Drosophila Ryks and their roles in axon and muscle guidance

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Homodimerization of the Wnt Receptor DERAILED Recruits the Src Family Kinase SRC64B

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ABSTRACT

Ryk pseudokinase receptors act as important transducers of Wnt signals, particularly in the nervous system. Little is known, however, of their interactions at the cell surface. Here, we show that a *Drosophila* Ryk family member, DERAILED(DRL), forms cell surface homodimers and can also heterodimerize with the two other fly Ryks, DERAILED-2 and DOUGHNUT ON 2. DERAILED homodimerization levels increase significantly in the presence of its ligand, WNT5. In addition, DERAILED displays ligand-independent dimerization mediated by a motif in its transmembrane domain. Increased dimerization of DRL upon WNT5 binding or upon the replacement of DERAILED's extracellular domain with the immunoglobulin Fc domain results in an increased recruitment of the Src family kinase SRC64B, a previously identified downstream pathway effector. Formation of the SRC64B/DERAILED complex requires SRC64B's SH2 domain and DERAILED's PDZ-binding motif. Mutations in DERAILED's inactive tyrosine kinase-homologous domain also disrupt the formation of DERAILED/SRC64B complexes, indicating that its conformation is likely important in facilitating its interaction with SRC64B. Finally, we show that DERAILED's function during embryonic axon guidance requires its Wnt-binding domain, a putative juxtamembrane extracellular tetrabasic cleavage site, and the PDZ-binding domain, indicating that DERAILED's activation involves a complex set of events including both dimerization and proteolytic processing.

INTRODUCTION

Wnts are secreted intracellular signaling proteins acting in many tissues during development (1). They have roles, among others, in axon guidance, nervous system cell fate determination, and the formation and maintenance of synapses (reviewed in references 2–6). Five distinct Wnt pathways and their associated receptors have been described to date. Several of them involve the Wnt ligands interacting with the Frizzled family of receptors. The first and most studied pathway is the so-called canonical Wnt pathway (reviewed in reference 7). It is activated by Wnt binding to the Frizzled and low-density lipoprotein (LDL) receptor-related protein (LRP) families of coreceptors, resulting in the cytosolic stabilization and nuclear translocation of β-catenin. Together with the T cell factor/Lef transcription factors, β-catenin regulates transcription of specific target genes. Wnt binding to Frizzled receptors can also activate pathways regulating cell mobility and planar cell polarity (PCP) (8) and a Ca^{2+}-dependent pathway regulating transcription (9).

Two other families of Wnt receptors have also been reported, the Ryk and Ror proteins (reviewed in references 10 and 11). Little is yet known bout their downstream pathways. While distinct from each other, Ryks and Rors, unlike the Frizzleds and LRPs, belong to the receptor tyrosine kinase (RTK) superfamily (12). The Ryks in particular, although not functioning exclusively in the nervous system (13), have been shown to play important roles there (reviewed in reference 10).

Ryk proteins are highly conserved during metazoan development and have several recognizable domains: an extracellular Wnt inhibitory factor (WIF) domain (14) and a putative juxtamembrane tetrabasic cleavage (TBC) site, both present in the extracellular domain (ECD), a single-pass transmembrane (TM) domain, and an intracellular domain (ICD), which consists of a tyrosine kinase-homologous domain with a putative postsynaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (Dlg1), and zonula occludens 1 protein (ZO-1) (15) binding domain (PDZ-BD) at the carboxy terminus. Although there is a single *Ryk* gene in mammals, the *Drosophila* genome bears three, *derailed (drl)*, *Derailed-2 (Drl-2)*, and *Doughnut on 2 (Dnt)*.

While Ryk was uncovered in mammals by its homology to the tyrosine kinases (16), the first indications of Ryk's roles *in vivo* came from studies of the *Drosophila drl* gene. *drl* was identified both as a gene controlling axon guidance in the developing embryonic central nervous system (CNS) (17) and as a gene required for wild-type learning and memory in adult flies (18). DRL is displayed on and controls the trajectories of axons that cross the embryonic ventral midline in the anterior-most of two anterior commissures (AC) present in each hemisegment (19). The absence of DRL causes these axons to misroute, leading to

incompletely separated commissures. Ectopic expression of DRL in posterior commissural (PC) axons, which normally do not express DRL, causes them to cross in the adjacent anterior commissure. DRL thus acts during embryogenesis as a repulsive axon guidance receptor. A subsequent study demonstrated that the Wnt protein WNT5, previously implicated in embryonic axon guidance (20), acts as a repulsive ligand for the DRL axons (21). In wild-type *Drosophila* embryos, WNT5 is expressed predominantly by PC axons (22) and repulses DRL axons, causing them to cross in the AC. Supporting this model, ectopic expression of WNT5 at the AC ventral midline results in the failure of the AC to form (21, 22). Both the commissure switching by PC axons ectopically expressing DRL and the disruption of AC formation by ectopic expression of WNT5 provide powerful genetic assays for *Drosophila* Wnt/Ryk signaling *in vivo*. Other studies established that the *drl* adult mutant learning and memory phenotype reflects axon guidance defects in the central complex and mushroom bodies (MBs) (23, 24), two centers of the brain associated with learning and memory. Furthermore, *Drosophila* Ryks have been shown to have additional roles both in the CNS and elsewhere. DRL and DOUGHNUT ON 2 (DNT) act in a subset of muscles to appropriately target them to specific epidermal tendon cells (25, 26). DRL plays a role in maintaining the wild-type physiology of the larval neuromuscular junction (NMJ) (27). DRL has also been shown to act as a non-cell-autonomous Wnt-interacting receptor in the MBs (28) and in the antennal lobes of the fly olfactory system (29). A number of studies of the mammalian Ryk protein indicate that it also plays important roles in several aspects of nervous system development (30– 35). Finally, evidence has been provided that injury-induced upregulation of Wnt/Ryk signaling contributes to poor posttrauma axonal regeneration (36–39; reviewed in reference 40), further indicating the need to better understand the relatively poorly characterized interactions of Ryk at the cell surface and to identify members of its downstream pathway.

During embryonic axon guidance in the CNS (19) and in DRL's function at the larval NMJ (27), the cytoplasmic domain of DRL is required for its function, indicating that DRL acts to transduce the WNT5 signal to as yet unknown cytoplasmic and nuclear targets. DRL however, like the other Ryks, is thought to be catalytically inactive due to a constellation of amino acid substitutions in conserved residues of the kinase domain (16), raising the question of how it might signal across the membrane. Supporting the hypothesis that DRL is not an active kinase, DRL encoded by a gene bearing a mutation in the codon for an invariant lysine (K371A) in the tyrosine kinase-homologous domain, which is required for catalytic activity, displayed wild-type function *in vivo* in both dominant gain-of-function and rescue assays in the *Drosophila* embryonic nervous system and musculature (41). Furthermore, DRL's purified cytoplasmic domain does not display catalytic phosphor transfer activity (16) and does not detectably bind ATP (F. Shi and M. Lemmon, personal communication). Our previous findings that DRL forms a complex with the Src family kinase (SFK) SRC64B, as do

their mammalian orthologs, Ryk and c-Src, indicate at least one mechanism by which Ryks might transduce an intracellular signal (42).

Here, we demonstrate that binding of WNT5 to DRL increases the level of DRL's homodimerization above the basal levels mediated by a motif in the TM domain. Homodimerization by the binding of WNT5 to wild-type DRL or upon replacement of DRL's extracellular domain with the dimerizing immunoglobulin Fc domain results in an increased recruitment of SRC64B. These results suggest that ligand-dependent dimerization acts to increase DRL/SRC64B interaction. Furthermore, we identify DRL's PDZ-BD and SRC64B's SH2 domain as being required for DRL/ SRC64B complex formation. Strikingly, point mutations in the inactive DRL tyrosine kinase-homologous domain block its interaction with SRC64B, indicating a likely requirement for its ability to adopt a specific conformation in order to form complexes with SRC64B. Finally, we show that DRL requires both its extracellular and intracellular domains, as well as a conserved juxtamembrane tetrabasic cleavage site in the extracellular domain, for its role in repulsive axon guidance *in vivo*.

MATERIALS AND METHODS

Constructs, transfection, immunoblotting, and immunoprecipitation.
Tagged (hemagglutinin [HA], FLAG, MYC, and V5)-actin promoter-driven or upstream activation sequence (UAS) promoter-driven wild-type DRL and mutant DRL (ICD-only, ECD-only, PDZ-BD, TBC, WIF, ICD [the last four of which lack the PDZ-BD, TBC, WIF, and ICD domains, respectively], and kinase domain mutations), wild-type DRL-2, wild-type DNT, wild-type SRC64B, and mutant SRC64B (SH2, SH3 and K312R kinase dead) expression plasmids were constructed by open reading frame (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). The Fc-DRL construct was generated by PCR and standard cloning techniques starting with an Fc ORF-containing plasmid generously provided by J. Thomas. The UAS-DRL constructs were cotransfected with pAc-GAL4 to drive expression of DRL. S2 cell transfections were performed using Effectene (Qiagen). Lysates were prepared using a high-stringency buffer (50 mM Tris-HCl [pH 8.0], 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM sodium orthovanadate, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.4 mM EDTA, and 10% glycerol) containing protease inhibitors (Roche). Cell lysate immunoprecipitations were performed using rabbit anti-FLAG (Sigma), rabbit anti-V5 (Sigma), rabbit anti-HA (AbCam), or mouse anti-FLAG antibody-coated beads (Sigma). Immunoblots were incubated with mouse anti-MYC (AbCam), rabbit anti-MYC (Upstate/Millipore), mouse anti-HA (Sigma), rabbit anti-HA (AbCam), mouse anti-FLAG (Sigma), rabbit anti-FLAG (Sigma), rabbit anti-V5 (Sigma), or horseradish peroxidase (HRP)-conjugated mouse anti-V5

(Sigma) antibodies to detect the tagged SRC64B and DRL species. Anti-*Drosophila* ribosomal protein P3 (43), kindly provided by M. Kelley, was used to control for equivalent loading of cell lysates on blots. Bound multiple-label-grade HRP-conjugated secondary antibodies (Jackson ImmunoResearch) were detected with enhanced-chemiluminescence (ECL) reagent (GE Healthcare). Blots shown are representative of three or more experiments.

Mammalian two-hybrid constructs and procedure.

The Checkmate mammalian two-hybrid system (Promega) was used to assay SRC64B-DRL interactions in SFK-deficient SYF cells (44) (LGC; PromochemATCC), which were transfected using Fugene (Roche). Coding sequences for wild-type and mutant cytoplasmic domains of DRL were cloned in frame with that for the GAL4 DNA-binding domain in the pBind vector, and the full-length wild-type or mutant SRC64B ORFs were cloned in frame with the VP16 activation domain in the pACT vector.

Cell surface biotinylation of DRL.

Cell surface biotinylation experiments were performed on transfected S2 cells using sulfo-N-hydroxysuccinimide (NHS)–LC– biotin (Pierce) at a final concentration of 2 mM for 30 min at room temperature. The reagent was quenched with three wash steps using 1 x phosphate-buffered saline (PBS) containing 100 mM glycine. Double immunoprecipitations were performed as follows. Cell lysates were first immunoprecipitated with rabbit anti-HA in the highstringency buffer plus protease inhibitors described above. Washed immune complexes were denatured by boiling in SDS, then diluted into Triton X-100-containing buffer, followed by immunoprecipitation with rabbit anti-FLAG antibodies. Proteins were separated by SDS-PAGE and detected on immunoblots with mouse anti-HA, mouse anti-FLAG, and streptavidin-HRP antibodies (Invitrogen).

TOXCAT assays.

TOXCAT assays were performed as previously described (45). The expression vector pccKAN and its derivates pccGpa-WT and pccGpa-G831, encoding the wild-type glycophorin A (Gpa) TM domain (residues Leu 75 to Thr 87) and the nondimerizing G831 Gpa mutant, respectively, were kindly provided by J. Mendrola and M. Lemmon. Oligonucleotides encoding DRL TM domains were annealed and ligated in frame into pccKAN as NheI/BamHI fragments, thus generating ToxR=– (DRL TM)–maltose-binding protein [ToxR=–(DRL TM)–MBP] chimeric open reading frames. The constructs were confirmed by DNA sequencing. The expression of the ToxR chimera was verified by immunoblotting using anti-MBP antisera (New England BioLabs), and the proper membrane insertion of the chimera was verified by a maltose complementation assay described previously (46). For chloramphenicol acetyltransferase (CAT) assays, MM39 *Escherichia coli* lysates were prepared as described previously (46). CAT assays were performed using a CAT enzyme assay system (Promega) according to the manufacturer's instructions.

Fly stocks and immunohistochemistry.

The *UAS-DRL-MYC; EG-GAL4* stock was used as previously described (42) as a sensitized background in which to perform the commissure switching assays. Axons were visualized by diaminobenzidine (DAB) staining with rabbit anti-MYC and HRP-conjugated secondary antibodies (Upstate/Millipore).

RESULTS

DRL forms homodimers, and the three *Drosophila* Ryks form heterodimers.

Receptor dimerization is a mechanism frequently associated with the activation of signaling pathways (47). To evaluate whether DRL forms homodimers, we coimmunoprecipitated proteins from lysates derived from *Drosophila* S2 cells transiently cotransfected with plasmids expressing differentially tagged DRL species, DRL-FLAG and DRL-HA. Cell lysates were immunoprecipitated with anti-FLAG, and DRL-HA was detected by anti-HA immunoblotting. DRL-HA was precipitated by anti-FLAG in the presence of DRL-FLAG but not in its absence (Fig. 1). We also examined whether the three *Drosophila* RYK orthologs, DRL, DRL-2, and DNT, can interact with each other. All pairwise combinations of the three Drosophila Ryk family members formed heterodimeric complexes (Fig. 1). These data indicate that DRL forms homodimeric complexes and that the three Ryk proteins are capable of interacting with each other. DRL homodimers are displayed on the cell surface. To evaluate whether DRL homodimers can be detected at the cell surface, we performed cell

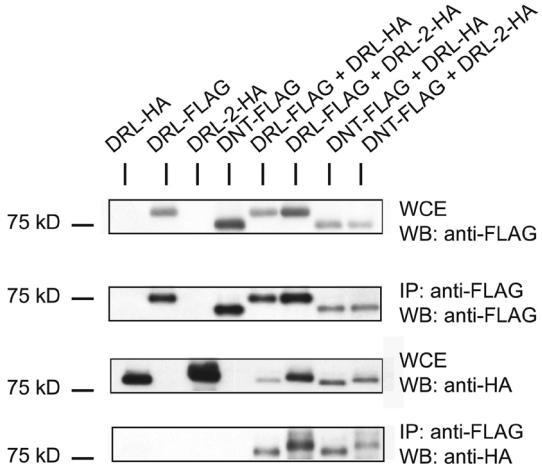

Fig 1 DRL forms homodimers and heterodimers with DRL-2 and DNT. *Drosophila* S2 cells were transiently transfected with the indicated expression constructs, and lysates were immunoprecipitated (IP) with antibody specific to tagged DRL (anti-FLAG) and immunoblotted (WB) with the reciprocal antibody (anti-HA) to detect coimmunoprecipitation of the other tagged protein. Expression of DRL, DRL-2, and DNT was confirmed by immunoblotting of whole-cell extracts (WCE). DRL-HA coimmunoprecipitated with DRL-FLAG, and all pairwise combinations of Ryks also formed immunoprecipitable complexes, indicating that the three *Drosophila* Ryks form heterodimers.

surface biotinylation coimmunoprecipitation experiments. In brief, S2 cells were transiently transfected with DRL-HA and DRL-FLAG and cell surface proteins were biotinylated with a cell-nonpermeable biotin cross-linking reagent at 3 days posttransfection. A cytoplasmic green fluorescent protein (GFP)-expressing construct was also cotransfected to control for the cell surface specificity of the biotinylation treatment. Expression of the DRL constructs and the biotinylation of proteins were confirmed in cell lysates by antitag and streptavidin-HRP immunoblotting, respectively (Fig. 2A). Lysates were first immunoprecipitated with anti-HA, and proteins were dissociated by boiling in SDS. DRL-FLAG in the complex was then immunoprecipitated with anti-FLAG and detected on separate immunoblots using anti-FLAG, anti-HA, and streptavidin-HRP to confirm immunoprecipitation of DRL-FLAG and the lack of immunoprecipitation of DRL-HA and to detect biotinylated DRL-FLAG. DRL-FLAG that coimmunoprecipitated with DRL-HA was also detected by streptavidin-HRP (Fig. 2B, bottom panel), indicating that DRL homodimers are present at the cell surface. We did not observe biotinylation of GFP, consistent with the expectation that only cell surface proteins were labeled (Fig. 2C).

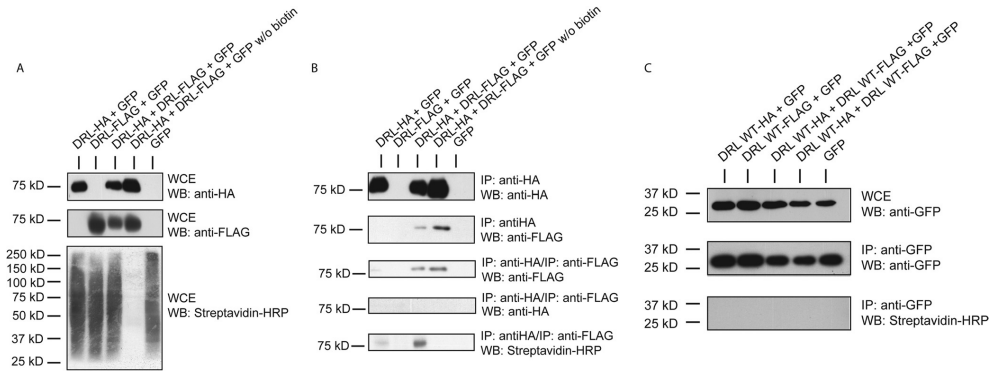

Fig 2 DRL homodimers are present at the cell surface. (A) S2 cells were transiently transfected with the indicated expression constructs and treated with a membrane-impermeable cell surface biotinylation reagent except as otherwise noted in the figure. Cell lysates were first immunoprecipitated with anti-HA to precipitate DRL-HA-containing complexes; then complexes were washed, disrupted by boiling, and reprecipitated with anti-FLAG to precipitate DRL-FLAG. All samples were immunoblotted (WB) with the appropriate antibodies to detect immunoprecipitation of DRL-HA and potential coimmunoprecipitation of DRL-FLAG and with streptavidin-HRP to detect biotinylated proteins. The expression of DRL wild-type (WT) variants and the efficiency of biotinylation were confirmed by immunoblotting of the whole-cell extract (WCE). (B) The initial anti-HA immunoprecipitates were similarly analyzed, establishing efficient precipitation of the HA-tagged species and coimmunoprecipitation of the FLAG-tagged species (top two panels). Immunoblotting of the doubly immunoprecipitated (anti-HA followed by anti-FLAG) proteins revealed that, while the FLAG-tagged species was precipitated, the HA species was no longer detectable and that the FLAG-tagged species that initially coimmunoprecipitated with the HA-tagged species was detected with streptavidin-HRP (bottom three panels). (C) The lack of biotinylation of simultaneously expressed cytoplasmic GFP confirmed the cell surface specificity of the biotinylation. Thus, we conclude that DRL dimers are present at the cell surface.

WNT5 increases DRL homodimerization in a WIF domain dependent fashion.

We next evaluated how DRL homodimerization is influenced by the presence of its ligand, WNT5. S2 cells express low, but clearly detectable, levels of WNT5 (Fig. 3A); therefore, we compared the effects of overexpressing WNT5 with those of a reduction of its expression by preincubation of the cells with double-stranded RNA (dsRNA) targeting the *wnt5* transcript (48). *gfp*-targeting dsRNA was used as a control for nonspecific effects. The dsRNA treatment was highly effective, as indicated by its ability to suppress the expression of endogenous WNT5 (Fig. 3A). Reduced expression of WNT5 significantly decreased DRL homodimerization relative to that of the *gfp*-dsRNA control (Fig. 3B, second panel from bottom). Overexpression of WNT5 did not significantly increase homodimerization, relative to that of the *gfp*-targeting control, indicating that there was sufficient endogenous WNT5 to saturate DRL. The results from similar experiments done with differentially tagged DRL species lacking the Wnt binding WIF domain (WIF) indicated that reduction of WNT5 expression did not decrease the levels of homodimerized WIF DRL below those of the dsRNA-*gfp* control (Fig. 3C, second panel from bottom). Thus, the presence of WNT5 increases wild-type DRL homodimerization; however, DRL retains the ability to homodimerize in a ligand-independent fashion in the absence of its WNT5-binding domain.

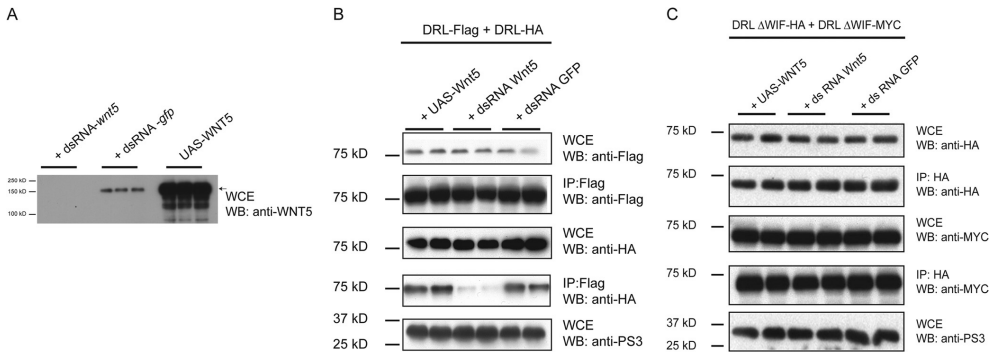

Fig 3 DRL homodimerization is increased upon WNT5 binding in a WIF domain-dependent fashion. (A) S2 cells were pretreated with *wnt5*-targeting dsRNA or control *gfp*-targeting dsRNA or transfected with UAS-WNT5 and pAc-GAL4 to overexpress WNT5. Highly efficient WNT5 knockdown and overexpression were observed. (B) The pretreated cells were then transfected in duplicate with DRL-HA and DRL-FLAG, and lysates were immunoprecipitated (IP) with anti-FLAG and immunoblotted (WB) with anti-HA to detect coimmunoprecipitation. Expression of the differentially tagged DRL species was confirmed by WCE immunoblotting. DRL homodimerization was dependent on WNT5 expression, and endogenous levels of WNT5 were sufficient to mediate the dimerization. (C) S2 cells pretreated as above were transfected in duplicate with DRL ΔWIF-HA and DRL ΔWIF-MYC expression constructs. Cell lysates were immunoprecipitated with anti-HA and immunoblotted with anti-MYC to detect coimmunoprecipitation of the differentially tagged species. The expression of both DRL species was confirmed by WCE immunoblotting. DRL ΔWIF formed homodimers that, unlike the wild-type protein, are resistant to the effects of *wnt5* knockdown.

Transmembrane domain contributions to DRL homodimerization.

To evaluate which domain of DRL is required for the formation of homodimers, we then performed coimmunoprecipitations from lysates derived from cells pretreated with *wnt5*-targeting dsRNA and cotransfected with plasmids expressing full-length DRL (DRL-FLAG) and MYC-tagged DRL species lacking either the carboxyterminal PDZ-BD (ΔPDZ-BD), a putative extracellular tetrabasic cleavage site (ΔTBC), the WIF domain (ΔWIF), or the entire intracellular domain (ΔICD). Each of the mutant DRL species retained the ability to interact with full-length DRL (Fig. 4A, bottom panel). These results indicated that the sequences

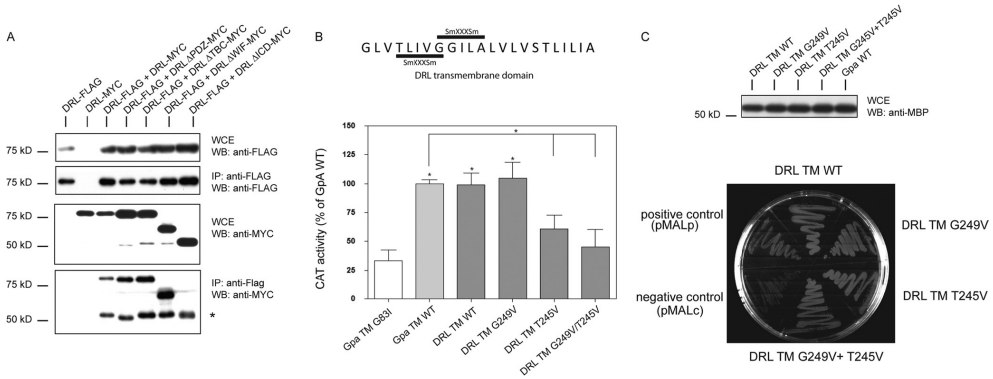

Fig 4 DRL homodimerization is mediated by a motif in the transmembrane domain. (A) S2 cells were pretreated with dsRNA targeting the wnt5 transcript as described above and then transiently transfected with the indicated wild-type and DRL mutant expression constructs, and lysates were immunoprecipitated (IP) with anti-FLAG and immunoblotted with anti-MYC to detect coimmunoprecipitation. The expression of DRL WT and the various DRL truncation mutants was confirmed by WCE immunoblotting. Fragments corresponding to the DRL intracellular domain (indicated by an asterisk), evident in the anti-MYC blots, increase in intensity during the immunoprecipitation, presumably due to the presence of proteases resistant to the inhibitors included. Our unpublished mass spectroscopy data indicate that they result from cleavage at or near the putative tetrabasic cleavage site (data not shown). DRL ΔPDZ-BD, DRL ΔTBC, DRL ΔWIF, DRL ΔICD all coimmunoprecipitated with DRL WT, indicating that the sequences mediating complex formation lie in the TM region. (B, top) The sequence of the wild-type DRL TM domain and the locations of small amino acid (Sm)-X-X-X-small amino acid motifs. The wild-type and T245V and G249V mutant TM domains were cloned singly and as a T245V G249V double mutant into the pccKAN vector and transformed into *E. coli*, and quantitative TOXCAT assays were performed on cell lysates. Comparison was made to a negative control (pccKAN without a TM domain), a positive control (encoding a fusion bearing the known homodimerizing glycophorin A [Gpa] TM domain), and another negative control (the Gpa TM domain with a mutation [G83I] which abolishes homodimerization). CAT activities were expressed as percentages in comparison to that for the Gpa-TM chimera (100% activity). The data shown are the means from three independent experiments, each performed in triplicate,standard deviations (SD) (,P0.05). The TM domain of DRL displayed robust interaction comparable to that for the Gpa control, and the T245V, but not the G249V, mutation was found to reduce the formation of DRL-TM homodimers (lower panel). (C) To determine that the TOXCAT fusion proteins were the expected size, we prepared lysates of E. coli transformed with the indicated plasmids and analyzed them by anti-MBP immunoblotting. All expression plasmids gave rise to proteins of the anticipated size (top). To confirm the appropriate insertion of the fusion proteins into the periplasmic membrane, transformed *E. coli* cells were grown on maltose as the sole carbon source; their viability requires the localization of MBP to the periplasmic space. While bacteria transformed with the negative control (pMALc) failed to grow, the others displayed robust growth (bottom). Thus, the TOXCAT test plasmids employed in these studies generated proteins of the anticipated size which were appropriately localized to the periplasmic space.

facilitating ligandindependent DRL homodimerization likely resided in the TM region, which was present in each of these mutant proteins tested. Dimerization through TM region interactions has been reported for a number of receptors (47) and is usually dependent on small structural motifs with a consensus sequence of small amino acid-X-X-X-small amino acid, where X represents any amino acid (49). We identified two such motifs in the DRL transmembrane domain (Fig. 4B, top panel), TLIVG and GGILA. To evaluate their roles in DRL homodimerization, we used a well-established bacterial assay for quantifying DRL TM domain self-interaction, TOXCAT (46). In brief, the *E. coli* codon-optimized DRL TM domain open reading frame was cloned into a vector allowing its expression at the periplasmic membrane as a fusion protein with a transcriptional regulator, ToxR. Dimerization of ToxR, which is required for its activity, increases if the tested TM domain homodimerizes. Transcription factor activity, reflecting TM domain-mediated ToxR dimerization, is read out by quantitative assay of the activity of chloramphenicol acetyltransferase (CAT), whose gene's transcription is under ToxR control. We mutated the sequences encoding the first amino acid in both of the DRL TM motifs to encode valine and compared them with the wildtype sequence in the TOXCAT assay. All constructs generated MBP fusion proteins of the appropriate sizes, as indicated by immunoblotting; the proteins were correctly inserted into the membrane, as indicated by the ability of transformed cells to grow on maltose as the sole carbon source (Fig. 4C).

Homodimerization mediated by the wild-type DRL TM domain was comparable to that observed with the glycophorin A (Gpa) TM domain, a previously reported homodimerizing sequence (45). Mutation of the first motif to VLIVG (T245V), but not that of the second motif to VGILA (G249V), resulted in significantly reduced homodimerization (Fig. 4B, bottom panel). Thus, we conclude that DRL's ligand-independent homodimerization at the cell surface is likely mediated primarily by the TLIVG motif.

DRL dimerization in the presence of WNT5 or its forced dimerization mediated by the immunoglobulin Fc domain results in increased SRC64B recruitment.

To evaluate the effect of WNT5 binding to DRL on the recruitment of SRC64B by DRL (42), we determined the levels of SRC64B coimmunoprecipitating with DRL in cells that either overexpressed WNT5 or had reduced expression of WNT5 due to their preincubation with *wnt5*-targeting dsRNA as described above. Less SRC64B immunoprecipitated with DRL in the presence of *wnt5*-targeting dsRNA than in the presence of control *gfp*-targeting dsRNA (Fig. 5). As was observed in the DRL homodimerization experiments, there was no effect of overexpressing WNT5, presumably due to its already saturating endogenous levels. Thus, WNT5 binding to DRL results in increased recruitment of SRC64B. We reasoned that, if dimerization is involved in DRL receptor activation, increased dimerization should result in increased recruitment of SRC64B. To force DRL dimerization, we constructed a plasmid

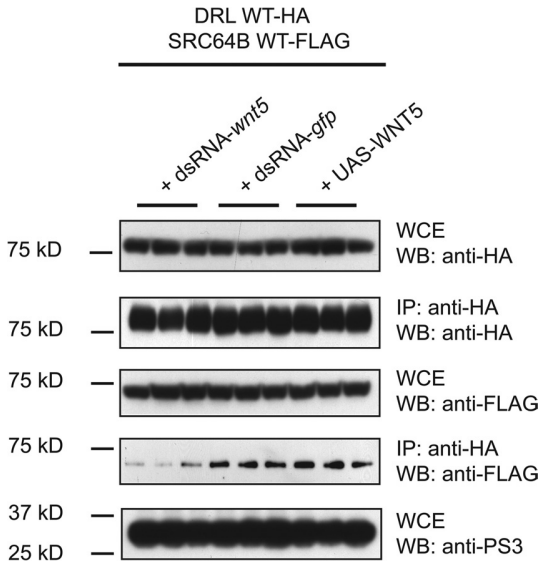

Fig 5 WNT5 binding results in increased SRC64B recruitment. S2 cells were pretreated as described for Fig. 3 and transfected in triplicate with DRL WT-HA and SRC64B WT-FLAG. Cell lysates were immunoprecipitated with anti-HA and immunoblotted with anti-FLAG to detect coimmunoprecipitating SRC64B. The expression of DRL WT and SRC64B WT was confirmed by immunoblotting the whole-cell extract (WCE). Pretreatment of cells with *wnt5*-targeting but not *gfp*-targeting dsRNA resulted in reduced SRC64B recruitment, but overexpression of WNT5 did not increase recruitment above that for the *gfp*-targeting control dsRNA, indicating that endogenous levels of WNT5 cause maximal ligand-dependent recruitment of SRC64B by DRL.

encoding a fusion protein of DRL with its extracellular domain replaced by the Ig Fc region (Fc-DRL-V5), a previously used dimerization domain (see, for example, references 50 and 51). We established that, as expected, Fc-DRL species form dimers to a larger extent than wild-type DRL, as assayed by coimmunoprecipitation of differentially tagged otherwise-identical proteins (Fig. 6A, second panel from bottom). Wild-type DRL (DRL-WT-V5) and a TM species lacking the extracellular domain (DRL-ECD-V5) served as controls (Fig. 6B). The DRL-encoding plasmids were individually cotransfected into dsRNA-*wnt5*-treated cells with a tagged SRC64B-encoding plasmid, lysates were prepared and immunoprecipitated with an antibody specific to tagged DRL, and the SRC64B precipitating with the DRL species was detected by antibody specific to tagged SRC64B. We observed increased SRC64B recruitment by Fc-DRL relative to that for both the wild-type and membrane-bound intracellular domain-only proteins (Fig. 6C, lower right panel).

SRC64B's SH2 domain and its catalytic activity are needed for its interaction with DRL. We then performed mammalian two-hybrid and coimmunoprecipitation assays to identify the domains of SRC64B required for its interaction with DRL. SRC64B has three major domains, the SH3, SH2, and kinase domains (Fig. 7A). The SH3 and SH2 domains serve to mediate the intra- and intermolecular interactions that regulate kinase activity as well as the interaction of SFKs

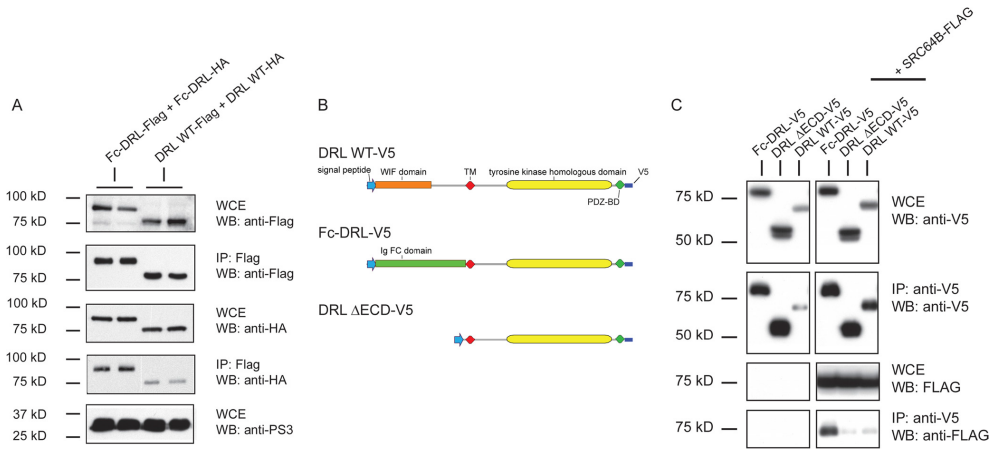

Fig 6 Forced dimerization of DRL increases SRC64B recruitment. (A) We first established that the replacement of DRL's extracellular domain by IgG Fc increases the level of DRL homodimerization. The indicated expression constructs were cotransfected into S2 cells in duplicate, and anti-Flag immunoprecipitations were performed, followed by immunoblot detection with anti-HA. A clear increase in homodimerization levels, relative to the wild-type (WT) control, was observed for Fc-DRL (second panel from bottom). (C) To evaluate the effects of forced dimerization of DRL on SRC64B recruitment, dsRNA-wnt5-treated S2 cells were transfected as indicated with SRC64B WT-FLAG and individual V5-tagged expression constructs encoding Fc-DRL (where DRL's extracellular domain was replaced by the IgG-Fc domain), DRL WT, and Drl ΔECD (a TM species with the wild-type cytoplasmic and TM domains of DRL lacking the extracellular domain) (panel B shows schematic representations of these proteins). DRL species were immunoprecipitated with anti-V5, and complexes were subsequently immunoblotted with anti-FLAG to detect coimmunoprecipitation of SRC64B. The expression of the DRL variants and SRC64B WT was confirmed by WCE immunoblotting. SRC64B was recruited to a much larger extent by FC-DRL than by wild-type DRL or DRL ΔECD (bottom right panel).

with their substrates (reviewed in reference 52). We generated plasmids encoding SRC64B species lacking either the SH3 or SH2 domain or bearing the kinase activity-destroying K312R point mutation (the equivalent of the mammalian K298R mutation) and tested their abilities to physically interact with DRL. To avoid complications due to DRL homodimerization, we evaluated the interactions of the various SRC64B species with a cytoplasmically localized non-membrane-tethered wildtype DRL intracellular domain (DRL-ICD-HA). The results from the mammalian two-hybrid assays indicate that SRC64B's SH2 domain, but not its SH3 domain, is required for DRL interaction (Fig. 7B). Furthermore, they confirm our previous finding (42) that this kinase-inactivating mutation inhibits SRC64B/DRL interaction, indicating that SRC64B kinase activity is required for the recruitment of SRC64B to DRL's cytoplasmic domain. Coimmunoprecipitation experiments confirmed these results (Fig. 7C, second panel from bottom). Thus, SRC64B interacts with DRL via its SH2 domain and apparently either must be an active tyrosine kinase or must assume a certain conformation, which is prevented by the K312R mutation, to bind DRL. We also investigated whether kinase-inactive SRC64B could bind to DRL in the presence of active SRC64B e.g., once DRL is phosphorylated by active SRC64B, can the kinase-inactive species bind via its SH2 domain? Cells were cotransfected with a mix of three differently tagged

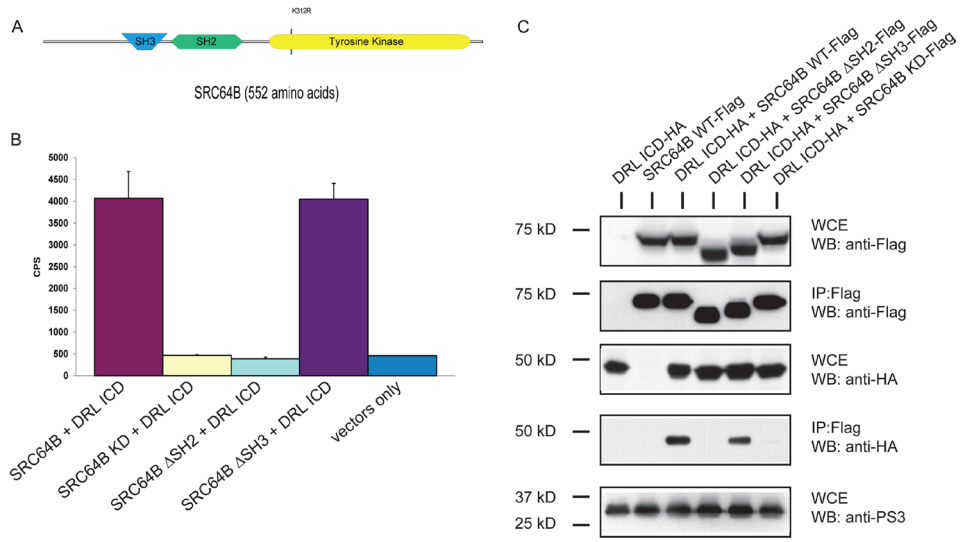

Fig 7 SRC64B's SH2 domain and a kinase domain amino acid required for catalytic activity are needed for formation of the SRC64B/DRL complex. (A) Schematic representation of SRC64B with its SH2, SH3, and tyrosine kinase domains and the location of the kinase-dead K312R mutation. (B) To determine the SRC64B domains required for its interaction with DRL, we performed mammalian two-hybrid assays. The indicated fusion protein-encoding constructs were transfected with a luciferase reporter gene in triplicate into SYF (SFK-deficient) cells, and luciferase activity was measured 48 h posttransfection and plotted normalized to an internal control. SRC64B WT and SRC64B ΔSH3, but not Src64B ΔSH2 or SRC64B kinase dead (KD), interact with the intracellular domain of DRL (DRL ICD). (C) To confirm the two-hybrid results, S2 cells were transfected with the indicated expression constructs and lysates were immunoprecipitated (IP) with antibody specific to the SRC64B variants (anti-FLAG) and immunoblotted (WB) with the reciprocal antibody (anti-HA) to detect coimmunoprecipitation of DRL. Expression of DRL ICD, SRC64B WT, and SRC64B mutants was confirmed by immunoblotting the whole-cell extract (WCE). Both the SRC64B ΔSH2 and KD, but not the ΔSH3, species display significantly reduced complex formation with DRL relative to the wild-type control.

plasmids encoding DRL-ICD-HA, SRC64B-WT-MYC, and SRC64B-kinase-dead–FLAG, and lysates were prepared and immunoprecipitated with anti-HA or anti-FLAG. The SRC64B or DRL species in the complex were detected with tag-specific antibodies on immunoblots. We found that the presence of SRC64B-kinase-dead did not result in a decrease in the amount of active SRC64B that coimmunoprecipitated with DRL-ICD (Fig. 8A). Conversely, the presence of active SRC64B did not increase the amount of DRL-ICD coimmunoprecipitating with SRC64B-kinase-dead (Fig. 8B). We conclude from this lack of competition between kinase-active and -inactive SRC64B that individual SRC64B molecules binding to DRL must either possess tyrosine kinase activity or adopt a conformation precluded by the K312R mutation.

The PDZ-binding domain of DRL and specific amino acids in its tyrosine kinase-homologous domain are required for its interaction with SRC64B.

We then evaluated the requirement for DRL's cytoplasmic domains in its physical interaction with SRC64B. The two clearly identifiable domains in DRL's intracellular domain are the

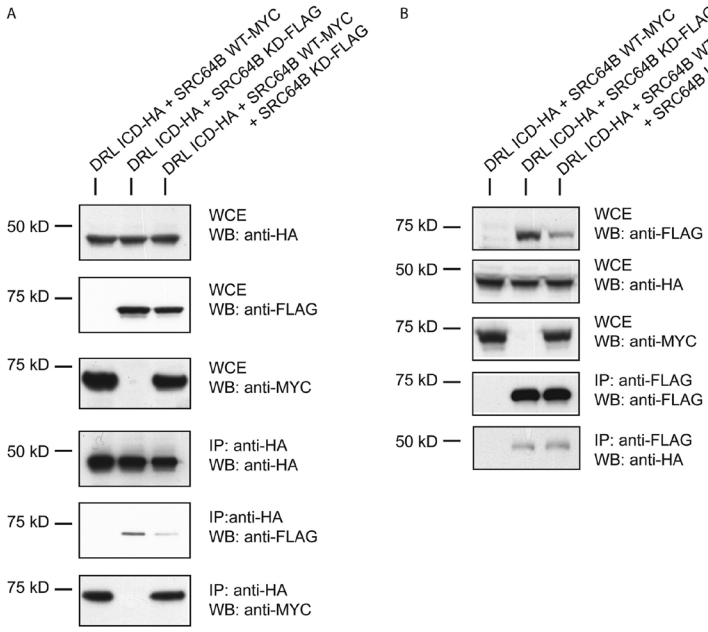

Fig 8 Kinase-dead SRC64B does not compete with active SRC64B for binding to DRL. (A) To ascertain whether the presence of kinase-dead SRC64B interferes with the ability of active SRC64B to interact with DRL, plasmids encoding the proteins indicated were cotransfected into S2 cells. Expression levels of the various species (WCE) and the efficiency of immunoprecipitation are shown (upper five panels). The presence of kinase-dead SRC64B (SRC64B KD-FLAG) did not diminish the amount of active SRC64B coimmunoprecipitating with DRL (bottom panel). (B) In a complementary experiment, we addressed whether or not the presence of active SRC64B would increase the amount of DRL coimmunoprecipitating with kinase-dead SRC64B. Plasmids encoding the proteins indicated were cotransfected into S2 cells. Expression levels of the various species (WCE) and the efficiency of immunoprecipitation are shown (upper four panels). The presence of active SRC64B did not appreciably increase the amount of DRL coimmunoprecipitating with kinase-dead SRC64B (bottom panel).

inactive kinase domain and the carboxy-terminal PDZ-BD (Fig. 9A). We therefore generated a DRL expression construct bearing two mutations in the tyrosine kinase-homologous domain, K371A and D486A, which mutate conserved amino acids in RTK subdomains II and VII (53), respectively, which are required for catalytic phosphotransfer (52). We assayed this mutant and another lacking the PDZ-BD for their abilities to interact with SRC64B in mammalian two-hybrid and coimmunoprecipitation experiments. Both mutants failed to interact with SRC64B in either assay (Fig. 9B and C), indicating a requirement for the PDZ-BD and for the ability of the DRL cytoplasmic domain to assume a conformation which is precluded by these specific mutations. We then evaluated whether the DRL T245V mutation, which as shown above inhibits ligand-independent DRL TM domainmediated dimerization as determined by TOXCAT assay, affected the ability of DRL to recruit SRC64B. Full-length DRL bearing this mutation displayed reduced complex formation with SRC64B in the presence of endogenous WNT5, relative to the wild-type DRL control, in coimmunoprecipitation assays (Fig. 10). Thus, impairing TM-mediated dimerization reduces DRL's ability to recruit SRC64B.

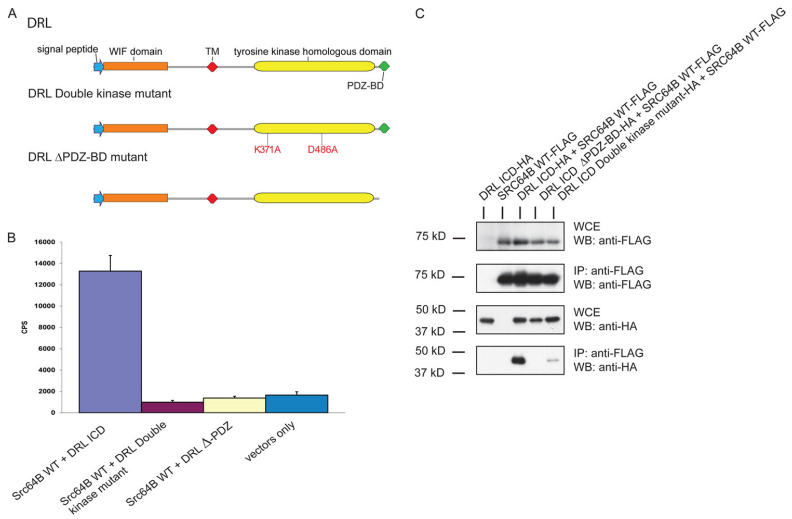

Fig 9 DRL's PDZ-BD and amino acids in its tyrosine kinase-homologous domain mediate its interaction with SRC64B. (A) Schematics of DRL displaying its domains and the DRL mutants (double kinase domain mutant and ΔPDZ-BD) used in the following assays. (B) To ascertain the requirement for DRL's intracellular domains, we performed mammalian two-hybrid assays. The indicated fusion protein constructs were transiently transfected into SYF (SFK-deficient) cells in triplicate, and luciferase activity was measured 48 h posttransfection and plotted, normalized to an internal control. DRL, but not the double kinase or ΔPDZ-BD mutant, interacts with SRC64B. (C) To confirm the mammalian two-hybrid results, we performed coimmunoprecipitation experiments. S2 cells were transiently transfected with the indicated expression constructs, and lysates were immunoprecipitated (IP) with antibody specific to SRC64B (anti-FLAG) and immunoblotted (WB) with the reciprocal antibody (anti-HA) to detect coimmunoprecipitation of the DRL species. The expression of DRL ICD variants and SRC64B was confirmed by immunoblotting the whole-cell extract (WCE). The DRL double kinase domain and ΔPDZ-BD mutants display significantly less interaction with SRC64B than the wild-type control.

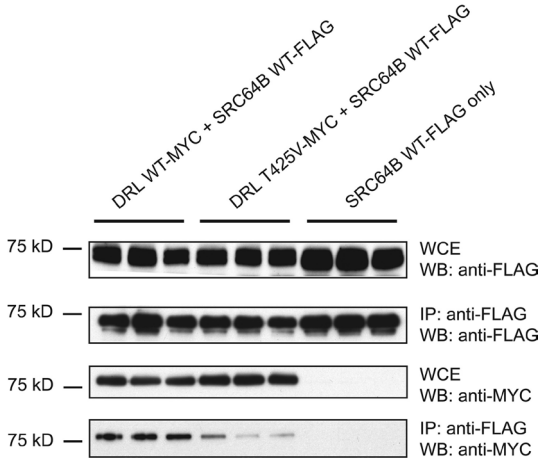

Fig 10 Reduced recruitment of SRC64B by DRL bearing the TM T245V mutation. S2 cells were transfected with the indicated plasmids, and lysates were prepared and immunoprecipitated with anti-FLAG (SRC64B). Expression of the DRL and SRC64B species was confirmed by anti-FLAG and antiMYC immunoblots of whole-cell lysates (WCE), and the efficiency of immunoprecipitation was confirmed with anti-FLAG. Significantly less DRL T245V immunoprecipitated with SRC64B, as detected by anti-MYC immunoblots of the anti-FLAG immunoprecipitates, than the wild-type DRL control.

***In vivo* requirements for DRL's WIF domain, tetrabasic cleavage site, and cytoplasmic domain in an axon commissure switching assay.**

To evaluate the roles of the various extra- and intracellular domains of DRL, we generated UAS-MYC-tagged transgenes of mutant DRL ORFs (ΔWIF, ΔTBC, ΔICD, and ΔPDZ-BD) by random P-element insertion and generated a collection of roughly expression-matched inserts by performing quantitative anti-MYC immunoblotting of dissected third-instar larval central nervous systems expressing the UAS transgene under the control of a panneural driver (data not shown). The transgenes were then evaluated in a previously described (19) assay for DRL function, specifically as described above, for their ability to cause commissure switching of a subset of EG-GAL4$^+$ neurons which normally cross the ventral nerve cord midline in the more posterior of the two commissures found in each hemisegment. We performed this assay in a genetic background sensitized by the presence of one copy of wild-type UAS-DRL, which is not sufficient to cause commissure switching by itself (42) (Fig. 11). Quantitation of the switching events indicates that DRL's WIF, TBC, and ICD domains are required to force commissure switching, while the Δ-PDZ-BD mutation decreases DRL's activity in this assay by approximately one-third (Fig. 11). Unexpectedly, the mutation affecting TM homodimerization in the TOXCAT assay, DRL T245V, had no apparent effect (discussed below). Thus, we conclude that DRL function during embryonic CNS development requires

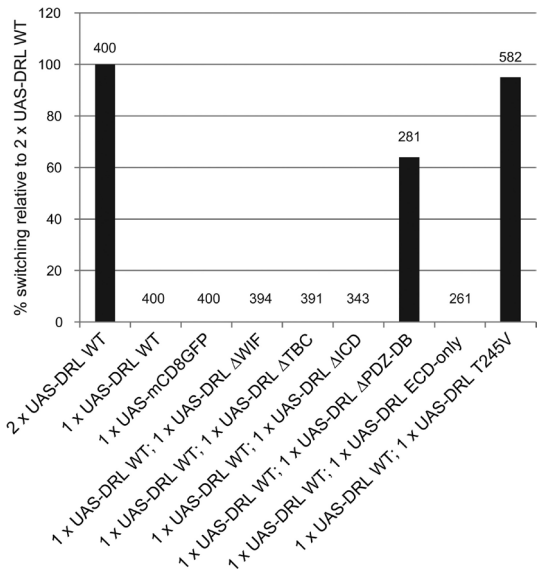

Fig 11 DRL's WIF, TBC, ECD, and ICD and the PDZ-BD are required to effect axon commissure switching in a sensitized background. Stage 16 *Drosophila* embryos of the indicated genotypes were stained with anti-MYC to label EG$^+$ neurons traversing the midline in their stereotypic patterns in both the AC and PC, and the numbers of hemisegments indicated above the bars were scored for axon commissure switching. The numbers of hemisegments with switched axons, normalized to 2UAS-DRL, which was set at 100%, are plotted. Expression of DRL PDZ-DB results in 36% less switching than 2 UAS-DRL WT. UAS-DRL T245V causes switching essentially as well as wildtype DRL, while expression of DRL-ΔWIF, -ΔTBC, -ECD-only, -ΔCYTO, and -ΔICD did not cause the EG$^+$ PC axons to switch to the AC.

its ability to bind the WNT5 ligand, its extracellular juxtamembrane TBC site, and signal transduction mediated by the cytoplasmic region, possibly via the PDZ-BD.

DISCUSSION

Signaling through the Ryk family of catalytically inactive tyrosine kinase-homologous receptors has recently been found to play important roles in nervous system development (10). Here, we have provided evidence that activation of the WNT5/DRL pathway occurs via dimerization of DRL molecules at the cell surface. While this mechanism has not been previously reported for the Ryk family of transmembrane Wnt receptors, it is a common theme in receptor-mediated signal transduction (12). Extracellular ligand induced receptor dimerization of catalytically active receptors generally results in the juxtaposition of their cytoplasmic domains and transphosphorylation of tyrosine residues via their intrinsic kinase activity, resulting in the binding of downstream pathway members. Dimerization of catalytically inactive tyrosine kinases can result in the recruitment of cytosolic kinases which effect signal transduction. While our data are most readily explained by direct homo- or heterodimeric interaction of Ryk proteins, we cannot exclude the possibility that they associate indirectly as part of a larger complex or that other proteins stabilize their direct interaction.

The degree of DRL dimerization is increased by the presence of WNT5 in a manner dependent upon DRL's WIF Wnt-binding domain. Increased dimerization, either by replacement of DRL's extracellular domain with the IgG Fc domain or upon WNT5 binding results in increased recruitment of the SFK SRC64B. We have previously shown that SRC64B is required *in vivo* for WNT5/ DRL-dependent axon repulsion in the embryonic central nervous system (42). Whether this interaction results in localizing SRC64B close to its phosphorylation targets or in titrating SRC64B away from particular parts of the growth cone to steer the axon is at present unclear.

We also demonstrated that the three *Drosophila* Ryks are capable of forming heterodimers in transfected cells, indicating that they may do so *in vivo*. While all of the tissues where pairwise combinations of DRL, DRL-2, and DNT may be coexpressed have not been reported, we have previously shown that DRL and DNT act at least partially redundantly in a subset of muscle fibers to target them to their correct epidermal tendon cell attachment sites (26). Mutants homozygous for either of the associated genes show a phenotype of partial penetrance of a muscle attachment site bypass; penetrance increases to essentially 100% in the doubly homozygous mutants. Thus, during myotube guidance, these two Ryks may form functional signaling heterodimers.

DRL also exhibits a basal level of dimerization in the absence of a ligand. Coimmunoprecipitation experiments revealed that none of the defined extracellular or intracellular domains of DRL is

required for ligand-independent homodimerization. These results caused us to examine the potential involvement of sequences in the wild-type TM domain, which was still present in all of the other domain-specific mutants. Previous studies have revealed that TM domain-mediated dimerization of proteins is often mediated by small amino acid-X-X-X-small amino acid motifs, where X represents any amino acid (49).

Our analyses of mutations in each of the two such sequences present in DRL's TM domain indicate that only one of them, VLIVG, mediates significant levels of homodimerization in the TOXCAT assay, indicating that it may help to facilitate DRL's *in vivo* ligand-independent homodimerization. Supporting such a role for Ryk TM domain interactions in dimerization is a previous report that the wild-type TM sequence of Ryk, as well as those of many other human RTK-related proteins, showed significant activity in the TOXCAT assay (54). These results indicate that such interactions are an evolutionarily conserved general feature of the RTKs. The specific sequences mediating the likely TM-dependent homodimerization of other Ryks have not been determined, but we note that DRL-2 has six such motifs, DNT has three, and human Ryk bears two in their TM domains (data not shown).

We observed that, although the protein bearing the mutation (T245V) that reduced DRL TM activity in the TOXCAT assay displayed reduced SRC64B recruitment, DRL T245V enhanced axon commissure switching *in vivo* to the same extent as the wildtype control. One interpretation of this difference is that, while the coimmunoprecipitation assay allows the observation of increased recruitment of simultaneously overexpressed SRC64B in tissue culture cells, WNT5-dependent dimerization of the overexpressed T245V protein *in vivo* may result in sufficiently high recruitment of SRC64B, which is present at wild-type levels, to elicit full signaling activity.

We have demonstrated that DRL's PDZ-BD is involved in DRL's interaction with SRC64B and contributes to DRL's role during embryonic axon guidance. PDZs and PDZ-DBs are frequently found protein structures which facilitate protein-protein interactions (reviewed in reference 55). The interaction of the PDZ-BD with SRC64B is unlikely to be direct since SRC64B does not contain an obvious PDZ domain. While we have not addressed here the identity of the protein(s) interacting with DRL's PDZ-BD, studies of mammalian Ryk have shed some light on its PDZ-BD interactions. Ryk's PDZ-BD has been shown to interact with Dishevelled (Dvl) (56), a component of all Wnt signaling pathways uncovered to date (reviewed in reference 57). More-recent studies have also identified the PCP pathway member Vang as a PDZ-BD interactor (58, 59). Thus, Dishevelled, Vang, or other proteins may contribute to the stability of the DRL/SRC64B complex.

Both mammalian two-hybrid and coimmunoprecipitation experiments confirmed our earlier report (42) that SRC64B's kinase activity is required for its interaction with DRL. Here, we investigated whether DRL phosphorylated by active SRC64B could bind to the kinase-dead SRC64B. We found that, even in the presence of active SRC64B, the kinase-dead species interacts very weakly with DRL and does not effectively compete active SRC64B out of its complex with DRL. Although we cannot rule out the possibility that, once active SRC64B phosphorylates DRL, it binds sufficiently tightly that exchange with the kinase-dead species is infrequent, it seems probable that individual SRC64B molecules must possess kinase activity to interact with DRL. Alternatively, the K312R mutation somehow precludes SRC64B from attaining a particular conformation required for its interaction with DRL. We conclude based on these data that SRC64B must be able to autophosphorylate or phosphorylate DRL to effect its binding to DRL.

We observed that mutations in DRL's tyrosine kinase-homologous domain interfered with its ability to interact with SRC64B despite DRL's inability to bind to ATP and catalyze phosphotransfer. Although formal proof will require determination of the structure of DRL's intracellular domain in a complex with SRC64B, we speculate that these data indicate that DRL's intracellular domain must adopt a particular conformation in order to interact with and regulate the localization or activity of SRC64B. Such allosteric interactions between pseudokinases and their signaling partners have been the subject of recent interest (60–62). One such example is the STRAD pseudokinase, which, in combination with scaffolding protein MO25, regulates the LKB1 tumor suppressor protein kinase (63, 64). Activation of LKB1 requires that STRAD adopt a "closed" conformation, one associated with active protein kinases. It has thus become clear that at least some pseudokinases do not act merely as passive scaffolds but must assume specific conformations in order to bind or activate downstream pathway members.

Our *in vivo* data indicate that the conserved extracellular juxtamembrane TBC site, in addition to the Wnt-binding WIF domain, cytoplasmic domain, and PDZ-BD, is required for DRL's full activity in a dominant gain-of-function axon commissure switching assay. TBC sites are short peptide sequences recognized and cleaved by subtilisin-like proteases (65). DRL's TBC site is required for rescue of the *drl* mutant MB phenotype (J.-M. Dura, personal communication). DRL's non-cell-autonomous role in the MBs indicates that its extracellular Wnt-binding domain is shed from MB-extrinsic neurons to play a role in MB axon guidance (28). The role of the TBC motif in signal transduction during embryonic axon guidance, where DRL transduces a signal via its cytoplasmic domain, is presently less obvious. Possibly, it is involved in the proteolytic processing of DRL prior to intramembrane cleavage to release its intracellular domain for transit to the nucleus as has been reported for mammalian Ryk (33). WNT5 signaling through DRL, therefore, likely involves a complex set of events, including dimerization, proteolytic cleavage

at the TBC site, and the interaction of DRL's cytoplasmic domain, via both its tyrosine kinase-homologous domain and PDZ-BD, with SRC64B and other proteins yet to be identified.

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