4. Discussion

It is a fundamental problem in developmental biology to understand the cellular and molecular changes, which drive the formation of different organs. Here, I focused on elucidating the molecular and cellular mechanisms that underlie pre-neuromast formation within, and modes of migration of, the zebrafish migratory epithelial posterior lateral line primordium (pllp).

4.1 Cellular rearrangements, membrane changes and tissue organization within the pllp during migration and neuromast deposition

The pllp is comprised of about one hundred cells and migrates through the horizontal myoseptum towards the tip of the tail. In order to visualize tissue dynamics within the pllp, I performed time-lapse analysis of its migration, using the vital membrane dye BODIPY ceramide (Figure 4). This analysis revealed that the migrating pllp undergoes dynamic tissue re-organization. Cells within the leading edge of the pllp undergo cellular rearrangements while being progressively positioned towards the trailing end of the pllp. The cells acquire bottlenecked shapes and arrange into epithelial rosettes.

The rosette is the unit of separation, meaning that the detachment from the rest of the pllp occurs within the interneuromasts, lying between the rosettes, and never within the rosette itself. BODIPY ceramide labeling together with immunohistochemical stainings, specific for actin, PRKC and E-cadherin (Figure 3), reveals that cellular rearrangements, which result in rosette formation along the pllp, involve changes in membrane size and structure. These changes include basal membrane alterations, which may be a result of basal membrane growth, and apical membrane constriction, so that the overall geometry of the cells is modified. This spatial conformation allows the adjoining of several cells,

at the point where the apical membrane is constricted, to form a rosette. During *Drosophila* germ band axis elongation, epithelial cells are known to undergo rosette formation, which later resolve, thus resulting in convergence and extension of a cellular array. These cells undergo cellular rearrangements and membrane changes, which allow them to form and to resolve the rosette (Blankenship et al., 2006). In accordance with the observations in my study, rosette formation during tissue morphogenesis appears to require, cell membrane changes, which include size and structure adjustments, as well as cellular rearrangement and intercalation.

4.2 Dynamic regulation of cytoskeletal and junctional proteins during rosette formation

The results of this study suggest that constriction of apical membranes and enrichment of apical proteins generates selective adhesion of cells within prospective neuromast rosettes. During the process of rosette formation, the localization of actin rich adhesion complexes, within the leading edge, is changed from being aligned with the direction of tissue movement to being enriched within the center of rosettes (Figure 3). Actin accumulation within the center of a forming rosette was identified previously in a study on liver parenchyma tissue (Song et al., 1998). In accordance with my observations, regarding the significance of actin involvement, the author's conclusion was that F-actin might play an essential role in the formation of rosette-like structures. In a study performed on germband elongation in *Drosophila* (Blankenship et al., 2006), transient rosette-like structure formation is a result of germband cell polarization. This polarization is achieved through a sequence of events leading to the asymmetric distribution of actin and Myosin II, which hence temporarily accumulate in the center of the rosette. Prior to rosette formation, cell form changes from hexagonal to non-hexagonal, allowing cells to intercalate and to form rosettes. The authors suggest that actin-myosin networks could coordinate

interface contraction in adjacent pairs of cells to create multicellular rosette structures. One well-studied example is the *C.elegans*, where establishment of cell shape changes and cell polarization, has been shown to be regulated by acto-myosin machinery, in response to sperm entry. Acto-myosin contractility is a process in which the cell cortex undergoes contraction that produces regions with high acto-myosin density, resulting in cell polarity (Munro et al., 2004; Schonegg and Hyman, 2006; Cowan and Hyman, 2007). Even though, I could not obtain conclusive results concerning the role of Myosin II in the process of rosette formation, it may be proposed, based on my results obtained with actin labeling in *WT*, that during the transition of leading edge cells to the trailing end within the pllp, constriction of apical membranes and accumulation of actin, may be a result of acto-myosin contractility machinery, which forces the apical membrane to shrink. The reduction in apical membrane size results in the joining of several cells to form a rosette. In the *Drosophila* retina, similar conclusions were obtained for rosette formation and adherence junction protein (E-Cadherin and beta-catenin) accumulation (Brown et al., 2006).

To my knowledge, my data provides the first evidence that the tight junction protein ZO-1 and the polarity regulator proteins PRKCs, which are epithelial markers, are recruited to newly forming rosette-junctions during epithelial tissue morphogenesis (Figure 3), where they may influence the formation or stabilization of cell-cell contacts. ZO-1 is important for creating a belt-like structure that joins epithelial cells at the intersection of their apical and lateral surfaces, thereby creating a selective permeability barrier. A study on MDCK cells suggests that normal localization of ZO-1 to the plasma membrane requires cell-cell contact (Siliciano and Goodenough, 1988). Furthermore ZO-1 localization to the tight junction and tight junction formation is regulated by PRKC, as demonstrated in MDCK cells and zebrafish (Suzuki et al., 2001; Gao et al., 2002; Gopalakrishnan et al., 2007; Rohr et al., 2006). Also in this study, ZO-1 was found to be diminished in morphant pllps (Figure 15), probably due to the loss of cell-cell contacts and reduced PRKCi activity.

Previous studies implicated classical (type 1) cadherins in the development of the posterior lateral line organ (pllo). Gene expression analysis showed that two cadherins, -1 and -2, are expressed in the pllo (Lio et al., 2003), and loss of function experiments have shown that cadherin-2 is essential for pllo formation (Kerstetter et al., 2004). In this study, E-Cadherin accumulation was not detected in the center of the rosette, suggesting that E-Cadherin is not involved in the apical membrane constriction during rosette formation (Figure 3). This could be confirmed by analyzing formation of apical membrane constrictions in the absence of E-Cadherin, within the pllp, using gene knockdown technique. Moreover, E-Cadherin localization was not seen to be altered in the *lgl2* or *prkci* morphant pllps, nor in pllps, which over-express the constitutively active form of Lgl2 or dominant inhibitory form of PRKCi (Figure 16). This could indicate that a failure of the pllp to undergo rosette formation in the absence of Lgl2 or PRKCi is independent of E-Cadherin regulation. Thus, the involvement of E-Cadherin in pllo formation may be mediated by a different mechanism than the one implicated in this study.

4.3 Conservation of Lgl2 and PRKCi signaling pathway in tissue organization of the pllp

Delay or failure to generate presumptive neuromast rosettes, within the pllp caused by interference with Lgl2 or PRKCi activity (Figures 15,16,17), results in reduced neuromast deposition and, hence, severe truncations of the posterior line organ (Figures 11,12). In a variety of cellular contexts, PRKCi regulates Lgl2 function by phosphorylation events within the central Lgl2 domain of the protein, which render Lgl2 inactive (Betschinger et al., 2003; Planet at al., 2003; Yamanaka et al., 2003; Betschinger et al., 2003). In this study I demonstrate, for the first time, the possible interaction between penner/Lgl2 and PRKCi in the context of pllo formation in zebrafish. This interaction is probably mediated by a similar phosphorylation event. The similarity in phenotypes, obtained by a

constitutively active form of Lgl2 and a dominant inhibitory form of PRKCi, support this possibility. Further biochemical experiments will have to be carried out. The possible phosphorylation of Lgl2 by PRKCi suggests that during pllo formation, Lgl2 could be displaced from the apical membrane, and thus establishes polarity within the pllp cells. Hence, it may be deduced that the defects observed in pllo formation, due to over-expression of a constitutively active form of Lgl2, are probably caused by apical localization of this protein, due to the inability of this form to undergo phosphorylation. The ineffectiveness of a Lgl2-autoinhibitory form (Lgl^{S3E}, data not shown), to disrupt pllo formation or to rescue the *lgl2* morphant phenotype, indicates that this form is probably inactive, apparently due to an inability to associate with the membrane (Betschinger et al., 2005).

The resemblance between the *myo VIa* phenotype and the *lgl2* phenotype (Figure 12) correlates with data obtained from a study on *Drosophila* neuroblasts, in which *Drosophila* Myosin VI, Jaguar interacts with Lgl2 in the context of asymmetric division of the neuroblast (Petritsch et al., 2003). Myo VI is important for coated and un-coated vesicles endocytosis by moving along actin filaments (Buss et al., 2004, Hasson, 2003, Aschenbrenner et al., 2004), and for the anchoring of stereocilia plasma membrane to actin filaments (Self et al., 1999, Frank et al., 2004, Seiler et al., 2004). Apical membrane constriction could be a result of endocytosis, in which apical membrane parts are removed in the form of vesicles. Membrane constriction could also be a consequence of membrane anchoring to the actin filaments. Whether Myo VI is associated, directly or indirectly, with the proposed machinery for apical membrane constriction, as described above, is to be elucidated by further experiments.

4.4 Involvement of the epithelial polarity machinery in cell proliferation of the pllp

Drosophila Lgl2 has been characterized as a neoplastic tumor suppressor gene, a term which refers to the fact that homozygous mutants acquire abnormal overgrowth of the presumptive adult optic centers and the imaginal discs (Baek, 1999). In *lgl1* knock-down mice, a large proportion of neural progenitor cells fail to exit the cell cycle and to differentiate. Instead, they continue to proliferate and finally undergo apoptosis (Klezovitch et al., 2004). In zebrafish *lgl2/penner* mutants, basal epidermal cells were shown to undergo hyper-proliferation (Sonawane et al., 2005). Similar to the hyper-proliferation observed in zebrafish *lgl2/penner* mutant, over-proliferation also occurs within the *lgl2* morphant pllp, in particular loss of proliferation-control was observed in the region within the trailing half of the pllp (Figure 20, Table 1). Taken together, it may be proposed that cells within *lgl2* morphant pllps lose the ability to polarize and to form rosettes, and instead, they become hyper-proliferative. Loss of epithelial cell polarity is a hallmark of cancer (Bilder et al., 2000), and in this study, loss of polarity, which is a result of loss of the polarity regulators Lgl2 or PRKCi, could be the rationale for the hyper-proliferation phenotype. Evidence, for a loss of cell polarity in the trailing half of the pllp, is provided by the loss of ZO-1 and PRKC accumulation within the apical compartment of the cells, as well as a loss of apical localization of the centrosomes and basal localization of the nuclei. Loss of polarity in the trailing half of the pllp is observed in both *lgl2* and *prkci* morphants (Figures 15,16,17).

4.5 The cell polarity machinery (Lgl2 signaling) does not affect Notch/Delta prepattern during pre-neuromast formation

In this study, I show for the first time that Delta D positive aggregates, which presumably correspond with presumptive hair cells, associate but do not co-localize with apical focal points of the rosette in the migrating pllp (Figure 19). The hair cell is surrounded by supporting cells, which together compose the future neuromast. Further experiments will have to be conducted to elucidate the nature of these Delta D aggregates, their precise localization within the cells of the pllp, and the molecular interactions they may have.

The findings in this study suggest that Lgl2 is required for formation of apical focal points and rosette clustering rather than for controlling Delta/Notch-mediated hair cell specification. During *Drosophila* sensory-organ precursor cell divisions, Lgl2 inhibits Notch signaling within the anterior daughter cell (Justice, 2003, Langevin, 2005). Furthermore, in the zebrafish pllp, Notch and Delta were suggested to act antagonistically with regard to hair cell specification, based on gene expression data (Itoh and Chitnis, 2001). Their study suggests that a hair cell, which expresses Delta, inhibits its neighbor cells, which express Notch, from adopting the same fate. Therefore, in contrast to Notch inhibition by Lgl2 in *Drosophila*, Lgl2 has apparently no effect on Notch/Delta signaling in the context of the zebrafish pllp.

4.6 Myosin II is necessary for pllp migration

Epithelial cell migration is crucial for tissue morphogenesis (Gumbiner, 1996), and Myosin II has been implicated, in several studies, to be important for cell migration (Nakayama et al., 2005; Schaar and McConnell, 2005; Koppen et al. 2006). Moreover, Myosin II and Lgl2 have been previously proposed to interact

with each other (Strand et al., 1994, Kalmes et al., 1996). This interaction could play a role in pllp migration and hence, in neuromast formation. However, the results of this study suggest that Myosin II, rather than Lgl2, affects pllp migration. In the lgl2 morphants, pllp migration speed was decreased, without blocking pllps from reaching the tip of the tail (section 3.12.1). In the case of Myosin II inhibition, pllps failed to reach the tip of the tail at some point of their migration (Figure 23). These results may indicate that in the context of pllp migration, Myosin II is involved in control of cell migration and that Lgl2 does not affect this involvement. A possible mechanism, which may be employed by Myosin II could involve the SDF1-like chemokine and CXCR4-like chemokine receptor (David et al., 2002; Li et al., 2004; Haas and Gilmour, 2006). However, this remains to be determined by further experiments.

4.7 Future outlook

It remains to be determined what mechanism alters the localization of actin rich adhesion complexes from an organization aligned with the direction of tissue movement, within the leading edge region, to one, within rosettes, where actin rich adhesion complexes are directed toward a central Delta expressing cell. One possibility is that Delta/Notch interactions have an active role in re-directing actin distribution and tissue morphogenesis. An alternative possibility is that morphogenesis of rosettes which is controlled by the epithelial cell polarity regulators Lgl2 and PRKCi and definition of a central Delta expressing cell surrounded by Notch expressing cells are independent processes (Figure 25). In that case, it will be interesting to identify the mechanism that links these two self-organizing events.

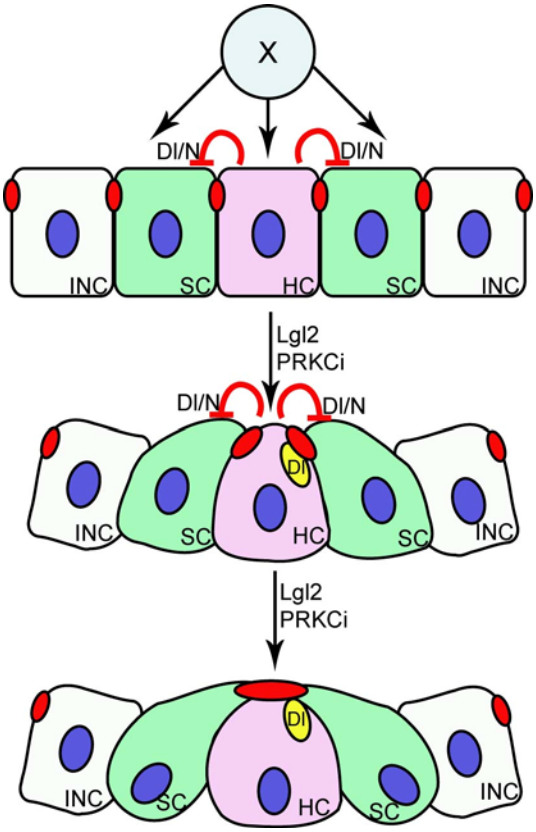

Figure 25: Possible model for rosette formation

Possible scenario illustrating the process of rosette formation. Delta/Notch activity (Dl/N) singles out a Delta D positive hair cell progenitor (HC, pink) among a group of presumptive support cells (SC, green). Delta D-positive aggregates (yellow oval) associate with but do not co-localize with actin rich adhesion complexes (red oval). Each rosette is surrounded by a group of interneuromast cells (INC, white) which do not undergo apical clustering. Activity of the cell polarity regulators Lgl2 or prkci is required for apical clustering of actin rich adhesion complexes. It is not known what mechanism directs the actin rich adhesion complexes towards a central Delta expressing cell. One possibility is that an unknown factor (X) links tissue morphogenesis regulated by Lgl2 and PRKCi with the Dl/N interaction involved in hair cell specification.