1. Introduction

In this study, I investigated the molecular and cellular mechanisms involved in cell-cell rearrangement and cell membrane changes of cells within a migratory tissue, the zebrafish posterior lateral line primordium (pllp). Within this tissue, I characterized the cell biology of tissue morphogenesis. Using a developmental genetics approach, I analyzed the molecular pathways contributing to the patterning of this tissue and the subcellular remodeling of individual cells underlying tissue morphogenesis. These changes, within the tissue, are crucial for the formation of rosette-like arrangements of cells that will later become neuromasts, which are the sensory units of the lateral line organ in fish and amphibians. In this chapter, I will introduce two processes: cell migration and cell polarity. These processes are fundamental for tissue morphogenesis and establishment of the lateral line organ. I will also introduce molecular regulators of cell polarity, which were found to be important for pllp morphogenesis and establishment of cell polarity. Finally, I will introduce the zebrafish and the lateral line organ.

1.1 Cell migration and tissue re-organization

Cell migration is a fundamental process during development of an organism. Within the developing organism, cells can migrate individually. Examples are neural crest cells, hematopoietic stem cells and germ cells. Alternatively, they can migrate as a coherent tissue. Within migrating cell clusters and migrating tissues, cells exhibit dynamic interactions with each other and with the microenvironment surrounding them. A well-studied example of tissue migration is the *Drosophila* border cell cluster, which is comprised of 6–10 specialized somatic follicle cells that perform a stereotypic migration during oogenesis (King,

1970; Montell, 2001; Rorth, 2002). During mid-oogenesis, border cells delaminate from the anterior follicular epithelium and migrate in-between the nurse cells towards the oocyte. The migrating cluster is composed of two central cells (known as polar cells) and a variable number of outer cells. After reaching the oocyte, they turn and migrate dorsally towards the germinal vesicle (Montell et al., 1992). Different genes regulate the migration and cell-to-cell interaction of border cells. The *Drosophila* ligand of the PDGF/VEGF family (PVF1) and its receptor, PDGF/VEGF Receptor (PVR), are important for the first phase of migration by affecting actin accumulation within follicle cells through Myoblast city (Mbc), a Rac activator, and Rac, a Ras-like GTPase of the Rho family (Duchek et al., 2001). The EGF receptor (EGFR), another guidance receptor, is important for the second phase of border cell migration (Duchek and Rorth, 2001).

The dynamic regulation of the adhesion between the migrating cells plays an essential role, thus demonstrating that adhesion molecules, such as Cadherins and Catenins, are important for this process. Both border and nurse cells express *Drosophila* E-Cadherin, and a lack of DE-Cadherin in either cell type blocks migration (Pacquelet and Rorth, 2005; Oda et al., 1997; Niewiadomska et al., 1999). Adhesion between border and nurse cells is polarized with the highest level of DE-Cadherin at the front of the cluster, which generates increased affinity of the leading border cells and their nurse cell substrate. Conversely, expression of DE-Cadherin of trailing border cells is reduced and therefore adhesion between these cells and nurse cells decreases, which allows the border cluster to move forward (Pacquelet and Rorth, 2005).

Recent work has revealed that border cells within the cluster change their positions relative to each other during migration. Outer cells that are initially at the front of the cluster, at the beginning of migration, are located at lateral or rear positions of the cluster during migration and may be at the front of the cluster when migration ceases. In contrast, the two polar cells remain in the center of the cluster throughout migration (Prasad and Montell, 2007). These findings raise the problem of how cells in the front of the cluster that express high levels of DE-

Cadherin, relocate to the rear of the cluster during migration unless the levels of DE-Cadherin are changing according to location within the cluster.

Among the factors, involved in fixing the position of cells within the cluster, is the apical protein Bazooka. Genetic mosaics analysis in which *bazooka* (*baz*) mutant border cells were generated within a wild type environment revealed abnormal arrangements of rosette and central polar cells within the migrating border cell cluster. Such cell clusters were often elongated with individual cells trailing each other rather than migrating as a group of closely attached cells. Thus *baz* appears to be required for correct cell adhesion within the migrating cluster (Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004).

Tissue re-organization within a migratory tissue is characterized by dynamic morphogenetic changes. Axis elongation in the *Drosophila* germband epithelium is an example that could shed a light over the process, involved during re-organization. The cells change position with each other while maintaining a coherent epithelial sheet by a network of adherens junctions. Moreover, the process of cell intercalation requires the remodeling of adhesion between the neighboring cells, which is govern by the polarized localization of Myosin II at the interfaces between anterior and posterior germband cells. Also actin exhibits same localization patterning. In addition, the adherens junction proteins DE-Cadherin, Armadilo/ -catenin and Bazooka/PAR-3 are enriched in the dorsal-ventral interfaces (Bertet et al. 2004; Zallen and Wieschaus, 2004; Blankenship et al. 2006). During elongation, germband cells change neighbor cells continiously to create new interactions in their immediate enviroment (Zallen and Zallen, 2004; Blankenship et al. 2006). The formation of rosette structure that could bring up to 11 cells to cennect through a single point, and the following resolution of the rosette is a proposed mechanism, which allow efficient intercalation of the germband cells (Blankenship et al. 2006; Zallen, 2007).

Within the context of individually migrating cells, vertebrates neural crest cells (NCCs), are a migratory population of cells that arise at the border of neural plate and epidermis, at the time of neural tube closure. Neural crest cells contribute to the formation of neural and non-neural structures within vertebrates.

For example, most cells of the peripheral nervous system, melanocytes and smooth muscle cells of the heart outflow tract are neural crest derived (Le Douarin and Dupin, 2003; Barembaum and Bronner-Fraser, 2005). FoxD3, a winged-helix transcription factor, is required for the delamination of NCCs from the chicken neuroepithelium. FoxD3 initiates changes in cell-cell adhesion properties by down-regulating the cell adhesion molecule N-Cadherin and by up-regulating the mesenchymal markers Integrin-β1 and Laminin. These changes in cell-cell adhesion, regulated by FoxD3, are important for the migration of the NCCs (Cheung et al., 2005). A subpopulation of NCCs are cardiac neural crest cells (CNCs), which migrate to the heart and play two major roles in the patterning of the cardiovascular system: Firstly, CNCs participate in the patterning of the pharyngeal arches and their derivatives, including the aortic arch arteries, which will become the great arteries of the thorax and secondly, a subpopulation of CNCs migrates into the cardiac outflow tract and participates in the formation of the outflow septum (Kirby and Waldo, 1995). Connexin 43 (Cx43α1), a gap junctions protein, was shown to be necessary for the migration of CNCs to the heart (Huang et al., 1998). Cx43α1 was also shown to be necessary for other types of cell migration, including primordial germ cell migration (Juneja et al., 1999). These findings demonstrate the importance of cell-cell connections, mediated by Cx43α1, for the correct migration of cell populations. Cx43α1 regulates the actin cytoskeletal organization within CNCs (Xu et al., 2006), thus affecting retraction of trailing edge cells and contraction of the cell body (Etienne-Manneville, 2004) and hence regulating cell motility.

1.2 Cell polarity

Cell polarity is a fundamental property of living cells and it is required for the proper function of cells and the correct development of the embryo. Loss of cell polarity is a hallmark of cancer. In epithelial cells, polarization is characterized by a differential distribution of plasma membrane proteins and lipids to distinct membrane compartments, namely the apical and baso-lateral domains, and by

the differential distribution of cytoplasmic organelles and the cytoplasmic and cortical cytoskeleton. In addition, polarized epithelial cells confine tight junctions, which separate the apical and baso-lateral membrane domains and which also form barriers to ion and macromolecules diffusion (Rodriguez-Boulan and Nelson, 1989). Epithelial cells are organized with their apical side facing an external lumen, and their basal side facing either the extracellular matrix or adjacent cells. Several junctional structures are present along apico-basal membranes that function in cell-cell adhesion, cell communication, or as paracellular diffusion barriers. Most apically, the vertebrate tight junction (TJ) (equivalent to the *Drosophila* subapical region) provides a tight seal towards the external lumen and this structure is adjacent to the adherens junction (AJ)(*Drosophila* zonula adherens (ZA)), which serves in cellular adhesion. The AJ and ZA belong to a family of actin-associated cell junctions (Geiger and Ginsberg, 1991) and their functionality results from the Cadherin-Catenin homophilic adhesion system. The Cadherins are transmembrane glycoproteins that interact through their cytoplasmic domain with α-, β- and γ- Catenin and P120catenin (Magee and Buxton, 1991). Along basolateral membranes, desmosomes and hemidesmosomes are present. In *Drosophila*, the basolateral septate junction (SJ) is functionally similar to the vertebrate TJ (Tepass et al., 2001).

In *Drosophila* genetic screens, several genes were identified to be required for epithelial apico-basal polarity. Functional genetics combined with biochemical analysis revealed that many of the proteins encoded by these genes, as well as their vertebrate homologs, are components of three protein complexes.

The most apical protein complex which localizes to the vertebrate TJ or the *Drosophila* subapical region contains the transmembrane protein Crumbs (Crb), the homolog of zebrafish Oko meduzy (Omori and Malicki, 2006), which associates with the first Postsynaptic Density Protein 95 (PSD 95)/Disc Large/Zona Occludens (ZO-1) (PDZ) domains of Discs lost (DLT), the homolog of mammalian PATJ (Bhat et al., 1999) and of the membrane-associated

guanylate kinase (MAGUK) protein Stardust (Sdt), the homolog of mammalian Pals1 and of zebrafish Nagie oko (Bachmann et al., 2001; Wei and Malicki, 2002). Recent studies in zebrafish revealed that the FERM domain protein Mosaic Eyes binds the FERM domain-binding site of Crb (Hsu et al., 2006).

A second apical protein complex contains *Drosophila* atypical Protein Kinase C (DaPKC) (Wodarz et al., 2000), which is the homolog of vertebrate PRKC including zebrafish Heart and Soul (Has)/PRKC iota (PRKCi)) (Horne-Badovinac et al., 2001; Peterson et al., 2001), Bazooka (Baz) which is the homolog of vertebrate Par-3/ASIP (Muller and Wieschaus, 1996; Kuchinke et al., 1998) and the PDZ-domain protein Dm Par-6 or vertebrate Par-6, which can bind directly to Bazooka or Par-3/ASIP, respectively (Petronczki and Knoblich, 2001; Yamanaka et al., 2001; Suzuki et al., 2001). The Par protein complex is partially overlapping with the vertebrate AJ and ZA in *Drosophila*. Studies in *Drosophila* and in the chicken neural tube show that Bazooka and Par-3/ASIP localize independently from Par-6 and PRKC to the adherens junction, basally from their apical partner proteins within the PAR complex (Harris and Peifer, 2005; Afonso and Henrique, 2006; Mirouse et al., 2007). The Par complex is important for the establishment and maintenance of the vertebrate AJ and *Drosophila* ZA (Horne-Badovinac et al., 2001; Muller and Wieschaus, 1996).

A third protein complex that contains proteins with overlapping localization patterns, localizes to the *Drosophila* SJ or basolateral membranes in vertebrate epithelial cells, and includes Lethal Giant Larvae2 (Lgl2), the homolog of zebrafish penner (Sonawane et al., 2005). Computer predictions of *Drosophila* Lgl2 protein reveal 2 beta-propellers, two low-complexity regions and a Tomosyn homology region (THR) within the protein (Betschinger et al., 2005). Moreover, Lethal Discs Large (Dlg), a MAGUK protein with multiple PDZ domains (Woods and Bryant, 1991) and Scribble (Scrib), which is also a multi PDZ domain protein that belongs to the Leucine-rich repeats and PDZ domains (LAP) subfamily (Bilder and Perrimon, 2000), are included in the third protein complex and function as neoplastic tumor suppressor proteins in *Drosophila* (Bilder, 2004). The loss, of the third complex, leads to cell polarity defects that include the

mislocalization of apical and ZA markers, which spread basally (Bilder et al., 2000).

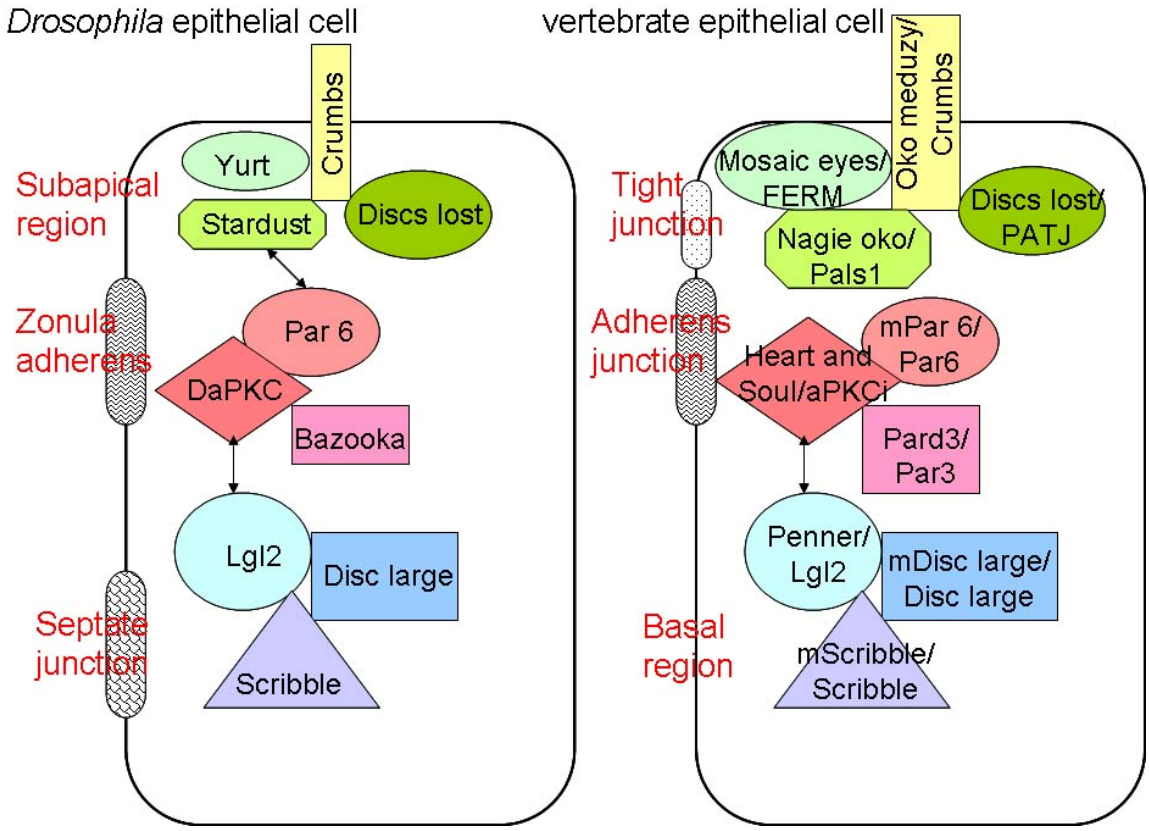

Figure 1: Depicted are the three polarity complexes in the *Drosophila* and vertebrate epithelial cell. The Crumbs complex, which is apical to the *Drosophila* ZA (vertebrates AJ), the PAR complex, which is apical and partially overlapping with the *Drosophila* ZA (vertebrates AJ) and the Lgl complex, which is localized baso-laterally.

Next, I will discuss in detail a representative component protein for each of the three polarity complexes mentioned above: Penner/Lgl2, Heart and Soul/PRKCi and Stardust/Mpp5/Pals1/Nagie oko. In addition, I will also introduce Myosin VI which has relevance to the results presented in this study.

1.2.1 Lethal giant larvae 2

The *Drosophila lethal giant larvae2* (*lgl2*) mutation was first discovered by Bridges and Brehme in the 1930's and was recognized later, in the late 1960's, as a recessive oncogene. In *Drosophila*, homozygous mutations lead to abnormal overgrowth of the presumptive adult optic centers and the imaginal discs, which results in late larval or early pupal lethality (Baek, 1999). Loss of both maternal and zygotic Lgl2 in *Drosophila* leads to a failure of embryogenesis and to the loss of cell shape (Manfruelli, 1994). Further studies in *Drosophila* showed that Lgl2 acts as a cell polarity regulator, which affects ZA formation (Bilder et al., 2000). In *Drosophila* neuroblasts, Lgl2 and Dlg are required to localize the cell fate determinants proteins Prospero and Numb and their adaptor proteins Miranda and Partner of numb (Pon) to the basal cortex. However, apical localization of proteins including Inscuteable and Partner of Inscuteable (Pins) is not affected (Peng et al., 2000).

DaPKC and PRKC phosphorylate several conserved serine residues of Lgl2, in *Drosophila* neuroblasts and vertebrate Madin-Darby canine kidney (MDCK) cells, respectively (Betschinger et al., 2003, Plant et al., 2003, Yamanaka et al., 2003). In *Drosophila* neuroblasts, phosphorylation of Lgl2 by DaPKC inactivates Lgl2 and thus releases it from the apical cell cortex (Betschinger et al., 2003). In mammalian cells, the mammalian orthologue of Lgl2, Mlgl, was shown to be important for cell polarization in a rat astrocytes wounding assay, in which polarization is characterized by re-orientation of the Golgi, cell elongation and emergence of stress fibers perpendicular to the wound edge. Similar to the situation in *Drosophila* neuroblasts, in this wounding assay, Mlgl phosphorylation by PRKC is important for cell polarization (Plant et al., 2003). Lgl2 inactivation by phosphorylation is an autoinhibition process (Figure 2), in which the phosphorylated protein is folding, allowing the N-terminus to interact with the C-terminus. The N-terminus domain contains WD-40 repeat motifs, which are important for protein-protein interactions. The non-phosphorylated form of Lgl2 is an active and unfolded form, which allows the C-

terminal end to associate with cytoskeletal proteins (Betschinger et al., 2005). Lgl2 associates with non-muscle Myosin II heavy chain, which disassociates upon phosphorylation of Lgl2 (Strand et al., 1994, Kalmes et al., 1996). Myosin II is an actin-associated protein, but experiments have shown that association of Lgl2 C-terminus to the actin-cytoskeleton is not dependent only on Myosin II but also on other actin-associated protein/proteins (Betschinger et al., 2005).

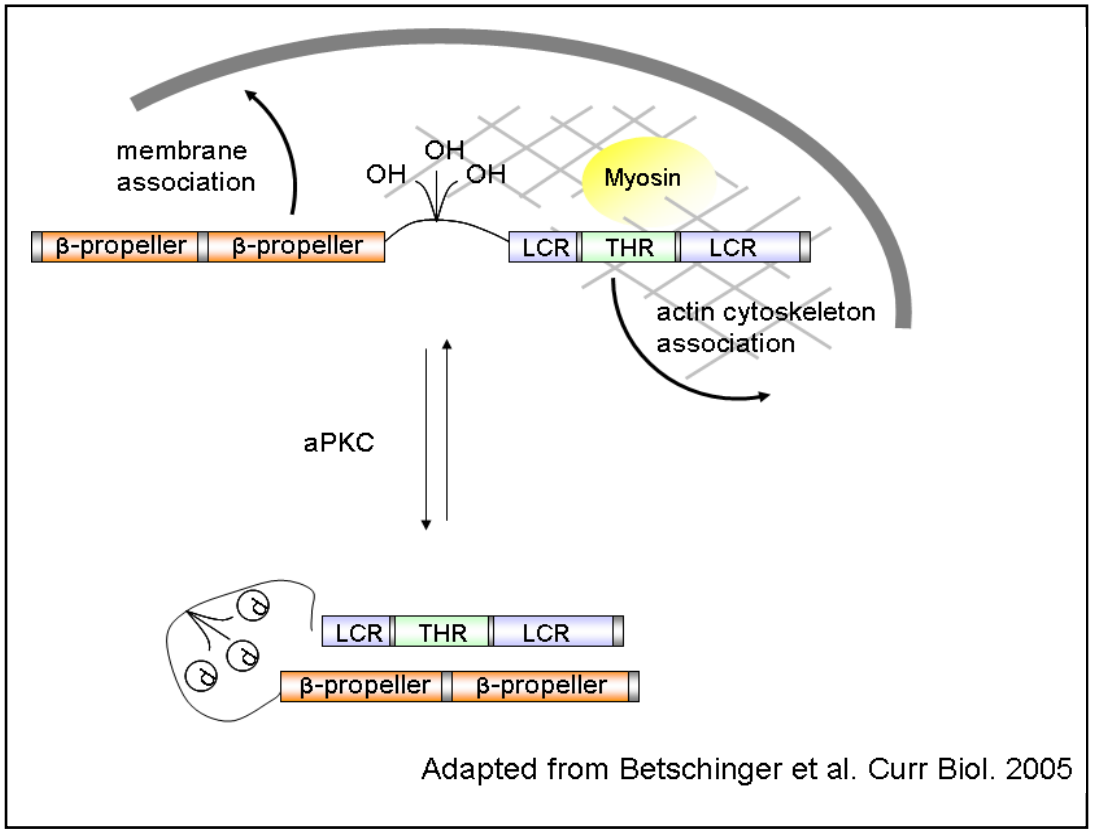

Figure 2: Depicted is the autoinhibition process of Lgl2 that results from the phosphorylation by PRKC. In the phosphorylated form, the N-terminus of Lgl2 binds to the C-terminus resulting in an autoinhibition of Lgl2 and thus release of Lgl2 from the membrane to the cytoplasm.

In addition to DaPKC and PRKCs restricting the activity of Lgl2, studies in *Drosophila* have shown that DaPKC and Lgl2 have antagonistic genetic interactions. A weak *lgl* phenotype could be suppressed by reducing PRKC levels in neuroblasts, leg and wing imaginal discs (Rolls et al., 2003). In the *Drosophila* sensory precursor (pI) cells, Lgl2 was found to regulate the asymmetric distribution of the fate determinants Numb and Neuralized during pI division and to inhibit the plasma membrane localization of Sanpodo, thus regulating Notch signaling in the anterior pI daughter cell (Langevin et al., 2005). In *Drosophila* epithelial cells, Lgl complex proteins interact antagonistically with Crumbs complex proteins, to define apical and basolateral membrane compartments. This interaction is also important for the formation of the zonula adherens early in development (Tanentzapf and Tepass, 2003).

Studies in yeast revealed another potential function for Lgl2 in establishing cell polarity. The yeast homologs of Lgl2, Sro7p and Sro77p serve conserved functions, which was confirmed by a complementation assay of salt sensitivity defects in *sro7/sro77* mutants, using mammalian and *Drosophila lgl2* (Kim et al., 2003, Larsson et al., 1998). *sro7/77* double-mutants show defects in exocytosis and fusion of the post-Golgi vesicles with the plasma membrane. Sro7p and Sro77p bind to the plasma membrane t-SNARE Sec9p, which is important together with other SNARE members, for the docking of vesicles to the plasma membrane. Vesicle fusion at the budding tip is required for asymmetric cell division of budding yeast (Lehman et al., 1999). Moreover, Sro7/77p bind to Exo84p, a component of the exocyst complex, which is essential for targeting vesicles to specific sites of the plasma membrane for exocytosis (Zhang et al., 2005).

Currently, not much is known about the developmental role of Lgl proteins in vertebrates. Studies in mice revealed that there are two *lgl* genes known as *lgl1* and *lgl2*. Loss of murine *lgl1* causes formation of neuroepithelial rosette-like structures and severe hydrocephalus (Klezovitch et al., 2004). A large proportion of neural progenitor cells fail to exit the cell cycle and to differentiate and instead, continue to proliferate and finally undergo apoptosis. In zebrafish, *lgl2/penner*

was discovered in a genetic screen for skin mutants and was shown to be necessary for hemidesmosome formation in basal epidermal cells (Sonawane et al., 2005). Hemidesmosomes contain multi-protein complexes, which are essential for stable adhesion between basal epidermal cells and the underlying basement membrane formed of extracellular matrix. In *penner* mutant larvae, basal epidermal cells migrate to ectopic places, and hyper-proliferate.

Two genes, which participate in the core planar cell polarity (PCP) pathway, interact with Lgl2. PCP refers to the polarization of epithelial cells within the plane of the tissue layer. PCP can operate in non-motile tissue as well as in motile populations and was recently shown to control, among other developmental processes, two steps of convergence and extension of gastrulating cells in zebrafish and Xenopus. The core PCP pathway consists of the cell-surface proteins Frizzled (Fzd), Strabismus (Stbm), and Flamingo (Fmi) and the cytoplasmic proteins Dishevelled (Dsh), Prickle (Pk) and Diego (Dgo). These proteins are considered core proteins, since in *Drosophila* they are most noticeably required for PCP in epithelial tissues that generate structures of the adult (Zallen, 2007). Moreover, Dsh, a cytoplasmic protein and the ten known members of the Fzd family of transmembrane proteins also participate in canonical Wnt signaling, which involves the binding of Wnt ligands to Fzd receptor family members and a resulting cascade, which includes the translocation of β-catenin to the nucleus and the formation of a transcriptional enhancer complex composed of LEF-TCF DNA binding proteins. Dsh and Fzd participate also in the non-canonical pathway, which is often described as representative of two distinct pathways, the Wnt/Ca2+ and Wnt/planar cell polarity. The non-canonical pathway involves also G-proteins, phospholipase C (PLC), protein kinase C (PKC), c-jun kinase (JNK) and Ca2+/calmodulin-dependent protein kinase II (CaMKII). The Wnt/Ca2+ pathway includes the hydrolysis of phosphatidylinositol biphosphate (PIP2) by PLC to produce diacylglycerol (DAG) and inositol triphosphate (IP3), which in turn promotes intracellular release of Ca2+. The PCP pathway includes the activation of PKC, which regulates Dsh protein, which subsequently stimulates JNK activity

(Eisenberg and Eisenberg, 2007). Dsh was found to interact with Lgl2, in a yeast two-hybrid assay. Studies performed in *Xenopus* showed that Dsh regulates the localization and stability of Lgl2. In addition, also Fzd8 was shown to affect Lgl2 localization and activity (Dollar et al., 2005). Since PKC participates in the non-canonical pathway and Lgl2 is a phosphorylation target of aPKC/PRKC, it appears likely that Dsh and Fzd8 interaction with Lgl2 is part of the non-canonical pathway.

Therefore, Lgl2, which has been discovered as a tumor suppressor, has different other functions like regulating apico-basal cell polarity in *Drosophila* and vertebrates as well as regulating asymmetry formation in yeast and controlling Hemidesmosomes formation in zebrafish. The interaction with PCP proteins may implicate on an additional function of Lgl2.

1.2.2 Atypical Protein Kinase C

Protein Kinases of the C type (PKCs) are serine/threonine kinases, the first of which was described as a histone kinase in the rat brain (Takai et al., 1977). There are at least 11 PKC isoforms that have been identified in mammalian tissues to date. The distinct PKC isoforms are grouped into three subfamilies: conventional or “classical” PKCs (cPKCs), “novel” PKCs (nPKCs) and “atypical” PKCs (aPKCs or PRKCs) (Yamaguchi et al., 2006).

The PKCs are involved in several pathways. Cell polarity regulation is one of the processes in which PKCs are involved. In *Caenorhabditis elegans*, PKC-3, the homolog of *Drosophila* PRKC, was found to associate with Par-3, the homolog of *Drosophila* Bazooka (Baz) and vertebrate Par-3/ASIP, and shown to be essential for proper asymmetric cell division (Tabuse et al., 1998; Izumi et al., 1998). In *Drosophila*, DaPKC was found to bind Baz in a yeast two-hybrid assay. DaPKC and Baz are mutually dependent on each other for their apical localization (Wodarz et al., 2000; Nagai-Tamai et al., 2002; Benton and St Johnston, 2003) and both genes are required for the maintenance of cell polarity in epithelial cells and neuroblasts (Kuchinke et al., 1998; Schober et al., 1999;

Wodarz et al., 2000; Rolls et al., 2003). In a primary rat astrocytes cell migration assay, Cdc42 activates Par-6-PKCzeta, leading to phosphorylation and inactivation of GSK-3β, which in turn affects adenomatouos polyposis coli (Apc) association with the plus ends of microtubules. This cellular signaling cascade results in the establishment of cell polarity (Etienne-Manneville and Hall, 2003) and indicates its importance for cell migration. Studies made on neuronal precursor cell migration in chicken support this observation. Cdc42 activity was found to be crucial for both generating the monopolar morphology and the formation of the protrusion in rhombic lip neuronal precursors. Moreover, the Par protein complex was found to regulate the orientation of cellular protrusions required for directional cell migration (Sakakibara and Horwitz, 2006).

PRKCs are also implicated in cellular survival. In the pheochromocytoma cell line PC12, PKC iota was shown to mediate nerve growth factor (NGF)-cell survival by phosphorylating IKK beta kinase, which in turn phosphorylates Iκ B thus activating NF-κB (Wooten et al., 2000). Another phosphorylation target for the PKC isozyme, PKC delta, in the context of cell survival, which was found in NGF-treated PC12 cells, is ErbB3-binding protein (Ebp1). Ebp1 phosphorylation stimulates its binding to phosphorylated nuclear anti-apoptotic serine/threonine kinase Akt and in turn the Ebp1/Akt complex interacts with caspase-activated deoxyribonuclease (CAD) and inhibits its DNA fragmentation activity (Ahn et al., 2006). Further investigations in transformed cells and tumor cells containing activated p21 (RAS) oncoprotein verified that PKC delta induces its anti-apoptotic activity through the anti-apoptotic serine/threonine kinase Akt (Xia et al., 2007).

PRKCs are regulators of cell proliferation. Upon cell proliferation, cells reenter cell cycle and the GTP/GDP binding GTPase, Ras, activity is required for the G_0/G_1 transition (Cai et al., 1990, Feig and Cooper, 1988, Dobrowolski et al., 1994). In order for Ras, to exert its activity, upregulates Ras cyclin D_1 concentration (Filmus et al., 1994, Albanese et al., 1995). The activation of cyclin D1 by Ras appears to be mediated by several pathways and several PKC isozymes are proposed to mediate these pathways. aPKC lambda is suggested to act upstream of the Rho family GTPase (Rac), between Ras and Rac in

mediating upregulation of cyclinD1. In contrast, aPKC zeta and nPKC epsilon are suggested to act downstream of Rac to activate extracellular signal-regulated kinases (ERKs) for signal transmission to the cyclin D1 promoter. Another pathway in which Ras employs ERKs in order to upregulate cyclin D1 is via mitogen-activated protein (MAP)/ERK kinase (MEK1), in which aPKC zeta functions downstream of MEK-1. nPKC epsilon was also shown to be involved in this pathway, although its position in the cascade is unclear (Kampfer et al., 2001). Studies in *Drosophila* neuroblasts and epithelial cells of the eye imaginal discs also showed that PRKC is necessary to promote cell proliferation (Rolls et al., 2003). Reduction in the function of mammalian atypical PKC zeta can suppress Rac1/cdc42 induced over-proliferation, thus implying aPKC zeta in the promotion of cell proliferation (Qiu et al., 2000). In INS-1 cells, aPKC zeta is activated by two growth factors, hepatocyte growth factor (HGF) and parathyroid hormone-related protein (PTHrP) to promote cell proliferation (Vasavada et al., 2007).

In the context of cell polarity regulation, PKCs have sereval known phosphorylation targets. As discussed above, Baz, together with DaPKC, is important for establishing cell polarity and it was shown that mammalian ASIP/Par-3 is phosphorylated by aPKC (Hirose et al., 2002; Nagai-Tamai et al., 2002). In primary rat astrocytes, PKC zeta is necessary for the phosphorylation of GSK-3β, resulting in its inactivation, and thus the establishment of cell polarity (Etienne-Manneville and Hall, 2003). The Par-1 protein kinase, which directs anterior-posterior asymmetry in the one-cell stage *C.elegans* embryo and the *Drosophila* oocyte (Pellettieri and Seydoux, 2002) as well as in regulating cell polarity in mammelian epithelial cells (Bohm et al., 1997), is another phosphorylation target. Human Par-1b (hPar-1b) and Par-1a (hPar-1a) are phosphorylated by aPKC zeta. hPar-1b phosphorylation, by aPKC zeta, results in a negative regulation of its kinase activity and disassociation from plasma membrane (Hurov, 2004). In MDCK cells, mammalian Par-1b (Suzuki et al., 2004) and xPar-1 in Xenopus (Kusakabe and Nishida, 2004) are also phosphorylation targets of aPKC. The phosphorylation of a conserved threonin in

mammalian Par-1b and two conserved residues, a threonin and serine residues, in xPar-1, enhances binding of 14-3-3/Par-5 to Par-1b/xPar-1, thus induces Par-1b/xPar-1 dissociation from the lateral membrane (Suzuki et al., 2004; Kusakabe and Nishida, 2004). Another phosphorylation target of DaPKC is Crumbs (Crb) (Sotillos et al., 2004). DaPKC binds directly to Crb and Pals-1 associated TJ protein (Patj) of the Crb protein complex during the formation of the ZA and the establishment of the apical domain. The *C. elegans* homolog of aPKC, PKC-3, phosphorylates Par-2, thus restricting its localization away from the anterior cortex (Hao et al., 2006). As mentioned within the previous section, DaPKC and PRKC phosphorylate Lethal Giant Larvae 2 (Lgl2) in *Drosophila* and mammalian cells, respectively, thus establishing apico- basal polarity (Betschinger et al., 2003; Plant, 2003; Yamanaka et al., 2003). In *Drosophila* sensory precursor (pI) cells, Numb is directly phosphorylated by DaPKC, which regulates its asymmetric localization (Smith et al., 2007). MacMARCKS, a member of the myristoylated alanine-rich C-kinase substrate (MARCKS) family (Bhat et al., 1991), is also a phosphorylation target of PKC (Li and Aderem, 1992). In polarized MDCK epithelial cells MacMARCKS is found at the basolateral membrane and displaced from its location into the cytosol upon phosphorylation by PKC (Myat et al., 1998). Similar results were obtained for MARCKS phosphorylation by PKC in rat oligodendrocyte progenitor cells (Baron et al., 1999).

Interaction between different protein complexes could indicate the involvement of these complexes in similar pathways. In *Drosophila* photoreceptor rhabdomeres, the Par complex proteins, including DaPKC, associate with Crumbs complex proteins. Discs-lost directly interacts with Par-6 (Nam and Choi, 2003). The interaction between these two complexes may explain phenotypic similarities observed in zebrafish mutants, *heart and soul* (*has*) and *nagie oko* (*nok*) (Malicki et al., 1996). In zebrafish, *has* encodes the homolog of *Drosophila* DaPKC. *has* mutants exhibit defects in heart tube assembly, maintenance of the retinal pigmented epithelium, lamination of the neural retina, inflation of brain ventricles and body curvature. *has* affects the formation and maintenance of AJ within the polarized retinal neuroepithelium (Horne-Badovinac et al., 2001).

1.2.3 Stardust/Mpp5/Pals1/Nagie oko

Nagie oko (nok) is the zebrafish homologue of mammalian protein associated with Lin-7 (Pals1) and *Drosophila* Stardust (Sdt). Pals1 was discovered in an assay identifying binding partners proteins for the amino terminus of murine mLin-7 (a MAGUK protein) and was named protein associated with Lin-7 (PALS). Pals1 is a MAGUK protein that contains a PDZ domain, an SH3 domain, a GUK domain and an mLin-7 binding domain (Kamberov et al., 2000). Pals1 interacts directly with Pals1-associated tight junction (PATJ) via PDZ domain of PATJ. Human Crb2 was found to interact with PATJ indirectly by binding to the Pals1 PDZ domain (Roh et al., 2002). *Drosophila stardust* mutants exhibit severe disruption in apico-basal polarity and ZA formation, causing multi-layering, tissue disintegration and defects in cuticle formation (Muller and Wieschaus, 1996; Grawe et al., 1996; Bachmann et al., 2001; Hong et al., 2001). At later stages, SJ fail to develop, and both Dlg and Scrib are mislocalized (Hong et al., 2001). In contrast to the importance of Stardust in epithelial cell polarity, neuroblast polarity does not require Stardust (Bachmann et al., 2001; Hong et al., 2001).

The zebrafish *nok* gene is widely expressed throughout all tissues during embryogenesis and restricted to the retina and neural tube at 72hpf. In *nok* mutants, retinal lamination is disrupted and ectopic cell rosettes are forming (Wei and Malicki, 2002). In addition to pigmentation defects, this mutant exhibits an abnormal brain shape, curved body axis and heart morphogenesis defects (Wei and Malicki, 2002; Rohr et al., 2006). The *nok* mutant phenotype is similar to phenotypes caused by *has* and *oko meduzy* (*ome*) mutants, the latter encoding a Crb type II protein (Omori and Malicki, 2006).

1.2.4 Myosin VI

Myosins are motor molecules that hydrolyse ATP to move along actin filaments and microtubules. Whereas most Myosins move towards the (+) end of the actin filaments, Myosin VI is an exception, since it moves towards the (-) end (Wells et al., 1999). Myosin VI was discovered in *Drosophila* as an F-actin binding protein and was classified as unconventional Myosin heavy chain. The N-terminal domain contains ATP-binding, actin-binding and calmodulin/Myosin light chain-binding motifs (Kellerman and Miller, 1992). Myosin VI homo-dimerizes via its coiled-coil domain (Frank et al., 2004). The ability of Myosin VI to stay bound to actin for long periods of time and to take multiple steps along an actin filament before detaching is thought to be a critical feature for cargo transport, but this function has not been clarified yet for Myosin VI (Frank et al., 2004). In *Drosophila*, Myosin VI catalyses transport of cytoplasmic particles, with this function being an actin-based and ATP-dependent mechanism (Mermall et al., 1994). In a *myo VI* mutant cell line, Golgi size and secretion are found to be reduced, suggesting that Myosin VI transports vesicles from the Golgi complex, along actin filaments, onto microtubules, for longer distance transport within the cell (Warner, 2003).

In *Drosophila* border cells, Myosin VI is required for cell migration and it is expected to be a downstream target of *Drosophila* transcription factor CCAAT enhancer binding protein (CERP). CERP is encoded by the *slbo* gene, which regulates border cell migration, and it was shown that in *slbo* mutants myosin VI expression is reduced. Armadillo (*Drosophila* beta-catenin) and E-Cadherin expression, which are known to interact with each other, were reduced in myosin VI-depleted border cells. Moreover, migration defects, which were observed in myosin VI-depleted cells, could be rescued upon E-Cadherin overexpression. In the context of border cells, myosin VI has been suggested to be in a complex with armadillo and E-Cadherin, which is important for their stability and protection from degradation and which is required for border cell migration (Geisbrecht and

Montell, 2002). In *Drosophila* neuroblasts, Myosin VI interacts physically with and affects the basal localization of the adaptor protein Miranda. Furthermore, Myosin VI is necessary for correct spindle orientation in *Drosophila* neuroblasts, thus connecting Myosin VI to asymmetric division of neuroblasts (Petritsch et al., 2003).

In mammals, Myosin VI is important for the maintenance of hair cell integrity within the inner ear of the mice. In Snell's waltzer mice, which have a mutation in Myosin VI, stereocilia fuse together and are disorganized (Avraham et al., 1995, Self et al., 1999). In the Striped bass fish (*Morone saxatilis*), two isoforms of Myosin VI were identified in a degenerate PCR screen of retinal cDNA (*myo VIa* and *myo Vib*) (Breckler et al., 2000). *myoVIb* is specifically expressed in the hair cells of the inner ear and lateral line organ. In contrast, *myoVIa* is broadly expressed with high expression in the brain, gut and kidney (Kappler et al., 2004; Seiler et al., 2004). In a screen for mutations affecting zebrafish hair cell development, a *myoVIb ru920/satellite* mutation was identified (Kappler et al., 2004; Seiler et al., 2004). The mutation does not affect hair cell number, but rather the mechanoelectrical transduction ability of the hair cells (Kappler et al., 2004). Hair bundles in the inner ear of the *ru920/satellite* mutant were found to be malformed, shorter or thinner (Seiler et al., 2004).

1.3 Zebrafish as a model organism

The zebrafish (*Danio rerio*) is a tropical fish belonging to the minnow family (Cyprindae). The zebrafish is native to the Ganges region in Eastern India and also to Pakistan, Bangladesh, Nepal and Myanmar. The fish is omnivorous and feeds on various small aquatic insects, crustaceans, worms and plankton. It grows to about 3.8 cm in length and lives for around 5 years. The fish is named for its five uniform pigmented, horizontal blue stripes on the side of the body.

The zebrafish is considered a genetically amenable vertebrate model system due to rapid embryonic development, passing from egg to larvae stage in less then three days, which gives the zebrafish an advantage over other

vertebrates. Moreover, the transparency of the zebrafish larvae allows the identification of many mutations during genetic screens. Already in the 1930s the zebrafish was used as a developmental model organism, due to the optical clarity of the embryos and larvae. The establishment of genetic techniques such as cloning, mutagenesis, transgenesis and mapping, combined with the isolation of thousands of developmental mutants found by genetic screening, allowed the use of the zebrafish as a tool for vertebrate developmental analysis.

Occasionally, zebrafish mutants have relevance to human diseases, for example, the Huntington's disease gene homolog in zebrafish (Karlovich et al., 1998), the zebrafish *vhnf1* gene, which is orthlogous to the human homeobox gene, *vHnf1*, in which, mutations are associated with polycystic kidney disease (PKD) and maturity-onset diabetes of the young, type V (MODY5) (Sun and Hopkins, 2001), as well as the zebrafish genes *presenilin* (*ps*)*-1* and *apolipoprotein E* (*apoE*), which have both been implicated in familial Alzheimer's disease (FAD) (Leimer et al., 1999; Monnot et al., 1999). Thus, the mature zebrafish has a tremendous potential, not only as a developmental model, but also as a model for adult and late life human diseases.

There are different ways of producing mutations in zebrafish. One way is to expose male fish to the mutagen ethylnitrosourea (ENU), a potent alkylating mutagen that induces point mutations with high efficiency (Driever et al., 1996; Haffter et al., 1996). Zebrafish are relatively resistant to high levels of ENU toxicity, which allow higher levels of mutagenesis and specific locus hit rates than can be achieved with other vertebrate models. Another way is using retroviral methods, but with lower efficiency than by chemical mutagenesis (Lieschke and Currie, 2007). Generation, of transgenic fish, is done by DNA-constructs injection into one-cell stage embryos. The DNA constructs integrate into the fish genomic DNA, and thus allow the study of specific proteins in the fish (Stuart et al., 1988). Exogenous overexpression of different proteins ubiquitously in the embryo, by mRNA injection into the yolk of one cell stage embryos allows the study of subcellular localization patterns and the interactions with other proteins. A recently used method to investigate the role of different genes in the

development of the fish is the knockdown method using Morpholino antisense oligonucleotides (MO), which blocks translation of target mRNAs. Animals that develop from the injected eggs are referred to as “morphants” (Nasevicius and Ekker, 2000; Draper et al., 2001).

Another method, used in zebrafish, to investigate gene function, is genetic mosaics. This method addresses questions such as, what are the phenotypic consequences when some cells, in an animal, carrying a *WT* gene and the other cells are homozygous mutants. Another question is, which cell or cells in an animal must carry the *WT* gene, within cells that are homozygous mutants, in order to produce a wild-type phenotype. A gene is considered to act cell-autonomously when the phenotype of a given cell, in a mosaic animal, is depended solely on whether or not the cell has the *WT* or the mutated gene and is unaffected by the genotypes of other cells. A gene is considered to act cell-nonautonomously when a cell carrying the *WT* gene or a cell is homozygous mutant, in a mosaic animal, exhibits a mutant phenotype or a *WT* phenotype, respectively. Cell-nonautonomy implicates cell-cell interactions, and mosaic analysis can be used to identify the responsible interacting cells (Yochem and Herman, 2003). Mosaic analysis in zebrafish is performed by transplantation of cells taken from a *WT* or mutant donor of the late blastula stage into the same stage mutant or *WT* host embryo, respectively (Ho and Kane, 1990). The transplantations are being carried out according to a fate map. During the course of embryonic development, in vertebrates, cells become allocated into separate lineages of which each displays a limited and stereotyped set of cell fates. It is important to know how early cell positions relate to later fates and this information is contained in the fate map (Kimmel et al., 1990).

1.4 Development of the posterior lateral line organ

The lateral line organ (llo) is a mechanosensory system present in fish and amphibians. The functional unit of this organ, called the neuromast, is comprised of a ring of support cells surrounding a central cluster of sensory hair cells. The llo is composed of two parts, the anterior lateral line organ (allo) and the posterior lateral line organ (pllo) (Gompel et al., 2001). The pllo derives from a primordium, which is formed just caudal to the otic vesicle and starts migration along the horizontal myoseptum at about 20 hpf until the tip of the tail. The migrating primordium consists of about 100 cells and during its migration it deposits clusters of cells, the pre-neuromasts, which will eventually differentiate to neuromasts. The primordium completes its migration at about 42 hpf, after depositing 5-6 neuromasts along its migratory path and another 2-3 neuromasts at the tip of the tail (Metcalfe, 1985; Kimmel et al., 1995). The neuromast is the functional unit of the pllo and is an individually innervated rosette of hair cells, surrounded by supporting cells in an arrangement similar to that of the sensory epithelia in the inner ear. The neuromast is important for schooling, rheotaxis, and the detection of predators and prey (Kappler et al., 2004). At about 48 hpf, a second primordium starts to migrate from the cephalic region, much like the first primordium, depositing neuromasts between the first array of deposited neuromasts (Sapede et al., 2002). Another source for neuromast formation are interneuromasts cells, which are deposited between the neuromasts by the migrating primordium (Grant et al., 2005).

The second component of the llo is the lateral line nerve system. Growth cones of posterior lateral line sensory neurons are found within the premigratory primordium when it is adjacent to the ganglion. During migration of the primordium, growth cones of these sensory neurons are found within the primordium (Metcalfe, 1985).

The migration of the primordium is dependent on the presence of an SDF1-like chemokine, which is expressed along the migration path and a CXCR4-like

chemokine receptor that is expressed by the migrating cells and down-regulated at the trailing edge, which allows for the deposition of neuromasts there (David et al., 2002; Li et al., 2004; Haas and Gilmour, 2006).

Another independent mechanism that controls neuromasts deposition involves the receptor tyrosine kinase Met and its ligand, hepatocyte growth factor (Hgf). Down-regulation of the *met* gene within the trailing edge of the migrating primordium is correlated with the periodic deposition of neuromasts (Haines et al., 2004).

The migratory primordium is prepatterned into clusters of presumptive neuromasts (Gompel et al., 2001, Itoh and Chitnis 2001). The proneural gene *atonal* homolog, *zath1*, is expressed in centrally located neuromast hair cells and within clusters within the primordium, identifying the presumptive hair cells of the presumptive neuromast. The neurogenic genes *deltaA* and *deltaB* show the same expression. In contrast, the neurogenic gene *notch3* (*notch5*) expression is excluded from the presumptive hair cell and it is expressed in the presumptive supportive cells (Itoh and Chitnis 2001). The Delta ligand and Notch receptor interact to generate instructive signals that mediate lateral inhibition, in which a cell that adopts the hair cell fate inhibits its proximal neighbors from adopting the same fate (Muskavitch, 1994). What cellular and molecular mechanisms underlie the arrangement of the cells within the migrating pllp to form a cluster of presumptive neuromast and how this presumptive neuromast partitions from the rest of the pllp are fascinating questions. In this study I will propose cellular and molecular mechanisms, which may elucidate parts of this puzzle.