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Drosophila Ryks and their roles in axon and muscle guidance

Liza Lahaye

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Drosophila Ryks and their roles in axon and muscle guidance

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1

Introduction

In order for an organism to develop, grow, mature and function properly the cells within that organism need to communicate with each other and their environment. *wnt* genes encode highly conserved secreted proteins that are essential for cell-to-cell communication during the development of a variety of organisms. They play diverse roles in nervous system development, adult homeostasis, regeneration and stem cell specification. When misregulated they can cause various diseases such as cancer, tetra-amelia, bone density defects and vascular defects. Unraveling the signaling cascades mediated by these extracellular signals provides a greater comprehension of how organisms develop and function, and moreover offers opportunities to manipulate these signals for therapeutic benefit.

1.1. WNT SIGNALING IN THE CNS

The first member of the Wnt family (WNT1) was discovered in a retroviral insertion mutagenesis screen by Nusse and Varmus in 1982 and cloned as a candidate proto-oncogene (1,2). Over the years many more *wnt* genes were identified in a number of species: there are 19 mammalian Wnt family homologues (3,4). This family of secreted lipid-modified glycoproteins (reviewed by (5,6)) is highly conserved amongst vertebrates; all Wnts contain a signal sequence for secretion, a number of highly charged amino-acid residues and many potential glycosylation sites. They also have 22 cysteine residues that are invariably spaced within Wnt proteins, suggesting that protein folding requires the formation of multiple intramolecular disulfide bonds (7). Their hydrophobic character came to light when trying to purify active Wnt protein. Analyses of the Wnt proteins by use of mass spectroscopy unveiled that the first absolutely conserved cysteine residue within the Wnt protein family is modified by the attachment of a palmitate moiety (8). Accordingly, most Wnts were found to be associated to cell membranes and the extracellular matrix (ECM) (9). Up till now there have been two papers on the crystal structure of the interaction between Wnt and a receptor (10,11) that will certainly aid in elucidating binding specificities and provide insights for designing specific inhibitors to fight cancer.

Despite these similarities Wnts are very divergent in the functions they can perform, due to specific sequence variations between family members, but also due to their highly divergent temporal and spatial expression patterns. Remarkably we find a diverse Wnt protein collection already present in Cnidarians, an ancient metazoan phylum containing over 10,000 species amongst which are fresh water polyps and sea anemones (12). Besides the fact that this indicates the early time point for *wnt* gene diversity in evolution, more importantly it denotes that it has remained essential for the proper development of multicellular organisms (13). The intricacy and complexity of Wnt-signaling is significantly enhanced by the plethora of potential Wnt-receptors, enabling the Wnt-family to activate a number of different signaling

pathways. Emerging data suggests there is considerable crosstalk between the different Wnt pathways, implying that these pathways are not autonomous and therefore not to be thought of as separate, linear pathways. In this chapter I will very briefly discuss the different Wnt-pathways known to date and introduce our model system; *Drosophila melanogaster*. Both the nervous system and musculature of *Drosophila*/fruit fly were employed to study Wnt-signaling through RYK which is the subject of the work presented in the remainder of this thesis.

1.1.1 The Canonical WNT Pathway

The canonical Wnt pathway is the most intensely studied Wnt pathway (reviewed by (14). A number of the components downstream of the canonical Wnt ligand have been identified and their roles in development and their involvement in a variety of cancers elucidated. In the absence of Wnt-signaling the complex of Axin, Adenomatous polyposis coli (APC),

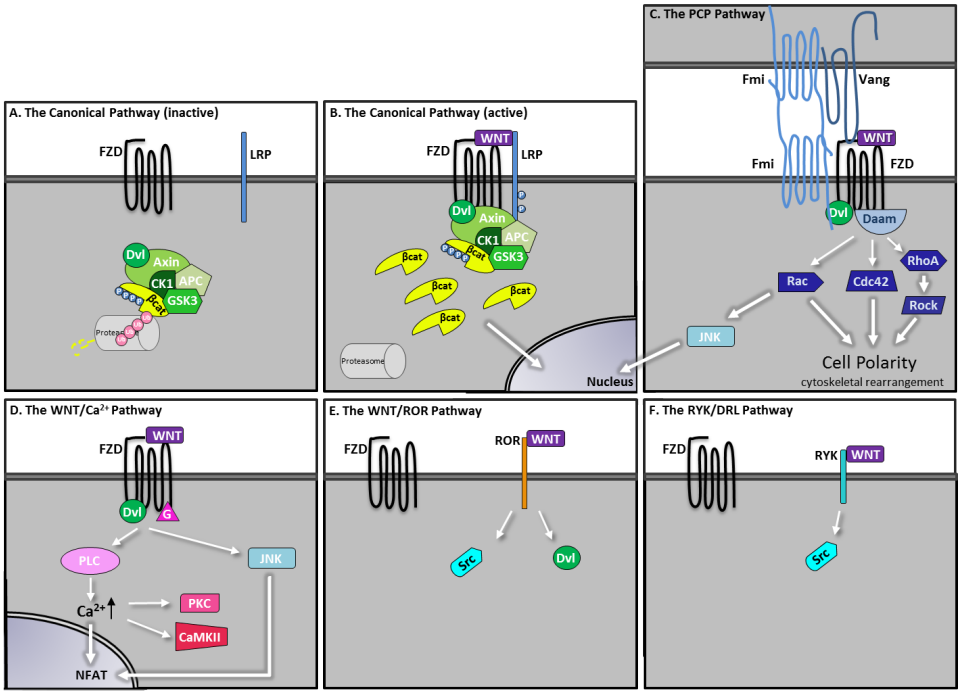


Fig 1. Schematic representation of the known Wnt-pathways. The inactive (A) canonical pathway is portrayed and ends with the degradation of β-catenin (βcat). When Wnt binds the complex of Frizzled (FZD) and Low Density Lipoprotein (LDL) receptor related protein (LRP), β-catenin accumulates and translocates to the nucleus where it regulates targeted gene expression. In the Planar Cell Polarity pathway (C) Wnt binds to another complex of FZD with Flamingo (Fmi) and Van Gogh (Vang) to ultimately guide cell polarity and cytoskeletal rearrangement. The Calcium pathway (D) activates transcription factors through another cascade of proteins. In (E) and (F) two other receptors are shown that are receptors for Wnt and provide a downstream signal upon ligand-binding. All of the above pathways will be addressed in more detail below.

Casein Kinase 1α (CK1α) and glycogen synthase kinase 3 (GSK3) is responsible for the degradation of cytosolic β-catenin, keeping cytoplasmic levels low (15–17). In the presence of the canonical Wnts (Wnt1, Wnt3, Wnt3a, Wnt8 or Wnt8a) β-catenin accumulates in the cytosol and subsequently translocates to the nucleus, where it affects transcription. Many studies have focused on the interactions of Wnt ligands with their canonical receptors, the Frizzled proteins (18,19). Once Wnts are secreted they can bind to the extracellular Cysteine Rich Domain (CRD) of the Frizzled (FZD) protein family (20,21) that is in a complex with the low density lipoprotein (LDL) receptor related protein (LRP5/6) (22–25). FZDs are sevenpass-transmembrane receptors of which 10 family members are found in mice and humans and 4 family members are present in *C. Elegans* and *Drosophila*. When Wnt binds the receptor complex of FZD and LRP this induces phosphorylation of LRP that allows for the subsequent recruitment of Axin (26–28). Another protein called Dishevelled (Dsh) is also phosphorylated in a FZD dependent fashion (29,30) and inhibits the complex formed by Axin, APC and GSK3. This inhibition leads to the prevention of GSK3-dependent phosphorylation of β-catenin (31), resulting in the accumulation of β-catenin in the cytosol and translocation into the nucleus. Nuclear β-catenin regulates target gene expression by displacing Groucho and binding to a family of transcription factors named T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) (32–35) and induces the transcription of Wnt-target genes. A comprehensive overview of Wnt target genes known up to date have been listed by Roel Nusse on his website; http://www.stanford.edu/group/nusselab/cgi-bin/Wnt/target_genes.

1.1.2. The WNT/Planar Cell Polarity Pathway

The polarity of cells is essential for developing embryos, allowing cells to align correctly with respect to each other and orient specifically with respect to tissue axes within the plane of a sheet. The Wnt/Planar Cell Polarity (PCP) pathway regulates different cellular behaviors such as cell motility and polarity that are mediated by cytoskeletal remodeling (reviewed in (36,37). A number of the components of the PCP pathway were first identified in *Drosophila* using genetic screens, molecular cloning and functional analyses of the patterning of the adult wing, abdomen, notum and eye (38–41). FZD was found to be an essential component of PCP signaling (42–44). The extracellular domain (ECD) of FZD is able to interact with the transmembrane protein Van Gogh (Vang) that resides at the plasma membrane (PM) of a neighboring cell (45). This intercellular contact between FZD and Vang is facilitated by homophilic interactions of two Flamingo (Fmi) proteins that bridge the two neighboring cells and interact with both FZD and Vang on either side (46–49). However, the ligand interacting with FZD to activate the *Drosophila* Wnt/PCP pathway remains to be identified. It has been shown, that Wingless (Wg) and DWNT4 can modulate the interactions between FZD and Vang (50). Furthermore, there is evidence that the vertebrate PCP signaling is established with the help of Wnt5a and Wnt11 (51–55). Nonetheless, it is thought that downstream of FZD

through Dsh, two parallel pathways lead to the activation of the small GTPases Rho and Rac (41,56–59). The small GTPase Rac can activate the c-Jun N-terminal kinase (JNK) (60,61), the other small GTPase Rho recruits Dishevelled associated activator of morphogenesis (DAAM1) (62), leading to the activation of Rho associated kinase (ROK) (63–66). Both parallel pathways mediate the reorganization of the actin cytoskeleton that is necessary to assume the correct orientation and position of the individual cells within a tissue.

1.1.3. The WNT/Ca^{2+} Pathway

Activation of the Wnt/Ca^{2+} pathway via the FZD receptor leads to an increase in the concentration of Ca^{2+} that subsequently leads to a modulation of the expression of target genes, resulting in cellular responses, such as changes in cell movement and cell adhesion. There are reports that show an increase in the release of intracellular calcium ions for WNT4, WNT5A and WNT11 (67–71). It is thought that after FZD activation, signaling is transduced via heterotrimeric G proteins that can activate phosphodiesterase 6 (PDE6) and Phospholipase C (PLC), of which PLC gives rise to Inositol triphosphate (IP_3) and Diacylglycerol (DAG). IP_3 induces Ca^{2+} release from the ER which together with the abundantly present calmodulin activates CamKII (72). Whereas DAG activates protein kinase C (PKC) (73), both CamKII (67–71) and PKC can induce the nuclear transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB). PKC is also able to induce another transcription factor; cAMP response element-binding protein (CREB), and the released Ca^{2+} can activate the widely expressed protein phosphatase calcineurin (Cn) that induces Nuclear Factor of Activated T-cells (NFAT) (74)], a third transcription factor. These transcription factors can modulate the expression of several target genes thereby altering cellular behavior.

1.1.4. The WNT/ROR pathway

In contrast to the above described Wnt pathways the WNT/ROR pathway does not act via the FZD receptor, but signals through the receptor tyrosine kinase-like orphan receptor (ROR) (75–78). Interestingly, a number of examples have been described in which this pathway feeds back into the canonical Wnt pathway and the PCP pathway (79–82). There is also a study that shows signaling of WNT5a through ROR that is independent of FZD as a co-receptor (83).

ROR is a member of the receptor tyrosine kinase family of proteins. A number of organisms express two structurally related members, ROR1 and ROR2. *Drosophila* has two Ror orthologues named ROR and NRK. They are conserved amongst species and their extracellular domains (ECDs) contain an immunoglobin (Ig) domain, a Frizzled-like cysteine-rich domain (CRD) and a Kringle (Kr) domain (84). The CRD is crucial for the interaction with Wnt ligands (75–77,85–88). The functions for the Kringle domain have not yet been resolved,

but are presumed to bind other Wnt regulatory proteins (89,90). The Ig-like domains, found in the N-terminal extracellular regions, are present in most species, except for *Drosophila* ROR and NRK. The Ig-like domains can be found in many more proteins and seem to be involved in protein-protein and protein-ligand interactions and are implicated in cell adhesion.

The intracellular domain possesses a tyrosine kinase domain; a proline-rich domain (PRD) that is surrounded by two Ser/Thr-domains; Ser/Thr1 and Ser/Thr2 (84). The combination of a PRD and two Ser/Thr-domains have not been found in any other protein, but are thought to interact with signaling mediators. The PRD domain of ROR2 was found to associate with the actin binding protein filamin A (FLNa) and this interaction is essential in filopodia formation and Wnt5a-induced cell migration (91). The PRD domain was also found to be responsible for the recruitment of SRC leading to the phosphorylation of mROR2 causing activation of the receptor (92). Furthermore, the ROR cytoplasmic domain contains potential phosphorylation sites and several consensus motifs for protein-protein interactions, such as the WW domain, the SH3 motif, and the SH2 motif. These domains are likely required for the association of Ror-family members with adaptors and signaling molecules in the signaling transduction cascade.

In vertebrates, the data so far suggests that Ror signaling is mediated via the non-canonical Wnt, WNT5a. WNT5a/ROR2 signaling has, amongst others, been shown to play a crucial role in inhibiting WNT3a mediated canonical Wnt signaling at the level of TCF/LEF-mediated transcription (76,93,94). The downstream signaling partners in the Ror pathway that have been elucidated so far are Dsh, that is phosphorylated in a WNT5a/ROR dependent fashion (83) and SRC that has been shown to phosphorylate mROR2 (92)(reviewed in Petrova et al, 2014 (78)).

The expression of Rors during development has been addressed in several papers (see review (78,95). *ror2* mutant mice die neonatally likely due to respiratory dysfunction and cyanosis, just as observed in *wnt5a* mutants and *ror1* mutants. The phenotypes of *ror2* and *wnt5* mutant mice are similar and exhibit dwarfism, facial anomalies, short limbs and tails (76,96–98). Although *ror1* mutant mice die like their *wnt5a* and *ror2* mutant counterparts they do not display any of the apparent morphological phenotypes and their size is comparable to wild-type mice (99).

In humans, mutations within *ror2* gene cause autosomal dominant brachydactyly type B (BDB) (100,101), and an autosomal recessive form of Robinow syndrome (RRS), (102,103) reviewed in (104,105). Increased expression of ROR2 has been observed in squamous cell carcinoma of the oral cavity in which expression levels correlate to the degree of the

malignancy (106). Activation of non-canonical WNT5a/ROR2 signaling causes osteosarcoma cell lines to demonstrate invasive properties in vitro (107). ROR1 overexpression is associated with hematopoietic malignancies of B-cell lineage in humans (108–112). More research is needed to elucidate the signaling cascade of Rors.

1.1.5 The WNT/RYK/DRL Pathway

One of the more recently identified Wnt driven pathways is employing RYK as a receptor (reviewed in (113)). The first report published that RYK is a receptor for WNT5 was in 2003

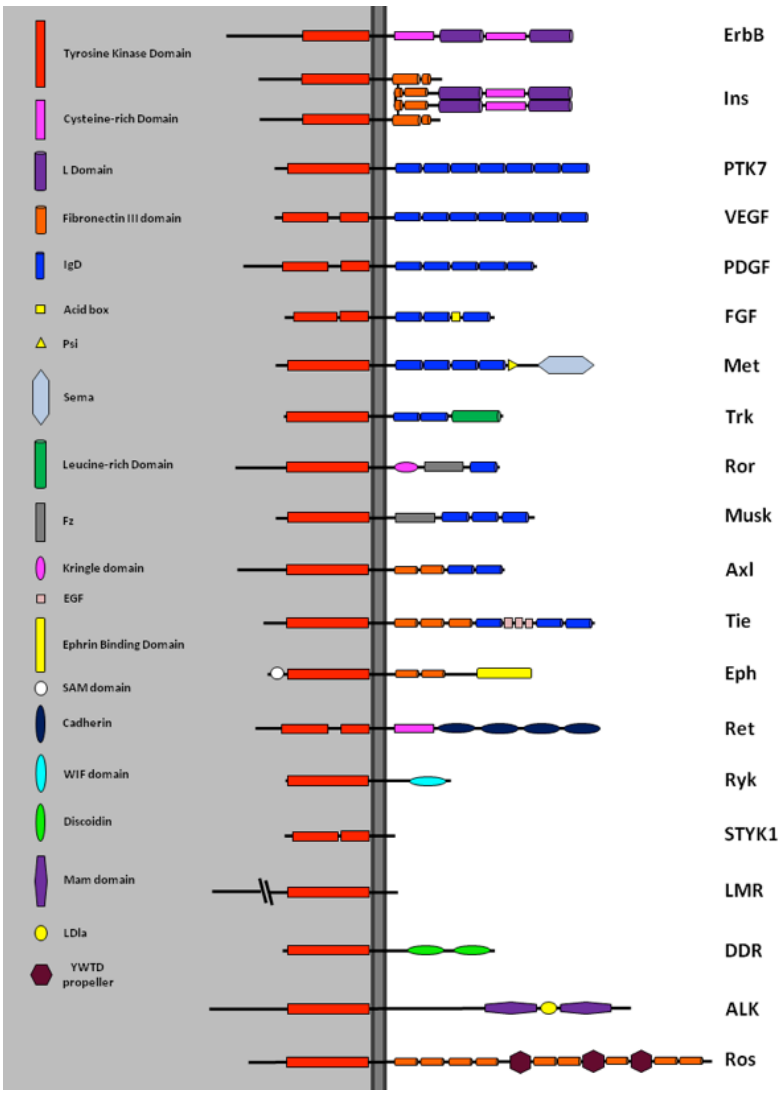


Fig 2 A schematic representation of the 20 subfamilies that make up the receptor tyrosine kinase family. The red rectangles illustrate the intracellular tyrosine kinase domain(s).

in *Drosophila* (114) that showed that WNT5 repels RYK expressing axons, playing a role in axon guidance in the ventral nerve cord during embryogenesis. In mice it was also shown that RYK expressing axons could be repelled by WNT5a as well as WNT1 (115,116). In zebrafish the interaction between WNT5b and RYK was found to regulate the directional cell movement during gastrulation (117) which was also affected in the African clawed frog (118) yet here RYK employed besides WNT11 also co-receptor Frizzled 7. RYKs are members of the receptor tyrosine kinase (RTK) family. This family is composed of 58 receptors that span the membrane once, have an extracellular ligand binding domain that can bind growth factors, cytokines and hormones with high affinity and transduce signals via their cytoplasmic tyrosine kinase domain (reviewed in (119,120)). These signals regulate a range of important cellular responses but can also play a critical role in the development and progression of cancer.

The RTK family can be subdivided in different classes according to function, of which class XVI describes receptor related to tyrosine kinase (RYK) that was uncovered by a PCR-based screen for novel RTKs in 1992 (121,122). The conservation of RYK between species is relatively high: comparison between human and mouse RYK indicates that the homology at the amino acid level is 97%. Single RYK orthologues have also been identified in other organisms, like *Danio rerio*, *Xenopus laevis*, *Caenorhabditis elegans*, however *Drosophila melanogaster* has three orthologues; Derailed (DRL), Derailed-2 (DRL-2) and Doughnut on 2 (DNT) (exhibiting 60% amino acid (aa) identity to DRL) (113,123). Downstream of DRL it was shown that DRL can interact with kinase SRC in the *Drosophila* nervous system (124). Before the WNT5/RYK/DRL pathway is examined in more detail, the model system *Drosophila melanogaster* is introduced in the next paragraphs.

1.2. *DROSOPHILA MELANOGASTER* AS A MODEL ORGANISM

For over a century *Drosophila* has been used in scientific research. Initially, it was the ease of forward genetics that made the fruit fly an attractive model and enabled the discovery of genes that are involved in body patterning, organogenesis, nervous system development, muscle development, but also genes required for learning/memory, pain, audition, olfactory, vision and courtship (125). The *Drosophila* generation time is about ten days when kept at 25°C: the *Drosophila* life-cycle starts with the embryonic stage followed by three larval instar stages (molds) and a pupa stage giving ultimately rise to an adult fly. Genetic manipulation and the generation of transgenic flies became technically possible with the development of the P-element mediated germ line transformation technique established by Spradling and Rubin in 1982 (126). A further important advance for the study of gene function was made by Andrea Brand and Norbert Perrimon in 1993 (127) who developed a method which

makes use of a bipartite yeast Gal4/UAS system to allow stable differential spatial-temporal transgenic expression of any gene of interest in the fly genome. The Gal4 gene encodes the yeast transcription protein that specifically binds the UAS (upstream activating sequence), a short section of the promoter region. Furthermore there are many tissue specific Gal4 drivers publicly available, that allows expression of your gene of interest in a temporal/spatial pattern in a specific genetic background. The *Drosophila* genome was fully sequenced and published in 2000 which greatly added the implementation of novel genome wide reverse genetic approaches to uncover gene function. In 2001 Rong and Golic (128) introduced homologous recombination in the fruit fly, and more recently, a publicly available genome-comprehensive transgenic RNAi library has been developed for knock down expression in specific tissues. This is especially useful for mutations that cause lethality, since dsRNA against your gene of interest can be expressed with the Gal4/UAS system in a restricted pattern. The genetic knock down in a desired spatial/temporal pattern allows the study of its consequences where that is impossible in a complete knock out that could be potentially lethal. As of 2012 the CRISPR/Cas system (clustered regularly interspaced short palindromic repeat/CRISPR-associated) is the newest genetic editing tool. In origin it is a bacterial defense system that protects bacteria against invading viruses and plasmids. It has been adapted to create targeted double strand break(s) (DSB) in the genome. A single DSB will lead to small deletions and insertions by non-homologous end joining at the target site, whereas a double cut can lead to bigger deletions of the target area. In addition, homologous recombination with a desired template allows even more possibilities to edit the genome. Interestingly, of all known genes that cause human disease about 75% of them have a conserved orthologue in the genome of *Drosophila* (129).

1.2.1 The *Drosophila* Central Nervous System

The brain and spinal cord, which make up the Central Nervous System (CNS), receive and interpret signals from the peripheral nervous system. Neurons in the brain extend their axons to their targets throughout development. During this growth they interact with specific guidance cues through cell adhesion molecules (CAMs) to direct them to their target region and to establish appropriate connections called synapses (130). In order to facilitate correct navigation and migration the milieu for growing axons is requiring temporal and spatial control of many and often dynamic cues. Some of these axons can traverse large distances (many centimeters or even meters), creating an elaborate and precise network throughout the nervous system. The known proteins that are involved in axon guidance during development are in many cases conserved amongst a large variety of vertebrate and invertebrate species (reviewed in (131,132)).

The *Drosophila* brain develops from the neuroectoderm that gives rise to either neuroblasts or progenitor cells of the epidermis (133). Approximately 500 neuroblasts progress deeper into the embryo to produce a central neural primordium. The progenitors of the epidermis give rise to the epidermal sheet as well as to the peripheral nervous system (PNS). An overview of the embryonal nervous system situated within the embryo around stage 16 can be seen in Fig 3A. The BP102 antibody highlights all CNS axons present within the embryo. The top of the is the anterior part of the embryo containing the developing brain hemispheres and the ventral nerve cord (VNC) can be seen running down from the brain hemispheres to the posterior

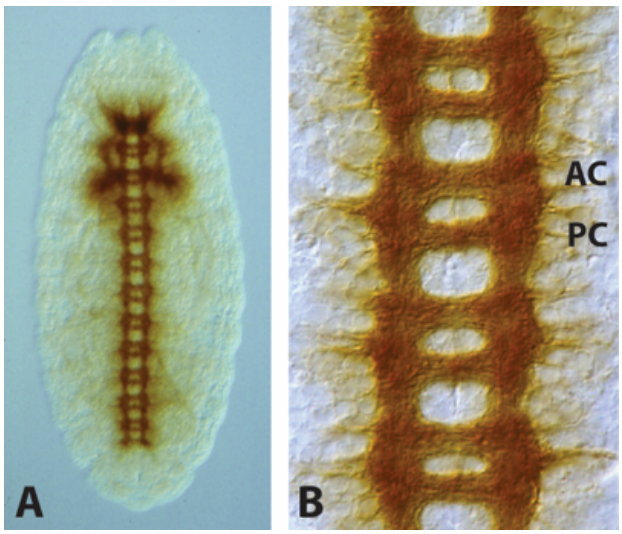


Fig 3 The nervous system of a *Drosophila* embryo at stage 16 (3A) with two bilateral brain lobes and the ventral nerve cord in situ, with two longitudinal tracts that run along either side of the midline. The zoom in (B) on four embryonal segments provides more detail for the anterior commissures (AC) and the posterior commissures that create an environment in each segment where axons traverse the midline. Anterior is up.

part of the embryo. The neurons send their axons down the VNC in the embryo along two longitudinal tracts that run alongside the midline. In each embryonal segment axons can cross the midline through either the anterior commissure (AC) or the posterior commissure (PC) (Fig 3B) and continue to their respective target site and establish a synaptic contact.

During embryogenesis the neuroblasts form three neuromeres/neural segments: the protocerebrum, deutocerebrum and the tritocerebrum which make up the brain hemisphere at the larval stage. This brain hemisphere of the larvae becomes the supraesophageal ganglion of the adult (133), whereas the neuromeres of the mandible, maxilla and labium (three gnathal segments) give rise to the subesophageal ganglion. The eye-antennal disc

does not develop till the third instar larvae stadium, generating the antennal and optic lobes. The major brain centers that ultimately make up the adult *Drosophila* brain are depicted in the figure 4. Two brain structures, the antennal lobes and the mushroom bodies have been the subject of intense study and have been shown to be required for proper behavior and

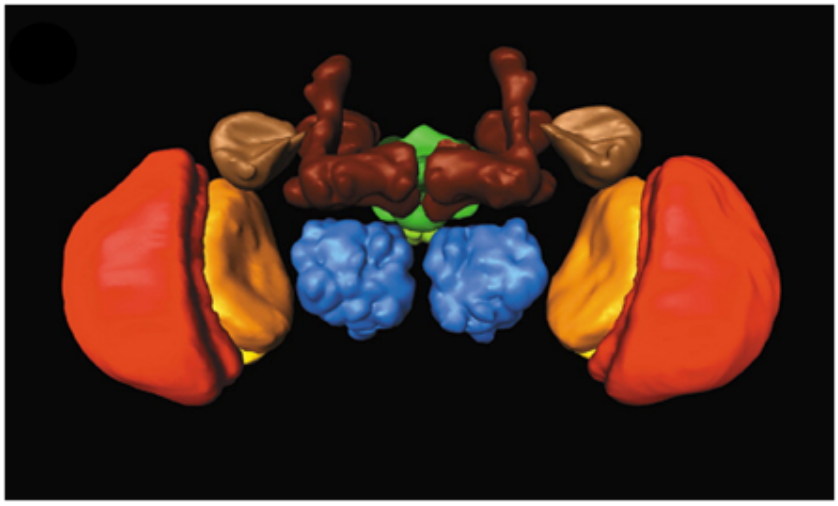


Fig 4 A polygonal brain model of an adult brain generated from an individual labeled data set. Colour code: Red: medulla; orange: lobula; yellow lobula plate; chocolate brown: mushroom body; beige; lateral horn; blue: antennal lobe; olive green: noduli; dark green: ellipsoid body; light green: fan-shaped body; grayish green: protocerebral ridge (not visible, behind fan-shaped body). Taken from: Rein, K; Zöckler, M et al (2002) *Current biology: **CB*** vol. 12 (3) p. 227-31 with permission from last author.

learning and memory of the adult fly (reviewed in (134,135)). These structures are discussed in greater detail below.

1.2.2 The antennal lobes and mushroom bodies of *Drosophila*

In 1850 the French biologist Félix Dujardin first discovered the Mushroom Body (MB) and it is thought to be common to all arthropods (136). The Mushroom Bodies (MB) are some of the most clearly distinguishable neuropile structures in the insect brain consisting of a dense network of glia, dendrites and axons. It receives information amongst others from the olfactory system. Odor is first detected by olfactory receptor neurons (ORNs) in the antennae and the maxillary palp (137,138) and conducted to the antennal lobe (Fig. 5). The antennal lobes - are the primary olfactory centers of the brain (139) consisting of about 50 glomeruli of which each glomerulus receives input from specific ORNs. Subsequently the antennal lobe launches projection neurons through three antennal cerebral tracts that in turn project to the lateral horn in the *Drosophila* brain however the inner antennal cerebral tracts sends collaterals to the MB

There are three compartments that make up the MB; the calyx, the pedunculus and the lobes (140–143) and can be seen as two bilateral symmetrical clusters (Fig. 5). The cell bodies of the MB intrinsic neurons (also known as Kenyon cells reside in the dorsal posterior brain and form a cluster, sending their dendritic branches in the anterior direction (the calyx), which converge to form the pedunculus. From this pedunculus the axons bifurcate at its anterior end producing vertical lobes (α' and α) and medial lobes (β', β and γ).

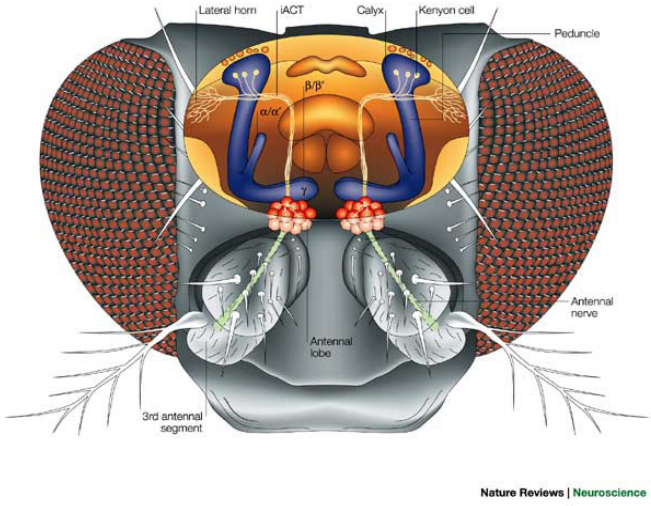


Fig 5 The position of the Antennal lobes in red, the Mushroom Body (in blue) and the central complex is outlined in the middle and back of the *Drosophila* head. Taken from: Heisenberg, Martin (2003) *Nature reviews. Neuroscience* vol. 4 (4) p. 266-75 with permission from author.

The MB is regarded as an integrative center for a multitude of sensory information and is shown to be specifically important for olfactory learning and memory (144–146). A detailed description of other tasks the MB could be involved in, like sleep, place, memory and others can be found in Tanaka et al; 2008 (147). The MB have also been shown to be dispensable for certain experience-independent olfactory related behaviors (148–151). After critical evaluation of the literature, Ito et al 1998 (152) and Tanaka et al 2008 (147) postulate that the mushroom body itself might not be the center for learning and memory, it can equally be considered as a preprocessor of sensory signals on their way to "higher" protocerebral regions. Still little is known about the exact function and identity of neural circuits that are responsible for specific behaviors.

Furthermore, still much needs to be learned about how other sensory information is relayed to the MB and to which brain regions output neurons project to. Assigning functions to defined brain regions is the place to start since it has proven to be exceedingly difficult to integrate the

individual contributions of brain regions into a behavioral model of the brain. The insect brain is relatively small (about 200.000 neurons) and there are some straight-forward behavioral paradigms available that allow the dissection of circuits underlying complex behavior. It is of interest to extrapolate the information gained from the *Drosophila* MB to circuit function in a vertebrate brain. So far, it is found that the genes that are expressed early in the development of the MB share considerable homology with genes involved in the early development of specific regions in the mammalian prosencephalon (142).

1.2.3 The embryonic musculature of *Drosophila*

The *Drosophila* musculature has been used as a model to identify genes that are needed for the different stages of muscle formation, such as mesoderm specification, myogenesis, myoblast fusion, muscle fiber specification and guidance of muscle fibers to their attachment sites. Many genes that are required for muscle formation during embryogenesis are highly conserved in sequence and function from insects to vertebrates. The process of myogenesis in *Drosophila* takes about 5.5 hours to complete. Muscles stem from a germ layer named the mesoderm, which is first specified at the blastoderm stage as the most ventral cells. These cells are formed under the control of the gene dorsal that is maternally contributed and establishes the dorsoventral axis in the embryo (153). During gastrulation, invagination



Fig 6 Displays the muscles of a living *Drosophila* embryo, which nicely presents the reiterated pattern of all segments. Downloaded from: http://www.biochem.mpg.de/schnorrer/research/index.html, with permission of Frank Schnorrer.

of these specified cells takes place along this dorsoventral axis and they continue to migrate dorsally, eventually lining the ectoderm of the embryo (154). Meanwhile, transcription factors twist and snail are upregulated under control of dorsal in all mesodermal cells. Since the mesoderm gives rise to several different tissues, i.e. the visceral mesoderm, somatic mesoderm, heart and fat body (155), the uniform mesoderm is regionally and temporally specified during embryonic development. An important developmental regulator of dorsal versus ventral structures in the embryo is the morphogen Decapentaplegic (Dpp), while the Twist protein is an important determinator of muscle fate. Twist expression is first detected uniformly at stage 5, low twist expression is maintained in cells that express the segmentation gene evenskipped (156). High twist expression is maintained, till stage 11, by cells that express another transcriptionfactor named; sloppy paired (slp), which is under control of Wg (157). These cells eventually produce the somatic mesoderm (158), among them are the body wall muscle fibers.

The embryonic somatic musculature forms an intricate network of about 600 individual muscle fibers that are arranged beneath the epidermis in a stereotypical segmentally reiterated pattern (159), that is visualized in figure 6. This network is sustained by the fact that all muscles attach at both ends to a tendon-like cell that is part of the exoskeleton, thereby providing the integrity needed to withstand the contractile forces exerted by the muscles. Each hemisegment consists of 30 muscles and each muscle is initiated by a single muscle founder cell. All 30 fibers are defined by size, position, orientation, attachment sites and innervation (reviewed in (160)). The muscle founder cell undergoes a series of processes leading to the fusion of the muscle founder cell with a number of surrounding fusion competent myoblasts (FCMs). This fusion process creates multinucleated myofibers whose two leading edges subsequently migrate towards clusters of tendon cell progenitors in the epidermis (161–163). The initial determination of the tendon cell progenitors in *Drosophila* is provided by segment polarity genes such as *wingless* and *hedgehog* (156). They are responsible for activating the early growth response (Egr)-like transcription factor Stripe in segmentally-reiterated clusters of epidermal cells (164). Stripe expression is both necessary and sufficient to promote muscle migration towards these clusters of tendon cell progenitors (165–167). The approaching myofiber itself, however, induces final differentiation of the single selected tendon cell by secreting the protein vein (reviewed in Schweitzer, et al 2010 (162)). The neuregulin-like ligand Vein accumulates at the muscle tendon junction to activate the Epidermal Growth Factor (EGF) pathway solely in the tendon cell progenitor that is contacted by the muscle fiber (168) (Fig. 7).

This pathway upregulates Stripe expression, which further activates other proteins, like guidance cues and terminal differentiation factors for the tendon cell. The tendon precursor

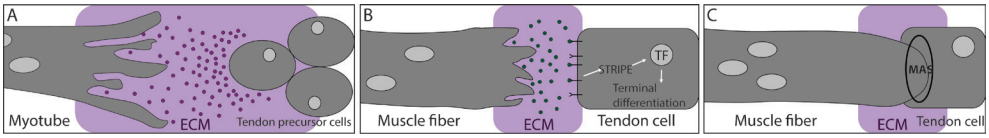


Fig 7 Schematical representation of myotube guidance. In (A) the approaching myotube is attracted by unknown factors to the tendon precursor cells. In close proximity of the tendon cells (B) the muscle fiber starts expressing Vein that induces tendon cell specification and ends in the establishment of a myotendinous junctions.

cells that are not contacted by a muscle fiber cease to express Stripe and do not differentiate into a tendon cell. The final stage of tendon cell determination is defined by the association of muscle specific αPS2βPS integrin with tendon-secreted ECM-component Thrombospondin (Tsp). The tendon specific αPS1βPS integrin binds to the extracellular Laminin (Lam). Both muscle and tendon cell secrete several factors to the ECM, like Tiggrin (169) and Slowdown (Slow; a regulator of Tsp) respectively. Many proteins mediate the formation of a myotendinous junction. Both muscle and tendon specific Integrins are associated with the actin cytoskeleton in each cell, enabling the myotendinous junctions (MTJs) to withstand the mechanical forces that occur during larval locomotion.

The basic morphology of the body wall musculature of the embryo is carried over into the three larval molds however muscle size increases about a hundred fold (170). After the third larval stage, *Drosophila* undergoes pupal metamorphosis during which the larval somatic musculature degenerates and the presumptive adult musculature, where multiple fibers are bundled into single muscles, will be formed.

One can consider three distinct phases in *Drosophila* myotube guidance; unidirectional migration, bidirectional migration and attachment, which are also employed in vertebrate myotube progression. There are some significant differences in the mechanisms of muscle formation in vertebrates and invertebrates, especially during the earlier phases. Whereas *Drosophila* muscle founder cells are programmed from the start, the vertebrate myoblasts receive their specification when they contact their respective tendons. This suggests that the more complex vertebrate musculature has acquired more flexibility during myoblast specification to increase the plasticity of fiber formation in a complex muscle field. But the final stages of *Drosophila* muscle guidance and attachment show many similarities to these operating in vertebrates on a cell biological level. The molecules that are involved in establishing the musculature are only just emerging (171); work done in *Drosophila* can provide a steady stepping stone for understanding this process in vertebrates. In addition, there is accumulating evidence that a number of conserved molecules, such as Slit and DRL,

are important players not only in the guidance of muscles to their tendon cells but also in guidance of axons to their target cells.

1.3 BIOLOGICAL ROLES OF WNT/RYK SIGNALING

The generation of the *ryk* knockout mice in 1992 gave important insights into the function of RYK in mammals (172). Without RYK present mice die perinatally, likely as a result of cardiovascular impairments (172,173). During embryogenesis the *ryk*$^{-/-}$ embryos present with craniofacial and skeletal defects, which include cleft palate and significant shortening of the long bones of the fore- and hindlimbs respectively (172). Besides its roles for correct development of a number of tissues during development, RYKs are also of importance in the adult, since misregulation of RYK is associated with human disease in later life (174), predominantly cancer (175–180).

ryk expression is observed in many different mammalian tissues during development as well as in adults (see review Serfas and Tyner, 1998 (181)), but in the fruit fly DRL, DRL-2 and DNT seem to be restricted to distinct subsets of cells in the nervous system and the musculature. *drl* was initially identified in a screen for genes that are required for axon pathfinding in the *Drosophila* embryo (182) and for learning and memory in the adult fly (183,184). Recently, reports have been published on describing roles for DRL as an axonal guidance cue operating in the mushroom bodies (185), the antennal lobes (186) and as a protein important for synapse formation at the neuromuscular junction (NMJ) (187). Outside the CNS DRL has been involved in correct muscle guidance of the lateral transverse muscles (188) and in the formation of the salivary glands (189). A role for RYK in tissues other than the nervous system has also been uncovered in *C. elegans*, in which embryonic cell fate patterning in the vulval cell lineage is regulated by Wnt induction of RYK (190).

In the embryonal ventral nerve cord of *Drosophila* there are two major routes available to cross the ventral midline; the anterior commissure (AC) and the posterior commissure (PC) as aforementioned in section 1.2.1. In the wildtype embryo the axons that express DRL traverse the midline via the AC where the PC neurons that do not express DRL cross the midline through the PC (191,192). However when DRL is ectopically expressed in the PC neurons, the PC neurons switch to project through the AC (114,193). This misrouting can be suppressed by heterozygosity for *wnt5*. This result can be explained to mean that WNT5 which is normally expressed at the PC acts as a repellent for DRL expressing neurons (114,193). And indeed, when WNT5 is misexpressed in the midline glia (Sim-Gal4) the DRL expressing neurons do no longer cross the ventral nerve cord (VNC), hence the AC is not formed and the neurons are stalled at the intersections. The penetrance of this midline overexpression dominant gain of function phenotype is dependent on the level of WNT5 expression. The assay can be used

to screen for genes that are required for WNT5 signaling, i.e. candidate genes are evaluated for their ability to suppress this induced phenotype (114,124,193).

In Chapter 2 we use an *in vivo* **axon guidance assay** developed by the Thomas lab (114,192) that is named; the *drl*-mediated commissure switching assay (Fig 8). This assay is based on the ability of WNT5 to repel DRL$^+$ axons. When *drl* is ectopically expressed in all *eg*$^+$ neurons using the *eg-Gal4* driver and a *UAS-drl* transcript the neurons that normally cross the midline through the PC are forced to cross the midline through the AC (Fig 8, right panel). The penetrance of this phenotype depends on the *drl* expression levels, allowing amongst others the assessment of different *drl* mutants in a biological context. Both the commissure

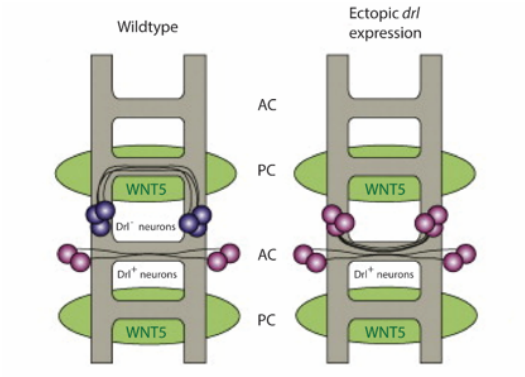


Fig 8 Illustration of the *drl*-mediated commissure switching assay. The crossing of *drl*$^-$ neurons via the PC in the wildtype is depicticted in the left panel. When *drl* is expressed ectopically (right panel) these neurons are repelled by WNT5 and proceed to cross the midline through the AC.

switching after ectopic overexpression of DRL and the midline WNT5 overexpression assay have been successfully used by our lab and others to identify novel members of the WNT5/DRL pathways such as the non-receptor kinase SRC64B (124).

In contrast to the roles of DRL as a signaling receptor in the embryonic VNC, there is also evidence to show that DRL can sequester its ligand; WNT5, acting as a sink to titrate out the amount of DRL that can interact with an alternative, signaling receptor. This was shown in the mushroom body (MB) where the *drl* mutant was also found to display a phenotype: the αα' vertical lobes are reduced and the β lobes can cross the midline in the *drl* mutant, see fig 9 (185,194). However the specific expression of DRL in the MB in an otherwise *drl null* background does not rescue the phenotype implying that DRL is not intrinsically present in the MB (194). Furthermore it was shown that this *drl* mutant phenotype was rescued by expressing DRL without its intracellular domain (ICD). This dual mechanism of DRL action; acting as a sink vs a signaling receptor, is probably determined by co-receptor context.

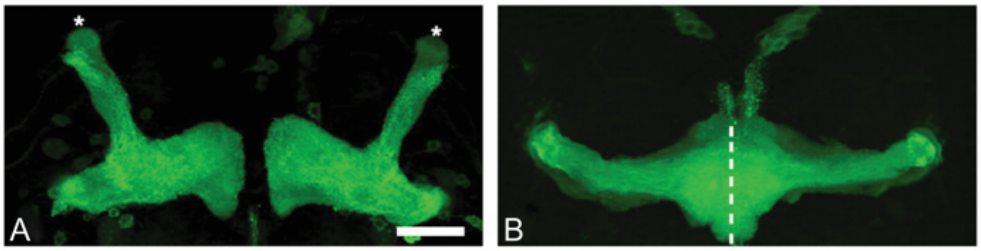


Fig 9 The wildtype mushroom body is displayed in (A) where the asterisks indicate the αα′ vertical lobes that are present in the wild-type situation or reduced in the mutant situation (B). The midline is shown by a dashed line and midline crossing can also observed in the *drl* mutant. Scale bars: 50 μm in A. Adapted from *Grillenzoni N, et al Development 2007* (194) with permission.

DRL can be in a complex where it is able to transduce a signal through Src kinase (see section 1.3.3) or it can be in a different setting where its only role could be to down regulate the amount of WNT5 by its sequestration. The role of DRL during the MB development will be addressed in Chapter 3 (this thesis). Despite the many questions remaining on the biochemical mechanisms by which DRL functions, there are important roles for RYK/DRL during the development and in the adult *Drosophila* nervous system and in its musculature (114,182–188,193,195,196).

The phenotype of the musculature in the *drl* mutant was first described by Callahan et al 1995 (188). Normally the lateral transverse muscles 21-23 (LTMs 21-23) present in the embryonal muscle segment (schematically represented in Fig 10A) can be seen to attach ventrally at a tendon cell at the level of muscle 12 whereas in the *drl* mutant we find that muscle 21 or occasionally 23 overshoots its normal attachment site and establishes an ectopic attachment site beyond muscle 13 (Fig10B). This phenotype is not fully penetrant and therefore we used this **muscle guidance assay** in Chapter 4 to assess genetic interactions that are either

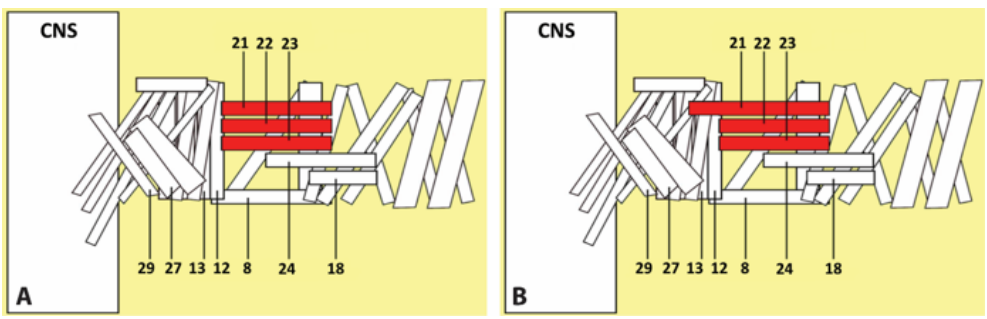


Fig 10 This representation of a single muscle segment demonstrates most of the 30 muscles that make up a muscle segment. The LTMs 21-23 are portrayed in red in the wildtype (A) and in the *drl* mutant (B). The bypassing muscle phenotype in the *drl* mutant is illustrated by LTM 21 that bypasses its normal muscle attachment site and attaches further ventral beyond muscle 13.

capable of phenotypic rescue or increasing the penetrance of the phenotype.

The Wnt-receptor RYK was also found to be expressed in many tumor cell types in cancer; neuroblastomas, primitive neuroectodermal tumors (PNETs), Wilm's tumors and melanoma (197). H-RYK is also overexpressed in malignant ovarian tumors (177), and overexpression of H-RYK causes transformation *in vitro* and tumor formation *in vivo* (178). And there are implications for RYK, through the interaction with for instance EphB2 and EphB3, to possibly mediate tumour migration, invasion and metastasis (123). Recently, an article was published indicating a role for WNT5a-RYK signaling in hematopoietic stem cell proliferation and repopulation (198).

1.3.1 RYK/DRL Protein Structure

The protein encoded by the *ryk/drl* gene is an atypical member of the RTK family because of the lack of conservation of certain amino acid residues (see section 1.3.2). The ECD of RYK/DRL contains about 250 amino acids, which is considerable less than the average of 400 amino acids present in other ECD of RTKs. These 250 amino acids have five potential

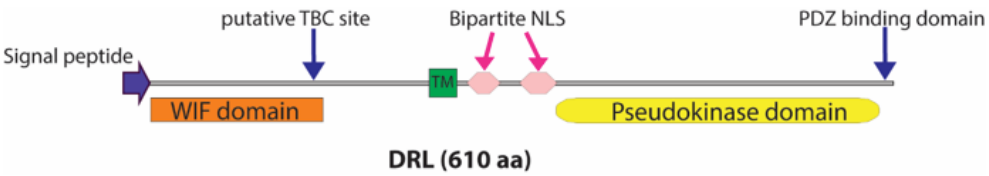


Fig 11 Illustration of the domains present within DRL that starts at the N-terminus with the signal peptide. The ECD contains the WIF domain that also holds the putative TBC site. In green the transmembrane region (TM) is portrayed, followed by the bipartite nuclear localization signal (NLS) that resides in the ICD of DRL. The ICD also includes the pseudokinase domain as well as the PDZ binding domain.

N-glycosylation sites, a putative tetra-basic protease cleavage (TBC) site and importantly there are two leucine-rich motifs near the N-terminus that bear homology to the secreted Wnt inhibitory factor (WIF), illustrated in Fig 11. It has been established that RYK can bind Wnts

(WNT1, 3, 3a and 5a) via its WIF domain (114,173,199–201). The TBC site (KRRK) can be recognized by the furin family of proteases and therefore allows posttranslational modification. After cleavage an α- and β-subunit of the RYK receptor can theoretically be formed which can be linked via a disulfide bond on the cell surface, a mechanism that is experimentally shown for some integrin and insulin receptors (123). Within the ECD of RYK/DRL there is a cysteine available on either side of the TBC motif that could potentially cause the disulfide bond formation linking the two subunits (202). The transmembrane domain (TMD) of RYK also contains two cysteines that allow the formation of disulfide bonds. However, *Drosophila* DRL and DRL-2 do not contain cysteines in the TMD, whereas DNT has a single cysteine residue

residing within the TMD. This could be functionally significant since cysteines as well as other amino acid motifs can play a role in dimerization and activation of transmembrane proteins. It was established that the disulfide-linked insulin receptor is able to form homodimers without being bound by its ligand (203–205). The preformed homodimer is believed to undergo a conformational change upon ligand binding that causes transphosphorylation of both ICDs and activates a signaling cascade. This mechanism could also be operating for RYK and DRL activation as they too belong to the RTK family and are disulfide linked.

The ICD amongst others contains a PDZ-binding domain (TRYV at the very end of the DRL-ICD sequence) (Fig 11 and 12) and could therefore potentially interact with proteins containing a PDZ domain (206). The most likely candidate would be Dsh (207), since this PDZ containing protein has been implicated to play a role in most pathways that are activated by Wnts. In addition, it has been shown that the noncanonical WNT11/ FZD7-mediated endocytosis of Dsh requires RYK, both RYK and FZD7 are needed to bind WNT11 ultimately regulating the convergent extension (CE) movements in *Xenopus leavis* (118).

Furthermore, it has been reported that when cells are stimulated with WNT3 this results in proteolytic cleavage of RYK. The first cleavage event takes place at the TBC motif of RYK. A second cleavage event within RYK's TMD is mediated by γ-secretase and results in a cytosolic fragment that is stabilized and transported to the nucleus by interacting with Cdc37 (208,209). This nuclear localized RYK ICD most likely functions as a transcriptional regulator as published for ErbB4 (210,211), but it does not seem to activate the TCF-luciferase reporter (208). This RYK processing is required for neural progenitor cell differentiation (208).

The examination of the homology for DRL and its siblings DRL-2 and DNT reveals high homology especially for the WIF- and pseudokinase domain that are present in all three proteins. The homology is also maintained for the putative TBC site, KRKK in DRL, and that resides within the WIF domain. However, the bipartite nuclear localization signal (NLS), highlighted in purple in the DRL sequence only (see Fig 12), shows relatively low homology compared to DRL-2 and DNT and even between DRL-2 and DNT. Suggesting, that both DRL-2 and DNT have lost their NLS in the genomic duplication event. The final four amino acids, TRYV in DRL, represent the PDZ binding domain which is identical in DNT yet altered in DRL-2. Although the preservation of the second to last residue, a tyrosine, could be sufficient to maintain its PDZ binding capacity.

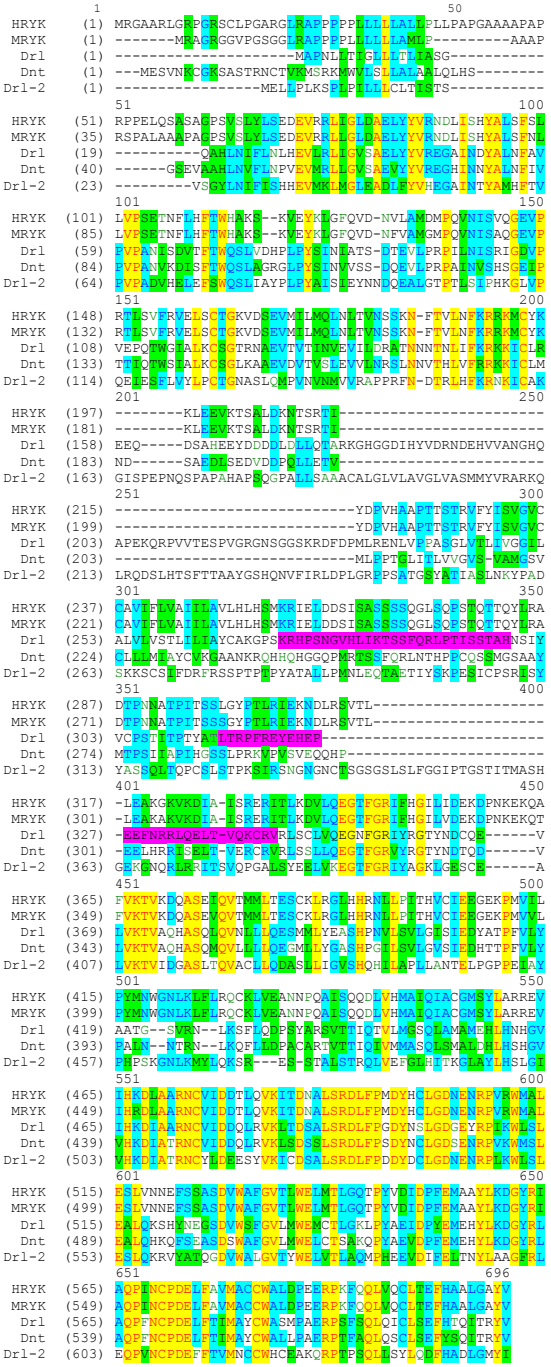


Fig 12 Alignment of the amino acid sequence of human RYK (HRYK), murine RYK (MRYK) and their *Drosophila* counterparts Derailed (DRL), Doughnut (DNT) and Derailed-2 (DRL-2). The yellow highlights represent the amino acids (aa) that are conserved amongst these species, whereas blue displays the aa that are most conserved yet not within all proteins. The green color highlights aa that are either conserved or similar and could perform the same function within a protein structure. The purple highlights in the DRL sequence only represents the bipartite nuclear localization signal.

1.3.2 Are RYKs Pseudokinases?

The intracellular domain (ICD) of RYK contains a number of subdomains that are hallmarks of the larger family of protein kinases (212). Yet a number of conserved phosphotyrosine kinase (PTK) domains seem to harbor amino acid substitutions, rendering them to be likely catalytically inactive as a kinase (see Table 1).

Table 1 The left column contains the species and the three *Drosophila* family members. The other columns display the different conserved PTK domains that are established for RTKs and that are not conserved in RYK/DRL.

Conserved PTK domain	Subdomain I GXGXXG	Subdomain II VAVK	Subdomain VIb IHRDLAARN	Subdomain VII DFG	Subdomain VIII KWMAPE
HRYK	**Q**EGTFG	**AF**VK	IH**K**DLAARN	D**NALS**	**R**WMA**L**E
MRYK	**Q**EGTFG	**TF**VK	IHRDLAARN	D**NALS**	**R**WMA**L**E
DRL	**Q**EGNFG	V**L**VK	IH**K**D**I**AARN	D**SALS**	KW**LSL**E
DRL-2	**K**EGTFG	**AL**VK	**T**H**K**D**I**A**T**RN	D**SALS**	KW**LSL**E
DNT	**Q**EGTFG	V**L**VK	**T**H**K**D**I**A**T**RN	D**SSLS**	KWM**SL**E

Within the kinase domain the glycine-rich loop with consensus sequence GxGxxG together with subdomain II constitutes the putative ATP binding site in conjunction with subdomain VII and is one of the most conserved sequence motifs in protein kinases (212,213). In RYK as well as DRL, the first nonpolar glycine of subdomain I is replaced by a polar glutamine, which could interfere with the binding of ATP. Furthermore, subdomain II and VII contain significant amino acid substitutions. Although the aspartate residue (D) for subdomain VII is present, it is anticipated that the subsequent residue alterations could disrupt the interaction with ATP. Thus the putative ATP binding site of RYK/DRL is therefore structurally different from those found in other PTKs and these aa sequence changes could prevent an interaction with ATP.

Furthermore, an aspartate (D) in another catalytic loop (IHRDLAARN) is in both RYK and DRL not preceded by an arginine residue (R); they can therefore be included in an expanding subfamily of proteins that have recently been considered non-RD kinases (214). The distinguishing feature of these assumed non RD-kinases or pseudokinases is that their potential activation domain does not appear to be phosphorylated, in direct contrast to the RD kinases (i.e., MAPK, IRK, and FGFR kinase) (215). However it has to be noted that, in murine RYK, subdomain VIb does match the conserved kinase domain, whereas in human and *Drosophila* RYK it does not. In mice, RYK could therefore theoretically function as a RD kinase, which one needs to take into account when extrapolating RYK data from mice to human. The other motifs presented in Table 1 also contain amino acid substitutions believed to interfere with the kinase activity of RYK/DRL. Nonetheless, there are other PTK domains that are conserved in RYK/DRL, leaving the possibility open for kinase activity within the cytoplasmic domain.

Several studies have been done to address the kinase activity of RYKs PTK domain experimentally. For that purpose a RYK antibody was produced and phosphorylation of RYK was detected in an *in vitro* kinase assay of immunoprecipitated RYK (121). In contrast, a FLAG:RYK fusion protein in bacteria was used and no phosphorylated tyrosines could be detected. These conflicting results might be explained by the possibility that the RYK phosphorylation, that is detected, is performed by another kinase that is associated with RYK. It has to be further taken into account that both experiments were done in the absence of a ligand which could be necessary to stimulate the intrinsic kinase activity of RYK. In another study, a chimeric receptor approach using the nerve growth factor (NGF) receptor TrkA was used to analyze the function of H-RYK (216). This chimeric TrkA:RYK receptor was made by fusing the ECD of TrkA to the transmembrane and cytoplasmic domains of the H-RYK receptor. The authors were unable to detect autophosphorylation of the chimeric receptor after stimulation with NGF, suggesting that H-RYK is catalytically inactive under these conditions. Alternatively, one could interpret this result to mean that the interaction between NGF and the ECD of TrkA does not mimick the interaction between RYK and a specific WNT. If a conformational switch is needed to bring the ICDs in proximity of each other, one could argue that the chimeric receptor is unable to induce a conformational change and therefore no autophosphorylation will be detected. It was observed, however, that NGF stimulation of the chimeric receptor was able to activate the mitogen-activated protein kinase (MAPK) pathway. More recently, it has been found that RYK binds another kinase; SRC and therefore, the above mentioned results might be explained by the recruitment of this other kinase.

In order to address the catalytic capacity of RYK/DRL more directly, a *drl* mutant was made that is regarded as catalytically inactive/kinase dead. Since the lysine in subdomain II at amino acid position 371 is thought to be involved in the phosphotransfer reaction and has been shown to be essential for catalytic activity of tyrosine kinases (217), this lysine was mutated to an alanine. This DRL K371A mutant was expressed in *Drosophila* embryos and was able to rescue the muscle bypass phenotype in a DRL mutant background. In addition, when DRL K371A was misexpressed in PC neurons, the PC neurons switched their axonal projections to the AC, just as is observed when DRL wildtype protein is misexpressed in the PC neurons. We can conclude that the substitution of K371 to an alanine does not interfere with the downstream signaling cascade of DRL. However, it does not exclude phosphorylation of DRL via an alternate route, such as the recruitment of another kinase.

1.3.3 SRC as a functional binding partner for RYK/DRL

An indication for such an interaction with another kinase came from the observation that the RYK ICD has been found to interact with SRC (124). Src family kinases (SFKs) that belong to the nonreceptor tyrosine kinases and are highly conserved amongst species. These allosteric

enzymes can exist in at least two conformational states and both states can be stabilized. What state is stabilized depends on the interactions with other proteins of two different domains (218–220). The first domain through which the SFKs can bind other proteins is the phosphorylation-dependent SH2 domain and the second is the phosphorylation-independent SH3 domain. Figure 13 demonstrates that in the inactive state SRC is phosphorylated on residue Y527 (221) and interacts with SH2 while the SH3 domain interacts with the kinase domain rendering it inactive. Upon dephosphorylation of Y527 the conformation of both SH2 and SH3 open up (Fig. 13) and autophosphorylation of Y416 causes Src-activation(222–224).

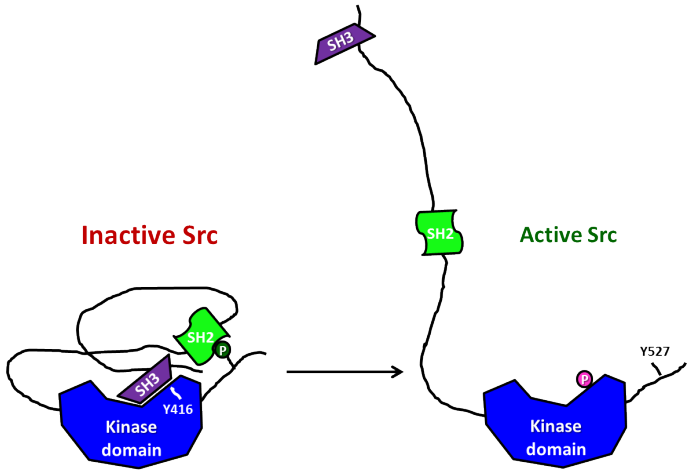


Fig 13 Schematic of the activational opening of SH2 and SH3 generating an active Src molecule.

The *Drosphila* genome has two SFKs; SRC64B (225) and SRC42A, both of which are able to interact with DRL (124,226). It has also been shown that SRC42A has at least partial redundancy to SRC64B (124,189,227,228). The same complex can be formed in mammalian cells between RYK and c-SRC, therefore the interaction between RYK and SRC seems to be evolutionary conserved (124).

1.4 GENERAL OUTLINE

In **Chapter 1** a general overview of Wnt signaling is presented which is followed by a more in-depth review of what is known about a specific non-canonical Wnt-receptor, RYK/DRL. DRL acts as a transmembrane receptor for the *Drosophila* Wnt family member, WNT5. The implications of this conserved interaction during development of vertebrates, invertebrates and in human disease will be addressed. Finally, our model organism *Drosophila melanogaster* is introduced with which most of the research presented in this thesis has been executed, with special attention for the CNS and musculature. This will provide the essential

background for the work presented in the following thesis chapters.

Chapter 2 focuses on the biochemical properties of RYK/DRL. We show that DRL can form homodimers and can also from heterodimers with two DRL protein orthologues, DRL-2 and DNT. We proceed to show that dimerization is dependent on its transmembrane domain and increased upon stimulation with WNT5. The downstream kinase that is associated with DRL, i.e. SRC64B, and the DRL/SRC64B interaction is discussed into detail and the specific domains required for their interaction presented. Furthermore, we show the functional relevance of these domains *in vivo*, specifically, we show their requirements for axonal guidance in the *Drosophila* ventral nerve cord during embryogenesis.

Chapter 3 describes an important function for DRL in the patterning of the mushroom body, a learning and memory center within the brain of *Drosophila*. DRL-2 is one of two family members of DRL that can also bind WNT5, providing a repulsive cue for the growing axon. Previously, DRL, DRL-2 and WNT5 have been shown to play a role in antennal lobe patterning. The authors showed that DRL in the antennal lobe is able to sequester WNT5 from DRL-2, thus preventing signaling through DRL-2. In contrast, we show here that extrinsic DRL binds its ligand WNT5, this extracellular complex is subsequently cleaved and the WNT5/DRL cleaved extracellular complex is presented as a repulsive ligand for DRL-2 expressing mushroom body axonal growth cones. The DRL, DRL-2, WNT5 ternary complex described here in the developing adult brain is, to our knowledge, novel to the Wnt-field.

In **Chapter 4** we show that not only axon guidance is affected in the WNT5 and DRL mutants, but also a subset of muscles fibers is misguided and overshoots their normal targets. Specifically, the lateral transverse muscles 21-23 are affected in this phenotype, one out of the three muscles does not attach at the appropriate muscle attachment site (MAS). We also show that DNT, another family member of DRL displays the same phenotype. Furthermore the new ectopic MASs are fully functional throughout embryonic and larval stages of development. These data reveal a remarkable evolutionary conservation and re-employment of the molecular machinery for both early initial guidance of axons in the embryonic CNS and for guidance of individual muscle fibers to their final attachment targets in the embryonic epidermis.

The results presented in this thesis and the future plans that emanate from this work are discussed in **Chapter 5**.

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2

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

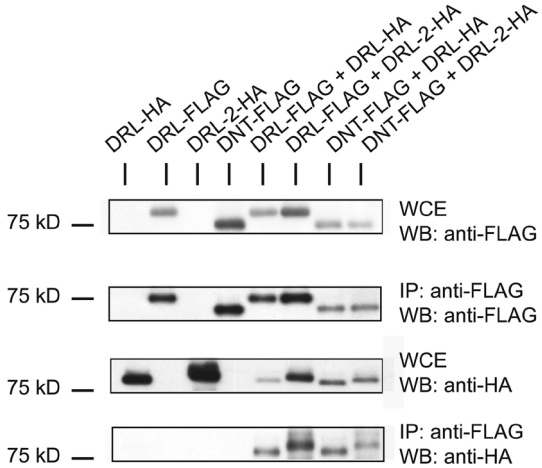


Fig 1 DRL forms homodimers and heterodimers with DRL-2 and DNT. *Drosophila* S2 cells were transiently transfected with the indicated expression constructs, and lysates were immunoprecipitated (IP) with antibody specific to tagged DRL (anti-FLAG) and immunoblotted (WB) with the reciprocal antibody (anti-HA) 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).

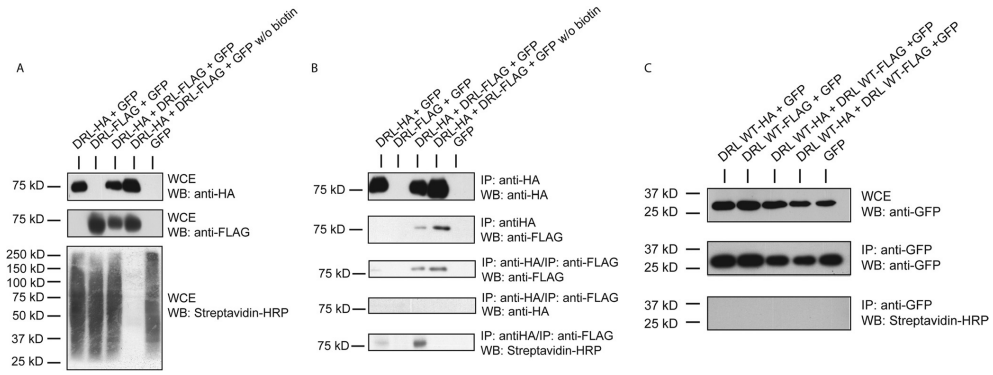


Fig 2 DRL homodimers are present at the cell surface. (A) S2 cells were transiently transfected with the indicated expression constructs and treated with a membrane-impermeable cell surface biotinylation reagent except as otherwise noted in the figure. Cell lysates were first immunoprecipitated with 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.

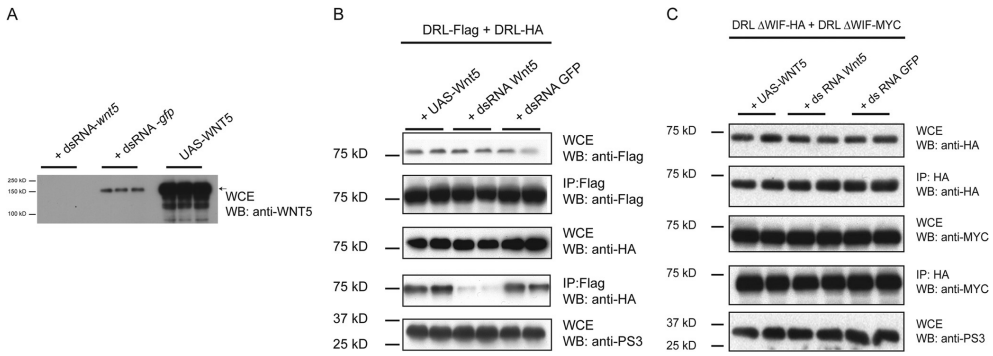


Fig 3 DRL homodimerization is increased upon WNT5 binding in a WIF domain-dependent fashion. (A) S2 cells were pretreated with *wnt5*-targeting dsRNA or control *gfp*-targeting dsRNA or transfected with UAS-WNT5 and pAc-GAL4 to overexpress WNT5. Highly efficient WNT5 knockdown and overexpression were 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

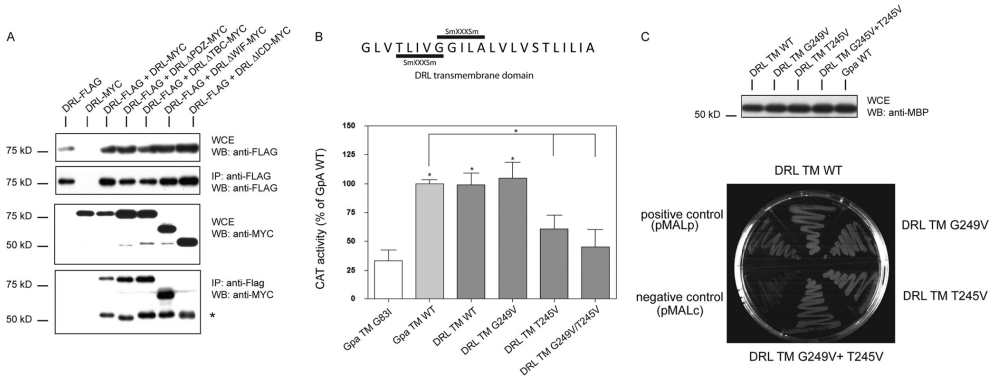


Fig 4 DRL homodimerization is mediated by a motif in the transmembrane domain. (A) S2 cells were pretreated with dsRNA targeting the wnt5 transcript as described above and then transiently transfected with the indicated wild-type and DRL mutant expression constructs, and lysates were immunoprecipitated (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

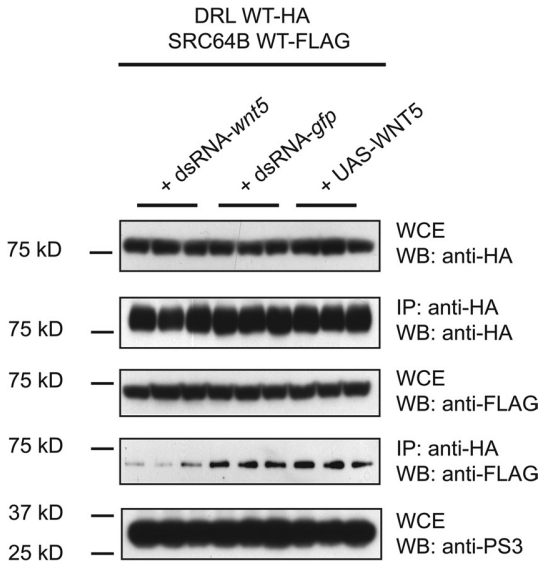


Fig 5 WNT5 binding results in increased SRC64B recruitment. S2 cells were pretreated as described for Fig. 3 and transfected in triplicate with DRL WT-HA and SRC64B WT-FLAG. Cell lysates were immunoprecipitated with anti-HA and immunoblotted with anti-FLAG to detect coimmunoprecipitating SRC64B. The 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

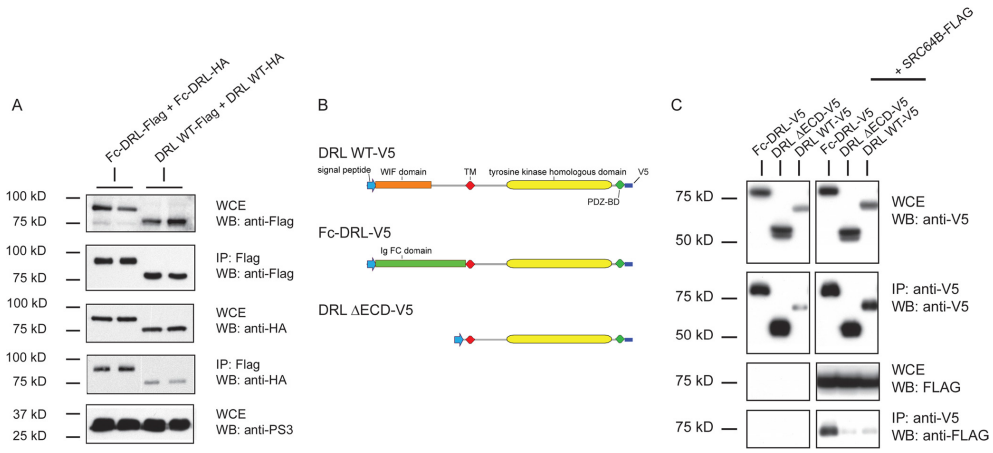


Fig 6 Forced dimerization of DRL increases SRC64B recruitment. (A) We first established that the replacement of DRL's extracellular domain by IgG Fc increases the level of DRL homodimerization. The indicated expression constructs were cotransfected into S2 cells in duplicate, and anti-Flag immunoprecipitations 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

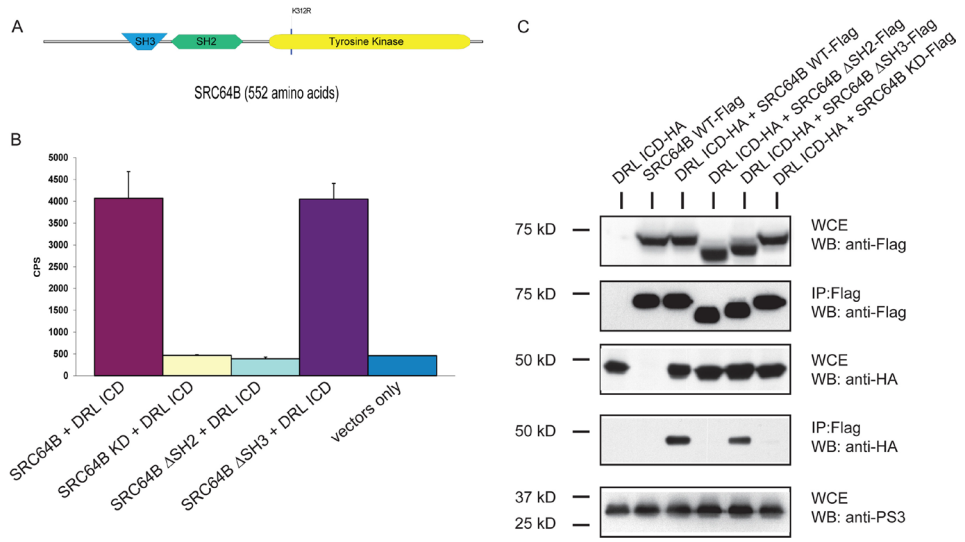


Fig 7 SRC64B's SH2 domain and a kinase domain amino acid required for catalytic activity are needed for formation of the SRC64B/DRL complex. (A) Schematic representation of SRC64B with its SH2, SH3, and tyrosine kinase domains and the location of the kinase-dead K312R mutation. (B) To determine the SRC64B 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

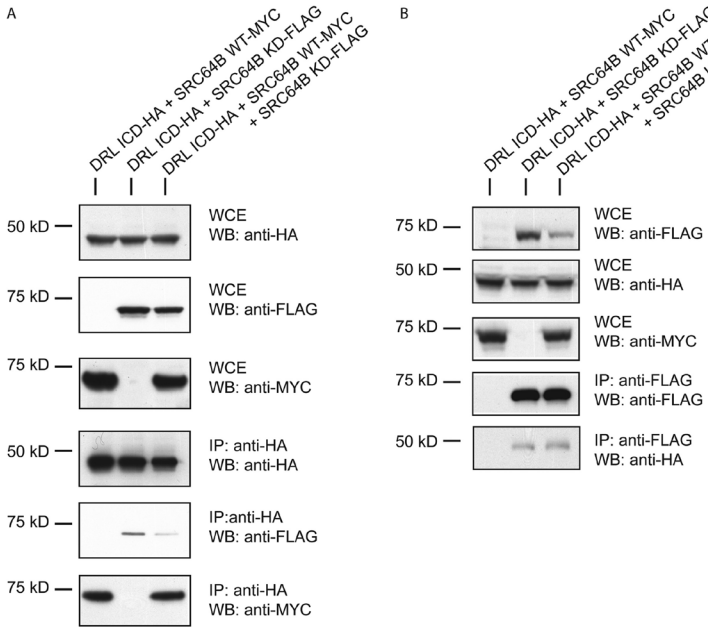


Fig 8 Kinase-dead SRC64B does not compete with active SRC64B for binding to DRL. (A) To ascertain whether the presence of kinase-dead SRC64B interferes with the ability of active SRC64B to interact with DRL, plasmids encoding the proteins indicated were cotransfected into S2 cells. Expression levels 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.

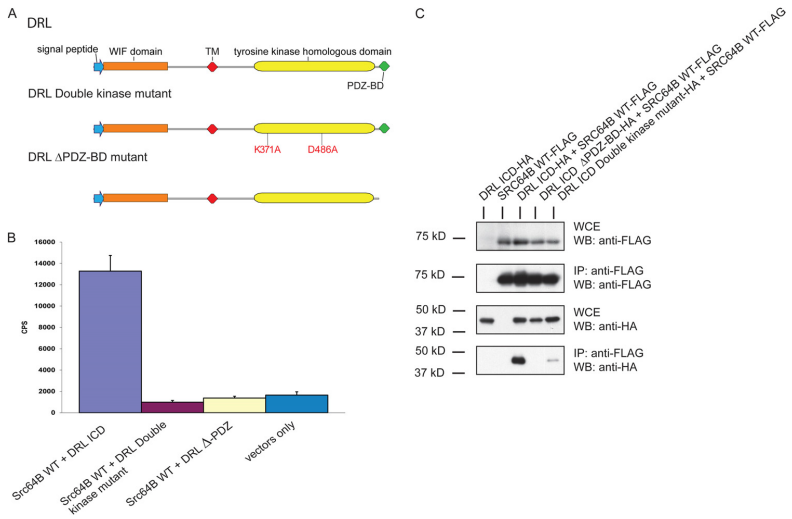


Fig 9 DRL's PDZ-BD and amino acids in its tyrosine kinase-homologous domain mediate its interaction with SRC64B. (A) Schematics of DRL displaying its domains and the DRL mutants (double kinase domain mutant and ΔPDZ-BD) used in the following assays. (B) To ascertain the requirement for DRL's intracellular 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.

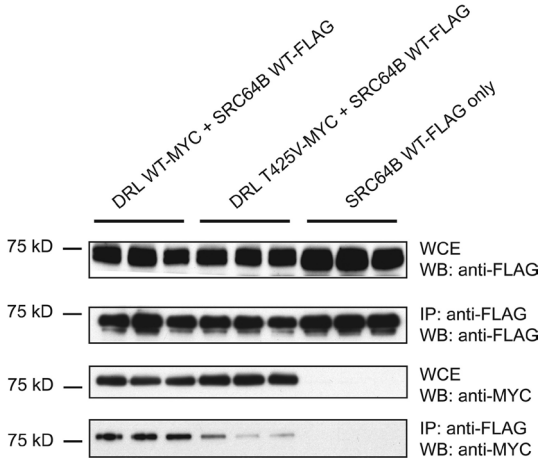


Fig 10 Reduced recruitment of SRC64B by DRL bearing the TM T245V mutation. S2 cells were transfected with the indicated plasmids, and lysates were prepared and immunoprecipitated with anti-FLAG (SRC64B). Expression of the DRL and SRC64B species was confirmed by anti-FLAG and antiMYC immunoblots of whole-cell 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

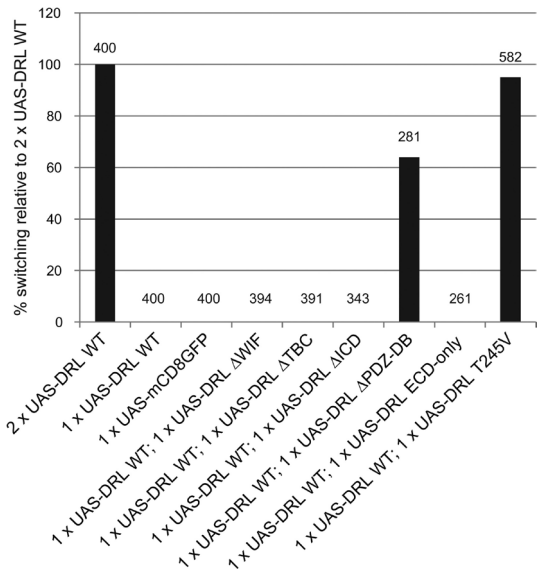


Fig 11 DRL's WIF, TBC, ECD, and ICD and the PDZ-BD are required to effect axon commissure switching in a sensitized background. Stage 16 *Drosophila* embryos of the indicated genotypes were stained with anti-MYC to label EG$^+$ neurons traversing the midline in their stereotypic patterns in both the 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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3

Guidance of *Drosophila* mushroom body axons depends upon DRL-Wnt receptor cleavage in an adjacent brain structure

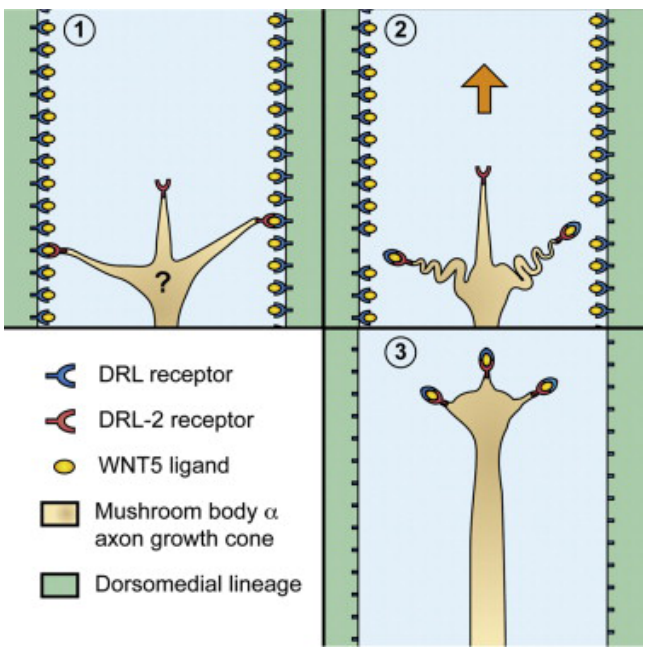
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SUMMARY

In vivo axon pathfinding mechanisms in the neuron-dense brain remain relatively poorly characterized. We study the *Drosophila* mushroom body (MB) axons whose α and β branches connect to different brain areas. We show that the Ryk family WNT5 receptor, DRL (derailed) which is expressed in the dorsomedial lineages, brain structure precursors adjacent to the MBs, is required for MB α branch axon guidance. DRL acts to capture and present WNT5 to MB axons rather than transduce a WNT5 signal. DRL's ectodomain must be cleaved and shed to guide α axons. DRL-2, another Ryk, is expressed within MB axons and functions as a repulsive WNT5 signaling receptor. Finally, our biochemical data support the existence of a ternary complex composed of the cleaved DRL ectodomain, WNT5 and DRL- 2. Thus, the interaction of MB-extrinsic and -intrinsic Ryks via their common ligand acts to guide MB α axons.



INTRODUCTION

The MBs are structures in the insect brain implicated in learning and memory (reviewed in Heisenberg, 2003). Each MB arises from 4 neuroblasts which give rise sequentially to three types of neurons: γ neurons (late embryonic and early larval stage), α'β' neurons (late larval stage) and αβ neurons (pupal stage). Each αβ neuron projects an axon that branches to send an α branch dorsally, which contributes to the formation of the α lobe, and a β branch medially, which contributes to the formation of the β lobe (Lee et al., 1999). The α lobe plays specific roles in long-term aversive memory in the *Drosophila* adult brain (Pascual and Preat, 2001; Yu et al., 2006). Different guidance cues are likely required for the α and β branches. For instance, mutations in the *Eph* and *Hiw* genes result in specific effects on α branch versus β branch guidance, respectively (Boyle et al., 2006; Shin and DiAntonio, 2011). The *drl* gene encodes a receptor tyrosine kinase-related protein, which plays roles with its ligand WNT5 in MB development and was first isolated on its role in olfactory memory (Dura et al., 1993; Grillenzoni et al., 2007).

drl' s first described role in axon guidance was axon commissure choice in the embryonic nerve cord (Bonkowsky et al., 1999; Callahan et al., 1995; Yoshikawa et al., 2003). Each ventral nerve cord segment has two commissures, one anterior and one posterior, where the axons that project contra-laterally cross the midline. The *drl*$^{+}$ expressing neurons send their axons in the anterior commissure because of the presence of WNT5, a repulsive ligand, in the posterior commissure. Here, we report that *drl* is required during brain development for MB α branch guidance, but a lack of DRL does not affect branching of the αβ axons. We confirm our previous report (Grillenzoni et al., 2007) that *drl* is neither expressed nor required within the αβ neurons and demonstrate that, rather, it is expressed by a neural cell lineage adjacent to, but distinct from, the extending MB axons. Interestingly, DRL's cytoplasmic domain, and hence its intracellular signaling activity, is not required for correct α axon guidance. The cytoplasmic domain of another Ryk, DRL-2 which is expressed by MB neurons, is however required for α axon guidance indicating that it acts as an MB axon-intrinsic WNT5 receptor. Furthermore, we demonstrate that DRL's conserved putative tetrabasic cleavage (TBC) site, whose cleavage results in the extracellular shedding of DRL's Wnt-binding domain, is required for MB axon guidance. Finally, we find that the shed DRL extracellular domain forms a complex, via WNT5, with transmembrane DRL-2. Our data indicate a novel mechanism regulating Wnt signaling where a shed extrinsic receptor serves to guide brain axons.

RESULTS

α axons are misguided in *drl* and *wnt5* mutants

Here, we show that *drl* is required for appropriate MB α axon guidance. By examining visualization MARCM (Lee and Luo, 1999) MB neuron clones in the drl^{null} mutant brain, we found that branching of the α and β branch axons occurs normally but that α axons extend inappropriately along the medial trajectory and display aberrant midline crossing (Figures 1A and 1B). Notably, the separation angle between the wild-type α and β axon branches is still observed in *drl* mutants (wild-type: 118.4° ± 5.7°; *drl*: 128.1° ± 16.6°; P = 0.60; t test, results are means ± s.e.m. with n = 5 in each case) indicating that initial appropriate separation between the branches occurs. We observed abnormal midline crossing previously in drl^{null} mutant MBs (Grillenzoni et al., 2007). These two defects, α axon misguidance and midline crossing, are independent since we found one or the other in *drl* hypomorphs (drl^{hypo}; an incomplete loss-of-function allele) (Figures 1C and 1D and Table S1). In this study we focus on α axons since their trajectory, and not that of the β's, is altered in the *drl* mutant. We scored for axon growth defects where the axon stops soon after the branching point. Notably, we did not observe α axon growth defects in the 36 single- and two-neuron null clones analyzed, but 35 out of these 36 clones (97%) displayed α misguidance (Figure 1G and Table S2). These results demonstrate that the *drl* receptor is required for MB α axon guidance. αβ axons extend individually and asynchronously from newly-born αβ neurons which are derived from continuously dividing neuroblasts for most of the pupal stage (circa 5 days at 25°C). α axon misguidance was observed in drl^{null} animals as soon the adult αβ axons can be visualized with a specific pioneer αβ GAL4 line (*c708a*-GAL4 (Zhu et al., 2006)) (data not shown). The *c708a*-GAL4 was not expressed strongly enough in the early pupae to visualize the pioneer αβ at that stage but the misguidance observed in the adult brain likely reflects earlier guidance errors in the developing brain.

The WNT5 protein acts as a repulsive axon guidance ligand for the DRL receptor in the embryonic central nervous system (Yoshikawa et al., 2003) and is involved in MB development (Grillenzoni et al., 2007). Thus, we evaluated the effects of the loss of *wnt5* on α axon guidance in the MBs. The analysis of *wnt5* mutant brains with *c739*-GAL4 line (Figure S1) revealed absence of α lobe (circa 30% of the MBs), absence of α and β lobe (circa 50% of the MBs) as well as wild-type MBs (circa 20%). If we take into account only where α axons are affected, examination of visualization MARCM clones in $wnt5^{null}$ brains, revealed misguidance in 60% of them (n=20) (Figure 1E) while the remainder had growth defects (Table S2) similarly to what was previously reported (Shimizu et al., 2011). Both α and β guidance were also observed to be affected in the same neurons (Figure S1E and H). Altogether, 51% of the $wnt5^{null}$ clones (n=47) displayed α axon misguidance (Figure 1G and

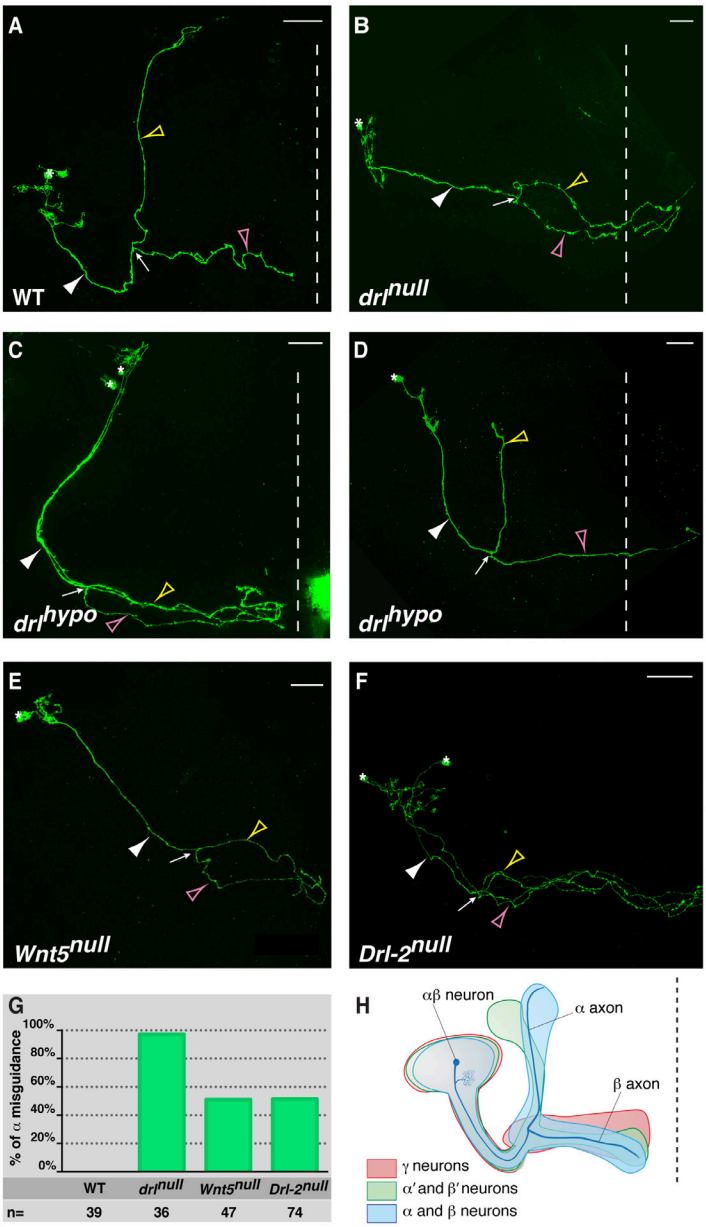


Figure 1. DRL, WNT5 and DRL-2 are required for MB α branch guidance (A) A single αβ neuron clone in a wild-type brain. (B) A single αβ neuron clone in a *drl*^{null} brain displaying α misguidance and inappropriate midline (dotted line) crossing of both the α (yellow arrowhead) and β (pink arrowhead) axons. (C and D) Neuron clones in *drl*^{hypo} individuals reveal the uncoupling of the α-misguidance and midline crossing phenotypes. (E and F) Neuron clones in *wnt5*^{null} (E) and *drl-2*^{null} (F) brains display α misguidance. In all images, the white arrow indicates the αβ branch point and the white arrowhead indicates the peduncle. (G) Quantitation of the penetrance of the α misguidance phenotype in the different mutant and control neuron clones. n=number of clones analyzed. See genotypes and other details in Supplemental Information for Figure 1. (H) Schematic representation of an αβ neuron in the context of the MB lobes. See also Figures S1, S2 and Tables S1, S2. 26

Table S2), indicating that WNT5 is involved in α axon branch guidance.

DRL is not detectably expressed within the MBs nor does *UAS-drl* expression driven by MB-specific GAL4 drivers rescue the *drl*^{null} phenotype (Grillenzoni et al., 2007). DRL, therefore, is unlikely to be an intrinsic α branch WNT5 receptor. To further rule out the possibility that DRL expression is required within the MBs, we used the *MB247-GAL80* (*MB-GAL80* (Krashes et al., 2007) transgene to suppress GAL4 activity in the MBs while expressing *drl* in all neurons with *elav-GAL4*. Expression of *MB-GAL80* suppressed the GAL4-driven pan-neural expression of a *mCD8-GFP* (*mGFP*) reporter to undetectable levels specifically only in the MBs both at 48 h APF and in the adult (data not shown) indicating its effectiveness. Pan-neural expression of *UAS-drl* in all non-MB neurons rescued the *drl*^{null} mutant MB phenotype to the same extent as when *drl* was expressed in all neurons (Figure 2A). Thus, DRL is required outside of, not within, the MB axons to ensure correct α branch guidance.

DRL-2 acts as an MB-intrinsic signaling receptor for α guidance

What is the intrinsic MB receptor that interacts with the WNT5 ligand to guide α axons? DRL-2 and DNT (doughnut) are the two other *Drosophila* Ryks (Fradkin et al., 2010) and therefore represented plausible candidates. Homozygous *dnt*^{null} mutants (Lahaye et al., 2012) did not display any MB phenotype (data not shown). Conversely, *drl-2*^{null} mutant neurons displayed α axon misguidance (Figure 1F). The analysis of *drl-2* mutant brains with *c739- GAL4* line (Figure S2) revealed absence of α lobe (circa 60% of the MBs) as well as wildtype MBs (circa 35%). Altogether, 51% (n=74) of the *drl-2*^{null} visualization MARCM clones displayed α misguidance (Figure 1G and Table S2). If we take into account only where α axons are affected, examination of visualization MARCM clones in *drl-2*^{null} brains revealed that α misguidance occurred in 90% of them (n=41) while the other 10% exhibited growth defects (Figure S2 and Table S2). Strikingly, *drl-2* acts non-cell-autonomously in *drl-2*$^{-/-}$ regular MARCM αβ MB neuroblast clones in otherwise *drl-2* heterozygous animals. These clones displayed wild type α guidance (data not shown). Similar non-cell-autonomous Wnt/Planar Cell Polarity-mediated MB axon branch extension defects were previously described for the membrane receptors *frizzled* and *strabismus* (Ng, 2012; Shimizu et al., 2011). Protein perdurance could be an alternative explanation of the absence of mutant phenotype displayed by *drl-2* regular MARCM clones. Nevertheless, two pieces of data strongly argue against the perdurance of DRL-2. First, the neuroblast clones are induced in L1, days before the birth of the αβ neurons. Second, the DRL-2 protein seems to be actively degraded (Figure S3). It is likely that mutant axons surrounded by wild type axons correctly pathfind by other mechanisms probably involving axon-axon interactions. Nevertheless, mutant rescue experiments with specific MB GAL4 lines led to the notion of MB-autonomy for these membrane receptors (Ng, 2012; Shimizu et al., 2011). Importantly, we were able to rescue *drl-2*^{null} α misguidance by expressing a *UAS-*

drl-2 transgene under the control of the MB αβ neuron-specific *c739-GAL4* driver (Aso et al., 2009) but not by expressing *drl-2* in all non-MB neurons or by inhibiting MB GAL4 expression from *c739-GAL4* by expression of GAL80 in the MB's (Figure 2B). Also, *201Y-GAL4* as well as *c305a-GAL4*, strongly expressed into the γ and α'β' neurons respectively (Aso et al., 2009) failed to rescue the *drl-2*^{null} α misguidance phenotype when associated with a *UAS-drl-2* transgene (data not shown). These results indicate that *drl-2* plays an MB-autonomous role in α branch guidance. This MB axon-specific rescue supports our conclusion that *drl-2* is an MB axon-intrinsic receptor involved in α guidance.

Does DRL-2 transduce an intracellular signal in the MB axons? We generated a *UAS-drl-2Δcyto* transgene and it failed to rescue the loss of the α lobe, indicating that DRL-2 likely transduces the WNT5 signal in MB axons (Figure 2B). Supporting our identification of DRL-2 as an MB-intrinsic WNT5 receptor, DRL-2 protein was detected in the growing α branch at 48 hours after puparium formation (APF) in wild type, but not in *drl-2*^{null} mutant, brains (Figure S3). No apparent difference in the levels of DRL-2 between the α and β branches were detected making it unlikely that DRL-2 localization determines why the b axon trajectories are unaffected in the *drl-2*^{null} mutant background. We then determined whether DRL-2 interacts with WNT5. Epitope-tagged DRL-2 bound WNT5, while DRL-2 lacking its Wnt-binding WIF domain did not, indicating that DRL-2 binds WNT5 via its WIF domain (Figure S4). Therefore, we performed further genetic experiments to determine whether *wnt5* interacts with *drl-2* to guide α axons.

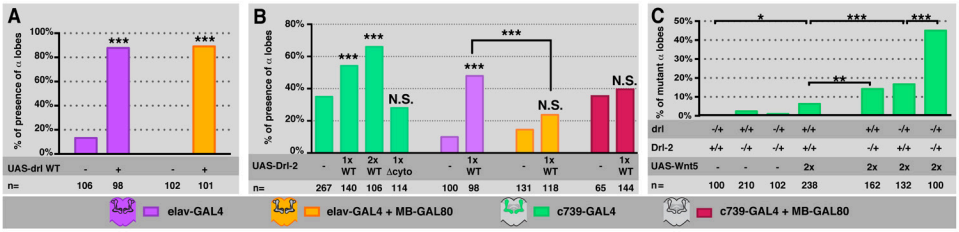


Figure 2. DRL and DRL-2 interact with WNT5 and are required to guide α branch axons (A) Rescue of the *drl*^{null} mutant phenotype by pan-neural expression of *UAS-drl WT* driven by *elav-GAL4* (purple) versus expression of *UAS-drl WT* in all non-MB neurons in the *elav-GAL4*; *MB-GAL80* background (orange). (B) Rescue of the *dl-2*^{null} mutant phenotype by *UAS-drl-2* but not by *UAS-drl-2Δcyto*, driven by the αβ specific *c739-GAL4* driver (green). Rescue of *drl-2*^{null} by *UAS-drl-2* driven in all neurons by *elav-GAL4* (purple) but not in all non-MB neurons by *elav-GAL4; MBGAL80* (orange). *c739 MB-GAL80* failed to rescue the *drl-2*^{null} MB phenotype when associated with a *UAS-drl-2* transgene by inhibiting MB GAL4 expression from *c739-GAL4* (red). (C) *drl*, *drl-2* and *wnt5* genetically interact during α branch guidance. For all panels, n=number of MBs analyzed, and ***: P <0.001, **: P <0.01, *:P <0.05, N.S.: not statistically different by χ^2 test. See genotypes and other information in Supplemental Information for Figure 2. See also Figures S3 and S4.

drl-2, *wnt5* and *drl* interact genetically during α branch guidance

Next we examined whether DRL-2 could act as an axon-repulsing WNT5 receptor in another context. Ectopic expression of wild-type *drl*, under control of the *eg-GAL4* driver, in *Drosophila* embryonic posterior commissure (PC) axons, which normally do not express DRL, causes them to cross in the adjacent anterior commissure due to their repulsion by WNT5 which is predominantly expressed by PC neurons (Bonkowsky et al., 1999). We found that expression of two copies of *UAS-drl-2* driven by *eg-GAL4* resulted in >95% axon commissure switching (Figure S4) in the wild type background. DRL-2-dependent switching was essentially completely suppressed by the absence of WNT5. Together, these results indicate that DRL-2 can act as a WNT5 axon-repulsing guidance receptor. We conclude that DRL-2 is likely an intrinsic MB receptor, which mediates a repulsive WNT5 signal required for α axon guidance.

Do *wnt5*, *drl-2* and *drl* genetically interact during α branch guidance? We were not able to detect genetic interactions even in the triple heterozygous condition ($wnt5^{+/-}$; $drl^{+/-}$ $drl\text{-}2^{+/-}$ 100% WT MBs, n = 102). Nevertheless, we noticed that when *wnt5* was strongly overexpressed in the MBs a modest but significant fraction (less than 10%) of the MBs showed α lobe misguidance (Figure 2C). The simplest interpretation for this phenotype could be that an excess of WNT5 emanating from the MBs is binding to DRL-2 receptor but does not provide a guidance cue because it is not bound and localized by extrinsic DRL (see below). Thus, the amount of available DRL-2 receptor would be decreased. In this situation, reducing the amount of DRL would further increase the amount of free WNT5 and reducing the dose of *drl-2* will further decrease the amount of available DRL-2. Indeed, when *drl* or *drl-2* is heterozygous ($drl^{+/+}$ or $drl\text{-}2^{+/+}$) in the WNT5 over-expressing background, α misguidance significantly increased relative to the controls (Figure 2C). Finally, we observed a dramatic increase in α misguidance in $drl^{+/+}$; $drl\text{-}2^{+/+}$ brains overexpressing WNT5 (Figure 2C), indicating that *drl*, *wnt5* and *drl-2* interact to guide α axons.

DRL is expressed by the lineages giving rise to the central complex and localizes WNT5

Where is DRL expression required to control α axon guidance? We tested a number of brain GAL4 drivers, which do not express in the MBs, for their ability to rescue the drl^{null} phenotype (data not shown). We identified *distalless (Dll)-GAL4* which is expressed in the dorsomedial (DM) lineages in the postembryonic brain (Izergina et al., 2009). DM neuroblast lineages contribute to the developing central complex but not the MB (Bayraktar et al., 2010; Izergina et al., 2009). Indeed, we did not observe *Dll-GAL4* expression in the developing MBs from the third larval instar to adult stages (data not shown and Figure S5) confirming previous reports. At the third larval instar stage, DRL is expressed in six large groups of cells at the DM margins of the brain hemispheres (Figure 3A). Upon double-labeling brains expressing

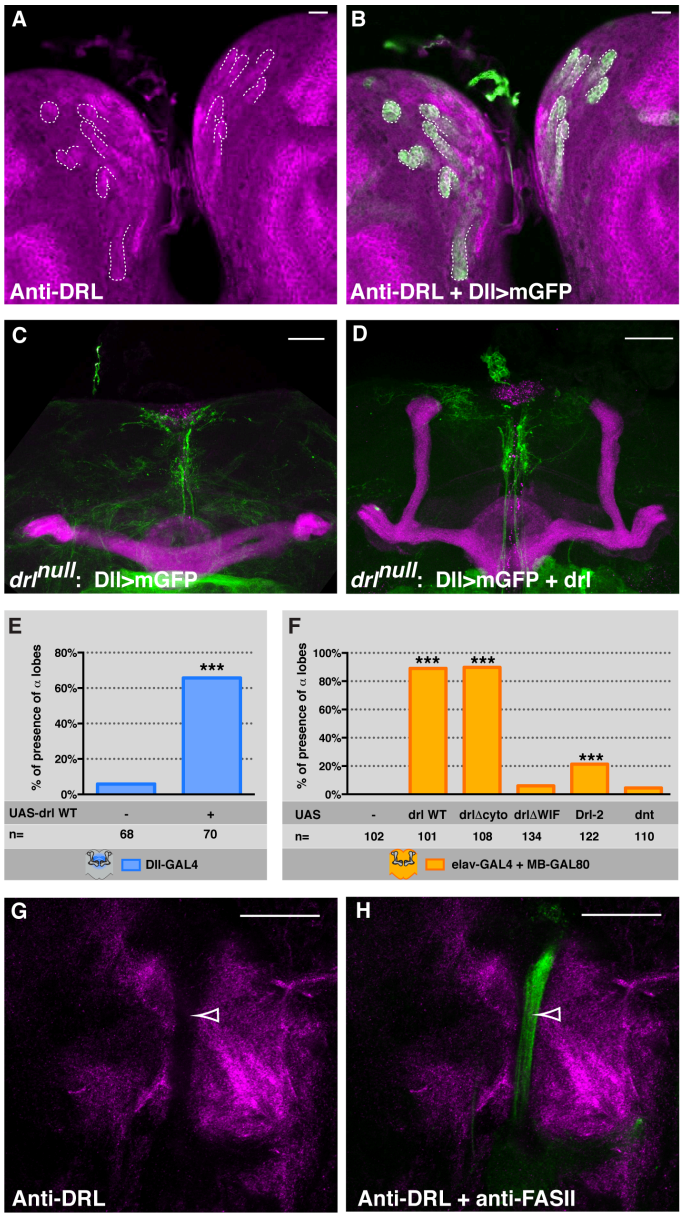


Figure 3. DRL is expressed in the dorsomedial lineages, precursors of the central complex (A) DRL (magenta) is expressed in six large groups of cells at the DM margins of the 3rd instar brain hemispheres (dotted outlines). (B) These cells are identified as DM lineage neurons by co-localization of DRL and GFP in brains expressing mGFP (green) driven by *Dll-GAL4*. (C and D) Anti-FASII staining (magenta) reveals the absence of the α lobes in a drl^{null} brain (C) which is rescued by expression of *UAS-drl WT* driven by *Dll-GAL4* (green; D). (E) Quantitation of α lobe rescue by *Dll-GAL4* (blue). (F) Quantitation of rescue of the drl^{null} phenotype by *drl WT, drl Δcyto* or *drl-2*, but not by *drl ΔWIF* or *dnt*. All constructs are driven by *elav-GAL4; MB-GAL80* (orange). n=number of MBs analyzed and ***: $P < 0.001$ (χ^2 test). (G and H) 24 hours APF WT brains. DRL (magenta) is expressed around, but not in (white arrowhead) the FASII positive α branch (green). See genotypes and details in Supplemental Information for Figure 3. See also Figure S5.

mGFP driven by *Dll-GAL4* with anti-GFP and anti-DRL, we observed colocalization of DRL and GFP in these cells (Figure 3B). Expression of DRL in the DM lineages rescued the *drl*^{null} phenotype (Figures 3C-3E). The spatial relationship between the *Dll-GAL4* neurons and the MBs was studied from 0 hours to 48 hours APF and revealed a close proximity of the *Dll-GAL* expressing neurons and the developing MBs (Figure S5). Expression of DRL lacking its cytoplasmic domain (*UAS-drlΔcyto*), but not DRL lacking its Wnt-binding WIF domain (*UAS-drlΔWIF*), in all non-MB neurons rescued the mutant phenotype to the same extent as the *UAS-drl WT* (Figure 3F). Therefore, although DRL must bind WNT5 to act, signaling through DRL is not required for α branch guidance. DRL's expression in the cells surrounding the MBs at 24 hours APF, but not in them (Figures 3G and 3H), is consistent with a MB-extrinsic role for DRL in α axon guidance. Does extrinsic DRL act to properly localize WNT5 to guide α axons? WNT5 is broadly expressed in the developing brain but a clear WNT5-free channel is present at the level of the α MB lobes (Shimizu et al., 2011). We found that WNT5 was mis-expressed in this region in *drl*^{null} brains as early as 24 hour APF (Figure 4). This result indicates that WNT5 distribution in the brain is controlled, at least in part, by the DRL receptor. WNT5 expression appeared globally increased in the *drl* mutant brain. Indeed, *wnt5* transcript levels, quantitated by qRT-PCR of 3rd instar brain RNA, were increased 1.5-fold in *drl* mutant brains, relative to controls (data not shown). Therefore in the developing brain, DRL acts to regulate *wnt5* mRNA levels in addition to its role in localizing the WNT5 protein.

Cleavage and release of DRL's ectodomain is required for α branch guidance

DRL's extracellular domain (ECD) was detected at 48 hours APF on the tips of the MB lobes (Figures 5A-5F). Interestingly, DRL was found at significantly higher levels at the α lobe tip than at the β lobe tip (Figure 5G). This is the only clear molecular difference between the α and the β lobes thus far reported and might be relevant to the *drl* mutant phenotype when only the α trajectory is affected. Importantly, the intracellular domain of DRL tagged by a C-terminal MYC epitope tag was not found at the tip of the wild-type α lobe when a *UAS-drl-WT-MYC* transgene was overexpressed in the DM lineages (Figure 6), indicating that the cytoplasmic domain of DRL is not localized to the α lobe tip. Thus we conclude that the DRL species present at the α lobe tip consists of only the Wnt-binding ECD.

Since *drl* expression is not required in the MBs, yet the DRL ECD was localized to the MB lobe tips, we tested the hypothesis that DRL's ECD is released by proteolysis and shed from expressing cells to guide α axons. DRL has a putative tetrabasic cleavage (TBC) site whose cleavage would result in the extracellular shedding of DRL's ECD bearing the intact Wnt-binding domain. We mutated the TBC site (KRKK>AAAA) to generate a *UAS-drlΔTBC* transgene. Although one copy of wild-type transgene strongly rescued, even two copies of the *UAS-drlΔTBC* transgene failed to rescue the *drl*^{null} α lobe misguidance phenotype (Figure

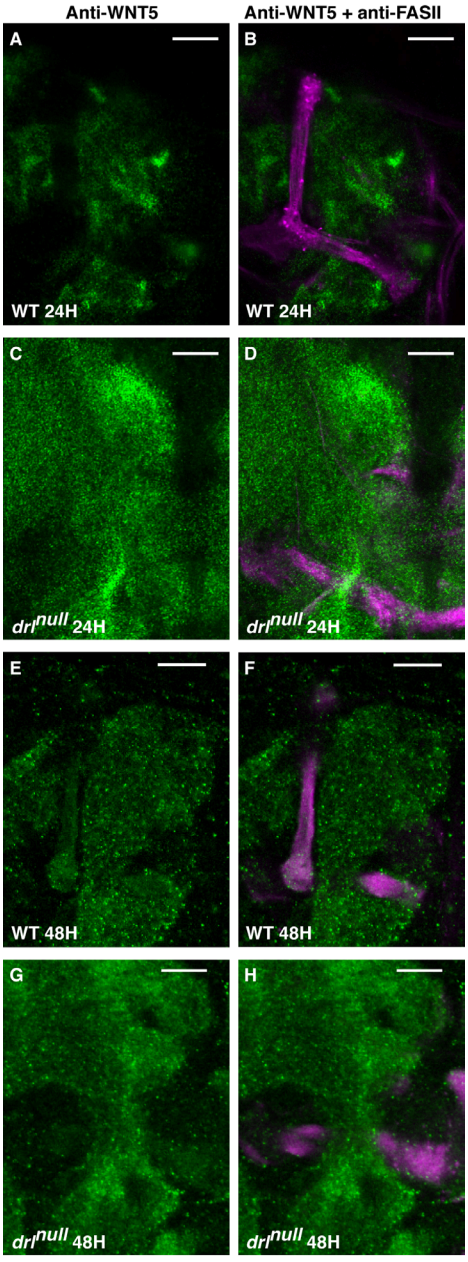


Figure 4. DRL restricts WNT5 expression in the brain (A, B, E, and F) Wild-type 24 h APF (A and B) and 48 h APF brains (e,f) stained with anti-WNT5 (green) and anti-FASII (magenta) revealed a WNT5-free channel at the level of the α 28 MB lobes. At 48 h APF WNT5 is expressed within the MB lobes but the channel was still apparent. (C, D, G, and H) *drl*^{null} 24 h APF (C and D) and 48 h APF brains (G and H) where WNT5 was mis-expressed in the channel region. The confocal laser and acquisition and processing settings were the same for the wild-type and mutant. At 24 h APF, 6 MBs were analyzed in both cases (WT and *drl*^{null}) with 0 mutant and 6 wildtype MBs for WT and 4 mutant and 2 wild-type for *drl*$^{null.}$ At 48 h APF, 6 MBs were analyzed for WT and 12 MBs for *drl*^{null} with 0 mutant and 6 wild-type MBs for WT and 10 mutant and 2 wild-type for *drl*$^{null.}$ Genotypes: (WT) *w* ^{1118}/*y w*^{67c23} (24 h APF), *w* ^{1118} (48 h APF). (*drl*^{null}) *w* ^{1118} /*w*^{1118} ; *lio*^{2} /*drl*^{R343}. Scale bars represent 20 μm. Images are single confocal sections.

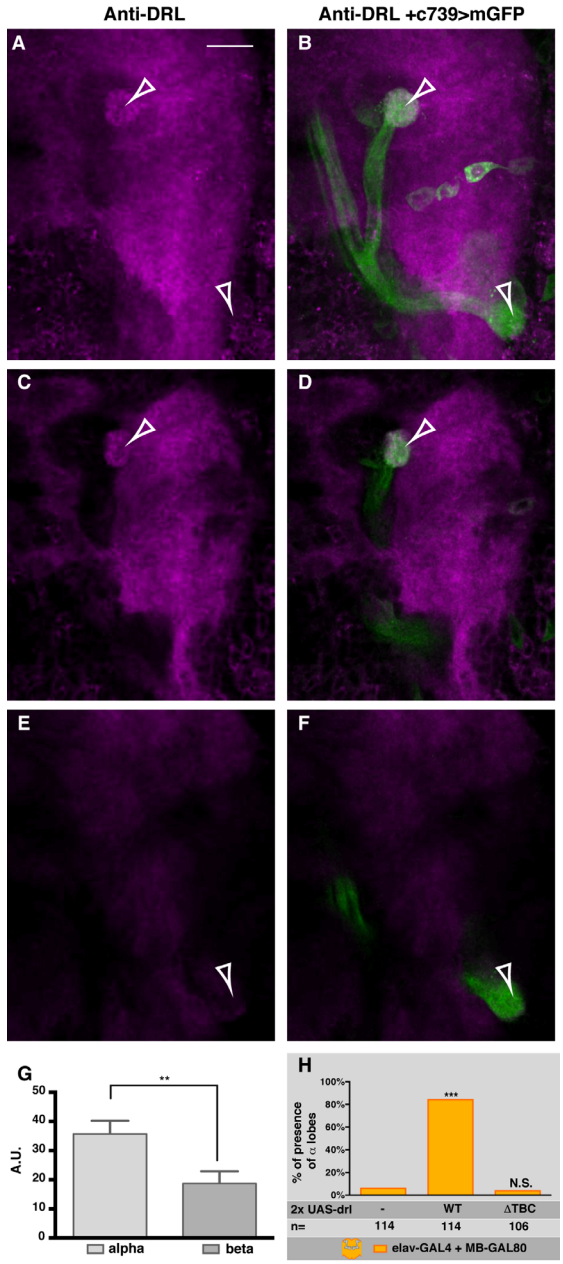


Figure 5. DRL is present at the tips of the MB lobes and DRL's TBC site is required to rescue the mutant phenotype (A-F) *c739-GAL4 UAS-mCD8GFP* 48 h APF brain. DRL (magenta), detected with an antibody recognizing the ECD, is present at the tip (arrowheads) of the MB lobes (GFP in green). (A and B) 40 confocal sections. (C-F) 1 confocal section. (G) Quantitation of the intensity of the DRL signal, normalized to that of GFP, in arbitrary units (A.U.). Results are means +/- SEM. n=5 MBs analyzed. DRL was present significantly more at the α lobe tip than at the β lobe tip. **: $P < 0.01$ (paired t test). (H) DRL's TBC site is required for MB-extrinsic guidance of the α lobe. n=number of MBs analyzed, and ***: $P < 0.001$, NS: not statistically different (χ^2 test). See genotypes and details in Supplemental Information for Figure 5.

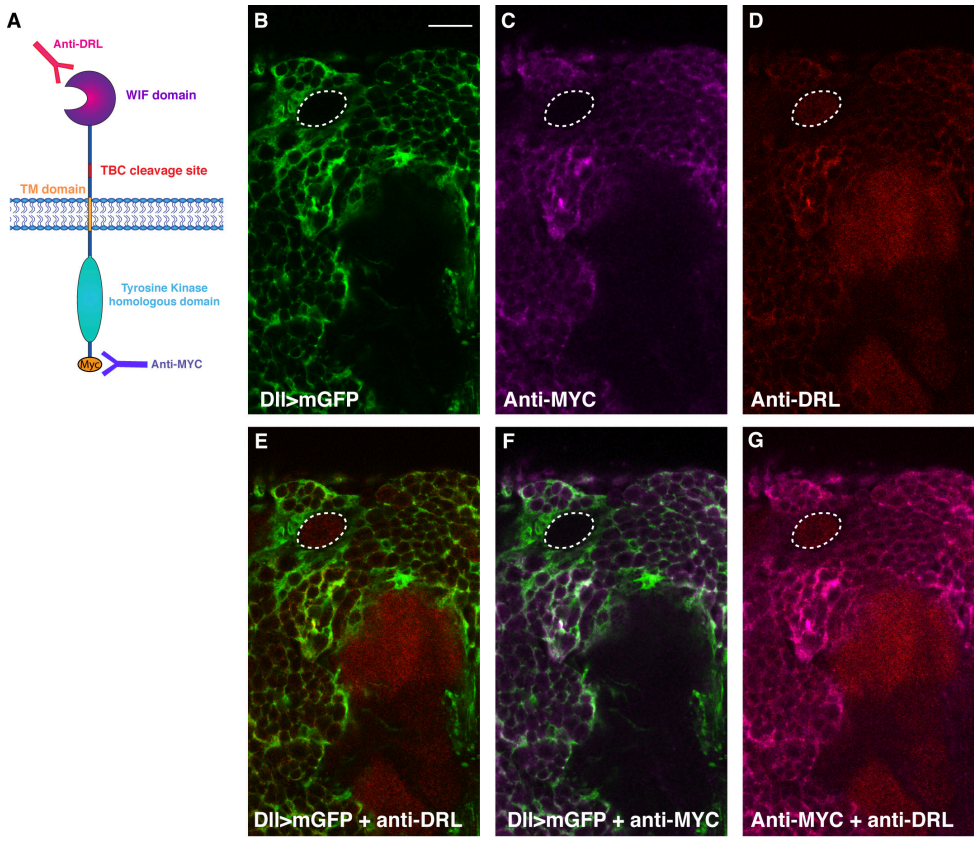


Figure 6. The cleaved DRL ECD is present at the tip of the MB α lobe (A) Schematic representation of the DRL ECD and intracellular domain recognized respectively by the anti-DRL and by the anti-MYC antibody. (B-G) All images shown are of 48 h APF *UAS-mCD8GFP Dll-GAL4* brains. Green represents *Dll-GAL4* driven GFP, red indicates anti-DRL and magenta anti-MYC. The DRL ECD, revealed by the anti-DRL antibody (red), was found at the tip of the MB α lobe (dotted circle in D, E, and G). The intracellular domain of DRL tagged by a carboxyterminal MYC epitope tag (magenta) was not found at the tip of the α lobe when a *UAS-drl-WT-MYC* transgene was overexpressed under *Dll-GAL4* driver control (dotted circle in C and F). Thus, we conclude that the DRL species present at the α lobe tip consists of only the Wnt-binding ECD. Genotype: *UAS-drl WT-MYC/y w* $^{67c23;}$ *UAS-mCD8GFP Dll-GAL4 /+*. Scale bars represent 20 µm. Images are single confocal sections. See also Figures S6 and S7.

5H and Figure S6 for the sub-cellular specificity of *UAS-drlΔTBC* transgene expression). We also observed that DRL ECD displayed limited diffusion throughout the brain when the TBC site was mutated (Figure S7) further supporting the hypothesis that DRL's ECD is released by cleavage at the TBC site. Finally, anti-MYC immunoblot analyses of 3rd instar brains expressing either DRL-WT-MYC or DRL-ΔDTBC-MYC revealed the presence of a MYC-tagged species corresponding in size to the expected intracellular cleavage product only from animals expressing wild type DRL (Figure S7I). This further reinforces the likelihood that DRL is cleaved at the TBC site *in vivo*.

The DRL ECD forms a WNT5-dependent complex with DRL-2

WNT5 is enriched at the tips of the lobes, in the 48 hours APF MBs (Shimizu et al., 2011) (and data not shown). The presence of both WNT5 and DRL's ECD at the tips of the 48 hours APF α lobes raised the question as to whether the DRL's ECD might form a ternary complex with WNT5 and the MB-intrinsic DRL-2. To investigate this possibility, we transfected Schneider S2 cells with DRL-2 and WNT5 expression constructs. After 48 hours, cells were harvested, washed and resuspended in serum-free media in the presence of soluble Fc-DRL-ECD, to mimic the DRL ECD species liberated by cleavage at the TBC site, or control human IgG. Fc-containing complexes were captured on Protein A agarose, washed and then subjected to SDS-PAGE and immunoblotting for DRL-2. We found that the Fc-DRL-ECD precipitated DRL-2 only when WNT5 was also expressed (Figure 7). We conclude that DRL's ECD interacts with DRL-2 in a WNT5-dependent manner.

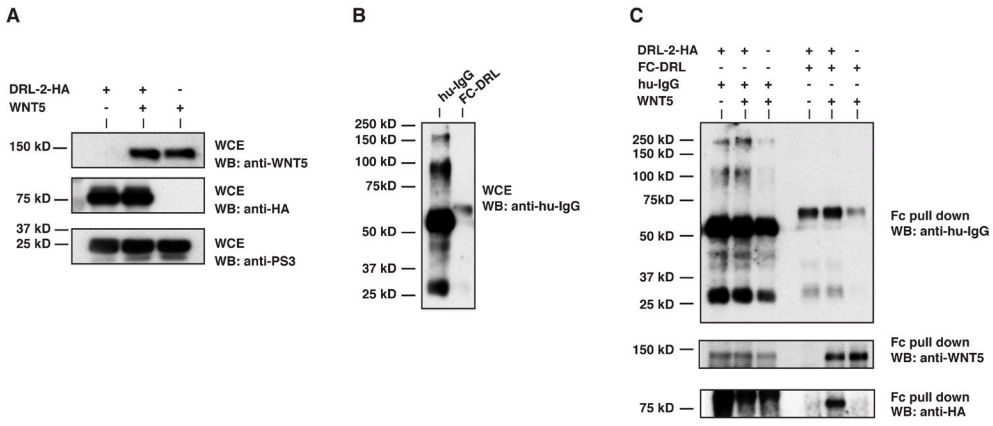


Figure 7. The DRL ECD forms a WNT5-dependent complex with transmembrane DRL-2 (A) Immunoblots of whole cell extracts (WCE). (B and C) Immunoblots of the Fc immunoprecipitations. S2 cells were transfected with the indicated expression constructs. 48 hours post-transfection, cells were washed and transferred to serum-free media containing either Fc-DRL ECD or control human IgG. Fc-containing complexes were captured on Protein A beads, washed and proteins analyzed by SDS-PAGE and immunoblot with anti-WNT5 and anti-HA antibodies to detect co-immunoprecipitating WNT5 and DRL-2, respectively. DRL-2 precipitated with the DRL ECD only in the presence of WNT5 indicating that these proteins form a ternary complex. The bands in the human IgG immunoprecipitations on the lower two blots are nonspecific as evidenced by their presence in all three samples. The data shown are representative of three experiments.

DISCUSSION

Here, we have shown that the wild-type guidance of the MB α axons results from an interplay between two *Drosophila* Ryks. DRL, expressed outside of, but near the MBs, interacts with DRL-2, which is expressed on MB axons, via their common ligand, WNT5. These interactions between DRL, WNT5 and DRL-2 during α axon guidance contrast with those described for the patterning of the antennal lobes (ALs). DRL in the ALs likely sequesters WNT5 and prevents it from signaling through DRL-2 (Sakurai et al., 2009; Yao et al., 2007). Loss-of-function alleles of all three genes display MB α axon misguidance indicating that these proteins, in contrast to their roles in the ALs, act together, rather than antagonistically, to guide α axons. Strikingly, while the β axon trajectories are unaffected in the *drl* and *drl-2* mutants, these axons often fail to stop at the midline indicating roles for DRL and DRL-2 in the cessation of β axon extension. Further studies will be required to understand the control of β axon extension and to identify the other mechanisms that guide them medially.

We suggest that the DM lineage DRL expression domain in the pupal brain surrounds the growing α MB lobe where transmembrane DRL captures WNT5 and limits its diffusion. Supporting this is our observation that WNT5 invades this region in the absence of DRL. DRL-bound WNT5 repulses the extending DRL-2-expressing α axon growth cones, preventing their medial migration, thus causing them to navigate dorsally. Our observation that DRL's TBC site is required for α axon guidance indicates a likely need for DRL's ECD to be shed to effect α axon guidance. We cannot visualize the DRL ECD/WNT5 complex on single growing neurons but the presence of DRL ECD and WNT5 at the tips of the α branch axons at 48 hours APF of MB development likely reflects the history of the α axon growth cone interaction with the DRL ECD/WNT5 complex. The continued presence of this complex at the a lobe tip raises the interesting question as to whether this complex stably modulates DRL-2 signaling. Resolving this question will require the identification of MB signaling pathway members downstream of DRL-2.

The role of axon guidance receptor guiding axons in which it is not expressed is not unprecedented. In *Drosophila*, the *frazzled* (*fra*) receptor guides specific embryonic central nervous system axons and lamina layer-specific targeting of photoreceptor axons by controlling the distribution of its ligand, Netrin (Hiramoto et al., 2000; Timofeev et al., 2012). The embryonic axons apparently employ an unidentified intrinsic receptor which is not FRA (Hiramoto et al., 2000) while the Netrin receptor expressed by the incoming photoreceptor is FRA itself (Timofeev et al., 2012). Unlike DRL, FRA at the lamina target site requires its cytoplasmic domain for its own proper localization and function in localizing Netrin (Hiramoto et al., 2000). No evidence that FRA needs to be proteolytically-processed for its roles in either

tissue has been presented. Strikingly, a recent report of the structure of Netrin complexed with two of its receptors revealed that Netrin has two distinct receptor binding sites (Xu et al., 2014). Wnt protein binding has been shown to oligomerize the Frizzled and LRP co-receptors expressed on the same cell surface likely by binding to both receptors (Cong et al., 2004). Existing biochemical and structural data, however, do not explicitly address whether monomeric Wnt proteins can simultaneously bind to two receptors as does Netrin or whether Wnts act in complexes containing multiple Wnt proteins such as aggregates or by being displayed on the surface of exosomes (Beckett et al., 2013; Gross et al., 2012). Nonetheless, such a ligand-dependent *trans*-interaction between two Wnt receptors on different cells, as we have shown here for DRL and DRL-2 has not been previously reported to our knowledge.

The regulation of axon guidance receptors by proteolytic processing has also been documented for several axon guidance molecules, although in most cases only *in vitro* data are available (reviewed in (Bashaw and Klein, 2010)). Extracellular domains of transmembrane proteins, including cell surface signaling receptors, are often shed by regulated intramembrane proteolysis (reviewed in (Brown et al., 2000)) to effect extracellular roles or simply as a byproduct of a requirement to release the intracellular domain. In most cases proteolytic processing triggers cytoplasmic signaling pathways. The interaction of membrane-bound Ephrin ligand with the Eph receptor (Hattori et al., 2000) provides an example of the role of a ligand-bound extracellular domain superficially similar to that of WNT5/DRL ECD. Regulated proteolytic cleavage of the membrane-bound ligand enhances growth cone retraction. A more recent study of the role of EphA4 receptor cleavage during spinal motor neuron guidance demonstrated that EphA4 cleavage in the mesenchymal target is required to allow the target-derived ephrinA ligand to interact with EphA4 present on the axon (Gatto et al., 2014). Cleavage-resistant EphA4 sequesters target-derived ephrinA preventing it from repulsing the axon. In contrast, our data support a model where WNT5-binding and cleavage of the DRL ECD is required to facilitate WNT5 signaling through DRL-2. Another clear difference between Eph and DRL mechanisms is that, unlike ephrin, WNT5 is a secreted non-membrane bound protein. Finally, although the individual steps, ligand-localization and ectodomain shedding, that we have demonstrated to be necessary for DRL's role in a axon guidance have precedents, to our knowledge this combination of steps has not been previously reported for an axon guidance receptor.

The DRL ECD may act similarly to the secreted Wnt-binding signaling modulators such as the sFRPs (secreted Frizzled-related proteins) and Dickkopf proteins (reviewed in (Cruciat and Niehrs, 2013)). However, the difference between WNT5 bound to membrane-anchored DRL versus to a secreted Wnt-modulatory protein is the specificity of their spatial localization. DRL-bound WNT5 could provide a localized repulsive cue to guide axons, while it is unlikely

that a widely-expressed freely-diffusing secreted protein could provide a directional signal. Is this axon guidance mechanism conserved? *Drosophila* expresses three Ryks, however, all other higher eukaryotes express only one. Our finding that *drl* can rescue the *drl-2* mutant phenotype when expressed in MB neurons (data not shown) indicates that a single Ryk expressed in axons and structures adjacent to them would suffice to guide them. This is further supported by the invariant conservation of the TBC site in all Ryks. Strikingly, DRL's TBC site is also required for its role during embryonic pathfinding (Petrova et al., 2013) where its cytoplasmic domain, and hence likely signal transduction, is required (Petrova et al., 2013; Yoshikawa et al., 2001). This indicates that cleavage at the juxtamembrane site, likely in addition to an intramembrane cleavage, is possibly necessary for receptor endocytosis or freeing the intracellular domain for transport to the nucleus, the latter having been reported for mammalian Ryk (Lyu et al., 2008).

In both the *Drosophila* embryonic nerve cord and in the developing MBs, localized WNT5 acts as a Ryk guidance cue. WNT5 localization is, however, achieved by two different mechanisms. During embryogenesis, WNT5 is preferentially expressed by posterior commissural neurons since DRL represses *wnt5* transcription in anterior commissural neurons (Fradkin et al., 2004). In the MBs, we have shown that WNT5 is localized in a para-MB pattern via the interaction of WNT5 with extrinsic DRL. We also demonstrated that the DRL ECD is shed and forms a ternary complex with WNT5 and the axon-intrinsic DRL-2 receptor. The capture and localization of a widely-expressed ligand to the surfaces of cells nearby axons to guide those axons as well as the formation of a ternary complex by a shed ECD, the ligand and an axon-intrinsic receptor may likely prove to be conserved developmental strategies.

EXPERIMENTAL PROCEDURES

Drosophila stocks

All crosses were maintained on standard culture medium at 25°C. The following alleles were used, *lio2* , drl^{R343} , $wnt5^{400}$ and $drl\text{-}2^{E124}$. Except where otherwise stated, all alleles have been described previously (http://flystocks.bio.indiana.edu/). To examine the effects of homozygosity for drl^{null} and for $drl\text{-}2^{null}$, we generated lio^{2} /drl^{R343} and $drl\text{-}2^{E124}$/Df(2R)Exel8057 animals, respectively, to minimize the effects of the genetic backgrounds of homozygosity for the individual alleles.

Brain dissection, MARCM mosaic analysis and visualization

Pupal brain dissection and immunostaining

Brains were dissected and treated as previously described (Timofeev et al., 2012). They were incubated in PBS with 0.5% Triton X-100 (PBT) and 5% normal horse serum (blocking solution) at room temperature for 30 minutes, followed by overnight incubation at 4°C with

primary antibodies diluted in blocking solution. Brains were then washed three times in PBT for 20 min, followed by 30 min in the blocking solution, and then addition of the secondary antibodies with incubation for 2 h at room temperature. Brains were then washed in PBT for 2 h and were mounted with Vectashield (Vector Laboratories). Rabbit anti-DRL, guinea pig anti-DRL-2, mouse anti-MYC and rabbit anti-WNT5 were pre-absorbed with 10 *y w 67c23* heads and thoraxes in the blocking solution at the final dilution (1:2000, 1:1000, 1:1000 and 1:150, respectively). The pre-absorbed anti-DRL-2 was also pre-absorbed a second time using *drl-2*^{null} mutant 48 h APF brains. The following secondary antibodies were used at a dilution of 1:500: anti-rabbit Cy3 (Jackson ImmunoResearch) and anti-guinea pig Cy3 (Jackson ImmunoResearch). Anti-Fasciclin II (mAb 1D4 from DSHB) was used at 1:50 dilution followed by anti-mouse Cy3 (Jackson ImmunoResearch) at a dilution of 1:300. For 24 h and 48 h APF anti-WNT5 immunostaining, dissected brains were incubated with anti-WNT5 (1:150) in PBS at 4°C for 2 h 30 min, washed in 1x PBS and fixed in PLP for 1 h at room temperature, then the protocol above was followed.

Adult brain dissection and immunostaining

Fly heads and thoraxes were fixed for 1 h in 3.7% formaldehyde in PBS. Brains were dissected in PBS. They were then treated for immunostaining as previously described (Boulanger et al., 2011; Lee and Luo, 1999). Primary antibody used was anti-Fasciclin II (mAb 1D4 from DSHB) at 1:50 dilution followed by anti-mouse Cy3 (Jackson ImmunoResearch) at 1:300.

Presence of α lobes

An α lobe was considered as present when either an apparently complete wild-type lobe (more than 80% of the cases) or thinner lobe (less than 20% of the cases) with an estimated width ≥ 40% of that of wild-type lobe width, was seen using the FIJI software.

MARCM Clonal analysis

To generate clones in the MB, we used the Mosaic Analysis with a Repressible Cell Marker (MARCM) technique (Lee and Luo, 1999). For single- and two-cell clones, 48 h APF pupae were heat-shocked at 37°C for 15 min. For neuroblast clones, first instar were heat shock at 37°C for 1 h. Adult brains were fixed for 15 min in 3,7% formaldehyde in PBS before dissection and staining. We used the term “visualization MARCM clones” when homozygous mutant clones were examined in a homozygous mutant background and “regular MARCM clones” when homozygous mutant clones were examined in a heterozygous background.

Axon commissure switching assay

The assay was performed essentially as described previously (Callahan et al., 1995) except *UAS-mCD8-GFP* was included to allow visualization of the *eg*$^{+}$ axons by staining with anti-GFP (Roche) and anti-CD8 (Life Sciences)

Quantitative qRT-PCR

RNA from 3rd instar brains was prepared using RNAeasy (Qiagen) according to the manufacturer's specifications and reversed transcribed using the IScript cDNA Synthesis Kit (Biorad). cDNA was amplified on a CFX384 Real Time PCR System (BioRad) using Power SYBR Green PCR Master Mix (Applied Biosystems) and intron-spanning primers hybridizing to the *wnt5* gene and *RP49* control. *wnt5* RNA levels are reported in arbitrary units normalized to *RP49* levels

Microscopy and image processing

Images were acquired at room temperature using a Zeiss LSM 780 laser scanning confocal microscope (MRI Platform, Institute of Human Genetics, Montpellier, France) equipped with a 40x PLAN apochromatic 1.3 oil-immersion differential interference contrast objective lens. The immersion oil used was Immersol 518F. The acquisition software used was Zen 2011. Contrast and relative intensities of the green (GFP) and magenta (Cy3) channels were processed with Imaris and FIJI software. The angles between α and β axon branches were measured using the angle tool of FIJI software.

Constructs, transgenic flies, transfections, immunoprecipitation and immunoblotting

HA-tagged actin promoter-driven wild-type *drl-2* and *drl-2* lacking its WIF domain (*ΔWIF*) and MYC-tagged UAS wild-type *drl* and *drl-2* and their mutants lacking the cytoplasmic or WIF domain 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%20 vectors.html). Fc-DRL ECD was constructed by appending DRL ECD-coding sequences in frame to the FC ORF (kindly provided by John Thomas) and the fusion protein ORF was subsequently transferred into the pDEST10 baculovirus vector. Recombinant Fc-DRL ECD expressing baculovirus were generated using the Bac-to-Bac system (Invitrogen) and FcDRL-ECD protein was purified by Protein A chromatography from infected Sf9 cell culture supernatants. All constructs were verified by DNA sequencing. S2 cell transfections were performed using Effectene (Qiagen). MYC-tagged *drl-* and *drl-2* expressing transgenic fly lines (UASs) were generated by BestGene and MYC expression-matched lines (DRL species) and transgenes inserted into the same *attP* site (DRL-2) were subsequently used. Western blot analyses indicate that the *UAS-drl* (WT, Δcyto and ΔWIF) species are similarly expressed when driven by *elav-GAL4* in the 3rd instar larval brain. Lysates were prepared using a high-stringency buffer (50mM Tris-HCl, pH 8.0; 150 mM sodium chloride ; 1% NP40; 0.5% sodium deoxycholate; 0.1% SDS; 0.2 mM sodium orthovanadate 10 mM sodium fluoride; 5 mM sodium pyrophosphate; 0.4 mM EDTA; 10% glycerol) containing protease inhibitors (Roche). For the immunoblot presented in Figure S7, lysates were prepared from

10 L3 brains homogenized in 1X NuPAGE LDS sample buffer (Invitrogen) containing 0.5 M DTT. Immunoprecipitations were performed using rabbit anti-HA (AbCam) and mouse anti-human Fc (Jackson Immunoresearch). Immunoblots, prepared by standard procedures, were incubated with mouse anti-HA (Sigma), rabbit anti-WNT5 (Fradkin et al., 2004) and mouse anti-MYC (DSHB). Anti–*Drosophila* ribosomal protein P3 (Kelley et al., 2000), kindly provided by M. Kelley and anti-mouse a-TUBULIN (Sigma) were used to control for equivalent gel loading. Bound multiple-label grade HRP-conjugated secondary antibodies (Jackson ImmunoResearch) were detected with enhanced ECL reagent (GE Healthcare).

Statistics

Comparison between groups expressing a qualitative variable was analyzed for statistical significance using the $\chi 2$ test. Comparison of two groups expressing a quantitative variable was analyzed using the two-tailed Student's t test. Comparison of the distribution of the ratios was analyzed using the Wilcoxon rank sum test. Values of $P < 0.05$ were considered to be significant.

Supplemental Information

Supplemental Information includes Supplemental Information for Figure 1-3 and 5, seven figures and two tables.

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SUPPLEMENTARY MATERIAL

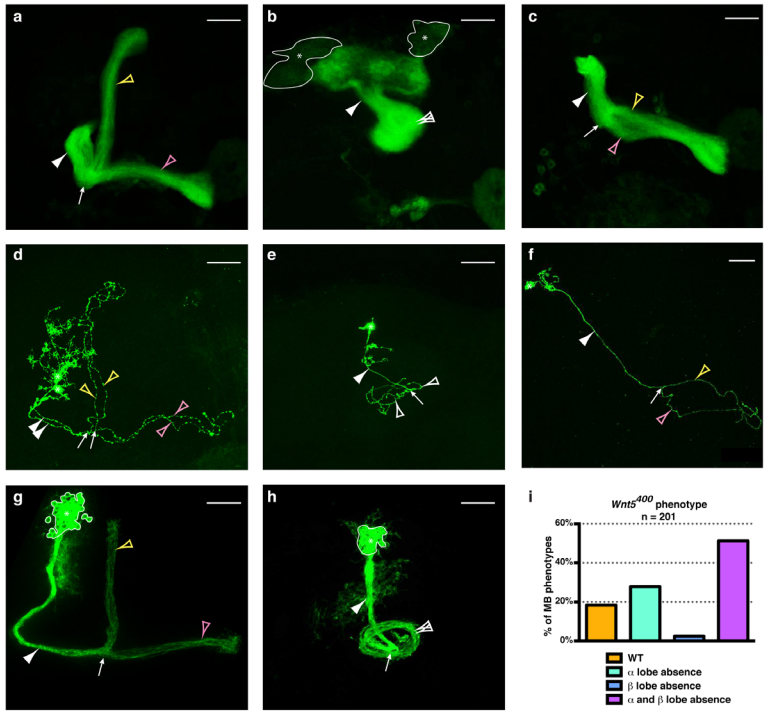


Figure S1, related to Figure 1. WNT5 is involved in α branch axon guidance

All the images shown are of *wnt5*^{null} individuals expressing *mGFP* driven by *c739-GAL4* to specifically label adult αβ neurons. An asterisk indicates the neuronal cell body, a white arrowhead and a white arrow represent the peduncle and the branch point, respectively. The α lobe or branch (yellow arrowhead) projects vertically and the β lobe or branch (pink arrowhead) projects toward the midline. (A, D, and G) In a *wnt5*^{null}, when total MBs are visualized 18% appear wild-type (A). As representative clones of this wild-type class; a two-cell clone (D) and a multiple cell clone (G) are shown. (B, E, and H) 51% of the MBs display a ball-shaped phenotype (B), due to the misguidance of both the α and β axons (empty white arrowhead) as shown in a single neuron clone (E) or in a multiple cell clone (H). In this class, β branch axons were also misguided indicating that *wnt5*, possibly via another receptor than *drl,* might be involved in the β branch guidance. (C, F) 28% of the MBs lack the dorsal lobe (C) which is likely caused by misguidance of the α axons, as is shown in the single cell clone (F). Note that this panel is also presented as Figure 1E. (I) Graph representing the distribution of MB phenotypes in *wnt5*^{null} hemizygous males. Genotypes: (A, B, C, and I) *w*^{1118} *wnt5*^{400} *FRT19A/Y; c739-GAL4 UAS-mCD8GFP/+.* (D-H) *w*, hs-FLP, tubP-GAL80, wnt5*^{400} *FRT19A/w*^{1118} *wnt5*^{400} *FRT19A; c739-GAL4 UAS-mCD8GFP/UAS-mCD8GFP*. Scale bars represent 30 μm. Images are composite confocal stacks. See also Table S2.

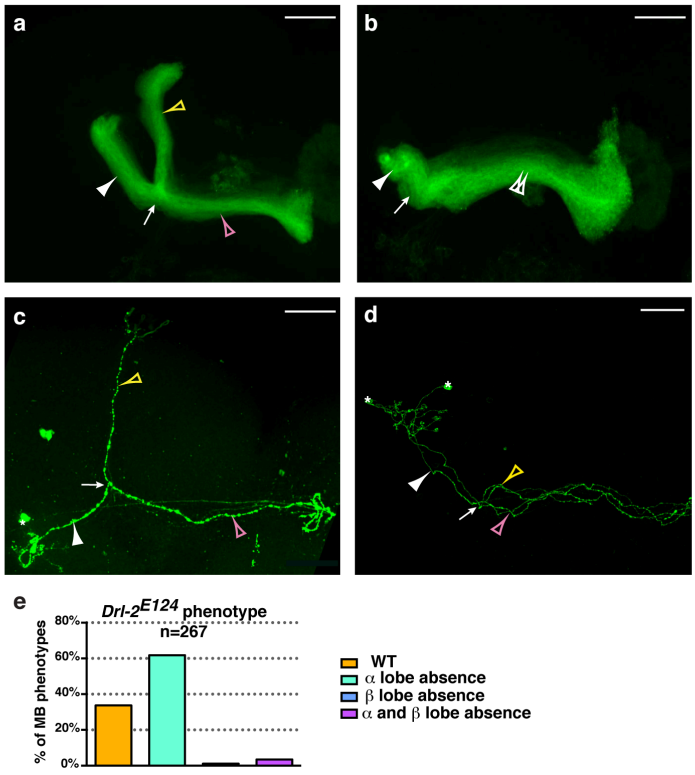


Figure S2, related to Figure 1. DRL-2 is involved in α branch axon guidance

All the MB neurons shown are from *drl-2^{null}* individuals. *GFP* expression driven by *c739- GAL4* specifically labels the adult αβ neurons. An asterisk indicates the neuronal cell bodies, a white arrowhead and a white arrow represent the peduncle and the branch point, respectively. In the *drl-2^{null}* mutant, ~ 34% of MBs appear wild-type when total MBs were visualized (A). A representative single cell clone of this class is shown (C). More than 60% of the MBs lack the dorsal lobe which is likely caused by α branch misguidance as is observed in a two-neuron clone (D). Note that this panel is also presented as Figure 1F. (E) Graph representing the distribution of MB phenotypes in *drl-2^{null}* individuals. Genotypes: (A, B, and E) *w^{1118}/y w 67c23; c739-GAL4 UAS-mCD8GFP, Drl-2^{E124}/Sp UAS-mCD8GFP, Df(2R)Exel8057*. (C and D) *w*, hs-FLP122, tubP-GAL80, FRT19A/w sn FRT19A; c739-GAL4 UAS-mCD8GFP, Drl- 2^{E124}/UAS-mCD8GFP, Df(2R)Exel8057*. Scale bars represent 30 μm. Images are composite confocal stacks. See also Table S2.

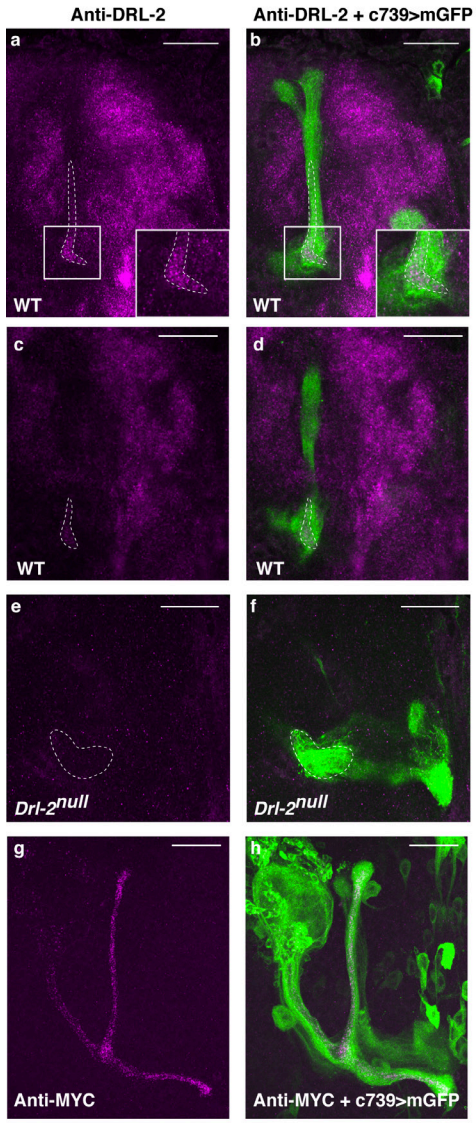
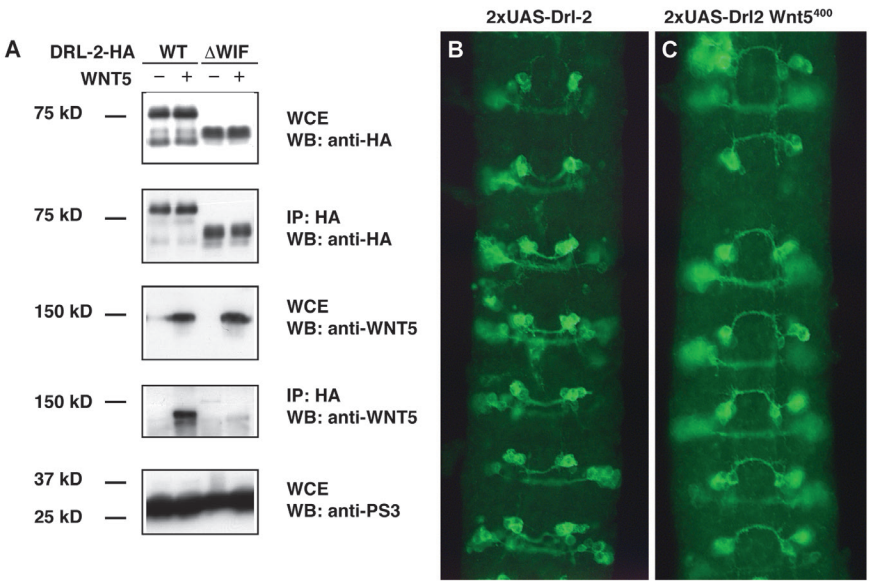


Figure S3, related to Figure 2. DRL-2 is expressed in the developing MB αβ neurons

All images shown are of 48 h APF *c739-GAL4 UAS-mCD8GFP* brains. DRL-2 (magenta) was expressed in the αβ axons (dotted area) of the wild-type MB (A, C). The overlap between MB neuron-expressed GFP (green) and DRL-2 is shown (B, D). Inserts in the lower right hand part of A and B panel show an enlargement of the area indicated in the main images by a white square. (E and F) 48 h APF *c739-GAL4 UAS-mCD8GFP drl-2^{null}* brain. DRL-2 was undetectable in a *drl-2^{null}* brain showing the specificity of the antibody. (G and H) 48 h APF *c739-GAL4 UAS-mCD8GFP; UAS-Drl-2-MYC* brains. MYC (magenta) was expressed in the αβ axons and MYC staining is apparently equivalent in the α and β branches. MYC expression is restricted to the core region and not in all of the GFP (green)-expressing αβ axons. This indicates that the DRL-2-MYC protein may be actively degraded and therefore is present only in the most recently extended axons. Genotypes: (A-D*) y w 67c23/y w 67c23; c739- GAL4 UAS-mCD8GFP/+*. (E and F) *y w^{67c23}/y w^{67c23}; c739-GAL4 UAS-mCD8GFP, Drl- 2^{E124}/Df(2R)Exel8057*. (G and H) *y w^{67c23}/y w^{67c23}; c739-GAL4 UAS-mCD8GFP/+; UAS-Drl- 2-MYC/+*. Scale bars represent 30 μm. Identical confocal settings were used in (A-F) images. Images are composite or single (C and D) confocal stacks.



D

Genotype	% No switch	% Semi-switch	% Switch	n
2xUAS-Drl-2 / Y ; UAS-mCD8-GFP / + ; eg-GAL4 / +	0.5	6	93	376
2xUAS-Drl-2 Wnt5400 / Y ; UAS-mCD8-GFP / + ; eg-GAL4 / +	99	1	0	255

Figure S4, related to Figure 2. DRL-2 interacts with WNT5

(A) DRL-2 interacts with WNT5 via its WIF domain. *Drosophila* S2 cells were transiently transfected with the indicated expression constructs, lysates were immunoprecipitated (IP) with antibody specific to tagged DRL-2 (anti-HA) and subsequently immunoblotted (WB) with anti-WNT5 to detect co-immunoprecipitation of WNT5. Expression of DRL-2 and WNT5 was confirmed by immunoblotting of the whole cell extract (WCE). DRL-2 lacking its WIF domain (ΔWIF), unlike the wild-type receptor, does not interact with WNT5. (B-D) DRL-2 acts as a Wnt5-dependent axon repulsion receptor. Two copies of *UAS-Drl-2* in the wild-type (B) versus w*nt5*^{400} (C) backgrounds were driven by *eg-GAL4* in the presence of *UAS-mCD8-GFP* to visualize the *eg+* axons. Representative pictures and the quantitation (D) are shown.

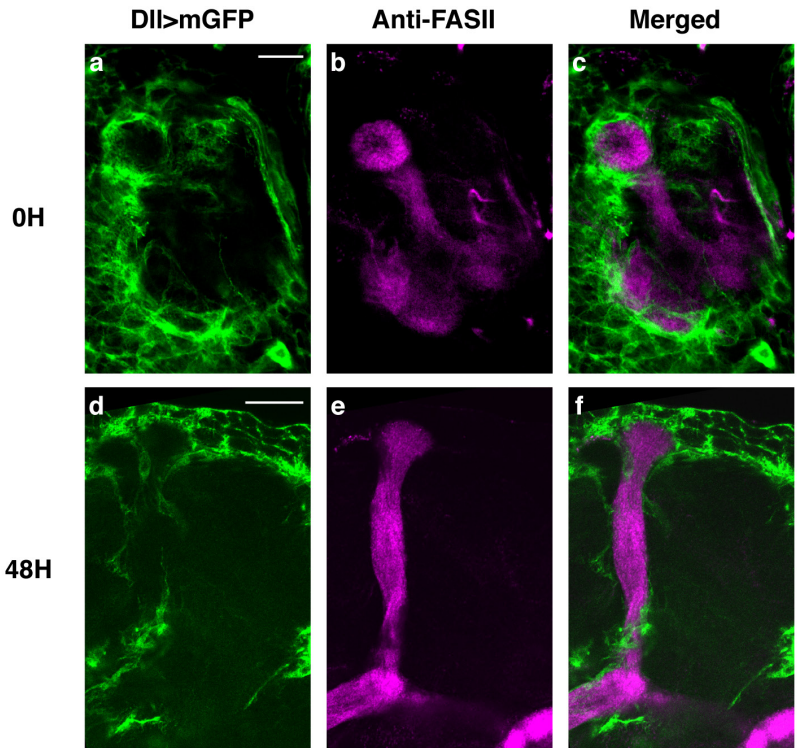


Figure S5, related to Figure 3. Close proximity of the Dll-GAL4 expressing neurons and the developing MBs

(A-C) 0 h APF brain *UAS-mCD8GFP Dll-GAL4* brain. (D-F) 48 h APF *UAS-mCD8GFP Dll-GAL4* brains. Green represents *Dll-GAL4* driven *GFP* and magenta corresponds to antibody against FASII showing the MB lobes. The spatial relationship between the *Dll-GAL4+* neurons and the MBs was examined from 0 hours to 48 hours APF brains and revealed a close proximity of the *Dll-GAL*-expressing neurons to the developing MBs. Genotype: *y* w^{67c23}*/y* w^{67c23}*; UAS-mCD8GFP Dll-GAL4 /+*. Scale bars represent 20 µm. Each image is a single confocal section.

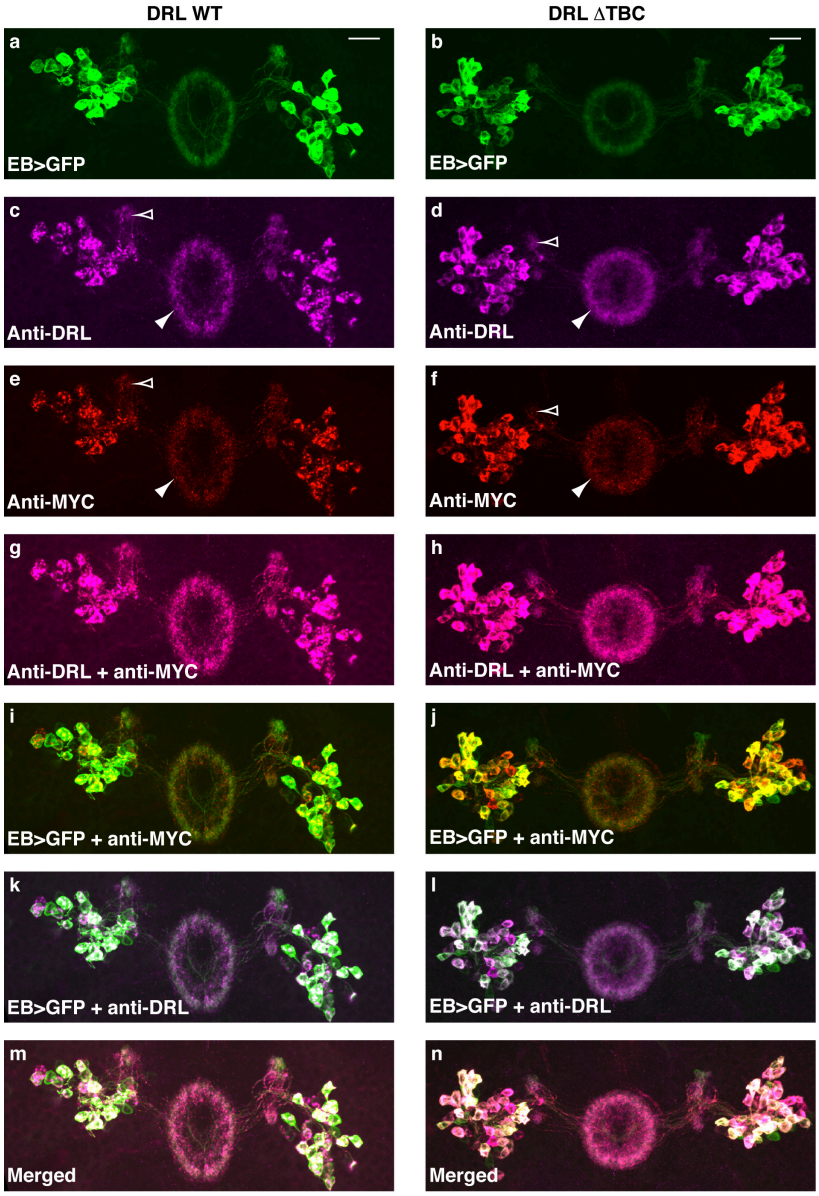


Figure S6, related to Figure 6. Sub-cellular specificity of *UAS-drlΔTBC-MYC* transgene expression

All images shown are of 48 h APF *drl^{null}, EB + c232-GAL4* brains. Green represents ellipsoid-body *GAL4*-driven *GFP* (*EB-GAL4 + c232-GAL4*), magenta indicates anti-DRL antibody and red corresponds to antibody against MYC. The stainings were done in a *drl^{null}* mutant background to avoid visualizing endogenous DRL expression. The ellipsoid body, which is not visibly affected by the lack of DRL, was used as a reference point for the localization of the transgene-driven proteins. The *UAS-drlΔTBC-MYC* transgene expression (B, D, F, H, J, L, and N) appeared very similar, if not identical, to that of the wild-type transgene (A, C, E, G, I, K, and M), either at the dendrite (empty arrowhead) or at the axon (arrowhead) levels. Genotypes: DRL WT: *UAS-drl WT-MYC/y w^{67c23}; drl^{R343}/lio^{2} UASmCD8GFP; (EB + c232)-GAL4/+*. DRL ΔTBC*: y w^{67c23}/y w^{67c23}; drl^{R343}/lio^{2} UASmCD8GFP; (EB + c232)-GAL4/2xUAS-drlΔTBC-MYC.* Scale bars represent 20 µm. Images are composite confocal sections

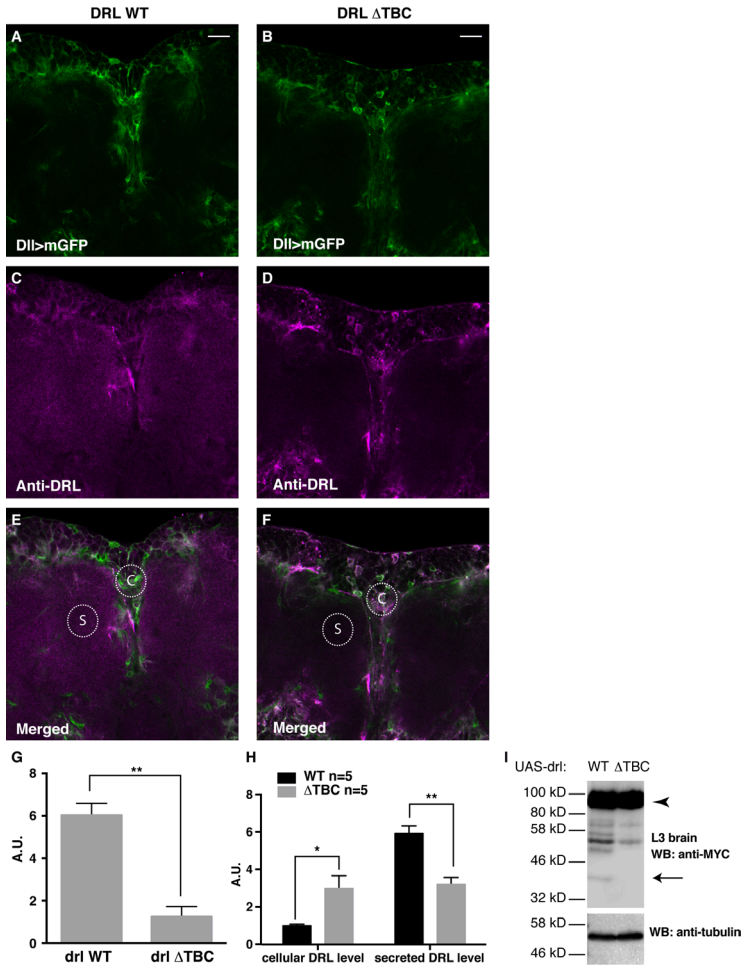


Figure S7, related to Figure 6. DRL ECD diffusion throughout the brain is significantly suppressed when the TBC site is mutated

(A-F) All images shown are of 48 h APF *drl*^{null} *UAS-mCD8GFP Dll-GAL4* brains. Green represents *Dll-GAL4*-driven GFP and magenta indicates anti-DRL antibody. (G) Ratio of the shed to cell membrane-associated DRL in *UAS-drl WT* (WT) compare to *UAS-drlΔTBC* (ΔTBC) calculated from the quantitation shown in (H) **: P < 0.01 (Wilcoxon rank sum test). (H) Quantitation of the intensity of the DRL signal in arbitrary units (A.U.). Intensity measurements of DRL were made with the Plot Profile tool of the FIJI software using identical settings for the two samples and normalized to GFP expression. Cell-associated DRL was measured in the region "C" and the shed DRL ECD in the region "S" shown in E and F. The data were subsequently analyzed using Prism software to calculate the means +/- SEM. n=5 brains analyzed in each case. There is more cell-associated DRL in the ΔTBC brain than in the WT brain because there are two ΔTBC and only one WT transgenes *: P < 0.05 (t test). Nevertheless, there is more secreted DRL in the WT brain than in the ΔTBC brain **: P < 0.01 (t test). Thus, DRL ECD diffusion from the DRL-expressing cells requires a wild-type TBC site. Genotypes: *UAS-drl WT-MYC/y w*^{67c23}*; drl*^{R343}*/lio*^{2} *UAS-mCD8GFP Dll-GAL4*. y *w*^{67c23}*/y w*^{67c23}*; drl*^{R343}*/lio*^{2} *UAS-mCD8GFP; Dll-GAL4; 2xUAS-drlΔTBCMYC/+*. (I) Western blot analysis of *elav-GAL4* L3 brain lysates (10 brains/lane) expressing either 2x*UAS-drl-Myc* (drl WT) or 2x*UAS-drlΔTBC-Myc* (drl ΔTBC). Anti-MYC revealed a dominant band of ~90 kD (arrowhead) and a small band of ~40 kD (arrow). The dominant band corresponds to full length DRL. The small band corresponds to the expected size of the carboxyterminal region of the protein liberated by cleavage at the TBC site present in drl WT but not in drl ΔTBC. Scale bars represent 20 μm. Images are composites of 10 confocal sections.

Table S1, related to Figure 1. Quantitation of neuroblast and 2-cell/single-cell clone phenotypes in the WT and *drl* mutant

Clones		WT	α mutant		α and β guidance	WT+ MLC	α mutant + MLC		β guidance	n=
			guidance	growth			guidance	growth		
WT	NB	78% 32	0% 0		0% 0	22% 9	0% 0		0% 0	41
	2/1-cell	90% 35	0% 0	0% 0	0% 0	10% 4	0% 0	0% 0	0% 0	39
***drl*^{null}**	NB	4% 1	22% 6		0% 0	0% 0	74% 20		0% 0	27
	2/1-cell	0% 0	6% 2	0% 0	0% 0	3% 1	91% 33	0% 0	0% 0	36
***drl*^{hypo}**	NB	57% 30	4% 2		0% 0	29% 15	10% 5		0% 0	52
	2/1-cell	34% 4	8% 1	8% 1	0% 0	42% 5	8% 1	0% 0	0% 0	12

WT: wild-type, NB: neuroblast clones, 2/1-cell: 2-cell/single-cell clones, MLC: midline crossing, n: number of clones analyzed. The number in blue in the lower right-hand corner of the cells corresponds to the number of clones analyzed in each category.

Table S2, related to Figure 1 and to Figures S1 and S2. Quantitation of neuroblast and 2-cell/single-cell clone phenotypes in WT, *drl*, *Wnt5* and *Drl-2* mutants

Clones		WT	[α] guidance	[α] growth	[α and β] guidance	[β] guidance	n=
WT	NB	100% 41	0% 0		0% 0	0% 0	41
	2/1-cell	100% 39	0% 0	0% 0	0% 0	0% 0	39
***drl*^{null}**	NB	4% 1	96% 26		0% 0	0% 0	27
	2/1-cell	3% 1	97% 35	0% 0	0% 0	0% 0	36
***Wnt5*^{null}**	NB	29% 5	47% 8		24% 4	0% 0	17
	2/1-cell	31% 15	26% 12	17% 8	26% 12	0% 0	47
***Drl-2*^{null}**	NB	25% 30	70% 83		3% 4	2% 2	119
	2/1-cell	42% 31	50% 37	5% 4	1.5% 1	1.5% 1	74

WT: wild-type, NB: neuroblast clones, 2/1-cell: 2-cell/single-cell clones, n: number of clones analyzed. The number in blue in the lower right-hand corner of the cells corresponds to the number of clones analyzed in each category. The WT and *drl*^{null} [α] numbers correspond to those in Table S1 under [α] and [α] + MLC but are here now pooled because MLC is not taken into account in this table.

4

WNT5 Interacts with the Ryk Receptors Doughnut and Derailed to Mediate Muscle Attachment Site Selection in *Drosophila melanogaster*

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ABSTRACT

In recent years a number of the genes that regulate muscle formation and maintenance in higher organisms have been identified. Studies employing invertebrate and vertebrate model organisms have revealed that many of the genes required for early mesoderm specification are highly conserved throughout evolution. Less is known about the molecules that mediate the steps subsequent to myogenesis, e. g. myotube guidance and attachment to tendon cells. We use the stereotypic pattern of the *Drosophila* embryonic body wall musculature in genetic approaches to identify novel factors required for muscle attachment site selection. Here, we show that *wnt5* is needed in this process. The lateral transverse muscles frequently overshoot their target attachment sites and stably attach at novel epidermal sites in *wnt5* mutant embryos. Restoration of WNT5 expression in either the muscle or the tendon cell rescues the mutant phenotype. Surprisingly, the novel attachment sites in *wnt5* mutants frequently do not express the Stripe (SR) protein which has been shown to be required for terminal tendon cell differentiation. A muscle bypass phenotype was previously reported for embryos lacking the WNT5 receptor Derailed (DRL). *drl* and *wnt5* mutant embryos also exhibit axon path finding errors. DRL belongs to the conserved Ryk receptor tyrosine kinase family which includes two other *Drosophila* orthologs, the Doughnut on 2 (DNT) and Derailed-2 (DRL-2) proteins. We generated a mutant allele of *dnt* and find that *dnt*, but not *drl-2*, mutant embryos also show a muscle bypass phenotype. Genetic interaction experiments indicate that *drl* and *dnt* act together, likely as WNT5 receptors, to control muscle attachment site selection. These results extend previous findings that at least some of the molecular pathways that guide axons towards their targets are also employed for guidance of muscle fibers to their appropriate attachment sites.

INTRODUCTION

The establishment of the musculature in higher organisms is a multistep process involving myoblast specification and fusion, followed by guidance of the myotubes towards the muscle attachment sites (MAS) (reviewed in [1]). Final differentiation of both the muscle and the attachment sites is initiated when the multinucleated fiber attaches to the tendon cell. Intercellular communication between the myofiber and the tendon cells mediated by secreted or transmembrane proteins is essential to ensure a stable muscle attachment resistant to contraction-induced detachment (reviewed in [2]). Only a few molecules that regulate these different stages of muscle pattern formation have been identified so far, but most characterized factors show a remarkable degree of functional conservation between vertebrates and invertebrates. The *Drosophila* embryonic body wall musculature with its stereotyped pattern and amenability to genetic analysis has been an excellent model to unravel the cellular and molecular mechanisms underlying this process [2,3,4,5,6,7].

The *Drosophila* somatic musculature is established into a stereotypical segmentally reiterated pattern during embryonic development. Pattern formation starts at 7.5 hours after egg laying (AEL) and is completed 5.5 hours later when the muscle fibers form stable contacts with the epidermal tendon cells in the insects' exoskeleton (reviewed in [6]). Muscles persist through the larval stages until the pupal stage when they degenerate and are replaced by the adult musculature [8]. Initially, each embryonic somatic muscle fiber is formed by the fusion of a muscle founder cell and a number of fusion-competent myoblasts [9]. The fusion process creates multinucleated myofibers whose two leading edges subsequently migrate towards clusters of tendon cell progenitors in the epidermis [1,2,7].

The initial determination of the tendon cell progenitors in *Drosophila* is provided by segment polarity genes such as *wingless* (*wg*) and *hedgehog* that activate the early growth response (Egr)-like transcription factor Stripe (SR) in segmentally-reiterated clusters of epidermal cells [10]. Once SR is activated these cells become tendon cell progenitors and SR expression is both necessary and sufficient to promote muscle migration towards these cells [11,12,13]. However, final differentiation of the single selected tendon cell requires direct interaction with a muscle fiber (reviewed in [2]).

Upon muscle attachment, Vein, a neuregulin-like ligand secreted from muscle, accumulates at the muscle-tendon junctionto activate the Epidermal Growth Factor pathway only in the tendon cell progenitor that is contacted by the muscle fiber [14]. This signal maintains SR expression and results in the differentiation of the progenitor into a mature tendon cell. The precursor cells that are not contacted by a muscle fiber cease to express SR and do not differentiate into tendon cells. SR, in turn, induces the expression of both the Slit [15] and

Leucine-rich tendon-specific proteins [16]. These proteins then act as positive and negative guidance cues, respectively, for the muscle fibers. The final stage of tendon cell determination is defined by the association of αPS2/ βPS Integrin (at the muscle tip) with Thrombospondin (TSP; at the extracellular matrix of the tendon cell) mediating the formation of a myotendinous junction at the attachment site [17,18]. This junction withstands the mechanical forces that occur during larval locomotion.

Several proteins expressed in the muscle or tendon cells have been shown to control muscle guidance and attachment. The Roundabout (ROBO) protein which is expressed in a subset of muscle fibers, acts as the guidance receptor for Slit produced by the tendon cells [15]. ROBO and Slit interactions are also needed for guidance of axons across the *Drosophila* embryonic ventral midline in the central nervous system (CNS) [19,20]. The Kon-tiki (KON) protein is expressed on the tips of a subset of growing myotubes and is needed, in a pathway involving the Glutamate receptor binding protein protein, for their guidance to the correct attachment site [21]. The tendon cell-derived ligand of KON remains to be identified.

Another molecule shown to act as a guidance receptor both for axons and myotubes is DRL (reviewed in [22]). It was initially identified in screens for genes required for axon pathfinding in the *Drosophila* embryo [23,24] and for learning and memory in the adult [25]. It was also shown to be required for the correct tendon cell attachment of a subset of the lateral transverse muscles (LTMs 21–23) [24].

DRL is a member of the conserved transmembrane receptor tyrosine kinase Ryk family [26,27] which bears an extracellular Wnt-binding WIF domain and an intracellular tyrosine kinase homologous domain. *C. elegans*, zebra fish and mammals have a single Ryk ortholog while three Ryk proteins, DRL, DRL-2 and DNT, are encoded in the fruit fly genome. DRL-2 and DNT share 35% and 60% amino acid identity, respectively, with DRL.

The *Drosophila* Wnt family member WNT5 acts as a ligand for DRL in the nervous system and both genes are required for the proper axon guidance leading to correct formation of the embryonic ventral cord commissures [28,29]. Wnt proteins are highly conserved secreted molecules that play roles in diverse signaling pathways acting during normal development and are perturbed during oncogenesis [30]. Wnt genes are also important for the development and function of the nervous system throughout the animal kingdom. They have roles in neuronal differentiation, axon extension, axon guidance and neural circuit assembly in both vertebrates and invertebrates (reviewed in [31,32]).

A number of at least partially distinct Wnt signaling pathways have been uncovered and, of these, the canonical Wnt pathway is most extensively studied (reviewed in [33]). Wnt binding

to the Frizzled and LRP co-receptors results in the accumulation of bcatenin in the cytosol and its translocation to the nucleus where it activates TCF/LEF-dependent transcription of target genes. There are also alternative, non-canonical, Wnt pathways, e. g., the Ca^{2+} , Planar Cell Polarity (PCP) and Ryk pathways (reviewed in [22,30,34,35]). The least is known about the downstream signaling components of the Wnt pathway that acts via the RYK receptor.

Wnt/Ryk interactions are essential both in *Drosophila* and mammals for normal nervous system development (reviewed in [22,31]). Ryks have been characterized as ''dead''-tyrosine kinases based on the observation that they contain amino acid substitutions on sites in the potential kinase domain that would likely render them inactive as protein kinases [26,27]. However, it is still unclear whether Ryk's kinase domain might be active under certain conditions [36]. During mammalian brain neurogenesis, Ryk has been reported to be cleaved at a conserved sequence in the transmembrane domain resulting in the translocation of the cytoplasmic domain to the nucleus where it may regulate transcription [37]. WNT5/DRL signaling during formation of the *Drosophila* embryonic nervous system requires the Src family non-receptor tyrosine kinase, SRC64B [38], indicating that it may be involved in actively transducing an intracellular signal upon WNT5 binding to DRL.

Here, we present evidence that *wnt5* is required for appropriate attachment of a subset of embryonic muscles, the LTMs 21, 22 and 23. In animals lacking WNT5, myotubes overshoot their normal attachment sites and form ectopic contacts. This bypass phenotype was previously observed in *drl* mutant embryos that lack DRL which is normally expressed in the LTMs [39,40]. DRL was found to be required in the muscle fiber to rescue the guidance defect [39]. We find that WNT5, a secreted protein, expressed in either the tendon cells or the muscle fiber is sufficient to restore correct muscle attachment in *wnt5* mutant embryos. The majority of the novel ectopic attachment sites in *drl* and *wnt5* mutants do not express SR, indicating that it is not needed to form or maintain these novel muscle attachments to the body wall. The ectopic attachment sites persist through larval stages and accumulate Fasciculin2 (FAS2), a cell adhesion protein that is normally present in both the muscle and the tendon cells at the myotendinous junction. Finally, we generated a mutant allele of *dnt*, and found that it is also required for correct LTM attachment, while the third *Drosophila* Ryk family member, *drl-2*, is not.

RESULTS

The embryonic muscles 21 through 23 often overshoot their attachment sites when WNT5 is absent

The somatic mesoderm gives rise to a stereotypic segmentallyreiterated set of body wall muscles during *Drosophila* embryonic development. The muscle pattern of the abdominal

hemisegments A2 to A6 consists of 30 fibers that attach at both sides to tendon cells in the epidermis [6]. The DRL receptor is required for the correct attachment site selection by a subset of these muscles, the LTMs (muscles 21–23 [39]). In wild type embryos, the LTMs extend ventrally towards the dorsal border of muscle fiber 12 at which site they attach to a tendon cell in the epidermis (Figs. 1A and 1G). However, in the *drl* mutant, the LTMs frequently overshoot their target and extend further ventrally passed muscle 13 or 6 to muscle fiber 7 and attach to an ectopic epidermal attachment site ([39]; Figs. 1C and 1G). Usually, only one of the three muscles per hemisegment exhibits this phenotype.

DRL also serves as an axon guidance receptor for the WNT5 protein during embryonic ventral nerve cord commissure formation [29]. We wondered whether WNT5 possibly also acts during formation of the embryonic musculature. We therefore examined the morphology of the embryonic muscle pattern in *wnt5* mutants using an antibody against the Muscle Myosin protein which labels all somatic muscles (Materials and Methods). Most muscles develop normally and attach at their correct position to the body wall in $wnt5^{400}$ null mutant embryos. However, one or more of the muscle fibers 21, 22 and 23 extends far more ventrally than in the wild type to attach at the epidermis beyond the ventral border of muscle 13 in 17% of the hemisegments scored (Fig. 1B and Table 1). Occasionally, either muscle 6 or muscle 7 is absent or not correctly attached in these mutants, but we did not observe a correlation between the absence of muscles 6 or 7 and the bypass phenotypes of the LTMs. In the *drl* mutant, this bypass phenotype is more penetrant and is observed in 36% of the hemisegments, while this phenotype was never observed in wild type control embryos (Fig. 1C and Table 1; [39]). Since, the degree of myotube overextension varies somewhat; we scored a fiber as overshooting its target only when it extended ventrally and attached ectopically beyond muscle 13 at the end of embryogenesis (stage 17, 18 hours AEL).

The number of LTMs that bypass their normal attachment sites when DRL alone or when DRL and WNT5 are both absent are the same (Table 1), suggesting that DRL and WNT5 are in one pathway controlling attachment site selection of the LTMs. However, the significant difference in the numbers of overextended LTMs in *wnt5* mutants (17%) versus that of *drl* mutants (36%) indicates that DRL may bind multiple ligands to mediate muscle guidance. We therefore investigated whether two other *Drosophila* Wnt family members, Wnt4 and Wnt2, which are expressed in the epidermis or the mesoderm and for which mutant alleles exist, exhibit bypass phenotypes. In the *Wnt2, Wnt4* double mutant no overshooting occurs (Table 1) indicating that these Wnt proteins are unlikely to be involved in LTM guidance and attachment.

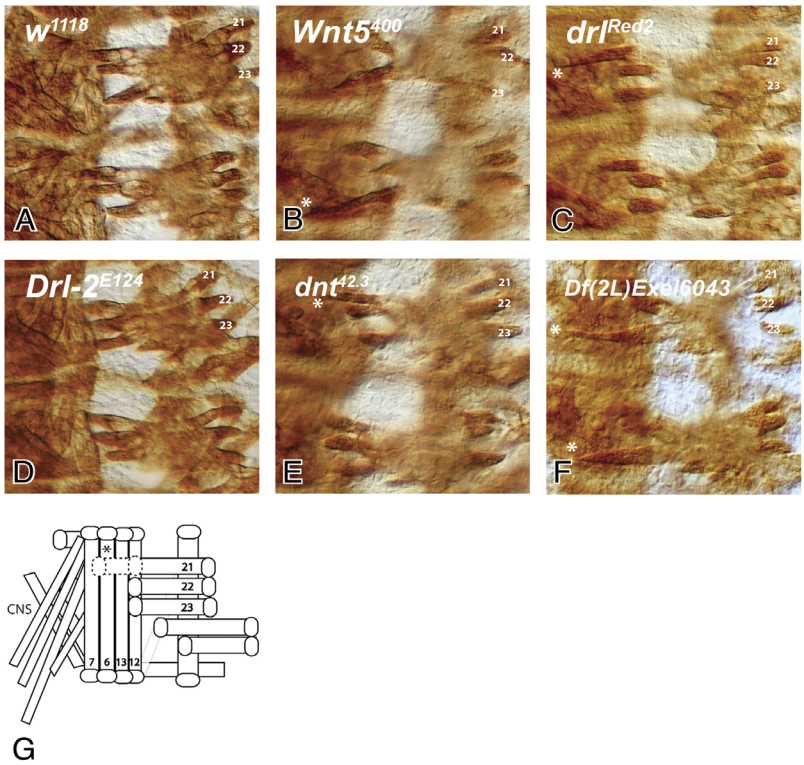


Fig 1 LTM muscle fibers 21, 22 and 23 frequently overshoot their attachment sites in *wnt5*, *drl* and *dnt* mutant embryos. Stage 16 embryo body wall muscle preparations stained with anti-Muscle Myosin are shown for the wild type control (w^{1118}) (A), $wnt5^{400}$ (B), drl^{Red2} (C), *drl- 2*^{E124} (D), $dnt^{42.3}$ (E) and *Df(2L) Exel6043* (F). Two hemisegments are displayed for each genotype with one set of muscles 21–23 labelled. In *wnt5*, *drl* and *dnt* mutants, LTMs frequently bypass their normal attachment at the epidermis at muscle 12 and instead extend ventrally beyond muscle 13 and attach at a novel epidermal site located close to muscle fiber 7. Df(2L)Exel6043 mutant embryos, that lack both DNT and DRL, display this phenotype in all hemisegments of the homozygous animals. The penetrance of these phenotypes is shown in Table 1. The muscle bypass phenotype is schematically shown in panel (G). The * indicates the location of the novel, ectopic epidermal attachment in panels (B), (C), (E), (F) and (G). Anterior is up and ventral is left.

Generation of a *dnt* mutant and establishing its role in LTM attachment site selection

In *Drosophila*, there are two other Ryk proteins in addition to DRL, DRL-2 and DNT (reviewed in [22]). Since *drl* mutants were previously reported to display a partially penetrant muscle bypass phenotype [39], we investigated whether the other two Ryk family members are also required for attachment site selection. DRL-2 mutants exhibit defects in axon guidance in the antennal lobe [41] and synaptic target specificity at the neuromuscular junction [42] and characterized *dnt* mutant alleles have not been reported. We did not observe any bypass phenotypes in the muscle pattern of *drl-2*^{E124} mutants (Fig. 1D; Table 1). Furthermore, the numbers of LTMs bypassing their normal attachment site does not increase beyond those observed in the *drl* mutant in the *drl*, *drl-2* double mutant (Table 1).

Table 1. LTM muscle bypass phenotype in Wnt5, drl and dnt mutants and restoration of attachment when Wnt5 is present in tendon cells or muscle.

Genotype	% hemisegments with bypassing LTM muscles	n=number of hemisegments counted
w^{1118}	0%	464
Wnt5^{400}	17%	330
Wnt5^{400}; UAS-*Wnt5*/+	12%	392
Wnt5^{400}; 24B-GAL4/+	11%	395
Wnt5^{400}; UAS-*Wnt5*/24B-GAL4	0%	391
Wnt5^{400}; mef2-GAL4/+	10%	375
Wnt5^{400}; UAS-*Wnt5*/mef2-GAL4	0%	334
Wnt5^{400}; sr-GAL4/+	20%	365
Wnt5^{400}; UAS-*Wnt5*/sr-GAL4	2%	394
Wnt5^{400}; UAS-*Wnt5*/ P{GawB}tey^{5053A}	13%	97
drl^{Red2}	36%	363
Drl-2^{E124}	0%	185
drl^{Red2},*Drl-2*^{E124}	35%	322
Wnt5^{400}/+; *drl*^{Red2}/+	0%	200
dnt$^{42.3}$	8%	363
drl^{Red2}/Df(2L)Exel6043	50%	355
drl^{Red2}/Df(2L)ED1231	50%	354
dnt$^{42.3}$/Df(2L)Exel6043	8%	385
Df(2L)ED1231 (*drl*, *dnt*)	94%	49
Df(2L)ED1231/+	0%	100
Df(2L)Exel6043 (*drl*, *dnt*)	96%	183
Df(2L)Exel6043/+	0%	100
Wnt5^{400};*drl*^{Red2}	36%	382
Wnt5^{400}; Df(2L)Exel6043/+	27%	185
Wnt5^{400}/+; Df(2L)Exel6043/+	16%	95
Wnt2^{L},*Wnt4*^{C1}	0%	190
Wnt5^{400};*Wnt2*^{L},*Wnt4*^{C1}	21%	374
Wnt5^{400} 3rd instar larvae	8%	192
drl^{Red2} 3rd instar larvae	16%	192

We used a P-element mobilization strategy starting with a P element, *P{EP}dntEP2158*, inserted 350 base pairs upstream of the *dnt* ATG translational initiator codon to generate mutants in this gene (Materials and Methods). A mutant allele of *dnt*, *dnt*$^{42.3}$, was obtained by imprecise excision of the P-element resulting in a deletion of 2322 base pairs uncovering most of the first exon of the *dnt* transcript. This deletion removes the ATG initiator codon, the first 15 amino acids of the Wnt-binding WIF domain and the 39 splice donor site (Fig. 2A). RNA *in situ* analyses of the *dnt*$^{42.3}$ line indicates that the mutant embryos have no detectable *dnt* transcript (compare Figs. 2B and 2C). Mutants are viable as homozygotes and analysis of their embryonic musculature indicates that a LTM bypass phenotype is observed in 8% of the hemisegments in the absence of *dnt* (Table 1), while the rest of the muscle pattern appears normal.

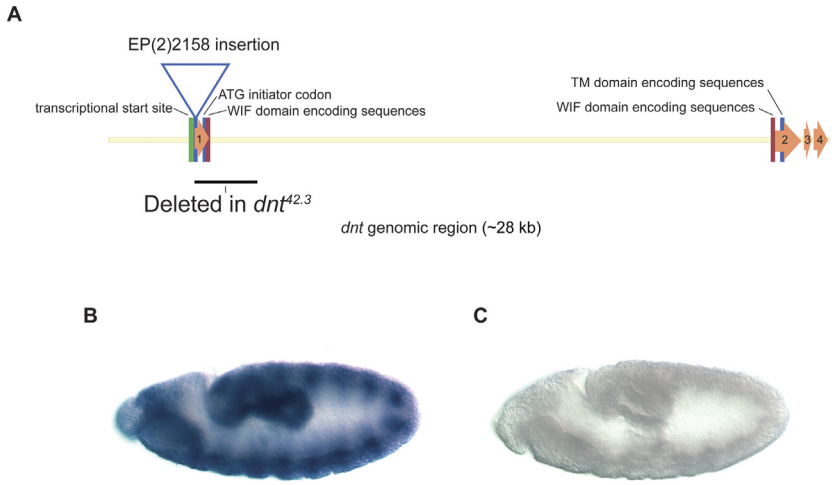


Fig 2 Generation of a *dnt* mutant by imprecise excision of an adjacent P-element. The *P{EP}dntEP(2)2158* insert, situated 350 bp upstream of the *dnt* ATG initiator codon, was mobilized by providing a source of transposase and imprecise excisions were selected for by loss of the w^+ marker in the P-element insert and molecularly characterized by sequencing cloned genomic PCR products (Materials and Methods). The locations of the insertion, the extent of the deletion in the $dnt^{42.3}$ allele and gene landmarks, e.g., exons and the location of the WIF encoding segments, are shown in (A). The $dnt^{42.3}$ allele displays dramatically decreased expression of *dnt* mRNA. Stage 11 wild type (B) and contemporaneously processed $dnt^{42.3}$ mutant (C) embryos hybridized with a dnt antisense probe are shown. Anterior is to the left and dorsal is up in panels (B) and (C).

WNT5 likely acts through DRL and DNT to mediate muscle attachment site selection

The proximity of the *drl* and *dnt* genes to each other precluded us from recombining a *drl* allele with $dnt^{42.3}$ to make a mutant line lacking both proteins. Therefore, in order to investigate whether *drl* and *dnt* function redundantly in LTM attachment site selection, we employed two independently generated deficiency chromosomes that uncover both genes. Deficiency *Df(2L)ED1231* has breakpoints at 37C5 and 37E3 while the smaller deficiency *Df(2L)Exel6043* at 37C5 and 37D7 (http://flybase.org/). Transheterozygotes bearing one copy of either deficiency and one copy of the *drl* mutation show an increase in penetrance of the bypass phenotype to 50% (as compared to 36% in the *drl* homozygous mutant), while $dnt^{42.3}$*/Df(2)Exel6043* embryos have a phenotypic penetrance of 8% (Table 1). Virtually all hemisegments display one or more bypassing LTMs (96%) in embryos homozygous for either deficiency (Fig. 1F, Table 1). These results indicate that *drl* and *dnt* likely act together to mediate appropriate attachment of the LTMs.

We generated animals bearing the *Df(2L)Exel6043* deficiency in the *wnt5* mutant background to determine whether *wnt5* interacts with *drl* and *dnt*. Neither *Wnt5* nor the deficiency heterozygotes display the muscle bypass phenotype, however females which

are heterozygous for *wnt5*, *drl* and *dnt* display a penetrance of 16% (Table 1). Furthermore, males which are hemizygous for *wnt5* and heterozygous for the deficiency display bypassing muscles in 27% of hemisegments (Table 1), a significant increase over the 17% observed in *wnt5* mutant homozygotes. Thus, we conclude that WNT5 likely signals via both DNT and DRL during muscle attachment site selection.

LTM ectopic attachments in the *wnt5* and *drl* mutants are stable and persist through the larval stages

We next addressed whether the ectopic attachments formed during embryogenesis are maintained to later stages of development. At stage 17 of embryonic development motoneurons innervating the body wall musculature become electrically active, enabling the larvae to use its muscles to push out through the vitelline membrane. The surface area of the larval musculature increases by approximately a 100-fold in the ensuing stages of larval development. We examined LTM attachment in *wnt5* and *drl* mutants in 3rd instar larvae just before puparation (5 days AEL) by staining the muscle fibers and their attachments with an antibody against the cell adhesion protein FAS2 (Materials and Methods). The larval LTMs were found to frequently extend beyond their normal attachment sites. 8% and 16% of hemisegments contained bypassing LTMs in the *wnt5* and *drl* mutant larvae, respectively (compare Fig. 3A with Figs. 3B and 3C; Table 1). These percentages are roughly half of what is observed at late embryogenesis suggesting that mutant larvae may have decreased survival rates due to defects in the nervous system [28] or other tissues. Clearly, however, a number of the ectopic attachment sites withstand the mechanical stress of hatching and the vigorous locomotion associated with larval feeding behavior.

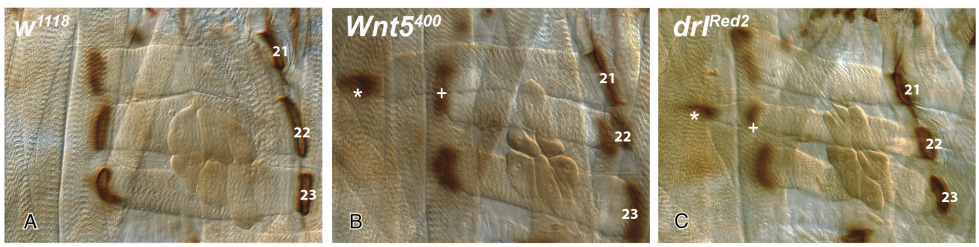


Fig 3 Muscle attachment defects persist from the embryonic to larval stages in *wnt5* and *drl* mutants. Third instar larval body walls of w^{1118} (A), $wnt5^{400}$ (B) and drl^{Red2} (C) mutant larvae are stained with anti-FAS2 (mAb 1D4). $wnt5^{400}$ larvae and drl^{Red2} larvae frequently bypass their normal attachment sites and extend ventrally where they form new stable attachments. The original and ectopic tendons cells are indicated by + and *, respectively. FAS2 protein is evident at both sites. The penetrance of the bypass phenotypes is indicated in Table 1. Anterior is up and ventral is left.

wnt5 protein and mRNA are present in LTMs and in tendon cells

To examine whether WNT5 is needed in either the muscle or the tendon cells for correct attachment we first determined the developmental patterns of WNT5 expression using anti-WNT5 antisera (Materials and Methods). WNT5 protein expression is first detected at stage 12 (approximately 10 hours AEL) in the CNS [28], throughout the epidermis with some accumulation in ventraland dorsal clusters of cells (Fig. 4A) and in the somatic mesoderm (Fig. 4B). This is the stage when founder cells fuse with myoblasts and generate the first extending myotubes. WNT5 can be detected in most muscle fibers, including the LTM muscle fibers 21 to 23, and in the tendon cells at early stage 16 when the individual muscle fibers are formed (Fig. 4C). WNT5 expression levels are significantly reduced at the end of embryonic development (stage 17) (Fig. 4D). A very similar temporal and spatial pattern of expression was observed for *wnt5* mRNA (Figs. 4E–H), suggesting that secreted WNT5 protein is present on or close to the cells in which it is produced. No *wnt5* protein or mRNA was detected in the $wnt5^{400}$ mutant embryos (data not shown; [28]).

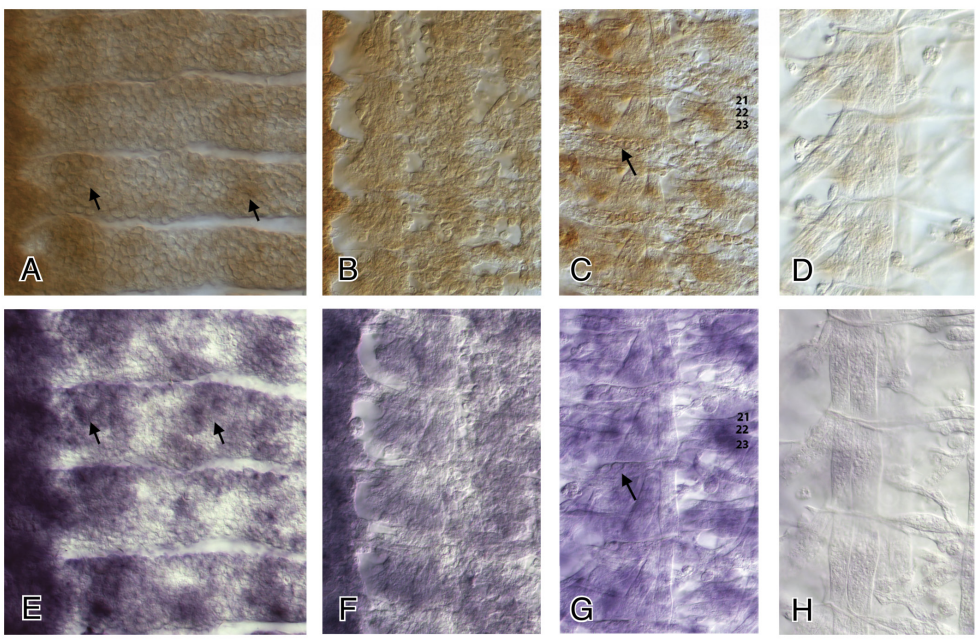


Fig 4 Wnt5 protein and mRNA expression domains in epidermis, muscle and tendon cells during embryonic development. WNT5 is predominantly expressed in subsets of neurons in the CNS from stage 12 onwards throughout embryonic development (data not shown; [28]). However, there is also strong expression from this stage onwards in the epidermis and the musculature. At stage 12, *wnt5* protein (A, B) and *wnt5* mRNA (E, F) expression is observed in the epidermis, most prominently in two clusters (arrows), and throughout the somatic mesoderm that will give rise to the body wall musculature. Later in embryonic development at early stage 16 WNT5 protein and *wnt5* mRNA are present in the attachment sites (arrows in panels C and G) and at low levels in most muscle fibers including the LTMs 21, 22 and 23 (C, G). At the end of embryonic development at late stage 17, *wnt5* protein (D) and *wnt5* mRNA (H) are almost undetectable in the somatic mesoderm. In all panels anterior is up and ventral is left.

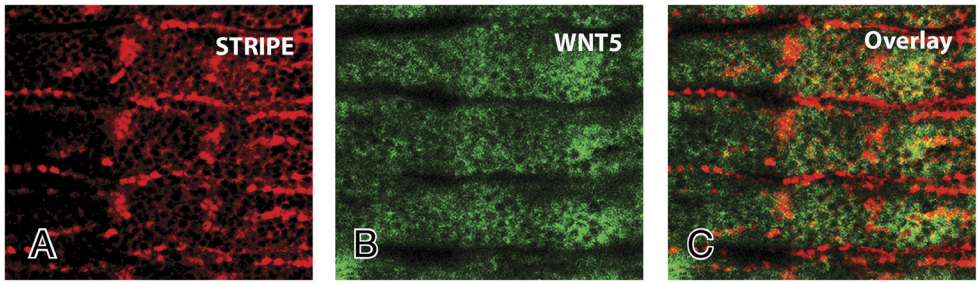


Fig 5 The WNT5 and SR epidermal expression domains partially overlap. Wild type embryos of stage 13 were double labeled with anti-SR and anti-WNT5 antibodies and visualized by confocal microscopy. SR protein (red) is present in a number of tendon precursor cells (A). The WNT5 epidermal expression domains (green) in these same embryos are shown in (B). The overlay of these panels is shown in (C). The SR expression domains partially overlap with the larger WNT5 domains. Anterior is up and ventral is left.

DRL expression in the mesoderm is first detectable around 10 hours AEL and is predominantly concentrated in the developing LTMs and its expression diminishes significantly by stage 16 when the fibers have made their attachments to the tendon cells [39]. DRL is expressed very early in development from 6 hours AEL onwards in reiterated stripes in the epidermis. DRL expression is also observed in clusters that partially colocalize with the SR expression domains at stage 13 [39]. We observe a similar partial co-localization of the SR and WNT5 protein domains in the early tendon cell precursors (Fig. 5). As development continues SR expression becomes confined to the epidermal tendon cells contacted by a muscle fiber. They are located at the segmental borders for the longitudinal muscles, at ventrally and dorsally located cells for attachment of the ventral and dorsal muscles, and in a few lateral groups where the LTMs attach (Fig. 6; [12]).

Expression of WNT5 in either tendon cells or muscle fibers is sufficient to establish correct LTM attachment

We used the yeast UAS-GAL4 transactivation system ([43]; Materials and Methods) to determine whether WNT5 is required by the approaching myofiber or the tendon cell for correct attachment site selection. We expressed WNT5 specifically in the developing muscle fibers (mef2-GAL4), the tendon cells (sr-GAL4) or both (24B-GAL4) in the Wnt5 mutant background. mef2-GAL4 drives expression from early mesoderm formation onwards (stage 10) and in the somatic muscle throughout embryonic development [44]. 24B-GAL4 also drives expression in mesoderm and somatic muscle from stage 10 onwards but is also present at the muscle attachment sites [43]. sr-GAL4 expression follows the endogenous expression pattern of the stripe promoter and is expressed in tendon cells and its epidermal precursors [45]. We found that expression of WNT5 in all muscle or in the tendon cells or in both, rescues the bypass phenotype in the otherwise Wnt5 mutant background (Table 1).

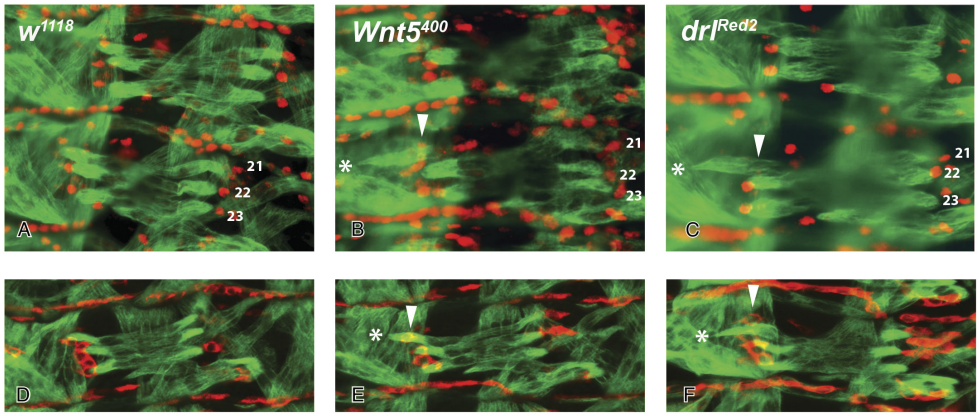


Fig 6 The new attachment sites of the bypassed muscle fibers in *wnt5* and *drl* mutants frequently do not express SR, while the bypassed attachment sites do. Double labeled stage 16 embryos are shown of w^{1118} (A), $wnt5^{400}$ (B) and drl^{Red2} (C) with anti-Muscle Myosin in green and anti-SR in red (Material and Methods). Asterisks mark the novel attachment sites of the overshooting LTM muscles; white arrowheads mark the locations of the original attachment sites. In *wnt5* mutants the novel target sites do not express SR in 65% of the segments containing overshooting muscles, while the bypassed attachment sites usually express SR. The SR positive, original tendon cell is also present in drl^{Red2} mutants, but is partly masked by the overshooting muscle fiber in panel (C), but clearly visible in panel (F)). These results were confirmed in embryos that express Tau-MYC under the control of a stripe promoter in both *wnt5* and *drl* mutants (data not shown). The following genotypes are shown, the control *UAS-Tau-MYC; sr-GAL4* embryos (D), $wnt5^{400}$*; UAS-Tau-MYC/sr-GAL4* (E) and drl^{Red2}*; UAS-Tau-MYC/sr-Gal4* (F). Anti-Muscle Myosin is shown in green and anti-MYC in red. No MYC protein is observed in the ectopic attachment sites. The photographs in Panels (A–C) were taken on a compound microscope and those in Panels (D–F) on a confocal microscope. Anterior is up and ventral is left.

Wnt5 expression in a single ventral longitudinal muscle (muscle 12) that is located in the region into which the bypassed muscle extends, does not inhibit extension of the bypassed muscle fibers (genotype: $wnt5^{400}$*; UAS-Wnt5/P{GawB}tey5053A*, Table 1).

We confirmed the earlier report [39] that when DRL is ectopically expressed in muscle only (genotype: drl^{Red2}*; mef2- GAL4/UAS-drl*) the bypass phenotype is fully rescued, while no rescue occurs when DRL is expressed only in the attachment sites (genotype: drl^{Red2}*; sr-GAL4/UAS-drl*) (data not shown). These results indicate that DRL expression is required in the muscle fiber while WNT5 can either be expressed in certain muscles or in the tendon cells to restore correct attachment of LTMs.

The bypassed tendon cells continue to express the SR protein while the novel attachment sites do not in the majority of the hemisegments

The failure of the LTMs in *drl* and *wnt5* mutant embryos to recognize their correct attachment sites in the epidermis might be a consequence of alterations in the fate or the formation of the tendon cells. The presence of SR in these cells is both necessary and sufficient for tendon

cell fate [11,13]. Early in development (stage 12/13) the epidermal clusters of tendon cell precursors labeled by SR protein in *wnt5* and *drl* embryos are similar in size and location to the wild type clusters (data not shown). Later in development, when muscle fibers and tendon cells are fully differentiated, we co-labeled the muscle fiber (with anti-Muscle Myosin) and the tendon cells (with the anti-SR antibody) in *wnt5* and drl^{Red2} mutant embryos and again did not observe any apparent obvious changes in SR expression patterns. More specifically, we find that the original bypassed attachment sites continue to express SR (Figs. 6A–C). However, the novel epidermal attachment sites that connect the bypassing muscle to the exoskeleton do not express SR in 65% of the hemisegments scored (Figs. 6A–C).

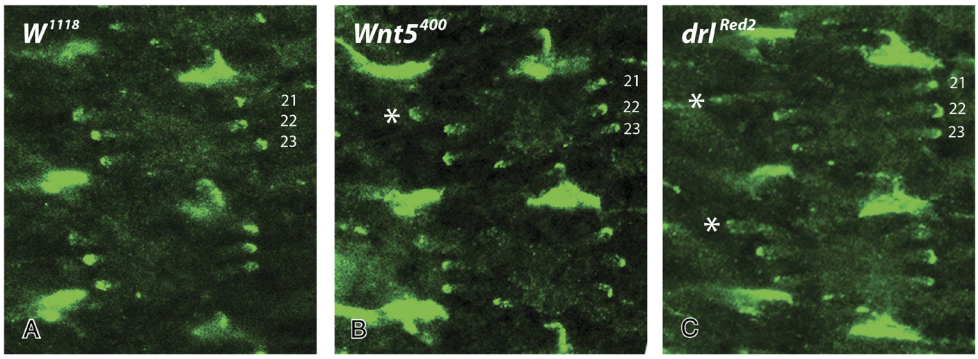


Fig 7 The new attachment sites of the bypassed muscle fibers in *wnt5* and *drl* mutants express βPS-integrin. Wild type (A), $wnt5^{400}$ (B) and drl^{Red2} (C) embryos were labelled with anti-βPS Integrin. Muscles 21–23 do exhibit an accumulation of βPS-Integrin protein at the tip of the overshooting fibers (white asterix).

We confirmed these results by examining embryos of the genotypes $wnt5^{400}$*; UAS-Tau-MYC/sr-GAL4* and drl^{Red2}*; UAS-Tau-MYC/sr-GAL4* for Myc and Muscle Myosin expression (Figs. 6D–F). We conclude that the presence of the SR protein in the bypassed tendon cell indicates that overshooting by the muscle fiber is a result of a defect of muscle guidance in *drl* and *wnt5* mutant embryos, rather than due to alterations in the fate or formation of the appropriate tendon cell. βPS integrin, a protein associated with the myotendinous junction formed at the end of tendon cell determination, accumulates at the tip of the overshooting muscle in the *wnt5* and *drl* mutant embryos (Fig. 7).

DISCUSSION

The development of the intricate muscle pattern of higher organisms requires the coordinate expression of numerous cellular factors regulating the specific fate, differentiation, orientation

and attachment of the individual muscle fibers. The first steps of muscle formation likely occur autonomously, but guidance of myofibers towards and attachment to their appropriate tendon cells are, at least in part, controlled by secreted and transmembrane proteins emanating from both the target cell and the approaching muscle fiber. Here, we have shown that, in *Drosophila*, the secreted WNT5 protein and the Ryk transmembrane receptor family members, DRL and DNT, are essential for guidance of a subset of embryonic body wall muscle fibers to their tendon cells.

There are three Ryk orthologs in *Drosophila*, DRL, DNT and DRL-2 (reviewed in [22]). We find that 36%, 8%, 0% of hemisegments display a LTM muscle bypass phenotype when *drl*, *dnt* or *drl-2* is absent, respectively. Homozygosity for relatively small deficiencies that uncover both *drl* and *dnt* results in the bypass phenotype in virtually all hemisegments (96%). Embryos which completely lack DRL and are heterozygous for a mutant allele of *dnt* display intermediate penetrance of the phenotype (50%). Embryos lacking DNT and are heterozygous for *drl* have bypassing muscles in 8% of their hemisegments. These results suggest that the Ryk family members, *dnt* and *drl*, coordinately regulate the attachment of the LTM muscle fibers to tendon cells with *drl* being the dominant player. The decrease in penetrance in the animals lacking both copies of *drl* and one copy of *dnt* (50%), relative to those completely lacking both genes (96%), indicates that *dnt* can at least partially compensate for the absence of *drl*. Consistent with this is the reported ability of the expression of *dnt* in the LTMs to partially rescue the *drl* mutant bypass phenotype [46].

Does WNT5 signal through DNT and DRL? Our genetic studies indicate that this is likely the case. Female embryos simultaneously heterozygous for *wnt5* and a deficiency which uncovers both *drl* and *dnt* display the bypass phenotype while those heterozygous for either *wnt5* or the deficiency alone do not. Furthermore, male *wnt5* mutant hemizygotes, display increased penetrance when single copies of *drl* and *dnt* are removed. Thus, we conclude that *wnt5* genetically interacts with *drl* and *dnt*, likely indicating that the WNT5 protein acts as a ligand for these two Ryk family members during muscle attachment site selection.

DRL is specifically expressed at the muscle tips of fibers 21–23 while they are in the process of extending towards their attachment sites [39]. The protein is also expressed early in development from 6 hours AEL (stage 10) onwards in reiterated stripes in the epidermis and at stage 12 in clusters of epidermal tendon precursor cells, partially overlapping with the SR expression domain [39]. Rescue of the *drl* mutant LTM bypass phenotype was only achieved when DRL was restored in the muscle and not the attachment sites. At present, the role of the early expression of *drl* in the tendon precursor cells is not clear.

dnt mRNA is also expressed in stripes in the epidermis associated with invaginating cells [46,47]. This transcript is also present at a low level in many embryonic tissues including the somatic musculature. Like DRL, DNT is likely required in the muscle fiber since transgenic expression of *dnt* in the LTMs rescues the *drl* phenotype. DRL-2 is most predominantly expressed in the central nervous system (data not shown), suggesting that it was unlikely to have a role in LTM guidance, as was shown in this study. While almost all hemisegments display overshooting LTMs in the absence of DRL and DNT, only one or two of the three LTM fibers, usually muscles 21 and/or 23, exhibit this phenotype. This result indicates that other non Ryk-dependent mechanisms are required to guide these three muscles to their attachment sites. Alternatively, these two muscles may experience fewer physical barriers blocking their ventral extension beyond muscle 12. In addition, the overshooting of the appropriate tendon cells by these muscles is only observed at the ventral and not the dorsal attachment sites, indicating that guidance mechanisms differ for the two ends of the muscle.

WNT5 has an important role in guidance of embryonic central nervous system commissural axons [28,29] and the salivary glands [48] and acts as a ligand for DRL in these tissues. When we investigated LTM trajectories in *wnt5* mutant embryos we found that one or more LTMs overshoot their normal tendon cells in only 17% of the hemisegments compared with 36% in the *drl* mutant. This result suggests that there are likely other DRL ligands in addition to WNT5. Possible other candidates include the other six wnt genes present in *Drosophila*, *wg*, *wnt2*, *wnt4*, *wnt6*, *wnt8* and *wnt10* (reviewed at ''The Wnt Home page'' (www. stanford. edu/group/nusselab/cgi-bin/wnt)). Segmentation defects during early embryogenesis in *wg* mutants and the lack of available mutants for *wnt6* and *wnt10* precludes further analyses of muscle pattern formation in the absence of these genes. Furthermore, WNT8 is not detectably expressed in the somatic mesoderm [49,50]. Since both WNT2 and WNT4 had been previously implicated in diverse stages of muscle formation and function [42,51] we analyzed LTM trajectories in a *wnt2/wnt4* double mutant. We did not observe any bypassing LTMs in the double mutant embryos, nor in the singly homozygous mutants, indicating that these two Wnt genes are not likely involved in regulating LTM attachment. WNT10 is the most probable alternative ligand for DRL and DNT in muscle since its mRNA is expressed in the developing somatic mesoderm [52], however evaluation of its potential roles awaits the generation of a mutant allele.

In which cells is WNT5 expressed and required? We found that *wnt5* mRNA and protein are expressed at low levels in all somatic muscles while they are extending, in mature attachment sites and also during early development in a subset of the tendon cell precursors and in the epidermis. Furthermore, rescue of the bypass phenotype is seen when a *wnt5* transgene is expressed in either of these two tissues. Since WNT5 is a secreted factor and rescue of

the *wnt5* phenotype is observed with restoration in either the muscle or the tendon cells, it is difficult to conclude unambiguously in which tissue it is needed. Restoring expression of WNT5 in muscle fiber 12 only does not rescue the bypass phenotype. This result suggests that it is not simply sufficient to have a high source of WNT5 in the muscle close to the original attachment sites for appropriate inhibition of LTM extension. It is more likely, that WNT5, which is widely expressed in the epidermis and musculature, is modified in some way to become locally activated as a specific LTM repulsive guidance cue. Support for this hypothesis comes from previous observations that WNT5 is proteolytically-processed [53]. Furthermore, WNT5 expressed by anterior commissural midline glial cells, but not in all neurons, blocks anterior commissure formation [28] due to the repulsion of DRL^+ axons, indicating that elevated local expression of WNT5 can have different outcomes depending on the cell types which express it. Finally, although WNT5 is observed to be widely expressed in the larval/adult brain, it acts specifically to guide mushroom body α-lobe axons [54] indicating that an apparently ubiquitously-expressed ligand can act as a directional cue. Alternatively, WNT5 may be sequestered from some regions of the extending muscle fiber by so-called ''extrinsic receptors'' [55] which results in a directional cue received by the leading edge of the muscle.

There is mounting evidence that the final differentiation of the *Drosophila* tendon cell, in particular the secretion of an elaborate extracellular matrix, is tightly coupled to the arrival of the muscle fiber (reviewed in [2]). The resulting myotendinous junction is essential for force transmission and counteraction of muscle contraction by tendon cells. Similar junctions exist in vertebrates where tendons attach the muscles to the bone. In *Drosophila*, it consists of hemi-adherens junction formed by the association of integrin receptor heterodimers on the muscle tip and the tendon cell with the intercalating ECM proteins [2] such as Laminin and TSP secreted from the tendon cells and Tiggrin from the muscle cell. The myotendinous junction is not functional when integrin, TSP or laminin are absent resulting in dissociation of fibers from their attachment sites which leads to lethality. The signals allowing recognition of the appropriate tendon cell, arrest of muscle fiber extension and the formation of the myotendinous junction remain unclear. However, genetic phenotypic analyses indicate that changes in local integrin receptor accumulation on muscle tips and differential responses to TSP presented on the tendons might slow down and stop muscle migration prior to the initiation of myotendinous junction formation (reviewed in [2]). A functional myotendinous junction is formed at the novel attachment site of *wnt5* and *drl* mutants as evidenced by our observation that βPS integrin accumulates at this site. We do not observe βPS integrin expression at the original attachment site indicating that the interaction of the muscle tip with the bypassed site, if it occurs at all, is not of sufficient duration to initiate attachment site maturation.

The observation that the initial outgrowth and guidance of the LTMs are normal in *wnt5* and *drl* mutants suggests that these proteins act during the recognition of the target cell and not earlier during muscle extension. Wnt/Ryk signaling may be required for induction of a localized ''stop'' signal for the LTM at its normal attachment site. In this scenario DRL and DNT present on muscle fibers 21–23 would bind activated WNT5 secreted from their normal attachment sites. This interaction might then result in the transcription of genes encoding extracellular matrix proteins in the muscle fiber which are required to increase adhesiveness between the muscle and tendon cell, slowing down the fibers extension. When either WNT5 or DRL/DNT is absent this signal is not appropriately received by the approaching fiber and it overshoots its target and attaches relatively randomly to a more distant epidermal cell.

In the *Drosophila* embryonic CNS, DRL acts as a repulsive guidance receptor on growth cones of anterior commissural axons to steer them away from the posterior commissural axons which express WNT5. It seemed thus possible that DRL/DNT also acts in the muscle as a repulsive receptor upon binding of WNT5. However, we did not observe any clear muscle guidance defects when WNT5 was ectopically expressed on either specific muscle fibers or in the tendon cells (data not shown). As mentioned above, it is possible that WNT5 has to be locally modified and activated or differentially sequestered to function as a guidance cue in this tissue.

We found that the novel attachment site for the overshooting muscle in embryos and larvae is an epidermal cell and not another muscle. The normal LTM attachment site that is not recognized by the bypassing muscle is present in *wnt5* and *drl* mutants as visualized by its ability to express SR, a transcription factor that is both necessary and sufficient to drive tendon cell fate. Therefore, this tendon cell follows important early stages of normal tendon cell differentiation, but does not bind the fiber.

In contrast, only 35% of the ectopic tendon cells express SR suggesting that SR expression is not obligatorily required for formation of a stable myotendinous junction. At present, we do not know whether the novel attachment site expressed SR earlier in development or whether, despite its stability against contractioninduced damage, the ectopic myotendinous junction is different in some manner from the normal junction as to not allow maintenance of SR expression. We find that the FAS2 protein that is normally expressed at the muscle tip and the tendon cell to which it attaches, is present at both the original and the novel attachment sites in *drl* and *wnt5* mutant larvae. This result indicates that the muscle ''filopodia'' likely transiently interact with its normal tendon cell target but does not cease extension. This further supports the notion that Wnt/Ryk signaling may increase the stability of muscle/tendon cell interactions.

It is too early to evaluate whether the molecular mechanisms of muscle attachment site selection are conserved between vertebrates and invertebrates because of the paucity of knowledge about the molecules required for tendon differentiation and its connections to muscle and skeletal tissues in vertebrates. Components of Integrin-mediated adhesion complexes, e. g., talin 1 and talin 2 and several laminin integrin receptors were, however, recently shown to be essential for the formation of the vertebrate myotendinous junction [56], as has been observed for their orthologs in *Drosophila* (reviewed in [2]). In the coming years, as more becomes known about the mechanisms that mediate the connections between muscles and tendons, it will be apparent whether other aspects of muscle guidance and target site selection are also conserved.

MATERIALS AND METHODS

Drosophila stocks

All *Drosophila* stocks were grown on standard cornmeal medium at 22uC. The following mutant alleles, GAL4 drivers and UAS reporter lines were obtained from their originating laboratories or the Bloomington *Drosophila* Stock Center and used in this study: w^{1118}, $wnt5^{400}$ [28], drl^{Red2} [23], $drl\text{-}2^{E124}$ [42], $dnt^{42.3}$ (this study), *sr-GAL4* [45], *UAS-wnt5* [28], *24B-GAL4* [43], *mef2-GAL4* ([44], *UAS-Tau-MYC* [57], *P{GawB}tey5053A* [58], the $wnt2^{-}$, $wnt4^{C1}$ double mutant and the *Df(2L)ED1231* and *Df(2L)Exel6043* deficiencies [59].

Generation of *dnt* mutants

A mutant allele of *dnt*, $dnt^{42.3}$, was generated by imprecise excision of the P-element insertion *P{EP}dntEP(2)2158* obtained from the Szeged Stock Center following a standard P-element mobilization strategy [60]. Sequences of the primers used to identify the deletion are available upon request. The $dnt^{42.3}$ mutant line is viable and has a deletion of 2322 base pairs from positions 19360852 to 19363174 of chromosome 2L (accession number GB:AE014134). This deletion uncovers most of the first exon of the *dnt* transcript including the ATG initiator codon, the first 15 amino acids of the Wnt-binding WIF domain and the splice donor site. RNA *in situ* analysis of the *dnt* mutant line reveals that the mutant embryos do not detectably express the *dnt* transcript (compare Figs. 2B and 2C).

RNA *in situ* hybridization

Embryo collections for RNA *in situ* hybridization were carried out at 22°C. RNA *in situ* hybridization and staging of embryos were performed as described [28]. *dnt* RNA and *wnt5* RNA antisense probes were hybridized to paraformaldehyde fixed embryos. The *dnt* probe included positions 386–1225 of the *dnt* RA transcript (accession number NM_057993). The *wnt5* antisense probe was generated by SP6 polymerase transcription of EcoRV-linearized pOT2-LD22614, which bears the full *wnt5* open reading frame.

Immunohistochemistry

All embryo collections for immunohistochemistry were carried out at 22°C. Antibody labelings were performed as described [38]. The following primary antibodies were used on formaldehyde-fixed embryos or third instar larval body walls: anti-Muscle Myosin mAb (Invitrogen), anti-FAS2 (1D4, Developmental Studies Hybridoma Bank (DSHB)), anti-βPS Integrin (CF.6G11; DSHB), guinea pig anti-SR (gift from T. Volk; [11]), rabbit-anti-GFP (Upstate), anti-Sex-Lethal ([61]; DSHB), rabbit anti-MYC (Upstate) and affinity-purified rabbit anti-WNT5 [28]. Secondary antibodies used were: HRP-conjugated goat anti-mouse or anti-rabbit (Jackson Laboratories) and AlexaFluor-488-conjugated and AlexaFluor-568-conjugated goat anti-mouse and goat anti-rabbit, respectively (Invitrogen). HRP staining was visualized by a standard DAB reaction. After antibody staining, embryos were stored in 70% glycerol in PBS and then dissected and imaged with an Axioplan2 microscope fitted with an Axiocam digital camera (Zeiss) or using an LCS (Leica) confocal microscope.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: LLL LGF JNN. Performed the experiments: LLL AWMD RRW LGF JNN. Analyzed the data: LLL LGF JNN. Wrote the paper: LLL LGF JNN.

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5

Discussion

DISCUSSION

In *Drosophila melanogaster* (fruit fly) WNT5 is the ligand of *Drosophila* Ryk's; DRL, DRL-2 and DNT that upon their interaction are able to transduce a signal that guides axonal growth cones and very likely provides the lateral transverse muscles (LTMs) with a stop signal amongst others. In adult mice expression of both WNT5 and RYK is induced upon axonal injury; preventing axonal regeneration. The fruit fly is an excellent model to study the consequences of WNT5 signaling through DRL and its family members.

The homodimerization of DRL can be induced through overexpression of WNT5, subsequently leading to an increase in the recruitment of downstream kinase; SRC64B. This signaling cascade is employed within the developing fruit fly embryo to guide a subset of growth cones to their destination. We demonstrate that SRC64B requires its SH2 domain to interact with DRL in S2 cells, while DRL in turn requires its PDZ-binding domain (PDZ-BD) to interact with SRC64B. In the *drl*-mediated commissure switching assay (**Chapter 1**, this thesis) the DRLΔPDZ-BD is however not fully active, this might be explained by the possibility that SRC64B does not bind directly to the DRL PDZ-BD but that the interaction between SRC64B and DRL is stabilized by another protein binding to the PDZ-BD, since SRC64B does not contain a PDZ domain. This idea is supported by the observation that mammalian RYK can bind Dsh through its PDZ-BD (1). We therefore hypothesize that Dsh might potentially stabilize the interaction between SRC64B and DRL. Another potential stabilizer could be Vang, a member of the PCP pathway, and also shown to interact with RYK through its PDZ-BD (2).

Based on what we reported previously about the interaction between SRC64B and DRL and in **Chapter 2** (this thesis) we suggest that the WNT5-DRL interaction induces a conformational change of DRL that in turn allows it to form a complex with SRC64B, we haven't however confirmed whether increased phosphorylation of DRL and/or SRC64B accompanies the conformational change. Nor did we determine whether DRL's tyrosine kinase domain, thought to be inactive, might become active upon binding to SRC64B. This could be addressed by co-expressing *wnt5*, *drl* and *src64B* in S2 cells, and performing an immunoprecipitation (IP) for SRC64B and analyze the immunoprecipitated species using mass spectrometry to identify DRL amino acids that have been post-translationally modified due to complex formation with SRC64B. This could be done for SRC64B as well by performing the reciprocal IP. In a personal communication between Bonkowsky and Thomas and Katso, Russel and Ganesan, (Katso, et al; 1999 (3)), it was reported that the potential tyrosine kinase activity of DRL is not required for muscle attachment site selection. DRL K371A, which is predicted to be catalytically-inactive is able to rescue the muscle attachment site phenotype and the drl^{null}

phenotype in the mushroom body (4). The observation that the DRL K371A mutant can rescue these phenotypes suggests that consensus RTK signaling is probably not involved in these processes. This, of course, does not exclude the possibility that DRL signals through another mechanism, such as via SRC64B and, possibly Dsh recruitment or by translocation of the ICD to the nucleus. Unpublished preliminary results of the Noordermeer/Fradkin lab indicate that DRL carries a bipartite nuclear localization signal (NLS) that allows the cleaved intracellular DRL fragment to translocate to the nucleus and repress WNT5 expression. Interestingly, the interaction of WNT5 with DRL inhibits the nuclear translocation of the DRL ICD, possibly constituting a negative feedback loop. DRL ICD nuclear import is dependent on the importins β11 and α2 in S2 tissue culture cells. While these results need confirmation in the living fly, current results indicate that DRL's NLS is required to rescue the DRL phenotype seen in boutons of neuromuscular junctions. Earlier studies also established that the intracellular domain of RYK, the mammalian equivalent of DRL, also translocates to the nucleus (5,6). These observations lend themselves for new insight and designing new experiments to tease out the different pathways that DRL can induce depending on its context.

Creating mutations for DRL rendering the NLS sequence, the TBC motif and its WIF domain inactive could be used to identify downstream signaling components via immunoprecipitations and mass spectrometry. The next generation RNA sequencing should permit us to evaluate the DRLΔNLS, ΔTBC and ΔWIF mutants in an otherwise *drl*^{null} background. Mutating the NLS, TBC motif or the WIF domain would enable us to distinguish between a direct effect of the nuclearly-localized cytosolic DRL fragment versus possible nuclear targets downstream of the DRL/SRC64B signaling cascade. The results that are obtained using the next generation sequencing technique could also be compared to *wnt5* mutant and *w*^{1118} embryos to confirm potential transcriptional targets of the WNT5/DRL pathway. Preliminary results indicate that this approach confirms our earlier results (Fradkin et al., 2004) that the WNT5 gene is a transcriptional target of the WNT5/DRL pathway. The results from this approach should be confirmed using other techniques like immunofluorescence microscopy which would allow evaluation of protein colocalization as well as, potentially, visualizing the real-time translocation of DRL's cytosolic domain from the plasma membrane to the nucleus.

The amino acids that mediate DRL homodimerization reside within its transmembrane domain. Previous reports described a specific motif within the TM region of proteins that facilitates homodimerization (7,8). This motif was found in a small screen that was performed within the seven residues (LIxxGVxxGVxxT) which mediate the homodimerization of Glycophorin A (GpA) (9). The consensus sequence of this motif is: GXXXG, where X stands for any amino acid and G for a small amino acid. The homodimerization of two TM domains of GpA are thought to involve right-handed crossing of two straight α-helices that are unified on a

single GXXXG-related motif (7,8). A database analysis showed that this motif can be found in transmembrane domains as the most highly biased sequence motif (10). The TM domain of DRL harbours two such motifs;TLIVG and GGILA and we find that TLIVG mediates ligand-independent homodimerization. In S2 cells, overexpressed DRL T245V shows a marked decrease in binding SRC64B, relative to wild type DRL. However *in vivo* results do not display the expected decrease in the switching assay for this mutant. The result is somewhat unexpected but could be explained by the fact that we ectopically overexpress DRL in the switching assay and that only a relative little amount of homodimerization and subsequent SRC64B recruitment may be necessary to activate the pathway. Another explanation could be that WNT5 is more abundantly present *in vivo* and is able to force dimerization through its interaction with DRL's extracellular domain and cause a subsequent conformational change that is proposed to take place, bypassing the requirement for the transmembrane motif. The data in **Chapter 2** also demonstrates that DRL can form heterodimers with its orthologs; DRL-2 and DNT. This finding is of importance since it is likely that DRL receptor context, e.g., in homo- versus heterodimers, may have consequences for its function *in vivo*. We show in **Chapter 3** that DRL is able to rescue the DRL-2 phenotype MB phenotype and, conversely, ectopic DRL-2 expression during embryogenesis causes the PC neurons to switch to the AC (95%). Importantly these results indicate that both DRL and DRL-2 can act as WNT5-responsive repulsive guidance cues.

In **Chapter 3** we employed the MARCM system to observe individual neurons of the mushroom body in *drl* ^{null} mutants, confirming previous results for the requirement of DRL in the guidance of α branch axons in the mushroom body (MB). 97% of the DRL clones present the α axon misguidance whereas the *wnt5* clones display the same phenotype for 51% of the clones. However we find that *drl-2* in our MARCM system displays the α axon misguidance in 51% of the clones, resembling the WNT5 phenotype incidence. We then showed that DRL is expressed in dorsomedial neuroblast (DM) lineages and recruits WNT5 at specific sites surrounding the MB via its WIF domain. DRLΔ-Cyto, expressed in the DM lineages, also rescues the mutant phenotype providing evidence that DRL is not likely involved in transducing a WNT5 signal. We go on to show that DRL-2 is expressed on α axons and requires its WIF domain and ICD to perform its biological role. Our results suggest that the complex of WNT5-DRL is cleaved on the surface of the DM lineage cells and interacts with DRL-2 expressed at the α axons, forming a ternary complex. This prevents the α axons from migrating medially, causing them to navigate dorsally. To the best of our knowledge, this is the first time that an extrinsic receptor fragment has been implicated in ligand-dependent guidance of axons expressing a different receptor. DRL requires its TBC motif for WNT5-DRL ECD cleavage, this motif is conserved in the mammalian RYK orthologue, suggesting that this mechanisms of ligand binding and complex shedding could provide a signalling cue for

neurons expressing a different Ryk family member, or maybe even a third receptor, in many different evolutionary contexts.

In **Chapter 4** we use the lateral transverse muscles of *Drosophila* embryos at stage 16/17 to address the function of DRL. We show that the *wnt5* mutant reproduces the muscle bypass phenotype as seen in the *drl* mutant. However for *drl* mutant embryos, the penetrance of the muscle bypass phenotype is 36% whereas for *wnt5* it is 17%. Reiterating the proportional difference we detected in the commissural phenotype 97% in *drl* mutants vs. 67% in *wnt5* and the α axon misguidance in the MB can be observed in 97% of *drl* clones vs 51% in *wnt5* clones. The fact that this discrepancy between *drl* and *wnt5* mutant phenotypes can be found in all tissues examined is striking and it would be interesting to investigate whether the WNT5 independent role of DRL is mediated through the same molecule(s) in the above mentioned tissues. The discrepancy between *drl* and *wnt5* mutant phenotypes led us to start our investigation with the possibility of another ligand for DRL. We tested *wnt2*- and *wnt4* mutants but we did not observe a muscle bypass phenotype for either of them separately nor in the double mutant. Furthermore, the *wnt5* mutant muscle bypass phenotype was not enhanced when both *wnt2* and *wnt4* were also mutated. However this does not exclude them from interacting with DRL or DRL-2 in the nervous system. The rest of the Wnts in *Drosophila* are not expressed in the mesoderm except for Wnt10, which remains poorly characterized. It would be very informative to investigate whether other Wnts are capable of interacting with DRL, DRL-2 and DNT.

We then examined whether there could be a function for DRL-2 and DNT in the muscle. The *drl-2*^{E124} mutant did not display the muscle bypass phenotype by itself nor did the penetrance of the phenotype increase in the double mutant for *drl* and *drl-2*. This allows us to exclude a role for DRL-2 in the guidance of the LTMs during the *Drosophila* embryonic stages. We created a *dnt* mutant in the lab, by imprecise excision of a local P-element, and found the LTM bypass phenotype in 8% of the segments. The generation of a double mutant for *drl* and *dnt* was not possible due to the proximity of *drl* and *dnt* in the genome. However, we made use of two independently-generated deficiency fly lines; *Df(2L)ED1231* and *Df(2L) Exel6043* whose overlap results in the deletion of both genes. The transheterozygous deficiency line displayed an increase of the muscle bypass phenotype to 94% and 96% respectively, indicating that DRL and DNT act together to guide the growing muscle fiber to its appropriate MAS. However we cannot rigorously exclude the involvement of other genes within the deleted genomic region. To address this issue we also crossed fly lines to obtain either deficiency over *drl*, which resulted in a muscle bypass phenotype of 50%, whereas *Dnt/Df(2L)Exel6043* displays the muscle bypass phenotype in 8% of the segments. The increase of the muscle bypass phenotype when either deficiency is placed over a *drl* mutant

chromosome further strengenths our evidence that DRL and DNT function redundantly in the LTMs during embryonic myogenesis. We also show that WNT5, DRL and DNT function in the same pathway since males hemizygous for *wnt5* and heterozygous for the deficiency display an increase in the muscle bypass phenotype to 27% and females that are heterozygous for *wnt5* and the deficiency show a muscle bypass phenotype of 16% versus a 0% rate for the *wnt5* heterozygote embryos.

In the wild-type embryo the approaching growth cone and the prospective tendon cell exchange a number signals of which Stripe expression is well described (11). Stripe expression is upregulated in the tendon cells through the interaction of Vein with the EGFR on the PM of the tendon cell. Vein is secreted by the advancing myofiber, this coupling of actions ensures that there is a tendon cell available for each myofiber on both ends. The other tendon cells in the adjacent area stop expressing Stripe and therefore do not differentiate into tendon cells. We wanted to address this tendon cell differentiation for the ectopic muscle attachment site. We examined this by performing an antibody staining on the w^{1118}, *wnt5* and *drl* mutants (see Figure 6 Chapter 4). This experiment allows us to suggest that the bypassing myofiber seems to interact with its prospective tendon cell but that it does not stop and proceeds to attach to an epidermal cell in the body wall. It seems likely that the signaling of WNT5 through DRL results in some kind of stop signal for the muscle. Unravelling the basis of this stop signal would greatly enhance our knowledge of muscle fiber development.

Besides the ectopic MAS our interest lies as well with receptor context, which is highly significant in the developing *Drosophila* embryo. We and others have previously shown that DRL positive axons are repulsed by WNT5 at the midline of the embryonic ventral nerve cord (VNC) and require their intracellular domain and its interaction with SRC64B to do so, whereas DRL does not need its ICD to guide α axons in the developing mushroom bodies (12–17). All these experiments make a case for DRL being able to induce different signals which will very likely depend on receptor context. Regarding receptor context it has been shown that RYK is able to interact with Frizzled 7 in *Xenopus laevis* (18). *Drosophila* has five Fz proteins of which Fz and Fz2 are implicated in canonical signaling and have been shown to act redundantly (19–27). We set out to investigate *Fz* and *DFz2* in the drl^{red2} mutant; the two single mutants did not display the muscle bypass phenotype. Yet when we examined the double mutant; *Fz,DFz2* we did not observe the muscle bypass phenotype but instead a novel muscle phenotype where we find two LTMs instead of three in 23% of the segments. From these unpublished results, we conclude that Fz and DFz2 act redundantly to facilitate formation of the *Drosophila* embryonic musculature.

Both hRYK and mRYK are detected in skeletal muscle (28). Yet, there are no reports of muscle phenotypes in mammalian *ryk* mutants to date. The conservation of the RYK protein structure between flies and vertebrates does suggest, however, a possible role for RYK in similar processes in mouse as well as man. The first study that showed functional conservation of the WNT/RYK pathway in axon guidance was by Liu et al 2005 (29). They demonstrate that RYK is present on corticospinal tract (CST) axons and these axons can be repelled by gradients of either the WNT1 or WNT5a proteins. This WNT/RYK-dependent chemo repulsion of the CTS axons is required for their posterior migration down and along the postnatal spinal cord. The authors continue to show that the repellence of RYK$^+$ CST axons can be counteracted by the administration of anti-RYK antiserum. Other research groups confirmed the repellence of RYK$^+$ axons by WNTs in other contexts, the corpus callosum, a forebrain commissure in mice, and in the optic lobe (30,31). Lastly, evidence is accumulating that RYK can act as an important suppressor of axonal regeneration after spinal cord injury (32–34).

RYK function is dependent on its specific cellular context: it is reported to be found in a complex with WNT, DVL and FZ in dorsal root ganglia explants where it is able to *attract* (as opposed to repel) neurites upon WNT3a stimulation (35). Our findings in Chapter 2, 3 and 4 address the roles that *Drosophila* DRL exercises during different stages of development and in diverse tissues. The functions of DRL-2 and DNT described there can possibly lead to new insights into the mechanisms employed by vertebrate RYK. In particular, the findings presented in Chapter 4, where WNT5/DRL-ECD acts as a guidance cue for DRL-2$^+$ axons, are intriguing in relation to what it is known about RYK's roles in mammals. If a WNT member forms a complex with the shed ECD of RYK it could provide an additional cue in the milieu of outgrowing axons in mammals, maybe in parallel to the translocation of RYK's ICD to the nucleus mediating WNT3 induced neuronal differentiation (5).

There are quite a few proteins that have a function in the guidance of different tissues in *Drosophila* among these is the family of Slit proteins. Initially, Slit was discovered for its role in the nervous system where it functions through its receptor Roundabout (Robo) (36,37). Later on it was found that it also controls leukocyte chemotaxis (38–40) and is involved in muscle guidance (37,41). This conservation of function throughout different tissues and contexts suggests that the guidance of distinct types of somatic cells depends, at least partially, on Slit and the interaction with its receptor Robo (42). We already drew a parallel between Slit and DRL in the introduction, because both are needed for guidance of different tissues by means of repellence. In the discussion we would like to suggest some proteins that could be of interest because they have been implicated to interact with the Slit/Robo pathway. It is conceivable that the WNT5/DRL- and Slit/Robo pathway might use similar proteins to transduce axon-repellent signals.

For instance we would like to determine if Kuzbanian (Kuz), a metalloprotease belonging to the Adam family and shown to genetically interact with *slit* (43), is involved in processing of DRL. Interestingly, Figure 4E of Albrecht et al (44) indicates the presence of LTM overshooters in the *kuzbanian* mutant. The same report also showed that Kuz cleaves the ECD of Robo in *Drosophila* cells and that this cleavage is required to maintain normal repellent activity and is even responsible for the recruitment of its downstream signaling protein Son of Sevenless (Sos) (43). It would be interesting to investigate whether Kuzbanian or one of its relatives can cleave DRL and play a role in the downstream signaling.

In vertebrates WNT5a and Wnt11 are involved in the PCP signaling, Wnt11 has no obvious orthologue in *Drosophila,* to date, however, *Drosophila* WNT5 is considered to be the orthologue of WNT5a. In addition, WNT5 signaling in *Drosophila* is likely via a PCP-like non-canonical pathway. Therefore *Drosophila* orthologues of the vertebrate proteins downstream of the WNT5a PCP receptor could play a role downstream of WNT5 in the fruit fly. One interesting candidate would be Syndecan (Sdc), a transmembrane heparan sulphate proteoglycan (HSPG) of which there is a single orthologue in *Drosophila.* In *Xenopus* embryos it has been shown that Sdc4 can bind to R-Spondin 3 (Rspo3) ultimately leading to the activation of the PCP pathway (45). The R-spondin family is able to modulate Wnt signaling and has been shown to play a role in development and disease (46–51). Currently little is known about their modes of action. It is thought that Rspo3 induces clathrin-mediated endocytosis that is dependent on Sdc4 resulting in the internalization of the Wnt-receptor complex (45). The endocytosis of the Wnt receptor complex is essential for PCP signal transduction and likely mediated by R-spondins. Furthermore, a genetic interaction between Sdc4 and Vangl2 has been demonstrated in mice (52). The same article demonstrates that Vangl2 regulates the steady-state protein levels of Sdc. Mice mutant for *Sdc4* display defects in skeletal muscle development and regeneration (53). Moreover there are indications that RTKs are involved in transducing Sdc signaling events (54–56). In *Drosophila,* Sdc has been found to suppress Slit/Robo2 signaling in tracheal cells; guiding tracheal migration and branch fusion (57). These observations taken together, Sdc would be an interesting candidate to further pursue.

The cleft palate phenotype seen in *ryk* mutant mice is also seen in other knockout mice including those lacking family members of the Eph-family. The Eph-family belongs to the receptor tyrosine kinase (RTK) family and their ligands are known as Ephrins. They play an important role in the organization of many tissues. *Drosophila* has a single Eph and Ephrin that have been implicated in neuronal development (58,59) and specifically in the development of the MBs; guiding specific axon branches of individual MB neurons (60), which is reminiscent of DRL's roles in the MBs.

Furthermore it has been shown that murine RYK can bind to EphB2 and EphB3 and can be phosphorylated by the ephrin receptors EphB2 and EphB3 (61). Another article also demonstrates the association between human RYK, EphB2 and EphB3 but fails to detect any phosphorylation of RYK by the Eph receptors (62). It would be nice to address this association in *Drosophila*, where determining biological functions of proteins is made easier by the available powerful genetic approaches.

WNT5a encodes two isoforms with distinct functions in cancers (63) a long (L) and short (S) isoform. WNT5a-L has been found to inhibit proliferation of tumor cell lines whereas WNT5a-S actually promotes their growth. Could this finding help us to promote axonal regeneration after spinal cord injury? RYK is a potent inhibitor of axonal regeneration after nerve injury (32,34). The knowledge that is gained by further studying the *Drosophila* Ryk signaling pathway(s) should certainly contribute to finding a way to block the action of RYK itself or that of downstream signaling members of these pathways in patients to effect better axon regeneration after spinal cord injury (64,65).

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SUMMARY

In the last decade it has become clear that a number of the molecular mechanisms that are required for proper navigation of axons in complex nervous systems are also employed to guide muscles to their appropriate attachment sites. Examples of gene families that mediate these diverse processes are the highly conserved Slit and the Roundabout gene families, and the Ryk family, the latter being the topic of this thesis. The fruit fly (*Drosophila melanogaster)* has three RYK members: DRL, DRL-2 and DNT. The aim of our research is to advance the understanding of the mechanisms by which the *Drosophila* RYKs operate in the nervous system and the musculature using biochemical and genetic approaches.

In **Chapter 2,** we demonstrate that DRL can form homodimers and heterodimers with its orthologues DRL-2 and DNT in a *Drosophila* cell line, suggesting that the partly overlapping temporal and spatial expression patterns of the different Ryk family members *in vivo* have important consequences for their function. RYKs act as Wnt receptors in mammals and in invertebrates. We find that the *Drosophila* WNT5 protein is able to significantly increase homodimerization of DRL causing a subsequent increase in the recruitment of SRC64B, a non-receptor tyrosine kinase, previously identified as a downstream effector of DRL. We further show that ligand independent homodimerization of DRL is mediated by a TLIGV motif in its transmembrane domain. The formation of the DRL/SRC64B complex requires the DRL PDZ-binding motif and the SH2 domain of SRC64B. In addition, several mutations in the putative inactive DRL kinase domain prevent formation of the DRL/SRC64B complex indicating that WNT5 induced conformational changes are likely essential for their interaction. Using an *in vivo* axon guidance assay we find that that the DRL Wnt-binding domain, a juxtamembrane extracellular tetrabasic cleavage (TBC) site and the PDZ-binding domain are needed for axon repulsion by DRL. These results suggest that activation of DRL as an axon guidance receptor involves a number of molecular events including dimerization and proteolytic processing.

In **Chapter 3** we study the function of *Drosophila* RYKs in the mushroom body (MB): a brain structure implicated in learning and memory acquisition. The α- and β-axons of MBs connect different regions of the brain required for these processes. We show that DRL is needed during *Drosophila* brain development to guide the MB α-branch in order to connect to its target neurons. However, DRL is not expressed by MB neurons themselves but instead can be detected in a neuronal cell lineage that adjoins the outgrowing MB α-branch. We find that the MB α-branch expresses DRL-2, another *Drosophila* RYK orthologue, and that correct MB α-branch guidance is dependent on its presence. The DRL-2 cytoplasmic domain is required for this function. This result indicates that DRL-2 can act as an axon-intrinsic guidance receptor

in this cellular context. Interestingly, the DRL cytoplasmic intracellular domain is not needed in the adjacent cells for a-branch guidance. DRL receptor function necessitates its cleavage at the TBC site for appropriate a-branch guidance, resulting in the extracellular shedding of DRL's Wnt binding domain. DRL, therefore, acts extrinsically to capture and present WNT5 to MB axons that express DRL-2 on their surface and transduce a subsequent intracellular signal. This model is consistent with our biochemical data supporting the existence of a ternary complex consisting of the shed DRL ectodomain, WNT5, and transmembrane DRL-2. Therefore, we conclude that MB-extrinsic and –intrinsic RYKs guide MB-α axons via their common ligand WNT5.

Drosophila RYKs do not only act as guidance receptors for axons; they are also involved in the guidance of the lateral transverse muscles (LTMs) to their final muscle attachment site (MAS) at the body wall in the *Drosophila* embryo. In **Chapter 4,** we show that in an embryo lacking *wnt5*, as has been shown for its receptor *drl,* these muscles often overshoot their target and attach more ventrally at an ectopic site in the body wall. Rescue experiments demonstrate that WNT5 expressed in either the muscle or the tendon cell can restore muscle attachment but that DRL has to be present at the approaching muscle fiber. Strikingly, *dnt*, but not *drl-2*, mutants also exhibit the muscle bypass phenotype. Moreover, in absence of both *drl* and *dnt* the number of overshooting fibers dramatically increases, indicating that *drl* and *dnt* act together to guide the LTMs to its appropriate MAS. We also show that *wnt5*, *drl* and *dnt* are likely in the same pathway since removing a copy of *wnt5* enhances the *drl/dnt* phenotype significantly. The approaching muscle fiber and the prospective tendon cell exchange a number of molecular signals enabling the formation of a stable extracellular matrix-mediated myotendinous junction. Surprisingly, the majority (65%) of the novel ectopic MASs do not express Stripe, a nuclear protein postulated to be both necessary and sufficient for tendon cell differentiation. Furthermore, we provide evidence that the overshooting fibers interact extensively with their prospective Stripe-positive tendon cell yet proceed to attach more ventrally. We conclude that an interplay of DRL, DNT and WNT5 is responsible for generating a stop signal to allow the muscle attachment to be established. Why and how attachment does occur at an ectopic ventrally located epidermal cell is not clear at this moment and future studies will be needed to identify the actual stop signal.

The studies described in this thesis enhance our understanding of the basic biological roles and operating mechanisms of Ryks in such diverse processes as axon guidance and muscle attachment site selection. We hope that this work will also contribute to future therapies for diseases in which these proteins are likely implicated such as cancer and poor regeneration after nervous system trauma.

SAMENVATTING

Het afgelopen decennium is duidelijk geworden dat de moleculaire mechanismen die nodig zijn voor de correcte navigatie van axonen in het complexe zenuwstelsel ook kunnen worden aangewend om spieren te begeleiden naar hun aanhechtingspunt. Voorbeelden van gen families die deze diverse processen mediëren zijn de goed geconserveerde Slit en Roundabout gen families en de Ryk familie. Deze laatst genoemde familie is het onderwerp van dit proefschrift. De fruitvlieg (*Drosophila melanogaster*) bezit drie Ryk familieleden: DRL, DRL-2 en DNT. Het doel van ons onderzoek is een beter begrip te verkrijgen van de mechanismen die de *Drosophila* Ryk's ontplooien in het zenuwstelsel en de spieren met behulp van biochemische en genetische benaderingswijzen.

In **hoofdstuk 2** demonstreren we dat DRL homodimeren kan vormen in een *Drosophila* cellijn en ook heterodimeren kan creëren met zijn orthologen DRL-2 en DNT. Dit suggereert dat de gedeeltelijke overlapping van de temporele en spatiële expressie patronen van de verschillende Ryk familieleden *in vivo* belangrijke consequenties heeft voor hun functie. Ryks functioneren als receptoren voor hun ligand Wnt in zoogdieren en invertebraten. Wij tonen aan dat het *Drosophila* WNT5 eiwit in staat is om de homodimerisatie van DRL significant te verhogen, wat ook leidt tot een toename in de rekrutering van SRC64B, een non-receptor tyrosine kinase, voorheen geïdentificeerd als een downstream effector van DRL. Verder laten we zien dat ligand onafhankelijke homodimerisatie van DRL wordt gemedieerd door het TLIGV motief in het transmembraan domein van DRL. De formatie van het DRL/SRC64B complex vereist de aanwezigheid van DRL's PDZ-binding motief en het SH2 domein van SRC64B. Bovendien hebben we verscheidene mutaties geïntroduceerd in het vermeende inactieve kinase domein van DRL die de formatie van het DRL/SRC64B complex voorkomen. Dit indiceert dat een WNT5 geïnduceerde conformatieverandering wellicht essentieel is voor hun interactie. Gebruikmakend van een *in vivo* axon geleidingsanalyse onderbouwen we dat DRL zijn Wnt bindingsdomein, juxtamembraan extracellulaire klief-motief en zijn PDZ bindingsdomein nodig heeft voor zijn rol in axon repulsie. Deze resultaten impliceren dat DRL een aantal moleculaire veranderingen moet ondergaan om zijn functie als een axon begeleidende receptor te kunnen vervullen. Tot deze veranderingen behoren onder meer de dimerisatie en proteolytische verwerking van DRL.

In **hoofdstuk 3** bestuderen we de functie van *Drosophila* RYK's in het mushroom body (MB): een hersenstructuur die in verband gebracht wordt met kennis en geheugen acquisitie. The α- en β axonen van het MB verbinden verschillende hersenregio's die nodig zijn voor deze processen. Wij tonen aan dat DRL gedurende de hersenontwikkeling nodig is om de α axonen te begeleiden zodat ze contact kunnen maken met hun targetneuronen. DRL wordt echter niet

tot expressie gebracht in de α MB neuronen zelf maar kan daarentegen gedetecteerd worden in een neuronale cellijn die grenst aan de uitgroeiende α axonen. Onze bevindingen laten zien dat deze α axonen DRL-2, een *Drosophila* RYK ortholoog, tot expressie brengen en dat de correcte uitgroei van de α axonen wordt begeleid door, en afhankelijk is van, DRL-2. Het cytoplasmatische domein van DRL-2 is noodzakelijk voor zijn functie. Deze resultaten geven weer dat DRL-2 kan optreden als een axon-intrinsieke begeleidingsreceptor in deze cellulaire context. Het cytoplasmatische domein van DRL daarentegen is in de aangrenzende cellen niet vereist voor α axon begeleiding. Om de α axonen te kunnen begeleiden hangt de functie van de DRL receptor volledig af van zijn klieving in het tetrabasic klief motief. Deze klieving heeft tot gevolg dat het extracellulaire deel van DRL, het Wnt bindingsdomein, vrijkomt. Dit impliceert dat DRL extrinsiek functioneert om WNT5 af te vangen en te presenteren aan de α axonen die DRL-2 tot expressie brengen aan hun celoppervlak en dit signaal intracellulair doorgeven. Dit model is consistent met onze biochemische data en ondersteunt het bestaan van een ternair complex dat bestaat uit het gekliefde ectodomein van DRL, WNT5 en de DRL-2 receptor. Daarom concluderen we dat MB-extrinsieke en –intrinsieke RYK's de MB-α axonen begeleiden via hun gemeenschappelijke ligand WNT5.

Drosophila RYK's functioneren niet alleen als begeleidingsreceptor voor axonen. Ze zijn ook betrokken bij de begeleiding van de laterale transversale spieren (LTMs) naar hun uiteindelijke aanhechtingspunt (MAS) in de lichaamswand van het *Drosophila* embryo. In **hoofdstuk 4** beschrijven we dat in een embryo waar *wnt5* ontbreekt, zoals eerder is aangetoond voor zijn receptor *drl*, deze LTM's regelmatig voorbij schieten aan hun aanhechtingspunt en zich verder ventraal hechten aan een ectopische plek in de lichaamswand. We demonstreren dat als WNT5 tot expressie gebracht wordt in de spieren of de MAS's de spieraanhechting hersteld wordt, terwijl DRL specifiek aanwezig moet zijn in de spier om zijn functie te vervullen. Opvallend heeft de *drl-2* mutant dit fenotype niet, maar de *dnt* mutant heeft ook het fenotype waarbij de spier verder ventraal een ectopisch aanhechtingspunt vindt. Daarbij zien we tijdens de afwezigheid van zowel *drl* als *dnt* het aantal doorschietende spieren dramatisch toeneemt. Dit laat zien dat *drl* en *dnt* samenwerken in de begeleiding van de LTM's naar hun MAS. We tonen ook aan dat *wnt5*, *drl* en *dnt* zeer waarschijnlijk in dezelfde pathway opereren, omdat het verwijderen van een copy van *wnt5* het fenotype van de *drl,dnt* mutanten significant verhoogt. De uitgroeiende spier en zijn beoogde MAS wisselen een aantal moleculaire signalen uit, die ze in staat stellen om een stabiele aanhechting tussen spier en MAS tot stand te brengen. Deze aanhechting wordt gefaciliteerd door de extracellulaire matrix. We vinden tot onze verrassing dat de meerderheid van de nieuwe ectopische MAS's geen Stripe tot expressie brengen; een nucleair eiwit waarvan verondersteld werd dat het zowel noodzakelijk als voldoende is voor de differentiatie van de MAS. Verder leveren we bewijs voor de interactie tussen de doorschietende spier en de voorbestemde MAS,

ook al maakt de doorschietende spier een ectopisch contact elders met een epidermale cel in de lichaamswand. We concluderen dat de wisselwerking van DRL, DNT en WNT5 verantwoordelijk is voor het genereren van een stopsignaal dat herkenning en aanhechting van de spier met de correcte MAS mogelijk maakt. Waarom en hoe de aanhechting met de ectopische epidermale cel tot stand komt is op dit moment niet duidelijk. Verder onderzoek is nodig om het nog onbekende stopsignaal te identificeren.

Het hier gepresenteerde onderzoek vergroot ons begrip van de fundamentele biologische rol en de werkingsmechanismen van Ryk's tijdens aansturing van axonen en spieren naar hun target cellen. Wij hopen dat dit werk ook zal bijdragen aan toekomstige therapieën voor ziektebeelden waar deze eiwitten bij betrokken zijn, waaronder kanker en regeneratie na trauma van het zenuwstelsel.

ABBREVIATION LIST

aa	amino acids
AC	Anterior Commissure
AEL	After Egg Laying
AL	Antennal Lobe
APC	Adenomatous Polyposis Coli
APF	After Puparium Formation
BDB	Brachdactyly type B
CAMKII	Ca2+/calmodulin-dependent protein kinase II
CAT	Chloramphenicol AcetylTransferase
CE	Convergent Extension
CK1α	Casein Kinase 1α
Cn	Calcineurin
CNS	Central Nervous System
CRD	Cysteine Rich Domain
CREB	cAMP response element-binding protein
CRISPR/Cas	Clustered regularly interspaced short palindromic repeat/CRISPR-associated
CST	Corticalspinal Tract
DAAM1	Dishevelled Associated Activator of Morphogenesis
DAG	Diacylglycerol
Dlg1	Disc large tumor suppressor
Dll	Distalless
DM	Dorsomedial
Dnt	Doughnut
Dpp	Decapentaplegic
DRL	Derailed
DRL-2	Derailed-2
DSB	Double stranded breaks
Dsh	Dishevelled
ECD	Extracellular Domain
ECL	Enhanced-ChemiLuminescence
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
Egr	Early Growth Response
EphB2	Ephrin receptor B2
EphB3	Ephrin receptor B3
FasII	Fasciclin II
FCM	Fusion Competent Myoblasts
FGFR	Fibroblast growth factor Receptor
FLNa	Filamin A
FMI	Flamingo
FRA	Frazzled
FZD	Frizzled
GAL4	Yeast transcription activator protein
Gpa	Glycophorin A
GSK3	Glycogen Synthase Kinase 3

HA	Hemagglutinin
HRP	Horseradish Peroxidase
ICD	Intracellular Domain
Ig	Immunoglobin
IP_3	Inositol triphosphate
IRK	Insulin kinase receptor
JNK	c-Jun N-terminal Kinase
Kr	Kringle
Lam	Laminin
LDL	Low Density Lipoprotein
LH	Lateral Horn
LRP	LDL receptor Related Protein
mAb	mouse Anti-body
MAPK	Mitogen-Activated Protein Kinases
MAS	Muscle Attachment Site
MB	Mushroom Body
Mew	Multiple edematous wing
MTJ	Myotendinous Junction
NFAT	Nuclear Factor of Activated T-cells
NF-KB	Nuclear transcription factor kappa-light-chain-enhancer of activated B-cells
NGF	Nerve Growth Factor
NMJ	Neuromuscular Junction
ORF	Open Reading Frame
ORN	Olfactory Receptor Neuron
PC	Posterior Commissure
PCP	Planar Cell Polarity
PDE6	Phosphodiesterase 6
PDZ-BD	zonula occludens 1 protein binding domain
PKC	Protein Kinase C
PLC	Phospholipase C
PM	Plasma Membrane
PN	Projection Neurons
PNETs	Primitive neuroectodermal tumors
PNS	Peripheral Nervous System
PRD	Proline Rich Domain
PSD95	Postsynaptic density protein
PTK	Phosphotyrosine Kinase
RGD	Arg-Gly-Asp
ROBO	Roundabout
ROCK/ROK	Rho associated Kinase
ROR	Receptor tyrosine kinase-like Orphan Receptor
RRS	Robinow syndrome
RTK	Receptor Tyrosine Kinase
RYK	Receptor Related to Tyrosine Kinase
Ser	Serine
SFK	Src family kinases
sFRPs	secreted Frizzled-Related Proteins

SH2	Src Homology 2
SH3	Src Homology 3
Slp	Sloppy-paired
Smo	Smoothened
Sr	Stripe
STAR	Signal Transduction and Activation of RNA
TBC	Tetra-Basic protease Cleavage
TCF/LEF	T cell-Specific transcription Factor/Lymphoid Enhancer-binding Factor
Thr	Threonine
TMD	Transmembrane domain
UAS	Upstream Activating Sequence
Vang	Van Gogh
VNC	Ventral Nerve Cord
WCE	Whole Cell Extracts
Wg	Wingless
WIF	Wnt Inhibitory Factor
ZO-1	zonula occludens 1

CURRICULUM VITAE

On December 20th 1976 Liza Lucia Lahaye was born in Gouda, The Netherlands. She studied Biomedical Sciences at the University of Utrecht and obtained the Bachelor as well as Master diploma. The Master internships were taken at the department of Cell Biology, Utrecht University and the Department of Pediatrics, Cell Biology and Physiology Washington University School of Medicine in St. Louis, USA. In Utrecht she worked in the lab of Prof. Dr. G.J.A.M. Strous and worked on the Growth Hormone Receptor and its dimerization capacity and maturation. In the USA, the amino acids that are responsible for the endocytosis rate of another receptor, LRP1B, were studied in the lab of Dr. G. Bu. To complete the Master's, the Master thesis was written at the Erasmus Medical Center at Rotterdam at the Department of Molecular Immunology in the lab of Dr. F.J.T. Staal and concerned the possible involvement of Wnt-signalling in abnormal haematopoietic development that results in B-cell Leukaemogenesis. The subsequent PhD project was executed in the lab of Prof. Dr. J.N. Noordermeer and Dr. L.G. Fradkin at the department of Molecular Cell Biology at the Leiden University Medical Center. Currently, she is holding a position as a postdoctoral researcher at the department of Cell Biology at the University Medical Center Groningen in the lab of Prof. Dr. O. Sibon.

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