Identification and functional characterization of PTK7 ligands in *Xenopus laevis*

PhD Thesis
in partial fulfillment 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 Brest, Belarus

March 2011

Affidavit

Herewith I declare that I prepared the PhD thesis "Identification and functional characterization of PTK7 ligands in *Xenopus laevis*" on my own and with no other sources and aids than quoted.

31.03.2011 _____

Submission date Hanna Peradziryi

List of Publications

Peradziryi H*, Kaplan N*, Podleschny M, Liu X, Wehner P, Borchers A[#], Tolwinski N[#] (2011) PTK7/Otk interacts with Wnts and inhibits canonical Wnt signaling. **Embo J**, advance online publication Jul 19. [#] co-last authors. *These authors contributed equally

Wagner G*, **Peradziryi H***, Wehner P, Borchers A (2010) PlexinA1 interacts with PTK7 and is required for neural crest migration. Biochem Biophys Res Commun. 402(2):402-7. * These authors contributed equally.

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Acknowledgements

First of all, I would like to thank my supervisor Dr. Annette Borchers for giving me an interesting project to work on and for all the guidance, help and especially for the encouragement I have got during my work in her group.

I would like to thank Prof. Tomas Pieler for giving me the opportunity to perform my thesis in his department and the members of my Thesis Committee, Prof. Andreas Wodarz and Prof. Reinhard Schuh for the helpful discussions and advices I got during our meetings.

I would like to thank all my colleagues from the Developmental Biochemistry team, especially Martina Podleschny, Peter Wehner, Ilona Wunderlich and Iryna Shnitsar for the help and the wonderful atmosphere in the lab.

I would like to thank the International MSc/PhD program in Molecular Biology and especially Dr. Steffen Burkhardt for giving me an opportunity to study at the Göttingen University.

My special thanks go to my parents and husband for all the care and support.

Abstract

Wnt signaling pathways are evolutionary conserved and regulate numerous processes in both developing and adult organisms. The canonical Wnt signaling pathway regulates cell proliferation and differentiation through changes in gene transcription. Non-canonical or Planar cell polarity (PCP) Wnt signaling pathway regulates cell polarization and migration through changes in the cytoskeleton organization. These pathways have both shared players like Wnt, Frizzled (Fz) and Disheveled (Dsh) and pathway-specific players. However, it is still unclear, how a cell distinguishes between different Wnt pathways.

PTK7 (Protein tyrosine kinase 7) is a regulator of the PCP Wnt signaling pathway, which is required for neural tube closure and inner ear hair cell polarity in vertebrates. Several intracellular interaction partners of PTK7 have been described. For example, it has been shown that PTK7 recruits Dsh to the plasma membrane to regulate *Xenopus* neural crest migration, but upstream signals or PTK7 ligands have so far not been identified.

Here we show that PTK7 can interact with canonical Wnt ligands through Frizzled, suggesting that PTK7 may also regulate canonical Wnt signaling. Indeed, PTK7 can inhibit canonical Wnt signaling in both *Xenopus* and human cell culture. Furthermore, epistasis experiments show that PTK7 inhibits canonical Wnt signaling upstream of Dsh on the level of Wnt/Fz level. The knock-down of PTK7 activates canonical Wnt signaling. The neural tube closure defects induced by knock-down of PTK7 are partially rescued by the inhibition of canonical Wnt signaling, further confirming the inhibitory effect of PTK7 on canonical Wnt pathway. In addition to suppression of canonical Wnt signaling, PTK7 induces an ATF2-mediated transcription in *Xenopus*, indicating that it activates non-canonical Wnt signaling. In summary, we suggest that PTK7 in *Xenopus* promotes non-canonical Wnt signaling by inhibiting canonical Wnt signaling through the interaction with Wnt and Fz.

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1. Introduction

1.1 Wnt signaling pathways

Wnt signaling pathways are involved in numerous aspects of developmental processes and adult tissues homeostasis maintenance. During development Wnt signaling pathways regulate such vital events as axis specification, gastrulation and neural development (Marikawa 2006; Weaver and Kimelman 2004; Montcouquiol et al. 2006; Petersen and Reddien 2009). In an adult organism Wnt pathways remain essential for the regulation of cell proliferation and specification during the renewal of gut epithelium, hair follicle establishment, bone mass regulation as well as the regeneration of injured tissues (Logan and Nusse 2004; Clevers 2006). The impairment of the Wnt signaling may result in neurodegenerative diseases and different types of cancer like colon cancer, hair follicle tumors and leukemia (Cadigan and Nusse 1997; Logan and Nusse 2004; Clevers 2006; MacDonald et al. 2009).

The β-catenin Wnt signaling is the best studied Wnt signaling pathway. The β-catenin Wnt signaling pathway is also referred to as canonical Wnt signaling pathway, because it was the first Wnt pathway discovered, while the Wnt pathways discovered later are known as non-canonical Wnt signaling pathways. Among non-canonical Wnt pathways, Planar cell polarity (PCP) signaling is relatively better characterized.

The Wnt pathways include common components and pathway-specific players. The core components shared by Wnt pathways are Wnt ligands, Frizzled (Fz) receptors and cytoplasmic protein Dishevelled (Dsh). Wnt ligands bind its receptor Fz resulting in the activation of Dsh, which is the branching point of canonical and non-canonical Wnt pathways (Figure 1) (Wharton 2003; Huang and Klein 2004; Gao and Chen 2010). Although the upstream players of canonical and PCP Wnt signaling pathways are shared, the downstream effects of these signaling pathways are distinct. Canonical Wnt signaling regulates cell proliferation and differentiation through changes in transcription of β-catenin target genes, while PCP signaling pathway regulates cell polarization and migration through regulation of the small GTPases Rho and Rac involved in cytoskeleton remodeling (Figure 1) (Montcouquiol et al. 2006; Angers and Moon 2009). However, this description is very simplified and Wnt pathways include numerous shared and pathway-specific regulators. The important question, which remains mainly unanswered, is how the mechanism allowing the cell to distinguish between different Wnt pathways functions.

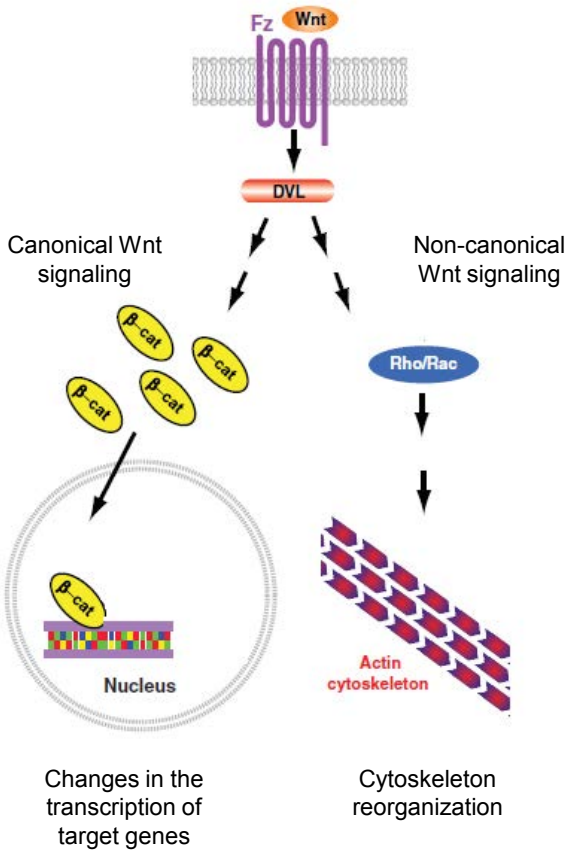

Figure 1. A simplified overview of canonical and non-canonical Wnt signaling pathways. Wnt ligands, Fz receptors and the cytoplasmic protein Dsh are the molecular components of both canonical and non-canonical Wnt signaling pathways. Wnt ligands bind to Fz receptors resulting in the activation of Dsh, which is the branching point of canonical and non-canonical Wnt pathways. The downstream effects of Dsh activation are distinct for different Wnt pathways. Canonical Wnt signaling regulates cell proliferation and differentiation through the changes in transcription of β-catenin target genes, while PCP signaling pathway regulates cell polarization and migration through the regulation of the small GTPases Rho and Rac involved in cytoskeleton remodeling (modified from Montcouquiol et al. 2006).

1.2 Key regulators of canonical and PCP Wnt signaling pathways

Wnt ligands, Frizzled (Fz) receptors and cytoplasmic protein Dishevelled (Dsh) are the key components of Wnt signaling, which are important for proper functioning of the canonical and the PCP signaling pathways (Figure 1). Vertebrates have a complex composition of Wnt ligands, Fz receptors and several additional co-receptors for Wnts like LRP6, Ror2 or Ryk (Nusse 2008; van Amerongen and Nusse 2009). The complex interactions between these proteins activate different Wnt pathways resulting in various cellular responses. However, our current knowledge is insufficient to fully explain how the complex responses are achieved.

1.2.1 Wnt proteins

The family of *Wnt* genes includes more than 100 members with high sequence homology. Wnt genes encode secreted glycoproteins with a highly conserved cystein-rich domain consisting of 22 to 24 cystein residues (Croce and McClay 2008; Angers and Moon 2009). Additionally Wnt proteins are palmitoylated on conserved cystein residues and hydrophobic in nature (Willert et al. 2003). The mutations of conserved cystein residues lead to a total loss of protein activity, indicating that palmitoylation is essential for biological function (Willert et al. 2003; Komekado et al. 2007). The glycosylation is shown to be required for the efficient secretion of Wnts (Komekado et al. 2007).

The two first independently identified *Wnt* genes are *wingless (wg)* in *Drosophila* (Cabrera et al. 1987; Rijsewijk et al. 1987) and *wnt-1* (originally called *int-1*) in mouse (Nusse and Varmus 1982; van Ooyen and Nusse 1984). Later *Wnt* genes have been found in a wide variety of metazoans but appear to be absent from plants, fungi and protists (Croce and McClay 2008). Multicellular animals have a complex composition of *Wnt* genes, which arose early in evolution. Already the sponge *Oscarella*, a primitive metazoan, has four *Wnt* genes (Nichols et al. 2006). cnidarians possess 14 *Wnt* genes, *Drosophila* has seven and humans nineteen (Croce and McClay 2008). The presence of multiple *Wnt* genes, which are often expressed in the same spatial-temporal pattern, indicates that Wnt ligands may induce very complex and specific effects on target tissues.

Wnt proteins can be subdivided in canonical and non-canonical ones depending on their ability to induce a second axis in *Xenopus* and their oncogenic properties. Canonical Wnt proteins are able to induce the formation of ectopic second axis in *Xenopus* and can morphologically transform mouse mammary cells, indicating an activation of canonical Wnt signaling, while non-canonical Wnt proteins lack these qualities and activate mostly non-canonical Wnt signaling (Wong et al. 1994; Du et al. 1995). Prototypic canonical Wnts are Wnt3a and Wnt8 and the prototypic non-canonical Wnts are Wnt5a and Wnt11. However, recent studies demonstrate that, depending on the proper receptor context, non-canonical Wnt proteins might be involved in canonical Wnt signaling activation. For example, the overexpression of Wnt5a does not induce axis duplication in *Xenopus*, however, if Wnt5a is overexpressed together with human Fz5 receptor, the formation of second axis takes place (He et al. 1997). These results prove that inability of Wnt5a to cause axis duplication in *Xenopus* is not caused by intrinsic properties of Wnt5a but rather by the lack of a proper Fz receptor. Additionally, Wnt11, a non-canonical Wnt signaling regulator in fish and frog (Heisenberg et al. 2000; Smith et al. 2000; Tada and Smith 2000;

Marlow et al. 2002), is involved in the axis formation in *Xenopus* embryos inducing local accumulation of nuclear β-catenin, which is a hallmark of the canonical Wnt signaling activation (Tao et al. 2005).

1.2.2 Frizzled receptors

Frizzled (Fz) proteins are Wnt receptors with seven membrane-spanning domains and an extracellular part with a conserved cystein-rich domain (CRD), which binds Wnts with high affinity (Figure 2) (Bhanot et al. 1996; Hsieh et al. 1999). The intracellular part of Fz has a Lys-Thr-x-x-x-Trp motif (where X is an any amino acid), which is important for the interaction with the cytoplasmic protein Dishevelled (Dsh) (Umbhauer et al. 2000; Wong et al. 2003).

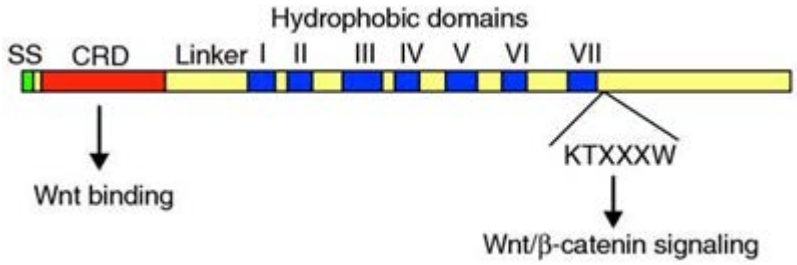

Figure 2. The structure of Frizzled. SS – signal sequence, CRD – cystein-rich domain. CRD is an extracellular domain essential for Wnt binding. The intracellular domain has a KTXXXW motif, which is required for the interaction with Dsh and the activation of canonical Wnt signaling. (modified from Huang and Klein 2004).

The first *Fz* gene was identified in *Drosophila* as a gene that disrupts the polarity of the fly epidermis (Vinson and Adler 1987). There are 10 *Fz* genes in humans, which are, similarly to *Wnt* genes, widely and dynamically expressed during development and often appear to be functionally redundant (Huang and Klein 2004). Both the properties of the Fz receptors and the availability of certain ligands and co-receptors may coordinate an activation of a specific Wnt signaling cascade (van Amerongen and Nusse 2009). For example, *Drosophila* Fz and Fz2 activate distinct branches of Wnt signaling because of different sequences of the intracellular domains. In *Drosophila* Fz is mainly involved in the establishment of PCP, while Fz2 activates canonical Wnt signaling (Bhanot et al. 1996; Tomlinson et al. 1997). However, the exchange of the cytoplasmic tails between Fz and Fz2 reverses their ability to activate certain Wnt signaling branches (Boutros et al. 2000). The specificity for a certain branch of Wnt signaling may also depend on the presence of

certain Wnt protein and Fz co-receptor. For instance, Fz activates canonical Wnt signaling together with low-density lipoprotein receptor-related protein 6 (LRP6) co-receptor in response to Wnt3a and non-canonical Wnt signaling together with receptor tyrosine kinase-like orphan receptor 2 (Ror2) co-receptor in response to Wnt5a (Grumolato et al. 2010).

1.2.3 Dishevelled

Dishevelled (Dsh) is a cytoplasmic protein, which is the branching point between different Wnt signaling cascades. *Dsh*, like the vast majority of PCP regulators, was originally identified in *Drosophila* as a gene important for proper polarity of wing and body hairs, and later the highly conserved homologues have been found in vertebrates (Wallingford and Habas 2005; Gao and Chen 2010). Structurally, Dsh protein has three conserved domains – the N-terminal DIX domain (Dishevelled-Axin), the central PDZ domain (Postsynaptic density 95, Discs Large, Zonula occludence-1) and the C-terminal DEP domain (Dishevelled, Egl-10, Plekstrin) (Figure 3) (Wallingford and Habas 2005; Gao and Chen 2010).

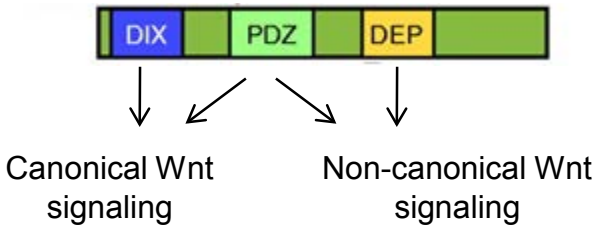

Figure 3. The structure of Dishevelled. Dsh has the N-terminal DIX domain, the central PDZ domain and the C-terminal DEP domain. The DIX domain activates the canonical Wnt signaling, the DEP domain activates the non-canonical Wnt signaling, the PDZ domain activates both signaling pathways (modified from Wallingford and Habas 2005).

Numerous Dsh interaction partners have been described, suggesting that Dsh acts as a scaffold protein. Dsh is able to selectively activate the required Wnt signaling branch through different domains (Wharton 2003; Gao and Chen 2010). The DIX domain is involved in the activation of canonical Wnt signaling, the DEP domain activates non-canonical Wnt signaling and the PDZ domain is required for both Wnt branches (Figure 3). The DIX domain mediates the formation of Dsh polymers, which are linked to the activation of canonical Wnt signaling (Schwarz-Romond et al. 2005). The PDZ domain interacts with the cytoplasmic tail of the Fz receptor and is essential for the activation of

canonical and PCP pathways (Wong et al. 2003; Punchihewa et al. 2009). The PDZ domain also mediates the PCP pathway by activating the small GTPase Rho (Habas et al. 2001). The DEP domain interacts with the small GTPase Rac to activate the PCP pathway (Habas et al. 2003; Rosso et al. 2005).

1.3 Molecular mechanism of canonical Wnt signaling pathway

Canonical Wnt signaling controls cell specification and proliferation through the regulation of the transcription factor β-catenin, which activates the expression of numerous genes (MacDonald et al. 2009).

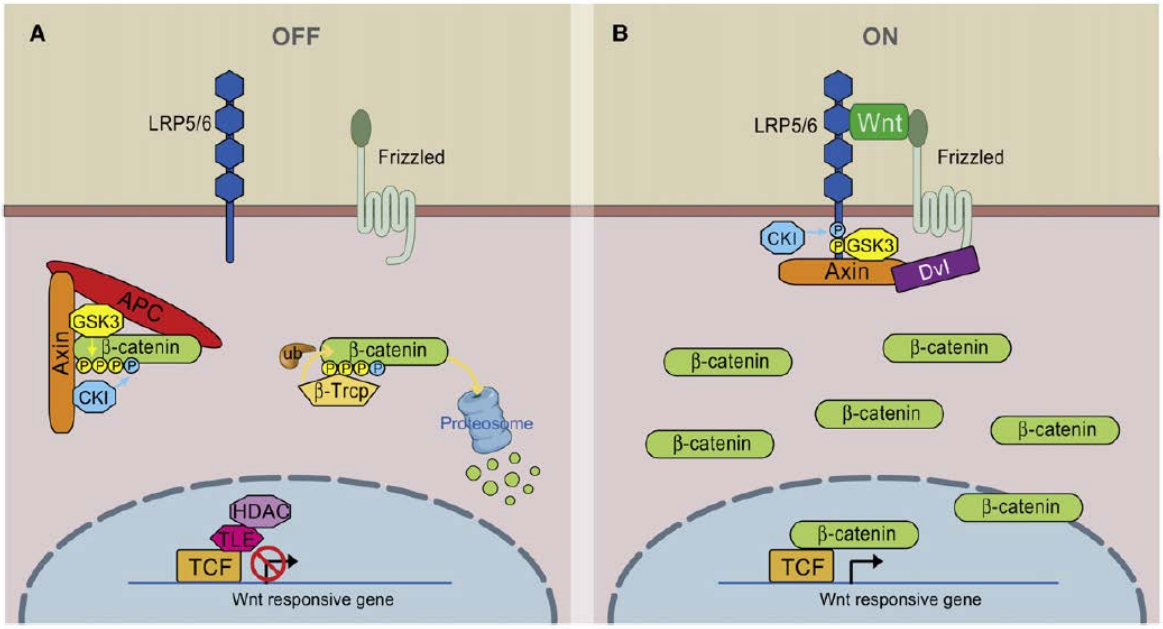

Figure 4. Overview of canonical Wnt signaling. (A) In the absence of Wnt, cytoplasmic β-catenin forms a complex with Axin, APC, GSK3 and CK1 and is phosphorylated by GSK3 and CK1. Phosphorylated β-catenin is targeted to proteosomal degradation by β-Trcp E3 ubiquitin ligase. In the nucleus β-catenin-responsive genes are repressed by the transcriptional repressors TCF/LEF, co-repressor Groucho/TLE and histone deacetylases HDAC. (B) In the presence of a Wnt ligand, a Fz/LRP receptor complex is formed. Dsh (Dvl) is recruited to the plasma membrane by Fz inducing LRP phosphorylation and axin recruitment to the plasma membrane, which results in the disruption of the destruction complex and the stabilization of β-catenin in the cytoplasm. Stabilized β-catenin enters the nucleus, where it serves as a co-activator for TCF/LEF to induce the transcription of responsive genes (from MacDonald et al. 2009).

When canonical Wnt signaling is inactive a group of proteins named the destruction complex promotes β-catenin degradation resulting in the lack of β-catenin-inducible genes expression. The destruction complex consists of the product of the *adenomatous polyposis*

coli gene (APC), Axin, glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1). Serine/threonine kinases CK1 and GSK3β phosphorylate β-catenin, which is subsequently ubiquitinated by β-Trcp E3 ubiquitin ligase resulting in its degradation (Figure 4 A) (Yost et al. 1996; Amit et al. 2002; Liu et al. 2002; Yanagawa et al. 2002). Axin is a scaffold protein that brings together CK1, GSK3β and β-catenin (Hart et al. 1998; Kishida et al. 1998). APC, like axin, is a tumor suppressor protein with a not fully understood function. Several models explaining the role of APC in the canonical Wnt signaling exist. It has been proposed that APC protects phosphorylated β-catenin from phosphatases enhancing its proteosomal degradation (Su et al. 2008). The second model suggests that APC competes with axin for β-catenin binding, removing phosphorylated β-catenin from the destruction complex and therefore promoting a next round of β-catenin phosphorylation (Xing et al. 2003). It was also proposed that APC may function in the nucleus by promoting the export of β-catenin to the cytoplasm leading to the inhibition of canonical Wnt signaling (Henderson and Fagotto 2002).

The canonical Wnt signaling pathway is activated when a Wnt ligand binds to its receptor Fz and co-receptor low-density lipoprotein receptor-related protein (LRP) (Figure 4 B). It has been shown that Wnt, Frizzled and LRP can form a triple complex *in vitro* (Cong et al. 2004) and that Frizzled/LRP chimeric protein can activate canonical Wnt signaling without Wnt protein (Tolwinski et al. 2003; Holmen et al. 2005). These results suggest that the main function of Wnt is to induce the formation of the Fz/LRP complex. The intracellular domain of Fz interacts with Dsh, which is essential for canonical Wnt signaling activation (Wallingford and Habas 2005). Despite very intensive studies, the function of Dsh still remains enigmatic. It is known that both Fz and Dsh are involved in Wnt-dependent LRP6 phosphorylation (Bilic et al. 2007; Zeng et al. 2008). When Wnt is present, the Fz/Dsh complex recruits the destruction complex component Axin together with GSK3β and CK1 to the plasma membrane where GSK3β can phosphorylate LRP6 at five conserved PPPSPxS motifs (Cliffe et al. 2003; Zeng et al. 2008). This step is called the initiation of canonical Wnt signaling (Figures 4 B, 5). Phosphorylated LRP6 in turn recruits more Axin/GSK3β complex to the plasma membrane leading to the signal amplification (Figure 5) (Baig-Lewis et al. 2007). Microtubule actin cross-linking factor 1 (MACF1) plays a role in the translocation of the Axin/GSK/CK1 complex to the plasma membrane (Chen et al. 2006) When axin is recruited to the plasma membrane, β-catenin is released from the destruction complex and it can enter the nucleus (Figure 4 B). In the nucleus β-catenin binds TCF/LEF transcription factors (Behrens et al. 1996; Molenaar et

al. 1996; Van de Wetering et al. 1996). Without β-catenin, TCF/LEF interacts with Groucho (Transducin-like enhancer protein, TLE) and histone deacetylase (HDAC) and the transcription of target genes is inhibited (Cavallo et al. 1998; Billin et al. 2000). However, when β-catenin enters the nucleus, it displaces Groucho, recruits different co-activators and the transcription is induced (Figure 4 B) (Hecht and Kemler 2000).

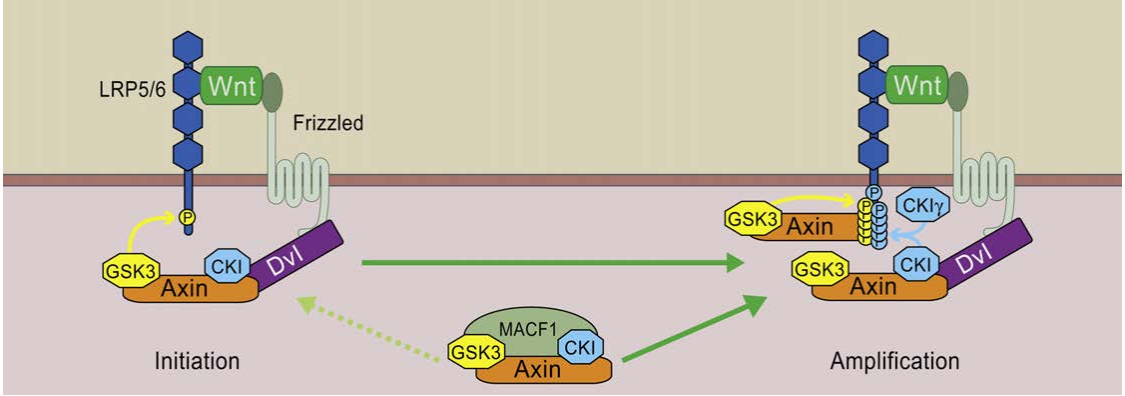

Figure 5. Initiation and amplification steps of canonical Wnt signaling. Wnt ligand forms a complex with Fz and LRP at the plasma membrane and Fz recruits Dsh (Dvl). Dsh in turn recruits the Axin/GSK/CK1 complex and GSK phosphorylates LRP at PPPSP motifs (Initiation). Being phosphorylated, LRP induces recruitment of more Axin/GSK/CK1 complexes to the plasma membrane, which robustly phosphorylates many LRP molecules at PPPSP motifs (Amplification). MACF1 plays a role in the translocation of Axin/GSK/CK1 complexes to the plasma membrane (from MacDonald et al. 2009).

1.4 Role of canonical Wnt signaling in early *Xenopus* development

Canonical Wnt signaling controls numerous developmental processes in multicellular organisms. During early embryogenesis it is a key regulator of body axis induction, embryo patterning and organogenesis (Logan and Nusse 2004; Weaver and Kimelman 2004; Marikawa 2006; Petersen and Reddien 2009).

1.4.1 Establishment of the dorsal-ventral axis in *Xenopus*

Xenopus has been used as a classical model to study the role of canonical Wnt signaling in early body plan formation. In frog canonical Wnt signaling is activated at the onset of gastrulation in the dorsal part of the embryo or Spemann organizer. The dorsal-ventral (D-V) axis in frog is established immediately after fertilization (Moon and Kimelman 1998; Weaver and Kimelman 2004). When a sperm enters an egg it induces the rapid polymerization of microtubules, which in turn leads to the shift of the cortical cytoplasm to the future dorsal side of the embryo (Figure 6) (Vincent and Gerhart 1987;

Elinson and Rowning 1988). As a result of cortical rotation, the activators of canonical Wnt signaling Wnt11, Dsh and GBP (GSK3β-binding protein), which are enriched in the cortical cytoplasm, are placed to the future dorsal part of the embryo (Marikawa et al. 1997; Miller et al. 1999; Weaver et al. 2003; Tao et al. 2005). At the onset of gastrulation Wnt11, GBP and Dsh stabilize β-catenin thereby inducing the transcription of β-catenin-responsive genes *Siamois* and *Twin*. Siamois and Twin proteins are homeobox transcription factors, responsible for induction of various genes, which promote the formation of the Spemann organizer (Lemaire et al. 1995; Laurent et al. 1997). The organizer dorsalizes the surrounding tissue, induces the formation of the neural tube and becomes dorsal mesoderm itself. Depletion of β-catenin results in ventralized embryos, which lack body axis (Figure 7) (Heasman et al. 1994). Similarly, if microtubular polymerization is blocked by UV irradiation, the embryos become ventralized and develop no dorsal structures (Figure 7) (Gerhart et al. 1989). In contrast, the overexpression of activators of canonical Wnt signaling like Wnt8, Dsh or β-catenin in the future ventral part of the embryo results in *Xenopus* axis duplication (Figure 7) (Sokol et al. 1991; Funayama et al. 1995; Rothbacher et al. 1995; Sokol et al. 1995).

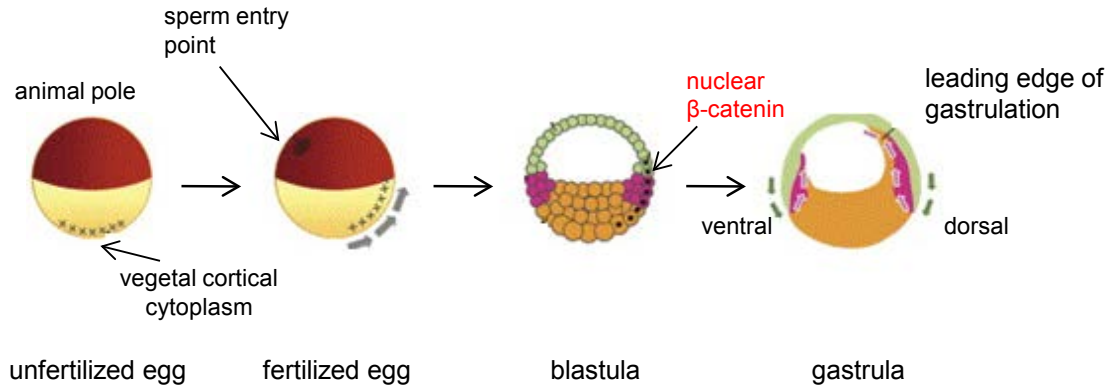

Figure 6. Establishment of dorsal-ventral axis in frog. The D - V axis in *Xenopus* is established during fertilization. The entry of a sperm induces the cortical rotation, a shift of cortical cytoplasm towards the point of a sperm entry. The cortical cytoplasm contains activators of canonical Wnt signaling, which due to cortical rotation are placed in the future dorsal side of an embryo. At the onset of gastrulation canonical Wnt signaling is induced at the dorsal side of an embryo to promote the formation of dorsal structures (from Marikawa 2006).

The ability of *Xenopus* embryos to develop a second axis in response to the activation of canonical Wnt signaling is used in a second axis assay to study the canonical Wnt signaling. This assay substantially contributed in our understanding of canonical Wnt signaling. It provides very fast and easy readout for canonical Wnt signaling. Already at

the four-cell stage the future dorsal and ventral blastomeres can be distinguished visually by size and color. The injection of activators of canonical Wnt signaling into the future ventral side induces the formation of second axis in the embryo (Figure 7) and, vice versa, the inhibitors of canonical Wnt signaling induce the formation of ventralized embryos lacking dorsal structures (Figure 7) (De Robertis and Kuroda 2004). Dorsal and ventral injections allow to analyze rapidly if a candidate induces ectopic axis or inhibits the axis formation. Additionally, it can be tested if a protein of interest enhances or represses active Wnt signaling when its mRNA is co-injected together with *Wnt*, *Dsh*, *β-catenin* or other canonical Wnt signaling activator mRNA ventrally.

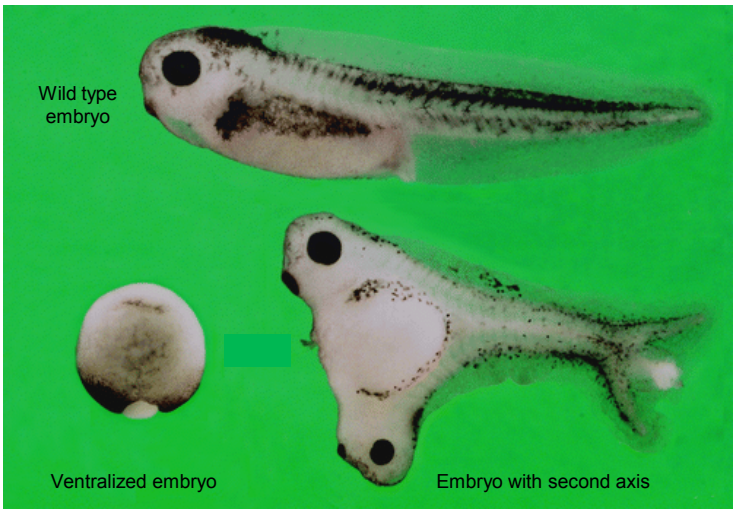

Figure 7. A wild type *Xenopus* embryo, a ventralized embryo and an embryo with second axis. Normal wild type embryo is shown on the top, the ventralized embryo without dorsal structures is shown in the bottom at left, the embryo with two axes is shown in the bottom at right (from De Robertis and Kuroda 2004).

1.4.2 Anterior-posterior patterning of the neural tube in *Xenopus*

Canonical Wnt signaling regulates patterning of the anterior-posterior (A - P) axis in diverse model systems like sea urchin, planarians, *Zebrafish*, *Xenopus* and mouse (Petersen and Reddien 2009).

Neuroectoderm in *Xenopus* gives rise to forebrain, midbrain, hindbrain and spinal cord precursor tissues, which are induced at the late gastrula stage. At this stage Wnts are produced at the posterior region of the neural plate and Wnt inhibitors like Dickkopf (Dkk), Frisbee (Frzb) and Cerberus are produced in the anterior region of neural plate, so A-P gradient of β-catenin activity is created in the neuroectoderm (Christian and Moon 1993; McGrew et al. 1995; Leyns et al. 1997; McGrew et al. 1997; Glinka et al. 1998;

McGrew et al. 1999; Kazanskaya et al. 2000). The gradient of β-catenin activity is required for the proper A-P neuroectoderm patterning, namely, Wnt signaling must be repressed anteriorly and activated posteriorly (Kiecker and Niehrs 2001). Kiecker and Niehrs overexpressed different amounts of Wnt8 or Wnt inhibitor Frzb1 in *Xenopus* embryos and compared anterior (*Bf1*, marks forebrain), middle (*Otx2*, marks fore- and midbrain) and posterior (*Krox20*, marks midbrain/hindbrain boundary) neural tube markers in injected and control embryos (Figure 8 A - C) (Kiecker and Niehrs 2001). These and other experiments showed that an increased canonical Wnt signaling activity posteriorizes neuroectoderm and the expression of posterior markers is expanded towards anterior pole, while the anterior markers are lost (Figure 8 B) (Fredieu et al. 1997; Kiecker and Niehrs 2001). In contrast, when canonical Wnt activity is decreased, neuroectoderm cells acquire the anterior identity and anterior markers will expand and shift more posteriorly (Figure 8 C) (Wang et al. 1997; Glinka et al. 1998; Kiecker and Niehrs 2001). In conclusion, dose-dependent Wnt signaling is necessary and sufficient for the A-P patterning of neural tube (Kiecker and Niehrs 2001).

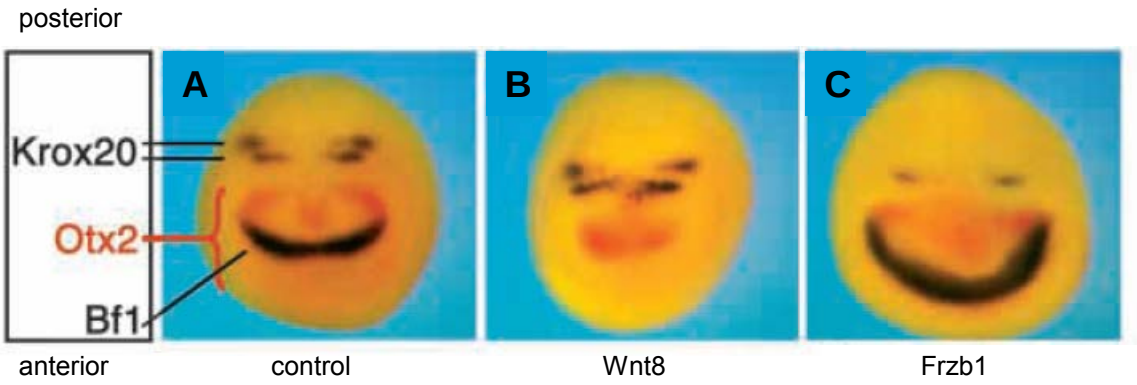

Figure 8. Wnt signaling pathway regulates anterior-posterior patterning of *Xenopus* neural tube. (A - C) Modulating the level of Wnt signaling changes the expression of regional-specific markers of the neural plate. *Bf1* – forebrain marker, *Otx2* - fore- and midbrain marker, *Krox20* - midbrain/hindbrain boundary marker. (A) Control embryos showing normal distribution of *Bf1*, *Otx2* and *Krox20*. (B) Overexpression of Wnt8 posteriorizes the neural plate: *Bf1* disappears, *Otx2* is reduced and *Krox20* shifts anteriorly. (C) Overexpression of Wnt inhibitor Frzb1 anteriorizes the neural plate: *Bf1* and *Otx2* are expanded at the expense of *Krox20* (from Kiecker and Niehrs 2001).

1.5 Developmental processes regulated by PCP signaling pathway

PCP signaling pathway regulates the establishment of cell polarity in the plain of an epithelium. It was first identified in *Drosophila* as a pathway regulating the polarization of

adult cuticular structures and eyes (Vinson and Adler 1987; Adler 1992). For example, *Fz* mutant flies are viable but have typical PCP phenotypes like disturbed orientation of wing hairs and bristles on notum and legs, as well as disturbed orientation of ommatidia in the eyes (Vinson and Adler 1987; Adler 1992; Zheng et al. 1995).

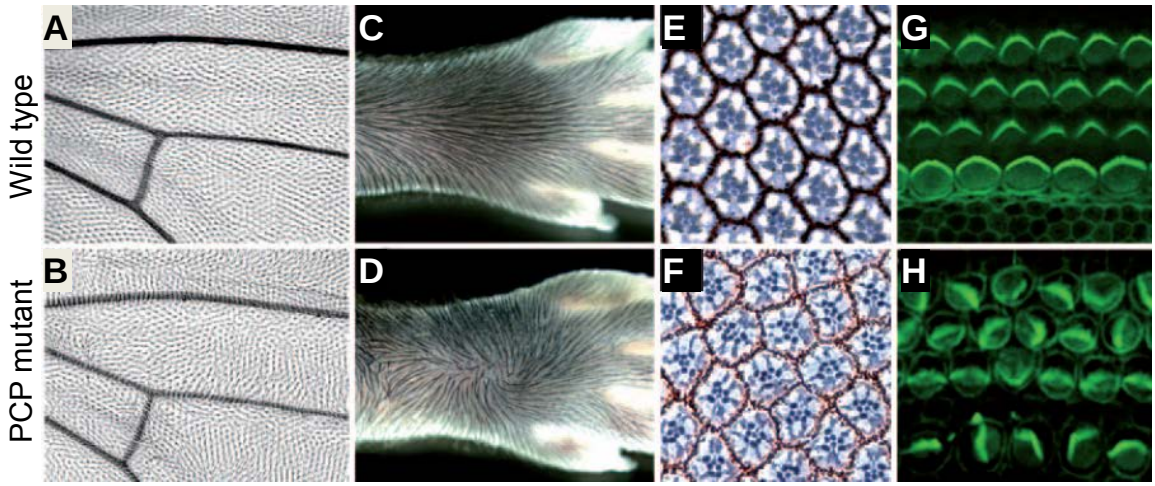

Figure 9. Processes regulated by the PCP signaling pathway in different systems. (A, B) Proximal-distal orientation of *Drosophila* wing hairs. (A) In wild type wing actin hairs point distally. (B) In PCP mutants wing hairs have disrupted orientation and form swirls and waves. *Fz* mutant is shown. (C, D) Proximal-distal orientation of mouse hairs. (C) In wild type mouse hairs uniformly point in distal direction. (D) In PCP mutant mouse hairs form swirls and waves. *Fz6* mutant mouse paw is shown. (E, F) Photoreceptor orientation in *Drosophila* eye ommatidia. (E) Ommatidia in the *Drosophila* eye are arranged in a specific pattern. Each ommatidium in the *Drosophila* eye has eight photoreceptor cells, which are also specifically arranged. (F) In *Stbm* PCP mutant the arrangement of photoreceptors in each ommatidium and the arrangement of ommatidia in the whole eye are disrupted. (G, H) Sensory hair cells in mouse inner ear generate bundles of actin-based stereocilia polarized in an organized pattern (actin is labeled in green through phalloidin staining). (G) In wild type mouse inner ear actin bundles are polarized. (H) In *Vangl2* PCP mutant mouse inner ear actin bundles are randomly distributed and the polarity is lost (from Seifert and Mlodzik 2007).

In the fruit fly wing each epithelial cell forms a hair pointing in distal direction so wing hair cells are aligned with each other (Figure 9 A). In the PCP mutant wing hair patterning is disrupted and hairs point in different directions (Figure 9 B). *Drosophila* has a complex eye build from repeating building blocks ommatidia. Each building block has eight photoreceptor cells arranged in specific pattern (Figure 9 E). In the PCP mutants this pattern is lost (Figure 9 F).

In mouse the most prominent PCP phenotype is craniorachischisis, a severe neural tube closure defect when the neural tube remains open from the midbrain/hindbrain boundary throughout the whole spinal cord (Figure 10 A - D). In mouse the mutants of the

core PCP genes *Vangl*, the mouse ortholog of *Vang* (Kibar et al. 2001), *Dvl1/2* and *Dvl2/3*, the mouse ortholog of *Dsh* (Hamblet et al. 2002), *Celsr1*, the mouse ortholog of *Fmi* (Curtin et al. 2003), *Fz3/6* (Wang et al. 2006) and *PTK7* (Lu et al. 2004) show craniorachischisis. Additionally, mouse PCP mutants have misorientated inner ear sensory hair cells. Sensory cells in the wild type inner ear form polarized bundles of actin-based stereocilia (Figure 9 G). In the PCP mutant the orientation of the stereocilia is randomized (Figure 9 H). Misorientation of hair follicle cells in the skin is also an indication for PCP defects. Normal mice have aligned hairs (Figure 9 C), while in the mutant hairs usually do not point in one direction and form swirls (Figure 9 D).

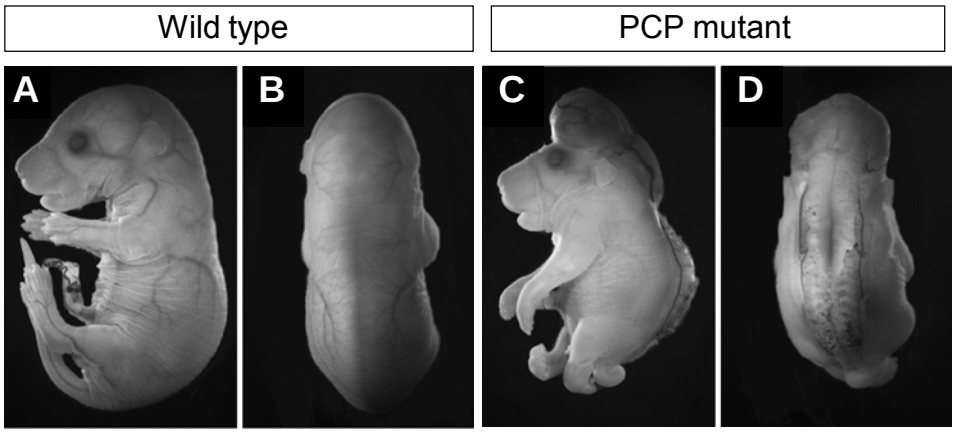

Figure 10. Mouse PCP mutants have open neural tube (craniorachischisis). (A, B) A lateral and a dorsal views of wild type mouse embryos respectively. In the wild type mouse neural tube is fully closed. (C, D) A lateral and a dorsal views of PCP mutant mouse embryos respectively. In the PCP mutant mouse neural tube remains open the midbrain/hindbrain boundary throughout the whole spinal cord from . *Fz3/Fz6* mutant is shown (from Wang et al. 2006).

Convergent extension (CE) is a process of tissue rearrangement during which the tissue narrows in one direction and extends in another due to cell intercalation (Figure 11 A, B). This process is required during fish and frog development for proper gastrulation, neurulation and organogenesis to shape a body (Roszko et al. 2009). PCP regulates cell shape changes and the formation of cellular protrusions during CE. *Zebrafish* PCP mutants *trilobite* (*Zebrafish Vang*) (Jessen et al. 2002), *silberblick /Wnt11* (Ulrich et al. 2003) and *pipetail* (*Wnt5*) (Kilian et al. 2003) show CE defects. The *Zebrafish* PCP mutants fail to extend their anterior-posterior axis properly, because cells do not migrate and intercalate effectively, leading to shortened and broadened embryos (Figure 11 C - F).

In *Xenopus* during gastrulation the dorsal mesodermal cells undergo CE. If components of the PCP pathway Fz, Dsh or Pk are knocked-down, these movements are

disrupted (Wallingford et al. 2002). To determine an effect of a molecule on CE so called Keller sandwich explants are widely used (Petersen et al. 2008). Keller explants are cells of the dorsal marginal zone, which normally undergo CE movements. When dissected, they continue to narrow and elongate (Figure 12 A, B). The injection of molecules that alter PCP signaling, like dominant-negative Dsh, disrupts the elongation (Figure 12 C) (Wallingford et al. 2000; Wallingford and Harland 2001).

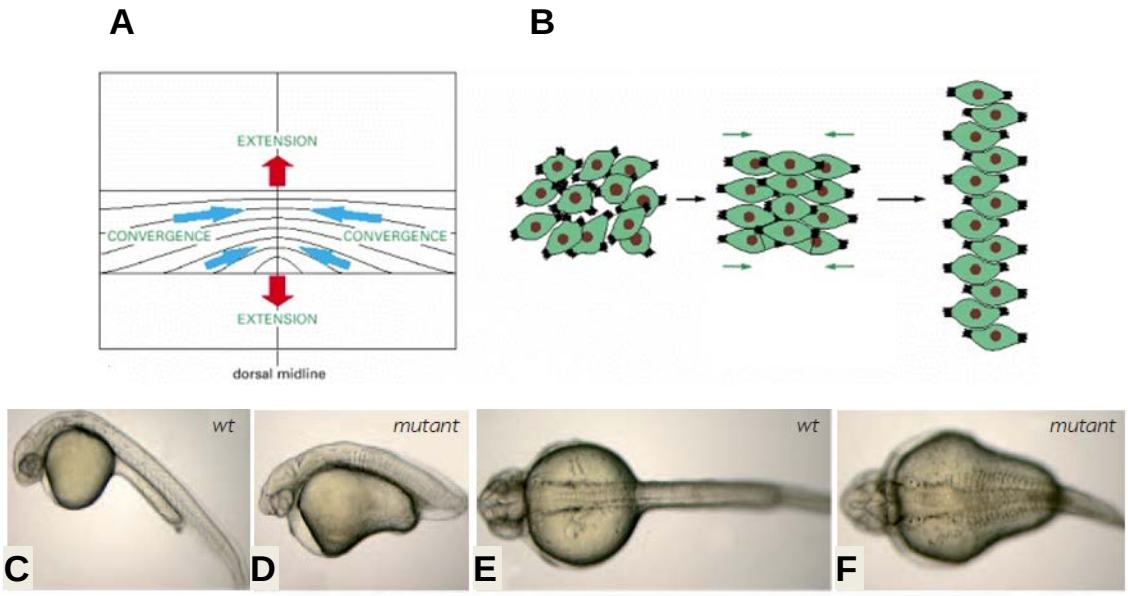

Figure 11. Convergent extension movements. (A) The pattern of convergent extension during gastrulation, dorsal view. Blue arrows represent convergence toward the dorsal midline, red arrows represent extension of the anterior-posterior axis. (B) Schematic diagram of the cell behavior during convergent extension. The cells form lamellipodia and attempt to crawl over one another. Alignment of the movements along a common axis leads to convergent extension (from Molecular Biology of the Cell. 4th edition). (C–F) Convergent extension movements in *Zebrafish* embryos are required for proper gastrulation and neurulation. *Zebrafish* PCP mutants fail to extend their anterior-posterior axis properly, because cell migration and intercalation processes are misregulated. (C, E) A lateral and a dorsal views of wild type *Zebrafish* embryos respectively. A wild type embryo is properly elongated. (D, F) A lateral and a dorsal views of the PCP mutant *Zebrafish* embryos respectively. The PCP mutant *Zebrafish* embryo has shortened and broadened body. *Trilobite/Stbm* mutant is shown (from Seifert and Mlodzik 2007).

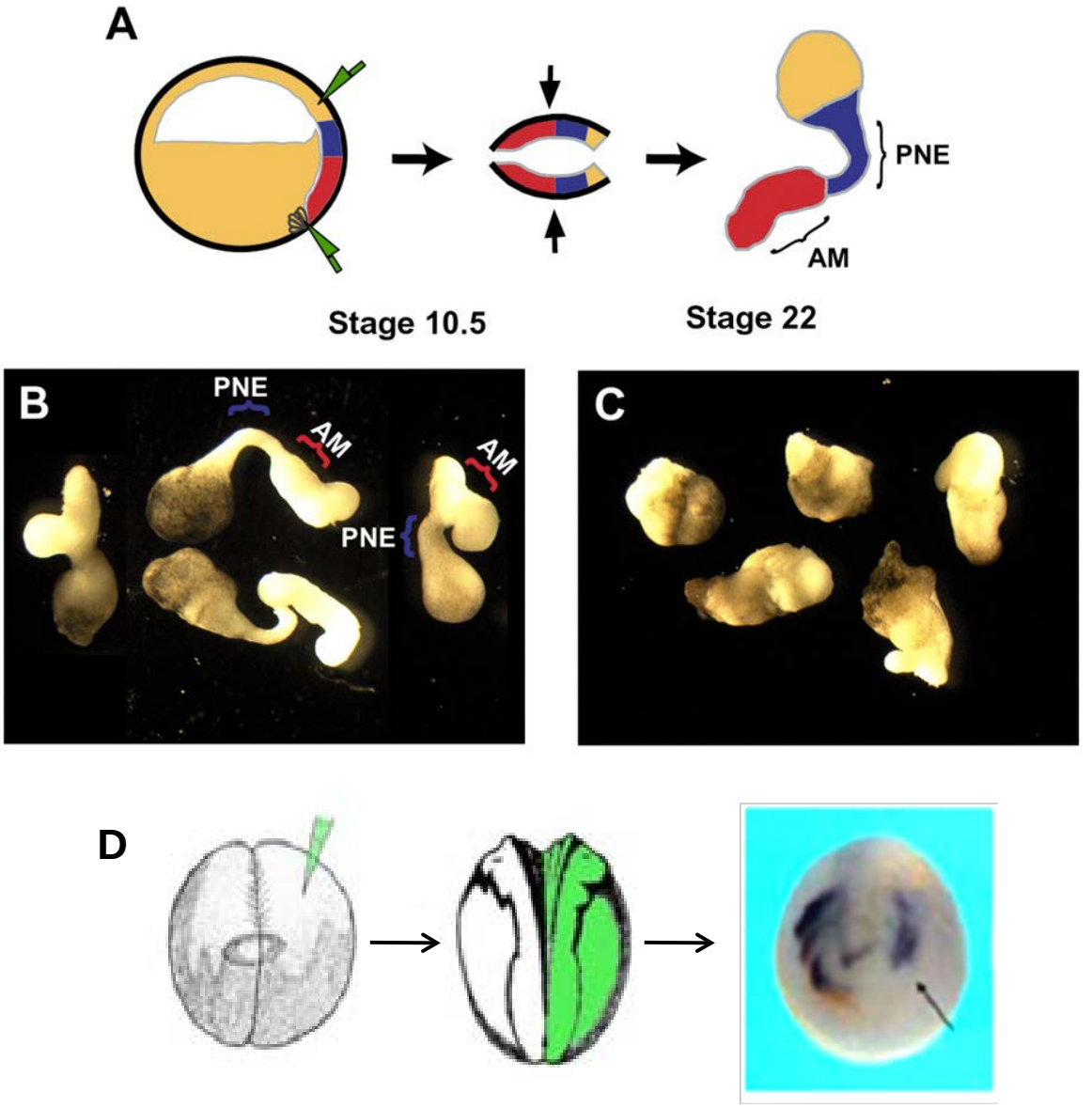

Figure 12. PCP signaling regulates convergent extension movements and migration of neural crest cells in *Xenopus*. (A – C) PCP signaling regulates CE extension movements in *Xenopus*. (A) To prepare Keller sandwich, dorsal marginal zones, which consists of dorsal axial mesoderm (AM, red), posterior neural ectoderm (PNE, blue), and some anterior ectoderm (yellow) of two gastrulating embryos are dissected and pressed together to form a sandwich-like structure. Dorsal marginal zone tissues continue to undergo CE movements resulting in the narrowing and extension of the Keller sandwich explants. (B) Keller sandwich explants from control embryo showing normal CE movements. (C) Keller sandwich explants from the embryos injected with dominant-negative Dsh construct, which blocks PCP signaling, showing disrupted elongation and extension (from Wallingford and Harland 2001). (D) PCP regulates migration of neural crest in *Xenopus*. Schematic representation of microinjection experiment followed by *Twist* WISH at the neurula stage. The migration of CNC cells is inhibited at the injected side (marked by an arrow) while the non-injected side has three branches of normally migrating CNC cells. The embryo was injected with Morpholino against PCP regulator *PTK7* (modified from Shnitsar and Borchers 2008).

Another developmental process, which is regulated by PCP signaling in *Xenopus*, is the migration of neural crest cells (De Calisto et al. 2005; Carmona-Fontaine et al. 2008). Cranial neural crest cells (CNC) in *Xenopus* are migrating in three distinct branches: mandibular, hyoid and branchial (Mayor et al. 1999). These cells express specific CNC markers like transcription factor *Twist* (Hopwood et al. 1989). The injection in one blastomere at the two-cell stage affects only one half of the embryo allowing to use the second half as an endogenous control. Using whole mount *in situ* hybridization (WISH) against CNC markers like *Twist* the migration of the cells can be compared between the injected and the non-injected sides (Figure 12 D) (De Calisto et al. 2005; Shnitsar and Borchers 2008). The injection of mRNA coding for dominant-negative Dsh or dominant-negative Wnt11, both of which inhibit PCP signaling, results in the inhibition of CNC cells migration at the injected side (De Calisto et al. 2005).

The main effect of PCP signaling pathway is the cytoskeletal rearrangement, but it can also control the transcription of several genes, namely *Xenopus paraxial protocadherin* gene (*Xpapc*) and transcription factor *atf2* gene trough JNK kinase cascade (Schambony and Wedlich 2007). This property has been used to create a luciferase reporter construct containing firefly luciferase gene under the control of *atf2* gene promoter (van der Sanden et al. 2004). This construct provides a fast and quantitative way to monitor PCP signaling and allows to distinguish activators and inhibitors of PCP (Ohkawara and Niehrs 2011). However, it can serve as a read-out only for Rho-dependent branch of PCP since it activates only the JNK but not the Rac-dependent one branch of this signaling pathway.

1.6 Molecular mechanism of the Planar cell polarity pathway

PCP pathway and its basic mechanism were first identified in *Drosophila* and later it was discovered that the components of PCP pathway are highly conserved through animal kingdom from *Drosophila* to vertebrates (Seifert and Mlodzik 2007; Vladar et al. 2009). The PCP pathway shares Wnt, Fz and Dsh with canonical Wnt signaling pathway but it is β-catenin independent. It also includes a large set of proteins like Van Gogh (Vang or Strabismus, Stbm), Prickle (Pk), Flamingo (Fmi or Starry night) and Diego (Dgo), which are specific for PCP pathway (Figure 13) (Montcouquiol et al. 2006; Vladar et al. 2009; McNeill 2010).

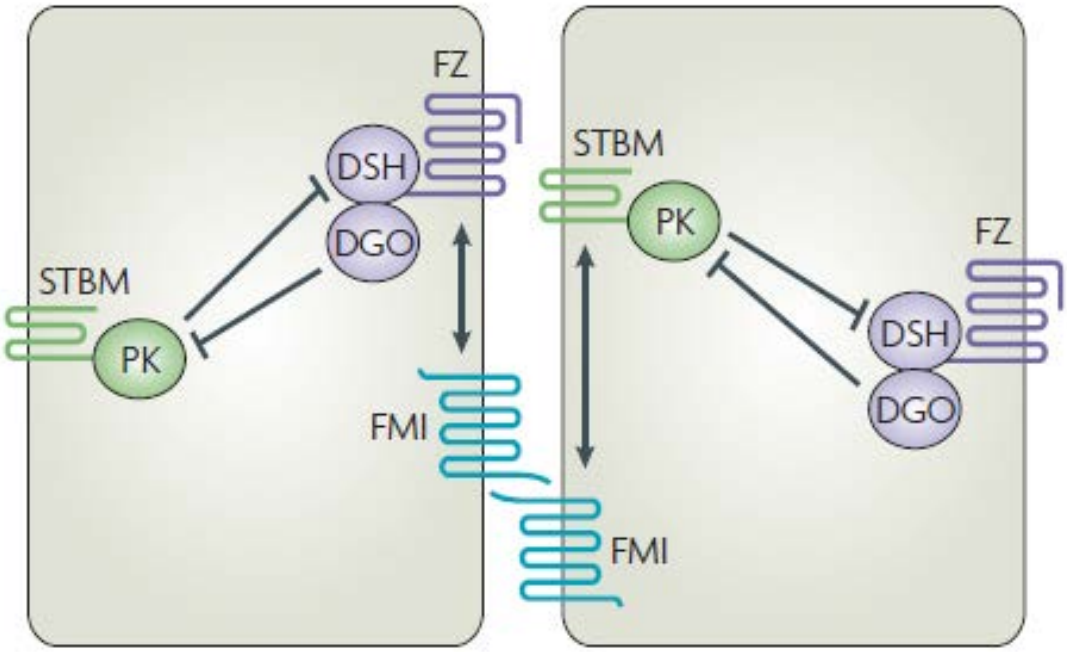

Figure 13. Molecular interactions between PCP core proteins. The figure depicts an apical view of two cells with PCP. Fz/Dsh/Dgo and Stbm/Pk complexes are localized on opposite cellular poles and mutually inhibit each other. The cytoplasmic domain of Fz activates Dsh by recruiting it to the plasma membrane, while Stbm and Pk bind Dsh to inhibit its recruitment to the membrane antagonizing Fz/Dsh/Dgo complex. Fmi co-localizes with both Fz/Dsh/Dgo and Stbm/Pk complexes and binds Fz and Stbm (from Seifert and Mlodzik 2007).

All players of this pathway can be subdivided into so called "core" PCP genes and tissue-specific PCP genes. The core PCP genes are highly evolutionary conserved and seem to be required in all cells with PCP to establish general planar polarity, while the tissue-specific PCP genes function downstream of core PCP genes in establishing tissue-specific properties of polarized cells (Seifert and Mlodzik 2007; Vladar et al. 2009). The core PCP genes are *Fz* (Vinson et al. 1989; Theisen et al. 1994), *Dsh* (Theisen et al. 1994), *Fmi* (Chae et al. 1999; Usui et al. 1999), *Vang* (Taylor et al. 1998; Wolff and Rubin 1998), *Pk* (Gubb et al. 1999) and *Dgo* (Feiguin et al. 2001; Das et al. 2004). The asymmetrical localization of core PCP proteins is a prerequisite for changes in cell morphology (Vladar et al. 2009). In all *Drosophila* tissues analyzed, Fz/Dsh/Dgo and Stbm/Pk complexes are localized on opposite cellular poles and mutually inhibit each other making a cell asymmetric (Figure 13) (Strutt and Strutt 2005; Seifert and Mlodzik 2007; Vladar et al. 2009). The recruitment of Dsh to the plasma membrane is a hallmark of PCP activation. The cytoplasmic domain of Fz activates Dsh by recruiting it to the plasma membrane (Axelrod et al. 1998; Rothbacher et al. 2000; Wong et al. 2003), while Stbm and Pk bind Dsh to inhibit its recruitment to the membrane, thereby antagonizing Fz/Dsh/Dgo complex

(Bastock et al. 2003; Jenny et al. 2003; Bellaiche et al. 2004; Ciruna et al. 2006). Dgo competes with Pk for Dsh binding and promotes Dsh activation (Das et al. 2004; Jenny et al. 2005). Being recruited to the plasma membrane, Dsh becomes activated and promotes changes in the cytoskeleton through activation of the small GTPases Rho and Rac (Habas et al. 2001; Habas et al. 2003; Rosso et al. 2005). The mechanism of this activation is poorly understood. It is known that Dsh associates with the cytoplasmic protein Daam to promote the activation of the small GTPase Rho (Habas et al. 2001). Rho is able to induce ROCK kinase, which is involved in actin cytoskeleton rearrangements (Habas et al. 2001). Additionally, Dsh may induce cytoskeletal rearrangements through the activation of the small GTPase Rac, which in turn stimulates the activity of c-jun N-terminal kinase (JNK) (Habas et al. 2003; Rosso et al. 2005).

Some core PCP proteins do not have an asymmetric localization. The atypical cadherin Fmi is a seven-pass transmembrane protein with a homophilic binding property (Chae et al. 1999; Usui et al. 1999). Fmi co-localizes with both Fz/Dsh/Dgo and Stbm/Pk complexes and binds Fz and Stbm (Figure 13) (Usui et al. 1999; Das et al. 2002; Chen et al. 2008). The function of this protein is not understood yet. It is proposed that Fmi is required on both sides of the cell to transmit the information about polarity between cells (Strutt and Strutt 2005).

It is not understood how PCP is initially established and what kind of signals promote the instructive cue for the cell orientation. The most promising candidate proteins, which could function upstream of transmembrane PCP regulators to establish cell asymmetry, are Wnt proteins. However, in *Drosophila* loss of function or overexpression of neither Wingless nor any other Wnt or combinations of several Wnts display a PCP phenotype (McNeill 2010). In contrary, there are evidences in both *Xenopus* and *Zebrafish* that Wnts are the permissive signals for PCP establishment. Wnt5a and Wnt11 are involved in convergent extension movements in fish and frog (Heisenberg et al. 2000; Tada and Smith 2000; Kilian et al. 2003). Wnt11 and Wnt11r regulate the migration of neural crest cells in *Xenopus* (De Calisto et al. 2005; Matthews et al. 2008). Both convergent extension movements and neural crest migration are regulated by PCP signaling (Carmona-Fontaine et al. 2008; Matthews et al. 2008; Roszko et al. 2009). Additionally, *Wnt5* knock-out mice also have a PCP phenotype in the cochlea (Qian et al. 2007).

1.7 Protein tyrosine kinase 7 (PTK7)

Protein tyrosine kinase 7 (PTK7) or colon carcinoma kinase 4 (CCK4) is an important regulator of PCP in vertebrates. PTK7 knock-out mice have typical PCP phenotypes – disrupted neural tube closure and misorientated hairs of the inner ear (Figure 14 B) (Lu et al. 2004). Additionally, during mouse gastrulation PTK7 regulates convergent extension and is essential for polarized cell motility, elongation and alignment (Yen et al. 2009). Similarly to the mouse data, in *Xenopus*, knock-down of PTK7 by Morpholino nucleotides results in neural tube closure and convergent extension defects (Figure 14 C) (Lu et al. 2004). In *Xenopus* PTK7 regulates cranial neural crest cell migration, a process, which depends on PCP signaling (Figure 14 D) (Shnitsar and Borchers 2008). In *Zebrafish* PTK7 knock-down also disrupts convergent extension movements leading to the formation of embryos with shortened axis (Golubkov et al. 2010).

The *Drosophila* PTK7 ortholog Off track (Otk) can interact biochemically and genetically with PlexinA to regulate axon guidance (Winberg et al. 2001). Plexins are single-pass transmembrane receptors for Semaphorin guidance cues involved in the regulation of cell shape and cell motility during axon guidance, vascular growth, immune cell regulation and tumor progression (Kruger et al. 2005). Otk/PlexinA1 interaction has been implicated in Semaphorin repulsive signaling during axon guidance in *Drosophila* (Figure 15) (Winberg et al. 2001). Similarly, in chick Otk interacts with PlexinA1 to mediate Semaphorin6D inhibitory effect during cardiac morphogenesis (Toyofuku et al. 2004).

PTK7 was first isolated from colon carcinoma tissue (Mossie et al. 1995) and the expression of *PTK7* is frequently misregulated in cancer tissues (Easty et al. 1997; Endoh et al. 2004; Muller-Tidow et al. 2004).

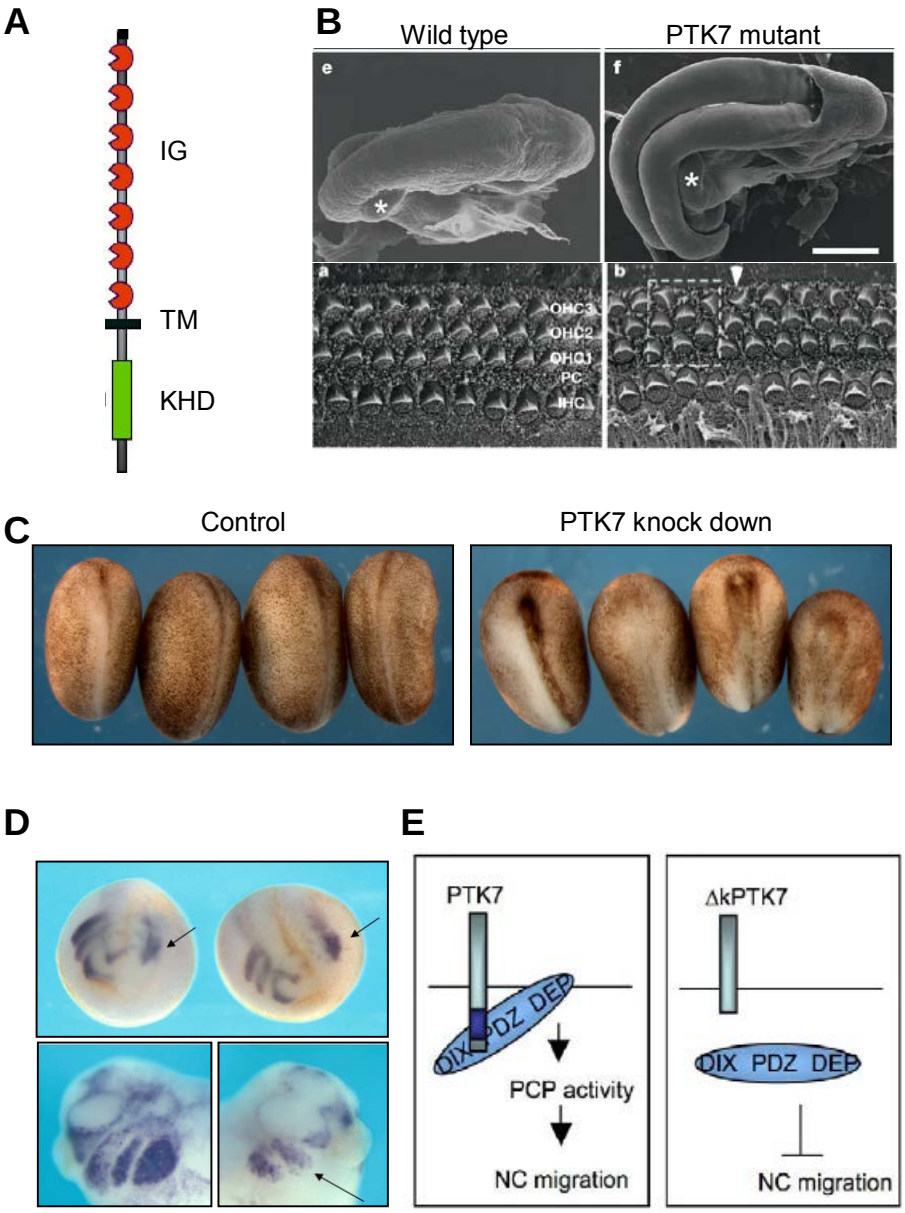

Figure 14. PTK7 regulates PCP in vertebrates. (A) Structure of PTK7 protein. IG immunoglobulin domain, TM transmembrane domain, KHD kinase homology domain. (B) PTK7 knockout mice have open neural tubes and misorientated inner ear hair cells (from Lu et al. 2004). (C) PTK7 knock-down by antisense oligonucleotides injection in *Xenopus* embryos. Embryos injected with control Morpholino have closed neural tubes, while the neural tubes of embryos injected with Morpholino against PTK7 remain open (from Lu et al. 2004). (D) PTK7 knock-down by antisense oligonucleotides injection in *Xenopus* embryos. Neural crest cells at the injected side do not migrate (marked by arrows) (from Shnitsar and Borchers 2008). (E) Model of the role of PTK7 in neural crest migration. PTK7 recruits Dsh to the plasma membrane, leading to the activation of PCP signaling and enabling neural crest migration. Deletion of the conserved kinase domain of PTK7 inhibits membrane localization of Dsh and neural crest migration (from Shnitsar and Borchers 2008).

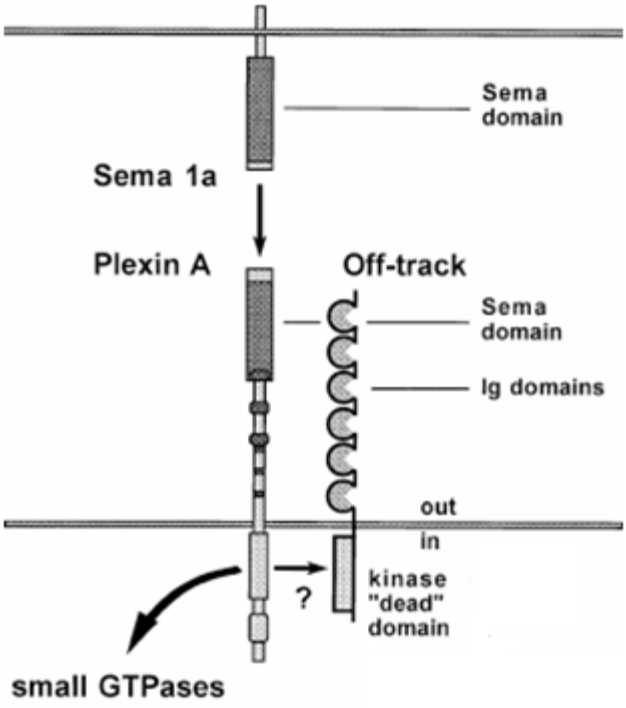

Figure 15. Off-track/PlexinA1 interaction is implicated in Semaphorin repulsive signaling in *Drosophila*. Off-track interacts with PlexinA1 to mediate the response to Sema1a to regulate axon guidance in *Drosophila* (from Winberg et al. 2001).

PTK7 is an evolutionary conserved gene found from Hydra (where it is called *lemon*) to human. PTK7 is reminiscent of tyrosine kinase. It is a transmembrane protein with seven extracellular immunoglobulin-like domains and an intracellular kinase-homology domain (Figure 14 A) (Mossie et al. 1995; Park et al. 1996). However, an intracellular kinase homology domain of PTK7 has mutated DFG triplets, which are necessary for catalytic activity, indicating that PTK7 lacks a kinase activity. Indeed, so far no kinase activity has been shown for any PTK7 ortholog (Chou and Hayman 1991; Miller and Steele 2000). Despite the lack of kinase activity, the intracellular domain of PTK7 is highly conserved suggesting additional functions (Chou and Hayman 1991; Miller and Steele 2000). Indeed, it was shown that the recruitment of Dsh to the plasma membrane through the intracellular kinase homology domain of PTK7 is important for the regulation of neural crest migration (Figure 14 E) (Shnitsar and Borchers 2008). However, in *Xenopus* PTK7 does not interact with Dsh directly and adaptor protein RACK1 is required for this function (Wehner et al. 2011). RACK1 and PTK7 interaction is involved in the regulation of neural tube closure (Wehner et al. 2011).

1.8 Role of endocytosis in Wnt signaling

Endocytosis can influence both canonical and non-canonical Wnt signaling and might be involved in the choice between Wnt signaling pathways (Kikuchi et al. 2009).

Endocytosis plays a crucial role in many signaling pathways. It is mostly required for the removal of receptors from the plasma membrane to switch off signaling pathways (Sorkin and von Zastrow 2009). However, recent data indicate that in some cases endocytosis is crucial for promoting the pathway activation with a signaling taking place in endosomes (Seto et al. 2002). Furthermore, there is an evidence that the endosomal localization of receptors may selectively activate specific signaling outcomes. For example, Epidermal growth factor receptor (EGFR) is involved in the activation of both MAP kinases cascade and Shc effector protein (Vieira et al. 1996). If internalization of EGFR is blocked, the downstream effector Shc is still activated, but MAP kinase phosphorylation is attenuated (Vieira et al. 1996).

The role of endocytosis in Wnt signaling is actively studied now. The endocytosis of Wnt ligand/receptor complex is important for both canonical and non-canonical Wnt signaling, however, depending on the ligand-receptor combination and cellular context, it may either promote or inhibit the signaling (Gagliardi et al. 2008; Kikuchi et al. 2009). There are two types of endocytotic routes: clathrin-mediated and caveolin-mediated, which are mediated through clathrin-coated pits and through caveolae, a flask-shaped invaginations of plasma membrane, respectively. The Wnt receptors may be internalized through either a clathrin- or caveolin-dependent endocytotic route (Kikuchi et al. 2009).

1.8.1 Endocytosis in canonical Wnt signaling

The first indication that endocytosis might be involved in the regulation of β-catenin-dependent signaling came from studies in fruit fly. In *Drosophila* embryos Wnt signaling establishes proper segmentation. Wnt signaling is upregulated in the anterior part of the segment and downregulated in the posterior one (Howes and Bray 2000). It has been shown that *Drosophila* Wg is internalized and targeted to lysosomal degradation (Dubois et al. 2001). Moreover, this degradation was more active in the posterior part of each segment than in the anterior one. It was proposed that endocytosis of Wg with subsequent degradation restricts canonical Wnt signaling activity to the anterior region of the segment (Dubois et al. 2001).

However, later it has been discovered that endocytosis may promote canonical Wnt signaling. Blitzer and Nusse showed that interfering with clathrin-mediated endocytosis by dominant-negative dynamin or various chemical compounds blocks Wnt3a or Wg-dependent activation of canonical Wnt signaling in L cells (Blitzer and Nusse 2006).

The function of Dsh is essential for clathrin-mediated endocytosis of Wnt signaling components. Dsh interacts with the clathrin adaptor β-arrestin, essential for clathrin endocytosis protein, and the knock-down of β-arrestin suppresses Dsh phosphorylation and stabilization of β-catenin by Wnt3a (Chen et al. 2001; Bryja et al. 2007). β-arrestin is involved in the endocytosis of G-protein coupled receptors (GPCR) through the clathrin pathway (Ma and Pei 2007). Seven transmembrane domains protein Fz structurally resembles GPCR, however, it does not interact with β-arrestin directly, but through Dsh (Chen et al. 2001; Bryja et al. 2007).

To summarize, these results indicate that clathrin-dependent endocytosis is involved in activation of canonical Wnt signaling through Dsh and β-arrestin action (Kikuchi et al. 2009).

Additionally, the Wnt co-receptor LRP6, one of the key molecules of the canonical Wnt signaling pathway, can be endocytosed through both clathrin- and caveolin-dependent endocytosis. In response to Wnt3a LRP6 is internalized through caveolin-positive vesicles in HEK293 cells and this internalization is necessary for canonical Wnt signaling activation (Figure 16 A) (Yamamoto et al. 2006; Yamamoto et al. 2008). Knock-down of caveolin by siRNA or block of endocytosis by dominant-negative dynamin in HEK293 cells abolishes LRP6 internalization and stabilization of β-catenin induced by Wnt3a treatment (Yamamoto et al. 2006; Yamamoto et al. 2008). Alternatively, LRP6 can be internalized through clathrin-dependent route in response to Dkk1 ligand (Figure 16 B). Dkk1 is a secreted Wnt antagonist, which binds LRP6 and inhibits canonical Wnt signaling (Niehrs 2006). Dkk1 can bind receptor Kremen and form triple complex with Kremen and LRP6 leading to an inhibition of canonical Wnt signaling (Mao et al. 2002). It has been discovered recently that the binding of Dkk1 to Kremen and LRP6 induces the rapid internalization of the ternary complex through clathrin-positive vesicles, resulting in the removal of LRP6 from plasma membrane and the suppression of canonical Wnt signaling (Mao et al. 2002; Yamamoto et al. 2008). The existing model proposes Wnt3a and Dkk1 force LRP6 to enter different endocytotic routes leading either to caveolin-dependent activation of canonical Wnt signaling or clathrin-dependent inhibition of it respectively (Figure 16 A, B) (Yamamoto et al. 2008; Kikuchi et al. 2009).

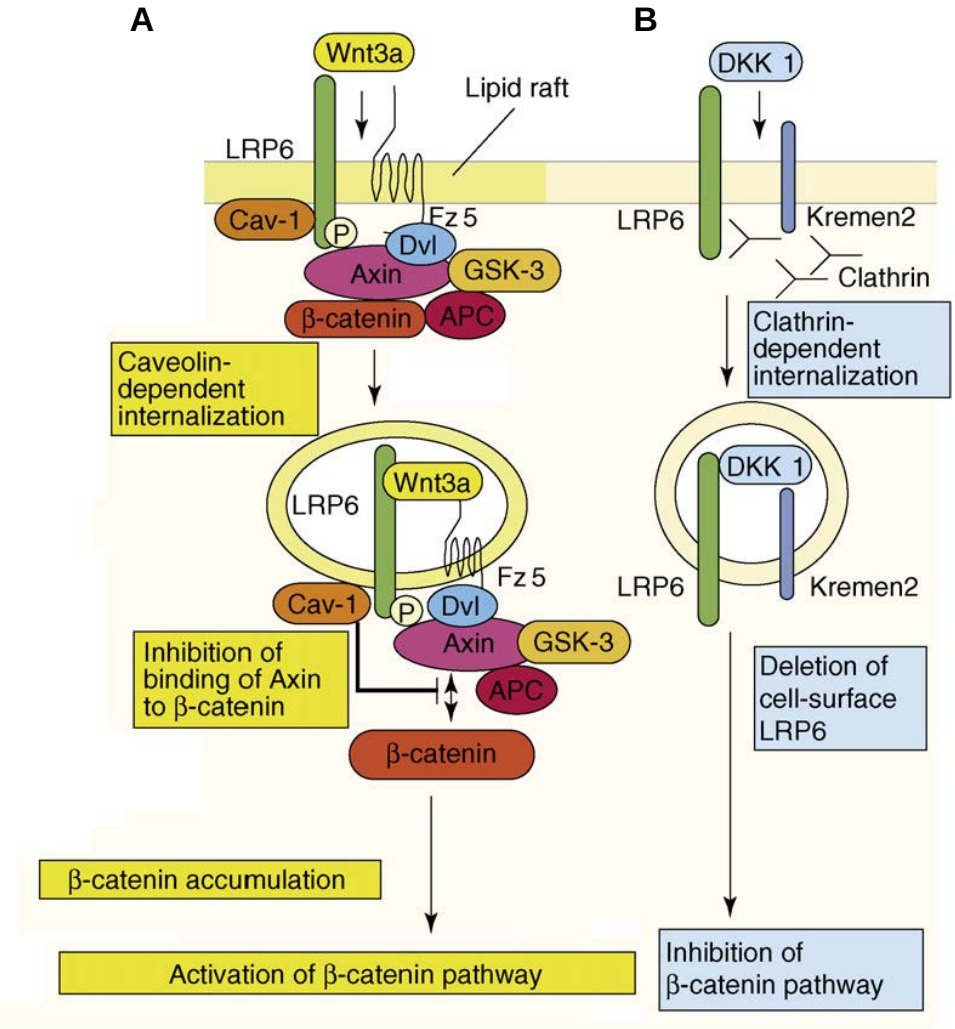

Figure 16. Wnt3a and Dkk1 regulate distinct internalization pathways of LRP6 to tune the activation of β-catenin signaling. (A) In response to Wnt3a LRP6 is endocytosed together with Fz through caveolin-dependent endocytotic route, leading to the activation of canonical Wnt signaling. (B) In response to Dkk1 LRP6 is endocytosed together with Kremen through clathrin-dependent endocytotic route leading to the inhibition of canonical Wnt signaling (from Kikuchi et al. 2009).

1.8.2 Endocytosis in non-canonical Wnt signaling

Endocytotic processes are important for the regulation of not only canonical, but also non-canonical Wnt signaling pathway. The crucial step in PCP activation is the recruitment of Dsh to the plasma membrane by the cytoplasmic domain of Fz (Axelrod et al. 1998; Rothbacher et al. 2000; Wong et al. 2003). It has been shown that Dsh interacts with different components of the endocytotic machinery to induce the internalization of Fz and to promote the activation of PCP signaling pathway (Kikuchi et al. 2009). First, it was demonstrated that Dsh can bind β-arrestin to regulate canonical Wnt signaling (Chen et al. 2001; Bryja et al. 2007). However, Dsh/β-arrestin interaction is also important for the

proper functioning of non-canonical Wnt signaling. In HEK293 cells, upon the stimulation with PCP activator Wnt5a, β-arrestin is recruited to the Fz cytoplasmic tail through Dsh and promotes the endocytosis of Fz through clathrin-coated vesicles to activate non-canonical Wnt signaling (Figure 17) (Chen et al. 2003). Depletion of β-arrestin by antisense Morpholino oligonucleotides causes typical convergent extension defects in *Xenopus* proving the functional relevance of Dsh/β-arrestin interaction for PCP signaling (Kim and Han 2007). Moreover, β-arrestin interacts with Rho and mediates Dsh-dependent activation of Rho but not Rac (Kim and Han 2007).

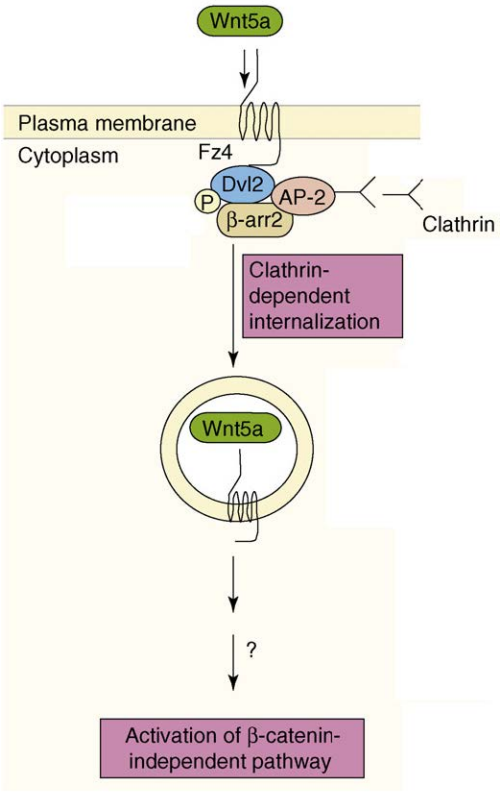

Figure 17. Activation of non-canonical Wnt signaling pathway through clathrin-dependent endocytosis. Upon the stimulation with Wnt5a, β-arrestin is recruited to the Fz cytoplasmic tail through Dsh and promotes the endocytosis of Fz through clathrin-coated vesicles to activate non-canonical Wnt signaling. Dsh can also interact with AP2 complex, which recruits the endocytosed proteins within clathrin-coated vesicles (from Kikuchi et al. 2009).

Another study shows that Dsh can interact with another component of the endocytotic machinery, μ2 adaptin, a subunit of clathrin adaptor complex 2 (AP2) (Figure 17) (Yu et al. 2007). The AP2 complex recruits the endocytosed proteins to clathrin-coated vesicles (Kirchhausen 1999). Dsh and μ2 adaptin interaction cause the internalization of Fz4 in *Xenopus* animal caps (Yu et al. 2007). A Dsh mutant with deleted AP2 binding

region cannot activate PCP in *Xenopus,* but retains the ability to promote canonical signaling, suggesting that AP2-dependent internalization of Fz plays an exclusive role in the non-canonical but not in the canonical Wnt signaling (Yu et al. 2007). However, in *Drosophila* no data support the importance of endocytosis in PCP establishment. The AP2 or fly arrestin *Kurz* mutants develop no PCP phenotype (Berdnik et al. 2002; Mukherjee et al. 2005)

It is clear that both clathrin- and caveolin-dependent types of endocytosis are important for regulation of Wnt signaling in vertebrates. However, the outcome of endocytosis is highly dependent on the ligand/receptor complex and cellular context.

1.9 Caveolin proteins

Caveolin proteins serve as molecular markers for caveolae, bottle-shaped invaginations of plasma membrane up to 100 nm in diameter (Figure 18). Caveolae originate from lipid rafts, cholesterol- and sphingolipid-rich microdomains of the plasma membrane with enriched localization of signaling molecules (Parton and Simons 2007). Caveolins form a coat around caveolae. In addition to membrane and caveolae localization, caveolin proteins may be found in Golgi apparatus, transport vesicles and as soluble cytoplasmic proteins (Williams and Lisanti 2004). There are three caveolin family member genes – *caveolin1* (*cav1*), *caveolin2* (*cav2*) and *caveolin3* (*cav3*), which have been identified in *Caenorhabtitis elegans* and in many vertebrates. Cav1 is expressed ubiquitously, but the level of expression varies depending on the tissue with the highest levels detected in the epithelial and endothelial cells, adipose tissue, smooth muscle cells and fibroblasts, Cav2 is co-expressed with Cav1 and it requires Cav1 for proper membrane localization (Scherer et al. 1997; Parolini et al. 1999), while Cav3 is expressed mainly in striated muscle cells (Song et al. 1996; Tang et al. 1996).

Cav1 has hairpin membrane spanning structure and both N- and C-terminal parts of Cav1 face the cytoplasm (Figure 18) (Williams and Lisanti 2004). Cav1 is able to form oligomers consisting from 14 - 16 monomers (Sargiacomo et al. 1995).

Caveolins have diverse functions like vesicle trafficking, cholesterol homeostasis, cell migration, signal transduction and tumor suppression, and they have been linked with Wnt signaling and cell migration (Fra et al. 1995; Williams and Lisanti 2004). However, how exactly these proteins mediate so many processes is unknown.

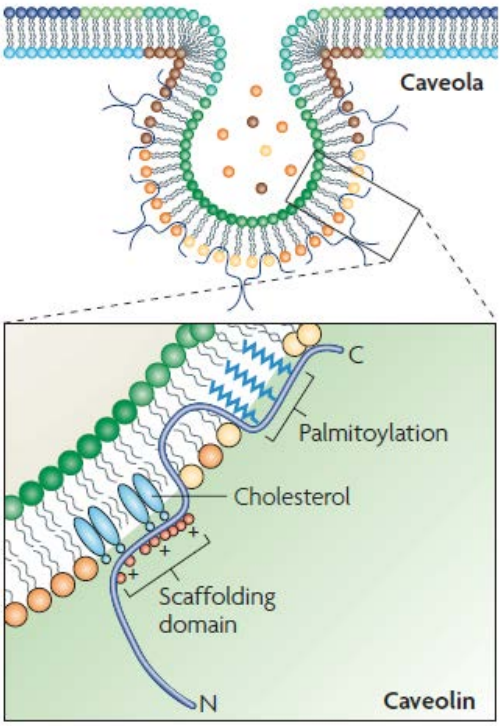

Figure 18. Caveolae and caveolin structures. Caveolae are bottle-shaped invaginations of plasma membrane, coated with caveolins. Caveolin has a hairpin membrane spanning structure with both N- and C-terminal parts facing cytoplasm (from Parton and Simons 2007).

There are contradictory data concerning the role of caveolin in Wnt signaling. Wnt3a-dependent internalization of LRP6 through caveolin-dependent endocytotic route activates canonical Wnt signaling in HEK293 cells (Yamamoto et al. 2006; Yamamoto et al. 2008). If HEK293 cells are transfected with siRNA against *cav1*, the activation of canonical Wnt signaling by Wnt3a is attenuated (Yamamoto et al. 2006; Yamamoto et al. 2008). Clearly, caveolin activates canonical Wnt signaling in HEK293 cells. However, in NIH 3T3 cells and in *Zebrafish* embryos caveolin inhibits canonical Wnt signaling by binding β-catenin and recruiting it to membrane caveolae domains and consequently preventing it from entering the nucleus (Galbiati et al. 2000; Mo et al. 2010). Overexpression of Cav1 inhibited the activation of canonical Wnt signaling by Wnt1 or β-catenin in NIH 3T3 cells (Galbiati et al. 2000). Cav1 is required for proper dorsal-ventral patterning in *Zebrafish*. Overexpression of Cav1 resulted in typical ventralized embryos, while downregulation of Cav1 by antisense Morpholino oligonucleotides cause the expansion of dorsal marker, demonstrating the inhibition of canonical Wnt signaling by Cav1 in this system (Mo et al. 2010). Moreover, *cav1* knockout mice have upregulated canonical Wnt signaling (Sotgia et al. 2005). The molecular mechanism of the inhibition of

canonical Wnt signaling by Cav1 in mice remains unknown, but in NIH 3T3 cells and *Zebrafish* embryos it appears to be endocytosis-independent.

Being regulators of membrane dynamics, cytoskeletal remodeling and interactions of the cell surface with the extracellular matrix, caveolins are involved in the cell migration (Navarro et al. 2004). Downregulation of Cav1a results in loss of focal adhesion sites and adhesion causing inhibition of cell migration (Wei et al. 1999).

1.10 Aims

PTK7 (Protein tyrosine kinase 7) is a regulator of the PCP Wnt signaling pathway in vertebrates. PTK7 is a transmembrane protein with seven extracellular immunoglobulin-like domains and an intracellular kinase-homology domain. Considering the structure of PTK7, it is likely that it may function as a receptor. However, no PTK7 extracellular interaction partners were described. The goal of the PhD project was to elucidate the signaling mechanism of PTK7 by identifying PTK7 ligands and verifying *in vivo* relevance of them in *Xenopus* development.

2. Materials and Methods

2.1 Model Organism

The African clawed frog *Xenopus laevis* (*X. laevis*) was used as a model organism during this study. Adult frogs were purchased from Nasco (Ft. Atkinson, USA).

2.2 Bacteria

E.coli strain XL1-Blue (RecA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac[F'proAB, ZΔM15, Tn10(Tetr)]^{c} (Stratagene)) was used during this study.

2.3 Cell lines

The following cell lines were used:

HEK293 – human embryonic kidney cells immortalized by adenovirus transfection. ATCC^{R} number CRL-1573^{TM}.

L cells – mouse fibroblasts. L cells were used to obtain control conditioned medium for comparison with Wnt3a and Wnt5a conditioned medium. ATCC^{R} number CRL-2648^{TM}.

L Wnt3a cells – were obtained by stable transfection of L cells with Wnt3a expression vector (R. Moon laboratory). The cells secrete biologically active mouse Wnt3a protein. ATCC^{R} number CRL-2647^{TM}.

L Wnt5a cells– were obtained by stable transfection of L cells with Wnt5a expression vector (R. Moon laboratory). The cells secrete biologically active mouse Wnt5a protein. ATCC^{R} number CRL-2814^{TM}.

2.4 Chemicals, Buffers and Media

2.4.1 Chemicals

The chemicals were purchased from the following companies: Roth (Karlsruhe), Sigma (Munich), Biomol (Hamburg), Applichem (Darmstadt) and Biochrom (Berlin).

2.4.2 Buffers and Media

The buffers were prepared using deionized water (MiliQ).

Alkaline phosphatase buffer (APB): 100 mM Tris-HCl (pH 9.5), 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween20

Blocking solution: 1x PBS, 5% non fat dry milk and 0.1% Tween20

CoIP buffer I: 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mM EGTA, 0.5% (v/v) NP-40, 10% (v/v) glycerol, protease inhibitor cocktail (1 tablet per 50 ml of the buffer, Roche)

CoIP buffer for II: 50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 0.5% NP-40, protease inhibitor cocktail (1 tablet per 50 ml of the buffer, Roche)

Cystein solution: 2% L-Cystein hydrochloride, pH 8.0

Danylchik's for Amy (DFA) medium: 53 mM NaCl, 5 mM Na_2CO_3, potassium gluconate 4.5 mM, sodium gluconate 32 mM, $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, BSA 0.1%

Hybridization mix (Hyb-mix): 50% (v/v) Formamid, 5xSSC, 1 mg/ml Torula RNA (Sigma), 100 µg/ml Heparin, 1x Denhards, 0.1% (v/v) Tween20, 0.1% (w/v) CHAPS (Sigma)

Injection buffer: 1x MBS, 2% Ficoll 400 (Sigma)

Laemmli running buffer (10x): 250 mM Tris-base, 2.5 M Glycine, 0.1% SDS

Laemmli loading buffer (6x): 350 mM Tris-HCl pH 6.8, 9.3% Dithiotreit, 30% (v/v) Glycerol, 10% SDS, 0.02% Bromphenolblue

Luria-Bertani (LB)-Medium: 1% (w/v) Bacto-Trypton (DIFCO), 0.5% (w/v) yeast extract (DIFCO), 1% (w/v) NaCl, pH 7.5

LB-Agar: 1.5% (w/v) agar (DIFCO) in liquid LB-medium

MAB: 100 mM Maleinic acid; 150 mM NaCl, pH 7.5

MBS Buffer (1x): 10 mM Hepes pH 7.4, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.2 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.66 mM KNO_3

MEM: 100 mM MOPS, 2 mM EGTA, 1 mM $MgSO_4$

MEMFA: 1x MEM with 3.7% (v/v) Formaldehyde

Nile blue: 0.01% (w/v) Nile blue in 0.1x MBS

PBS (10x): 8% (w/v) NaCl, 2% (w/v) KCl, 65 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4

Ptw buffer: 1xPBS with 0.1% Tween20

SSC: 150 mM NaCl, 15 mM Sodium citrate, pH 7.4

TAE (Tris/Acetat/EDTA): 40 mM Tris-Acetate (pH 8.5), 2 mM EDTA

TE-Buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA

Tris-HCl (pH 6.8, 7.5, 8.2, 8.8, or 9.5): 1 M Tris-HCl, pH adjusted with 37% HCl

Western blotting buffer: 3.03 g Tris-base, 14.4g Glycine, 200 ml methanol, 800 ml H_2O

X-gal staining solution: 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$

2.5 Constructs

2.5.1 Vectors

pCS2+ is an expression vector, which can be used in the *Xenopus* model system. It contains a strong promoter/enhancer region (simian CMV IE94), polylinker and SV40 viral polyadenilation signal. The SP6 viral promoter allows *in vitro* transcription of sense polyadenilated mRNA for microinjections. The T7 viral promoter allows *in vitro* transcription of antisense RNA for *in situ* hybridization (Rupp et al. 1994).

pCS2+/MT is a pCS2+ vector variant with 6x Myc tag sequence inserted into BamHI/ClaI sites for the expression of tagged proteins (Klisch et al. 2006).

pCS2+/HA is a pCS2+ vector variant containing HA tag sequence inserted via Xba1 site for the expression of tagged proteins (Damianitsch et al. 2009).

pCS2+/GFP is a pCS2+ vector variant containing GFP sequence inserted into XbaI/XhoI sites for the expression of GFP-tagged proteins (this study).

pCMV-Sport6 is an eukaryotic expression vector (Invitrogen).

pGEM-T is a cloning vector (Promega).

pEGFP-C1 is a vector for the expression of GFP-fusion proteins in cell culture. It contains the variant of wild-type GFP, was optimized for brighter fluorescence (Clontech).

pSP64T is an expression vector containing SP6 RNA polymerize-binding site, which can be used for *in vitro* RNA transcription (Promega).

pGEM2 is a cloning vector containing SP6 and T7 RNA polymerize sites, which allow *in vitro* RNA transcription (Promega).

pBluescript is a phagemid vector usable for DNA cloning. It contains phage f1 intergenic region required for packaging of DNA into bacteriophage particles (Fermentas).

pGL3 vector contains firefly luciferase gene. The vector is designed for analysis of factors, which can regulate mammalian gene expression like promoters, enhancers and DNA-binding proteins (Promega).

pRL-TK vector has the herpes simplex virus thymidine kinase (HSV-TK) promoter to provide Renilla luciferase expression (Promega).

2.5.2 Expression constructs and luciferase reporters

The expression constructs and luciferase reporters used in this study are presented in Table 1. The constructs were linearized and *in vitro* transcribed as indicated in Table 2.

Table 1 Expression constructs and luciferase reporters

Name	Vector	Insert	Cloning strategy
PTK7	pCMVSport6	full length *Xenopus laevis PTK7* (AAH60500)	(Shnitsar and Borchers 2008)
PTK7-HA	pCS2+/HA	full length *Xenopus laevis PTK7* with HA tag	(Shnitsar and Borchers 2008)
PTK7-MT	pCS2+/MT	full length *Xenopus laevis PTK7* with Myc tag	(Shnitsar and Borchers 2008)
PTK7-GFP	pCS2+/GFP	full length *Xenopus laevis PTK7* with GFP tag	Myc tag was cut off from PTK7-MT construct with ClaI and XbaI. GFP sequence amplified from pEGFP-C1 plasmid with primers Gfp1F ClaI and Gfp1R XbaI was inserted into the same sites.
ΔkPTK7-MT	pCS2+/MT	*Xenopus laevis PTK7* without intracellular kinase-homology domain with Myc tag	(Shnitsar and Borchers 2008)

Name	Vector	Insert	Cloning strategy
ΔkPTK7 -GFP	pCS2+/GFP	*Xenopus laevis PTK7* without intracellular kinase-homology domain with GFP tag	Myc tag was cut off from ΔkPTK7 -MT construct with ClaI and XbaI. GFP sequence amplified from pEGFP-C1 plasmid with primers Gfp1F ClaI and Gfp1R XbaI was inserted into the same sites instead of Myc tag.
exPTK7-MT	pCS2+/MT	extracellular part of *Xenopus laevis PTK7* with Myc tag	extracellular piece of *PTK7* was amplified from PTK7-MT using primers exPTK7MT F and exPTK7MT R ClaI. PCR product was cut with BamHI and ClaI and inserted into the same sites of pCS2+/MT vector.
exPTK7-HA	pCS2+/HA	extracellular part of *Xenopus laevis PTK7* with HA tag	extracellular part of *PTK7* was amplified from PTK7 using primers secPTK7F BamHI and secPTK7R ClaI. PCR product was cut with BamHI and ClaI and inserted into the same sites of pCS2+/HA vector.
ΔexPTK7-HA	pCS2+/HA	*Xenopus laevis PTK7* without extracellular domain with HA tag	(Shnitsar and Borchers 2008)
Wnt8	pCS2+	full length *Xenopus laevis Wnt8*	*Wnt8* was cut from MT-Wnt8 using EcoRI and XhoI and inserted into the same sites of pCS2+
dnWnt8	pCS2+	dominant-negative Wnt8, *Xenopus Wnt8* with truncated C terminus	(Hoppler et al. 1996)
Wnt3a	pGEM2	full length *Xenopus laevis Wnt3a*	(Wolda et al. 1993)
MT-Wnt8	pCS2+/MT	full length *Xenopus laevis* Wnt8 with Myc tag	(Damianitsch et al. 2009)

Name	Vector	Insert	Cloning strategy
MT-Wnt3a	pCS2+/MT	full length *Xenopus laevis* Wnt3a with Myc tag	(Damianitsch et al. 2009)
MT-Wnt5a	pCS2+/MT	full length *Xenopus laevis* Wnt5a with Myc tag	(Damianitsch et al. 2009)
MT-Wnt11	pCS2+/MT	full length *Xenopus laevis Wnt11*with Myc tag	(Damianitsch et al. 2009)
Xcaveolin1a	pCMVSport6	full length *Xenopus laevis caveolin1a*	Purchased from RZPD. Catalogue number IRBMp990B0725D
caveolin1a	pCS2+	full length *Xenopus laevis caveolin1a*	*caveolin* sequence was amplified from Xcaveolin1a with primers Cav1aF EcoRI and Cav1aR XhoI. PCR product was cut with EcoRI and XhoI and inserted into the same sites of pCS2+ vector.
caveolin1a-GFP	pCS2+/GFP	full length *Xenopus laevis caveolin1a* fused with GFP	*GFP* sequence was amplified from pEGFP-C1 vector using primers Gfp1F XhoI and Gfp1R XbaI. PCR product was cut with XhoI and XbaI and inserted into the same sites of caveolin1a construct.
caveolin1a-HA	pCS2+/HA	full length *Xenopus laevis caveolin1a* with HA tag	*caveolin* was amplified from Xcaveolin1a with primers Cav1aF EcoRI and Cav1aR XhoI. PCR product was cut with EcoRI and XhoI and inserted into the same sites of pCS2+/HA vector.
Frizzled7	pCS2+	full length *Xenopus laevis Frizzled7*	(Medina et al. 2000)
Frizzled7-MT	pCS2+/MT	full length *Xenopus laevis Frizzled7* with Myc tag	(Winklbauer et al. 2001)

Name	Vector	Insert	Cloning strategy
Frizzled7-HA	pCS2+/HA	full length *Xenopus laevis Frizzled7* with HA tag	*Frizzled7* sequence was amplified from Frizzled7 with primers Fz7-HA F EcoRI and Fz7-HA R XhoI. PCR product was cut with EcoRI and XhoI and inserted into the same sites of pCS2+/HA vector.
exFz7-MT	pCS2+/MT	extracellular domain of *Xenopus Frizzled7* with Myc tag	(Winklbauer et al. 2001)
lacZ	pCS2+	bacterial *β-galactosidase*	(Smith and Harland 1991)
xTwist	pGEM-T	full length *Xenopus laevis twist*	(Hopwood et al. 1989)
Dsh-MT	pCS2+/MT	full length *Xenopus laevis disheveled* with Myc tag	(Sokol 1996)
β-catenin-MT	pCS2+/MT	full length human *β-catenin* with Myc tag	(Behrens et al. 1996)
hFrizzled5	pCS2+	full length human *Frizzled5*	(He et al. 1997)
En-2	pBluescript	full length *Xenopus laevis* engrailed2	(Hemmati-Brivanlou et al. 1991)
Krox20	pGEM-T	full length *Xenopus laevis krox20*	(Bradley et al. 1993)
HoxB9	pBluescript	full length *Xenopus laevis* homeobox protein-coding gene *hoxB9*	(Godsave et al. 1994)
Siamois	pGL3	contains *Xenopus laevis* Siamois promoter upstream of Firefly *luciferase* ATG	(Brannon et al. 1997)

Name	Vector	Insert	Cloning strategy
Renilla	pRL-TK	contains Renilla *luciferase* gene downstream of herpes simplex virus thymidine kinase (HSV-TK) promoter	Promega
ATF2	pGL3	contains *atf2* gene promoter and Firefly luciferase gene	(van der Sanden et al. 2004)
TOP Flash	pGL3	contains 7 TCF/LEF binding sites and firefly *luciferase* gene	(Korinek et al. 1997)
PlexinA1	pCMVSport6	full length *Xenopus laevis Plexin A1*	obtained from ImaGenes, catalogue number IRBHp990B1170D
PlexinA1-HA	pCS2+/HA	full length *Xenopus laevis Plexin A1*with HA tag	*PlexinA1* sequence was amplified from PlexinA1 construct with primers Plx-HA F ClaI and Plx-HA R XhoI. PCR product was cut with ClaI and XhoI and inserted into the same sites of pCS2+/HA vector
rPlexinA1-HA	pCS2+/HA	full length *Xenopus laevis Plexin A1* with HA tag without PlexinA1 Morpholino binding sites	*PlexinA1* sequence was amplified from PlexinA1 construct with primers primers rPlxA1 F ClaI and Plx-HA R XhoI. PCR product was cut with ClaI and XhoI and inserted into the same sites of pCS2+/HA vector

Table 2 Linearization of DNA constructs and *in vitro* transcription

Construct name	sense RNA		antisense RNA	
	cut	polymerize	cut	polymerize
PTK7	NotI	SP6	SexA1	T7
PTK7-HA	NotI	SP6		
PTK7-MT	NotI	SP6		
PTK7-GFP	NotI	SP6		
ΔkPTK7-MT	NotI	SP6		
exPTK7-MT	NotI	SP6		
exPTK7-HA	NotI	SP6		
ΔexPTK7-HA	NotI	SP6		
Wnt8	NotI	SP6		
Wnt3a	NotI	SP6		
MT-Wnt8	NotI	SP6		
dnWnt8	NotI	SP6		
MT-Wnt3a	NotI	SP6		
MT-Wnt5a	NotI	SP6		
MT-Wnt11	NotI	SP6		
Xcaveolin1a	NotI	SP6	SalI	T7
caveolin1a	NotI	SP6	SalI	T7
caveolin1a-GFP	NotI	SP6		
caveolin1a-HA	NotI	SP6		
Frizzled7	NotI	SP6		
Frizzled7-MT	NotI	SP6		
lacZ	NotI	SP6		
xTwist			EcoRI	T7
Dsh-MT	NotI	SP6		
β-catenin-MT	NotI	SP6		
hFrizzled5	AvaIII	SP6		
Krox20			EcoRI	T7
En-2	XhoI	T7	XbaI	T3
HoxB9			BamHI	T7
PlexinA1-HA	NotI	SP6		
rPlexinA1-HA	NotI	SP6		

2.6 Oligonucleotides

The oligonucleotides (primers) were purchased from Sigma-Aldrich Chemie.

2.6.1 Sequencing primers

The sequencing primers used during this study are listed in Table 3.

Table 3. Sequencing primers

Construct/vector	Primer name	Primer sequence 5'→3'
pCS2+	SP6	TTAGGTGACACTATAGAATAC
	T7	TCTACGTAATACGACTCACTATAG
	T3	AATTAACCCTCACTAAAGGG
PTK7	AB0	AGAACTAGGAGTCGATGC
	AB1	TTCAGAGATGGGACGCCGTTA
	AB2	TGGAGGTGGTATTCAAACCC
	AB3	AGGGATGCTGGGAACTACAC
	AB4	CCATTGTTCTCTCTGTGGTTG
	AB5	GCATCTGGCTAACAGTCG
	AB7	TCCTATTGCCCTTAGTAGTGC
pCMVSport6	SP6	CTATTTAGGTGACACTATAG
	T7	TAATACGACTCACGTATAGGG

2.6.2 Cloning primers

The sequences of cloning primers are indicated in 5'→3' direction (the digestion enzyme site is underlined).

Gfp1F ClaI AT<u>ATCGAT</u>TGGTGAGCAAGGGCGAG

Gfp1R XbaI AT<u>TCTAGA</u>CTTGTACAGCTCGTCCATGC

exPTK7MT F BamH1 TT<u>GGATCC</u>ATGGGGCCGATTGTGCTC

exPTK7MT R Cla1 TT<u>ATCGAT</u>TCTGGATGAGTTTGTATGGGGAA

secPTK7 F BamHI	AGTGAGAGGATCCGGGGCAGGAAC
secPTK7R ClaI	CGATCGATGACCGCAGCAACCACAGAGAGAACAATG
Cav1aF EcoRI	TTGAATTCAGCATGTCTGGTGGCAAATACATAG
Cav1aR XhoI	TTCTCGAGCACTTCTTTGCGTAAGGAA
Gfp1F XhoI	ATCTCGAGGTGAGCAAGGGCGAGGAG
Fz7-HA F EcoRI	TTGAATTCATGTCCTCTACAGTCTCGCTGC
Fz7-HA R XhoI	TTCTCGAGCACCGCAGTCTCCCCTTTGCTGCC
Plx-HA F ClaI	TTATCGATATTGTTCTAATTATTATTAGGGAAGATGC
Plx-HA R XhoI	TTCTCGAGACTACTCTGCGCCATTGTGTCGATGACC
rPlxA1 F ClaI	TTATCGATATGCTCCTGCACGCGGGCAAACCTCTTCTGTTCCATTTATGGAC

2.6.3 Morpholino oligonucleotides

Antisense Morpholino oligonucleotides (Morpholinos, MO) were purchased from Gene Tools, LLC (Philomath, USA). Morpholinos were dissolved in RNAse-free water to a 1μM concentration. The sequences of the Morpholinos used in this study are presented in Table 4.

Table 4. Antisense Morpholino oligonucleotides

Morpholino name	Target gene	Sequence 5'→3'	Working concentration
PTK7MO2	*Xenopus laevis PTK7*	TGCATCGCGGCCTCTCCCCTCAC	5-10 ng/embryo
PTK7MO3	*Xenopus laevis PTK7*	TTCCTGCCCCGGATCCTCTCACTGC	5-10 ng/embryo
PTK7mmMO2	mutated PTK7MO2, which does not bind target sequence	TGgATCcCGcCCTCTgCCgTCA	5-10 ng/embryo
PTK7mmMO3	mutated PTK7MO3, which does not bind target sequence	TTgCTcCCCCGcATCCTgTCAgTGC	5-10 ng/embryo

Morpholino name	Target gene	Sequence 5'→3'	Working concentration
PlxA1MO1	*Xenopus laevis PlexinA1*	AGGGAGGTTATATTTCCAAGTTGAC	5-10 ng/embryo
PlxA1MO2	*Xenopus laevis PlexinA1*	GGCCTCTCGGCGTGGAGCAGCATCT	5-10 ng/embryo
control Mo	no target	CCTCTTACCTCAGTTACAATTTATA	5-10 ng/embryo

2.7 DNA methods

2.7.1 Plasmid DNA preparations

Isolation of plasmid DNA in analytical amounts was performed using Illustra^{TM} Plasmid Prep Mini Spin Kit (GE Healthcare). For the isolation of plasmid DNA in preparative amounts Illustra^{TM} Plasmid Prep Midi Flow Kit (GE Healthcare) was used. The DNA isolation was performed according to the manufacturer's instructions. DNA concentrations were measured using the ND-1000 Spectrophotometer, Coleman Technologies Inc.

2.7.2 DNA restriction digestion

DNA restriction digestion was performed with restriction endonucleases purchased from MBI Fermentas according to manufacturer's instructions.

2.7.3 Agarose gel electrophoresis

DNA or RNA fragments were separated in a horizontal electrical field into agarose gel (Sharp et al. 1973). The electrophoresis was run in the standard TAE-running buffer at 100-120 V in horizontal electrophoresis chamber. Depending on the expected sizes of DNA/RNA fragments, 0.8 to 2% (w/v) agarose gels were prepared in TAE buffer. Gels always contained 0.5 μg/ml ethidium bromine to visualize nucleic acids. Before loading samples into the gel slots, nucleic acids were mixed with DNA loading dye (6x, Ambion). After the electrophoresis, DNA bands were visualized with the UV-transilluminator (Herolab) and documented with the ChemiDoc video documentation system (EASY view).

Standard DNA ladders were used to determine the sizes of DNA fragments (High, Middle or Low Range, Fermentas).

2.7.4 Purification of DNA fragments from agarose gel or restriction digestion mixture

The purification of DNA fragments from agarose gels or restriction digestion mixture was performed with the Illustra^{TM} GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to manufacturer's instructions.

2.7.5 Polymerase chain reaction (PCR)

A standard PCR reaction was used to amplify desired DNA fragments (Mullis et al. 1986).

The PCR reaction mixture:

Matrix DNA – 50 ng

10x buffer – 5 µl

dNTP (10mM each, Fermentas) – 2 µl

DNA polymerase – 1 µl

10 µM forward primer – 1 µl

10 µM reverse primer – 1 µl

HPLC water (Roth) – up to 50 µl

DreamTag^{TM} polymerase (5 U/µl) (Fermentas) was used for analytical PCR. High fidelity *Pfu* polymerase (2.5 U/µl, Fermentas) was used for the PCR followed by molecular cloning of the amplified fragments.

The following program was used for DNA fragments amplification:

1 cycle:

Initial denaturation step – 95°C, 2 min

30 cycles:

Denaturation – 95°C, 45 sec

Primer annealing – the temperature depends on primer GC content, 45 sec

Elongation step – 68°C for *Pfu* polymerase, 72°C for DreamTag^{TM} polymerase, 1min/1000bp of target DNA fragment

1 cycle:

Final elongation step – 68°C or 72°C depending on the polymerase used, 10 min

2.7.6 DNA ligation

T4 DNA ligase (Fermentas) was used according to manufacturer's instructions. For the ligation a vector and an insert were taken in 1 to 3 molar ratio. The total amount of vector DNA was 50 ng. For 20 µl of a single reaction mixture 2 µl of T4 DNA ligase (5 U/µl) was used. The ligation was performed 1 hour at room temperature for inserts shorter than 1000 bp or overnight at 16°C for inserts longer than 1000 bp. The ligase was inactivated 10 min 65°C before the transformation of *E.coli* Xl1blue strain.

2.7.7 Chemical transformation and electrotransformation of bacterial cells

For chemical transformation 100 µl of chemically competent *E.coli* XL1blue cells were thawed on ice, mixed with 1 or 2 µl of the ligation mix or 100-500 ng of plasmid DNA, incubated for 10 min on ice and heat-shocked for 2 min at 41°C, then left for 2 min on ice. 1 ml of warm LB medium was added to the cells and the mixture was incubated at 37°C for 1 hour (Mandel and Higa 1970).

For electrotransformation 40 µl of electrocompetent cells were thawed on ice, mixed with 1 or 2 µl of the ligation mix, transferred into a yellow 2 mm electroporation cuvette (Thermo Electron) and incubated for 1 min on ice. After application of an electrical pulse of 1.8 kV and 25 µF, the transformation reaction was mixed with 0.5 ml of LB-medium (Dower et al. 1988).

Bacterial pellets were seeded on LB agar plates supplemented with appropriate antibiotics for the selection of transformed cells (0.1 mg/ml ampicillin (Biomol), 0.05 mg/ml kanamycin (Biomol). Colonies were grown overnight at 37°C (Mandel and Higa 1970).

2.7.8 DNA sequencing analysis

Dye-termination sequencing method, which is the modification of Sanger chain-termination sequencing, was used (Sanger et al. 1977). The Big Dye^{TM} Terminator Kit (Applied Biosystems) was used for preparation of the sequencing PCR according to the manufacturer's instructions.

The sequencing PCR mixture:

DNA matrix – 200-400 ng

Seq mix – 1.5 µl

Seq buffer – 1.5 µl

Primer – 8 pmole

HPLC water – up to 10 µl

The sequencing PCR

25 cycles:

96 °C, 10 sec

55°C, 15 sec

60°C, 40 sec

To purify the sequencing reaction the following components were added to the PCR mixture: 1 µl of 125 mM EDTA (pH 8.0), 1µl of 3 M sodium acetate (pH 5.4) and 50 µl 100% ethanol. The sample was incubated 5 min at room temperature, then centrifuged 15 min at 14000 rpm. The pellet was washed with 70 µl of 70% ethanol, dried and diluted in 15 µl of HiDi™ buffer (Applied Biosystems).

The automated sequencing was performed by in-house sequencing lab using the ABI 3100 Automated Capillary DNA Sequencer (Applied Biosystems).

2.8 RNA methods

2.8.1 *In vitro* synthesis of capped sense mRNA

In vitro synthesis of sense capped mRNAs for microinjections into *Xenopus* embryos was performed with the SP6 or T7 mMessage mMachine kits™ (Ambion) according to the manufacturer's protocol. For a 20 µl reaction 0.5 - 1 µg of linearized DNA template was used. The reaction took place 2 - 3 hours at 37°C. It was followed by the 15 min treatment with 5 units of TURBO DNAseI (Ambion) to remove the template DNA. Synthesized RNA was purified with Illustra™ RNAspin Mini RNA Isolation Kit (GE Healthcare). RNA concentration measurement was performed using the ND-1000 Spectrophotometer, Coleman Technologies Inc.

2.8.2 *In vitro* synthesis of labeled antisense RNA

The following reaction mixture was used for the synthesis of labeled antisense RNA for whole mount *in situ* experiments:

5 µl of a 5x transcription buffer (Fermentas)
1 µl each 10 mM rATP, rCTP, rGTP 0.64 µl 10 mM rUTP (Boehringer)
0.36 µl digoxigenin-rUTP (Boehringer)
1 µl 0.75 M DTT
0.5 µl RNAseOut (Invitrogene),
200 - 1000 ng linearized DNA template
1 µl T3, T7 or Sp6 polymerase (20U/µl, Fermentas)
RNAse-free water to the final volume of 25 µl

The reaction took place 2 - 3 hours at 37°C. It was followed by 15 min treatment with 5 units of TURBO DNAseI (Ambion) to remove the template DNA. Synthesized RNA was purified with RNeasy^{TM} Mini Kit (Quiagen) according to manufacturer's instructions.

2.9 *Xenopus* embryo culture, micromanipulations and microinjections

2.9.1 Preparation of *Xenopus laevis* testis

The *Xenopus* male frog was sacrificed by submerging it in 0.05% benzocaine for 30 min at room temperature. The frog was decapitated, the skin flap was removed from the belly and an incision into the muscle was performed. The testes were removed through the incision together with fat body and detached from it. The testes were washed 3 times with and stored in the 1x MBS buffer at 4°C.

2.9.2 Embryo microinjections and culture

Xenopus laevis female frogs was induced to lay eggs by human chorionic gonadotropin (hCG, Sigma Aldrich) injections into the dorsal lymph sac (1000 units hCG approximately 12 hours before desired egg-laying). Laid eggs were *in vitro* fertilized with minced testis in 0.1x MBS. Fertilized embryos were treated with 2% cyteine hydrochloride, pH 8.0 to remove the jelly coat. The embryos were cultured in 0.1 X MBS at 12.5 - 18°C. Albino embryos were stained with Nile Blue vital dye for 10 min prior to injection to allow to distinguish animal and vegetal poles, as well as different stages of development. Injections were performed in injection buffer on a cooling plate (14°C). The solutions for microinjections were loaded into the glass needles prepared on Leitz Needle puller. The Microinjector 5242 (Eppendorf) was used during this study. The mRNA and Morpholino oligonucleotides were injected animally to the prospective ectodermal tissues. For different purposes injections at 1-, 2-, 4- or 8-cell stages were performed. The injection volumes varied from 5 to 10 nl. Injected embryos were kept for at least 1 hour in the injection buffer to allow the heeling of injection opening and then transferred into 0.1x MBS. The developmental stages were defined according to Nieuwkoop and Faber Normal Table of *Xenopus laevis* (Daudin).

2.9.3 Second axis assay

The second axis assay was performed as described (Kuhl and Pandur 2008) Pigmented *Xenopus* embryos were injected into the marginal zone of one ventral blastomere at the four-cell stage. Axis duplication was induced by injection of 3 pg *Wnt3a*, 10 pg *Wnt8*, 250 pg *Dsh* and 50 pg *β-catenin* mRNA. 500 pg of *PTK7* or *lacZ* mRNA were co-injected. The second axis induction was scored at tadpole stages (stages 22 - 25).

2.9.4 *Xenopus* ectodermal explants (animal caps)

To investigate the intracellular localization of selected proteins the animal cap assay was employed (Wallingford and Harland 2001). Animal caps were excised with forceps from stage 8 embryos on 1% agarose-coated Petri dishes in 0,8x MBS buffer. At stage 10.5 - 14 animal caps were fixed 45 min in MEMFA and immunostained.

2.10 Whole-mount *in situ* hybridization (WISH) and X-gal staining

2.10.1 X-gal staining

ß-galactosidase (*lacZ*) mRNA was co-injected as a lineage tracer for whole-mount *in situ* hybridization (WISH) experiments. The X-gal staining allows the visualization of the *lacZ* RNA-injected regions of the embryo (Hardcastle et al. 2000). The embryos were fixed 1 hour in MEMFA before staining, then washed 3 times in 1x PBS and stained with X-gal solution in the dark until the desired level of staining was achieved. The X-gal solution was washed away 3 times with PBS and the embryos were re-fixed at least 2 hours in MEMFA. The embryos were transferred in 100% ethanol for long-term storage.

2.10.2 Whole-mount *in situ* hybridization (WISH)

Whole-mount *in situ* hybridization (WISH) was performed as described (Harland 1991). All the steps were performed at room temperature with mild agitation.

WISH day 1.

Rehydration of embryos

Prior to WISH embryos were rehydrated, as it is described in the Table 5

Table 5. Rehydration of embryos

Step Number	Solution	Incubation time
1	100% ethanol	3 min
2	75% ethanol in water	3 min
3	50% ethanol in water	3 min
4	25% ethanol in PTw	3 min
5	PTw	3 min

Proteinase K treatment

To make the embryos accessible for RNA probes, they were treated with proteinase K (10 µg/ml) in PTw. The proteinase K incubation time was chosen depending on the embryo stage (Table 6).

Table 6. Proteinase K treatment procedure

Developmental stage of *Xenopus* embryos	Incubation time (min)	Temperature
9 - 10.5	6 - 8	room temperature
14 - 16	8 - 10	room temperature
20 - 25	15 - 18	room temperature
36	22 - 25	room temperature
40	17 - 20	37°C
42 - 43	27 - 30	37°C
46	32 - 35	37°C

Acetylation and refixation

Acetylation of embryos was performed as described in the Table 7.

Table 7 Acetylation of *Xenopus* embryos

Step Number	Buffer	Incubation time
1	1M Triethanolamine chlorid, pH 7.0 (TEA)	2x 5min
2	1M TEA with 0.3% acetic anhydride	5 min
3	1M TEA with 0.6% acetic anhydride	5 min
4	PTw	5 min

Upon acetylation, embryos were fixed for 20 min in PTw containing 4% (v/v) formaldehyde and washed 5 times with PTw buffer.

Hybridization

After the last washing step approximately 1 ml of PTw was left in the tubes and 250 µl Hyb-Mix was added. The solution was replaced immediately by 500 µl of fresh Hyb-Mix and incubated for 10 minutes at 60°C. Hyb-Mix was exchanged again and embryos were incubated 4 - 5h at 60°C. The Hyb-Mix was replaced with the desired labeled RNA probe, diluted in Hyb-Mix solution. The hybridization took place overnight at 60°C.

WISH day 2.

Washing and RNAse treatment

To remove unbound RNA probes, the samples were washed and digested with RNAse A (10 µg/ml) and RNAse T1 (10 U/ml) as described in the Table 8.

Table 8. Washing and RNAse treatment of *Xenopus* embryos

Step Number	Solution	Incubation temperature and time
1	Hyb Mix	60°C, 10 min
2	2x SSC	60°C, 3x 15 min
3	RNAses in 2x SSC	37°C, 60 min
4	2x SSC	room temperature, 5 min
5	0.2x SSC	60°C, 2x 30 min
6	MAB	room temperature, 2x 15 min

Blocking and antibody reaction

Embryos were blocked with MAB buffer, containing the Boehringer Mannheim Blocking Reagent (BMB) and horse serum, and incubated with Sheep Alkaline phosphatase-coupled anti-Dig antibody (Sigma) according to the Table 9.

Table 9. Blocking and antibody incubation

Step Number	Solution	Incubation temperature and time
1	MAB/2% BMB	room temperature, 10 min
2	MAB/2% BMB/20% Horse serum	room temperature, 30 min
3	MAB/2% BMB/20% Horse serum 1:5000 α-DIG antibodies	room temperature, 4 hours
4	MAB	room temperature, 3x 10 min
5	MAB	4°C, overnight

WISH day 3.

Staining reaction

The alkaline phosphatase staining reaction was performed as described in Table 10.

Table 10. Alkaline phosphatase staining reaction

Step Number	Solution	Incubation time
1	MAB	5x 5 min, room temperature
2	APB	3x 5min, room temperature
3	APB with 80 µg/ml NBT, 175 µg/ml BCIP	Up to three days, 4°C

Upon the staining, albino embryos were fixed in MEMFA, washed with PTw, documented and stored in 100% ethanol. Pigmented embryos were bleached as described in section 2.10.3 to remove the pigment, which can interfere with the specific WISH signal.

2.10.3 Bleaching

The bleaching of pigmented embryos was performed as described in Table 11.

Table 11. Bleaching of pigmented *Xenopus* embryos

Step Number	Solution	Incubation time
1	2x SSC	3x 5 min
2	2x SSC with 50% formamide, 1% H_2O_2,	Until embryos loose pigment
3	MEMFA	30 min
4	PTw	3x 5 min

Bleached embryos were documented and stored in 100% ethanol.

2.11 Cell culture techniques

2.11.1 Propagation of cell lines

Cells were cultured in DMEM medium (Biochrom) supplemented with 10% fetal calf serum (FCS), 100 units/µl penicillin and 100 µg/ml streptomycin (full DMEM). The media for L Wnt3a cells and L Wnt5a cells were also supplemented with 0.4 and 0.6 mg/ml G418 (Invitrogen) respectively. Cell lines were maintained at 37°C, 95 % humidity, 5 % CO_2.

2.11.2 Subculturing of cell lines

The cells in 75 cm^2 flask were rinsed with 1x PBS and incubated with 2 - 3 ml of 0.25% (w/v) trypsin 10 - 20 min at 37°C until the cell layer was dispersed. The reaction was stopped with 5 ml of DMEM medium. The appropriate number of cells was transferred into a fresh 75 cm^2 flask containing 15 ml of fresh full DMEM medium.

2.11.3 Wnt3a and Wnt5a conditioned medium collection

The conditioned medium was obtained according to LGC Promochem recommendations. L Wn3a, L Wnt5a or control L cells were splitted 1:10 in 75 cm^2 flasks

and maintained in full DMEM. After 4 days the first batch of conditioned medium was collected. Fresh full DMEM medium was applied on the cells and after 3 days the second batch of the conditioned medium was collected. The medium batches were mixed together, sterile filtered, aliquoted and stored at - 20°C.

2.11.4 Transfection of HEK293 cells

HEK293 cells were transfected with Lipofectamine2000™ (Invitrogen) according to the manufacturer's instructions. Cells were plated 24 hours before transfection in DMEM containing 10% FCS but no antibiotics. The amounts of DNA and Lipofectamine2000™ as well as the seeding density of HEK293 cells are presented in Table 12. DNA and Lipofectamine2000™ were diluted in OptiMem (Gibco), incubated 5 minutes at room temperature, then DNA and Lipofectamine samples were combined and incubated 15 min at room temperature. The mixture was applied on the cells for 4 hours, after which the plating medium was changed for fresh full DMEM.

Table 12. Transfection of HEK293 cells with Lipofectamine2000™

Plate type	Seeding density (cells per well)	Amount of DNA per well	Volume of lipofectamine per well	Volume of OptiMem
96 well plate	8000	200 ng	0.5 µl	12.5 µl
24 well plate	75000	800 ng	2 µl	50 µl
6 well plate	300000	3200 ng	8 µl	200 µl
10 cm^2 Petri Dish	2250000	24 µg	60 µl	1500 µl

2.12 Protein techniques

2.12.1 Antibodies

The antibodies used during this study are presented in Table 13.

Table 13. Antibodies

Name	Company, catalogue number	Description	Dilution		
			WB	IP	IF
HA.11	Covance, MMS-101P	Primary mouse monoclonal antibodies. recognizes HA tag (peptide CYPYDVPDYASL)	1:1000	1:150	1:100
HA.11-FITC	Covance, FITC-101L	HA.11 antibody directly coupled with FITC fluorescent dye			1:100
9E10 MT	Sigma, M4439	Primary mouse monoclonal antibodies, recognize myc tag (peptide corresponding to residues 408-439 of the human cMyc protein)	1:5000	1:500	
9E10 MT-Cy3	Sigma, C6594	9E10 MT antibody directly coupled with Cy3 fluorescent dye			1:100
MT	Abcam ab19234	Primary goat polyclonal against myc tag (recognizes peptide EQKLISEEDL)	1:10000		
GFP	Roche, 11814460001	Primary mouse monoclonal against GFP	1:1000		
GFP 1	Abcam, ab290	Primary rabbit polyclonal against GFP			1:1000
actin C4	Millipore, MAB1501	Primary mouse monoclonal against actin	1:10000		
Wnt3a	Cell Signaling, 2721	Primary rabbit against mouse Wnt3a	1:1000		
Wnt5a	Cell Signaling, 2392	Primary rabbit against mouse Wnt5a	1:1000		
LRP6	Cell Signaling, 2560	Rabbit IgG against human LRP6	1:1000		
pLRP6	Cell Signaling, 2568	Rabbit IgG against Ser1490 phosphorylated human LRP6	1:1000		
α-mouse-Alexa 488	Invtrogen, A11029	Secondary goat anti mouse IgG coupled with Alexa 488 dye			1:200

Name	Company, catalogue number	Description	Dilution		
			WB	IP	IF
α-mouse-Alexa 596	Invitrogen, A11005	Secondary goat anti mouse IgG coupled with Alexa 596 dye			1:200
α-rabbit-FITC	Sigma, F7367	Secondary goat anti rabbit IgG coupled with FITC dye			1:200
α-rabbit-HRP	Cell Signaling, 7074	Secondary anti rabbit IgG coupled with HRP		1:2000	
α-mouse-HRP	Santa Cruz, sc-2005	Secondary goat anti mouse IgG coupled with HRP		1:5000	
α-goat-HRP	Santa Cruz SC-2020	Secondary donkey anti goat IgG coupled with HRP		1:10000	

2.12.2 Protein electrophoresis under the denaturating conditions (SDS-PAGE)

The proteins were separated by SDS polyacrylamid gel electrophoresis (Laemmli 1970). Gels of the different acrylamid percentages were used for the analysis of the proteins with distinct molecular weights according to the Table 14.

Table 14. The sizes of separated proteins and the percentages of the correspondent acrylamid gels

Protein size, kDa	% of acrylamide
36 - 205	5%
24 - 205	7.5%
14 - 205	10%
14 - 66	12.5%
10 - 45	15%

The protein samples were diluted 1:5 with 6x Laemmli loading buffer and boiled for 5 min at 95°C, then applied on the gel. The gel running was performed in the BioRad™ gel chambers in 1x Laemmli running buffer. At first the voltage of 70 V was applied, and once the bromphenol-blue front reaches the separating gel, the voltage was raised to 120 V.

2.12.3 Harvest of HEK293 cells for Western Blotting

HEK293 cells were scraped from cell culture surface with a cell scraper and centrifuged 10 min at 3000 rpm. The CoIP lysis II cells was added to the pellet (100 and 200 µl of buffer for 1 well of 12- and 6-well plate respectively). The cells were lysed 5 times through insulin syringe and centrifuged 15 min, 14000 rpm at 4°C. The supernatant was diluted 1:5 with 6x Laemmli loading buffer, boiled for 5 min at 95°C and applied on the gel.

2.12.4 Lysis of *Xenopus* embryos for Western Blotting

The embryos were lysed through insulin syringes in CoIP lysis buffer I. For one embryo 10 µl of CoIP buffer lysis buffer I was used. Lysates were centrifuged for 15 min at 16000 g at 4°C and supernatants were diluted 1:5 with 6x Laemmli loading buffer, boiled for 5 min at 95°C and applied on the gel.

2.12.5 Western Blotting

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (0.45 µm, Schleicher & Schuell) in the BioRad Transfer chamber using the wet blotting method (Sambrook et al. 1989). The transfer was performed in Western Blotting (WB) buffer for 70 min at 110 V at 4°C.

After the transfer, the membrane was blocked with a blocking solution. The incubation with primary antibody solution was carried out overnight at 4°C in the blocking solution. Next morning the membrane was washed 3 times 15 min in blocking solution and the secondary antibodies coupled with horse reddish peroxidase (HRP) were applied in blocking solution for 1 - 2 h at room temperature. Then, the membrane was washed again with blocking solution 2 times for 15 min and one time in Ptw for 15 min. The HRP signals were detected with the ECL Kit Super Signal Dura^{TM} West Kit (Pierce) on X-ray detection film (Amersham).

2.12.6 Co-immunoprecipitation in *Xenopus* embryos

Co-immunoprecipitation (CoIP) in *Xenopus* embryos was performed as described (Steinbeisser and Swain 2008). *Xenopus* embryos were injected with mRNAs coding tagged proteins animally into both blastomeres at the two cell stage and cultured until stage 10.5 – 11. 50 embryos were lysed through an insulin syringe in CoIP lysis buffer I. The lysates were centrifuged for 15 min at 16000 g at 4°C and the supernatants were incubated for 30 min with 30 µl Protein A Sepharose (Amersham) at 4°C for pre-clearing. After the centrifugation supernatants were transferred into fresh tubes and incubated with antibodies (anti-HA.11 (Convance) or anti-MT 9E10 (Sigma)) for two hours at 4°C, followed by two hours incubation with 30 µl Protein A Sepharose (Amersham) at 4°C. Sepharose beads were washed 3 times for 5 min with CoIP lysis buffer I at 4°C, boiled for 3 min in 6x Laemmli loading buffer and analyzed by Western blotting.

2.12.7 Co-immunoprecipitation in HEK293 cells

HEK293 cells were transfected 48 hours before co-immunoprecipitation (CoIP). One 10 cm^2 Petri dish was used for each sample. The cells were scraped from the dish, washed ones in ice-cold PBS. The pellets were resuspended in 1 ml of CoIP lysis buffer II and lysed 5 times through an insulin syringe. Lysates were centrifuged for 15 min at 16000 g at 4°C and the following steps were the same as for CoIP in *Xenopus* embryos (see previous section).

For the CoIP experiments with HEK293 cells supernatants, exFz7-MT, exPTK7-HA or empty vector DNAs were transfected in HEK293 cells and the cell supernatants containing secreted proteins were collected after three days. The cell supernatants were mixed in a 1:1 ratio (1 ml total volume), 400 ng rhWnt3a (RαD Systems) was added and the antibodies were applied. Antibody reaction and the following steps were the same as for CoIP in *Xenopus* embryos (see previous section).

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2.13 Luciferase assay

2.13.1 Luciferase assay in *Xenopus* embryos

The two-cell stage embryos were injected animally into both blastomeres with 50 pg Siamois (S0 1234, (Brannon et al. 1997)) or 100 pg ATF2 (van der Sanden et al. 2004) and

10 pg Renilla (Promega) luciferase reporter DNA in combination with the respective mRNAs. The following mRNAs concentrations were used: 50 pg of *β-catenin*, 500 pg *Dsh*, 1 ng *LRP6*, 250 pg *Fz7*, 100 pg *Wnt3a*, 100 pg *Wnt8* and 250 - 500 pg *PTK7* RNA. Six samples were used for one injected construct combination. Each sample contained lysates from six stage 11 embryos. The Dual Luciferase Assay Kit (Promega) was used for the measurement of Firefly and Renilla luciferase activity according to the manufacturer's instructions. The measurements were performed on a Centro LB 960 luminometer (Berthold Technologies).

2.13.2 Luciferase assay in HEK293 cells

HEK293 cells were transfected in 96 well plates with 38 ng TOPflash (obtained from R. Moon), 6 ng Renilla (Promega) and 156 ng of rest DNA per one well. Three to six independent wells were transfected for one construct combination. The conditioned Wnt3a or control medium was applied in 24 hours after transfection for another 24 hours. The cells were lysed in Passive Lysis Buffer (Promega) and the Dual Luciferase Assay Kit (Promega) was used for the measurement of Firefly and Renilla luciferase activity according to the manufacturer's instructions. The measurements were performed on a Centro LB 960 luminometer (Berthold Technologies).

2.14 Immunofluorescent staining

2.14.1 Immunofluorescent staining of *Xenopus* animal caps

Animal caps and CNC explants were fixed for 45 min in MEMFA, washed 3 times for 15 min in Ptw, blocked for 1 hour in Ptw containing 10% FCS and incubated with primary antibody in Ptw overnight at 4°C. The unbound antibodies were washed away 3 times for 15 min in Ptw and then secondary antibodies were applied if required for 2 hours in Ptw at room temperature. The unbound secondary antibodies were washed away 3 times for 15 min in Ptw buffer. The protein localization was analyzed by laser scanning microscopy on LSM 510Meta, Zeiss.

2.14.2 Immunofluorescent staining of HEK293 cells

HEK293 cells were stained 24 hours after transfection. The cells were fixed for 10 min in 4% PFA, washed 3 times for 15 min in PBS, permeabilized for 10 min in PBS containing 0.1% TritonX100, blocked for 1 hour with PBS with 10% BSA. The antibody staining was performed the same as for *Xenopus* animal caps (section 2.14.1) but PBS buffer was used instead of Ptw.

3. Results

3.1 PTK7 interacts with canonical Wnt proteins

The main aim of this work was to identify the extracellular ligands of PTK7. PTK7 is a transmembrane protein with a single membrane-spanning domain, seven extracellular immunoglobulin-like domains and an intracellular part containing a kinase-homology domain, which is a typical structure of a receptor tyrosine kinase. However, so far no extracellular interaction partners of PTK7 have been identified. To search for PTK7 ligands a candidate approach was used. As PTK7 regulates non-canonical Wnt signaling and is a part of Fz-Dsh complex (Lu et al. 2004; Shnitsar and Borchers 2008), we asked if PTK7 can interact with Wnt proteins.

Co-immunoprecipitation (CoIP) experiments between PTK7 and different Wnt proteins were performed in *Xenopus* embryos. mRNAs coding for myc-tagged Wnt and HA-tagged PTK7 were injected into *Xenopus* embryos at the two-cell stage and the embryos were used for CoIP with the antibodies against HA tag at gastrula stages (Figure 19 A). Indeed, myc-tagged Wnt3a and Wnt8 were co-immunoprecipitated together with PTK7-HA (Figure 19 B, C), but Wnt5a and Wnt11 were not (Figure 19 D, E). Interestingly, Wnt3a and Wnt8 activate canonical Wnt signaling in *Xenopus*, while Wnt5a and Wnt11 are involved in the activation of PCP (Wong et al. 1994; Du et al. 1995). It was a surprising result that PTK7, a known regulator of non-canonical Wnt signaling, interacts with canonical Wnts, but not with non-canonical ones.

To confirm the interaction CoIP experiments were performed in the reverse direction. The *MT-Wnt3a* or *MT-Wnt8* mRNA was injected together with *exPTK7-HA* mRNA, which codes an extracellular domain of PTK7, at the two-cell stage *Xenopus* embryos. At the gastrula stages the embryos were used for CoIP with the antibodies against myc tag. Indeed, exPTK7-HA was co-immunoprecipitated together with myc-tagged Wnt3a and Wnt8 (Figure 20 A, B).

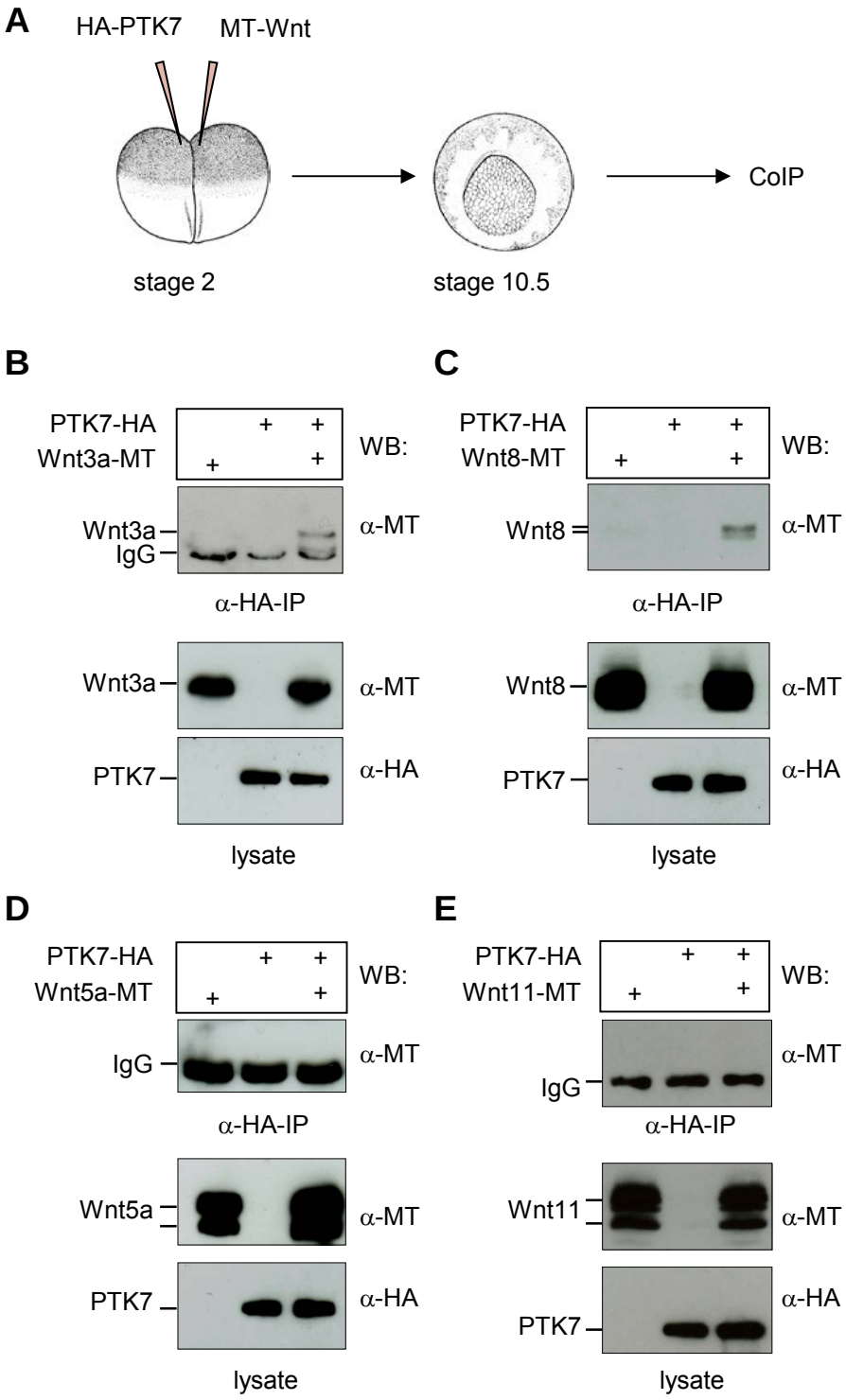

Figure 19. PTK7 interacts with Wnt3a and Wnt8 but not with Wnt5a and Wnt11 proteins. (A) *Xenopus* embryos were injected at the two-cell stage with 1 ng *HA-PTK7* and 80 pg *MT-Wnt* mRNAs and lysed at the gastrula stage for CoIP. (B-E) CoIP experiments showing co-precipitated Wnt proteins in the upper panel and *Xenopus* lysates used for CoIP in the lower panels. (B) PTK7 co-precipitates MT-Wnt3a. (C) PTK7 co-precipitates MT-Wnt8. (D) PTK7 does not co-precipitate MT-Wnt5a. (E) PTK7 does not co-precipitate MT-Wnt11.

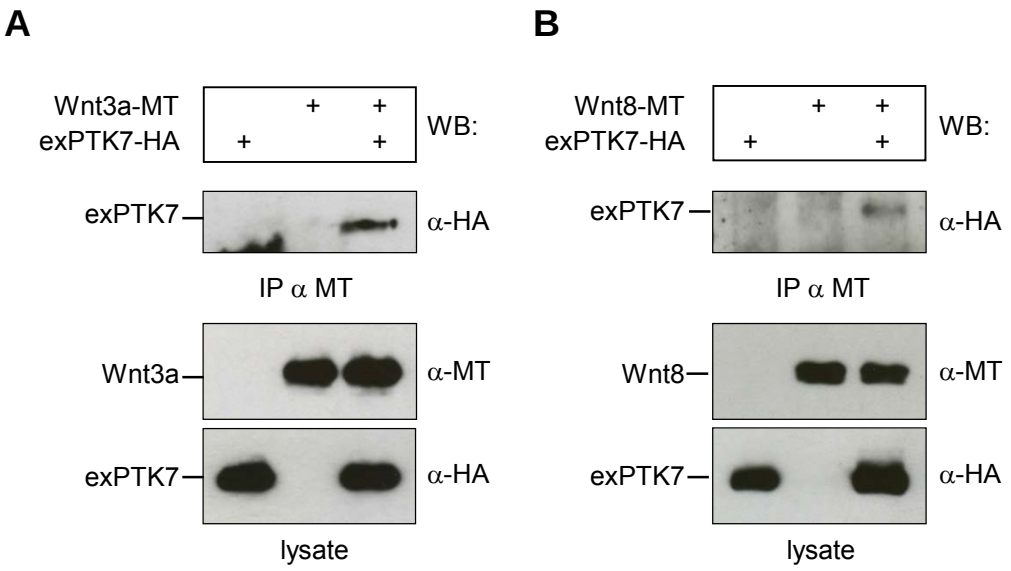

Figure 20. Wnt3a and Wnt8 co-precipitate exPTK7, a deletion mutant of PTK7 consisting of only extracellular domain. The co-precipitated exPTK7-HA is shown in the upper panel, the *Xenopus* lysates used for CoIP are shown in the lower panels. *Xenopus* embryos were injected at the two-cell stage with 800 pg *exPTK7-HA* and 80 pg *MT-Wnt3a* or *MT-Wnt8* mRNAs and lysed at the gastrula stages for CoIP. (A) Wnt3a-MT co-precipitates exPTK7-HA. (B) Wnt8-MT co-precipitates exPTK7-HA.

Our data indicated that PTK7 selectively interacts with canonical, but not with non-canonical Wnt proteins.

3.2 PTK7 interacts with Wnt ligands through Frizzled

As PTK7 can interact with *Xenopus* Fz7 (M. Podleschny, unpublished), we asked if PTK7 interacts with Wnts directly or through Fz receptor. Constructs coding for an extracellular part of PTK7 (exPTK7-HA) or an extracellular domain of Fz7 (exFz7-MT) were transfected in HEK293 cells and the cell culture supernatants were collected after three days. Supernatant containing exPTK7-HA was incubated with recombinant human Wnt3a (rhWnt3a) protein in the presence or absence of exFz7-MT and anti-HA immunoprecipitates were analyzed using Western Blotting. CoIP of rhWnt3a with exFz7-MT was used as a positive control (Figure 21). PTK7 can co-precipitate rhWnt3a only when exFz7 was present in the mixture, indicating that PTK7/Wnt3a binding is mediated through the extracellular domain of Frizzled protein (Figure 21).

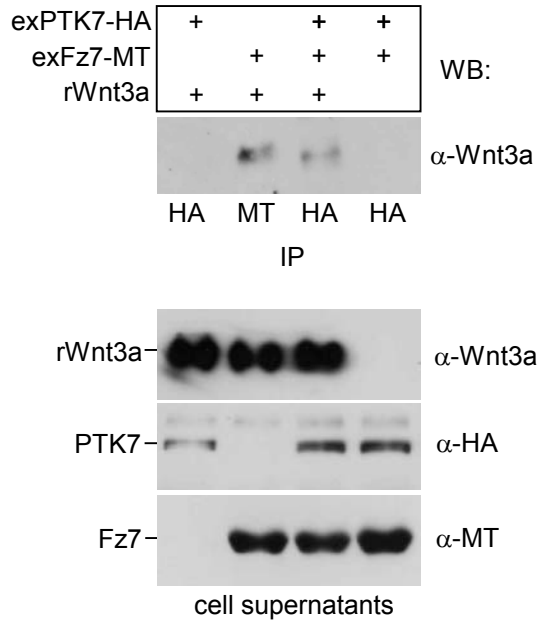

Figure 21. PTK7 interacts with Wnt3a through an extracellular domain of Fz. exFz7-MT, exPTK7-HA or empty vector DNAs were transfected in HEK293 cells and the cell supernatants containing secreted proteins were collected after three days. The cell supernatants were mixed in a 1:1 ratio (1 ml total volume), 400 ng rhWnt3a was added as indicated in the upper panel and CoIP with the indicated antibodies was performed. Co-precipitated Wnt proteins are shown in the upper panel and the cell supernatants used for CoIP are shown in the lower panels. exFz7-MT co-precipitates rhWnt3a (positive control), however, exPTK7-HA co-precipitates rhWnt3a only when exFz7-MT is present.

3.3 PTK7 inhibits canonical Wnt signaling

The interaction between PTK7 and canonical Wnt proteins and Fz7 indicated that PTK7 may affect canonical Wnt signaling. To test this, PTK7 was used in second axis assays and Siamois luciferase assays in *Xenopus* embryos.

Canonical Wnt proteins are able to induce a second axis in *Xenopus* when they are overexpressed in the future ventral part of embryo (Figure 22 A). This property allows to study the effect of different proteins on canonical Wnt signaling in so called second axis assay. The mRNA coding for Wnt3a or Wnt8, both of which are able to induce second axis formation in *Xenopus*, was injected ventrally together with mRNA coding for PTK7 or lacZ. *LacZ* RNA was used as a control to have the equal amount of RNA injected in every embryo. PTK7, when overexpressed together with Wnt3a and Wnt8, significantly inhibited the second axis formation (Figure 22 B, C), showing that PTK7 negatively regulates canonical Wnt signaling.

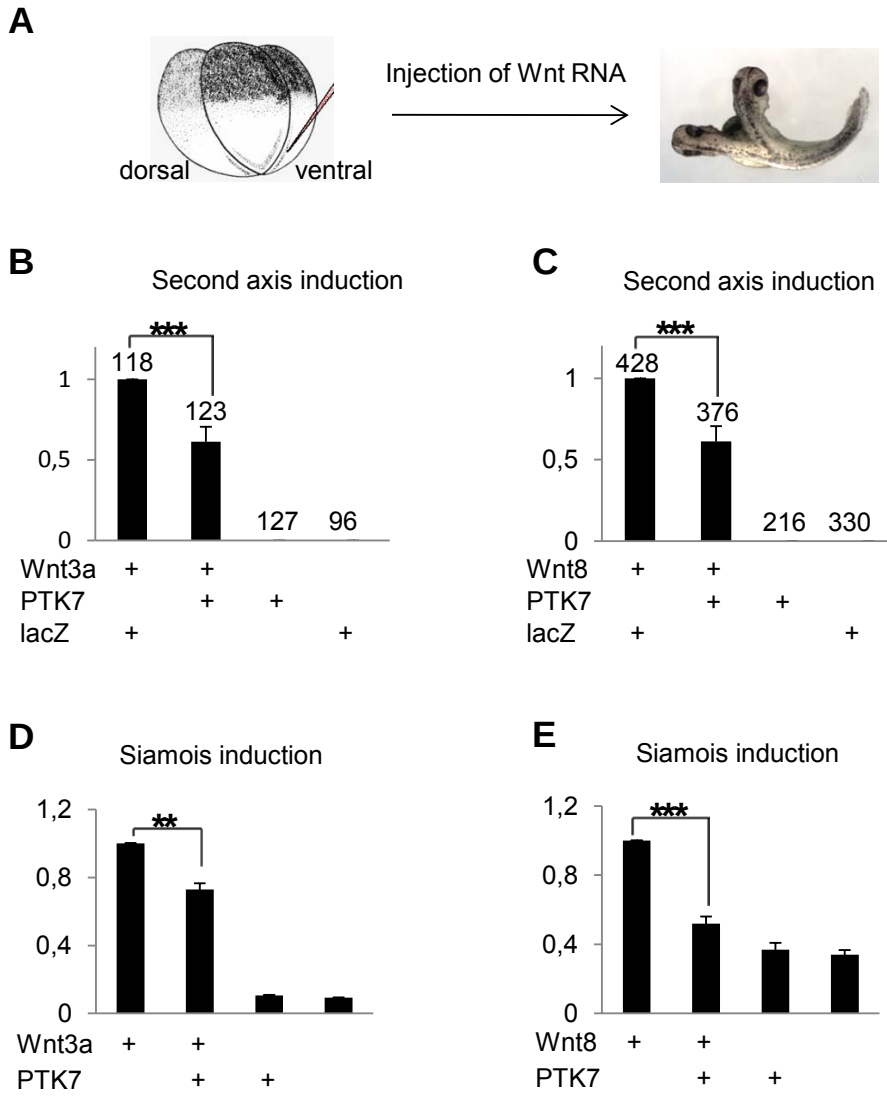

Figure 22. PTK7 inhibits Wnt3a and Wnt8 in second axis and Siamois luciferase assays. (A) Canonical Wnts overexpressed at the future ventral side induce second axes in *Xenopus* embryo. (B, C) Injection of 500 pg *PTK7* mRNA inhibits second axis formation induced by 3 pg *Wnt3a* or 10 pg *Wnt8* mRNAs. The numbers of injected embryos and standard errors are indicated for each column. The graphs summarize the results of five independent experiments. The second axis inducing activity of Wnts was set to 1. (D, E) Injection of 500 pg *PTK7* mRNA inhibits the induction of Siamois reporter by 100 pg *Wnt3a* or *Wnt8* mRNAs. The graphs summarize the results of three independent experiments + standard errors. The Wnt inducing activity was set to 1. Siamois reporter induction was normalized to Renilla reporter induction. *** p values in a Student t-test < 0.001, **p<0.01.

To confirm these results luciferase assays using a Siamois reporter construct were performed. *Siamois* gene is strongly induced by β-catenin (Carnac et al. 1996). The Siamois reporter construct has a firefly luciferase gene under the control of a *siamois* promoter, which contains several β-catenin binding sites (Brannon et al. 1997). The Siamois and a Renilla reporter constructs were injected together with mRNAs coding for

canonical Wnts with or without *PTK7* mRNA. The Siamois reporter construct is activated by canonical Wnt signaling, while the Renilla reporter construct activation does not depend on Wnt signaling activity and it is used as an internal control reporter. *Wnt3a* and *Wnt8* strongly induced the activation of Siamois reporter and, similarly to the second axis assay results, *PTK7* significantly inhibited both *Wnt3a*- and *Wnt8*- dependent activation of a Siamois reporter (Figure 22 D, E). These data show that PTK7 can inhibit Wnt-induced canonical Wnt signaling in *Xenopus*.

3.4 Knock-down of PTK7 leads to an activation of canonical Wnt signaling in *Xenopus*

As the overexpression of PTK7 inhibits canonical Wnt signaling, the downregulation of PTK7 should have an opposite effect. To knock-down PTK7 antisense Morpholino oligonucleotides, which block PTK7 translation by binding to the 5' untranslated region of *PTK7* mRNA, were used (PTK7MO). PTK7 mismatch Morpholino oligonucleotides (PTK7mmMO), which can not bind to the *PTK7* mRNA and do not block the PTK7 translation were used as a control (Wehner et al. 2011). PTK7MO or PTK7mmMO were injected alone or in combination with *Wnt8* RNA in *Xenopus* embryos at the one-cell stage and ectodermal explants were cut at the blastula stage. The explants were cultured till the neurula stage to achieve efficient knock-down of PTK7, which is predominantly expressed during this stage. It was necessary to perform the experiment in ectodermal explants, because the whole embryos injected with *Wnt8* mRNA and PTK7MO did not survive till the desired stage. The luciferase assay showed that PTK7MO activates Wnt signaling and acts synergistically with Wnt8 (Figure 23 A, B). This effect could be rescued by co-injection of *PTK7* mRNA, which lacks the Morpholino binding site, excluding the possibility of unspecific Morpholino effects (Figure 23 B).

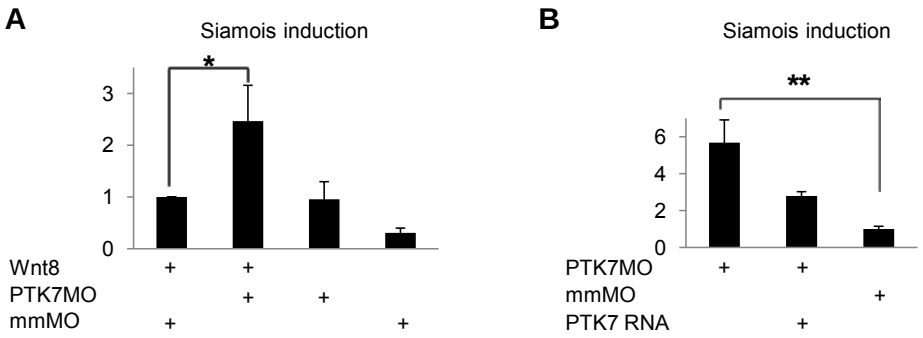

Figure 23. PTK7 loss of function induces canonical Wnt signaling in *Xenopus* ectodermal explants. (A) Injection of 20 ng PTK7MO significantly increased the activation of the Siamois reporter induced by 100 pg *Wnt8* compared to the explants from embryos injected with 20 ng of control PTK7mmMO. The graph summarizes three independent experiments + standard errors. Siamois reporter induction was normalized to Renilla reporter induction. (B) The injection of 20 ng PTK7MO significantly increased the Siamois reporter activation in *Xenopus* ectodermal explants compared to 20 ng of control PTK7mmMO. This effect was rescued by co-injection of 700 pg *PTK7* mRNA lacking the Morpholino binding site. The graph summarizes three independent experiments + standard errors. Siamois reporter induction was normalized to Renilla reporter induction. ** p values in a Student t-test < 0.01, * p<0.05.

3.5 The extracellular and transmembrane domains of PTK7 are required for the inhibition of canonical Wnt signaling

To determine which domains of PTK7 are required for the inhibition of canonical Wnt signaling, different PTK7 deletion mutants were tested in Siamois luciferase assay (Figure 24 A). Deletion of the extracellular domain (ΔexPTK7) abolished the ability of PTK7 to inhibit β-catenin signaling induced by *Wnt8* mRNA (Figure 24 B). However, overexpression of the extracellular domain of PTK7 alone (exPTK7) was not sufficient to inhibit canonical Wnt signaling (Figure 24 C). The kinase homology domain of PTK7 is evolutionary conserved and required for the Dsh recruitment to the plasma membrane (Shnitsar and Borchers 2008). This domain is not necessary for the inhibition of canonical Wnt signaling, because a deletion construct lacking the intracellular kinase-homology domain (PTK7Δk) can inhibit Wnt8 in Siamois luciferase assay (Figure 24 D). These data show that both extracellular and transmembrane domains are required for PTK7 function in canonical Wnt signaling.

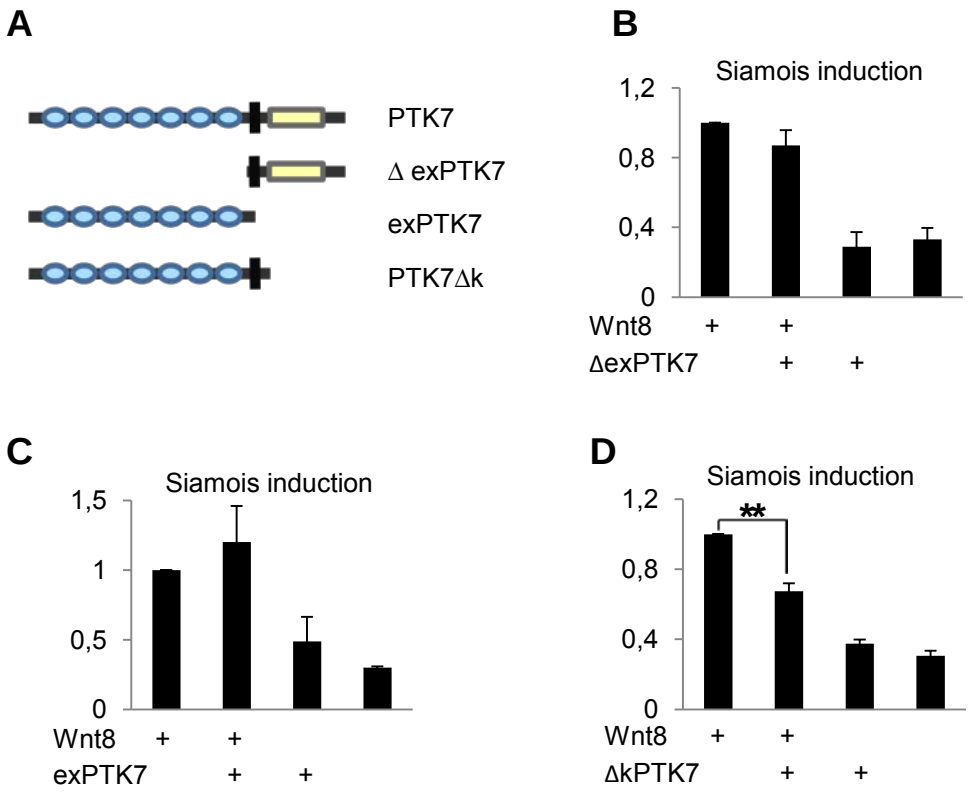

Figure 24. PTK7Δk deletion mutant inhibits canonical Wnt signaling while exPTK7 and ΔexPTK7 deletion constructs do not. (A) PTK7 mutants used for luciferase assay. (B-D) Results of luciferase assay. The graphs summarize the results of three independent experiments + standard errors. The Wnt8 inducing activity was set to 1. Siamois reporter induction was normalized to Renilla reporter induction. (B, C) Co-injection of 500 pg of Δ*exPTK7* or *exPTK7* mRNA does not inhibit canonical Wnt signaling in Siamois luciferase reporter assays. (D) Co-injection of 500 pg *PTK7Δk* inhibits *Wnt8* (100 pg of mRNA injected) in Siamois luciferase reporter assays. ** p values in a Student t-test < 0.01.

3.6 PTK7 inhibits canonical Wnt signaling in the receiving but not in the sending cell

In both luciferase and second axis assays the *PTK7* and *Wnt* mRNAs were co-injected together. This means that the proteins were expressed in the same cells and the possibility that PTK7 may affect the production, modification or secretion of Wnt proteins exists. Thus, it was necessary to check if PTK7 can inhibit exogenous Wnt proteins. To address this issue, PTK7 was transfected together with TOPflash luciferase reporter, which is activated by β-catenin, and Renilla reporter, which is used as an endogenous control reporter, in HEK293 cells. The cells were treated with either Wnt3a conditioned medium or control conditioned medium. The cells were used for luciferase assay 24 hours after the application of the media. Both full length PTK7 and PTK7Δk could reduce the TOPflash reporter activation by Wnt3a, consistent with the results obtained in *Xenopus* embryos

(Figure 25). This experiment shows that PTK7 inhibits canonical Wnt signaling in the receiving, but not in the sending cell and that the data obtained in *Xenopus* embryos are consistent in human cell culture.

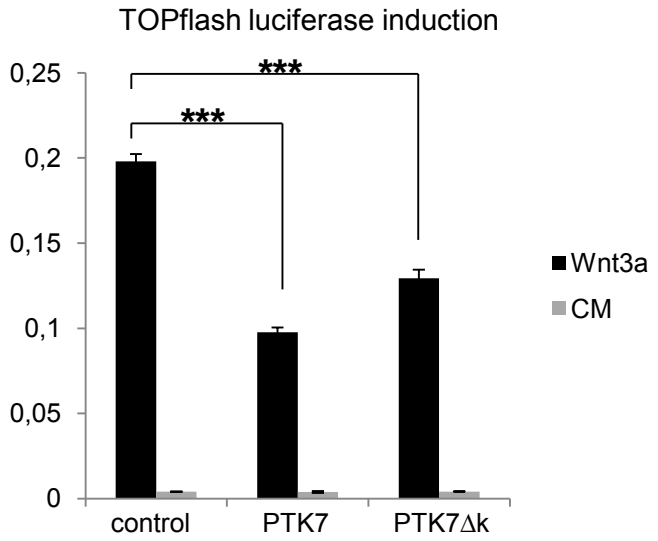

Figure 25. PTK7 inhibits canonical Wnt signaling in HEK293 cells. To activate canonical Wnt signaling HEK293 cells transfected with full length PTK7 or PTK7Δk were treated with Wnt3a conditioned medium or control conditioned medium (CM). The cells transfected with PTK7 constructs have lower TOPflash reporter activation. The mean value of 5 independently transfected cell samples + standard deviations is shown in the graph. The TOPflash reporter activity was normalized to Renilla reporter activity. *** p values in a Student t-test < 0.001.

3.7 PTK7 inhibits canonical Wnt signaling upstream of Dsh and β-catenin

PTK7 inhibits canonical Wnt ligands in both luciferase and second axis assays, however, it is not clear at which level PTK7 interferes with canonical Wnt signaling. To answer this question the ability of PTK7 to inhibit the downstream players of Wnt signaling Dsh and β-catenin was tested. Both *Dsh* and *β-catenin* mRNAs are able to induce ectopic second axis in *Xenopus* embryos when injected ventrally. PTK7 co-injection does not influence the second axis induction by these proteins (Figure 26 A, B). To confirm these results luciferase assays with β-catenin-inducible Siamois reporter were performed in *Xenopus* embryos. Indeed, PTK7 does not inhibit Dsh and β-catenin in Siamois reporter assay (Figure 26 C, D). However, the co-injection of *PTK7* mRNA could weakly inhibit LRP6 in Siamois luciferase assay (Figure 26 E).

These data show that PTK7 inhibits canonical Wnt signaling upstream of Dsh.

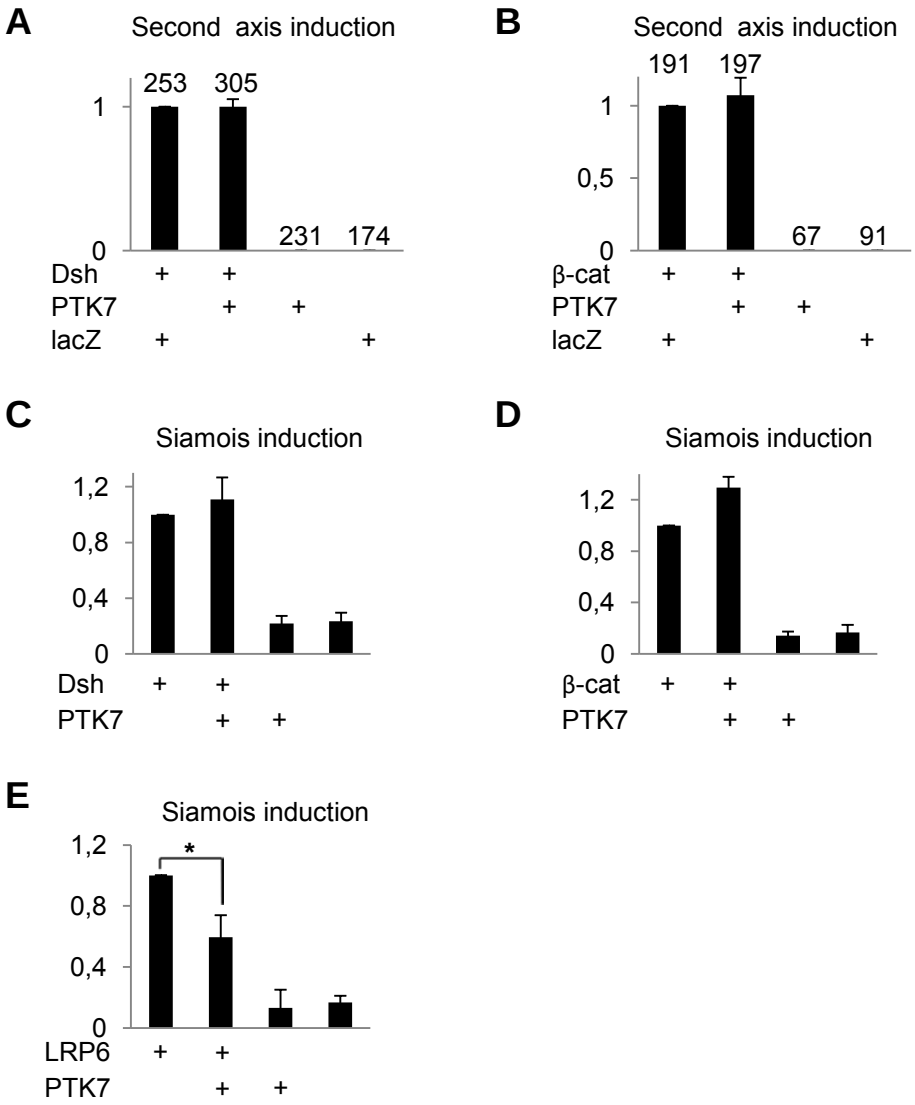

Figure 26. PTK7 inhibits canonical Wnt signaling upstream of Dsh and β-catenin. (A, B) The co-injection of 500 pg *PTK7* does not inhibit second axis induction by *Dsh* (250 pg mRNA injected) or *β-catenin* (50 pg injected). The second axis induction was analyzed at early tadpole stages. The graphs summarize the results of three independent experiments + standard errors. The numbers of injected embryos are indicated for each column. The second axis inducing activity of Dsh and β-catenin were set to 1. (C, D) Co-injection of 500 pg *PTK7* does not inhibit the induction of the Siamois reporter by 500 pg *Dsh* or 40 pg *β-catenin* mRNA. The graphs summarize the results of three independent experiments + standard errors. The Dsh or β-catenin inducing activity was set to 1. Siamois reporter induction was normalized to Renilla reporter induction. (E) The co-injection of 500 pg *PTK7* inhibits *LRP6* (1 ng of RNA injected) in Siamois luciferase assay in *Xenopus* embryos. The graph summarizes the results of three independent experiments + standard errors. The LRP6 inducing activity was normalized to1. Siamois reporter induction was normalized to Renilla reporter induction. * p values in a Student t-test < 0.05.

3.8 PTK7 does not affect LRP6 phosphorylation

Phosphorylation of LRP6 at Ser1490 by CK1 in response to Wnt protein is one of the first steps in the activation of canonical Wnt signaling (Tamai et al. 2004). As PTK7 acts

upstream of Dsh and weakly inhibits LRP6 in Siamois luciferase assay, it probably may affect LRP6 phosphorylation. HEK293 cells transfected with PTK7 or an empty vector were treated with control conditioned medium (CM) or Wnt3a CM. The cell lysates were used for Western Blotting with the antibodies against phospho-LRP6 and total LRP6. Actin was used as a loading control. Wnt3a treatment induces phosphorylation of LRP6 at Ser1490. However, the co-transfection of PTK7 does not influence this process (Figure 27).

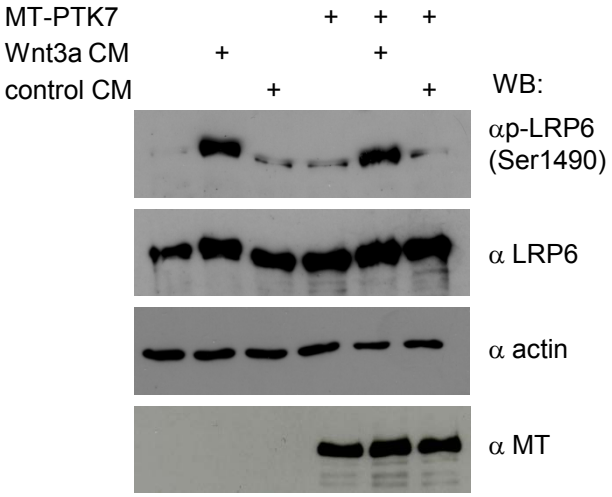

Figure 27. PTK7 does not affect LRP6 phosphorylation at Ser1490. HEK293 cells were transfected either with PTK7-MT or empty vector and treated with Wnt3a CM or control CM for 6 hours. Overexpression of PTK7 does not inhibit Wnt3a-induced LRP6 phosphorylation at Ser1490 site.

3.9 PTK7 inhibits canonical Wnt signaling by interfering with Fz receptors

PTK7 recruits Dsh to the plasma membrane in *Xenopus* and is part of a Fz/Dsh complex (Shnitsar and Borchers 2008). PTK7 inhibits canonical Wnt signaling upstream of Dsh. These data suggest that PTK7 might inhibit canonical Wnt signaling at the level of the Frizzled receptor. Indeed, PTK7 could inhibit human Fz5 (hFz5), which induces canonical Wnt signaling in *Xenopus*, in Siamois luciferase assay (Figure 28 A). Moreover, the inhibition of Wnt8 by PTK7 is rescued by co-injection of low doses of Fz7 (Figure 28 B). When overexpressed in low doses, Fz7 does not induce canonical Wnt signaling, but can efficiently rescue the inhibitory effect of PTK7. Additionally, PTK7 could interact with Fz7 receptor in HEK293 cells (by M. Podleschny). These data suggest that PTK7

inhibits canonical Wnt signaling through interference with Fz signaling and by supplying more Fz the effect of PTK7 might be overcome.

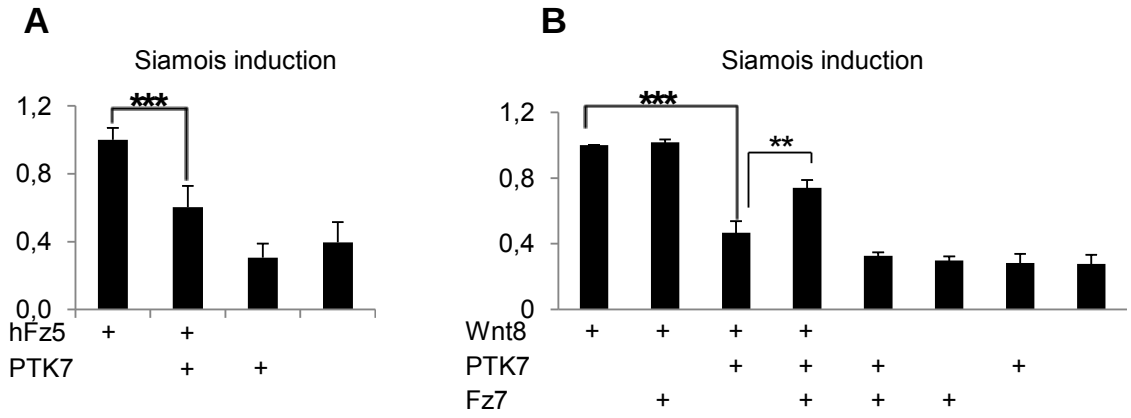

Figure 28. PTK7 inhibits canonical Wnt signaling at the level of Frizzled receptor. (A) PTK7 inhibits the activation of canonical Wnt signaling by hFz5. The injection of 500 pg *PTK7* mRNA inhibits the induction of Siamois reporter by 1000 pg of *hFz5* mRNA. The graph shows the result of one representative experiment. Six samples containing 10 embryos each were used. Standard deviations between the samples are shown for each column. The hFz5 inducing activity was set to 1. Siamois reporter induction was normalized to Renilla reporter induction. (B) Fz7 rescues the inhibition of Wnt8 by PTK7 in luciferase assay. 100 pg of *Wnt8*, 250 pg of *PTK7* and 500 pg of *Fz7* mRNAs were used. The graphs summarize the results of three independent experiments + standard errors. The Wnt8 inducing activity was set to 1. Siamois reporter induction was normalized to Renilla reporter induction. *** p values in a Student t-test < 0.001, **p<0.01.

3.10 PTK7 overexpression affects neural plate patterning

Canonical Wnt signaling regulates anterior-posterior (A – P) neural plate patterning in *Xenopus* (Kiecker and Niehrs 2001). Wnt8 and Wnt3a posteriorize the neural plate while the inhibition of canonical Wnt signaling anteriorizes it (Kiecker and Niehrs 2001). As PTK7 is expressed in the neural plate (Shnitsar and Borchers 2008) and inhibits canonical Wnt signaling, we decided to check if PTK7 can affect the neural plate patterning in *Xenopus* embryos. *PTK7* or *PTK7Δk* mRNA together with mRNA for lineage tracer *lacZ* were injected in one blastomere at the two-cell stage *Xenopus* embryos. The embryos were cultured till the neurula stage and the expression patterns of the neural plate markers *Krox20* (rhombomere 3 and 5 marker) and *Engrailed2* (midbrain-hindbrain boundary marker) were analyzed. Indeed, overexpression of PTK7 and PTK7Δk causes the posterior shift of *Krox20* and *Engrailed2* at the injected side in comparison to the non-injected side suggesting that PTK7 overexpression anteriorizes neural plate (Figure 29 A-D). These data support the finding that PTK7 and PTK7Δk can inhibit canonical Wnt signaling. However, the knock-down of PTK7 by Morpholino oligonucleotides does not posteriorize neural

plate (data not shown), suggesting that the A – P patterning of neural plate is not an endogenous function of PTK7.

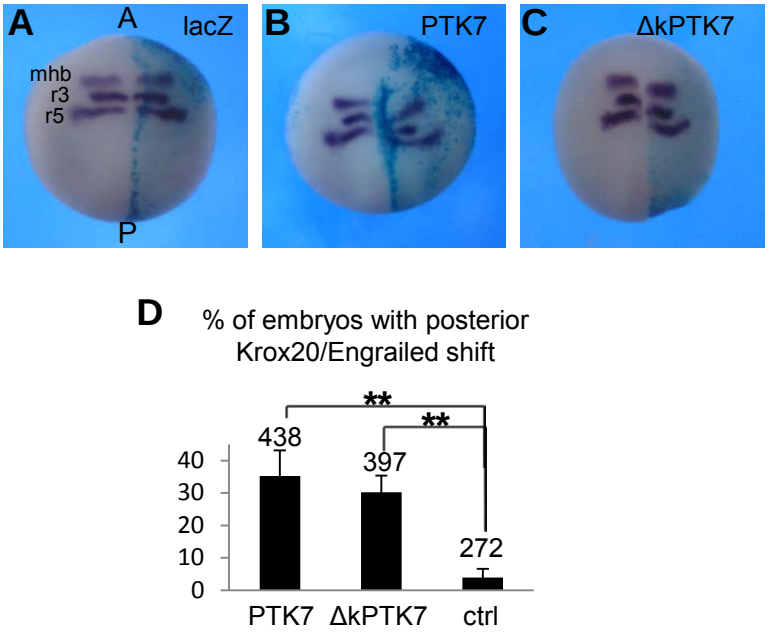

Figure 29. PTK7 overexpression affects the anterior-posterior patterning of *Xenopus* neural plate. (A-C) 1 ng of *PTK7* or 250 pg of *PTK7Δk* were injected together with *lacZ* lineage tracer in one blastomere at the two-cell stage and *Krox20/Engrailed2* expression was analyzed at the neurula stage. The injected lacZ-positive side is shown on the right. Mhb – midbrain/hindbrain boundary (*Engrailed2* expression), r3, r5 – rhombomeres 3 and 5 (*Krox20* expression). A – anterior, P – posterior. (A) Control embryos show normal positioning of *Engrailed2* and *Krox20*. (B, C) PTK7 and PTK7Δk cause a posterior shift of *Engrailed2* and *Krox20*. (D) The graph shows the percentages of embryos with posterior shift of *Engrailed2* and *Krox20*. The graph summarizes the results of three independent experiments + standard deviations. The numbers of injected embryos are indicated for at the top of each column. ** p values in a Student t-test < 0.01

3.11 PTK7 activates Planar cell polarity

It is known that PTK7 is an important regulator of PCP (Lu et al. 2004; Shnitsar and Borchers 2008; Yen et al. 2009). However, so far the ability of PTK7 to modulate PCP signaling has not been analyzed quantitatively. Moreover, it was not even clear if PTK7 activates or inhibits PCP, because both the overactivation and the inhibition of this pathway often lead to the same phenotypes. An ATF2 luciferase reporter assay (Ohkawara and Niehrs 2011), which allows to analyze PCP activity in *Xenopus*, has been used to address this question. The ATF2 luciferase reporter construct is used to monitor JNK-dependent activation of PCP. *Xenopus* embryos were injected with mRNAse coding for full length PTK7 and the deletion mutants of PTK7 together with ATF2 reporter and

Renilla reporter, which is used as an endogenous control reporter. Gastrula stage embryos were used for luciferase assay. Full length PTK7 was able to activate the ATF luciferase reporter dose-dependently, PTK7Δk could activate it weakly but exPTK7 and ΔexPTK7 mutants could not (Figure 30). Wnt5a, a known activator of PCP signaling in *Xenopus*, was used as a positive control. These data indicate that PTK7 is indeed activating PCP and only the full length molecule can do it effectively.

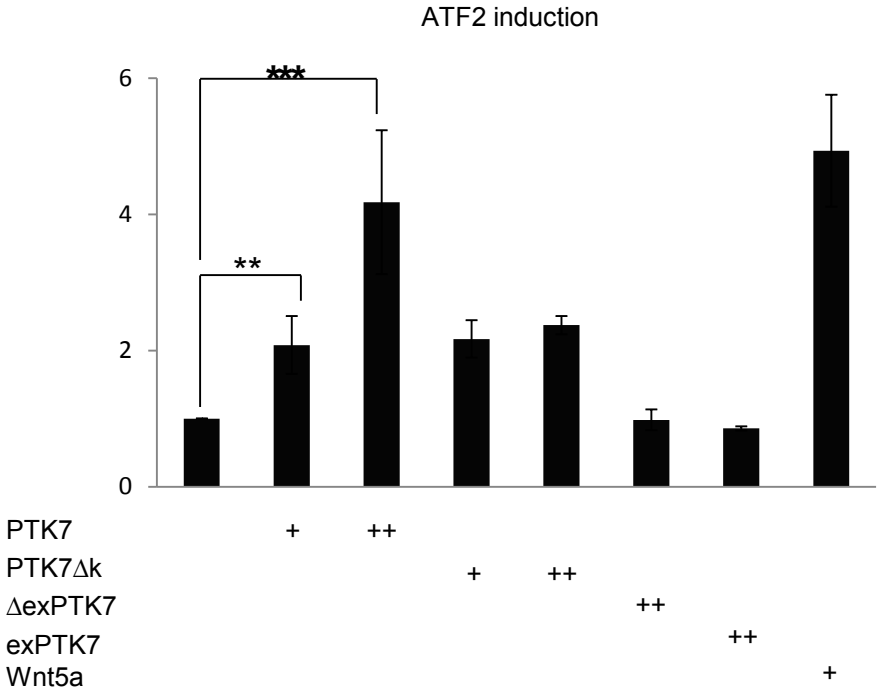

Figure 30. PTK7 activates PCP signaling in ATF2 luciferase reporter assay. PTK7 activates ATF2 reporter, PTK7Δk has a weak effect, while ΔexPTK7 and exPTK7 do not activate ATF2 reporter. 500 or 1000 pg of *PTK7* or its deletion mutants mRNAs were injected marginally at the two-cell stage. 500 pg of *Wnt5a* was used as a positive control. The graph summarizes the results of three independent experiments + standard deviations. ATF2 reporter induction was normalized to Renilla induction. Negative control was normalized to 1. *** p values in a Student t-test < 0.001, ** p<0.01.

3.12 Wnt8 does not inhibit the activation of PCP signaling by PTK7

As PTK7 can activate PCP and interacts with canonical Wnt proteins, it was interesting to analyze if canonical Wnt ligands can influence the PTK7-dependent activation of ATF2 reporter. ATF2 luciferase assay in *Xenopus* shows that Wnt8 does not inhibit the activation of PCP signaling by PTK7, suggesting that the overactivation of canonical signaling does not affect the ability of PTK7 to activate non-canonical Wnt signaling (Figure 31).

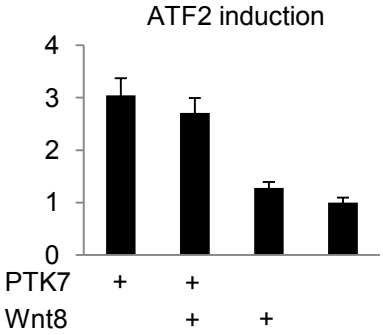

Figure 31. Wnt8 does not inhibit PTK7-induced activation of ATF2 reporter. The co-injection of 100 pg of *Wnt8* mRNA does not inhibit the induction of ATF2 reporter by 750 pg of *PTK7* mRNA. The negative control was set to 1. The graph shows the result of one representative experiment. Five samples containing 8 embryos each were used. Standard deviations between the samples are shown for each column. ATF2 reporter induction was normalized to Renilla reporter induction.

3.13 PTK7 co-localizes with caveolin in HEK293 cells

PTK7 interacts with Wnt proteins and inhibits canonical Wnt signaling. Wnt receptors like Fz and LRP6 may change their cellular localization when they bind Wnt ligands (Yamamoto et al. 2006; Yamamoto et al. 2008). It is also possible that PTK7, being a transmembrane PCP regulator and a Wnt binding partner, may change its localization in the response to Wnt proteins. To check this hypothesis, HEK293 cells were transiently transfected with PTK7-GFP and treated with Wnt3a, Wnt5a or control conditioned medium (CM). The localization of PTK7-GFP was analyzed using confocal microscopy. Upon CM treatment more vesicular-like bodies were detected in the cells treated with Wnt3a CM in comparison to the cells, which were treated with Wnt5a or control CM. However, upon Wnt3a treatment, PTK7 was still localized mostly to the plasma membrane, so no robust PTK7 re-localization has been detected (Figure 32 A-D). To prove that the PTK7 is indeed localized to endocytotic vesicles, the co-localization experiments with fluorescent dextran were performed. When dextran is added to the cell culture medium, it can enter the cell only through endocytotic vesicles. PTK7-GFP transfected HEK293 cells were treated with Wnt3a CM containing fluorescent dextran for half an hour, then fixed and analyzed with confocal microscope. Indeed, a portion of PTK7-containing vesicles co-localized with dextran, indicating that PTK7 is endocytosed (Figure 32 E).

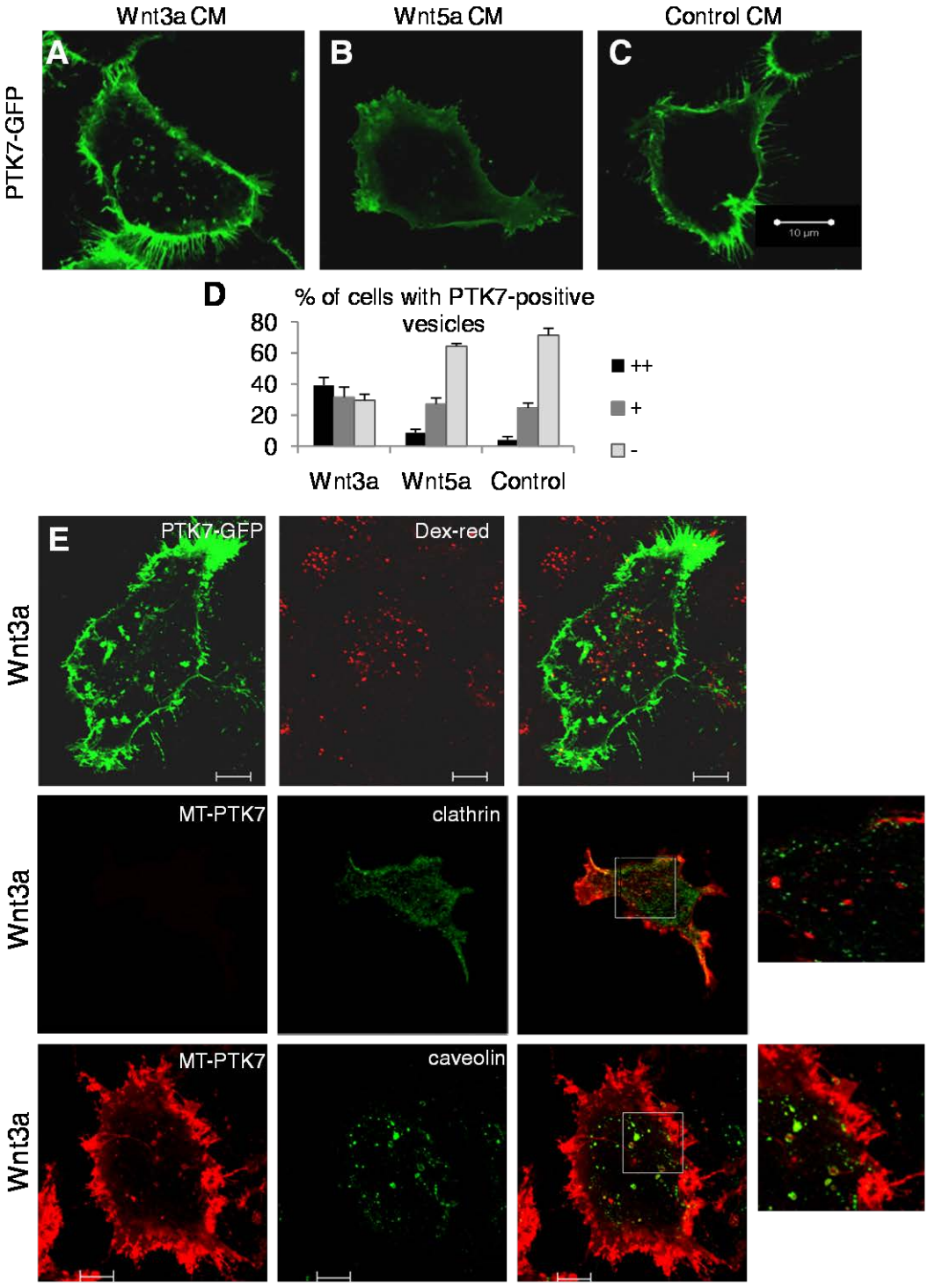

Figure 32. PTK7 is endocytosed through caveolin but not with clathrin route in HEK293 cells. (A-D) HEK293 cells transfected with PTK7-GFP were treated with Wnt3a CM, Wnt5a CM or control CM for 1 hour, then fixed and analyzed using a confocal microscope. (A) PTK7-GFP is localized to vesicular-like bodies in the cells treated with Wnt3a CM. (B, C) PTK7-GFP – positive vesicles are not formed when cells are treated with Wnt5a or control CM. (D) The quantification of PTK7-positive vesicles. Each graph summarizes the results of three independent experiments + standard deviations. (E) PTK7-GFP overexpressed in HEK293 cells co-localizes with dextran red (Dex-red) upon Wnt3a CM treatment. (F) PTK7-MT overexpressed in HEK293 cells does not co-localize with clathrin upon Wnt3a CM treatment. (G) PTK7-MT overexpressed in HEK293 cells co-localizes with caveolin upon Wnt3a CM treatment.

To check the nature of PTK7-positive vesicles HEK293 cells transiently transfected with PTK7-MT were treated with Wnt3a CM for 3 hours, then fixed and immunostained against MT and endogenous clathrin or caveolin. PTK7-MT was partially co-localized with caveolin, but not with clathrin (Figure 32 F, G). To conclude, PTK7 is endocytosed through a caveolin-mediated endocytotic pathway.

3.14 PTK7 co-localizes and interacts with caveolin1a in *Xenopus*

Xenopus animal cap assay was used to find out if PTK7 and caveolin1a (Cav1a) can co-localize in *Xenopus* cells like they do in HEK293 cells. Animal caps are ectodermal explants of blastula stage *Xenopus* embryos that allow to analyze the intracellular localization of overexpressed tagged proteins. Embryos at the one-cell stage were injected with *PTK7-MT* and *Cav1a-GFP* mRNAs with or without *Wnt8* mRNA. Later at the blastula stage animal caps were excised, cultured until the gastrula stage, fixed, immunostained and analyzed using a confocal microscope. It was observed that PTK7-MT co-localized with overexpressed Cav1a and this co-localization was increased in presence of canonical Wnt8 (Figure 33 A – B).

To check if PTK7 biochemically interacts with Cav1a, *Xenopus* embryos were injected with *PTK7-MT* or *PTK7Δk-MT*, a PTK7 mutant, which lacks intracellular kinase-homology domain, mRNA in combination with *Cav1a-HA* mRNA, cultured until the gastrula stages and CoIP against MT was performed. Both PTK7-MT and PTK7Δk-MT co-precipitated Cav1a-HA, indicating that the intracellular kinase-homology domain of PTK7 is not required for the interaction with Cav1a (Figure 33 C).

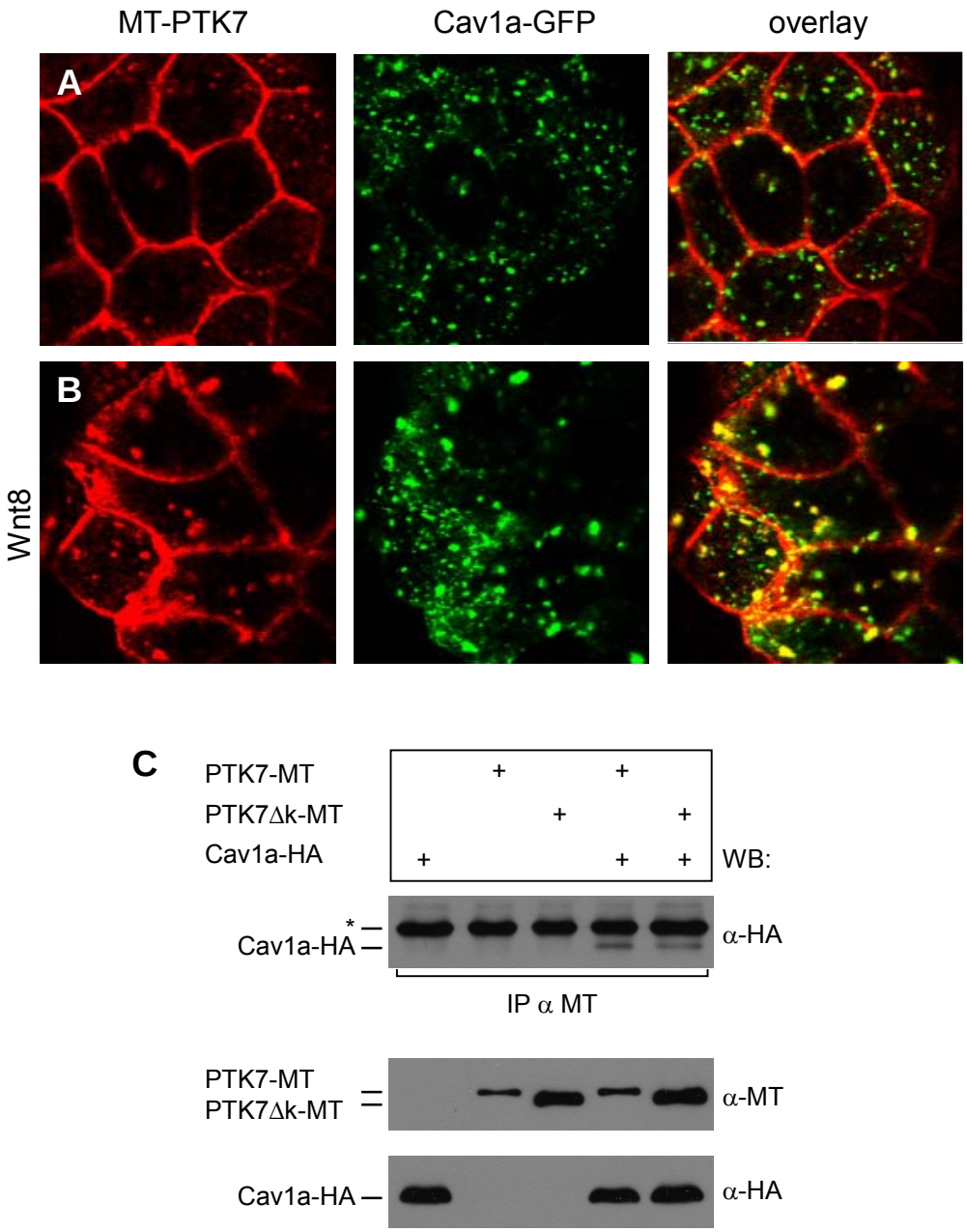

Figure 33. PTK7 co-localizes and interacts with caveolin1a in *Xenopus*. (A, B) PTK7 co-localizes with Cav1a in *Xenopus* animal caps. 700 pg *PTK7-MT*, 100 pg *Cav1a-GFP* and 100 pg *Wnt8* mRNAs were injected animally at the one-cell stage *Xenopus* embryos. Animal caps were cut at the blastula stage, cultured until the gastrula stages, fixed, immunostained and analyzed by confocal microscopy. (A) PTK7-MT weakly co-localizes with Cav1a-GFP. (B) PTK7-MT strongly co-localizes with Cav1a-GFP in presence of Wnt8. (C) PTK7-MT and PTK7Δk-MT can co-precipitate Cav1a-HA from *Xenopus* embryos. The embryos were injected with 800 pg *PTK7-MT*, *PTK7Δk-MT*, 100 pg *Cav1a-HA* and lysed at the gastrula stages. CoIP experiments showing co-precipitated Cav1a-HA in the upper panel and *Xenopus* lysates used for CoIP in the lower panels. * - antibody heavy chain.

3.15 Endocytosis is not required for PTK7-dependent inhibition of canonical Wnt signaling

As PTK7 binds Wnt ligands, is endocytosed in response to Wnt ligands and inhibits canonical Wnt signaling, we hypothesized that PTK7 might be endocytosed together with Wnt resulting in the attenuation of the canonical Wnt signaling. If this hypothesis is true, the inhibition of endocytosis might lead to the loss of PTK7-mediated inhibition of canonical Wnt signaling. To block endocytosis a dominant-negative dynamin (dnDyn) construct was used. dnDyn blocks the formation of endocytotic vesicles and it has recently been shown to inhibit gastrulation movements in *Xenopus* (Lee and Harland 2010). dnDyn itself did not affect the activation of canonical Wnt signaling and the overexpression of dnDyn did not affect the ability of PTK7 to inhibit canonical Wnt signaling (Figure 34). These results indicate that PTK7 inhibits canonical Wnt signaling not through Wnt endocytosis.

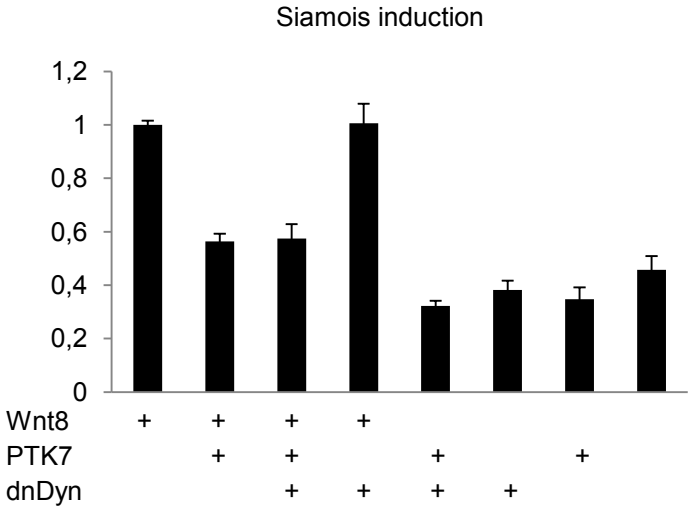

Figure 34. Dominant-negative dynamin does not influence PTK7-dependent inhibition of canonical Wnt signaling. (A) dnDyn does not affect inhibition of canonical Wnt signaling by PTK7 in Siamois luciferase reporter assay. *Xenopus* embryos were injected with 100 pg *Wnt8*, 500 pg *PTK7*, 500 pg *dnDyn* RNAs. The Wnt8 inducing activity was set to 1. The graph shows the result of one representative experiment. Six samples containing 10 embryos each were used. Standard deviations between the samples are shown for each column. Siamois reporter induction was normalized to Renilla reporter induction.

3.16 Caveolin inhibits canonical Wnt signaling in *Xenopus* embryos

There are controversial data about the role of caveolin1 in canonical Wnt signaling in the different systems. Caveolin1 activates Wnt signaling in HEK293 cells but inhibits it in

NIH 3T3 cells and *Zebrafish* embryos (Galbiati et al. 2000; Yamamoto et al. 2006; Yamamoto et al. 2008; Mo et al. 2010). To analyze how Cav1a influences canonical Wnt signaling in *Xenopus*, Siamois luciferase assays were performed. *Xenopus* Cav1a inhibited the activation of the Siamois reporter by Wnt8 and by β-catenin in *Xenopus* (Figure 35 A, B). These data are similar to those obtained in the *Zebrafish* model system. Moreover, Cav1a inhibited canonical Wnt signaling similarly to PTK7 and the overexpression of both constructs had an additive effect (Figure 35 A). These data indicate that PTK7 and Cav1a may act together to inhibit canonical Wnt signaling.

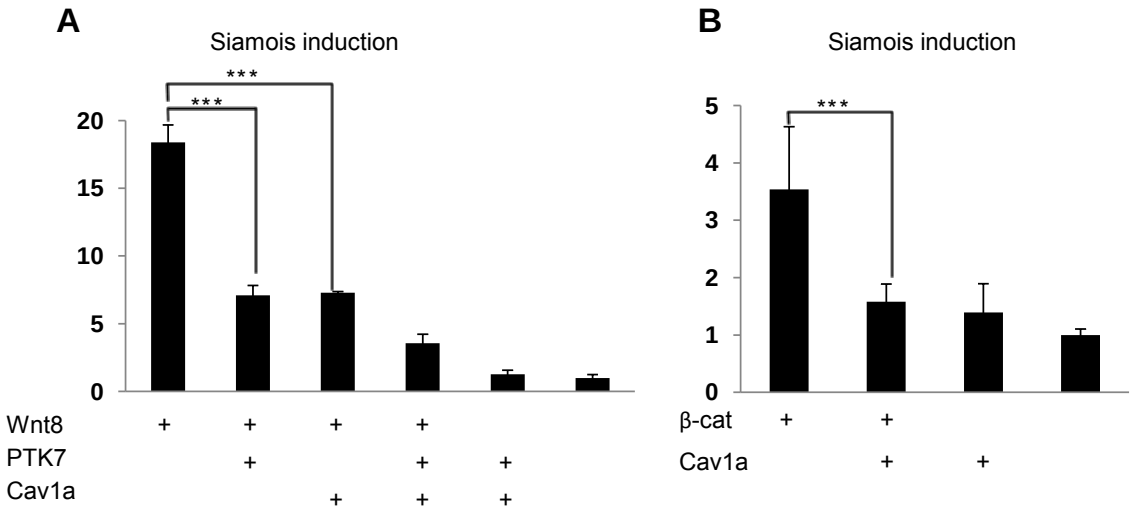

Figure 35. Caveolin1a inhibits canonical Wnt signaling in Siamois luciferase assay. (A) Cav1a and PTK7 inhibit Wnt8-induced canonical Wnt signaling additively in Siamois luciferase assay. The one-cell stage embryos were injected with 100 pg *Wnt8*, 150 pg *Cav1a*, 250 pg *PTK7* RNAs. The graph shows a single representative experiment. Three samples containing 20 animal caps each were used for each measurement. Standard deviations between the samples are shown for each column. Siamois reporter induction was normalized to Renilla reporter induction. (B) Cav1a inhibits β-catenin induced canonical Wnt signaling in Siamois luciferase assay. The one-cell stage embryos were injected with 50 pg *β-catenin* and 500 pg *Cav1a* RNAs. The graph shows a single representative experiment. Six samples containing 10 embryos each were used. Standard deviations between the samples are shown for each column. Siamois reporter induction was normalized to Renilla reporter induction.

3.17 Overexpression of caveolin1a inhibits migration of *Xenopus* neural crest cells

As caveolins are often involved in the regulation of cellular migration (Navarro et al. 2004), we analyzed if the overexpression of Cav1a might affect the migration of cranial neural crest (CNC) cells in *Xenopus* embryos. *Cav1a* mRNA was injected into one

blastomere at the two-cell stage to overexpress Cav1a only in one half of *Xenopus* embryo. *LacZ* mRNA was used as a lineage tracer. The embryos were cultured till tadpole stages and the migration of CNC cells was visualized by whole mount *in situ* hybridization (WISH) against the CNC marker *Twist*. *Cav1a*-injected embryos displayed an inhibition of CNC cells migration at the injected side in comparison to the non-injected side (Fig 36 A, C), while control embryos injected with *lacZ* mRNA alone had normally migrated CNC cells at both sides (Fig 36 B, C). These data suggest that Cav1a might regulate cellular migration in *Xenopus*.

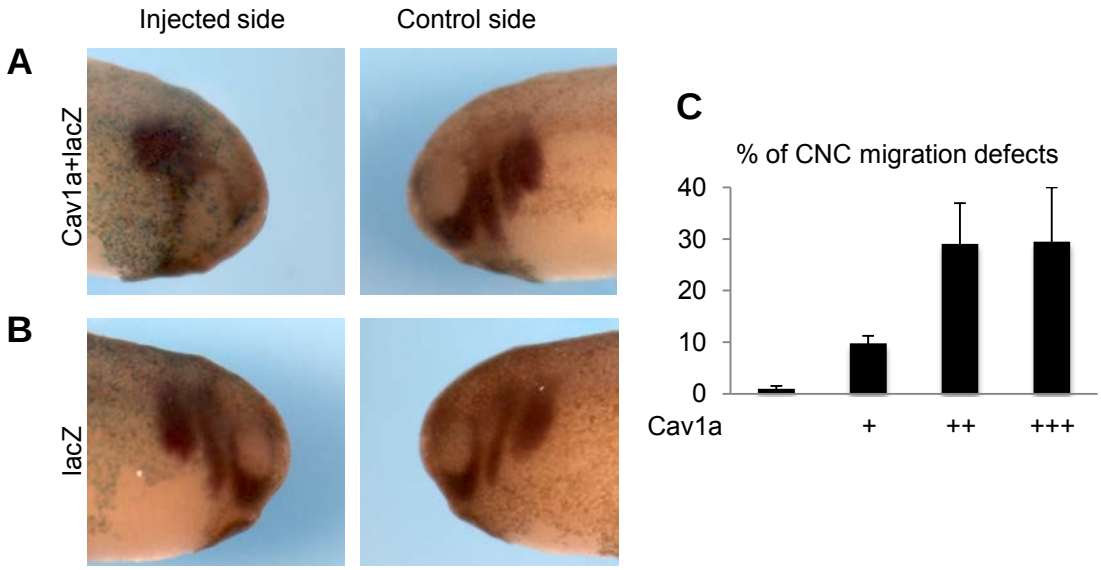

Figure 36. Overexpression of caveolin1a inhibits migration of *Xenopus* cranial neural crest cells. (A–B**)** 125 pg, 250 pg or 500 pg of *Cav1a* and 75 pg *lacZ* (lineage tracer) mRNAs were injected into one blastomere at the two-cell stage *Xenopus* embryos to overexpress mRNAs only in one half of an embryo. The embryos were cultured until tadpole stages and the migration of CNC cells was visualized by WISH against the CNC marker *Twist*. (A) *Cav1a*-injected embryo showing disrupted migration of CNC cells at the injected side. (B) *LacZ*-injected control embryo showing normal migration of CNC at both sides. (C) The graph shows the percentages of embryos with disrupted CNC cells migration. The graph summarizes the results of three independent experiments + standard deviations.

3.18 Neural tube closure defects, induced by PTK7 loss of function, can be partially rescued by the inhibition of canonical Wnt signaling

PTK7 knock-down induces neural tube (NT) closure defects, which is a typical PCP phenotype (Lu et al. 2004; Wehner et al. 2011). PTK7 overexpression activates non-canonical Wnt signaling and inhibits canonical Wnt signaling in *Xenopus*. As canonical and non-canonical Wnt pathways are connected and PTK7 regulates both of them, we

asked if the NT closure defects caused by PTK7 knockdown by Morpholino oligonucleotides (PTK7 MO) injection are associated not only with PCP misregulation, but, at least partially, with the overactivation of canonical Wnt signaling. In this case, the PTK7 MO NT closure phenotype should be rescued by the inhibition of canonical Wnt signaling. To inhibit canonical Wnt signaling, dominant-negative Wnt8 (dnWnt8), which blocks the activity of canonical Wnt proteins, was used (Hoppler et al. 1996). The injection of control mismatch MO (mmMO) induced less than 10% of neural tube closure defects (Figure 37 A, E) and the injection of *dnWnt8* RNA induced no neural tube closure defects, but the embryos closed neural tubes slower than the control ones (Figure 37 B, E). PTK7 MO induced severe NT closure defects (more than 60%) (Figure 37 C, E) and the co-injection of *dnWnt8* RNA significantly improved the PTK7 loss of function phenotype, where only about 20% of embryos injected with both PTK7 MO and *dnWnt8* displayed the NT closure phenotype (Figure 37 D, E).

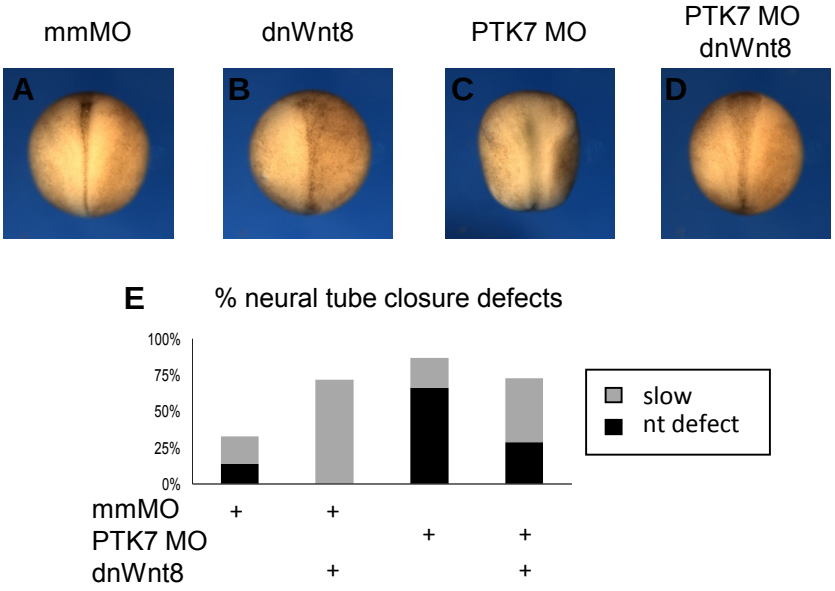

Figure 37. Dominant-negative Wnt8 (dnWnt8) partially rescues the PTK7 loss of function neural tube closure defect. (A-D) *Xenopus* embryos were injected with 20 ng of PTK7 MO or its mismatch control (mmMO) in combination with 200 pg *dnWnt8* RNA as indicated. The embryos were analyzed at the late neurula stage. (A, B) Embryos injected with mmMO or *dnWnt8* RNA show normal tube closure. (C) Embryos injected with PTK7 MO have open neural tubes. (D) Co-injection of *dnWnt8* rescues neural tube closure defects induced by PTK7 MO. (E) The graph shows the percentages of embryos with disrupted neural tube closure. Injection of *dnWnt8* leads to a delayed neural tube closure phenotype, which was categorized as "slow". Severe defects, when neural tube remains open, were categorized as "defect". The graph shows one representative experiment.

3.19 PlexinA1 and PTK7 interact in biochemical and phenotypical assays in *Xenopus*

PTK7 is an important regulator of PCP signaling in many organisms (Lu et al. 2004; Shnitsar and Borchers 2008; Yen et al. 2009; Golubkov et al 2010, Wehner et al. 2011). However, PTK7 functions not only in Wnt pathway, but also in Plexin/Semaphorin signaling. PTK7 interacts with Plexin in *Drosophila* to regulate Semaphorin repulsive signaling during axon guidance and in chick to regulate cardiac morphogenesis (Winberg et al. 2001; Toyofuku et al. 2004). We asked, if this interaction is conserved in *Xenopus*. Indeed, full length PTK7 could be co-immunoprecipitated with full length PlexinA1 in *Xenopus* embryos (by G. Wagner). Next we tested if the interaction between PTK7 and PlexinA1 plays a role in *Xenopus* cranial neural crest (CNC) cells migration, because PlexinA1 and PTK7 are both expressed in CNC cells and interact biochemically (Shnitsar and Borchers 2008; Wagner et al. 2010). *PTK7* and *PlexinA1* mRNAs were injected alone or in combination into one blastomere together with lineage tracer *lacZ* at the two-cell stage *Xenopus* embryos to overexpress mRNAs in one half of an embryo. When the embryos reached tadpole stages, they were fixed and used for whole mount *in situ* hybridization against CNC marker *Twist*. In control and PTK7-injected embryos no CNC migration phenotypes were observed, while the overexpression of PlexinA1 had a mild effect (Figure 38 A - C, E). However, the embryos injected with both PTK7 and PlexinA1 had significant CNC migration defects (Figure 38 D, E). These data indicate that PTK7 and PlexinA1 interact to regulate the migration of CNC cells in *Xenopus*.

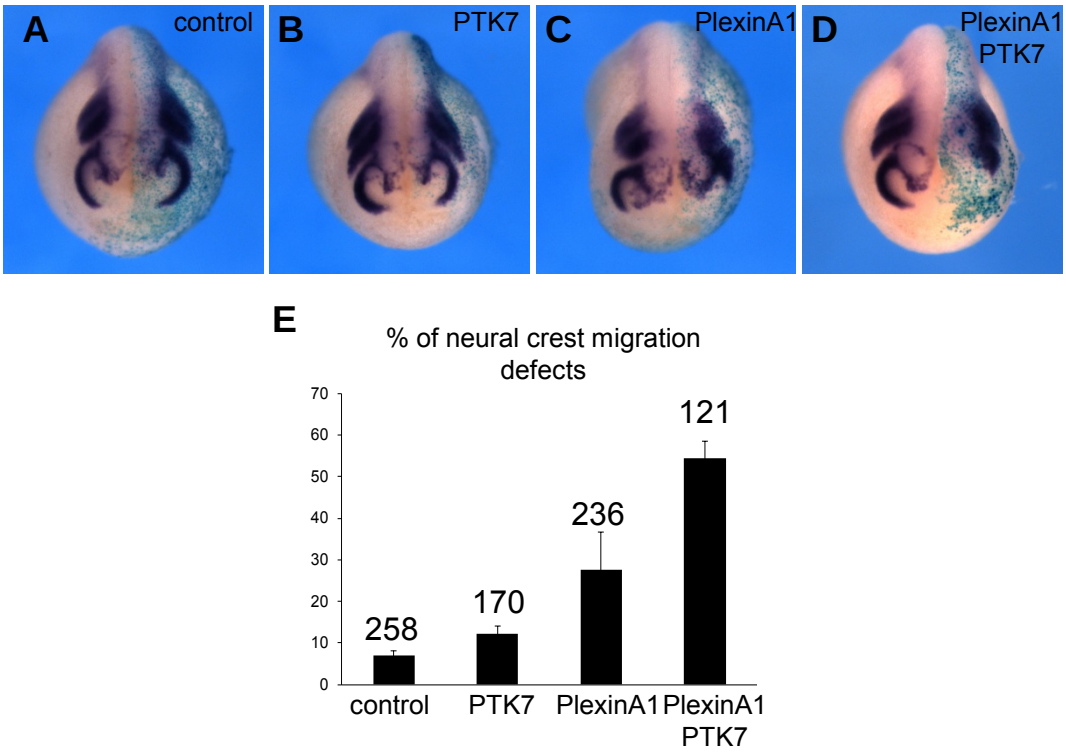

Figure 38. Co-expression of PlexinA1 and PTK7 leads to an increase in cranial neural crest migration defects. (A-D) Embryos were injected with the indicated mRNA constructs in combination with 75 pg *lacZ* RNA in one blastomere at the two-cell stage. *GFP* RNA was co-injected to have the equal amounts of mRNAs injected. Whole mount *in situ* hybridization against neural crest marker *Twist* was performed at stage 22 to analyze the migration of neural crest cells. The injected lacZ-positive side is blue. (A, B) Control and *PTK7* injected embryos show normal migration of neural crest cells. (C) *PlexinA1* injected embryo shows affected neural crest migration at the injected side. (D) Embryo injected with both *PlexinA1* and *PTK7* shows strongly affected migration of neural crest cells at the injected side. (E) Graph summarizing the percentage of NC migration defects of three independent experiments + standard deviations. Number of injected embryos is indicated for each column.

3.20 PlexinA1 is required for neural crest migration

Loss of function analysis was performed to determine if PlexinA1 is required for cranial neural crest (CNC) cells migration. To knock-down PlexinA1 the combination of two antisense Morpholinos, which bind 5'UTR and the beginning of *PlexinA1* gene, was used. The two-cell stage *Xenopus* embryos were injected into one blastomere with 20 ng of PlexinA1 Morpholino 1 and 2 mixture (PlxA1 MO) together with *lacZ* as a lineage tracer. When the embryos reached tadpole stage they were used for whole mount *in situ* hybridization (WISH) against the CNC marker *Twist*. The knock-down of PlexinA1 resulted in dose-dependent inhibition of migration of CNC cells in comparison to the embryos injected with the same concentrations of control MO (Figure 39 A, B, E).

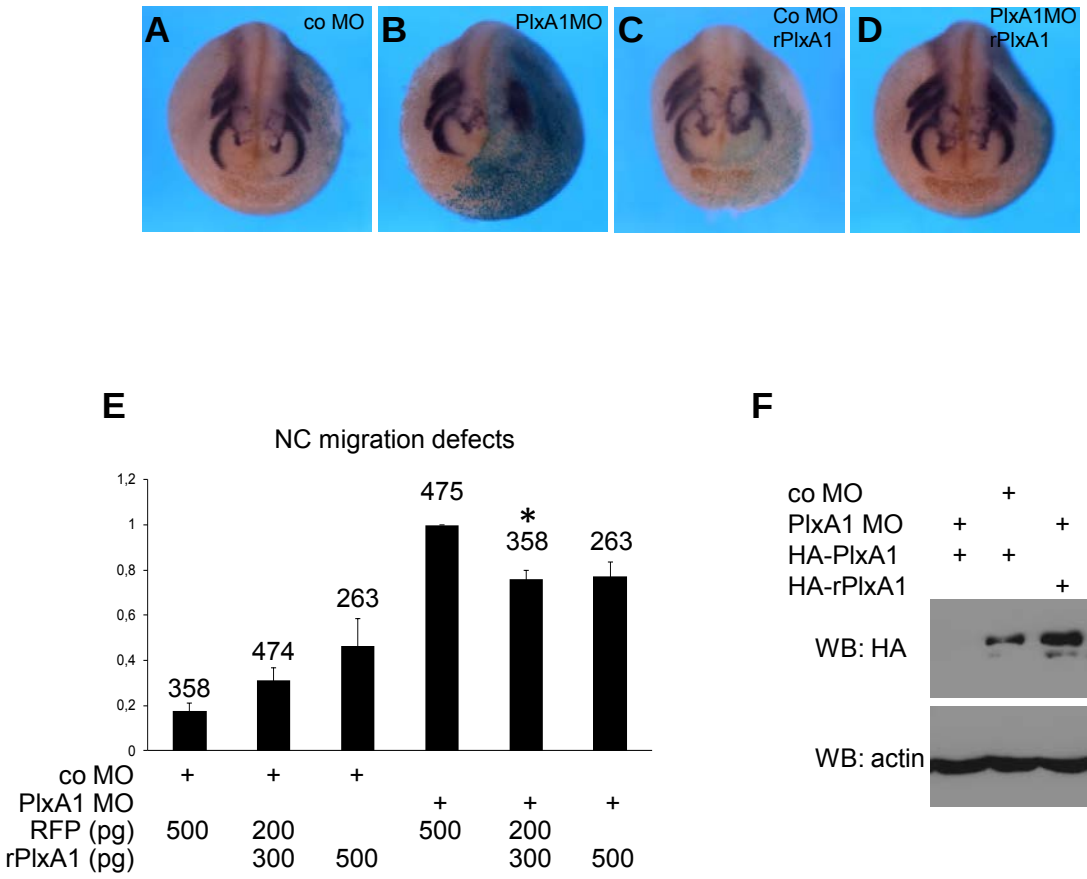

Figure 39. Knock-down of PlexinA1 results in inhibition of cranial neural crest migration. (A–E) Embryos were injected with the indicated Morpholinos and mRNA constructs in combination with 75 pg *lacZ* mRNA in one blastomere at the two-cell stage. *RFP* RNA was co-injected to have equal amounts of mRNAs injected. Whole mount *in situ* hybridization against the neural crest marker *Twist* was performed at stage 22 to analyze the migration of neural crest cells. The injected lacZ-positive side is blue. (A) Embryo injected with control MO (co MO) shows normal migration of neural crest cells. (B) Embryo injected with PlxA1 MO has disrupted migration of neural crest cells on the injected side. (C) *rPlexinA1* mRNA injected embryo shows affected neural crest migration at the injected side. (D) Co-injection of *rPlexinA1* mRNA rescues PlexinA1 MO phenotype. (E) Graph summarizing the percentage of NC migration defects of three independent experiments + standard deviations. Number of injected embryos is indicated for each column. * p values in a Student t-test < 0.05. (F) Western Blot showing that the expression of a rescue construct, which lacks both MO binding sites (HA-rPlxA1), is not affected by PlxA1 MO or the control MO. In contrast, the expression of a construct containing the PlexinA1 MO binding site (HA-PlxA1) is inhibited by the PlexinA1 MO. The expression of the HA-tagged Plexin constructs was detected by HA antibody staining (upper panel). Actin was used as a control (lower panel).

To prove that the effect of Morpholinos is specific, rescue experiments were performed. The rescue construct *rPlexinA1*, which lacks the binding sites for both PlexinA1 MOs was generated. Using Western Blotting it was confirmed that *rPlexinA1*

mRNA translation is not affected by the co-injection of PlxA1 MO, while the translation of *PlexinA1* construct, which has intact MO binding sites, was downregulated (Figure 39 F). Although the overexpression of rPlexinA1 alone resulted in mild CNC migration phenotypes (Figure 39 C, E), it could partially rescue the effect of PlxA1 MO (Figure 39 D, E). These results suggest that PlexinA1 is required for the migration of CNC cells in *Xenopus* embryos.

4 Discussion

Wnt signaling pathways regulate diverse cellular responses during embryonic development and in an adult organism (Cadigan and Nusse 1997; Logan and Nusse 2004; Clevers 2006; Chien et al. 2009; MacDonald et al. 2009; Petersen and Reddien 2009; van Amerongen and Nusse 2009; Vladar et al. 2009). Canonical Wnt signaling regulates the proliferation and differentiation of cells through transcriptional activation of target genes (Behrens et al. 1996; Molenaar et al. 1996; Moon 2005; Nusse 2005; MacDonald et al. 2009). Non-canonical Wnt signaling pathways regulate processes like cell shape formation and migration through reorganization of cellular cytoskeleton (Montcouquiol et al. 2006; Seifert and Mlodzik 2007; Angers and Moon 2009; Roszko et al. 2009; Vladar et al. 2009). Interestingly, canonical and non-canonical PCP Wnt signaling pathways, which mediate diverse outcomes, share core players like Wnt ligands, Fz receptors and cytoplasmic effector Dsh. The big question, which remains mainly unanswered, is how the mechanism allowing the cell to distinguish between different Wnt pathways and provide distinct responses functions. It is possible that diverse outputs are generated on the different levels of Wnt signaling pathways. This might be the level of Wnt proteins or the level of Wnt receptors and co-receptors. However, recent studies support the hypothesis that the complex of transmembrane receptors rather than the specificity of a ligand defines the signaling outcome (He et al. 1997; Tao et al. 2005; van Amerongen et al. 2008; van Amerongen and Nusse 2009; Grumolato et al. 2010).

Both *Wnts* and *Frizzleds* are the members of multigene families. For example, there are 19 Wnts and 10 Fz receptors in humans (Huang and Klein 2004; Croce and McClay 2008). This complexity allows a great number of possible ligand/receptor combinations, which might lead to various cellular responses. To make the situation even more complex, several transmembrane co-receptors for Wnt ligands like LRP or Ror2 exist and are often involved in the choice of preferred Wnt signaling pathway (Nusse 2008; van Amerongen and Nusse 2009; Grumolato et al. 2010).

Traditionally Wnt proteins can be subdivided into two classes, canonical Wnts and non-canonical Wnts depending on their ability to activate canonical Wnt signaling. Canonical Wnt proteins are able to induce the formation of ectopic second axis in *Xenopus* and can morphologically transform mouse mammary cells indicating an activation of canonical Wnt signaling (Wong et al. 1994; Du et al. 1995). Non-canonical Wnt proteins

lack these qualities and activate non-canonical Wnt signaling (Wong et al. 1994; Du et al. 1995). These outcomes could be explained by the differences in the structure of canonical and non-canonical Wnts. However, recent studies demonstrate that the different signaling outcomes are the result of the presence of different receptors in the system. For example, non-canonical Wnt5a, which alone does not induce axis duplication in *Xenopus*, may induce it when it is overexpressed together with human Fz5 receptor (He et al. 1997). Wnt11, a non-canonical Wnt signaling regulator in fish and frog (Heisenberg et al. 2000; Smith et al. 2000; Tada and Smith 2000; Marlow et al. 2002), is involved in the axis formation in *Xenopus* embryos inducing local accumulation of nuclear β-catenin (Tao et al. 2005). These results support the hypothesis that the receptor context regulates the activation of different Wnt pathways.

Additionally, it has been shown that Ror2 and LRP6, Fz co-receptors for Wnt, are involved in the activation of different branches of Wnt signaling. Fz activates canonical Wnt signaling together with LRP6 co-receptor in response to Wnt3a and non-canonical Wnt signaling together with Ror2 co-receptor in response to Wnt5a (Grumolato et al. 2010). Interestingly, Ror2 antagonizes canonical Wnt signaling (Mikels and Nusse 2006; Li et al. 2008; Lee et al. 2010; Yuan et al. 2011) similarly to PTK7.

PTK7 could join the list of the co-receptors for Fz involved in the choice of preferred Wnt signaling branch. PTK7 is a known regulator of PCP Wnt signaling in vertebrates. PTK7 knock-out mice have disrupted neural tube closure and misorientated hairs of inner ear (Lu et al. 2004). During mouse gastrulation PTK7 is important for polarized cell motility and convergent extension movements (Yen et al. 2009). It also regulates convergent extension movements in *Zebrafish* (Golubkov et al. 2010). Similarly to mouse data, in *Xenopus* knock-down of PTK7 by antisense Morpholino nucleotides results in neural tube closure and convergent extension defects (Lu et al. 2004; Wehner et al. 2011). In *Xenopus* PTK7 recruits Dsh to the plasma membrane to regulate cranial neural crest cell migration, the process, which depends on PCP signaling (Shnitsar and Borchers 2008). Here we show that PTK7 selectively interacts with Wnt proteins and Fz7 in *Xenopus*, activates PCP Wnt signaling and inhibits canonical Wnt signaling. We propose that PTK7 forms a complex with Fz7 and Wnt to support the activation of PCP signaling at the expense of canonical Wnt signaling (Figure 40).

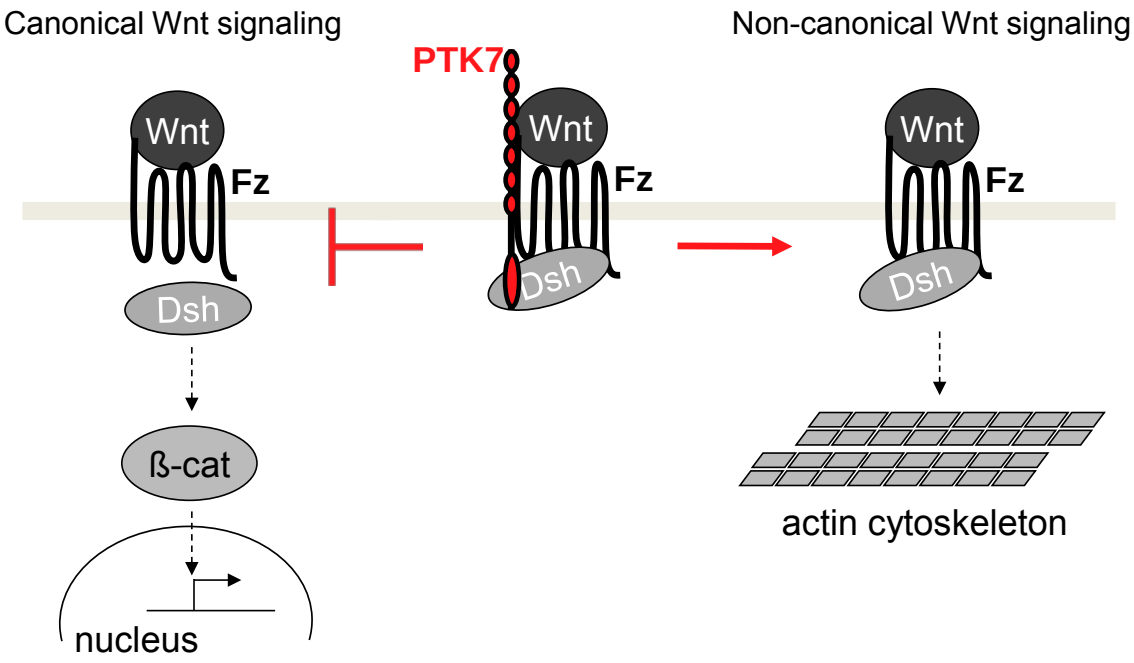

Figure 40. A model for PTK7 function in Wnt signaling. PTK7 interacts with both Wnt and Fz to inhibit canonical Wnt signaling and with Fz and Dsh to promote non-canonical Wnt signaling.

4.1 PTK7 is a Frizzled co-receptor for Wnt ligands

The aim of this work was to identify the extracellular ligands of PTK7. Since PTK7 can regulate non-canonical Wnt signaling and it is a part of Fz-Dsh complex (Lu et al. 2004; Shnitsar and Borchers 2008) we asked if PTK7 can interact with Wnt proteins.

Co-immunoprecipitation experiments between PTK7 and different Wnt proteins in *Xenopus* embryos showed that Wnt3a and Wnt8, but not Wnt5a and Wnt11, can be co-immunoprecipitated together with PTK7. However, this binding is mediated through Fz7 extracellular domain, suggesting that PTK7 is a co-receptor of Fz7. Additionally, PTK7 interact with full length Fz7 independently from the presence of Wnt (by M. Podleschny). Thus, biochemical assays suggest that PTK7 might be a Fz co-receptor, which is able to interact selectively with different Wnts through Fz7 extracellular domain. Fz7 is a Wnt receptor, which can be involved in the activation of both canonical and non-canonical Wnt signaling depending on the cellular context (Medina et al. 2000; Winklbauer et al. 2001; Abu-Elmagd et al. 2006). We propose that PTK7 and Fz7 form a receptor complex, which interacts with distinct Wnt ligands.

4.2 PTK7 inhibits canonical Wnt signaling and activates PCP Wnt signaling

PTK7 is an important regulator of PCP signaling pathway (Lu et al. 2004; Shnitsar and Borchers 2008; Yen et al. 2009; Golubkov et al. 2010; Wehner et al. 2011). PTK7 knock-out mice have typical PCP phenotypes: misorientated hairs of inner ear cells and craniorachischisis (Lu et al. 2004). In *Xenopus* knock-down of PTK7 leads to an open neural tube and defects in the migration of neural crest cells (Shnitsar and Borchers 2008; Wehner et al. 2011). However, the ability of PTK7 to modulate PCP signaling was not analyzed quantitatively. Moreover, it was not even clear if PTK7 activates or inhibits PCP because both the overactivation and the inhibition of this pathway may cause the same phenotypes. Experiments with ATF2 luciferase reporter construct, which is used to monitor JNK-dependent activation of PCP, show that full length PTK7 is able to activate ATF2 reporter dose-dependently, PTK7Δk, which lacks intracellular kinase-homology domain, could activate it weakly but exPTK7, which consists of only an extracellular domain, and ΔexPTK7 mutant, which lacks extracellular domain, could not. These data indicate that PTK7 is indeed activating PCP and only full length molecule can do it effectively. However, PTK7 has an opposite effect on canonical Wnt signaling. The overexpression of PTK7 inhibits canonical Wnt signaling in Siamois luciferase assay, second axis assay and during neural plate patterning in *Xenopus* and the knock-down of PTK7 activates canonical Wnt signaling in Siamois luciferase assay. Epistasis experiments suggest that PTK7 inhibits canonical Wnt signaling upstream of Dsh on Wnt/Fz level. So PTK7 activates non-canonical and inhibits canonical Wnt signaling (Figure 40). Interestingly, some non-canonical Wnt signaling activators like Wnt5a receptor Ror2 are able to antagonize canonical Wnt signaling (Mikels and Nusse 2006; Li et al. 2008; Lee et al. 2010; Yuan et al. 2011).

It has been reporter recently that PTK7 interacts with β-catenin to promote canonical Wnt signaling (Puppo et al. 2011). Puppo and colleagues showed that PTK7 knock-down by Morpholino oligonucleotides leads to the loss of organizer genes induction, suggesting the inhibition of canonical Wnt signaling in the knock-down situation. However, our data do not support this statement. We see the inhibition of canonical Wnt signaling by PTK7 in luciferase and second axis assays and the activation of the canonical Wnt signaling by PTK7 Morpholino.

Full length PTK7 activates PCP by the recruitment of Dsh to the plasma membrane (Shnitsar and Borchers 2008). However, the ability of PTK7Δk to weakly activate PCP signaling is quite surprising, because it cannot recruit Dsh to the plasma membrane (Shnitsar and Borchers 2008), suggesting that there is some additional mechanism contributing in the ability of PTK7 to activate PCP independently from the activity of intracellular kinase-homology domain. Probably the inhibition of canonical Wnt signaling by PTK7 is required for the activation of PCP signaling because of two supporting observations. First, the activation of PCP signaling by different PTK7 mutants correlates well with their ability to inhibit canonical Wnt signaling. Full length PTK7 inhibits canonical Wnt signaling and activates non-canonical PCP signaling, PTK7Δk has weak inhibitory effect on canonical Wnt signaling and weakly activates non-canonical one, while exPTK7 and ΔexPTK7 do not influence neither canonical nor non-canonical Wnt signaling. Second, PTK7 knock-down phenotype in *Xenopus*, an open neural tube, can be rescued by dominant-negative Wnt8, which inhibits canonical Wnt signaling. So typical PCP phenotype caused by PTK7 can be partially rescued by the inhibition of canonical Wnt signaling. Thus, PTK7 might activate non-canonical Wnt signaling at the expense of canonical Wnt signaling and furthermore, the inhibition of canonical Wnt signaling is probably required for the activation of non-canonical Wnt signaling.

However, it is unclear how PTK7 inhibits canonical Wnt signaling. There are several possible mechanisms. PTK7 selectively interacts with Wnt3a and Wnt8 but not with Wnt5a and Wnt11. First, PTK7 might bind and sequester canonical Wnt ligands inhibiting canonical Wnt signaling. However, PTK7 interacts with Wnts not directly but through the extracellular domain of Fz7. Also PTK7 and Fz7 interact with each other independently on Wnt presence. Most probably, binding the canonical Wnts to the PTK7/Fz7 complex prevents Fz7 from binding to the activators of canonical Wnt signaling like, for example, LRP6 and non-canonical Wnt signaling is preferably activated while the canonical one is attenuated. Indeed, PTK7 can also inhibit LRP6-induced canonical Wnt signaling. Additionally, the inhibition of canonical Wnt signaling by PTK7 can be rescued by the overexpression of Fz7. Also the interaction between PTK7 and caveolin might contribute in the inhibition of canonical Wnt signaling (see below).

In summary, we propose that PTK7 acts as a Fz7 co-receptor, which favors the participation of Fz7 in non-canonical over canonical Wnt signaling pathway (Figure 40).

4.3 PTK7 interacts with caveolin1a

In this study caveolin1a (Cav1a) was identified as a novel interaction partner of PTK7. PTK7 interacts with Cav1a biochemically in *Xenopus* embryos. Both PTK7 and Cav1a are expressed in the migrating neural crest cells suggesting that they indeed may function together (Razani et al. 2002; Shnitsar and Borchers 2008). Furthermore, both proteins co-localize in *Xenopus* animal caps and HEK293 cells. However, the functional importance of this interaction is not clear and several possibilities exist. Caveolins are components of bottle-shaped invaginations of plasma membrane called caveolae and they are important for various cellular functions including vesicular trafficking, signal transduction and cell migration (Fra et al. 1995; Navarro et al. 2004; Williams and Lisanti 2004; Kikuchi et al. 2009). Caveolin1 is indeed an important regulator of Wnt signaling, but its effect strongly depends on the cellular context. Endocytosis through caveolin-dependent endocytotic route activates canonical Wnt signaling in HEK293 cells and the caveolin1 knock-down attenuates the signaling in this system (Yamamoto et al. 2006; Yamamoto et al. 2008; Kikuchi et al. 2009). However, in *Zebrafish* system and mouse NIH 3T3 cells caveolin1 inhibits canonical Wnt signaling by binding of β-catenin and preventing it from entering the nucleus (Galbiati et al. 2000; Mo et al. 2010). It was interesting to clarify what function does caveolin fulfill in *Xenopus* embryos.

Our results suggest that in *Xenopus* Cav1a acts similarly to *Zebrafish* system where Cav1a inhibits β-catenin signaling. Cav1a can inhibit both Wnt8 and β-catenin – dependent activation of canonical Wnt signaling in *Xenopus* embryos. Moreover, double overexpression of Cav1a and PTK7 results in the stronger inhibition of canonical Wnt signaling in comparison to the overexpression of PTK7 or Cav1a alone. Interestingly, recently it was reported that PTK7 can bind β-catenin (Puppo et al. 2011). As caveolin1 interacts with β-catenin in several systems (Galbiati et al. 2000; Mo et al. 2010), it is possible that PTK7, Cav1a and β-catenin form a triple complex, which retains β-catenin in cytoplasm and inhibits canonical Wnt signaling.

PTK7 can be endocytosed through caveolin-dependent route in both human cell culture and *Xenopus* animal caps and the number of PTK7-positive vesicles is increased in the presence of canonical Wnts. Endocytosis of the ligand/receptor complex is a common mechanism of the attenuation of the signaling pathway (Sorkin and von Zastrow 2009). It is possible that the endocytosis of Wnt or Fz or both of them together with PTK7 results in the attenuation of canonical Wnt signaling. However, the inhibition of endocytosis by dominant-negative Dynamin, which blocks the budding of endocytotic vesicles, does not

influence the effect of PTK7 on canonical Wnt signaling. This shows that PTK7 endocytosis through caveolin route is not involved in the inhibition of canonical Wnt signaling by PTK7. It is also possible that the endocytosis of PTK7 through caveolin-dependent route might be involved in the activation of PCP signaling by PTK7, because endocytosis of ligand-receptor complex is required for the activation of PCP signaling in various systems (Chen et al. 2003; Kim and Han 2007; Yu et al. 2007; Kikuchi et al. 2009; Yu et al. 2010). Additionally, Cav1a overexpression in *Xenopus* results in the disruption of CNC cells migration, which might be associated with PCP signaling misregulation. More experiments clarifying the role of Cav1a in non-canonical Wnt signaling should be performed.

4.4 PTK7 interacts with PlexinA1 to regulate neural crest migration

PTK7/Otk interacts with PlexinA in *Drosophila* and chick to regulate Semaphorin repulsive signaling during axon guidance and cardiac morphogenesis respectively (Winberg et al. 2001; Toyofuku et al. 2004). This interaction is conserved in *Xenopus* where PTK7 and PlexinA1 interact biochemically and functionally. PlexinA1 and PTK7 are both expressed in CNC cells and interact to regulate the migration of CNC cells in *Xenopus* (Shnitsar and Borchers 2008; Wagner et al. 2010). Also at least 12 Semaphorins, including known interaction partners of PlexinA1, are expressed in the region of migrating CNC in *Xenopus* (Koestner et al. 2008) and might potentially regulate the migration of neural crest cells through PTK7/PlexinA1 complex. The knock-down of PlexinA1 results in the inhibition of CNC cells migration similarly to the knock-down of PTK7. As PTK7 also forms a complex with Fz7, a PlexinA1 co-receptor might be also involved in the functioning of this complex. However, it seems that PlexinA1 is not a regulator of Wnt signaling in *Xenopus* – it neither activates nor inhibits canonical Wnt signaling in Siamois luciferase assay and also it does not affect the recruitment of Dsh to the plasma membrane by PTK7 (data not shown). However, PlexinA1 might support the activation of PCP by PTK7, because Plexins can also activate small GTPases of Rho family, which also mediate PCP signaling (Kruger et al. 2005; Schlessinger et al. 2009). It is also possible that PTK7 plays a dual role – one as Fz7 co-receptor recruiting Dsh to the plasma membrane and inhibiting canonical Wnt signaling, and second as PlexinA1 co-receptor regulating the response to Semaphorin repulsive signaling.

5. Conclusions

The main aim of this study was to find the ligands of PTK7. It was demonstrated that PTK7 is able to interact with Wnt3a and Wnt8 but not with Wnt5a and Wnt11. The interaction between PTK7 and Wnt proteins is mediated through Fz receptor.

As Wnt3a and Wnt8 are the activators of canonical Wnt signaling, the new question about the function of PTK7 in the canonical Wnt signaling appeared. It was shown that the overexpression of PTK7 inhibits canonical Wnt signaling and the knock-down of PTK7 activates it. The extracellular and transmembrane domains of PTK7 are required for this function. Epistasis experiments showed that PTK7 inhibits canonical Wnt signaling upstream of Dsh on the Wnt/Fz level. Moreover, PTK7-mediated inhibition of canonical Wnt signaling could be rescued by the co-injection of Fz receptor, suggesting that PTK7 most probably attenuates canonical Wnt signaling on the Fz level.

Next, it was shown that PTK7 can activate PCP signaling pathway and an extracellular, a transmembrane and an intracellular domains are required for this function.

In this study caveolin1a was identified as a novel interaction partner of PTK7. PTK7 interacts with caveolin1a biochemically in *Xenopus* embryos and both proteins co-localize in *Xenopus* animal caps and HEK293 cells. It was shown that caveolin1a can inhibit Wnt8 - dependent activation of canonical Wnt signaling in *Xenopus* embryos, similarly to PTK7. It was also demonstrated that PTK7 can be endocytosed through caveolin-dependent route in both human cell culture and *Xenopus* animal. However, the endocytosis through caveolin route is not involved in the inhibition of canonical Wnt signaling by PTK7.

It was also shown that PTK7 and PlexinA1 interact biochemically and functionally in *Xenopus* to regulate the migration of neural crest cells. Also PlexinA1, as well as PTK7, is required for the migration of cranial neural crest cells in *Xenopus*.

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