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# Different Wnt signals act through the Frizzled and RYK receptors during *Drosophila* salivary gland migration

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Guided cell migration is necessary for the proper function and development of many tissues, one of which is the *Drosophila* embryonic salivary gland. Here we show that two distinct Wnt signaling pathways regulate salivary gland migration. Early in migration, the salivary gland responds to a WNT4-Frizzled signal for proper positioning within the embryo. Disruption of this signal, through mutations in *Wnt4*, *frizzled* or *frizzled 2*, results in misguided salivary glands that curve ventrally. Furthermore, disruption of downstream components of the canonical Wnt pathway, such as *dishevelled* or *Tcf*, also results in ventrally curved salivary glands. Analysis of a second Wnt signal, which acts through the atypical Wnt receptor Derailed, indicates a requirement for *Wnt5* signaling late in salivary gland migration. WNT5 is expressed in the central nervous system and acts as a repulsive signal, needed to keep the migrating salivary gland on course. The receptor for WNT5, Derailed, is expressed in the actively migrating tip of the salivary glands. In embryos mutant for *derailed* or *Wnt5*, salivary gland migration is disrupted; the tip of the gland migrates abnormally toward the central nervous system. Our results suggest that both the *Wnt4-frizzled* pathway and a separate *Wnt5-derailed* pathway are needed for proper salivary gland migration.

**KEY WORDS: Derailed, WNT5, WNT4, RYK, Migration, Salivary gland**

## INTRODUCTION

Wnt signaling plays a crucial role in many developmental processes, including cell fate determination, cell proliferation and cell migration. Many of these processes are carried out through the Frizzled family of receptors. Of the four *Drosophila* Frizzled genes, the best characterized are *frizzled* (*fz*) and *frizzled 2* (*fz2*), which are redundant in segmentation of the early embryo (Bhanot et al., 1999). Three different branches of Wnt signaling lie downstream of Frizzled receptors. In what is called the canonical Wnt pathway, the presence of a Wnt signal stabilizes cytoplasmic β-catenin, leading to its nuclear translocation and the activation of the transcription factor TCF (Pangolin – FlyBase) or LEF (Logan and Nusse, 2004). The other two branches, which do not require β-catenin, are referred to as the planar cell polarity (PCP) and calcium pathways (Cadigan and Liu, 2006; Logan and Nusse, 2004).

A completely different type of Wnt signaling uses related-to-tyrosine-kinase (RYK) receptors rather than Frizzled receptors. Members of the RYK subfamily have unusual, but highly conserved, amino acid substitutions in their intracellular kinase domains that eliminate kinase activity (Callahan et al., 1995; Halford et al., 1999; Hovens et al., 1992; Oates et al., 1998). In addition, RYKs have an extracellular Wnt inhibitory factor (WIF) domain or Wnt-binding domain that allows RYKs to function as Wnt receptors. In *Drosophila*, WNT5 has been shown to bind to the WIF domain of the RYK receptor Derailed (DRL) (Yoshikawa et al., 2003). Much like Frizzled receptors, RYKs are important for a variety of developmental processes, including axon guidance, organogenesis and craniofacial development (Bonkowsky et al., 1999; Fradkin et al., 2004; Halford et al., 2000; Inoue et al., 2004; Lu et al., 2004).

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These two different Wnt pathways are both known for their roles in cell migration and axon guidance (Bovolenta et al., 2006; Kamitori et al., 2005; Nelson and Nusse, 2004). In addition, we find that they are required for salivary gland migration. Much of our knowledge about cell migration in general has been gained by studying single motile cells in culture. While these studies have contributed greatly to our understanding of the mechanics of cell movement, they do not provide us with a very clear picture of cell migration in the three-dimensional context of a living organism. Furthermore, in many cases, cells do not migrate alone within the embryo, but rather migrate as part of a larger tissue (Lecaudey and Gilmour, 2006). Migration of the salivary glands in *Drosophila* embryos offers an opportunity to explore these processes.

The *Drosophila* embryonic salivary glands provide a morphologically simple system in which to study collective cell migration. The salivary gland anlage is specified by the homeotic gene, *Sex combs reduced* (*Scr*), which activates several factors, including the transcription factor *fork head* (*fkh*) in the salivary placodes (Panzer et al., 1992). During early stage 11, the circular salivary placodes form and are visible as two groups of cells on either side of the ventral midline in parasegment 2 (Fig. 1A,B). The salivary gland cells invaginate into the interior of the embryo at a 45° angle during stage 12 until they contact the visceral mesoderm (Fig. 1C,D). The tubular salivary glands then turn toward the posterior, continuing their migration until all of the salivary gland cells have internalized (Fig. 1E,F). During this phase of migration, the salivary gland tip cells extend lamellipodial protrusions and, using integrin-based motility, actively travel along the visceral mesoderm. The substrate for this movement is the circular visceral mesoderm (CVM) that will ultimately form the inner layer of the gut muscle (Bradley et al., 2003; Kerman et al., 2006; Vining et al., 2005). During their migration, the glands are guided by the chemoattractant Netrin and the chemorepellent Slit to arrive at their correct position within the embryo (Kolesnikov and Beckendorf, 2005). By stage 14, the longitudinal visceral mesoderm (LVM), which will form the outer layer of the gut muscle, migrates from the posterior part of the embryo anteriorly over the CVM and separates

the distal tip of salivary glands from the CVM (Fig. 1J). Separation of proximal portions of the salivary gland from the CVM occurs as the gut contracts posteriorly. After the migration has finished, the distal tip of the salivary gland maintains contact with the LVM (Fig. 1G,H,K) (Vining et al., 2005).

Here we show that the proper positioning of the *Drosophila* salivary glands requires two separate Wnt pathways. First, the canonical Wnt pathway, activated by WNT4, is required early in the migration of the salivary gland. Disruption of either *fz* or *fz2*, as well as additional downstream components of the Wnt pathway such as *dishevelled* (*dsh*), results in misguided salivary glands. Second, the atypical Wnt receptor *derailed* responds to WNT5 late in salivary gland development. Mutations in *drl* or *Wnt5* lead to a guidance defect that specifically affects the cells located at the leading tip of the migrating salivary glands. Furthermore, we provide evidence that Src kinases may be acting downstream of DRL. This study provides novel insights into the guidance mechanisms governing salivary gland migration.

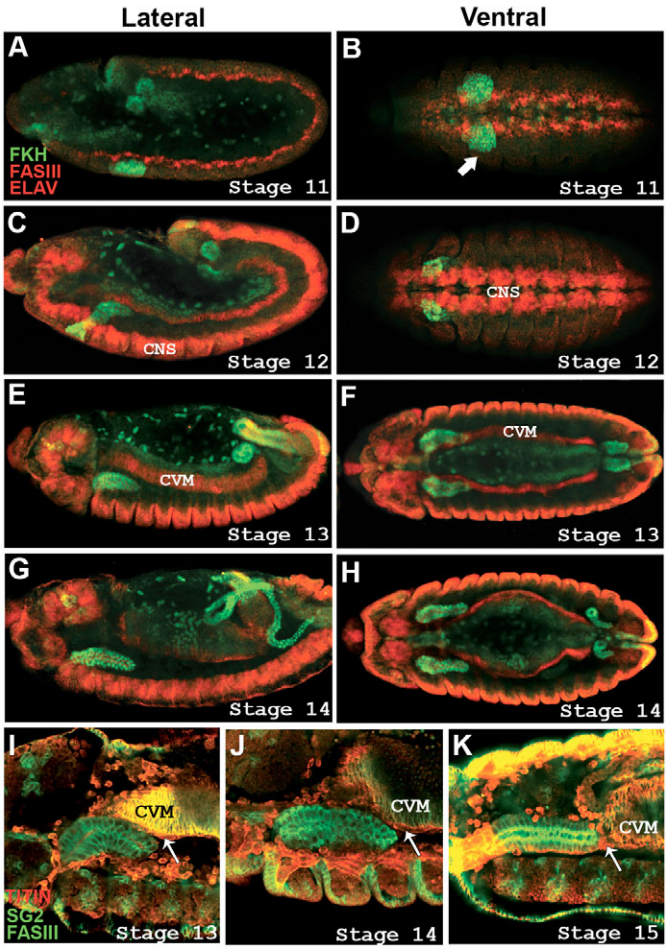


**Fig. 1. Morphogenesis of the *Drosophila* salivary glands.** (**A-H**) Salivary gland cells (arrow) are stained for FKH in green, the CNS is visualized in red by ELAV staining and the CVM is stained for FASIII, also in red. (A,C,E,G) Lateral and (B,D,F,H) corresponding ventral views of embryos, stages 11 through 14. (A,B) Salivary glands begin as a pair of single-layered epithelial disks, the salivary placodes that invaginate by apical constriction to form slender tubes. (C,D) As they leave the surface these tubes extend dorsally and posteriorly at a 45° angle on either side of the CNS until they reach the visceral mesoderm. (E,F) Then they change paths and migrate actively along the mesoderm until they lie horizontally within the embryo, dorsal and lateral to the CNS. (G,H) By stage 14, the glands encounter the longitudinal visceral mesoderm (not shown) and separate from the circular visceral mesoderm. (**I-K**) Lateral views of embryos stage 13-15 stained with the salivary gland marker SG2 in green, the mesodermal marker Titin in red and the CVM marker FASIII also in green. In these panels the CVM appears yellow due to FASIII-Titin co-staining; LVM (arrows) is red due to Titin, but not FASIII, staining. (I) At the end of stage 13, cells of the LVM migrate anteriorly and displace the distal end of the salivary gland from the CVM. (J,K) From stage 14, the tip of the salivary gland remains in contact with the LVM.

## MATERIALS AND METHODS

### Fly strains

The following alleles were used: $drl^{R343}$, $drl^{3.765}$, $Drl\text{-}2^{E124}$, $Wnt5^{D7}$ (Bonkowsky et al., 1999; Yoshikawa et al., 2003), $Src64^{P1}$ (Dodson et al., 1998), $Src42^{E1}$ (Tateno et al., 2000), $dnt^{e00722}$, $fz^{1}$, $sli^{2}$, $w^{1118}$, $Wnt2^{L}$ and $Wnt4^{EMS23}$ (from the Bloomington Stock Center).

The following GAL4 and UAS lines were used: *UAS-drl*, $UAS\text{-}drl^{\Delta i}$ (Yoshikawa et al., 2001; Yoshikawa et al., 2003), *UAS-Wnt5* (Fradkin et al., 2004), *UAS-Wnt4* (Cohen et al., 2002), *UAS-fzN*, *UAS-fz2N* (Zhang and Carthew, 1998), $UAS\text{-}dsh^{DIX}$, $UAS\text{-}dsh^{bPDZ}$ (dominant-negative constructs for canonical Wnt pathway) and $UAS\text{-}dsh^{\Delta DEP+}$, $UAS\text{-}dsh^{DEP+}$ (dominant-negative construct for PCP pathway) (Axelrod et al., 1998), *UAS-TCFΔN* (Bloomington Stock Center), *fkh-GAL4* (B. Zhou, PhD thesis, University of California, 1995), and *bap-GAL4* (Weiss et al., 2001).

### Immunocytochemistry and in situ hybridization

Embryo fixation and staining were performed as described (Chandrasekaran and Beckendorf, 2003). The salivary gland, apical-specific antibody used was mouse anti-Crumbs (CRB) (Cq4; Developmental Studies Hybridoma Bank, University of Iowa) at 1:25 and the salivary gland nuclear-specific antibody used was rabbit anti-FKH (1:1000). In addition, rabbit anti-SG2 (PH4αSG2 – FlyBase) was used at 1:3000 to visualize the salivary glands (Abrams et al., 2006). Rat anti-Titin (Sls – FlyBase) was used at 1:500 to visualize the LVM (Machado et al., 1998). Rabbit anti-β-galactosidase antibody (Roche) was used at 1:1000. The mouse anti-FASIII (FAS3 – FlyBase) (7G10) antibodies were all obtained from the Hybridoma Bank and used at 1:10. Alexa Fluor 546 and 488 (Molecular Probes) secondary antibodies were used at 1:500. Fluorescent in situ hybridization was performed as described (Tautz and Pfeifle, 1989) with modifications (Harland, 1991) using antisense digoxigenin-labeled probes. Mouse anti-DIG (Roche) was used at 1:100 with the Alexa Fluor 546 (Molecular Probes) secondary antibody at 1:250. After washing, embryos were cleared with 50% glycerol, then 70% glycerol and visualized on a Zeiss 510 confocal microscope.

## RESULTS

### *derailed* is expressed in the distal tip cells of the salivary glands

Using whole-mount RNA in situ hybridization to determine the patterns of gene expression during *Drosophila* embryogenesis, the Berkeley *Drosophila* Genome Project has created a searchable database (http://www.fruitfly.org/cgi-bin/ex/insitu.pl) of gene expression patterns. This database has allowed us to identify over 250 genes that are expressed in the embryonic salivary glands and ducts. One such gene encodes the receptor *drl*. Although previous studies described *drl* RNA and protein expression in embryos, salivary gland expression was not reported (Yoshikawa et al., 2003). We found that *drl* RNA expression commenced at stage 11 in the dorsoposterior portion of the salivary placodes, encompassing the initial site of invagination and thus the cells that will later form the distal tip of the salivary gland (Fig. 2A). During stage 12 *drl* RNA expression decreased, but an enhancer trap for *drl*, $drl^{3.765}$, showed β-galactosidase expression, which suggests that DRL persists at the tip of the migrating salivary gland through stage 16 (Fig. 2B).

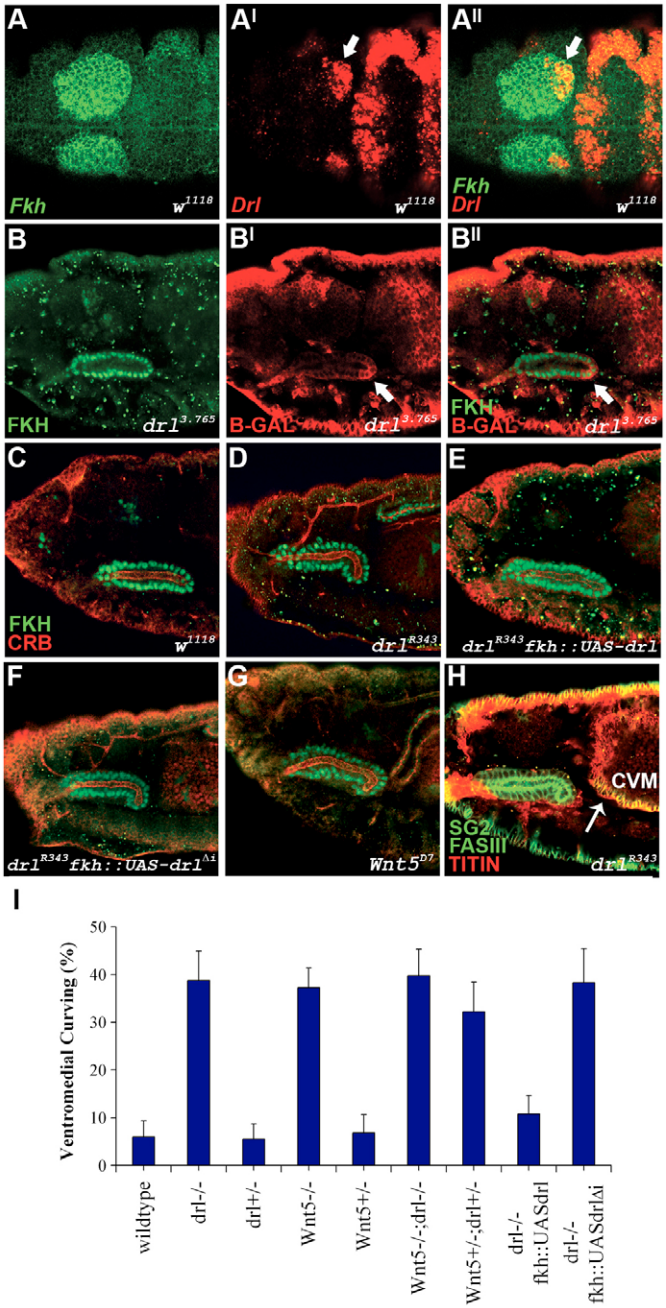


**Fig. 2. *drl* and *Wnt5* are required for proper positioning of the tip of the salivary gland.** (**A-A″**) Wild-type *Drosophila* embryos were hybridized in situ with *fkh* and *drl* probes. Ventral view of a stage 11 embryo. The *drl* receptor is expressed in the dorsoposterior of the salivary placode (arrow), which later corresponds to the salivary gland cells that lead invagination into the embryo. (**B-B″**) Lateral view of stage 15 embryo with an enhancer trap insertion at the *drl* locus. β-galactosidase expression at the salivary gland is confined to the distal tip. Arrows denote the location of *drl* expression in the salivary gland. (**C-H**) Lateral view of stage 15-16 embryos stained for FKH and the apical cell marker CRB. (**I**) Graphical representation of phenotypic penetrance in embryos scored for salivary gland migration defects at stages 14-16. (C) Wild-type control. (D,I) In *drl*-null embryos, the salivary glands curve ventromedially. (E,I) The $drl^{R343}$ mutant phenotype can be rescued by *UAS-drl* using a salivary-gland-specific GAL4 driver, *fkh-GAL4*. (F,I) The intracellular domain of *drl* is important for *drl* function, as *UAS-*$drl^{\Delta i}$ does not rescue the salivary gland migration phenotype in $drl^{R343}$ mutant embryos. (G,I) In embryos lacking *Wnt5* the tip of the salivary gland is bent toward the CNS. (H) Lateral view of a stage 15 embryo stained with the salivary gland marker SG2 in green, the mesodermal marker Titin in red and the CVM marker FASIII also in green. The salivary glands of $drl^{R343}$ mutant embryos do not maintain contact with LVM (compare with Fig. 1K).

## Expression of *drl* in the salivary placode is dependent on *Scr* and *fkh*

Since *drl* RNA expression in the salivary placodes begins at stage 11, later than primary *Scr* target genes, *drl* might be indirectly activated by *Scr* through one of these target genes. As expected, we found that *drl* expression was absent in *Scr* mutant embryos (Fig. 3B, compare with A). Among the primary *Scr* targets, we tested three transcription factors that are required for salivary gland development. In embryos mutant for *huckebein* (*hkb*) or *trachealess* (*trh*), *drl* expression remained unchanged (data not shown). In contrast, *fkh*-mutant embryos lacked *drl* expression in the salivary placodes, although its expression in the epidermis and central nervous system (CNS) was unaffected (Fig. 3C, compare with A).

## WNT5-DRL signaling is required at the tip of the salivary glands

In the CNS, *drl* and *Wnt5* control axon pathfinding by repelling growth cones that attempt to choose the incorrect commissure as they cross the midline (Bonkowsky et al., 1999). The conspicuous expression of *drl* at the tip of the salivary gland, along with its previously reported role in axon guidance, suggested that *drl* might be required for salivary gland guidance. We found that in 40% of *drl*-null embryos the salivary glands curved ventromedially toward the CNS, instead of lying parallel to the midline of the embryo as in wild type (Fig. 2D,I). Interestingly, this abnormal curvature affected only the distal tip of the salivary gland, the cells that specifically express *drl*, and was first seen in mutant embryos at stage 14. This is the time that the tip cells normally detach from the CVM and associate with the LVM. We found that the *drl*-mutant glands did not maintain contact with the LVM. Instead they curved ventromedially, and the tip frequently adhered to the somatic mesoderm (Fig. 2H, Table 1).

To test whether DRL is required autonomously in the salivary glands, we utilized the GAL4-UAS system (Brand and Perrimon, 1993) to express full-length DRL throughout the salivary glands of *drl* mutants. The gland guidance defects of *drl* mutants were completely rescued in embryos carrying the *fkh-GAL4* and *UAS-drl* constructs (Fig. 2E,I). In contrast, we were unable to rescue the *drl* mutant phenotype using *UAS-*$drl^{\Delta i}$, a construct lacking the intracellular domain, and thus the atypical kinase domain, of *drl* (Yoshikawa et al., 2003) (Fig. 2F,I).

During embryonic development, WNT5 is expressed predominantly in the CNS (Fradkin et al., 2004; Yoshikawa et al., 2003). *Wnt5* RNA expression begins during stage 12 and WNT5 protein starts to accumulate primarily in the posterior commissures during stage 13 (Fradkin et al., 2004). The CNS, and thus the expression domain for *Wnt5*, is adjacent to the salivary glands during their posterior migration, making an interaction between the ligand in the CNS and its receptor in the salivary gland tip possible.

**Table 1. Attachment to the longitudinal visceral mesoderm is disrupted in *drl* mutant embryos**

| | Uncurved glands | | Curved glands | | Percent total | | Number of embryos scored |
|---|---|---|---|---|---|---|---|
| | Unattached to LVM | Attached to LVM | Unattached to LVM | Attached to LVM | Unattached to LVM | Attached to LVM | |
| $drl^{R343}$ | 2 | 23 | 14 | 1 | 40 | 60 | 40 |
| $w^{1118}$ | 2 | 37 | 1 | 2 | 7 | 93 | 42 |

In embryos lacking *Wnt5*, the tips of the salivary glands migrated ventromedially, just as they do in *drl*-mutant embryos (Fig. 2G). The $Wnt5^{D7}$; $drl^{R343}$ double mutant showed the same phenotype with similar penetrance as either of the single mutants alone, suggesting that *drl* and *Wnt5* act in the same pathway (Fig. 2I). Embryos heterozygous for both *drl* and *Wnt5* also showed the ventral curving defect with a slightly lower penetrance (Fig. 2I).

There are two other *Drosophila* members of the RYK subfamily, *doughnut on 2* (*dnt*) and *Derailed 2* (*Drl-2*), that might participate in *Wnt5* signaling. DNT can partially rescue a muscle attachment defect of *drl* mutant embryos, suggesting that DNT and DRL may have significant overlapping biochemical activities (Oates et al., 1998). *Drl-2*, like *drl*, has been implicated in axon guidance (S. Yoshikawa and J. B. Thomas, personal communication). Since *drl*-null mutations caused only a partially penetrant salivary gland phenotype, we tested whether the other two *Drosophila* RYKs might be acting redundantly with *drl*. By themselves, both *dnt* and *Drl-2* mutant embryos showed qualitatively similar phenotypes to those of *drl* mutants, but with significantly lower penetrance (Fig. 4B,C,E). We also discovered dominant genetic interactions between mutations in *drl* and *Drl-2*, suggesting that these two genes might be acting in the same process (Fig. 4D,E). As embryos lacking both *drl* and *Drl-2* did not show an enhancement over either of the single mutants with regard to salivary gland migration, it remains unclear whether these genes act redundantly in salivary gland positioning.

Taking the results in this section together, we conclude that WNT5, probably emanating from the CNS, acts through the DRL receptor at the tip of migrating salivary gland to keep the gland on track and prevent it from bending toward the CNS. The DNT and DRL-2 receptors may play a minor role in this WNT5 signaling.

## WNT4 signaling is also required for salivary gland migration

Since relatively little is known about the interactions between *drl* and its ligands, we wanted to test whether additional Wnt ligands might be needed for salivary gland migration. Of the seven known Wnt homologs in the *Drosophila* genome (Adams et al., 2000; Rubin et al., 2000), we tested *wg*, *Wnt2*, *Wnt4* and *Wnt5* mutants for salivary gland defects. We were unable to ascertain whether *wg* is needed for salivary gland guidance due to its earlier requirement for

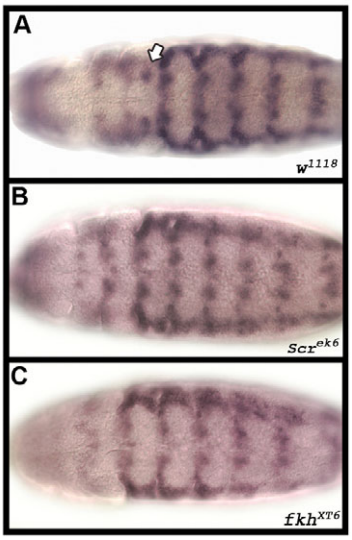


**Fig. 3. Expression of *drl* in the salivary gland is dependent on *Scr* and *fkh*.** (**A-C**) In situ hybridizations for *drl* were performed on stage 11 wild-type (A) $Scr^{ek6}$ (B) and $fkh^{XT6}$ (C) mutant *Drosophila* embryos. *drl* expression in the salivary placode is absent in both *Scr* and *fkh* mutants, indicating that *drl* is a transcriptional target of FKH. Arrow in A denotes the location of *drl* in wild-type salivary placodes.

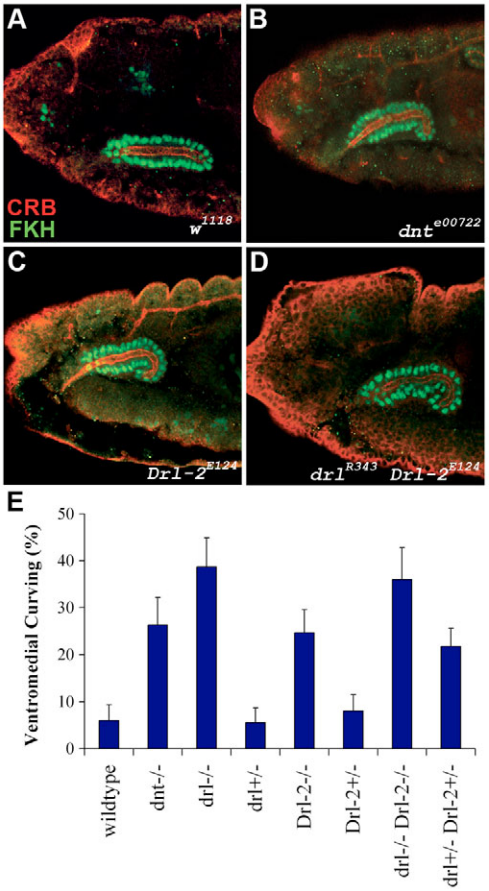


**Fig. 4. Salivary gland positioning is disrupted in *dnt* and *Drl-2* mutant embryos.** (**A-D**) Lateral views of stage 15-16 *Drosophila* embryos stained with FKH and CRB. (**E**) Graphical representation of phenotypic penetrance in embryos scored for salivary gland migration defects at stages 14-16. (A) Wild-type control. (B) $dnt^{e00722}$ mutant embryos have salivary glands that curve ventrally at the tip of the salivary gland. (C) Similarly, *Drl-2*-null embryos also show a *derailed*-like phenotype. (D,E) Salivary gland curving is also seen in $drl^{R343}$ $Drl\text{-}2^{E124}$ double mutants, but the penetrance of the phenotype is not enhanced compared to either of the single mutants.

segmentation. *Wnt2* mutant embryos did not exhibit salivary gland defects. However, lack of *Wnt4* did disrupt salivary gland guidance. Embryos mutant for *Wnt4* displayed a ventral curving phenotype that is similar to *drl* and *Wnt5* mutants, except that the entire gland, rather than just the tip, was curved ventromedially (Fig. 5B). This positioning defect occurred earlier than the *drl* phenotype, disrupting salivary gland positioning as early as stage 12. At this stage in wild-type embryos, *Wnt4* is expressed ventral to the migrating salivary glands in narrow ectodermal stripes and in the assembling ventral nerve cord (Graba et al., 1995).

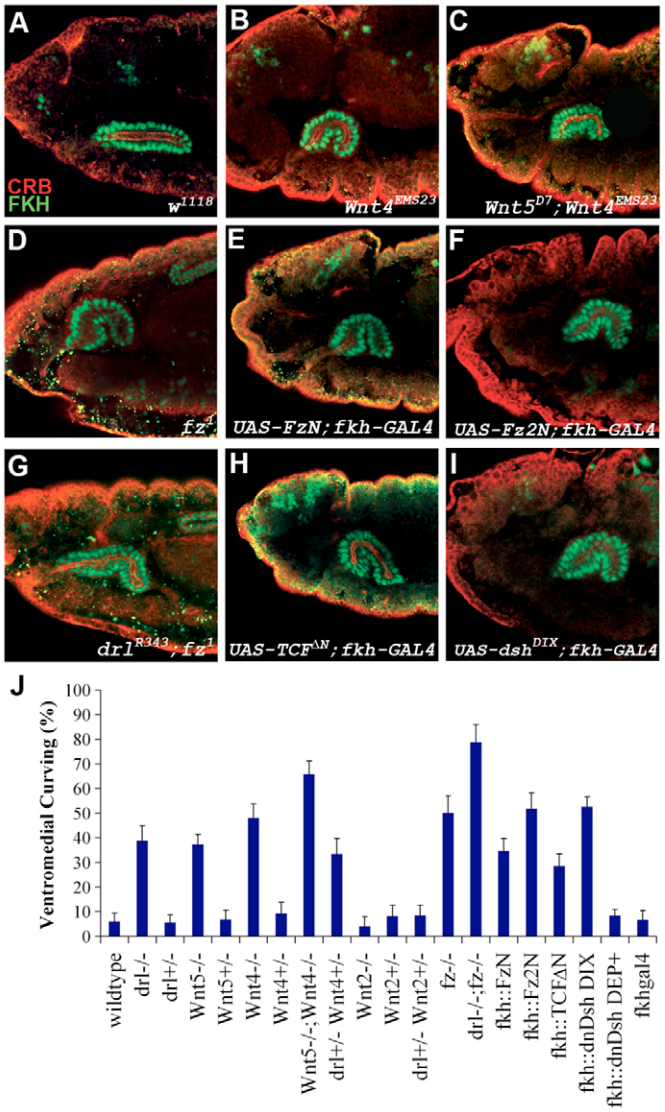


**Fig. 5. Two independent Wnt pathways regulate salivary gland positioning.** (**A-I**) Lateral views of stage 15-16 *Drosophila* embryos stained with FKH and CRB. (**J**) Graphical representation of phenotypic penetrance in embryos scored for salivary gland migration defects at stages 14-16. (A) Wild-type control. (B) *Wnt4*-null embryos have a ventral curving defect that affects a large portion of the salivary gland. (C,J) Embryos mutant for both $Wnt4^{EMS23}$ and $Wnt5^{D7}$ show an enhanced penetrance (68%) of the salivary gland guidance phenotype compared with the single mutants. (D) $fz^{1}$ mutant embryos show a phenotype very similar to that of $Wnt4^{EMS23}$. (E,F) Using the GAL4/UAS system, dominant-negative transgenes of either *fz* or *fz2* were ectopically expressed in the salivary gland using a salivary-gland-specific driver. The loss of *fz* signaling in the gland resulted in guidance defects similar to those seen in *Wnt4* mutants. (G,J) Furthermore, embryos mutant for both $drl^{R343}$ and $fz^{1}$ show a higher penetrance of salivary gland guidance defects than $drl^{R343}$ or $fz^{1}$ alone. (H,I) Expression of dominant-negative transgenes for *Tcf* or *dsh* in the salivary glands, using a salivary-gland-specific GAL4 driver, results in ventral curving of a large portion of the salivary gland.

## Ectopic WNT5 and WNT4 repel salivary glands

Since *Wnt5* is expressed in the CNS and mutations in *Wnt5* resulted in the tip of the salivary glands curving toward the ventral surface of the embryo, WNT5 might normally act as a repellent to keep salivary glands away from the CNS during their migration. If so, it should be possible to direct the glands away from an ectopic source of WNT5. Indeed, ectopic expression of WNT5 in the visceral mesoderm (dorsal to the gland) was sufficient to redirect the salivary glands ventrally, away from the visceral mesoderm (Fig. 6B). Interestingly, the ventral curving affected only the tip of the salivary gland, those cells that express *drl*. Thus, WNT5 is a strong chemorepellent, sufficient to repel the migrating salivary glands.

*Wnt4* is also expressed in the CNS and its mutant phenotype suggests that it might also be a repellant. Accordingly, *Wnt4*, ectopically expressed in the visceral mesoderm, forced the salivary glands away from the visceral mesoderm (Fig. 6C). Unlike *Wnt5*, misexpression of *Wnt4* rerouted the entire gland, not just the leading cells. This phenotype suggests that WNT4 does not act as a ligand for DRL; instead it may act as a ligand for either FZ or FZ2, both of which are broadly expressed in salivary glands (Bhanot et al., 1999; Park et al., 1994). This possibility is explored further in the next section.

## WNT5 and WNT4 act on discrete pathways to influence different stages of salivary gland placement

There are no data to support WNT4 acting as a ligand for DRL. In fact, previous studies have shown that, in contrast to *Wnt5*, *Wnt4* does not interact genetically with *drl* in the CNS and fails to bind to DRL (Yoshikawa et al., 2003). However, WNT4 does bind to two of the *Drosophila* Frizzled homologs, FZ and FZ2 (Wu and Nusse, 2002). Hence, WNT4 might work through the canonical Wnt pathway rather than through the WNT5-DRL pathway during salivary gland development. To test this, we expressed dominant-negative constructs of *fz* and *fz2* (Zhang and Carthew, 1998) specifically in the salivary glands. Expression of either *UAS-fzN* or

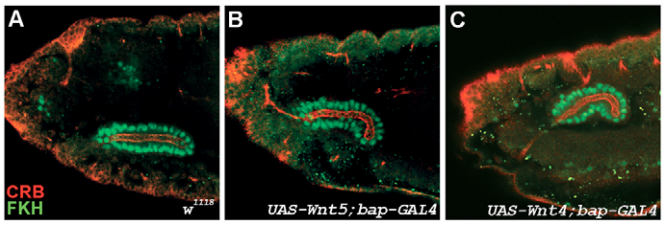


**Fig. 6. Ectopic expression of either *Wnt5* or *Wnt4* is sufficient to repel the salivary glands.** (**A-C**) Lateral views of stage 16 *Drosophila* embryos stained for FKH and CRB. (B) WNT5 was ectopically expressed in the visceral mesoderm, dorsal to the gland (located above the gland in these panels). The misexpression of WNT5 is sufficient to redirect the tip of the salivary gland ventrally. (C) When *Wnt4* is ectopically expressed in the visceral mesoderm, it is sufficient to repel the entire salivary gland away from the visceral mesoderm. These data support the hypothesis that both Wnt proteins act as repellents.

*UAS-fz2N* in the salivary glands resulted in curved salivary glands similar to those in *Wnt4*-mutant embryos; a large portion of the salivary gland curved toward the CNS and this curving began early, as the gland migrated along the circular visceral mesoderm (Fig. 5C,D). *fz*-mutant embryos show this same ventral curving phenotype, despite the presence of *fz2*, which acts redundantly with *fz* during segmentation of the embryo (Fig. 5E) (Bhanot et al., 1999). Furthermore, $Wnt5^{D7}$;$Wnt4^{EMS23}$ double mutants had a higher penetrance of salivary gland curving than either of the single mutants alone, emphasizing that two independent Wnt pathways are needed for proper salivary gland guidance (Fig. 5F,J). Similarly, the penetrance of ventral curving in *drl* mutant embryos was enhanced from 40 to 79% in the $drl^{R343}$;$fz^{1}$ double mutant (Fig. 5G,J). In both $Wnt5^{D7}$;$Wnt4^{EMS23}$ and $drl^{R343}$;$fz^{1}$ double mutants, a combination of phenotypes was seen: both ventral curving specific to the salivary gland tip and curving affecting a large portion of the salivary gland. Taken together, these data demonstrate that there are two Wnt pathways regulating salivary gland migration: a *Wnt4-fz/fz2* signaling pathway that is required throughout the gland, and a *Wnt5-drl* signaling pathway that specifically affects the tip of the migrating salivary gland.

Dominant-negative transgenes were used to investigate whether canonical Wnt pathway members that act downstream of Frizzled receptors are required for salivary gland guidance. Embryos expressing a dominant-negative TCF ($UAS\text{-}TCF^{\Delta N}$) in their salivary glands resembled *Wnt4* mutants, with ventral curving that affected a large percentage of the salivary gland (Fig. 5H). We also tested the effects of several *dsh* dominant-negative constructs that specifically disrupt either the canonical Wnt signaling pathway or the PCP pathway (Axelrod et al., 1998). The *dsh* dominant-negative transgenes that affect only PCP signaling ($UAS\text{-}dsh^{\Delta DEP+}$, $UAS\text{-}dsh^{DEP+}$) had no affect on salivary gland guidance; however, transgenes specific to the canonical Wnt pathway ($UAS\text{-}dsh^{DIX}$, $UAS\text{-}dsh^{bPDZ}$) caused ventrally curved salivary glands resembling *Wnt4*, *Tcf*, *fz* and *fz2* mutants (Fig. 5I,J). These results strengthen our conclusion that the canonical Wnt pathway, activated by the WNT4 ligand, is involved in the early stages of salivary gland migration.

## Src kinases genetically interact with *drl* in salivary glands

Our analysis indicates that WNT4 is most probably signaling through the canonical Wnt pathway, but what lies downstream of *Wnt5* and *drl* is less clear. Recent experiments have suggested that Src kinases might be involved. *Src64* (*Src64B* – FlyBase) binds to *drl* in a yeast two-hybrid screen and genetically interacts with *drl* in the developing nervous system (R. Wouda, J. N. Noordermeer and L. G. Fradkin, personal communication). In addition, *Src64* and *drl* have been shown to interact, either directly or indirectly, in *Drosophila* mushroom body development (Nicolai et al., 2003). We found that *drl* and Src kinase genes also interacted genetically in the salivary glands. *Src64* mutant embryos displayed ventral curving of salivary gland tips, similar to *drl* mutant embryos (Fig. 7B,G), as did *drl Src64* doubly heterozygous embryos (Fig. 7C). Placement of *Src64* downstream of *drl* is further supported by our finding that homozygous *drl Src64* double mutants had a similar frequency of guidance defects as either single mutant (Fig. 7G).

We tested whether the other *Drosophila* Src kinase, SRC42 (SRC42A – FlyBase) also interacts genetically with *drl*. We did find *drl-Src42* interactions, but they were more complex than the *drl-Src64* interactions. While *Src42* homozygous mutant embryos had salivary gland defects that included ventral curving at the tip of the salivary gland (Fig. 7D), they also displayed more generalized curving defects that occurred earlier than the *drl* phenotype (Fig. 7E). Similar to the *Src64* interaction, *drl Src42* doubly heterozygous embryos displayed guidance problems that closely resembled the *drl* mutant phenotype (Fig. 7F). Thus, it appears that *Src42* may be working downstream of *drl* late in salivary gland development, but may play a role in earlier salivary gland positioning as well.

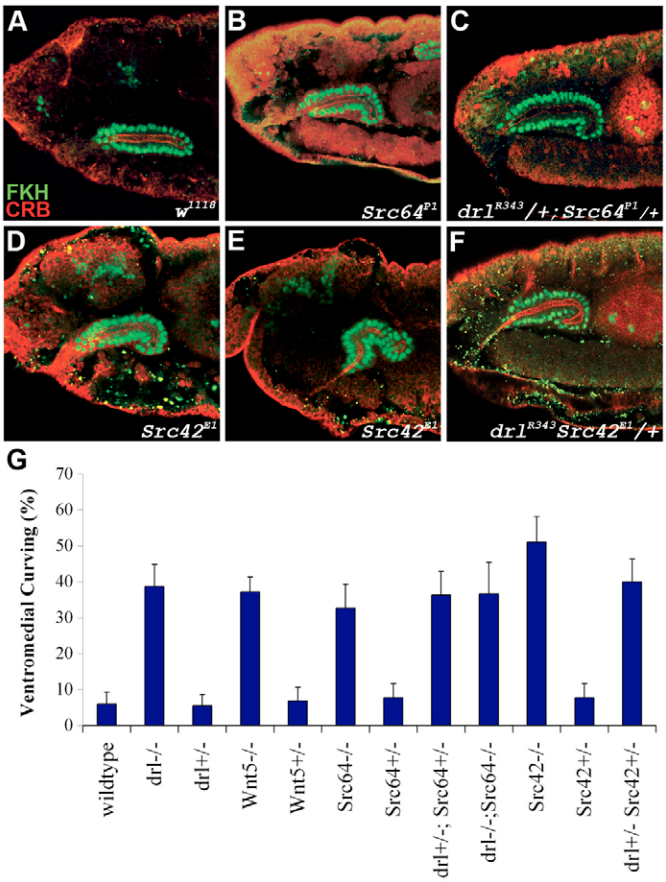


**Fig. 7. *drl* genetically interacts with *Src64* and *Src42* in the salivary glands.** (**A-F**) Lateral views of stage 15-16 *Drosophila* embryos stained with FKH and CRB. (A) Wild-type control. (B) $Src64^{P1}$ embryos closely resemble $drl^{R343}$ embryos. (C) $drl^{R343}$ and $Src64^{P1}$ doubly heterozygous embryos exhibit ventral curving of the salivary gland tips. (D,E) The salivary glands in $Src42^{E1}$ show a variety of defects, including ventral curving at the tip of the glands (D) and curving that affects a large portion of the length of the gland (E). Embryos heterozygous for both $drl^{R343}$ and $Src42^{E1}$ resemble $drl^{R343}$ embryos. (**G**) Graphical representation of phenotypic penetrance in embryos scored for salivary gland migration defects at stages 14-16.

# DISCUSSION

## Canonical Wnt signaling regulates early salivary gland migration

Salivary gland migration can be separated into three phases (Vining et al., 2005). In the first phase, the salivary glands invaginate into the embryo at a 45° angle, moving dorsally until they reach the visceral mesoderm. *fkh*, *RhoGEF2* and *18 wheeler* have been shown to regulate apical constriction of the salivary gland cells during this invagination process (Weigel et al., 1989; Myat and Andrew, 2000a; Nikolaidou and Barrett, 2004; Kolesnikov and Beckendorf, 2007). In addition, *hkb* and *faint sausage* are needed for proper positioning

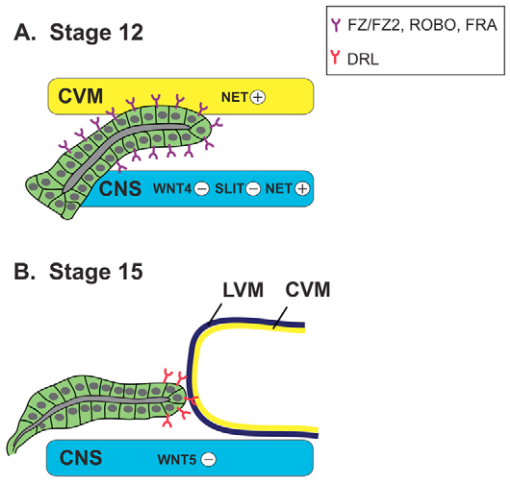


**Fig. 8. Model of salivary gland migration in *Drosophila*.** (**A**) As the salivary glands invaginate into the embryo during stage 12 they are attracted to the CVM by Netrin. The salivary glands also encounter both attractive (Netrin) and repulsive (WNT4 and Slit) signals from the CNS, which guide the salivary glands during their posterior migration. The receptors for these early signals are present throughout the entire salivary gland and appear to work, for the most part, independently of each other. (**B**) During stage 15, the salivary glands meet the LVM and rely on the WNT5 repulsive signal from the CNS to keep the distal tip of the salivary gland positioned so it can adhere to the longitudinal visceral mesoderm. This is accomplished through Derailed, which is present specifically in the cells at the tip of the salivary glands.

of the site of invagination (Myat and Andrew, 2000b). No guidance cues have been identified for this first phase of migration; it may be that the patterns of constriction and cell movements at the surface of the embryo are sufficient to direct the invaginating tube.

During the second phase of migration, as the salivary gland moves posteriorly within the embryo, two guidance cues, Netrin and Slit, guide salivary gland migration along the visceral mesoderm (Kolesnikov and Beckendorf, 2005). Netrin, which is expressed in the CNS and the visceral mesoderm, works to maintain salivary gland positioning on the visceral mesoderm. At the same time, Slit acts as a repellent from the CNS to keep the salivary glands parallel to the CNS. Here we have shown that there is a third guidance signal, WNT4, acting through FZ or FZ2 receptors, that is also required in the second phase of salivary gland migration (Fig. 8A). Loss of *Wnt4*, *fz* or *fz2* in the embryo resulted in salivary glands that were curved in a ventromedial direction. This curving affected a large portion of the salivary gland and may have resulted from the fact that the *fz* and *fz2* receptors, in contrast to *drl*, are expressed throughout the salivary gland. Furthermore, dominant-negative transgenes that disrupt the function of DSH or TCF caused the same phenotype, suggesting that transcription induced by the canonical Wnt signaling pathway is needed to maintain the proper migratory path of the salivary glands on the CVM. The migration along the CVM takes more than 2 hours for completion, which would leave adequate time for a transcriptional response.

Although *Wnt4* and *slit* are both required for the second phase of migration, and their mutants show similar, though distinguishable, phenotypes, we believe that they act independently. While most *slit*-mutant embryos have medially curving salivary glands, embryos lacking *Wnt4* had salivary glands that curved in a distinctly different, ventromedial, direction. Embryos doubly mutant for *Wnt4* and *slit* showed predominantly one or the other phenotype and neither phenotype increased in severity (data not shown). These results suggest, though they do not prove, that *Wnt4* and *slit* act in distinct pathways.

## Atypical Wnt signaling mediates final positioning of the salivary glands

After the entire salivary gland has invaginated, migrated posteriorly within the embryo and lies parallel to the anteroposterior axis of the embryo, the distal ends of the salivary glands come into contact with the LVM. We have shown that *drl* and *Wnt5* are required for this late phase of salivary gland positioning (Fig. 8B). Loss of either *drl* in the salivary gland or *Wnt5* in the CNS resulted in the distal tip of the salivary gland being misguided to a more ventromedial position. This change in the shape of the salivary gland was seen only after the salivary glands were no longer in contact with the CVM (after stage 13). Thus we propose that *drl* is required during the third phase of salivary gland migration, as the salivary gland detaches from the CVM and contacts the LVM.

The striking expression of *drl* at the tip of the salivary gland makes the leading cells uniquely different from the rest of the salivary gland cells. These cells project lamellipodia upon reaching the visceral mesoderm and beginning their posterior migration. They may act to both guide and pull the rest of the gland during migration (Bradley et al., 2003). Cells at the tip of a migrating organ are frequently specialized to guide migration. For example, the coordinated migration of the tracheal branches in *Drosophila* is achieved by induction of distinct tracheal cell fates within the migrating tips. This is illustrated by the fact that FGF (BNL – FlyBase) signaling becomes restricted to the tips of the tracheal branches soon after they begin to extend (Gabay et al., 1997; Sutherland et al., 1996). The migration and growth of *Drosophila* Malpighian tubules provide another clear example of specialized cells needed at the tip of a migrating tissue. One cell is singled out to become the tip cell, which directs the growth of the Malpighian tubules as well as organizes the mitotic response and migration of the other cells forming each tubule (Hoch et al., 1994). In other systems, such as *Dictyostelium* slugs, cells at the tip of a migrating group are required and solely able to guide migration (Dormann and Weijer, 2001). Our results establish that the leading cells of the migrating salivary glands have a specialized role to play in proper salivary gland positioning. First they are required to initiate invagination within the embryo, then they actively participate in migration along the CVM, and finally they ensure that the distal tip of the gland will remain associated with the LVM at the end of the migratory phase.

Despite the fact that we have firmly established *Wnt5* and *drl* as important for the final placement of salivary glands, the signaling pathways downstream are not well defined. Because salivary-gland expression of full-length *drl* can rescue the *drl*-mutant phenotype, but *drl* lacking the intracellular domain cannot, we are confident that the intracellular domain of DRL is important for signaling. Similarly, misexpression of full-length *drl* can misguide axons in the ventral nerve cord, but misexpression of *drl* lacking its intracellular domain cannot (Yoshikawa et al., 2003). The genetic interactions found in this study between *drl* and *Src64* support recent findings suggesting that *Src64* acts downstream of *drl* in the ventral nerve cord (R. Wouda, J. N. Noordermeer and L. G. Fradkin, personal communication). In addition, we have shown that the other *Drosophila* Src kinase, *Src42*, may be required at two stages, during salivary gland migration along the CVM and downstream of WNT5-DRL signaling as the gland moves onto the LVM.

Another intriguing finding of this study is the involvement of the two remaining *Drosophila* RYKs, *Drl-2* and *dnt*, in salivary gland development. The phenotypes of *Drl-2* and *dnt* mutants are less penetrant than *drl* mutants, but they are qualitatively very similar. Furthermore, embryos doubly heterozygous for *drl* and *Drl-2* have salivary glands that resemble those seen in *drl* mutant embryos. These three RYKs appear to act in a partially redundant fashion in the salivary glands, as none of the single gene mutations leads to completely penetrant phenotypes. However, we did not see an increase in penetrance of the *drl* phenotype in embryos lacking both *drl* and *Drl-2*. In addition, we were unable to detect transcripts for either *Drl-2* or *dnt* in the salivary gland. While it is possible that *dnt* and *Drl-2* are expressed at very low levels in the salivary gland, they might be acting non-autonomously.

## WNT5 and WNT4 signaling pathways operate independently of each other

An interesting dilemma in understanding RYK signaling is how inactive kinases propagate a signal into the cell. Recent mammalian studies have suggested that RYKs may associate with another catalytically active receptor, such as FZ or EPH, at the membrane (Halford et al., 2000; Lu et al., 2004; Trivier and Ganesan, 2002). In the mouse, the extracellular WIF domain of RYK interacts with FZD8, and it has been proposed that the two proteins may form a ternary complex with WNT1 to initiate signaling (Lu et al., 2004). However, data from flies and nematodes support the argument that DRL and its *Caenorhabditis elegans* homolog LIN-18 act independently of FZ. Genetic studies of cell specification in the nematode vulva suggest that LIN-18 acts in a parallel and separate pathway from the LIN-17/FZ receptor (Inoue et al., 2004). Similarly, reduction of *fz* and *fz2* gene activity in flies has no effect on a DRL misexpression phenotype in the ventral nerve cord (Yoshikawa et al., 2003). Here we have shown that double mutants for the *Wnt4* and *Wnt5* ligands and for the *fz* and *drl* receptors both show strong enhancements in comparison to the single mutants, reinforcing the conclusion that these two ligands are activating different pathways. In addition, we can separate the functions of these two pathways by phenotype. The *Wnt4-fz/fz2* phenotype becomes evident earlier and affects a larger portion of the salivary gland than the *Wnt5-drl* phenotype. Taken together, these results demonstrate that there are two independent Wnt pathways regulating salivary gland positioning. The early WNT4 signal appears to activate the canonical Wnt pathway, whereas there is a later requirement for WNT5 signaling through DRL and the Src kinases.

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