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Src family kinases are required for WNT5 signaling through the Derailed/RYK receptor in the *Drosophila* embryonic central nervous system

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Members of the RYK/Derailed family have recently been shown to regulate axon guidance in both *Drosophila* and mammals by acting as Wnt receptors. Little is known about how the kinase activity-deficient RYKs transduce Wnt signals. Here, we show that the non-receptor Src family tyrosine kinases, SRC64B and SRC42A, are involved in WNT5-mediated signaling through Derailed in the *Drosophila* embryonic central nervous system. Analysis of animals lacking SRC64B and SRC42A reveals defects in commissure formation similar to those observed in *Wnt5* and *derailed* mutants. Reductions in SRC64B expression levels suppress a *Wnt5*/*derailed*-dependent dominant gain-of-function phenotype, and increased levels of either SRC64B or SRC42A enhance *Wnt5*/*derailed*-mediated axon commissure switching. Derailed and SRC64B form a complex, which contains catalytically active SRC64B, the formation or stability of which requires SRC64B kinase activity. Furthermore, Derailed is phosphorylated in a SRC64B-dependent manner and coexpression of Derailed and SRC64B results in the activation of SRC64B. The mammalian orthologs of Derailed and SRC64B also form complexes, suggesting that Src roles in RYK signaling are conserved. Finally, we show that coexpression of WNT5 and Derailed 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/Derailed-mediated signaling.

KEY WORDS: Axon guidance, RYK, Src family kinase, Wnt, Signal transduction

INTRODUCTION

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

Whereas 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 (PC) commissure. 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 (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 might reflect changes in inter-axonal adhesion. Moreover, mutation of *drl* (also known as *linotte*) results in memory deficits (Dura et al., 1993), likely to be 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 that sometimes have members in common. 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/β-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 might 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 by Bovolenta et al., 2006; Keeble and Cooper, 2006) underlie 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).

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Members of the RYK family of 'dead' or 'fractured' RTKs 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 substitutions in highly conserved amino acid residues required for phosphotransfer that are likely to render them inactive as kinases. Although apparently lacking kinase activity, a human TRKA (NTRK1)-RYK fusion 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., 2007) 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 probably not transducing a signal in these contexts; instead, it apparently 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 proteins (Lu et al., 2004). Human RYK acts in a ternary complex with the Fz and Wnt proteins, signaling through the adaptor protein Dishevelled 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 in *drl* mutants (Yao et al., 2007), indicating that the *Wnt5* gene itself is a pathway target.

Although DRL function in the *Drosophila* embryonic CNS does not apparently involve intrinsic tyrosine kinase activity (Yoshikawa et al., 2001), the cytoplasmic domain of DRL is required for axon repulsion (Yoshikawa et al., 2003) and 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 probably interact with other proteins that transduce their signal.

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. Furthermore, the SFKs and DRL physically interact resulting in Src activation and DRL tyrosine phosphorylation. Mammalian SFK and RYK orthologs also co-immunoprecipitate from transfected tissue culture cell lysates indicating that RYK-SFK interactions are evolutionarily conserved.

MATERIALS AND METHODS

Fly stocks

The following alleles, GAL4 drivers and reporters were used in this study and are described at FlyBase: w^{1118}, UAS-WNT5^{11C}, SIM-GAL4, ELAV-GAL4, OK6-GAL4, UAS-SRC64B, UAS-SRC64BK312R, UAS-SRC64B-RNAi, UAS-SRC42A, $Src64B^{PI}$, $Src64B^{KO}$, $Src42A^{E1}$, $porc^{PB16}$, $Wnt5^{400}$, drl^{Red2}, UAS-DRL, SEMA2B-τ-MYC, UAS-NLS-β-Gal, UAS-GFP, UAS-mCD8GFP, DA-GAL4 and EG-GAL4. β-Gal or GFP balancer chromosomes were used to identify the appropriate progeny. Transgenic lines were generated using standard techniques in our laboratory and at BestGene. Two SEMA2B-GAL4 transgenic fly lines were generated (gift of B. Dickson, Research Institute of Molecular Pathology, Vienna, Austria). Expression of GAL4 is first detected at embryonic stage 13 in the dorsal vessel and gut, and from stage 15 onwards in the peripheral nervous system (chordotonal organs) and the CNS (subsets of AC neurons, see Fig. S1B in the supplementary material).

Immunohistochemistry and RNA in situ hybridization

Antibody labeling, RNA in situ hybridization 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 monoclonal antibody (mAb) BP102 (gift of C. Goodman, University of California, Berkeley, CA), rabbit anti-MYC (Upstate), rabbit anti-GFP (Invitrogen), mouse mAb anti-Wrapper (Noordermeer et al., 1998), mouse mAb anti-REPO (Alfonso and Jones, 2002) and rabbit anti-SRC64B (gift of T. Xu, Yale University, New Haven, CT). 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-driven or UAS promoter-driven wild-type and mutant DRL (HA) and SRC64B or SRC42A (MYC) and wild-type untagged WG and WNT5 expression plasmids were constructed by ORF PCR, oligonucleotide-mediated mutagenesis and Gateway-mediated recombination (Invitrogen) into appropriate destination vectors (provided by T. Murphey; http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html). Y. Zou and K-L. Guan, respectively, provided HA-tagged mouse RYK (Liu et al., 2005) and human c-SRC (Li et al., 2004) expression plasmids. The UAS-WRAPPER construct (Noordermeer et al., 1998) was co-transfected with pAc-GAL4 to express Wrapper. To generate an *Src64B*-specific RNA interference transgene, gene-specific inverted repeats (bp 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 reverse transcribed (RT)-PCR of first strand embryonic cDNA as described (see Fig. S1 in the supplementary material). Similar reductions in *Src64B* expression were observed with two different inserts. All plasmids were verified by sequencing.

S2 and Kc cell transfections were performed using Effectene (Qiagen) and 293T cell transfections with Fugene (Roche). Lysates were prepared using a high-stringency SDS-containing RIPA buffer (Muda et al., 2002) containing a cocktail of protease inhibitors (Roche). *Drosophila* cell lysate immunoprecipitations were performed using rabbit anti-MYC (Upstate) or rabbit anti-DRL or anti-Wrapper mAb. 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. Bound multiple-label grade HRP- (Jackson ImmunoResearch) or 800CW (Li-Cor)-conjugated secondary antibodies were 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 mAb (Jackson ImmunoResearch) was 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, diluted 1:10 into buffer containing 1% Triton X-100 and then DRL-HA immunoprecipitated with reagents from the Profound anti-HA Kit (Pierce).

Rabbit anti-DRL was as described (Yao et al., 2007). Anti-PY434SRC64B antiserum was commercially generated and purified (Eurogentec) against the SRC64B peptide RVIADDEYCPKQG and its phosphorylated version (RVIADDEpYCPKQG) as described (O'Reilly et al., 2006) and verified as phosphopeptide-specific by ELISA and by its lack of staining on $Src64B^{KO}$ mutant embryos (data not shown).

293T cell lysate immunoprecipitations were performed using anti-c-SRC mAb (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.

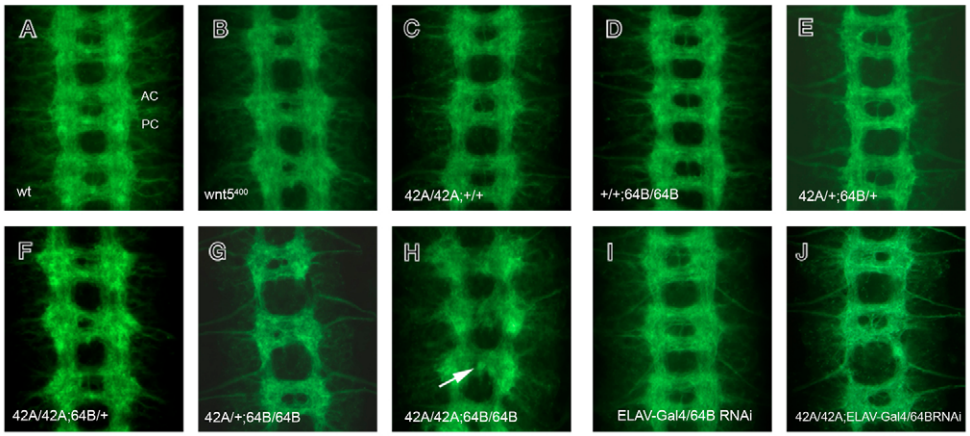

Fig. 1. SFKs play redundant roles during formation of the embryonic CNS commissures. Stage 16 *Drosophila* embryos of the indicated genotypes were stained with mAb BP102 to label all central axons. Anterior is up. (**A**) Wild type, (**B**) *Wnt5*^{400}, (**C**) *Src42A*^{E1}, (**D**) *Src64B*^{KO}, (**E**) *Src42A*^{E1}/+; *Src64B*^{KO}/+, (**F**) *Src42A*^{E1}; *Src64B*^{KO}/+, (**G**) *Src42A*^{E1}/+; *Src64B*^{KO}, (**H**) *Src42A*^{E1}; *Src64B*^{KO}, (**I**) ELAV-GAL4/UAS-RNAi-*Src64B* and (**J**) *Src42A*^{E1}; ELAV-GAL4/UAS-RNAi-*Src64B* are shown. Defects similar to those seen in *Wnt5*^{400}, namely '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 also in individuals of a *Src42A* mutant background when *Src64B* expression is reduced in the nervous system by RNA interference. The commissures are completely fused (white arrow in H) in the double homozygotes. See Table 1 for quantitation. AC, anterior commissure; PC, posterior commissure.

Moon]. Lysates were prepared and assayed using the Dual Luciferase Reporter Assay System (Promega) with normalization to internal Renilla controls. The Checkmate Mammalian Two-hybrid System (Promega) was used to assay SRC64B-DRL interaction in vivo. The cytoplasmic domain of DRL was cloned in frame with the GAL4 DNA-binding domain in the pBind vector and the full-length wild-type or kinase-deficient SRC64B ORF was cloned in frame with the VP16 activation domain in the pACT vector. SFK-deficient SYF cells (Klinghoffer et al., 1999) were obtained from LGC Promochem-ATCC and transfected using Fugene.

RESULTS

SFKs are required for embryonic commissure formation

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 SFK family (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, as 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). Embryos lacking SRC42A and pan-neuronally expressing *Src64B*-targeting double-stranded (ds) RNA (Fig. 1J, Table 1) displayed qualitative and quantitative phenotypes similar to those of *Src42A*; *Src64B*^{KO}/+ and *Src42A*/+; *Src64B*^{KO} mutant embryos. This result confirms a requirement for wild-type *Src64B* expression levels in SRC42A-deficient neurons for commissure formation. Altered commissure formation in the partial SFK mutants is unlikely, therefore, to be due to secondary effects of the loss of *Src64B* in other tissues. The failure of these animals to exhibit the full SFK-null commissural phenotype probably reflects incomplete knockdown of *Src64B* expression levels (see also below). Quantitation of these phenotypes is presented in Table 1. These results and the similar commissural phenotypes observed 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.

Table 1. Abnormal commissural axonal projections in SFK mutants

Genotype	Segments with abnormal commissural axonal projections (%)	Number of segments scored
w^{1118}	0	251
Wnt5^{400}	67	237
Src42A^{E1}	11.5	330
Src64B^{KO}	12.5	350
Src42A^{E1}/+; *Src64B*^{KO}/+	6.4	280
Src42A^{E1}/+; *Src64B*^{KO}	39	270
Src42A^{E1}; *Src64B*^{KO}/+	26	180
Src42A^{E1}; *Src64B*^{KO}	99	150
ELAV-Gal4/UAS-RNAi-*Src64B*	12	449
Src42A^{E1}; ELAV-Gal4/UAS-RNAi-*Src64B*	33	496

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

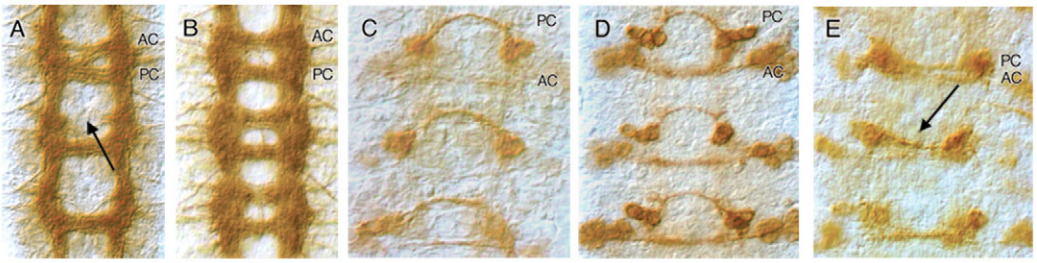

Fig. 2. *Src64B* is required for *Wnt5/drl*-mediated axon repulsion. (**A**) Ectopic WNT5 expression in the midline glia (SIM-GAL4, UAS-WNT5/+) results in frequent thinning or complete loss (arrow) of the AC. (**B**) Heterozygosity for *Src64B* suppresses the thinning/loss of the AC (SIM-GAL4, UAS-WNT5/+; *Src64B*^{Pl}/+). (**C**) EG^{+} neurons crossing in the AC and PC in a *Drosophila* embryo with one copy of UAS-DRL-MYC and one copy of UAS-NLS-β-Gal visualized by anti-MYC staining (UAS-DRL-MYC/+; UAS-NLS-β-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-SRC64B/+; EG-GAL4/+). Quantitation is presented in Tables 2 and 3. Stage 16 embryos are shown, anterior is up.

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 dsRNA 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 that project their axons to the contralateral side through the AC (Rajagopalan et al., 2000). *Src64B* mRNA levels in dsRNA-expressing animals were evaluated by semi-quantitative RT-PCR and found to be ~10% of wild-type levels (see Fig. S1A in the supplementary material). *Sema2b*$^{+}$ axons with decreased levels of *Src64B* misprojected or apparently stalled in a number of hemisegments (25%, *n*=383) (see Fig. S1B in the supplementary material). No apparent changes in *Sema2b*$^{+}$ cell fate or cell body position were observed. These data provide further support for SFK roles in commissure formation.

To address whether there were widespread CNS patterning defects in SFK mutant animals that might cause the aberrant commissure formation, we examined the midline and lateral glia in the various SFK mutant combinations by staining with anti-Wrapper (Noordermeer et al., 1998) and anti-REPO (Alonso and Jones, 2002) antibodies. The lateral glial cells appeared in their wild-type positions in all mutant combinations except the *Src42A*/+; *Src64B* and double-null mutants, whereas the midline glia appeared wild-type in all combinations except the double-null SFK mutant (see Fig. S2 in the supplementary material). The intermediate commissural phenotypes seen in the 'partial' SFK mutants are therefore unlikely to be caused by major CNS patterning and fate changes.

Wild-type *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 wild-type *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, ~16% of hemisegments displayed the absence or thinning of the AC (Fig. 2A, Table 2), owing to the repulsion of the DRL^{+} AC axons by WNT5 produced by the midline glia. This assay was previously used to establish that wild-type expression levels of the O-acyltransferase *porcupine* (*porc*; *por* – FlyBase) (Fradkin et al., 2004) and of *drl* (Yoshikawa et al., 2003) are required for the loss of the AC, thus 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 greater than 5-fold reduction in the loss of the AC (Fig. 2, compare A and B; Table 2). The extent of suppression observed in *Src64B* heterozygotes was similar to that seen in animals heterozygous for *drl* (Table 2). Heterozygosity for *Src42A*, by contrast, did not suppress the WNT5-dependent midline overexpression phenotype (Table 2). These data indicate that wild-type *Src64B* expression levels are required for this dominant gain-of-function phenotype and that *Src64B* is therefore likely to be a member of the *Wnt5*/*drl* signaling pathway.

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

We then evaluated 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 determine whether wild-type *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 3). Increased

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*^{Pl}/+	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 the pan-axonal BP102 monoclonal antibody.

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
1× UAS-mCD8-GFP	0	100	567
2× UAS-DRL-MYC	56	44	550
1× UAS-DRL-MYC; 1× UAS-NLS β-Gal	1	99	459
1× UAS-DRL-MYC; 1× UAS-SRC64B	34	66	510
1× UAS-mCD8-GFP; 1× UAS-SRC64B	0	100	418
1× UAS-DRL-MYC; 1× UAS-SRC64B K312R	3	97	566
1× UAS-DRL-MYC; 1× 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 2× 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.

expression of wild-type *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 1× UAS-DRL-MYC; 1× UAS-NLS β-Gal and 1× UAS-DRL-MYC; 1× UAS-SRC64B, respectively; Fig. 2E, Table 3). Enhancement of switching was also observed when *Src42A* expression levels were increased, but to a lesser extent than with overexpression of SRC64B (8% versus 34%, respectively; Table 3). Expression of a kinase-deficient *Src64B* transgene (gift of Willis Li, University of Rochester, Rochester, NY) in this sensitized background did not increase switching, indicating that the kinase activity of SRC64B is required for its interaction with DRL (Table 3). These data support the hypothesis that the SFKs are members of the *Wnt5/drl*-signaling pathway.

SRC64B and DRL are both expressed in AC axons

To determine whether *Src64B* and *drl* are normally coexpressed in neurons, we determined the expression domains of *Src64B* mRNA relative to those of *Wnt5* and *drl* in the embryonic CNS. *Src64B* mRNA was observed throughout the ventral nerve cord (Fig. 3A) and overlapped 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), whereas *Wnt5* mRNA is most predominantly found in neuronal cell bodies associated with the PC (Fig. 3C) (Fradkin et al., 2004). SRC64B protein was found in most, if not all, longitudinal and commissural axonal projections in the CNS (Fig. 3D) and was not readily detectable in the *Src64B^{P1}* mutant (Fig. 3E). To confirm that SRC64B was transported out along axons, a double labeling of third instar larval neuropiles coexpressing SRC64B and GFP in motoneurons was performed. SRC64B, like the mammalian (Maness et al., 1988) and *C. elegans* (Itoh et al., 2005) Srcs, was found to be efficiently transported within the axons (Fig. 3F). A similar localization of SRC42A has been reported previously (Takahashi et al., 2005) and DRL is also 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.

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

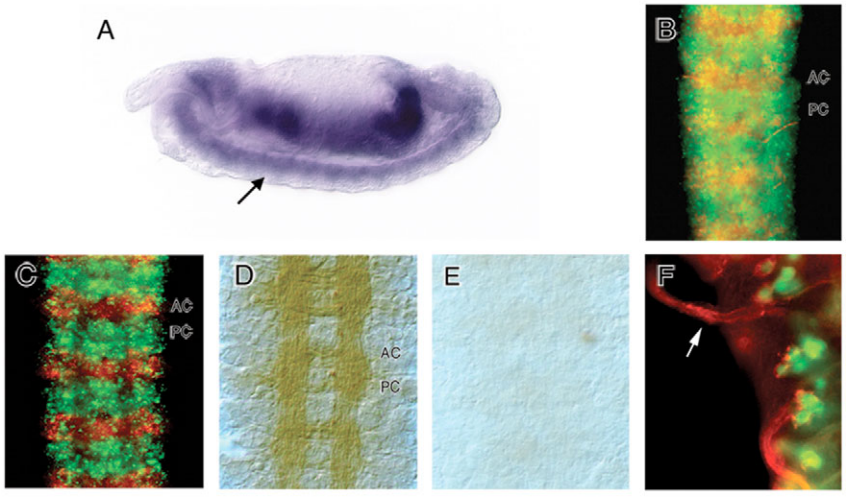

Fig. 3. *Src64B* mRNA expression overlaps with *drl* mRNA expression and SRC64B protein is present in axons. (**A**) Wild-type *Drosophila* embryo labeled with a *Src64B* antisense RNA probe shows *Src64B* expression throughout the ventral nerve cord (arrow) and in the gut. (**B**) Double RNA in situ 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 wild-type longitudinal and commissural axons. (**E**) Axons of a homozygous *Src64B^{P1}* mutant embryo are not stained by anti-SRC64B. (**F**) Fluorescent double-antibody labeling of a third instar larval neuropile ectopically expressing SRC64B (red) and GFP (green) in motoneurons (OK6-GAL4>UAS-GFP). The arrow indicates fasciculated SRC64B-expressing motoneuron axons.

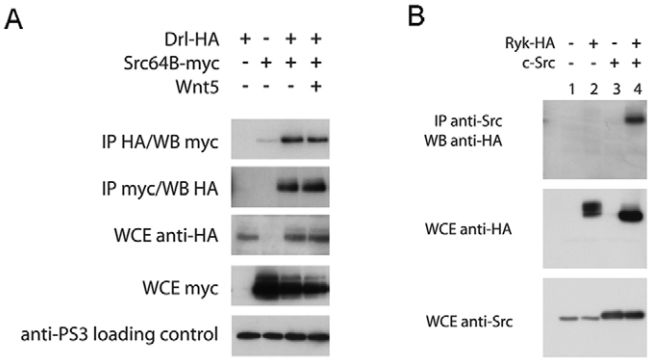

Fig. 4. SRC64B and DRL and their mammalian orthologs physically associate. (**A**) *Drosophila* Kc cells were transfected with the indicated expression constructs, lysates were immunoprecipitated (IP) with antibodies specific to DRL (anti-HA) or SRC64B (anti-MYC) and then immunoblotted (WB) with the reciprocal antibody to detect co-immunoprecipitation. The expression of DRL and SRC64B was confirmed by immunoblotting of the whole-cell extract (WCE). 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 human 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 owing to altered post-translational processing. Endogenous c-SRC protein is visible in lanes 1 and 2 of the lowermost blot.

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 with its mammalian orthologs, is 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 *Drosophila* S2 cells (data not shown). SRC42A also co-immunoprecipitated with DRL, but at only very low levels, despite its presumptive membrane localization (see Fig. S3 in the supplementary material). The inefficient co-immunoprecipitation of SRC64B with the membrane protein Wrapper (Noordermeer et al., 1998) from cells coexpressing these proteins further indicates the specificity of the DRL-SRC64B co-immunoprecipitation (see Fig. S4 in the supplementary material).

We investigated whether this apparent physical interaction between a *Drosophila* RYK and SFK could also be observed with their mammalian orthologs. HA-tagged mouse RYK and untagged human c-SRC expression constructs were co-transfected 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. RYK co-immunoprecipitated 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 that some post-translational modification of RYK takes place upon increased expression of c-SRC. Collectively, the results of these co-immunoprecipitation experiments and the axonal localization of both RYK/DRL and SFKs in mammals and *Drosophila*, make it likely that RYKs and Srcs interact in evolutionarily distant species.

Formation of the DRL-SRC64B complex results in DRL phosphorylation and increased SRC64B activation and requires SRC64B kinase activity

We then evaluated whether the kinase activity of SRC64B 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: 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 plasmids expressing a SRC64B and DRL intracellular domain fusion protein were transfected into SFK-deficient cells (Klinghoffer et al., 1999) to eliminate possible interference by the highly conserved endogenous mammalian SFKs. Coexpression of wild-type SRC64B and DRL intracellular domain fusion proteins led to significant increases in luciferase expression above that of the controls, indicating that these proteins physically interact (Fig. 5B). No significant expression of luciferase was observed when catalytically inactive SRC64B (KD) was coexpressed with DRL (Fig. 5B).

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 kDa protein(s) in the doubly transfected cells (Fig. 6A). The tagged DRL and SRC64B proteins both displayed apparent molecular weights of ~75 kDa on denaturing gels. Therefore, to investigate whether this species includes DRL, we initially immunoprecipitated the DRL-containing complex with anti-DRL, dissociated it by boiling and immunoprecipitated DRL with anti-HA (DRL). Anti-phosphotyrosine immunoblots revealed that DRL tyrosine phosphorylation is increased upon its coexpression with SRC64B (Fig. 6B).

SFKs are known to be differentially phosphorylated at specific tyrosine residues depending on their state of activation (reviewed by 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 (TBC) site or N-terminal PDZ-binding domain, are required for SRC64B activation (Fig. 6C and see Fig. S5 in the supplementary material). 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 that was associated with DRL included catalytically active molecules. Anti-DRL immunoprecipitation of lysates from cells co-

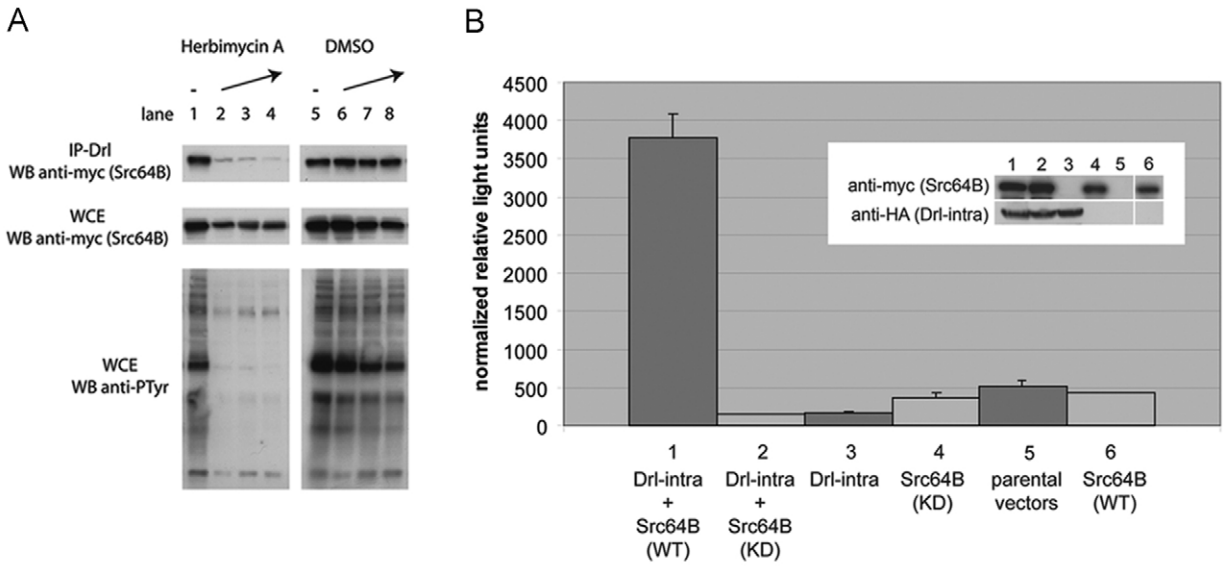

Fig. 5. SRC64B tyrosine kinase activity is required for the formation or stability of the DRL-SRC64B complex. (**A**) Herbimycin A treatment leads to significantly decreased amounts of SRC64B co-immunoprecipitating with DRL. Aliquots of Kc cells co-transfected with DRL-HA and SRC64B-MYC expression constructs were treated for 24 hours with increasing concentrations of herbimycin A or equivalent volumes of the DMSO carrier, WCEs prepared, DRL-SRC64B complexes immunoprecipitated with anti-DRL and then immunoblotted with anti-MYC (SRC64B) antibody (top panels). Lanes 1 and 5, untreated samples; lanes 2-4, herbimycin A-treated at 2.5, 5 and 10 μM final concentration, respectively; lanes 6-8, DMSO controls. Equal amounts of WCEs were evaluated for SRC64B-MYC expression (middle panels) and overall tyrosine phosphorylation levels (lower panels). (**B**) Wild-type (WT), but not kinase-dead (KD), SRC64B interacts with the intracellular domain of DRL (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.

transfected with SRC64B and DRL expression plasmids and immunoblotting with anti-PY434SRC64B revealed that at least some of the SRC64B protein bound to DRL is catalytically active (Fig. 6D).

WNT5/DRL-mediated signaling does not affect the TCF/LEF canonical Wnt target

As a previous study indicated that engagement of mammalian RYK by Wnt proteins results in the transduction of a canonical Wnt signal, culminating in increased TCF/LEF-dependent transcription (Lu et al., 2004), we evaluated whether or not their *Drosophila* orthologs also signal through this pathway. *Drosophila* S2 tissue culture cells were transfected with the indicated combinations of expression plasmids and a TCF/LEF-responsive luciferase reporter gene [Super8XTopFlash (Veeman et al., 2003] or a control reporter (Super8XFopFlash) and luciferase levels were measured 48 hours post-transfection. Transfection of a construct encoding a canonical Wnt protein, Wingless (WG), resulted in high TCF/LEF-dependent levels of luciferase expression. S2 cells express both *fz* and *fz2* as assayed by quantitative RT-PCR (data not shown), precluding the necessity of transfecting expression plasmids for either of the canonical Fz receptors. Transfection of a WNT5-expressing plasmid alone or in combination with a DRL-expressing plasmid did not result in expression of luciferase above control levels (Fig. 7), suggesting that *Wnt5*/*drl*-mediated signaling does not increase TCF/LEF activity.

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 those seen with WG alone (Fig. 7), suggesting that *Wnt5*/*drl*-mediated interactions do not apparently inhibit contemporaneous canonical Wnt signaling.

DISCUSSION

The genetic data presented in this report indicate that the *Drosophila* SRC64B and SRC42A SFKs play redundant roles in establishing the embryonic CNS commissures. Furthermore, we present evidence that SRC64B, and possibly SRC42A, act in commissure formation, at least in part as members of the WNT5/DRL signaling pathway. First, animals homozygous for a null allele of one of the SFKs and heterozygous for a null allele of the other display commissural phenotypes similar to those seen in *Wnt5* and *drl* mutants, including poorly separated commissures and longitudinal breaks. These phenotypes are even more severe in animals completely deficient for the SFKs; their commissures are fused and the longitudinal axon tracts are largely absent. SFK double-null phenotypes are significantly more severe than those of *Wnt5* or *drl* mutants, suggesting that a complete lack of the SFKs might affect other pathways involved in commissure formation as well. Indeed, the midline and lateral glia, although appearing wild-type in most SFK mutant combinations [as in the *Wnt5* mutant (Fradkin et al., 2004); data not shown], are mislocalized in the double-null SFK animals.

Second, we observe that wild-type levels of *Src64B* are required for a dominant gain-of-function phenotype of *Wnt5*; heterozygosity for a *Src64B*-null mutant resulted in a dramatic reduction 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*, established members of the *Wnt5*/*drl* signaling pathway. The other *Drosophila* SFK, SRC42A, which is also expressed in the embryonic CNS (Takahashi et al., 2005), plays partially redundant roles to those

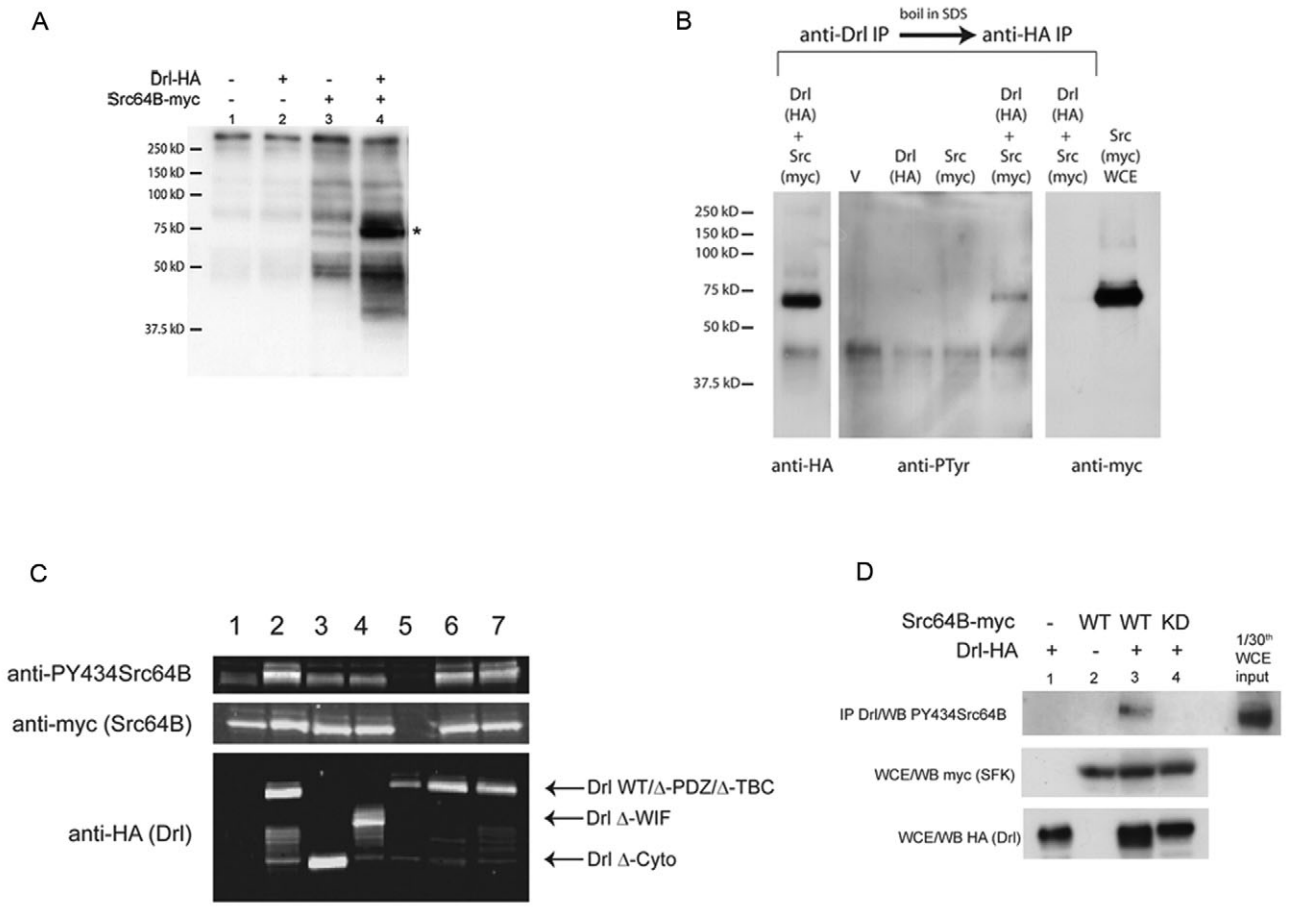

Fig. 6. DRL is tyrosine phosphorylated in a SRC64B-dependent manner and DRL coexpression activates SRC64B. (**A**) DRL and SRC64B co-transfected cell lysates contain a predominant protein species with increased tyrosine phosphorylation (asterisk). *Drosophila* S2 cells were transfected with the indicated plasmids and WCEs 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 reprecipitated with anti-HA antiserum and analyzed by anti-phosphotyrosine immunoblotting. V, vector alone. (**C**) Coexpression of DRL and SRC64B results in a WIF and cytoplasmic domain-dependent activation of SRC64B. S2 cells were transfected as follows: lane 1, SRC64B[WT] only; lane 2, DRL[WT] + SRC64B[WT]; lane 3, DRL[ΔCyto] + SRC64B[WT]; lane 4, DRL[ΔWIF] + SRC64B[WT]; lane 5, DRL[WT] + SRC64B[KD]; lane 6, DRL[ΔTBC] + SRC64B[WT]; and lane 7, DRL[ΔPDZ] + SRC64B[WT]. WCEs were immunoblotted to detect active SRC64B (anti-PY434SRC64B), pan-SRC64B (anti-MYC) and DRL (anti-HA). All transfections contained SRC64B-MYC except lane 5. Quantitation of a similar experiment performed in triplicate is shown in Fig. S5 in the supplementary material. (**D**) DRL-associated SRC64B is, at least in part, catalytically active. Lysates from cells transfected as indicated were immunoprecipitated with anti-DRL and analyzed by anti-PY434SRC64B immunoblotting. Control anti-MYC (SRC64B) and anti-HA (DRL) blots are shown.

of SRC64B in other tissues (Takahashi et al., 2005; Tateno et al., 2000; Harris and Beckendorf, 2007). Heterozygosity for *Src42A* did not suppress the *Wnt5* midline glial expression phenotype, possibly because SRC42A is not limiting under these conditions.

Third, 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* (Yoshikawa et al., 2003) mutants. The aberrant pathfinding of *Src64B* dsRNA-expressing *Sema2b*$^{+}$ neurons, which normally project through the AC, indicates that wild-type expression levels of *Src64B* are required for their correct routing. The incomplete penetrance of this phenotype possibly reflects the presence of wild-type levels of SRC42A, which we have shown is at least partially redundant with SRC64B. Alternatively, expression of *Src64B*-targeted dsRNA might not adequately reduce SRC64B expression levels to effect full penetrance.

Fourth, we demonstrate that *drl* and *Src64B*/*Src42A* interact synergistically in an axon-switching assay. Increased SFK neuronal expression levels alone could not force axons that normally traverse the PC to cross in the AC. Use of a DRL-expressing transgene facilitating only moderate switching showed that elevated *Src64B* or *Src42A* expression levels significantly increased switching. SFK catalytic activity is required to enhance DRL-dependent switching, as 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 wild-type 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).

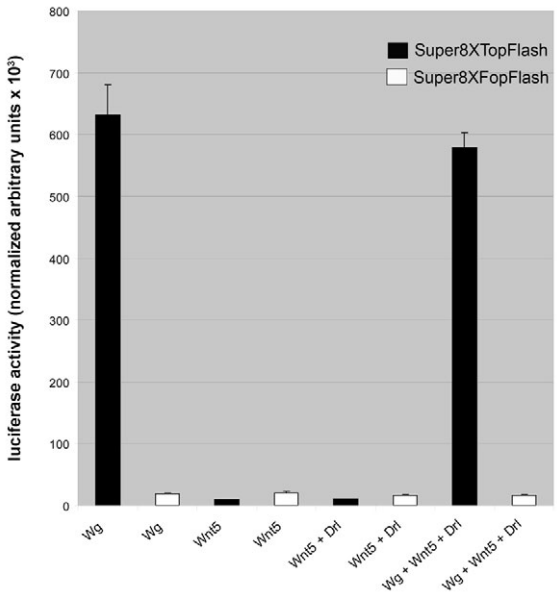

Fig. 7. The expression of WNT5 and DRL neither activates nor represses canonical TCF/LEF-dependent transcription. *Drosophila* S2 cells were transfected in triplicate with the expression plasmids indicated and either the TCF/LEF-dependent transcription reporter Super8XTopFlash (black bars) or the control Super8XFopFlash (white bars). Luciferase expression levels were determined, normalized to internal Renilla controls and plotted.

Supporting these previous observations and the genetic data presented here, we found that SRC64B and DRL physically interact, as assayed by co-immunoprecipitation. The formation or stability of this complex is apparently 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 this involvement of SFK catalytic activity was provided by our observation that wild-type, but not catalytically inactive, SRC64B physically interacts with the DRL cytoplasmic domain as assayed in two-hybrid experiments. RYK and c-SRC, like their *Drosophila* orthologs, co-immunoprecipitate from transfected cell lysates, suggesting that these interactions are evolutionarily conserved.

DRL phosphotyrosine content increases significantly when DRL is coexpressed with SRC64B in transfected tissue culture cells. Which of the 16 tyrosine residues within the DRL cytoplasmic domain is phosphorylated in a SRC64B-dependent manner and the function of this phosphorylation have yet to be determined. However, several of these residues are conserved between the three *Drosophila* RYK orthologs (DRL, DRL-2 and DNT) and mammalian RYK and a subset of these are predicted to be tyrosine kinase targets (data not shown). The levels of active SRC64B, as measured with an activation-specific anti-SRC64B antiserum, are significantly increased upon coexpression of SRC64B and DRL, and at least part of the active SRC64B population is physically associated with DRL. That the WIF domain, in addition to the cytoplasmic region, of DRL is required to activate SRC64B indicates that extracellular interactions of DRL contribute to its activation of SRC64B.

Unlike the in vivo assays implicating SFKs in the WNT5/DRL pathway presented here, 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 is possible, however, that endogenous expression levels of WNT5 or of 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. Our data, however, indicate that DRL-SRC64B interactions might be constitutive. Ligand-independent association of signal-transducing kinases with cell surface receptors is not, however, unprecedented. The mammalian Janus kinases are 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 Fz co-receptor have also been shown to interact in the absence of Wnt protein (Lu et al., 2004). RYKs might therefore possibly recruit at least a subset of their co-receptors and downstream effectors in a Wnt-independent manner. WNT5 binding to DRL might result in subtle conformational changes to pre-existing DRL-SRC64B complexes, which were 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).

Binding of Wnt protein to RYK stimulates TCF/LEF-dependent transcription via the Dishevelled 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 does not regulate TCF/LEF-dependent transcription. Transfection of *Drosophila* S2 tissue culture cells, which respond to the canonical WG ligand, with DRL and WNT5 expression constructs does not increase 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. The *Drosophila* WNT5 protein, however, can also signal via Fz family receptors to activate a non-canonical Wnt pathway (Srahna et al., 2006). Although our data render it unlikely that SRC64B is a member of the canonical Wnt signaling pathway, it might act in a pathway parallel to canonical Wnt signaling as has been reported for SFKs during convergent extension cell movement in zebrafish (Jopling and den Hertog, 2005) and during cell fate specification and cleavage orientation in *C. elegans* (Bei et al., 2002).

It is presently unclear whether the SFKs relay a WNT5/DRL signal, and if so, to what downstream pathway members and by what mechanisms. Our observations that increased *Src64B* and *Src42A* expression levels enhance *drl*-mediated commissure switching of axons in a sensitized 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. This hypothesis is attractive because the SFKs are widely expressed throughout the ventral nerve cord and are also likely to act downstream of other axonal receptors, the mammalian orthologs of which [e.g. the TRKB (NTRK2), ephrin A 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, DRL-dependent asymmetric localization or regulation of SFKs within the growth cone might mediate axon steering. Such localized changes in growth cone SFK activity effect axon turning in cultured *Xenopus* primary neurons (Robles et al., 2005).

Although the identification of the relevant SFK targets and of 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 potential SFK targets, through a combination of genetic and biochemical approaches should further reveal the mechanisms by which Wnt proteins signal through the RYKs.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/13/2277/DC1

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