The *Drosophila* ARF6-GEF Schizo controls commissure formation by regulating Slit

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Summary

The CNS of bilateral symmetric organisms is characterized by intensive contralateral axonal connections. Genetic screens in *Drosophila* have identified only a few genes required for guiding commissural growth cones toward and across the midline. Two evolutionarily conserved signaling molecules, Netrin and Slit, are expressed in the CNS midline cells. Netrin acts primarily as an attractive signaling cue, whereas Slit mediates repulsive functions. Here, we describe a detailed analysis of the *Drosophila* gene *schizo*, which is required for commissure formation. *schizo* leads to a commissural phenotype reminiscent of *netrin* mutant embryos. Double-mutant analyses indicate that Netrin and Schizo act independently. The *schizo* mutant phenotype can be suppressed by either expressing *netrin* in the CNS midline cells or by a reduction of the *slit* gene dose, indicating that the balance of attractive and repulsive signaling is impaired in *schizo* mutants. Overexpression of the *schizo* RNA in the CNS midline using the GAL4/UAS system leads to a *slit* phenocopy, suggesting that *schizo* primarily antagonizes Slit signaling. This is further supported by cell type-specific rescue experiments. The *schizo* gene generates at least two proteins containing a conserved Sec7 and a pleckstrin homology domain (PH) characteristic for guanine nucleotide exchange factors (GEF) acting on ARF GTPases, which are known to regulate endocytosis. In support of the notion that *schizo* regulates Slit expression via endocytosis, we found that block of endocytosis leads to a *schizo*-like phenotype. We thus propose that the balance of the two signaling cues Netrin and Slit can be regulated, controlling membrane dynamics.

Key words: Schizo, Slit, Endocytosis, *Drosophila*, Midline crossing, GEF

Introduction

A common feature of all bilaterally organized animals is that the left and right body halves need to be interconnected. The majority of neurons found in the central nervous system (CNS) project their axons on the contralateral body side. In order to do so they first of all need to interpret attractive long-range signals that guide them toward the CNS midline (Mueller, 1999; Song and Poo, 2001; Tessier-Lavigne and Goodman, 1996). Here, the growth cones change their growth behavior and after having crossed the midline they grow away from the attractive source and never cross the midline again.

Over recent years our knowledge of the molecular mechanisms underlying this process in a number of species has greatly advanced (Grunwald and Klein, 2002; Yu and Bargmann, 2001). An evolutionarily conserved family of proteins called Netrins mediates the initial attraction of growth cones toward the midline. Netrins are expressed by the CNS midline cells and are secreted into the extracellular space, where they presumably form a gradient that guides commissural growth cones toward the CNS midline (Kennedy et al., 1994; Serafini et al., 1994). In *netrin* mutants no attractive signal is generated and the majority of axons fails to cross the midline in the first place (Hamelin et al., 1993; Harris et al., 1996; Hedgecock et al., 1990; Mitchell et al., 1996; Serafini et al., 1996). A similar mutant phenotype is displayed by animals lacking the Netrin receptor Unc40/Dcc/Fra (Fazeli et al., 1997; Keino-Masu et al., 1996; Kolodziej et al., 1996). As soon as the midline has been crossed, the same Netrin signal can be interpreted as a repulsive signal by expression of an Unc40/Unc5 heterodimeric receptor (Hong et al., 1999; Leonardo et al., 1997).

Beside Unc5, which mediates repulsion in response to Netrin, there is a second repulsive system operating at the CNS midline. In *Drosophila*, this repulsive signal is encoded by the gene *slit* (Kidd et al., 1999). It ensures that ipsilateral projecting growth cones never cross the midline and keeps axons that have crossed the midline once away from the midline. In homozygous *slit* mutants, attractive signals predominate and all axons collapse at the CNS midline (Rothberg et al., 1988). Slit is a member of the Leucin Rich Repeat (LRR)-type family of secreted proteins. It contains four LRR type repeats, six epidermal growth factor (EGF)-like motifs and a C-terminal cystein-rich region and is proteolytically cleaved (Brose et al., 1999; Brose and Tessier-Lavigne, 2000; Rothberg and Artavanis-Tsakonas, 1992; Rothberg et al., 1990). Like Netrin, Slit is thought to be secreted into the extracellular space, but direct evidence supporting this notion is lacking. However, at least the C-terminal portion of the Slit protein is detected as being fixed to membranes of the secreting midline cells only. The nature

of the Slit receptor expressed by the midline glia is unclear but may involve heparane sulfate (Hu, 2001).

The prime function of Slit is to mediate repulsive growth (Kidd et al., 1999). However, in a number of cases Slit can act as an attractive guidance cue, too (Englund et al., 2002; Kramer et al., 2001; Schimmelpfeng et al., 2001). The different functions of Slit are mediated by a conserved family of transmembrane receptors related to the Roundabout protein (Battye et al., 1999; Brose et al., 1999; Kidd et al., 1999; Kidd et al., 1998a; Li et al., 1999). *Drosophila* possesses three different *roundabout* genes, *robo*, *leak* (also called *robo2*) and *robo3*, which are expressed in subsets of CNS neurons. The combined action of these *robo* genes provides a 'Robo-code', which helps to establish the organization of discrete axon fascicles in the longitudinal connectives (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b). Netrin and Slit signaling do not act independently of each other, and signaling pathways intersect (Stein and Tessier-Lavigne, 2001).

An alternative way to regulate the repulsive signaling activity is to modulate the cell surface expression of the Roundabout receptors. In *Drosophila* this function is mediated by *commissureless*. *commissureless* mutants are characterized by a complete loss of commissures resulting from an overactivation of the repulsive signaling (Georgiou and Tear, 2002; Keleman et al., 2002; Kidd et al., 1998b; Tear et al., 1996). Commissureless, for which no clear vertebrate homologs have been described yet, helps to target the Roundabout receptor into the endosome. Thus in *commissureless* mutants an excess of Robo is secreted into the cell membrane, resulting in an exaggerated repulsive signaling (Keleman et al., 2002; Myat et al., 2002).

Pan-neural expression of Slit leads to a *roundabout* phenocopy. Enhanced expression in the midline, however, does not lead to prominent phenotypes and it had been suggested that efficient post-transcriptional mechanisms tightly regulate the levels of secreted Slit (Battye et al., 1999; Kidd et al., 1999). Assuming that such mechanisms are needed to downregulate membrane-bound Slit, either by increasing endocytosis or decreasing the rate of membrane transport of Slit, we might expect reduced CNS midline crossing due to an enhanced expression of the repulsive signal.

A number of extensive phenotypic screens for mutations affecting CNS axon pattern formation were conducted in *Drosophila* (Hummel et al., 1999a; Seeger et al., 1993). Beside mutations in *frazzled*, *slit*, *roundabout* and *commissureless* (Seeger et al., 1993) we have identified mutations only in two additional genes, *weniger* and *schizo* (*siz* – FlyBase), which showed a prominent loss of commissural axon tracts (Hummel et al., 1999a). *netrin schizo* double-mutant analyses indicated that *schizo* acts in a pathway parallel to *netrin* directing commissural growth cones toward the midline or that *schizo* functions to repress the repulsive signaling originating from the CNS midline (Hummel et al., 1999a).

Here we report a further characterization of the *schizo* mutant CNS phenotype and demonstrate that *schizo* acts in the CNS midline glial cells. *schizo* mutant embryos are characterized by increased Slit activity, whereas overexpression of *schizo* in the CNS midline cells leads to a mild *slit* phenocopy. *schizo* encodes a protein homologous to vertebrate Arf6-GEF proteins, which have been shown to regulate membrane dynamics. Expression of a dominant negative Dynamin protein in the CNS midline cells leads to a block of endocytosis in these cells. Concomitantly a *schizo* phenocopy develops, supporting the model that the level of Slit expression is regulated by endocytosis.

Materials and methods

Drosophila lines and genetics

Two EMS-induced *schizo* alleles were identified in a phenotypic screen (Hummel et al., 1999a; Hummel et al., 1999b). A third *schizo* mutant was identified in a subsequent genetic screen (U. Lammel and C.K., unpublished). The $schizo^{3}$ mutation does not lead to an abnormal CNS phenotype and was considered to be a hypomorph. The following deficiency chromosomes were used to localize the *schizo* gene (breakpoints are indicated): Df(3L)ri-79c (77B7-9 – 77F1-5), Df(3L)ME107 (77F3 – 78C8-9), Df(3L)Pc-MK (78A2-78C8-9, Df(3L)ME14 (78A2-78B1), Df(3L)ME178 (78A7-B1; het) and Df(3L)Pc-cp2 (78B1-2 – 78D) (deficiencies were kindly provided by A. Carpenter or the Bloomington Stock Center). Df(3L)ME14, Df(3L)ME178, Df(3L)ME107 and Df(3L)Pc-MK did not complement both schizo alleles, placing schizo in the 78A/B interval flanking the gene knockout which is affected by Df(3L)Pc-cp2. The P-element induced lethal schizo mutation l(3)3 was generously provided by A. Carpenter and was mapped into the schizo locus.

Sema2b-τmyc flies were provided by B. Dickson, Wien; UAS-*netrinB* flies were provided by C. Goodman, Berkeley; UAS-$shibire^{DN}$ flies were obtained from the Bloomington Stock Center; the *loner* alleles T1032 and T1015 were provided by E. Olson.

The P-element EP(3)3375 is inserted in the 78A/B interval about 20 kb upstream of *schizo* and complements *schizo*. The P-element-induced *schizo* allele *P224* was generated by local hopping experiments (Tower et al., 1993).

Histology

Antibody staining was performed as described (Hummel et al., 1999a). Mab BP102 was obtained from the Hybridoma bank in Iowa. A polyclonal β-galactosidase antibody (Cappel) was used to visualize balancer carrying embryos and the AA142 expression in midline glia cells. In-situ hybridization was performed according to Tautz and Pfeifle (1989) using a digoxigenin-labeled RNA probe of the LP01489 cDNA.

Results

schizo affects commissure formation

Only four zygotically active genes were found in a screen for mutations affecting commissure formation (*frazzled*, *weniger*, *schizo* and the *netrin* gene complex) (Hummel et al., 1999a). Two EMS-induced *schizo* mutants ($schizo^{C1\text{-}28}$ and $schizo^{U112}$) were initially identified. Subsequently we identified two P-element induced *schizo* alleles ($schizo^{l(3)3}$ and $schizo^{P244}$). All these alleles led to a reduction in the number of commissural fibers crossing the CNS midline. Interestingly, the anterior commissures were affected more prominently (Fig. 1A-C, Fig. 3B). Not all neuromeres were equally affected and the strongest defects were generally observed in abdominal segments A1-A4. All CNS midline cells formed in normal number in the absence of *schizo* function (Fig. 1C and data not shown). However, as generally observed in mutants affecting formation of commissures, the midline glial cells migrated out laterally along the few remaining commissural fibers (Fig. 1C). In addition to the commissural phenotype, we noted defects in the longitudinal connectives (Fig. 1B,C arrowheads).

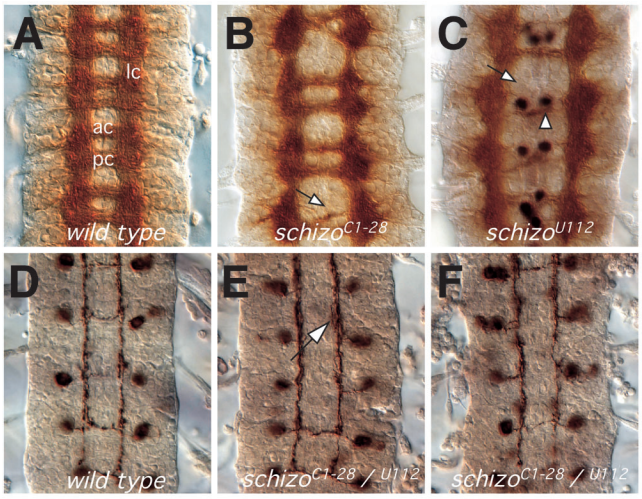

Fig. 1. *schizo* is required for commissure formation. Frontal views of dissected central nervous system (CNS) preparations of stage-16 embryos. (A-C) Stained for the presence of all CNS axons using Mab BP102. (D-F) Stained for the presence of the Myc epitope using Mab 9E10. (C) The midline glial cells are labeled by β-galactosidase expression using the *AA142* enhancer. Anterior is up. (A) Wild-type embryos are characterized by a regular arrangement of longitudinal connectives (lc) and segmental commissures (ac, pc). (B) In homozygous *schizo*$^{C1-28}$ mutant embryos the formation of commissures is reduced (arrow). The longitudinal connectives are thinner. (C) Homozygous *schizo*^{U112} embryos display a stronger commissural phenotype. Most frequently the anterior commissure is affected (arrow). In neuromeres with reduced commissures the midline glial cells migrate toward the connectives (arrowhead). (D) In wild-type embryos the sema-τmyc marker is expressed in only a few neurons in each hemineuromer. The corresponding axons cross the midline in one fascicle and turn anterior within the longitudinal connective. (E,F) In mutant *schizo*$^{U112/C1-28}$ embryos the sema-τmyc marker cannot be detected in about 50% of the commissures. Within the longitudinal connectives we noted a defasciculation of the Myc positive axon bundles (arrow).

In order to analyze the functions of *schizo* during axonal pathfinding more precisely, we used the Sema-τmyc marker. Here the Myc epitope is expressed in two neurons that send their axon across the midline in the posterior commissure and then follow a path within the longitudinal connective (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b) (Fig. 1D). To minimize background effects we analyzed *schizo*$^{C1-28}$/*schizo*^{U112} mutant embryos. Unlike wild type, *schizo* mutant axons frequently (>50% of the neuromeres, 20 embryos analyzed) did not cross the midline and showed fasciculation defects (Fig. 1E,F).

schizo interacts with *netrin* and *slit*

The most prominent function of *schizo* is its role in commissure development. Two major signaling cascades are known to control axonal growth across the midline. They are initiated by the signaling molecules Netrin and Slit, which are both secreted by the CNS midline glial cells in the *Drosophila* embryo (Battye et al., 1999; Harris et al., 1996; Kidd et al., 1999; Mitchell et al., 1996).

First genetic interaction studies of *schizo* and *frazzled* or *schizo* and *netrin* function demonstrated a much stronger commissural phenotype in double mutants than embryos mutant only for *schizo*, *frazzled* or *netrin* (Hummel et al., 1999a) (Fig. 2). The commissural phenotypes of the double-mutant embryos suggest that *schizo* is not acting within the Netrin signaling pathway but may be required for a Netrin-independent attractive pathway. Alternatively, *schizo* may be necessary for suppressing the perception or the generation of a repulsive signal normally generated by the CNS midline cells.

The balance between Slit and Netrin regulates commissural axon midline crossing

To further analyze the role of *schizo* in commissure formation, we attempted to rescue the *schizo* mutant phenotype by expression of a *netrin* transgene in the CNS midline cells. To induce *netrin* expression in the CNS midline cells we employed the GAL4 system (Brand and Perrimon, 1993). Following expression of UAS-*netB* (Mitchell et al., 1996) in the midline glial cells using a *sli*-GAL4 driver line (Scholz et al., 1997), the *schizo* mutant commissural phenotype is suppressed, whereas the connective phenotype of *schizo* mutant embryos was relatively unaffected by an increased *netrin* expression (Fig. 3A-C). Thus, enhanced *netrin* expression in the CNS midline is able to compensate for the loss of *schizo* function.

However, since genetic data indicated that *schizo* and *netrin* act independently and *netrin* RNA expression was found to be normal in *schizo* mutants (data not shown), one might speculate that a reduced repulsive function could compensate for the loss of *schizo*, too. The main axonal repulsive signal is encoded by *slit*. Slit is an LLR protein secreted by the CNS midline glial cells (Rothberg et al., 1990). When *schizo* function is normally required to downregulate repulsive signaling, either by affecting the generation of active Slit protein or by preventing signaling in the commissural growth cones, the mutant *schizo* phenotype could be explained by an upregulated Slit signaling. Thus, one might expect that the *schizo* commissural phenotype could be suppressed by a concomitant reduction in the dose of

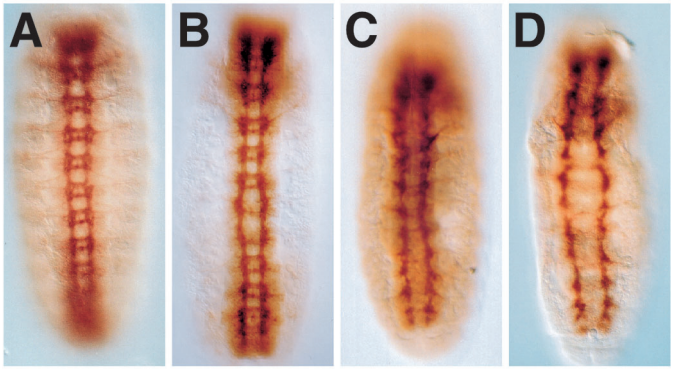

Fig. 2. *schizo* interacts with *frazzled* and *netrin*. Whole-mount antibody staining of stage-15/16 embryos using BP102 and subsequent HRP immunohistochemistry. Anterior is to the left. (A) Wild-type embryo. (B) Homozygous *schizo*^{U112} mutant embryos have reduced commissures. (C) *Df(1)netrin*/*schizo*^{U112} double-mutant embryo lacks most of the commissures compared with *schizo*^{U112} mutants. (D) In a *frazzled*^{23H9}/*schizo*^{U112} double-mutant embryo the axons cannot respond to the attractive signal secreted by the midline cells and almost all commissures are missing, indicating that *schizo* acts independently of the *netrin* pathway.

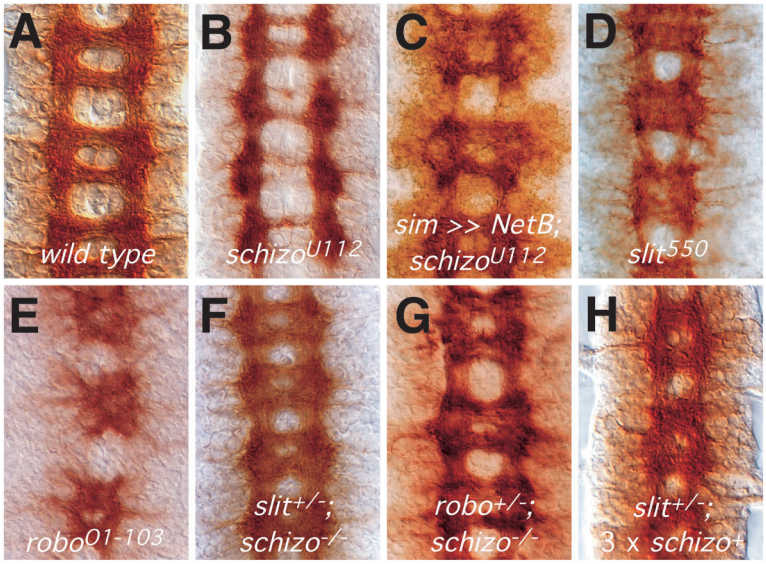

Fig. 3. *schizo* affects the balance between repulsive and attractive signaling. Frontal views of dissected central nervous system (CNS) preparations of stage-16 embryos stained for the presence of all CNS axons using Mab BP102. Anterior is up. (A) Wild-type embryo. Two commissures form in each neuromere. (B) *schizo* mutant embryo lacks many commissural tracts. (C) Expression of *netrinB* in all CNS midline cells of *schizo* mutant restores the commissural pattern. (D) Embryos homozygous for the hypomorphic $slit^{550}$ allele develop a characteristic fused commissure phenotype, which is similar to the loss of function *robo1* phenotype (E). (F,G) The *schizo* mutant phenotype is reverted by reducing the dose of *slit* or *robo*. (H) Overexpression of *schizo* using a translocation chromosome in a heterozygous *slit* mutant background also led to a fused commissure phenotype.

slit function. We have generated $slit^{-/+}$; $schizo^{-/-}$ as well as $robo^{-/+}$; $schizo^{-/-}$ embryos, and in both cases observed a suppression of the *schizo* CNS phenotype (Fig. 3D-G). Thus *schizo* might be required to negatively regulate Slit signaling.

If *schizo* is indeed a negative regulator of *slit* function, we may expect that an increase of *schizo* gene dose should result in a decrease of active Slit signaling. One might thus be able to enhance the mutant *slit* phenotype by using a *schizo* gene duplication. Following mapping of *schizo* to the base of the left arm of chromosome 3 we utilized a chromosomal translocation of the corresponding part of the third chromosome to the Y chromosome (*Tp(3;Y)A81*) to generate embryos with three copies of *schizo*. In an otherwise wild-type background, this triplication of the *schizo* region did not result in an abnormal CNS phenotype (data not shown). However, when we placed the *schizo* translocation in a heterozygous $slit^{-/+}$ background, we observed a *slit*-like phenotype that was never detected in heterozygous *slit* embryos (Fig. 3H).

schizo encodes an Arf-GEF

To further understand the function of *schizo*, we mapped the *schizo* locus to the chromosomal interval 78A/B using a series of deficiency chromosomes between the genes *poils aux pattes* and *knockout* (see Materials and methods). To identify the *schizo* gene in this chromosomal interval we used P-element-induced *schizo* alleles. The chromosomal insertions of the P-elements in *l(3)3* (kindly provided by A. Carpenter) and *P224* (generated in a local hopping experiment; see Materials and methods) were determined by inverse PCR and Southern analyses and suggested that *schizo* corresponds to *CG 32434* (Fig. 4A). The lethality associated with the P-element-induced *l(3)3 schizo* mutation could be reverted by precise excision of the P-element and mutant *l(3)3* embryos displayed a *schizo* phenotype with reduced commissures and defective fasciculation in the longitudinal connectives (not shown). Subsequent sequencing of cDNA clones LP01489, RE44556 and GH10594 isolated by the BDGP showed that the *schizo* locus encompasses 41 kb of genomic DNA. At least two different promoters direct the expression of two isoforms of 1325 amino acids (SchizoP1) and 1313 amino acids (SchizoP2) in length. Verification of the cloning of *schizo* was obtained by genetic rescue experiments (see below). These deduced *schizo* proteins correspond to the Iso1 and Iso2 variants of the *loner* gene, which was recently identified in a screen for mutations affecting mesoderm development (Chen et al., 2003). By contrast to Chen et al. (2003) we found that GH10594 is entirely contained within the LP01489 sequence and found no evidence for a third *schizo* protein isoform.

The deduced Schizo proteins share three conserved sequence modules. In the N-terminal region there is a so-called IQ domain, which is predicted to interact with calmodulin. Within the C-terminal third of the protein a Sec7 domain is directly adjacent to a PH-domain (Fig. 4B). Proteins characterized by such a domain signature are generally acting as guanine nucleotide exchange factors (GEFs). The *Anopheles* homolog is about 90% identical. The closest human homologs are EFA6 (Franco et al., 1999; Perletti et al., 1997), being 32% identical to Schizo, lacking the IQ domain, and ARF-GEP_{100} (Someya et al., 2001) showing a 40% identity to Schizo (Fig. 4B). Both human proteins were shown to act as ADP ribosylation factor 6 (ARF6)-GEFs (Franco et al., 1999; Someya et al., 2001) suggesting that *schizo* might have a similar function (see below).

The molecular identification of *schizo* allowed us to determine the expression pattern throughout development. *schizo* expression is already detected in the unfertilized egg, indicating a prominent maternal contribution (Fig. 4). *schizo* expression stays almost uniform until the end of stage 10. Within the developing nervous system, expression can be noted in the CNS midline cells. In addition, *schizo* expression can be detected in the epidermis and the visceral mesoderm (Fig. 4C-H).

schizo functions in the CNS midline to control commissural crossing

The commissural *schizo* phenotype does not allow the deduction of cell type in which Schizo normally acts. To test the cell-type requirement we employed the GAL4 system and established UAS-*schizo*P1 and UAS-*schizo*P2 transgenic flies. Expression of the different *schizo* proteins was directed in the CNS midline cells of mutant *schizo* embryos using the *sim-*

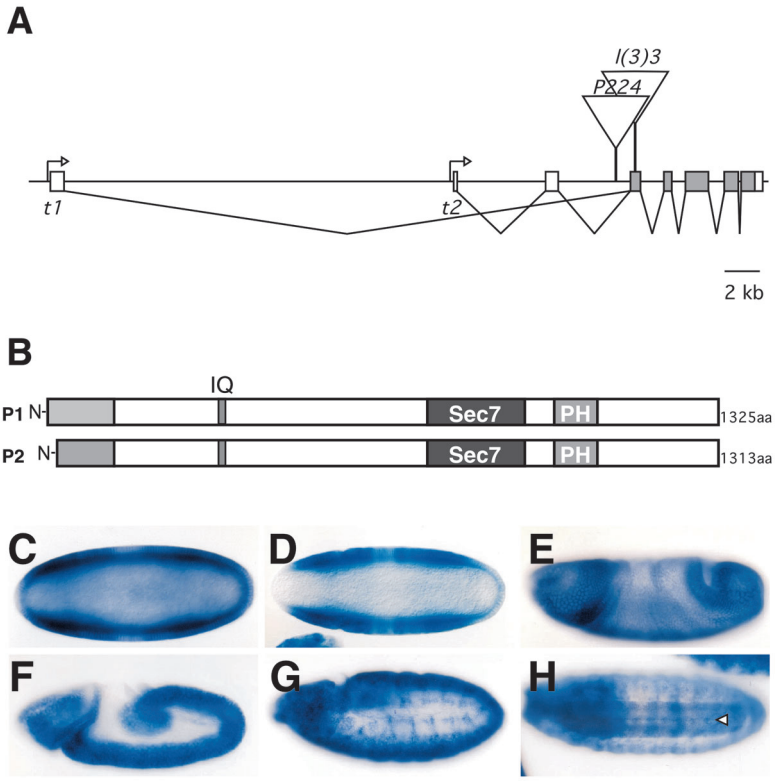

Fig. 4. *schizo* encodes two Arf-GEF-like proteins. (A) Schematic drawing of the *schizo* locus. Two transcripts are represented by EST clones identified by the BDGP (*t1* RE44556, *t2* LP01489). Transcription is from left to right. The location of two P-element insertions is indicated, both *l(3)3* and *P224* lead to a strong *schizo* mutant phenotype (data not shown). (B) The two deduced Schizo proteins are characterized by three sequence domains: an IQ domain, which might confer Calmodulin binding, a Sec7 and a Plecstrin homology (PH) domain, which are a signature of guanine exchange factors. (C) *schizo* is expressed maternally and appears to be negatively regulated by segmentation genes (D). (E,F) *schizo* is broadly expressed during early development. (G) At the extended germ band stage expression of *schizo* in the ectoderm is reduced. (H) *schizo* continues to be expressed in the central nervous system (CNS). The arrow denotes expression in the CNS midline.

GAL4 or *sli*-GAL4 driver strains (Scholz et al., 1997). In both cases expression could rescue the *schizo* mutant CNS phenotype indicating that Schizo acts in the midline glial cells, which express both Slit and Netrin (Fig. 5).

Schizo counteracts Slit function by promoting endocytosis

Genetic data indicated that *schizo* impairs Slit signaling in the CNS. This was further supported by overexpression of *schizo*. Whereas expression of *schizo* (P1 or P2) in all CNS midline cells of wild-type embryos did not evoke an abnormal phenotype, the same expression of *schizo* in heterozygous *slit* mutant embryos was able to induce a mild *slit* phenocopy (Fig. 6B). These results were similar to the ones obtained using chromosomal translocations, supporting the notion that Schizo acts in the CNS midline by regulating the level of Slit expression.

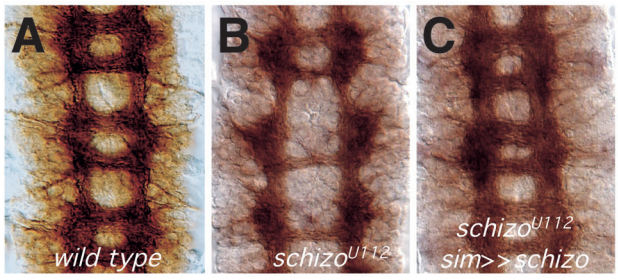

Fig. 5. *schizo* acts in the central nervous system (CNS) midline cells. Frontal views of dissected CNS preparations of stage-16 embryos stained for the presence of all CNS axons using Mab BP102. Anterior is up. (A) Wild-type embryo: note the regular arrangement of the Fas2-positive longitudinal fascicles. (B) The EMS-induced *schizo*^{U112} mutation leads to a lack of commissures. (C) Following expression of *schizo*t2 in all midline cells of a homozygous mutant *schizo* embryo, the mutant phenotype is rescued and commissures form normally.

To reduce Slit activity, *schizo* might suppress exocytosis of Slit-containing vesicles, or it might promote endocytosis of Slit-containing vesicles from the membrane. Work from vertebrate tissue culture models has shown that Arf-GEFs such as Schizo can activate endocytosis (Franco et al., 1999). To test whether endocytosis might be relevant for commissure formation we expressed a dominant negative Shibire protein that efficiently blocks endocytosis (Moline et al., 1999) specifically in the CNS midline cells using the *sim*-GAL driver. In about 60% of such embryos we observed a *schizo* phenocopy (Fig. 6C). When higher levels of the dominant negative Shibire protein were expressed using the *rho*-GAL driver all embryos developed a *schizo* phenocopy, suggesting that endocytosis participates in the regulation of Slit function in the CNS midline cells. To further support the notion that Schizo induces endocytosis of Slit we expressed the negative Shibire protein in a heterozygous *slit* mutant background. This indeed led to clear suppression of the Shibire-induced phenotype (Fig. 6D). Schizo and its vertebrate homologs exert at least part of the function through the small GTPase Arf6 (Franco et al., 1999; Someya et al., 2001; Chen et al., 2003). Arf6 mRNA is supplied maternally and is expressed ubiquitously during embryonic development (data not shown). To determine whether Schizo acts via Arf6 to control endocytosis of Slit by the midline glial cells we expressed a dominant negative Arf6 construct (Chen et al., 2003). Following expression in the midline cells using the *sim*-GAL driver we observed no mutant phenotype. Following expression of higher levels of Arf6^{DN} using the *rho*-GAL driver, about

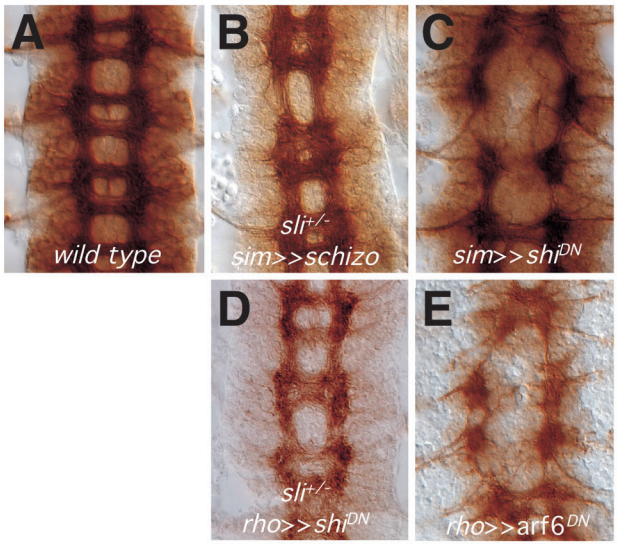

Fig. 6. Commissure formation requires endocytosis. Frontal views of dissected central nervous system (CNS) preparations of stage-16 embryos stained for the presence of all CNS axons using Mab BP102. Anterior is up. (A) Wild-type embryo. (B) Overexpression of *schizo* in all midline cells of a heterozygous *slit* embryo leads to a fused commissure phenotype. (C) Expression of a dominant negative form of Shibire in all CNS midline cells blocks endocytosis and leads to a *schizo*-like CNS phenotype. (D) Expression of a dominant negative form of Shibire in all CNS midline cells of a heterozygous slit animal does not impair commissure formation. (E) Expression of a dominant negative Arf6 protein in all CNS midline cells lead to *schizo* phenocopy.

15% of the embryos developed a *schizo* phenocopy (Fig. 6E), supporting the notion that Schizo acts via Arf6-regulated endocytosis to control the level of Slit expression on the midline glial cells.

Discussion

Here we have reported a functional characterization of the *Drosophila* gene *schizo*, which has been initially isolated, based on its requirement for commissure formation. Loss-of-function mutations in the *schizo* locus result in a reduced formation of commissures, suggesting that *schizo* either acts to attract growth cones toward the midline or to suppress the repulsive signals emanating from the CNS midline cells. Our genetic and molecular data support a model in which *schizo* negatively regulates the expression of Slit in the CNS midline cells.

Two major signaling molecules guide commissural growth cones

Over the last years a number of elegant genetic and biochemical studies have revealed the key components guiding commissural growth cones toward and across the CNS midline (Dickson, 2002). In all complex metazoan organisms two conserved major signaling molecules are involved. Netrin proteins are secreted by the CNS midline cells, from where they presumably form a gradient that helps to orient the growth of commissural axons toward the CNS midline (Tessier-Lavigne and Goodman, 1996). In addition, the CNS midline cells express a repulsive signal that is encoded by the gene *slit* (Brose and Tessier-Lavigne, 2000; Wong et al., 2002). Like Netrin, Slit is supposed to be secreted; however, antibodies directed against the C-terminal part always detect Slit on the membrane of the midline glial cells. Possibly the N-terminal portion of Slit may be released from the CNS midline to form a gradient similar to the one proposed for Netrin (Brose et al., 1999; Brose and Tessier-Lavigne, 2000; Schimmelpfeng et al., 2001). Given the phenotypic analyses, such a gradient appears probable (Murray and Whitington, 1999; Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b). Both signaling molecules act on conserved receptor systems, the activity of which is tightly regulated in a coordinated fashion (Stein and Tessier-Lavigne, 2001). Furthermore, both systems share downstream components, indicating that Netrin and Slit function is closely interwoven (Yu et al., 2002).

Regulation of *slit* expression

The expression of guidance cues is often dynamic and has to be developmentally regulated, either on a transcriptional or a post-transcriptional level. Recent data have identified transcriptional factors regulating the expression of Slit in the CNS midline cells. The initial formation of these cells requires the function of the PAS bHLH transcription factor *single-minded* (*sim*), which is also involved in the regulation of *slit* expression (Wharton and Crews, 1993; Wharton et al., 1994). In addition, the *slit* promoter also contains binding sites for the SOX HMG domain protein Fish-hook (Fsh) and the POU domain protein Drifter (Dfr) (Ma et al., 2000). All three genes have been shown to act in concert to regulate Slit expression (Ma et al., 2000).

An independent level of regulation of *slit* expression appears to be mediated by the transcription factor Lola, which affects the expression level of *slit* (Crowner et al., 2002). Similarly, the Slit receptors, which are encoded by the Robo gene family are subject to intense transcriptional control (Crowner et al., 2002; Zlatic et al., 2003).

Recently the relevance of post-transcriptional regulation of Roundabout by Commissureless was shown (Keleman et al., 2002; Myat et al., 2002). Commissureless was shown to act as a cytoplasmic sorting receptor for Roundabout, diverting it from the synthetic to the late endosomal compartment (Keleman et al., 2002). In the absence of Commissureless, all Roundabout is transported to the cell surface, where it binds Slit to induce repulsive signaling. Thus the *commissureless* mutant phenotype, which is characterized by a loss of axon commissure, can be explained by an increased repulsive signaling originating from the CNS midline.

Here we have shown that *schizo* acts in a rather similar way to *commissureless*; however, rather than affecting the Roundabout receptor, *schizo* appears to act on the expression of the Slit ligand. First we found that the triplication of the *schizo* gene interfered with *slit* function and that reduction of *slit* expression in *schizo* mutant embryos rescued the *schizo* mutant phenotype. Finally, expression of a *schizo* transgene in the Slit-expressing CNS midline cells (1) was able to rescue the *schizo* mutant phenotype and (2) could induce a *slit* phenocopy when expressed in wild-type embryos. The deduced nature of the Schizo protein suggests that it affects Slit expression by post-transcriptional mechanisms.

Schizo, an Arf6-GEF, regulates ligand presentation

Guanine-nucleotide exchange factors (GEFs) help to convert the inactive GDP-bound form of small GTPases into a GTP-bound active form. Schizo is a new Sec7 domain containing GEF, which shows 40% homology to human Arf-GEP$_{100}$. Arf-GEP$_{100}$ localizes to endosomal membranes (Someya et al., 2001) and promotes GDP/GTP exchange on ARF6. The small GTPase ARF6 is a plasma membrane-localized protein and functions in the regulation of membrane ruffling, cell motility, aspects of endocytosis and exocytosis, membrane recycling, reorganization of the cortical actin cytoskeleton and activation of phospholipase D (Kondo et al., 2000; Radhakrishna et al., 1999; Randazzo et al., 2000; Turner and Brown, 2001). In *Drosophila* Arf6 is remarkably well conserved, being more than 96% identical to the human counterpart (not shown).

One aspect that might hint at how Schizo regulates Slit expression is the role of ARF6 in endocytosis and exocytosis. The function of ARF6 in endocytosis is twofold. It either regulates clathrin-mediated endocytosis at the apical surface of polarized epithelial cells (Altschuler et al., 1999; Palacios et al., 2001) or it is able to regulate non-clathrin-mediated endocytosis and the recycling pathway in non-polarized cells (Brown et al., 2001). ARF6 has also been postulated to play a role in Ca^{2+}-activated dense core vesicle (DCV) exocytosis by regulating phosphatidylinositol(4,5) biphosphate (PIP_2) (Aikawa and Martin, 2003). Overexpression of a UAS-ARF6 construct in midline glia cells does not result in a *schizo*-like phenotype (data not shown), whereas expression of a dominant negative form of Arf6 results in a phenocopy of several phenotypes associated with the *schizo* mutant (Fig. 6) (Chen et al., 2003). This suggests that Arf6 might also be involved in the regulation of Slit expression.

Coordinated expression of Slit and Netrin

In-vivo Slit and Netrin are both expressed by the same CNS midline cells and their expression needs to be in an intricate balance. The importance of this balance and not the individual expression levels is highlighted by the fact that we were able to rescue the *schizo* mutant phenotype by both increased Netrin expression or reduced Slit expression. Within the midline glia, however, Schizo appears to primarily affect Slit expression either by inducing its endocytosis and subsequent degradation or by blocking exocytosis and thus release of Slit.

The latter case would suggest that Slit and Netrin are brought to the membrane of the midline glial cells in distinct vesicle populations, whereas the former case would require a specific membrane receptor for the Slit protein expressed by the CNS midline glia. Given the fact that the secreted Slit protein is found at very high levels at the midline glial cell membrane, this appears probable. Moreover, expression of a dominant negative Shibire protein in the midline glia leads to a *schizo* phenocopy. *shibire* encodes the *Drosophila* dynamin and is required for endocytosis and a block of *shibire* function leads to a block of endocytosis (Moline et al., 1999), which might result in higher levels of Slit expression. Thus, regulation of membrane dynamics appears crucial in controlling the function of the signaling molecule Slit.

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