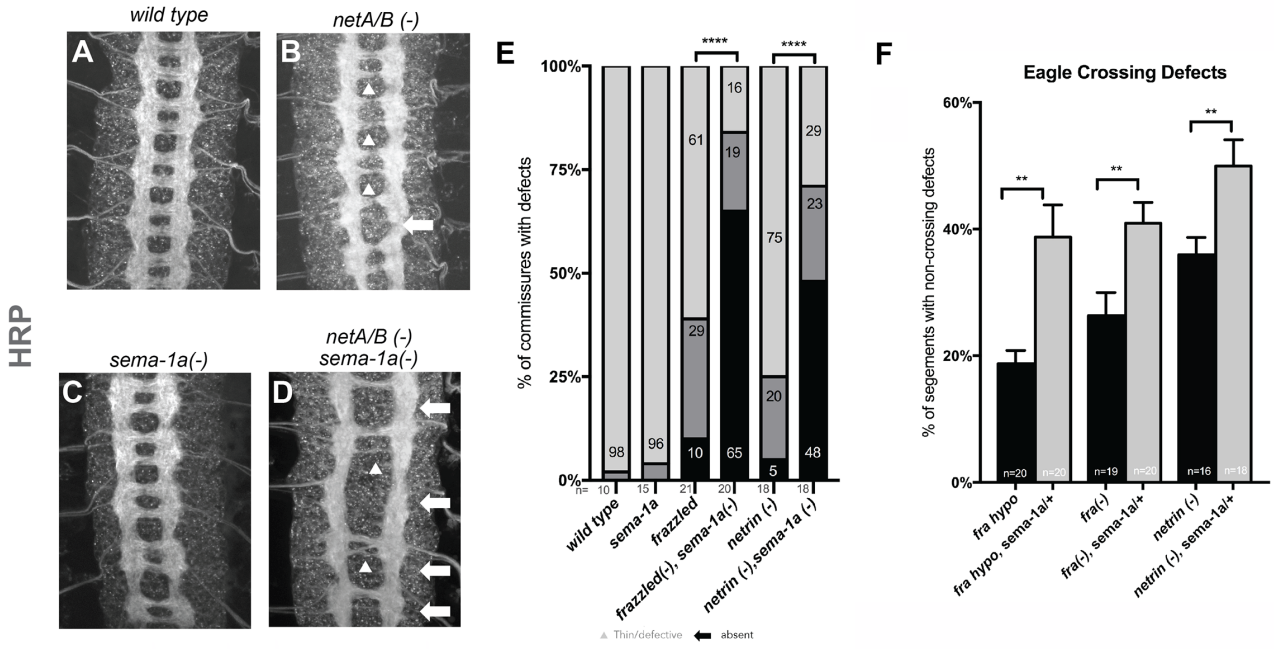
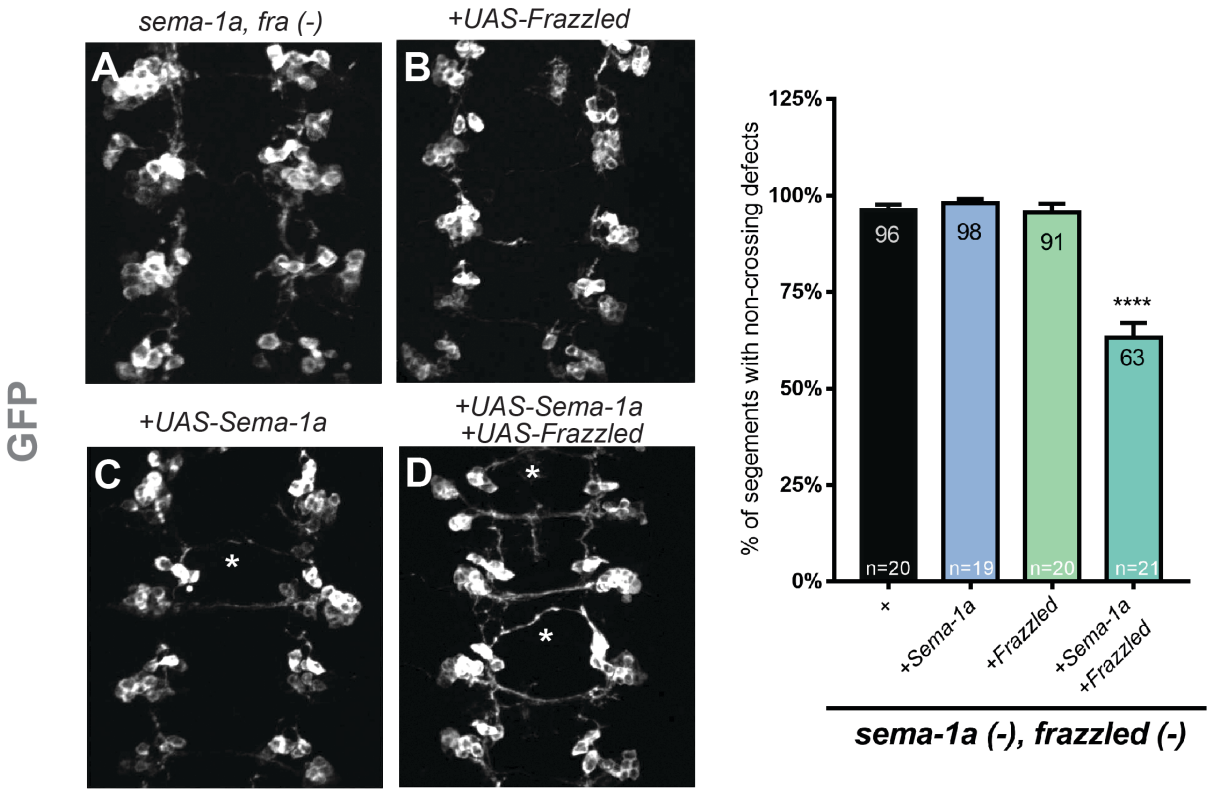
Supplemental Figure 1



Supplemental Figure 1: Sema-1a dominantly enhances the eagle crossing defects in both *fra* and *netAB* mutants. Related to Figure 2.

(A–D) Stage 16 embryos of the indicated genotypes stained with anti-HRP. Arrowheads indicate thin/defective commissures, arrows indicate missing commissures and asterisks indicate rescued commissures. (A) In wild type embryos, thick anterior and posterior commissures are formed as axons cross the midline in every segment. (B) *netAB* mutants show thin (20%) and missing commissures (5%). (C) Sema-1a mutants show no obvious signs of commissural defects. (D) *NetAB, sema-1a* double mutants show a 48% loss of commissures. (E) Histogram quantifies commissural defects as absent (black bar), thin/defective (dark gray) or wild-type (light grey) in the genotypes shown in (A–D). Scores for *fra* and *fra, sema-1a* double mutants are included as a reference. Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using ANOVA (****p<0.0001). (F) Heterozygosity for *sema-1a* significantly enhances the EW crossing defects in *fra* hypomorphs (fra^3/fra^6) to 38%. Loss of one copy of *sema-1a* also enhances crossing defects in *fra* single mutants (fra^3/fra^4) from 24% to 43%. EW crossing defects in *NetAB* mutants (34%) are also increased when a single copy of *sema-1a* is removed (50%). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed using ANOVA (**p<0.01).

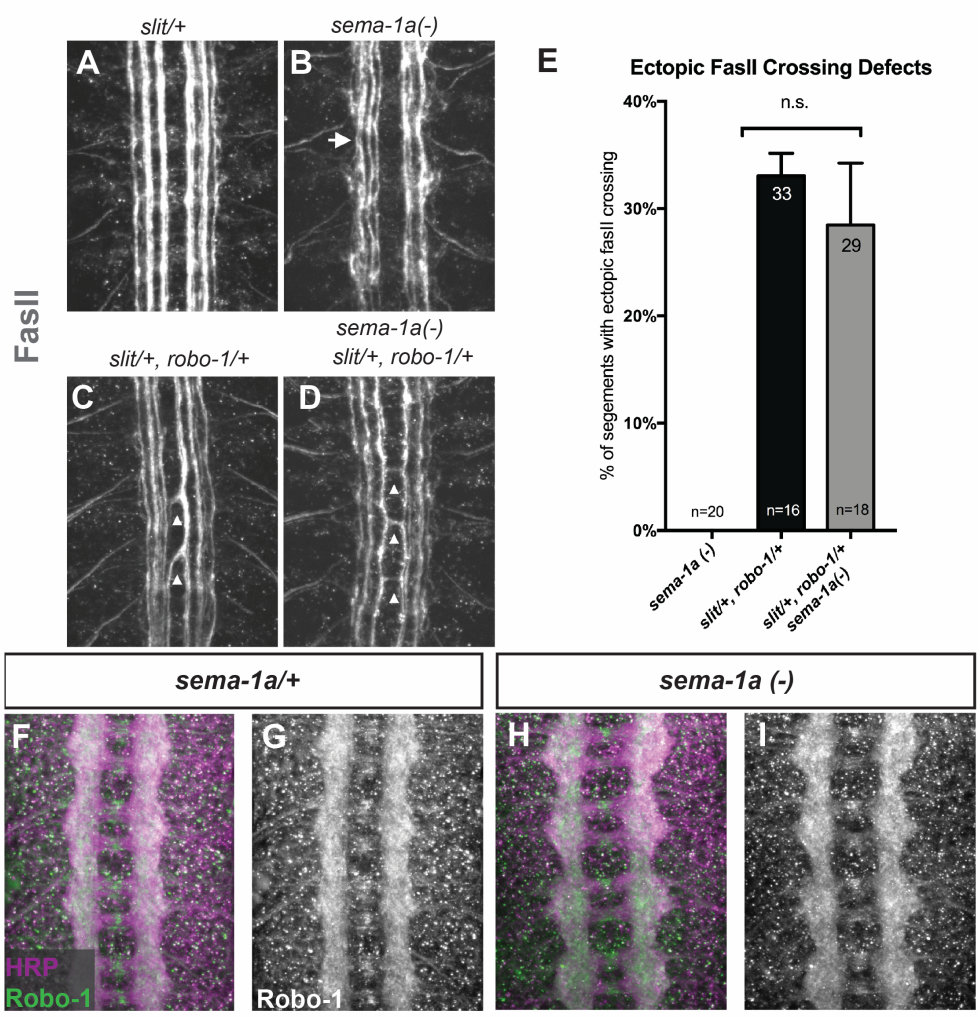
Supplemental Figure 2



Supplemental Figure 2: Sema-1a and Fra function additively to promote midline crossing. Related to Figure 2.

(A–D) Stage 16 embryos of the indicated genotypes stained with anti-GFP. Asterisks indicate rescued EW commissures. (A) Commissure formation is impaired in *sema-1a, fra* double mutants and EW axons fail to cross the midline in 96% of segments. (B) Expression of UAS-Sema-1a with eagleGal4 fails to rescue crossing defects in *sema-1a, fra* double mutants. EW neurons fail to cross in 98% of segments. (C) Expression of UAS-Fra with eagleGal4 fails to rescue crossing defects in *sema-1a, fra* double mutants. EW neurons fail to cross in 91% of segments. (D) Combined expression of UAS-Sema-1a and UAS-Fra transgenes partially rescues the crossing defects and commissure formation is significantly improved from double mutants. EW neurons fail to cross in only 63% of segments. Quantification of commissural defects. Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using ANOVA (****$p<0.0001$).

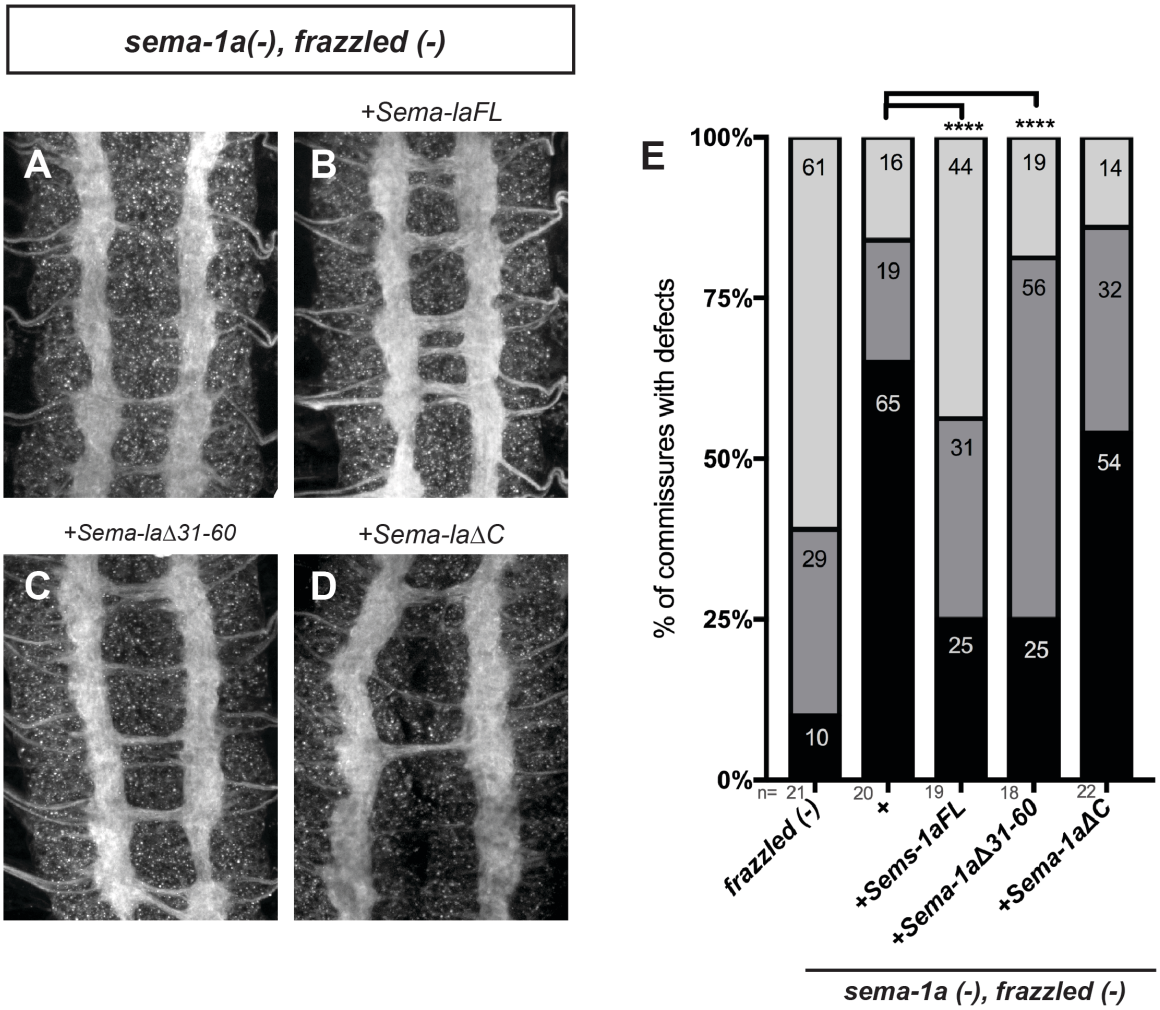
Supplemental Figure 3



Supplemental Figure 3: Loss of *sema-1a* does not suppress ectopic FasII crossing defects or alter Robo-1 expression. Related to Figure 2.

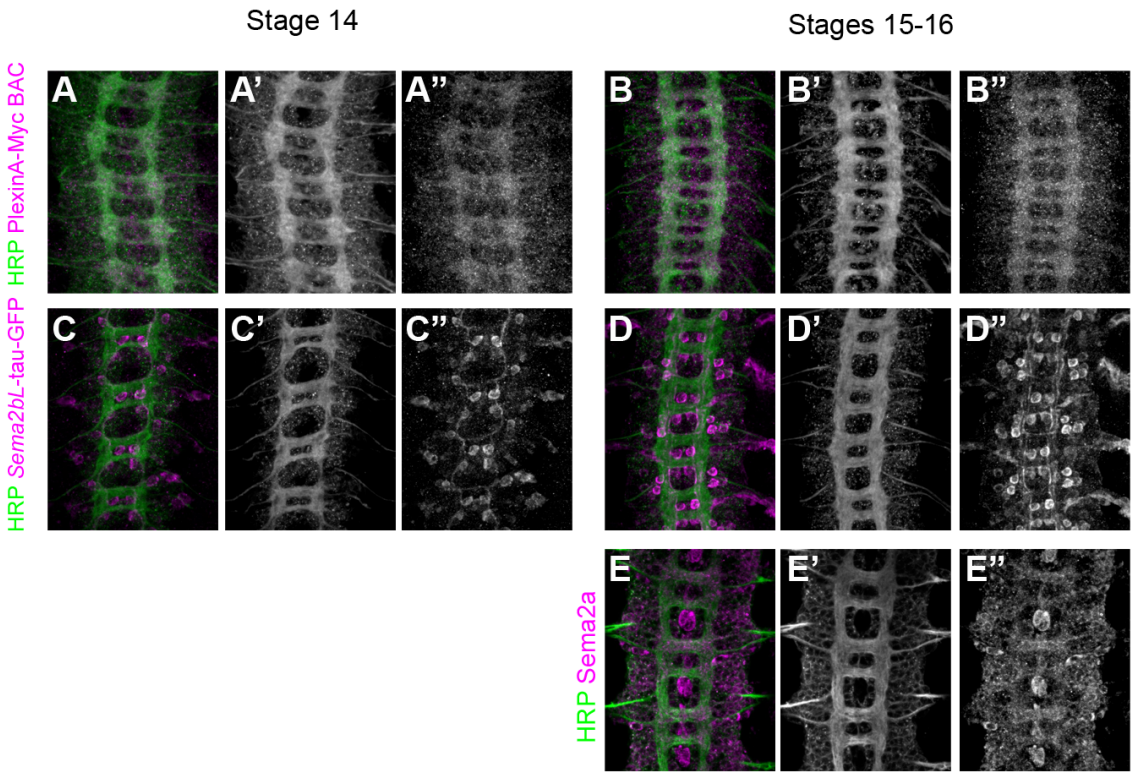
(A–D) Stage 17 embryos of the indicated genotypes stained with the Anti-FasciclinII (FasII). Anti-FasII labels three longitudinal tracts of ispilateral axons. Arrowheads indicate segments with ectopic crossing of FasII axons. (A) Embryos heterozygous for *sema1a* display intact longitudinals and FasII positive neurons never cross the midline. (B) *sema-1a* mutants show longitudinal breaks (arrow) but FasII axons never cross the midline (C*)* Embryos heterozygous for *robo-1* and *slit* show ectopic crossing defects (33%) due to reduced repulsion from the midline (D). These ectopic crossing defects are not significantly suppressed when *sema-1a* is also mutant suggesting that Sema-1a does not act as a negative regulator of Robo-1 repulsion. (E) Quantification of ectopic FasII crossing defects in the genotypes shown in (A–D). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by ANOVA. (F-I) Stage 15–16 embryos of the indicated genotype stained with anti-HRP and anti-Robo-1. (F) Embryos heterozygous for *sema-1a* exhibit the normal distribution of Robo-1 protein expression (G) Robo-1 is normally excluded from the commissural segment of axons (H) *sema-1a* mutant embryos do not show any qualitative elevation of Robo-1 protein expression (I) and Robo-1 protein is still restricted from commissural segments in s*ema-1a* mutants, suggesting that Sema-1a does not regulate Robo-1 protein expression at the midline.

Supplemental Figure 4



Supplemental Figure 4: The cytoplasmic domain of Sema-1a is required to promote midline crossing. Related to Figure 4.
(A–D) Stage 16 embryos of the indicated genotypes stained with anti-HRP. (A) Commissure formation is impaired in *sema-1a, fra* double mutants and 65% of commissures are absent. (B) Pan-neural expression of full length Sema-1a with elavGal4 can partially rescue crossing defects in *sema-1a, fra* double mutants reducing the number of missing commissures to 25%. (C) A Sema-1a transgene lacking a small region of the cytoplasmic domain (from aa31-60) does not rescue the midline crossing phenotype as well as wild-type, suggesting this region is important for promoting midline crossing. (D) A Sema-1a transgene lacking the cytoplasmic domain fails to rescue the crossing defects and commissure formation is not significantly different from double mutants. (E) Quantification of commissural defects as absent (black bar), thin/defective (dark gray) or wild-type (light grey) in the genotypes shown in (A–D). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using ANOVA (****p<0.0001).

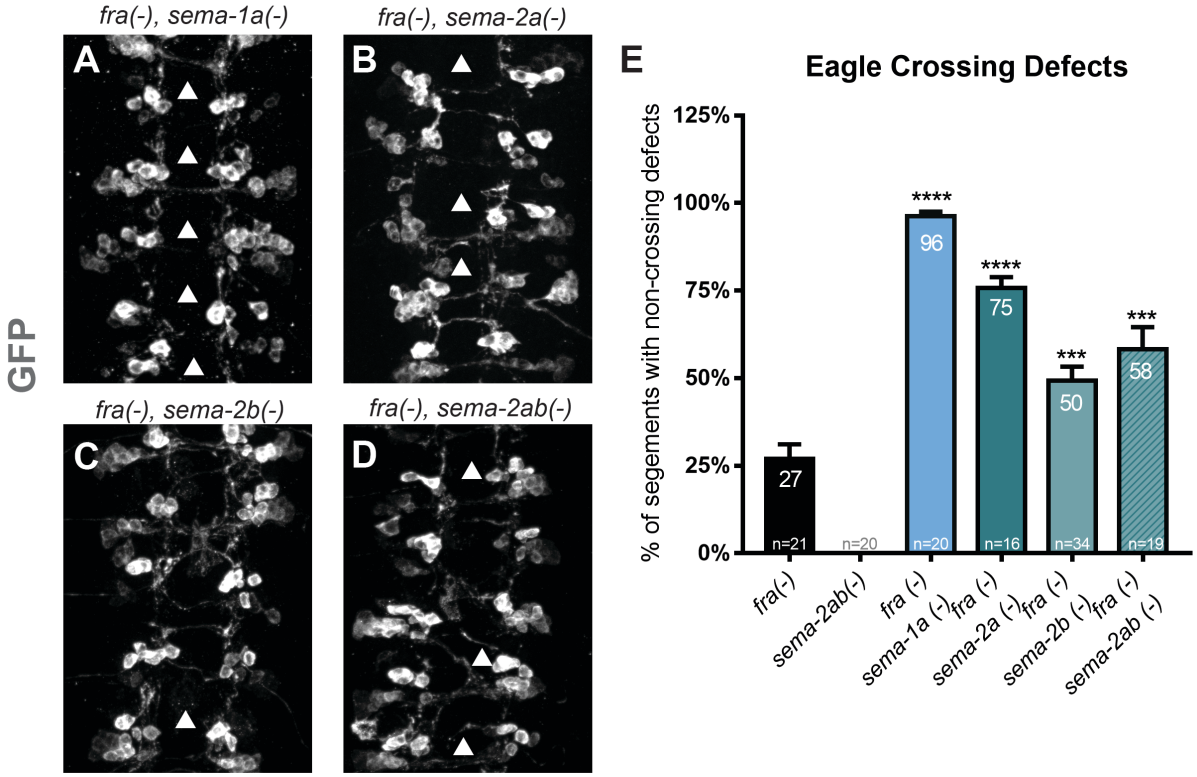
Supplemental Figure 5



Supplemental Figure 5: Expression patterns of Sema-2s and PlexA during midline crossing. Related to Figure 6.

(A–B) The PlexinA Myc-tagged BAC labels plexinA-expressing neurons and their axonal trajectories. Embryos of the indicated stages stained with anti-Myc. (A) PlexA is expressed broadly throughout the CNS at stage 14 and (B) stage 15-16. (C-D) The Sema2bL-tGFP reporter labels Sema-2b-expressing neurons and their axonal trajectories. Embryos of the indicated stages stained with anti-GFP. (C) Expression of Sema-2b is highly restricted in cell bodies close to the midline at stage 14 (D) Sema-2b expression increases during stage 15-16. (E) Embryos stained with anti-Sema-2a antibodies at stage 15-16. Sema-2a expression is very restricted with a strong enrichment at the midline.

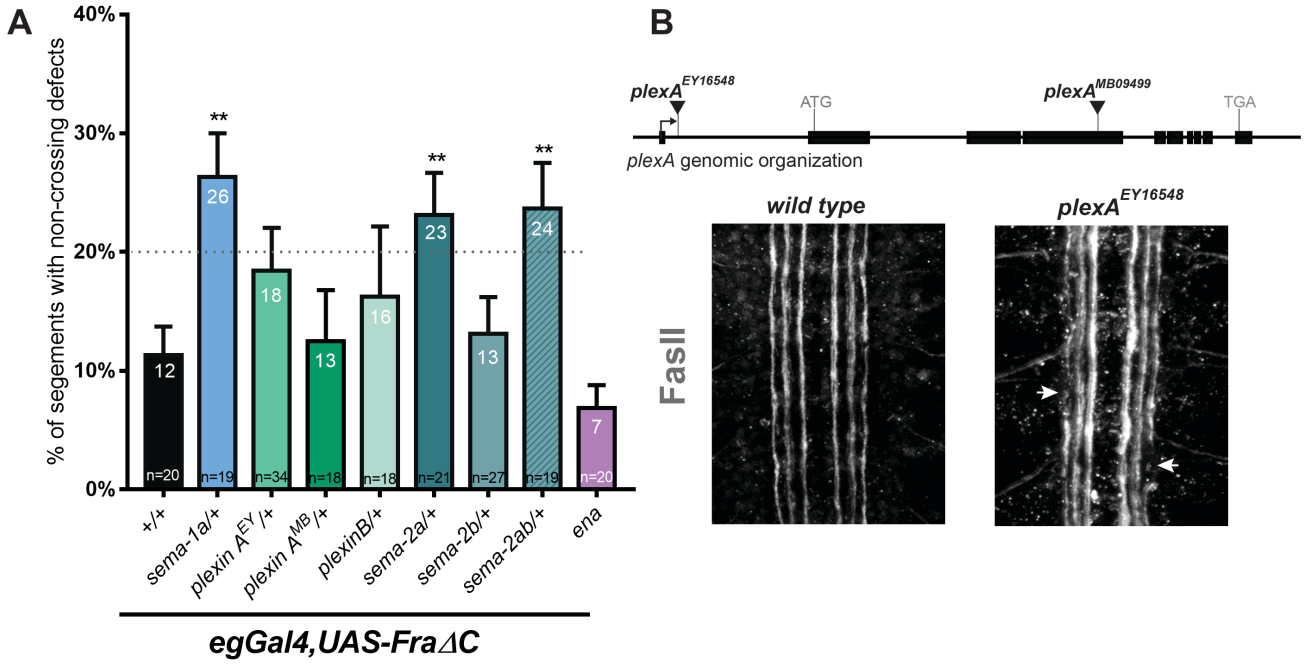
Supplemental Figure 6



Supplemental Figure 6: The secreted *sema-2s* enhance crossing defects in *fra* mutants. Related to Figure 6.

(A–D) Stage 15–16 embryos of the indicated genotypes carrying eg-GAL4, and UAS-taumycGFP transgenes, stained with anti-GFP antibodies. Anti-GFP labels cell bodies and axons of the eagle neurons (EG and EW) in these embryos. Arrowheads indicate segments with non-crossing EW axons. (A) *fra, sema-1a* double mutants display strong EW crossing defects (arrowheads 97%). (B) Embryos mutant for both *fra* and *sema-2a* show increased crossing defects (75%) when compared to *fra* single mutants. (C*) fra, sema-2b* double mutants also show a significant increase in EW crossing defects (50%) suggesting *sema-2b* also promotes midline crossing. (D) Unexpectedly, triple mutants exhibit slightly lower EW crossing defects (58%) than the *fra, sema2a* double mutants. This is in contrast to the effects observed when all axons are examined (Figure 6), where double and triple mutants were indistinguishable. (F) Histogram quantifies EW midline crossing defects in the genotypes shown in (A–D). Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using ANOVA (****p<0.0001, ***p<0.001).

Supplemental Figure 7



Supplemental Figure 7: A candidate screen of genes implicated in Sema/Plexin signaling reveals other potential factors involved in Sema-1a mediated midline crossing. Related to Figure 6.

Candidate genes that are implicated in Sema-1a signaling were screened for dominant enhancement of the FraΔC phenotype by scoring the crossing defects in EW neurons. In this case, we used a different insert of the FraΔC transgene that produces a milder phenotype (12% non-crossing). Dotted line indicates screening threshold for enhanced phenotype. (A) Quantification of EW midline crossing defects in the FraΔC screening background. Heterozygosity for *sema2a*, but not *sema2b*, was found to significantly enhance the crossing defects in eagle neurons to 23%. Heterozygosity for both sema-2a and sema-2b resulted in a similar increase in crossing defects (24%). No significant enhancement in crossing defects was observed for p*lexA* or p*lexB* heterozygotes. Heterozygosity for *ena* failed to enhance crossing defects. Data are represented as mean+SEM. n, number of embryos scored for each genotype. Significance was assessed by multiple comparisons using ANOVA (****p<0.0001, ***p<0.001, **p<0.01, *p<0.05). (B) Model of functional responses of Sema-1a reverse signaling through downstream effectors. (B) Schematic of *plexin A* genomic locus in D. melanogaster with P element mutations. The PlexAEY^{16548} allele is the result of an insertion in the untranslated region. The PlexA^{MB09499} allele was generated by an insertion following amino acid residues 1398 (Bellen et al., 2011; Jeong et al., 2012). PlexAEY^{16548} homozygous mutants exhibit breaks in the longitudinal fascicles (arrows) similar to defects observed in plexA deficiencies.

Supplemental Table 1: List of genotypes analyzed in each figure. Related to Figures 1-7, Figures S1-S4 and Figures S6 and S7.

The table lists the full genotypes that correspond to the abbreviated genotypes presented in the main and supplemental figures. Please see associated Microsoft Excel spreadsheet.

Supplemental Experimental Procedures

Phenotypic quantification: For EW commissural neuron axon crossing phenotypes, whole-mount or filleted embryos were analyzed at Stages 15 and 16. Eight abdominal segments were analyzed per embryo when possible, and for each embryo, the percentage of non-crossing segments was calculated. A segment was considered non-crossing when both clusters of EW axons (six axons per segment) failed to reach the midline. For quantification of phenotypes using HRP, both posterior and anterior commissures were scored. A commissure was considered absent if it was not continuous or distinguishable from the other commissure in the segment. Commissures were scored as thin/defective if they were substantially thinner than those in wild-type embryos or were observed to be excessively defasciculated.