen (brown). (A) Wild type. *aft* is expressed at high levels in DB1 (arrow) and other terminal cells. DB, dorsal branch; DT, dorsal trunk. (B) *branchless* homozygote. In the absence of the *branchless* FGF, the DB and other primary branches fail to form and *aft* expression is not induced. (C) *breathless* homozygote. Absence of the *breathless* FGF receptor causes a similar phenotype. (D) *pointed* homozygote. In the absence of the pointed ETS domain transcription factor, a downstream effector in the Branchless pathway, the DB and other primary branches form but *aft* expression is not induced (arrow). The weak early expression of *aft* in all tracheal cells persists in the mutants (arrowheads). (E) UAS*bnl*B4-2; hsGAL4 embryo. Additional DB cells (arrow) express *aft* after ubiquitous expression of *branchless*. Bar for A-E, 10 µm.

all cells of the GB and cerebral branches but not other branches, only the lead cells of branches that target the CNS would express the proper combination of regulators and only at the right time just before entering the CNS.

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