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

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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 branching: 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 juxta-membrane 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

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in vitro and that an uncleavable 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: $comm^{\Delta e39}$, $robo^{GA285}$, $robo^{z1772}$, $slit^{2}$, kuz^{e29}, kuz^{H143}, kuz^{l12}, kuz^{420} and kuz^{2583}. All *robo*, *slit* and *kuz* alleles were balanced over *CyWgβGal*. To generate transgenic fly strains, *UASKuzHA*, *UASSlitU* and *UASRoboU* were transformed into w^{1118} flies using standard procedures. The Gal4-UAS system was used to express transgenes in the apterous 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 uncleavable Slit, 27 base pairs encoding HNMISMMYP 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). The following secondary antibodies were used: rabbit anti-HRP and mouse anti-HRP (Jackson Laboratories). Blots were developed with the ECL Plus Western Blotting Detection System (Amersham).

Double-stranded RNA interference

dsRNA was made using standard techniques. PCR amplification of *Drosophila kuz* was conducted using primers that included 5′ T7 promoter regions: 5′-TTAATACGACTCACTATAGGGAGACACCAGTACTCC-GTT-3′ (forward) and 5′-TTAATACGACTCACTATAGGGAGT-TCGCTATGTGGCGCCA-3′ (reverse). Reverse transcription was performed using the MEGAscript T7 High Yield Transcription Kit (Ambion). Single-stranded sense and anti-sense RNAs were incubated in 100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4), 2 mM magnesium acetate for 4 minutes at 95°C, followed by 10 minutes at 70°C, then slowly cooled to 4°C. S2R+ cells were transfected and processed as described above and 5 μl of the double-stranded RNA was added at the time of transfection.

Immunohistochemistry

HRP immunohistochemistry was performed as previously described, and images were obtained using a Zeiss Axiocam and Openlab software (Improvision). For fluorescence staining, the following antibodies were used: mouse 1D4/FasII [Developmental Studies Hybridoma Bank (DSHB), 1:100], mouse MAb BP102 (DSHB, 1:100), rabbit anti-Myc (Sigma-Aldrich, 1:500), mouse anti-Slit (DSHB, C555.6D, 1:25), mouse anti-βgal (DSHB, 40-1a, 1:250), rabbit anti-GFP (Molecular Probes, 1:500), mouse anti-Robo (DSHB, 1:50), mouse anti-HA (BabCO, 16B12, 1:1000), and mouse anti-Myc (9E10, 1:250). Stacks of images were obtained using a Leica DMIRE2 confocal and a 63× oil immersion objective. A maximum projection of the stacks was generated with NIH Image/ ImageJ software.

Cell immunofluorescence

293T cells were seeded on poly-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% paraformaldheyde/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 AlexaFluor488, 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.,

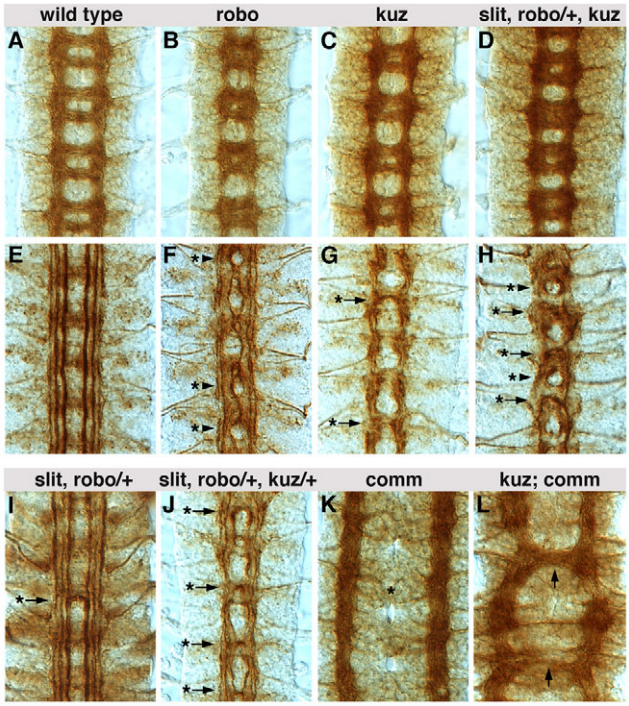

Fig. 1. Genetic interactions between *kuz*, *slit*, *robo and comm*. (**A-L**) Stage 16 embryos were stained with MAb BP102 (A-D,K,L) or MAb FasII (E-J) to reveal all CNS axons or subsets of ipsilateral axons, respectively. Anterior is up. (A) A wild-type embryo exhibiting the characteristic ladder-like axon scaffold. (B) A *robo* mutant embryo; note the reduction in thickness of the longitudinal connectives and the commensurate thickening of the commissures. (C) *kuz* mutants also show thinning of the longitudinals and thickening of the commissures. (D) In *kuz* mutants that are simultaneously heterozygous for *slit* and *robo*, the thickening of the commissures is qualitatively enhanced. (E) A wild-type embryo has three bundles of FasII-positive axons that do not cross the midline. (F) In *robo* mutant embryos, the medial bundle of FasII-positive axons wanders back and forth across the midline (arrowheads with asterisks). (G) *kuz* zygotic mutants have a milder crossing defect (arrows with asterisks), a phenotype that is enhanced when *slit* and *robo* are also heterozygous (H). (I) A *slit*, *robo*/+ embryo with a mild midline crossing defect (arrow with asterisk). (J) An example of a *slit, robo*/+, *kuz*/+ embryo showing dominant enhancement (arrows with asterisks). (K) *comm* mutant embryos completely lack axon commissures, whereas *kuz; comm* double mutants reveal a partial restoration of commissure formation (arrows in L).

2005), and mutations in *comm* result in a complete absence of axon commissures because of excess Robo repulsion (Fig. 1K) (Kidd et al., 1998b). As *comm, robo* double mutants resemble *robo* single mutants (Seeger et al., 1993), we reasoned that, if *kuz* promotes Robo repulsion, then at least some axons should be able to cross the midline in *kuz; comm* double mutants. This is indeed the case; significant restoration of commissure formation was observed in double mutants, although it was apparent that most axons still failed to cross the midline (Fig. 1L). Here, we note that this degree of suppression probably underestimates the role of *kuz*, as there is a significant maternal contribution. Together, these genetic results support the idea that Kuz functions in the Slit-Robo pathway to regulate midline repulsion.

We next sought to more closely characterize the behavior of a smaller group of ipsilateral neurons, the Apterous (ap) neurons (Lundgren et al., 1995). Removing either one copy of *kuz* or one copy of *slit* resulted in a mild ectopic crossing defect, whereas simultaneously limiting *kuz* and *slit* 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 *slit* or *robo* heterozygotes (Fig. 2C,D; see also Table S1 in the supplementary material). These data solidify the idea that *slit* 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 *slit* 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 *slit* 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 *slit* mutants just as well as expression of wild-type Slit-FL (Fig. 4D,H). Several independent transgenic lines gave

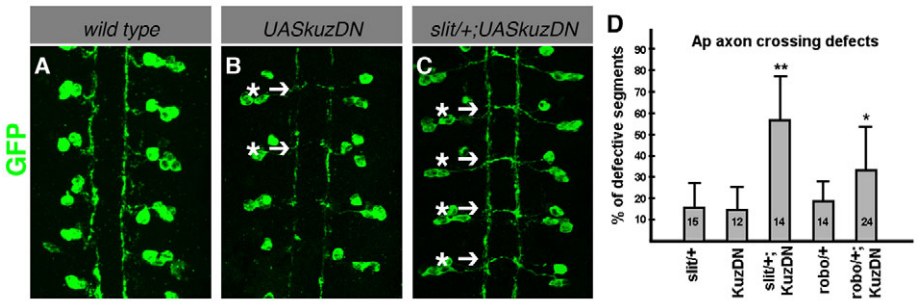

Fig. 2. Misexpression of *UASkuzDN* results in ectopic crossing of apterous neurons. (**A-C**) Stage 16 *apGal4, UASTau-Myc-GFP* embryos were stained with a polyclonal antibody against GFP. Anterior is up. In wild-type embryos (A) the ap axons do not cross the midline, whereas misexpression of *UASKuzDN* driven by *apGal4* (B) results in ectopic crossing (arrows with asterisks). (C) Removing one copy of *slit* 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.

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).

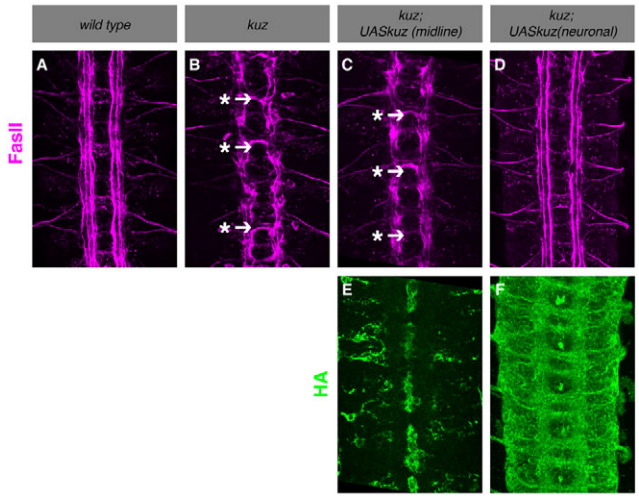

Fig. 3. *kuz* is required in neurons. (**A-F**) Stage 16 embryos were stained with a mAb to FasII (1D4). Anterior is up. (A) A wild-type embryo stained with mAb 1D4. (B) Ipsilateral axons from the medial and intermediate fascicles aberrantly cross the midline in *kuz* mutants (arrow with asterisk). (C,E) 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 in greater than half of all segments (107 out of 135 or 79% of segments show ectopic crossing, *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).

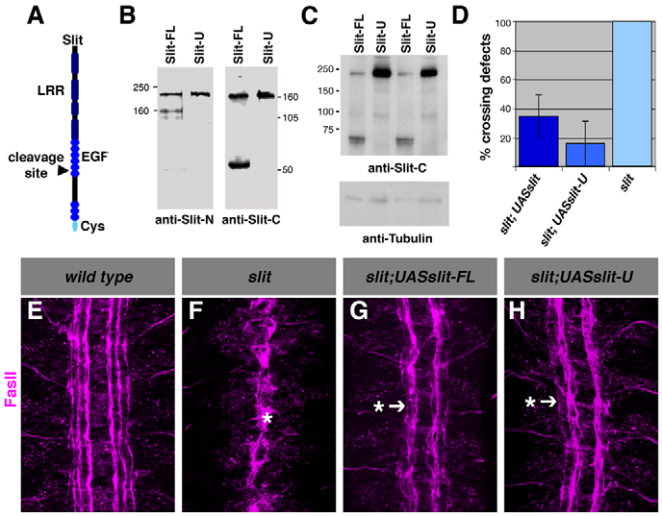

Fig. 4. Slit-U rescues the *slit* mutant phenotype. (**A**) Schematic drawing of Slit. The arrow indicates the cleavage site. Slit-U or Slit-FL were expressed in HEK293T cells. Total lysates of the cells were harvested and subjected to polyacrylamide gel electrophoresis (PAGE). (**B**,**C**) Western 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 *slit* 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 *slit* embryo mostly rescues the aberrant midline crossing phenotype, but does not restore proper lateral positioning of the FasII-positive fascicles (arrow with asterisk).

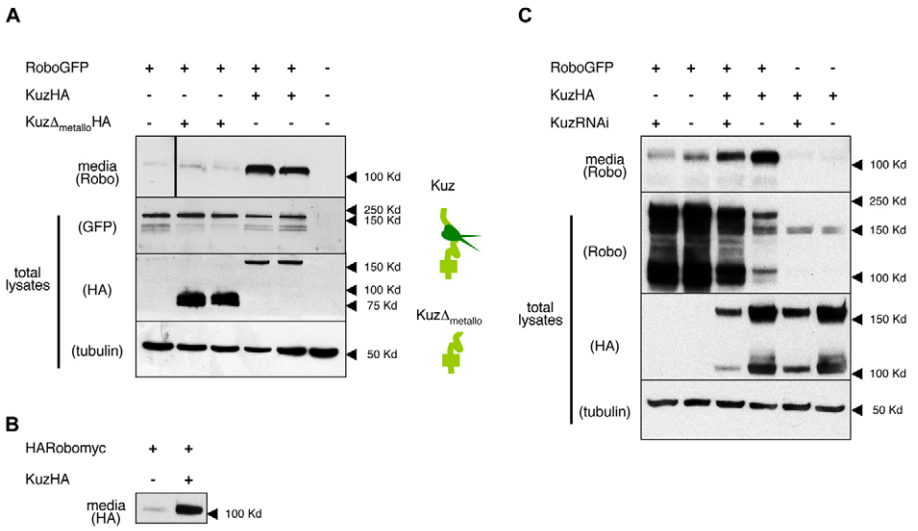

Fig. 5. Kuz promotes Robo cleavage in vitro. *UASroboGFP* with or without *UASkuzHA* were transfected into *Drosophila* S2R+cells. (**A**) Western blotting of separated proteins harvested from the media with antibodies to a N-terminal epitope of Robo reveals a significant increase in the amount of Robo 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 *UASkuz*^{Dmetallo} 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 Kuz$^{\Delta metallo}$, 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.

We next tested whether co-expression of Kuz could influence the production of this cleavage fragment. Indeed, in cells co-transfected with Kuz and Robo, there was an increased amount of Robo-ECTO detected in the media (Fig. 5A, lanes 4 and 5), suggesting that Robo could be a substrate of Kuz in vitro. Expression of a form of Kuz that lacks the protease domain (KuzΔmetalloprotease) had no influence on the accumulation of Robo-ECTO (Fig. 5A, lanes 2 and 3). Antibody staining of these cells revealed comparable levels of membrane-localized Kuz or KuzΔmetalloprotease, as well as similar degrees of colocalization with Robo (see Fig. S3 in the supplementary material). These data demonstrate that Robo-ECTO shedding can be enhanced by Kuz in vitro.

To further demonstrate the Kuz dependency of Robo-ECTO shedding, we performed RNA interference experiments to show that knockdown of Kuz prevents the enhanced shedding (Fig. 5C). Treatment with *kuz* double-stranded RNA (dsRNA) significantly reduced the levels of KuzHA when compared with those of untreated cells (Fig. 5C, lanes 3-6). Comparison of Robo-ECTO levels in the media of cells treated with *kuz* dsRNA (lanes 1 and 3) with levels in untreated cells (lanes 2 and 4) showed that attenuation of *kuz* expression results in a marked decrease in Robo-ECTO in the media, both in the presence and in the absence of transfected Kuz (Fig. 5C). We were unable to unambiguously demonstrate that *kuz* dsRNA treatment knocks down expression levels of endogenous Kuz because there is no antibody specific to *Drosophila* Kuz. However, the observation that *kuz* dsRNA treatment results in a decrease in Robo-ECTO shedding is highly suggestive that this treatment is able to knock down endogenous Kuz levels (Fig. 5C, lanes 1 and 2).

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 commissures (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).

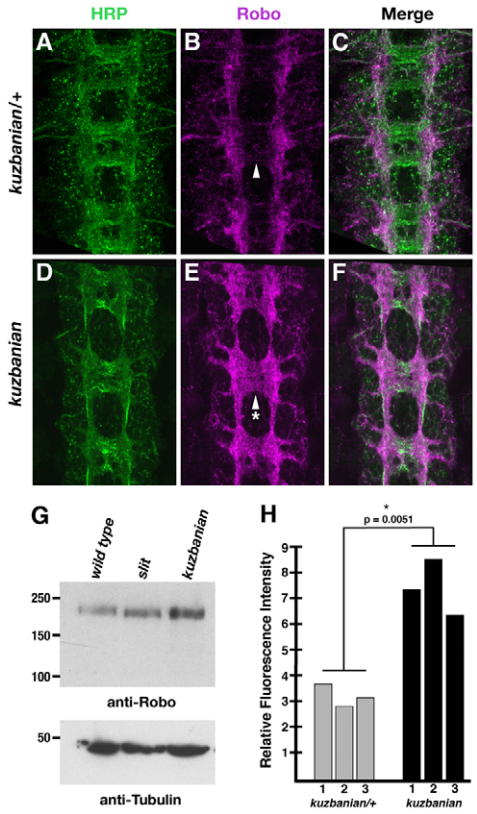

Fig. 6. Surface levels of Robo are increased and mislocalized in *kuz* mutants. (**A-F**) Stage 15 *kuz*/+ heteroygotes (A-C) or *kuz* mutant (D-F) embryos were dissected and stained live to reveal Robo surface expression. Anterior is up. Embryos were stained together on the same slide and micrographs were generated using identical confocal settings. (A-C) A *kuz*/+ heterozygous embryo stained with anti-HRP to reveal all axons (A) and with anti-Robo to visualize Robo distribution (B). Robo is enriched on longitudinal connectives and almost absent from axon commissures (compare B and C). (D-F) A *kuz* mutant embryo exhibits increased levels of Robo expression (compare E and B) and Robo protein is no longer restricted from the axon commissures (E,F). (**G**) A western blot from embryonic extracts of the indicated genotypes reveals increased Robo expression levels. Anti-tubulin was used as a loading control. (**H**) Quantification of Robo fluorescence intensity reveals a significant increase in Robo surface expression in *kuz* mutants. Each bar in the histogram represents an individual embryo. Fluorescence intensity was calculated as previously reported (Yang and Bashaw, 2006).

It is important to note that the region of Fra used to replace the three Fn domains of Robo does not include the Netrin binding domain, therefore the activity of Robo should not be affected by the presence of Netrin in vivo. We chose Fra as a swapping partner to create Robo-U because it has a similar structural organization to Robo. In addition, we did not detect Kuz-dependent shedding of the Fra ECTO domain in Fra-transfected cells with an antibody raised against the N-terminus of Fra (Fig. 7B, lanes 4 and 8). We were unable to detect shed ectodomain in the media of cells expressing Robo-U, either with or without added Kuz, suggesting Robo-U is not cleaved in this assay (Fig. 7B, lanes 3 and 7). We were also interested in determining whether Fra-Ro (a chimeric receptor consisting of the ectodomain of Fra and the transmembrane and intracellular domains of Robo) would undergo ectodomain shedding, because previous studies have shown that it is capable of mediating Netrin-dependent repulsive signaling in 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. 7F-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

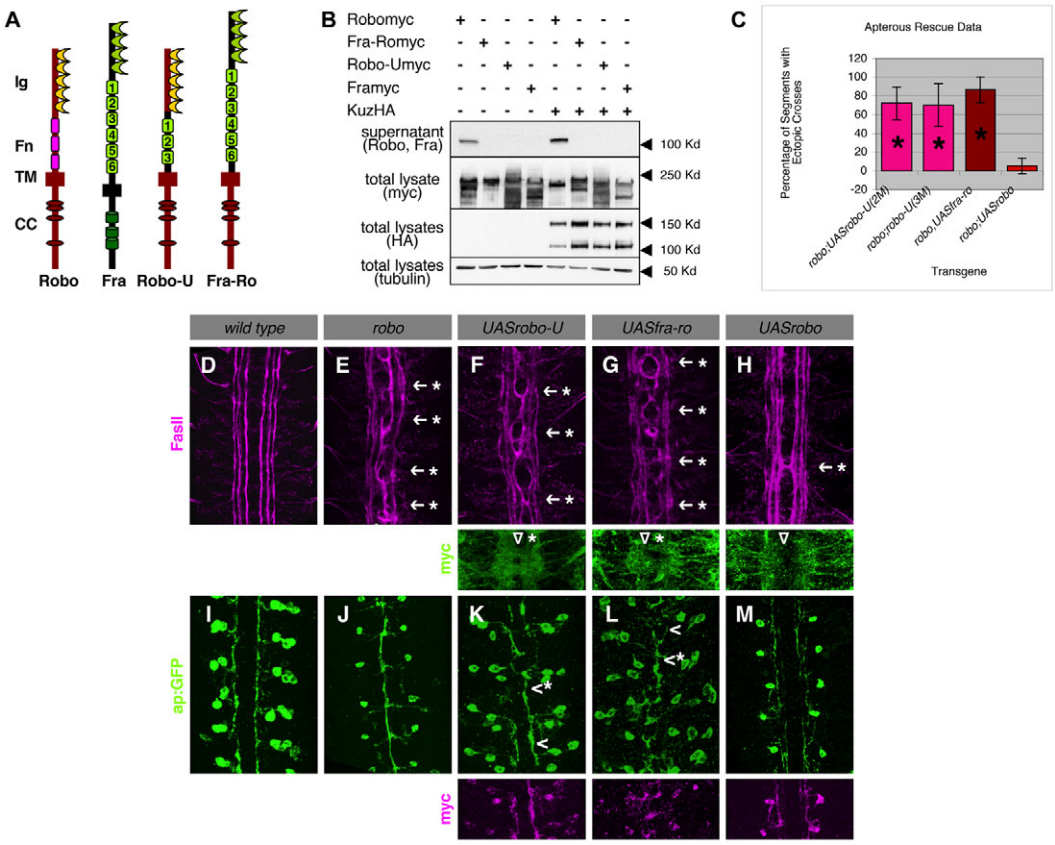

Fig. 7. Expression of an uncleavable form of Robo does not rescue *robo* mutants. (**A**) Schematic depicting the receptors used in the rescue experiment. In Robo-U the Fn domains of Robo are swapped for the first three Fn domains of Fra. The Fra-Ro receptor has the ectodomain of Fra and the transmembrane and intracellular domains of Robo. (**B**) The media and total lysates of S2 cells expressing the receptors depicted in A, with or without added *UASkuzHA*, were subjected to PAGE and subsequent western blotting as previously described. No ectodomain shedding was detected from cells expressing Fra-RoMyc, Robo-UMyc, or FraMyc. Western blotting of the total lysates with antibodies directed against Myc, HA and tubulin show the relative levels of Myc-tagged receptor, KuzHA and tubulin, respectively. (**C**) Quantification of the number of segments in which ap axons ectopically cross the midline in *robo* mutants expressing different forms of the Robo receptor under the control of *apGal4*. Shown is the percentage of segments in which the ap axons cross the midline. **P*<0.0001 (unpaired Student's *t*-test) compared to ap axons expressing wild-type Robo. Error bars indicate s.d. (**D-H**) Stage 16 embryos were stained with a MAb to FasII (1D4). Anterior is up. (D) A wild-type embryo stained with mAb 1D4. (E) In *robo* mutants, the medial-most fascicles cross the midline (arrows with asterisks). In *robo* embryos expressing Robo-UMyc (F) or Fra-RoMyc (G) under the control of the *elavGal4* driver, the medial-most fascicles still cross the midline in many segments (arrows with asterisks). (H) Pan-neural (*elavGal4*) expression of wild-type Robo provides significant rescue of the *robo* phenotype, although one can still observe infrequent ectopic crossing of FasII-positive axons (arrow with asterisk). The small panels below the FasII panels indicate the expression levels and patterns of the Myc-tagged receptors. Arrowheads indicate the midline; asterisks indicate Myc-tagged receptor expression on crossing axons. (**I-M**) Stage 16 embryos were stained with a polyclonal antibody directed against GFP to detect the ap neurons. Anterior is up. (I) In wild-type embryos the ap neurons never cross the midline. (J) In *robo* mutants, the ap neurons collapse upon the midline. In *robo* mutants expressing Robo-U (K) or Fra-Ro (L) driven by *apGal4*, the ap axons from either side of the midline are fused and collapsed on the midline in some segments (<*), but are correctly routed on either side of the midline in others (<). (M) Expression of wild-type Robo in ap neurons in a *robo* mutant background rescues the ectopic crossing phenotype.

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 *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). *UASFra-Ro* misexpression showed a similar phenotype. Misexpression of *UASRobo-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 repellant activity in the context of midline guidance; however, we cannot exclude the possibility that *Robo-U* could be deficient for other non-cleavage dependent functions.

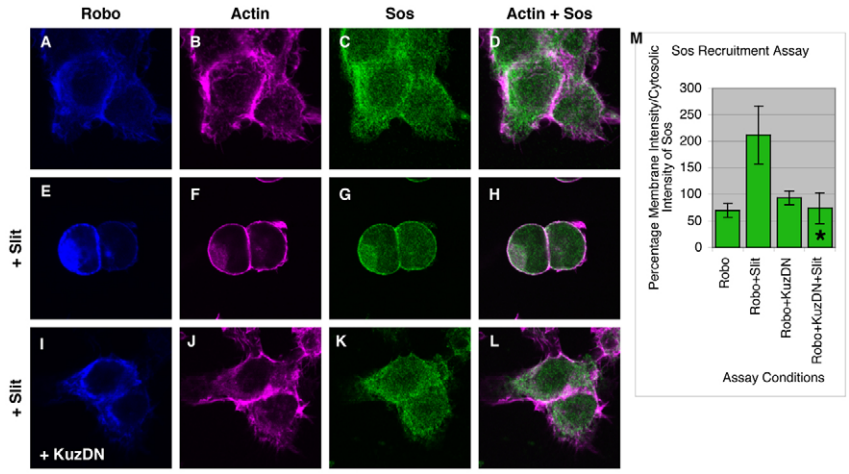

Fig. 8. Expression of mouse KuzDN blocks Slit-dependent recruitment of Sos to the plasma membrane. HEK293T cells were transiently transfected with human Myc-His-ROBO1. Endogenous human SOS is visualized by rabbit anti-mSos1 antibodies (green), human ROBO1 is visualized by mouse anti-Myc antibodies (blue), and F-actin is visualized by rhodamine-conjugated phalloidin (magenta). A single confocal section is shown. (**A-D**) A ROBO1-expressing cell treated with control medium. The morphology of the cell is elongated and SOS is predominantly localized in the cytoplasm. (**E-H**) A ROBO1-expressing cell treated with SLIT2-conditioned medium for five minutes. The cell morphology has become remarkably rounded and SOS proteins are recruited to the membrane, partially colocalizing with ROBO1 (white in H). (**I-L**) Cells expressing both ROBO1 and mouse KuzDN treated with SLIT2 medium. The cells retain their elongated morphology and endogenous SOS localization is cytosolic. (**M**) Quantification of the ratio of the average pixel density of SOS staining colocalized with the membrane to the average pixel density of cytosolic SOS. Shown is the average of the ratio of membrane to cytosolic SOS. **P*<0.0001 (unpaired Student's *t*-test) compared with ROBO1-expressing cells treated with bath application of SLIT2. Error bars indicate s.d.

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

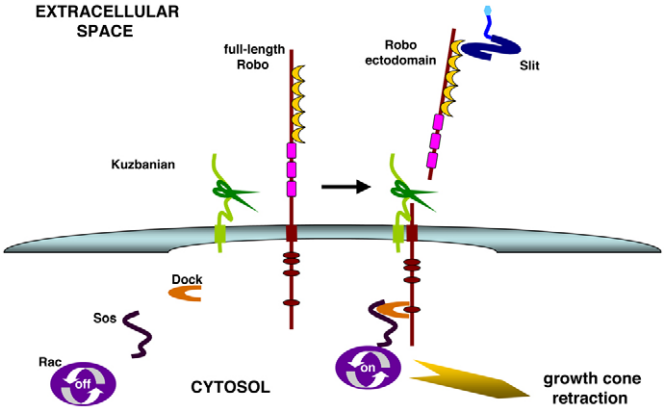

Fig. 9. Model of Kuz function in Slit/Robo repulsion. Our data suggest a model for Kuz function in the Slit/Robo pathway in which Slit binding to the Robo receptor results in cleavage of Robo by Kuz. Release of the Robo ectodomain could cause a conformational change in the remainder of the membrane-bound Robo receptor that strengthens its interaction with the Dock and recruits Sos to the plasma membrane. Sos is then properly localized to the plasma membrane, where it activates Rac to promote growth cone repulsion.

as can wild-type Slit. What then is the significance of Slit cleavage? Although the cleavage fragments are clearly present in western blots of total embryonic protein, it is not known where in the embryo this cleavage is occurring. Proteolysis might be important for developmental events other than axon guidance that involve Slit; for instance, muscle migration or attachment (Kidd et al., 1999; Kramer et al., 2001), or formation of the heart (Qian et al., 2005; Santiago-Martinez et al., 2006). Future experiments might shed light on the significance, if any, for Slit proteolysis in these contexts. Even though Slit-FL and Slit-U appear to be largely interchangeable in the experiments presented here, it cannot be ruled out that Slit proteolysis plays a role in fine-tuning axon guidance. Slit-FL does not fully rescue *slit* mutant axon guidance defects, which leaves open the question of whether cleavage is important for those guidance events that are not rescued; for example, the fine-tuning of the lateral positioning of axons.

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 ectodomain 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. GPI-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.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Table S1. Genetic interactions between *kuz, slit and robo*

A. Fas II axon guidance defects

Genotype	Number of segments scored (embryos)[1]	+	++	+++	FasII axon circling	% of FasII crossing defects[2]	% of FasII circling defects[3]	Statistics (unpaired *t*-test)
w^{1118}	110 (10)	0	0	0	0	0	0	–
kuz^{H143}/+	66 (6)	0	0	0	0	0	0	–
$slit^{1}$, $robo^{5}$/+	132 (12)	9	19	2	0	22.7	0	–
$slit^{1}$, $robo^{5}$/+, kuz^{H143}	143 (13)	27	39	8	ND	51.7	ND	$P<0.0001$
$slit^{1}$, $robo^{5}$/+, kuz^{112}	143 (13)	23	42	18	ND	58	ND	$P<0.0001$
$slit^{1}$, $robo^{5}$/+, kuz^{e29}	154 (14)	21	50	36	ND	69.5	ND	$P<0.0001$
kuz^{H143}	110 (10)	ND	ND	ND	4	50	3.6	–
kuz^{e29}	77 (7)	ND	ND	ND	2	76.6	2.6	–
$slit^{1}$, $robo^{5}$, kuz^{H143}/+, +, kuz^{H143}	110 (10)	ND	ND	ND	31	100	28.2	$P<0.0011$
$slit^{1}$, $robo^{5}$, kuz^{H143}/+, +, kuz^{e29}	110 (10)	ND	ND	ND	43	100	48.9	$P<0.0001$

B. Apterous axon crossing defects[4]

Genotype	Number of segments scored (embryos)[1]	Ap crossing defects	% of Ap crossing defects	Statistics (unpaired *t*-test)
w^{1118}	96 (12)	0	0	–
kuz^{H143}/+	104 (13)	20	19.2	–
$slit^{2}$/+	120 (15)	20	16.7	–
$slit^{2}$, +/ kuz^{H143}, +	288 (36)	117	40.6	$P<0.0001$
$slit^{2}$, +/ kuz^{2583}, +	224 (28)	92	41.1	$P<0.0001$
$slit^{2}$, +/ kuz^{112}, +	360 (45)	112	31.3	$P<0.0001$
UASKuzDN	96 (12)	13	13.5	–
$slit^{2}$/+;*UASKuzDN*	112 (14)	65	58	$P<0.0001$
robo/+	112 (14)	22	19.6	–
robo/+;*UASKuzDN**	192 (24)	61	31.8	$P<0.05$

Stage 16-17 embryos stained with anti-FasII MAb were scored as follows: +, thinner than normal fascicle ectopically crossing the midline; ++, normal fascicle ectopically crossing the midline; +++, thicker than normal fascicle ectopically crossing the midline.

[1]Eight abdominal and three thoracic segments were scored in each animal for a total of eleven segments.

[2]Percentage of FasII crossing defects is defined as total number of defects divided by segments scored.

[3]Percentage of FasII circling defects is defined as total number of defects divided by segments scored.

[4]Eight abdominal segments were scored in each embryo.

ND, not determined.