The Adam family metalloprotease Kuzbanian regulates the cleavage of the roundabout receptor to control axon repulsion at the midline

Hope A. Coleman, Juan-Pablo Labrador*, Rebecca K. Chance and Greg J. Bashaw†

SUMMARY
Slits and their Roundabout (Robo) receptors mediate repulsive axon guidance at the Drosophila ventral midline and in the vertebrate spinal cord. Slit is cleaved to produce fragments with distinct signaling properties. In a screen for genes involved in Slit-Robo repulsion, we have identified the Adam family metalloprotease Kuzbanian (Kuz). Kuz does not regulate midline repulsion through cleavage of Slit, nor is Slit cleavage essential for repulsion. Instead, Kuz acts in neurons to regulate repulsion and Kuz can cleave the Robo extracellular domain in Drosophila cells. Genetic rescue experiments using an uncleavable form of Robo show that this receptor does not maintain normal repellent activity. Finally, Kuz activity is required for Robo to recruit its downstream signaling partner, Son of sevenless (Sos). These observations support the model that Kuz-directed cleavage is important for Robo receptor activation.

KEY WORDS: Axon guidance, Midline, Repulsion, Slit, Robo, Adam, Kuzbanian, Drosophila, Metalloprotease

INTRODUCTION
Slit ligands and their Robo receptors play conserved roles in regulating repulsive axon guidance during nervous system development (Dickson and Gilestro, 2006; Garbe and Bashaw, 2004; Nguyen-Ba-Charvet and Chedotal, 2002). At the midline in Drosophila, Slit is secreted by midline glia and, through the activation of Robo receptors, prevents abnormal crossing of the midline by ipsilateral axons and re-crossing by commissural axons (Kidd et al., 1999; Kidd et al., 1998a). Loss-of-function mutations in either slit or robo lead to axon misrouting at the midline: slit mutations result in the complete collapse of all CNS axons, whereas mutations in robo result in a milder phenotype in which axons cross and re-cross the midline many times (Battye et al., 1999; Kidd et al., 1999; Kidd et al., 1998a).

Slit proteins are large secreted proteins consisting of leucine-rich repeats (LRRs), seven to nine EGF repeats and a C-terminal cysteine knot (Brose and Tessier-Lavigne, 2000; Rothberg et al., 1990). In both fly and vertebrate systems, Slit proteins undergo proteolytic processing to generate a large N-terminal fragment (Slit-N) and a smaller C-terminal fragment (Brose et al., 1999; Wang et al., 1999). Experiments using cultured rat dorsal root ganglion or olfactory bulb neurons indicate that different fragments of Slit have different properties (Nguyen-Ba-Charvet et al., 2001). Full-length Slit (Slit-FL) and Slit-N bind Robos and repel axons, but the two forms have opposite effects on axon crossing: Slit-N stimulates branching and Slit-FL inhibits branching (Nguyen-Ba-Charvet et al., 2001).

What molecules might contribute to Slit processing? Kuzbanian (Kuz) was originally identified in Drosophila for its role in regulating Notch signaling during neurogenesis (Pan and Rubin, 1997; Rooke et al., 1996). Kuz is a single-pass transmembrane metalloprotease belonging to the Adam family that is widely expressed throughout development in the Drosophila central nervous system (Fambrough et al., 1996). It is expressed at the cell surface where it recognizes and cleaves its substrates within their extracellular domain, resulting in ecto-domain shedding. Although several Kuz substrates have been identified – including Notch, APP (amyloid precursor protein; also known as Appl – FlyBase) and ephrins – protein sequence analysis has not revealed a signature cleavage or Kuz association site, making it difficult to predict Kuz substrates by sequence alone (Becherer and Blobel, 2003; Gomis-Ruth, 2003). Kuz processes substrates whose functions range from cell fate specification to axon guidance and these processing events are essential for the correct development of the CNS (Yang et al., 2006).

In the context of axon guidance, the mammalian Kuz homolog ADAM10 has been shown to regulate the cleavage of Ephrin A2 ligands during Ephrin-dependent contact repulsion (Hattori et al., 2000). ADAM10 is constitutively associated with the GPI-linked Ephrin A2 ligand. Upon binding of the ligand by the EphA2 receptor, Kuz cleaves Ephrin A2 in the extracellular juxtamembrane region, releasing it from the membrane and allowing the growth cone of the EphA2 expressing cell to retract (Hattori et al., 2000). In addition, expression of a dominant-negative form of Kuz in Drosophila midline glia results in ectopic crossing of ipsilateral axons, and there are dose-dependent genetic interactions between kuz and slit, suggesting that Kuz might regulate the cleavage of Slit during midline axon repulsion (Schimmelpfeng et al., 2001).

Here, we report that, in a sensitized screen for genes involved in Slit-Robo repulsion, we have identified several alleles of kuz. We find no evidence that Kuz is involved in the processing of Slit, nor does Slit proteolysis appear to be required for the normal repulsive guidance function of Slit. Genetic rescue experiments demonstrate that although Kuz is normally expressed in both midline glia and CNS neurons, neuronal expression of Kuz completely rescues kuz mutant phenotypes, whereas midline expression does not. We also present evidence that Kuz promotes cleavage of the Robo receptor...
in vitro and that an unceavable form of the Robo receptor (Robo-U) is unable to rescue the robo mutant phenotype, suggesting that it does not maintain normal Robo repulsive activity. Finally, we show that ADAM10/Kuz function is required for the Slit-dependent recruitment of the Sos (Son of Sevenless) Ras/Rac GEF to the Robo receptor, suggesting that Kuz is important for Robo receptor activation. Together, these observations support the hypothesis that Kuz-directed cleavage of Robo is important for axon repulsion at the midline.

**MATERIALS AND METHODS**

**Genetics**

The following mutant alleles were used in this study: comm<sup>1339</sup>, robo<sup>11283</sup>, robo<sup>1777</sup>, slit<sup>1</sup>, kuz<sup>229</sup>, kuz<sup>2112</sup>, kuz<sup>420</sup> and kuz<sup>273</sup>. All robo, slit and kuz alleles were balanced over CyWgsGal. To generate transgenic fly strains, UASKuzHA, UASSlitU and UASRoboU were transformed into w<sup>118</sup> flies using standard procedures. The Gal4-UAS system was used to express transgenes in the apertural ipsilateral neurons (apGal4), in the eagle commissural neurons (egGal4), in all neurons (elavGal4), or in midline glia (slitGal4 and simGal4). All crosses were conducted at 25°C.

**Molecular biology**

To generate unceavable Slt, 27 base pairs encoding HNHSIMMYP between the fifth and sixth EGF repeats were deleted using standard procedures. All other constructs were made using standard molecular biology techniques.

**Biochemistry**

S2R+ cells on poly-L-lysine coated plates were transfected at 40% confluency using Effectene (Qiagen) and induced 24 hours later with 0.5 mM copper sulfate. Twenty-four hours after induction, the culture media was harvested and the cells were lysed in 1× PBS, 0.5% Triton X-100, 1× protease inhibitor (Roche) for 20 minutes at 4°C. Proteins were resolved on SDS-PAGE gels and blotted with rabbit anti-GFP (Molecular Probes, 1:500), mouse anti-Robo (DSHB, 1:50), mouse anti-HA (BabCO, 16B12, 1:1000) or mouse anti-Myc (9E10, 1:250). Stacks of images were obtained using a Bio-Rad M.D. system with a 40X objective. A maximum projection of the stacks was generated with NIH Image/Imagel software.

**Cell immunofluorescence**

293T cells were seeded on poly-L-lysine coated coverslips and transfected at 40% confluency using Effectene (Qiagen). Twenty-four hours after transfection, cells were starved in serum-free DMEM medium for 12-16 hours and then stimulated with conditioned medium of hSlit2-stably-expressing 293T cells (a gift from Dr Y. Rao, Peking University School of Life Sciences, Beijing, China) for 5 minutes. Treated cells were washed with 1×PBS once and immediately fixed in 4% paraformaldehyde/1×PBS for 20 minutes. Fixed cells were permeabilized in 0.1% Triton X-100/1×PBS for 2 minutes and blocked with 3% BSA/1×PBS for 5 minutes. Cells were then incubated with primary antibody (rabbit anti-hSos, 1:50; mouse anti-Myc, 1:1000; rat anti-HA, 1:1000) overnight at 4°C and then secondary antibody (rabbit Alexa Fluor®488, mouse Cy3, rat Cy5 secondary antibody) for 30 minutes at room temperature. For each group of cells, at least 10 cells were randomly selected for quantification. Average fluorescence intensity was calculated using NIH Image J software, as previously described (Yang and Bashaw, 2006).

**RESULTS**

**Genetic interactions between kuz, slit and robo**

In wild-type *Drosophila* embryos, staining with the BP102 monoclonal antibody (MAB) revealed a ladder-like structure of axons composed of longitudinal connectives that are bridged by anterior and posterior commissures in each segment (Fig. 1A). The majority of axons cross the midline once and only once, while a smaller set of ipsilateral axons stay on their own side of the midline (Dickson and Gilestro, 2006). Both *kuz* and *robo* mutants showed thinning of the longitudinal connectives paired with thickening of the commissures (Fig. 1B,C). In *robo* mutants, this phenotype results from loss of midline repulsion, which allows axons to abnormally re-enter the midline (Kidd et al., 1998a; Seeger et al., 1993). The similarity of phenotypes observed in *kuz* and *robo* mutants suggests that Kuz might be involved in midline repulsion.

In wild-type embryos, Fasciclin II (FasII)-positive axons project longitudinally and never cross the midline (Fig. 1E), whereas mutations in either *robo* or *kuz* result in differing degrees of abnormal midline crossing (Fig. 1F,G). We took advantage of the mild midline crossing defects associated with simultaneous 50% reduction of the *slit* and *robo* genes (Fig. 1I) to perform a genetic screen to identify additional components of the repulsive guidance pathway. We screened a collection of several hundred mutants that were previously isolated in a large screen for genes that regulate the formation of axon commissures (Seeger et al., 1993). Several alleles of *kuz* were found to dominantly enhance the *slit, robo* transheterozygous phenotype (Fig. 1J; see Fig. S1 and Table S1 in the supplementary material), suggesting that *kuz* is involved in midline repulsion and might be a positive regulator of *slit-robo* signaling. In addition, removing all of the zygotic *kuz* in *slit, robo* trans-heterozygotes enhanced the *kuz* mutant phenotype, whereas *kuz, robo* double mutants exhibited a midline crossing phenotype that was no stronger than that of *robo* mutants alone (Fig. 1D,H; see also Table S1 in the supplementary material; data not shown).

Here, it is important to note that *kuz* zygotic mutants maintain significant levels of *kuz* function owing to maternally deposited genes/proteins and that they do not exhibit cell fate defects (see Fig. S2 in the supplementary material) (Fambrough et al., 1996); the loss of both maternal and zygotic *kuz* leads to *notch* mutant cell fate specification phenotypes (Rooke et al., 1996).

To obtain further evidence for a role of *kuz* in Slit-mediated repulsion, we analyzed embryos that were mutant for *kuz* and *commissureless* (*comm*). *Comm* normally functions to inhibit Robo repulsion by preventing delivery of the Robo receptor to the growth cone plasma membrane (Keleman et al., 2002; Keleman et al.,...
copy of slt resulted in a mild ectopic crossing defect, whereas simultaneously limiting kuz and slt significantly enhanced this phenotype (see Fig. S1C in the supplementary material). Additionally, restricted misexpression of a dominant-negative kuz transgene (Pan and Rubin, 1997) in ap neurons caused ectopic crossing of axons in about fifteen percent of segments (Fig. 2B,D), and this phenotype was significantly more severe in slt or robo heterozygotes (Fig. 2C,D; see also Table S1 in the supplementary material). These data solidify the idea that slt and kuz interact genetically, and also suggest that kuz function is required in neurons.

Kuz function is required in neurons to regulate repulsion

Previous reports have shown that kuz mRNA is broadly expressed throughout the developing embryonic CNS, including in neurons and midline glia (Fambrough et al., 1996). Because we observed a discrepancy between previous studies suggesting that Kuz protease activity is required in midline glia (Schimmelpfeng et al., 2001) and our finding that Kuz function is required in the ap neurons, we sought to further elucidate in which cells kuz expression is necessary. To test where kuz is required for midline guidance, we performed genetic rescue experiments using the Gal4-UAS system (Brand and Perrimon, 1993). Expression of UASKuz or tagged UASKuzHA in all postmitotic neurons using elavGal4 completely rescued the ectopic midline crossing of FasII-positive axons in kuz mutants (Fig. 3A,B,D,F; data not shown) (Fambrough et al., 1996). By contrast, expression of UASKuz or UASKuzHA in midline glia using slitGal4 did not rescue ectopic midline crossing (Fig. 3C,E). Although it is difficult to directly compare levels of expression in the two different cell types, antibody staining to detect transgenic UASKuzHA revealed that the two drivers appear to express comparable levels of Kuz (Fig. 3E,F). Taken together with the observation that expression of dominant-negative Kuz in the ap ipsilateral neurons causes ap axons to ectopically cross the midline (Fig. 2B), these rescue data further support the idea that Kuz is required in neurons for repulsion. Although these observations point to a potential cell autonomous role for kuz in regulating Robo repulsion, it is also possible that Kuz could act non-autonomously by cleaving substrates in trans on adjacent cells. Indeed, ADAM10 has been shown to act non-autonomously to cleave Ephrin A5, in trans, on adjacent cell surfaces (Janes et al., 2005).

Proteolytic processing of Slit is not required for midline repulsion

In order to directly test the importance of Slit processing for midline repulsion, we deleted the nine amino acids that make up the spacer region between the fifth and sixth EGF repeats to generate an uncleavable version of the protein- Slit-U (Fig. 4A). Biochemical analysis using antibodies directed against either the N- or C-terminus of Drosophila Slit revealed that indeed Slit proteolysis in cultured 293T cells and in Drosophila embryos is abolished by this deletion (Fig. 4B,C). We next compared the function of Slit-U with that of wild-type Slit (Slit-FL) in a transgenic rescue assay. In slt mutant embryos, all axons collapsed on the midline (Fig. 4D,F). Restoration of Slit-FL expression in the midline glia using slitGal4 provided a strong rescue of the ectopic crossing phenotype seen in slt mutant embryos, but did not appear to restore proper lateral position of the FasII-positive fascicles (Fig. 4D,G). Surprisingly, we found that midline expression of Slit-U was able to rescue the midline guidance phenotypes of slt mutants just as well as expression of wild-type Slit-FL (Fig. 4D,H). Several independent transgenic lines gave
similar results in our rescue assay (data not shown). The fact that the effects of Slit-U in rescue experiments were indistinguishable from those of Slit-FL suggests that the cleavage of Slit is not essential for its role in midline repulsion.

### Kuz promotes Robo cleavage

Because, in the case of Notch signaling, Kuz has been shown to promote the cleavage of both the Delta ligand and the Notch receptor (Pan and Rubin, 1997; Qi et al., 1999; Six et al., 2003), we next investigated the possibility that the Robo receptor might be a substrate for Kuz. Given that Kuz is a transmembrane protein with extracellular protease activity, we first tested whether we could observe the release of the Robo extracellular domain (Robo-ECTO) into the media from Robo-transfected cells and found that we could readily detect low levels of an approximately 100-kDa Robo immunoreactive fragment in the culture media (Fig. 5A, lane 1). Importantly, this fragment was not observed in untransfected cells (Fig. 5A, lane 6). To further verify that this fragment corresponds to Robo-ECTO, we generated HA-tagged Robo and assayed the culture media by immunoblotting with anti-HA. Again, we detected a 100-kDa fragment, indicating that indeed this represents the Robo-ECTO that is shed from the cell surface (Fig. 5B, lane 1).

---

**Fig. 2. Misexpression of UASkuzDN results in ectopic crossing of apterous neurons.** (A-C) Stage 16 apGal4, UASTau-Myc-GFP embryos were stained with a polyclonal antibody against GFP. Anterior is up. In wild-type embryos (A) the ap axons do not cross the midline, whereas misexpression of UASKuzDN driven by apGal4 (B) results in ectopic crossing (arrows with asterisks). (C) Removing one copy of slt in embryos misexpressing UASKuzDN enhances the crossing phenotype. (D) Quantification shows the percentage of segments in which the ap axons cross the midline. **P<0.0001, *P<0.05 (unpaired Student’s t-test). Error bars indicate s.d.

---

**Fig. 3. kuz is required in neurons.** (A-F) Stage 16 embryos were stained with a mAb to FasII (1D4). Anterior is up. (A) A wild-type embryo stained with mAb 1D4. (B) Ipsilateral axons from the medial and intermediate fascicles aberrantly cross the midline in kuz mutants (arrow with asterisk). (C,E) Midline expression of UASKuzHA using a slitGal4 driver does not rescue the kuz phenotype. One hundred percent of kuz mutant embryos with midline kuz overexpression show ectopic midline crossing of FasII-positive axons (n=15 embryos). (D,F) Pan-neural expression of UASKuzHA provides complete rescue of the kuz mutant phenotype (0 out of 144 segments show ectopic crossing, n=16 embryos).

---

**Fig. 4. Slit-U rescues the slt mutant phenotype.** (A) Schematic drawing of Slit. The arrow indicates the cleavage site. Slit-U or Slit-FL were expressed in HEK293T cells. Total lysates of the cells were harvested and subjected to polyacrylamide gel electrophoresis (PAGE). (B,C) Western blotting with antibodies to either the N- or the C-terminus of Slit indicates that Slit-U is not processed in 293T cells (B) or in embryos (C). (D) Quantification of segments in which FasII-positive neurons cross the midline. Error bars indicate s.d. (E-H) Stage 16 embryos were stained with a mAb to FasII (1D4). Anterior is up. (E) A wild-type embryo stained with mAb 1D4. (F) A slt mutant embryo. All axons collapse on the midline and there are no longer any distinct fascicles (asterisk). Expression of UASslit-FL (G) or UASslit-U (H) driven by slitGal4 in a slt embryo mostly rescues the aberrant midline crossing phenotype, but does not restore proper lateral positioning of the FasII-positive fascicles (arrow with asterisk).
Kuzbanian cleaves Robo

We next sought in vivo evidence for a role of Kuz in regulating Robo processing. Specifically, we examined the surface expression levels and localization of Robo in the embryonic CNS of live-dissected kuz mutant and sibling embryos (Fig. 6). In contrast to kuz/+ heterozygotes, in which surface Robo expression was restricted to the longitudinal portions of CNS axons (Fig. 6A–C), kuz mutants showed elevated expression of Robo and a marked mislocalization of Robo on the surface of axon commisures (Fig. 6D–F). These observations are consistent with a previous report showing mislocalization of Robo in fixed and permeabilized kuz loss-of-function embryos (Schimmelpfeng et al., 2001). Quantification of Robo surface levels revealed a significant increase in expression that is consistent with an increase in the detection of full-length Robo by western blot (Fig. 6G,H). Despite the increase in Robo expression levels, we were unable to consistently detect the presence of ectodomain fragments in wild-type embryos, potentially because of their rapid degradation or because of our assays were not sensitive enough. This limitation precluded the analysis of the effects of kuz mutations on the in vivo cleavage of Robo. Nevertheless, together with our genetic interaction data and in vitro biochemical data, these observations further suggest that Kuz-dependent cleavage of Robo is important in vivo for midline repulsion.

**An uncleavable form of the Robo receptor does not rescue robo mutants**

Both our genetic and biochemical evidence suggest that Kuz processing of Robo is important for Robo repulsive function in the context of midline guidance. In order to directly test this hypothesis in vivo, we created an uncleavable form of the Robo receptor (Robo-U). Our initial efforts to create an uncleavable Robo via small insertions were not successful; however, we were able to create an uncleavable Robo receptor by swapping the first three Fibronectin type III (FnIII) domains from the attractive Netrin receptor Frazzled (Fra) for the three Fn domains of Robo (Fig. 7A).

**Fig. 5. Kuz promotes Robo cleavage in vitro.** UASroboGFP with or without UASKuzHA were transfected into Drosophila S2R+ cells. (A) Western blotting of separated proteins harvested from the media with antibodies to a N-terminal epitope of Robo reveals a significant increase in the amount of Robo ectodomain in the media of cells co-transfected with UASKuzHA compared with that of cells with no ectopic expression of kuz (lanes 1, 4 and 5). Co-transfection of UASrobo and UASKuzDmetallo did not increase the amount of Robo ectodomain detected in the media (lanes 1, 2 and 3). A lane between the first and second lane shown was excised from the media blot. Western blotting of the total lysates with antibodies directed against GFP, HA and tubulin show the relative levels of RoboGFP, KuzHA or KuzDmetallo, and tubulin, respectively. (B) Western blotting of protein harvested from the media of cells transfected with UASHAroboMyc with or without UASKuzHA using an antibody directed against HA. (C) Western blots of proteins harvested from dsRNA-treated S2 cells. Cells expressing UASroboGFP, UASKuzHA, or both were treated with kuz dsRNA. The level of Robo ectodomain detected in the media from cells expressing both robo and kuz is lower in cells treated with kuz dsRNA (lanes 3 and 4). Treatment with kuz dsRNA also reduces the amount of Robo ectodomain detected in the media of cells with no transfected kuz (lanes 1 and 2). Western blotting of the total lysates with antibodies directed against Robo, HA and tubulin show the relative levels of RoboGFP, KuzHA and tubulin, respectively.
is capable of mediating Netrin-dependent repulsive signaling in vivo gain-of-function assays (Bashaw and Goodman, 1999). Interestingly, Fra-Ro does not seem to undergo ectodomain shedding in vitro, suggesting that its in vivo repellent activity might be independent of Kuz function (Fig. 7B, lanes 2 and 6). All four of the receptors assayed in the ectodomain shedding experiment were properly expressed at the plasma membrane of Drosophila S2 cells, suggesting that they are normally trafficked (see Fig. S3 in the supplementary material). In addition, we confirmed that Robo-U retains Slit binding activity that is qualitatively similar to that of wild-type Robo using a cell overlay binding assay (see Fig. S3 in the supplementary material).

Having determined that Robo-U is not cleaved in our in vitro assay, we next sought to characterize the activity of this receptor in vivo. Loss of Robo function resulted in a severe phenotype in which the FasII-positive ipsilateral axons repeatedly crossed the midline (Fig. 7E). This phenotype could be almost completely rescued by expressing UASRobo with the pan-neural driver elavGal4 (Fig. 7H) (Fan et al., 2003; Garbe and Bashaw, 2007). We reasoned that if Robo-U maintained its signaling function in vivo, expressing it in robo mutant embryos would rescue the ipsilateral crossing phenotype, as wild-type Robo does. However, pan-neural expression of UASRobo-U in robo mutant embryos provided only modest rescue of the mutant phenotype, with many ipsilateral axons still crossing the midline (Fig. 7F), which suggests that Robo processing is important for its repulsive signaling function at the midline. Several independent inserts of the Robo-U transgene gave similar results (Fig. 7C). Fra-Ro also provided only a very slight rescue of the robo phenotype (Fig. 7G), suggesting that despite its ability to mediate repulsive activity when misexpressed in a gain-of-function assay (Bashaw and Goodman, 1999), it cannot substitute for endogenous robo in midline repulsion. Importantly, transgenic expression levels of Robo-U, Fra-Ro and Robo were comparable, as assessed by immunostaining for the Myc epitope tag (Fig. 7F-H, bottom panels; see also Fig. S4 in the supplementary material). Interestingly, although transgenic wild-type Robo was cleared from commissures as endogenous Robo was, Robo-U was expressed along the entire length of the commissural axons (Fig. 7E-H, arrowheads; see also Fig. S4 in the supplementary material). It is possible that proteolysis of Robo is important for its exclusion from the commissural portion of contralateral axons. This observation is also consistent with our surface staining experiments (Fig. 6), as well as with a previous report showing that crossing axons in kuz loss-of-function embryos express Robo protein (Schimmelpfeng et al., 2001).

In order to quantify the level of rescue, we counted the number of segments in which the ipsilateral ap axons ectopically crossed the midline. In robo embryos the ap axons completely collapsed on the midline (Fig. 7J). Transgenic expression of either Robo-U or Fra-Ro in ap neurons showed comparably low levels of rescue with respect to ectopic midline crossing when compared with the near complete rescue seen in robo embryos expressing Robo (Fig. 7C,K-M). However, in contrast to the complete collapse of ap axons in robo embryos, expression of these chimeric receptors did seem to partially restore midline repulsion, with some segments having ap axon tracts on both sides of the midline (Fig. 7K-M). The three transgenic receptors assayed each showed comparable levels of expression in the ap neurons (Fig. 7K-M, bottom panels).

Although the inability of Robo-U to restore proper repulsion at the midline supports the hypothesis that processing of Robo is important for this process, it is also possible that this chimeric receptor has simply lost its signaling ability as a result of...
misfolding or some other alteration of function due to exchanging the Fn repeats. To test whether Robo-U is able to mediate repulsion in vivo, we misexpressed this receptor in a small subset of contralateral neurons, the eagle (eg) neurons. In wild-type embryos, the eg neurons crossed the midline in both the anterior and posterior commissures of each segment (see Fig. S5A in the supplementary material). Misexpression of \textit{UASRobo} in eg neurons prevented the posterior subset of eg axons from crossing the midline because of the high levels of repulsion mediated by excess Robo signaling (see Fig. S5D in the supplementary material). Misexpression of \textit{UASSRO-U} was also able to repel the posterior eg neurons from the midline (see Fig. S5B in the supplementary material), suggesting that it maintains repulsive signaling activity in vivo. This repulsive activity is seemingly independent of Robo processing and is likely to be initiated by a different mechanism to that used to activate endogenous Robo. It is possible that over or misexpression of these receptors causes them to behave in a way that they would not normally in vivo. We believe that our rescue assay is a better determinant of how these transgenic receptors act in the context of normal development, as we are directly measuring their ability to substitute for the endogenous receptor. These rescue experiments suggest that cleavage of Robo is important for its repellent activity in the context of midline guidance; however, we cannot exclude the possibility that \textit{Robo-U} could be deficient for other non-cleavage dependent functions.
ADAM10/Kuz function is required for recruitment of Sos to the Robo receptor

If Robo processing by Kuz is important for Robo activation, disruption of Kuz activity might prevent the association of downstream signaling molecules with the cytoplasmic domain of Robo. Sos is a Ras/Rac guanine nucleotide exchange factor (GEF) that associates with the Slit-bound Robo receptor in a ternary complex with the SH3-SH2 adaptor protein Dreadlocks to regulate Rac activity (Yang and Bashaw, 2006). Studies in mammalian cells show that the Rac-GEF activity of Sos is highly dependent upon its precise subcellular localization (Innocenti et al., 2002), suggesting that if Sos recruitment to the plasma membrane was blocked, its activity would be disrupted. In order to determine whether Kuz regulates the Slit-dependent recruitment of Sos to the plasma membrane, we used the mammalian HEK293T system. In the absence of the ligand Slit, endogenous Sos was located diffusely in the cytosol in cells transfected with human ROBO1 (hROBO1), and the cultured cells had a splayed-out flattened morphology (Fig. 8A-D). Bath application of human SLIT2 (hSLIT2) to hROBO1-transfected cells induced a recruitment of Sos protein to the membrane and a distinct rounded morphology (Fig. 8E-H). By contrast, expression of a dominant-negative form of mammalian ADAM10 was able to block the Slit-induced relocalization of Sos and its associated change in cell morphology (Fig. 8I-L; see also Fig. S6 in the supplementary material), suggesting that Kuz processing of the Robo receptor is an important step in the initiation of its signaling cascade. Importantly, ADAM10DN was appropriately expressed at the plasma membrane (see Fig. S6 in the supplementary material). Expression of ADAM10DN in the absence of hSLIT2 treatment did not affect the gross morphology of the cells, demonstrating that expression of this protein does not interfere with overall cell health (see Fig. S6 in the supplementary material). These data provide evidence that limiting Kuz/ADAM10 activity in Robo-expressing cells results in a significant reduction in the ability of Robo to initiate downstream signaling events in response to Slit stimulation and further suggest that Kuz/ADAM10 regulation of Robo signaling is conserved in humans.

DISCUSSION

Both our genetic and biochemical findings support the hypothesis that cleavage of the Robo receptor—rather than its Slit ligand—by the metalloprotease Kuz is important in the context of midline guidance. Loss of Kuz protease activity or of the cleavage site of Robo in vivo results in ectopic crossing of ipsilateral axons because of the loss of Robo-mediated repulsion, whereas an uncleavable form of Slit is able to rescue guidance defects in slit mutants as well as does Slit-FL. Furthermore, biochemical analyses have demonstrated that Robo is a substrate of KuzADAM10 in vitro. Finally, our Sos recruitment assay demonstrates that reduction of endogenous Kuz protease activity attenuates Slit-dependent relocalization of Sos to the plasma membrane, where it acts as a regulator of actin cytoskeletal rearrangement and, presumably, growth cone retraction.

Our data suggest a model in which Kuz promotes Robo ectodomain shedding as a mechanism of Robo activation (Fig. 9). We propose that Kuz cleavage of Robo is initiated by binding of Slit, and that the release of the ectodomain of Robo causes a conformational change in Robo that allows its cytoplasmic domain to associate with Sos via the SH3-SH2 adaptor protein Dreadlocks. Sos is then properly localized in order to exert its effect on cytoskeletal rearrangement.

What is the function of Slit processing in Drosophila?

In light of the strong evidence from vertebrate studies indicating that the different Slit cleavage products have distinct properties (Nguyen-Ba-Charvet et al., 2001), we were surprised to find that an uncleavable form of Slit can rescue slit mutants as effectively
Kuzbanian cleaves Robo

How does Kuz function in Robo-mediated growth cone retraction?

Although it seems evident that Kuz activity is important for Robo-mediated growth cone retraction, it is unclear how Robo ectodomains shedding is involved in the repulsive process. Both Notch and Ephrins are known to be substrates of Kuz, but the role that Kuz plays in their signaling is very different. GPl-linked Ephrin A2 forms a stable complex with ADAM10, although ADAM10 proteolytic activity is only initiated when EphA3, the transmembrane Eph receptor, is present. ADAM10 cleavage of Ephrin A2 can be considered a permissive event, in that it releases the strong Eph-ephrin tether that attaches the two cell surfaces, thereby allowing the EphA3-expressing growth cone to retract (Hattori et al., 2000). The role of Kuz in Notch signaling is more directly linked to Notch activation (Mumm and Kopan, 2000). Although the genetic data we present cannot distinguish whether Kuz acts in a permissive or an activating capacity with respect to Robo signaling, the observation that the expression of dominant-negative ADAM10 blocks Slit-induced recruitment of Sos to the plasma membrane suggests that Kuz/ADAM10 is likely to be important for the association of Robo with its signaling effectors. In other words, it appears that Kuz/ADAM10 contributes to the initiation of Robo signaling events.

How is Kuz activity regulated?

If Kuz is indeed playing an activating role in Robo signaling, it should be regulated in a way to prevent continuous repulsive signaling. The most parsimonious explanation for regulation of Kuz activity is that it is Slit dependent. Indeed Notch and Ephrin proteolysis by Kuz is known to be dependent upon ligand binding. Additionally, other studies have demonstrated that ADAM10 substrates, including APP and Notch, are cleaved upon receptor-ligand binding (Beel and Sanders, 2008). We tested whether Kuz proteolysis of Robo was also dependent upon ligand binding, but unfortunately we were not able to detect a Slit-induced effect on Kuz-dependent Robo ectodomain shedding in vitro (data not shown). However, these experiments were performed in Drosophila S2 cells in which both Robo and Kuz were overexpressed, and the normal regulation of cleavage might not be maintained in this context. The possibility also exists that Kuz processing of Robo might be regulated by calcium influx, differential substrate glycosylation events, or substrate oligomerization, as is observed with some ADAM10 substrates (Beel and Sanders, 2008). In the future, it will be important to determine if Robo proteolysis is dependent on Slit binding, perhaps by examining, both in mammalian cells and in vivo, the processing of a Robo receptor that cannot bind Slit.

Acknowledgements

We thank Kim Bland and Corey Goodman for generating the uncleavable Slit construct, John Flanagan for the mouse ADAM10 reagents, D. J. Pan for the Kuzbanian dominant-negative transgenes and the Bloomingston Stock Center for Drosophila strains. We are grateful to members of the Bashaw lab for thoughtful discussion and to Andy McClelland for assistance with the quantification of the Sos recruitment assay. This work was supported by a Whitehall Foundation Research Grant, a Burroughs Wellcome Career Award, and NIH grants NS046333 and NS054739 to G.J.B. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.047993/-/DC1

References


