5

Discussion

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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