Rhomboid 3 orchestrates Slit-independent repulsion of tracheal branches at the CNS midline

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Accepted 27 April 2004

Development 131, 3605-3614
Published by The Company of Biologists 2004
doi:10.1242/dev.01242

Summary

EGF-receptor ligands act as chemoattractants for migrating epithelial cells during organogenesis and wound healing. We present evidence that Rhomboid 3/EGF signalling, which originates from the midline of the *Drosophila* ventral nerve cord, repels tracheal ganglionic branches and prevents them from crossing it. *rho3* acts independently from the main midline repellent Slit, and originates from a different sub-population of midline cells: the VUM neurons. Expression of dominant-negative Egfr or Ras induces midline crosses, whereas activation of the Egfr or Ras in the leading cell of the ganglionic branch can induce premature turns away from the midline. This suggests that the level of Egfr intracellular signalling, rather than the asymmetric activation of the receptor on the cell surface, is an important determinant in ganglionic branch repulsion. We propose that Egfr activation provides a necessary switch for the interpretation of a yet unknown repellent function of the midline.

Key words: *Drosophila*, *ru*, *Egfr*, Epithelial migration, VNC midline

Introduction

Cell migration is an essential process in epithelial morphogenesis. During organogenesis, epithelial cells adhere to specific substrates and move along distinct tracks to give rise to the characteristic form and shape of tissues. Cell-cell signalling and interactions with the extracellular matrix control several different steps in the migration process: the polarisation and extension of cellular protrusions, the generation of propelling force, and the selective adhesion to neighbouring cells and the extracellular matrix (Ridley et al., 2003). Epidermal Growth Factor receptors (Egfrs) regulate cell migration in a variety of systems (Blay and Brown, 1985; Chen et al., 1994; Li et al., 1999; Tokumaru et al., 2000; Caric et al., 2001; Duchek and Rorth, 2001). For example, they mediate chemotactic migration in the mouse telencephalon (Caric et al., 2001), help attract keratinocytes to the sites of wound healing (Tokumaru et al., 2000) and mediate attraction for a variety of cultured cells (Wells, 2000). In isolated cells, ligand-bound Egfr can be directly visualised at the source side of a cell exposed to a gradient of EGF, while the cell is moving towards the source of ligand (Bailly et al., 2000). The single Drosophila *Egfr* is required for the attraction of a group of specialised follicle cells, the border cells, towards the oocyte (Duchek and Rorth, 2001; Duchek et al., 2001). Egfr activation in border cells leads to the extension of long, actin-rich processes towards the source of the Gurken ligand coming from the oocyte, and to the subsequent movement of the cells towards the oocyte (Duchek and Rorth, 2001; Fulga and Rorth, 2002). Thus, Egfr mediates chemoattraction in a large variety of systems where it acts by direct modulation of the dynamic cytoskeletal organisation of the migrating cells (Rorth, 2002; Montell, 2003). We describe an unexpected role for the *Drosophila Egfr* in mediating repulsion of epithelial cells of the tracheal system.

The morphogenesis of the embryonic tracheal (respiratory) network depends on the charted migration of ~2000 epithelial cells deriving from 20 epidermal invaginations. These cells undergo three successive rounds of branching to generate a tubular network that extends along stereotyped paths towards specific target tissues. The last branching event produces thin, unicellular terminal branches that associate with distinct organs (Samakovlis et al., 1996a; Manning and Krasnow, 1993). The ventral nerve cord (VNC) is invaded by 20 ganglionic branches (GBs), which sprout from the lateral trunk of the trachea. GB migration towards and inside the CNS is highly stereotyped and has been described in detail elsewhere (Englund et al., 1999). Each GB initially tracks along the inter-segmental nerve and towards the CNS. GB1, the leading cell of the ganglionic branch, enters the nerve cord and changes substrate to track along the segmental nerve, proceeding ventrally on top of the longitudinal fascicles and towards the CNS midline. Finally, after reaching the midline, GB1 takes a sharp turn and migrates dorsally through the dorsoventral channel and then turns posteriorly on the dorsal side of the VNC. At the end of embryogenesis, GB1 will have trailed a remarkable 50 μm inside the CNS. Genetic analysis has uncovered a number of factors that are necessary for this fixed migratory path: the FGF homolog Branchless is required to guide the GBs towards the CNS and to induce them to enter it (Sutherland et al., 1996), in part by inducing the expression of the nuclear protein Adrift

(Englund et al., 1999). Once inside the CNS, Slit (Rothberg et al., 1988), the main repulsive cue for axons at the midline, becomes a key guiding cue for the migrating GBs. Slit controls several, distinct aspects of ganglionic branch pathfinding into the CNS: it is first required to attract GBs towards the CNS, an effect mediated by its receptor Robo2, and then to prevent GBs from crossing the midline once they reach it, which is mediated by Robo (Englund et al., 2002).

To identify additional signals that steer GB1 migration, we screened a collection of P-element insertions for GB pathfinding phenotypes. One of the strains recovered showed a specific GB1 midline-cross phenotype reminiscent of *robo* or *slit* mutants, but unlike mutants in the *slit* pathway, had no defects in axonal pathfinding. The mutation was found to affect *roughoid/rhomboid 3* (*ru* – FlyBase) an intramembrane protease that activates Egfr ligands (Wasserman et al., 2000). Our analysis indicates that Rhomboid 3 defines a new signalling centre for tracheal repulsion from the midline. Rho3 is expressed by the VUM midline neurons, where it activates an Egfr ligand. *Egfr* and *Ras* but not *Raf*, *yan* or *mbc*, are required in GB1 for its turn away from the midline. The analysis of loss-of-function and overactivation phenotypes suggests that EGF itself is not a chemorepellent for GBs, instead it appears to provide a necessary activation switch for the interpretation of a yet unknown, Slit-independent, repellent function of the midline.

Materials and methods

Fly strains and genetics

The *inga* P-element insertion line is from the third chromosome collection from the Department of Genetics (University of Szeged, Hungary) (Deak et al., 1997). Ganglionic branch phenotypes identical to those seen in *inga* were also observed in *inga/Df(3L)Ar14-8* (breakpoints 61C5-8, 62A8), in the hypomorphic allele $rough oid^{1}$ (Lindsley and Zimm, 1985) and in $rho3^{pllb}$, a molecularly defined deletion that removes the entire *rho3* locus (Wasserman et al., 2000). The phenotypic analysis and all genetic experiments were performed on $rho3^{pllb}$. Other strains used include: $slit^{GA20}$, a protein-null *slit* allele (Battye et al., 2001); $robo^{GA285}$ [also known as $robo^{1}$ (Kidd et al., 1998)]; and *P{LacW}spi*^{s3547}, $mbc^{Dll.2}$, mbc^{C1}, sl^{2} and sl^{3} (all described in FlyBase). Genetic interaction experiments were performed by balancing the relevant alleles to CyO ftz-*lacZ* and crossing these strains to $rho3^{pllb}$ or to wild type (Oregon-R). Antibody staining against β-Gal allowed the identification of genotypes in embryos. The following GAL4 and UAS strains were used: SRF-Gal4 (Jarecki et al., 1999); sim-Gal4 (Menne et al., 1997); slit-Gal4 (Battye et al., 1999); elav-Gal4 (Luo et al., 1994); UAS-NLS:lacZ and UAS-EgfrDN (Freeman, 1996); UAS-RasN17 (Lee et al., 1996); UAS-RasV12 (Halfar et al., 2001); UAS-RafDN (de Celis, 1997); UAS-YanAct (Rebay and Rubin, 1995); UAS-Rho1 (Bier et al., 1990b); UAS-Rho3 (Wasserman et al., 2000); UAS-λ top 4.2 and UAS-λ top 4.4, two independent insertions of the same transgene, in which the Egfr extracellular domain is substituted by the λ-repressor dimerisation domain) (Queenan et al., 1997); UAS-tor^{4021}, a constitutively active form of the Egfr (Lai et al., 1995); UAS-Δp60, a deletion variant of the adaptor protein p60 which has dominant negative effect on PI3-K activity (Weinkove et al., 1999); and UAS-Ricin (Hidalgo et al., 1995). All Gal-4/UAS experiments where performed at 29°C to optimise Gal4 activity. Typically, embryos were collected for 8 hours at room temperature and then staged for 10 hours at 29°C. Embryos were scored by classifying each ganglionic branch in one of the following classes: migrating normally, not entering the CNS, arrested inside the CNS, arrested at the midline, misrouted and crossing the midline.

Molecular identification of the *inga* locus

The *inga* P-element line contains a single P-element, as analysed by Southern blot. Genomic DNA flanking the P-element was obtained by plasmid rescue in *E. coli* after cleavage of the genomic DNA with *Eco*RI or *Bam*HI. This DNA was sequenced and used to search the databases. The search identified also a cDNA clone that was obtained from Research Genetics: LP02893. The *inga* P-element was inserted at position 137648 of the Celera contig AE003741 and 345 bp upstream of the first exon of the cDNA LP02893.

Antibodies, embryo staining and whole–mount in situ hybridisation

Embryo fixation, antibody staining, light and confocal fluorescence microscopy were performed as described previously (Samakovlis et al., 1996b). Primary antisera were anti-β-galactosidase (diluted 1:1500, Cappel), mAb1D4 against Fasciclin 2 [1:10 (Van Vactor et al., 1993)], mAb BP102 that labels all CNS axons (1:50), mAb2A12 against tracheal lumen (1:5), mAb22C10 labelling a subset of CNS and PNS axons [1:20 (Zipursky et al., 1984)], anti-Repo (1:5), mouse anti-Wrapper [1:10 (Noordermeer et al., 1998)], mouse anti-Robo [1:10 (Kidd et al., 1998)] and mouse anti-Slit [1:10 (Rothberg et al., 1990)], each obtained from the Developmental Studies Hybridoma Bank at The University of Iowa. Secondary antibodies included biotin (1:300), Cy2 (1:200), Cy3 (1:300) and Cy5 (1:200) conjugates (Jackson Laboratories), and Alexa Fluor-594 (1:400) and -488 (1:200) conjugates (Molecular Probes). When necessary, the signal was developed using Vectastain Elite ABC Kit (Vector Laboratories). Embryos were visualised with a Zeiss Axioplan2 microscope under Nomarski optics or a Zeiss confocal microscope. Confocal stacks were processed using the Volocity software (Improvision) to obtain three dimensional reconstructions. Whole-mount in situ hybridisation was performed using random-primed, digoxigenin-labelled *roughoid/rho3* cDNA (LP02893, Research Genetics) as a probe; embryo staging was according to (Campos-Ortega and Hartenstein, 1985).

Results

roughoid/rhomboid 3 is required for repulsion of ganglionic branches from the VNC midline

A collection of 2640 P-element insertions (Deak et al., 1997) was screened for mutants affecting the pathfinding of the ganglionic branch (GB) into the CNS. One of the recovered mutants, which we named *inga* (from *ingen återvändo*, meaning ‘no turning back’ in Swedish) was characterised by a specific midline-cross phenotype: at stage 16.3-4, upon approaching the CNS midline, a significant number of *inga* GBs failed to turn posteriorly and dorsally at the midline and crossed to the other side, or remained lingering on it. We could not detect any other defect in the tracheal system of *inga* embryos. Sequence analysis of the genomic region surrounding the transposon in *inga* mutants showed that the P-element was inserted in the previously described *roughoid*/*rho3* locus (Wasserman et al., 2000) (see Materials and methods), and all available *ru*/*rho3* alleles as well as *inga*/Df(3L)Ar14-8 embryos (a chromosomal deficiency removing the 61-62 region), showed the same tracheal phenotype as *inga*. Therefore, we concluded that *inga* is an allele of *rho3* and focused our subsequent analysis on a previously characterised null allele, $rho3^{pllb}$ (Wasserman et al., 2000). In $rho3^{pllb}$ embryos, penetrance of the midline cross phenotype was close to complete, so that 45 of the 48 embryos analysed had at least one midline cross. On average, two midline crosses where observed per embryo, in total 10% of the GBs failed to turn at

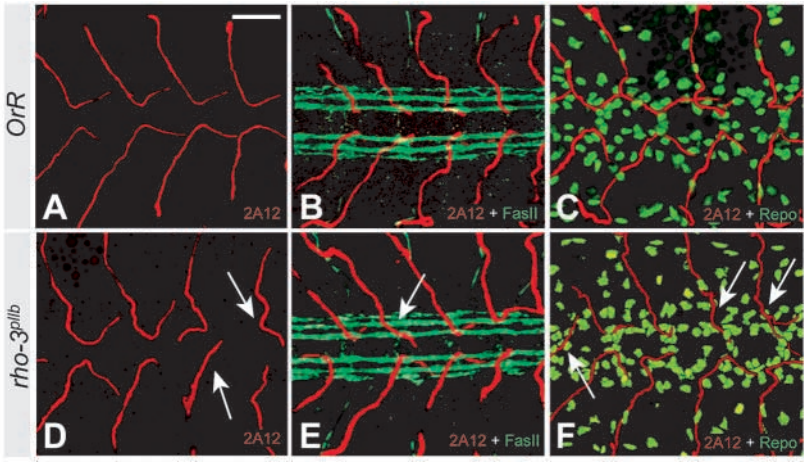

Fig. 1. *rho3* affects CNS midline repulsion of tracheal ganglionic branches (GBs). (A-F) Three dimensional confocal reconstructions of late stage16 embryos double-stained to reveal the tracheal lumen (by mAb2A12, A-F in red) and longitudinal fascicles (by mAb1D4, B, E in green) or CNS glia (with the exception of midline glia, by anti-Repo C, F in green). All panels show ventral views, anterior towards the left. In wild-type embryos, GBs (A, red) and longitudinal fascicles (B, green) never cross the midline. In *rho3*, an average of two GBs per embryo cross the midline (D-F, arrows). In the same embryos the longitudinal fascicles appear unaffected (green in E and compare with B), as does the pattern of glial cells (F, compare with C). Scale bar: 20 μm.

the midline (*n*=960, Fig. 1). No midline crosses were ever found in wild type (Oregon R, *n*=837). Additionally, 3% of GB1s stalled upon reaching the midline in the mutant. An important function of midline cells is to provide repellent signals for the migration of the axons of the longitudinal connectives. In *rho3*^{pllb} we did not detect any midline crosses of the longitudinal axons, stained for Fasciclin 2 (Fig. 1E). The route of GB1 inside the VNC is demarcated by extensive contacts with different groups of glial cells (Englund et al., 2002), and we therefore determined the position and integrity of these landmark cells with two different markers in *rho3* mutants. The transcription factor Repo is expressed in most of the glial cells (including exit, longitudinal and subperineural glia) (Halter et al., 1995). Midline glia, however, selectively express the cell surface protein Wrapper (Noordermeer et al., 1998). Repo and Wrapper staining of *rho3*^{pllb} mutant embryos were analysed by confocal microscopy and three-dimensional reconstruction and did not reveal any alterations in the expression patterns of Repo and Wrapper (Fig. 1 and data not shown). We conclude that rho3 is specifically required for the pathfinding of the ganglionic branches at the VNC midline.

rho3 and *slit*, act in parallel to prevent ganglionic branches from crossing the midline

Rhomboid 3 is an intramembrane protease required for the activation of the Spitz Egfr ligand during eye development (Wasserman et al., 2000). It is unclear if Rhomboid type proteases are restricted to Egfr signalling in flies or if they may be able to process other targets, and the tracheal *rho3*^{pllb} phenotype is similar to the GB1 midline crosses of *slit* and *robo* mutants (Englund et al., 2002). For this reason, we set out to determine whether *rho3* and *slit* may act in the same pathway during GB guidance. We first asked whether the amount or localisation of Slit protein might be affected at the VNC midline of *rho3* mutants. Wild-type and *rho3* embryos were stained with an antibody against Slit (Rothberg et al., 1988) and analysed by confocal microscopy. We did not detect any changes in the staining pattern of Slit in *rho3* mutants, suggesting that *rho3* is not required for Slit expression (Fig. 2). In order to establish if *rho3* may be part of Slit signalling or act in parallel to Slit in GB repulsion, we made a double mutant of *rho3* and a *slit* null *allele* (*slit*^{GA20}) and quantified the ganglionic branch migration phenotypes. In *slit* mutants, 46% of the ganglionic branches cross the midline, the rest of the GBs either stalled outside (11%) or appeared misrouted inside the VNC (Fig. 2). In *slit*; *rho3* double mutants, the number of branches crossing the midline was significantly increased (66%, *n*=260) but the proportion of branches arrested outside the VNC did not change (12%, Fig. 2). Thus, the GB1 phenotypes of *rho3* and *slit* were additive, arguing that the EGF and Slit signalling pathways act independently on GB repulsion.

rho3 is expressed by VUM midline neurons

To investigate the role of *rho3* in midline repulsion of GBs, we analysed its site of expression in relation to GB migration inside the VNC. *rho3* expression has been previously described only in larval eye-antennal imaginal disc (Wasserman et al., 2000). We analysed the expression of *rho3* in embryos by whole-mount in situ hybridisation with a *rho3* cDNA probe (LP02893). *rho3* was dynamically expressed in embryos, in a subset of cells of the peripheral nervous system (not shown) and, more weakly, in the CNS. The expression in the VNC became detectable from stage 13 in a segmental pattern at the midline (not shown). Until the end of stage 15, weak but reproducible expression could be seen in group of a few midline cells positioned ventrally in each segment of the VNC (Fig. 3B, arrow). By stage 16, the CNS expression appeared more diffuse (not shown). To define the cell type expressing *rho3*, and its spatial relationship to the source of Slit, we made use of the enhancer trap insertion *rho3*^{inga}, which harbours the β-galactosidase reporter. The enhancer trap essentially confirmed the pattern observed by in situ: β-gal was detected mainly in the lateral chordotonal organs of the peripheral nervous system (not shown) and in the CNS. Within the nerve chord, β-gal expression was detected in a distinct cluster of midline cells in each segment, positioned ventrally to the commissures and, more weakly, in the tip cell of each ganglionic branch (Fig. 3A, Fig. 5, arrowheads). Three-dimensional reconstructions of confocal stacks derived from staining with cell type-specific markers revealed the identity of the *rho3*-expressing cells as the ventral unpaired group of midline neurons (VUM). VUMs cells bodies are identifiable by the expression of the cytoskeletal protein Futsch, recognised by 22C10 (Fig. 3). VUMs cell bodies are placed ventral and slightly posterior to midline glia (visualised with Wrapper in

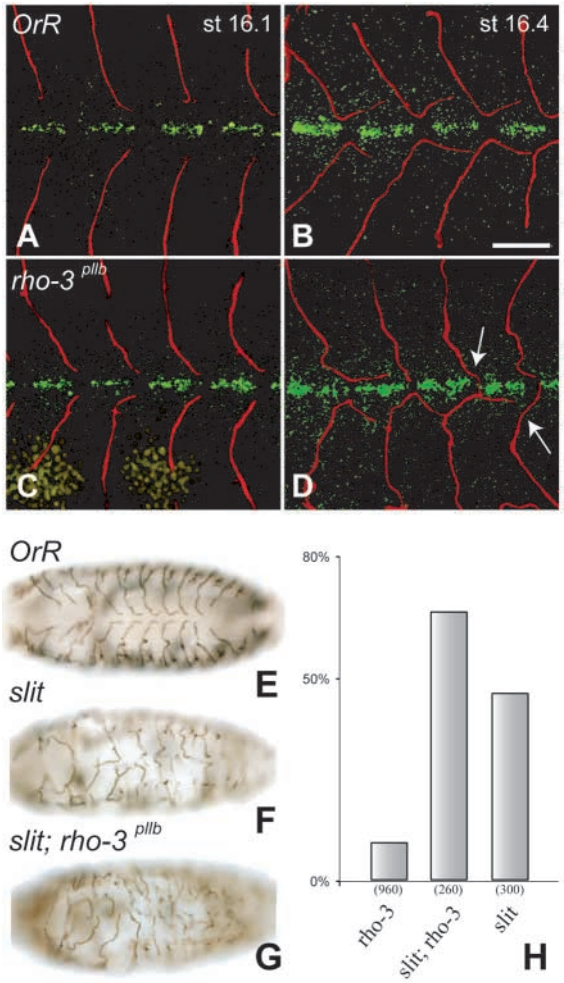

Fig. 2. *rho3* acts independently of *slit*. (A-D) Slit protein levels are not changed in *rho3* mutants. An anti-Slit antibody (in green; red is the tracheal lumen, mAb2A12) detects similar levels of Slit in early (C) and late (D) *rho3* stage 16 embryos when compared with wild type (A,B), even in the vicinity of branches that cross the midline in the mutant (D, arrows). A-D are ventral views, anterior towards the left. Scale bar in B: 20 μm. (E-G) A ventral view of late stage 16 embryos stained for the tracheal lumen (by mAb2A12) reveals GB pathfinding defects in *slit* mutants (F) and *slit, rho3* double mutants (G) when compared with wild type (E, anterior is towards the left). (H) Quantification of the midline cross phenotype shows that the effect of the two mutations is additive.

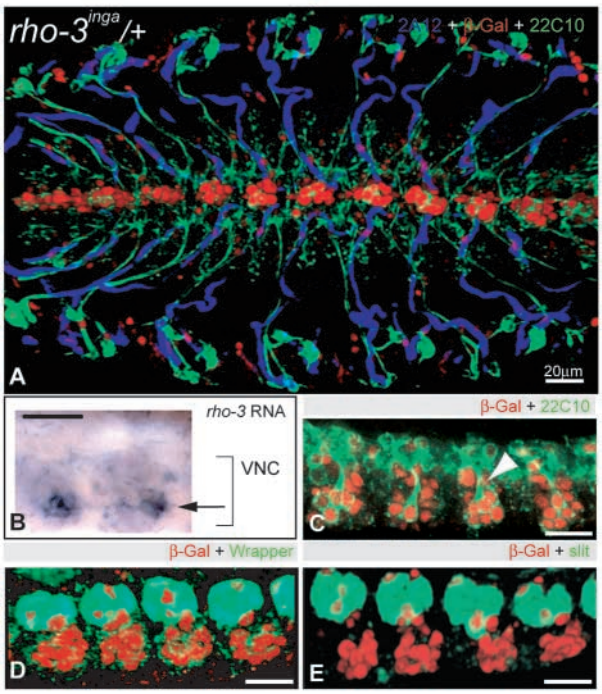

Fig. 3. *rho3* is expressed by the ventral unpaired group of midline neurons (VUM). (A) Ventral view of late stage 16 *inga* heterozygote embryo triple stained to reveal the tracheal lumen (mAb2A12 in blue), a subset of PNS and CNS axons (mAb22C10 in green), and the expression of β-Gal in the *rho3*^{inga} enhancer trap (anti-β gal in red). The panel shows a 3D reconstructions deriving from a confocal stack, anterior is towards the left. Strong β-gal expression is detected in each segment in ventral clusters of cells at the midline. (B) A similar expression pattern is detected by in situ hybridisation with a specific *rho3* cDNA probe at stage 15, in clusters of cells at the VNC ventral midline (B, arrow; B-E are lateral views of the VNC, anterior towards the left, ventral downwards; B is a stage 15 embryo, C-E are late stage 16). (C-E) 3D confocal reconstruction allows the identification of the *rho3*-expressing cells (in red, stained by anti-β-gal) as the VUM neurons. VUM cell bodies are readily stained by mAb22C10 (C, in green), which also allows identification of the characteristic VUM axonal tract (C, arrowhead). Slit staining (E, in green) and Wrapper staining (D, in green) of midline glia, shows little overlap with β-gal expression in *rho3*^{inga} (D,E, in red). Scale bars: 20 μm.

Fig. 3), which are the source of Slit within the midline (Fig. 3) (Rothberg et al., 1988). Frequently, one or two midline glia also seemed to express variable levels of β-gal (Fig. 3).

The site of expression of *rho3* defines the source of a midline repellent signal for GB1. Given the known role of *rho3* as an Egfr ligand activator in vivo and in vitro (Wasserman et al., 2000; Campbell, 2002; Urban et al., 2002), we were prompted to investigate whether this repulsive signal may be one of the *Drosophila* Egfr ligands.

Egfr signalling is required in GBs for midline repulsion

Egfr signalling is known to be required at several steps in the development of the CNS, including the specification and survival of the midline glia (Klambt et al., 1991), which then provide a source of signals that guide neuronal and tracheal migration inside the VNC. The early functions of EGF signalling make the analysis of mutations in components of the pathway difficult to interpret in the context of the migration of GB1 at late stage 16. As an example, a hypomorphic mutation in the main Egfr ligand, *spitz* (Rutledge et al., 1992) resulted in severe GB midline crossing defects (Fig. 4A), but also caused major defects in axonal migration (Fig. 4C), as Spitz is essential for both midline glia differentiation and survival (Klambt et al., 1991). To overcome the problem of secondary effects, we made use of a Gal4 strain that exclusively activates gene expression in the terminal cells of all tracheal branches (SRF>Gal4) (Jarecki et al., 1999) (Fig. 4E), to express a series of dominant-negative and activated constructs of Egfr pathway components. Expression of SRF (*pruned*, *blistered*) (Affolter

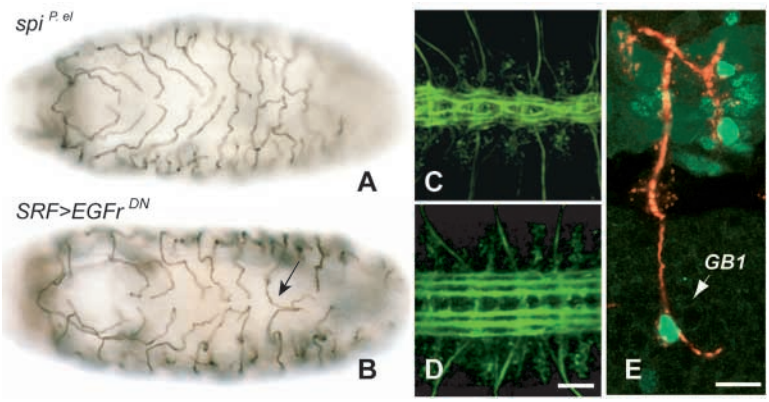

Fig. 4. The Egfr mediates midline repulsion of GB1. (A,B) A ventral view of late stage 16 embryos stained for the tracheal lumen (by mAb2A12). Many GB midline crosses are observed in *spi* mutants (A). (C,D) *spi* mutants also lack a functional midline. (C) In *spi*, longitudinal fascicles collapse on the midline (stained by mAb1D4; compare with D, wild type; all panels show ventral views, anterior towards the left). (B,E) SRF-Gal4 drives gene expression specifically in tracheal tip cells. (E) A single GB is shown that expresses UAS-NLS:*lacZ* in the GB1 nucleus (arrow) under the control of SRF-Gal4 (mAb2A12 stains the tracheal lumen in red, anti-β-gal is green). (B) A ventral view of a late stage 16 embryo expressing dominant-negative Egfr, showing misroutings and midline crosses.

et al., 1994; Guillemin et al., 1996) is activated at stage 14 and is a marker for GB1 terminal differentiation; therefore, the expression of transgenes under the control of its promoter should not interfere with earlier cell specification events in GB1.

SRF>Gal4 driven expression of a dominant negative form of the EGF receptor (Freeman, 1996) had a profound effect on the migratory behaviour of GBs. Forty-two percent of the GBs were affected (*n*=240), including 9% of stalled and 25% of misrouted branches. Importantly, 5% of GBs crossed the midline (Fig. 6E) and an additional 3% arrested upon reaching it (not shown). The range of phenotypes observed upon expression of the dominant-negative receptor suggested that Egfr signalling might have an additional, earlier, role in GB guidance towards the midline that is independent from *rho3*. The midline crossing phenotype induced by the terminal cell-specific expression of the dominant-negative construct suggested that *rho3* expression VUMs activates an Egfr ligand, which guides GBs away from the midline through the activation of the EGF receptor.

A potential cell-autonomous role for *rho3* in GB1

Egfr signalling in GB1 determines the turning response of the tracheal cells at the midline. A number of observations prompted us to test the potential role of the migrating GBs as signalling source. The *inga* enhancer trap in *rho3* showed a weak but reproducible expression of *lacZ* in GB1 from stage 15 (Fig. 5A). In addition, the overexpression of Rho1 in the terminal cells produced a strong phenotype in tracheal migration (see below), implying that GB1 is endowed with all the components required for the secretion of an active EGF signal.

To explore whether part of the *rho3* mutant phenotypes are due to its expression in the tracheal cells, we attempted to ablate the GB1s by the expression of Ricin A. If signalling deriving from GB1s was important for their own migration then the ablation of some or most GB1s might result in misrouting of the few remaining ones that escape ablation. However, if GB1 signals were important for the VNC cells, then the ablation of GB1 might cause abnormalities in the pattern and migration of the glia and neurons.

Embryos expressing UAS-RicinA (Hidalgo et al., 1995) under the control of SRF-Gal4 showed a normal appearance of glial populations and axonal tracts (as detected by Repo, Wrapper and Fasciclin 2, respectively Fig. 5). Thus, if any signalling occurs from GBs to CNS cell populations, it appears unnecessary for the patterning of the latter. Moreover, the few GB1s that escaped ablation, presumably owing to mosaic Gal4 expression, migrated normally (Fig. 5, arrows), implying that GBs are unlikely to signal to each other during their migration in the CNS.

To address the potential tissue-specific requirement of *rho3* during GB migration, we expressed the close relative Rho1 in different populations of CNS cells and in GB1s, and assayed the extent of rescue of the *rho3* tracheal phenotype. SRF-directed expression of Rho1 was not sufficient to significantly rescue the midline cross phenotype of *rho3* ganglionic branches (Fig. 5E). By contrast, the same transgene expressed under the control of three CNS-specific Gal4 strains provided a significant rescue of the GB phenotypes (Fig. 5E). *single minded*-Gal4 is initially expressed by all midline cell precursors, but becomes later restricted to midline glia (Scholz et al., 1997); *slit*-Gal4 is limited to midline glia (Scholz et al., 1997). Both *sim*- and *slit*-Gal4 directed expression of Rho1 approximately halved the occurrence of GB midline crosses in the *rho3* mutant (Fig. 5E). *elav*-Gal4 is expressed in all post-mitotic neurons but not in midline glia (Lin and Goodman, 1994). Strikingly, *elav*-Gal4 directed expression of Rho1 suppressed the *rho3* GB midline cross phenotype to 1% (Fig. 5E). We conclude that Rhomboid signalling is required in the CNS, rather than in the GB1 itself, to prevent ganglionic branch midline crossing. The weak *rho3* expression in GB1 might be part of a positive feedback loop, a common feature of Egfr signalling in flies.

Ras, but not Raf or Yan, mediate GB repulsion from the midline

Egfr signalling is mediated by a number of downstream effectors in different cell types. In order to determine which one is used in GB1 pathfinding, we analysed a panel of mutants and dominant-negative constructs of known downstream effectors for their effect on GB migration. *myoblast city* (*mbc*) (Rushton et al., 1995) is a conserved adaptor necessary for the chemo-attractant function of Gurken during border cell migration in the ovary (Duchek et al., 2001). *mbc* alleles had no defects in GB pathfinding (data not shown). As *mbc* has negligible maternal contribution and is not readily detected in tracheal tissues (Erickson et al., 1997), we conclude that it is unlikely to have a role in Egfr-mediated GB repulsion from the midline. We also tested two additional effectors that have been

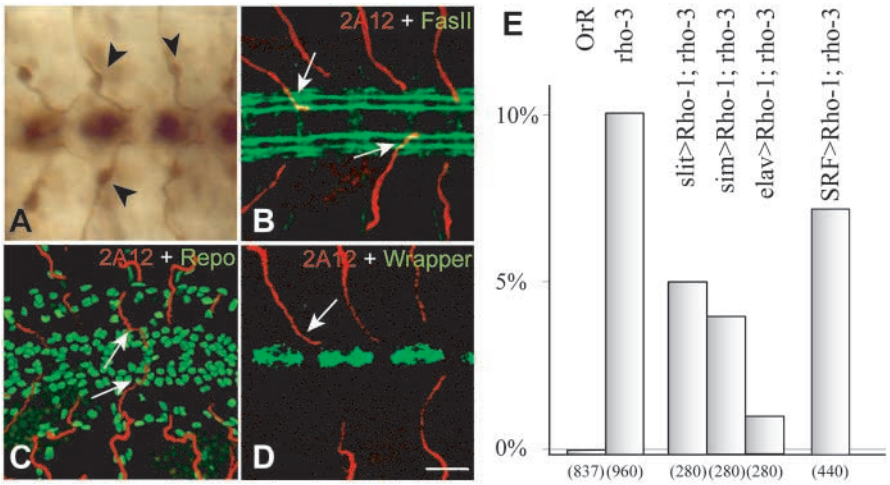

Fig. 5. Rhomboid is required in the CNS to prevent GB midline crossing. (A) The *inga* enhancer trap showed weak, but reproducible, reporter gene expression in GB1. Ventral view of an *inga*/+ embryo, double stained for β-gal and the tracheal lumen (mAb2A12, both in brown). GB1 expression is shown (arrowheads in A). (B-D) RicinA ablation of most of all GB1 cells (in a SRF>RicinA embryo) did not affect longitudinal fascicles (stained by anti-Fas2 in green, B) and glial populations (green in C,D; stained by anti-Repo and anti-Wrapper respectively, all panels show ventral views). Scale bar in D: 20 μm. In addition, isolated surviving GBs migrate correctly (B-D arrows). (E) Rhomboid expression by CNS cells, but not by GB1, rescues the *rho3* GB midline cross phenotype. The table represents the frequency of midline crosses for the different genotypes. Expression of Rho1 in midline glia (by *slit*-Gal4) or in midline cells (by *sim*-Gal4) halved the frequency of GB midline crosses in *rho3*, whereas expression in all CNS neurons (by *elav*-Gal4) substantially rescued the *rho3* midline cross phenotype. Expression of the same transgene in GB1 (by SRF-Gal4), did not produce convincing rescue.

implicated in Egfr-elicited migratory responses in other systems, PLCγ and PI3K (reviewed by Schlessinger, 2000). The fly PLCγ is encoded by the *small wing* (*sl*) locus (Thackeray et al., 1998). *small wing* embryos had extra terminal sprouts emanating from the primary tracheal branches but showed no specific defects in GB migration inside the VNC (data not shown). Δp60 is a deletion variant of the adaptor p60, which has dominant-negative effects on PI3K activity in vivo and in vitro (Rodriguez-Viciana et al., 1997; Kodaki et al., 1994; Weinkove et al., 1999). SRF-Gal4 driven expression of Δp60 resulted in a stalling phenotype of 19% of the GBs (n=280) but not midline crosses (Fig. 6D). This may reflect a requirement of PI3-K in the early extension of the GBs towards the midline, which was also impaired by the expression of the dominant-negative form of Egfr in GB1 (Fig. 4B).

The activation of Ras is a necessary step in many of the cellular responses induced by of Egfr signalling in Drosophila (Rommel and Hafen, 1998). It leads to the activation of Raf (Li et al., 1998), and culminates with activation of the Ets-transcription factor Pointed and the nuclear export of Yan, another Ets protein, which antagonises Pnt in the activation of target genes (O'Neill et al., 1994; Tootle et al., 2003). SRF-Gal4-directed expression of a dominant-negative form of Ras (Lee et al., 1996) resulted in stalled branches inside or outside the VNC (52%, n=240). Importantly, a significant number of GBs was grossly misrouted (8%) or crossed the midline (4%, Fig. 6A, arrow) suggesting that Ras is required in the GB1cells for their turn away from the midline. The large proportion of arrests in cell migration observed in these experiments might reflect a broader requirement for these common effectors in tracheal cell migration and sprouting.

To analyse whether Egfr mediated repulsion of GB1 from the midline requires Raf or downstream pathway components, we expressed a dominant-negative form of Raf (de Celis, 1997) and an activated form of Yan (Rebay and Rubin, 1995) under the control of SRF-Gal4 (Fig. 6). These constructs caused many of the branches to stall or misroute but in neither case could we find any branches that crossed the VNC midline (n>200). As an example, expression of the activated Yan construct stalled the migration of 45% of the GBs, and misrouted an additional 7% (n=300), but not a single midline cross was observed.

In summary, activation of Ras appears to be required for repulsion of GB1 from the midline, whereas the remaining components of the pathway are required for tracheal cell extension inside the VNC but not for the decision to cross the midline barrier.

Rho3 midline crosses are due to decreased Egfr/Ras signalling in GB1, rather than to the lack of a directional cue from the midline

An important issue for cell migration in complex landscapes is the discrimination between signals that directly provide spatial information and others that facilitate the interpretation of different instructions. To find out whether the expression of Rho3 generates a spatial cue for the migrating GB1, we first increased the amount of Egfr ligand secreted from the midline by expressing the closely related protease Rho1 (Bier et al., 1990a; Urban et al., 2001) in midline cells, with the sim-GAL4 driver (Scholz et al., 1997). Rhomboid 1 is a functionally redundant partner of Rho3 during eye and leg development (Wasserman et al., 2000; Campbell, 2002) and has been shown to have similar biochemical specificity to Rho3 (Urban et al., 2002). In these embryos, 18% of the GB1s turned posteriorly prematurely, before reaching their characteristic positions close to the midline. Thus, an increase in the amount of ligand from the midline can repel GB1s from its source. If the Egfr ligands provided a spatial guiding signal, overactivation of Egfr signalling in the GB1 either by the expression of activated forms of the receptor, Ras or the Rho1 activator, should disturb the asymmetric response to it, and might cause a random misrouting phenotypes, crossings of the midline or a prominent early arrest of its migration. We expressed two different forms of activated Egfr (Queenan et al., 1997; Lai et al., 1995) (see Materials and methods), Rhomboid 1 (Bier et al., 1990a), Rhomboid 3 (Wasserman et al., 2000) and activated Ras in the terminal cells, and analysed the pathfinding phenotypes in GB1. In all cases, overactivation of Egfr signalling in GB1 resulted in misroutings but no significant midline crossing. Notably, the overexpression

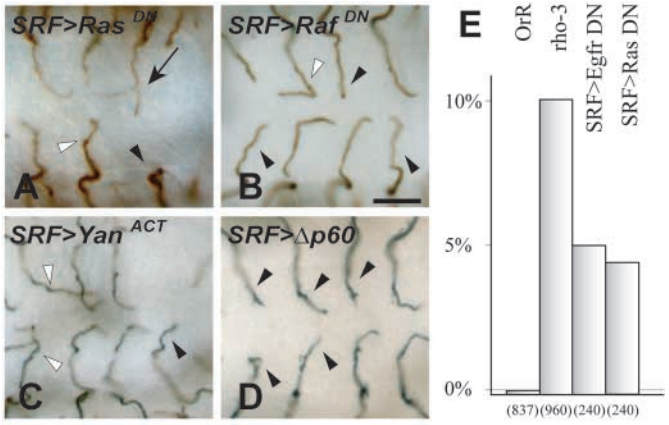

Fig. 6. Dominant-negative Ras, but not Raf or activated Yan, can cause GB1 midline crosses. (A-D) SRF driven expression of dominant-negative Egfr and Ras, but not dominant-negative Raf, PI3K or activated Yan produced midline crosses. Ventral views of embryos expressing dominant-negative constructs stained by mAb2A12. Dominant-negative Ras (A), Raf (B) and PI3K (Δp60, see Materials and methods, C) or activated Yan (Yan^{ACT}, D) had similar effects on GB migration, causing many GBs to fail to enter the VNC, or to arrest (arrowheads in A-D) or meander inside the VNC (white arrowheads in A,B,D). Only dominant-negative Ras caused midline crosses (arrow in A). In all panels, anterior is towards the left. Scale bars: 20 μm. (E) Quantification of the frequency of midline crosses for the different genotypes.

of all constructs in the terminal cells induced GBs to turn prematurely before reaching the midline (‘early turns’; Fig. 7). This phenotype was generated both by the overexpression of activated Egfr, activated Ras and Rhomboid 3 (Fig. 7), and became more prominent in embryos overexpressing Rho1 in their terminal cells, probably owing to higher levels of activity provided by this transgene. In Rho1-overexpressing embryos, 60% of the affected branches (26% of the total, *n*=300) were turning posteriorly before reaching the midline (Fig. 7). The GB1 expression of non-cell autonomous Egfr activators, such as Rhomboid, runs the risk of affecting the surrounding neurons and glia, as well as the migrating GB. Nevertheless, the longitudinal fascicles of the CNS appeared unaffected in these Rho1-overexpressing embryos (Fig. 7), suggesting that the overexpression of Rho1 in the terminal cells did not substantially influence the patterning and migrations of neurons and glia. The premature turning phenotypes generated either by increasing the amounts of active Egfr ligand deriving from the midline, or by raising the levels of Egfr signalling in GB1 are similar. These results, coupled with the analysis of dominant-negative constructs in the trachea (see above), suggest that the midline crossing phenotype in *rho3* mutants is due to decreased levels of Egfr signalling in GB1, rather than to the lack of spatial information. Instead, the high level of Egfr activation, which is normally reached at the midline, appears important for the interpretation of a yet unidentified directional signal.

Discussion

The multiple roles of Egfr at the VNC midline

The essential components of the Egfr signalling pathway have been associated with ventral nerve cord development soon after their discovery. *rhomboid*, *spitz* and *pointed* mutants were originally identified for their effect on the ventral ectodermal region (Mayer and Nusslein-Volhard, 1988). Egfr signalling was also found to play a central role in the development of the VNC midline, where it is first required for cell differentiation and positioning of midline glia and later for their survival during the late stages of embryogenesis (Klambt et al., 1991; Stemerdink and Jacobs, 1997; Hidalgo et al., 2001; Bergmann et al., 2002).

The expression of *rho3* in VUMs and its function in GB1 guidance away from the midline identifies a new role of Egfr signalling in the VNC. Unlike *rho1*, *rho3* mutants have a normal VNC pattern in which longitudinal connectives and glial populations appear normal, suggesting that *rho3* is specifically required for GB1 guidance. Expression of dominant-negative forms of the EGF receptor or Ras in GB1 phenocopied the *rho3* guidance phenotype. In addition, overactivation of Egfr signalling in the trachea was sufficient to redirect GB1 and induce early turn phenotypes. Finally, *rho3* is required in parallel to *slit*, the main repulsive cue deriving from midline glia (Kidd et al., 1999). Taken together, these results suggest that *rho3* mutant GB1s are misrouted because of reduced levels of Egfr/Ras signalling in GB1 cells, rather than to indirect, subtle defects of midline patterning or signalling capacity in *rho3* mutants. This leads us to propose a simple model in which Rho3 activates one or more Egfr ligands secreted by the midline cells. Reception of this signal by migrating GBs is mediated by Egfr and Ras, and promotes turning away from the midline.

Three *Drosophila* Egfr ligands are activated by Rhomboid proteases: Gurken, which is only present in oocytes, Spitz and Keren (Ghiglione et al., 2002; Reich and Shilo, 2002; Urban et al., 2002), the latter expressed in embryos below the detection level of in situ hybridisation or antibody staining (Reich and Shilo, 2002; Urban et al., 2002). Thus, the ligand activated by Rho3 to guide GB1 migration is very likely Spitz, as it is expressed and is functional at the midline (Golembo et al., 1996) (data not shown), but we cannot formally exclude a contribution by Keren.

How does Rho3 guide ganglionic branch migration?

The mammalian EGF receptors regulate migration in a variety of contexts, but in all known examples they appear to promote responses to chemoattractants. They do so by directly affecting cytoskeletal organisation, mainly through the PI3K, PKC or PLC pathways. The proper activation of the fly Egfr is also necessary for the migration of border cells towards the source of Gurken in the dorsal part of the oocyte (Duchek and Rorth, 2001). During this migration Egfr activation is coordinated with the activation of the fly PDGF/VEGF receptor homologue and requires the conserved adaptor protein Mbc (Dock 180/CED-5) (Duchek et al., 2001). Mbc provides a link to activated Rac and actin re-arrangements, which lead to the stereotyped attraction of the border cells towards the oocyte (Duchek and Rorth, 2001; Duchek et al., 2001). It is, however, unclear whether Egfr provides the necessary spatial information for border cells during their pathfinding, or if it is required for the interpretation of positional cues provided by Pdgf/Vegfr or other receptors (Montell, 2003).

There are substantial differences in the ways by which Egfr controls migration in GB1 and in border cells. Our analysis

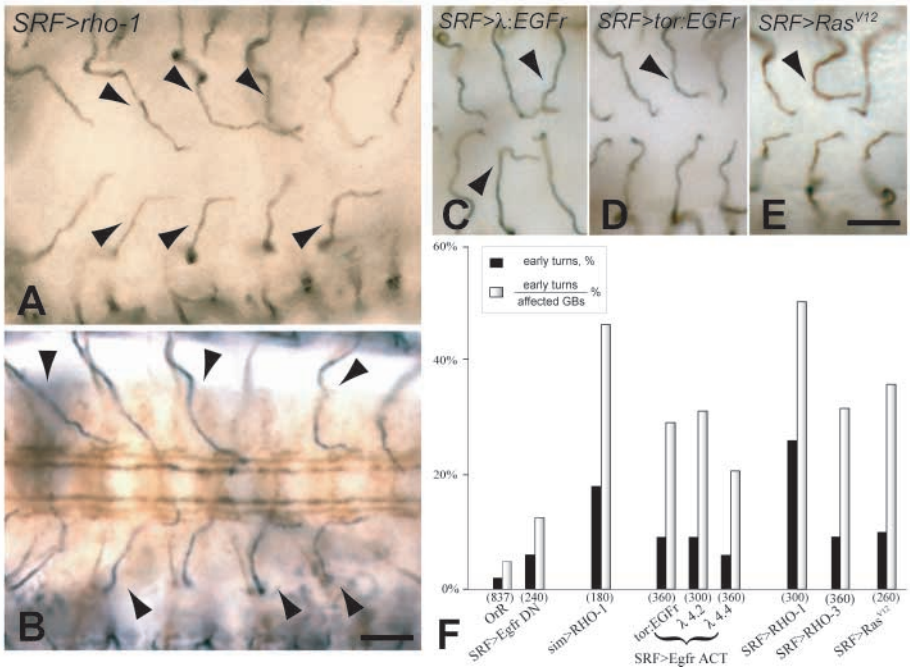

Fig. 7. Egfr pathway overactivation in GB1 causes premature turns. (A-E) Ventral views of late stage 16 embryos stained for tracheal lumen (mAb2A12) and double-stained for lumen and longitudinal fascicles (B, by mAb2A12 in black and anti-Fas2 in brown, respectively). SRF driven expression of Rho1 (A,B), two different forms of activated Egfr (C,D) and activated Ras (E) caused GBs to turn posteriorly prematurely, before reaching the midline (arrowheads in A-E). (F) The overall frequency of early turns for each genotype (black bar) and the fraction of affected branches that was classified as early turns (white bar). As an example, dominant-negative Egfr had a strong effect on GB migration but only about 10% of the affected branches classified as early turns (F). We interpret those early turns as a result of randomised migration. By contrast, close to 30% of the branches affected by activated Egfr were early turns (F). In SRF-Rho1 50% of the GBs were turning prematurely (F), yet the longitudinal fascicles appear unaffected (B, anti-Fas2 in brown).

indicates that Egfr signalling is not a chemotactic cue for tracheal pathfinding, it rather reveals a surprising role in mediating repulsion from the signalling source. In addition, *mbc* mutants did not show any midline crossing phenotypes that would resemble the phenotypes of *rho3* or the ones generated by inactivation of the receptor. Furthermore, the increase of signalling levels in GB1, either by the expression of Rho1, activated receptor or activated Ras, resulted in a significant phenotype opposite of that of the *rho3* mutants: induced GBs to turn early before reaching the midline. This suggests that at the appropriate distance from the midline, Egfr activation becomes a switch to initiate the turn of GB1 away from it. Hence, an experimental increase of signalling levels can shift the crucial switch further away from the midline, while decreased signalling causes midline crosses. In essence, migrating GBs use Egfr activation to efficiently compute their relative distance from the midline, fine-tuning their response to the repulsive and attractive cues originating from it.

Rho3 signalling in GB1 may provide a switch that changes attraction to repulsion

Migration in general, and axonal pathfinding at the midline in particular, is known to rely on a number of guidance signals, at times redundant ones (Dickson, 2002; Montell, 2003). The major midline repulsive signal for GB1 is Slit, yet a genetic test showed that *rho3* acts in parallel to Slit. We hypothesize that Egfr works in an analogous manner by activating a second, yet undiscovered, signalling system for GB repulsion. Such a guidance cue may be specific for GB1 migration, as axonal fascicles are not affected in *rho3* mutants. Alternatively, the activation of Egfr in GB1 provides an epithelial specific regulation of a common repulsive signal used by both axons and GB1.

What could this repulsive signal be? Likely candidates fall in the short list of conserved signals repelling axons and non-neural cells in different systems: Netrins, Semaphorins and Ephrins (reviewed by Dickson, 2002). Netrins are involved in the repulsion of motor axons in both vertebrates and invertebrates (Harris et al., 1996; Mitchell et al., 1996; Keleman and Dickson, 2001) and both *Drosophila* Netrins are expressed at the CNS midline, where they mediate attraction of commissural axons (Mitchell et al., 1996). Semaphorins and Ephrins are also capable of repelling axons and non-neural cells in different contexts (Dickson, 2002; Van Vactor and Lorenz, 1999; Mellitzer et al., 2000), and they therefore represent possible guiding cues for GBs. Intriguingly, each family uses receptor tyrosine kinases as receptors (in the case of Ephrin) or co-receptors (in the case of Semaphorins). Most of these signals are bi-functional, they can elicit both attractive and repulsive responses on the receiving cells depending on context. Egfr activation in GB1 may lead to the post-translational modifications that activate a repellent receptor or inactivate an attractant one and may represent a general 'switch' mechanism for changing the orientation of cell migration depending on the strength of RTK signalling.

We thank M. Freeman and T. Schupbach for fly strains. The work was supported by grants from the Swedish research council (VR) and the Swedish foundation for strategic research (SSF) to C.S.

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