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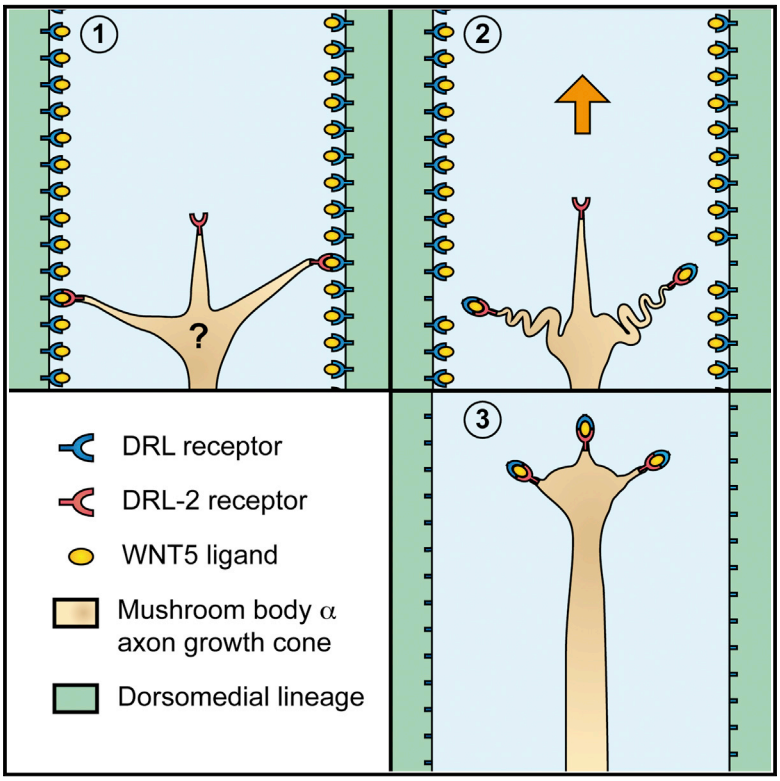
Graphical Abstract



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In Brief

Reynaud et al. find that the DRL receptor, which is not expressed in mushroom body axons, guides them during *Drosophila* development by localizing WNT5 ligand on an adjacent brain structure. There, WNT5, in complex with the shed DRL ectodomain, guides axons via repulsion through their intrinsic DRL-2 receptor.

Highlights

- The DRL receptor positions the WNT5 ligand around the growing mushroom body (MB)
- Localized WNT5 repulses MB axons via their intrinsic DRL-2 receptor
- DRL ectodomain cleavage and shedding is necessary for MB axon guidance
- A DRL ectodomain/WNT5/DRL2 ternary complex is formed

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Guidance of *Drosophila* Mushroom Body Axons Depends upon DRL-Wnt Receptor Cleavage in the Brain Dorsomedial Lineage Precursors

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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 mushroom bodies (MBs) are structures in the insect brain implicated in learning and memory (reviewed in Heisenberg, 2003). Each MB arises from four 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 Préat, 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. However, the cytoplasmic domain of another Ryk, DRL-2, which is expressed by MB neurons, is 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 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 mosaic analysis with a repressible cell marker (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

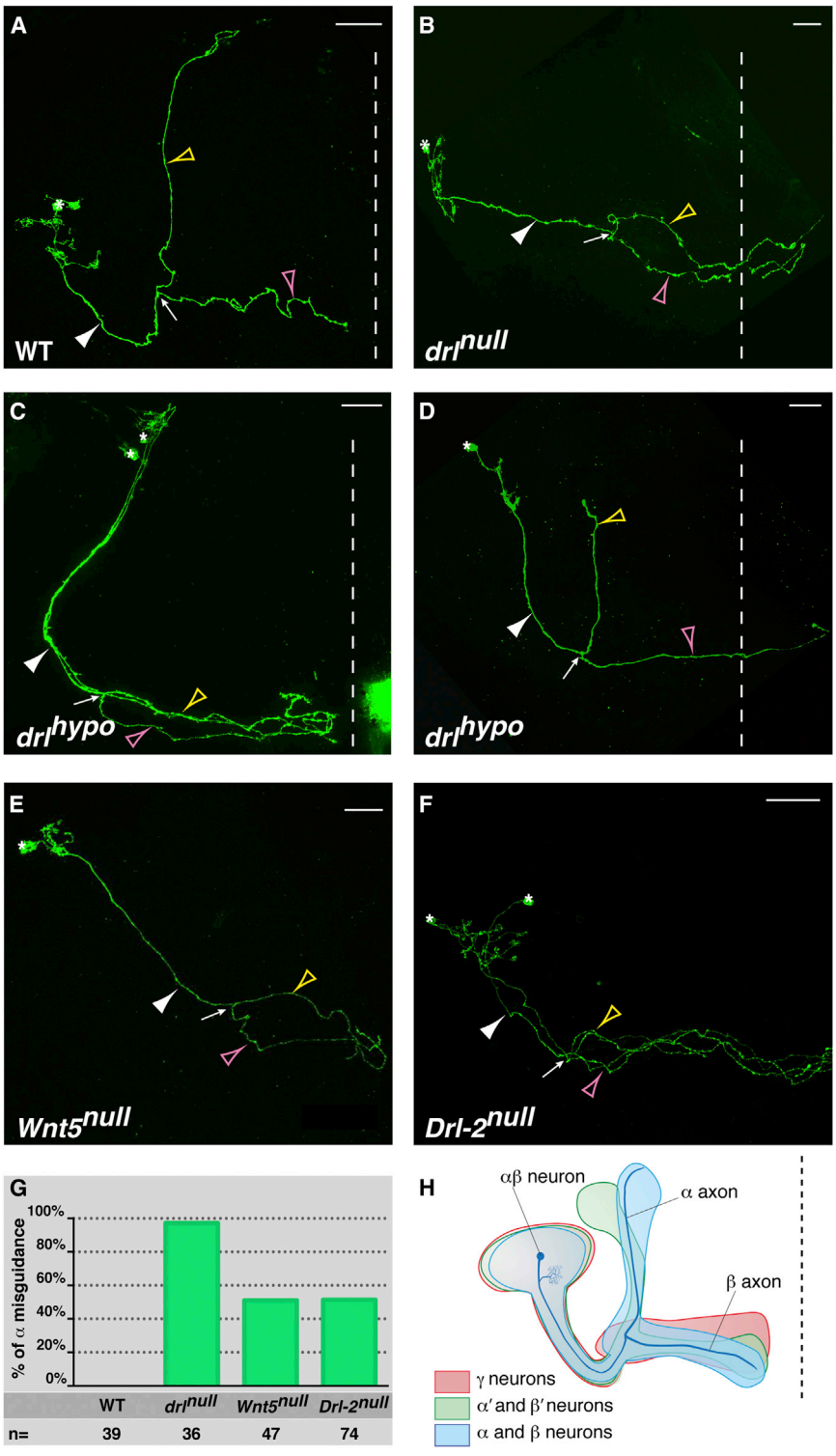

Figure 1. DRL, WNT5, and DRL-2 Are Required for MB α Branch Guidance

(A) A single αβ neuron clone in a wild-type (WT) 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 and S2 and Tables S1 and S2.

display aberrant midline crossing (Figures 1A and 1B). Notably, the separation angle between the wild-type (WT) α and β axon branches is still observed in *drl* mutants (WT: 118.4° ± 5.7°; *drl*: 128.1° ± 16.6°; p = 0.60, t test; results are means ± SEM 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; 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; 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.

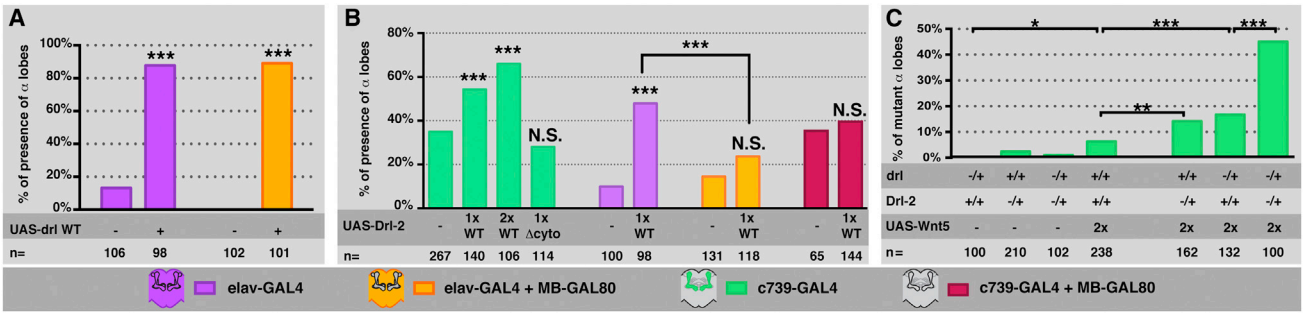

Figure 2. DRL and DRL-2 Interact with WNT5 and Are Required to Guide α Branch Axons

(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 *Drl-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*; *MB-GAL80* (orange). *c739-GAL4 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 ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and N.S. indicates not statistically different by χ^2 test.

See genotypes and other information in Supplemental Information for Figure 2.

See also Figures S3 and S4.

The WNT5 protein acts as a repulsive axon guidance ligand for the DRL receptor in the embryonic central nervous system (CNS) (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 (~30% of the MBs), absence of α and β lobe (~50% of the MBs), and WT MBs (~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), similar to what was previously reported (Shimizu et al., 2011). Both α and β guidance were also observed to be affected in the same neurons (Figures S1E and S1H). Altogether, 51% of the *Wnt5*^{null} clones (n = 47) displayed α axon misguidance (Figure 1G; 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 hr 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 (~60% of the MBs) as well as WT MBs (~35%). Altogether, 51% (n = 74) of the *Drl-2*^{null} visualization MARCM clones displayed α misguidance (Figure 1G; 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; 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 WT 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 WT 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 MBs (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 hr after puparium formation (APF) in WT, 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 was detected, making it unlikely that DRL-2 localization determines why the β 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.

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 WT *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 WT 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-2$^{+/-}$* 100% WT MBs, n = 102). Nevertheless, we noticed that when *Wnt5* was strongly overexpressed in the MBs, a modest but significant fraction (<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-2$^{-/+}$*) in the WNT5-overexpressing background, α misguidance significantly increased relative to the controls (Figure 2C). Finally, we observed a dramatic increase in α misguidance in *drl$^{-/+}$*; *Drl-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-instar larval to adult stages (data not shown; Figure S5), confirming previous reports. At the third-instar larval 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 *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 hr to 48 hr 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 hr APF, but not in them (Figures 3G and 3H), is consistent with an 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 misexpressed in this region in *drl^{null}* brains as early as 24 hr 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 third-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 hr APF on the tips of the MB lobes (Figures 5A–5F). Interestingly, DRL was

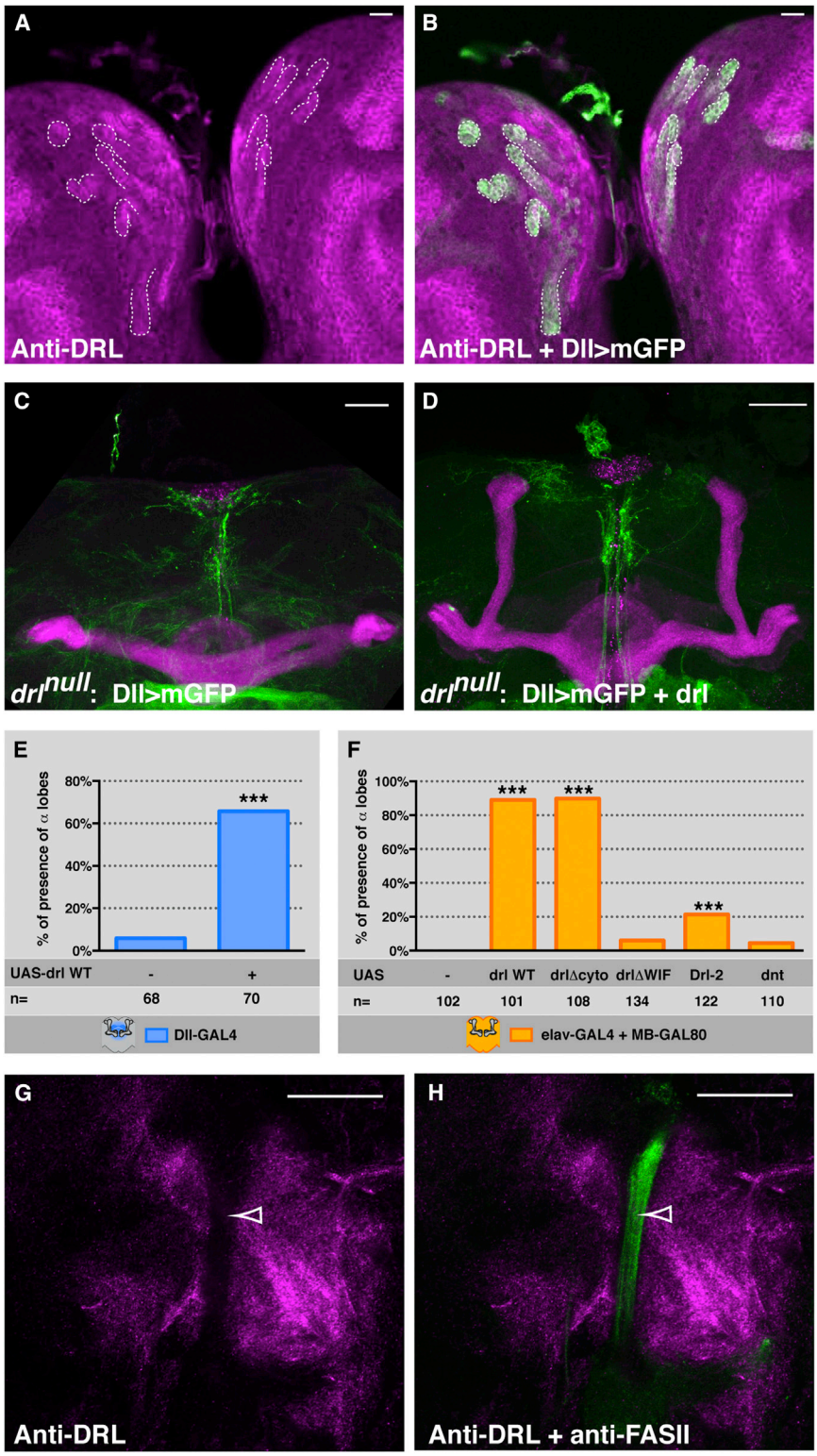

Figure 3. DRL Is Expressed in the Dorsomedial Lineages, Precursors of the Central Complex

(A) DRL (magenta) is expressed in six large groups of cells at the DM margins of the third-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. ***$p < 0.001$ (χ^2 test).

(G and H) 24-hr-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.

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 WT 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 WT transgene strongly rescued, even two copies of the *UAS-drlΔTBC* transgene failed to rescue the drl^{null} α lobe misguidance phenotype (see Figures 5H and 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

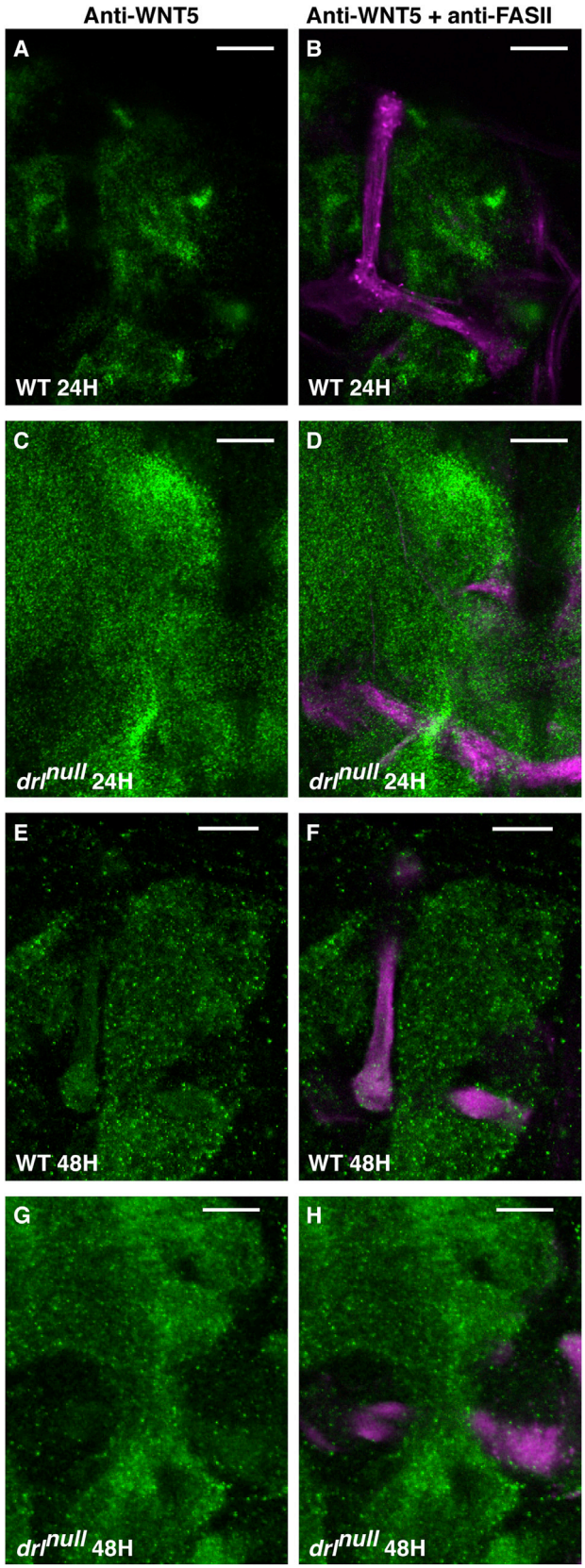

Figure 4. DRL Restricts WNT5 Expression in the Brain

(A, B, E, and F) Wild-type (WT) 24-hr-APF (A and B) and 48-hr-APF brains (E and F) stained with anti-WNT5 (green) and anti-FASII (magenta) revealed a WNT5-free channel at the level of the α MB lobes. At 48 hr APF, WNT5 is expressed within the MB lobes, but the channel was still apparent.

(C, D, G, and H) drl^{null} 24-hr-APF (C and D) and 48-hr-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 WT and mutant.

At 24 hr APF, six MBs were analyzed in both cases (WT and drl^{null}), with zero mutant and six WT MBs for WT and four mutant and two WT for drl^{null}. At 48 hr APF, six MBs were analyzed for WT and 12 MBs for drl^{null}, with 0 mutant and 6 WT MBs for WT and 10 mutant and 2 WT for drl^{null}. Genotypes: (WT) w^{1118}/ y w^{67c23} (24 hr APF), w^{1118} (48 hr APF). (drl^{null}) w^{1118} /w^{1118} ; lio^{2}/drl^{R343}. Scale bars represent 20 μm. Images are single confocal sections.

the hypothesis that DRL's ECD is released by cleavage at the TBC site. Finally, anti-MYC immunoblot analyses of third-instar brains expressing either DRL-WT-MYC or DRL-ΔTBC-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-hr-APF MBs (Shimizu et al., 2011) (data not shown). The presence of both WNT5 and DRL's ECD at the tips of the 48-hr-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 hr, 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 immunoglobulin G (IgG). Fc-containing complexes were captured on protein A agarose, washed, and 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.

DISCUSSION

Here, we have shown that the WT 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 among 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

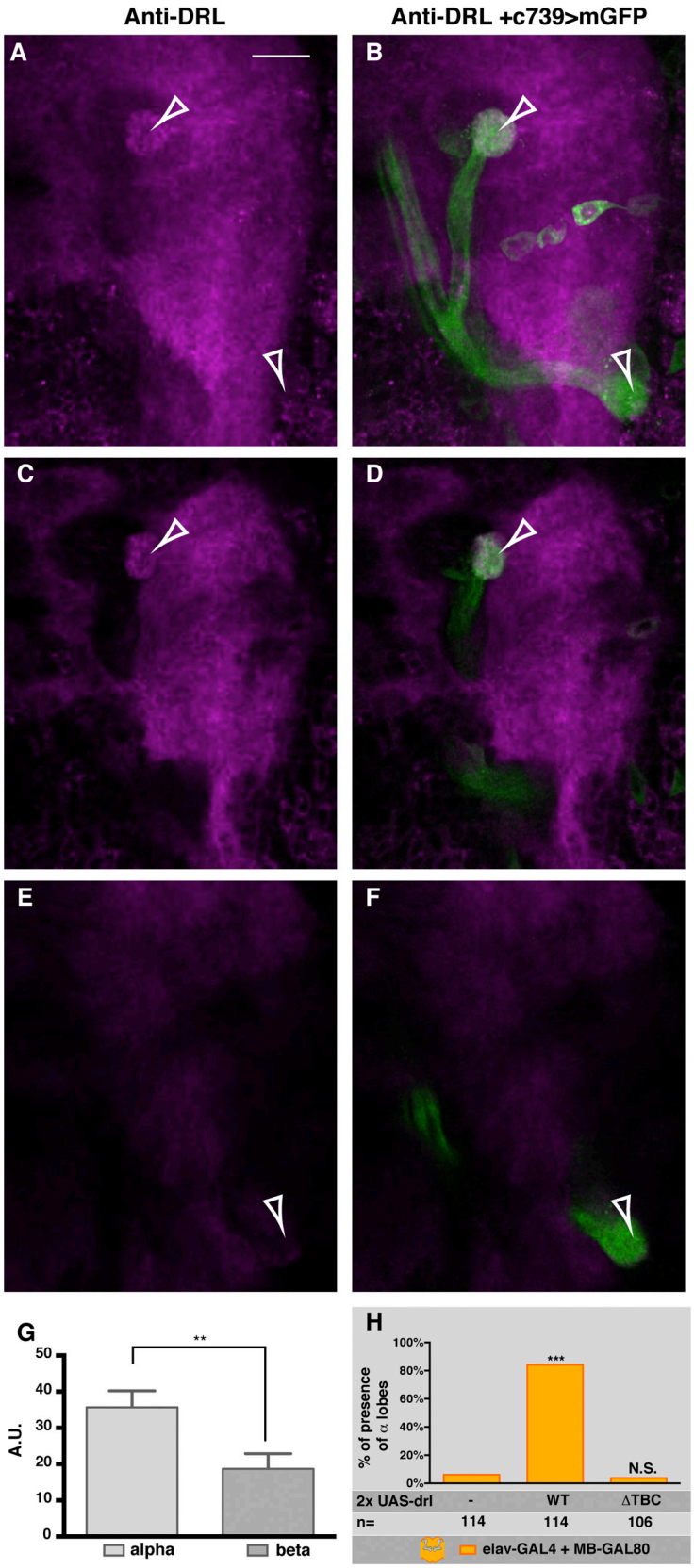

Figure 5. DRL Is Present at the Tips of the MB Lobes, and DRL's TBC Site Is Required to Rescue the Mutant Phenotype

(A–F) *c739-GAL4 UAS-mCD8GFP* 48-hr-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. $^{***}p < 0.001$; NS, not statistically different (χ^2 test).

See genotypes and details in Supplemental Information for Figure 5.

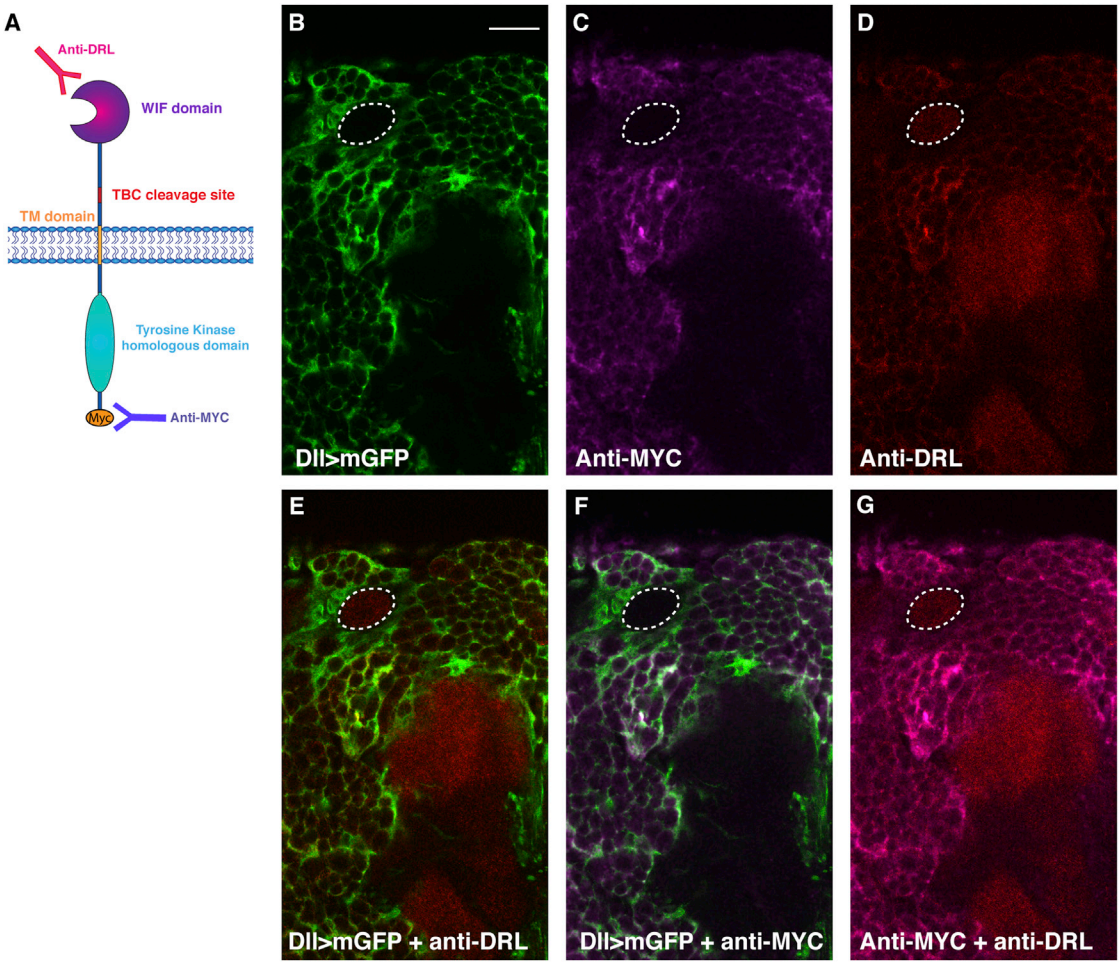

Figure 6. The Cleaved DRL ECD Is Present at the Tip of the MB α Lobe

(A) Schematic representation of the DRL ECD and intracellular domain recognized by the anti-DRL and anti-MYC antibody, respectively.

(B–G) All images shown are of 48-hr-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 carboxy-terminal 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.

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 hr 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 α 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 CNS 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 that is not FRA (Hiramoto et al., 2000), while the Netrin receptor expressed by the incoming

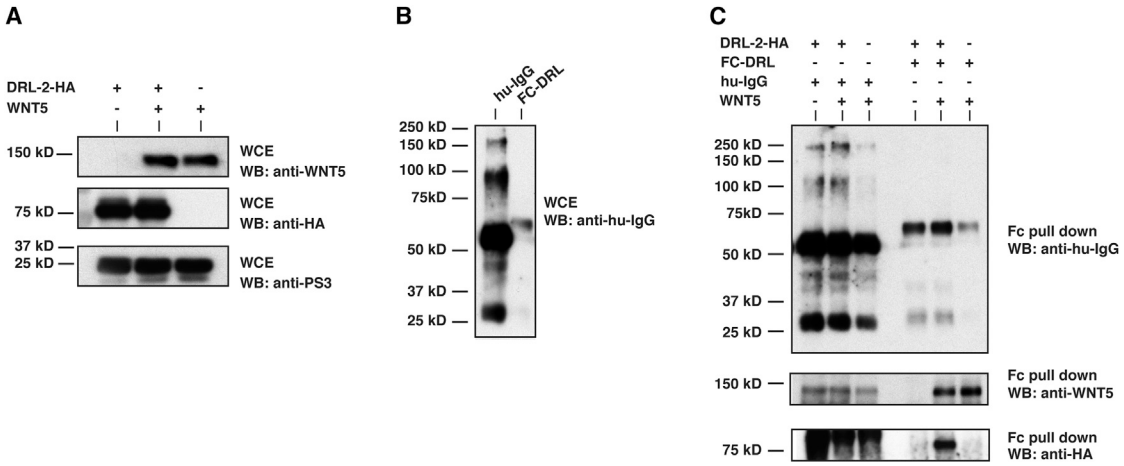

Figure 7. The DRL ECD Forms a WNT5-Dependent Complex with Transmembrane DRL-2

(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 hr 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 and washed, and proteins were 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 non-specific, as evidenced by their presence in all three samples. The data shown are representative of three experiments.

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). We described here a ligand-dependent *trans* interaction between two Wnt receptors, DRL and DRL-2, on different cells.

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, we have demonstrated that ligand localization and ectodomain shedding are necessary for DRL's role in α axon guidance.

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, whereas 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: lio^{2}, 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 min, 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 hr at room temperature. Brains were then washed in PBT for 2 hr 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 (1:2,000, 1:1,000, 1:1,000, and 1:150, respectively). The pre-absorbed anti-DRL-2 was also pre-absorbed a second time using $Drl\text{-}2^{null}$ mutant 48 hr 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 hr and 48 hr APF anti-WNT5 immunostaining, dissected brains were incubated with anti-WNT5 (1:150) in PBS at 4°C for 2 hr 30 min, washed in 1× PBS and fixed in PLP for 1 hr at room temperature, then the protocol above was followed.

Adult Brain Dissection and Immunostaining

Fly heads and thoraxes were fixed for 1 hr 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 WT lobe (>80% of the cases) or thinner lobe (<20% of the cases), with an estimated width ≥40% of that of WT lobe width, was seen using Fiji software.

MARCM Clonal Analysis

To generate clones in the MB, we used the MARCM technique (Lee and Luo, 1999). For single- and two-cell clones, 48-hr-APF pupae were heat-shocked at 37°C for 15 min. For neuroblast clones, first-instar larvae were heat-shocked at 37°C for 1 hr. 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).

qRT-PCR

RNA from third-instar brains was prepared using RNAeasy (QIAGEN) according to the manufacturer's specifications and reversed transcribed using the IScript cDNA Synthesis Kit (Bio-Rad). cDNA was amplified on a CFX384 Real Time PCR System (Bio-Rad) 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 40× 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

Hemagglutinin (HA)-tagged actin promoter-driven WT *Drl-2* and *Drl-2* lacking its WIF domain (*ΔWIF*) and MYC-tagged UAS WT *drl* and *Drl-2* and their mutants lacking the cytoplasmic or WIF domain 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). 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 Fc-DRL-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 third-instar larval brain. Lysates were prepared using a high-stringency buffer (50 mM 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, and 10% glycerol) containing protease inhibitors (Roche). For the immunoblot presented in Figure S7, lysates were prepared from 10 L3 brains homogenized in 1× 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 α-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 χ^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 additional information about Figures 1, 2, 3, and 5, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.035.

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REFERENCES

Aso, Y., Grübel, K., Busch, S., Friedrich, A.B., Siwanowicz, I., and Tanimoto, H. (2009). The mushroom body of adult Drosophila characterized by GAL4 drivers. J. Neurogenet. *23*, 156–172.

Bashaw, G.J., and Klein, R. (2010). Signaling from axon guidance receptors. Cold Spring Harb. Perspect. Biol. *2*, a001941.

Bayraktar, O.A., Boone, J.Q., Drummond, M.L., and Doe, C.Q. (2010). Drosophila type II neuroblast lineages keep Prospero levels low to generate large clones that contribute to the adult brain central complex. Neural Dev. *5*, 26.

Beckett, K., Monier, S., Palmer, L., Alexandre, C., Green, H., Bonneil, E., Raposo, G., Thibault, P., Le Borgne, R., and Vincent, J.P. (2013). Drosophila S2 cells secrete wingless on exosome-like vesicles but the wingless gradient forms independently of exosomes. Traffic *14*, 82–96.

Bonkowsky, J.L., Yoshikawa, S., O'Keefe, D.D., Scully, A.L., and Thomas, J.B. (1999). Axon routing across the midline controlled by the Drosophila Derailed receptor. Nature *402*, 540–544.

Boulanger, A., Clouet-Redt, C., Farge, M., Flandre, A., Guignard, T., Fernando, C., Juge, F., and Dura, J.M. (2011). ftz-f1 and Hr39 opposing roles on EcR expression during Drosophila mushroom body neuron remodeling. Nat. Neurosci. *14*, 37–44.

Boyle, M., Nighorn, A., and Thomas, J.B. (2006). Drosophila Eph receptor guides specific axon branches of mushroom body neurons. Development *133*, 1845–1854.

Brown, M.S., Ye, J., Rawson, R.B., and Goldstein, J.L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell *100*, 391–398.

Callahan, C.A., Muralidhar, M.G., Lundgren, S.E., Scully, A.L., and Thomas, J.B. (1995). Control of neuronal pathway selection by a Drosophila receptor protein-tyrosine kinase family member. Nature *376*, 171–174.

Cong, F., Schweizer, L., and Varmus, H. (2004). Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP. Development *131*, 5103–5115.

Cruciat, C.M., and Niehrs, C. (2013). Secreted and transmembrane wnt inhibitors and activators. Cold Spring Harb. Perspect. Biol. *5*, a015081.

Dura, J.M., Preat, T., and Tully, T. (1993). Identification of linotte, a new gene affecting learning and memory in Drosophila melanogaster. J. Neurogenet. *9*, 1–14.

Fradkin, L.G., van Schie, M., Wouda, R.R., de Jong, A., Kamphorst, J.T., Radjkoemar-Bansraj, M., and Noordermeer, J.N. (2004). The Drosophila Wnt5 protein mediates selective axon fasciculation in the embryonic central nervous system. Dev. Biol. *272*, 362–375.

Fradkin, L.G., Dura, J.M., and Noordermeer, J.N. (2010). Ryks: new partners for Wnts in the developing and regenerating nervous system. Trends Neurosci. *33*, 84–92.

Gatto, G., Morales, D., Kania, A., and Klein, R. (2014). EphA4 receptor shedding regulates spinal motor axon guidance. Curr. Biol. *24*, 2355–2365.

Grillenzoni, N., Flandre, A., Lasbleiz, C., and Dura, J.M. (2007). Respective roles of the DRL receptor and its ligand WNT5 in Drosophila mushroom body development. Development *134*, 3089–3097.

Gross, J.C., Chaudhary, V., Bartscherer, K., and Boutros, M. (2012). Active Wnt proteins are secreted on exosomes. Nat. Cell Biol. *14*, 1036–1045.

Hattori, M., Osterfield, M., and Flanagan, J.G. (2000). Regulated cleavage of a contact-mediated axon repellent. Science *289*, 1360–1365.

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. Nat. Rev. Neurosci. *4*, 266–275.

Hiramoto, M., Hiromi, Y., Giniger, E., and Hotta, Y. (2000). The Drosophila Netrin receptor Frazzled guides axons by controlling Netrin distribution. Nature *406*, 886–889.

Izergina, N., Balmer, J., Bello, B., and Reichert, H. (2009). Postembryonic development of transit amplifying neuroblast lineages in the Drosophila brain. Neural Dev. *4*, 44.

Kelley, M.R., Xu, Y., Wilson, D.M., 3rd, and Deutsch, W.A. (2000). Genomic structure and characterization of the Drosophila S3 ribosomal/DNA repair gene and mutant alleles. DNA Cell Biol. *19*, 149–156.

Krashes, M.J., Keene, A.C., Leung, B., Armstrong, J.D., and Waddell, S. (2007). Sequential use of mushroom body neuron subsets during Drosophila odor memory processing. Neuron *53*, 103–115.

Lahaye, L.L., Wouda, R.R., de Jong, A.W., Fradkin, L.G., and Noordermeer, J.N. (2012). WNT5 interacts with the Ryk receptors doughnut and derailed to mediate muscle attachment site selection in Drosophila melanogaster. PLoS ONE *7*, e32297.

Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451–461.

Lee, T., Lee, A., and Luo, L. (1999). Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development *126*, 4065–4076.

Lyu, J., Yamamoto, V., and Lu, W. (2008). Cleavage of the Wnt receptor Ryk regulates neuronal differentiation during cortical neurogenesis. Dev. Cell *15*, 773–780.

Ng, J. (2012). Wnt/PCP proteins regulate stereotyped axon branch extension in Drosophila. Development *139*, 165–177.

Pascual, A., and Préat, T. (2001). Localization of long-term memory within the Drosophila mushroom body. Science *294*, 1115–1117.

Petrova, I.M., Lahaye, L.L., Martiáñez, T., de Jong, A.W., Malessy, M.J., Verhaagen, J., Noordermeer, J.N., and Fradkin, L.G. (2013). Homodimerization of the Wnt receptor DERAILED recruits the Src family kinase SRC64B. Mol. Cell. Biol. *33*, 4116–4127.

Sakurai, M., Aoki, T., Yoshikawa, S., Santschi, L.A., Saito, H., Endo, K., Ishikawa, K., Kimura, K., Ito, K., Thomas, J.B., and Hama, C. (2009). Differentially expressed Drl and Drl-2 play opposing roles in Wnt5 signaling during Drosophila olfactory system development. J. Neurosci. *29*, 4972–4980.

Shimizu, K., Sato, M., and Tabata, T. (2011). The Wnt5/planar cell polarity pathway regulates axonal development of the Drosophila mushroom body neuron. J. Neurosci. *31*, 4944–4954.

Shin, J.E., and DiAntonio, A. (2011). Highwire regulates guidance of sister axons in the Drosophila mushroom body. J. Neurosci. *31*, 17689–17700.

Timofeev, K., Joly, W., Hadjieconomou, D., and Salecker, I. (2012). Localized netrins act as positional cues to control layer-specific targeting of photoreceptor axons in Drosophila. Neuron *75*, 80–93.

Xu, K., Wu, Z., Renier, N., Antipenko, A., Tzvetkova-Robev, D., Xu, Y., Minchenko, M., Nardi-Dei, V., Rajashankar, K.R., Himanen, J., et al. (2014). Neural migration. Structures of netrin-1 bound to two receptors provide insight into its axon guidance mechanism. Science *344*, 1275–1279.

Yao, Y., Wu, Y., Yin, C., Ozawa, R., Aigaki, T., Wouda, R.R., Noordermeer, J.N., Fradkin, L.G., and Hing, H. (2007). Antagonistic roles of Wnt5 and the Drl receptor in patterning the Drosophila antennal lobe. Nat. Neurosci. *10*, 1423–1432.

Yoshikawa, S., Bonkowsky, J.L., Kokel, M., Shyn, S., and Thomas, J.B. (2001). The derailed guidance receptor does not require kinase activity in vivo. J. Neurosci. *21*, RC119.

Yoshikawa, S., McKinnon, R.D., Kokel, M., and Thomas, J.B. (2003). Wnt-mediated axon guidance via the Drosophila Derailed receptor. Nature *422*, 583–588.

Yu, D., Akalal, D.B., and Davis, R.L. (2006). Drosophila alpha/beta mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. Neuron *52*, 845–855.

Zhu, S., Lin, S., Kao, C.F., Awasaki, T., Chiang, A.S., and Lee, T. (2006). Gradients of the Drosophila Chinmo BTB-zinc finger protein govern neuronal temporal identity. Cell *127*, 409–422.