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A novel Dbl family RhoGEF promotes Rho-dependent axon attraction to the central nervous system midline in *Drosophila* and overcomes Robo repulsion

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The key role of the Rho family GTPases Rac, Rho, and CDC42 in regulating the actin cytoskeleton is well established (Hall, A. 1998. *Science*. 279:509–514). Increasing evidence suggests that the Rho GTPases and their upstream positive regulators, guanine nucleotide exchange factors (GEFs), also play important roles in the control of growth cone guidance in the developing nervous system (Luo, L. 2000. *Nat. Rev. Neurosci*. 1:173–180; Dickson, B.J. 2001. *Curr. Opin. Neurobiol*. 11:103–110). Here, we present the identification and molecular characterization of a novel Dbl family Rho GEF, GEF64C, that promotes axon attraction to the central nervous system midline in the embryonic *Drosophila* nervous system. In sensitized genetic backgrounds, loss of GEF64C function causes a phenotype where too few axons cross the midline. In contrast, ectopic expression of GEF64C throughout the nervous system results in a phenotype in which far too many axons cross the midline, a phenotype reminiscent of loss of function mutations in the Roundabout (Robo) repulsive guidance receptor. Genetic analysis indicates that GEF64C expression can in fact overcome Robo repulsion. Surprisingly, evidence from genetic, biochemical, and cell culture experiments suggests that the promotion of axon attraction by GEF64C is dependent on the activation of Rho, but not Rac or Cdc42.

Introduction

During development, neuronal growth cones interpret a balance of attractive and repulsive cues present in the extracellular environment to find their correct targets. Many phylogenetically conserved ligands and receptors that control axon guidance decisions have been discovered (Tessier-Lavigne and Goodman, 1996). For example, in the embryonic *Drosophila* central nervous system (CNS),* midline glia cells secrete Netrin and Slit; Netrin attracts axons across the midline, whereas Slit repels axons, preventing them from crossing more than once (Harris et al., 1996; Mitchell et al., 1996; Kidd et al., 1999). Netrin attraction is mediated by deleted in colo-rectal carcinoma (DCC) receptors and Slit repulsion is mediated by Roundabout (Robo) receptors (Keino-Masu et al., 1996; Kolodziej et al., 1996; Kidd et al., 1998a). Chimeric receptors, generated by exchanging the cytoplasmic domains of the attractive Netrin receptor DCC and the repulsive Slit receptor Robo, have shown that the sign of the growth cone response is encoded in the cytoplasmic domains of these receptors (Bashaw and Goodman, 1999).

Results and discussion

To identify signaling molecules involved in controlling axon guidance decisions, we have used chimeric receptor overexpression phenotypes to perform a sensitized genetic screen. Overexpression of the Robo-DCC chimeric receptor (Robo's extracellular domain fused to DCC's cytoplasmic domain) leads to dose-dependent CNS axon guidance defects in which axons abnormally cross the CNS midline, and also results in reduced viability. We screened the EP collection (a collection of P-element inserts that allow GAL4-dependent misexpression of flanking genes [Rorth et al., 1998]) for genes that, when overexpressed pan-neurally in combination with Robo-DCC, would enhance the viability defects of the chimera. Such genes could play a role in DCC-mediated at-

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*Abbreviations used in this paper: CNS, central nervous system; DCC, deleted in colo-rectal carcinoma; fra, frazzled; GEF, guanine nucleotide exchange factor; GST, glutathione *S*-transferase; PH, pleckstrin homology; Robo, Roundabout.

Key words: axon guidance; attraction; repulsion; Rho; GEF

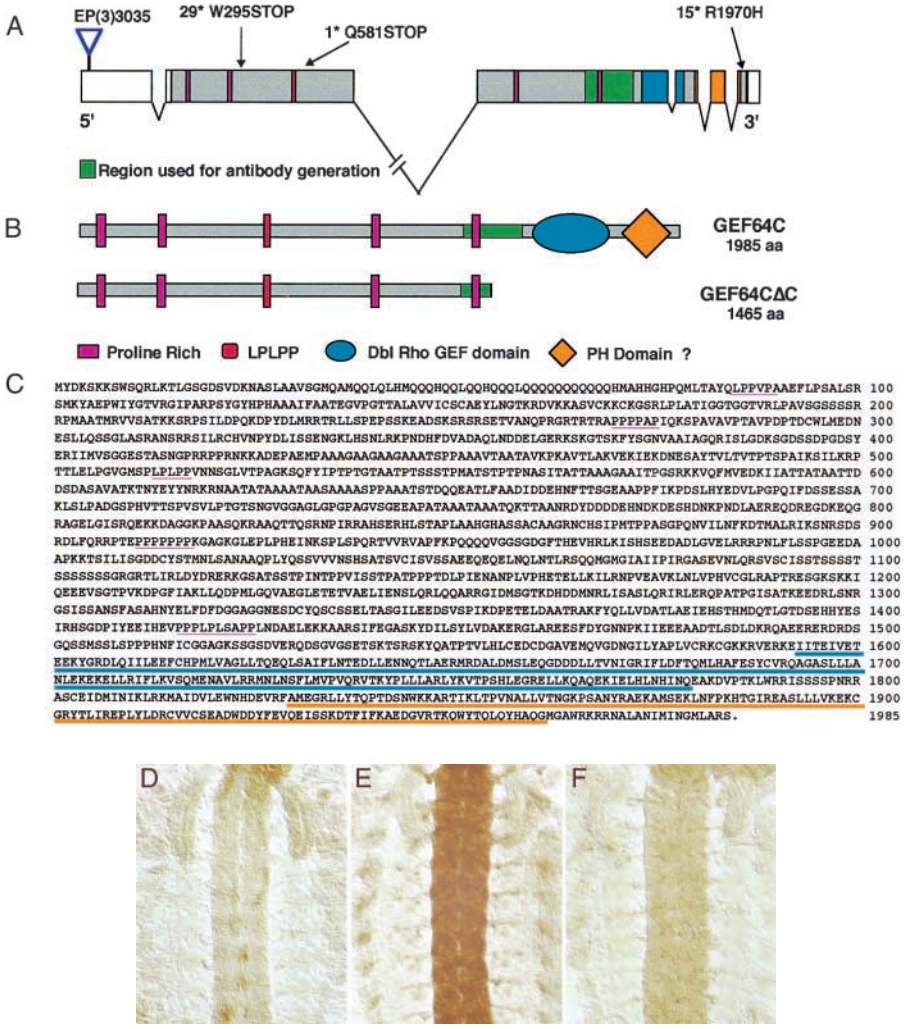

Figure 1. **Molecular characterization and expression of *GEF64C*.** (A) Genomic organization of the *GEF64C* locus. The location of the EP insert, the sequenced mutant alleles, and the region used for antibody generation are indicated. Coding sequences are represented by filled rectangles, UTRs by open rectangles. Colored regions of the coding sequence are as indicated in B. (B) Schematic diagram of the GEF64C and GEF64CΔC proteins. Individual domains are as indicated. The PH domain was identified by the SMART sequence analysis program, but had a very low significance score (10^{e-1}). Since all known Dbl domain proteins have PH domains flanking the Dbl, we have included the PH domain with a small question mark next to it. (C) Amino acid sequence of GEF64C. Sequences of identified domains are underlined in the color corresponding to each domain as indicated in B. (D–F) Stage 15–16 embryos stained with anti-GEF64C antibody. Anterior is up. (D) Wild-type. (E) *UASGEF64C/ElavGal4*. (F) *UASGEF64CΔC/ElavGal4*. These sequence data are available from EMBL/Genbank/DDBJ under accession no. AY064174.

tractive axon guidance, or alternatively could function in parallel attractive-signaling pathways. Here we present the characterization of one of the genes identified in this screen.

Expression of *EP3035* dramatically enhances the axon guidance defects of the Robo-DCC chimera, leading to a significant increase in ectopic midline crossing (unpublished data). Molecular characterization of the genomic region adjacent to *EP3035* revealed a large transcription unit that encodes a novel member of the Dbl family of guanine nucleotide exchange factors (GEFs) (Cerione and Zheng, 1996) specific for the Rho family of small GTPases (Fig. 1), *GEF64C*. In addition to the canonical Dbl and pleckstrin homology (PH) domains, *GEF64C* also contains several proline-rich motifs, including a sequence similar to the Enabled EVH1 domain binding site (LPLPP) (Niebuhr et al., 1997) (Fig. 1). RNA in situ analysis on *EP3035/ElavGal4* embryos confirms that *EP3035* drives overexpression of the *GEF64C* transcript. In addition, the genetic enhancement of *Robo-DCC* by *EP3035* can be phenocopied by expressing a UAS *GEF64C* transgene, confirming that the enhancement is due to *GEF64C* expression (unpublished data). Protein expression analysis in wild-type embryos, using an mAb to *GEF64C*, reveals broad, low level expression of this GEF, with some enrichment in the CNS (Fig. 1 D). The specificity of the *GEF64C* mAb is demonstrated by comparing embryos expressing full-length *UASGEF64C* under control of *elavGAL4*, with those expressing a COOH-terminal truncation, *UASGEF64CΔC,* which removes the mAb epitope (Fig. 1 B). Robust CNS expression can be seen in animals with the wild-type transgene, while only the low-levels characteristic of wild-type expression can be seen in animals with the truncated transgene (Fig. 1, E and F).

Since *GEF64C* was identified in a gain of function screen, we wanted to assess the consequence of loss of *GEF64C* function on midline axon guidance. We generated point mutations in the GEF and sequenced three independent alleles (see Materials and methods). Two of these alleles, $GEF64C^{1}$ and $GEF64C^{29}$, result in premature stop codons, while a third generates a missense mutation at the COOH terminus of the protein (Fig. 1). Embryos carrying mutations in *GEF64C* were examined with an antibody that labels all CNS axons (mAb BP102). No major defects were discovered in the *GEF64C* mutants: the longitudinal connectives and commissural axon bundles were comparable to those seen in wild-type animals (Fig. 2, A and B). RNA interference using a fragment of *GEF64C* double-stranded RNA also failed to reveal strong axon guidance defects, arguing against maternal contribution as an explanation for the absence of a mutant phenotype (unpublished data).

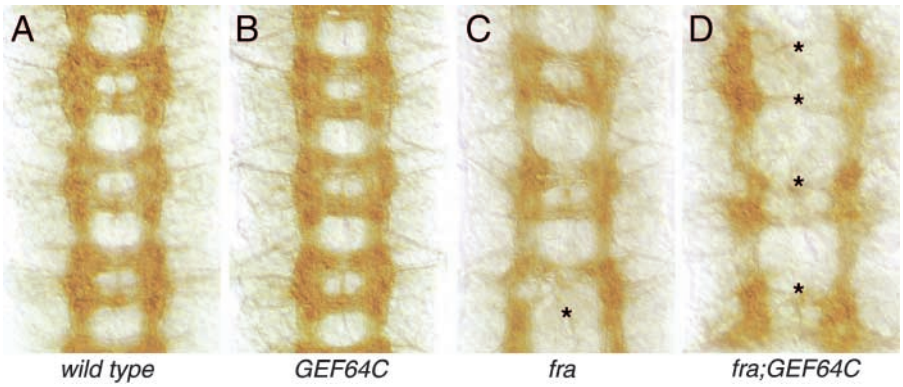

Figure 2. ***GEF64C* loss of function.** Stage 16 embryos stained with mAb BP102 to label all CNS axons. Anterior is up. Genotypes are shown below each panel. Abnormally thin or absent commissures are indicated by arrows with asterisks.

Genetic redundancy could explain the modest consequences of removing *GEF64C*; indeed, examination of the *Drosophila* genome reveals that there are ~22 GEFs specific for Rho family GTPases, a number of which appear to be expressed in the embryonic CNS (unpublished data). This raises the possibility that multiple GEFs function during midline guidance and that disrupting just one has limited effect. For example, mutations in *Drosophila trio*, another Rho GEF with well-established roles in regulating axon outgrowth, cause only minor disruptions in the CNS axon scaffold, whereas they have more profound effects in combination with other mutations that affect midline axon guidance (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000). Alternatively, it is possible that *GEF64C* mutations do cause defects in subsets of CNS neurons, but that these defects are not readily apparent when all axons are visualized simultaneously.

To further investigate a potential requirement for *GEF64C* in midline axon guidance, we looked at the effects of removing *GEF64C* function in animals that carried mutations in the *frazzled (fra)* gene, which encodes the *Drosophila* homologue of the DCC-attractive Netrin receptor (Kolodziej et al., 1996). Mutations in *fra* cause a range of defects in CNS axon guidance consistent with its role in attracting commissural axons to the midline (Fig. 2 C). *fra; GEF64C* double mutant embryos exhibit a marked enhancement of the guidance defects typically observed in *fra* mutants; there is a substantial reduction in commissure thickness and a greater number of segments where commissures fail to form (Fig. 2 D). Thus, in the *fra* mutant background where normal axon attraction to the midline is partially defective, loss of *GEF64C* exacerbates these defects, suggesting an endogenous role for *GEF64C* in attractive guidance at the midline. It should be noted that this double mutant analysis does not provide evidence of *GEF64C*'s involvement in DCC signaling, nor does it preclude such a role. Dose-sensitive genetic and biochemical interactions between *fra* and *GEF64C*, which could suggest a direct involvement in *DCC* signaling, have not thus far been observed.

In contrast to the modest effects of loss of *GEF64C* function, pan-neural overexpression of *GEF64C* (using *EP3035* or *UASGEF64C*) results in a dramatic, dose-dependent, gain of function phenotype, in which many axons abnormally project across the midline. The commissures are thicker and there is a commensurate reduction in the longitudinal axon tracts (Fig. 3 A). This phenotype suggests that *GEF64C* expression promotes axon attraction to the midline. The point mutations in *GEF64C* were introduced on the *EP3035* chromosome, allowing for GAL4 overexpression of the mutant alleles. None of the mutant alleles, nor the *UASGEF64CΔC* transgene (a deletion of the Dbl and PH domains), were capable of generating the gain of function phenotype, indicating that the abnormal midline crossing is due to *GEF64C* expression, and that this effect requires the intact Dbl and PH domains. Examination of gain of function embryos with antibodies to Wrapper (Noordermeer et al., 1998), a marker for midline glia, indicates that the guidance defects caused by *GEF64C* overexpression are not a secondary consequence of nonautonomous perturbations of midline glial cell survival or migration (unpublished data).

The *GEF64C* overexpression phenotype is qualitatively similar to the phenotype of mutations in the Robo receptor, raising the possibility that *GEF64C* promotes attraction to the midline by interfering with Robo repulsion. Several observations argue against this idea. First, there are significant differences between the *GEF64C* gain of function and *robo* loss of function phenotypes: *robo* mutations have more profound effects on the growth cones that pioneer the ipsilaterally projecting FasII-positive posterior corner cell (pCC) pathway than does *GEF64C* overexpression (unpublished data). Second, overexpression of *GEF64C* does not appear to affect Robo protein expression or localization (unpublished data). The third observation relates to genetic predictions based on the function of *commissureless* (*comm*). Comm downregulates Robo receptors on commissural axons (Tear et al., 1996; Kidd et al., 1998b). In *comm* mutants no axons cross the midline; in *robo;comm* double mutants the phenotype is like *robo* (Seeger et al., 1993). Thus, if *GEF64C* overexpression were blocking *robo* function, the *GEF64C* gain of function should be at least partially epistatic to mutations in *comm*; this is not the case (unpublished data). For these reasons, we believe that *GEF64C* overexpression exerts its effects through stimulation of an attractive signaling pathway, rather than through inhibition of Robo repulsion.

The *GEF64C* gain of function phenotype suggests that by increasing the expression of an attractive signaling molecule, it is possible to overcome the normal repulsive signals that are present at the midline. To determine whether *GEF64C* expression would also allow axons to cross the midline in genetic backgrounds where axons are biased toward being repelled, we coexpressed *GEF64C* with a hyperactive mutant

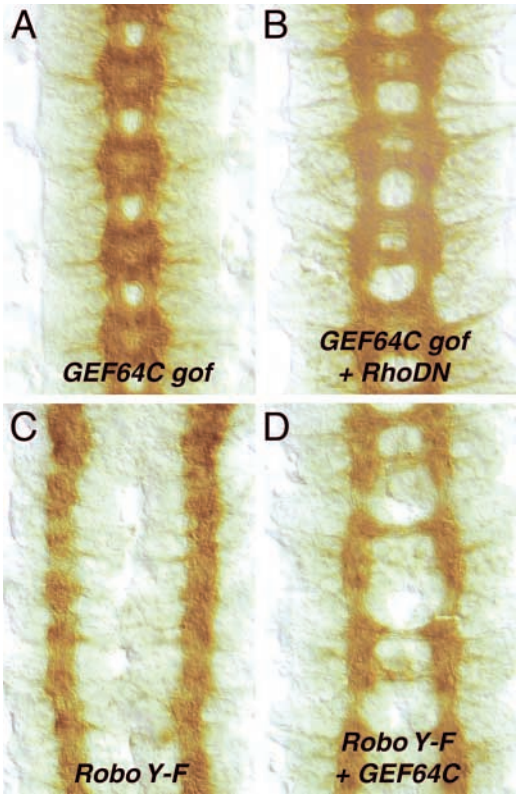

Figure 3. ***GEF64C* gain of function overcomes Robo repulsion and is suppressed by the *RhoA* dominant negative.** Stage 16 embryos stained with mAb BP102 to label all CNS axons. Anterior is up. Genotypes are shown below each panel. Note the more wild-type appearance of commissural and longitudinal axon bundles in the embryo coexpressing *GEF64C* and the *RhoA* dominant negative (B), relative to *GEF64C* alone (A).

form of the Robo receptor: RoboY-F (Bashaw et al., 2000). Pan-neural expression of *UASroboY-F* results in a commissureless phenotype, in which no axons cross the midline (Fig. 3 C). If in this *roboY-F* background we simultaneously drive *GEF64C* expression, many commissural axons are now able to cross the midline, and some segments appear to be nearly wild-type (Fig. 3 D). Thus, even in this artificially repulsive background, *GEF64C* can allow significant axon growth to and across the midline, raising the exciting possibility that finding ways to stimulate the activity of functionally homologous mammalian GEFs could promote regrowth of injured axons in the adult CNS.

How does expression of *GEF64C* promote axon attraction? One likely scenario is that it exerts its effects by specifically activating one or more of the Rho-family GTPases. There are six RhoGTPases in the fly genome: Rac1, Rac2, Mtl, RhoA, RhoL, and Cdc42 (Dickson, 2001). We made use of the *GEF64C* gain of function phenotype and dominant negative GTPase transgenes for Rac1, RhoA, and Cdc42 to determine which, if any, of these GTPases are the downstream target(s) of *GEF64C*, reasoning that genetically limiting the downstream target should suppress the *GEF64C* gain of function phenotype. Based largely on the differential effects of Rac and Rho on neurite extension (Rac promotes extension and Rho promotes retraction), it has been proposed that during axon guidance Rac could play a role in attractive responses, whereas Rho could stimulate repulsion (Dickson, 2001). We therefore predicted that the *GEF64C* gain of function phenotype would depend on Rac activity, but not on Rho. Surprisingly, the opposite appears to be true; the RhoA dominant negative strongly suppresses the *GEF64C* gain of function, whereas the Rac1 and Cdc42 dominant negatives have little or no effect (Fig. 3 B and unpublished data). This observation argues against the simplest form of the model that Rac mediates attraction, and Rho mediates repulsion.

To test if the specificity of *GEF64C* for RhoA seen in our genetic experiments is also observed in independent assays for GEF64C function, in vitro binding, and guanine nucleotide exchange assays were performed. Glutathione *S*-transferase (GST) pull down experiments indicate that GEF64C can bind equally well to Rac1, RhoA, and Cdc42 (unpublished data), whereas GEF64C acts as an in vitro exchange factor for Rac and Rho (exhibiting a modest preference in catalyzing the exchange of GDP for GTP on Rho, relative to Rac) but does not have exchange activity for Cdc42 (Fig. 4 D). Such promiscuity in the in vitro association of GEFs with small GTPases has been observed for many RhoGEFs, including Vav and Trio (Van Aelst and D'Souza-Schorey, 1997). To further examine the function of GEF64C, its effects on the actin cytoskeleton in cultured fibroblasts were determined (Fig. 4, A–C). Microinjection of a GEF64C expression vector into quiescent, serum-starved Swiss 3T3 cells resulted in a dramatic stimulation of actin stress fiber formation relative to control cells (Fig. 4, A and B), a phenotype indicative of Rho activation (Hall, 1998). Coinjection of GEF64C and C3 transferase, a protein inhibitor specific for Rho (Ridley and Hall, 1992), completely blocked GEF64C's ability to induce stress fibers, arguing further that GEF64C functions by activating Rho (Fig. 4 C).

The reciprocal loss and gain of function genetic data presented here support a role for *GEF64C* in promoting axon attraction to the CNS midline. Overexpression of *GEF64C* can overcome the normal repulsive signals present at the midline, and can even drive attraction to the midline in a background where Robo repulsion is abnormally strong. Surprisingly, genetic and cell culture evidence suggest that these attractive effects are meditated through the activation of RhoA, but not Rac. These findings present a paradox. Previous evidence from a number of different experimental systems is consistent with the general idea that Rac and Cdc42 are positive regulators of neurite outgrowth and that Rho is a negative regulator (reviewed in (Luo, 2000)). These observations on axon outgrowth have been extended to axon guidance, suggesting that Rac and Cdc42 would mediate attractive guidance responses and Rho would mediate repulsion, and have led to the investigation of the role of the Rho GTPases in the regulation of axon guidance. For example, Ephexin, a GEF for RhoA, has been implicated in the repulsive responses mediated by Eph receptors (Shamah et al., 2001), and the repulsive effects *Drosophila* Plexin B, a member of the Semaphorin receptor family, also appear to be mediated by RhoA (Driessens et al., 2001; Hu et al., 2001).

Our findings suggest that the opposite is also possible; namely, that RhoA may also play a role in attraction, and ar-

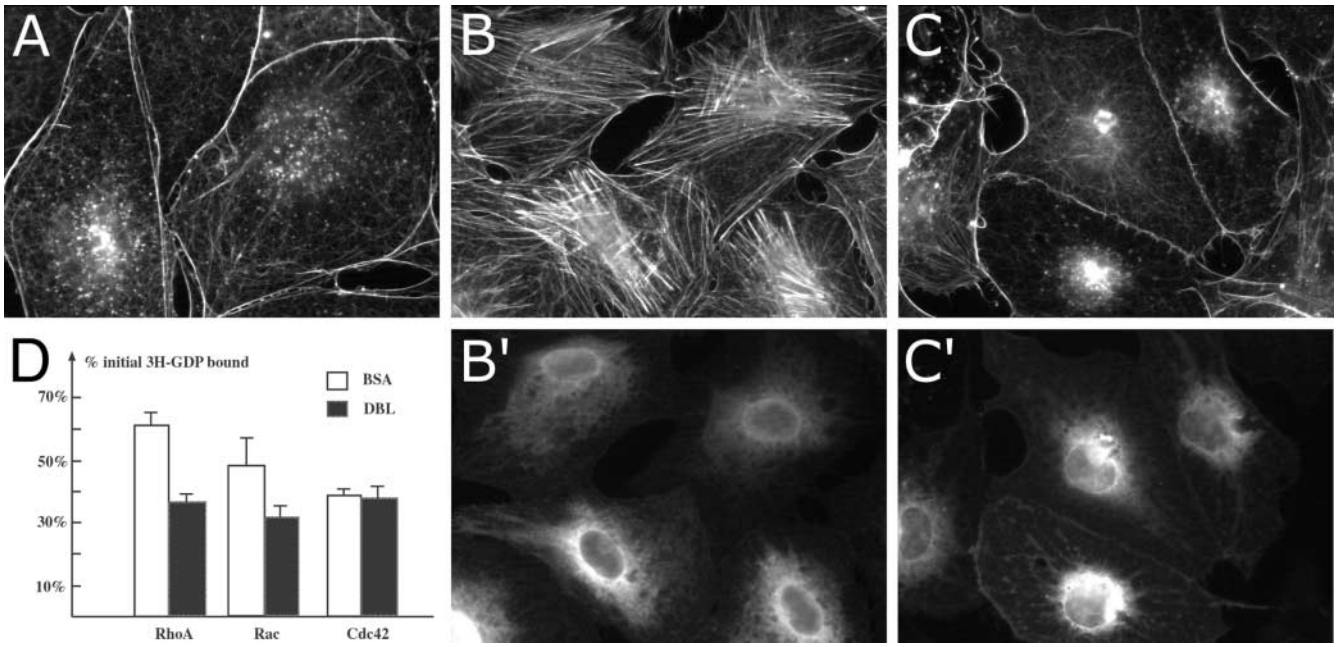

Figure 4. **GEF64C promotes RhoA-dependent actin stress fiber formation in fibroblasts.** (A) Uninjected control cells. (B) Cells injected with a GEF64C expression construct show striking actin stress fiber formation. (B′) Injection marker for cells shown in B. (C) Cells coinjected with the GEF64C expression construct and C3 transferase protein. C3 strongly inhibits GEF64C-induced stress fiber formation. (C′) Injection marker for cells shown in C. (D) GEF exchange assays for Rac, Rho, and Cdc 42. Histogram columns are as indicated. Activity is expressed as the percent of initial $^{[H3]}$GDP remaining bound after 25 min. The relatively weak, but significant exchange activity that we observe could be attributable to the fact that the PH domain was not included in these assays, as fusion proteins containing both the Dbl and PH domains were poorly expressed.

gue against a single generalizable function for the Rho GTPases in axon guidance. The simplest interpretation of our data is that GEF64C promotes midline attraction through the stimulation of an attractive signaling pathway. However, it should be noted that until GEF64C function is linked to a known receptor/ligand system, it remains a formal possibility that GEF64C expression could exert its effects through enhancing the activity of an unknown repellant present in the lateral CNS. Because such a model would predict strong defects in the longitudinal extension of FasII-positive axons, something that we do not observe; we favor the idea that GEF64C activates an attractive pathway. Nevertheless, discovering how the Rho GTPases can elicit different and even opposite axon guidance responses in different contexts is a major challenge for the future, and promises to enrich our understanding of the molecular mechanisms of axon guidance in the developing nervous system.

Materials and methods

Genetics

The EP insert in *GEF64C* (EP3035) came from a screen of the ~2300 line EP collection provided by the Rubin lab. Flies from each EP line were crossed to flies that constitutively express the Robo-DCC chimera in all neurons: *UASRobo-DCC, ElavGAL4/TM3*. A primary screen was performed for lines that were lethal when coexpressed with the Chimera. Lines that were lethal independent of the chimera were eliminated, and the remainder were screened anatomically for those that enhanced Robo-DCC's axon guidance defects. EMS point mutations in *GEF64C* were isolated in an F1 selection screen for suppressors of the lethal interaction of *EP3035* and *UASRobo-DCC*: *EP3035/TM3* males were mutagenized with EMS and then crossed to *UASRobo-DCC,ElavGAL4/TM3*. Non-TM3 escapers were isolated, retested for suppression, and lines were established. Several lines which failed to give the *GEF64C* gain of function phenotype were selected for sequencing. The following fly stocks were also used: (a) *ElavGAL4*, (b) Df(3L)10H, a deficiency for the GEF64C region, (c) *fra/CyoWgβgal*, (d) *comm,EP3035/TM3βgal*, (e) *comm,ElavGAL4/*TM3β*gal*, (f) *UASRoboY-F*, (g) *UASRacN17*, (h) *UASRhoN19*, (i) *UASCdc42N17*, (j) *fra/CyoWgβgal; GEF64C^{29}/*TM6β*gal*, (k) *fra/CyoWgβgal; Df(3L)10H/TM6βgal*, and (l) *EP3035,ElavGAL4/TM3βgal*.

Molecular biology and biochemistry

Genomic DNA flanking *EP3035* was isolated and used to screen the LD embryonic cDNA library (BDGP) using standard procedures. cDNA and mutant allele sequencing was performed on an ABI sequencer. RNA in situ analysis and RNA interference were performed using standard procedures. The full-length *GEF64C* cDNA was subcloned into pCDNA3.1 (Invitrogen) and pUAST for expression in mammalian cells and transgenic flies, respectively. *UASGEF64CΔC* was derived from the full-length *UASGEF64C* and transgenic lines of each construct were established. The pharmacia pGEX system was used for GST fusions of the GEF64C Dbl and Dbl-PH domains: regions of interest were amplified by PCR and subcloned into pGEX. Analogous GEF64C constructs were made in Novagen's pCite vector for in vitro translation. All constructs were sequenced. GST fusions of Rac, Rho, and CDC42 were from Liqun Luo. GST proteins were prepared according to the manufacturer's instructions. In vitro binding experiments were performed as described previously (Bashaw et al., 2000). GEF exchange assays were performed as described previously (Self and Hall, 1995).

Immunohistochemistry and antibody production

Embryo staining and monoclonal antibody production procedures (GEF64C amino acids 1316–1580) were as described previously (Kidd et al., 1998a).

Cell culture

Quiescent, serum-starved Swiss 3T3 cells were prepared as described previously (Nobes and Hall, 1995). Cells were microinjected with an expression vector (pcDNA3) encoding GEF64C into the nucleus at a concentration of 200 μg/ml. Injected cells were marked by coinjection of biotin-conjugated lysinated dextrans (Molecular Probes) at 2 mg/ml, which was detected in fixed cells using Alexa 350 Streptavidin (Molecular

Probes). Rho activity was inhibited by injection of C3 transferase protein with cDNAs into the cell nucleus at a concentration of 200 μg/ml. After 2.5 h, cells were fixed, without washing, in 4% paraformaldyhyde/0.2% gluteraldyhyde/PBSA for 10 min at room temperature. Cells were permeabilized with 0.2% Triton X-100/PBS for 5 min and blocked with sodium borohydride (0.5 mg/ml in PBS) for 10 min at room temperature. Cells were stained for filamentous actin structures by incubating for 20 min with TRITC-conjugated phalloidin (0.1 μg/ml).

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