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Evolutionary conservation of midline repulsion by Robo family receptors in flies and mice

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Evolutionary conservation of midline repulsion by Robo family receptors in flies and mice

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Introduction

Slit and Robo regulate midline crossing and repulsion

As the nervous system develops in animal embryos, neuronal axons are guided to their synaptic targets by extracellular cues that signal through axon guidance receptors expressed on the surface of the axon (Battye et al., 1999). In animals with bilateral symmetry, one of the important decisions made by nearly every axon in the embryonic nervous system is whether to stay on its own side of the body, or to cross the midline and connect to cells on the opposite side. Failure of axons to properly cross the midline can result in severe defects. The Roundabout (Robo) family is an evolutionarily conserved group of axon guidance receptors that regulate midline crossing in a wide range of animal groups by signaling midline repulsion in response to their ligand Slit. Mutations in the Slit-Robo pathway has been identified in association with neurological diseases such as Parkinson's Disease and Horizontal Gaze Palsy with Progressive Scoliosis (Engle, 2010; Lin, 2009).

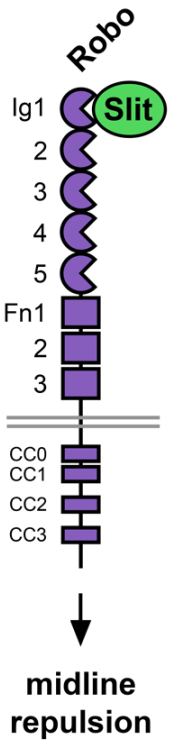

Figure 1. The Robo1 receptor in Drosophila is characterized by its distinct domains. Five immunoglobin-like (Ig) domains and three fibronectin (Fn) domains make up the ectodomain of Robo, and the four aptly named conserved cytoplasmic (CC) domains reside in the cytoplasm of the axon. The CC domains are thought to carry out midline repulsion signaling inside the neuron once Slit binds to the Ig1 domain on the cell surface.

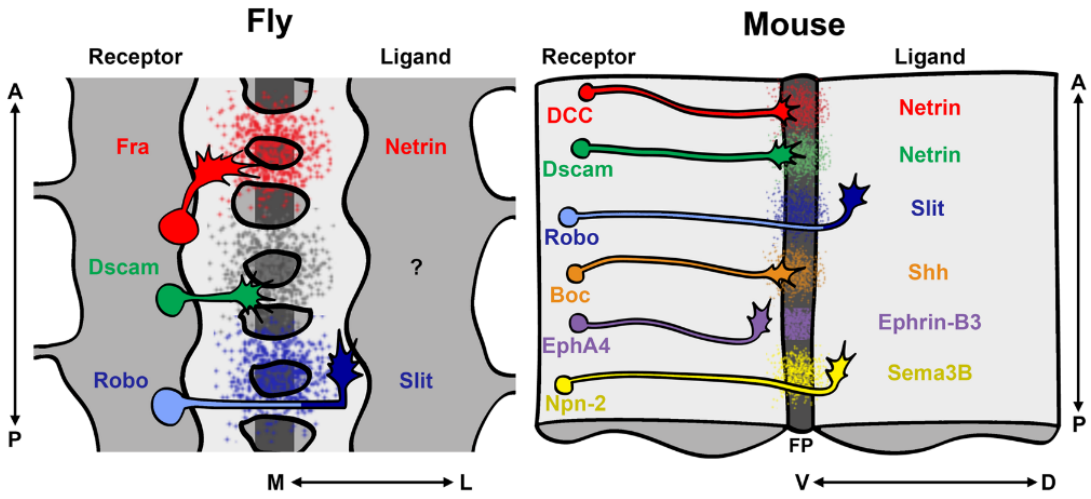

Figure from Evans and Bashaw, Curr Opin Neurobiol 20, 79-85.

Figure 2. Expression of axon guidance ligands and their receptors in the embryonic fly (*Drosophila*) and mouse central nervous system (CNS). Several conserved cues regulate nerve cord midline crossing of axons in the insect and vertebrate nerve cords, including the ligand Slit, which induces midline repulsion when it binds to its Robo family receptors. Robo receptors regulate both precrossing and postcrossing midline repulsion, as commissural axons cross the midline and must be regulated in ventral or lateral positioning.

Slit-Robo mechanism in *Drosophila*

Robo is expressed on growth cones of commissural axons, along with other receptors that regulate midline crossing. The Slit ligand, located at the midline of the bilateral nerve cord, can bind the Ig1 domain of Robo1 in *Drosophila,* which induces midline repulsion in that particular axon. Previous studies show that Ig1 deletion prevents any Slit-Robo interaction, proving the integral role played by Slit in midline repulsion (Brown et al., 2015). In normal *Drosophila* embryos, the Comm receptor prevents excess midline repulsion by limiting Robo presence until necessary contralateral axons have crossed (Howard et al., 2017).

Gene duplications and conserved Robo structure

The last common ancestor of insects and vertebrates possessed one ancestral Robo gene that was integral to midline repulsion. As the evolutionary paths diverged, the Robo gene was duplicated twice in the lineage that led to *Drosophila,* while three duplications occurred in the mammalian lineage. The duplicated Robo receptors in *Drosophila,* Robo2 and Robo3, play minor roles in midline repulsion. The mouse Robo2 receptor (mRobo2) is in many ways redundant to the properties of mRobo1, but it does play a complementary role in antagonizing post-midline crossing (Evans and Bashaw, 2010). The two isoforms of mRobo3 are identical except for an alternatively-spliced exon at the C-terminus in the fourth conserved cytoplasmic (CC) motif. mRobo3.1 in fact promotes initial midline crossing in the rostral direction of the nerve cord. mRobo3.2 complements mRobo3.1 by antagonizing secondary midline crossing, much like mRobo2. However, evidence from previous studies indicated that the two isoforms of mRobo3 most likely do not bind Slit (Chen et al., 2008; Zelina et al., 2014).

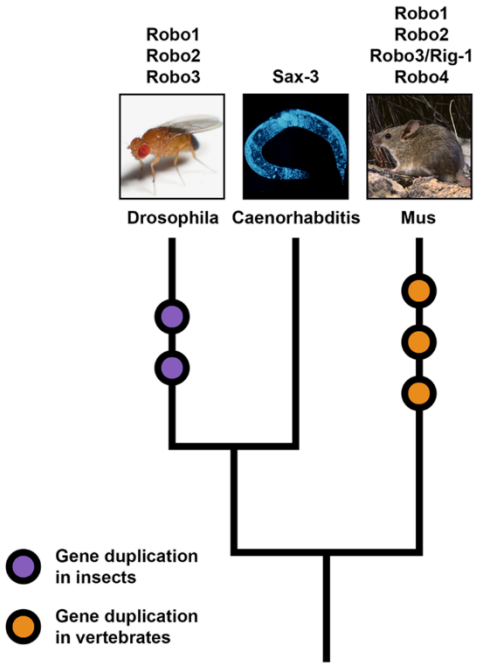

Figure 3. The ancestral *robo* gene was duplicated multiple times in the insect and vertebrate lineages, producing multiple Robo family members in flies and mice. These Robo receptors are unique to these lineages and are not present in unrelated species, such as the nematode C. elegans.

The Robo receptors in mice and *Drosophila* are remarkably similar, containing the same number and size of domains. Robo receptors in both species interact with the ligand Slit, although mRobo3 does not appear to bind. The function of midline crossing prevention when bound is conserved as well. (Reichert et al., 2016).

The extent of conservation in the Robo mechanism from *Drosophila* to mice

Despite their strong evolutionary conservation, it is unknown if the mechanisms of Robo signaling are conserved across different species. Can Robo receptors from mice regulate axon guidance decisions in Drosophila embryos, or do species-specific differences exist in the cellular signaling mechanisms by which Slit and Robos regulate midline crossing? To investigate the evolutionary conservation of Robo signaling

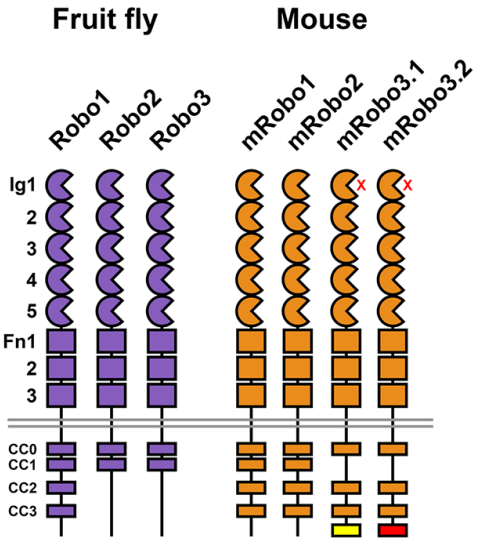

Figure 4. Robo1 is the main midline repulsive receptor in *Drosophila*, along with Robo2 and Robo3, both of which play an accompanying role in midline repulsion. Each duplicate Robo receptor in both flies and mice has conserved the five immunoglobulin (Ig) domains and three fibronectin (Fn) domains, all of which reside outside of the axon membrane. Robo2 and Robo3 in *Drosophila* do not have the conserved cytoplasmic (CC) regions of Robo1, which is a possible explanation for their reduced role in midline repulsion. While the mouse Robo2 (mRobo2) receptor conserves the domain structure of mouse Robo1 (mRobo1), the Robo3 gene (mRobo3) diverges slightly more. Mouse Robo3 encodes two Robo3 isoforms (mRobo3.1 and mRobo3.2), and recent evidence suggests that mRobo3 is not a Slit receptor.

mechanisms, we used two techniques in Drosophila to express Robo receptors from mice in fly neurons during embryonic development: We used the GAL4/UAS system to express mouse Robos at high levels in all embryonic neurons, and we used a robo1 rescue transgene to express mouse Robos in a pattern that reproduces fly robo1's normal

expression pattern and expression levels. We find that mouse Robo receptors are able to signal midline repulsion in Drosophila neurons when expressed at high levels, indicating that Robo signaling mechanisms are evolutionarily conserved. However, the mouse Robo receptors are not as effective at enacting midline repulsion as fly Robo1, suggesting some degree of evolutionary divergence between the receptors of the two species.

Methods

Gain-of-function mouse Robo expression

We crossed flies expressing elav-GAL4 with four lines of flies expressing mRobo1, mRobo2, mRobo3.1, and mRobo3.2. We placed the male and female flies together in a cage. Their offspring were collected in the embryonic stage of development every twenty-four hours, fixed in formaldehyde, and stored in methanol. These embryos were stained with primary antibodies m1D4 (dilution ratio 1:100) and goat anti-HRP FITC (1:100), as well as secondary antibody goat anti-mouse cy3 (1:1000). Anti-HRP antibodies label all axons in the ventral nerve cord, and m1D4 recognizes FasII proteins that are expressed in a subset of longitudinal axon pathways.

We also crossed flies carrying the eg-GAL4 gene and UAS-TMG transgenes with those carrying UAS-mRobo1, UAS-mRobo2, UAS-mRobo3.1, and UAS-mRobo3.2. We caged these crosses in a similar manner, allowing them to mate and produce embryos. These embryos were collected, fixed, and stained with primary antibodies mouse anti-HA (1:1000), Rb anti-GFP (1:500), and goat anti-HRP 647 (1:100). Secondary antibodies used were goat anti-mouse cy3 (1:1000) and goat anti-Rb 488 (1:500). The nerve cords of these eg-GAL4/UAS-TMG flies, when dissected, allowed for quantification of ectopic

crossing, which was performed under a confocal microscope.

Mouse Robo rescue of robo1 mutants

We then performed a rescue of flies with mutated robo receptors by crossing them with flies expressing the elav-GAL4/UAS transgene. We made four crosses, one for each type of mouse Robo, and we made a positive control rescue, using elav-GAL4/UAS to express *Drosophila* Robo1 in all neurons of flies with robo1 mutations.

We caged these crosses to reproduce for embryo collection. The embryos were collected, stained, and scored in a similar manner. Primary antibodies used were 1D4 (dilution 1:100), βgal (1:150), and goat anti-HRP FITC (1:100). The secondary antibody used was goat anti-mouse cy3 (1:1000).

Replacement of mRobo Ig1 domains with *Drosophila* Robo1 Ig1

Furthermore, we created chimeric Robo receptors, combining the Slit-binding domain, Ig1, of *Drosophila* Robo1 with mRobo receptors 1, 2, and 3.2. The fragments of *Drosophila* Robo and mouse Robo DNA were generated via PCR reaction and assembled via Gibson reaction with a pAW vector backbone. These chimeric plasmids were transformed into competent E. coli cells and grown into cultures. Sequences were verified by Simple Sequence with the corresponding primers.

The effectiveness of the chimeric receptor was tested in another *Drosophila* robo1 rescue. Offspring from these rescues were again caged for embryo collection and stained with primary antibodies 1D4 (dilution 1:100), βgal (1:150), goat anti-HRP 488 (1:200), and secondary antibody goat anti-mouse cy3 (1:500).

Results

Mouse Robo1 and 2 receptors can signal midline repulsion when expressed in all neurons

The elav-GAL4 transgene is used to express Robo receptors in all neurons, instead of just normal levels of expression. When elav-GAL4 is present and active, it activates upstream activation sequence (UAS) genes, in this case mouse Robo genes. (Berger et al., 2007) We assume that elav-GAL4 expression of Robo overrides regulation by Comm.

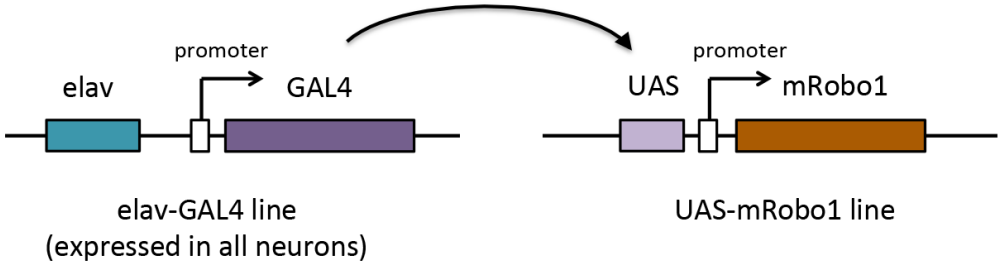

Figure 5. Expression of Robo receptors in all neurons is carried out through the elav-GAL4/UAS mechanism. In embryos carrying the elav transgene and the mouse Robo transgene downstream of an upstream activator sequence (UAS), elav activates GAL4 in order to activate the mRobo transgene via UAS. Thus, the Robo receptor is expressed in all neurons.

We crossed two fly lines, one with the elav-GAL4 transgene, the other line containing UAS-mRobo transgenes. Their offspring expressed mRobo receptors in all neurons. This cross was repeated three times in order to include all three mouse Robo gene duplications, mRobo1, mRobo2, and the two isoforms mRobo3.1 and mRobo3.2.

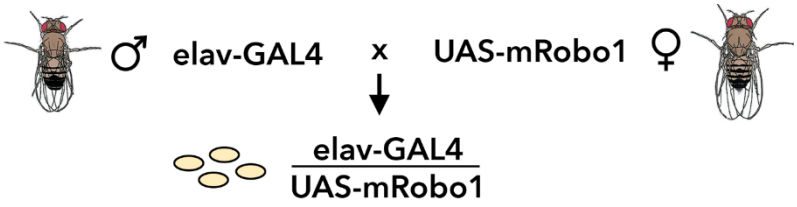

Figure 6. Expression of mRobo receptors in *Drosophila* using the elav-GAL4/UAS system was achieved by a simple cross between males expressing elav-GAL4 and females expressing mRobo receptors via the UAS system. Visible mutated traits engineered by previous crosses were used as indicators for carriers of each gene. Offspring from this cross were placed in a cage stock to mate and create embryos for further examination.

The flies from the elav-GAL4/UAS-mRobo crosses expressed mouse Robo

receptors in all neurons. Those that expressed mRobo1 and mRobo2 exhibited high levels of midline repulsion, similar to a positive control cross in which the elav-GAL4/UAS system was utilized to express *Drosophila* Robo1 in all neurons. However, although mRobo3.1 and mRobo3.2 were also expressed in all neurons, midline crossing was normal. The nerve cords in these lines more closely resembled those of flies with wild-type Robo1 receptors. This suggests that the two isoforms of mRobo3 do not effect midline repulsion, most likely because they do not bind Slit.

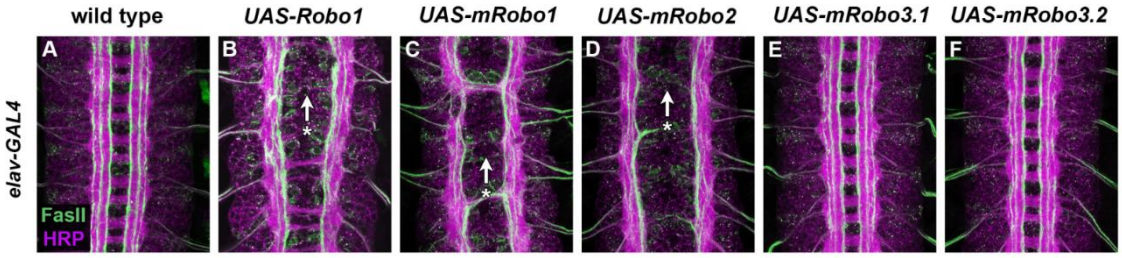

Figure 7. Images of embryonic nerve cords expressing various types and levels of Robo family receptors explicate each type's effectiveness in *Drosophila* midline repulsion. Fluorescent antibody staining highlights neuronal subsets In wild type *Drosophila* embryos **(A)**, axons cross the midline unhindered. This is due to normal levels of Robo expression and unhindered regulation of Robo expression by Comm. In embryos expressing high levels of the *Drosophila* Robo1 receptor, mRobo1, or mRobo2 **(B-D)**, the axons experience significant midline repulsion (lack of crossing indicated by arrow with asterisk), suggesting mRobo1 and mRobo2 function similiarly to *Drosophila* Robo1. In embryos expressing mRobo3.1 and mRobo3.2 **(E-F)**, axons cross the midline unhindered, which suggests, again, that neither isoform of mRobo3 binds Slit.

Simultaneously, we crossed a line of flies containing the eg-GAL4 and UAS-TMG transgenes with four lines with UAS-mRobo, expression of which allows for antibody highlighting of a specific subset of neurons (EW). The offspring from this cross allowed for antibody staining and quantification. When scored, we found that mRobo1 and mRobo2 were about 15% less effective in enacting midline repulsion than *Drosophila* Robo1 expressed at the same level. Again, we found mRobo3.1 and mRobo3.2 to exhibit 100% ectopic crossing, indicating that the presence of these mouse Robo genes are ineffectual in midline repulsion, even when expressed in all neurons via the elav-

GAL4/UAS genes.

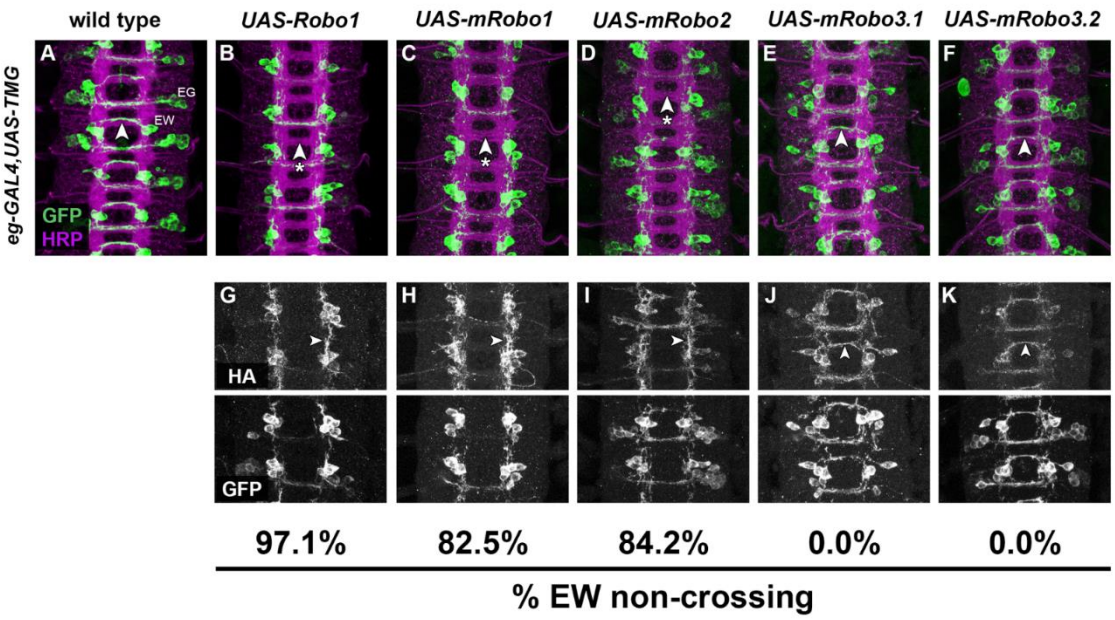

Figure 8. Images of embryos expressing the eg-GAL4, UAS-TMG transgene, which allows GFP antibody highlighting of a specific subset of neurons. Percentages of midline repulsion from each cross are displayed below each image. In wild type *Drosophila,* EW neurons cross the midline unhindered **(A, indicated by arrow)**. *Drosophila* Robo1 prevents midline crossing **(B, arrow with asterisk)**; therefore EW neurons grow along the nerve cord rather than across. Embryos expressing mRobo1 and mRobo2 exhibit midline repulsion similar to *Drosophila* Robo1 **(C-D, arrow with asterisk)**, but with about 15% less accuracy. mRobo3.1 and mRobo3.2 do not prevent midline crossing of EW axons **(E-F, arrow)**, which implies yet again that mRobo3 is not a Slit receptor. HA staining demonstrates that each Robo receptor is expressed on EW axons, either as they repelled from the midline **(G-I, arrow)** or as they cross the midline **(J-K, arrow)**.

mRobo2 can partially rescue midline repulsion in robo1 mutants

We performed four rescue crosses of mutated *Drosophila* Robo receptors (robo1) using elav-GAL4/UAS to express mouse Robo1, 2, 3.1, and 3.2 in all neurons. In addition, we performed a rescue of robo1 mutants with *Drosophila* Robo1 expressed via the elav-GAL4/UAS system. Offspring from these crosses produced embryos for collection, staining, and scoring. The UAS-Robo1 positive control cross produced embryos with 0% ectopic crossing and visibly extreme midline repulsion, indicating a successful rescue. We then found that of the four mouse Robo crosses, mRobo2 was the most successful in rescuing for mutated *Drosophila* receptors; however, embryos from the mRobo2 rescue still exhibited approximately 40% ectopic crossing, so the rescue

could only be deemed partial.

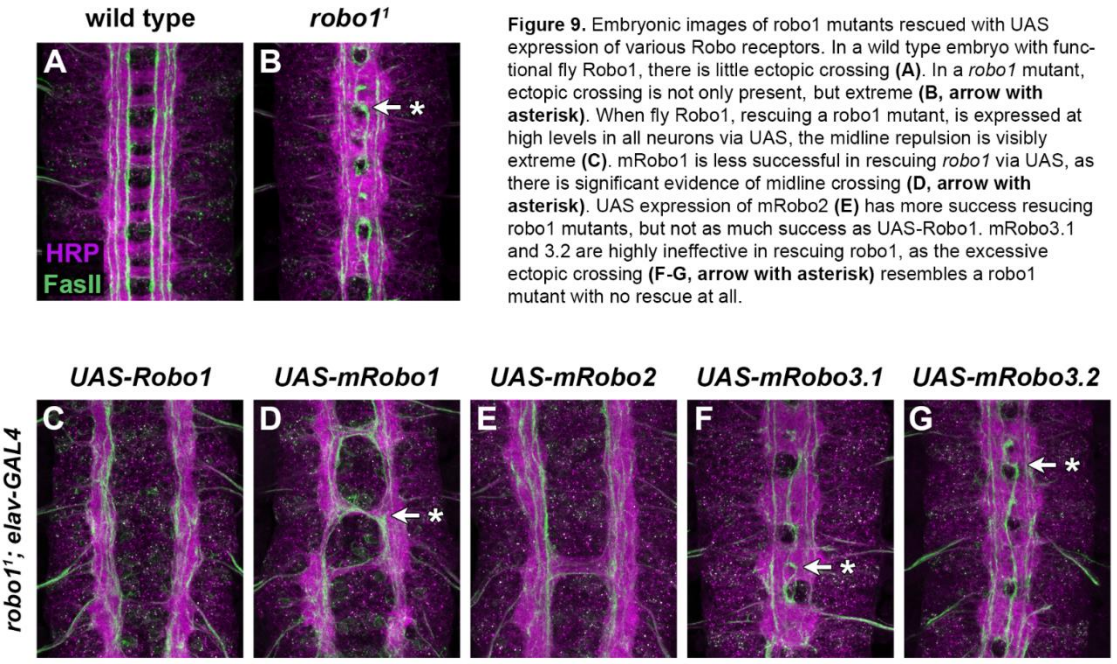

Figure 9. Embryonic images of robo1 mutants rescued with UAS expression of various Robo receptors. In a wild type embryo with functional fly Robo1, there is little ectopic crossing **(A)**. In a *robo1* mutant, ectopic crossing is not only present, but extreme **(B, arrow with asterisk)**. When fly Robo1, rescuing a robo1 mutant, is expressed at high levels in all neurons via UAS, the midline repulsion is visibly extreme **(C)**. mRobo1 is less successful in rescuing *robo1* via UAS, as there is significant evidence of midline crossing **(D, arrow with asterisk)**. UAS expression of mRobo2 **(E)** has more success resucing robo1 mutants, but not as much success as UAS-Robo1. mRobo3.1 and 3.2 are highly ineffective in rescuing robo1, as the excessive ectopic crossing **(F-G, arrow with asterisk)** resembles a robo1 mutant with no rescue at all.

We found that mRobo1 was less successful in rescuing, with embryos from this cross displaying over 90% ectopic crossing. Nonetheless, the effects of mRobo1 were visibly present. In contrast, robo1 mutant embryos rescued with either mRobo3.1 or mRobo3.2 displayed 100% ectopic crossing. The nerve cords in these crosses most closely resembled those of robo1 mutants with no rescue. This supports our earlier findings that mRobo3.1 and mRobo3.2 cannot successfully effect midline repulsion in

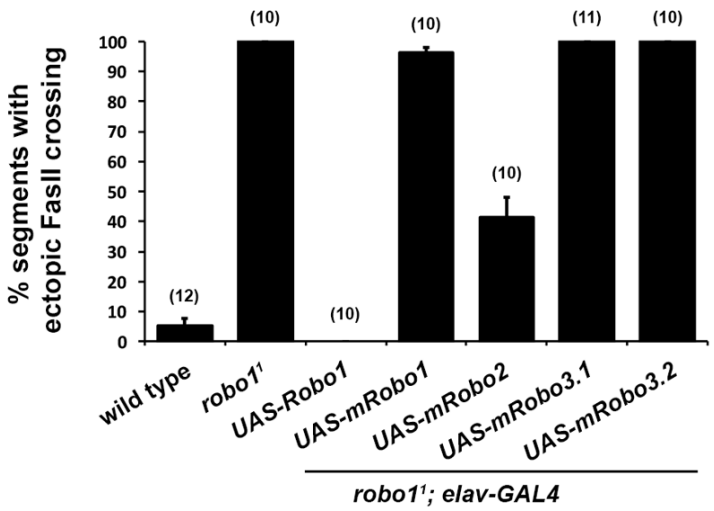

Figure 10. Graph of robo1 rescue embryo scoring. Percentage of embryonic segments with ectopic crossing negatively correlates with the success of the rescue. The rescue of robo1 with UAS expression of *Drosophila* Robo1 scored 0% ectopic crossing, indicating a complete rescue. The rescue with UAS expression of mRobo2 scored approximately 40% ectopic crossing, although this percentage is still notably higher than that of ectopic crossing in wild-type Robo1 embryos. Embryos rescued with mRobo1, mRobo3.1, and mRobo3.2 scored at nearly 100% ectopic crossing, which closely resembles the scoring of a null robo1 mutant with no rescue.

Drosophila.

Mouse Robos cannot rescue robo1 mutants in same expression pattern as fly Robo1

A parallel series of experiments expressed the four mouse Robos via a rescue construct that regulated expression in the same pattern as endogenous robo1. When expressed at the same level as fly Robo1, none of the mRobo receptors were able to rescue for robo1. This indicated a difference in rescue ability between mouse and fly Robos.

Mouse and *Drosophila* difference in rescue ability does not depend on Slit-binding domain

We created chimeric receptor genomes combining the Ig1 domain of *Drosophila* Robo1 with all other domains of mouse Robo 1, 2, and 3.2. Once their sequences were confirmed, each of the three chimeric receptors were inserted into a line of flies and crossed with robo^{GA285} mutants to test their effectiveness as a rescue.

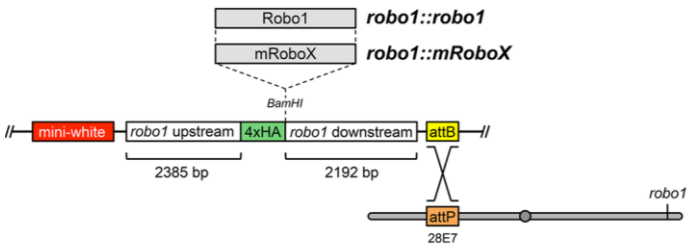

Figure 11. Schematic of chimeric receptor rescue construct. Constructs carrying a mRobo chimeric receptor with a *Drosophila* Ig1 domain were inserted into the Bam-HI restriction site of the robo1 sequence following the 4xHA tag. Meiotic recombination with robo1 mutants ensured that the offspring selected carried both the robo1 mutation and the rescue construct on one chromosome.

This rescue was conducted by crossing flies carrying the chimeric transgene with flies carrying a robo^{GA285} mutation. Meiotic recombination ensured that the offspring used for embryo collection and staining carried both the robo mutation and the

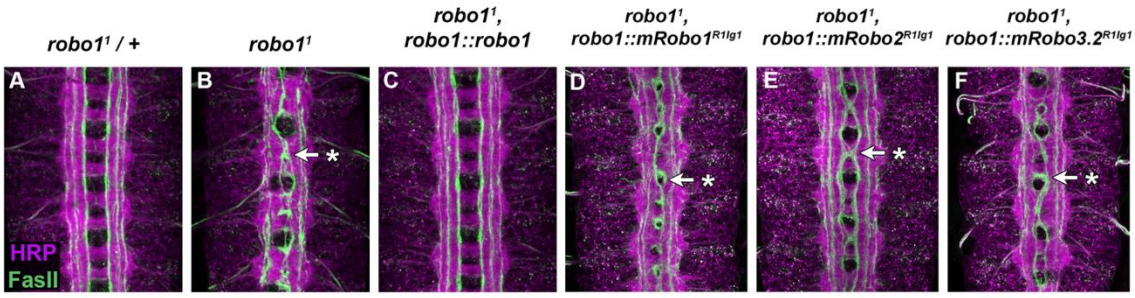

Figure 12. Images of embryos with robo1 mutations expressing various rescue constructs of Robo and mRobo receptors. In a heterozygous mutant *robo1* embryo, expression is relatively normal, resembling wild type **(A)**. In a mutant *robo1* embryo with no rescue, there is excessive ectopic crossing in the total absence of Robo **(B, arrow with asterisk)**. When a mutant *robo1* is rescued by fly Robo1, the embryo again resembles a wild type in terms of midline crossing **(C)**. When chimeric receptors containing a fly Ig1 domain and all other mouse Robo domains rescues a mutant *robo1*, the embryos exhibit ectopic crossing similiar to mutant *robo1* **(D-F, arrow with asterisk)**, indicating that these chimeric receptors cannot effectively rescue *robo1* mutants. However, in a qualitative sense, the mRobo2 chimeric receptor seems marginally more effective in terms of midline repulsion **(E)**.

transgenes. All three chimeric genomes exhibited ectopic crossing in 100% of embryonic segments, indicating an unsuccessful rescue. However, embryos expressing mRobo2 with a *Drosophila* Ig1 domain exhibited a qualitative difference in the extremity of ectopic crossing. Mouse Robo2 embryos had noticeably less intense crossing, which might indicate low levels of midline repulsion effected by the chimeric receptor. Overall, we found the mRobo^{R1Ig1} chimeras to be unsuccessful in rescuing *Drosophila* robo1 mutants.

Discussion

We found that mouse Robo receptors 1 and 2, when expressed in all neurons, can repel axons from the midline in *Drosophila* embryos. This suggests that the mechanisms by which *Drosophila* Robo receptors signal midline repulsion are conserved in mouse Robo receptors. However, the two isoforms of mouse Robo3, mRobo3.1 and mRobo3.2, are ineffective in *Drosophila* midline repulsion. These findings are corroborated by earlier studies that suggest that mRobo3.1 and 3.2 do not bind Slit, therefore nullifying their role in midline repulsion. This supports the previously studied conclusion that the

mechanism of midline repulsion was lost with the gene duplications that created mRobo3.1 and 3.2, although it remains to some extent in mRobo1 and 2.

We also found that mRobo2, when expressed in all neurons and without the presence of functional Robo1, can partially rescue midline repulsion in robo1 mutants. Although the mRobo2 rescue was not as successful as the Robo1 rescue, there was a marked difference between the effectiveness of the mRobo2 rescue and that of mRobo1, 3.1, and 3.2. This suggests that the mRobo2 gene has the most evolutionary conservation of the midline repulsion mechanism of *Drosophila.* The mechanism is only partially conserved, since *Drosophila* Robo1 rescues robo1 mutants 60% more effectively accuracy than mRobo2.

Again, the lack of midline repulsion in the mRobo3.1 and 3.2 rescues supports the hypothesis that these two isoforms of mRobo3 do not bind Slit and the evolutionary mechanism of midline repulsion has been lost. And while the mRobo1 rescue embryos exhibit nearly 100% ectopic crossing, there is a stark qualitative difference in the axons of these embryos and those without rescue. The evolutionary mechanism has been somewhat conserved in mRobo1, but it is not enough to rescue Robo1 mutants.

The three chimeric receptors, mRobo1, 2, and 3.2, each with the Ig1 domain of *Drosophila* Robo1, further support these conclusions. Even when expressed in all neurons, the mRobo1 and mRobo3.2 chimeras cannot rescue robo1 mutants. Although the mRobo2 chimera also exhibits 100% ectopic crossing, images of the nerve cords of the embryos from this rescue reveal slightly less extreme crossing. This suggests that the mRobo2 chimeric receptor does exhibit a weak level of midline repulsion, again supporting the conclusion that the evolutionary mechanisms of the Robo gene is most

strongly conserved in mouse Robo2.

We also conclude that replacing the Ig1 domain of the mRobo receptors with the *Drosophila* Robo1 Ig1 domain does not increase mRobo receptors' ability to effect midline repulsion. Therefore the partial loss of evolutionary conservation does not lie in the Slit-binding domain of the mouse Robo receptors.

Conclusion

From these discussions we can conclude that while the mechanism of midline repulsion is somewhat conserved from flies to mice, the ability of mouse Robo receptors to effect midline repulsion in *Drosophila* is significantly diminished. If the structure of mouse Robo1 and 2 is so strikingly similar to that of *Drosophila* Robo, yet mRobo receptors are significantly less effective in *Drosophila,* then perhaps the structure of Robo receptors, most importantly the Ig1 domain, is not the most significant effector of midline repulsion.

We must consider in the implications of these results that there may be more complexity to the Slit-Robo mechanism than previously thought. We must also take into consideration the potential difficulty of mouse Robo receptors to bind Slit ligands endogenous to flies. In the future, we may investigate other aspects of midline repulsion as effected by *Drosophila* Robo receptors, discovering some other factor than Slit-binding that is not present in mouse Robo receptors.

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