The transmembrane receptors Otk and Otk2 function redundantly in *Drosophila* Wnt signal transduction

PhD Thesis

in partial fulfilment of the requirements for the degree
"Doctor rerum naturalium (Dr. rer. nat.) "
in the Molecular Biology Program at the
Georg August University Göttingen,
Faculty of Biology

submitted by
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born in
Braunschweig, Germany

2012

AFFIDAVIT

I hereby declare that I prepared the thesis "The transmembrane receptors Otk and Otk2 function redundantly in *Drosophila* Wnt signal transduction" on my own with no other sources and aids than quoted.

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Day of PhD examination: January 23rd, 2013

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ACKNOWLEDGMENTS

It is a pleasure to express my gratitude to all those who supported me during the completion of this project.

First of all, I would like to thank Prof. Andreas Wodarz for giving me the exciting opportunity to join his department and for the supervision during these years. Thank you for the advice and support during the many turns of the project and for giving me the possibility to develop my own ideas and work independently.

Furthermore, I want to thank Prof. Annette Borchers and Prof. Reinhard Schuh for being members of my thesis committee and for helpful comments during thesis committee meetings. I also want to thank Gregor Bucher for discussion and help on phylogenetic analysis.

Huge thanks to all current and most of the former lab members of the Stem Cell Biology department for good times, much fun and making these years highly enjoyable! Thanks to the “Clonator” Mona as well as Katja and Claudia for the excellent technical support and keeping the lab running, Patricia for guidance through paperwork, Hamze for being a great friend and sharing the ups and downs of PhD life, Caro, Katja, Jaffer, Manu and Sascha for the fun times, my Bachelor and Master students Laura and Emma and the former lab members Ieva, Marilena, Gang, Nils and Tobi for many unforgettable moments.

I also would like to thank the members of the “Kafferunde” for having this tradition and for getting the day off to a good start.

I want to acknowledge the IMPRS for Molecular Biology for offering financial support in terms of a travel grant, which allowed me to visit an exciting EMBO meeting on Wnt signaling. In particular, I am grateful to Dr. Steffen Burkhardt, Kerstin Grüninger and Ivana Jurik for the enormous support of students and for the excellent organization of the program.

Many thanks to Birgit, Marie and Nils for proofreading and valuable comments.

During my study and PhD times in Göttingen many friendships arose and I feel very fortunate having such great people around me.

Finally, I am very grateful to my family for their truly unconditional support and to Nils and Paula for being at my side, great times together and letting life never become boring.

ABSTRACT

Canonical and non-canonical Wnt signaling pathways have in common Frizzled (Fz) as the core Wnt receptor element. Recent findings implicate that not only the choice of Wnt ligands, but also the presence of additional components in the receptor complex determine signaling pathway specificity.

Vertebrate PTK7 (protein tyrosine kinase 7) encodes a catalytically inactive receptor-tyrosine kinase and is required for the control of planar cell polarity (PCP) in frogs and mice by acting as Fz co-receptor and inhibiting canonical Wnt signaling. Mutation of a Drosophila homolog of PTK7, the gene *off-track* (*otk*), was reported not to cause PCP phenotypes in the fly, but is suggested to block canonical Wingless signaling in embryonic patterning. We found that in contrast to previous reports, flies homozygous for a complete knock-out of *otk* are viable and fertile and indeed do not show PCP phenotypes. However, we discovered an *otk* paralog (*otk2*, *CG8964*). Otk and Otk2 are co-expressed throughout embryonic and larval development. They are highly expressed in the visceral mesoderm as well as in the nervous system and enriched at anterior commissures. Otk and Otk2 interact biochemically and possibly function redundantly in Wnt signal transduction.

Surprisingly, flies homozygous for a double knock-out of *otk* and *otk2* are viable as well and neither show PCP nor Wingless signaling phenotypes. However, *otk,otk2* double mutants are male sterile due to an obstruction in the ejaculatory duct and this is possibly linked to disturbed Wnt2 signaling. Overall defects in the nervous system cannot be observed, most likely because only a subset of neurons is affected. This could be explained by the abundance of receptors and co-receptors acting in a redundant manner. Indeed, expression data suggest a possible connection to Wnt5/Drl signaling. Furthermore, genetic and biochemical studies revealed that Otk/Otk2 genetically and biochemically interact with *fz1*, indicating that Otk/Otk2 might also function as Fz1 co-receptors in the signal transduction of Wingless or other members of the Wnt family under certain circumstances.

These results suggests that Otk and Otk2 function as redundant receptors in several Drosophila Wnt signaling pathways, including Wnt2 as well as Wnt5/Drl signaling.

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1 INTRODUCTION

1.1 Wnt signaling

Wnt signaling pathways are evolutionarily conserved and regulate many aspects of important biological phenomena, ranging from embryogenesis to cell behaviour and maintenance of adult tissue homeostasis.

During development Wnt signaling regulates processes such as cell fate determination, axis specification, gastrulation and neural development (Wodarz & Nusse, 1998; Logan & Nusse, 2004). In an adult organism, Wnt signaling remains indispensable for the regulation of tissue maintenance, stem cell self-renewal as well as the regeneration of injured tissue (Logan & Nusse, 2004; Reya & Clevers, 2005; Clevers, 2006). The pathway is strictly kept in a homeostatic range, as exceeding signaling levels lead to diseases like cancer, whereas low levels of Wnt signaling could be one of the reasons for many degenerative conditions (Fig. 1) (Angers & Moon, 2009).

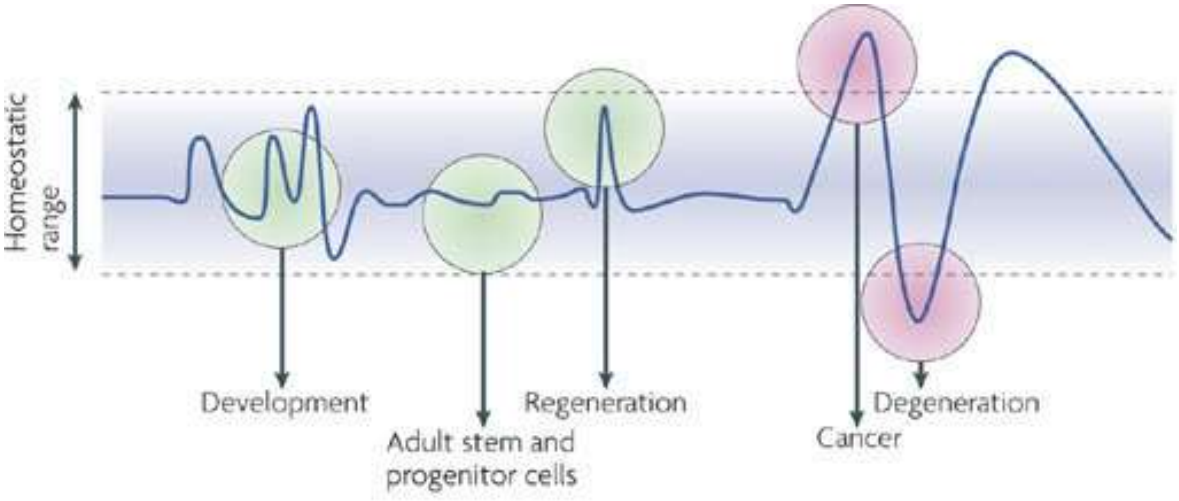

Fig. 1: Homeostatic range of Wnt signaling during development and disease.
Wnt signaling is dynamically regulated, but kept in a homeostatic range during development, stem cell maintenance and regeneration. Upregulated Wnt signaling leads to cancer and conversely low levels of Wnt signaling are one of the reasons for degenerative conditions. Taken from (Angers & Moon, 2009).

Historically, the nomenclature "Wnt" was established after the Drosophila w*ingless* (*wg*) and the mouse *int-1* oncogene, originally identified as the integration site for the mouse mammary tumor virus (Nusse & Varmus, 1982), were shown to be homologous (Baker, 1987; Cabrera *et al*, 1987; Rijsewijk *et al*, 1987). This exciting

discovery was one of the first examples of a gene implicated in cancer as well as in embryogenesis. Further studies in *Xenopus* revealed that ectopic expression of *int1* induces duplication of the dorsal axis, suggesting a conserved role for this gene as an organizer and signal between germ layers that leads to pattern formation (McMahon & Moon, 1989). The affiliation of *wingless* to the segment polarity group of genes paved the way for discovering the functional mechanism of *int1*/*wingless* by studying genetic and biochemical interactions with other genes in this group (Nüsslein-Volhard & Wieschaus, 1980).

In the following years, the mammalian Wnt family, consisting of 19 members of secreted glycoproteins, became classified according to their ability to induce secondary dorsal-ventral axes in *Xenopus* embryos and to morphologically transform C57MG mouse mammary epithelial cells. For example, overexpression of Wnt1, Wnt3a and Wnt8 is sufficient to induce a double axis (Du *et al*, 1995) and transform C57MG cells (Wong *et al*, 1994; Olson & Papkoff, 1994; Shimizu *et al*, 1997), whereas other Wnts such as Wnt4, Wnt5a and Wnt11 do not show this property. The ability of the above mentioned members of the Wnt family to transform mouse mammary epithelial cell lines and to induce a secondary axis in *Xenopus* embryos can be deduced from their ability to evoke an increase in the cytoplasmic levels of the protein β-catenin. This pathway is therefore referred to as the Wnt/β-catenin or canonical pathway, because it was the first Wnt pathway identified.

On the other hand, for example Wnt5a and Wnt11 are required for correct convergent extension movements in *Xenopus* and zebrafish embryos, independent of β-catenin function (Heisenberg *et al*, 2000; Wallingford *et al*, 2001; Kilian *et al*, 2003; Veeman *et al*, 2003). Based on the grouping of Wnt family members into a canonical and a non-canonical class, the view of canonical (β-catenin-dependent) pathways in contrast to non-canoncial (β-catenin-independent) pathways emerged. Non-canonical pathways include the so far most characterized Wnt-calcium pathway and the planar cell polarity (PCP) pathway, which requires Wnt-c-Jun N-terminal kinase (JNK) activity (Fig. 2) (Montcouquiol *et al*, 2006). However, the degree to which these pathways overlap is presently unclear, but should not be underestimated.

Interestingly, the different Wnt pathways comprise both common components as well as pathway-specific players. The core components shared by all pathways mentioned are Wnt ligands, which act as morphogens, Fz transmembrane receptors and the cytoplasmic protein Dishevelled (Dsh/Dvl). Wnt binding to Fz results in the activation

of Dsh, which can be seen as the branching point of canonical and non-canonical pathways (Fig. 2) (Gao & Chen, 2010). Canonical Wnt signaling controls cell proliferation and differentiation through influence on the transcription of β-catenin target genes, while PCP signaling regulates cell polarization and migration through regulation of the small GTPases Rho and Rac involved in remodeling of the actin cytoskeleton.

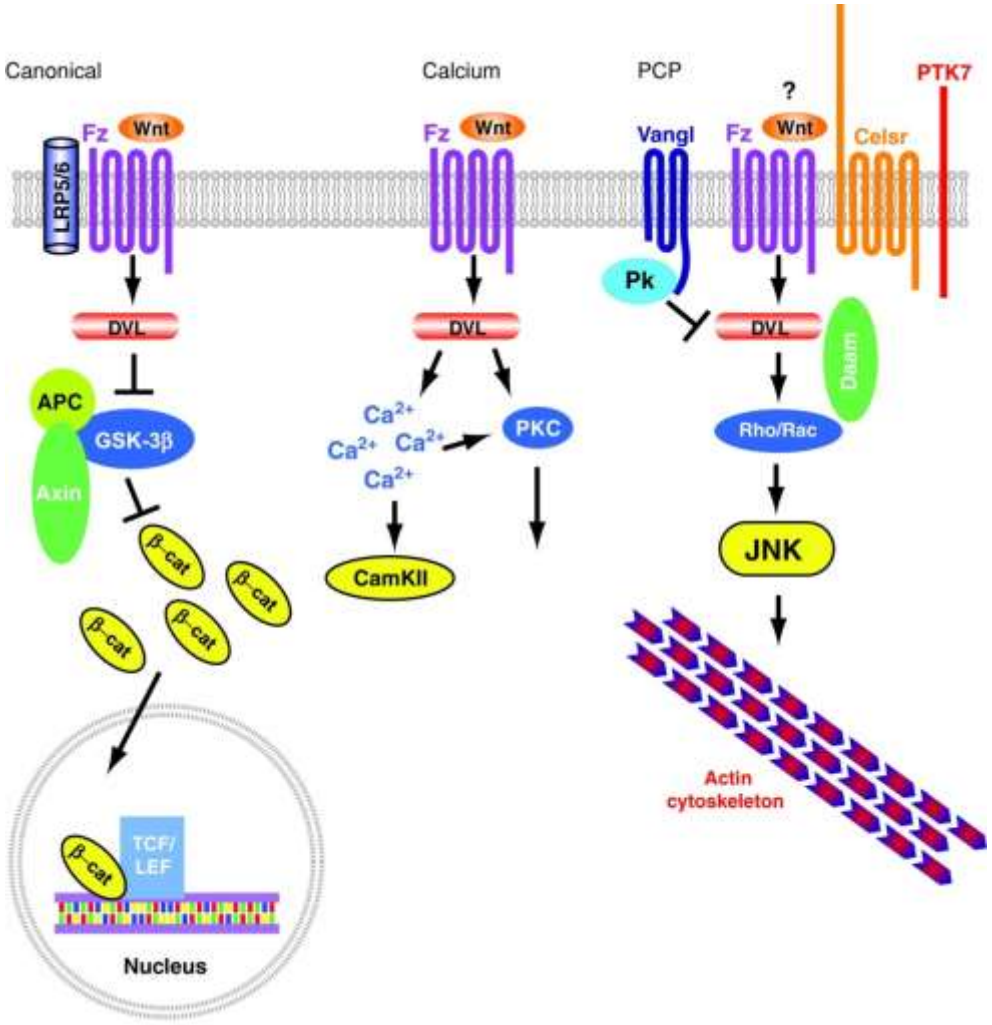

Fig. 2: Canonical and non-canonical Wnt signaling pathways.
In the canonical pathway, Wnt binding to Fz and its co-receptor LRP5/6 leads to activation of Dishevelled and this in turn inhibits β-catenin degradation. Increased cytoplasmic β-catenin can subsequently translocate to the nucleus and bind to members of the TCF/LEF family, activating transcription. An increase in the levels of intracellular calcium is the central event in the Wnt-Calcium pathway. This leads to the activation of Protein kinase C (PKC) as well as Calmodulin kinase II (CamKII) and each of these factors is then able to mediate intracellular responses. In the PCP pathway, Fz becomes activated, leading to Dvl activation. This then signals via the small GTPases Rho and Rac as well as C-Jun N-terminal kinase (JNK) to modulate the actin cytoskeleton. The PCP pathway also involves other transmembrane proteins like Vangl, Celsr and PTK7. Taken from (Montcouquiol *et al*, 2006), vertebrate nomenclature of the proteins is used.

1.2 Canonical / β-catenin-dependent signaling

Mutations in the *Drosophila* genes *dishevelled (dsh)* and *armadillo* (*arm*, *Drosophila* homolog of β-catenin) showed similar cuticle defects as *wingless* mutant embryos (Perrimon & Mahowald, 1987; Wieschaus & Riggleman, 1987). In contrast, mutations in shaggy/zeste-white 3 (*Drosophila* homolog of Glycogen Synthase Kinase-3, GSK-3) cause the completely opposite phenotype (Siegfried *et al*, 1992). Epistasis studies uncovered that these genes were indeed core members of the same newly identified signaling pathway (Noordermeer *et al*, 1994). Consistently, the ability to induce a secondary axis in *Xenopus* embryos, as described for canonical Wnts, was also reported for Dsh, β-catenin and a dominant negative version of GSK-3 (Guger & Gumbiner, 1995; He *et al*, 1995; Dominguez *et al*, 1995).

These combined observations from vertebrate and invertebrate model systems provided the first draft of the highly conserved canonical/β-catenin-dependent signaling pathway. The critical event in this "classic" model of Wnt signaling is the stabilization and accumulation of cytoplasmic β-catenin, which can then translocate to the nucleus and activate transcription. In the absence of Wnt, the cytoplasmic levels of β-catenin are tightly regulated by a destruction complex consisting of amongst others Axin, Casein kinase 1α (CK1α), adenomatous polyposis coli (APC) and GSK-3 (Gordon & Nusse, 2006). CK1α and GSK-3 are responsible for β-catenin phosphorylation (Amit *et al*, 2002; Liu *et al*, 2002), which in turn results in ubiquitylation and subsequent proteasomal degradation (Aberle *et al*, 1997) (Fig. 3).

Pathway activation occurs upon Wnt ligand binding to the seven pass-transmembrane Frizzled (Fz) receptor (Yang-Snyder *et al*, 1996; Bhanot *et al*, 1996; Bhat, 1998) and its co-receptors LRP5 or -6 (*Drosophila* Arrow) (Pinson *et al*, 2000; Tamai *et al*, 2000; Wehrli *et al*, 2000). Wnt binding to Fz triggers the phosphorylation of Dsh, which weakens the destruction complex (Yanagawa *et al*, 1995; Willert *et al*, 1997). Furthermore, GSK-3 and CK1α phosphorylate LRP, which then recruits Axin to the plasma membrane, resulting in inactivation of the destruction complex (Davidson *et al*, 2005; Zeng *et al*, 2005). As a consequence, β-catenin is no longer degraded, accumulates in the cytoplasm and enters the nucleus, where it interacts with the DNA-binding proteins of the TCF/Lef family (T-cell factor and Lymphoid enhancer factor) (Behrens *et al*, 1996; Molenaar *et al*, 1996). β-catenin binding converts the TCF proteins into potent transactivators by displacing transcriptional co-repressors

like Groucho (Cavallo *et al*, 1998; Roose *et al*, 1998) (Daniels & Weis, 2005) and recruiting co-activators like Bcl9/Pygopus and Legless (Kramps *et al*, 2002; Hoffmans *et al*, 2005; Städeli & Basler, 2005) (Fig. 3).

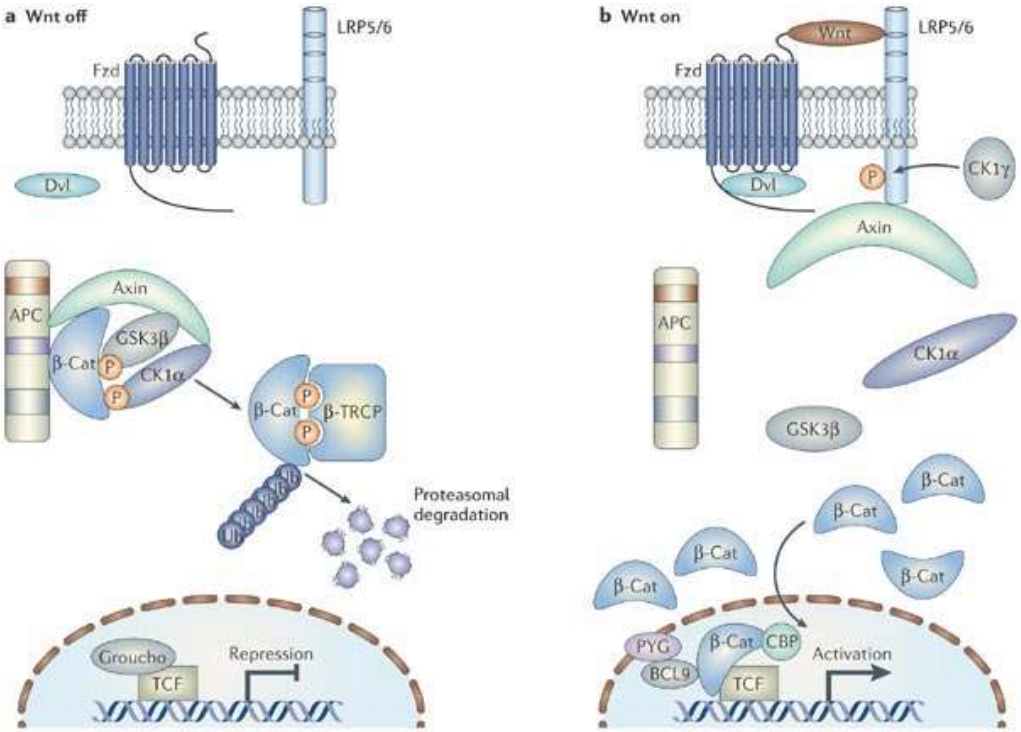

Fig. 3: Overview of canonical/β-catenin dependent Wnt signaling.
(A) In the absence of Wnt ligands, β-catenin is held in a destruction complex by Axin and APC, phosphorylated by GSK3β and CK1α and as a result degraded. Nuclear DNA binding proteins of the TCF/Lef family recruit transcriptional co-repressors like Groucho and thereby actively repress target genes. **(B)** Upon Wnt ligand binding, Fz binds to Dvl and LRP5/6 is phosphorylated by CK1α. This recruits Axin to the membrane and the destruction complex disassembles. β-catenin accumulates in the cytoplasm and translocates to the nucleus. It displaces the transcriptional co-repressors and instead recruits activators proteins like Bcl9/Pygopus and Legless, leading to the activation of TCF target genes. Taken from (Barker & Clevers, 2006)

1.3 Non-canonical / PCP signaling

The best characterized non-canonical Wnt pathway is the so-called planar cell polarity (PCP) pathway. It was initially described in *Drosophila*, where it is responsible for establishing polarity in the plane of an epithelium perpendicular to the apico-basal axis of polarity. Flies mutant for PCP genes have typical phenotypes like the disturbed orientation of wing hairs and bristles on thorax and legs, as well as the disturbed orientation of ommatidia in the eyes. In vertebrates, PCP signaling

controls convergent extension movements, tissue organization and collective cell movement (Zallen, 2007).

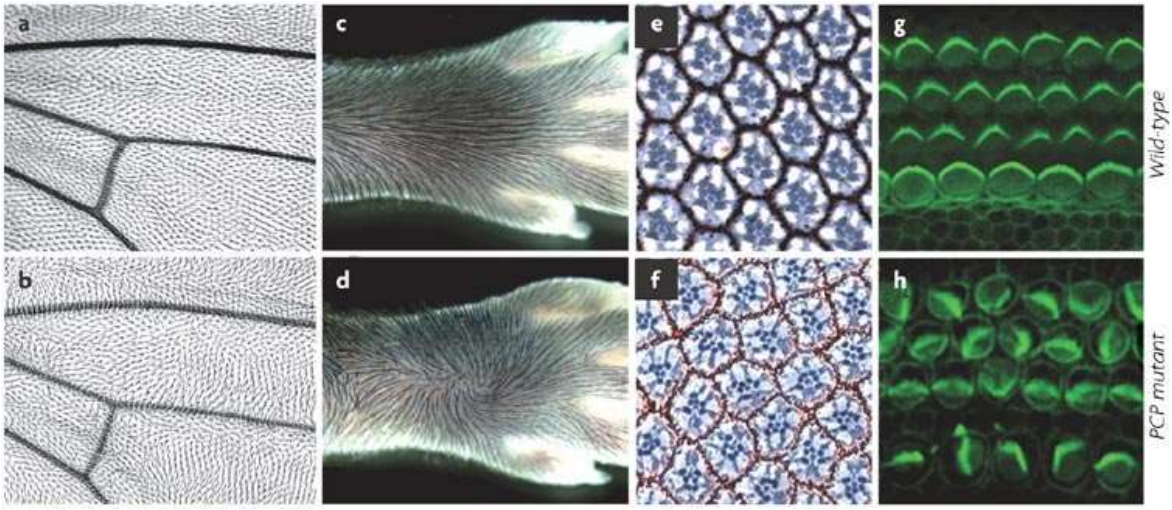

Fig. 4: PCP signaling regulates tissue organization in different systems.
(A) *Drosophila* wings are covered with hairs which all point distally. **(B)** Mutations in PCP genes like *frizzled* lead to a disruption of this organization, creating swirls and waves on the wing. **(C,D)**, Similarly, the pattern of mammalian fur is disturbed in PCP mutants **(D)**. **(E,F)** The ommatidia in the Drosophila eye each contain eight photoreceptors, arranged in a characteristic pattern, which becomes disorganized in PCP mutants. **(G)** Stereocilia in the sensory hair cells of the mammalian cochlea are polarized, this polarity is disturbed in PCP mutants **(H)**. Taken from (Seifert & Mlodzik, 2007).

Frizzled and Dsh are key players in this pathway and mutants display clear PCP phenotypes. The pathway diverges downstream of Dsh in that it does not involve Axin, GSK-3 or β-catenin, which are core components of canonical signaling.
Dsh contains three conserved domains – a DIX, PDZ and DEP domain and the latter one was described to be required for Drosophila and vertebrate PCP signaling by associating with the small GTPase Rac, which then activates JNK (Boutros *et al*, 1998; Axelrod *et al*, 1998; Heisenberg *et al*, 2000; Wallingford & Habas, 2005). Other components that were shown to act both in Drosophila and vertebrate PCP signaling include Strabismus/Van Gogh-like, Prickle, and Flamingo/Celsr (Veeman *et al*, 2003).
Strikingly, in *Drosophila* no Wnt protein could be shown to actively participate in PCP signaling. This is in contrast to the situation in vertebrates, where both Wnt5a and Wnt11 have been implicated to be essential for correct convergent extension in mouse (Qian *et al*, 2007) and Zebrafish (Heisenberg *et al*, 2000).

1.4 Drosophila Wnt family

Wnt proteins are a family of secreted glycoproteins that play pivotal roles in development and disease (Logan & Nusse, 2004). While the mammalian genome encodes 19 Wnt proteins, *Drosophila* has seven *Wnt* genes (Table 1), but only one member of the family, Wingless, is well characterized. None of the other *Wnt* family members was discovered in screens for mutant phenotypes, but rather by their molecular homology. Furthermore it should be mentioned that no Wnt has so far been shown to be required for PCP signaling in *Drosophila*.

Table 1: *Drosophila* Wnt family members and their vertebrate homologs.

Drosophila	Vertebrates
Wingless	Wnt1
Wnt2	Wnt7
Wnt3/5	Wnt5
Wnt4	Wnt9
Wnt6	Wnt6
Wnt8	-
Wnt10	Wnt10

1.4.1 Wingless (Wg)

As already mentioned, the segment polarity gene *wingless* (*wg*) is the *Drosophila* homolog of the mouse *int1*-gene (Rijsewijk, 1987; Cabrera 1987; Baker, 1987). The term "segment polarity gene" was coined for one class of mutants retrieved in a genetic screen that shared a similar patterning phenotype during embryogenesis (Nüsslein-Volhard & Wieschaus, 1980). Another mutant allele of the *wingless* gene leading to the loss of wing tissue had been identified earlier (Sharma & Chopra, 1976).

The segmental subdivisions of the *Drosophila* embryo are controlled by the temporal expression of the maternal, gap and pair rule classes of genes. Genes of the segment polarity class, which includes *wingless*, are required in each segment for correct pattern formation (for review, see (Ingham, 1988)). Mutations in *wg* and other segment polarity genes cause the posterior portion of each segment to be deleted and replaced by a mirror-image duplication of the anterior portion. As a consequence, the segmentation of the embryonic epidermis, which is normally covered with naked

cuticle and denticle belts in an alternating way, is severely disturbed in *wg* mutant embryos, leading to a complete lawn of denticle (Nüsslein-Volhard & Wieschaus, 1980).

During germ band extension of early *Drosophila* embryonic development, *wg* is expressed as a continuous stripe in the posterior region of each of the fourteen parasegments (Baker, 1987; van den Heuvel *et al*, 1989), next to the expression domain of the segment polarity gene *engrailed* (*en*), which comprises the anterior part of each parasegment (DiNardo *et al*, 1985). *wg* is required for formation of both the segment boundary and the parasegment groove, which forms at the junction of *wg*- and *en*-expressing cells (Perrimon & Mahowald, 1987) (Fig. 5).

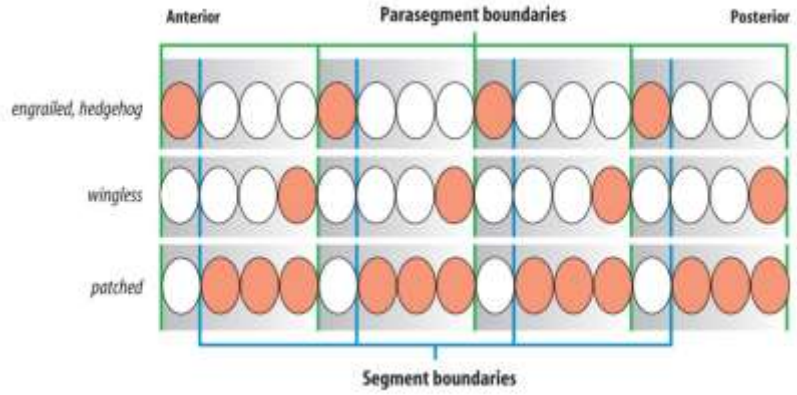

Fig. 5: Expression domains of segment polarity genes.
During the cellular blastoderm stage, *wingless* starts to be expressed at the posterior margin of each parasegment, whereas *engrailed* is expressed at the anterior margin together with *hedgehog*. The segment boundaries lie posteriorly to the *engrailed* expression domain. Taken from (Wolpert *et al*, 2007).

During embryonic development, *wg* is necessary for multiple phases of patterning of the segmented trunk ectoderm (Bejsovec & Martinez Arias, 1991; Dougan & DiNardo, 1992; Bejsovec & Wieschaus, 1993) as well as for proper development of the head (Schmidt-Ott & Technau, 1992). Subsets of neuroblasts in each segment of the early central nervous system require non-autonomous *wg* activity for determination and subsequent delamination (Chu-LaGraff & Doe, 1993). Furthermore, *wg* is needed for patterning of the developing midgut (Immerglück *et al*, 1990; Thüringer & Bienz, 1993; Bienz, 1994) as well as for growth of the malphigian tubules (Skaer & Martinez Arias, 1992). These studies revealed that Wg is a protein secreted both within and across tissues and affects patterning also in neighboring cells (Klingensmith & Nusse, 1994). Analysis of the central nervous system of *wg* null mutant embryos has revealed only subtle defects, indicating only a minor role in neurogenesis during late

embryonic development (Patel *et al*, 1989). After embryogenesis, *wg* is involved in patterning of imaginal discs as well as several other adult structures (reviewed in Klingensmith & Nusse, 1994).

1.4.2 Wnt2

Wnt2 was discovered as a member of the *Drosophila* Wnt family of genes (Russell *et al*, 1992) and is required for the development of the male reproductive tract (Kozopas *et al*, 1998). *Drosophila* testes are surrounded by a sheath consisting of outer male-specific pigment cells and an inner muscle layer (Kozopas *et al*, 1998; Susic-Jung *et al*, 2012). *Wnt2* mutant flies are viable, but male-sterile due to both the absence of pigment cells and defects in development of the inner muscle layer of the testis sheath. Furthermore, ectopic expression of *Wnt2* in females results in the formation of normally male-specific pigment cells, indicating that *Wnt2* controls sexually dimorphic development (Kozopas *et al*, 1998). This was supported by another study showing that male-specific embryonic gonadal Wnt2 expression is necessary and sufficient to induce the formation of pigment cells (DeFalco *et al*, 2008a).
Further analyses revealed that Wnt2 is also required for the correct attachment of direct flight muscles to their epithelial sites, leading to flightlessness in homozygous mutant animals (Kozopas & Nusse, 2002).

1.4.3 Wnt4

The *Wnt4* gene maps close to *wingless (wg)* (30 kb upstream) and was isolated from a library of genomic fragments that associate *in vivo* with Ultrabithorax proteins. Wnt4 is expressed in a segment polarity-like pattern and in the visceral mesoderm, thereby partially overlapping with Wingless expression (Graba *et al*, 1995). Their co-expression was ascribed to the sharing of cis-regulatory elements (Gieseler *et al*, 1995). Functionally, Wnt4 antagonizes Wingless signaling both in the *Drosophila* ventral epidermis as well as in a heterologous system, the *Xenopus* embryo, most likely upstream of the activation of transcriptional targets (Gieseler *et al*, 1999). In contrast to the ventral epidermis, *wg* and *Wnt4* are not co-expressed in the dorsal epidermis and produce distinct responses in these cells. Moreover, *Wnt4* requires Hedgehog to elicit its effects, whereas *wg* acts independently of *hedgehog* (Buratovich *et al*, 2000).

In contrast to *Drosophila* embryogenesis, when *wg* and *Wnt4* can have either antagonistic or distinct functions, they induce similar cellular responses during imaginal development in that overexpression of Wnt4 or Wg leads to notum-to-wing transformation and alters the morphogenesis of adult appendages (Gieseler *et al*, 2001).

1.4.4 Wnt5 (DWnt3)

The *Wnt5* gene, which has also been described as *DWnt3*, encodes an unusual Wnt protein that contains a long amino terminal extension without any known conserved domains. Its molecular mass of 112 kDa is almost three times larger than the one of the other Wnt family members (Eisenberg *et al*, 1992; Russell *et al*, 1992). Wnt5 protein localizes to the CNS during late embryogenesis and overexpression leads to malformation of commissures from stage 13 on, most likely due to a reduction of axon bundles crossing the midline (Fradkin *et al*, 1995). Wnt5 was identified as a ligand for Derailed (Drl), the *Drosophila* Ryk homolog, in a screen for mutations that suppress the ability of Drl to switch axons to the anterior commissure (AC) when misexpressed by neurons in the posterior commissure (PC) (Yoshikawa *et al*, 2003). *Wnt5* mutant flies are homozygous viable and fertile, but uncoordinated. When Drl was misexpressed in a *Wnt5* mutant background, its PC-to-AC switching activity was strongly suppressed, suggesting that Drl requires Wnt5 to switch axons (Fig. 6) (see 1.5.4). High levels of *Wnt5* mRNA were found in the posterior commissures, whereas the protein was enriched on both commissures compared to the longitudinal tracts. Based on these findings Wnt5, was proposed to act as a repulsive ligand for Drl-expressing axons that cross along the AC (Yoshikawa *et al*, 2003). Another study discovered that Wnt5 is required for the separation and defasciculation of early axonal projections that will form the mature commissural and longitudinal connectives. Furthermore, in *Wnt5* mutant embryos, no separation of midline-crossing axons into two distinct commissures occurs and consequently no axons cross along the AC (Fradkin *et al*, 2004).

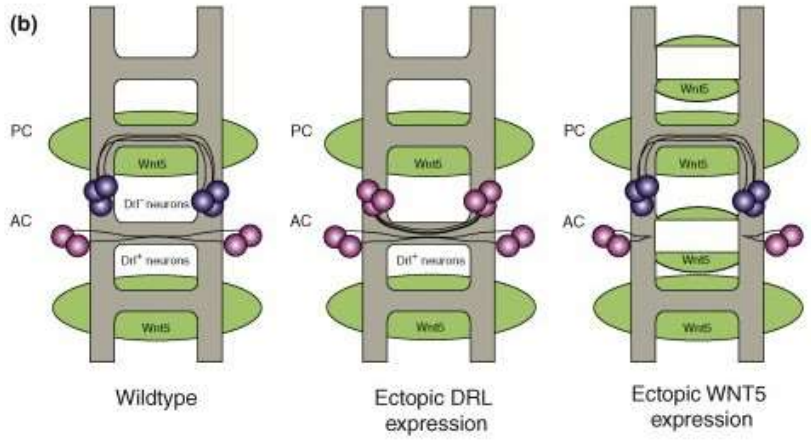

Fig. 6: Wnt5/Drl-mediated axon guidance during *Drosophila* embryonic CNS development.
Wnt5 is expressed in the posterior commissure (PC) and repels Drl-expressing axon, which therefore cross the midline across the anterior commissure (AC). Upon ectopic expression of Drl in PC neurons, these also cross along the AC. Ectopic Wnt5 expression along the midline leads to a loss of the AC. Depicted are the axons of eagle-expressing neuronal lineage. Taken from (Fradkin *et al*, 2010).

In contrast to its role in embryonic axon guidance, genetic analyses of Wnt5/Drl signaling in two Drosophila postembryonic brain centers, the mushroom body (MB) and the antennal lobes (ALs), suggest that Drl can also act to sequester Wnt5 to prevent it from interacting with other receptors (Moreau-Fauvarque *et al*, 1998; Grillenzoni *et al*, 2007; Yao *et al*, 2007; Sakurai *et al*, 2009). In *Drosophila*, odor information received by olfactory receptor neurons, located in the antennal and maxillary palps, is processed by glomeruli organized in the antennal lobe to form synapses with projection neurons which then relay olfactory information to the mushroom body calyx and the lateral horn. In *drl* mutants, the MB dorsal lobes are lost and the medial lobes are fused across the midline (Moreau-Fauvarque *et al*, 1998; Grillenzoni *et al*, 2007). Overexpression of Drl phenocopies the *Wnt5* mutant phenotype and overexpression of Wnt5 leads to a *drl* mutant-like phenotype, suggesting that *Wnt5* and *drl* act antagonistically during MB development (Grillenzoni *et al*, 2007). Two studies revealed that Drl plays a similar Wnt5 sequestration function during AL development (Yao *et al*, 2007; Sakurai *et al*, 2009).

1.4.5 Other Wnt family members

Wnt6 and *Wnt10* are located on the second chromosome within 100 kb to *Wnt4* and *wingless* (*wg*), however due to a lack of loss of function data, little is known about their functions. The expression of *Wnt6* is weak in embryos, but identical to that of *wg* in imaginal discs, suggesting that either both genes are under the control of the same

enhancer or Wnt6 is induced by earlier Wg expression (Janson *et al*, 2001). Wnt10 is expressed in the embryonic mesoderm, central nervous system and gut (Janson *et al*, 2001).

Another Wnt family member, WntD (Wnt inhibitor of Dorsal; Wnt8 in Llimargas & Lawrence, 2001), acts as a feedback inhibitor of the *Drosophila* NF-κB homolog Dorsal during both embryonic patterning and the Toll-mediated innate immune response (Gordon *et al*, 2005; Ganguly *et al*, 2005; Gordon *et al*, 2008).

1.5 Wnt co-receptors

The identification of Frizzled transmembrane proteins as Wnt receptors in *Drosophila* cell culture (Bhanot *et al*, 1996) provided the essential link between extracellular Wnts and the intracellular components of Wnt signal transduction. However, in the past decade, other transmembrane receptors have been shown to bind Wnt ligands, resulting in the activation of diverse intracellular signaling pathways. Apart from members of the Frizzled receptor family, Wnt receptors include the low-density lipoprotein receptor-related protein (LRP, *Drosophila* Arrow), the protein tyrosine kinase 7 (PTK7) as well as the receptor tyrosine kinase-like orphan receptor (Ror) and the related to tyrosine kinase (Ryk) families. Wnt interactions with these receptors often lead to cellular effects that are unrelated to β-catenin, possibly mediating a variety of non-canonical signaling pathways (Van Amerongen *et al*, 2008) (Fig. 7).

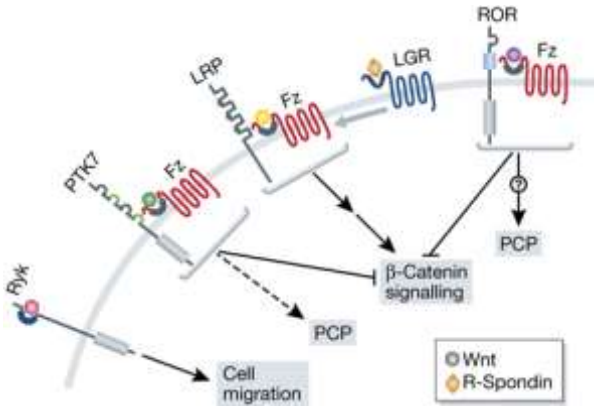

Fig. 7: Modulation of Wnt signaling by many receptors.
Frizzled transmembrane proteins are considered to be the main Wnt receptors; however, the final result of Wnt-Frizzled interaction is influenced by co-receptors. Wnt/Fz/LRP signaling activates canonical signaling, whereas PTK7 and Ror have been shown to inhibit β-catenin-dependent signaling. Ryk can function independently of Fz. Taken from (Vincent & Beckett, 2011).

1.5.1 Modulation of Wnt signaling outcome by (co-)receptors

Apart from the originally identified Frizzled receptors, other transmembrane Wnt receptors have been identified and subsequently shown to be involved in modulating the signaling outcome (Fig. 8). For example, Wnt5a and Wnt11, typically known as regulators of non-canoncial Wnt signaling, can also induce canonical signaling depending on receptor context (He *et al*, 1997; Tao *et al*, 2005; Mikels & Nusse, 2006; Cha *et al*, 2008). Thus, receptors can be bound by different Wnts and these in turn can activate multiple receptors. This suggests that Wnt signaling outcomes are dictated largely by the nature of the Wnt ligand as well as the nature of the Wnt receptor expressed in a tissue (Mikels & Nusse, 2006; van Amerongen *et al*, 2008; van Amerongen & Nusse, 2009; Vincent & Beckett, 2011).

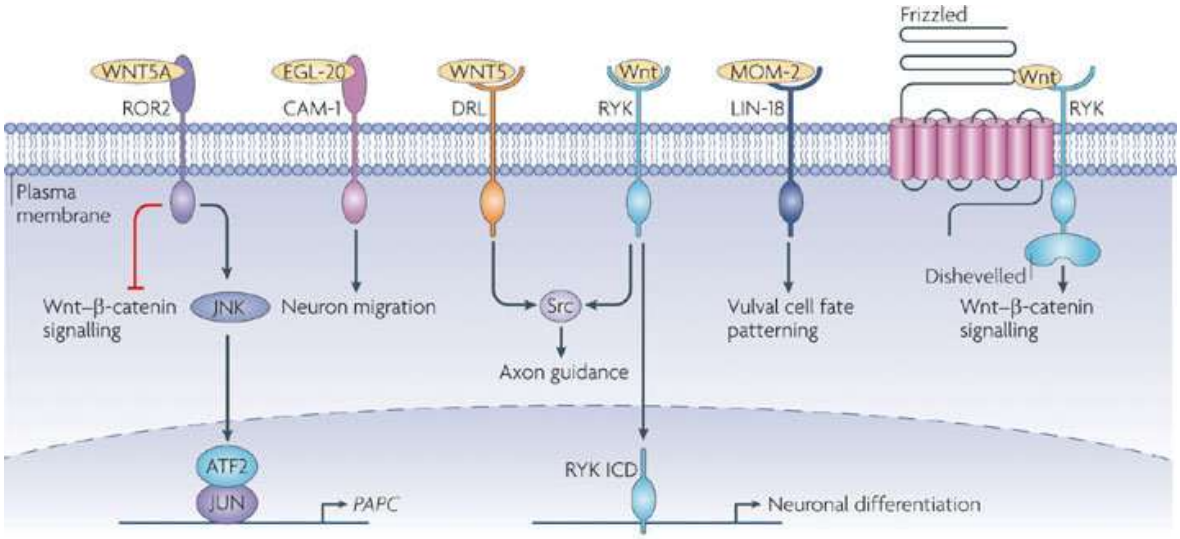

Fig. 8: Non-Frizzled receptors for Wnt ligands.
Wnt signals can be transduced by other transmembrane receptors apart from the Frizzled proteins. This signal transduction might regulate Wnt signaling positively or negatively and thereby modulate the biological outcome. Vertebrate Ror-2 and its *C. elegans* homolog Cam-1 interact with Wnt5 or Egl-20, respectively via their CRD domains. This activates JNK signaling in vertebrates or neuronal migration in *C. elegans*. Vertebrate Ryk, *Drosophila* Drl and *C. elegans* Lin-18 interact with their respective Wnt ligands via WIF domains. Drl and Ryk control axon guidance via recruitment of Src family kinases. In vertebrates, the cleaved intracellular domain (ICD) of Ryk can translocate to the nucleus and regulate neuronal differentiation. Additionally, Ryk can form a complex with Fz and Dsh and this might be important for β-catenin-dependent signaling. Taken from (Angers & Moon, 2009).

1.5.2 LRP / Arrow

Members of the LRP (low density lipoprotein receptor-related protein; LDL-related protein) family are highly important Frizzled co-receptors for canonical signaling. For example, Arrow, the *Drosophila* homolog of LRP, is essential for Wingless signal transduction (Wehrli *et al*, 2000). Similarly, LRP6 was shown to function as a co-receptor for Wnt signal transduction in *Xenopus* (Tamai *et al*, 2000) and mice (Pinson *et al*, 2000). So far, no modulation of PCP signaling was observed for Arrow/LRP. Therefore, this class of co-receptors is believed to act strictly canonical (Vincent & Beckett, 2011).

1.5.3 Ror

Ror (Receptor tyrosine kinase-like orphan receptor) proteins belong to the Receptor tyrosine kinase family (RTK), a superfamily of transmembrane glycoproteins involved in many critical processes like proliferation and differentiation as well as cell migration (Lemmon & Schlessinger, 2010). Ror-family RTKs are evolutionarily conserved in invertebrates and vertebrates and the members in vertebrates are Ror1 and Ror2 (Minami *et al*, 2010). Structural characteristics of vertebrate Ror proteins are their extracellular Frizzled-like cysteine-rich domain (CRD), which binds to Wnt ligands, and their Kringle domain, which is membrane-proximal. In addition to the CRD and Kringle domain, most vertebrate Ror-family members possess extracellular immunoglobulin (Ig)-like domains (Minami *et al*, 2010). The intracellular region of Ror contains a conserved tyrosine kinase domain, as well as a proline-rich (PRD) and two serine/threonine-rich domains (S/TRD1 and S/TRD2) (Fig. 9). The functions of the different protein domains are diverse and extensively reviewed in (Forrester, 2002; Yoda *et al*, 2003; Minami *et al*, 2010). *Drosophila* has two Ror family members and they neither possess extracellular Ig-like domains nor intracellular S/TRD and PRD domains (Fig. 9). *Drosophila* Ror is the homolog of vertebrate Ror1 and its closest ortholog in the *Drosophila* genome is Dnrk (*Drosophila* Neurospecific receptor kinase), which is most similar to vertebrate MuSK (Muscle-specific kinase) (Green *et al*, 2008).

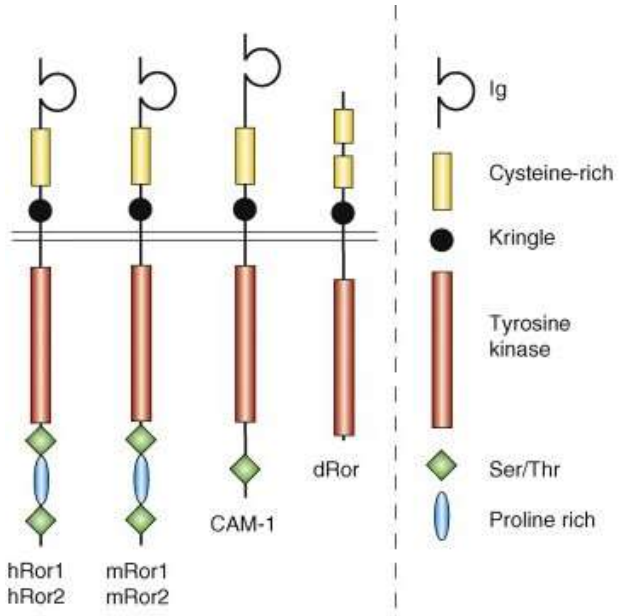

Fig. 9: Structure of Ror receptor tyrosine kinases (RTKs) in different species.
The extracellular region of human (hRor1 hRor2), mouse (mRor1, mRor2), *C. elegans* (CAM-1) and *Drosophila* (dRor) contains a cystein-rich as well as a Kringle domain. The cytoplasmic region is characterized by a tyrosine kinase domain, two serine/threonine-rich domains and a proline-rich domain. In contrast to its homologs, dRor does neither have extracellular Ig domains nor intracellular serine/threonine-rich and proline-rich domains. Taken from (Green *et al*, 2008).

The function of Ror proteins in development has been extensively studied in different organisms. Disruptions of human *Ror2* leads to skeletal defects like brachydactyly (Oldridge *et al*, 2000; Schwabe *et al*, 2000) and dwarfism (Bokhoven *et al*, 2000; Afzal *et al*, 2000). Similar phenotypes were observed in mice mutant for *Ror2* and the phenotype is enhanced in *Ror1*,*Ror2* double knock-outs (Ho *et al*, 2012). *Xenopus Ror2* (*Xror2*) transcripts were detected in gastrula and neurula embryos (Hikasa *et al*, 2002) and a *Xror2* loss of function mutation results in defects in convergent extension movements (Schambony & Wedlich, 2007). Although Ror proteins are highly expressed in the developing nervous system of many species, its precise role in neuronal development remains unclear (Green *et al*, 2008). However, *Cam-1*, the *C. elegans* homolog of *Ror2* is expressed in the nervous system and vulval precursor cells and is needed for neuronal migration and axonal development as well as vulval development (Forrester *et al*, 1999; Green *et al*, 2007). The *Drosophila* homologs *Dror* and *Dnrk* are exclusively expressed in the nervous system, however, their precise function is still unknown (Wilson *et al*, 1993; Oishi *et al*, 1997). *Dnrk* has been demonstrated to genetically interact with the Dystrophin glycoprotein complex consisting of Dystroglycan and Dystrophin (Kucherenko *et al*, 2008; Kucherenko *et al*,

2011) and plays a role in axon pathfinding and rhabdomere length in *Drosophila* eye development (Marrone *et al*, 2011).

Although Ror proteins were initially given that name because the associated ligand was unknown, it has been shown in the past years that Ror proteins interact with several Wnt ligands via their CRD (Hikasa *et al*, 2002; Oishi *et al*, 2003; Billiard *et al*, 2005; Mikels & Nusse, 2006a; Green *et al*, 2007). Furthermore, the CRD is required for interacting with other co-receptors like Frizzled (Oishi *et al*, 2003). In mammals, it has been demonstrated that Ror2 acts as a receptor or co-receptor for Wnt5a and this interaction inhibits canonical signaling (Oishi *et al*, 2003; Mikels & Nusse, 2006a). Similarly, Cam-1, the *C. elegans* Ror protein, functionally inhibits Egl-20, a *C. elegans* Wnt protein (Kim & Forrester, 2003; Forrester *et al*, 2004). In addition to suppressing canonical signal transduction, these co-receptors were shown to be able to actively promote non-canonical signaling in a Frizzled-dependent manner (Grumolato *et al*, 2010).

1.5.4 Ryk / Derailed (Drl)

Interaction between Wnt signaling and Ryk (for related to tyrosine kinase) transmembrane proteins was initially implicated to function as an axon guidance pathway (Fradkin *et al*, 2010). Ryks belong to the transmembrane receptor tyrosine kinase (RTK) family, however their protein kinase activity has shown to be inactive (Hovens *et al*, 1992; Stacker *et al*, 1993). Ryk family proteins have, among several other common protein motifs, an extracellular Wnt-binding Wnt-inhibitory-factor-1 (WIF) domain (Patthy, 2000) that has been shown to interact with Wnt5, but not Wnt4 or Wingless (Yoshikawa *et al*, 2003), (see also 1.4.4).

In *Drosophila* the first functional relevance of Ryk was uncovered in a screen for genes involved in neuronal pathway selection (Callahan *et al*, 1995). Neurons in flies homozygous mutant for the Ryk homolog *derailed* (*drl*) project along inappropriate paths (Callahan *et al*, 1995). Next, Derailed was demonstrated to be expressed exclusively by neurons that project in the anterior of the two commissures (anterior commissure, AC) present in each hemisegment and absence of *drl* leads to abnormal crossing of these neurons into the posterior commissure (PC). Conversely, ectopic expression of Drl in PC neurons forces them to cross into the AC (Bonkowsky *et al*, 1999). In *drl* mutants somatic muscles attach abnormally within the epidermis,

indicating that similar mechanisms control muscle attachment site selection and axon pathfinding (Callahan *et al*, 1996).

Furthermore, the *drl* gene was identified in a screen for adult Drosophila learning and memory mutants; here described as *linotte* (*lio*) (Dura *et al*, 1993; Dura *et al*, 1995). *lio* mutants of the *drl* gene display structural brain defects in two adult brain centers implicated in learning and memory, the central complex and the mushroom bodies (Moreau-Fauvarque *et al*, 1998; Simon *et al*, 1998).

In vertebrates, Fz and Ryk can form a ternary complex with Wnt-1 and Wnt3a during neurite extension and axon fasciculation (Lu *et al*, 2004a). The *C. elegans* Ryk homolog Lin-18, which is required to establish polarity of the secondary vulval lineage, genetically interacts with Lin-17, the homolog of Frizzled (Sternberg & Horvitz, 1988; Inoue *et al*, 2004). Furthermore, it was shown that Wnt3a-induced nuclear translocation of the Ryk intracellular domain is important for neural progenitor cell differentiation (Lyu *et al*, 2008). Other recent studies suggest that Ryk and Fz also function together during *Xenopus* convergent extension (Kim *et al*, 2008) as well as mammalian cortical axon repulsion (Li *et al*, 2009). In essence, these studies revealed that Ryk not only acts as a Wnt receptor but also together with Frizzled in a variety of canonical and non-canonical pathways (Fradkin *et al*, 2010). The outcome of several studies characterizing different aspects of Wnt/Ryk signaling in the developing mammalian brain is that they mediate repulsive axon guidance, similarly to Drl in the embryonic *Drosophila* central nervous system (CNS) (Liu *et al*, 2005; Keeble *et al*, 2006; Schmitt *et al*, 2006).

Whereas Wnt/Ryk play a role in axon guidance during development, inappropriate activation of this pathway after injury leads to poor axon re-growth (Fradkin *et al*, 2010). Neither Wnts nor Ryk are expressed in the healthy adult spinal cord. However, its injury results in the induction of Wnt1 and Wnt5a expression in the gray matter as well as increased Ryk expression in damaged axons, resulting in poor regeneration (Liu *et al*, 2008; Miyashita *et al*, 2009).

1.5.5 Protein Tyrosine Kinase 7 (PTK7)

The human homolog of mouse Protein tyrosine kinase 7 (PTK7) was first cloned from colon carcinoma tissue and therefore initially named Colon carcinoma kinase-4 (CCK-4) (Mossie *et al*, 1995). PTK7 is a transmembrane protein with seven extracellular immunoglobulin-like domains, a single transmembrane region and a

cytoplasmic kinase homology domain. Structurally, PTK7 therefore belongs to the receptor tyrosine kinase family; however, the molecule is catalytically inactive in all species tested so far due to a mutation in the active DFG triplet required to coordinate ATP binding during phosphorylation (chicken Kinase-like gene (KLG) (Chou & Hayman, 1991); human PTK7 (Mossie *et al*, 1995); hydra Lemon (Miller & Steele, 2000); mouse PTK7 (Jung *et al*, 2004)).

Loss of function of mouse PTK7 leads to defects in neural tube closure and misorientation of stereociliary bundles in the inner ear - both characteristic planar cell polarity (PCP) defects. Similarly, knockdown of PTK7 in *Xenopus* embryos also results in neural convergent extension and neural tube closure defects (Lu *et al*, 2004; Yen *et al*, 2009; Paudyal *et al*, 2010). Furthermore, PTK7 is expressed in Xenopus neural crest cells and PTK7 knockdown impairs correct neural crest cell migration (Shnitsar & Borchers, 2008). Analysis of different transcription factor knock-out phenotypes suggested the caudal type homeobox transcription factors (Cdx) Cdx1 and Cdx2 as well as the forkhead transcription factor FoxF to be the transcriptional regulators of PTK7 during mouse development (Jakobsen *et al*, 2007; Savory *et al*, 2011).

Further characterization of PTK7 function revealed that in contrast to mouse (Yen *et al*, 2009), it is required for Dsh localization to the plasma membrane in *Xenopus* (Shnitsar & Borchers, 2008) and that this process requires the adaptor proteins receptor of activated C kinase (RACK1) and protein kinase Cδ1 (PKCδ1) (Wehner *et al*, 2011). Two recent studies identified PTK7 to interact with β-catenin in *Xenopus,* leading to its stabilization (Puppo *et al*, 2011), or with Wnt proteins in both *Xenopus* and *Drosophila,* which in contrast was shown to inhibit canonical Wnt signaling (Peradziryi *et al*, 2011). Based on these findings, the current model for PTK7 function is that it inhibits β-catenin dependent signaling, thereby promoting PCP signaling (Fig. 10). This was further supported by an RNA interference study that identified the *Drosophila* PTK7 homolog *off-track* as a regulator of JUN NH_2-terminal kinase (JNK) (Bakal *et al*, 2008).

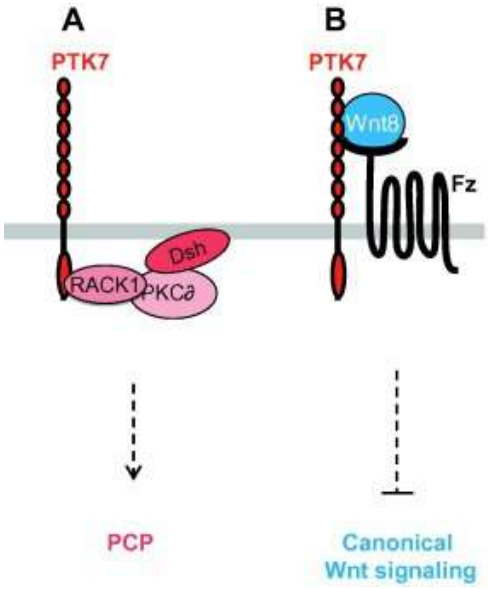

Fig. 10: Model for PTK7 function in canonical and PCP signaling.
(A) *Xenopus* PTK7 activates PCP signaling by recruiting Dsh to the membrane. This recruitment is mediated via interaction with RACK1 and PKCδ1. **(B)** PTK7 also functions as a Fz co-receptor in binding of canonical Wnt ligands. This interaction inhibits canonical signaling, possibly by trapping canonical Wnts. Taken from (Peradziryi *et al*, 2012).

1.5.6 Off-track (Otk) – *Drosophila* homolog of PTK7

PTK7 has a *Drosophila* homolog, the gene *off-track* (*otk*), which encodes for a glycoprotein and was first described as a neural cell adhesion molecule expressed in several areas of the developing nervous system during *Drosophila* embryogenesis (Pulido *et al*, 1992). In contrast to its homolog Ptk7 in mouse and *Xenopus*, mutations in this gene were not reported to display any planar cell polarity phenotypes. Instead, the otk^{3} mutant allele, removing parts of the first exon including the start codon, was reported to be homozygous lethal and to cause defects in axon pathfinding and defective targeting of photoreceptor axons to the brain (Winberg *et al*, 2001; Cafferty *et al*, 2004). Otk was demonstrated to associate with the Semaphorin 1a receptor Plexin A and therefore proposed to mediate the repulsive signaling response of Plexin A to its ligand (Winberg *et al*, 2001). Furthermore, it was shown that Otk is expressed in the *Drosophila* visual system and required for lamina-specific targeting of R1-R6 axons (Cafferty *et al*, 2004). Recently, it was suggested that Otk acts as a receptor for *Drosophila* Wnt4 in inhibiting canonical signaling (Peradziryi *et al*, 2011).

1.6 Scope of the thesis

The importance of other Wnt transmembrane receptors apart from Frizzled has been appreciated within the past decade, but is so far not completely understood. Which pathway becomes activated is at least in part determined by the choice of Frizzled co-receptors (Angers & Moon, 2009). Frizzled co-receptors like LRP/Arrow activate canonical signaling exclusively, while other co-receptors such as Ror and Ryk/Derailed control non-canonical signaling. PTK7/Otk also seems to belong to this group of co-receptors required for pathway selectivity (Peradziryi *et al*, 2012), however, the exact molecular mechanism as well as its conservation between different species remain to be clarified.

The aim of this work therefore was to investigate the role and function of Otk in *Drosophila* development. To this end I generated null alleles of both Drosophila Otk paralogs *otk* and the so far undescribed gene *CG8964*/*otk2* and analyzed the effects of Otk loss as well as overexpression on general viability and with regard to different Wnt signaling pathways. According to the function of vertebrate PTK7, Otk/Otk2 could act as receptors of Wnt ligands and might regulate canonical or non-canoncial Wnt signaling. To investigate this possibility, the localization of Otk as well as the effect of *otk/otk2* deletion was analysed with regard to different Wnt ligands as well as receptors. In this context it was intriguing to determine whether Otk might also be a target of Wnt signaling. For this purpose I analysed the expression of Otk in different *wnt* mutant backgrounds. Furthermore, genetic interaction experiments with other Wnt receptors were performed to study if receptors might act redundantly.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals were of analytical grade and were purchased from Biomol (Hamburg, Germany), Bio-Rad (Munich, Germany), Biozym (Oldendorf, Germany), Difco (Detroit, USA), Fluka (Buchs, Switzerland), Gibco/BRL Life Technologies (Karlsruhe, Germany), Merck (Darmstadt, Germany), Polysciences (Eppelheim, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), Sigma-Aldrich (Steinheim, Germany), Macherey-Nagel (Düren, Germany) if not stated otherwise. Solutions were prepared in distilled water and sterilized by autoclaving or sterile filtration.

2.1.2 Enzymes

Enzymes for molecular biology methods were purchased from Bioline (Luckenwalde, Germany), Fermentas (St. Leon-Rot, Germany), Genecraft (Lüdingshausen, Germany), Promega (Madison, USA), Roche (Mannheim, Germany) and used with the buffers supplies.

2.1.3 Bacterial strains

In this study, different *Escherichia coli* (*E. coli*) strains were used for the amplification of plasmid DNA and for the production of recombinant GST-fusion proteins. They are listed in Table 2. *E. coli* was grown in either lysogeny broth (LB) medium (10 g/l tryptone peptone, 5 g/l yeast extract, 5 g/l NaCl) or 2xYTA medium (16 g/l tryptone peptone, 15 g/l yeast extract, 5 gl NaCl). For production of agar plates, 2 % agar-agar was added to the LB medium. It was autoclaved at 120 °C for 30 min and after cooling to 60 °C, antibiotics were added (100 µg/ml ampicillin, 50 µg/ml kanamycin).

Table 2: Bacterial strains used in this study.

Strain	Genotype	Application
DH5α	Φ80*lac*ZΔM15, Δ*lac*ZYA-*argF)*U169, *deoR*, *recA1*, *endA1, hsdR17*(r_k-, m_k+), *phoA*, *supE44,* λ-*, thi-1, gyrA96, relA1*	Amplification of plasmid DNA
BL21	F-, *omp*T, *hsdS*$_B$(r_B-, m_B-), *dcm*, *gal*, λ(DE3)	Expression of recombinant proteins

TOP10	*F´[lacIq, Tn10(TetR)]mcrAΔ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15, ΔlacX74, recA1, araD139, Δ(araleu), 7697 galUgalKrpsL (StrR) endA1 nupG*	Cloning of PCR fragments in pENTR vector
XL1-Blue	*endA1, gyrA96(nal^R)*, *thi-1, recA1,relA1, lac, glnV44*, F'[*Tn10 proAB+lacI^q Δ(lacZ)M15*], *hsdR17(r_k-, m_k+)*	Site-directed mutagenesis

2.1.4 Oligonucleotides

All oligonucleotides were designed using DNA-Star Lasergene V6 software (DNASTAR Inc., Madison, USA) and synthesized by Metabion (Martinsried, Germany). Common considerations for primer design were taken into account, like e.g. a base composition of 50-60 % (G+C), no primer self-complementarity and primer lengths between 17-28 bp if possible. All primers used in this study are listed in Table 3.

Table 3: Oligonucleotides used in this study.

Primer name	Sequence 5' → 3'	Description
General sequencing primer for cloning		
M13 for	GTAAAACGACGGCCAG	pENTR forward
M13 rev	CAGGAAACAGCTATGAC	pENTR reverse
ACTf	GAGCATTGCGGCTGATAAGG	pAWG and pAWM forward
SVr	GGCATTCCACCACTGCTCCC	pAWM reverse
HSPf	TATAAATAGAGGCGCTTCGT	pTWG and pTWR forward
EGFP-N-rev	CGGACACGCTGAACTTGTG	pAWG, pTWG and pPWG-attB reverse
RFPr	GGACAGCTTCAAGTAGTCGG	pTWR reverse
UASPf	GGCAAGGGTCGAGTCGATAG	pPWG-attB forward
Otk primer for cloning		
Otk_seq1	GTGGAGGGCGCCTCAGG	Sequencing Otk constructs in pENTR
Otk_seq2	CACCCTAAGCTTTGCCAGC	
Otk_seq3	AGCTACGGCTGCACAATTGG	
Otk_seq4	AGGCAGCGGAAGTGGAAGC	
Otk_seq5	GAGTGCATCCAGGAAGACG	
otkExDomBamHI_for	CGAATGGTATCGCGGATCCGAGAAACTTAGC	Introduction of a BamHI site in the extracellular domain of Otk
otkExDomBamHI_rev	GCTAAGTTTCTCGGATCCGCGATACCATTCG	

otkExDomEcoRI_for	GCAGAATGGCACGAATTCCTTTGCCAGC	Introduction of an EcoRI site in the extracellular domain of Otk
otkExDomEcoRI_rev	GCTGGCAAAGGAATTCGTGCCATTCTGC	
Otk2 primer for cloning		
CG8964_for_CACC	CACCATGGGGCTGAACGGAAGACG	Cloning of Otk2 in pENTR
CG8964_rev+Stop	TCACACAATATCGTCGGCCACC	
CG8964_rev-Stop	CACAATATCGTCGGCCACCTGC	
Other cloning primers		
fz1_gate_for	CACCATGTGGCGTCAAATCCTGTTTA	Cloning of Fz in pENTR
fz1_gate_rev	GACGTACGCCTGCGCCCGGGTCCG	
fz1_SEQ_for	CTGCATGACTGTGGAGCCCCATG	
fz1_gate_STOP_rev	CTAGACGTACGCCTGCGCCCG	
fz2_gate_for	CACCATGAGACACAATCGACTGAAG	Cloning of Fz2 in pENTR
fz2_gate_rev	TACGTGGCTGGCCGCCGGCTGCTTGA	
fz2_SEQ_for	CCTCAACGAAGCCGTGCCGCGGACG	
fz2_gate_STOP_rev	TCATACGTGGCTGGCCGCCGGCTGC	
Ror_gate_for	CACCATGAACAAATACTCGGCAT	Cloning of Ror in pENTR
Ror_gate_rev	CATTTCTGGATTACTGGCCTTAAAGT	
Ror_SEQ_for	TATCCTTGGGCATCACCATCG	
Ror_gate_STOP_rev	TTACATTTCTGGATTACTGGCCTTA	
Primer for characterization of transdeletion lines		
Otk_Intron1_rev1	GAATCGTACTGCCTGCCAAGC	Characterization of P-element lines used for the deletion of *otk* and afterwards for characterization of the successful *otk* deletion
Otk_Intron1_for1	GCTTGGCAGGCAGTACGATTC	
Otk_Intron1_rev2	GCTGTCCAAAGTTGCGTGTCC	
Otk_Intron1_for3	CGACGAGGGTAAGTGGAAGAATG	
Otk_Intron1_rev3	CATTCTTCCACTTACCCTCGTCG	
Otk_Exon2_rev1	TGAGCTGGCCAAAACGGAAGC	
Otk_Exon2_rev2	GATTCGCACTCGAATTTCACG	
Otk_Start_for	CGGTTATACATATACGCAGATGACTGC	
Otk_Start_rev	GCAGTCATCTGCGTATATGTATAACCG	

UpstreamEP2017_for1	CTCACGATGATCATCTTAAGTTGGTTGC	
UpstreamEP2017_for2	AGCTGATTCTTGCTTTGGTCATTAAGACG	
UpstreamEP2017_for3	GACTGTCTTAGATGCACAGCAATAGG	
Otk_Exon1_for1	GGATGATCTCAATATGCGGCCTGG	
Otk_Exon1_rev1	CCAGGCCGCATATTGAGATCATCC	
Otk_Exon3_rev1	CAGTTTGGCGGGCGGAGACG	
Otk_Exon4_rev1	GACCTGGAATGGGCGCCGAGC	
Otk_Exon5_rev1	CTCCCTTGTGCGAGATGGC	
d01360L1	CTTTAAAATTCCACTTCAAACTTGC	
e03992R3	GATTCAACTTTTTGTTGGACTCG	
e03992L1	CACAGATAAATTGCACATCACATTACG	
e03992L2	CACAGATAAATTGCACATCACATTACG	
e03992R1	GTATTTATTACACTGAGCGGAAGG	Characterization of P-element lines used for the deletion of *otk* and afterwards for characterization of the successful *otk* deletion
e03992R2	TGTGTGGCCCAGAGGTGAAAGC	
otk_Start_for2	CATATACGCAGATGACTGCTAGG	
otk_Intron1_rev4	GTTGCCTGTGTTAGTGAGCTCG	
otk_Intron1_rev5	GAATCTATTAAAACAGAACG	
otk_Exon1_for2	CTGGTCATGGCTTTGATGATGG	
otk_5UTR_for1	CTAATTGAGACCAAAGGTGTCG	
otk_Intron1_rev6	GTCTCACTTCAAGTGGGTATCG	
otk_Exon2_for1	CTTCCGTTTTGGCCAGCTCATCG	
otk_5UTR_for2	GTATCAGTTCGTGTGTTCTCGG	
otk_Intron1_for2	GGACACGCAACTTTGGACAGC	
otk_Intron1_for4	GCTCCTGCCCTTGCAGAGCG	
otk_Intron4_rev1	GGAAGCGGTCGAAATGAAGACC	
otk_Intron3_rev1	CAAGCAAAGACAGATTTGCTGTCG	
otk_Intron5_rev1	CAATTGGATCTATGAAAATGC	
otk_Intron6_rev1	GATTATATCAGTAGTTTGTCAGC	
UP_inverse	CATGATGAAATAACATAAGGTGGTCCCGTC	
PBac5’	CAATTTTACGCAGACTATCTTTCTAGGG	Additional primers for characterization of the *otk* deletion
PBac3’	CGTACGTCACAATATGATTATCTTTCTAGG	

FRT seq1	CGTGACTGTGCGTTAGGTCCTCC	
XP for	AATGATTCGCAGTGGAAGGCT	
FRT seq3	CCAATTCGCCCTTGAAGATCTAGC	Additional primers for characterization of the *otk* deletion
PBac_seq1	GATATATGAAACCAATCAAAACTCG	
PBac_seq2	GAAACCTTTACATGAGCCTGACG	
PBac_seq3	CCTCGATATACAGACCGATAAAAC	
CG8889Exon4_rev1	CCAATATCATTGTCACCCGGCAC	
CG8889Exon3_rev1	GTATCTGGGGATCAGCAATAAGCAGC	
CG8889Exon5_rev1	GTCGAGACGCGGTTATAGAGC	Characterization of P-element lines used for the deletion of *otk2* and *otk,otk2*
UpstreamCG8889_for1	CATGTTTGTTTTGCTTTGAGC	
UpstreamCG8889_for2	GTACATGATTGCATTTCAGC	
UpstreamCG8889_for3	GTGCGCCCTGAAAGCGATGATGC	
Otk5'_rev	GCAAACTCGGCTAAACAAAACG	
CG8964_Exon2_rev1	TGACTTGTGGAGCAGGCGTTCC	
CG8964_Start_for	GAAATGGGGCTGAACGGAAGACG	
CG8964_Exon1_for	CCTGATCTTCTGCTCTTACG	
CG8964_Exon3_for1	GACCCATCGAGGTCGCAGAAGC	
CG8964_Exon3_for2	GACAAGGATCTCACCTACTTGAACG	
CG8964_Intron1_for1	GTGAGTTAAGTCGGCCGACTGACTCA	Characterization of *otk2* and *otk,otk2 deletions*
CG8964_Intron2_rev1	GTAAGTATTAAATGTTGCATCATGC	
CG8964_Intron2_for1	CTTACATATAATTCAATCCTTCC	
CG8964up5'UTR_for	CAGCGAAGTCGCTCTCATCC	
CG8964Intron1_rev1	GAAGAAAAGATGAGTCAGTCG	
CG8964down3'UTR_rev	GAGCAGCGGAGGAAGTTTACG	
PBac_seq4	CTAGTGAAGTTCCTATTCC	
CG7739for1	TACACCAAGCAGAACCCACGGAG	Control primer for *CG7739*
CG7739rev2	CCTGAGTAGTGGTACTGTAGGTG	

2.1.5 Vectors and constructs

Several constructs were generated in this study for production of fusion proteins in *Escherichia coli* or for transfection of cells. The basic vector backbones, to which the others go back, are listed in Table 4. The constructs that were used in this study are listed in Table 5.

Table 4: Vectors used in this study.

Vector name	Description	Reference
pENTR/D-TOPO	Entry vector for Gateway cloning, Kanamycin resistance	Invitrogen, Carlsbad, Germany
pAWG	Expression vector, Actin5C promoter, C-terminal GFP tag, Ampicillin resistance	Murphy lab, Baltimore, USA
pAWM	Expression vector, Actin5C promoter, C-terminal Myc tag, Ampicillin resistance	Murphy lab, Baltimore, USA
pTWG	Expression vector, UASt promoter, C-terminal GFP tag, Ampicillin resistance	Murphy lab, Baltimore, USA
pTWR	Expression vector, UASt promoter, C-terminal RFP tag, Ampicillin resistance	Murphy lab, Baltimore, USA
pPWG-attB	Expression vector, UASp promoter, C-terminal GFP tag, donor attB sequence for ΦC31-mediated integration, Ampicillin resistance	Murphy lab, Baltimore, USA
pGEX4T1	Vector for expression of GST fusion proteins in *E. coli*, Ampicillin resistance	Amersham Pharmacia Biotech, England

Table 5: Constructs used in this study.

Construct name	Description	Cloning strategy / Reference
Otk constructs		
Otk-pENTR	Full length Otk in pENTR	Iryna Shnitsar, unpublished
OtkΔC-pENTR	Otk lacking the cytoplasmic domain (aa 776-1033) in pENTR	Iryna Shnitsar, unpublished
OtkΔEx-pENTR	Otk lacking the extracellular domain (aa 2-474) in pENTR	Iryna Shnitsar, unpublished
Otk-pAWG	Full length Otk, Actin promoter, C-terminal GFP-tag	LR recombination of Otk-pENTR in pAWG
Otk-pAWM	Full length Otk, Actin promoter, C-terminal Myc-tag	LR recombination of Otk-pENTR in pAWM
OtkΔC-pAWM	Otk lacking the cytoplasmic domain (aa 776-1033), Actin promoter, C-terminal Myc-tag	LR recombination of OtkΔCy-pENTR in pAWM
OtkΔEx-pAWM	Otk lacking the extracellular domain (aa 2-474), Actin promoter, C-terminal Myc-tag	LR recombination of OtkΔEx-pENTR in pAWM
Otk-pPWG-attB	Full length Otk, UASp promoter, C-terminal GFP tag, attB donor site	LR recombination of Otk-pENTR in pPWG-attB
OtkΔC-pPWG-attB	Otk lacking the cytoplasmic domain (aa 776-1033), UASp promoter, C-terminal GFP tag, attB donor site	LR recombination of OtkΔCy-pENTR in pPWG-attB

OtkΔEx-pPWG-attB	Otk lacking the extracellular domain (aa 2-474), UASp promoter, C-terminal GFP tag, attB donor site	LR recombination of OtkΔEx-pENTR in pPWG-attB
Otk-pTWG	Full length Otk, UASt promoter, C-terminal GFP tag	LR recombination of Otk-pENTR in pTWG
Otk-pENTR+BamHI	Otk-pENTR with BamHI site introduced into extracellular domain	Mutagenesis of Otk-pENTR with otkExDomBamHI_for/rev
Otk-pENTR+BamHI+EcoRI	Otk-pENTR with BamHI and EcoRI sites introduced into extracellular domain	Mutagenesis of Otk-pENTR +BamHI with otkExDomEcoRI_for/rev
OtkEx-pGEX4T1	Extracellular domain of Otk (aa 159-338) in pGEX4T1	Otk-pENTR+BamHI+EcoRI cut with EcoRI and BamHI, fragment ligated in pGEX4T1
Otk-pOT2	cDNA clone containing full length Otk, binding sites for SP6 and T7	LP17455 (DGRC)
Otk2 constructs		
Otk2-pENTR	Full length Otk2 in pENTR	Amplification of Otk2 coding sequence with CG8964_for_ CACC / CG8964_rev-Stop and introduction of PCR product in pENTR
Otk2-pAWG	Full length Otk2, Actin promoter, C-terminal GFP-tag	LR recombination of Otk2-pENTR in pAWG
Otk2-pAWM	Full length Otk2, Actin promoter, C-terminal Myc-tag	LR recombination of Otk2-pENTR in pAWM
Otk2-pPWG-attB	Full length Otk2, UASp promoter, C-terminal GFP tag, attB donor site	LR recombination of Otk2-pENTR in pPWG-attB
Otk2-pTWR	Full length Otk2, UASt promoter, C-terminal RFP tag	LR recombination of Otk2-pENTR in pTWR
Otk2-pFLCI	cDNA clone containing full length Otk, binding sites for T3 and T7	DGRC (RE41180)
Other constructs		
Fz1-pENTR	Full length Fz1 in pENTR	Amplification of Fz1 coding sequence with fz1_gate_for/ rev and introduction of PCR product in pENTR
Fz1-pAWM	Full length Fz1, Actin promoter, C-terminal Myc-tag	LR recombination of Fz1-pENTR in pAWM
Fz2-pENTR	Full length Fz2 in pENTR	Amplification of Fz2 coding sequence with fz2_gate_for/ rev and introduction of PCR product in pENTR
Fz2-pAWM	Full length Fz2, Actin promoter, C-terminal Myc-tag	LR recombination of Fz2-pENTR in pAWM

Ror-pENTR	Full length Ror in pENTR	Amplification of Ror coding sequence with Ror_gate_for/ rev and introduction of PCR product in pENTR
Ror-pAWM	Full length Fz1, Actin promoter, C-terminal Myc-tag	LR recombination of Ror-pENTR in pAWM

2.1.6 Antibodies

Antibodies used in this study are listed in Table 6 and Table 7 and were used at the indicated dilutions.

To raise polyclonal antibodies against Off-track (Otk), a GST fusion protein corresponding to aa 159-338 of the extracellular domain was purified and injected into guinea pigs (Eurogentec, Seraing, Belgium). The final bleed of guinea pig 336 was used for all experiments described in this study. To generate peptide antibodies against Off-track2 (Otk2, CG8964), the peptides VELGRMDSTTSEPQLE (aa 93-98, internal fragment, EP104043) and ESTILEQESQVADDIV (aa 418-433, at C-terminus, EP104042) were used to inject into rabbits (Eurogentec, Seraing, Belgium). The serum affinity purified against the C-terminal peptide EP104042 was used for all experiments described in this study.

Table 6: Primary antibodies used in this study.
IF - Immunofluorescence, IP - Immunoprecipitation WB - Western Blot, DSHB – Developmental Studies Hybridoma Bank, University of Iowa, USA.

Epitope	Animal	Application	Dilution	Designation	Source
Actin	Rabbit	WB	1:2000	A2066	Sigma
Bazooka	Rabbit	IF	1:1000	DE99646-2	Wodarz *et al*, 2000
BP 102	Mouse	IF	1:50	BP 102	DSHB
β-Galactosidase	Mouse	WB,IP	1:200	JIE7	DSHB
C-Myc	Mouse	WB,IP	1:200	9E10	DSHB
DE-Cadherin	Rat	IF	1:5	DCAD 2	DSHB
Elav	Mouse	IF	1:50	9F8A9	DSHB
Fasciclin III	Mouse	IF	1:20	7G10	DSHB
GFP	Mouse	IF,WB	1:1000	A11120	Molecular Probes
GFP	Rabbit	IF,WB	1:1000	A11121	Molecular Probes
GST	Rabbit	WB	1:20000	G7781	Sigma
Miranda	Guinea Pig	IF	1:1000	DE02120	Wodarz, unpublished
Otk	Guinea Pig	WB,IF	1:1000	DE11243 SAC336	This work
Otk2	Rabbit	WB,IF	1:100	EP104042	This work

Tubulin α	Mouse	WB	1:1000	12G10	DSHB
Vasa	Rabbit	IF	1:2000		Ruth Lehmann
Wingless	Mouse	IF	1:20	4D4	DSHB
Wnt5	Rabbit	IF	1:150		Fradkin *et al*, 2004

Table 7: Secondary antibodies used in this study.
IF - Immunofluorescence, WB - Western Blot.

Epitope	Conjugate	Application	Dilution	Origin	Source
Guinea Pig IgG	HRP	WB	1:10000		Dianova
Mouse IgG	HRP	WB	1:10000	Goat	Dianova
Rabbit IgG	HRP	WB	1:10000	Goat	Dianova
Guinea Pig IgG	Cy2, Cy3	IF	1:200	Donkey	Dianova
Guinea Pig IgG	Cy5	IF	1:200	Goat	Dianova
Mouse IgG	Cy2, Cy3	IF	1:200	Donkey	Dianova
Mouse	Cy5	IF	1:200	Goat	Dianova
Rabbit IgG	Cy2, Cy5	IF	1:200	Goat	Dianova
Rabbit IgG	Cy3	IF	1:200	Donkey	Dianova

2.1.7 Fly stocks

Table 8: Fly stocks used in this study.
BL - Bloomington Stock Center.

Stock	Genotype	Description	Reference
wild type oregonR	*wild type*	Red eyes	Stock collection Wodarz
white-	w^{1118}	White eyes	BL 5905
Gla/CyO(ftz::lacZ)	*Gla/CyO, P(ftz::lacZ)*	Balancer 2nd chromosome	Stock collection Wodarz
TM3(ftz::lacZ)/ TM6B	*w;;TM3(ftz::lacZ)ce, Ser/TM6B, e, Tu, Ser*	Balancer 3rd chromosome	Stock collection Wodarz
If/CyO; MKRS/TM6B	*w; If/CyO; MKRS/TM6B*	Balancer 2nd and 3rd chromosome	Stock collection Wodarz
da-Gal4	*w[*]; P{w[+mW.hs] =GAL4-da.G32}UH1*	Gal4 driver line, ubiquitous expression in daughterless pattern, 3rd chromosome	BL 5460
PhiC31 86FB	P{ry[+t7.2]=hsp70-flp}1, y[1] w[*]; M{3xP3-RFP.attP}ZH-86Fb; M{vas-int.B}ZH-102D	phiC31 integrase expressed from the vasa promoter; 3rd chromosome attP site	BL23648
ywhsFlp;Sco/CyO	P{ry[+t7.2]=hsFLP}12, y[1] w[*]; sna[Sco]/CyO	Heat shock FLP stock used for transdeletion	BL1929

otk^{CPTI000252}	*PBac{602.P.SVS-1}otk*^{CPTI000252}	GFP trap otk	Kyoto stock center
CG8964^{SH1639}	*P{lacW}l(2)SH1639*^{SH1639}	LacZ enhancer trap CG8964	Kyoto stock center
P(XP)d01360	*P{XP}d01360*	P-element insertion upstream of *otk*	Exelixis collection, Harvard
PBac(PB)c01790	*PBac{PB}Mppe*^{c01790}	P-element insertion upstream of *otk2*	Exelixis collection, Harvard
PBac(RB)e03992	*PBac{RB}e03992*	P-element insertion downstream of *otk*	Exelixis collection, Harvard
Df(3R)BSC39	Df(2R)BSC39, cn[1] bw[1]/SM6a, bw[k1]	chromosomal deletion removing *otk* and *otk2*	BL 7145
Df(3R)BSC199	w[1118]; Df(2R)BSC199/CyO	chromosomal deletion removing *otk* and *otk2*	BL 9626
otk^{A1}	*w; otk*^{A1}	Null alele *otk*	This work
otk^{C26}	*w; otk*^{C26}	Null allele *otk2*	This work
otk,otk2^{D72}	*w;otk,otk2*^{D72}*/CyO*	Null allele *otk* and *otk2*	This work
UASp::Otk-GFP29	*w;; UASp::Otk-GFP29*	Transgenic line after injection of Otk-pPWG-attB in BL 23648	This work
*UASp::Otk*ΔC-GFP14	*w;; UASp::Otk*ΔC-GFP14	Transgenic line after injection of OtkΔC-pPWG-attB in BL 23648	This work
*UASp::Otk*ΔEx-GFP20	*w;; UASp::Otk*ΔEx-GFP20	Transgenic line after injection of OtkΔEx-pPWG-attB in BL 23648	This work
UASt::OtkGFP	*w;; UASt::OtkGFP*	Transgenic line after injection of Otk-pTWG in *white-*	This work
UASp::Otk2-GFP37	*w;; UASp::Otk2-GFP37*	Transgenic line after injection of Otk2-pPWG-attB in BL 23648	This work
UASt::Otk2-RFP	*w;; UASt::Otk2-RFP*	Transgenic line after injection of Otk2-pTWR in *white-*	This work
wg^{CX4}	*wg[l-17] b[1] pr[1]/CyO*	*wg* null allele	BL 2980
wnt4^{EMSS23}	*wnt4[EMS23] bw[1]/CyO, P{ry[+t7.2]=HB-lacZ}GS1*	*wnt4* null allele	BL 6650
wnt4^{C1}	*w[1118]; Wnt4[C1]/CyO*	*wnt4* null allele	BL 6651
wnt2^{L}	*w[1118]; Wnt2[L]/ CyO, amos[Roi-1]*	*wnt2* null allele	BL 6909

wnt2^{O}	*Wnt2[O]/CyO, amos [Roi-1]*	*wnt2* null allele	BL 6958
wnt2^{I}	*Wnt2[I]/CyO, amos [Roi-1]*	*wnt2* null allele	BL 6960
wnt2^{RJ}	*Wnt2[RJ]/CyO, amos[Roi-1]*	*wnt2* null allele	BL 6959
Df(2L)DE	*w[1118]; Df(2L)DE/CyO, P{ry[+t7.2]=ftz/lacB}E3*	chromosomal deletion removing *wg* and *wnt4*	BL 6653
wnt4^{C1},wnt2^{L}	*y[1] w[1118]; Wnt4[C1] Wnt2[L]/CyO-Df(2R) B80, y[+]*	deletion of *wnt4* and *wnt2*	BL 6907
wg^{CX4} ,wnt2^{L}	*wg[l-17] Wnt2[L]/CyO*	deletion of *wg* and *wnt2*	BL 6908
wnt5^{400}		*wnt5* null allele	Fradkin *et al*, 2004
dsh^{1}	*yw dsh^{1}/Y/C(1)*	*dsh^{1}* allele, disrupts tissue polarity but not Wg signaling	Stock collection Wodarz
fz^{J22}	*fz^{J22}/TM6C*	*fz* allele, autonomous	Paul Adler
fz^{H51}	*fz^{H51} th st/TM6C*	*fz* allele, non-autonomous	Paul Adler
fz^{R52}	*fz^{R52} th st/TM6C*	*fz* allele, non-autonomous	Ken Cadigan
fz^{P21}	*fz^{P21} th st/TM6C*	*fz* allele, non-autonomous	Paul Adler
fz^{R52}Df(3L)Dfz2	*w;;fz^{R52}Df(3L)Dfz2 P[w+FRT2A]/TM6*	*fz,Dfz2* double mutant	Ken Cadigan
ProtB-EGFP	*w;;protamineB-eGFP*		Raja & Renkawitz-Pohl, 2005

2.2 Molecular biology methods

If not indicated otherwise, standard procedures were carried out as described in the manufacturer's protocol or in "Molecular Cloning: A Laboratory Manual" (Sambrook & Russell, 2000).

2.2.1 Polymerase chain reaction (PCR)

Polymerase chain reaction allows the specific amplification of DNA fragments *in vitro*. In general, PCR reactions were done in 50 µl total reaction volume. 1 µl plasmid DNA (corresponding to 20 – 50 ng) or 5-10µl fly genomic DNA (corresponding to 200 ng) were mixed with 400 nM of forward and reverse primer (Table 3), 250 µM of each dNTP (Bioline) and 1 U polymerase in the corresponding buffer (see Table 9 for a standard pipetting scheme). The primers used in this study are listed in Table 3. PCR

reactions were performed with Taq polymerase (Genecraft) or Pfu polymerase (Bioline). For PCR reactions in a smaller final volume the reaction volumes were adjusted accordingly (Table 9). A standard PCR program is depicted in Table 10.

Table 9: Standard PCR.

Template DNA	1 µl (20-50 ng plasmid DNA) 5 µl (200 ng genomic DNA)	2 µl (200 ng genomic DNA)
Forward Primer (10 µM)	2 µl	0,5 µl
Reverse Primer (10 µM)	2 µl	0,5 µl
dNTPs (25 mM each)	0,5 µl	0,2 µl
10X polymerase buffer	5 µl	2 µl
Polymerase	0,5 µl	0,2 µl
ddH2O	ad **50 µl**	ad **20 µl**

Table 10: Standard PCR program.

Step	**Temperature**	**Duration**	
1) Initial denaturation	95°C	5 min	
2) Denaturation	95°C	30 sec	
3) Annealing	52-65 °C	30 sec	5-10°C below primer Tm
4) Elongation	72°C	1,5 min/kb (Pfu) 0,5-1 min/kb (Taq)	
	Repeat steps 2-4 34 times		
5) Final elongation	72°C	5 min	
	4°C	Hold	

The annealing temperature was chosen based on the melting temperature as indicated by the supplier.

2.2.2 Restriction digestion of DNA

Plasmid DNA or PCR products were subjected to site-specific restriction digestion in order to obtain DNA fragments containing the desired sequence. Restriction digestion was carried out according to manufacturer's instructions.

2.2.3 Agarose gel electrophoresis

DNA fragments obtained by PCR or restriction digestion products were separated electrophoretically on ethidium bromide-containing agarose gels (1 % w/v agarose, 40 mM Tris, 10 mM EDTA, 0,5 µg/ml ethidium bromide). DNA samples were mixed with 1/6 volume of DNA loading buffer (Fermentas), loaded and separated at 100 V for 20 – 30 min. To determine the size of separated DNA fragments, GeneRuler 1kb

DNA Ladder (Fermentas) was loaded in a separate pocket. DNA bands were visualized with UV light.

2.2.4 DNA extraction from agarose gels

DNA was extracted from agarose gels using the NucleoSpin Extract II Kit (Machery-Nagel) according to manufacturer's instructions.

2.2.5 Ligation of DNA fragments

DNA inserts and vector backbones that have been digested with suitable restriction enzymes were ligated using T4 DNA ligase (Fermentas). If only one enzyme was used for cloning, vector backbones were incubated with calf intestine phosphatase (CIAP) (Fermentas) for 30 min at 37 °C, followed by a purification step. Calf intestine phosphatase removes 5' phosphate groups from DNA and thus prevents vector self-ligation.

2.2.6 Generation of expression constructs by Gateway cloning technology

To express full length and partially deleted versions of genes of interest in S2r+ cells and transgenic flies, the corresponding coding regions were amplified with primers indicated in (Table 3). The PCR products were cloned into pENTR/D-TOPO vector (Invitrogen) according to manufacturer's instructions. The inserts of the corresponding pENTR constructs were recombined into destination vectors of the Drosophila Gateway Vector Collection (Carnegie Institution of Washington, Baltimore, MD) by λ phage mediated LR recombination. The LR recombination was performed with LR Clonase Enzyme Mix (Invitrogen) according to the manufacturer's protocol, using 100 ng of pENTR, 150 ng of destination vector and 0,5 µl LR Clonase mix.

2.2.7 Transformation of bacteria

For plasmid DNA amplification, the plasmid or the ligation/pENTR/Clonase reaction was used to transform chemically competent bacteria. *E. coli* strains used in this work and their applications are listed in Table 2. Briefly, competent cells were thawed on ice, approximately 100 ng of plasmid DNA was added, gently mixed and incubated on ice for 30 min. Then the cells were heat-shocked at 42 °C for 45 sec, followed by incubation on ice for 2 min and recovery with warm SOC medium (2% tryptone, 0,5% yeast extract, 10mM NaCl, 2,5mM KCl, 10mM MgCl2, 10mM MgSO4, 20mM glucose) at

37 °C for 30-60 min. The cells were streaked on LB agar plates (1% tryptone, 0,5% yeast extract, 1% NaCl, 1% agar) containing antibiotic for selection (see 2.1.3) of transformed cells and cultured overnight at 37 °C.

2.2.8 Plasmid purification from bacteria

To purify plasmid DNA from *E. coli* cells in smaller amounts, plasmid DNA was purified according to a modified plasmid purification method from Qiagen, Germany. Briefly, a single colony of transformed E. coli cells was inoculated in 2 ml LB medium containing the appropriate antibiotic for selection (100 µg/ml ampicillin, 50 µg/ml kanamycin). After incubation overnight at 37 °C with constant agitation, the cells were pelleted by centrifugation for 1 min at 13000 rpm. The pellet was resuspended in 200 µl of buffer P1 (50mM Tris-HCl, pH 8,0. 10mM EDTA, 100 µg/ml RNase A). Afterwards 200 µl of buffer P2 (200mM NaOH, 1% SDS) was added, the mixture was mixed by inverting 5-6 times and incubated for 5 min at room temperature. After addition of 200 µl of buffer P3 (3M K acetate, pH 5,5) the mixture was inverted 5-6 times and centrifuged for 20 min at 13000 rpm, 4 °C. The supernatant was transferred to a fresh centrifuge tube and DNA was precipitated with 400 µl of isopropanol for 30 min at 13000 rpm, 4 °C. The resulting DNA pellet was washed with 200 µl ice-cold 70% ethanol and subsequently air-dried at room temperature. Purified plasmid DNA was dissolved in 20 µl ddH_2O.

To obtain large amounts of pure DNA for transfection and injection of flies, plasmid DNA was purified with Nucleobond X100 kit (Macherey-Nagel) according to the manufacturer's procedure.

2.2.9 Long-term storage of bacteria

E. coli can be stored in glycerol stocks at -80 °C for several years. Glycerol stocks were prepared by adding 200 µl 99 % glycerol to 800 µl of a logarithmic-phase *E. coli* culture in a cyrotube vial. They were mixed by inverting them, frozen in liquid nitrogen and stored at -80 °C. For recovery, parts of the stocks were scratched with a fresh pipette tip and cultured in LB medium containing the appropriate antibiotic.

2.2.10 Site-directed mutagenesis

Mutageneses were performed to create point mutation in a sequence of interest. In brief, mutagenesis was done by allowing Pfu polymerase to synthesize the complete

vector based on two mutagenesis primers containing the desired base pair exchanges. Pfu polymerase exhibits 3'→ 5' proofreading activity. Therefore whole vectors can be synthesized with high fidelity. The primers are complementary to opposite strands of the vector and the newly synthesized vector will contain the mutation. The PCR program used is shown in Table 11. Methylated parental DNA was digested with *Dpn*I endonuclease. The remaining mutation-containing DNA was transformed into chemically competent bacteria as described in 2.2.7.

Table 11: PCR program for site-directed mutagenesis.

Step	Temperature	Duration
1) Initial denaturation	95°C	30 sec
2) Denaturation	95°C	30 sec
3) Annealing	55 °C	1 min
4) Elongation	68°C	1 min/kb
	Repeat steps 2-4 12-18 times	
5) Final elongation	37°C	Hold

Point mutation: 12 cycles; single amino acid change: 16 cycles; deletion or insertion of multiple amino acids: 18 cycles.

2.2.11 Sequencing of DNA

Sequencing reations were set up as indicated in Table 12. The PCR program for the sequencing reaction is shown in Table 13. After completion, DNA was precipitated. Briefly, 1 µl of 125 mM EDTA, 1 µl of 3 M NaAc and 50 µl of 100% ethanol was added to the sample and incubated for 5 min at room temperature. Then the sample was centrifuged for 15 min at 13000 rpm, the supernatant was removed and the pellet washed with 70% ethanol and subsequently air-dried. Afterwards the pellet was dissolved in 15 µl of HiDi (Applied Biosystems). Analysis of sequencing reactions was done by an in-house sequencing service in the Department of Developmental Biochemistry, Ernst-Caspari-Haus, GZMB, Göttingen.

Table 12: Sequencing PCR.

Template DNA	300 ng plasmid DNA	20-30 ng PCR
Forward Primer (10 µM)	0,8 µl	0,8 µl
SeqMix	1,5 µl	1 µl
SeqBuffer	1,5 µl	1 µl
ddH2O	ad 10 µl	ad 10 µl

Table 13: Sequencing PCR program.

Step	Temperature	Duration
1) Initial denaturation	96°C	2 min
2) Denaturation	96°C	20 sec
3) Annealing	55 °C	30 sec
4) Elongation	60°C	4 min
	Repeat steps 2-4 26 times	
5) Final elongation	12°C	Hold

2.2.12 Extraction of genomic DNA from flies

30 male flies were collected in one eppendorf tube and snap frozen in liquid nitrogen. 400 µl of lysis buffer containing 100 mM TrisHCl, pH 7,5, 100 mM EDTA, pH 8,0, 100 mM NaCl and 0,5% SDS was added into the tube. Frozen flies were homogenized with a biovortexer. The lysate was incubated at 65°C for 15-30 min. Afterwards, 228,4 µl of 5 M KAc and 571,6 µl of 6 M LiCl were added into the lysate and incubated on ice for 15 min before being centrifuged at 13,000 rpm for 15 min. 1 ml of supernatant was transferred into a new tube. 600 µl of isopropanol was added and the mixture was centrifuged again at 13000 rpm for 15 min. DNA pellet was washed with 70% ethanol and dissolved in 150 µl of sterile water.

2.2.13 Extraction of genomic DNA from single flies

For genotyping a large amount of fly stocks, DNA was isolated from single flies. Briefly, one male fly was snap-frozen in liquid nitrogen. Subsequently, the fly was squished in 50 µl squishing buffer (10 mM TrisHCl pH 8,2, 1 mM EDTA, 25 mM NaCl) supplemented with 0,5 µl Proteinase K (20 mg/ml) (Fermentas) using a pipette tip. The mixture was incubated for 20-30 min at 37 °C. Afterwards, Proteinase K was inactivated by incubation at 95 °C for 5 min. The debris was pelleted and the supernatant could be directly used for PCR (2.2.1).

2.2.14 Long-template PCR

Expand Long Template PCR System (Roche) was used for the characterization of gene deletions in *otk* and *otk2* mutant alleles. Components of PCR reaction and thermal cycles were set up according to the instructions. The PCR reaction mix used to amplify *otk* and *otk2* genomic regions and the corresponding PCR program for long template PCR are shown in Table 14 and Table 15.

Table 14: Long-template PCR.

	Buffer 1	Buffer 2	Buffer 3
Genomic DNA	3 µl	3 µl	3 µl
Forward Primer (10 µM)	2 µl	2 µl	2 µl
Reverse Primer (10 µM)	2 µl	2 µl	2 µl
dNTPs (25 mM each)	0,7 µl	1 µl	1 µl
10X buffer	5 µl	5 µl	5 µl
Enzyme mix	0,75 µl	0,75 µl	0,75 µl
ddH2O	ad 50 µl	ad 50 µl	ad 50 µl

Table 15: PCR program for long-template PCR.

Step	Temperature	Duration
1) Initial denaturation	93°C	2 min
2) Denaturation	93°C	10 sec
3) Annealing	60°C	30 sec
4) Elongation	68°C	4-8 min depending on length of expected fragment
	Repeat steps 2-4 10 times	
5) Denaturation	93°C	15 sec
6) Annealing	60°C	30 sec
7) Elongation	68°C	4-8 min + 20 sec for each cycle
	Repeat steps 5-7 10 times	
8) Final elongation	68°C	7 min
9)	4°C	Hold

2.3 Biochemical methods

2.3.1 Culture and transfection of Schneider S2R+ cells

Schneider S2R+ cells, an immortalized culture of Drosophila embryonic cells, which expresses Frizzled2 (Schneider, 1972), were used for experiments in the cell culture system in this study. The cells were grown at 25 °C in *Drosophila* S2 medium (Invitrogen) supplemented with serum and antibiotics. Cells were counted using a Neubauer improved counting chamber. FuGene HD Transfection Reagent (Roche, Indianapolis, IN) was used for cell transfection according to the instructions. $2*10^6$ S2r+ cells were resuspended in 2 ml fresh S2 medium with supplements. Resuspended cells were seeded in one well of a 6-well plate. 2 µg of target plasmid was diluted in 100 µl of sterile water. 4 µl of FuGene transfection reagent was added

into the plasmid solution and vortexed for 2 sec. The mixture was incubated at room temperature for 15 min before pipetting into the cell culture.

2.3.2 Co-Immunoprecipitation (CoIP) in S2R+ cells

For Co-Immunoprecipitation (CoIP), two wells of a 6-well plate were were transfected. The cells were propagated to T25 or T75 flasks after two days and harvested after another 2-3 days. Cells were harvested by scraping, pelleted by centrifugation at 1000 g for 5 min and washed two times with PBS. Cell lysis was done in 1 ml freshly prepared cold CoIP buffer (50 mM TrisHCl pH7,5, 150 mM NaCl, 1 % NP40 with protease inhibitors (Pefabloc 200 µg/ml, Pepstatin 2 µg/ml, Aprotinin 2 µg/ml, Leupeptin 2 µg/ml (Roche)) by homogenization using a 26 G insulin syringe. Subsequently, the cells were disrupted by sonication with alternating bursts for 10 min. The lysates were then centrifuged at 13 000 rpm for 15 min (4°C) to pellet the cell debris and the supernatant was transferred into fresh tubes and pre-cleared with 20 µl of plain ProteinA/G Sepharose beads (BioCat) for 1 h on a rotator at 4°C. At the same time, 30 µl Protein A/G sepharose beads per sample in 300 µl lysis buffer were combined with 200 µl antibody (anti-Myc and anti-β-gal, see Table 6) and incubated for 1h on a rotator at 4°C. After pre-clearing, 20µl of each sample were kept as an input control and the rest was divided among the antibody-sepharose beads complexes. The antibody-antigen reaction took place for overnight on a rotator at 4°C. The beads were subsequently washed 5 times with 800 µl CoIP buffer (centrifugation at 3000 rpm, for 2 min each time, 4°C). 30 µl of 2X SDS buffer (Wodarz, 2008) were added to each sample followed by incubation at 95°C for 5 min for protein denaturation. The samples were stored at -20°C or used directly for SDS-PAGE and Western blot.

2.3.3 Protein extraction from embryos

Protein extraction under denaturing and non-denaturing conditions was performed according to standard procedures (Wodarz, 2008). Proteinase inhibitors were freshly added to the lysis buffer before use (see 2.3.2).

2.3.4 Determination of protein concentration

Protein concentration was determined according to Bradford method with Roti-Quant reagent (Roth, Karlsruhe, Germany). 800 µl ddH_2O were mixed with 200 µl of

the reagent and 2 µl of the protein lysate was added. 2 µl lysis buffer were used as blank. The absorption was measured at 600 nm with a photometer. As a rule of thumb, OD600 =1 corresponds to approximately 1 mg/ml of total protein.

2.3.5 SDS-polyacrylamide gel electrophoresis

Discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) allows the electrophoretic separation of proteins according to their size (Weber & Osborn, 1969). SDS-PAGE was performed as described in (Wodarz, 2008). PageRuler Prestained Protein Ladder (Fermentas) was loaded to determine the size of the separated proteins.

2.3.6 Western Blot

Proteins separated by SDS-PAGE (2.3.5) were transferred onto a nitrocellulose membrane by wet blotting according to standard procedures (Wodarz, 2008). Primary and secondary antibodies used for Western blot are listed in Table 6 and Table 7. If the membrane was to be incubated with another primary antibody derived from another species than the first antibody, the HRP on the membrane was inactivated by incubation with 0,01% sodium azide in blocking buffer.

2.3.7 Coomassie staining

Polyacrylamide gels were rinsed with ddH_2O and stained with Brilliant Blue R Concentrate (Sigma Aldrich) diluted 1:5 for 30-60 min at room temperature. Afterwards, the gel was rinsed with ddH_2O and destained in 10 % v/v acetic acid and 20 % v/v ethanol. Brilliant Blue R concentrate is a methanol based stain. The stain contains methanol and acetic acid so gels do not require fixing prior to staining.

2.3.8 GST fusion protein purification for antibody generation

100 ml of 2xYTA medium (see 2.1.3) was inoculated with 5 ml overnight culture of BL21 bacteria transformed with OtkEx-pGEX4T1 and incubated at 37 °C until the culture reached high density (OD600=2). 2 ml ethanol was added to induce chaperones. IPTG was added to a final concentration of 0,1 mM to induce the expression of recombinant protein. After incubation for 3-5 h at 20 °C bacteria were harvested by centrifugation at 7700 g for 10 min at 4°C. The pellet was resuspended

in 5 ml lysis buffer (50 mM TrisHCl pH7,5, 150 mM NaCl, 1 mM EDTA) and frozen at -80°C overnight to disrupt the bacteria.

The pellet was thawed in warm water and immediately proteinase inhibitors (see 2.3.3), 1 mM DTT and 1 mg/ml lysozyme were added. Bacteria were disintegrated by sonication with 10 sec bursts alternated with 10 sec of incubation on ice. 10% TritonX-100 was added to the lysate to a final concentration of 1%. The mixture was gently rotated for 30-60 min and ultra-centrifuged at 100 000 g for 1 h at 4°C. 100 µl of 50:50 slurry of Glutathione-Sepharose beads (GE Healthcare) was added to the supernatant and rotated for 30 min at room temperature. The beads were sedimented and washed three times with PBS. The bound protein was eluted from the beads by elution buffer containing 15 mM reduced glutathione in 50 mM TrisHCl, pH 8,0. Eluted protein was snap frozen and stored at -80 °C for later use.

Protein concentration was estimated by running the samples on a SDS gel together with standard samples containing known concentrations of BSA and subsequent Coomassie staining (2.3.7).

2.4 Immunohistochemistry

2.4.1 Fixation and immunofluorescent staining of embryos

Formaldehyde fixation and incubation of fixed embryos with primary and secondary antibodies was done according to standard procedures (Müller, 2008).

2.4.2 Fixation and immunofluorescent staining of larval tissues

Larval brains of wandering third instar larvae were dissected in PBS and then fixed in 4% formaldehyde in PBS for 20 min at room temperature on a rocking platform. The tissue was washed three times with PBTw (PBS, 0,1 % Tween) and thereafter permeabilized by treating with PBS containing 1 % Triton X-100 for 1 h at room temperature on a rocking platform. Subsequent steps were performed according to the protocol for antibody staining of embryos (2.4.1).

Imaginal discs were dissected in cold PBS and fixed in 4% formaldehyde in PBS for 20 min at room temperature on a rocking platform. Subsequent steps were performed according to the protocol for antibody staining of embryos (2.4.1).

2.4.3 Fixation and immunofluorescent staining of adult tissues

Adult testes were dissected in a drop of PBS and fixed in 4% formaldehyde in PBS for 20 min at room temperature on a rocking platform. Subsequent steps were performed according to the protocol for antibody staining of embryos (2.4.1). Actin was stained with Phalloidin Alexa Fluor 555 diluted 1:100 (Molecular Probes, A34055) together with the secondary antibodies.

2.4.4 Fluorescent in situ hybridization (FISH) of embryos

FISH was performed using the Tyramide Signal Amplification (TSA) Kit (Molecular Probes) according to the protocol provided. Fixation of embryos was performed as described in 2.4.1. For preparation of a Digoxigenin (DIG)-labelled RNA probe, 5 µg of template DNA was linearized in 30 µl final volume. The linearized template was then purified using the High Pure PCR Product Purification Kit (Roche), according to manufacturer's instructions except for the elution volume which was adjusted to 20 µl. In vitro transcription and incorporation of DIG was performed with the DIG RNA labeling kit (SP6/T7) (Roche) according to manufacturer's instructions. The labeled probe was purified using the RNeasy® Plus Mini Kit (Qiagen). Fragmenting of the probe was performed according to the instructions provided in the TSA Kit (Molecular Probes). The purified DIG labeled probe was mixed with hybridization solution to a final volume of 1,5 ml. At this point the probe could be stored at -20°C.

2.4.5 Mounting of adult wings

Wings were removed from adult flies and dehydrated in 100 % isopropanol for at least 5 min. Wings were placed on a glass slide and the isopropanol was allowed to evaporate. A small drop of Roti® Histokitt (Roth) was put onto the wing and they were covered with a cover slip for microscopic analysis.

2.4.6 Microscopy and image acquisition

Samples were examined using 25 X 0,8 NA Zeiss Plan-Neofluar and 63 X 1,4 NA Zeiss Plan-Apochromat oil immersion objectives on a confocal laser-scanning microscope (Carl Zeiss LSM 510 Meta). Pinholes were set to 1 airy unit for image acquisition. Images were captured by 1024 x 1024 pixels at the appropriate zoom using 2-line mean averaging. Brightfield images were acquired with an AxioImager.Z1 upright

microscope using 10 X 0,3 NA Zeiss Plan-Neofluar and 25 X 0,8 NA Zeiss Plan-Neofluar oil immersion objectives.

2.5 Genetic methods

2.5.1 Fly breeding and fly stocks

The fly stocks used in this work were kept at 18°C, 25°C or 29°C on standard medium (Ashburner, 2004). For collection of embryos flies were kept in egg collection cages on apple juice agar plates. To stimulate egg-laying, a small amount of yeast paste was applied at the centre of the apple juice plate.

Standard medium: 712 g cornmeal, 95 g soya flour, 168 g dry yeast, 450 g malt extract, 150 ml 10% Nipagin (700 ml 99% ethanol, 300 ml H_2O, 100 g Nipagin ($C_8H_8O_3$)), 45 ml propionic acid, 50 g agar, 400 g sugar beet sirup, add 9,75 l ddH_2O

Apple juice plates: 40 g agar, 1 l H_2O, 340 ml apple juice, 17 g sugar, 20 ml 10% Nipagin

2.5.2 UAS-GAL4 system

The UAS-GAL4 system (Brand & Perrimon, 1993) is used to ectopically overexpress the gene of interest in a time and tissue specific manner. The method makes use of the yeast transcription factor GAL4 that binds to an upstream activating sequence (UAS), thereby activating gene expression. In UAS reporter flies the gene of interest is placed under the control of UAS. Another fly stock, the so-called GAL4 driver line, contains the GAL4 gene under the control of a promoter or enhancer of a gene with a known expression pattern. When the two fly stocks are crossed, GAL4 activates the expression of the gene of interest. By choosing different GAL4 driver lines, the gene can be expressed in a wide variety of tissues and developmental stages. GAL4 driver lines and UAS reporter flies used in this work are described in Table 8.

2.5.3 Generation of transgenic flies with *white*- background

Different constructs inserted in P-element based vectors were used to generate transgenic flies. The vectors were injected into the posterior end of syncytial

blastoderm *white*- embryos together with another plasmid encoding transposase. This transposase activity mediates integration of the transgenic construct into the genomic DNA (Rubin & Spradling, 1982). Subsequently, transgenic flies that have successfully integrated the P-element could be recognized by red eye color, since the vectors used for injection contain the *mini-white* gene (Bachmann & Knust, 2008). Briefly, 20 µg of plasmid was mixed with 5 µg of transposase DNA in 50 µl injection buffer containing 5 mM KCl, 0.1 mM sodium phosphate, pH 6,8. *white*- flies were allowed to lay embryos on apple juice plates for 20 min at 18 °C. The embryos were dechorionated, aligned on cover slips and immersed in 10S Voltalef oil (Prolabo). The plasmid mixture was injected into the pole cells at the posterior ends of the embryos using a micromanipulator (InjectMan NI2, Eppendorf). After injection, embryos were kept in Voltalef oil at 18 °C for 48 h before the hatched larvae were collected. Flies were single-crossed to *w-;Gla/CyO(ftz::lacZ)* flies for the selection of positive transgene insertion and further insertion site analysis. The transgenic constructs used for injection are listed in Table 5.

2.5.4 Generation of transgenic flies using ΦC31-mediated integration

The Streptomyces phage ΦC31 is able to insert its own genomic DNA into that of bacterial genomes using an enzyme called integrase. Unlike transposase, which mediates genome-wide P-element insertion, the ΦC31 integrase operates in a sequence-specific and unidirectional manner. The P-element vectors contain both the transgene and a donor sequence (attB) and fly stocks used for injection have to carry an attP sequence which acts as the recipient site (Groth *et al*, 2004; Fish *et al*, 2007). Furthermore, the flies express ΦC31 integrase under control of the *vasa* promoter, providing an endogenous source of ΦC31 integrase (Bischof *et al*, 2007). This results in the site-specific insertion of the attB-containing transgene into the attP site. Hybrid sites (attL and attR) are formed during this process and prevent further integrase-catalyzed movement of the integrated transgene (Fish *et al*, 2007) (Fig. 11). Fly stocks used for ΦC31-mediated integration are listed in Table 8 and constructs used for injection in Table 5.

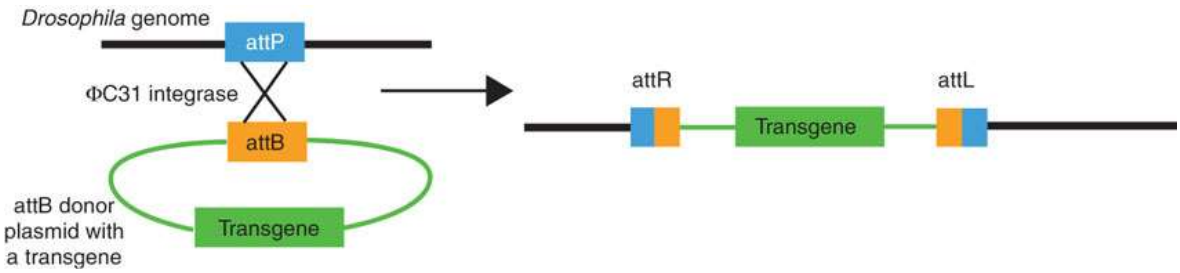

Fig. 11: Scheme of ΦC31 integrase-mediated trangene integration.
The preplaced attP sequence acts as the recipient site in the Drosophila genome. A P-element based plasmid contains both the transgene and a donor attB sequence and is injected into attP-containing recipient embryos. This results in site-specific insertion of the transgene into the attP site. The formation of hybrid attR and attL sites prevents further integrase-mediated movement of the already integrated transgene. Taken from (Fish *et al*, 2007).

2.5.5 FLP-FRT mediated gene deletion

To generate a deletions of *otk* and *otk2 rush*, FLP-FRT mediated gene deletion was employed (Parks *et al*, 2004). The method makes use of the ability of FLP recombinase to mediate recombination between two FRT sites located on homologous chromosomes (Golic & Lindquist, 1989). By this, a deletion of a genomic region flanked by two transposons that contain FRT sites can be achieved. Upon induction of FLP recombinase the genomic region between two FRT sites in *trans* is deleted, leaving a single transposon at the deletion site. Successful recombination between the two FRT sites in *trans* can be detected by loss of red eye color, if *white* genes are deleted together with the genomic region or by PCR (Fig. 12).

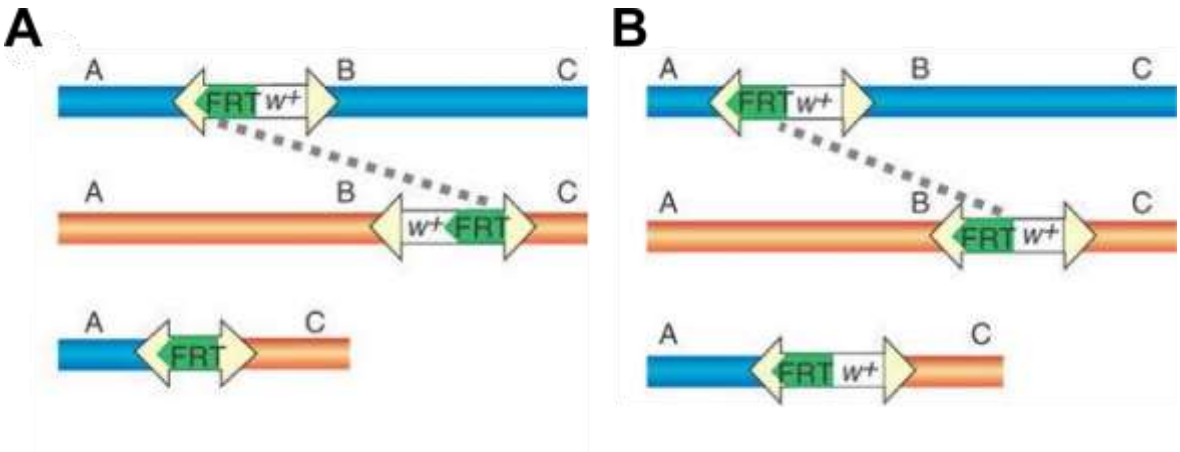

Fig. 12: Schematics of FLP-FRT recombination of two FRT containing transposons in *trans*.
The chromosomes are shown in orange and blue, the transposon insertion elements are indicated in light yellow and FRT elements in green. The order of theoretical markers A,B and C along the chromosome is indicated. FLP-FRT recombination allows the generation of *white*- deficiencies **(A)** or *white*+ deficiencies **(B)**, depending on the orientation of the white+ containing P-element. Modified from (Parks *et al*, 2004)

In case of *otk* and *otk2*, three suitable transposon insertion lines containing FRT sites were available from the Harvard stock collection. (Fig. 13A). The P(XP)d01360 element is located upstream of the 5'UTR of *otk*, while the *piggyBac* insertion line PBac(RB)e03992 element is inserted downstream of the 3'UTR of *otk.* Furthermore, the PBac(PB)c01790 element is located upstream of *otk2* in the second exon of *mppe* and P(XP)d01360 is downstream of the 3'UTR of *otk2* (Fig. 13A). Like this it was possible to generate null alleles of *otk*, *otk2* as well as a double knock-out of *otk,otk2*. The features of the *P*(XP) and *piggyBac* lines have been described (Thibault *et al*, 2004) and are depicted in (Fig. 13B).

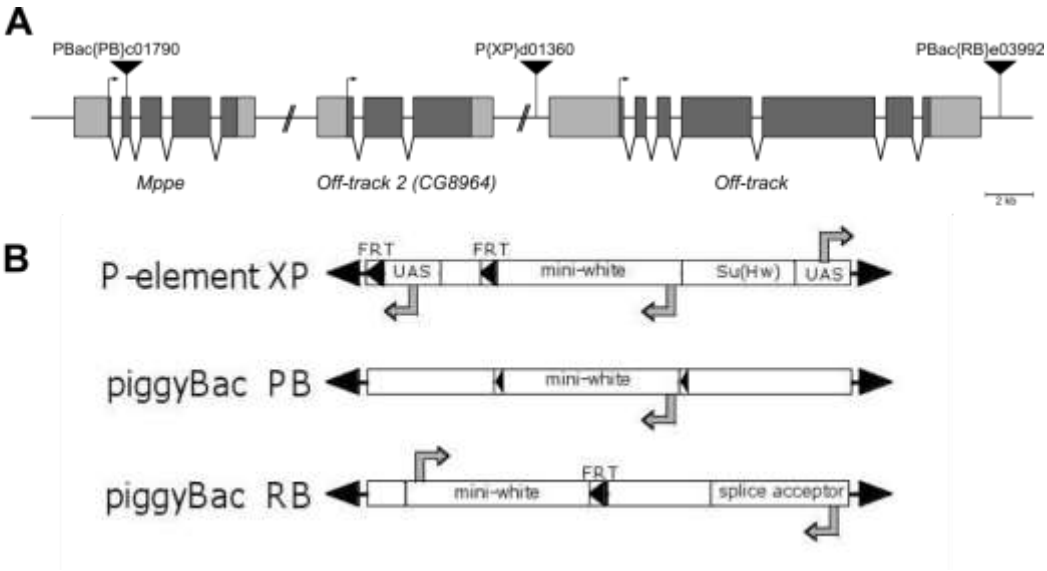

Fig. 13: P-elements used for FLP-FRT recombination mediated deletion of *otk* and *otk2*.
(A) Overview of the genomic region of *otk*, *otk2* and the neighbouring gene *Mppe* on chromosome 2R. Null alleles for *otk* and *otk2* alone as well as both *otk* and *otk2* were generated via FLP/FRT mediated recombination between FRT sites contained in P(XP)d01360 and PBac(RB)e03992 **(*otk* deletion)**, PBac(PB)c01790 and P(XP)d01360 **(*otk2* deletion)** or PBac(PB)c01790 and PBac(RB)e03992 **(*otk,otk2* deletion)**. **(B)** Schmatic representations of the P-elements utilized. The *piggyBac* vector (PB) comprises a *mini-white* gene flanked by short (48 bp) FRT sites, whereas PBac(RB) and P(XP) contain long (199 bp) FRT sites. Modified from (Thibault *et al*, 2004).

Genomic maps and crossing schemes to generate *otk* and *otk2* as well as *otk,otk2* deletions are provided in the appendix. In brief, a heat-inducible FLP recombinase was introduced on the first chrosmosome and L2 larvae were heat-shocked for two hours at 37 °C on two consecutive days. Mutant flies carrying the *otk* deletion were selected by the loss of the *mini-white* genes carried by both transposon lines and resulting in white eye colour. Flies carrying a potential deletion for *otk2* were

screened by PCR, since no selection based on eye-colour was possible. In case of the *otk,otk2* double knock-out, both eye colour phenoptypes were possible, depending which FRT site would be used for recombination. Flies selected based on white eye phenotype were screened first.

2.6 Viability and Life span measurements

2.6.1 Viability analysis

Viability was determined by aligning about 100 embryos on apple juice agar plates. The embryos were allowed to develop at 25 °C and hatching rates were recorded after at least 24 h.

2.6.2 Life span analysis

Survival curves were recorded as previously described (Wang *et al*, 2003). Cohorts of about 100 males or females were separated two days after hatching and were transferred to fresh vials. Subsequently, flies were transferred to fresh vials every four days and the number of dead flies was recorded.

2.7 Phylogenetic analysis

2.7.1 Phylogenetic trees

Phylogenetic analyses were conducted with Mega software version 5 (Tamura *et al*, 2011) using ClustalW alignment and neighbor-joining method with a gap opening penalty of 60.

2.7.2 Dot Plot Analysis

Dot Plot analysis was performed using DottupP with word size 6 (http://srs.ebi.ac.uk/).

3 RESULTS

3.1 *off track (otk)* and the neighboring gene *off-track2 (otk2, CG8964)* are paralogs evolved by gene duplication

The *off-track* (*otk*) gene is located on the right arm of the second chromosome (2R: 7,888,983 - 7,907,329(-)) and codes for a protein with a length of 1033 amino acids. I performed a protein BLAST search with the protein sequence of Otk to identify potential additional homologs in the *Drosophila* genome. Indeed, I found that the protein encoded by the annotated and so far not described gene *CG8964* is closely related to Otk (53 % identity over 427 amino acids, Fig. 14). *CG8964* is located upstream and right next to *otk* on the second chromosome (2R: 7,910,651 - 7,912,775(-)), suggesting that it is the result of a gene duplication. Therefore, the gene *CG8964* was named *off-track2* (*otk2*).

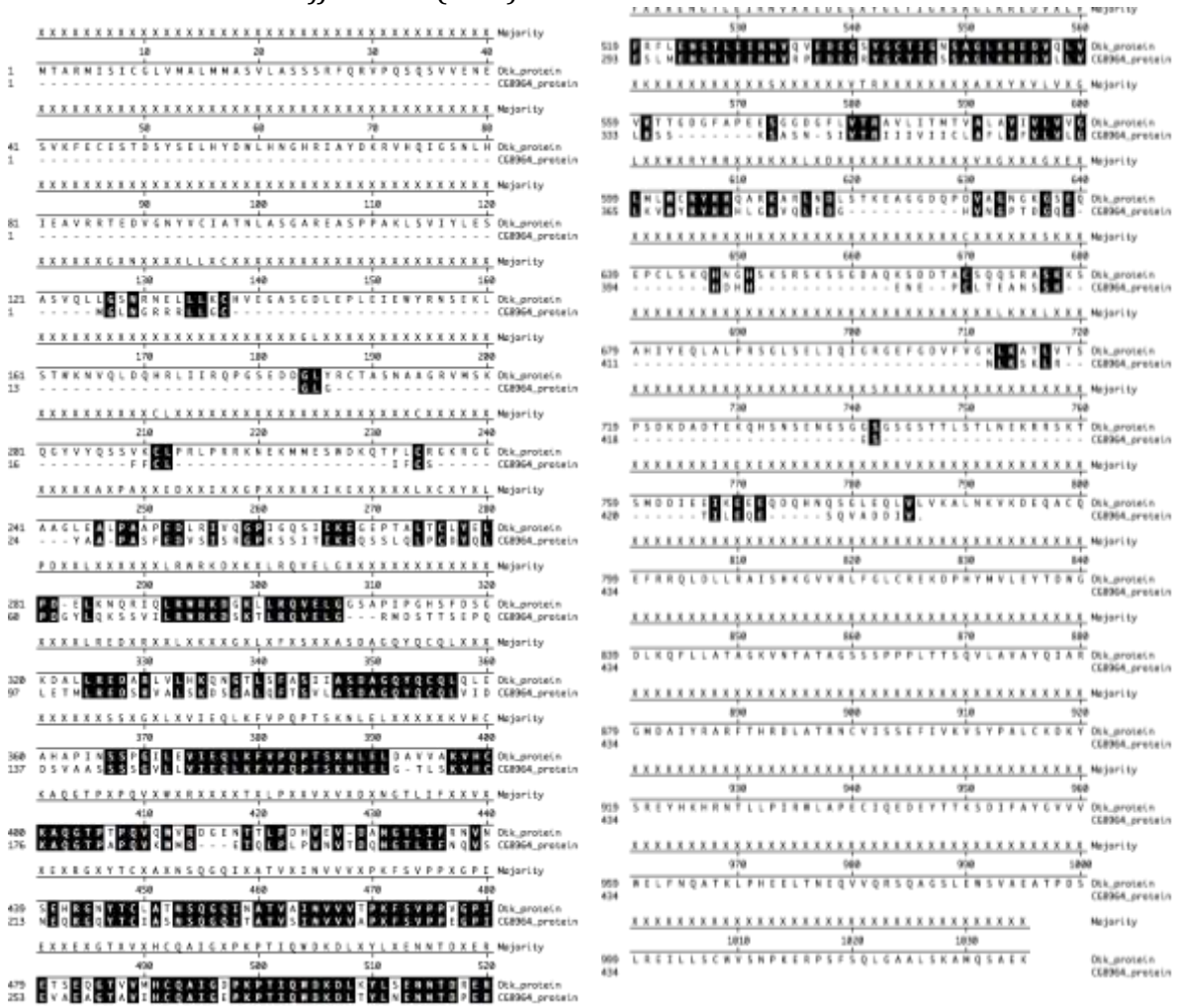

Fig. 14: Alignment of the protein sequences of Otk and the gene product of CG8964.

Phylogenetic analysis confirmed that *Drosophila otk* and *otk2* are indeed two paralogs of the single *PTK7* gene in mouse and human (Fig. 15 A). To test whether the gene duplication is specific for *Drosophila* or occurred also in other arthropods, the sequences from different arthropod species homologous to *Drosophila* Otk and Otk2 were analyzed. The resulting phylogenetic tree clearly shows that two Otk paralogs can only be found in *Drosophila* species, but not in other arthropod species (Fig. 15 B). Hence, a lineage specific duplication has generated two PTK7 homologs in *Drosophila*.

Structurally, Otk is a transmembrane molecule. It consists of five extracellular immunoglobuline-like domains, a single transmembrane domain and a kinase homology domain on the C-terminus (Fig. 16 A). Its paralog Otk2 only comprises three immunoglobuline-like domains and only a very short stretch of 69 amino acids intracellularly (Fig. 16 A). To answer the question which parts of the much shorter Otk2 sequence correspond to which parts of the Otk sequence, a dot plot was performed, visualizing matching residues in both sequences. This analysis demonstrated that the entire Otk2 sequence (except for the signal peptide) can be matched to a continuous stretch within the Otk sequence (Fig. 16 B), ranging from the third immunoglobulin-like domain until the beginning of the kinase homology domain.

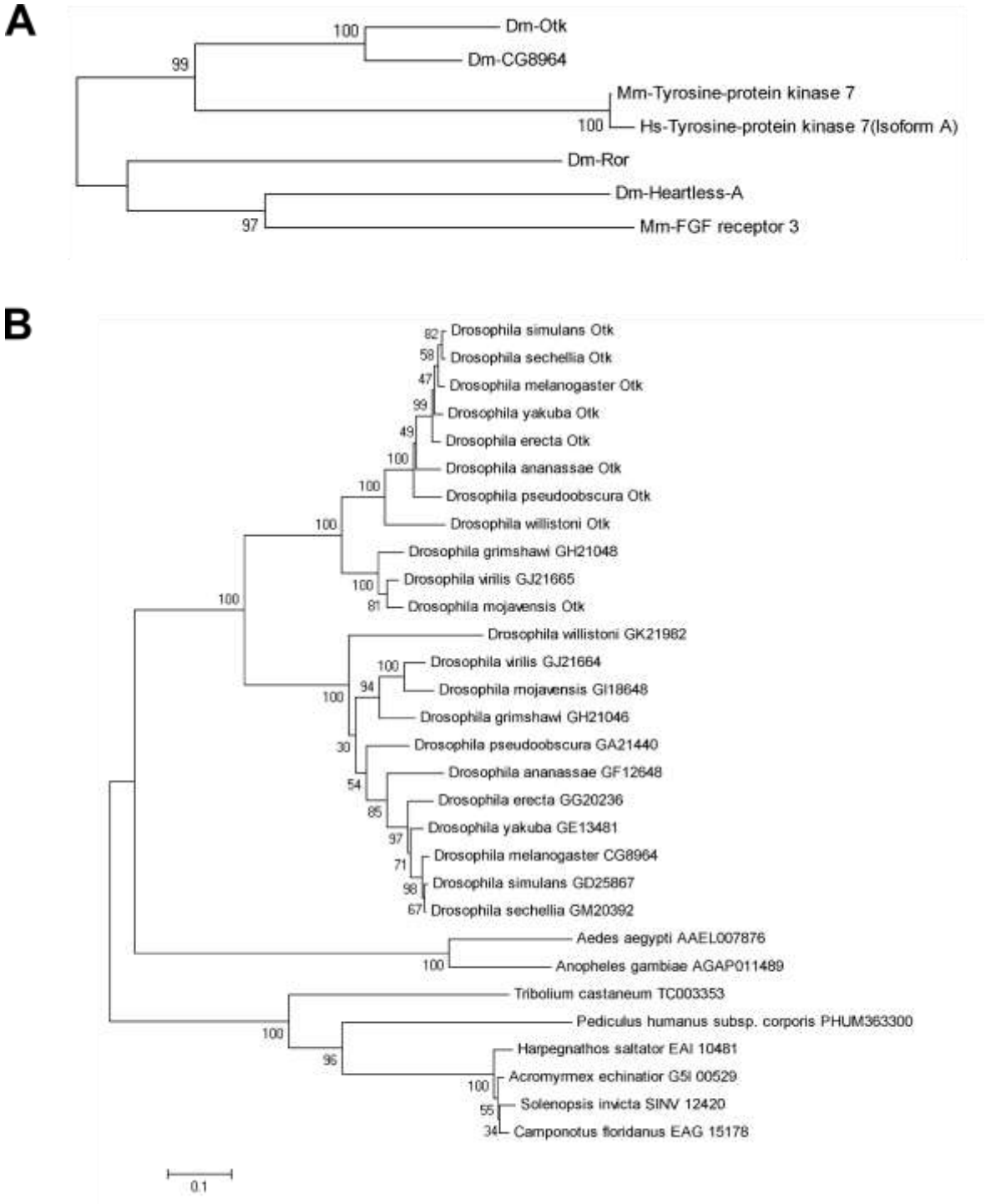

Fig. 15: Off-track (Otk) and Off-track2 (CG8964, Otk2) are paralogs evolved by gene duplication. **(A)** Phylogenetic tree representation of an alignment of sequences from different species homologous to *Drosophila* Otk. Blast-P of Dm-Otk in *Drosophila* returned Dm-CG8964 (Otk2) as the best hit; Dm-Ror and Dm-Heartless-A were the second and third hit, repectively. Blast-P of Dm-Otk in Mouse returned Mm-PTK7 as the best hit, while Mm-FGF receptor3 was the second best hit. Blast-P of Dm-Otk in Human returned Hs-PTK7 as the best hit. ClustalW alignment of these sequences and Neighbor-Joining tree confirms that the PTK7 branch is separated from the other proteins by a bootstrap value of 100. In this branch, two *Drosophila* genes, but only one in the other species can be found. Dm, *Drosophila melanogaster*; Mm, *Mus musculus*; Hs, *Homo sapiens*. **(B)** Phylogenetic tree of sequences from different arthropod species homologous to *Drosophila* Otk.

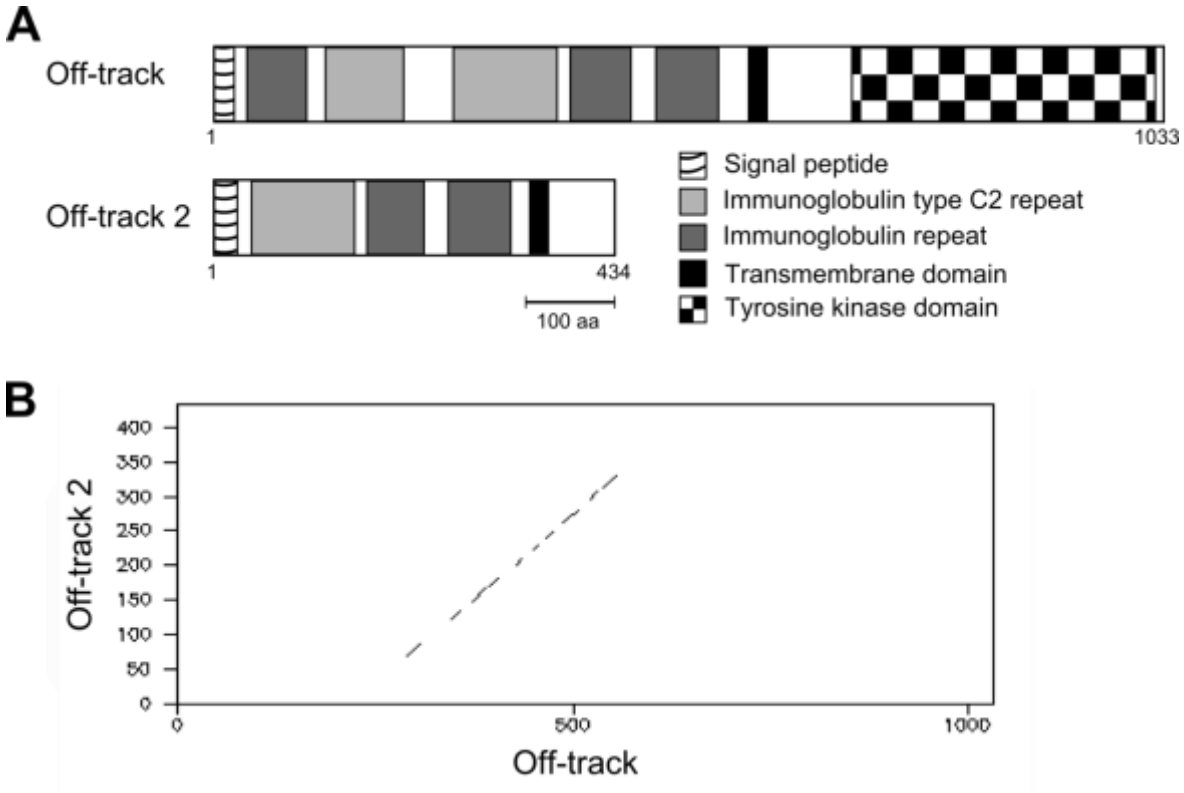

Fig. 16: Structural similarity between Otk and Otk2.
(A) Protein structures of Otk and Otk2. Domains were predicted using the SMART sequence analysis tool. **(B)** Dot plot comparing the protein sequences of Otk (x-axis) and Otk2 (y-axis) to visualize matching residues in both sequences.

As already mentioned, Otk2 lacks the entire tyrosine kinase homology domain. In other species it was shown for PTK7 that the tyrosine kinase domain does not possess any kinase activity (Chou & Hayman, 1991; Mossie *et al*, 1995; Miller & Steele, 2000; Jung *et al*, 2004). Interestingly, the short intracellular amino acid stretch of Otk2 (aa 366 – 434) contains a motif that is highly conserved between Otk2 and Otk (Fig. 17).

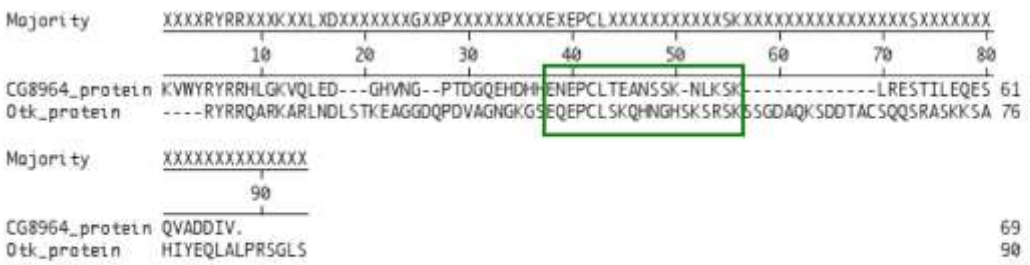

Fig. 17: Alignment of the intracellular carboxy terminus of Otk2 with the corresponding Otk sequence.

The existence of two closely related PTK7 homologs could mean that *otk* and *otk2* function redundantly and may explain why these genes have not been found in previous screens for planar cell polarity and Wnt signaling pathway phenotypes.

3.2 Biochemical interactions of Off-track and Off-track2

It is known for Xenopus PTK7 that the protein forms homodimers (Annette Borchers, personal communication). To test whether Otk and Otk2 also have the ability to interact with themselves or possibly also with each other, I performed immunoprecipitation experiments with transiently transfected S2r+ cells.

To test for homodimerization, Myc- and GFP-tagged Otk constructs under the control of an Actin promoter were co-overexpressed in S2r+ cells. Cell lysates were immunoprecipitated using anti-Myc antibody. Immunoblotting with anti-GFP antibody demonstrated that Otk-GFP could be co-precipitated with Otk-Myc. The same experiment was performed with Myc- and GFP-tagged Otk2 constructs and showed that Otk2-GFP co-immunoprecipitates with Otk2-Myc as well. As control, cells were transfected with GFP-tagged constructs only and with an empty vector (Fig. 18 A). Hence, both Otk and Otk2 appear to be able to form homodimers, as described for the vertebrate homologs.

The existence of the Otk paralog Otk2 in Drosophila raises the question whether the two proteins can also interact with each other. To test for heterodimerization, Myc-tagged Otk2 and GFP-tagged Otk constructs under the control of an Actin promoter were co-overexpressed in S2r+ cells. Cell lysates were subjected to anti-Myc IP. Immunoblotting with anti-GFP antibody demonstrated that Otk-GFP could be co-precipitated with Otk2-Myc. The reverse experiment was performed with Myc-tagged Otk and GFP-tagged Otk2 constructs and showed that Otk2-GFP co-immunprecipitates with Otk-Myc as well (Fig. 18 B). From these results it can be concluded that Otk and Otk2 most likely can form both homo- and heterodimers, however, it remains to be investigated whether other proteins are involved in this interaction.

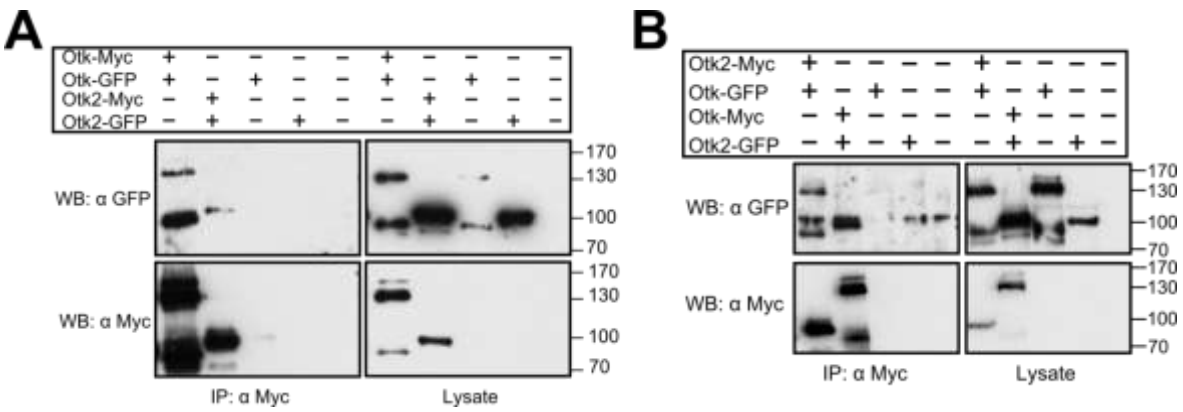

Fig. 18: Off-track and Off-track2 form homodimers and heterodimers.
(A and B) Otk-myc or Otk2-myc and Otk-GFP or Otk2-GFP expression vectors were transfected as indicated in Drosophila S2r+ cells. Cell lysates were immunoprecipitated and analyzed by Western Blot with the indicated antibodies. IP, Immunoprecipitation; WB, Western Blot.

As the previous results do not provide any information about the domains involved in the dimerization, a first approach using Otk deletion constructs was undertaken to further narrow down the regions required for homo- and heterodimerization. S2r+ cells were transiently transfected with Myc-tagged full length Otk or two deletion constructs of Otk in combination with GFP-tagged Otk or Otk2 and were subjected to anti-Myc IP with subsequent immunoblot against GFP. Both a version of Otk lacking the extracellular domain and a C-terminally truncated Otk protein were able to co-immunoprecipitate full length Otk as well as Otk2 (Fig. 19). This result suggests that the transmembrane domains could mediate their interaction.

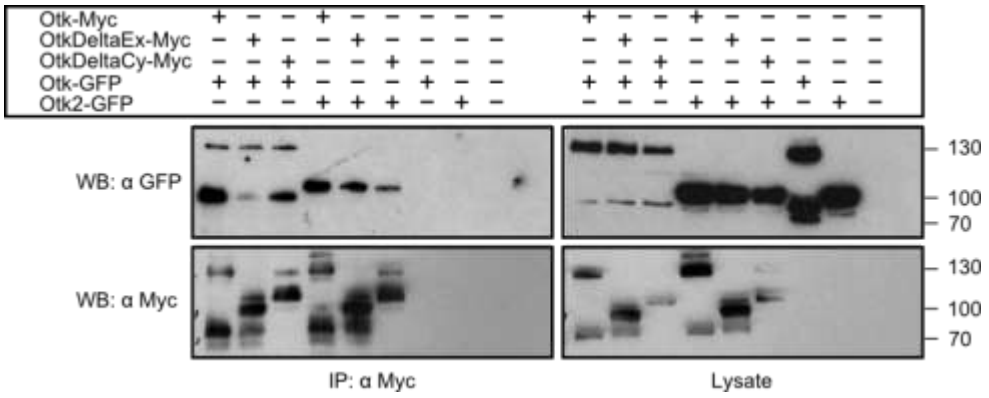

Fig. 19: Homo- and heterodimerization of Otk and Otk2 requires the transmembrane domain. Otk-myc or myc-tagged Otk deletion contructs and Otk-GFP or Otk2-GFP expression vectors were transfected as indicated in Drosophila S2r+ cells. Cell lysates were immunoprecipitated and analyzed by Western Blot with the indicated antibodies. OtkDeltaCy lacks the cytoplasmic domain (aa 776-1033) and OtkDeltaEx lacks the Extracellular domain (aa 2-474). IP, Immunoprecipitation; WB, Western Blot.

3.3 Expression of Off-track and Off-track2

Otk has been described 20 years ago to be dynamically expressed during *Drosophila* embryogenesis (Pulido *et al*, 1992). To verify the expression pattern of Otk and to thereby gain further insight into the potential function of the two proteins, several approaches were undertaken.

3.3.1 Expression of Off-track and Off-track2 reporter lines

A homozygous viable GFP enhancer trap line in the otk locus and a lacZ enhancer trap line for Otk2 were available. The transposon PBac{602.P.SVS-1}otk^{CPTI000252} (will be called *otk^{CPTI000252}* for simplification) is inserted in the first intron of the *otk* locus (position 2R:7,902,551 (+)) and encodes for a GFP gene trap. The line is not a functional protein trap and thus does not provide any insight into the subcellular localization, but is still useful as a reporter line. GFP expression could be observed in a segmental pattern during early embryonic development from stage 9 on (Fig. 20 A, B). It is possible that expression starts earlier, but is too weak to be detected. Later on, expression could be observed in the developing nervous system and in the developing gut (Fig. 20 C-F). Dissection of wandering L3 larvae revealed expression in the leg imaginal disc (Fig. 20 G) as well as in the brain (Fig. 20 H-J). No expression was observed in wing imaginal discs (data not shown), which was surprising regarding a

predicted function of Otk in planar cell polarity signaling. In the brain, strong reporter expression was seen in the optic lobes of the central nervous system (Fig. 20 H). Co-immunostaining with neuroblast markers showed that the Otk reporter is not expressed in neuroblasts but in the surrounding cells, which are most likely neuroblast progeny (Fig. 20 I). Furthermore, expression could be observed in a dorsal structure in the brain, which possibly is the developing mushroom body.

The transposon P{lacW}l(2)SH1639^{SH1639} (will be called CG8964^{SH1639} for simplification) is inserted in the 5'UTR of the *CG8964* locus (position 2R:7,912,740..7,912,810) and encodes for a lacZ enhancer trap. LacZ expression could be detected in segmental stripes in early embryonic development and later on in the developing gut as well as the developing nervous system (Fig. 20). Hence, the expression is very similar to that observed for *otk*^{CPTI000252}, indicating that the two proteins play a role in the same processes.

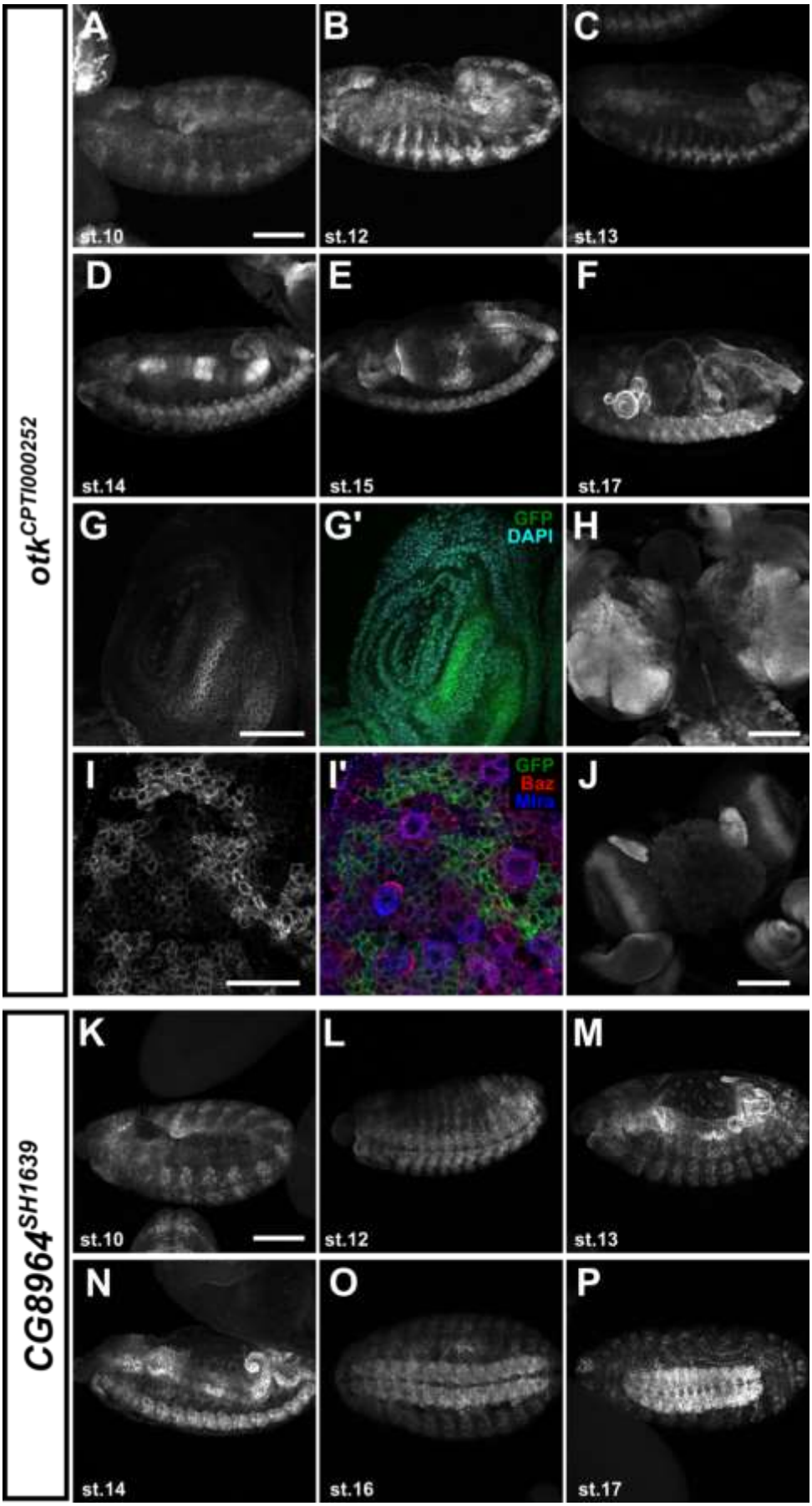

Fig. 20: Expression of Off-track and Off-track2 reporter lines.

Fig. 20 (previous page): (A-F) Transgenic embryos expressing a GFP gene trap in the *otk* locus were stained against GFP. Stages of embryonic development are indicated. **(G)** Leg imaginal disc from L3 larvae expressing otk GFP gene trap stained against GFP. **(H-J)** Brain from L3 larvae expressing otk GFP gene trap stained against GFP. **(H)** Dorsal view of the brain. **(I)** Magnification of the optic lobes. Miranda (Mira) and Bazooka **(Baz)** were co-stained to mark the neuroblasts. **(J)** Ventral view of the brain. **(K-P)** Transgenic embryos expressing a lacZ gene trap in the *otk2* locus were stained against beta-galactosidase. Stages of embryonic development are indicated. Anterior is to the left in A-F and K-P. Scale bars: A-F, H, J, K-P = 100 µm, G = 50 µm, I = 20 µm.

3.3.2 mRNA Expression of Off-track and Off-track2 during embryonic development

To confirm the results obtained from the analysis of the *otk* and *otk2* reporter lines and to gain further insight into the mRNA localization, fluorescent *in situ* hybridzation (FISH) was performed. Both Otk and Otk2 mRNA are expressed in segmental stripes in the process of germband extension (Fig. 21 A; Fig. 22A). During germband retraction, both mRNAs start to be expressed in the developing nervous system (Fig. 21 B; Fig. 22 B). From stage 12 on, mRNA is also expressed in the developing gut (Fig. 21 C-E; Fig. 22 C,D). In late embryos, Otk and Otk2 mRNA are strongly expressed in the gut as well as the nervous system (Fig. 21 E, F; Fig. 22 E,F).

The expression of both mRNAs in segmentally repeated stripes is reminiscent of the expression of the segment polarity regulator Wingless. To check for a potential co-localization, embryos hybridized with FISH antisense probes were co-stained against Wingless. This clearly demonstrated that both Otk and Otk2 mRNA co-localize with Wingless protein (Fig. 21 G-I; Fig. 22 G-I).

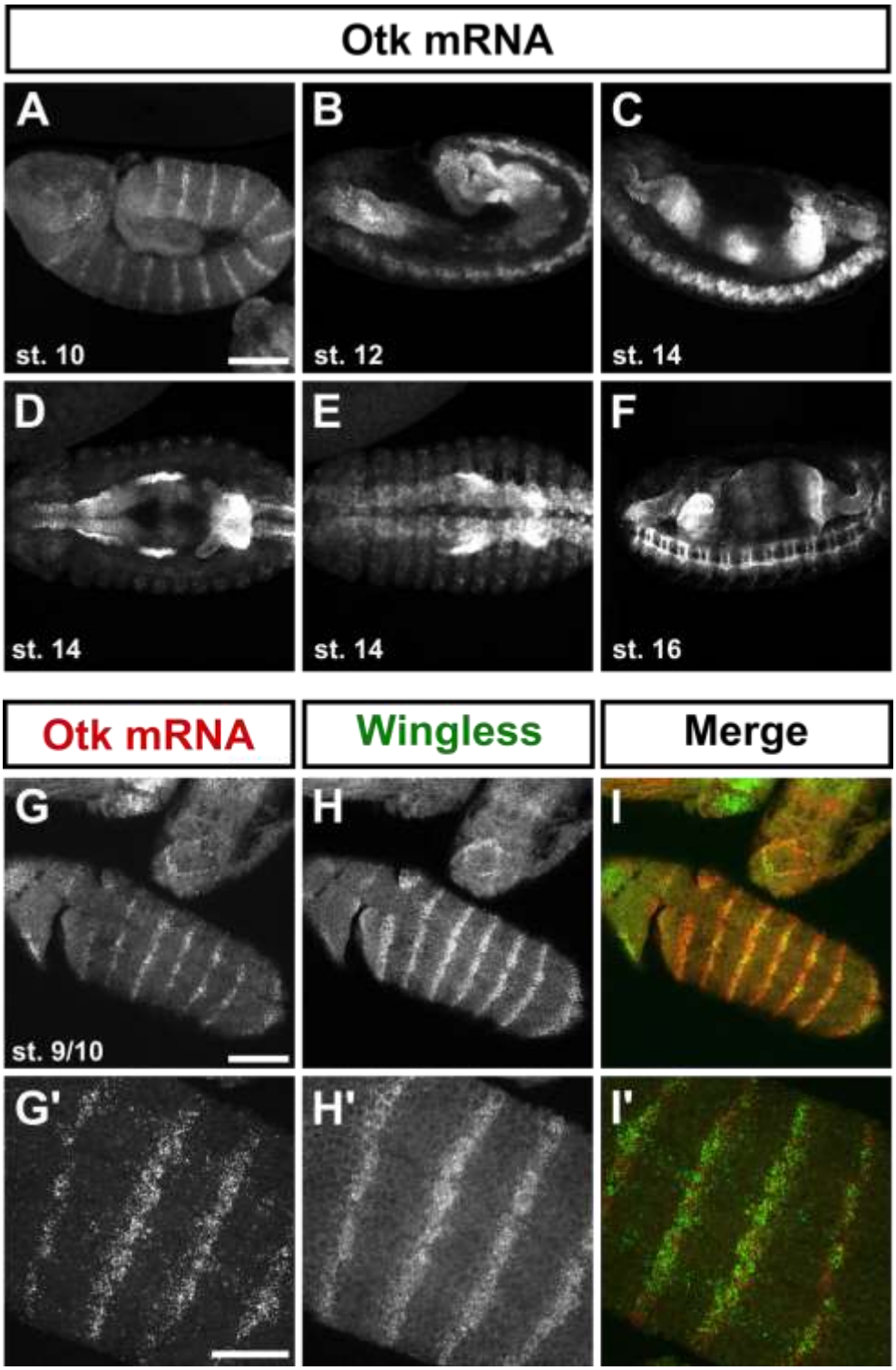

Fig. 21: mRNA expression of Off-track in embryos.
(A-F) Fluorescent *in situ* hybridization (FISH) with antisense probe against *otk* on *white*- embryos. **(G-I)** Embryos hybridized with an antisense probe against *otk* were co-stained against Wingless. Stages of embryonic development are indicated. Anterior is to the left. Scale bars: A-I = 100 µm, G'-I' = 50 µm.

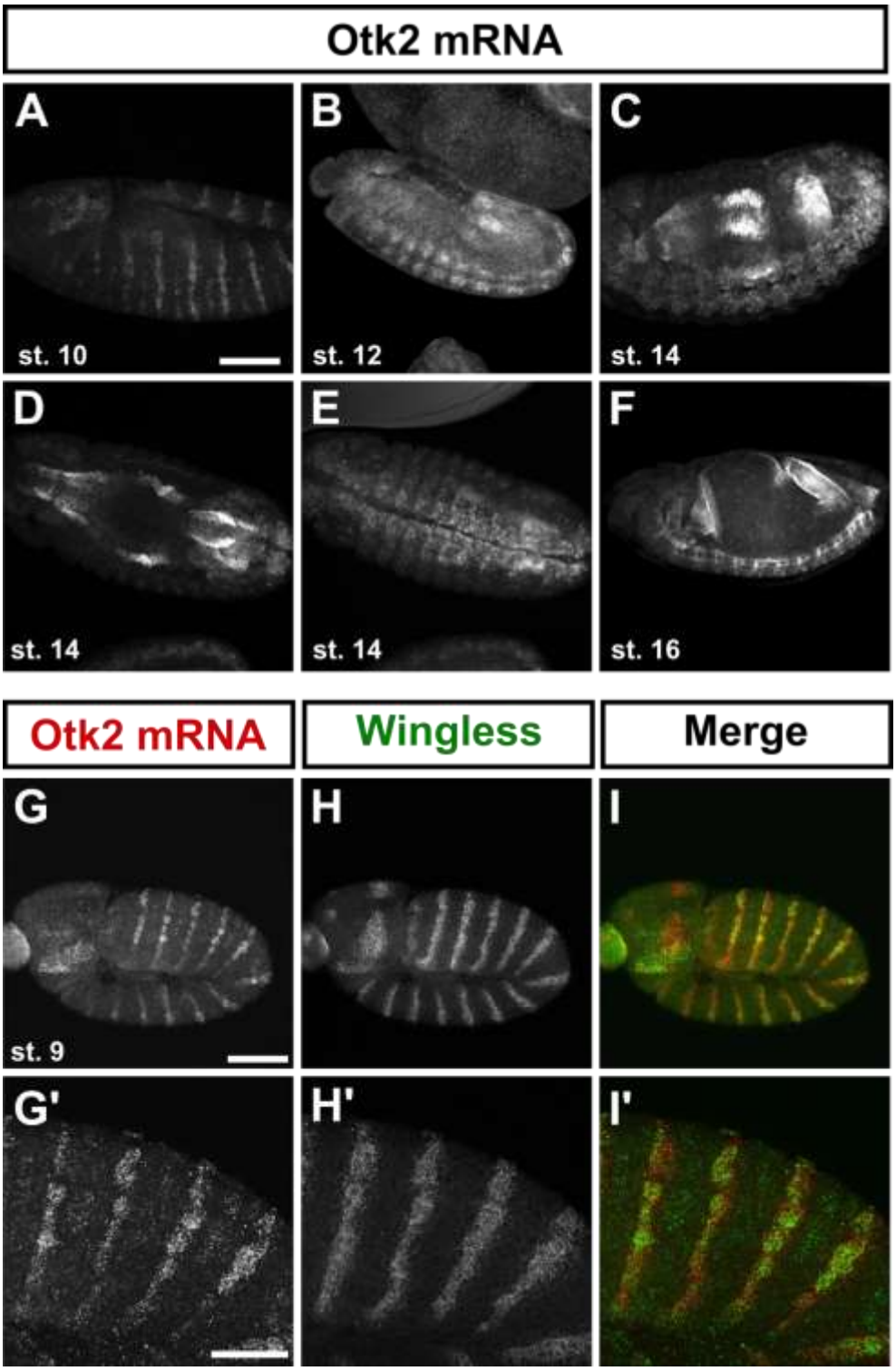

Fig. 22: mRNA expression of Off-track2 in embryos.

(A-F) Fluorescent *in situ* hybridization (FISH) with antisense probe against *otk2* on *white*- embryos. **(G-I)** Embryos hybridized with an antisense probe against *otk2* were co-stained against Wingless. Stages of embryonic development are indicated. Anterior is to the left. Scale bars: A-I = 100 µm, G'-I' = 50 µm.

3.3.3 Protein expression of Off-track and Off-track2 during embryonic development

To determine the expression and subcellular localization of Otk and Otk2 during embryonic development, antibodies were generated against the two proteins. Whole mount immunofluorescent staining with an antibody raised against the extracellular domain of Otk confirmed the previously obtained expression data from reporter lines and FISH analysis. Otk is expressed in segmental stripes, in the developing nervous system and in the gut. Interestingly, it seems to be enriched in three domains along the developing gut (Fig. 23 G, H). In late embryogenesis, high expression can be detected in the gut and the nervous system (Fig. 23 A-L). Corresponding results were obtained from stainings with a peptide antibody raised against Otk2 (Fig. 24 A-F).

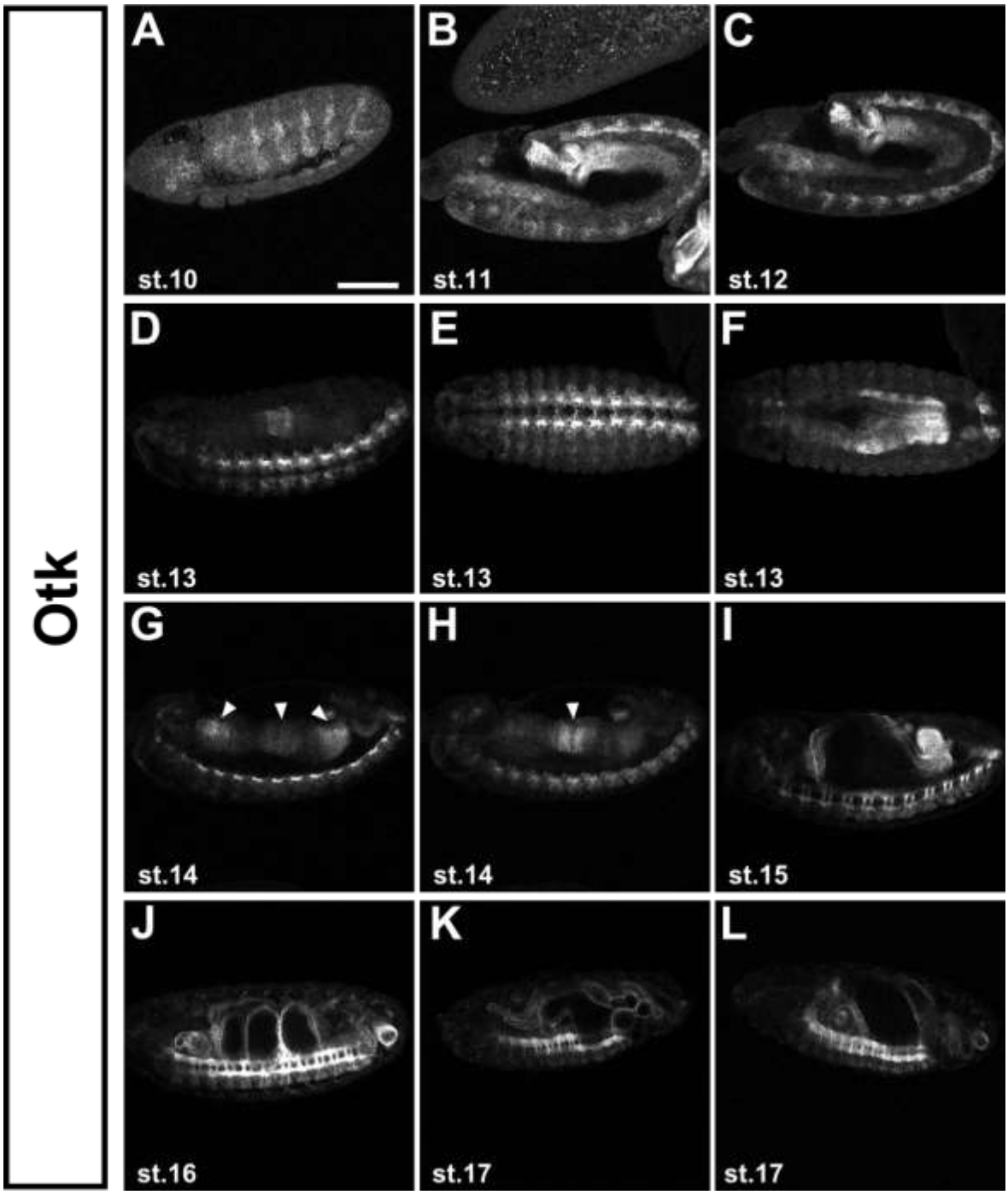

Fig. 23: Protein expression of Off-track in embryos.
(A-L) *white*- embryos were stained with an antibody against the extracellular domain of Otk. Stages of embryonic development are indicated. Anterior is to the left. Scale bar = 100 µm.

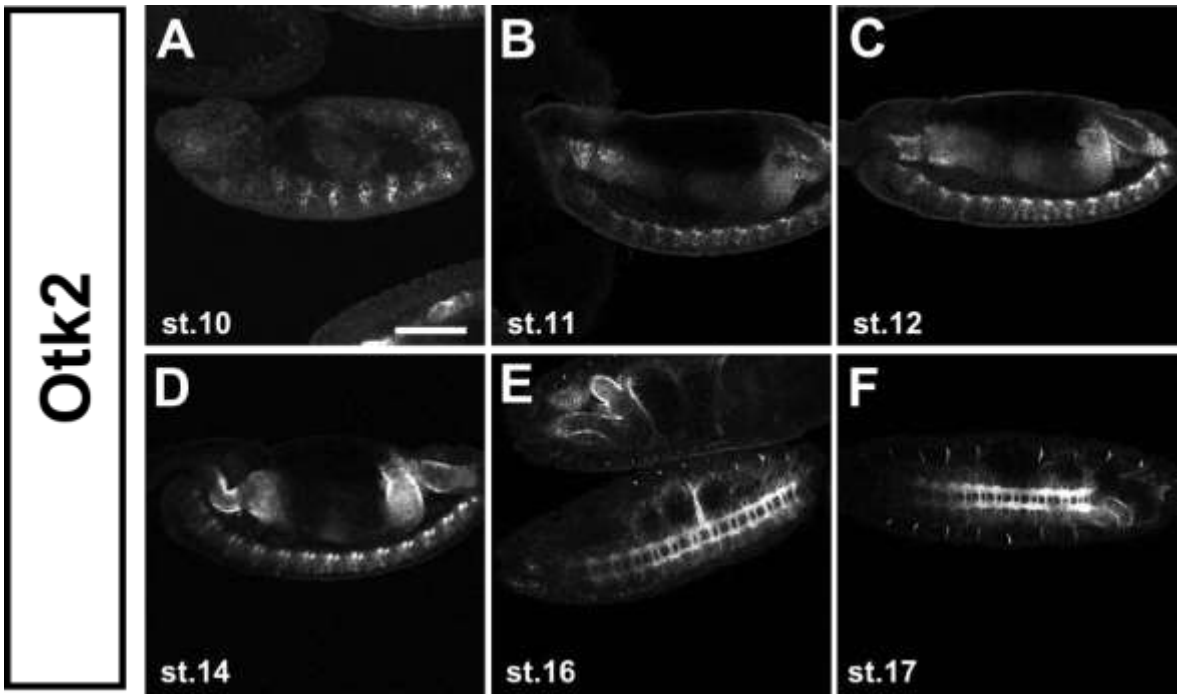

Fig. 24: Protein expression of Off-track2 in embryos.
(A-F) *white*- embryos were stained with a peptide antibody raised against Otk2. Stages of embryonic development are indicated. Anterior is to the left. Scale bar = 100 µm.

FISH analysis using antisense probes against Otk and Otk2 showed that the mRNA of both genes co-localizes with Wingless. To further validate this finding, co-stainings using antibodies against Otk and Wingless were performed (Fig. 25 A, B). These stainings showed that Otk is in fact expressed in cells located below the epithelial Wingless expressing cells (Fig. 25 B). Co-staining with the neuronal marker Elav revealed that Otk is expressed in segmentally repeated patches of neuroepithelial cells (Fig. 25 C). Remarkably, Otk seems to be expressed in the fraction of neuroblasts expressing Wingless and their neuronal progeny (Fig. 25 D).

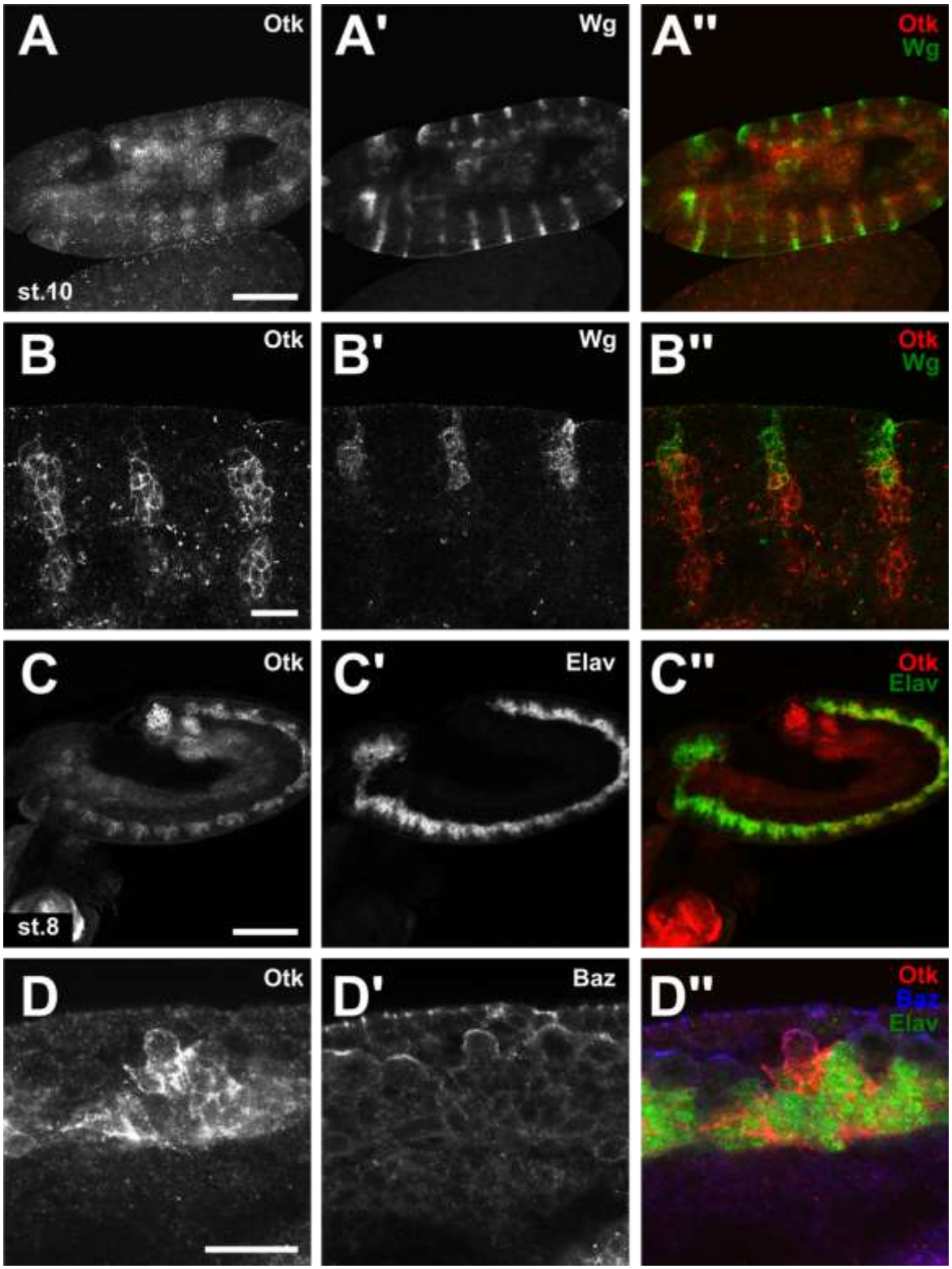

Fig. 25: Off-track is expressed in Wingless expressing neuroblasts and their neuronal progeny. **(A,B)** *white-* embryos were stained against Otk and Wingless. **(A)** Otk is expressed in segmental stripes that colocalize with Wingless expression. **(B)** Higher magnification reveals expression of Otk in cells located below the Wingless expressing cells, presumably neuroblast progeny. **(C,D)** *white-* embryos were stained against Otk and the neuronal marker Elav. **(C)** Otk is expressed in neuroblast progeny. **(D)** Otk is expressed in a subset of neuroblasts and their progeny. Anterior is to the left. Scale bars: A, C = 100 µm, B,D = 20 µm.

Wingless is not only expressed in the epithelium in segmentally repeated stripes, but also in parasegment 8 of the visceral mesoderm and the developing foregut and hindgut (Van den Heuvel *et al*, 1989). Interestingly, Otk is also highly expressed in the visceral mesoderm, partially co-localizing with Wingless (Fig. 26).

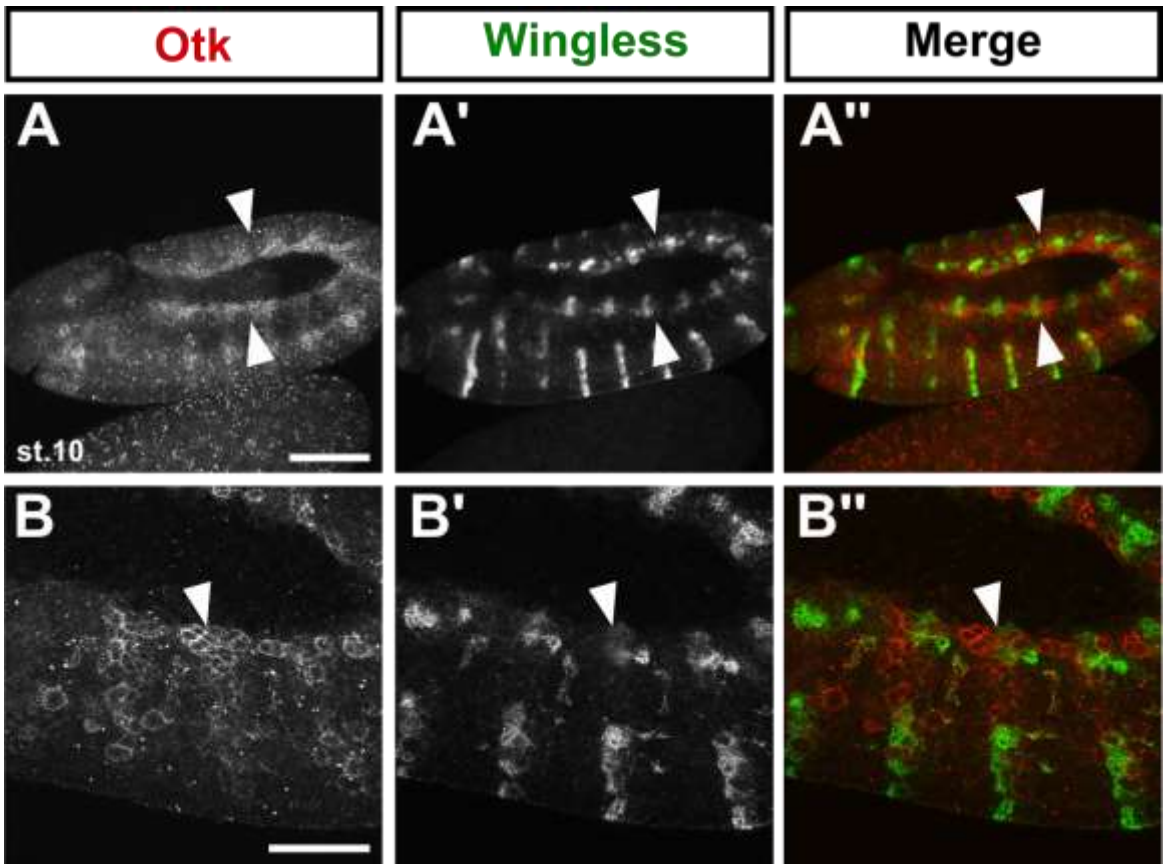

Fig. 26: Off-track is highly expressed in the visceral mesoderm.
white- embryos (stage 10) were stained against Otk and Wingless. **(A)** Wingless and Otk are expressed in the visceral mesoderm. **(B)** Otk is expressed along the entire visceral mesoderm and enriched in a segmental pattern. Anterior is to the left. Scale bars: A = 100 µm, B = 50 µm.

As mentioned already, Otk is expressed in three domains along the developing gut. This expression has been shown to be controlled by the transcription factor Biniou (vertebrate FoxF) (Jakobsen *et al*, 2007). Wingless has been described to be expressed in three domains in the developing gut as well, namely the proventriculus, the Malphigian tubules and the region that will form the second midgut constriction (van den Heuvel *et al*, 1989; Immerglück *et al*, 1990), indicating that there might be a connection between Otk and Wingless signaling in embryonic gut development. Whole mount immunofluorescent staining showed that the three expression domains of Wingless and Otk indeed overlap, however, Otk expression appears to be broader

in comparison to the restricted Wingless expression (Fig. 27). This might be explained by the fact that Wingless acts as a morphogen. Moreover, Otk expression appears to be stronger in the area adjacent to that of Wingless expression (Fig. 27 B-D).

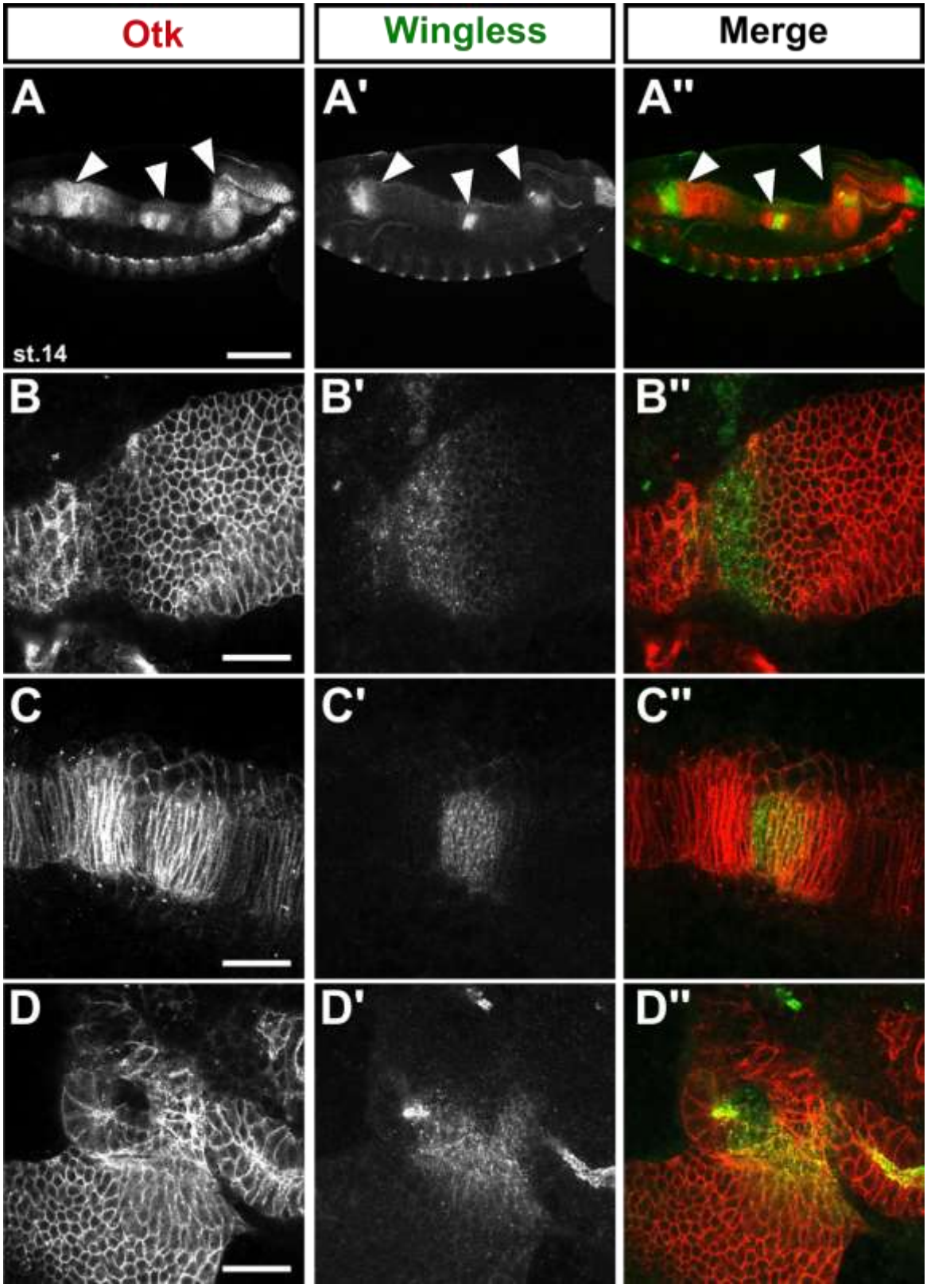

Fig. 27: Off-track is expressed in three regions in the embryonic gut.

Fig. 27 (previous page): *white*- embryos (stage 14) were stained against Otk and Wingless. **(A)** Wingless is expressed in three stripes in the developing embryonic gut. Otk is expressed in three domains in the developing gut that colocalize with Wingless expression but are broader than the distinct Wingless stripes. **(B-D)** Higher magnification of the anterior **(B)**, intermediate **(C)** and posterior **(D)** domain. Anterior is to the left. Scale bars: A = 100 µm, B = 20 µm.

Initial immunofluorescent stainings revealed a high expression of Otk and Otk2 in the late embryonic nervous system. The expression overlaps with the marker for CNS axons, Bp102 (Fig. 28 A). However, in the course of these analyzes it became obvious that Otk and Otk2 are enriched at the anterior commissures compared to the posterior commissures (Fig. 28 B-G). Wnt5 is known to be expressed in the posterior commissure and acts as a repellent ligand for Derailed-expressing neurons that cross the midline along the anterior commissure (Yoshikawa *et al*, 2003; Fradkin *et al*, 2004). In this study, I detected high levels of Wnt5 protein in the anterior commissures, colocalizing with Otk (Fig. 28 H, I) and most likely reflecting the bound pool of Wnt5 protein (Jasprien Nordermeer, personal communication).

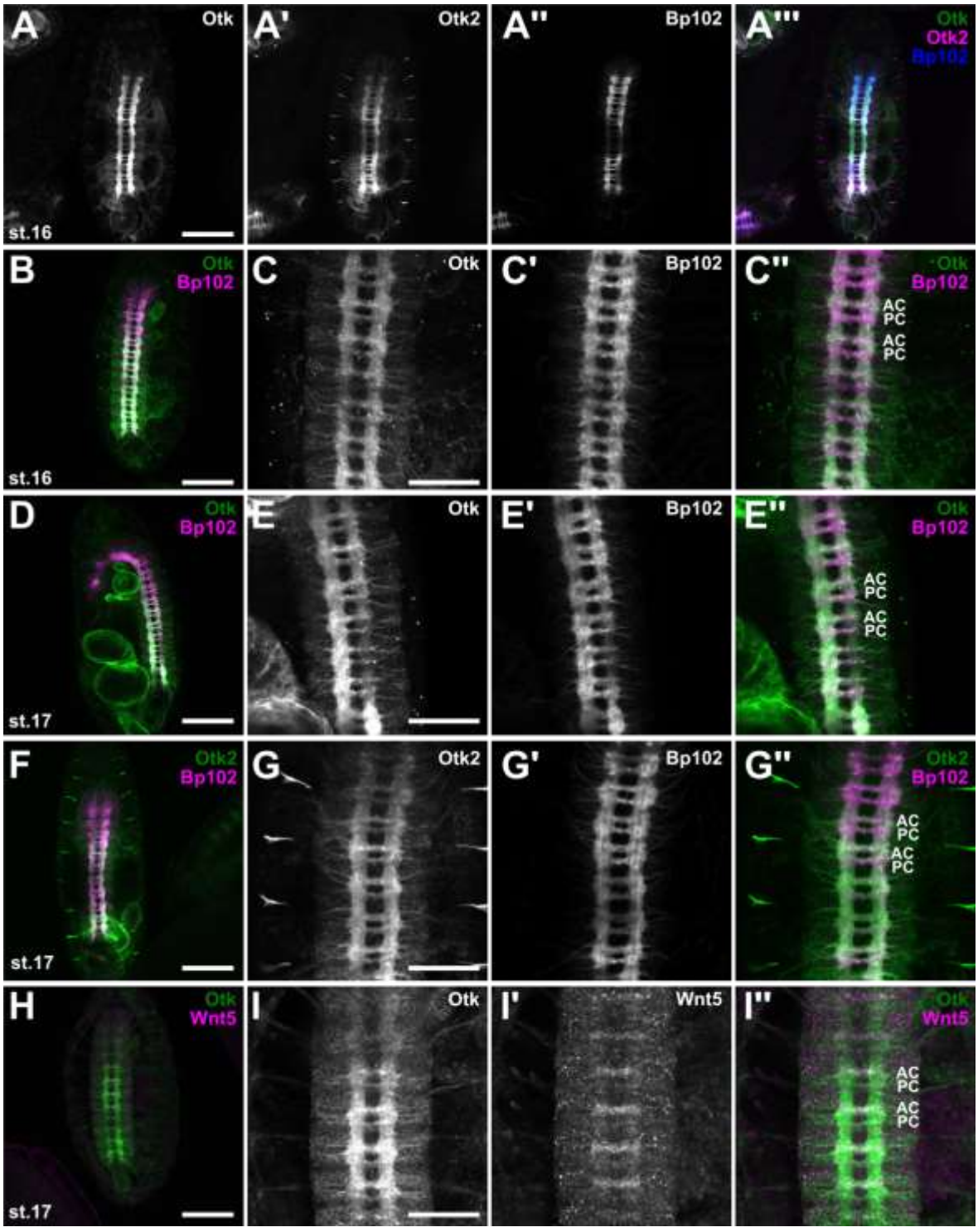

Fig. 28: Otk and Otk2 are expressed in the late embryonic nervous system and enriched at anterior commissures.

(A) *white-* embryos were stained against Otk, Otk2 and Bp102, an antibody against CNS axons. Otk and Otk2 are highly expressed in the nervous system and the expression colocalizes with Bp102 expression. **(B-D)** *white-* embryos were stained against Otk and Bp102. Higher magnifcation reveals an enrichment of Otk at anterior commissures at stage 16 **(C)**, which becomes even more prominent at stage 17 **(E)**. **(F,G)** *white-* embryos were stained against Otk2 and Bp102. Higher magnifcation reveals an enrichment of Otk2 at anterior commissures at stage 17 **(G)**. **(H,I)** *white-* embryos were stained against Otk and Wnt5. Wnt5 is enriched at anterior commissures **(I')**, clearly colocalizing with Otk **(I'')**. Stages of embryonic development are indicated. AC, anterior commissure; PC, posterior commissure. Anterior is to the top. Scale bars: A,B,D,F,H = 100 µm; C,E,G,I = 50 µm.

3.4 Expression of Off-track in *Drosophila* Wnt family mutants

To determine whether Otk expression itself might be a target of Wnt signaling, I compared Otk expression in different *wnt* mutant backgrounds (Fig. 29). Interestingly, Otk does not seem to be regulated by Wingless or Wnt4, since both the segmental localization in early embryos as well as the localization in the developing gut and nervous system were not affected (Fig. 29 A-D). Interestingly, the expression of Otk in the nervous system appeared to be slightly reduced in *wnt5* mutant embryos (Fig. 29 E) and overall Otk expression levels were strongly reduced in embryos homozygous mutant for *wnt2* (Fig. 29 F-H). This overall reduction did not become stronger in *wnt2,wg* and *wnt2,wnt4* double mutants, indicating that *wg* and *wnt4* indeed do not have any influence on Otk expression (Fig. 29 I,J).

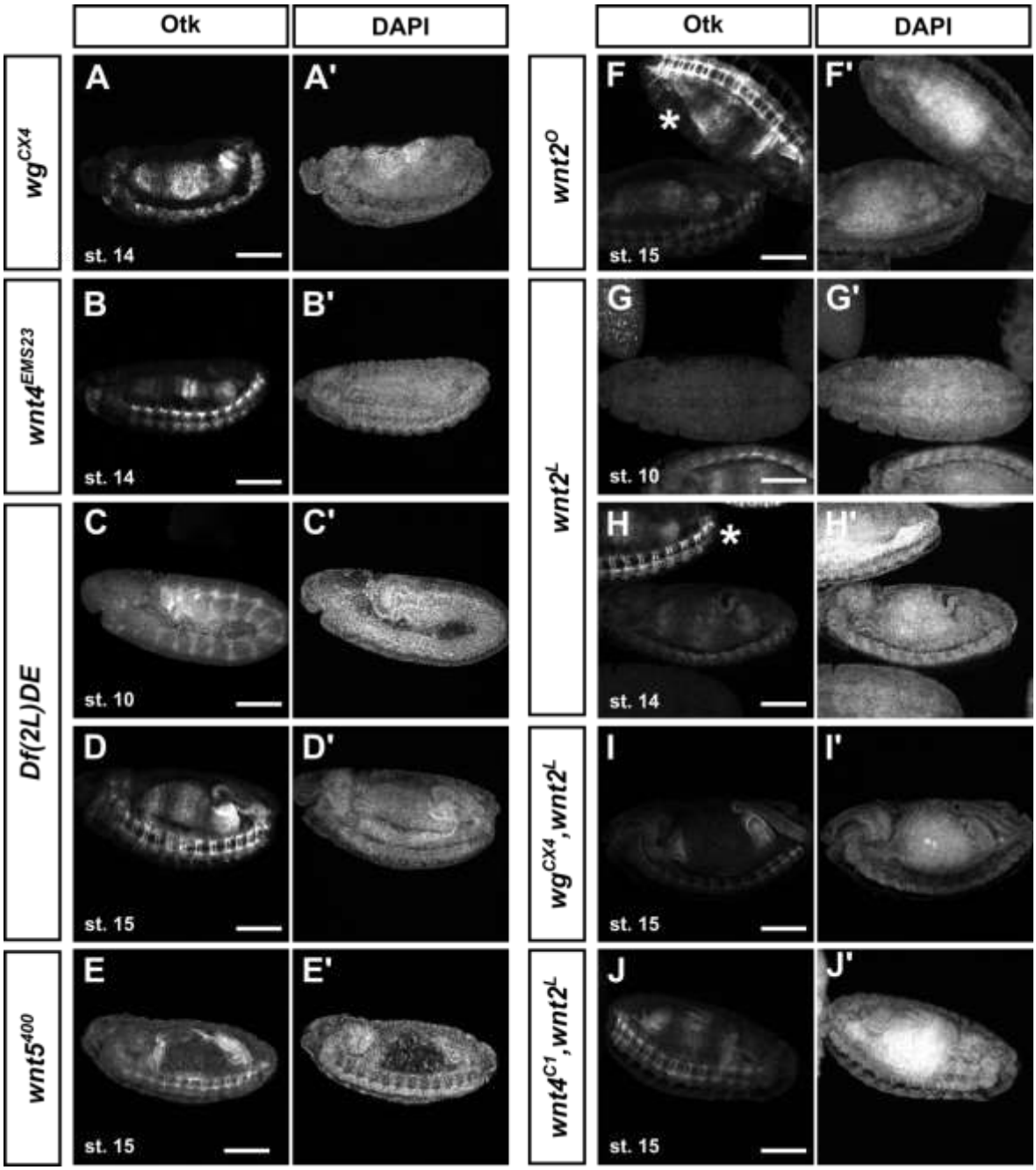

Fig. 29: Localization of Otk in embryos homozygous mutant for different Wnt family members. Homozygous mutant embryos of the indicated genotypes were stained against Otk. In embryos homozygous mutant for *wg* **(A)** and *Wnt4* **(B),** as well as a deficiency removing both *wg* and *wnt4* **(C,D)** the expression pattern of Otk is not changed. **(E)** In homozygous mutant *wnt5* embryos the expression in the nervous system is slightly reduced. **(F-H)** Otk expression is decreased in embryos homozygous mutant for *wnt2.* A homozygous compared to a heterozygous (asterisk) mutant embryo are depicted for comparison **(F,H)**. **(I,J)** Double mutants of the indicated genotypes display the same phenotype as the *wnt2* single mutant **(F-H)**. Anterior is to the left. Scale bars = 100 µm.

3.5 Generation of *otk* and *otk2* null alleles

To further investigate the function of Otk and Otk2, null alleles were generated for both genes as well as a double knock-out. For this purpose the full coding sequence of *otk* as well as *otk2* were removed via FLP/FRT-mediated excision (Parks *et al*, 2004). The peculiar chromosomal localization of both genes in tandem offers an easy way to generate a double knock-out for both genes using the same technique as for the *otk* and *otk2* single knock-out. This method utilizes the ability of FLP recombinase to induce recombination between two FRT sites positioned in *trans* on two complementary chromosomes. Three suitable transposon insertion lines containing FRT sites were available from the Harvard stock collection. The P(XP)d01360 element is located upstream of the 5'UTR of *otk* and the PBac(RB)e03992 element is inserted downstream of the 3'UTR of *otk.* The PBac(PB)c01790 element is located in the second exon of *Mppe*, a gene located upstream of *otk2* and P(XP)d01360 is downstream of the 3'UTR of *otk2* (Fig. 30 A). FLP recombinase-caused deletion of the genomic region between the FRT sites in P(XP)d01360 and PBac(RB)e03992 was used to remove the coding region of *otk*. Likewise, the genomic region of *otk2* was removed by recombination between the FRT sites located in P(XP)d01360 and PBac(PB)c01790. Finally, excision of the genomic region between the FRT sites in PBac(PB)c01790 and PBac(RB)e03992 deleted the genomic region of both *otk* and *otk2*.

Parts of the *Mppe* gene upstream of *otk2* were also removed during recombination using PBac(PB)c01790. *Mppe* is not an essential gene and encodes for a metallophosphoesterase that functions in Rhodopsin Rh1 deglycosylation (Cao *et al*, 2011).

Mutant flies potentially carrying one of the desired deletions were selected by the loss of the *mini-white* genes and/or PCR as described in 2.5.5. The following alleles could be recovered: otk^{A1} removes the genomic region of *otk* and $otk2^{C26}$ the genomic region of *otk2*, while $otk,otk2^{D72}$ is the double mutant of both genes (Fig. 30 A). As the three putative deletion lines were all homozygous viable, loss of the respective genes was tested by several methods. Each deletion was verified by PCR on adult genomic DNA (Fig. 30 B-D). First, PCRs were done on the transition from the genomic region to the ends of the P-elements to verify that the inner ends of the P-elements are deleted, while the outer ends still are left after recombination has taken place. Furthermore,

PCR was performed with primer pairs that encompass the entire deleted regions (Fig. 30 B-D). Subsequently, the resulting PCR bands were purified and sequenced.

To further prove that the obtained mutant lines indeed represent null alleles of the respective genes, I performed Western Blots with protein extracts from homozygous mutant embryos and the corresponding original transposon lines as well as chromosomal deficiencies as controls (Fig. 30 E). Detection with the antibody against the extracellular domain of Otk resulted in a band of approximately 120 kDa, corresponding to full-length Otk. As expected, this could be detected in all lanes except for otk^{A1}, $otk,otk2^{D72}$ and the deficiency lines. Interestingly, no protein could be detected in extracts from the original transposon line PBac(RB)e03992 either. Additional analysis will have to confirm if this line might represent an allele of *otk*. Detection with the peptide antibody against Otk2 resulted in a band of approximately 50 kDa, corresponding to full-length Otk2. Likewise, Otk2 protein could be detected in all lanes except for $otk2^{C26}$, $otk,otk2^{D72}$ and the deficiency lines (Fig. 30 E). The slight band in lane 7 is due to imprecise loading of the gel.

In addition, whole mount immunofluorescent stainings were performed on homozygous mutant embryos (Fig. 31 A-C). These analyses clearly demonstrated that no Otk protein can be detected in otk^{A1} embryos, while Otk2 localization is normal (Fig. 31 A). In agreement with this, no Otk2 protein could be detected in $otk2^{C26}$ embryos, while Otk localization is not changed (Fig. 31 B). Finally, neither Otk nor Otk2 could be detected in $otk,otk2^{D72}$ embryos (Fig. 31 C). I concluded that the obtained alleles otk^{A1}, $otk2^{C26}$ and $otk,otk2^{D72}$ are indeed null alleles for the respective genes.

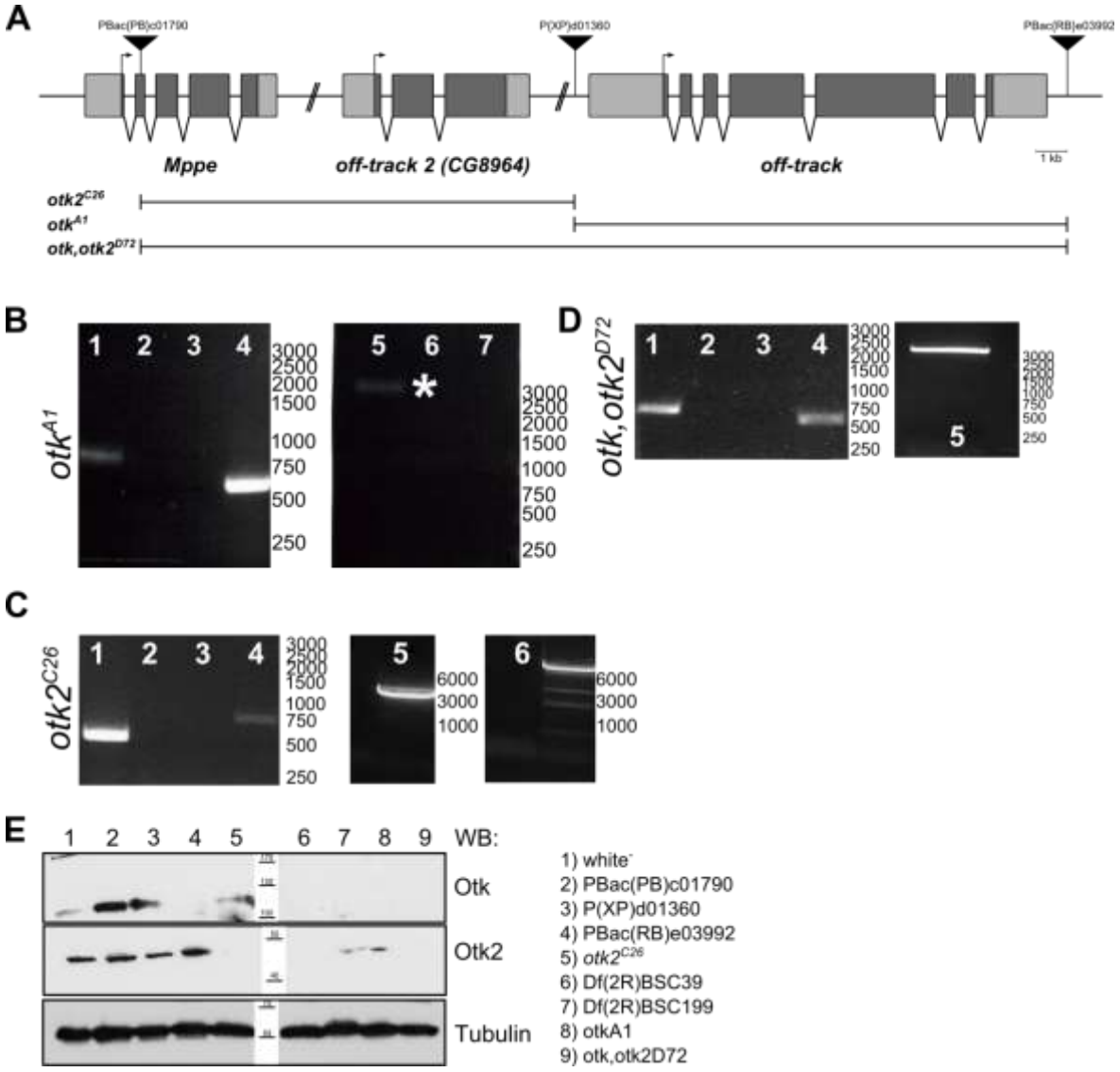

Fig. 30: Generation of *otk* and *otk2* null alleles.

(A) Overview of the genomic region of *otk*, *otk2* and the neighboring gene *Mppe* located on chromosome 2R. Insertion positions of the three P-element transposons utilized are shown. Null alleles for *otk* and *otk2* alone as well as both *otk* and *otk2* were generated via FLP/FRT mediated recombination between FRT sites contained in P(XP)d01360 and PBac(RB)e03992 **(otk^{A1})**, PBac(PB)c01790 and P(XP)d01360 **($otk2^{C26}$)** or PBac(PB)c01790 and PBac(RB)e03992 **($otk,otk2^{D72}$)**. **(B-D)** Verification of the three generated alleles by PCR. **(B)** Verification of the otk^{A1} deletion by PCR on genomic DNA from adult flies. Lane 1-4: The transition between the ends of the P-elements and the genomic region was amplified. PCR was done on the left (1) and right (2) end of P(XP)d01360 and on the left (3) and right (4) end of PBac(RB)e03992. Missing bands indicate the loss of the inner P-element ends. Lane 5 (asterisk): The deleted site was amplified with primers binding upstream and downstream of the whole deletion in the genomic region. The band obtained was purified and sequenced. Adult fly genomic DNA from P(XP)d01360 (lane 6) and PBac(RB)e03992 (lane 7) was used as control. Missing bands can be explained by the large size of the expected fragments. **(C)** Verification of the $otk2^{C26}$ deletion by PCR on genomic DNA from adult flies. Lane 1-4: The transition between the ends of the P-elements and the genomic region was amplified. PCR was done on the left (1) and right (2) end of PBac(PB)c01790 and on the left (3) and right (4) end of P(XP)d01360. Missing bands indicate the loss of the inner P-element ends. Furthermore, the deleted site was amplified with primers binding upstream of the deletion in the genomic region and the residual transposon (lane 5) as well as primers binding in the residual transposon and downstream of the deletion in the genomic region

(lane 6). The bands obtained were purified and sequenced. **(D)** Verification of the *otk,otk2^{D72}* deletion by PCR on genomic DNA from adult flies. Lane 1-4: The transition between the ends of the P-elements and the genomic region was amplified. PCR was done on the left (1) and right (2) end of PBac(PB)c01790 and on the left (3) and right (4) end of PBac(RB)e03992. Missing bands indicate the loss of the inner P-element ends. Lane 5: The deleted site was amplified with primers binding upstream and downstream of the whole deletion in the genomic region. The band obtained was purified and sequenced. **(E)** Protein extracts from embryos of the indicated genotype were analyzed by Western Blot with the antibodies shown. Protein extracts from embryos of the original transposon stocks (lane 2-4) as well as from homozygous embryos from chromosomal deficiency lines (lane 6 and 7) were used as controls. No Otk protein was detected in *otk^{A1}* (lane 8) and *otk,otk2^{D72}* (lane 9) homozygous mutant embryos as well as in embryos carrying the PBac(RB)e03992 P-element (lane 4). No Otk2 protein was detected in *otk2^{C26}* (lane5) and *otk,otk2^{D72}* (lane 9) homozygous mutant embryos. An antibody against tubulin was used as a loading control.

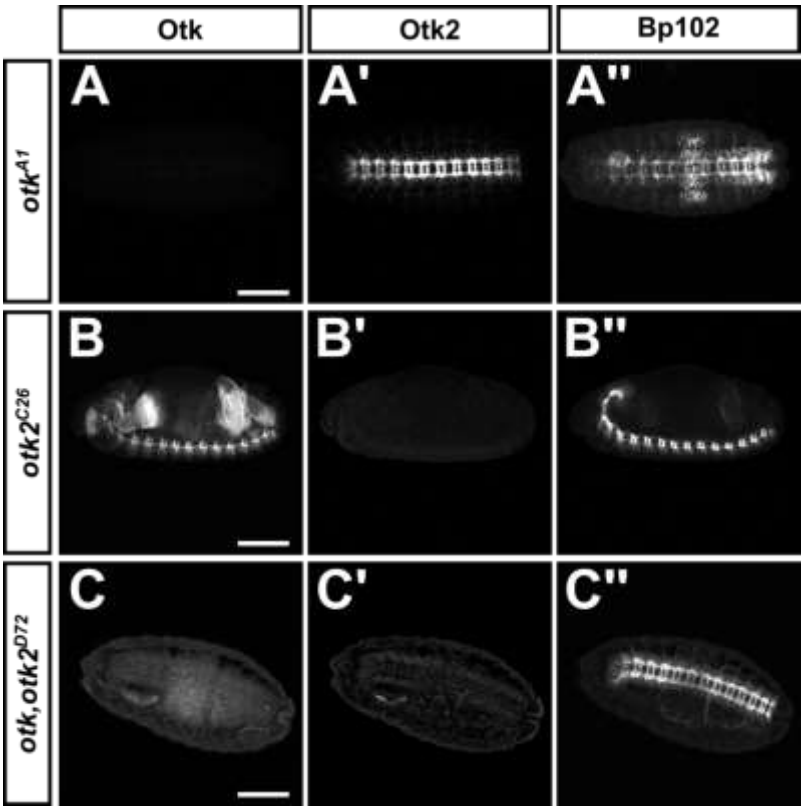

Fig. 31: Verification of *otk* and *otk2* null alleles.
(A-C) Verification of the three generated alleles by whole mount immunofluorescent stainings. Homozygous mutant embryos of the indicated genotypes were stained with antibodies against Otk, Otk2 and the CNS axon marker Bp102 as control. **(A)** In *otk^{A1}* homozygous mutant embryos no Otk protein can be detected, but the staining for Otk2 is normal. **(B)** In *otk2^{C26}* homozygous mutant embryos no Otk2 protein can be detected, but the staining for Otk is normal. **(C)** In *otk,otk2^{D72}* homozygous mutant embryos neither Otk nor Otk2 protein can be detected. Anterior is to the left. Scale bar = 100 µm.

3.6 Characterization of *otk* and *otk2* null alleles

Flies homozygous mutant for otk^{A1} or otk^{C26} were homozygous viable and did not show obvious phenotypic defects. This strongly disagrees with reports on the previously generated otk^{3} allele (Winberg *et al*, 2001; Cafferty *et al*, 2004; Peradziryi *et al*, 2011). Interestingly, also flies homozygous mutant for $otk,otk2^{D72}$ were viable, but male sterile and no homozygous stock could be established. To test whether loss of any of the genes leads to subtle defects several approaches were performed.

3.6.1 Novel *otk* and otk^{2} loss of function mutants are homozygous viable

To analyze if loss of either otk, otk2 or both genes leads to defects that cause a decrease in viability, I determined the hatching rate of embryos homozygous mutant for otk^{A1}, otk^{C26} and $otk,otk2^{D72}$. In case of the double deletion, maternally mutant embryos derived from homozygous mutant $otk,otk2^{D72}$ mothers and heterozygous fathers were used. The average viability of embryos homozygous for each of the deletions was comparable to that of *white-* embryos as well as the original transposon lines (Fig. 32).

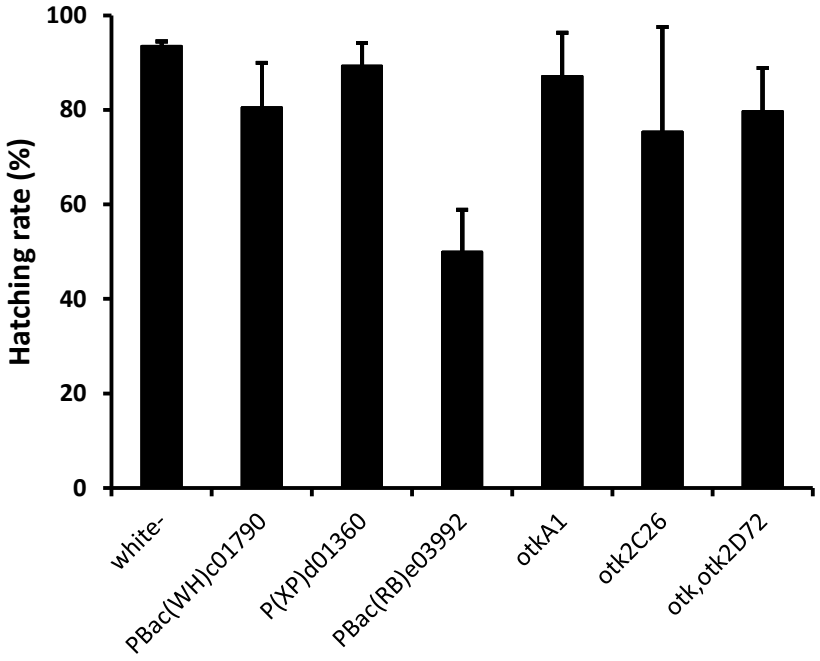

Fig. 32: **Novel *otk* and *otk2* loss of function mutants are homozygous viable and do not display any PCP or wing margin defects.**
(A) Lethality assays were performed with embryos of the indicated genotypes. Embryos were collected and allowed to develop for two days at 25 °C. The number of hatched embryos was determined. The experiment was repeated three times. Error bars represent the standard error of the mean.

3.6.2 Life span is not affected in novel *otk* and *otk2* loss of function mutants

To investigate if loss of either otk, otk2 or both genes alone is sufficient to extend or decrease life expectancy, flies homozygous mutant for otk^{A1}, otk^{C26} and $otk,otk2^{D72}$ were examined. No difference in life span could be seen for any of the genotypes observed (Fig. 33), suggesting that there is no relationship between Otk or Otk2 function and life expectancy.

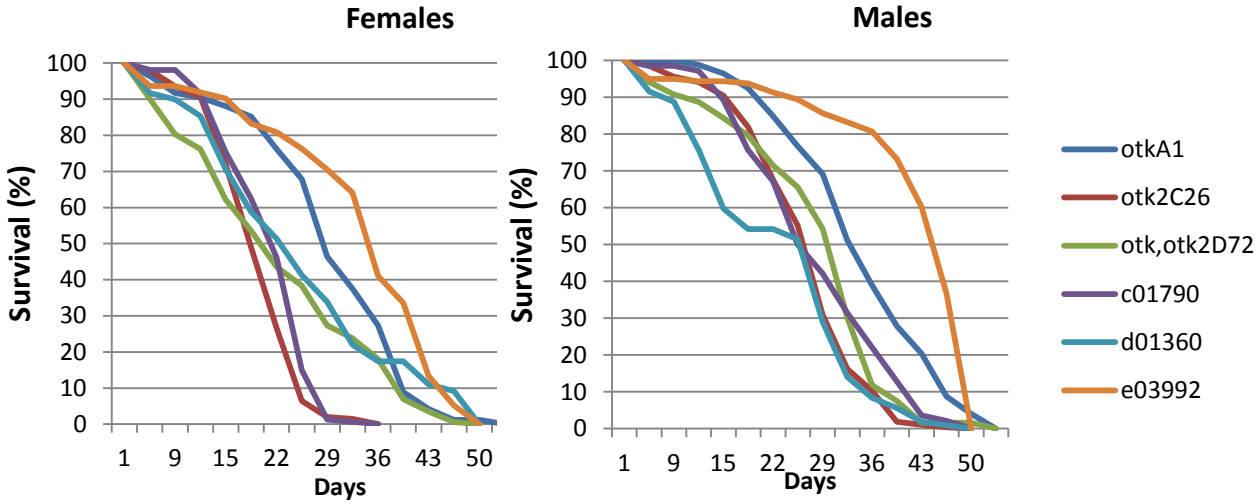

Fig. 33: Life span is not affected in novel *otk* and *otk2* loss of function mutants.
Survival rates of homozygous flies of the indicated genotypes were recorded. No difference can be observed between the deletion lines compared to the original transposon stocks.

3.6.3 Homozygous otk^{A1} and $otk,otk2^{D72}$ flies do not display any PCP or wing margin defects

Defects in planar cell polarity (PCP) signaling usually can be recognized by disorganization of wing hairs as well as thorax and leg bristles of adult flies (Axelrod & McNeill, 2002; Zallen, 2007). In contrast, failure in canonical Wingless signaling leads to wing margin defects (Couso *et al*, 1994; Zhang & Carthew, 1998). Furthermore, the photoreceptors in each ommatidium of the adult eye are arranged in an arrow-like manner with the arrow tips pointing towards the equator of the eye (Fig. 35 B). This organization is also frequently disturbed in PCP mutants (Axelrod & McNeill, 2002; Zallen, 2007).

To study if any of these defects can be found in *otk* or *otk2* mutants, wings of adult flies were examined (Fig. 34 A-E). In contrast to the function of PTK7 in vertebrate PCP signaling (Lu *et al*, 2004; Shnitsar & Borchers, 2008; Yen *et al*, 2009; Peradziryi *et al*, 2011), loss of neither *otk* or *otk2* alone (Fig. 34 D, data for *otk2* not shown) nor of both *otk* and *otk2* (Fig. 34 E) leads to any defects in wing hair orientation compared

to original P-element lines as controls (Fig. 34 B, C). In agreement with this finding, ommatidia organization was not disturbed either in *otk*^{A1}, *otk*^{C26}and *otk,otk2*^{D72} flies compared to wild type (Fig. 35 A-D, data for *otk2* not shown).

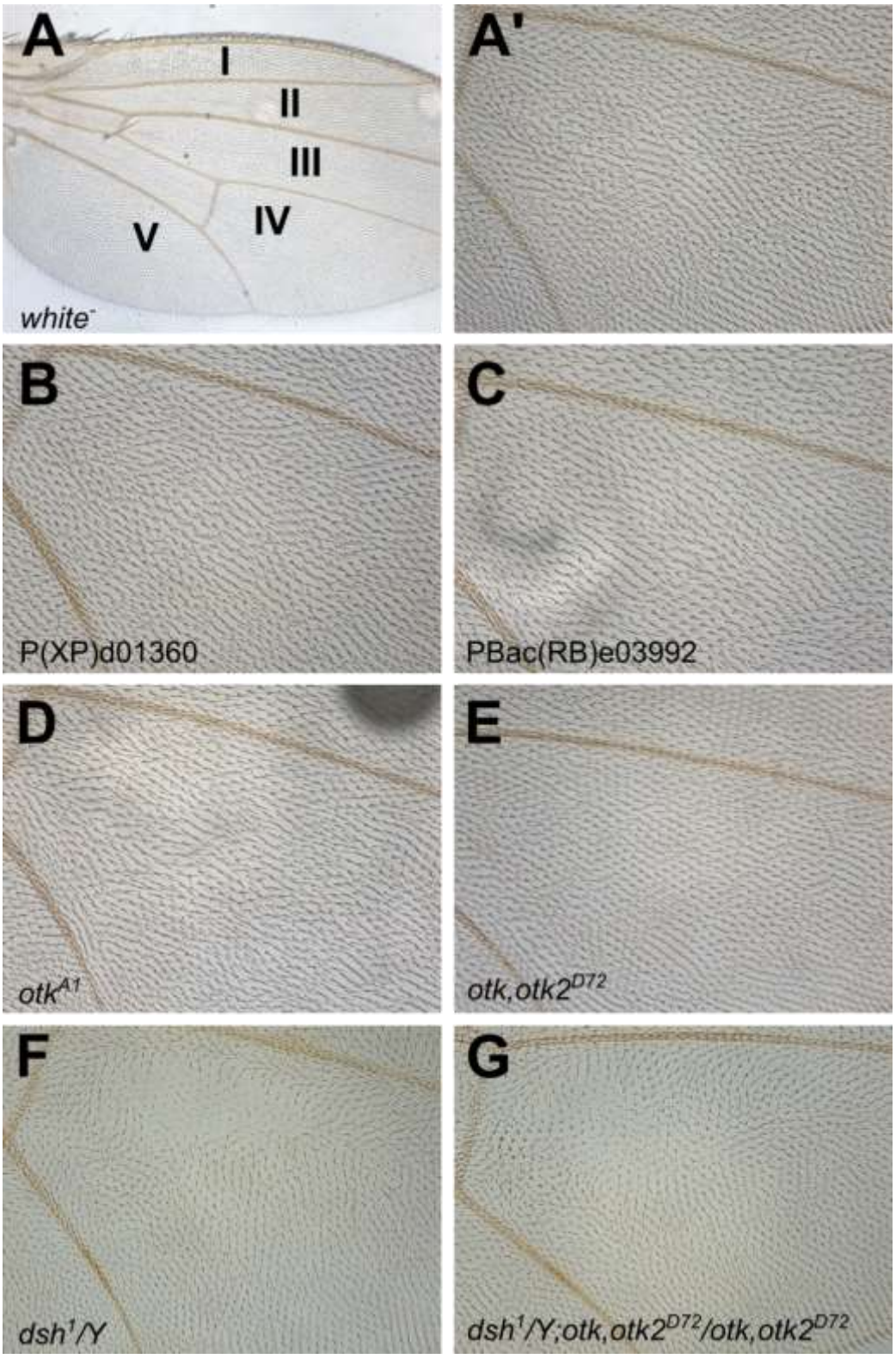

Fig. 34: Homozygous *otk*^{A1} and *otk,otk2*^{D72} flies do not display any wing PCP or margin defects. Wings from adult flies homozygous mutant for *otk*^{A1} **(D)** and *otk,otk2*^{D72} **(E)** (no maternal contribution) do not show any defects compared to *white-* flies or the original transposon lines **(A-C)**. **(F)** Wings from *dsh1/Y* flies as an example for a characteristic PCP phenotype. The phenotype is unchanged in

double mutants for *dsh1* and *otk,otk2^{D72}* **(G)**. **(A)** shows 10X magnifications of adult wings and the five regions of the wing are indicated. **(A',B-G)** display 25X magnifications of region IV of the respective wing. Anterior is up and proximal is to the left.

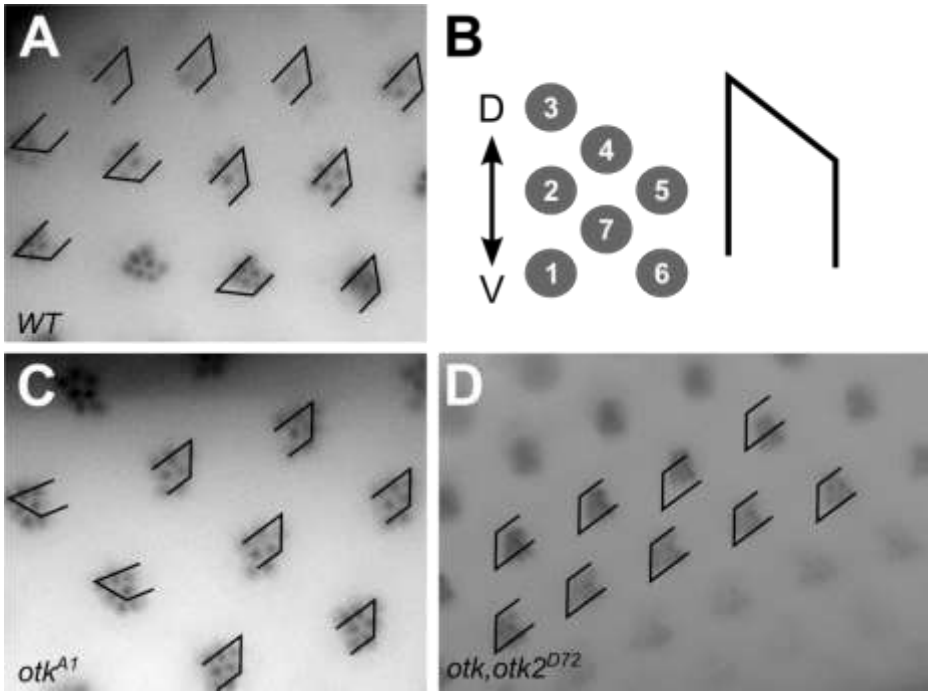

Fig. 35: Eyes of homozygous *otk^{A1}* and *otk,otk2^{D72}* flies do not display any PCP defects.
Eyes from adult flies homozygous mutant for *otk^{A1}* **(C)** and *otk,otk2^{D72}* **(D)** do not show any defects compared to wild type **(A). (B)** Schematic representation of the ommatidia structure of an adult eye.

3.6.4 *otk,otk2^{D72}* homozygous mutants flies are male sterile

As already mentioned, adult flies homozygous mutant for *otk,otk2^{D72}* were male sterile and this phenotype was fully penetrant. Apparently, both copies of both genes need to be removed, since transheterozygous *otk*,otk2^{D72}/*otk^{A1}* or *otk,otk2^{D72}/otk2^{C26}* males were fertile (Table 16). The sterility could be rescued by introduction of a full-length Otk-GFP transgene under UASp promoter, expressed by the ubiquitous driver daughterless::Gal4 (Table 17). In contrast, an Otk2-GFP transgene under UASp promoter did not rescue sterility. This suggests that even though the localization of the construct was normal (data not shown), the function of Otk2 is disturbed by the introduction of the C-terminal GFP tag.

Table 16: *otk,otk2^{D72}* homozygous mutant males are male sterile.

otk,otk2^{D72} / otk,otk2^{D72}	Male sterile
otk^{A1}/ otk^{A1}	Fertile
otk2^{C26}/ otk2^{C26}	Fertile
otk,otk2^{D72} / otk^{A1}	Fertile
otk,otk2^{D72} / otk2^{C26}	Fertile
otk,otk2^{D72} / Df(2R)BSC39	Male Sterile
otk,otk2^{D72} / Df(2R)BSC199	Male sterile

Table 17: Sterility *otk,otk2^{D72}* homozygous mutants males can be rescued by Otk transgenes.

otk,otk2^{D72} / otk,otk2^{D72}	Male sterile
otk,otk2^{D72} / otk,otk2^{D72} ; da / UASp::Otk-GFP29	Fertile
otk,otk2^{D72} / otk,otk2^{D72} ; da / UASp::OtkDeltaC-GFP14	Male sterile
otk,otk2^{D72} / otk,otk2^{D72} ; da / UASp::OtkDeltaEx-GFP20	Male sterile
otk,otk2^{D72} / otk,otk2^{D72} ; da / UASp::Otk2-GFP37	Male sterile
otk,otk2^{D72} / otk,otk2^{D72} ; da / UASt::Otk-GFP	not done
otk,otk2^{D72} / otk,otk2^{D72} ; da / UASt::Otk2-RFP	not done

To analyze whether sterility is caused by defects in sperm development, testes from males heterozygous and homozygous mutant for *otk,otk2^{D72}* were dissected and stained with Vasa, a marker for germline stem cells (Lasko & Ashburner, 1990) and FasciclinIII, which marks the hub (Gönczy & DiNardo, 1996) (Fig. 36 A-C). The hub consists of non-dividing stromal cells constituting the stromal niche for the germline stem cells and cyst stem cells (De Cuevas & Matunis, 2011). Both markers localize normally in testes homozygous mutant for *otk,otk2^{D72}* (Fig. 36 B,C), indicating that sterility is not caused by any defects in stem cell regulation. Furthermore, co-staining with the DNA marker DAPI revealed that all stages of sperm development, which can be distinguished by their characteristic packaging of the DNA, are present in testes from males homozygous mutant for *otk,otk2^{D72}* (Fig. 36 B''', C''').

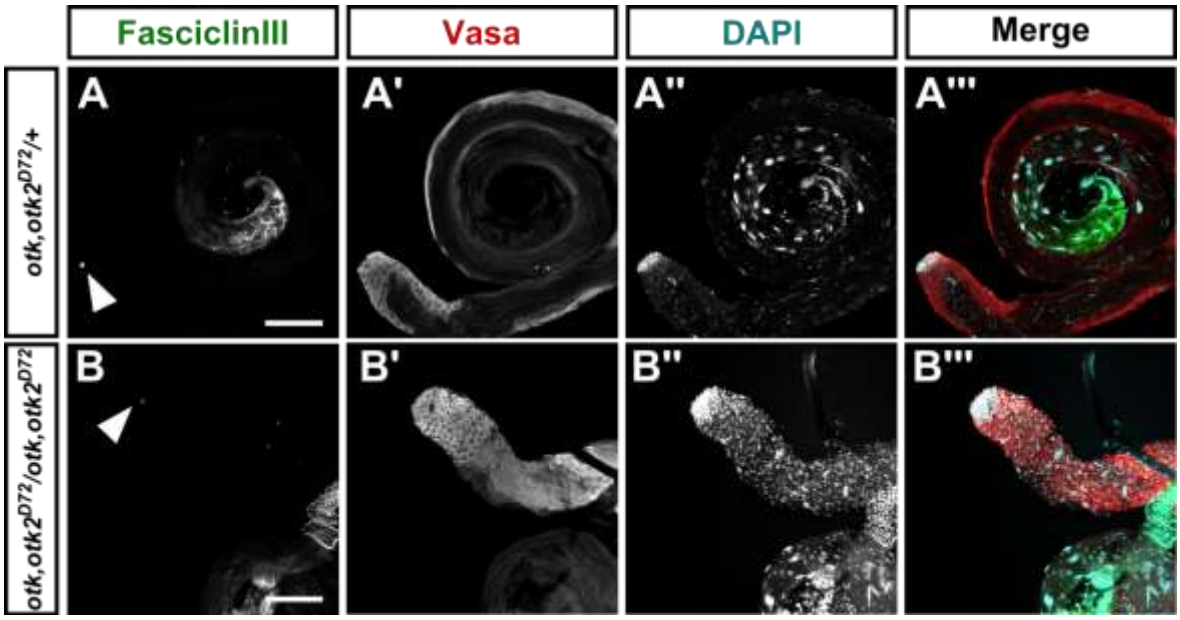

Fig. 36: Testes of *otk,otk2^{D72}* homozygous mutant males.
(A-C) Adult testes from males heterozygous **(A)** and homozygous **(B)** mutant for *otk,otk2^{D72}* were stained against FasciclinIII, which marks the hub (arrowhead in A and B) and terminal epithelium as well as Vasa, a marker for germline stem cells. Testis in (B) was unwound during the staining procedure. Scale bars: A-C = 100 µm.

To further confirm this, testes from males expressing a ProtamineB-eGFP (Raja & Renkawitz-Pohl, 2005) transgene were analyzed. During Drosophila spermatogenesis, histones are replaced by protamines to achieve sufficient chromatin condensation (Raja & Renkawitz-Pohl, 2005). Indeed, testes from males homozygous mutant for *otk,otk2^{D72}* contain all stages of development (Fig. 37Fig. 36 B, C), including late canoe stage (Fig. 36 B) and individualized spermatids with needle-shaped nuclei (Fig. 36 C), corresponding to testes from heterozygous control males (Fig. 36 A). Live imaging of mature sperm revealed that mature sperm from homozygous mutant males is motile as well (data not shown). I conclude that any late defects in spermatogenesis or sperm motility are unlikely to account for the observed sterility of *otk,otk2^{D72}/otk,otk2^{D72}* adult males.

However, after crossing of homozygous mutant *otk,otk2^{D72}* males expressing ProtaminB-eGFP to *white-* females, no sperm could be detected in the female reproductive tract in contrast to the control group with heterozygous mutant males (Appendix 1), even though the mating behavior was not affected (Caroline Ripp, personal communication). This finding strongly suggests that male sterility of *otk,otk2^{D72}/otk,otk2^{D72}* animals is caused by a structural or mechanical defect of the male reproductive tract.

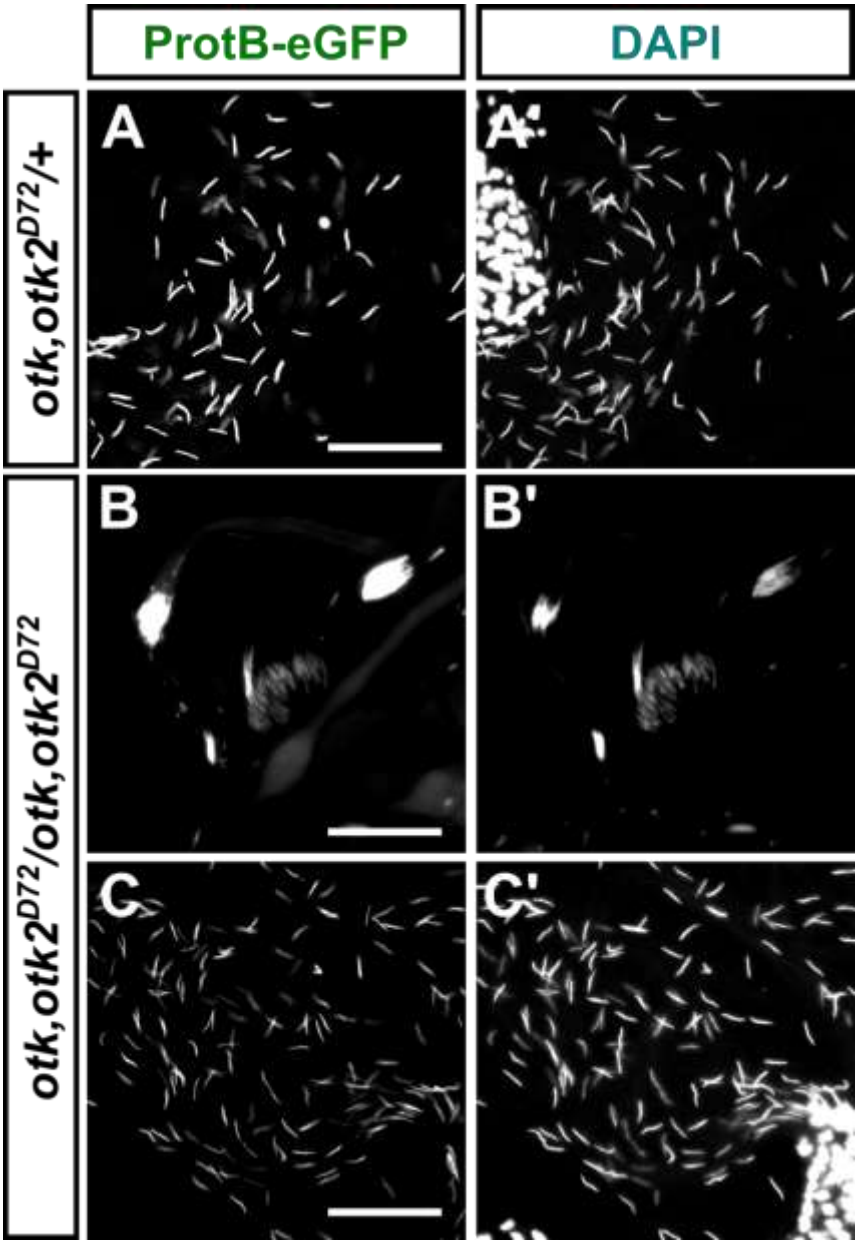

Fig. 37: Testes of *otk,otk2^{D72}* homozygous mutant males develop all stages of sperm development including motile sperm.
(A-C) Testes from adult males heterozygous **(A)** and homozygous **(B,C)** mutant for *otk,otk2^{D72}* carrying a ProtaminB-eGFP transgene. Images were taken by Caroline Ripp. Scale bars: A-C = 50 µm.

Wnt2 has been shown to be expressed in genital discs and to be involved in the attachment of the testes to the developing seminal vesicle as well as subsequent myoblast migration. Loss of *wnt2* results in male sterility due to defects in male reproductive tract formation (Kozopas *et al*, 1998). Next, I therefore analyzed the expression of Otk and Otk2 in genital discs. Indeed, Otk as well as Otk2 are expressed in both female and male genital discs as determined by antibody stainings and reporter expression (Fig. 38).

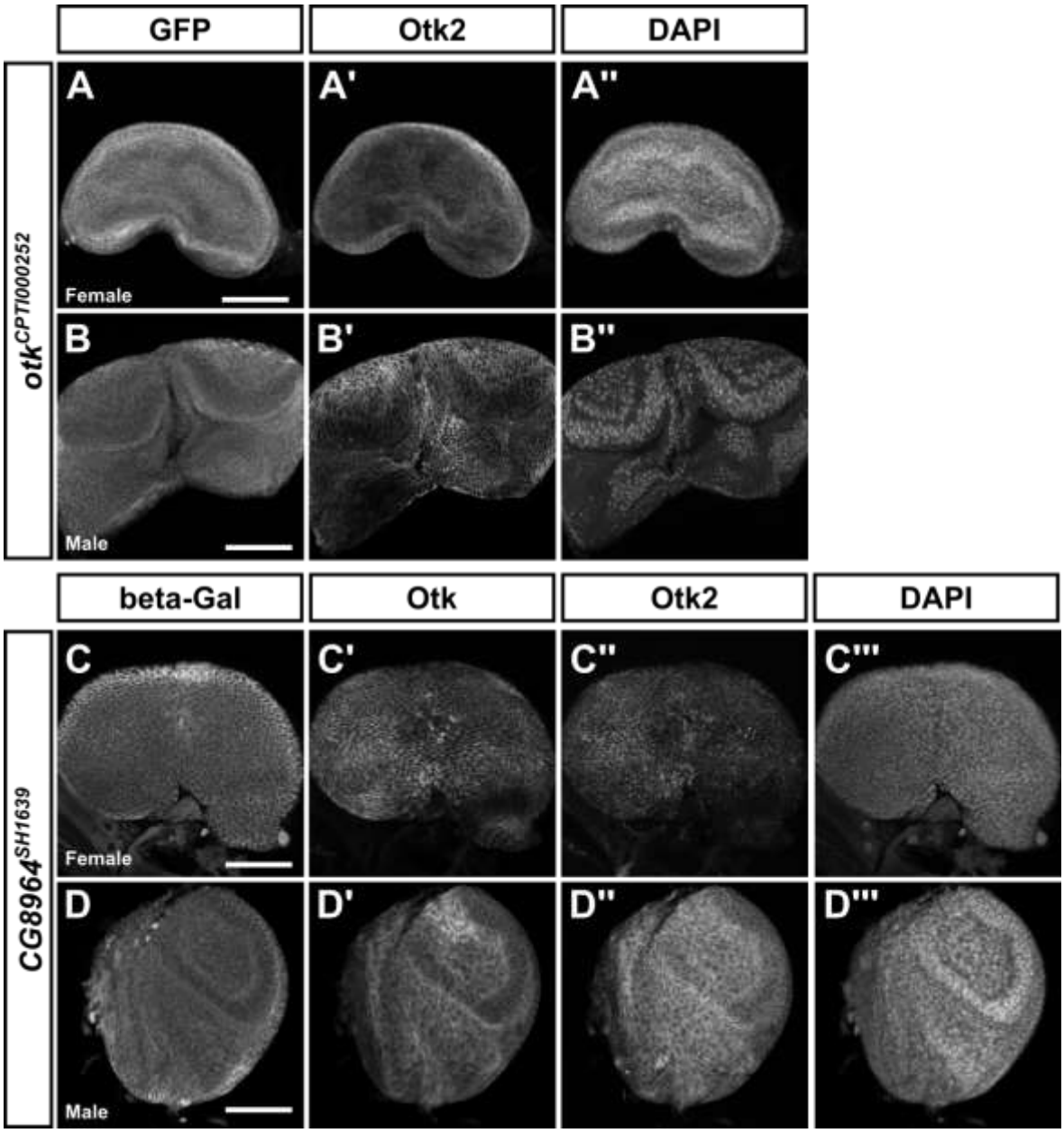

Fig. 38: Otk and Otk2 are expressed in both male and female genital discs.
(A,B) Genital discs from transgenic flies expressing a GFP gene trap in the *otk* locus were stained against GFP, Otk2 and DAPI. Otk staining could not be detected, indicating that the line is an allele of *otk*. **(C,D)** Genital discs from transgenic flies expressing a lacZ gene trap in the *otk2* locus were stained against beta-galactosidase, Otk, Otk2 and DAPI. Half of the male genital disc was lost during dissection in (D). Scale bar: 50 µm.

Since the testes of *wnt2* mutant males are not ensheathed by a continuous muscle layer (Kozopas *et al*, 1998), the muscle sheath of the male genital tract of *otk,otk2*^{D72} mutant males was analyzed. Surprisingly, the entire genital tract of *otk,otk2*^{D72} mutant males is surrounded by a complete muscle sheath (Fig. 39) and the filament organization of the single organs does not differ from that of heterozygous mutant control flies (data not shown). It was recently described that the seminal vesicle and

the sperm pump contain multinucleated striated muscles, whereas the paragonia and ejaculatory duct are enclosed by mononucleated striated muscle fibers. In contrast, the testes are encircled by smooth muscle fibers (Susic-Jung *et al*, 2012). All of these types of muscle fibers could be identified (Fig. 39) and no difference between *otk,otk2*^{D72} homo- and heterozygous mutant males was observed.

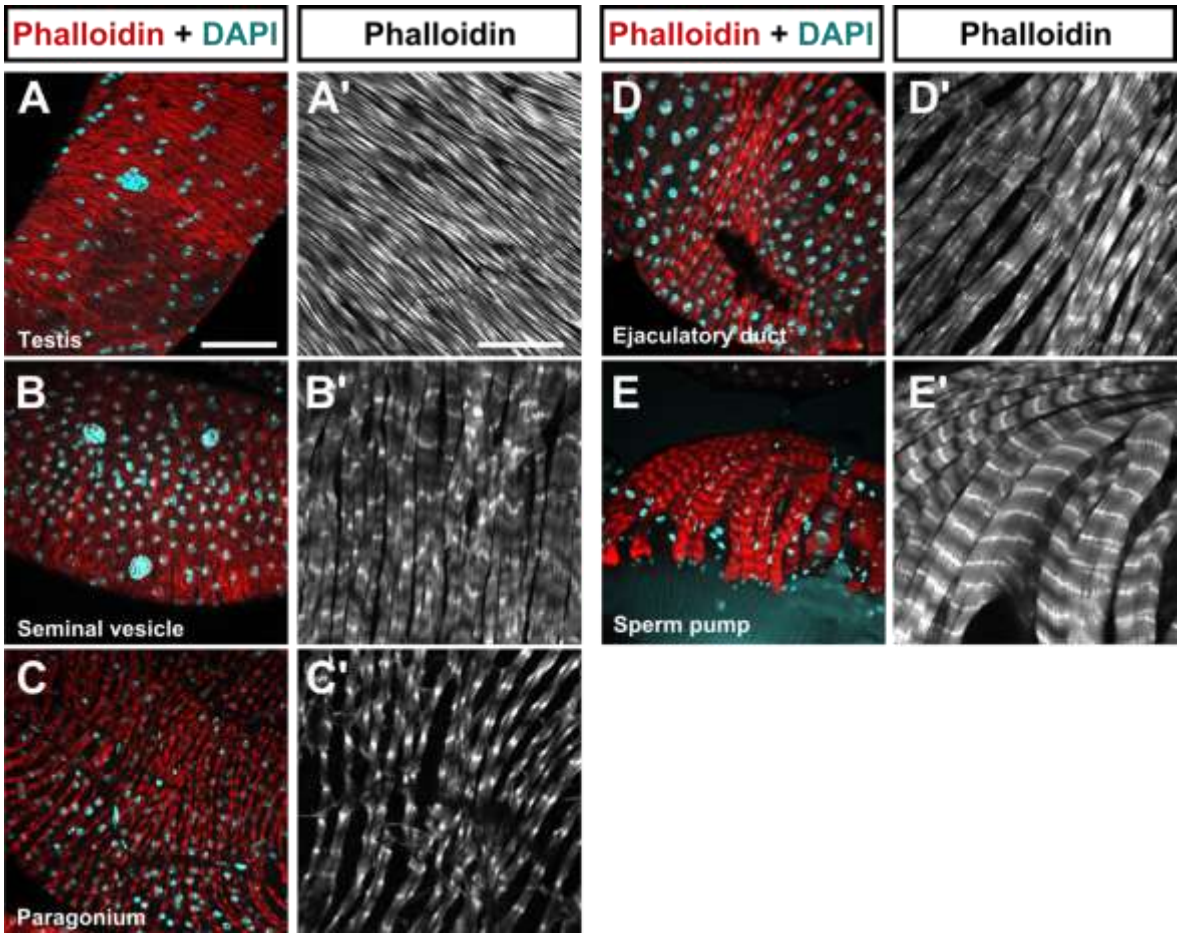

Fig. 39: The male reproductive system of homozygous *otk,otk2* mutants is surrounded by striated and smooth muscles.
The adult muscle sheaths of the different organs of the male reproductive tract of *otk,otk2*^{D72} homozygous mutant males was visualized with Phalloidin for F-actin detection. As described in (Susic-Jung *et al*, 2012) the testis **(A)** is surrounded by smooth muscle, whereas the seminal vesicles **(B)**, paragonia **(C)**, ejaculatory duct **(D)** and sperm pump **(E)** are ensheathed by striated musculature. Scale bars: A-E = 50 µm; A'-E' = 20 µm.

However, these analyzes revealed that the ejaculatory duct of homozygous mutant males is severely malformed. In contrast to the heterozygous control, the ejaculatory duct is much shorter and thickened (Fig. 40). The morphology of all the other organs of the reproductive tract is normal (Fig. 40). Further analysis of the muscle filaments of this organ uncovered that the ejaculatory duct indeed is severely obstructed (Fig.

41). Until the obstruction the actin filament organization is normal, however, from the obstruction site on it is strongly disorganized (Fig. 41).

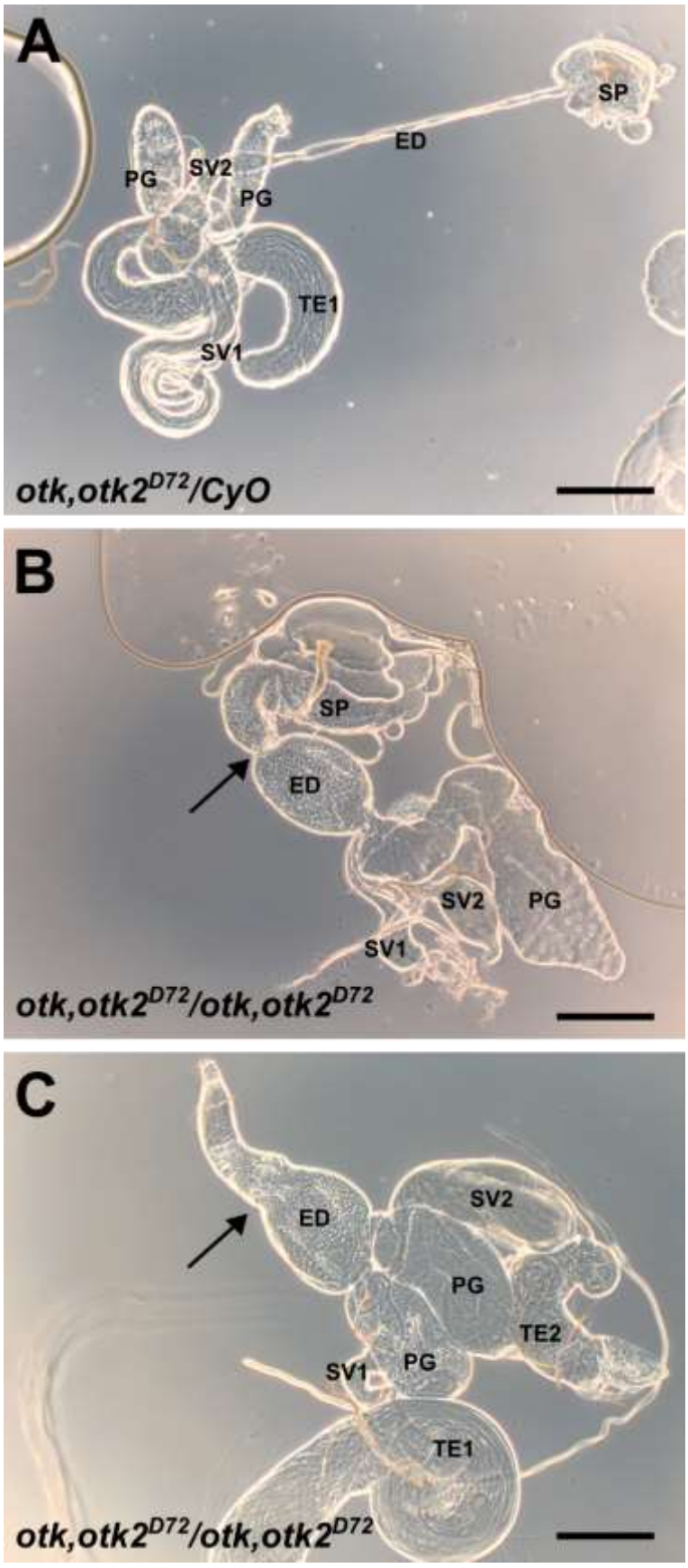

Fig. 40: *otk,otk2* loss of function leads to changes in the morphology of the ejaculatory duct.

Fig.40 (previous page): (A) Overview of the reproductive tract of males heterozygous for *otk,otk2^{D72}*. One testis was lost during dissection. **(B,C)** Overview of the reproductive tract of males homozygous for *otk,otk2^{D72}*. The arrow marks an obstruction in the ejaculatory duct, which is much shortened compared to (A). In (B) both testes were lost during dissection and in (C) the sperm pump was lost during dissection. ED, ejaculatory duct; PG, paragonium (accessory gland); SP, sperm pump; SV, seminal vesicle; TE, testis. Scale bars: 200 µm.

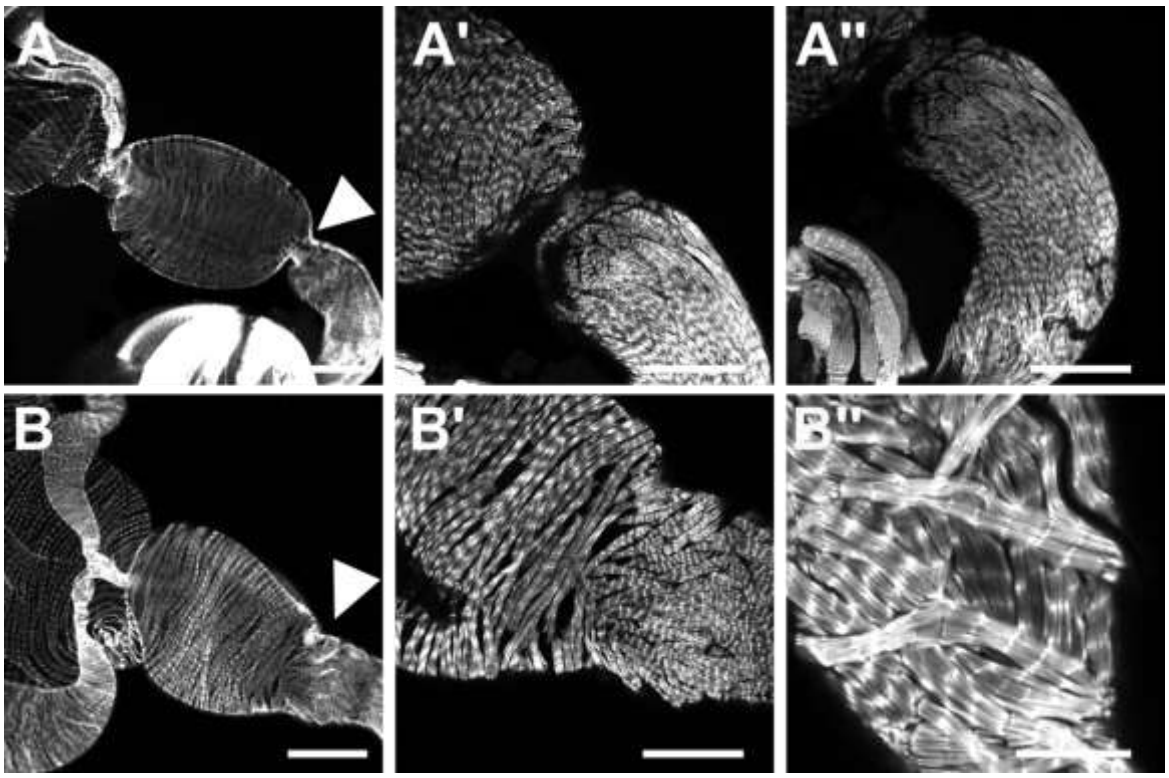

Fig. 41: Ejaculatory duct obstruction in *otk,otk2* homozygous mutant males.
The muscle sheath of the adult ejaculatory duct of *otk,otk2^{D72}* homozygous mutant males was visualized with Phalloidin for F-actin detection. **(A)** represents an enlarged view of the ejaculatory duct in Fig. 40 B and **(B)** of the one in Fig. 40 C. **A'** and **A''** as well as **B'** and **B''** are higher magnifications of the obstruction marked with an arrowhead in A and B, respectively. Scale bars: A,B = 100 µm; A', B', B'' = 50 µm; A'' = 20 µm.

3.7 *otk* and *otk2* interact with the Wingless receptor Frizzled

In *Drosophila*, Frizzled1 (Fz) and Frizzled2 (Dfz2) are functionally redundant receptors for Wingless, activating canonical signaling. Indeed, loss of both *fz* and *Dfz2* results in a phenotype that is virtually indistinguishable from *wingless* mutants (Bhat, 1998; Bhanot *et al*, 1999 ; Chen & Struhl, 1999; Müller *et al*, 1999). Additionally, Fz non-redundantly controls planar cell polarity signaling in *Drosophila* (Krasnow & Adler, 1994; Adler *et al*, 1997). In contrast, Dfz2 does not display any polarity signaling capacity and is most likely solely involved in canonical signaling. Its ligand

binding domain has a 10-fold higher affinity for Wg than the cysteine-rich domain of Fz (Zhang & Carthew, 1998; Chen & Struhl, 1999; Rulifson *et al*, 2000). I challenged the hypothesis that Otk and Otk2 could act as co-receptors for either Fz or Dfz2 by testing for both genetic and biochemical interactions.

Indeed, epistasis studies using different *fz* alleles indicated that otk,otk2 genetically interact, however, there were differences with regard to the combinations of *fz* alleles used (Table 18).

Table 18: *otk,otk2* genetically interacts with *fz*

otk^{A1} / otk^{A1}	viable
$otk2^{C26}$ / $otk2^{C26}$	viable
otk,$otk2^{D72}$ / otk,$otk2^{D72}$	viable (male sterile)
fz^{J22} / fz^{J22}	viable with PCP defects
fz^{H51}/ fz^{H51}	viable with PCP defects
fz^{R52} / fz^{R52}	lethal
fz^{P21} / fz^{P21}	lethal
fz^{R52} / fz^{J22}	viable with PCP defects
fz^{R52} / fz^{P21}	viable with PCP defects
fz^{R52}/ fz^{H51}	viable with PCP defects
fz^{J22} / fz^{P21}	viable with PCP defects
fz^{J22} / fz^{H51}	viable with PCP defects
fz^{H51} / fz^{P21}	viable with PCP defects
otk^{A1} / otk^{A1} ; fz^{J22} / fz^{J22}	viable with PCP defects
$otk2^{C26}$ / $otk2^{C26}$; fz^{J22} / fz^{J22}	viable with PCP defects
otk,$otk2^{D72}$ / + ; fz^{J22} / fz^{J22}	viable with PCP defects
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{J22} / +	viable
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{J22} / fz^{J22} (zygotic)	lethal
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{H51} / fz^{H51} (zygotic)	lethal
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{R52} / fz^{J22} (zygotic)	viable with PCP defects
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{R52} / fz^{P21} (zygotic)	viable with PCP defects
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{R52}/ fz^{H51} (zygotic)	viable with PCP defects
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{J22} / fz^{P21} (zygotic)	viable with PCP defects
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{J22} / fz^{H51} (zygotic)	lethal
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{H51} / fz^{P21} (zygotic)	viable with PCP defects
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{R52} / fz^{J22} (maternal)	viable with PCP defects
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{R52} / fz^{P21} (maternal)	lethal
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{R52}/ fz^{H51} (maternal)	lethal
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{J22} / fz^{P21} (maternal)	viable with PCP defects
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{J22} / fz^{H51} (maternal)	lethal
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{H51} / fz^{P21} (maternal)	lethal
otk^{A1} / otk^{A1} ; fz^{R52},Df(3L)DFz2 / +	viable
$otk2^{C26}$ / $otk2^{C26}$; fz^{R52},Df(3L)DFz2 / +	viable
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{R52},Df(3L)Dfz2 / +	viable
otk,$otk2^{D72}$ / otk,$otk2^{D72}$; fz^{R52},Df(3L)Dfz2 / fz^{R52},Df(3L)Dfz2	lethal

To verify the observed genetic interactions biochemically, GFP-tagged Otk and Otk2 and Myc-tagged Fz1 constructs under the control of an Actin promoter were co-overexpressed in S2r+ cells. Cell lysates were subjected to anti-Myc IP. Immunoblotting with anti-GFP antibody demonstrated that Otk-GFP as well as Otk2-GFP could be co-precipitated with Fz1-Myc (Fig. 42). The interaction between Otk-GFP and Fz1-Myc seemed to be quite weak, resulting in a rather faint band for Otk-GFP, however this was observed in several experiments (Fig. 42).

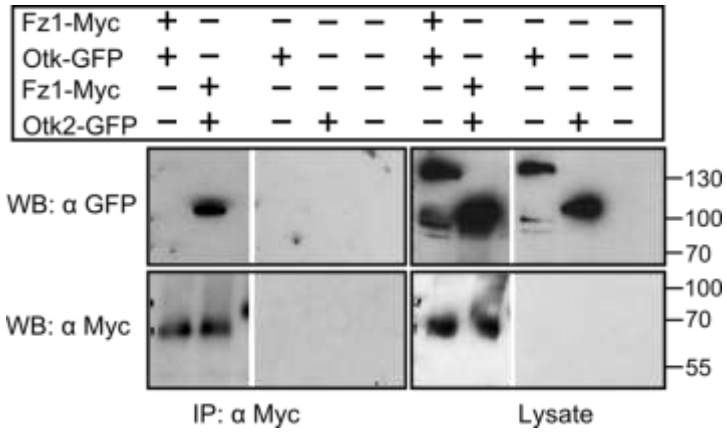

Fig. 42: Off-track and Off-track2 interact with Frizzled1.
Otk-GFP or Otk2-GFP and Fz1-Myc expression vectors were transfected as indicated in Drosophila S2r+ cells. Cell lysates were immunoprecipitated and analyzed by Western Blot with the indicated antibodies. IP, Immunoprecipitation; WB, Western Blot.

4 DISCUSSION

Despite their key role, Frizzled proteins are far from being the only receptors controlling Wnt signaling. The role of additional co-receptors and their emerging influence on signaling outcome has been appreciated within the past decade. Vertebrate PTK7 was described as a novel Wnt co-receptor that confers signaling specificity in response to different Wnt ligands. In this work, I characterized the *Drosophila* PTK7 homologs Off-track (Otk) and Off-track2 (Otk2) with regard to signaling mediated by different Wnt ligands present in *Drosophila*.

4.1 Expression of Otk and Otk2

Both RNA and protein expression analyses revealed that Otk and Otk2 are dynamically expressed during embryonic as well as larval development. Since only little is known about the precise role of *Drosophila* Otk so far, these analyses might give valuable hints regarding its function.

4.1.1 Otk and Otk2 co-localize with Wingless

During *Drosophila* embryonic development, Otk and Otk2 are expressed in segmental stripes that overlap with the position of Wingless (Wg) stripes (see 3.3.2, 3.3.3). This could mean that Otk and Otk2 are receptors for Wg in early embryonic development or that they are Wg targets. Since Otk expression is rather weak in early embryonic development and can only be detected from stage 9/10 on, whereas Wg expression is detectable much earlier, the latter possibility seems more likely. However, localization of Otk in segmental stripes was not changed in embryos homozygous mutant for *wg*.

Furthermore, Otk and Otk2 were found to be co-expressed with Wg in the visceral mesoderm (see 3.3.3). Wg expression in parasegment 8 of the visceral mesoderm as well as the developing fore- and hindgut was shown to be required for the formation of the second midgut constriction (Immerglück *et al*, 1990). The finding that Otk expression is broader than the region of Wg expression might be explained by the fact that Wg acts as a morphogen and induces expression of Otk over a distance. Apart from Wg, Wnt4 is expressed in the visceral mesoderm and developing gut as well.

Wnt4 transcripts were detected in the foregut, hindgut and two locations in the visceral mesoderm of the midgut, which correspond to the gastric caeca and the second midgut constriction (Graba *et al*, 1995). Formation of midgut constrictions was not affected in *otk* and *otk,otk2* mutants, indicating that *otk* and *otk2* most likely do not play a role in this process (data not shown). Furthermore, the localization of Otk in segmental stripes was not changed in embryos homozygous mutant for *wg* and *wnt4*, indicating that Otk expression most likely is not regulated by Wg or Wnt4.

A ChIP-on-chip approach aiming at elucidating the transcriptional network regulating smooth muscle development identified the *otk* enhancer to be a target of the transcription factor Biniou (homolog of vertebrate FoxF) in trunk visceral smooth muscle development (Jakobsen *et al*, 2007). In *biniou* mutant embryos, the visceral mesoderm expression of Otk was abolished, whereas expression in other regions of the embryo was not affected. Similarly, mouse PTK7 expression within the primitive streak was shown to depend on FoxF1 (Jakobsen *et al*, 2007). However, no direct connection between Biniou function and Wnt signaling, which might explain the peculiar expression pattern in the gut, was described so far.

4.1.2 Otk and Otk2 co-localize with Derailed

Otk is expressed within the *Drosophila* embryonic nerve chord, but shows enrichment in those axon tracts that cross the anterior commissure (AC). Interestingly, the Derailed (Drl) receptor tyrosine kinase is expressed exclusively by neurons that project in the anterior commissure. Drl was shown to be expressed in growth cones and axons of neurons as they enter the AC and extend through it, but it gets rapidly down regulated when they leave the AC (Callahan *et al*, 1995; Bonkowsky *et al*, 1999). Wnt5 on the other hand is expressed along the posterior commissure, where it acts as a repellent ligand for Derailed expressing neurons that cross the midline along the anterior commissure (Yoshikawa *et al*, 2003; Fradkin *et al*, 2004). Possibly, Otk/Otk2 and Drl interact in Wnt5-repulsive axon guidance along the AC.

4.1.3 Otk expression depends on Wnt2

To determine whether Otk expression might be controlled by Wnt signaling, I compared Otk expression in different *wnt* mutant backgrounds (3.4). Interestingly, Otk does not seem to be regulated by Wingless or Wnt4. Both the segmental localization in early embryos as well as the localization in the developing gut and

nervous system was not affected. However, the expression of Otk in the nervous system appeared to be slightly reduced in *wnt5* mutant embryos. Interestingly, overall Otk expression levels were strongly reduced in embryos homozygous mutant for *wnt2.* In particular, segmental stripe expression starting in stage 9/10 was abolished and expression in the developing gut as well as the nervous system was still detectable, but strongly reduced compared to heterozygous control embryos. This indicates that Otk is a target of Wnt2 signaling and that Wnt2 is required to maintain high Otk expression levels.

4.2 *otk* loss of function does not lead to lethality

Flies homozygous mutant for otk^{A1} were homozygous viable and did not show any phenotypic defects related to PCP or canonical signaling (see 3.6). This strongly disagrees with reports on the previously generated homozygous lethal otk^{3} allele (Winberg *et al*, 2001; Cafferty *et al*, 2004; Peradziryi *et al*, 2011). Since I have unambiguously shown that the complete *otk* locus is deleted in the otk^{A1} allele, this can only mean that the reported lethality of otk^{3} was due to a second site hit on the same chromosome and was not caused by mutation of *otk*.

Embryos mutant for otk^{3} were reported to display axon guidance defects in the CNS, but no data supporting this statement was shown in the respective publication (Winberg *et al*, 2001). In addition, the projection of SNa motor neurons to their muscle targets was demonstrated to be disturbed in 56% of otk^{3} homozygous mutants as assayed by Fasciclin II staining of motor projections. However, the underlying data seems rather weak since the representative image of an otk^{3} mutant embryo was taken at a different focal plane compared to the wild type control. Moreover, the authors claimed to have obtained similar defects upon Gal4-driven expression of short antisense Otk transcripts. Inconclusively, the UAS-containing gene-regulatory P-element utilized for this analysis is located even upstream of the *otk* 5'UTR (Winberg *et al*, 2001) and can therefore impossibly mediate the effects described.

Unfortunately, the finding that CNS longitudinal axon tracts display breaks and defasciculation defects in 15% of otk^{3} mutant embryos was not further commented on (Winberg *et al*, 2001). A similar phenotype was reported for *wnt5* mutant embryos

(Fradkin *et al*, 2004) and might hint at a possible connection between Otk and Wnt5/Drl signaling.

Furthermore, I tried to reproduce whether the otk^{A1} homozygous mutant animals display the photoreceptor targeting defects reported by another study (Cafferty et al., 2004). The ommatidia or single eye units in the adult *Drosophila* visual system each contain eight photoreceptor cells (R1-R8), which project into the optic lobes. Whereas R1-R6 cells connect to the lamina, R7 and R8 cells project to the medulla. In this study, Otk was shown to predominantly localize to the growth cones of R1-R6 photoreceptor axons and to be required for lamina-specific targeting of these axons. This conclusion was drawn from the observation that in otk^{3} mosaic animals expressing the R2-R5 marker *rough-τ-lacZ,* about one third of R2-R5 axons projected aberrantly into the medulla, instead of terminating in the lamina (Cafferty *et al*, 2004). Unfortunately, the origin of the reporter line was not referenced in the publication. However, inspection of a *rough-τ-lacZ* reporter line obtained from Christian Klämbt revealed that the wild-type reporter line showed the same phenotype as the otk^{3} mutants in (Cafferty *et al*, 2004) (data not shown). Therefore, the expression of *rough-τ-lacZ* in otk^{A1} mutant animals was not further analyzed.

A recent study reported a role for Otk in embryonic patterning by interaction with Wnt4 (Peradziryi *et al*, 2011). As described in 1.4.1and 1.4.3, patterning of the embryonic epidermis is mediated by segment polarity genes like *wingless*. Wingless is expressed at the posterior of each segment and specifies naked cuticle fate, whereas anterior cells express Engrailed and secrete denticles. Wnt4 has the capacity to oppose Wingless signaling in the ventral epidermis since ubiquitous overexpression of Wnt4 phenocopies loss of *wg* activity (Gieseler *et al*, 1999). However, *wnt4* loss of function does not influence embryonic patterning since homozygous mutant embryos hatch without obvious defects and die during larval stage (Cohen *et al*, 2002). otk^{3} mutant embryos published by (Winberg *et al*, 2001) were reported to have narrowed denticle belts and, conversely, overexpression of Otk led to ectopic denticles (Peradziryi *et al*, 2011). I could not reproduce any of these phenotypes me as neither otk^{A1} nor $otk,otk2^{D72}$ loss of function produced any cuticular defects (data not shown). Ubiquitous overexpression of one copy of full length Otk both under control of UASp (drives expression in the germline and somatic cells, this work) and UASt (drives expression in somatic cells, Winberg *et al*, 2001) resulted in viable flies, which did not display any obvious embryonic defects (data not shown). Strikingly, the previously

published conclusions were based on zoomed images of single segments and were not supported by any quantification (Peradziryi *et al*, 2011). Based on the finding that co-overexpression of Otk and Wnt4 leads to ectopic denticles, the authors concluded that Otk acts as a receptor for Wnt4 (Peradziryi *et al*, 2011). Yet, the same phenotype is already achieved by overexpression of Wnt4 alone and therefore might be mediated by any other receptor endogenously expressed in *Drosophila* embryos (Gieseler *et al*, 1999). Similarly, the destabilization of cytoplasmic Armadillo, which was ascribed to co-overexpression of Otk and Wnt4 (Peradziryi *et al*, 2011), has been previously described to be mediated by overexpression of Wnt4 alone (Gieseler *et al*, 1999). Moreover, loss of *otk* was shown to block the activity of overexpressed Wnt4, however, any quantification supporting this finding is lacking (Peradziryi *et al*, 2011). Finally, the authors claim that overexpression of Otk in wings leads to both (canonical) wing margin and planar cell polarity defects at the same time (Peradziryi *et al*, 2011) - such a phenotype cannot be explained by any existing signaling pathway model and could not be reproduced either (data not shown).

In conclusion, the misleading findings presented by Winberg, Cafferty and Peradziryi *et al*. did not contribute to elucidating the function of Drosophila *off-track*.

4.3 Drosophila Otks form homo- and heterodimers

Biochemical studies revealed that Otk and Otk2 can form homo- as well as heterodimers upon transient expression in S2r+ cells and that this interaction is most likely dependent on the transmembrane domain (see 3.2).

Homodimerization has been described for Xenopus PTK7 (Annette Borchers, personal communication) as well as for Drosophila Derailed (Jasprien Noordermeer, personal communication). Differential interaction of receptor subclasses might be a way to confer signaling pathway specificity. In this context, Drosophila Ryks similar to Otks were shown to form heterodimers with each other and interaction in both receptor classes seems to depend on the transmembrane domain (Jasprien Noordermeer, personal communication). It will be interesting to determine whether different Wnts are able to inhibit or increase dimerization and if this mechanism is required to recruit additional receptors to the membrane.

4.4 Otk and Otk2 might function as (co-)receptors in different Wnt signaling pathways

4.4.1 Possible role of Otk/Otk2 in Wnt2 signaling during male reproductive tract formation

Males homozygous mutant for *otk,otk2* are sterile. However, the morphology of testes including the germ cells and somatic cell types that support spermatogenesis are normal, suggesting that sterility is caused by structural and mechanical defects of the reproductive tract. This was supported by the finding that sperm from homozygous mutant males cannot enter the female reproductive tract even though no obvious changes in mating behaviour could be observed (Caroline Ripp, personal communication).

In *Drosophila*, the embryonic gonads give rise to the testes, whereas the genital disc gives rise to all other external genitalia and internal structures that connect to the testis, i.e. accessory glands, anal plate, ejaculatory duct and seminal vesicle (Estrada *et al*, 2003). During metamorphosis, the gonads and the somatic parts of the reproductive tract grow towards each other and finally join (Kozopas *et al*, 1998; Nanda *et al*, 2009). The testes, in which all stages of spermatogenesis take place, are surrounded by a basal membrane, a muscle layer and outermost pigment cells. Both outer pigment cells and the inner muscle layer are disturbed in Wnt2 mutants (Kozopas *et al*, 1998), leading to male sterility due to improper testis morphogenesis.

Since the results obtained for *otk,otk2* sterile males were very reminiscent of what is published for *Drosophila* Wnt2, I analyzed the sheath surrounding the male reproductive tract for the presence of pigment cells and the formation of the muscle sheath. In contrast to Wnt2 mutants, which completely lack pigment cells, the specification of these cells was not disturbed in *otk,otk2* homozygous mutant males. This can be explained by the recent finding that Wnt2 is expressed male-specifically in the somatic embryonic gonad and is necessary and sufficient for pigment cell precursor formation in the embryo (DeFalco *et al*, 2008b). In contrast, Otk expression could neither be detected in the embryonic somatic gonad nor the germ cells (data not shown) and is therefore unlikely to be involved in pigment cell formation.

Only little is known about the development and assembly of the muscle sheaths of the *Drosophila* male reproductive tract. Twist-expressing cells are associated with the genital disc and presumably are myoblast precursors. In *wnt2* mutant testes, myosin-

expressing tissue is present, even though covering the testes incompletely (Kozopas *et al*, 1998). This indicates that Wnt2 is most likely not required for muscle specification. Instead, Wnt2 seems to be involved in the attachment of the testes and the developing seminal vesicle. It was suggested that these muscle precursors migrate over the seminal vesicle onto the testis to form the muscle sheath and that Wnt2 might affect their migration or adherence (Kozopas *et al*, 1998). Migration defects in *wnt2* mutants might not be attributable to a loss of motility, but rather to a loss of the ability of cells to determine their final position. The entire genital tract of male sterile otk,otk2 mutant males is covered by a continuous muscle layer as determined by Phalloidin staining of actin filaments (3.6.4, Fig. 39), indicating that general muscle migration and attachment does not seem to be affected. However, the ejaculatory duct of these males is constricted and this obstruction most likely leads to the observed sterility. It is intriguing to re-investigate the phenotype of *wnt2* homozygous mutant males with regards to these findings.

Another study reported a requirement for Wnt2 for the correct patterning of direct flight muscles (DFMs), explaining the phenotype of held-out wings in *wnt2* mutant flies (Kozopas *et al*, 1998; Kozopas & Nusse, 2002). Such a phenotype could not be observed for *otk* or *otk,otk2* mutant flies, even when raised at higher temperatures (data not shown). Therefore it seems unlikely that Otk interacts with Wnt2 in DFM patterning.

Furthermore, Wnt2 was demonstrated to function in tracheal development together with Wingless (Llimargas & Lawrence, 2001). But since Otk is not expressed in the tracheal system, this signaling pathway was not further studied.

Interestingly, it should be mentioned in this context that *wnt4* loss of function also leads to female and male sterility. In *wnt4* mutant females the ovariolar sheath fails to migrate properly over the developing ovarioles. Wnt4 regulates cell movement during ovarian organogenesis by interaction with Dishevelled and Frizzled 2 (Dfz2), which was postulated to be the primary Wnt4 receptor in this process (Cohen *et al*, 2002). This was based on the observations that transheterozygous $Dfz2^{C2}/Dfz2^{C1}$ animals are sterile both as males and females (Chen & Struhl, 1999) and displayed the identical phenotype of folded ovarioles (Cohen *et al*, 2002). Dfz2-Dsh-Wnt4 signaling was also reported to regulate retinal axon guidance in the visual system (Sato *et al*, 2006). Since strong ubiquitous overexpression of UASp-Otk-GFP leads to female

sterility (data not shown) it will be interesting to investigate whether Otk might function in Dfz2-Wnt4 signaling as well.

4.4.2 Possible role of Otk/Otk2 in Wnt5/Drl signaling

Co-expression of Drl and Otk along the anterior commissure (3.3.3, Fig. 28, see also 4.1.2) might hint at a potential interaction between these receptor tyrosine kinases, which will have to be further characterized genetically and biochemically. Interestingly, both *wnt5* and *drl* mutants display subtle nervous system defects, but are homozygous viable (Dura *et al*, 1993; Callahan *et al*, 1995; Yoshikawa *et al*, 2003; Fradkin *et al*, 2004). This could be explained by the abundance of Wnt5 receptors and co-receptors acting in a redundant manner with Drl and one such candidate might be Otk. Preliminary experiments indicate that Otk/Otk2 enrichment at anterior commissures is abolished in drl mutant embryos (data not shown), supporting the hypothesis that Otk and Drl might indeed interact in guiding axons along the anterior commissure. Furthermore, Wnt5 and Drl act antagonistically in two postembryonic brain centers, the mushroom body (MB) and the antennal lobes (AL) (Moreau-Fauvarque *et al*, 1998; Grillenzoni *et al*, 2007; Yao *et al*, 2007; Sakurai *et al*, 2009). In these systems, the Wnt5 signaling is apparently mediated by other receptors and based on its expression and mild mutant phenotypes, Otk and Otk2 are possible candidates. Supporting this hypothesis, it RNAi knockdown of otk was reported to result in loss or reduction of MB α/β lobes (Shimizu *et al*, 2011).

4.4.3 Genetic interation of Otk/Otk2 with Fz

Both Otk and Otk2 were shown to interact biochemically with Fz, even though the Fz-Otk interaction seemed to be rather weak (3.7, Fig. 42). Furthermore, *otk,otk2;fz* triple mutants were homozygous lethal when the zygotic or maternal components of all three genes were removed (3.7). However, this interaction and the resulting lethality were dependent on the nature of *fz* alleles used (Table 18). The fz^{J22} mutant contains a mutation in the first intracellular loop and fz^{P21} harbors an insertion in the cysteine-rich domain. On the other hand, fz^{R52} and fz^{H51} contain a mutation in the third extracellular loop. Whereas fz^{P21}, fz^{R52} and fz^{H511} are cell non-autonomous, fz^{J22} was shown to act cell-autonomously (Jones *et al*, 1996; Povelones *et al*, 2005).

Possibly, Otk acts as a receptor for Wnts in some pathways and as a co-receptor together with Frizzled proteins in others, resulting in the effects observed. Similarly,

Ryk and Ror2 have been found to interact with Fz proteins even though they were shown to act as *bona fide* Wnt receptors in other systems (Lu *et al*, 2004a; Yamamoto *et al*, 2008). It is possible that these proteins function as co-receptors in a certain context. However, it is difficult to study, especially *in vivo*, whether proteins function as co-receptors in the same cell or as independent receptors on different cells. Likewise, Otk and Ror were found to interact biochemically as well (data not shown). Unfortunately, it is impossible to functionally classify these interactions as long as the main Otk/Otk2 ligands and potential signaling pathways have not been fully characterized.

4.5 Possible molecular mechanisms of Wnt/Otk signaling

The existence of a plethora of Wnt receptors suggests that they interact, leading to different signaling outcomes. Furthermore, it is not unlikely that many of them act redundantly. In the case of *Drosophila* Otk and Otk2 this is most probably the case since even a double knock out of both genes did not lead to any clear phenotypes in any of the Wnt pathways investigated. Based on previous expression and functional analysis one might speculate on potential molecular mechanisms regarding Wnt/Otk signaling.

4.5.1 Interaction with other receptors

Akin to its vertebrate homolog PTK7, Otk/Otk2 might be involved in the fine-tuning of signaling outcome. One model for PTK7 function is that a ROR2/Fz/Wnt complex could trigger PCP signaling, while the Lrp/Fz/Wnt complex could activate canonical Wnt signaling. Individual Wnts would favour the formation of one type of complex, thus explaining why some Wnts tend to activate PCP while others activate canonical signaling. In this context, PTK7, which binds the canonical Wnt3a and Wnt8 (Peradziryi *et al*, 2011), could inhibit canonical signaling by displacing LRP from the activating tripartite complex (Vincent & Beckett, 2011). It is proposed that PTK7 traps canonical Wnts in a non-canonical receptor complex thereby preventing them from activating canonical Wnt signaling (Peradziryi *et al*, 2011, 2012).

Regarding Wnt5-Drl/Ryk interaction regulating axon guidance in *Drosophila* and vertebrates as well as Mom-2/Lin-18 interaction controlling *C. elegans* vulval

development, the Ryk homologs are not considered to function as co-receptors but rather as bona fide Wnt receptors (Angers & Moon, 2009). In contrast, it was demonstrated for HEK293 cells that Wnt, Fz and Ryk form a ternary complex required for the activation of a β-catenin-TCF luciferase reporter (Lu *et al*, 2004a). Thus, Ryk seems to be both physically and functionally linked to canonical Wnt pathway components.

Similar models of function are also conceivable for Otk, however, it is urgently necessary to determine biochemically which *Drosophila* Wnts are bound by Otk/Otk2. Furthermore, it will be interesting to investigate whether Otk and Otk2 interact with other non-canonical receptors like Ror and Derailed and which signaling pathways might be influenced by these interactions. Unfortunately, no loss-of-function data are available for *Drosophila* Ror, making it impossible at the moment to perform genetic analysis which could determine whether Ror and Otk overlap in function or perform distinct roles.

In addition, it is likely that Otk/Otk2 also bind to receptors that are not directly associated with Wnt signal transduction. In this context, PTK7 and Otk were reported to interact with Plexins (Winberg *et al*, 2001; Wagner *et al*, 2010). This is a family of transmembrane receptors which bind ligands of the Semaphorin class and regulate axon guidance and general cell-cell communication (Peradziryi *et al*, 2012). In *Xenopus*, PTK7 and PlexinA1 affect cranial neural crest cell migration. Similarly, in *Drosophila*, Otk and PlexinA1 were reported to mediate Semaphorin1A-induced repulsive axon guidance, yet these results were based on the otk^{3} loss-of-function allele, which most likely carried a lethal second site hit, as discussed in 4.2. In light of a potential role of Otk/Otk2 in Wnt5/Drl-mediated repulsive axon guidance (see 4.4.2), it will be exciting to re-evaluate a possible connection between Otk/Otk2 and plexins.

4.5.2 Downstream signaling

The mechanism of vertebrate PTK7 as well as *Drosophila* Otk downstream signaling is still largely unknown. So far, no kinase activity could be reported for any of the homologs and, similarly to Derailed, they are believed to act as inactive kinases. The question arises, how apparently inactive Otk receptors would transduce Wnt signals. Different mechanisms are possible based on obervations in other systems.

Inactive Otk receptors might recruit catalytically active downstream effectors. In this context it was shown that *Xenopus* PTK7 is required for Dsh localization to the plasma membrane in (Shnitsar & Borchers, 2008) and this recruitment requires the adaptor proteins receptor of activated C kinase (RACK1) and protein kinase Cδ1 (PKCδ1) (Wehner *et al*, 2011). It remains to be investigated whether Otk is also involved in Dsh membrane recruitment and which other components might be required to achieve this.

Similarly, Drl lacking its C-terminal domain fails to misguide axons to the anterior path, indicating that Drl might also engage a cytoplasmic signaling pathway (Bonkowsky *et al*, 1999). Indeed, *drl* and *src64B*, a member of the Src family of tyrosine kinases, were found to interact during the formation of commissures during embryonic CNS developement (Wouda *et al*, 2008).

Another conceivable model of Otk action is protein cleavage and subsequent secretion or translocation of cleaved parts. Vertebrate PTK7 has recently been shown to be a target of membrane type-1 matrix metalloproteinase (MT1-MMP/MMP14). MT1-MMP recognizes a cleavage site in the seventh immunoglobulin domain of PTK7, thereby generating an extracellular fragment that is secreted and increases cell invasion. Since inhibition of MT1-MMP leads to similar convergent extension defects in zebrafish as does PTK7 loss of function, MT1-MMP-mediated proteolysis of PTK7 seems to be required for proper development (Golubkov *et al*, 2010; Peradziryi *et al*, 2012). Similarly, the introduction of an additional MT1-MMP cleavage site results in aberrant PTK7 proteolysis (Paudyal *et al*, 2010). These findings suggest that a tightly balanced ratio of membrane-bound and secreted fractions is indispensable for the proper function of vertebrate PTK7.

In case of Drl, it was shown that the Ryk sequence contains a γ-secretase cleavage site in its transmembrane region and that a C-terminal proteolytic fragment is released upon cleavage. Wnt3a promotes nuclear translocation of the Ryk intracellular domain, which is important for neural progenitor cell differentiation (Lyu *et al*, 2008).

5 SUMMARY AND CONCLUSIONS

Canonical and non-canonical Wnt signaling pathways have in common Frizzled (Fz) as the core Wnt receptor element. However, in the past decade, other transmembrane receptors have been shown to bind Wnt ligands, resulting in the activation of diverse intracellular signaling pathways. Apart from members of the Frizzled receptor family, Wnt receptors include the low-density lipoprotein receptor-related protein (LRP, *Drosophila* Arrow), the protein tyrosine kinase 7 (PTK7) as well as the receptor tyrosine kinase-like orphan receptor (Ror) and the related to tyrosine kinase (Ryk) families. Wnt interactions with these receptors often lead to cellular effects that are unrelated to β-catenin, possibly mediating a variety of non-canonical signaling pathways.

Vertebrate PTK7 (protein tyrosine kinase 7) encodes a catalytically inactive receptor-tyrosine kinase and is required for the control of planar cell polarity (PCP) in frogs and mice by acting as Fz co-receptor and inhibiting canonical Wnt signaling. PTK7 therefore also seems to belong to this group of co-receptors required for pathway selection, however, the exact molecular mechanism remains as well as its conservation between different species remain to be clarified.

In this work I describe the characterization of the Drosophila PTK7 homolog Off-track (Otk) and its paralog Otk2. Otk and its paralog Otk2 are co-expressed throughout embryonic and larval development. They are highly expressed in the visceral mesoderm as well as in the nervous system and enriched at anterior commissures. Otk and Otk2 interact biochemically and possibly function redundantly in Wnt signal transduction. Mutation of *otk* was reported not to cause PCP phenotypes in the fly, but is suggested to block canonical Wingless signaling in embryonic patterning. I found that in contrast to previous reports, flies homozygous for a complete knock-out of *otk* are viable and fertile and indeed do not show PCP phenotypes. Surprisingly, flies homozygous for a double knock-out of *otk* and *otk2* are viable as well and neither show PCP nor Wingless signaling phenotypes. However, *otk,otk2* double mutants are male sterile due to an obstruction in the ejaculatory duct and this is possibly due disturbed Wnt2 signaling. Overall defects in the nervous system cannot be observed, most likely because only a subset of neurons is affected. This could be explained by the abundance of receptors and co-receptors acting in a redundant manner. Indeed, expression data suggest a possible connection to Wnt5/Drl signaling. Furthermore,

genetic and biochemical studies revealed that *otk,otk2* genetically and biochemically interact with *fz1*, indicating that Otk/Otk2 might also function as Fz1 co-receptors in the signal transduction of Wingless or other members of the Wnt family under certain circumstances.

In summary, these results suggest that Otk and Otk2 indeed function as redundant receptors in several Drosophila Wnt signaling pathways, including Wnt2 as well as Wnt5/Drl signaling.

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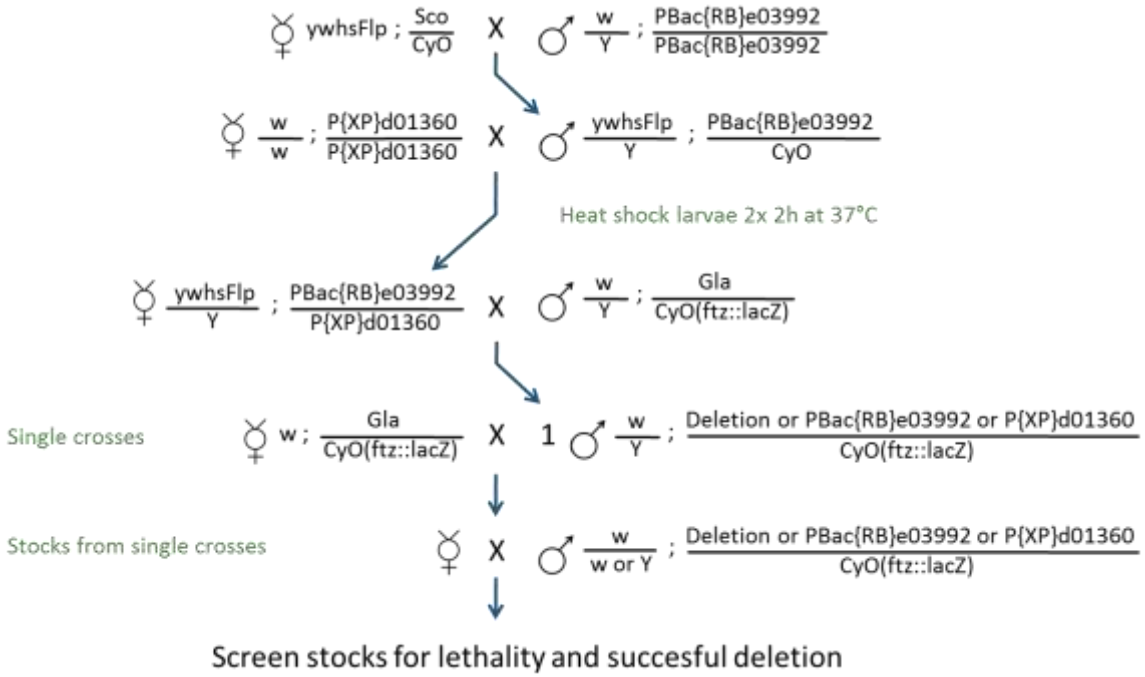
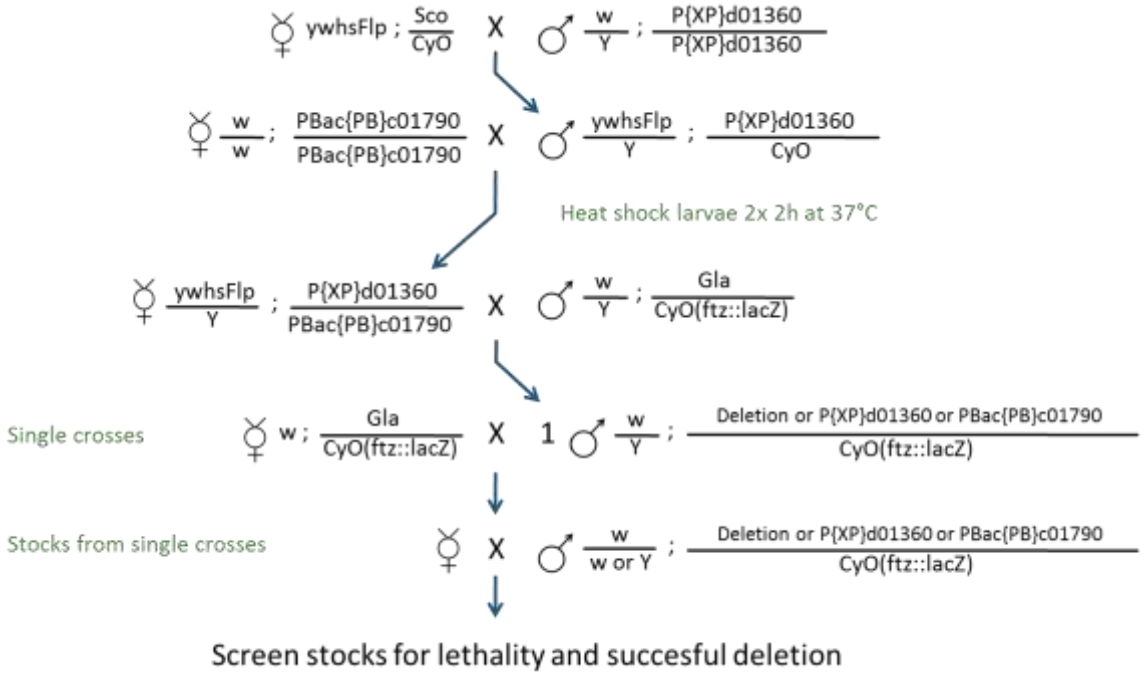
7 APPENDIX

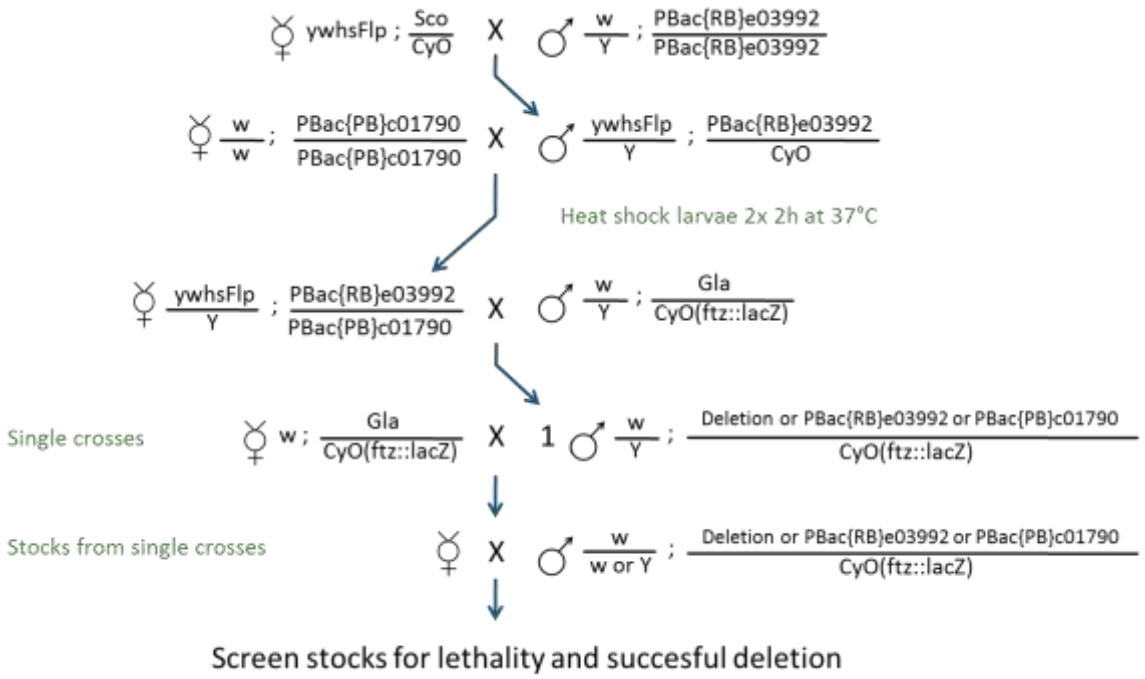
Appendix 1: Crossing scheme to obtain an *otk* deletion by FLP-FRT mediated recombination.



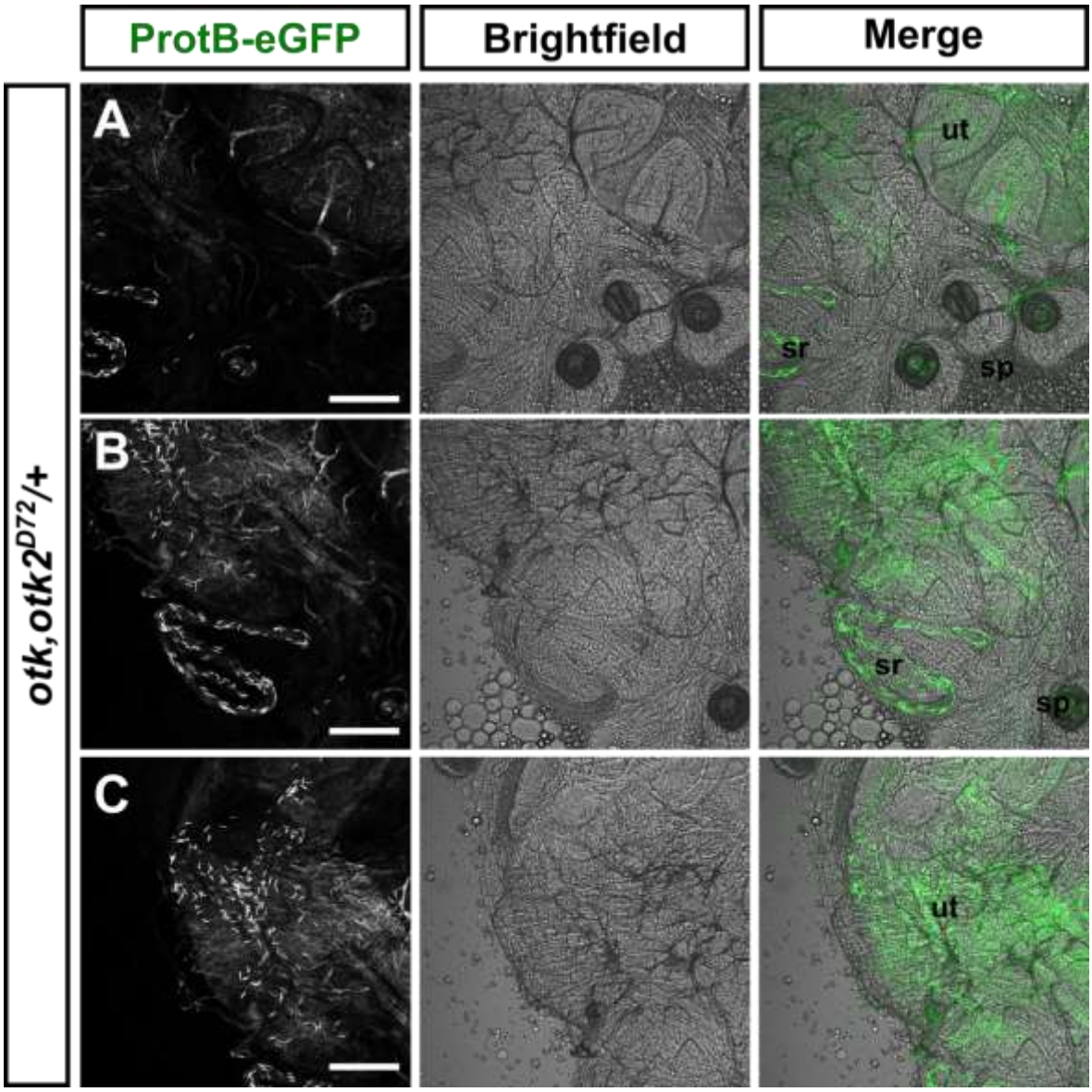
Appendix 2: Crossing scheme to obtain an *otk2* deletion by FLP-FRT mediated recombination.



Appendix 3: Crossing scheme to obtain an *otk,otk2* deletion by FLP-FRT mediated recombination.



Appendix 4: Sperm of *otk,otk2*^{D72} heterozygous mutant males enters the female reproductive tract. Reproductive apparatus from females after mating to adult males heterozygous mutant for *otk,otk2*^{D72} and carrying a ProtaminB-eGFP transgene. ut, uterus; sp, spermatheca; sv, seminal vesicle. Images were taken by Caroline Ripp. Scale bars = 100 µm.



Appendix 5: Vector maps and genomic regions of Otk and Otk2 (CD).