The role of Wnt5 during Axon guidance

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Chapter 3

Src Family Kinases are downstream effectors of Wnt5 Signaling through the Derailed/Ryk Receptor

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Summary

Members of the Ryk/Derailed (Drl) family have recently been shown to regulate axon guidance in both Drosophila and mammals by acting as Wnt protein receptors. Little is known about how the kinase activity-deficient Ryks transduce Wnt signals. Here, we show that Wnt5-mediated signaling through Drl in the *Drosophila* embryonic central nervous system involves the non-receptor Src family tyrosine kinases, Src64B and Src42A. Analysis of mutant animals lacking the Srcs reveal defects in commissure formation similar to those observed in *wnt5* and *drl* mutants. Reductions in Src64B expression levels suppress a *wnt5*/*drl*-dependent dominant gain of function phenotype and increased levels of either Src64B or Src42A enhance *wnt5*/*drl*-mediated axon commissure switching. Drl and Src64B form a complex, containing catalytically active Src64B, whose formation or stability requires Src64B kinase activity. Furthermore, Drl is phosphorylated in a Src64B-dependent manner and coexpression of Drl and Src64B results in the activation of Src64B which is dependent on both Drl's intracellular and WIF domains. Their mammalian orthologs also form a complex, suggesting that Src roles in Ryk signaling are likely conserved. Finally, we show that coexpression of Wnt5 and Drl has no apparent effect upon TCF/LEF-dependent transcription, suggesting that the Wnt5/Derailed signaling pathway is unlikely to directly regulate canonical Wnt pathway targets. Together, these findings indicate that the Src family kinases play novel roles in Wnt5/Drl-mediated signaling.

Keywords: Axon guidance/Ryk/Src/Wnt/Signal transduction

Introduction

During the development of the nervous system, axons are guided towards their targets by attractive and repulsive guidance cues (Dickson, 2002). The ventral midline of the *Drosophila* embryonic nervous system has proven an excellent model system in which to identify the molecules that control axon guidance (Araujo and Tear, 2003). A number of these proteins are encoded by highly conserved gene families, such as the *slits*, *roundabouts* and *netrins,* which have been shown to play roles, remarkably similar to their Drosophila orthologs, at the mammalian floorplate (Garbe and Bashaw, 2004).

While considerable knowledge has accumulated about the mechanisms controlling initial midline crossing, less is known about those controlling routing at intermediate choice points where extending axons may take alternative routes. An example of such a decision is commissure choice. *Drosophila* contralateral axons project stereotypically through one of the two major axon tracts in each hemisegment, the anterior (AC) or the posterior commissure (PC). An axon's projection through the AC is, at least in part, dictated by its repulsion away from the PC by the Wnt family member, Wnt5 acting through the Derailed (Drl) Ryk axonal receptor

Wnt family member, Wnt5 acting through the Derailed (Drl) Ryk axonal receptor (Bonkowsky et al., 1999; Callahan et al., 1995; Yoshikawa et al., 2003). *wnt5* (Fradkin et al., 2004) and *drl* (Callahan et al., 1995) mutants also display altered axon fasciculation which may reflect changes in intra-axonal adhesion. Moreover, mutation of *drl*, also known as *linotte*, results in memory deficits (Dura et al., 1993), likely caused by axon guidance defects in the larval brain (Moreau-Fauvarque et al., 1998; Simon et al., 1998).

Wnt family proteins signal through alternative receptors with distinct downstream pathways which sometimes share common members. In many tissues, Wnts signal by binding to the Frizzled (Fz) family of receptors in conjunction with LRP co-receptors (Cadigan and Nusse, 1997). Fzs can transduce Wnt signaling via a canonical armadillo/ -catenin pathway culminating in the regulation of TCF/LEF-dependent transcription or via non-canonical pathways (Widelitz, 2005), some involving the heterotrimeric GTPases (Katanaev et al., 2005; Katanaev and Tomlinson, 2006; Liu et al., 2001). Recently, the mammalian Wnt5a protein was shown to interact with the receptor tyrosine kinase (RTK), Ror, resulting in the repression of canonical Wnt signaling via an, as yet, uncharacterized mechanism (Mikels and Nusse, 2006). In these studies, Wnt5a was also shown to activate the canonical Wnt signaling pathway via interaction with a Fz family member, suggesting that pathway specificity may sometimes be determined by the Wnt receptor engaged and not solely by the specific Wnt itself.

In addition to their involvement in a number of diverse developmental processes (Logan and Nusse, 2004), Wnts play roles in various aspects of nervous system development, such as, cell fate determination, synapse formation, axon guidance and neurite outgrowth (Ciani and Salinas, 2005; Fradkin et al., 2005; Zou, 2004). Wnt-Ryk interactions (reviewed in Bovolenta et al., 2006 and Keeble and Cooper, 2006) underly the anterior-posterior guidance of subsets of axons in the mammalian spinal cord (Liu et al., 2005), cortical axon guidance across the corpus callosum (Keeble et al., 2006), establishment of the vertebrate retinotectal topographic map (Schmitt et al., 2005) and neurite outgrowth in vivo and in cultured primary cells (Lu et al., 2004).

Members of the Ryk family of “dead” or “fractured” RTKs were first cloned by degenerate PCR using primers targeted to motifs conserved in many RTKs and have been found in all metazoans examined (Halford and Stacker, 2001). The extracellular domain of Ryk members contains a Wnt-binding WIF domain (Patthy, 2000). Ryks bear amino acid substitutions in highly conserved amino acid residues required for phosphotransfer, rendering them likely inactive as kinases. Although apparently lacking kinase activity, a human TrkA-Ryk chimeric protein was shown to activate the MAPK pathway when bound by NGF (Katso et al., 1999), suggesting that Ryks transduce extracellular signals to downstream targets within the cell. Recent studies (Grillenzoni et al., 2007; Yao et al., in press) have, shown that Drl can also act to antagonize Wnt5 function in the Drosophila post-embryonic CNS. These functions require the WIF domain, but not the intracellular region of Drl. Therefore, Drl alone is unlikely to be transducing a signal in these contexts, instead it likely sequesters Wnt5, preventing it from interacting with other as yet unidentified receptors.

Like Drl (Yoshikawa et al., 2003), human Ryk has been shown to bind Wnt protein (Lu et al., 2004). It acts in a ternary complex with the Fz and Wnt proteins signaling through the adaptor protein Dishevelled (Dsh) to increase TCF/LEF-dependent transcription in transfected cells, suggesting that mammalian Ryk induces canonical Wnt pathway target genes (Lu et al., 2004). Little is known about the targets of *wnt5/drl*-mediated signaling; however, *wnt5* transcription increases in embryonic AC neurons in the absence of *drl* (Fradkin et al., 2004) and Wnt5 protein is ectopically displayed at the pupal brain midline (Yao et al., in press) in *drl* mutants, indicating that the *wnt5* gene itself is a likely pathway target.

While Drl function in the Drosophila embryonic CNS does not apparently involve intrinsic tyrosine kinase activity (Yoshikawa et al., 2001), Drl’s cytoplasmic domain is required for axon

repulsion (Yoshikawa et al., 2003) and also likely plays a regulatory role in Drl function during brain development (Taillebourg et al., 2005). In addition, mammalian Ryk lacking its cytoplasmic domain acts as a dominant negative protein (Schmitt et al., 2005). Thus, the kinase-deficient Drl/Ryk receptors likely interact with other proteins whose activities are more obviously compatible with roles in signal transduction.

Here, we show that the highly conserved Drosophila Src family non-receptor tyrosine kinases (SFKs), Src64B and Src42A, play roles in Wnt5/Drl signaling. *src64B* and *src42A* double mutant animals display commissural phenotypes similar to *wnt5* and *drl* mutants, suggesting that the SFKs play at least partially redundant roles. SFK gain and loss of function alleles enhance and suppress, respectively, phenotypes dependent on Wnt5/Drl signaling and the SFKs and Drl physically interact resulting in Src activation and Drl tyrosine phosphorylation. Mammalian SFK and Ryk orthologs also coimmunoprecipitate from transfected tissue culture cell lysates indicating that Ryk-SFK interactions are likely evolutionarily conserved.

Materials and methods

Fly stocks

The following alleles, Gal4 drivers and reporters were used in this study and are described on FlyBase: w^{1118}, UAS-Wnt5^{11C}, Sim-Gal4, Elav-Gal4, OK6-Gal4, UAS-Src64B, UAS-Src64BK312R, UAS-Src42A, $src64B^{PI}$, $src64B^{KO}$, $src42A^{E1}$, $porc^{PB16}$, $wnt5^{400}$, drl^{Red2}, UAS-Drl, Sema2B-τ-myc, UAS-NLS- -Gal, UAS-mCD8-GFP, Da-Gal4 and Eg-Gal4. β-Gal or GFP balancer chromosomes were used to identify the appropriate progeny. $src42A^{E1}$/CyO-GFP; $src64B^{KO}$/TM6-GFP individuals were crossed with the single mutants over marked balancers to generate progeny homozygous for one SFK and heterozygous for the other. UAS-Drl-myc, UAS-dsRNA-Src64B and Sema2b-Gal4 *Drosophila* lines were generated using standard techniques. A *sema2b* promoter- Gal4 P-element plasmid was kindly provided by B. Dickson.

Immunohistochemistry and RNA in situ hybridization

Antibody labelings, RNA *in situs*, and staging of embryos were performed as described (Fradkin et al., 1995; Fradkin et al., 2004). The following antibodies were used on formaldehyde-fixed embryos: mouse mAb BP102 (A. Bieber, N. Patel, C. S. Goodman, unpublished), rabbit anti-myc (Upstate), rabbit anti-GFP (Invitrogen) and rabbit anti-Src64B (T. Xu, unpublished). Rabbit anti-Src64B peptide antibody (Muda et al., 2002) was used to stain the larval neuropiles expressing Src64B in motoneurons.

Constructs, transfection, immunoblotting, and immunoprecipitation

Tagged actin promoter- or UAS-promoter-driven wildtype and mutant Drl (HA) and Src64B or Src42A (myc) and wildtype untagged Wg and Wnt5 expression plasmids were constructed by ORF PCR, oligonucleotide-mediated mutagenesis and Gateway-mediated recombination (Invitrogen) into appropriate destination vectors (kindly provided by T. Murphey; http://www.ciwemb.edu/ labs/murphy/Gateway%20vectors.html). Y. Zou and K-L. Guan, respectively, kindly provided HA-tagged mouse Ryk (Liu et al., 2005) and human c-Src (Li et al., 2004) expression plasmids. To generate a *src64B*-specific RNA interference transgene, gene-specific inverted repeats (basepairs 1363 to 1963 of accession number NM_080195) were cloned into a pUAST derivative bearing an intervening intron. Decreases in *src64B* mRNA levels were

determined by semi-quantitative RT (Reverse Transcribed)-PCR of first strand embryonic cDNA as described (Supplemental Fig. 1). Similarly reductions in *src64B* expression were observed with two different transgenic inserts.
S2 and Kc cell transfections were performed using Effectene (Qiagen) and 293T cell transfections with Fugene (Roche). Lysates were prepared using a high stringency RIPA buffer (Muda et al., 2002), containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% Desoxycholate, 0.1% SDS, 0.2 mM Na-orthovanadate, 10 mM NaF, 0.4 mM EDTA, 10% glycerol and a cocktail of protease inhibitors (Roche). Drosophila cell lysate immunoprecipitations were performed using rabbit anti-myc (Upstate) or rabbit anti-Drl. Immunoblots were incubated with mouse 9E10 anti-myc mAb or rabbit anti-myc (Upstate) and mouse anti-HA (Sigma) or rabbit anti-HA (AbCam) for the tagged Src64B and Drl species, respectively, with the 4G10 or PY20 anti-phosphotyrosine mAbs (Upstate and Sigma, respectively) or anti-PY434Src64B affinity-purified antiserum, followed by multiple-label grade HRP- (Jackson ImmunoResearch) or 800CW (Li-Cor)-conjugated secondary antibodies and visualized with enhanced ECL (Roche) or an Odyssey two color laser scanner (Li-Cor), respectively. The immunoprecipitation resin in the ExactaCruz kit (Santa Cruz Biochemicals) or a mouse anti-rabbit light chain monoclonal antibody (Jackson ImmunoResearch) were used to reduce the recognition of the rabbit anti-Drl antibodies used in immunoprecipitation on blots probed with rabbit antisera. For the double immunoprecipitation of Drl to assess its phosphotyrosine content, lysates were first precipitated with anti-Drl, washed immune complexes boiled in 1% SDS, then diluted 1:10 into 1% Triton X-100 containing buffer and Drl-HA immunoprecipitated with reagents from the Profound anti-HA kit (Pierce) according to the manufacturers protocol.

Rabbit anti-Drl was described (Yao et al., 2007) and anti-PY434Src64B antisera was commercially generated (Eurogentec) against the Src64B peptide RVIADDEpYCPKQG as described (O'Reilly et al., 2006). The Drl antiserum was affinity purified on immobilized GST-Drl and the phospho-specific anti-PY434Src64B antiserum was first depleted of non-phospho-specific antibodies by binding to a column bearing the non-phosphorylated peptide and subsequently positively selected on a column bearing the phosphorylated peptide.

293T cell lysate immunoprecipitations were performed using anti-C-Src monoclonal antibody (Upstate) and immunoblots were probed with anti-C-Src or anti-HA to detect HA-tagged Ryk. Luciferase assays were performed using the Super8XTop/FopFlash plasmids (Veeman et al., 2003), a kind gift from R. Moon. Lysates were prepared and assayed using the Dual Luciferase Reporter Assay System kit (Promega) following the manufacturer's instructions. Luciferase expression levels were normalized to internal Renilla controls. The Checkmate mammalian-two-hybrid system was used according to the manufacturer's instructions (Promega). The cytoplasmic domain of Drl was cloned in frame with Gal4 DNA-binding domain in the pBind vector and the full-length wildtype or kinase deficient Src64B ORF was cloned in frame with VP16 activation domain in the pACT vector. SFK-deficient SYF cells (Klinghoffer et al., 1999) were obtained from LGC Promochem-ATCC (Middlesex, UK) and transfected using Fugene (Roche) All expression plasmids were verified by sequencing.

Results

src64B is required for correct crossing of the ventral midline by AC axons

To identify members of the *wnt5*/*drl* signaling pathway in *Drosophila*, we analyzed candidate proteins in a directed yeast two-hybrid approach. Src64B, a member of the non-receptor tyrosine kinase Src family kinases (Thomas and Brugge, 1997), was found to interact with the Drl

intracellular domain bait fusion protein (data not shown). In order to evaluate possible roles of *src64B* in *wnt5*/*drl* -mediated signaling, we examined the ventral nerve cord commissures in embryos lacking *src64B*, *src42A*, or both. Homozygosity for null alleles of either *src64B* or *src42A* or simultaneous heterozygosity for both *src64B* and *src42A* resulted in only mild aberrations in commissural projections (Fig. 1C-E). However, embryos entirely lacking one of the SFK orthologs and heterozygous for a null allele of the other (Fig. 1F,G) displayed "fuzzy" commissures, longitudinal breaks and apparent axon stalling in the longitudinal pathways, previously reported for *wnt5* null mutant embryos (Fradkin et al., 2004) (Fig. 1B). Embryos entirely lacking both *src64B* and *src42A* displayed a highly penetrant severe commissural phenotype (Fig. 1H). Quantitation of these phenotypes is presented in Table I. These results and the similar commissural phenotypes visible in a previous study examining SFK roles during Drosophila embryonic development (Takahashi et al., 2005) suggest that the Drosophila SFKs play partially redundant roles in the formation of the embryonic commissures.

We also generated embryos that have reduced levels of Src64B in a lineage that crosses in the AC, the commissure most affected by the absence of *drl* or *wnt5*, by expressing *src64B*-specific double stranded (ds) RNA under control of a *Sema2b-Gal4* driver (Materials and Methods, Brand and Perrimon, 1993). *sema2b* is expressed in a small subset of segmentally reiterated neurons which project their axons to the contralateral side through the AC (Rajagopalan et al., 2000). *src64B* mRNA down-regulation by RNA interference was evaluated by semi-quantitative RT-PCR and found to be ~ 9-fold, relative to controls (Supplemental Fig. 1A). Sema2b^{+} axons with decreased levels of *src64B* were found to misproject or apparently stall in a number of hemisegments (25%, n= 383) (Supplemental Fig. 1B). No apparent changes in Sema2b^{+} cell fate or cell body position were observed. These data provide further support for SFK roles in the formation of the two distinct wildtype commissures.

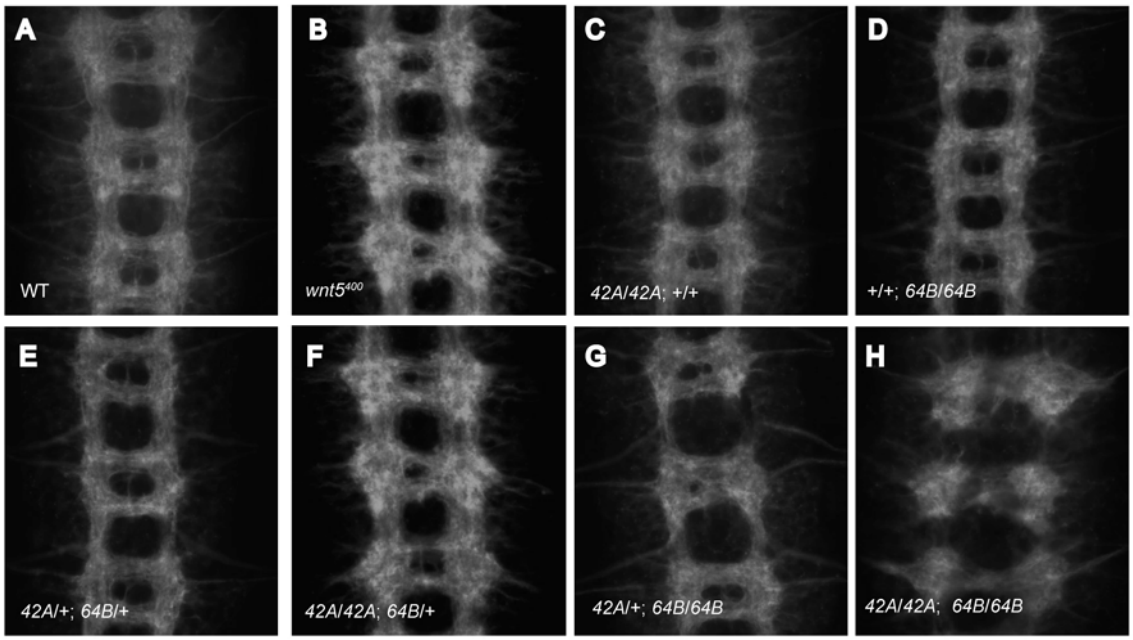


Figure 1. SFKs play redundant roles during formation of the embryonic CNS commissures. Stage 16/17 embryos of the indicated genotypes were stained with mAb BP102 to label all central neuron axons. Wildtype (**A**), $wnt5^{400}$ (**B**), $src42A^{E1}$ (**C**), $src64B^{KO}$ (**D**), $src42A^{E1}$/+; $src64B^{KO}$/+ (**E**), $src42A^{E1}$; $src64B^{KO}$/+ (**F**), $src42A^{E1}$/+; $src64B^{KO}$ (**G**) and $src42A^{E1}$; $src64B^{KO}$ (**H**) are shown. Defects similar to those seen in $wnt5^{400}$, "fuzzy" commissures and breaks in the longitudinal pathways, are observed in individuals homozygous for one of the SFK mutants and heterozygous for the other and become more severe in the double homozygotes. Quantitation is presented in Table I. Anterior is up.

Table 1 Abnormal commissural axonal projections in SFK mutants

Genotype	Segments with Abnormal commissural axonal projection (%)	Number of Segments scored
w^{1118}	0	251
$wnt5^{400}$	67	237
$src42A^{E1}$	10	330
$src64B^{KO}$	13	350
$src42A^{E1}$/+; $src64B^{KO}$/+	6.4	280
$src42A^{E1}$/+; $src64^{KO}B$	39	270
$src42A^{E1}$; $src64B^{KO}$/+	26	180
$src42A^{E1}$; $src64B^{KO}$	99	150

Embryos of the indicated genotypes were scored for thinning or loss of the commissures after staining with pan-axonal BP102 monoclonal antibody.

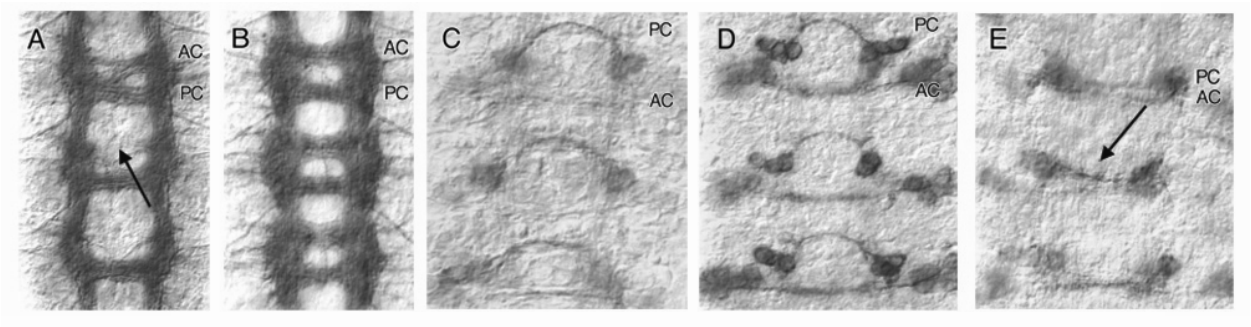


Figure 2. *src64B* is required for *wnt5/drl*-mediated axon repulsion. (**A**) Ectopic Wnt5 expression in the midline glia (sim-GAL4, UAS-Wnt5/+) results in frequent thinning or complete loss (arrow) of the AC. (**B**) Heterozygosity for *src64B* suppresses the thinning/loss of the AC (sim-GAL4, UAS-Wnt5/+; $src64B^{PI}$/+). (**C**) Eg^{+} neurons crossing in the AC and PC in an embryo with one copy of UAS-Drl-myc and one copy of UAS-NLS- -Gal visualized by anti-myc staining (UAS-Drl-myc/+;UAS-NLS- -Gal/+;Eg-Gal4/+). (**D**) Overexpression of Src64B in Eg^{+} neurons does not cause the PC axons to switch commissures (UAS-mCD8-GFP /UAS-Src64B; Eg-Gal4/+). (**E**) Simultaneous expression of Drl-myc and Src64B in the Eg^{+} axons significantly increases the number of the PC crossing lineages to switch to the AC (arrow) (UAS-Drl-myc/+; UAS-Scr64B/+; Eg-Gal4/+). Quantitation of these phenotypes is presented in Table III. Stage 16 embryos are shown, anterior is up.

Wildtype *src64B* expression levels are required for *wnt5*/*drl*-mediated axon repulsion

Next, we examined whether *src64B* genetically interacts with the *wnt5*/*drl*-mediated signaling pathway during embryonic nervous system development. We first evaluated whether wildtype *src64B* levels were required for a previously reported *wnt5* dominant gain of function phenotype (Fradkin et al., 2004; Yoshikawa et al., 2003). When *wnt5* was ectopically expressed from a single transgene in the midline glia using the Sim-Gal4 driver, approximately 16% of hemi-segments display the absence or thinning of the AC (Fig. 2A, Table II), likely due to the repulsion of the Drl^{+} AC axons by Wnt5 produced by the midline glia. We and others have previously used this or a similar genetic background to establish that wildtype O-acyltransferase *porcupine* (*porc*) (Fradkin et al., 2004) and *drl* (Yoshikawa et al., 2003) expression levels are

required for the loss of the AC, confirming that these genes are members of a Wnt5 signaling pathway. The removal of only a single copy of *src64B* from this genetic background resulted in a more than 5-fold reduction in the loss of the AC (compare Figs. 2A and B, Table I). The extent of suppression observed in *src64B* heterozygotes was similar to that seen in animals heterozygous for *drl* (Table II). Heterozygosity for *src42A*, in contrast, did not suppress the Wnt5-dependent midline overexpression phenotype (Table II), likely due to insufficient reduction of Src42A levels. These data suggest that wildtype *src64B* expression levels are required for this dominant gain of function phenotype and that *src64B* is therefore likely a member of the *wnt5*/*drl* signaling pathway.

Increased SFK expression enhances Drl-dependent PC to AC axon switching

We then asked whether increased SFK expression levels can force PC axons to cross in the AC, as was previously shown for *drl* (Bonkowsky et al., 1999; Yoshikawa et al., 2001; Yoshikawa et al., 2003). We found that increased expression of *src64B* driven by Eagle-Gal4 (Eg-Gal4) did not cause PC Eg-Gal4^{+} axons to switch to the AC (Fig. 2D). To evaluate whether wildtype *drl* expression levels might be limiting our ability to observe the effect of increased *src64B* expression, we used a UAS-Drl-myc transgene insert, which did not elicit substantial Eg-Gal4-dependent switching in single copy, but did when present in two copies (Table III). Increased expression of wildtype *src64B* in Eg-Gal4^{+} axons in the sensitized background (single copy of UAS-Drl-myc) resulted in significant enhancement in the number of switched axons (1% and 34% switched axons for 1X UAS-Drl-myc; 1X UAS-NLS -Gal and 1X UAS-Drl-myc; 1X UAS-Src64B, respectively; Fig. 2E, Table II). Enhancement of switching was also observed when *src42A* expression levels were increased (8%, Table III), but to a far lesser extent than with over expression of Src64B. Expression of a kinase-deficient *src64B* K312R transgene (J. Cooper, unpublished) in this sensitized background did not result in increased switching, indicating that SFK kinase activity is likely required for its interaction with Drl (Table III). These data support the hypothesis that the SFKs are members of the *wnt5/drl*- signaling pathway.

Table 2 Heterozygosity for *src64B* suppresses the *wnt5* midline glial overexpression phenotype.

Genotype	Loss or thinning of AC (%)	Loss or thinning of PC (%)	Number of segments scored
w^{1118}	0	0	251
sim-Gal4, UAS-Wnt5/+	16	0	240
sim-Gal4, UAS-Wnt5/+; $src64B^{P1}$/+	3	0	264
sim-Gal4, UAS-Wnt5/+; $src42A^{E1}$/+	17	0	256
sim-Gal4, UAS-Wnt5/drl^{red2}	3	0	248
$porc^{PB16}$/Y; sim-Gal4, UAS-Wnt5/+	2	0	380

Embryos of the indicated genotypes were scored for thinning or loss of the commissures after staining with pan-axonal BP102 monoclonal antibody.

***src64B* and *drl* are both expressed in AC axons**

To determine if *src64B* and *drl* are normally co-expressed in neurons, we determined the expression domains of *src64B* mRNA relative to those of *wnt5* and *drl* in the embryonic CNS. *src64B* mRNA is expressed throughout the ventral nerve cord (Fig. 3A) and overlaps with *drl* mRNA in the anterior part of each segment (Fig. 3B). *drl* RNA is expressed in neuronal cell

bodies that send their projections through the AC (Bonkowsky et al., 1999; Callahan et al., 1995; Yoshikawa et al., 2003), while *wnt5* mRNA is most predominantly found in neuronal cell bodies associated with the PC (Fig. 3C, Fradkin et al., 2004). Src64B protein is found in most, if not all, longitudinal and commissural axonal projections in the central nervous system (Fig. 3D) and is not readily detectable in the $src64B^{P1}$ mutant (Fig. 3E). To confirm that Src64B was transported out along axons, a double labeling of 3rd instar larval neuropiles co-expressing Src64B and GFP in motoneurons was performed. Src64B, like the mammalian (Maness et al., 1988) and *C. elegans* Srcs (Itoh et al., 2005), was found to be efficiently transported within the axons (Fig. 3F). A similar localization of Src42A was previously reported (Takahashi et al, 2005) and Drl was also previously shown to be present in AC axonal projections (Bonkowsky et al., 1999; Callahan et al., 1995). *src64B, src42A and drl*, are therefore expressed in AC axonal projections, supporting our observations that they functionally interact there.

Table 3 Increased SFK levels enhance commissure switching in a *drl*-sensitized background.

Genotype	PC to AC switching (%)	No Axons switching (%)	Number of segments scored
1X UAS-mCD8-GFP	0	100	567
2X UAS-Drl-myc	56	44	550
1X UAS-Drl-myc; 1X UAS-NLS βgal	1	99	459
1X UAS-Drl-myc; 1X UAS-Src64B	34	66	510
1X UAS-mCD8-GFP; 1X UAS-Src64B	0	100	418
1X UAS-Drl-myc: 1X UAS-Src64B K312R	3	97	566
1X UAS-Drl-myc; 1X UAS-Src42A	8	92	420

All embryos carry, besides the listed chromosomes, the Eg-Gal4 insert which was present in single copy in all genotypes except the 2X UAS-Drl-myc where it was present in two copies. Eg-$Gal4^{+}$ axons were visualized using anti-myc and scored for PC to AC switching when UAS-Drl-myc was present or by use of anti-GFP for UAS-mCD8-GFP.

Src64B and Drl and their mammalian orthologs physically interact

We then evaluated whether Src64B and Drl physically interact by ascertaining their ability to co-immunoprecipitate from transiently transfected tissue culture cell lysates. We expressed epitope-tagged Drl (Drl-HA) and Src64B (Src64B-myc) proteins in *Drosophila* Kc cells, which express little, if any, *wnt5* mRNA as assayed by quantitative RT-PCR and gene expression microarray analyses (our unpublished data and M. Fornerod, personal communication). Proteins were immunoprecipitated from cell lysates using antibodies specific for either Drl or the Src64B fusion protein and immunoblots of the immunoprecipitated proteins were probed with antibodies recognizing the reciprocal protein. Src64B, as its mammalian orthologs, is likely myristoylated and membrane-associated, so immunoprecipitations were performed under highly stringent conditions to disrupt membrane-protein interactions (Materials and Methods). Drl and Src64B were found to reciprocally co-immunoprecipitate both in the presence and absence of Wnt5 (Fig. 4A). Similar results were obtained using S2 cells (data not shown) and we observed that Src42A also co-immunoprecipitates with Drl, although less efficiently than Src64B (Suppl. Fig. 2).
We investigated whether this apparent physical interaction between a *Drosophila* Ryk and c-Src could also be observed with their mammalian orthologs. HA-tagged mouse Ryk and untagged human c-Src expression constructs were cotransfected into the 293T human embryonic kidney cell line. Cell lysate proteins were immunoprecipitated with anti-c-Src and immunoblots were probed with anti-HA antibody to visualize Ryk. These assays revealed that Ryk co-immunoprecipitates with c-Src (Fig. 4B), suggesting they form a complex. Ryk precipitated from

c-Src overexpressing cells migrated slightly faster on SDS-PAGE gels than the Ryk alone control, indicating the likelihood that some post-translational modification of Ryk takes place upon increased expression of c-Src. Together, the results of these coimmunoprecipitation experiments and the axonal localization of both Ryk/Drl and SFKs in mammals and Drosophila, indicate that Ryks and Srcs likely interact in evolutionarily distant species.

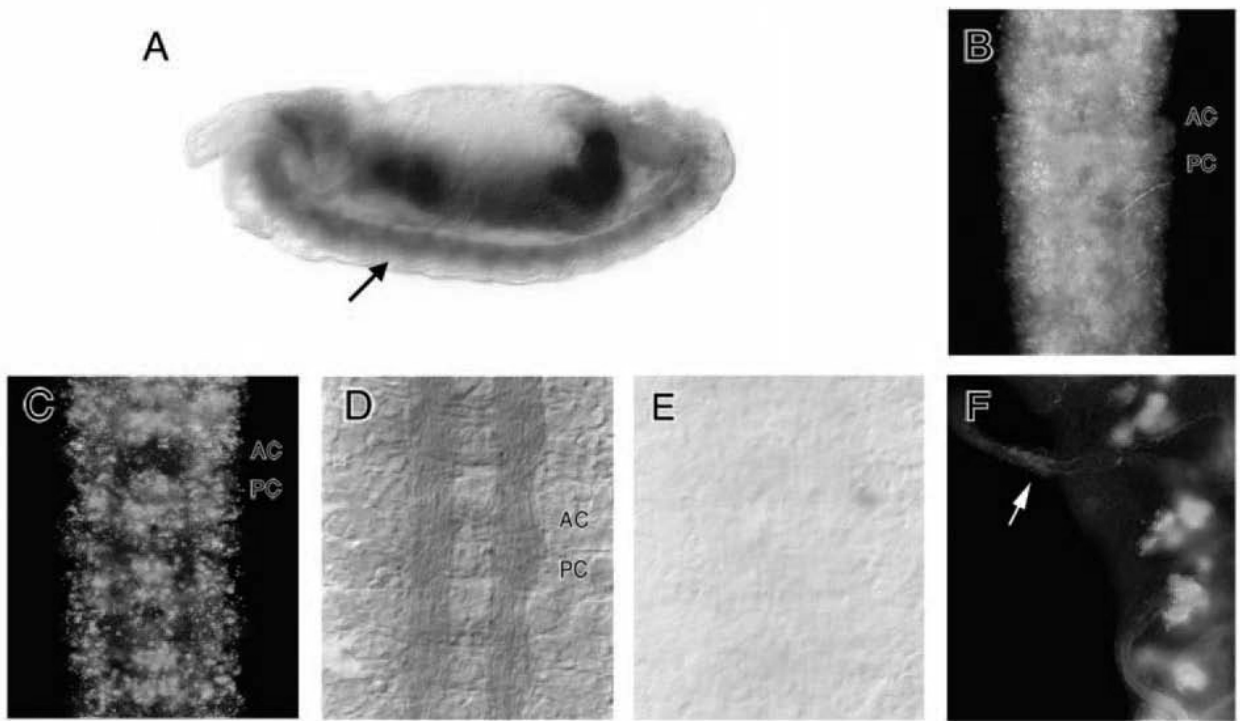


Figure 3. ***src64B*** **mRNA expression overlaps with drl mRNA expression and Src64B protein is present in axons.** (A) Wildtype embryo labeled with a *src64B* anti-sense RNA probe shows *src64B* expression throughout the ventral nerve cord (arrow) and in the gut. (B) Double RNA in situ staining for endogenous *src64B* mRNA (green) and *drl* mRNA (red) shows that *drl* and *src64B* overlap in the ventral nerve cord in the anterior portion of each segment. (C) Double RNA in situ staining for endogenous *wnt5* mRNA (green) and *drl* mRNA (red) shows that wnt5 is predominantly expressed in PC-associated neuronal cell bodies that do not express *drl*. (D) Src64B protein is expressed in the wildtype longitudinal and commissural axons. (E) Axons of a homozygous src64BPI mutant embryo are not stained by anti-Src64B. (F) Fluorescent double antibody labeling of a 3rd instar larval neuropile ectopically-expressing Src64B (red) and GFP (green) in motoneurons (OK6-Gal4 driver). The arrow indicates fasciculated Src64B-expressing motoneuron axons.

Tyrosine kinase activity is required for the formation or stability of the Drl/Src64B complex and Drl is phosphorylated in a Src64B-dependent manner

We then evaluated whether Src64B's kinase activity was required in the formation or stabilization of the Drl/Src64B complex. The physical association of Drl and Src64B was dependent on the kinase activity of Src64B or an associated tyrosine kinase since treatment of the co-transfected cells with herbimycin A, a tyrosine kinase-specific inhibitor, resulted in their reduced co-immunoprecipitation (Fig. 5A). To further assess the role of SFK kinase activity, we used a mammalian two-hybrid assay in which Src64B and Drl intracellular domain fusion protein expressing plasmids were transfected into SFK-deficient cells (Klinghoffer et al., 1999) to eliminate possible interference by the highly conserved endogenous mammalian SFKs. Coexpression of wildtype Src64B and Drl intracellular domain fusion proteins led to significant increases in luciferase expression above the controls indicating that these proteins physically

interact (Fig. 5B). No significant expression of luciferase was observed when catalytically inactive Src64B was co-expressed with Drl.

The requirement for tyrosine kinase activity in the formation or stability of the Src64B/Drl complex raised the question as to whether either Drl or Src64B displayed increased tyrosine phosphorylation upon coexpression. Evaluation of tyrosine phosphorylation of whole cell extract proteins derived from cells transiently transfected with Drl, Src64B or both expression constructs revealed a dramatic increase in the phosphorylation of a 75 kD protein(s) in the doubly-transfected cells (Fig. 6A). The tagged Drl and Src64B proteins both display apparent molecular weights of ~ 75 kD upon denaturing electrophoresis. Therefore, to investigate whether this species includes Drl, we first immunoprecipitated lysate proteins from cells transfected with either Drl-HA alone or Drl-HA and Src64B-myc using anti-Drl antiserum, denatured immune complexes by boiling in detergent, immunoprecipitated protein with anti-HA to enrich for Drl, separated proteins by SDS-PAGE and detected those containing phosphotyrosine by probing immunoblots with an anti-phosphotyrosine antibody. A control sample indicated that Src64B-myc was not immunoprecipitated from denatured extracts with anti-HA. This experiment revealed that Drl tyrosine phosphorylation is increased upon its coexpression with Src64B (Fig. 6B). Similar results were obtained when we performed an analysis of lysates derived from cells co-transfected with Src64B-myc and Drl-GFP expression constructs; tyrosine phosphorylation of Drl was observed only when Drl and Src64B were coexpressed (data not shown).

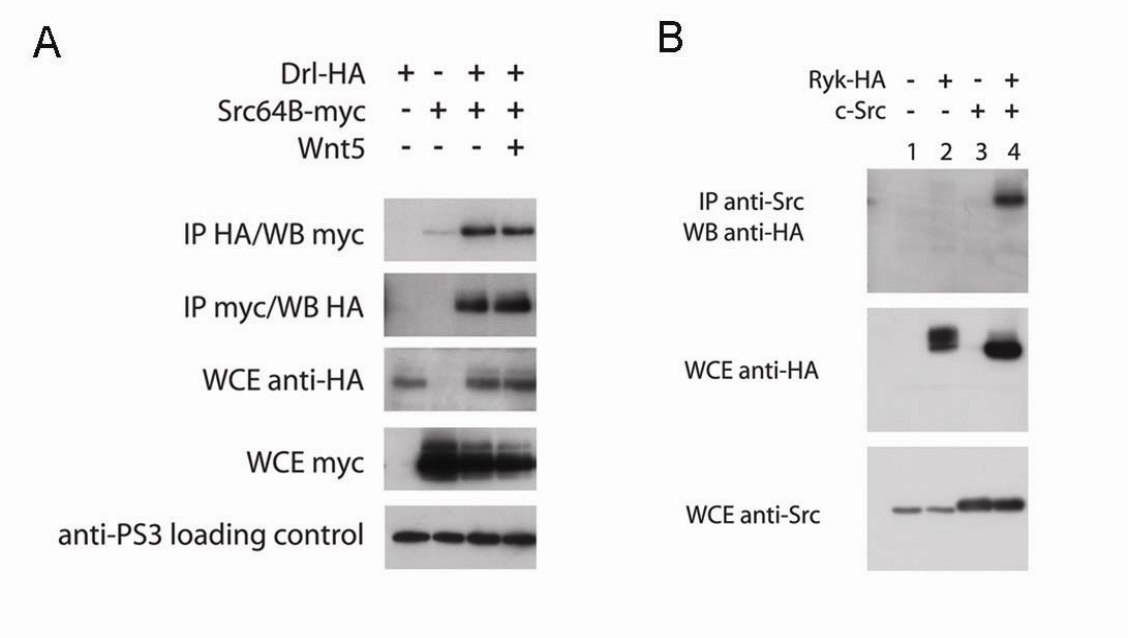


Figure 4. Src64B and Drl and their mammalian orthologs physically associate. (A) Drosophila Kc cells were transfected with the indicated expression constructs, lysates were immunoprecipitated (IP) with antibodies specific to Drl (anti-HA) or Src64B (anti-myc) and subsequently immunoblotted (WB) with the reciprocal antibody to detect co-immunoprecipitation. The expression of Drl and Src64B was confirmed by immunoblotting of the whole-cell lysates (WCE). The co-immunoprecipitation of Drl and Src64B specifically co-immunoprecipitate in the presence or absence of Wnt5 protein. (B) The mammalian orthologs of Drl and Src64B, Ryk and c-Src, physically interact as assayed by their co-immunoprecipitation from transfected 293T cell lysates. The expression of Ryk-HA and untagged c-Src was confirmed by WCE immunoblot. Ryk derived from c-Src-overexpressing cells migrates faster than control Ryk species presumably due to altered post-translational processing.

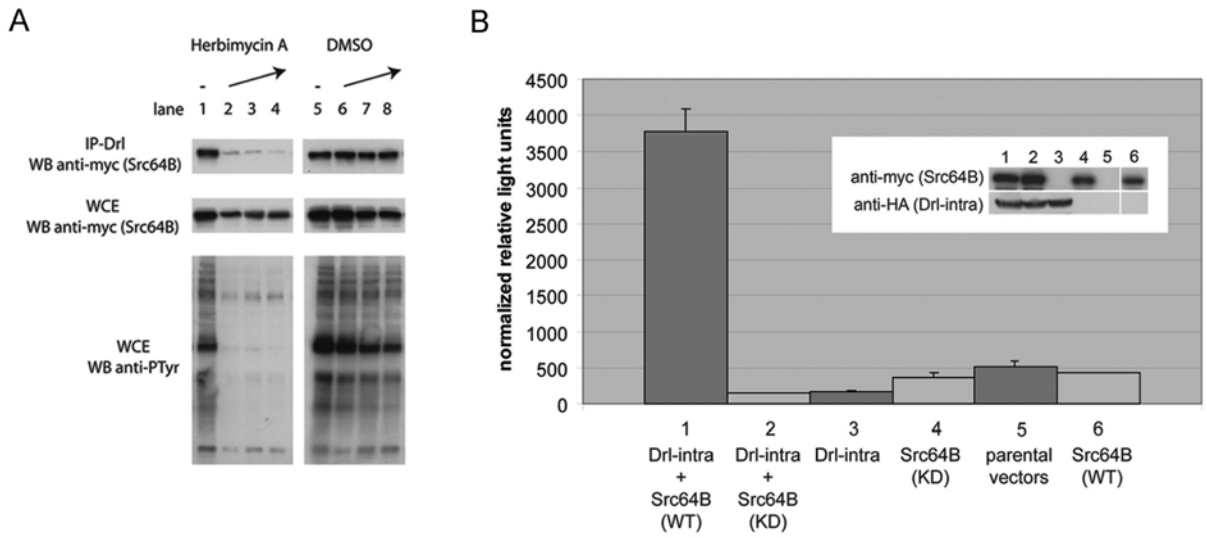


Figure 5. Src64B tyrosine kinase activity is required for the formation or stability of the Drl/Src64B complex. (A) Herbimycin A treatment leads to significantly decreased amounts of Src64B co-immunopreciptating with Drl. Aliquots of Kc cells cotransfected with Drl-HA and Src64B myc expression constructs were treated for 24 hours with increasing concentrations of herbimycin A or equivalent volumes of DMSO, WCEs prepared, Drl/Src64B complexes immunoprecipitated with anti-Drl and subsequently immunoblotted with anti-myc (Src64B) antibody (top panels). Lanes 1 and 5 are untreated samples, lanes 2-4 are herbimycin A-treated at 2.5, 5 and 10 M final concentration and lanes 6-8 are the DMSO-only controls. Equal amounts of WCEs were evaluated for Src64B-myc expression (middle panels) and overall tyrosine phosphorylation levels (lower panels). (B) Wildtype (WT), but not kinase-dead (KD) Src64B interacts with Drl's intracellular domain (Drl-intra) in a mammalian two-hybrid assay. The indicated fusion protein constructs were transfected into SFK-deficient cells and luciferase activity was measured 48 hours post-transfection and plotted normalized to an internal control. Immunoblotting for Drl and Src64B species (inset) indicates equivalent expression of the test plasmids. An irrelevant lane between lanes 5 and 6 was removed in preparing the panel.

While no significant changes in Src64B tyrosine phosphorylation levels, as assayed by anti-phosphotyrosine immunoblotting, were observed in the presence of Drl (data not shown), SFKs are known to be differentially phosphorylated at separate sites depending on their state of activation (reviewed in Roskoski, 2005). We therefore evaluated the degree of phosphorylation of the Src64B tyrosine at position 434, which is phosphorylated in catalytically active Src64B (O'Reilly et al., 2006). Anti-PY434Src64B immunoblot analysis of whole cell lysates derived from transfected cells revealed Drl-dependent activation of Src64B (Fig. 6C). The intracellular and WIF domains of Drl, but not its putative tetrabasic cleavage site or aminoterminal PDZ binding domain, are required for Src64B activation (Fig. 6C). Co-transfection of a plasmid expressing Wnt5 did not increase the amount of Src64B phosphorylation (data not shown) suggesting that the activation of Src64B by Drl is independent of Wnt5 under these conditions. Finally, we assessed whether or not the Src64B associated with Drl included catalytically active molecules. Anti-Drl immunoprecipitation of lysates from cells co-transfected with Src64B and Drl expression plasmids revealed that at least a subset of the Src64B protein bound to Drl is catalytically active (Fig. 6D).

wnt5/drl-mediated signaling is unlikely to affect the canonical Wnt target TCF/LEF

As the mammalian Wnt5a protein acting through the Ror receptor has been shown to inhibit canonical Wnt signaling (Mikels and Nusse, 2006), we evaluated whether or not Wnt5 interaction with Drl might similarly block canonical signaling. Coexpression of Wg, Wnt5 and Drl resulted in luciferase expression levels similar to that seen with Wg alone (Fig. 7),

suggesting that *wnt5*/*drl*-mediated interactions do not apparently inhibit contemporaneous canonical Wnt signaling.

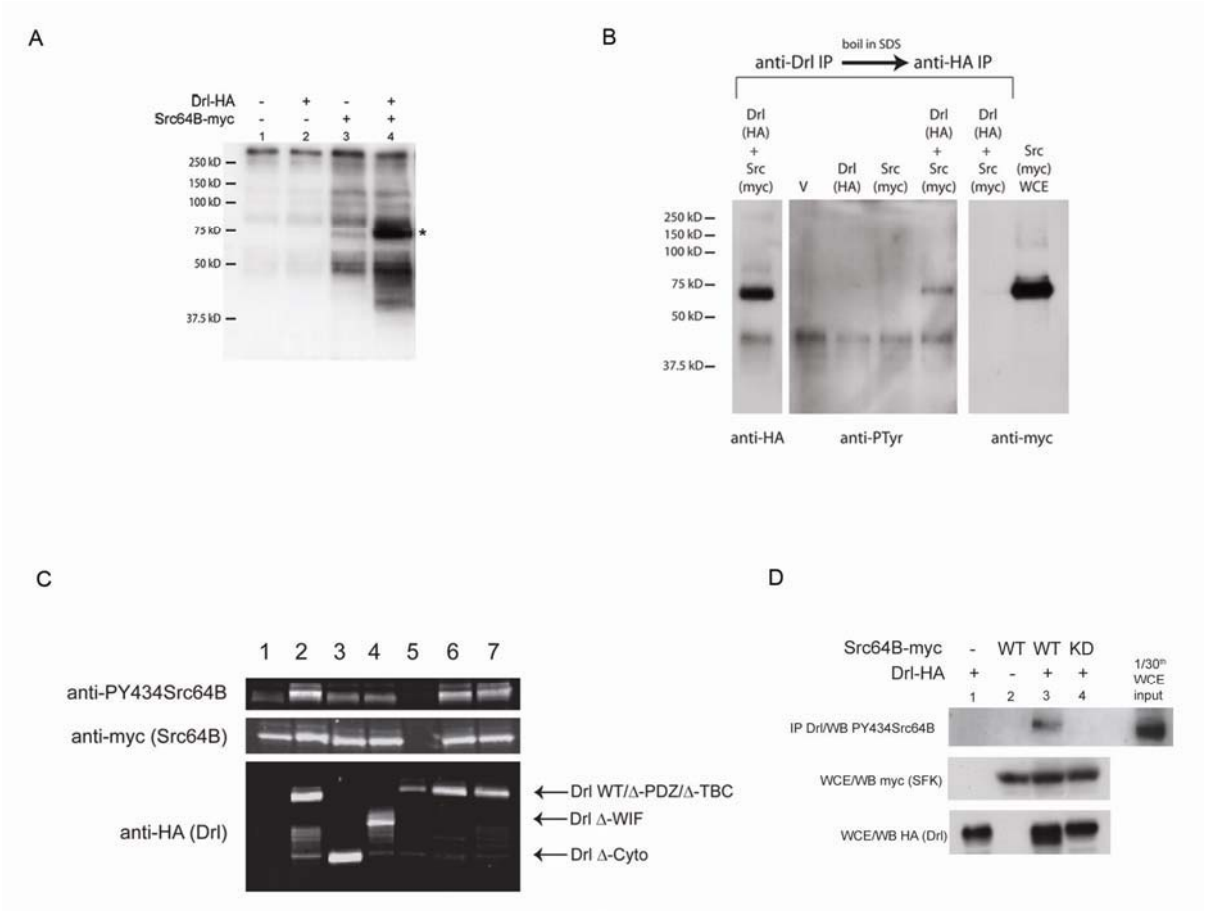
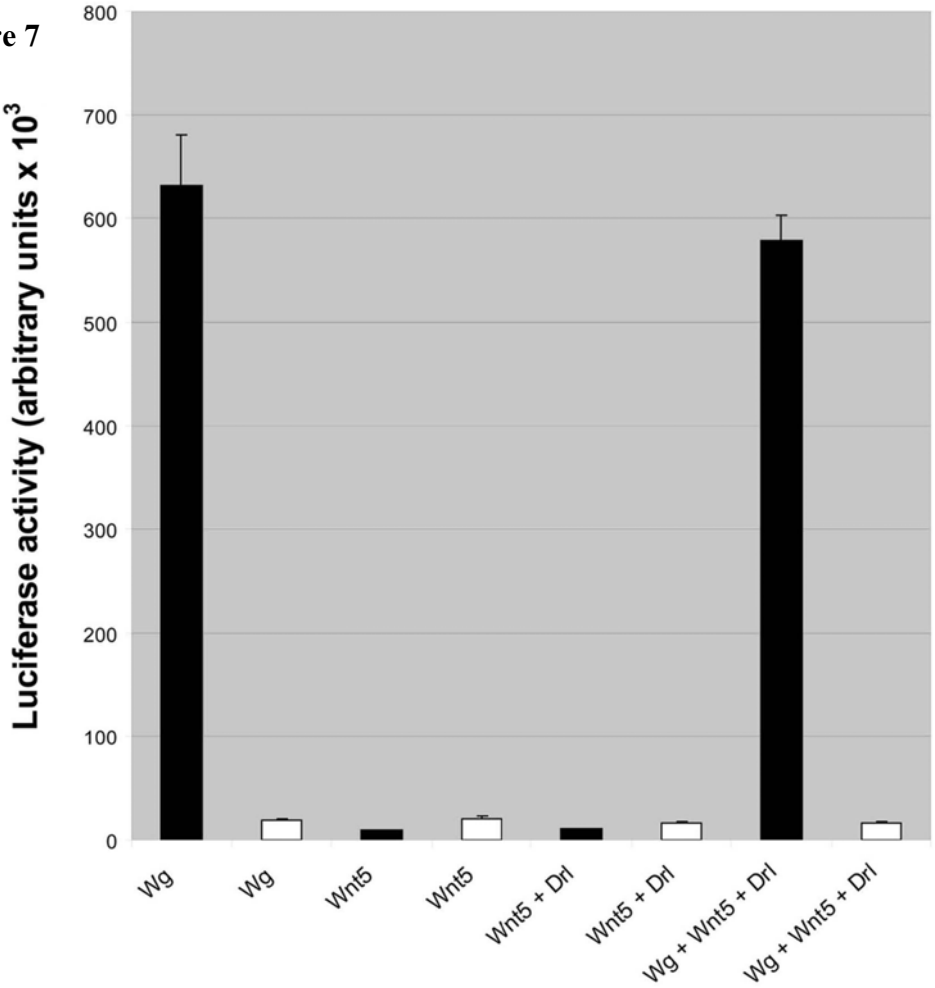
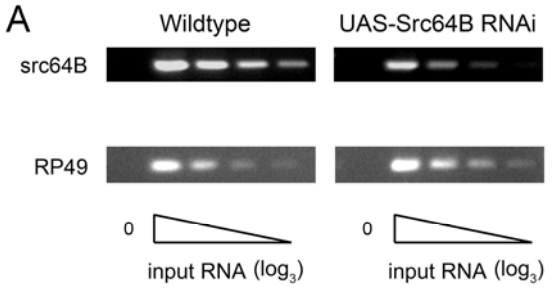


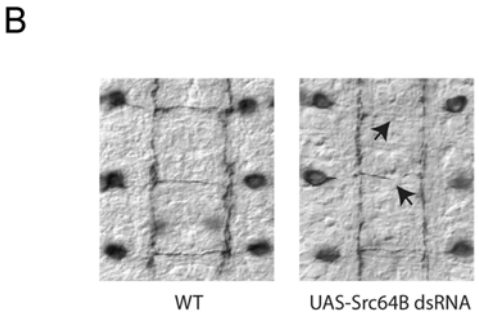
Figure 6. Drl is tyrosine phosphorylated in a Src64B-dependent manner and Drl coexpression activates Src64B. (A) Drl and Src64B co-transfected cell lysates contain a predominant protein species with increased tyrosine phosphorylation (asterisk). S2 cells were transfected with the indicated plasmids and WCE analyzed by immunoblotting with an anti-phosphotyrosine mAb. (B) Drl is phosphorylated in a Src64B-dependent manner. Lysates of S2 cells transiently-transfected with the indicated constructs were immunoprecipitated with anti-Drl antiserum, complexes washed, disrupted by boiling and Drl re-precipitated with anti-HA antiserum and analyzed by anti-phosphotyrosine immunoblotting. (C) Coexpression of Drl and Src64B results in a WIF and cytoplasmic domain-dependent activation of Src64B. S2 cells were transfected with wildtype or mutant tagged Drl and Src64B expression plasmids and WCEs immunoblotted to detect active Src64B (anti-PY434Src64B), pan-Src64B (anti-myc) and Drl (anti-HA). All transfections contained Src64B-myc except Lane 5. Lane 1: no addition; Lane 2: Drl-HA wildtype (WT); Lane 3: Drl-HA Δ-cytoplasmic region; Lane 4: Drl-HA Δ -WIF domain; Lane 5: Drl-HA WT; Lane 6: Drl-HA -PDZ; Lane 7: Drl-HA Δ -tetrabasic cleavage site (TBC). (D) Drl-associated Src64B is, at least in part, catalytically-active. Lysates from cells transfected with Drl-HA alone, Drl-HA and Src64B-myc (WT), Drl-HA and Src64B-myc (KD) and Src64B-myc (WT) alone, as indicated in the Figure, were immunoprecipitated with anti-Drl and analyzed by anti-PY434Src64B immunoblotting.

Figure 7. The expression of Wnt5 and Drl neither activates nor represses canonical TCF/LEF-dependent transcription. S2 cells were transfected in triplicate with the indicated expression plasmids and either the TCF/LEF-dependent transcription reporter Super8XTopFlash (black bars) or the control Super8XFopFlash (white bars). Luciferase expression levels were determined, normalized to internal Renilla controls and plotted.

Figure 7

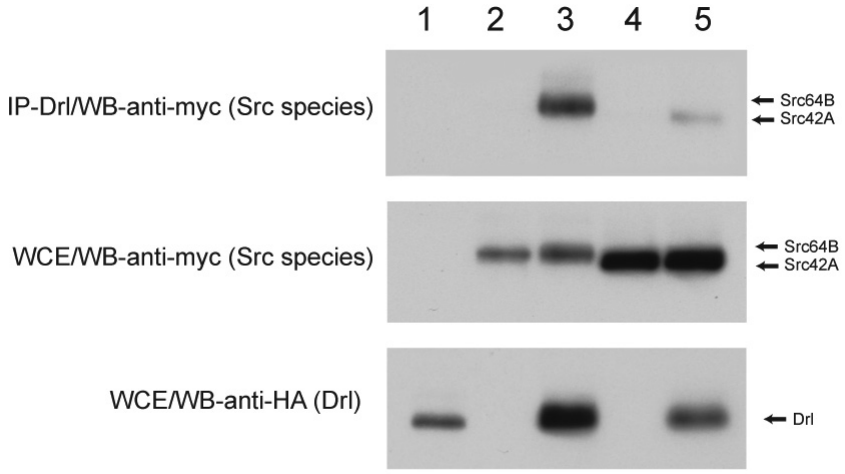






Supplemental Figure 1. Expression of the RNA interference UAS-RNAi-src64B transgene reduces the accuracy of axonal projection of the Sema2b+ neurons. (A)The UAS-RNAi-*src64B* transgene was expressed in vivo using the Da-Gal4 driver, total mRNA prepared from 0-24 hour old embryos and DNAse treated to reduce

genomic DNA contamination and *src64B* (upper panels) and control RP49 (lower panels) mRNA levels were estimated by RT-PCR of 3-fold serial dilutions of input first-strand cDNA. From this analysis, we conclude that expression of the RNAi-*src64B* transgene reduces *src64B* mRNA expression levels approximately 9-fold. Intron-spanning primer sets were used to allow the discrimination of cDNA amplification product from possible contaminating genomic DNA amplification product, the latter was not observed. No template was included in the PCRs in the first lane of each series. The following primers were used: src64B RTPCR Forward: AAATGCTGCAGCAAGCGACAGGA, src64B RTPCR Reverse: ACTCGAATGT-CGGCCGCTTCTC, RP49 RTPCR Forward: ATGACCATCCGCCCAGCA and RP49 RTPCR Reverse: TTGGGGTTGGTGAGGCGGAC. (B) AC projections do not cross the midline in approximately 25% of the segments in embryos with reduced Src64B expression (UAS-RNAi-Src64B/Sema2B-Gal4; Sema2b-τ-myc/+), Arrows indicate abnormal AC projections. AC is anterior commissure and PC is posterior commissure.



Supplemental Figure 2. Src42A co-immunoprecipitates with Drl. S2 cells were transfected as follows, lysates immunoprecipitated with anti-Drl and protein complexes immunoblotted with anti-myc to detect the SFK species. WCE were immunoblotted to confirm expression. Lane 1: Drl-HA only; Lane 2: Src64B-myc (WT) only; Lane 3: Drl-HA + Src64B-myc (WT); Lane 4: Src42A-myc (WT) only; Lane 5: Src42A-myc (WT) + Drl-HA.

Discussion

The genetic data presented in this report suggest that *the SFKs* play important roles in effecting *wnt5*/*drl*-mediated axon repulsion in vivo. First, we observe that wildtype levels of *src64B* are required for a dominant gain of function phenotype of *wnt5*; heterozygosity for a *src64B* null mutant resulted in a dramatic decrease in the loss of the AC caused by midline glial overexpression of *wnt5*. Similar levels of suppression are observed in embryos heterozygous for *drl* or *porc*, known members of the *wnt5*/*drl* signaling pathway. The sole other Drosophila Src family kinase, Src42A, is also expressed in ventral nerve cord embryonic axons (Takahashi et al., 2005) and has previously been shown to have at least partially redundant roles to those of Src64B in other tissues (Takahashi et al., 2005; Tateno et al., 2000, Harris and Beckendorf, 2007).

Second, we find that reduction of *src64B* expression levels in the Sema2B+ AC-crossing neurons by transgenic RNA interference results in axon pathfinding phenotypes similar to those seen in *wnt5* (Fradkin et al., 2004) and *drl* mutants (Yoshikawa et al., 2003). The aberrant pathfinding of *src64B*-dsRNA expressing Sema2b+ neurons, which normally project through the AC, indicates that *src64B* is required for their wildtype routing. The incomplete penetrance of this phenotype likely reflects the presence of wildtype levels of Src42A which we have shown is at least partially redundant to Src64B.

Third, we demonstrate that *drl* and *src64B*/*src42A* interact synergistically in an axon switching assay. Increased SFK neuronal expression levels alone could not force axons which normally traverse the PC, to cross in the AC. Use of a Drl-expressing transgene facilitating only moderate switching allowed the demonstration that elevated *src64B* or *src42A* expression levels significantly increased switching. SFK catalytic activity is required to enhance Drl-dependent switching, since kinase activity-deficient Src64B did not increase switching. Thus, catalytically active SFKs can synergize with limiting levels of Drl to induce commissure switching, and presumably act to control the wildtype trajectories of the AC axons. Furthermore, *src64B* and *drl* have previously been reported to interact genetically during pupal brain development (Nicolai et al., 2003) and it was also recently reported that *drl* genetically interacts with the SFK genes to control salivary gland migration (Harris and Beckendorf, 2007).
Supporting these previous observations and the genetic data presented here, we found that Src64B and Drl physically interact, as assayed by their co-immunoprecipitation. We observed that Ryk and c-Src, similarly to their *Drosophila* orthologs, co-immunoprecipitate from transfected cell lysates, suggesting that this interaction is evolutionarily conserved. The formation or stability of this complex is likely dependent upon SFK kinase activity as shown by the failure of the proteins to co-immunoprecipitate from lysates derived from cells treated with herbimycin A, a tyrosine kinase inhibitor. Further support for the involvement of SFK catalytic activity was provided by our observation that wildtype, but not catalytically inactive, Src64B interacts with the Drl cytoplasmic domain as assayed in two-hybrid experiments in SFK-deficient mammalian cells.

Drl phosphotyrosine content increases significantly when Drl is co-expressed with Src64B in transfected tissue culture cells. Which of the sixteen tyrosine residues within the Drl cytoplasmic domain is phosphorylated in a SFK-dependent manner and the function of this phosphorylation has yet to be determined. The levels of active Src64B, as measured with an activation-specific anti-Src64B antiserum, are significantly increased upon coexpression of Src64B and Drl. The requirement for Drl's WIF domain to activate Src64B, in addition to its cytoplasmic region, suggests that extracellular interactions of Drl, possibly with ligands or other proteins on the cell surface, contribute to its activation of Src64B.
Neither the physical association of Drl and Src64B in Drosophila or mammalian tissue culture cells nor the activation of src64B upon its coexpression with Drl displayed Wnt5-dependence under the conditions examined. It remains possible, however, that endogenous expression levels of Wnt5 or another Wnt capable of interacting with Drl are already saturating for Drl-dependent Src64B activation. Alternatively, higher than physiological expression levels of SFK/Drl might bypass Wnt5 binding-dependent recruitment of the SFKs by Drl. However, taken at face value, our data indicate that Drl/Src64B interactions are constitutive. Ligand-independent association of a signal transducing kinase with a cell surface receptor is not, however, unprecedented. The Janus kinases have been demonstrated to be constitutively and stably associated with the gp130 cytokine receptor (Giese et al., 2003; O'Shea et al., 2002). Furthermore, the mammalian Ryk protein and its apparent co-receptor, Fz, have also been shown to interact in the absence of Wnt protein (Lu et al., 2004). Ryks may therefore possibly recruit at least a subset of their co-receptors and downstream effectors in Wnt-independent manners. Wnt5/Drl interaction might therefore result in subtle conformational changes to preexisting Drl/Src64B complexes not detected in the assays employed here. Such changes might lead to alterations in tyrosine kinase target specificity, such as those demonstrated for the Src-interacting Na^+/K^+-ATPase (Tian et al., 2005).

Mammalian Ryk was previously shown to be phosphorylated on tyrosine when co-expressed with the Ephrin receptors, Ephb2 and Ephb3, but not Ephb7 (Halford et al., 2000). The dependence of Ryk phosphorylation upon the presence of an Ephrin ligand was not examined in this study. Interesting, SFKs are tightly associated with the Ephrin receptor cytoplasmic domains

(Knoll and Drescher, 2004; Vindis et al., 2004), raising the possibility that Ephrin receptor-dependent phosphorylation of Ryk might involve Src activity.
Studies of the mammalian Wnt/Ryk signaling pathway indicated that the binding of Wnt to Ryk stimulated TCF/LEF-dependent transcription via the Dsh adaptor protein in transfected cells, suggesting that the Ryk pathway overlaps with the canonical Wnt pathway (Lu et al., 2004). Our data indicate, however, that the *Drosophila* Wnt5/Drl signaling pathway is unlikely to regulate TCF/LEF-dependent transcription. Transfection of *Drosophila* S2 tissue culture cells with Drl and Wnt5 expression constructs does not result in detectable increases in TCF/LEF-dependent reporter gene expression. Furthermore, unlike the recently reported Wnt5A/Ror interaction (Mikels and Nusse, 2006), *wnt5*/*drl*-mediated signaling does not apparently block contemporaneous canonical Wnt signaling. Similarly though to its Ror-interacting mammalian ortholog, Wnt5A, the Drosophila Wnt5 protein can also apparently signal via Frizzled family receptors to activate a non-canonical Wnt pathway (Srahna et al., 2006). While our data render it unlikely that Src64B is a member of the canonical Wnt signaling pathway, it may act in a pathway parallel to canonical Wnt signaling as has been reported for convergent extension cell movement in zebrafish (Jopling and den Hertog, 2005) and cell fate specification and cleavage orientation in *C. elegans* (Bei et al., 2002).

It is presently unclear to what downstream pathway members the SFKs relay a Wnt5/Drl signal, and by what mechanisms, but the latter would seem likely to involve the tyrosine phosphorylation of specific target proteins. Our observations that increased *src64B* and *src42A* expression levels enhance *drl*-mediated commissure switching of axons in a sensitized, but not in a wildtype, background and that the SFKs and Drl physically interact, suggest the possibility that Drl dictates the target specificities of the bound SFKs by co-localizing them with potential targets. Such a mechanism is attractive since the SFKs are widely expressed throughout the ventral nerve cord and likely also act downstream of other axonal receptors whose mammalian orthologs, e. g., the TrkB, EphrinA and Netrin receptors, are known to interact with the SFKs (Iwasaki et al., 1998; Knoll and Drescher, 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004). Furthermore, the possible Drl-dependent asymmetric localization or regulation of SFKs within the growth cone might steer axons. Such localized changes in Src activity have recently been demonstrated to effect axon turning in cultured Xenopus primary neurons (Robles et al., 2005).

While the identification of the relevant SFK targets and other members of the Wnt5/Drl-mediated signaling pathway lies ahead, the data presented here indicate that the catalytically-active SFKs are required for Wnt5 -mediated axon repulsion via the catalytically inactive Drl receptor. Identification of other pathway members, including the anticipated SFK targets, through a combination of genetic, biochemical and phosphoproteomic approaches should further reveal the mechanisms by which Wnt proteins signal through the Ryks.

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