Identification of a dual role of the E3-ubiquitin ligase Mindbomb in the zebrafish neural tube morphogenesis

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THESE

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Identification d'un double rôle de l'E3-Ubiquitine ligase Mindbomb au cours de la morphogénèse du tube neural du poisson zèbre

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Summary

In this Ph.D. project, I study the functional link between epithelial polarity and Delta-Notch signaling in the context of zebrafish neural tube morphogenesis. Delta-Notch signaling is of primordial importance for embryonic development and adult tissue homeostasis. Accordingly, faulty Notch signaling is implicated in several pathologies. While it is well established that endocytic transport of Delta ligands is essential for Notch signaling, the reason for this requirement remains mysterious. Recent findings suggest that Delta undergoes two sequential rounds of endocytosis, the first of which promotes basal-to-apical ligand transcytosis. This might enable Delta to interact with apically localized Notch receptors and induce their activation through a second endocytosis-dependent process.

In the course of investigating the role of endocytosis in DeltaD trafficking, I inhibited the function of an E3-ubiquitin ligase Mindbomb (Mib) that is essential for endocytosis of DeltaD ligands and activation of Notch signaling. Surprisingly, I found that Mib loss-of-function led to a loss of apico-basal polarity in the neuroepithelium of the embryonic spinal cord. I further explored this phenotype and showed that the activity of the entire Notch signaling pathway is actually required for the establishment of apico-basal polarity in the zebrafish neural tube. Indeed, inhibition of Notch ligands and downstream transcriptional activators Rbpja and Rbpjb resulted in a disruption of apico-basal polarity. Moreover, ectopic activation of Notch ensued a complete rescue of apico-basal polarity in Mib loss of function embryos. Through a temporal analysis, I have further been able to show that Notch signalling is required for the earliest steps of establishment of neuroepithelial apico-basal polarity. Mib mutant embryos fail to upregulate the transcription of the apical polarity proteins Crumbs1 and Crumbs2a in the course of neural tube formation, suggesting that Notch signalling might act upstream of polarity complexes.

The apico-basal polarization of the zebrafish neural tube has been show to involve a special type of cell division, the so-called C-divisions that are oriented divisions and take place across the forming neural tube midline. C-divisions are known to be regulated by the planar cell polarity (PCP) pathway. Strikingly, we found that the Notch signaling component Mib affects C-divisions through an effect on PCP. Remarkably, this effect of Mib on PCP is independent of its role in Notch signaling. Zebrafish gastrulation are characterized to have highly oriented cell divisions along the anterior-postrior axis. These seterotypical-oriented cell divisions are PCP dependent.

To our surprise, Mib depleted embryos displayed alterations in their division orientation. These results indicate a novel role of Mib in the regulation of PCP signaling. Altogether, this study revealed a dual role of Mib in the epithelial morphogenesis of the zebrafish neural tube.

Keywords: Zebrafish, Neural tube morphogenesis, Delta-Notch signaling, and Planar cell polarity

Résumé

Au cours de ce projet de thèse, j'ai étudié le lien fonctionnel entre la morphogénèse épithéliale et la signalisation Delta-Notch, dans le cadre de la formation du tube neural chez le poisson-zèbre. La signalisation Delta-Notch est primordiale pour le développement embryonnaire et le maintien de l'homéostasie des tissus adultes. Ainsi, sa perte de fonction est associée à des pathologies variées. Tandis qu'il est établit que le transport endocytique du ligand Delta est essentiel à la signalisation Notch, la raison de cette nécessité demeure inconnue. Des découvertes récentes suggèrent que Delta subit deux cycles séquentiels d'endocytose, dont le premier permet une transcytose du ligand du pôle basal à l'apical. Cela permettrait à Delta d'interagir avec les récepteurs Notch situés à l'apical, et d'induire leur activation à travers le deuxième cycle d'endocytose.

Dans le but d'étudier le rôle de l'endocytose dans le transport de Delta D, j'ai inhibé la fonction de l'E3-ubiquitine ligase Mindbomb (Mib), essentielle pour l'endocytose du ligand Delta D et l'activation de la signalisation Notch. De façon inattendue, j'ai observé suite à la perte-de-fonction de Mib une perte de la polarité apico-basale dans le neuro-épithélium de la moelle épinière embryonnaire. L'analyse plus poussée de ce phénotype m'a ensuite permis de montrer que l'activité de l'intégralité de la signalisation Notch est requise pour l'établissement de la polarité apico-basale dans le tube neural de poisson-zèbre. En effet, l'inhibition des ligands de Notch et des activateurs transcriptionnels situés en aval, Rbpja et Rbpjb, résulte en l'interruption de la polarité apico-basale. De plus, l'activation ectopique de Notch entraîne un sauvetage complet de la polarité apico-basale dans les embryons déplétés pour Mib. Grâce à une analyse temporelle, j'ai été capable de montrer que la signalisation Notch est requise dans les étapes les plus précoces de l'établissement de la polarité apico-basale du neuro-épithélium du tube neural. Le mutant Mib échoue à activer la transcription de protéines de polarité apicale Crumbs1 et Crumbs2a au cours de la formation du tube neural, ce qui suggèrerait que la signalisation Notch agit en amont des complexes de polarité.

La mise en place de la polarité apico-basale dans le tube neural du poisson-zèbre a été montrée comme impliquant un type particulier de division cellulaire, la C-division. Celle-ci consiste en une division orientée, qui prend place perpendiculairement à la ligne médiane du tube neural en formation. Les C-divisions sont connues comme étant régulées par la voie de

signalisation de la polarité planaire (Planar Cell Polarity, PCP). De façon surprenante, nous avons montré que le composant de la signalisation Notch, Mib, affecte les C-divisions à travers la signalisation PCP. Cet effet de Mib sur la PCP est indépendant de son rôle sur la signalisation Notch. Un autre évènement bien décrit, dépendant de la signalisation PCP est l'orientation stéréotypique des divisions cellulaires au cours de la gastrulation. A notre surprise, les embryons déplétés pour Mib présentent une altération de l'orientation de leurs divisions dans ce cas. Ces résultats indiquent un rôle inédit de Mib dans la régulation de la signalisation PCP. Généralement, cette étude révèle un double-rôle de Mib dans la morphogénèse épithéliale du tube neural du poisson-zèbre.

Mots clés: Poisson zèbre, Morphogénèse, Tube neural, Signalisation Delta/Notch, Polarité planaire

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List of abbreviations

ADAM	A disintegrin and metalloproteinase
AJs	Adherence junctions
ANK	Ankyrin repeats
AP	Anterior-posterior
AP2	Adapter protein-2
aPKC	Atypical protein kinase C
ARP2/3	Actin-related protein 2/3
ASIP/Par3	Atypical PKC Isotype-Specific Interacting Protein/Partition defective-3
Atp1a1	ATPase Na+/K+ transporting alpha1 polypeptide
Baz	Bazooka
bHLH	Basic helix-loop-helix protein
CE	Convergent-extension
CNS	Central nervous system
CR	Cysteine-rich-region
Crb	Crumbs
CRIB	Cdc42/Rac interactive binding
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSL	CBF1/ Supressor of hair-less/Lag-1
DEP	Dishevelled, Egl-10 and Pleckstrin
Dgo	Diego
Div/Inv	Diversin/Inversin
Dlg	Discs-large
DLHPs	Dorsolateral hinge points
Dll	Delta-like
DOS	Delta and OSM-11-like
Ds	Dachsous
Dsh/Dvl	Dishevelled
DSL	Delta-Serrate-Lag2
DV	Dorsal-ventral
E(spl)-C	Enhancer of Split complex
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
ER	Endoplasmic reticulum
EVL	Enveloping layer
Fj	Four jointed

Fmi/Stan	Flamingo/Starry night
Ft	Fat
Fy	Fuzzy
Fz	Frizzled
GFAP	Glial fibrillary acidic protein
GlcNAc	N-acetyl glucosamine
Her4	Hairy enhancer of split related-4
Hes	Hairy/enhancer of split
In	Inturned
Jag	Jagged
JAM	Junctional Adhesion Molecule
JNK	Jun N-terminal Kinase
Kny	Knypek
Lfng	Lunatic Fringe
Lgl	Lethal giant larvae,
Lp	Looptail
MAGUK	Members Associated Guanylate Kinase homologues
Mam	Mastermind
MDCK	Madine-Darby Canine Kidney
Mfng	Manic Fringe
MHP	Median hinge point
Mib	Mindbomb
ML	medio-lateral
Msn	Misshapen
MT	Microtubule
NECD	Notch extracellular domain
Neur	Neuralized
Ngn1	Neurogenin1
NICD	Notch intracellular domain
NLS	Nuclear localization signal
Nok	Nagie oko
Nrarp	Notch regulated ankyrin repeat protein
NRR	Negative regulatory region
NT	N-terminal
Ofut-1	Protein O-fucosyl transferase-1
Ome	Oko meduzy
Pals1	Protein associated with Lin7

Papc	Paraxial protocadherin
PAR or Pard	Partitioning-defective
PATJ	Pals1 Associated- Tight Junction
PB1	Phox and Bem1D
PCP	Planar cell polarity
PDZ	PSD95-Dlg1-ZO1
Pk	Prickle
PtdIns(3, 4, 5)P3	Phosphatidyl-Inositol-(3, 4, 5)-phosphate
PtdIns(4, 5)P2	Phosphatidyl-Inositol-(4, 5)-phosphate
PTEN	Phosphatase and Tensin homologue
PTK	Protein tyrosine kinase
RAM	Rbpj-associated molecule
Rfng	Radical Fringe
Rok2	Rho kinase 2
Scrib	Scribble
Slb	Silberblick
SOP	Sensory organ precursor
TACE	TNF-α converting enzyme
TGF-β	Transforming growth factor-beta
TJs	Tight junctions
Tri	Trilobite
Vang/Stbm	VanGogh/Strabismus
ZO1	Zonula occludens-1

Introduction

The development of an organism requires tight coordination of many processes such as cell division, morphogenesis, and patterning. An enormous amount of research has been directed to identify the molecules that take part in these processes for proper embryonic development. Three important aspects of embryonic morphogenesis correspond to cell shape, cell to cell communication and cell movement, which are not just interrelated but their strict spatial and temporal coordination is essential for proper organ formation. In this Ph.D. thesis, I have explored a dual role of the Notch signaling component Mindbomb in the context of zebrafish neural tube morphogenesis. First, it takes part in the regulation apico-basal polarity of the neuroepithelium by promoting Delta/Notch signaling. Secondly, Mib plays a Notch-independent role in the regulation of planar cell polarity.

In the following section, I start by introducing Delta-Notch signaling and its major roles in developmental contexts. I then proceed to describe general mechanisms of tube formation and the specific morphogenesis of the zebrafish neural tube. Thereafter, I explain the establishment of apico-basal polarity and planar cell polarity.

1 Delta-Notch signaling

The Notch pathway is an evolutionarily conserved signaling pathway, which is crucial for proper embryonic development. Notch signaling regulates tissue homeostasis and maintenance of stem cells in adults (Artavanis-Tsakonas et al., 1995; Gridley, 1997). The pathway was originally identified in *Drosophila*, where the first mutant allele gave rise to a notched wing. Since then, proteins of the Notch pathway have been discovered in many species and studied extensively in flies, worms, and mammals. Various roles of Notch signaling have been discovered so far including cell fate specification, patterning, and morphogenesis (Bray, 2006; Fiúza and Arias, 2007). The importance of Notch signaling during development and in adults is highlighted by the findings that several diseases are associated with irregular Notch signaling. Mutation in the genes encoding Notch signaling components are linked to three inherited diseases in humans namely Alagille syndrome, spondylocostal dysostosis, and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (Gridley, 2003). Aberrant Notch signaling has also been studied in the context of various types of tumor pathogenesis such as human prostate cancer and cervical cancer (Allenspach et al., 2002).

1.1 Developmental functions of the Delta-Notch signaling pathway

Notch signaling allows a cell to communicate with its neighboring cells. The Notch signaling pathway is crucial for many developmental events such as neurogenesis and somitogenesis. Notch signaling does so through varied processes: lateral inhibition, binary cell fate decision, and by delimiting boundaries (Gridley, 1997).

1.1.1 Lateral Inhibition

One of the best-characterized functions of Notch is a process called lateral inhibition through which a fine pattern of distinct cell types is created. The term lateral inhibition is derived from the observation that during development, among a cluster of cells that have an equal potential to acquire certain cell fate, only one adopts the fate while it actively inhibits that acquisition of the same fate in its lateral neighbors (Figure 1).

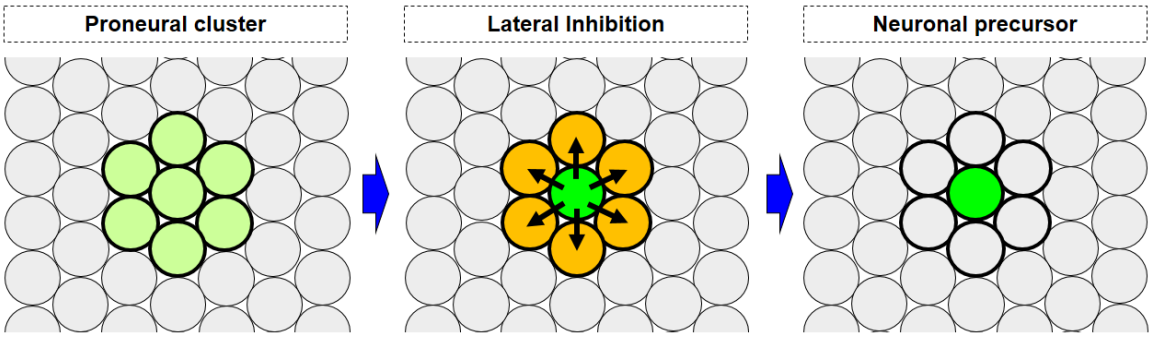


Figure 1: Lateral inhibition
Lateral inhibition in neurogenesis make sure that within a proneural cluster, a single cell will become a neuron (bright green) and inhibit its neighbouring cells from acquiring a neural fate.

Lateral inhibition has been well studied during *Drosophila* neurogenesis in sensory organ precursor (SOP) cells. During development, several groups of ectodermal cells with a neural potential emerge that are known as proneuronal clusters. In proneuronal clusters, lateral inhibition is mediated by few neurogenic genes. The primarily important neurogenic genes are Delta, Notch, Suppressor of Hairless, and the genes of the Enhancer of Split complex (E(spl)-C), which were first described in *Drosophila* for processing regulatory signals that is necessary for cell commitment to an epidermal rather than neural fate (Technau and Campos-Ortega, 1987). Notch signaling activity inhibits the future neural fate. One of the cells within the pro-

neuronal cluster acquires the neural fate by expressing higher levels of Delta that inhibits neighboring cells from acquiring the same fate. In *Drosophila*, patterns of bristles are created by lateral inhibition (Parks et al., 1997). When Notch signaling is impaired, cells failed to have lateral inhibition and henceforth, more and more cell acquire neurogenic fate, resulting in severe developmental defects (Itoh et al., 2003).

— **Molecular mechanism of lateral inhibition**

In vertebrates, Delta ligands are expressed in the neurogenic domain of the neural plate (Chitnis et al., 1995). In this neurogenic domain, cells express a transcription factor Neurogenin1 (Ngn1) that encodes a basic helix-loop-helix protein (bHLH). Expression of *ngn1* is modulated by Delta-Notch signaling that suggest that *ngn1* is the target of lateral inhibition. Indeed, misexpression of Ngn1 results in the formation of ectopic neurons in neuroectoderm (Blader et al., 1997). In addition, Ngn1 also modulates the expression of Delta. A vertebrate homologue of E(spl)-C proteins, Hairy enhancer of split related-4 (Her4), acts as a target of Notch to suppress neurogenesis (Takke et al., 1999 a zebrafish homologue of the Drosophila neurogenic gene E(spl), is a target of NOTCH signalling). Ngn1 activates Delta expression, which in turn activates Notch in neighboring cells; Notch activation further drives expression of *her4* that consecutively inhibit expression of *ngn1*. Therefore, when a cell express *ngn1*, it acquires the ability to inhibit *ngn1* and *delta* expression in neighboring cells, thus preventing them from differentiating as neurons.

1.1.2 Asymmetric cell divisions

Notch signaling controls binary cell fate choices that form patterns in the organism. Binary cell fate choices occur through two mechanisms. First, cells communicate with each other through an inhibitory feedback loop in proneural clusters during lateral inhibition (described above). Second, cells acquire a specific neural identity through asymmetric cell divisions that rely on cell polarization (Blader et al., 1997).

Asymmetric distribution of cell fate determinants during mitosis determines the cell fate of the daughter cells (Figure 2). During *Drosophila* neurogenesis, asymmetric distribution of Notch signaling-regulators such as Numb and Neur, determines the identity of the daughter cells as signal sending or signal receiving cell (Bardin et al., 2004). This asymmetric segregation of

regulators of Notch signaling is governed by polarity proteins *e.g.* Bazooka (Par3), par6, aPKC and Inscuteable (Schober et al., 1999).

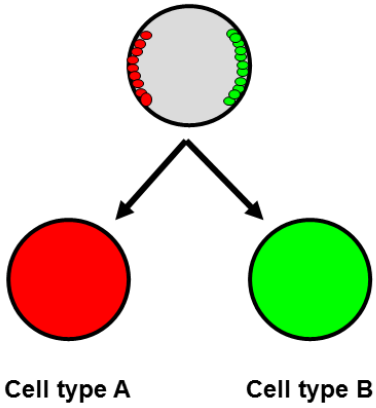


Figure 2: Asymmetric cell division
Asymmetric cell division distributes regulators of Notch signalling unequally between the two daughter cells. Because of this asymmetric segregation of Notch-regulators, one cell will present Notch ligands that will activate Notch receptors in its sibling. These daughter cells acquire different cell fate through binary cell fate decision.

In *C.elegans* and Sea urchin, Notch signaling is indispensable for cell fate specification during early embryogenesis and specification of the germ layers (Good et al., 2004; Sherwood and McClay, 1997). Another example of binary cell fate choice is the role of Notch in the maintenance of stem cell populations. Notch signaling mediates a decision whether a cell should differentiate or stay in a quiescent state in a given stem cell population (Chiba, 2006).

1.1.3 Delimiting Boundaries

Notch signaling is crucial for boundary formation in invertebrates and vertebrates. During the formation of the dorsal-ventral (DV) boundary of the *Drosophila* wing Notch activity is constrained to the DV boundary (de Celis and Bray, 1997). Notch loss-of-function results in the loss of wing marginal tissue, while ectopic activity of Notch results in extra wing tissue (Kim et al., 1996). Notch signaling also participates in boundary formation during vertebrate somitogenesis. Mesoderm is segmented into somites through a wave of oscillatory gene expression, called the segmentation clock. Several Notch components oscillate within the presomitic mesoderm. In the zebrafish, coordinated oscillatory expression of the bHLH repressors *her1* and *her7* is required for the segmentation of the paraxial mesoderm (Wahi et al., 2014). In mammals, *lunatic fringe*, which is a homologue of the *Drosophila* boundary-specific

molecule *fringe*, modulates Notch signaling to regulate inter-somitic boundary formation, as well as rostro-caudal patterning of the somites (Evrard et al., 1998). Another example of the role of Notch signaling in boundary formation is the segmentation of the Zebrafish hindbrain (Pasini et al., 2001).

1.2 Notch receptors

1.2.1 Structure of Notch receptors

Notch receptors are ~300-kDa single pass transmembrane proteins, which are composed of several functional units (Figure 3). Notch receptors are multidomain proteins that are conserved in virtually all metazoans. In mammals, there are four Notch receptors named Notch-1, Notch-2, Notch-3 and Notch-4. In Zebrafish, Notch receptors are referred to as Notch1 Notch 2a, Notch 2b and Notch3. Typically, Notch receptors are synthesized as large precursor glycoproteins, which are processed by a furin-like protease at the so-called S1 site into two non-covalently associated subunits during maturation.

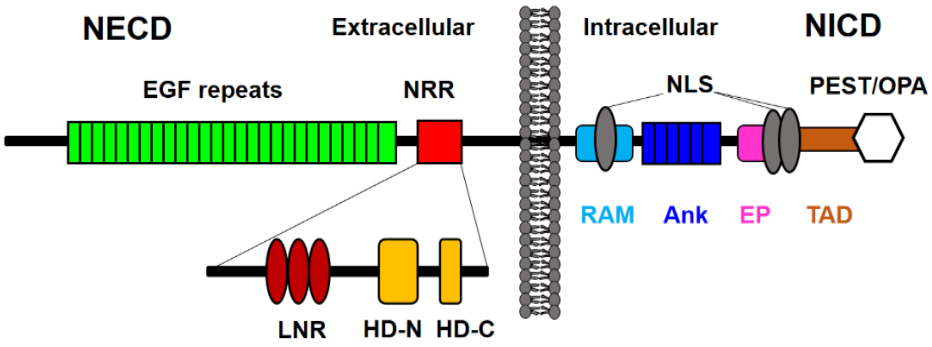


Figure 3: Domain organization of Notch receptors.
The transmembrane Notch receptor comprises mainly two domains: Notch extracellular domain (NECD) and Notch intracellular domain (NICD). Epidermal Growth Factor (EGF) repeats are shown in green, followed by the Negatively Regulatory Region (NRR) that consists of three Lin-Notch Repeats (LNR) and two heterodimerization domains (HD). The NRR maintains the receptor in its resting conformation when it is not bound to a ligand. NICD consists of the RBPJk-Associated Molecule (RAM) domain that binds CSL; a Nuclear Localisation Signal (NLS) with ankyrin repeats; the transactivation domain (TAD) and EP domain that bind the histone acetylase p300. The EP domain is included in the RE/AC (repression/activation) region that is responsible for the Notch receptor's ability to repress or activate target gene promoters. Finally, at the C-terminal end a destabilizing Proline Glutamate Serine Threonine-rich motif PEST and Glutamate rich sequence OPA motif are located.

The Notch extracellular domain (NECD) comprises of an N-terminal EGF repeats that are important for binding to the ligand. Notch receptors are usually resistant to activating proteolysis until ligand binding occurs. The EGF repeats are followed by the negative regulatory region (NRR) that ensures that Notch become activated if it is not bound to a ligand (Gordon et al., 2009). Two domains of NICD are involved in its interaction with the transcriptional mediator protein CSL: the Rbpj-associated molecule (RAM) domain and a cluster of ankyrin repeats (ANK). The Ank domain is a highly conserved motif that mediates binding with other proteins (Lubman et al., 2004).

1.2.2 Regulation of Notch receptor activity

1.2.2.1 Notch glycosylation

The activation of Notch signaling is highly regulated through various posttranslational modifications (Figure 4). Prior to localization at the cell membrane, the Notch receptor is cleaved at the S1 site in the extracellular domain by a calcium dependent furin-like protease to produce a mature heterodimeric Notch (hNotch) receptor in the *trans*-Golgi network (Logeat et al., 1998). The EGF-repeats in NECD are further glycosylated with glycans such as o-fucose, o-glucose glycans and N-glycans (Stanley, 2007). Several studies show that depletion of the protein O-fucosyl transferase-1 (Ofut-1) results in inactive Notch receptors and produces various defects associated with loss-of-Notch signaling in flies (Haines and Irvine, 2003), zebrafish (Appel et al., 2003), and in mammals (Lu and Stanley, 2006). O-fucosylation of Notch is therefore required for Notch signaling. A possible role of O-fucosylation could be that it promotes a stable cell surface expression of Notch receptors by modulating the trafficking of the receptor between the ER and the cell membrane (Sasamura et al., 2007).

The transfer of N-acetyl glucosamine (GlcNAc) to fucose on Notch is also required for Notch activity. In *Drosophila,* the glycosyl transferase responsible for this event is Fringe (Fng). In mammals, there are three Fringe genes, Lunatic Fringe (Lfng), Manic Fringe (Mfng) and Radical Fringe (Rfng) (Stanley, 2007). Studies in flies and mammals reveal that Fringe acts in the Golgi as a glycosyltransferase enzyme that modifies the EGF motifs of Notch and alters the ability of Notch to interact with its ligand Delta (Brückner et al., 2000; Moloney et al., 2000). Modification of *O*-fucose by Fringe affects the strength of Notch-ligand binding so that different Notch signaling outputs arise (Yang et al., 2005). For example, in the *Drosophila* wing disc Notch activation occurs at the dorsal-ventral interface by Fringe. Fringe inhibits Serrate signaling in the

dorsal cells and prevents it from signaling in ventral cells. However, Fringe promotes Delta signaling in ventral cells, allowing it to send signals to the dorsal side. This restricts Notch signaling to a stripe of cells at the dorsal-ventral boundary (Haines and Irvine, 2003). Similarly, in mammals, the vertebrate fringe homologue, lunatic fringe modulates somite segmentation and patterning (Evrard et al., 1998).

Modification of Notch by Fringe is followed by the addition of β1, 4-galactose to GlcNAcβ(1,3)Fuc-*O* dissacharide by Galactosyl-transferase (Moloney et al., 2000). Again, this modification of the Notch receptor regulates Notch activity since mice lacking β4GalT-1 have a poor expression of several Notch targets genes at mid-gestation and these embryos have an extra lumbar vertebra (Chen et al., 2006).

1.2.2.2 Notch endocytosis and trafficking

Notch is continuously internalized into early endosomes, further sorted to other endocytic compartments including recycling endosomes, multivesicular bodies/late endosomes, and lysosomes (Figure 4). These endocytic steps play a critical role in the regulation of Notch activity. The first evidence that endocytosis is crucial for the regulation of Delta/Notch signaling emerged from a study of Dynamin-dependent endocytosis in *Drosophila (Seugnet et al., 1997)*. Since then several molecules have been identified that are important for the endocytic trafficking of Notch receptors molecules such as Numb, a cytoplasmic protein, Sanpodo, a transmembrane protein and several ubiquitin interacting proteins.

- **Ubiquitination**

Several E3- ubiquitin ligase have been identified that are required for the Notch endocytosis and sorting. Deltex is a RING finger-E3 ligase that binds to NICD via Ank repeats. In the *Drosophila* wing, overexpression of Deltex led to the excessive accumulation of Notch in endocytic vesicles. In addition, Deltex is also been found to interact with Kurtz, the β-arrestin homologue in flies. It is suggested that Kurtz might facilitate the endocytosis of Notch-Deltex complex that further mediates the degradation of Notch receptor (Mukherjee et al., 2005).

In *Drosophila,* Notch signaling is restricted by the activity of two Nedd4 family proteins, Suppressor of deltex [Su(dx)] (Itch in mammals) and DNedd4. Su(dx) modulates the sorting of Notch within the early endosome towards an ubiquitin-enriched subdomain that is further transferred late endosomes (Wilkin et al., 2004). Another study in flies showed that Nedd4

antagonizes Notch signaling by promoting the degradation of Notch and Deltex (Sakata et al., 2004). These ubiquitin modifications may control the time that the receptor is located on the cell surface and hence remains accessible for ligands.

— **ESCRT-dependent regulation of Notch activity**

The Endosomal Sorting Complex Required for Transport (ESCRT) regulates the sorting of ubiquitinated membrane proteins from early endosomes into multivesicular bodies en route to the degradative compartment of late endosomes/lysosomes. *Drosophila* mutants lacking different ESCRT components display excessive cell proliferation defects due to excessive signaling from Notch proteins that could not be properly recycled or degraded (Thompson et al., 2005; Vaccari and Bilder, 2005). Lethal (2) giant discs (lgd) that encodes a C2-containing phospholipid binding protein that interacts with the ESCRTIII complex and regulates ligand-independent activation of Notch (Childress et al., 2006).

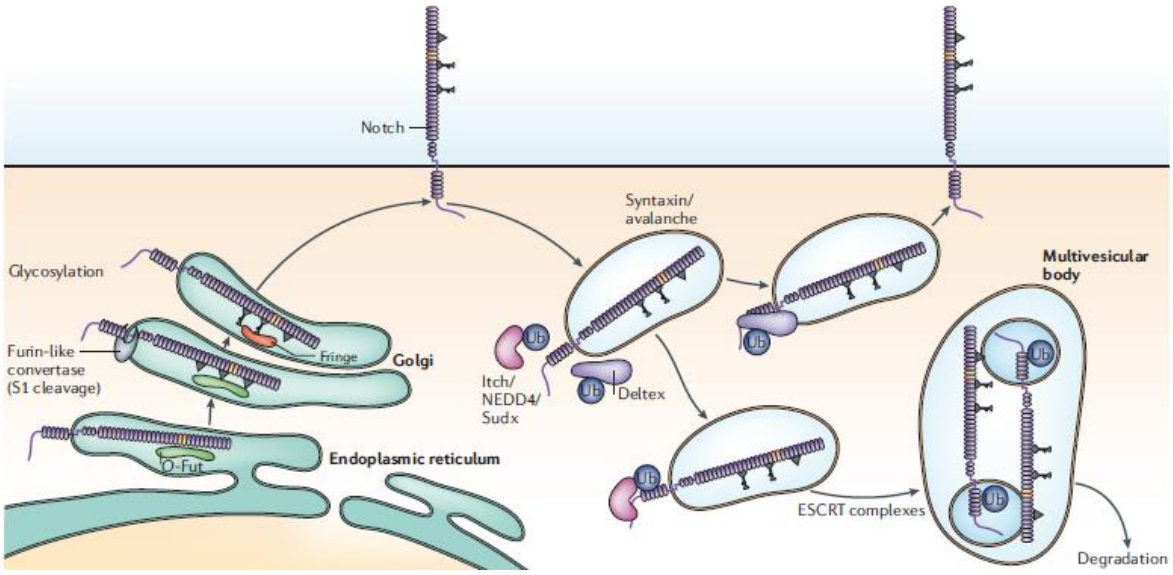


Figure 4: Processing and trafficking of Notch
Notch receptor molecules (purple) are produced in the endoplasmatic reticulum where they interact with O-fucosyl transferase (O-fut in green) before being transported to the Golgi. In the Golgi, Notch is further processed by Furin-like proteases that mediate the so-called S1 cleavage, glycosylated (e.g. by Fringe), and ultimately sent to the plasma membrane. Following its endocytosis Notch is trafficked through multivesicular bodied towards lysosomal degradation. Ubiquitin ligases such as Deltex and Itch/NEDD4/Su(dx), Syntaxins and ESCRT proteins regulate different aspects of Notch trafficking. (Bray, 2006).

— **Numb mediated endocytosis**

Numb, a protein-segregating determinant in flies has been shown to be involved in endocytosis. Numb interacts with α-adaptin, a component of the adapter protein-2 (AP2) complex that links cargoes to the clathrin coats of endocytic transport vesicles. Numb interacts with α-adaptin and Notch to recruit Notch into endocytic vesicles (Berdnik et al., 2002). In the context of the asymmetric cell divisions of the Drosophila SOP cells, Numb acts as a Notch-inhibitor that is asymmetrically segregated into one of two daughter cells. Numb has moreover been shown to interact with the transmembrane protein Sanpodo that is crucial for the Numb-mediated asymmetric trafficking of Notch (O'Connor-Giles and Skeath, 2003).

1.3 Notch ligands

1.3.1 Structure of DSL ligands

Notch receptors are activated through binding to type-1 cell–surface proteins of the Delta-Serrate-Lag2 (DSL) family of ligands (Figure 5). DSL ligands have a large extracellular domain with multiple tandem Epidermal Growth Factor (EGF) repeats. The N-terminal (NT) DSL domain and the first two EGF repeats are necessary for DSL ligands to bind Notch receptors (Parks et al., 2006; Shimizu et al., 1999). A conserved motif called Delta and OSM-11-like (DOS) domain within the first two EGF repeats contributes to ligand binding. The NT domain is further split into N1 and N2. N1 is a region with six conserved cysteine residues while N2 is a cysteine-free region.

The fly homologues of Delta and Serrate in mammals are called either Delta-like (Dll) or Serrate-like. There are three Delta-like or serrate like proteins: Dll1, Dll2 and Dll3. Additionally, there are two different serrate-like ligands in mammals referred as Jagged-1 and Jagged-2 (Jag-1 or Jag-2). The number of EGF repeats in Jag-1 and Jag-2 is almost doubled compared to Dll ligands. In addition, they carry an additional cysteine-rich-region (CR) which is absent in Dll ligands. In the zebrafish, there are four Delta ligands namely Delta A, Delta B, Delta C and Delta D. Usually, the intracellular parts of different DSL ligands share sequence homologies. Some ligands to however lack a C-terminal PDZ domain that is required to interact with cytoskeleton (D'Souza et al., 2010) and lysine residues that are potential sites for modification by E3-ubiqutin ligases (D'Souza et al., 2008).

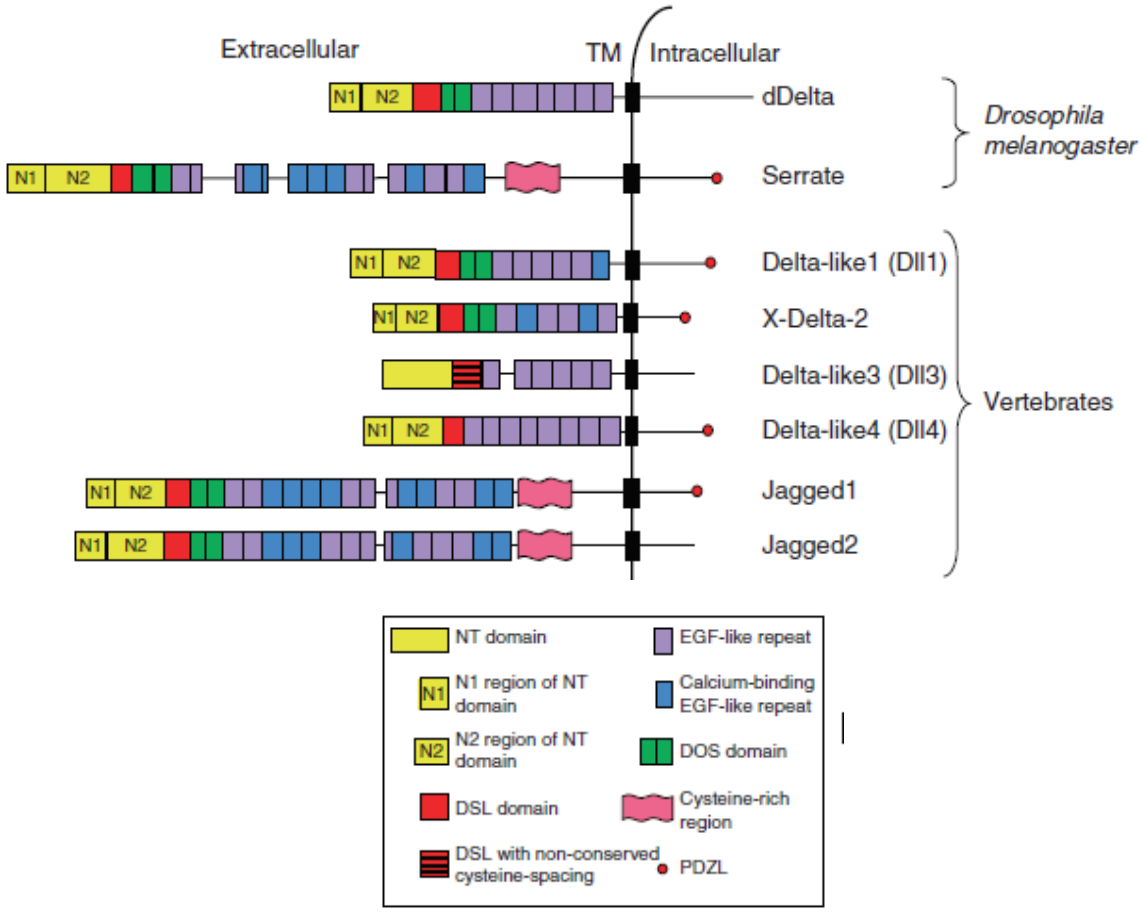


Figure 5: Protein structure of DSL ligands
The extracellular domain comprises an N-terminal (NT) domain (yellow) followed by a DSL domain (red) and several tandem EGF repeats (blue and purple). Additional DOS motifs (green) allows ligands to bind Notch. The NT domain is further subdivided in two N1 and N2 domains that do or do not contain cysteine residues respectively. Unlike Delta, Serrate and Jagged ligands have an extra cysteine-rich region (pink). The intracellular domain of few ligands comprises a carboxy terminal PSD-95/Dlg/ZO-1-ligand (PDZL) motif that has a Notch signaling independent role. (D'Souza et al., 2010)

1.3.2 Regulation of DSL ligand activity

Not only Notch receptors, but also their DSL ligands undergo proteolytic cleavages by ADAM metalloproteases and γ-secretase enzymes that modulate the strength and duration of Notch signaling and produce soluble intracellular domains of the ligands (Zolkiewska, 2008). After, ADAM-mediated processing, ligands are then targeted by presenilin dependent γ-secretase activity (Ikeuchi and Sisodia, 2003), which generates an intracellular DSL fragment. All DSL ligands contain positively charged residues that can serve as a nuclear localization signal

(NLS). Therefore, it is possible that the cleaved intracellular fragment then translocates to the nucleus, to regulate gene expression (Zolkiewska, 2008). Indeed, a study in mice reports that the soluble intracellular domain of Dll1 binds the transcriptional mediators of transforming growth factor-beta (TGF-β) signaling Smad2, Smad3 and Smad4. Thus, Dll1 mediates TGF-β signaling through binding to Smads and plays an important role for bi-directional Notch signaling (Hiratochi et al., 2007).

Moreover, posttranslational modification of Notch receptors via glycosylation also alters Notch-ligand activity (Stanley, 2007). Endocytosis and trafficking of DSL ligands within the signal-sending cell are also essential requirements for the activation of Notch receptor. In the absence of endocytosis, the ligands accumulate at the cell surface where they are unable to activate Notch (Itoh et al., 2003; Nichols et al., 2007; Parks et al., 2000). The endocytosis of DSL ligands is regulated by ubiquitination, which regulates ligand signaling activity and cell surface expression (Le Borgne and Schweisguth, 2003). The intracellular domains of Dll1, Dll4, Jag1 and Jag2 encompass several lysine residues that can provide potential ubiquitination sites. Several studies show that two structurally distinct RING-containing E3 ligases, Neuralized (Neur) and Mindbomb (Mib), promote ligand endocytosis by ubiquitylation (Itoh et al., 2003; Lai et al., 2005; Le Borgne et al., 2005), for details refer section 1.5.

1.4 Downstream signal transduction of Notch signaling

1.4.1 CSL-dependent Notch signalling

The Notch pathway mediates several processes such as nervous system development through juxtacrine signaling. The Notch receptor on the signal-receiving cell binds with a DSL ligand located on the signal-sending cell at its extracellular domain (NECD), which triggers a series of proteolytic cleavages of Notch. Firstly, a member of the disintegrin and metalloproteases (ADAM), TNF-α converting enzyme (TACE) cleaves Notch at the juxtamembrane region in the course of the so-called S2 cleavage. The intramembranous protease γ-Secretase then cleaves Notch within the transmembrane domain during an event known as the S3 cleavage. The S3-cleavage releases the Notch intracellular domain (NICD) from the plasma membrane, which then translocates into the nucleus. In the nucleus, NICD directly interacts with DNA binding proteins of the CBF1/ Supressor of hair-less/Lag-1 (CSL; in vertebrate called as Rbpj) family to drive the expression of Notch target genes (Artavanis-Tsakonas et al., 1995).

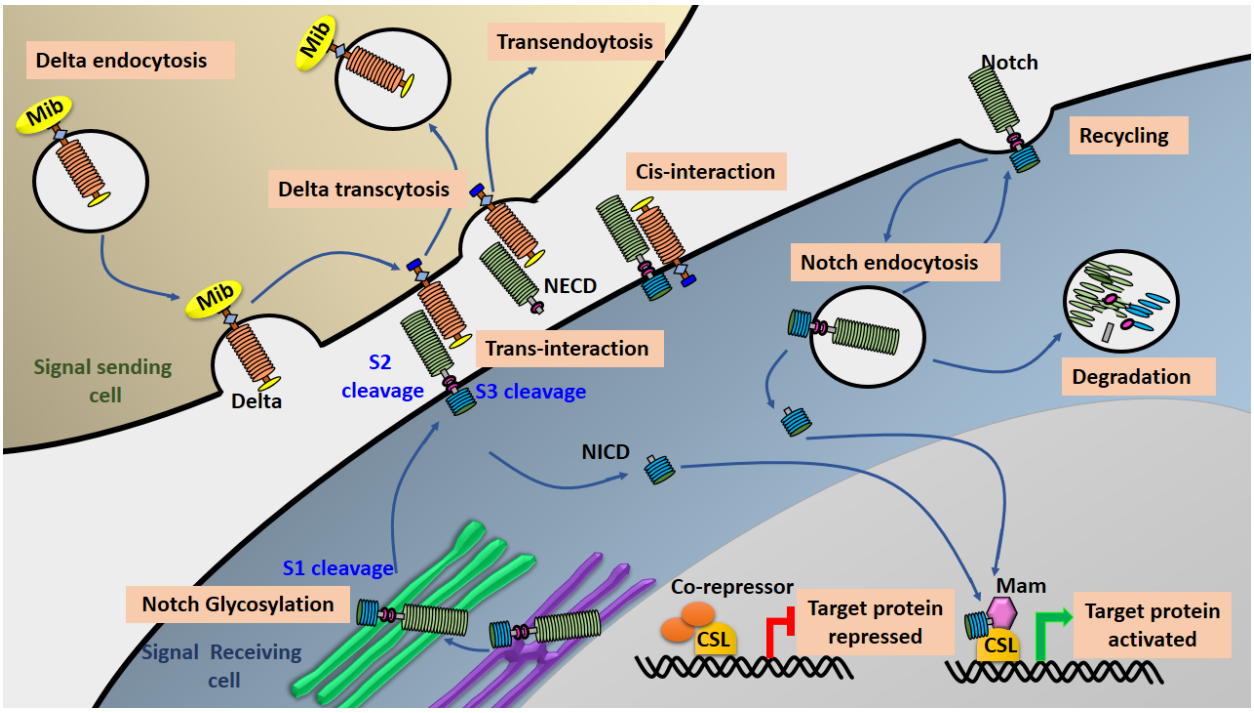


Figure 6: Overview of Notch signal transduction

The Notch receptor that is present on the signal receiving cell, binds a DSL family ligand that is present on the opposing signal sending cell through its extracellular domain. For the effective interaction with Notch, Delta requires internalization via E3-ubiquitin ligase Mindbomb (Mib) dependent endocytosis. Upon ligand binding, Notch undergoes a series of cleavages: the S2 cleavage by the ADAM protease TACE and the S3 cleavage by γ-secretase. The S3 cleavage releases the NICD, which is then transported to the nucleus. In the nucleus, NICD associates with CSL, Mastermind and transcriptional co-regulators to mediate the transcription of Notch target genes.

In the absence of NICD, CSL interacts with a histone deacetylase containing co-repressor complex to repress the transcription of Notch target genes (Kao et al., 1998). However, binding of NICD with CSL, replaces the co-repressor complex with a transcriptional activation complex that includes NICD, Mastermind (Mam) and a histone acetyltransferase such as p300. This transcriptional activation complex then turns on the activity of Notch target genes. Notch target genes include *notch regulated ankyrin repeat protein (nrarp) and bHLH* genes e.g. *hairy/enhancer of split (hes)* and *hes-related proteins (hey/hrt/herp)* (Artavanis-Tsakonas and Muskavitch, 2010; Lubman et al., 2004). A schematic overview of the CSL-dependent Notch pathway (canonical Notch signaling) is shown in Figure 6.

1.4.2 CSL-independent Notch signalling

There are growing evidences that Notch can signal in CSL-independent way, referred as noncanonical Notch signaling that can be either ligand-dependent or independent (Figure 7).

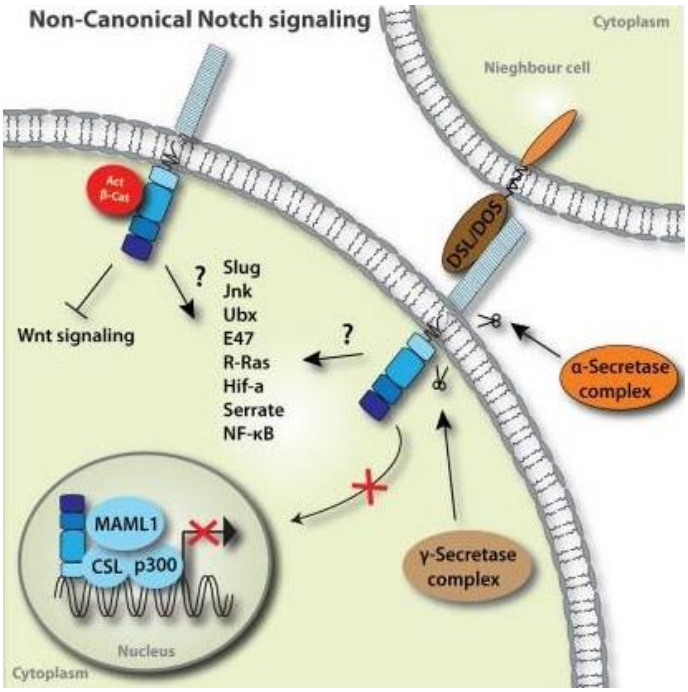


Figure 7: Noncanonical Notch signaling
Non-canonical Notch signaling occurs in a CSL-independent way. The Notch intracellular domain does not relocate into nucleus, instead binds other molecules to perform a special task (Andersen et al., 2012).

Unlike the canonical Notch pathway, mediators of non-canonical Notch signaling are poorly known. Some of the earliest evidence for non-canonical Notch signaling came from *in vitro* studies where increased Notch levels perturbed the differentiation of myoblast (C2C12) cells into muscle cells without interacting with CBF1 or upregulating endogenous HES-1 expression (Nofziger et al., 1999; Shawber et al., 1996). The most well studied and evolutionary conserved non-canonical Notch function is to regulate Wnt/ β-catenin signaling. Ligand/CSL-independent Notch signaling is frequently associated with an antagonism of Wnt/β-catenin signaling (Brennan et al., 1999). Few studies have also reported a function of Notch in regulating small GTPases such as R-Ras, independently of CSL-transcription (Hodkinson et al., 2007; Ohata et al., 2011).

1.5 The role of Delta-ligand endocytosis for Delta-Notch signaling

1.5.1 E3-ubiquitin ligase dependent endocytosis of DSL ligands

Over the past two decades, several studies have emphasized the role of ubiquitylation for the regulation of Notch signaling (Lai, 2004). The process of ubiquitylation occurs in several steps that ultimately result in the addition of the 76 amino-acid ubiquitin polypeptide to a substrate protein(Weissman, 2001). First, ubiquitin is activated in an ATP-dependent way through an ubiquitin-activating enzyme E1. Afterwards, an ubiquitin-conjugating E2 enzyme obtains the ubiquitin from E1. In final step, an E3 ubiquitin ligase that comprises an E2 docking site and a substrate recognition domain transfers ubiquitin from the E2 to the E3-defined substrate (Weissman, 2001).

Two structurally distinct RING-finger containing E3-ligases, Neuralized (Neur) and Mindbomb (Mib), have been shown to influence Notch signaling by mediating Notch-ligand endocytosis. Neuralized monoubiquitylates Delta to promote endocytosis of Delta. Neur-depleted cells accumulate Delta on the surface and fail to trigger lateral inhibition, suggesting a role of Neur-dependent Delta endocytosis in the activation of Notch receptors (Pavlopoulos et al., 2001). In zebrafish, *mindbomb (mib)* encodes for an E3-ubiquitin ligase that has Delta for its substrate. Mindbomb is recognized to have similar function in zebrafish that Neur has, as above mentioned, in *Drosophila*. Mib promotes the endocytosis of Delta ligands that is required for efficient activation of Notch. Mib loss-of-function embryos exhibit a neurogenic phenotype due to the failure of Notch signaling (Itoh et al., 2003).

In mammals, there are two Neur proteins, Neur1 and Neur2, and two Mib proteins, Mib1 and Mib2. Mib ubiquitination is responsible for DSL ligand endocytosis that activates Notch signaling. Neur functions downstream of Mib to direct lysosomal degradation of internalized ligands and thereby, regulate the level of ligand available for Notch activation (Song et al., 2006). In mice, Neur and Mib do not appear to be functionally alike in modulating Notch signaling. Only the disruption of Mib1 in mice produces the known Notch phenotypes such as defects in neurogenesis, somitogenesis, and skin morphogenesis and Notch embryonic lethality. Neur1 and Neur2 are dispensable for normal neurogenesis in mice, but Mib1 mutant embryos have a strong neurogenic phenotype in the brain and neural tube (Koo et al., 2005; Koo et al., 2007). A study in zebrafish suggests that Mib1 and Mib2 could potentially exert redundant activities (Zhang et al., 2007). Mib2 is however not strongly expressed during

embryonic development; therefore, Mib1 is essential in Notch-dependent embryonic processes (Koo et al., 2007). All these studies present evidence that endocytosis is essential for Delta-Notch signaling; however the reason for this requirement is still poorly known.

1.5.2 Why is Delta endocytosis required for Notch signalling?

Presently, the two models are proposed to explain the requirement of Delta endocytosis for Notch signaling: the pulling force model and the ligand activation model.

— **Ligand activation model**

The ligand activation model is based on the assumption that a newly synthesized ligand molecule brought to the plasma membrane is not competent to activate Notch and therefore, requires endocytosis, trafficking and recycling back to the cell surface to get ability to signal. The internalization of ligands may target them to a specialized Epsin-dependent endocytic compartment where they undergo an activating modification. Following recycling back to plasma membrane, the newly modified activated ligand can now effectively activate Notch (Emery et al., 2005; Wang and Struhl, 2004). Among the possibilities that have been envisaged for Delta-activating modifications are the clustering of ligands as well their re-insertion into specific membrane domain (Parks et al., 2000).

— **Pulling force model**

The pulling force model assumes that ligand binding alone is not enough to induce the activating proteolytic S2-cleavage of Notch and therefore, endocytosis of ligand is necessary to bring upon conformational changes in Notch (Gordon et al., 2008). Accordingly, endocytosis-defective ligands bind Notch, but are unable to activate Notch signaling (Nichols et al., 2007). The proteolytic S2-cleavage of Notch has moreover been shown to be accompanied the Delta-mediated trans-endocytosis of NECD into the signal-sending cell (Nichols et al., 2007; Parks et al., 2000). This suggest that ligand endocytosis could exert a physical force on Notch that triggers the release and internalization of NECD. In particular, it has been proposed that ligand endocytosis could be required to pull on Notch and expose the ADAM S2-cleavage site for activating proteolysis (Nichols et al., 2007). Accordingly, a recent study using optical tweezers has provided direct physical

evidence that clathrin/epsin/actin-dependent endocytosis exerts a mechanical pulling force on Notch receptors (Meloty-Kapella et al., 2012).

It is however important to note that the ligand activation and pulling force models may not be mutually exclusive. A study in human cell culture suggests indeed that Notch activation actually requires two subsequent rounds of endocytosis: A first round of ligand endocytosis and recycling would target Dll1 to a lipid-raft like microdomain where it could bind Notch receptor. A subsequent second ligand internalization would then actually lead to receptor activation maybe by exerting a physical force on the receptor molecule (Heuss et al., 2008).

1.5.3 Transcytosis of Delta ligands in epithelial cells

In the context of the development of the nervous system of both vertebrates and invertebrates, Delta/Notch signaling is often deployed in the context of apico-basally polarized neuro-epithelial cells. Most interestingly, a study by the group of Roland Le Borgne has led to the suggestion, that the requirement for Delta endocytosis is directly linked to the apico-basal transport of ligand molecules: In Neur mutants, when Delta endocytosis is inhibited, ligand molecules accumulate at the basolateral side of neuroepithelial cells, whereas Notch molecules localize apically. This led to the proposal that Delta endocytosis would be required to redirect Delta ligands from their initial baso-lateral site of secretion to the apical cell surface in order to allow them to interact with apically localized receptor molecules. In accordance with this model, Benhra and colleagues showed that Neur promotes basal to apical transcytosis of Delta in polarized Madine-Darby Canine Kidney (MDCK) cell culture cells (Benhra et al., 2010).

The importance of apico-basal ligand trafficking for Notch signaling is further underscored by the finding that the Actin-related protein 2/3 (ARP2/3) complex ensures a basal to apical transport of endocytosed Delta to an apical actin-rich structure (Rajan et al., 2009). In *Drosophila,* an endosomal recycling regulator Rab11 (Emery et al., 2005) and the exocyst component Sec15 (Jafar-Nejad et al., 2005) have moreover been shown to regulate Delta internalization and recycling. Interestingly, Rab11 and Sec15 have both been shown to affect basal-to-apical transcytosis of Delta ligands in mammalian epithelial cells (Oztan et al., 2007).

Altogether, these finding suggest that a first round of endocytosis may ensure the basal-to-apical transport of Delta ligands in accordance with the ligand activation model. This will allow to present Delta ligands on the apical surface where they can interact with Notch receptors. A

second round of endocytosis of ligands from the apical surface would then exert a pulling force on Notch receptors to induce proteolytic cleavage and activate Notch (Figure 8).

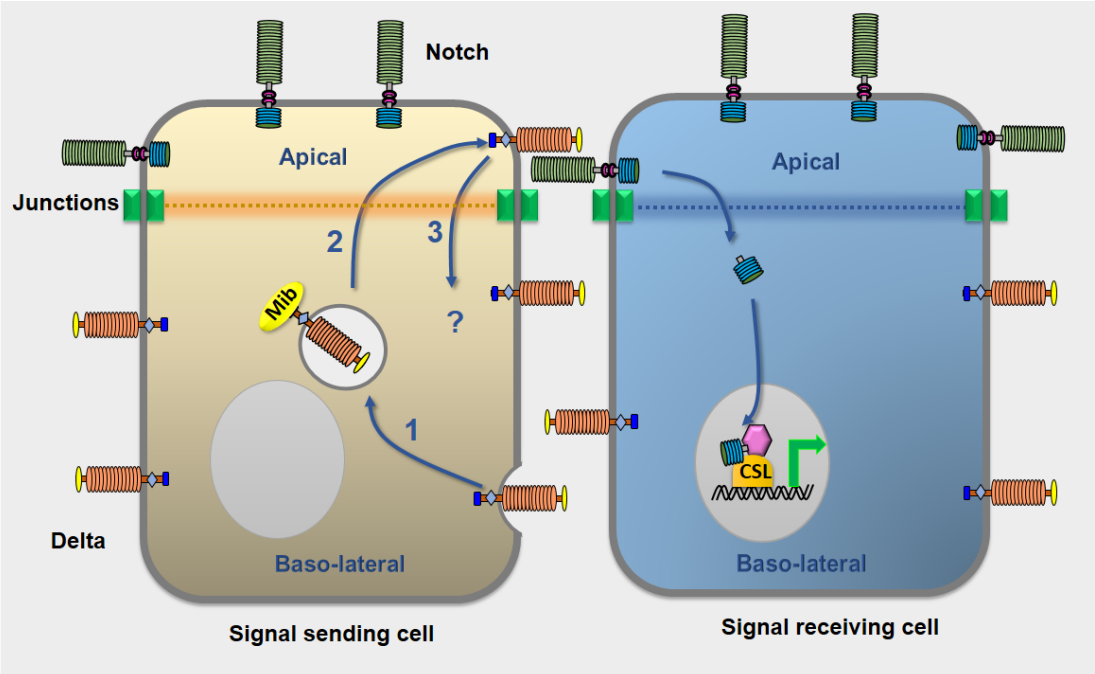


Figure 8: Epithelial transcytosis model
Transcytosis transports molecules via endocytic internalization, intracellular transport and exocytic re-secretion. The transcytosis of Delta molecules occurs in three steps: DeltaD molecules that are assumed to be localized at the basolateral membrane initially get internalized (1), the Delta carrying endosomes are then recycled to the apical membrane where apically located Notch can interact with the Delta molecule (2). In the final step (3), after the binding with Notch receptor, Delta may again be endocytosed and relocalized.

While Benhra *et al* (Benhra et al., 2010) have been able to provide evidence for Delta ligand trans-endocytosis in MDCK cell culture, it has not yet been possible to visualize the occurrence and dissect the physiological relevance of this process *in vivo*. The initial aim of my Ph.D. was to take advantage of novel live imaging assays that have been established in my host laboratory for the live imaging of endogenous Delta ligands (for details see Results section 1) to analyze the potential importance of Delta ligand trans-endocytosis for the regulation of neurogenic Delta/Notch signaling in the Zebrafish spinal cord.

While the initial aim of my work was hence to study the importance of apico-basal polarity for the regulation of Notch signaling, experiments performed right at the beginning of my Ph. D. led me to show that conversely, Notch signaling itself is required for the establishment of the apico-

basal polarity of the neuro-epithelium. In the light of this entirely unexpected novel finding, I devoted my PhD to the investigation of the role of Notch signaling in the regulation of apico-basal polarity in the context of the neural-tube morphogenesis. Therefore, in the following sections, I will first introduce the general mechanisms of epithelial tube morphogenesis and then describe the cell biological mechanisms involved in the establishment of both apico-basal and planar cell polarity.

2. Morphogenesis of tubular organs

Morphogenesis is the process of how an organism develops its shape. It is one of the fundamental aspects of embryonic development driving processes such as gastrulation, somite formation, and neurulation. In 1917, D'Arcy W. Thompson explained that certain animal body shapes are created by growth rates differing in directionality, e.g. the spiral shell of a snail (Thompson, 1917). Later, in 1952, Alan Turing studied the diffusion of activating and deactivating growth signals to set up patterns in development. Morphogenesis requires the coordinated movements of many cells (Turing, 1952); furthermore, these movements must be coordinated with the other fundamental processes of embryogenesis, such as proliferation, differentiation, and spatial patterning.

Epithelial cells can form tubes that are notably progenitors of alimentary canals and the central nervous system and generally an elementary unit of several organ designs. Morphogenesis of most tubes can be divided in five different categories *(*Figure 9*).* Wrapping and budding are the two mechanisms where the tube arises from a polarized epithelium. Wrapping occurs when a part of an epithelial sheet invaginates and twists until the edges of the invaginating tissue meet and fuse together, forming a tube that is parallel to the plane of the sheet such as during neural tube formation in the chick embryo (Colas and Schoenwolf, 2001).

In budding, cells move out in orthogonal direction from the plane of the epithelium and form a tube when this bud eventually grows. The formation of branches in human lungs and *Drosophila* tracheal system are typical examples of tubulation that occurs via budding (Metzger and Krasnow, 1999). In contrast to the above-mentioned mechanisms, the tube can also be generated from a cluster of cells (cavitation and cord hollowing) or an individual cell (cell hollowing) that may not be polarized but eventually polarize as the tube forms. During cavitation, cells organized in a thick cylindrical cluster form a central cavity by eliminating cells from the center of the mass. The mammalian female reproductive track and the amniotic cavity in vertebrates form typically by cavitation (Coucouvanis and Martin, 1995). In contrast, in cord hollowing, cells assembled in a thin cylindrical cord create a lumen between cells, without eliminating cells such as during gut formation in *C. elegans* (Leung et al., 1999). Cell hollowing however, is different from the other processes as the lumen forms within the cytoplasm of an individual cell rather than a group of cells, such as the formation of capillaries by the endothelial

cells in vertebrates and terminal cells of the *Drosophila* tracheal system (Lubarsky and Krasnow, 2003).

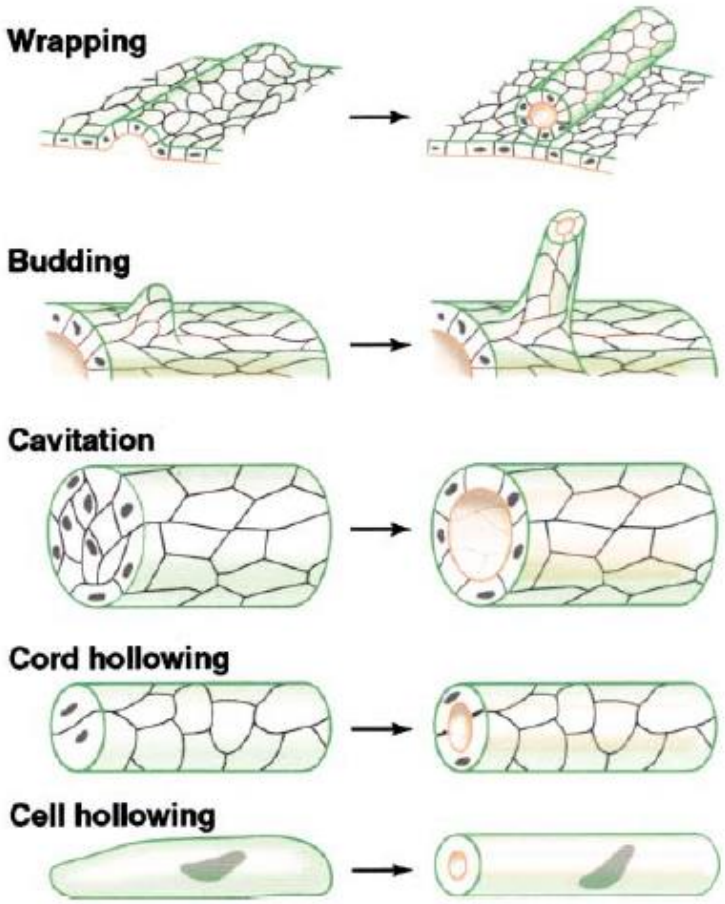


Figure 9: Morphological processes of tube formation
A tube can be formed by different mechanism: In wrapping, a part of an epithelial sheet invaginates and curls until the two ends meet and fuse together. During budding, a small group of cells moves out from an existing sheet and forms a tube as the bud grows. In cavitation and cord hollowing, a central cavity forms in a cylindrical mass of cells with or without elimination of cells from the centre respectively. Finally, in cell hollowing a cavity forms in an individual cell (Lubarsky and Krasnow, 2003).

2.1 Neurulation: forming a neural tube

One of very important morphogenetic event for embryonic development is neurulation, which generates the neural tube that is the precursor of the brain and spinal cord. Neural tube defects (NTD) account for major congenital anomalies in babies, which are the leading cause of death under one year of age. In humans, the most common NTD are myelomeningocele and anencephaly, which results from a failed neural-tube closure (Detrait et al., 2005). In mouse,

more than 80 mutant genes have been identified which disrupt neurulation and give rise to neural tube defects. The corresponding genes belong to several signaling pathways such as the planar cell polarity pathway and the sonic hedgehog pathway that regulate different cellular processes ranging from the initiation of neural tube closure, neural fold elevation, fusion of the neural folds to several other cranial neurulation specific events. This indicates that strict coordination between these different cellular processes is essential for proper neurulation (Copp and Greene, 2010; Copp et al., 2003). Hence, it is crucial to understand the morphogenetic mechanism underlying the formation of the neural tube.

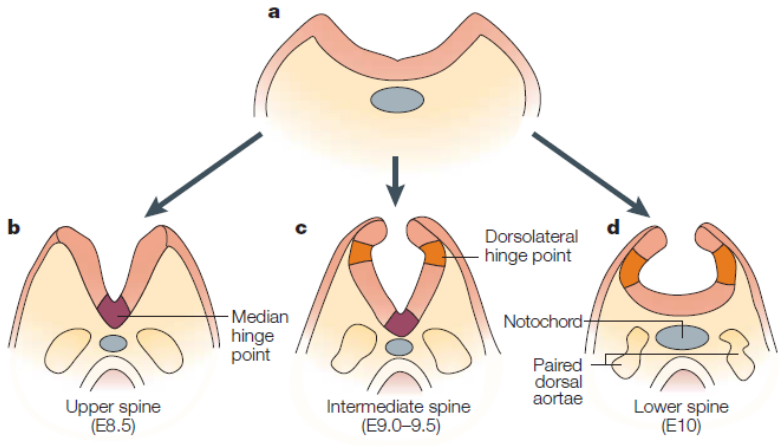


Figure 10: The early events of mouse neurulation along the developing spinal cord.
Neural fold elevation differs at different developmental stages and differs in morphology along the different levels of the body axis after the initial neural fold bending. (a) In the early stages of spinal neurulation at E8.5, the neural plate bends only at the Median hinge point (MHP) at the upper spine. (b), while at early E9.5, as closure progresses to thoracic level, bending occurs at the MHP and the DorsoLateral Hinge Point (DLHP). (c) At E10, when the lower spine is forming, MHP bending is lost and the neural plate bends solely at DLHPs (Copp et al., 2003).

Neurulation occurs through two distinctive primary and secondary phases. **Primary neurulation** creates the brain and the anterior part of the spinal cord from an epithelial cell sheet termed the neural plate. In mammals and several other vertebrates, the neural tube forms by the 'wrapping' mechanism where the neural plate creases inward until the edges meet and fuse together (Copp et al., 2003). In contrast, **secondary neurulation** creates the posterior part of the spinal cord from the tail bud in which a solid rod of mesenchymal cells transforms into an epithelial tube. The tube forms by 'hollowing' out of the interior of a solid cylindrical mass of precursor cells (Criley, 1969). Studies in mouse and several other vertebrates have shown that at the start of primary neurulation the neural plate bends and then continuously folds inwards at one point in

the plate. Further, the two edges of the neural plate meet at the dorsal midline, fuse with each other through epithelial adhesion and remodeling, and thus create a tube-like structure (Figure 10).

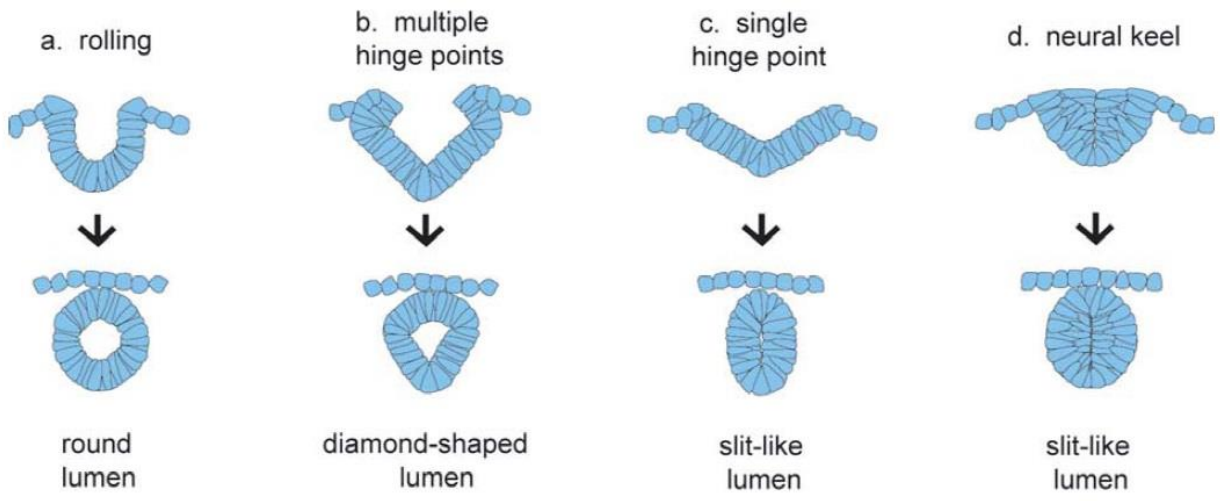


Figure 11: Variation of primary neurulation.
The neural plate may use different mechanisms to form the neural tube. (a) The neuroepithelium may roll to form the neural tube; e.g. *Xenopus* brain and a part of the mouse spinal cord develop typically via rolling; (b) and (c) Another strategy to form the neural tube is a bending of the neuroepithelium at defined hinge points (single or multiple), such as in the mouse and chick spinal cord. (d) Finally, the neural plate may sink inwards to form a solid structure of cells termed the neural keel. This is apparent in the *Xenopus* spinal cord and during Zebrafish neurulation (Lowery and Sive, 2004).

Different vertebrate species undergo different processes to generate neural tube as shown in Figure 11. Surprisingly, the same animal can make use of these different strategies at different stages or at different anterior-posterior locations of the neural tube. For example in mouse in the cranial region neural fold bending is different from that in the spinal region. In the cranial region, neural folds initially elevate at the median hinge point (MHP) and curl up to meet and fuse together. In contrast to cranial closure, spinal folds bending occurs at two points: the median hinge point (MHP) and then at dorsolateral hinge points (DLHPs), which brings the folds towards each other. Different combinations of bending points can be applied as the closure progresses along the spinal axis. For instance, during mouse neurulation the neural plate bends only at the MHP in the upper spine at E8.5 (Figure 10 a), as closure progresses to thoracic level, bending occurs at MHP and DLHP at early E9.5 (Figure 10 b). Further, at E10 stage, in the lower spine, MHP bending is lost and the neural plate bends only at DLHPs (Figure 10 c) (Shum and Copp, 1996).

2.2 Neural tube morphogenesis in the Zebrafish model system

2.2.1 Advantages of the model system

Danio rerio, commonly named zebrafish, is a small tropical fresh water fish from Southeast Asia. This species acquired the status of a model system to study many aspects of embryonic development when introduced by George Streisinger and colleagues at the university of Oregon in the early 1980s (Streisinger et al., 1981). Zebrafish is a vertebrate model system and therefore has more resemblance to humans than the other invertebrate model systems like flies and worms. Zebrafish gained popularity because of its ex-utero reproduction and optical transparency in early developmental stages. This allows studying the complex processes of organogenesis and gene expression with much more ease through imaging with single cell resolution. Moreover, this small fish is inexpensive and can be maintained easily under laboratory conditions. Its development is rapid, and a healthy female can lay 100s of eggs on a weekly interval.

Large-scale forward genetic screening techniques further made zebrafish a very successful model system for developmental genetics (Mullins et al., 1994). These screens generated a large number of mutants through insertional mutagenesis with retroviruses (Amsterdam et al., 1999) and chemical mutagens (Haffter et al., 1996). The availability of antisense morpholino-based gene inhibition procedures made zebrafish moreover suitable for targeted gene knockdown experiments. More recently, Hwang and colleagues established the use of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) / Cas9 nuclease system to induce targeted genetic modifications in zebrafish embryos (Hwang et al., 2013). The CRISPR/Cas9 system has been reported to target and modifie genomic loci in zebrafish with high efficiencies in somatic and germ cells (Jao et al., 2013) and can serve as a powerful tool for efficient reverse genetics (Shah et al., 2015).

2.2.2 General mechanism of the neural tube morphogenesis in zebrafish

Zebrafish neurulation appears different from neurulation in amphibians and amniotes. One reason is that a neural groove and neural folds are not formed; rather the neural plate sinks down to form the neural keel. Secondly, the lumen is formed through cavitation (Schmitz et al., 1993). The neural tube in the anterior trunk region originates from a multi-layered columnar neuroepithelium (Clarke, 2009). Neural plate cells at this stage of development are not

connected through junctional complexes, suggesting they may not be fully epithelial (Geldmacher-Voss et al., 2003). However, despite the lack of a clear epithelial architecture several studies suggest that to form the neural keel the neural plate cells moves inward as an organized cell layer just like in amniotes. Due to this inward folding, cells from the contralateral sides of the neural plate are juxtaposed at the midline. In addition, neural plate cells display an elongated epithelial-like morphology. Because the neural tube is generated from epithelial-like cells that undergoes a folding process, neurulation in the zebrafish is believed to incorporate few elements of 'primary neurulation' observed in other vertebrates (Lowery and Sive, 2004; Papan and Campos-Ortega, 1994). However, zebrafish neurulation exhibits the peculiarity that as cells of the neural keel and neural rod divide at the apical side of the neuro-epithelium, one of the daughter cells crosses the neural tube midline (i.e. the site of formation of the future lumen) and incorporates into the contralateral half on the neural tube, resulting in bilaterally distributed cell clones. These Crossing-divisions are therefore termed "C-divisions" (for details see Section 3.4.2 and 0). This clearly distinguishes zebrafish neurulation from other vertebrates (Kimmel et al., 1994).

The steps involved in the zebrafish neural tube formation are the following (Figure 12):

- The neural plate (Figure 12 a) sinks inward towards the dorsal-mid plane (Figure 12 b); cells interdigitate and intercalate to the collateral side of the neural plate, between 6 to 10 somite stages and form a solid structure termed the neural keel (Figure 12 c, d). At the neural keel stage, C-divisions at the dorsal midline give rise to a bilateral distribution of cell clones with mirror symmetric apico-basal polarity (Tawk et al., 2007).
- During 10-14 somite stages, the keel rounds up to form the neural rod, a cylindrical mass of cells that lacks a lumen (Figure 12 e).
- The neurocoel, lumen of the neural tube, forms through cavitation when the neuroepithelial cells retract their apical processes from the midline (Figure 12 f). Neurocoel formation starts at the 17-18 somite stage in the ventral neural tube, progresses to the dorsal side and is completed by 30 somites (Papan and Campos-Ortega, 1994).

Following neurocoel formation, the lumen must expand in order to complete neural tube formation. The osmoregulatory ion pump ATPase Na+/K+ transporting alpha1 polypeptide (Atp1a1) is critical for lumen expansion to create an osmotic gradient to drive water flow into

the closed brain ventricles after their morphogenesis (Lowery and Sive, 2005). A study showed that lumen expansion is driven by hydrostatic pressure where the Claudin family of transmembrane tight junction proteins and Na+/K+ ion channels together determine paracellular tightness. Zhang and colleagues showed that during brain ventricle expansion, Claudin5a seals the tight junctions of the neuroepithelium and maintains the fluid pressure that depends on Atp1a1 activity (Zhang et al., 2010). These studies also emphasize that the function of the Crumbs complex (An apical polarity complex, for description see section *3.1* below) is indispensable for lumen expansion via regulating epithelial integrity.

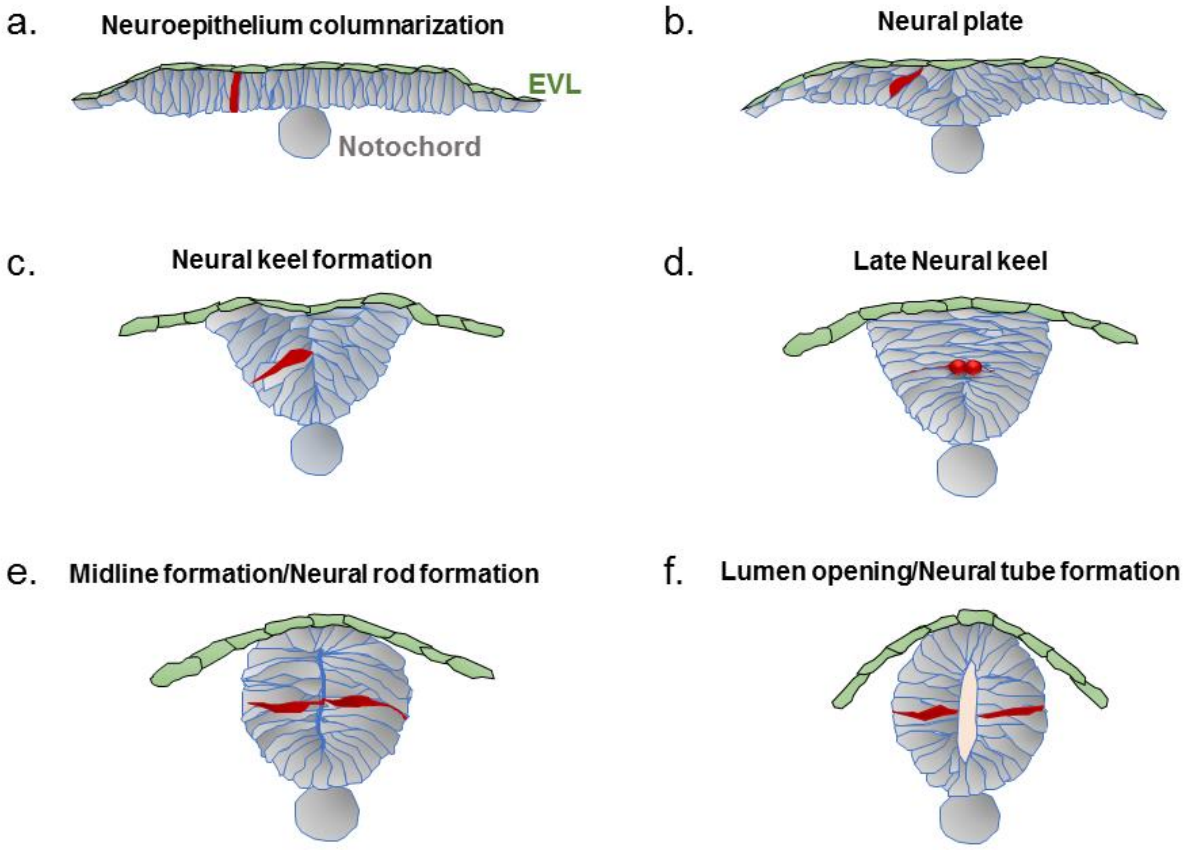


Figure 12: Zebrafish neurulation

Epithelial-like columnar cells form the neural plate (a). Neural plate cells further move inwards (b) and form the neural keel (c, d), which then transforms into the neural rod (e). After the formation of the apical luminal neural tube midline, the neural rod opens up to form a lumen and thus create the neural tube (f). A cell in red illustrates the cellular behaviour during midline-crossing C-divisions.

2.3 Cellular mechanisms of neural tube morphogenesis

During neurulation, neuroepithelial cells undergo several changes in their shapes. The elongation of neural plate cells can be considered the first change in cell shape. The neuroepithelial cells extend along their apico-basal axis to form a sheet of columnar cells (Schoenwolf and Powers, 1987). The microtubule cytoskeleton appears to drive cell elongation partly along with the actin-binding protein Shroom3. Especially, redistribution of γ-tubulin to the apical side is found to be important for correct apico-basal cell elongation (Lee et al., 2007).

The apical constriction of cells is a second way that brings changes in cell shape that has commonly been observed in the folding of epithelial sheets. In primary neurulation, bending at the MHP and DLHPs requires apical constriction of cells, which is regulated by Shroom (Haigo et al., 2003). In zebrafish, as junctional proteins are not present at the neural plate and keel stages, there are no typical apical constrictions of neuroepithelial cells. The closest process to apical constriction is an apical protrusion activity that allows cells to intercalate to the contralateral side of the neural rod. N-cadherin plays an important role in cellular rearrangements and intercalation during neural keel formation (Hong and Brewster, 2006).

Beyond the shape adopted by individual cells, cell division is critical for normal embryonic development. In mouse, several mutants have been identified that are related to abnormal cell cycle progression and cell proliferation, such as the *humpty dumpty* and the *curly tail* mutants. These mutants display different neural tube defects (Copp and Greene, 2010). During zebrafish neural tube development, specialized midline crossing C-divisions (refer *sections 3.4* and *4.4* for details) are important for the proper lumen formation along with the epithelial polarity

Another, crucial requisite for epithelial morphogenesis is the establishment of polarity. Epithelial cells exhibit apico-basal polarity, where the apical side faces the lumen of the tube. In addition, epithelial cells also exhibit planar cell polarity (PCP), in which specialized structures (for *e.g*. *Drosophila* wing hairs) are oriented within the plane of the epithelial sheet. In the following section, I will explain in detail the establishment of polarity *i.e.* apico-basal polarity exhibited by each cell individually (see *section 3*) and planar cell polarity that is the polarization of a cluster of cells within the plane of cell sheet (refer to section 4).

3 Apico-basal polarity

Cellular polarity is one of the fundamental necessities for morphogenesis. Polarity is defined as asymmetry in cell shape or function. Polarity can arise due to the polar distribution of molecules or uneven mechanical properties. Cellular polarity defines many crucial functions in different cell types, for instance axonal guidance in polarized neurons. Within the tissue, polarized cells form a highly organized array of cells that form a particular shape of the organ, which is important for the organ functionality and structure.

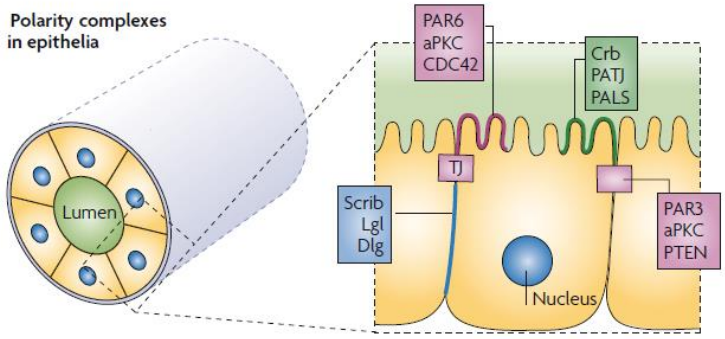


Figure 13: Cell polarization in a tubular epithelium
Epithelial tubes are made up of adjoining cells with strong adherence to each other. These cells display apico-basal polarity. At the interface between the apical and baso-lateral membrane domains, tight junctions and adherence junctions provide cell-to-cell adherence. The basal membrane interacts with the extracellular matrix while the apical membrane faces the lumen or the central canal (Bryant and Mostov, 2008).

An epithelium is a contiguous sheet of cells where each cell exhibits apico-basal polarity. The apical surface provides the luminal side of an organ, e.g. the central canal in the brain and spinal cord. In contrast, the basal lamina faces the basement membrane and extracellular matrix. The lateral side connects to adjacent cells through special junction proteins and cell-to-cell adhesion molecules (Figure 13) (Bryant and Mostov, 2008). Most embryonic epithelia consist of a single layer of cells and are usually classified based on cell shape: a squamous epithelium is composed of flattened cells, a columnar epithelium of elongated cells, and a cuboidal epithelium of cells of equal width and height.

Structurally, epithelial cells possess molecularly distinguished compartments, which allow them to differentiate between the interior and the exterior of the organism. This subcellular organization is referred to as apico-basal polarity. The integrity of epithelial apico-basal polarity is critical for many cellular processes and tissue architecture. In fact, the core polarity proteins lethal giant larvae (lgl), discs-large (dlg) and Scribble (Scrib) were first discovered as tumor

suppressor genes in *Drosophila* (Bilder et al., 2000). Several studies have shown that dysregulation of epithelial cell-to-cell adhesion is associated with many type of cancers (Nguyen and Massagué, 2007). Low levels of the tight junction molecules Zonula occludans-1 (ZO1) and Mupp-1 are associated with poor prognosis in patients with breast cancer (Martin et al., 2004). All these studies highlight the need for an in-depth understanding of epithelial establishment and maintenance.

3.1 Apico-basal polarity proteins and their localization

The polarity protein complexes along with junction proteins are required for cellular polarity in all epithelia. Each plasma membrane domain has a distinct protein composition that gives it distinct properties. The core polarity proteins form three main complexes named the Par complex, the Crumbs complex and the Scribble complex (Figure 14). The Par and Crumbs complexes usually localize at the apical domain whereas the Scribble complex localizes at the baso-lateral domain. On the apical side, adjacent cells connect to each other through intracellular junctions: tight junctions and adherence junctions.These polarity genes were first discovered in *C. elegans* (Kemphues et al., 1988) and *Drosophila* (Bilder et al., 2000; Tepaß and Knust, 1990). Later on, homologous proteins were discovered in many species including mammals (Goldstein and Macara, 2007).

3.1.1 The Par Complex

Partitioning-defective (PAR or Pard) proteins were first identified in *C. elegans* as determinants of asymmetric cell division, where *par* mutations leads to defects in the asymmetric localization and segregation of cytoplasmic P-Granules (i.e. germline-specific perinuclear RNA granules) during early germ cell divisions (Kemphues et al., 1988). The Par complex encompasses mainly four proteins: Par3, Par6, atypical protein kinase C (aPKC) and the small GTPase CDC42 (Figure 14). Par6 and Par3 carry several protein-protein interaction domains including a PDZ domain and a Cdc42/Rac interactive binding (CRIB) domain which allow these proteins to bind each other and several other proteins including the Crumbs complex proteins. Binding of Crumbs with Par6 through its PDZ domain allows recruiting Par6 at the apical membrane (Hurd et al., 2003). Par6 functions as an adaptor to link Par3 to aPKC and to the Cdc42 GTPase. However, Par6 can also directly bind aPKC through the Phox and Bem1D (PB1) domain. These

interactions are found to be important for the formation and maintenance of tight junctions in epithelial MDCK cells (Joberty et al., 2000).

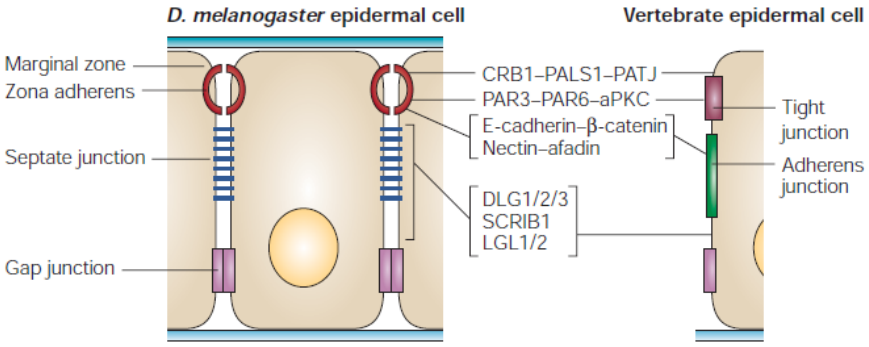


Figure 14: Localized assembly of apico-basal proteins in *Drosophila* and vertebrate epidermal cells

The three core cellular polarity complexes that are crucial for apico-basal polarity in epithelial cells are shown: the Par complex: Par3, Par6, aPKC (Cdc42 is not shown in figure); the Crumbs complex (Crumbs, PALS1 and PATJ) and the Scribble complex (Lgl1/2, Scribble and DLG). The Par complex localizes at the level of the tight junctions (TJ), the Crumbs complex localizes to or just above the TJs and the Scribble complex localizes at the lateral membrane (Macara, 2004).

Par3 acts as a scaffolding protein and through its PDZ domain makes a complex with Phosphatase and Tensin homologue (PTEN). This interaction is essential for the localization of PTEN at the junctional membrane (Feng et al., 2008). Another apical protein aPKC is a Serine/Threonine kinase and its phosphorylation of several proteins including Par3 is essential for downstream polarity events (Iden and Collard, 2008). Indeed, disruption of the complex results in severe damage to apico-basal polarity and tight junction structural integrity (Suzuki et al., 2002). Several studies have shown that Cdc42 which is a small GTPase belonging to the Rho family has a central role in cellular polarity in various systems. It regulates not just actin and microtubule cytoskeletons but also signalling pathways and endocytosis, all of which are crucial for the establishment and maintenance of epithelial organization and polarity (Etienne-Manneville, 2004).

3.1.2 The Crumbs complex

The Crumbs complex consists of three types of proteins: Crumbs (Crb), Protein associated with Lin7 (Pals1) and Pals1 Associated- Tight Junction (PATJ) (Figure 14 Localized assembly of apico-basal proteins in a *Drosophila* and vertebrate epidermal cell). Pals1 and Patj are both

cytoplasmic scaffolding proteins, whereas Crumbs is a transmembrane protein. Crb was first discovered in *Drosophila* as an important regulator of epithelial polarity (Tepaß and Knust, 1990).

The Crumbs complex is often located more apical than the Par complex, however it can interact with tight junctions (Schlüter et al., 2009). Likewise, Crumbs complex proteins interact with each other and target many proteins to the apical membrane, such as Occludins, ZO1 and aPKC, which then regulate tight junction biogenesis (Roh et al., 2003). It has been found that the Crumbs complex member Pals1 stabilizes the Par complex at the apical membrane by directly interacting with Par6 (Hurd et al., 2003). Conversely, the Par complex may also modify the Crumbs complex directly or indirectly via adherence junction proteins to stabilize it at the apical membrane (Tanentzapf and Tepass, 2003). Beyond establishing apical polarity, the Crumbs complex also helps to maintain epithelial integrity through its interaction with D-Moesin (an actin binding protein) and Actin to recruit Spectrin skeletal components to the apical membrane (Médina et al., 2002).

3.1.3 The Scribble Complex

The Scribble complex comprises mainly three proteins that are Lethal giant larvae (Lgl), Discs large (Dlg) and Sribble (Scrib). The Scribble complex proteins localize to the basal/lateral membrane and regulate molecular integrity and size of the apical membrane (Tepass et al., 2001). The main function of the Scribble complex is to antagonize the Par complex and therefore retain lateral identity (Tanentzapf and Tepass, 2003; Yamanaka et al., 2006). A fascinating connection of Lgl with Syntaxin (a SNARE protein) involves intracellular vesicular transport, which indicates that Lgl may contribute to epithelial polarity by regulating basolateral exocytosis (Müsch et al., 2002).

3.1.4 Junction proteins

The tight junctions are located at the apical side to form a barrier between cells and control paracellular flow. Tight junctions are composed of the transmembrane proteins Occludin and Claudin, the cytoplasmic scaffolding proteins ZO-1, 2, 3, and the Junctional Adhesion Molecule (JAM) family of proteins (Hartsock and Nelson, 2008). Occuludin and Claudin proteins have two main functions. First, to separate apical and basolateral proteins and secondly, to prevent mixing of paracellular fluids and allow selective ion movements between the cells by regulating

permeability (Tang and Goodenough, 2003; Tsukita et al., 2001). ZO (1, 2, and 3) are members of the MAGUK (Members Associated Guanylate Kinase homologues) family of proteins. These proteins contain PDZ and SH3 binding domains, which allow them to bind many proteins including both adherence junction and tight junction components and provide a link between these proteins. Additionally, these scaffolding proteins can also interact with the cytoskeleton of the cell (Hartsock and Nelson, 2008). In MDCK cells, JAM has been shown to be associated with the vertebrate orthologue of Par3, Atypical PKC Isotype-Specific Interacting Protein/Partition defective-3 (ASIP/Par3) (Itoh et al., 2001).

The adherence junctions lay a little basal to tight junctions and perform multiple tasks such as formation and stabilization of cell-cell adhesion, regulating the actin cytoskeleton and intracellular signaling. Adherence junction proteins include the transmembrane Ca^{2+} dependent Cadherins (E-cadherin and N-cadherin) and the members of the Catenin family p120-catenin, α- and β-catenin. E-cadherin is a single pass membrane protein that interacts with the other polarity proteins and mediates cell-to-cell adherence through its extracellular domain. The Catenins link the Cadherins to the actin cytoskeleton to stabilize cell-cell contact either directly or through Actin-binding proteins. An *in-vitro* study shows that β-catenin binds Dynein and forms a complex together with the adherence junction protein E-cadherin. This multimolecular complex allows a crosstalk between the microtubule and Actin networks that is important the epithelial integrity (Ligon et al., 2001). Adherence junctions are essential for epithelial integrity; however being dynamic, they allow rearrangement of cells within the sheet through various mechanisms like the interaction of Cadherin with the Actin-cytoskeleton, endocytosis, and recycling of Cadherins, and the cooperation of Cadherins with other adhesion proteins (Nishimura and Takeichi, 2009).

3.2 Establishment of cellular polarity

The establishment of cellular polarity is a fundamental attribute of many cell types that enables them to exert their specialized functions in an organ. The cells that are initially isotropic need to break their symmetry in order to attain the polarized structure. The process of symmetry breaking can be a random stochastic process or a highly organized event that is triggered by an external cue. Once the symmetry is broken several other factors play their part in a simultaneous fashion: Polarity proteins get assembled to their confined locations; the cytoskeleton is rearranged into a polarized network and several regulatory signalling molecules

are trafficked around the membrane domains to establish polarity (Macara, 2004). Another important requisite for proper structural and functional epithelia is the binding of individual cells in a sheet, a task performed by adhesion molecules between the adjacent cells. However, gluing the cells together is not enough. The formation of polarized cells also relies on their ability to grow and be retained within the sheet. This is achieved through a strict control over mitotic spindle orientation during cell division.

3.2.1 Symmetry breaking

The first step for the establishment of polarity is to generate asymmetry in the cell. This can be achieved by a cascade of extrinsic spatial cues that mediate cell-to-cell and cell-to-substratum adhesion. Adhesion between cells is primarily mediated by E-cadherin and adhesion of cells to the extracellular matrix (ECM) is mainly accomplished by Integrin adhesion receptors. The interaction between these adhesion receptors and their extracellular ligand molecules generates the primary spatial cues which determine the site of contact with the plasma membrane. These sites of contact and non-contact are nothing but the precursor areas of the basolateral and apical membrane domain respectively (Yeaman et al., 1999). Several studies have been carried out to understand these spatial cues, especially in 2-D and 3-D cell culture models. However, the 3-D cell culture system provides a better opportunity to understand the initial symmetry breaking event it more accurately mimics the *in vivo* conditions. The asymmetry can be generated in the 3-D cell culture system as early as the first cell division, as polarity determinants accumulate on the cleavage plane of dividing cells (Jaffe et al., 2008; Martin-Belmonte et al., 2007). Another example where cell-cell contact may provide the break in symmetry is shown in the calcium-dependent repolarization of epithelial cells. As E-cadherin is a calcium dependent adherence junction component, a calcium switch can control the assembly and disassembly of polarity proteins and the initial contact of cells (Ebnet et al., 2004).

Cells also interact with ECM components like Laminin and Collagens. MDCK cells, which are grown on a Collagen-coated surface, secrete Laminin from the basolateral side and make basement membrane in a sheet. A study in MDCK cells showed that the Laminin-dependent assembly of basement membrane induces the orientation of apico-basal polarity through interaction with β-Integrin and a small GTPase Rac1A. Here, β-Integrin lies upstream of Rac1. Inhibition of β-Integrin prevents Laminin organization at the basal membrane and thereby prevents orientation of polarity (Yu et al., 2005).

All cells have a fine meshwork of Actin cytoskeleton, which is composed of Actin filaments and molecular motors and various Actin-binding proteins. This dynamic structure is capable of fast assembly and disassembly of proteins at the cortex. Molecular motors such as myosin create a tension which keeps the cell in a certain shape. It is likely that any local changes in the tension and relaxation of the Actin-Myosin framework can drive the first step of symmetry breaking. This mechanical instability can occur spontaneously or in response to an external factor which gives rise to the initial polarization of the cell (Paluch et al., 2006). However, a stimulus that may trigger asymmetry can also be intracellular. One such example is in dividing yeast during cytokinesis, where astral microtubules send signals to the polar cortex to release cortical tension locally at the plane of division. This brings an asymmetry in mitotic spindle orientation, where equatorial astral microtubules are denser at the cleavage furrow that induces the cell polarization (Burgess and Chang, 2005).

There have also been studies indicating that the spontaneous stochastic variation in the stability and localization of polarity proteins can give rise to polarity (Sohrmann and Peter, 2003). In yeast, a membrane scaffolding protein Bem1p mediates assembly of Cdc42-GEF (Guanine exchange factor) and a Cdc42 effector kinase PAK. This complex amplifies clusters of Cdc42 at random sites in the cell to break symmetry and to induce polarization (Kozubowski et al., 2008).

3.2.2 Confining polarity proteins to their location

Once symmetry has been broken, the next step is to assemble polarity proteins at their specific sites. To understand the localized assembly of polarity proteins and to find out which proteins accumulate first and induce polarization, several studies have been done so far. Bilder and colleagues showed that during *Drosophila* embryogenesis, the polarity complexes function as a single regulatory unit to control apical polarity. They proposed that on one hand, Scrib prevents the Par3 homologue Bazooka (Baz) from promoting apical characteristics, and therefore allows basolateral development. On the other hand, Crb upholds Baz at the apical membrane by antagonizing Scrib. The fine tuning of the activities of the Crb and Scrib complexes therefore governs the establishment of proper polarity (Bilder et al., 2003).

In mammalian epithelial cells (MTD-1A), primordial adherence junctions form at the site of cell-to-cell contact where E-cad and ZO1 accumulate and further are followed by the assembly of Par3 and aPKC (Suzuki et al., 2002). In contrary to this, during *Drosophila* cellularization, Par3/Baz appears to be the first protein to accumulate at the apical domain. This apical

accumulation of Baz is independent of adherence junctions. Indeed, E-cad is then recruited to the cortex through Baz (Harris and Peifer, 2004). These studies suggest that despite the fact that polarity protein complexes are conserved among species the way they get confined to their landmark locations can be different. This variability accounts not just for the precise order of their assembly, but also for the mechanistic cues through which they are processed.

3.2.3 Reorganization of the cytoskeleton

The establishment of polarity in a cell also relies on cytoskeletal rearrangements. This not just reinforces the initial site of asymmetry, but also retains it. At cell-to-cell or cell-to-matrix contact sites tight junctions/adherence junctions (TJs/AJs) mediate localized assembly of specialized cytoskeleton and signalling molecules. Binding with the cytoskeleton strengthens adherence junctions. Besides, these local interactions can bring local or global changes in the spatial organizations of cortical proteins and microtubules (Drubin and Nelson, 1996). Several small GTPases play a very important part at this step such as Arp2/3 which modulates the Actin cytoskeleton at the landmark sites, resulting in Actin-polymerization and remodelling (Fukata and Kaibuchi, 2001).

The cytoskeleton plays a key role in both establishing and maintaining neuronal polarity. An active turnover of Actin filaments occurs in future axon in comparison to future dendrites. This initiates the establishment of polarity in a neuron much before the morphological polarization occurs (Tahirovic and Bradke, 2009). In wound healing assays, Integrins are activated by their interaction with the ECM at the leading edge of the cell, resulting in a cascade of signalling events. This further activates Cdc42 which then recruits the Par complex through the microtubule motors Dynein and thereby induces polarity (Etienne-Manneville and Hall, 2001).

3.2.4 Generating apico-basal domains

Another essential process subsequent to the stabilization of junctional proteins, localized assembly of polarity proteins and cytoskeletal rearrangements is the trafficking of proteins to specific membrane domains. Indeed, Par complex proteins are found to be one of the important regulators of endocytosis (Goldstein and Macara, 2007), therefore suggesting a potential mechanism for how different membrane domains are generated during the establishment of apico-basal polarity. For instance, the Par complex proteins interact with the endocytic protein Numb. aPKC has notably been shown to regulate Numb function by phosphorylating it and thus

contributing to the regulation of Numb-mediated Integrin endocytosis and membrane trafficking, which is important for the generation of polarity in migratory cells (Nishimura and Kaibuchi, 2007). Par3 may also play a crucial role in asymmetric segregation of Phophatidyl Inositides (PtdIns), which is important for cell polarization (Feng et al., 2008).

3.3 Maintenance of polarity

Once the apico-basal polarity of a cell is established, the next big thing is to maintain this status. In some cell types such as neurons and epithelial cells, it is absolutely essential to stabilize polarity to maintain their differentiation state and functional activity. Studies so far have revealed mainly two mechanisms that maintain cellular polarity. The first one is that the Actin cytoskeleton acts in a dynamic manner to allow endocytosis that helps membrane domains to keep the amount of certain molecules constant. Mutation in the proteins, which are implicated in endocytosis of polarity proteins, such as Syntaxin and Rab results in epithelial polarity defects (Lu and Bilder, 2005).

The second mechanism reveals the action of microtubules in reinforcing the initially established polarity by undergoing a dramatic rearrangement from a radial centrosomal array to non-centrosomal array. In columnar epithelia, non-centrosomal microtubules become aligned along the apico-basal axis, predominantly with minus ends at the apical pole and plus ends at the basal pole. In contrast to the basolateral proteins, apical proteins are trafficked in a microtubule-dependent fashion (Li and Gundersen, 2008).

In addition, apical and basolateral protein complexes antagonize each other to prevent the mixing of the components of each other's respective membrane domains. The Par complex protein aPKC phosphorylates the Scribble complex protein Lgl and therefore prevents its localization at the apical membrane (Betschinger et al., 2003). In a similar fashion, Lgl then competes with Par3 for binding to the Par6-aPKC complex. This inhibits the Par complex and thus facilitates the disassembly of apical proteins (Yamanaka et al., 2006). In flies, the Par3 homologue Baz functions redundantly with the Crumbs complex to maintain apical polarity at mid to late embryogenesis. In addition to this, Lgl and Crb also functions in a competitive manner to maintain the basolateral and apical domains respectively (Tanentzapf and Tepass, 2003). Such mutually exclusive interactions ensure that each polarity complex is confined to its actual domain only and does not overlap with the other ones.

Maintenance and establishment of apical and basolateral domains also depend on the activity of PTEN at the apical surface. PTEN is a lipid phosphatase which removes phosphate from Phosphatidyl-Inositol-(3, 4, 5)-phosphate (PtdIns (3, 4, 5)P3) and generates Phosphatidyl-Inositol-(4, 5)-phosphate (PtdIns(4, 5)P2) at the apical membrane. This apically segregated PtdIns(4, 5)P2 recruits Cdc42 to the apical membrane through the scaffold protein Annexin2. The newly recruited Cdc42 then assembles the Par complex, and via cytoskeletal rearrangements stabilizes the apical surface (Martin-Belmonte et al., 2007).

In addition to this, dedicated vesicular transport destined to apical and basolateral domains also contributes to maintain polarity. For example in cultured MDCK cells, the small GTPase molecule Rab8 has been found to be important for regulating the transport of polarity proteins to the basolateral membrane (Huber et al., 1993).

3.4 Apico-basal polarity in the context of zebrafish neural tube morphogenesis

As previously discussed, zebrafish can be a good model to study the morphgenesis of the neural tube. Epithelial polarity establishment has been described to be essential for neurulation. Several studies have revealed the importance of apico-basal polarity for proper neural tube lumen formation during zebrafish neurulation. One such study shows that *pard6-γb* mutants exhibit defects in mitotic spindle orientation in the forming neural tube (Munson et al., 2008). Similarly, zebrafish *aPKC* mutants display numerous defects in several organs including the digestive track, eye and neural tube. For example, mutants display the formation of multiple lumens in the neural tube and faulty spindle orientation in dividing cells of the retina (Belting and Affolter, 2007; Horne-Badovinac et al., 2001). The par complex protein Pard3 has also been shown to be crucial for the separation of eye field during retinal development (Wei et al., 2004) and for centrosome positioning during neurulation (Hong et al., 2010). Likewise, a *crumbs* gene homologue *oko meduzy (ome)* and related *crumbs* parologues are necessary for defining several features of apical membranes such as the size of the apical membrane in photoreceptor cells and cilia length in renal and otic vesicle epithelia (Omori and Malicki, 2006). Another study revealed that in zebrafish *n-cadherin* and *nagie oko* (*nok*, zebrafish Pals1 homologue) mutants, disruption of apico-basal polarity and junctional integrity perturbs retinal neurogenesis (Yamaguchi et al., 2010). Therefore, it is quite important to advance our knowledge about how apico-basal polarity in zebrafish neuroepithelium is established and understand how it contributes to neural tube lumen formation. In the following sections I will discuss our current

understanding of Zebrafish neurulation with a particular emphasis on the regulation of apico-basal polarity.

3.4.1 Localized assembly of polarity proteins during neural tube formation

The structure of the neural plate in amniotes is well characterized and known to be a single cell layered columnar epithelium (Colas and Schoenwolf, 2001). In early developmental stages, apical and basal polarity is inherited from the epiblast cells, where epithelial polarity was already present (Greene and Copp, 2009). In contrary to this, the zebrafish neural plate appears to lack typical apico-basal polarity until late neural keel stages when a central nervous system (CNS) midline (Box 1) is established.

During Zebrafish neurulation, a number of studies have shown that neuroepithelial cells polarize in a gradual way. Neural progenitors in zebrafish are known to undergo progressive epithelialization. The neural plate comprises two cell layers. Deep cells are columnar and attached with the basement membrane and superficial cells lie underneath the enveloping layer. During the neural plate/keel stages, as deep and superficial cells from the two sides converges towards dorsal midline, they generate active membrane protrusions that help cells to intercalate to the contralateral side of the midline. A study proposed that N-cadherin is required for the stabilization of these protrusions and loss-of-function of N-cadherin led to the failure of intercalations (Hong and Brewster, 2006).

Box 1: The CNS midline: an operational definition

In the context of the present manuscript, I will be using the term “midline” to refer to the apical side of the neuroepithelial cells where apical polarity determinants accumulate. The midline is the precursor of the future lumen. In wild-type tissue, the midline coincides with the geometric centerline where the two contralateral sides of the CNS meet.

Similarly, another study also showed that the tight junction-associated protein ZO1 and the adherence junction protein N-cadherin gradually become stabilized at 15 somite stage that is followed by another complex of Nok (the zebrafish Pals1 homologue) and Lin7c (a scaffolding protein associated with the Crumbs complex) proteins (Yang et al., 2009). The midline localization of these proteins is then further refined over time so that by the neural rod and neural tube stages these polarity proteins become localized to the emerging apical surface

(Geldmacher-Voss et al., 2003; Munson et al., 2008; Tawk et al., 2007; Yang et al., 2009). In contrast, the basal marker Glial fibrillary acidic protein (GFAP) is exclusively expressed at the basal extremes of neuroprogenitors cells (Tawk et al., 2007).

3.4.2 Polarized cell divisions during neurulation

In zebrafish neurulation, following mediolateral convergence, neural progenitors successively undergo a uniquely characterized and highly stereotyped medio-laterally oriented cell divisions. These divisions result in a deposition of one daughter cell on either side of the midline (Ciruna et al., 2006; Concha and Adams, 1998; Tawk et al., 2007). As one of the two daughter cells crosses the midline, these divisions are called midline crossing divisions or C-divisions. The daughter cell that remains on the ipsilateral side, stays connected to the basal membrane through a thin projection while the other daughter cell that crosses the midline has to establish the connection to the basal membrane of the contralateral side (Tawk et al., 2007).

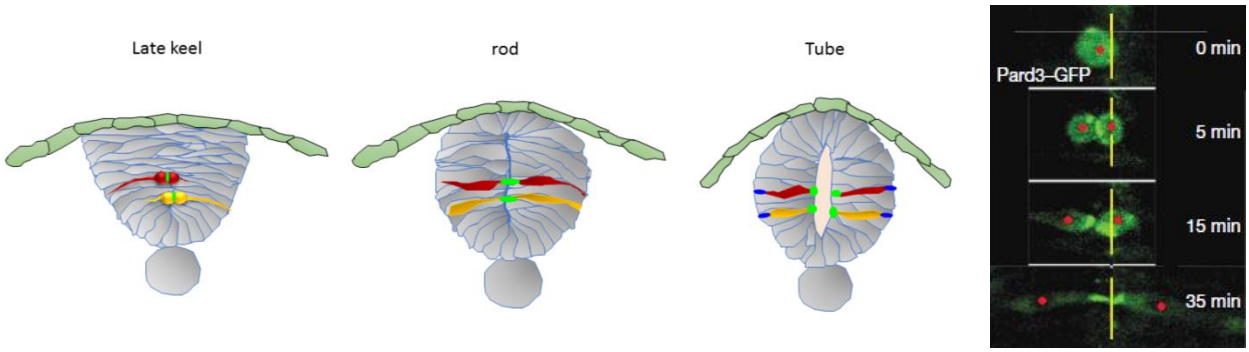


Figure 15: Subcellular distribution of Pard3 during neural tube morphogenesis
A schematic of transverse section of the zebrafish neural tube showing mirror-symmetric C-divisions during the morphogenesis of neural tube. Apical localization of Pard3 is shown in green and basal localization of GFAP is shown is blue. At the right side, a time-lapse sequence showing Pard3-GFP accumulation throughout a C-division; Pard3-GFP is distributed across the cleavage plane and inherited at the apical tips of the two daughter cells. The midline is shown in yellow. The right side image is adapted from (Tawk et al., 2007).

These specialized C-divisions were considered to be a driving force in the morphogenesis of the neural tube and for the establishment of apico-basal polarity. As, C-dividing cells generate mirror symmetric apico-basal polarity while integrating into the contralateral sides of the neural tissue. Tawk and colleagues showed that during C-divisions, Pard3 accumulates at the cleavage furrow of these dividing cells and after cytokinesis remains enriched at the apical end of the daughter cells *(*Figure 15*)*. Therefore, they suggested that C-divisions are crucial for the neural tube midline formation and the establishment of apico-basal polarity. In division-blocked

(over the period of C-divisions) wild-type embryos, however, cells are still able to generate a lumen with largely normal apico-basal polarity (Buckley et al., 2013; Ciruna et al., 2006). Hence, it appears that the localization of apical proteins at the forming midline can occur in a division-independent fashion. Therefore, to understand the molecular mechanisms through which neural progenitors cells acquire apico-basal polarity needs further investigation. It should however be noted that even if C-divisions are found to be dispensable for apico-basal polarity, they provide a morphogenetic advantage during lumen formation (Buckley et al., 2013).

Epithelial cells are also polarized within the plane of epithelium i.e. orthogonal to the apico-basal polarity, a polarisation termed planar cell polarity. An important study presented evidence that C-divisions are dependent on the Planar Cell Polarity (PCP) pathway that is crucial for the neural tube morphogenesis (Ciruna et al., 2006). In the next section, therefore, I will introduce the components and functions of the planar cell polarity pathway.

4 Planar cell polarity

Vertebrate embryogenesis incorporates numerous collective and individual cell movements. Neurulation and its associated tissue movements, for instance convergence and extension movements are among such processes. In addition, stereotypical oriented cell divisions contribute importantly to morphogenesis. The planar cell polarity (PCP) pathway has been found to be crucial for oriented cell movements and divisions. While studying the function of Notch signaling in the regulation of apico-basal polarity we observed that the loss-of-function of the Notch signaling component Mindbomb led to a PCP mutant like phenotypes. This observation led me to investigate a potential link of Notch signaling with PCP signaling. In the following section, I will discuss the components and function of the PCP pathway, with a particular emphasis on zebrafish neural tube development.

4.1 The Planar Cell polarity Pathway in *Drosophila*

In multicellular organisms, epithelial cells are not only polarized along the apico-basal axis, but also within the epithelial plane, a phenomenon called planar cell polarity (PCP). Two features describe PCP best: First, cells cooperatively align their respective individual polarity. Secondly, this polarized alignment occurs in a particular orientation with respect to the overall organization of the tissue, indicating the existence of global cues. The signaling molecules that are in charge of regulating the establishment and maintenance of planar cell polarity form the PCP signaling pathway.

Gubb and Garca-Bellido first described the PCP signaling pathway in the *Drosophila* adult wing as a small set of genes that controls the polarity of cuticular hairs and bristles(Gubb and García-Bellido, 1982). In the *Drosophila*, each cell has a hair that points posteriorly on the body surface and distally on the appendages (Figure 16) (Eaton, 2003). In *flies*, the abdomen, the eye and the bristles of the notum are among other well-studied systems for PCP. In the *Drosophila* eye, each ommatidium has eight photoreceptor cells that are arranged in an oriented pattern (Strutt, 2003).

PCP signaling pathways consist of three functional modules: a global directional cue module, a core module, and one of many tissue specific effector modules. *Table 1* gives a summary of the PCP signaling components and a model of establishing planar polarity is shown in Figure 16.

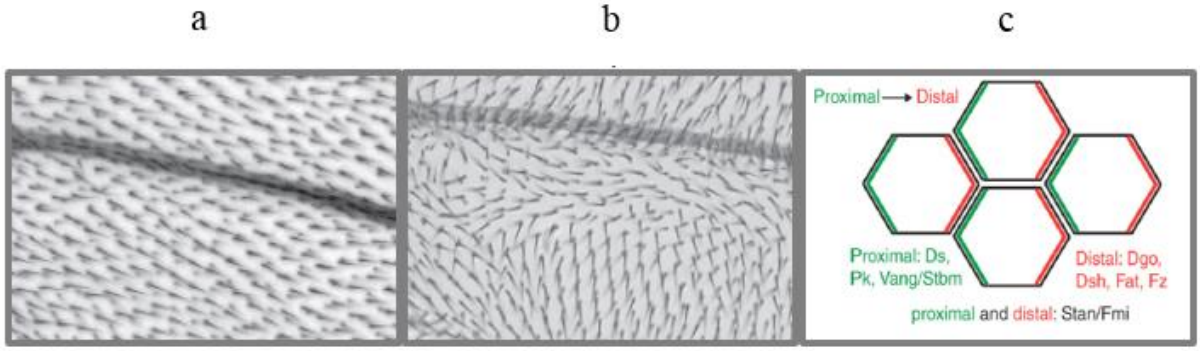


Figure 16: PCP in the *Drosophila* wing
(a). Wild-type wing showing nearly parallel alignment of wing hairs pointing distally; (b). Global disruption of wing hair alignment in VanGogh (Vang) mutant; (c). Subcellular localization of PCP proteins in the *Drosophila* wing epithelium. Proteins localize at proximal or distal faces (Wang and Nathans, 2007).

— A **global module** links the direction of polarization and the tissue axis. The main components belonging to this module are the atypical Cadherins Fat (Ft) and Dachsous (Ds), and the Golgi resident protein Four-jointed (Fj) (Figure 17). The primary function of the global module in PCP is to translate tissue-wide transcription gradients into subcellular gradients of one or more key signaling molecules along the tissue axis. Ds and Ft are expressed in oppositely oriented gradients that form heterodimers linking the surfaces of adjacent cells. This interaction provides directional information, which is then converted into oriented subcellular asymmetries that are interpreted by downstream signal transducers. The golgi resident protein Fj acts as an ectokinase on both Ft and Ds to make Ft a stronger ligand and Ds a weaker ligand. Therefore, the graded expression of Ft and Ds results in a larger fraction of Ft-Ds heterodimers in one orientation relative to the other. The mutant phenotype of Ft, Ds and Fj in *Drosophila*, includes cells that are capable to polarize with respect to their neighbors, but fail to acquire a global polarity within the tissue (Cho and Irvine, 2004).

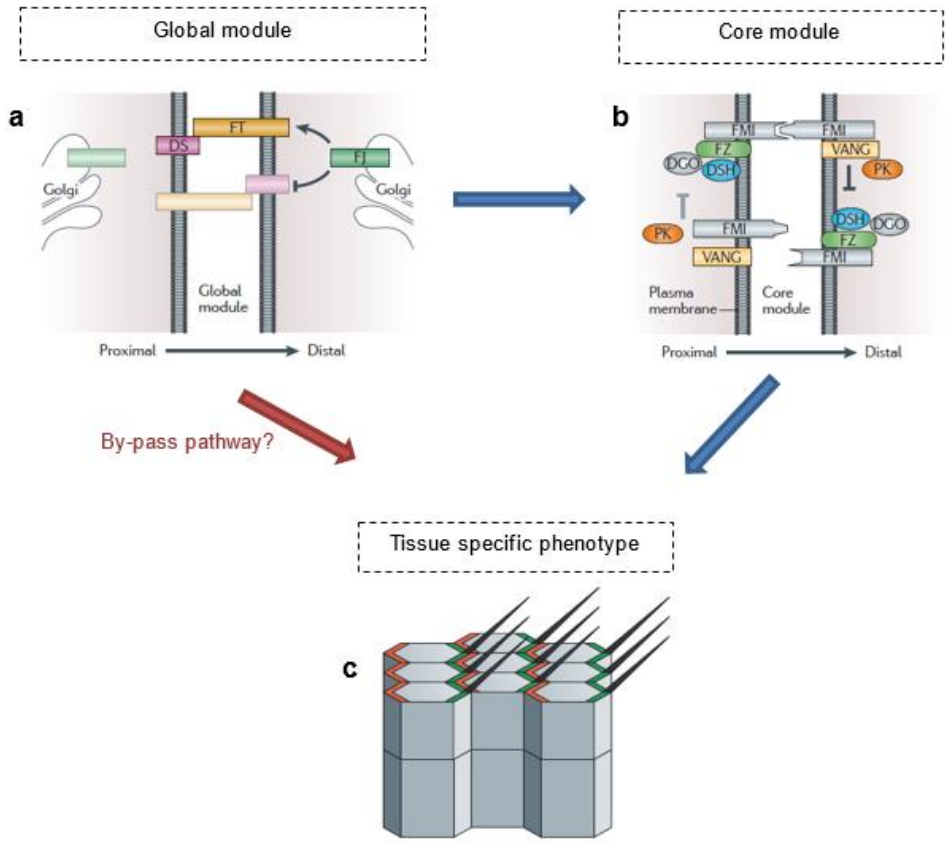


Figure 17: A modular model of PCP in *Drosophila*
The linear model (represented by blue arrows) postulates that the global module shown in (a). acts through the core module (b). that in turn acts at the tissue-specific level. Wing cells are depicted with properly oriented wing hairs (c). The bypass model (Red arrow) suggests that the global module can act independently from the core module, generating a signal that can be directly interpreted by the tissue (Bayly and Axelrod, 2011).

— A **core** module is responsible for generating sub-cellular asymmetry and acts to coordinate polarization between adjacent cells (Figure 17). Members of the core module mainly include the seven-pass-transmembrane protein Frizzled (Fz), the multi-domain protein Dishevelled (Dsh), the Lim domain protein Prickle (Pk), the four-pass-transmembrane protein Vang Gogh/Strabismus (Vang/Stbm), the Ankyrin repeat protein Diego (Dgo) and the seven-pass-transmembrane atypical Cadherin Flamingo/Starry night (Fmi/Stan). These proteins localize and communicate with each other at the cell boundaries, recruiting a group of interaction partners to the distal side and another to the proximal side and mutually excluding the oppositely oriented complexes, thereby aligning the polarity of adjacent cells. Fz and Dsh additionally function in the Wnt signaling pathway that involves GSK-3, Axin, and β-Catenin.

Therefore, the PCP pathway is also known as the non-canonical Wnt pathway, distinguished from the canonical Wnt/β-Catenin pathway (Cadigan and Nusse, 1997).

— There are several tissue specific **effector** modules that work downstream of the core and global modules. These downstream mediators receive the polarity information from the core PCP complexes to give rise to morphological asymmetries in the tissue.

Components	**Gene description/Function**
Core module	
Frizzled (Fz)	Seven pass transmembrane domains, Wnt receptor; recruits Dsh and Dgo to the membrane
Dishevelled (Dsh)	Cytoplasmic protein having scaffolding abilities; binds Fz, Pk, Vang and Dgo
Prickle (Pk)	Cytoplasmic protein, recruited to the membrane by Vang; interacts with Dsh, Vang and Dgo
Van Gogh/Strasbismus (Vang)	4-pass transmembrane protein, binds Pk, Dsh and Dgo
Diego (Dgo)	Cytoplasmic Ank repeat protein; binds Dsh, and Vang
Flamingo/Starry night (Fmi/Stan)	Cadherin with seven pass transmembrane receptor features, Homophilic cell adhesion
Global module	
Fat (Ft)	Atypical Cadherin, binds Ds
Four jointed Fat (Fj)	Type II transmembrane protein, Golgi resident luminal kinase
Dachsous (Ds)	Atypical cadherin, binds Ft

Table 1: PCP signaling pathway components in *Drosophila* (Maung and Jenny, 2011).

4.2 Planar cell polarity in vertebrates

In vertebrates, PCP signaling has been found to operate in several contexts, such as morphogenetic movements during anterior-posterior axis elongation (convergent-extension, CE), polarization of skin and hair follicles, and positioning of motile and sensory cilia. Convergent- extension movements during vertebrate gastrulation play a major role in carving the embryonic body. CE movements are essential for various polarized cell behaviors, including

directed cell migration and mediolateral/radial cell intercalation. In this section, I will explain first the molecules that regulate PCP-dependent processes in vertebrates, then go on to discuss the importance of CE movements, and oriented cell divisions for vertebrate embryonic development.

4.2.1 The Wnt/PCP pathway in vertebrates

Wnt signaling activates several pathways that are categorized as either canonical (Wnt- β-catenin pathway) or β-catenin independent noncanonical pathways. The Wnt/PCP pathway is one of the non-canonical Wnt pathways. While PCP has been studied extensively in *Drosophila,* no fly Wnt ligand has been found to be implicated in PCP signaling. In contrast, some Wnt ligands mediate PCP signaling in vertebrates. A summary of the most important Wnt/PCP core components in vertebrates is shown in Table 2.

— **Core components**

Like in *Drosophila,* Fz and Dvl are also core components of Wnt/PCP signaling in vertebrates. Fz2 and Fz7 act as Wnt5 and Wnt11 receptor during CE in zebrafish and *Xenopus* (Djiane et al., 2000; Heisenberg et al., 2000; Kilian et al., 2003). In *Xenopus*, a mutated Dishevelled (Dsh/Dvl) and a dominant negative form of Wnt11 (dn-Wnt11) revealed their functional role in the correct elongation of the body-axis. Additionally, fine-tuning of Dsh is required for the polarization of cells undergoing mediolateral cell intercalation during *Xenopus* CE movements (Wallingford et al., 2000). In zebrafish, silberblick (slb)/Wnt11 activity is required for cells to undergo correct convergent extension movements during gastrulation (Heisenberg et al., 2000).

Trilobite/Vangl2 and Pk are also found to serve in Wnt/PCP signaling in vertebrates. In mouse, Looptail (Lp) mutants exhibit neural plate closure defects. The Lp locus was identified as a homologue related to flies Vang/Stbm and therefore, named Vangl2 (Kibar et al., 2001). Trilobite (Tri) depletion causes CE defects in *Xenopus* (Goto and Keller, 2002) and zebrafish (Jessen et al., 2002). In zebrafish, *tri* mutants display a shortened body axis and a fusion of the eyes at pharyngula stages that is enhanced in *silberblick* (*slb)* (Heisenberg and Nüsslein-Volhard, 1997) and *knypek* (*kny*)(Marlow et al., 1998) mutants. Similarly, Pk loss-of-function and gain-of-function causes CE defects (Carreira-Barbosa et al., 2003).

Three *flamingo (fmi)* homologs in vertebrates belong to a class of adhesion G-Protein Coupled Receptors (GPCRs). Fmi comprises a Cadherin repeat containing extracellular domain and a seven pass transmembrane domain. The vertebrate Flamingo homologues Celsr1a and Celsr1b further regulate CE movements during zebrafish gastrulation (Curtin et al., 2003; Formstone and Mason, 2005).

— **Other components**

Ds and Ft homologues have been described in mice but their exact role has not yet been thoroughly investigated. In zebrafish and Xenopus, Glypican4, a protein belonging to the GPI-linked heparin-sulfate-proteoglycan family, is suggested to be a Wnt co-receptor along with Fz in the Wnt/PCP pathwayv(Ohkawara et al., 2003) and controls cell polarity during gastrulation movements in zebrafish (Topczewski et al., 2001). In addition, vertebrate homologues of *diego, diversin/inversin* (*div/inv)* have been also described (Simons et al., 2005). In mammals, an additional role of the basolateral polarity protein Scribble has been identified in the regulation of PCP, where transheterozygous scrib:vangl2 embryos exhibit a disruption in the polarization of stereocilia bundles in the mouse cochlea (Montcouquiol et al., 2003).

Components	Gene description/Function
Frizzled-7 (Fz7)	Seven pass transmembrane domains, Wnt receptor;
Disheveled (Dsh/Dvl)	Cytoplasmic scaffolding protein
Prickle1, Prickle 2 (Pk)	Cytoplasmic protein with LIM and PET domains
VanGogh like-2/Trilobite (Vangl2/Tri)	4-Pass transmembrane protein
Celsr1, Celsr2	Atypical Cadherin, seven pass transmembrane protein
Wnt11/Silberblick (Slb)	Secreted Wnt glycoprotein
Wnt5b/Pipetail (Ppt)	Secreted Wnt glycoprotein
Ryk	Wnt co-receptor
Diversin	Ankyrin repeat protein
Scribble	Scaffolding protein

Table 2: Major components of the PCP signaling pathway in vertebrates (Wang and Nathans, 2007)

4.2.2 Downstream effectors of vertebrate Wnt/PCP signaling

The Wnt/PCP pathway integrates polarity cues that are provided through core and global module proteins. These planar cues are transplated into tissue-specific readouts such as hair follicle orientation in *mammals* or convergent-extension movements during gastrulation in several vertebrates. The tissue specific readouts rely on genes that are commonly called secondary PCP genes or PCP effectors. These genes do not necessarily have a function that is restricted to the PCP pathway. Many of these genes act to modulate cytoskeleton machinery. Few examples of these tissue specific effector genes are discussed here.

Components	Description/function
Daam1	Formin-homology domain protein
RhoA	Small GTPase
Rho kinase 2 (Rok2)	Serine/Threonine Kinase, RhoA effector
Jun N-terminal kinase (JNK)	Serine/Threonine Kinase
Inturned (In)	Putative PDZ domain protein
Fuzzy (Fy)	4-pass transmembrane protein
Misshapen (Msn)	STE20-like protein kinase
Paraxial protocadherin (papc)	Protocadherin

Table 3: Wnt/PCP effectors in vertebrates (Wang and Nathans, 2007)

During vertebrate gastrulation, the scaffolding protein Dsh/Dvl acts in the Wnt/PCP pathway through its PDZ and/or Dishevelled, Egl-10 and Pleckstrin (DEP) domain. *daam1*, which encodes a formin homology protein binds Dsh through the PDZ and DEP domains and activates RhoA upon binding to it (Habas et al., 2001). In *Xenopus*, Daam1 controls CE movements by the activation of RhoA and Rac (Habas et al., 2003); however, Daam has not been found to be mandatory for PCP in flies (Matusek et al., 2006). Downstream of Wnt11 and Wnt5b, there is a specific activity of the Rho family of small GTPases in the regulation of CE movements. Studies in *Xenopus* and zebrafish have shown that Rok2 acts downstream of Wnt11 (Jessen et al., 2002), while Wnt5a needs the Jun N-terminal Kinase (JNK) (Yamanaka et al., 2002). This pathway may also involve the transcriptional activation of target genes such as *papc* (Medina et al., 2004).

Other effectors identified in vertebrates include *inturned (in), fuzzy (fy)* and *misshapen (msn)*. In zebrafish, In and Fy cooperate with the core module proteins Fz-Dvl to regulate ciliogenesis by

coordinating apical actin assembly in cells with motile cilia (Park TJ et al., 2006). Inhibition of msn, which encodes a STE20-like serine/threonine protein kinase, results in an epiboly defect. This is mainly caused by the depletion of actin cable formation at the leading edge of the enveloping layer (EVL) cells that is a major driving force for epiboly movements (Köppen et al., 2006). *Table* 3 below summarizes these and other effectors described in vertebrates.

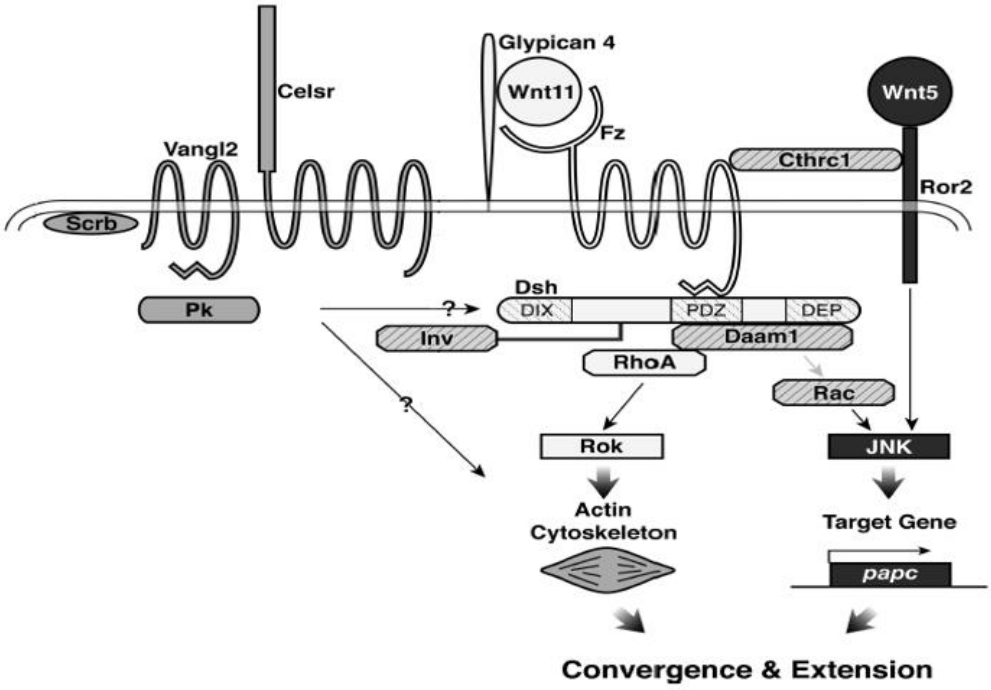


Figure 18: Model of the Wnt/PCP pathway during CE in Zebrafish and Xenopous
This model is composed of at least three major branches mediating CE. First, in the centre, the secreted ligand Wnt11, possibly aided by Glypican-4 binds the receptor Fz7 and activates Dsh. Daam1 utilizes the PDZ and DEP domains of Dsh to activate the downstream effectors RhoA and Rok to control the actin-cytoskeleton. Daam1 can also activate Rac which in turn induces JNK. Alternatively, as shown in the second branch (right side of the model), Wnt5 can bind the receptor Ror2 and activate JNK. Activated JNK, subsequently transcriptionally activates the protocadherin papc, a functional mediator for CE. The third branch (left side of the model) includes the other components of the core module. Our current knowledge is limited about this branch whether these proteins acts upon the cytoskeleton through their own effectors or whether they individually/collectively influence the other two streams. (Tada and Kai, 2009)

From a molecular point of view, the vertebrate PCP pathway is unlikely to be a linear pathway, especially in the context of gastrulation and neurulation. The description of a hypothetical model for CE movements in vertebrates shown in Figure 18 comprises at least three branches: (1) Wnt11-Fz-Dsh, (2) Wnt5-Ror2-JNK, and (3) Other core PCP components (Tada and Kai, 2009).

— The Wnt11 ligand binds the receptor Fz7 and activates Dsh. Glypican promotes Wnt11 binding. The PCP effector protein Daam1 binds Dsh through its PDZ and DEP domains. This interaction further activates the downstream effector RhoA that in turn stimulates Rok to regulate the actin-cytoskeleton that is important for CE movements. Alternatively, Daam1 can act upon Rac that further activates JNK. Alternatively, Wnt5b binds Ror2 that activates JNK. Activation of JNK induces the transcription of the target gene *papc* that encodes a protocadherin that acts as a functional mediator for CE movements.

— Other components of the core module including Vangl2, Celsr, Scrib, and Pk might interact with the Wnt/Fz-Dsh module. However, it is not well known whether they facilitate the Wnt-Fz-Dsh stream or directly influence the cytoskeleton. Inversin contributes to CE movements in *Xenopus* gastrulation while inhibiting the canonical Wnt pathway by targeting Dsh for degradation. Thus, Inversin (Inv) acts a molecular switch between different Wnt signaling cascades (Simons et al., 2005).

4.2.3 A new addition to the Wnt/PCP pathway: Ryk

In the course of my study, I observed that Mib (an E3-ubiquitin ligase that is an important component of the Notch pathway) loss-of-function embryos display PCP mutant-like phenotypes (see Results section for details). This raised the question, how Mib could be related to the PCP pathway. Recently a study in *C. elegans* reported that Mib interacts with the Receptor related to tyrosine kinase (Ryk) to promote its ubiquitination and to activate Wnt/β-catenin signaling (Berndt et al., 2011). This is an interesting link as previously several other studies from f*lies* to *mammals* have described Ryk as an important component of the PCP pathway. Ryk has been shown to act as a Wnt-co receptor, whose activity is likely to signal independently of Frizzled-activated pathways (Inoue et al., 2004; Lu et al., 2004; Yoshikawa et al., 2003).

Structurally, Ryk comprises a transmembrane domain, an extracellular domain with five potential N-linked glycosylation sites (similar to the Wnt inhibitory factor 1 Wif1) and an intracellular fragment with multiple domains including a protein tyrosine kinase (PTK) domain (Hovens et al., 1992). Ryk has been identified as key guidance receptor in axonal guidance in mice, where Wnt5a acts as a chemo-repulsive ligand for Ryk to drive callosal axons towards the contralateral brain hemisphere (Keeble et al., 2006).

Although most studies have proposed antagonizing roles of Ryk and Fz in Wnt-mediated developmental processes, such as *Wnt5b*-driven *Drosophila* salivary gland migration (Harris and Beckendorf, 2007), synergies between Ryk and Fz have also been reported. For example, Ryk promotes Fz-dependent signaling in *Xenopus*, where knockdown of Ryk leads to gastrulation defects with compromised Wnt11-induced Fz7 and Dvl endocytosis (Kim et al., 2008).

In zebrafish, Ryk acts downstream of Wnt5b. Ryk-deficient embryos display defects in Wnt5b-induced directional cell movements at gastrulation (Lin et al., 2010). The same study showed that Ryk, unlike Fz, needs a Wnt5b stimulation to recruit Dvl to the cell membrane, suggesting that Fz and Ryk act in separate pathways. Andre and colleagues revealed another important link between Ryk and the Wnt/PCP pathway. They showed that Ryk interacts with Vangl2 genetically and biochemically, and this interaction is induced by Wnt5a. In *vangl2* heterozygous mutant mouse embryos, Ryk depletion results in classic PCP defects including an open neural tube (Andre et al., 2012). Another study by Macheda and colleagues confirmed the potential role of Ryk in Wnt/PCP signaling during mammalian neuronal development (Macheda et al., 2012). Therefore, Ryk is an important candidate that could provide insights about how Mib is linked to PCP signaling.

4.3 Convergent-Extension and the Wnt/PCP pathway

The vertebrate Wnt/PCP pathway has been shown to regulate different morphogenetic movements such as convergent-extension (CE). CE is a crucial process through which tissues undergo narrowing along one axis and simultaneous extension along the orthogonal axis. These movements occur in both epithelial and mesenchymal tissues during embryogenesis in invertebrates and vertebrates, and play major roles in shaping the body plan (Irvine and Wieschaus, 1994). The first identified and best-studied example of CE movements in development is body axis elongation during *Xenopus* gastrulation (Keller, 2002). Generally, CE movements are characterized by collective movements of cells towards the dorsal side of the gastrula, promoting hence the elongation of the future antero-posterior body axis. The combination of collective cell movements and cell intercalations triggers a narrowing of the body-axis along its medio-lateral (ML) axis that is termed convergence, and a concomitant elongation along the anterior-posterior (AP) axis termed extension. CE thus includes two kinds of movements (Figure 19): First, a collective cell migration that is the coordinated movements of

a highly cohesive sheet of cells and involves no neighbor exchange. Here, the cells at the leading edge are highly polarized and give rise to various types of protrusions, such as lamellipodia and blebs. Second, cell intercalations promote the oriented exchange of neighboring cells.

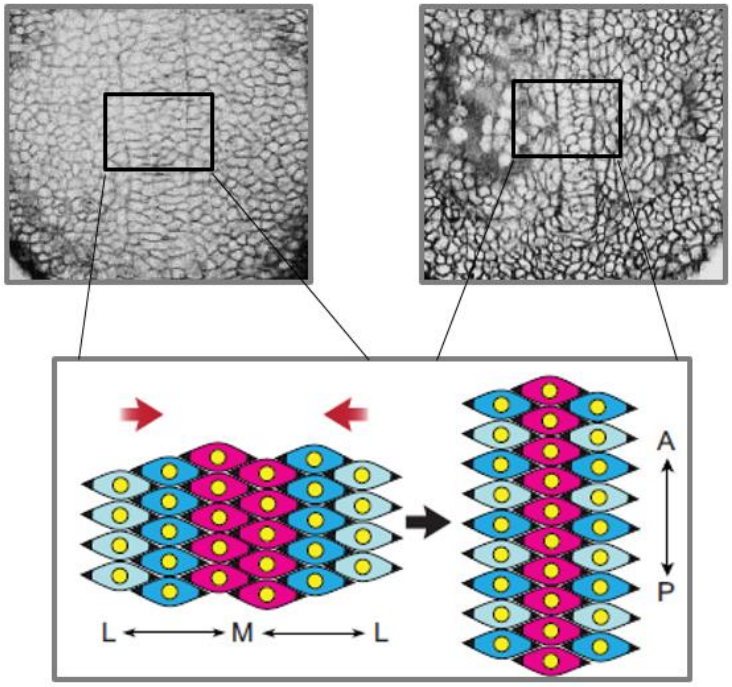


Figure 19: Convergent-extension movements in Zebrafish
During cell intercalation, cells orient along the Medio-Lateral (ML) axis and intercalate to redistribute their position along the Antero-Posterior (AP) axis of the tissue. At the left side, the forming notochord of a 9.5 hpf zebrafish embryo is shown, where cells along the ML axis undergo convergence. At the right side, the same embryo is shown at 10.2 hpf. While, convergence movements narrowed down the ML axis, the AP axis is elongated. Adapted from (Glickman et al., 2003; Tada and Heisenberg, 2012).

During early zebrafish gastrulation, prechordal plate progenitor cells undergo collective cell migration from the germ ring margin towards the animal pole, hence elongating the body axis. Moreover, convergence movements occur during gastrulation as mesendodermal progenitor cells collectively migrate towards the dorsal region. While getting closer to the forming body axis, the cells polarize along their ML axis and display progressively coordinated and oriented convergence movements. At the same time, mesendodermal cells near the notochord begin ML intercalations and radial cell intercalations that contribute to the elongation of the posterior body

axis. Thus, collective mesoendodermal cell migration and intercalation are both crucial features of CE movements during zebrafish gastrulation (Sepich et al., 2005).

Wnt/PCP signaling has been shown to be of prime importance for convergent-extension movements by controlling cell movement persistency and ML cell polarization during cell intercalations (Heisenberg et al., 2000; Jessen et al., 2002; Sepich et al., 2005; Topczewski et al., 2001). In addition, inhibition of Wnt/PCP signaling alters cell polarization and cohesion of prechordal plate progenitors (Ulrich et al., 2005).

4.4 Oriented cell divisions and Wnt/PCP signaling

4.4.1 Oriented cell divisions during zebrafish gastrulation

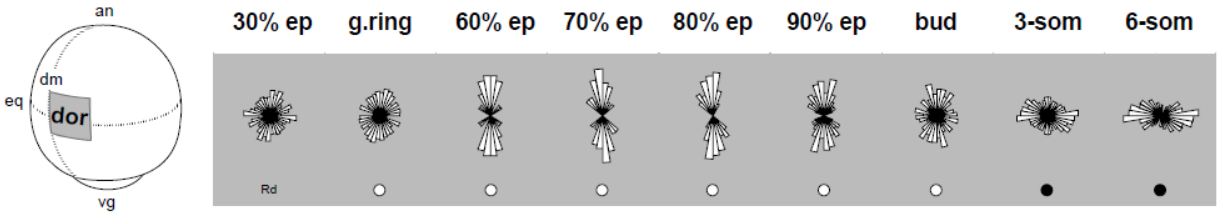


Figure 20: Cell division orientation during zebrafish gastrulation
During gastrulation and the beginning of neurulation, cell divisions are highly oriented. The orientation of cell division of the epiblast cells at the level of the dorsal midline is shown here. At the beginning of gastrulation, cell divisions occur at random directions that become more oriented towards the antero-posterior (AP) axis at around 60% epiboly. Later, as neurulation begins, the axis of cell division changes from AP to medio-lateral (Concha and Adams, 1998).

In *Drosophila* sensory bristle precursor cells, PCP is responsible for orienting asymmetric cell divisions (Bellaïche et al., 2004). Similarly, Wnt/PCP signaling has been shown to be important for orienting cell divisions in the zebrafish dorsal epiblast cells during gastrulation. These cells divide along the AP axis (Figure 20*)*, and this stereotypical orientation of dividing cells is compromised in Wnt/PCP mutant embryos (Gong et al., 2004). Another study showed that zebrafish maternal zygotic double mutants for Fz7a and Fz7b (MZFz7a/b) exhibit impaired CE movements and displayed defects in body axis elongation and gastrula stage cell division orientation (Quesada-Hernández et al., 2010). Recently, a study revealed that the Anthrax toxin receptor 2a (Antxr2a) interacts with the Wnt/PCP pathway via RhoA and its effector Rock2,

regulating hence the formation of a cortical actin-cap that is oriented along the AP axis (Castanon et al., 2013)

4.4.2 Oriented cell divisions during zebrafish neurulation

In zebrafish, during gastrulation cells divide parallel to the AP axis. When neurulation starts, cell division orientation shifts from AP to ML axis with a 90°rotation in the mitotic spindle (Geldmacher-Voss et al., 2003). This transition occurs at the bud stage and when the neural plate converges to form, the neural keel (3-6 somites) cells divide predominantly with parallel to the ML axis. During the progression of neural keel and neural rod formation, dividing cells also become restricted to the region close to the dorsal midline (Figure 21) (Concha and Adams, 1998). These divisions, also exhibit the unique characteristic that the dividing cells deposit one daughter cell on the contralateral side of the neural keel/neural rod. These cross-midline divisions of neural progenitors are therefore termed C-divisions.

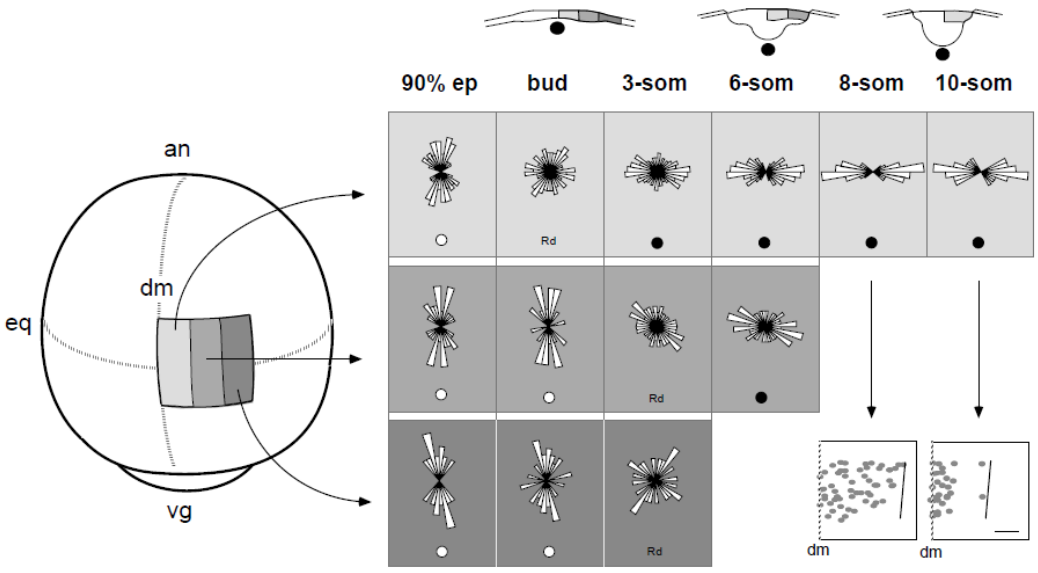


Figure 21: Cell division orientation during zebrafish neurulation
The orientation of cell division during neurulation vary as a function of both time and distance from the dorsal midline. During neurulation, the orientation of dorsal cell divisions switches from antero-posterior to medio-lateral. This is first visible at the neural plate stage, where cells divide primarily close to the dorsal midline (bud to 3 somites). Gradually divisions occur throughout the neural plate as the neural plate forms the neural keel. Subsequently, as neurulation continues, cell divisions become more restricted to the region close to the dorsal midline (dm) (Concha and Adams, 1998).

Orientation of C-divisions

As previously mentioned, Concha and Adams presented the first study describing the orientation of C-divisions during zebrafish neurulation (Concha and Adams, 1998). The rotation in the mitotic spindle is responsible for orienting the C-dividing neural progenitors along the ML direction (Geldmacher-Voss et al., 2003). Wnt/PCP signaling is critical for the stereotypical ML orientation of the C-divisions and for the neural rod midline formation. Quesada-Hernandez and colleagues revealed a role of Fz7 signaling for the orientation of C-divisions. They showed that maternal zygotic double mutant embryos for *fz7a* and *fz7b (MZfz7a/b),* display a significant reduction in the stereotypical orientation of dividing neural progenitors in the forming neural rod, resulting in a severely affected neural tube morphology with the branched midline and disorganized lumen (Quesada-Hernández et al., 2010).

Location of the C-divisions

As stated above, C-divisions occur across the forming midline parallel to the ML axis at the neural keel and neural rod stages. These developmental stages are characterized by the following two events:

— C-divisions occur parallel to the ML axis, across the forming midline. Interestingly this location also coincides with the apical domain of the dividing cells. Hence, there is an intriguing possibility that apico-basal polarity of neural progenitors and their C-divisions influence each other. (see section 3.4)

— A second event is that the neural plate converges towards the midline and elongates along the AP axis to form the neural keel and subsequently, the neural rod. These convergent-extension movements are PCP signaling dependent as described in section 4.3. As C-divisions occur at the time when the neural plate is undergoing convergent-extension, PCP signaling also influences C-divisions. Indeed, Ciruna and colleagues showed that polarization of dividing cells along the AP is required prior to mitosis, and that Wnt/PCP signaling is responsible for such polarization. They used a candidate approach to identify VangGogh-like 2/Strabismus (Vangl2/Stbm) as a key molecule involved in this process. They showed that in maternal zygotic *trilobite* mutant (MZ*tri*) embryos, neural keel cells fail to polarize, which further disrupts intercalation and bilateral distribution of (potentially) C-dividing daughter cells. This ultimately leads to defects in midline formation, in this case the

formation of two ectopic neural tube midlines, one in each lateral half of the folding neural plate (Ciruna et al., 2006). Similarly, Tawk and colleagues also showed that in MZtri mutants, delayed convergent-extension movements are responsible for defects in neural tube midline formation (Figure 22) (Tawk et al., 2007).

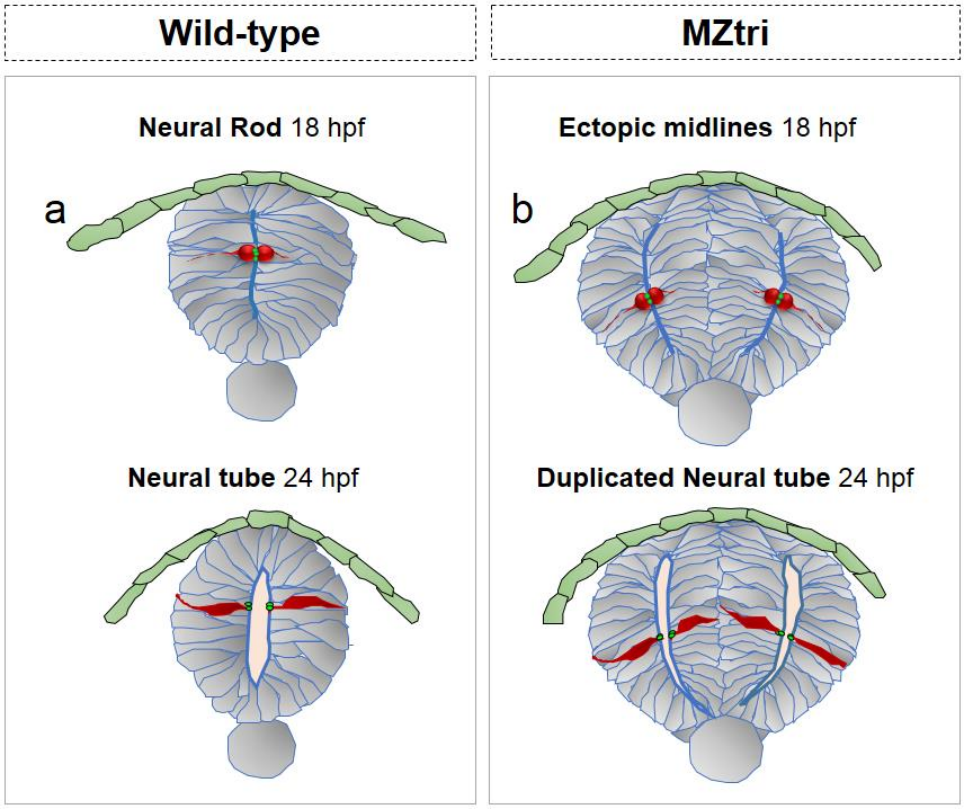


Figure 22: Double midline formation in zebrafish MZtri mutants.
In comparison to wild type (a), maternal zygotic trilobite mutants (*MZtri*) (b) exhibits delayed convergent-extension of neural plate cells. The delay in convergence causes the cells to undergo cell division in more lateral locations, rather than at the centre of the folding neural plate. This, results in two populations of C-dividing cells that give rise to two ectopic, more laterally situated midlines.

5 Scientific context of the study

Epithelial polarity on cellular (apico-basal polarity) and tissular (planar cell polarity) are the crucial factors for the proper morphogenesis of the neural-tube. How signalling pathways for instance Notch signalling pathway, contribute to the organ morphogeneis through regulating polarity is a fascinating field to be explored. Though, I started the project with the aim to understand the transcytosis of Delta ligands for the Notch signalling (Results-section 1). This study mainly culminated in two major findings: Firstly, Delta-Notch signalling is required to

establish apico-basal polarity in the zebrafish neural tube (Results-section 2). Secondly, the Notch signalling component Mindbomb plays an additional role in PCP signalling that is independent of its well known function (endocytosis of Delta ligand) in Notch signalling (Results-section 3). Furthermore, I attempt to understand that C-dividing cells might communicate with each other through Notch signalling (Results-section 4).

Results

While it is well established that Delta ligand endocytosis is required for Notch receptor activation, the actual reason for this requirement remains unknown. A number of recent studies have proposed that, in the context of apico-basally polarized neuro-epithelial cells, Delta endocytosis and intracellular transcytosis could be required to bring together basally secreted Delta ligands and apically localized Notch receptors (Benhra et al., 2010; Heuss et al., 2008; Wang and Struhl, 2004). Transcytosis transports molecules via endocytic internalization, intracellular transport, and exocytic re-secretion thus ensuring the intracellular translocation of membrane-bound molecules between the apical and baso-lateral compartments of epithelial cells. The initial aim of my Ph.D. project was to study the potential role of the intracellular transcytosis and apico-basal transport of Delta ligands for Notch signaling in the zebrafish nervous system. During the course of my study, I observed however that conversely, Notch signaling itself is important for the establishment of apico-basal polarity in the zebrafish neural tube. In addition, I surprisingly discovered a unique role of the Notch signaling component Mindbomb (Mib) in planar cell polarity (PCP) that is independent of its function of Notch signaling.

Below, I will first summarize my results regarding DeltaD trafficking and transcytosis. I will then describe the results related to apico-basal polarity and Notch signaling. Finally, I will show the results related to the role of Mib in planar cell polarity.

1 Trafficking of DeltaD ligands in the zebrafish nervous system

1.1 Live imaging of endogenous DeltaD ligand transcytosis

The proposed model of vectorial DeltaD transcytosis involves two subsequent trafficking steps: First, a basal-to-apical transendocytosis that translocates Delta ligands from their initial site of secretion at the baso-lateral membrane to the apical cell surface. Second, an endocytic internalization of Delta ligands from the apical cell surface that may potentially promote Notch receptor activation (Benhra et al., 2010). To study the trafficking of endogenous DeltaD ligands along the apico-basal axis a functional DeltaD transcytosis assay was developed in our lab (Figure 23).

In this assay, an unlabeled primary antibody recognizing endogenous DeltaD is first injected outside of the neural tube, i.e. facing the basolateral side of the epithelium. In a second step, a specialized fluorescent secondary antibody is then injected into the apical neural tube lumen. Following the baso-lateral internalization and the basal-to-apical transcytosis of DeltaD ligands, the piggybacked primary antibody becomes exposed to the luminal secondary antibody. Consequently, the second round of apical DeltaD endocytosis results in the internalization of the fluorescent secondary antibody into the cells of the neuro-epithelium.

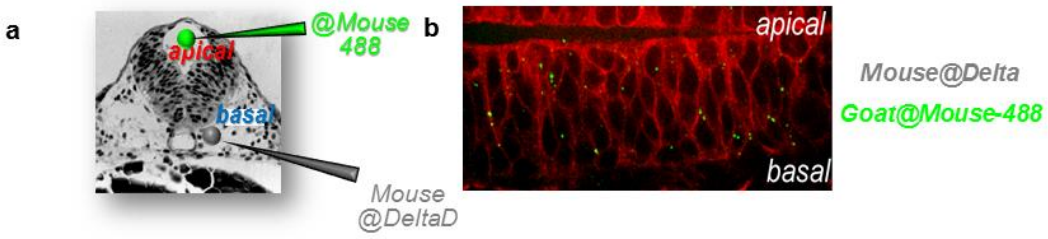


Figure 23: Visualizing transcytosis of DeltaD ligands *in vivo*
A DeltaD primary antibody is injected at the basal side of the neural tube and a fluorescently labelled secondary antibody at the apical side (a). The basal-to-apical transcytosis of DeltaD and a subsequent second round of apical ligand endocytosis result in the internalisation of the fluorescent secondary antibody into neuro-epithelial cells (b).

1.2 The initial site of secretion of DeltaD is limited to the basolateral domain in the zebrafish ear

According to the vectorial transcytosis model proposed by Benhra and colleagues, neuro-epithelial Delta ligands are initially localized at the basolateral domain, from where they would then be internalized and transported via Mindbomb (Mib) mediated endocytosis to the apical side to interact with apically localized Notch receptor molecules. The founding postulate of this model is therefore that Delta secretion is initially restricted to the baso-lateral cell surface. The aim of my first series of experiments was to test whether this does indeed apply to Zebrafish DeltaD. In order to identify the initial site of DeltaD secretion, I analyzed DeltaD localisation in embryos that lack the activity of the E3 Ubiquitin ligase Mindbomb (Mib) that is required for endocytic DeltaD internalization; in these embryos DeltaD can no more be internalized and ligands are therefore expected to accumulate a their initial site of secretion. DeltaD was detected in intracellular, potential endocytic, structures in the neuroepithelial cells of the ear in wild-type embryos, especially near to the apical membrane (Figure 24). When endocytosis of

DeltaD was inhibited using a morpholino against Mib (Itoh et al., 2003), DeltaD ligands accumulated on the basolateral membrane and no intracellular presence of DeltaD carrying endosomes were found (Figure 24 j-r). This shows that the initial site of secretion of DeltaD ligands in the otic vesicle neuro-epithelium is the basolateral membrane, in potential accordance with the vectorial transcytosis model.

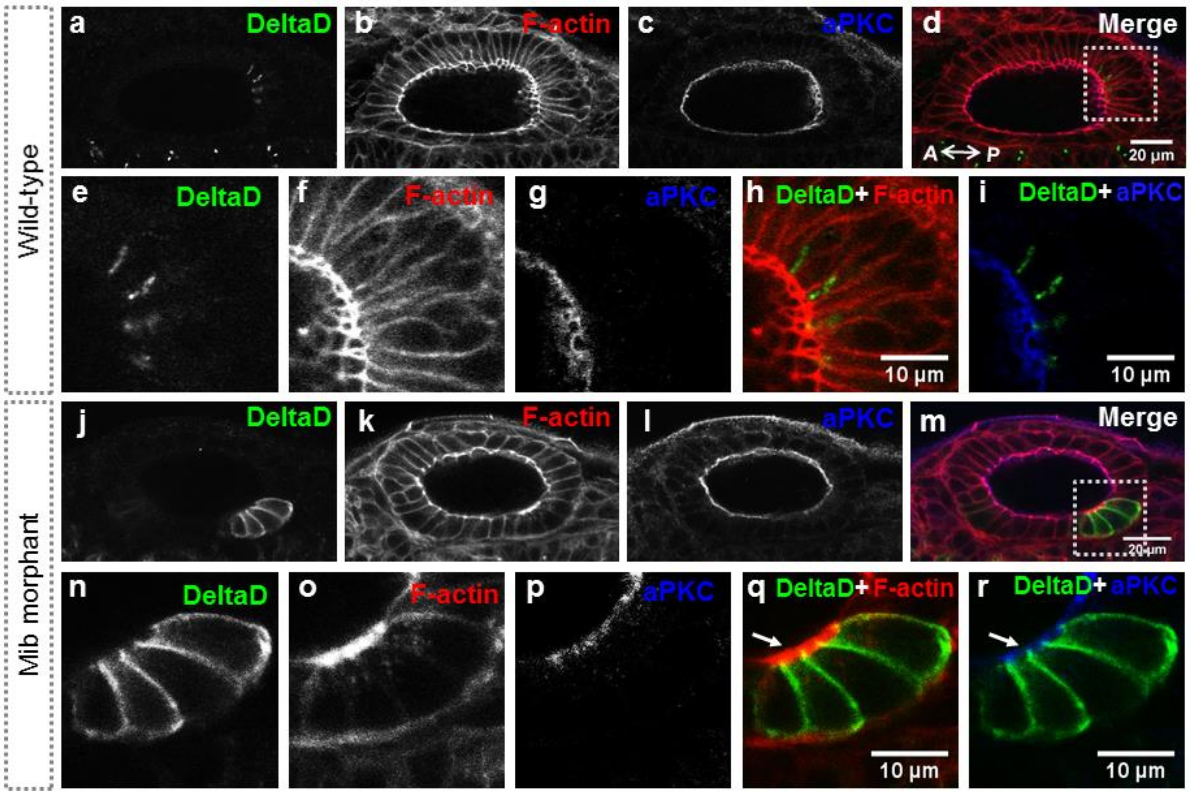


Figure 24: DeltaD trafficking in the zebrafish ear

Lateral view confocal images of 24 hpf wild-type (a-i) and Mib morphant (j-r) embryos at the level of the ear.
(a) DeltaD is detected in intracellular dots, near to the apical membrane.
(b) F-actin labelling shows the structure of the whole ear.
(c) Apical membrane is marked by aPKC.
(d) Merge. The box shows the area that is magnified in images e to i.
(e-g) A magnified view of DeltaD (e), F-actin (f) and aPKC (g) expressions.
(h-i) Merge images of F-actin (red)-DeltaD (green), and merge images of aPKC (blue)-DeltaD (green). No. of wild-type embryos were analyzed = 10
(j) Delta localized just at the basolateral membrane upon inhibition of Mib.
(k) f-actin staining in Mib morphant shows the normal architecture of the ear.
(l) Apical membrane is labelled with aPKC.
(m) Merge image. The box shows the area that is magnified in images n to r.
(n-p) A magnified view shows the expression of DeltaD at the basolateral membrane (n), F-actin staining (o), and aPKC localization at the apical membrane (p).
(q-r) Merged image of F-actin and DeltaD (q) shows that in Mib morphant, DeltaD localization is limited to the basolateral membrane. Merged image of aPKC and DeltaD (r) confirms that there is no colocalization between DeltaD and aPKC. No. of Mib morphant analyzed with the phenotype = 8/10

1.3 DeltaD secretion may not be restricted to the baso-lateral membrane in the zebrafish neural-tube

The functional Delta ligand transcytosis assay that has been developed in our lab (Figure 23) was initially developed in the context of the zebrafish neural tube. To further asses if the vectorial transcytosis model is also valid in this part of the nervous system, I further checked the subcellular localization of DeltaD molecules in the neural tube. In the zebrafish neural tube, DeltaD is detected in intracellular endocytic structures in the neuroepithelial cells of wild-type embryos (Figure 25 a-h) (Itoh et al., 2003; Kressmann et al., 2015). To find out the initial site of secretion of DeltaD ligands, I again inhibited the function of Mib.

To our surprise, we found that upon strong inhibition of Mib function, apico-basal polarity was lost in the neural tube (next section). Therefore, I reduced the dose of the morpholino and found that under hypomorphic conditions, apico-basal polarity was still intact. However, in contrast to the inner ear, DeltaD accumulated on both the apical and baso-lateral membranes of neural tube cells (Figure 25 e-j). In addition, a small amount of DeltaD is still observed in intracellular endocytic structures. The observation that DeltaD accumulates on both the apical and baso-lateral domain in Mib morphants, suggests that in the neural tube, the initial secretion of DeltaD may possibly not be limited to the basolateral domain. Interestingly, my observations in the inner ear and the neural tube suggest that the trafficking of DeltaD, and therefore potentially the regulation of Delta/Notch signaling are modulated differentially in a tissue context dependent manner.

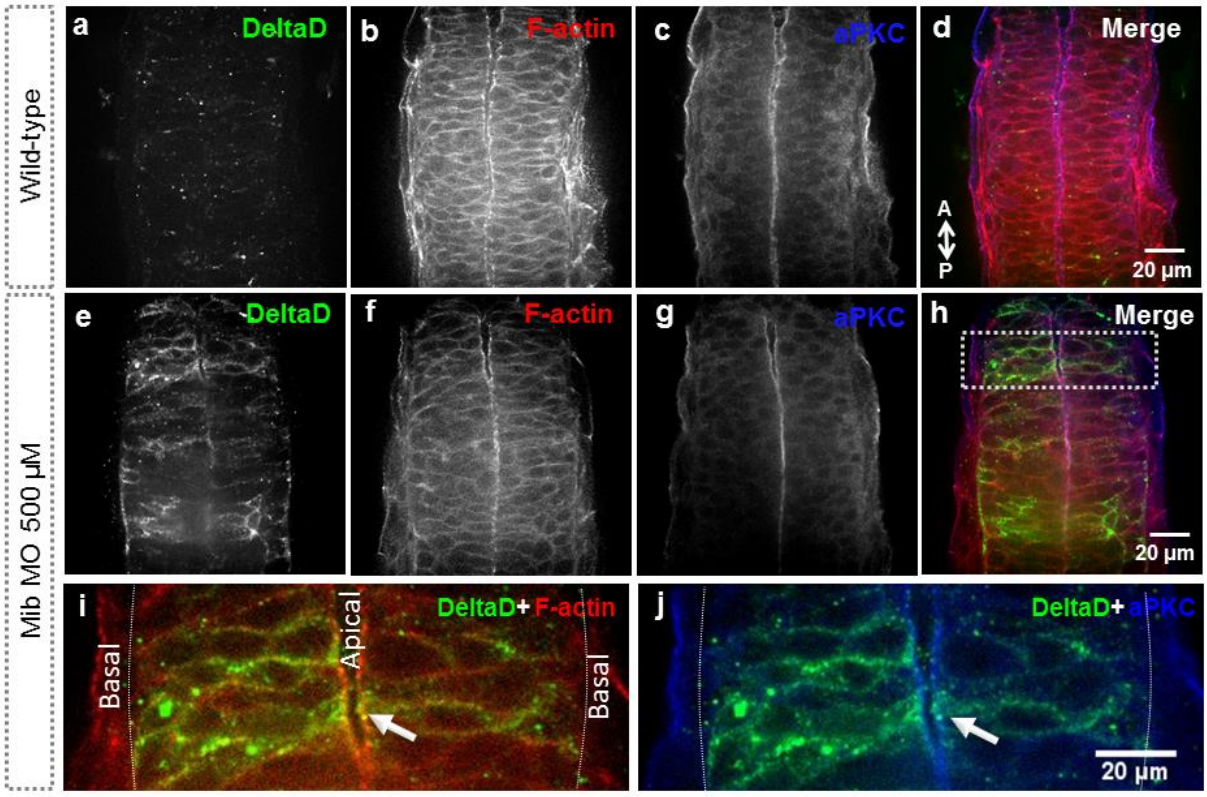


Figure 25: The initial site of secretion of DeltaD ligands is not restricted to the basolateral domain in the neural tube

Dorsal view confocal image of 24 hpf wild-type (a-d) and Mib morphant (e-j) embryos at the level of anterior spinal cord.
(a) DeltaD staining shows intracellular dots in the neuroepithelial cells in wild-type embryos.
(b) Phalloidin staining labels the F-actin to outline cells.
(c) The par complex protein aPKC labels the apical membrane in the spinal cord of wild-type embryos. aPKC labelling allows to show a uniform midline in the wild-type embryo.
(d) The merge image of WT embryo shows DeltaD in blue, F-actin in red and aPKC in green.
No. of wild-type embryos analysed=15
(e) Embryos injected with a low dose of Mib morphant (500 µM) embryo, a higher amount of DeltaD is expressed that localized mainly on the cell membrane and in small amount also in intracellular dots.
(f) F-actin staining shows the normal tissue morphology.
(g) aPKC localized to the apical membrane just like in wild-type embryo under these conditions.
(h) A box with dotted line shows the area that corresponds to the image (i) and (j).
(i) DeltaD (green) molecules colocalize with F-actin (red) at the neural tube midline in Mib morphants. The white arrow shows the colocalization of F-actin with DeltaD at the apical membrane.
(h) DeltaD (green) molecules colocalize with aPKC (blue) at the apical membrane in Mib morphant. DeltaD is present at the apical membrane (white arrow), overlapping with aPKC.
No. of Mib embryos analysed with the phenotype=18/20

2 Notch signaling regulates establishment of apico-basal polarity in the zebrafish neural tube

2.1 Loss-of-function of Mindbomb perturbs apico-basal polarity in the neural tube

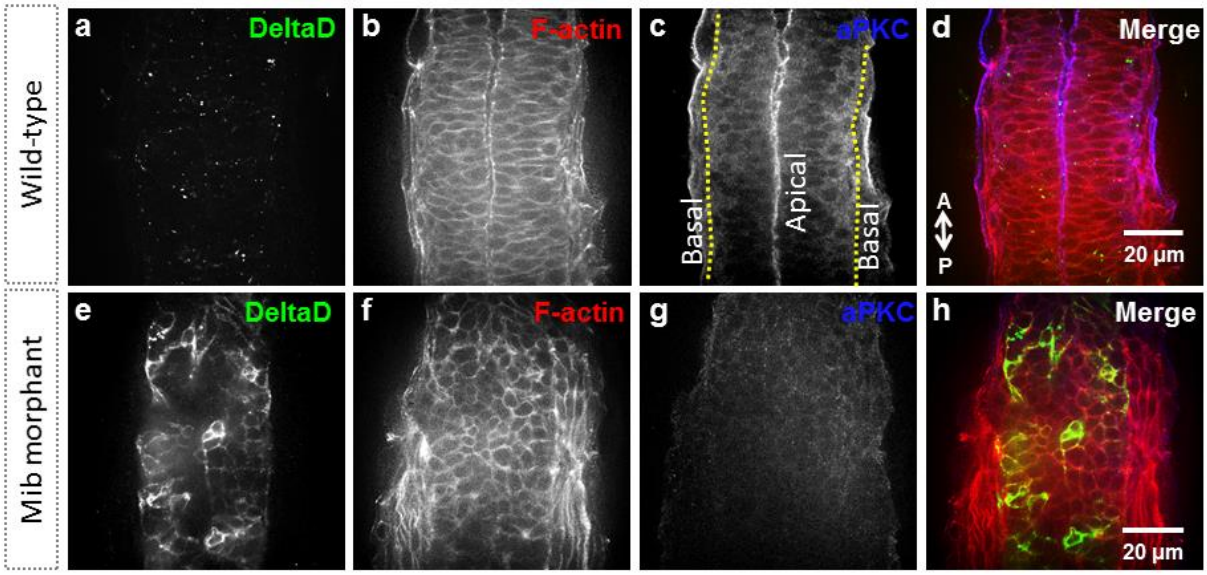


Figure 26: Embryo injected with higher dose of Mib morpholino exhibit loss of apico-basal polarity in the zebrafish spinal cord.

Dorsal view confocal image of 24 hpf wild-type (a-d) and Mib loss-of-function (e-h) embryos at the level of anterior spinal cord.
(a-d) DeltaD staining shows DeltaD containing intracellular dots in the neuroepithelial cells in wild-type embryos (a). Phalloidin staining labels the F-actin to outline cells (b). The par complex protein aPKC labels the apical membrane in the spinal cord of wild-type embryos. aPKC labelling shows a uniform midline in the wild-type embryo (c). The merge image of WT embryo (d) No. of wild-type embryos analyzed=15
(e-h) A Mib loss-of-function embryo that is injected with the morpholino against Mib at 1000 µM. DeltaD staining in this embryo shows a massive expression of DeltaD that is localized on cell membrane (e), F-actin staining in Mib embryos shows a disruption of the cellular morphology as compared to the wild-type embryo. The cells are more round as compared to the elongated cellular structures in wild-type cells. In addition, there is no midline visible here (f). The apical accumulation of aPKC is lost in Mib morphant (g), merged image (h). No. of Mib morphant showed apico-basal polarity defects=11/14

While investigating the role of endocytosis in DeltaD trafficking, I inhibited the function of Mib, which is essential for endocytosis of DeltaD ligands and activation of Notch signaling. To our surprise, we discovered that when a sufficiently high dose of a morpholino against Mib is used,

this led to the perturbation of apico-basal polarity in the spinal cord (Figure 26). These embryos exhibited a loss of apical accumulation of aPKC (Figure 26 g). The neuro-epithelial cells in the neural tube have a columnar shape, which is achieved by elongating the cell body along the apico-basal axis. We observed that in Mib morphants, neuro-epithelial cells were more roundish than elongated (Figure 26 f). Thus, Mib inhibition disrupts polarity and morphology of the neural tube (Figure 26 f).

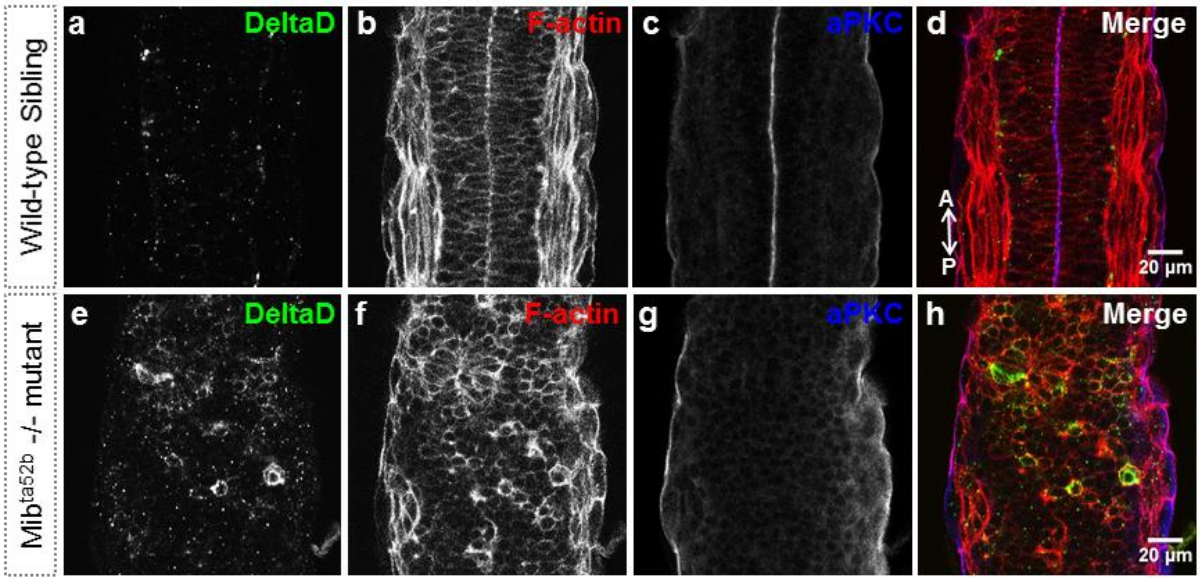


Figure 27: Mib mutant (Mib^{ta52b} -/-) exhibits loss of apico-basal polarity

Dorsal image of a 24 hpf Mib homozygous mutant and Wild-type sibling embryo.
(a-d) DeltaD expression in a wild-type sibling embro shows DeltaD carrying endosomes as intracellular dots (a). F-actin staining in sibling embryos displays a proper neural-rod formation and well-formed somites (b). aPKC localized at the apical membrane and the neural rod midline in sibling (c), Merged image (d). No. of wild-type sibling embryos analyed=15
(e-h) Mib^{ta52b} -/- embryo, DeltaD is massively overexpressed (e), F-actin staining shows the disruption of the neuroepithelial morphology in the spinal cord of the Mib^{ta52b} -/- embryo (f). In Mib^{ta52b} -/- embryo, apical expression of aPKC is lost (g). Merge image of Mib^{ta52b} -/- embryo (h). No. of Mib^{ta52b} embryos analysed with the loss of apico-basal polarity= 14/15

In recent years, there has been a concern regarding the use of morpholinos because of their potential off-target effects and toxicity (Kok et al., 2015). Therefore, it was important to repeat the experiment in Mindbomb mutants. I used the Mib^{ta52b} mutant strain that is having a point mutation in the Ring finger domain (van Eeden et al., 1996). This point mutation causes the substitution of an amino acid from Methionine to Arginine and gives a neurogenic phenotype (Itoh et al., 2003). I incrossed Mib^{ta52b} fish to get homozygous mutant embryos and then stained them for DeltaD, F-

actin and aPKC (Figure 27 e-h), along with their siblings (Figure 27 a-d). We found that Mib^{ta52b} homozygous embryo for mutant allele displayed a complete loss of apico-basal polarity where aPKC expression was missing (Figure 27 g) and the overall morphology of the neural tube was disrupted (Figure 27 f, h). Mib^{ta52b} homozygous mutant embryos were identified based on the DeltaD staining that was massively overexpressed in homozygous mutant (-/-) embryo as compared to the heterozygous mutant (+/-) or wild-type (+/+) siblings (Figure 27 a, d). These result shows that Mib is important for the apico-basal polarity of the zebrafish neural tube.

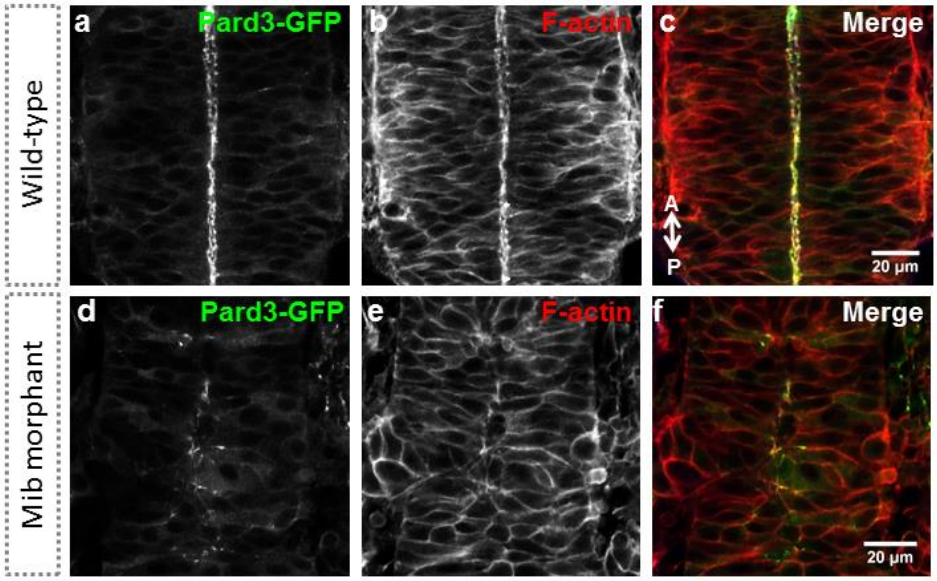


Figure 28: Pard3 expression in wild type versus Mib morphant embryos.
A GFP fusion construct of Pard3, an apical polarity protein, is injected in 24 hpf wild-type (a-d) embryo and in Mib morphant (e-h) embryos.
(a-c) The dorsal view of the spinal cord shows that Pard3-GFP localizes at the apical membrane in a wild-type embryo (a). F-actin staining (b) and Merge image (c). No. of wild-type embryos analyzed=8
(d-f) Pard3-GFP localization is disrupted at the apical membrane in Mib depleted embryo. The Pard3-GFP shows diffused cytoplasmic and a little amount of expression in form of intracellular and membrane dots (d). F-actin staining outlines cells (e). Merge image (f). No. of Mib morphant embryos analyzed with the defect in apical accumulation of Pard3-GFP =7/8

It has been shown that disruption of neuro-epithelial polarity result in the disruption of intercellular junctions and the mislocalisation of several apical polarity proteins that are members of the Par and the Crumbs complex (Cappello et al., 2006; Imai et al., 2006; Ohata et al., 2011). As we found that Mib is important for apico-basal polarity in the zebrafish neural tube, we next investigated the localisation of several polarity proteins in Mib-depleted conditions. Firstly, the expression of the Par complex protein Pard3 was observed by using a Pard3-GFP fusion

construct (Tawk et al., 2007). In Wild-type embryos, Pard3-GFP is enriched at the apical membrane (Figure 28 a-d). However, in Mib morphant embryo Par3-GFP failed to accumulate at the apical midline (Figure 28 e-h).

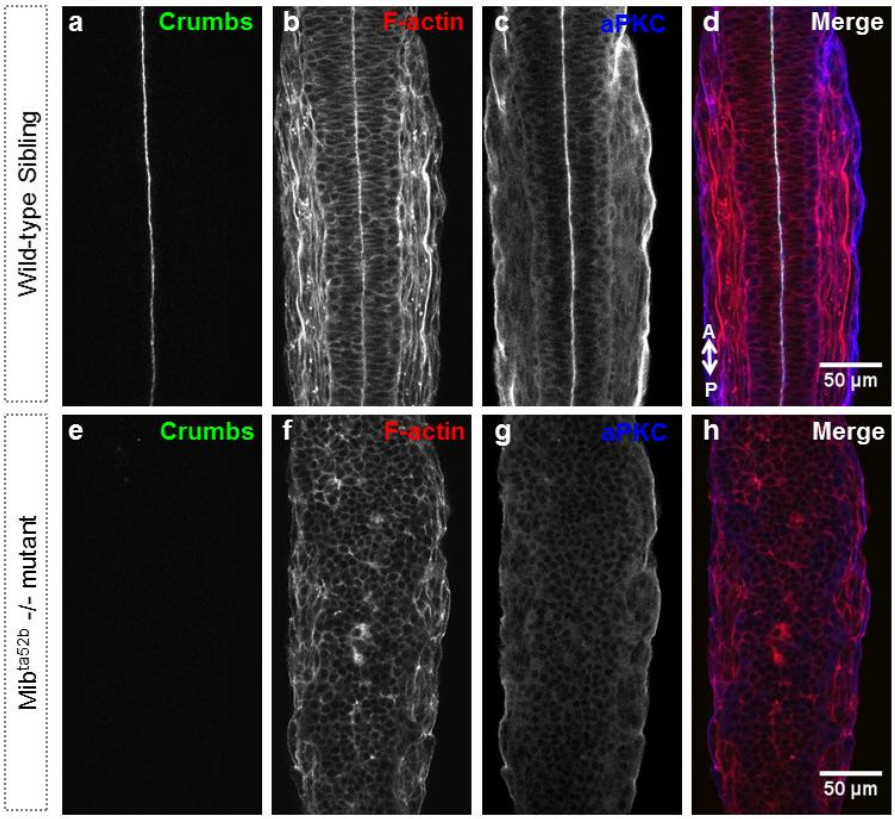


Figure 29: Crumbs expression is completely absent in Mib^{ta52b} homozygous mutant embryos.

Dorsal view images of 24 hrs embryos show the expression of the apical polarity protein Crumbs by antibody staining in Mib^{ta52b} homozygous mutant and wild-type sibling embryos.
(a-d) Crumbs expression at the apical membrane in wild-type sibling (a). F-actin staining (b). Similar to Crumbs, aPKC is localized at the apical membrane (c). A merge image is shown in (d). No. of wild-type sibling embryos =13
(e-h) In comparison to wild-type sibling, apical localization of Crumbs proteins is lost in Mib^{ta52b} homozygous mutant embryo (e). F-actin (f), and aPKC (g) expression in Mib^{ta52b} -/- mutant is shown. No. of Mib^{ta52b} -/- embryos with the loss of Crumbs=10/11

Previously, negative regulation of Notch by Crumbs protein has been genetically shown in *Drosophila,* where through a feedback loop Notch induces Crumbs (Herranz et al., 2006). Another sudy in zebrafish showed that Crumbs family proteins bind to the extracellular domain of Notch (Ohata et al., 2011). These studies show that Notch and Crumbs interact with each other. However, not much is known if Notch signaling acts upstream of the Crumbs complex to regulate apico-basal polarity. To explore this, firstly I checked the expression of Crumbs in loss-of-function

Mib embryos. For this purpose, I used an antibody against Crumbs, that allowed me to see the expression of endogenous Crumbs protein in the neural tube. In comparison to the wild-type sibling (Figure 29 a-d), Crumbs protein is not detected in the neural tube of Mib homozygous mutant (Figure 29 e-h).

In addition to the polarity complexes themselves, junctionals protein are very important for the establishment and maintenance of apico-basal polarity. I found that in Mib-depleted embryos, the adherence junction complex protein Zonula occludens 1 (ZO1) also lost its expression at the apical membrane (Figure 30). We next asked whether Mib has a Notch independent or Notch dependent role in the regulation of apico-basal polarity of the neural tube.

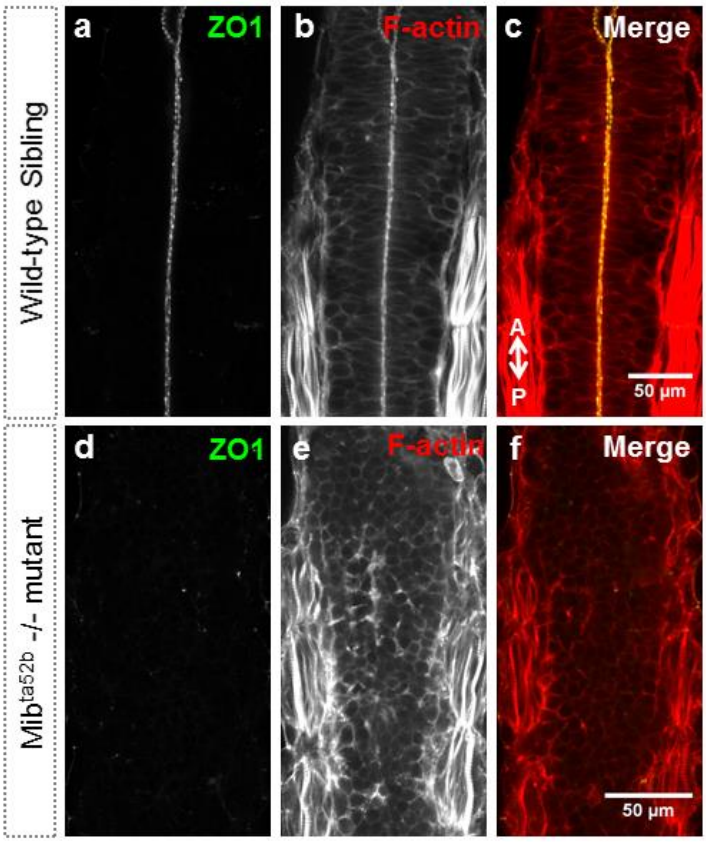


Figure 30: Mib homozygous mutant embryos lose the expression of an adherence-junction complex protein ZO1.
In comparison to a wild-type sibling (a), ZO1 staining shows the loss of ZO1 protein in a Mib^{ta52b} -/- embryo (d) at 24 hpf. F-actin staining allows visualizing cells and the overall neural tube structure in wild-type sibling (b) and Mib^{ta52b} homozygous mutant (e). Merge images shows F-actin in red and ZO1 in green for wild-type sibling (c) and Mib^{ta52b} -/- (f). No. of wild-type sibling embryos=5 and Mib^{ta52b} embryos showing the loss of ZO1=5/5.

2.2 Notch signaling is required for apico-basal polarity in the neural tube

A recent study in *Drosophila* suggests a distinct role of the E3-ubiquitin ligase Neuralized (Neur) in epithelial polarity that is independent of its known function in Notch signalling (Chanet and Schweisguth, 2012). Previously, it has been shown that Neur is required for the endocytosis of the Delta ligand in *Drosophila* (Pavlopoulos et al., 2001), similar to the function of Mib in zebrafish. To find out whether in the regulation of zebrafish neural tube apico-basal polarity, Mib plays a Notch-independent role, I inhibited different components of the Notch signaling pathway. I began with the double knockdown of DeltaA and DeltaD ligands, by injecting a morpholino against DeltaA in DeltaD (aei) homozygous mutant embryos (van Eeden et al., 1996). DeltaA/DeltaD depleted embryos showed apico-basal polarity defects and failed to form an apical midline (Figure 31).

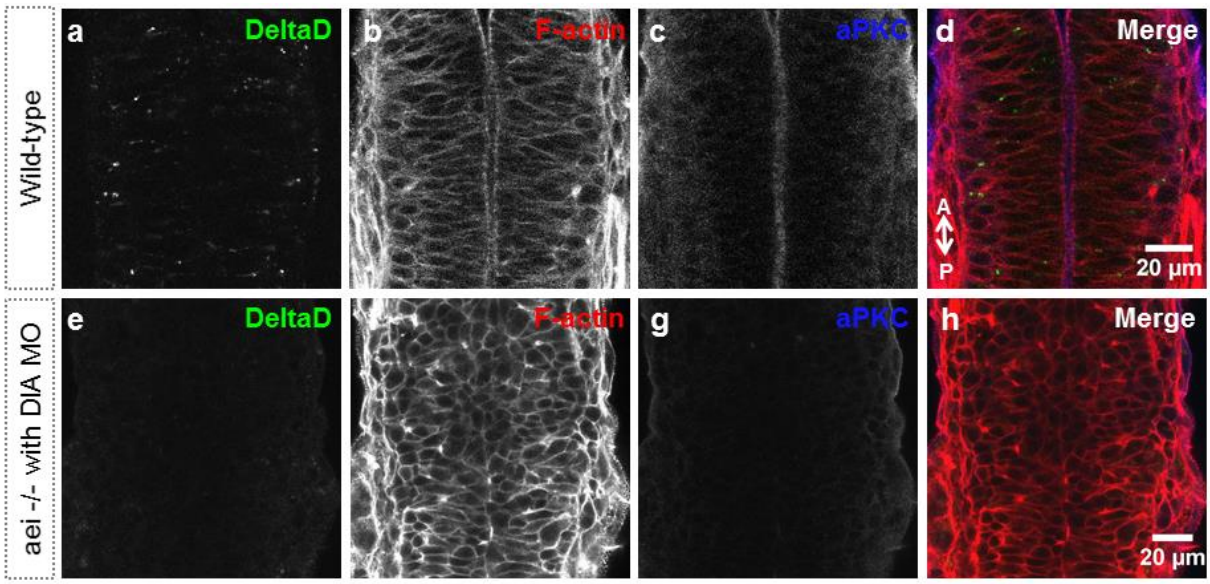


Figure 31: Notch ligands are indispensable for apico-basal polarity
Dorsal view of the spinal cord in a wild-type embryo at 24 hpf that is stained for DeltaD (a), F-actin (b), aPKC (c). Merge image (d). No. of wild-type embryos analyzed =15
Dorsal view of spinal cord in an aei -/- (DeltaD) mutant embryo injected with DeltaA (DlA) morpholino. DeltaD staining shows absence of DeltaD molecules (e). Staining for F-actin (f) and aPKC (g) shows the disrupted neuroepithelial morphology and loss of apico-basal polarity, No. of aei -/- with DlA morpholino embryos with the apico-basal polarity defects =13/15

We then blocked the function of γ-secretase by pharmacological inhibitors LY411575 (Fauq et al., 2007) and DAPT (Geling et al., 2002). Inhibition of γ-secretase prevents the S3-cleavage of

Notch receptor and therefore the release of NICD that is necessary for the activation of Notch signaling. We found that embryos with inhibited γ-secretase activity presented defects in apico-basal polarity. These embryos displayed a loss of the apical accumulation of aPKC and a perturbation of apical neural tube midline formation (Figure 32). These results confirm a potential role of Notch signaling in the regulation of epithelial polarity.

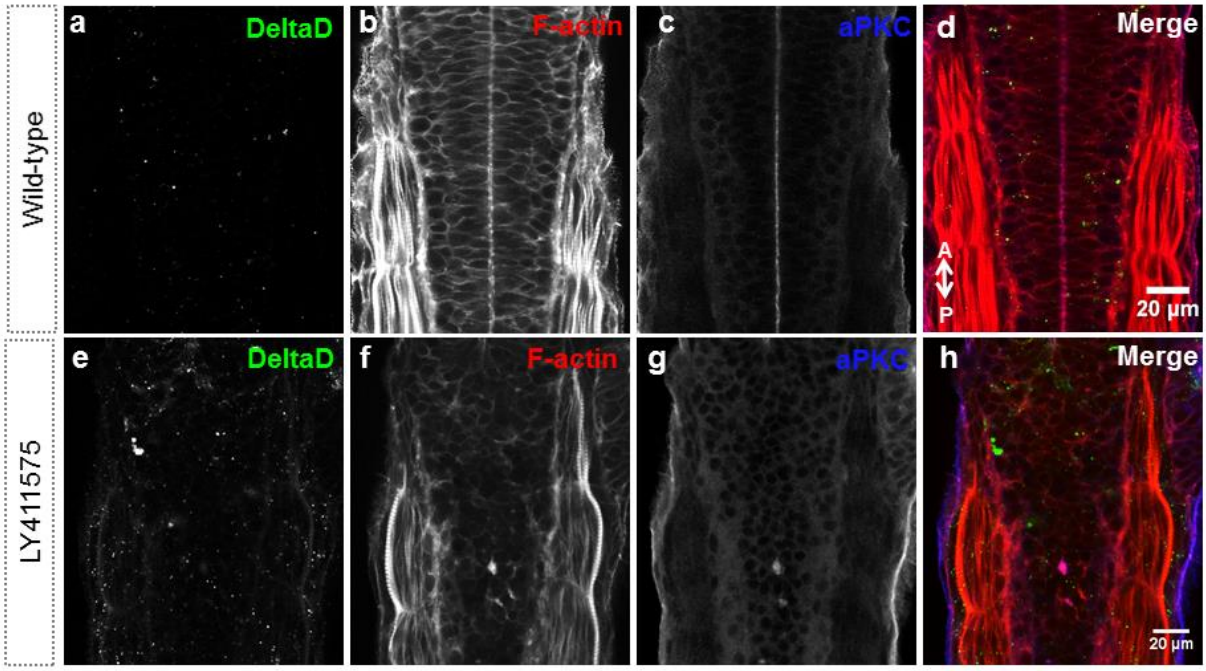


Figure 32: γ-Secretase is required for apico-basal polarity
A wild-type embryo with normal apico-basal polarity in the spinal cord, stained for DeltaD (a), F-actin (b) and aPKC (c) at 24 hpf. Merge is shown in (d). No. of wild-type embryos analyzed = 8
γ-secretase inhibitor LY411575 treated embryo shows a loss of apico basal polarity. DeltaD staining (e) shows no significant increase in DeltaD expression. F-actin (f) and aPKC (g) staining display the perturbation of apico-basal polarity upon inhibition of γ-secretase. Merge image is shown in (d). No. of LY411575 drug treated embryos with the apico-basal polarity defects =6/8
Experiment was performed by Li. Xiang.

Thereafter, we assumed that if Notch signaling regulates apico-basal polarity, then introducing a constitutively activate form of Notch (NICD) (Iso et al., 2001) in a Notch signaling impaired embryo should restore apico-basal polarity. Indeed, when I injected Mib morphant embryos with the Notch intracellular domain (NICD) RNA, it led to the complete rescue of apico-basal polarity (Figure 33).

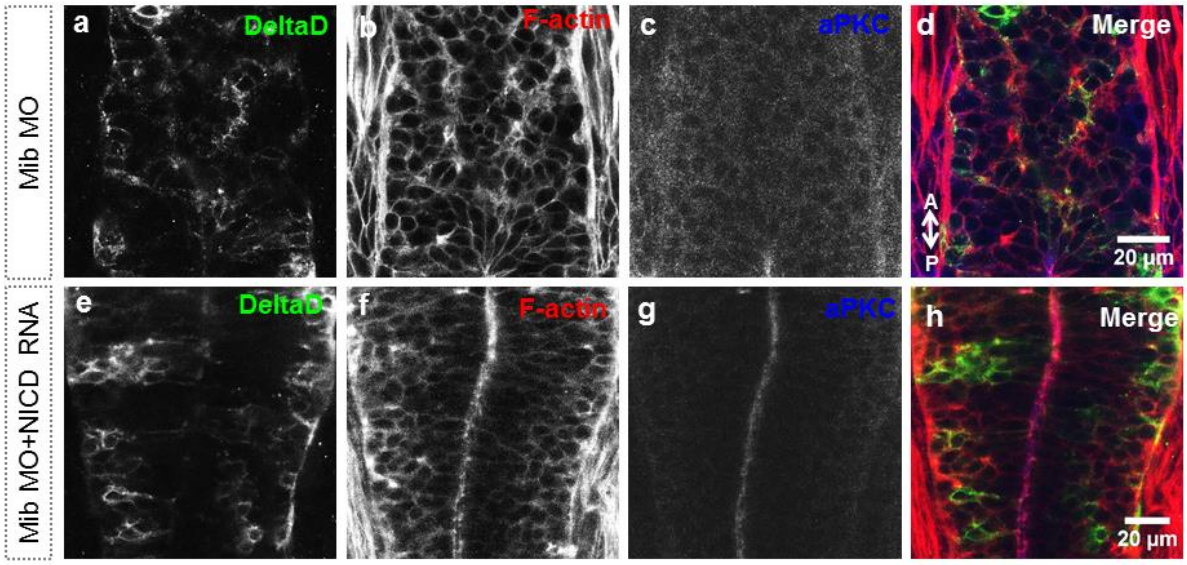


Figure 33: Activated Notch restores apico-basal polarity
A dorsal view confocal image at the level of anterior spinal cord of a 24 hpf Mib morphant embryos (a-d). Mib depleted embryo shows overexpression of DeltaD (a), and loss of apico-basal polarity (b-d). No. of Mib morphant embryos analysed = 10/10.
When activated Notch (NICD) is introduced in a Mib depleted embryo this leads to the complete rescue of apico-basal polarity (g). DeltaD expression is still upregulated (e) and F-actin staining is shown in (f). No. of rescued embryo=14/17

A previous study has reported that R-ras dependent noncanonical Notch signaling plays an important role in the maintenance of apico-basal polarity in the zebrafish neural tube (Ohata et al., 2011). Therefore, I next checked if canonical Notch signaling is required for apico-basal polarity in the zebrafish neural tube. To find out this, I made a double knockdown by using morpholinos against the transcriptional mediators of Notch signaling RbpjA and Rbpjb (Sieger et al., 2003) that are known as CSL in mammals and suppressor of hairless in *Drosophila*. Just like other Notch signaling components, loss-of-function of RbpjA and RbpjB gave apico-basal polarity defects where the accumulation of aPKC at the apical midline was perturbed (Figure 34).

Altogether, my results show that canonical Notch signaling is required for apico-basal polarity in the zebrafish neural tube. Next, we asked whether Notch signaling is required for the establishment or for the maintenance of apico-basal polarity.

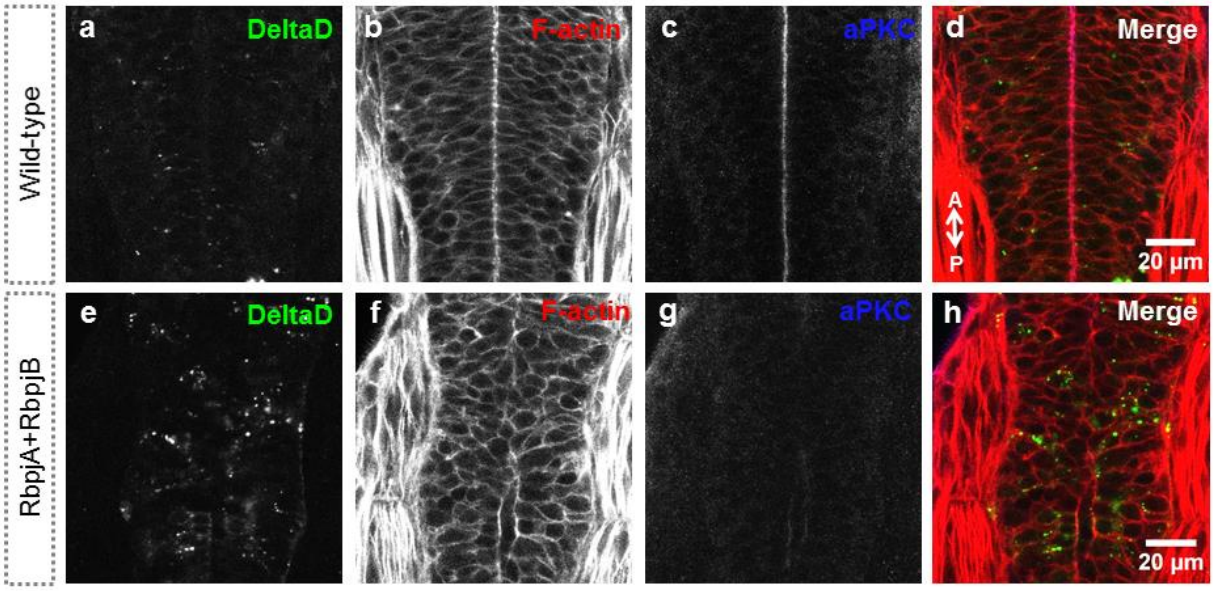


Figure 34: Transcriptional mediators of Notch signalling are required for apico-basal polarity.

Dorsal view of the spinal cord of Wild-type embryo at 24 hpf, stained with DeltaD (a), F-actin (b) and aPKC (c). Merge is shown (d). No. of wild-type embryo analyzed =8

Double knockdown of RbpjA and RbpjB via morpholinos causes a disruption of apico-basal polarity. DeltaD staining shows a slightly upregulated DeltaD (e). The neural tube is having partially disrupted morphology showed by F-actin staining (f) and loss of apical expression of aPKC (g). No. of double morphants analysed with the disruption of apico-basal polarity = 4/7

2.3 Notch signaling is required for the initial establishment of apico-basal polarity

Our previous observations show that Notch signaling is required for apico-basal polarity. Though how and when is Notch signaling required for apico-basal polarity, remain the questions. Previous studies in zebrafish and the embryonic stem cell-derived neural rosette system have suggested a role of Notch signaling in the maintenance of epithelial polarity (Main et al., 2013; Ohata et al., 2011). To see if Notch signaling is required for the maintenance or the establishment of the polarity, I performed long-term time-lapse imaging experiments to visualize the dynamic morphogenesis of the spinal cord. This allowed us to see the transition from the neural plate to the neural keel and the gradual formation of the neural rod (Figure 35). At the late keel stage, the two ends of the neural tissue converge towards each other, cells intercalate to the contralateral side of the forming neural rod, and the CNS midline starts to appear (Figure 35 d and e, Movie 1). In contrast, in Mib morphant embryos tissue only a stretch of neural tube midline becomes visible in the ventral neural tube but is then rapidly lost afterwards. (Figure 35 g, Movie 2).

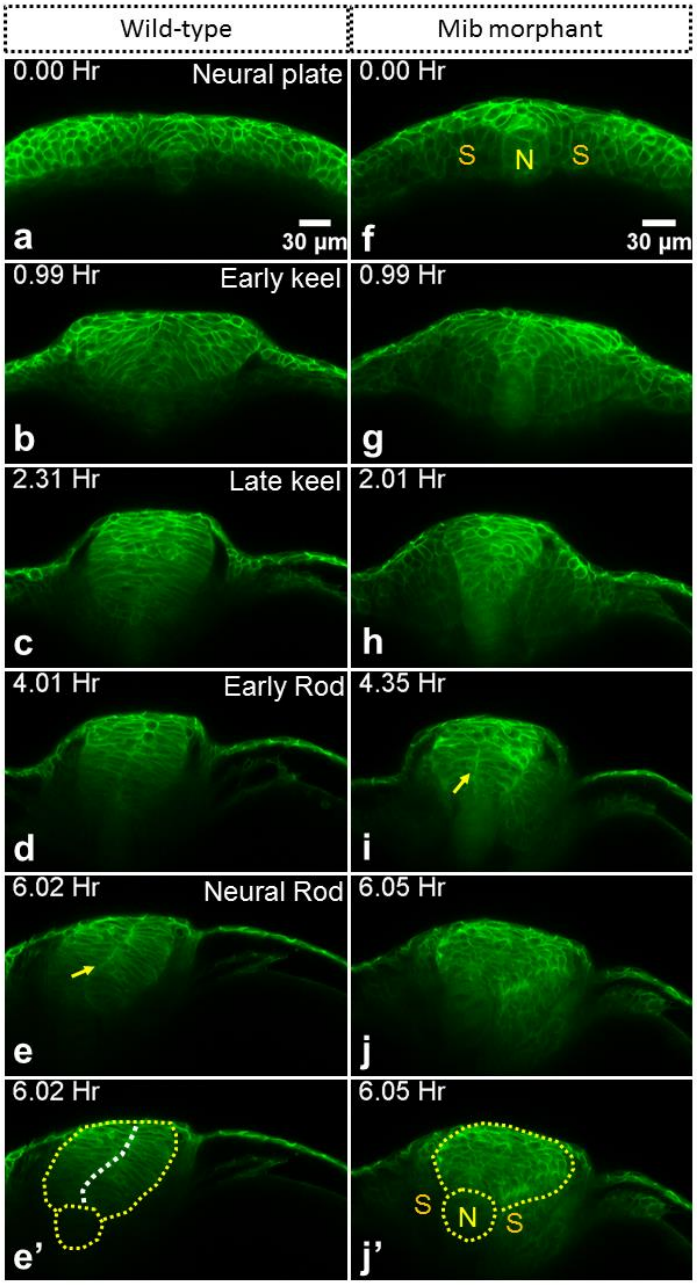
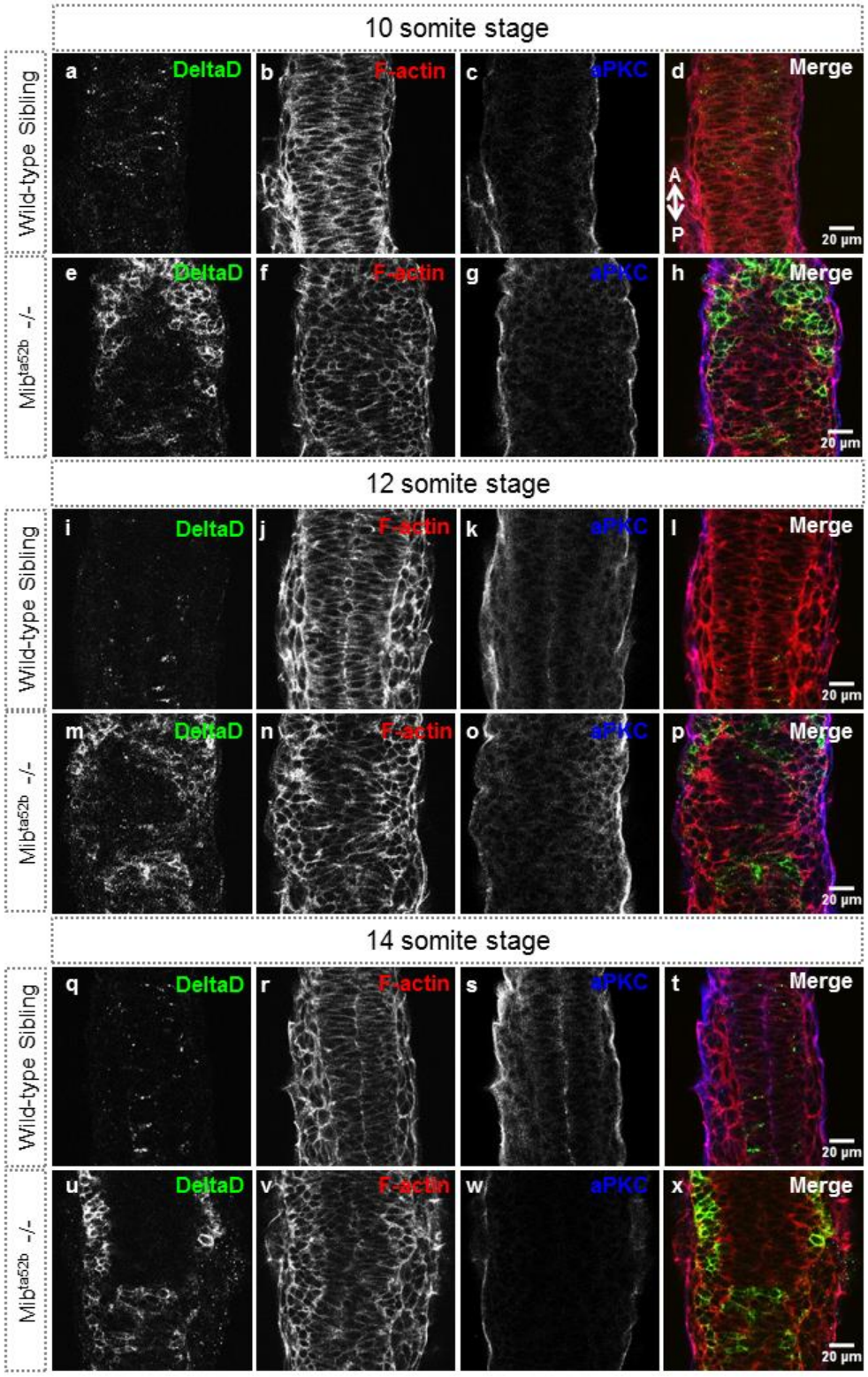


Figure 35: Morphogenesis from the neural plate to the neural rod in a wild-type versus Mib depleted embryo

Images from a time-lapse video to see the dynamic morphogenesis from the neural plate to the neural-rod at the level of anterior spinal cord. These transversal view images show the neural tissue (neural plate to neural rod), the underlying notochord (N) and the somites (S).
Morphogenesis of the neural-plate (a) into neural keel (b, c) and then into neural rod (d, e) in wild-type embryo. At the late neural keel, when the two sides of the neural tissue completely converge towards each other, a tissue midline starts to appear. Further, the neural rod is formed. In comparison to wild-type, Mib morphant embryo (f-j) shows the midline transiently on the ventral side shown by a yellow arrowhead (i). The midline never forms completely and disappears very soon. By the neural-rod stage, the morphology of the neural-rod is completely disrupted (j, j'). No. of wild-type time-lapse movies = 6 and Mib morphant with the loss of apico-basal polarity are=3/4.

There could be two explanations for this observation: First, apical-basal polarity is initially established (at least in the ventral neural tube) but in the absence of Notch signaling, maintenance of polarity is affected causing hence the collapse of apico-basal polarity. Secondly, as I used Mib morpholinos to inhibit Notch signaling, it is possible that it did not inhibit Notch signaling completely. Therefore, to explore this possibility, I did a careful temporal analysis of Mib^{ta52b} mutants (which display a stronger polarity phenotype when compared to mutants) and fixed them at different stages to analyze the appearance of markers of apico-basal polarity. In wild-type embryos, the apical neural tube midline appears around 12 somites (14 hpf) as polarity protein such as aPKC starts to accumulate at the midline, a process that is completed by the 14 somite stage (Figure 36). Contrary to this, in Mib homozygous mutant embryos, the midline never appears (Figure 36 e-h, n-p, u-x).

Figure 36: Notch signalling is required for the initial establishment of apico-basal polarity
Mib^{ta52b} mutant embryos and wild type sibling embryos are stained for DeltaD (a, e), F-actin (b, f) and aPKC (c, g) at 10 somites stage. The midline is not yet established and there is no expression of aPKC in homozygous mutant or wild-type sibling embryos. Merge mages are shown in (d) and (h).
At the 12 somite stage, F-actin staining shows the presence of an early midline in wild-type sibling embryo (j). In addition, aPKC starts to accumulate along the forming midline (k). However, the homozygous mutant embryo shows no sign of midline formation (m-p). Merge mages (l, p).
In a wild-type sibling embryos (q-t) the midline is completely formed by 14 somite stage (r), along with the accumulation of the aPKC at the midline (s). Mib^{ta52b} homozygous embryos, still at the 14 somites do not form the midline (v). There is no aPKC in the mutant embryo (w). Merge mages (t, x).


10 somite stage
Wild-type Sibling
Mib^ta52b -/-
a DeltaD
b F-actin
c aPKC
d Merge
A
P
20 µm
e DeltaD
f F-actin
g aPKC
h Merge
20 µm
12 somite stage
i DeltaD
j F-actin
k aPKC
l Merge
20 µm
m DeltaD
n F-actin
o aPKC
p Merge
20 µm
14 somite stage
q DeltaD
r F-actin
s aPKC
t Merge
20 µm
u DeltaD
v F-actin
w aPKC
x Merge
20 µm

A quantification of this experiments shows that none of the Mib homozygous mutants embryos ever display an occurrence of apical midline formation and accumulation of polarity proteins at the apical membrane (Figure 37), Therefore, in contrast to the previously mentioned studies, our results suggests that Notch signaling is actually necessary for the establishment of the neural tube apico-basal polarity. Our results suggest that Notch signaling is needed for the establishment of apioc-basal polarity. Henceforth, I investigated how Notch signaling regulate the establishment of apico-basal polarity by observing the transcript of polarity complex proteins.

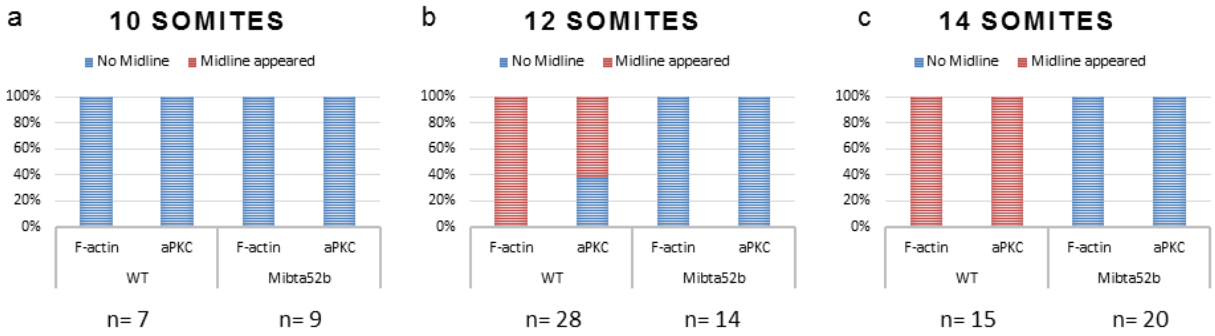


Figure 37: Mib mutants fail to establish apico-basal polarity

(a) Bar diagram showing that at 10 somites stage, none of the control (wild-type sibling) or mutant embryos is having midline as determined by the acpical enrichment of F-actin and aPKC. (b) At the 12 somites stage, 100% Wild-type sibling embryos have midline judged by apical accumulation of F-actin and 60% embryos also have apical accumulation of aPKC at the midline. None of the Mib homozygous mutant have a midline at 12 somites stage. (c) In all the 14 somites stage wild-type sibling embryos, midline is present and aPKC enriched apically. Mib^{ta52b} homozygous mutant fail to establish midline. n=no. of embryos that were analyzed.

2.4 Notch signaling regulates transcription of the *crumbs* genes

How does Notch signaling affect polarity proteins? Several studies previously showed interactions of polarity proteins with Notch receptors. In 2011, Ohata and colleagues showed that in zebrafish neural development, non-canonical Notch signalling maintains neuroepithelial polarity. In this context, the Crumbs complex protein Moe acts upstream of Notch and negatively regulate Notch by binding to its extracellular domain (Ohata et al., 2011).

In mammalian neural development, Notch is positively regulated by the PAR complex protein Pard3 and aPKC, promoting apical neuroepithelial identity (Bultje et al., 2009; Smith et al., 2007). These studies emphasize that Notch can be positively or negatively regulated by apico-basal polarity proteins.

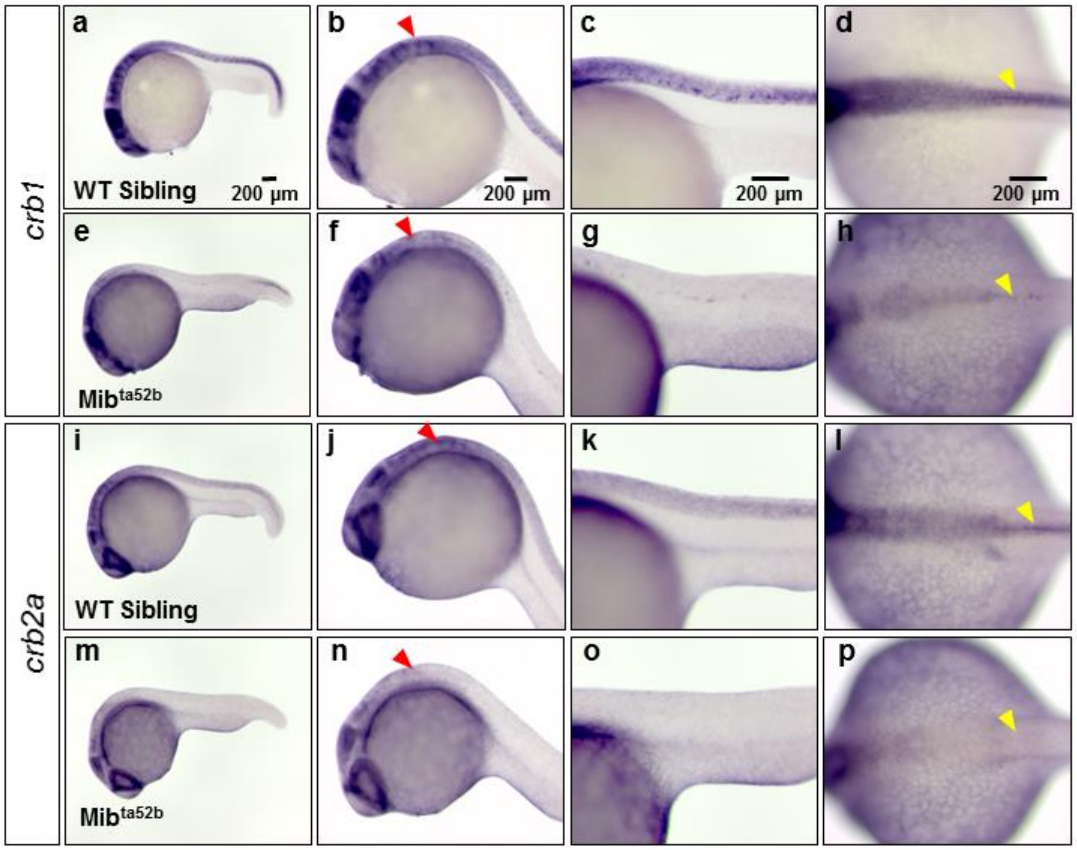


Figure 38: Mib loss-of-function affects crumbs complex transcription

In situ hybridization images of 24 hpf wild-type (WT) embryos shows the highly elevated *crb1* expression in the brain and spinal cord (a-d), which is diminished in the spinal cord of the Mib^{ta52b} mutant (e-h). No. of wild-type sibling embryos analyzed= 7, and No. of Mib^{ta52b} mutant with the loss of *crb1* transcription=6/6.

In comparison to the wild-type embryo (i-l), *crb2a* expression is lost in the spinal cord of the Mib^{ta52b} mutant embryos (m-p).Red arrowheads shows the expression of *crb1* and *crb2a* around hind brain, and yellow arrowheads indicate anterior spinal cord. No of wild-type sibling embryos=6, No. of Mib^{ta52b} mutant with the loss of *crb2a* transcription=6/6

Our results show that the polarity proteins Crumbs, aPKC and Pard3 are lost in Notch-depleted conditions. This suggests that Notch might function upstream of the apico-basal polarity machinery. To investigate this, I performed *in situ* hybridization assays to visualize the effect of Notch signalling depletion on the transcription of apico-basal polarity genes. In 24 hpf wild-type embryos, *crumbs1* (*crb1*) is expressed in the brain and all along the spinal cord (Figure 38 a-d). In Mib^{ta52b} mutant embryo *crb1* expression is greatly reduced in the spinal cord (Figure 38 e-h). Similarly, in 24 hpf wild-type embryos, *crb2a* is expressed in the eye, the brain, and the spinal cord (Figure 38 i-l). In Mib^{ta52b} mutant embryos, *crb2a* expression is reduced in the eyes and brain and lost in the spinal cord (Figure 38 m-p).

Next, I examine the expression of the Par complex proteins *pard6-gb* and *pard3*. I found that *pard3* is expressed ubiquitously in the nervous system of 24 hpf wild-type embryo (Figure 39 a-d), but was found to be heavily reduced in the brain and the spinal cord in Mib^{ta52b} mutant embryos (Figure 39D e-h). Finally, *pard6-gb* transcript is present in the eyes, the brain and the spinal cord in 24 hpf wild-type embryos (Figure 39 i-l). In mib loss-of-function embryos, *pard3* expression was severely reduced in the brain and the spinal cord (Figure 39 m-p).

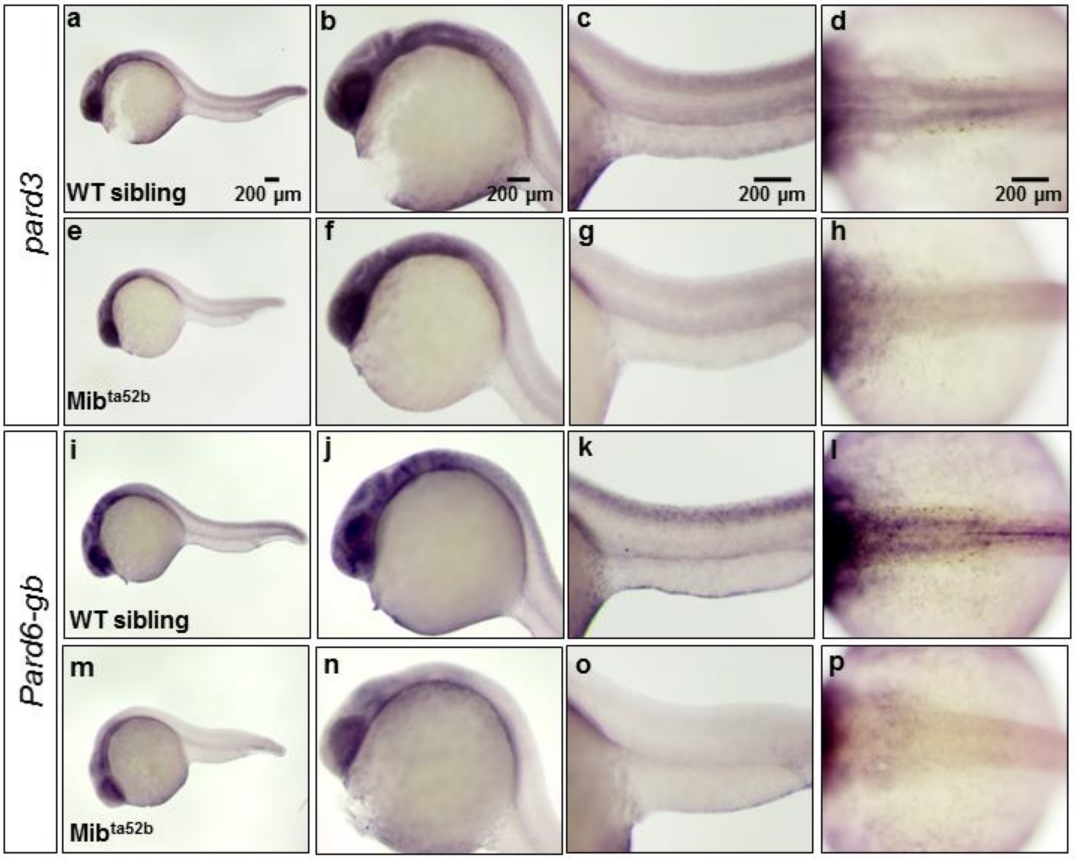


Figure 39: Mib Loss-of-function affects Par complex transcription
In situ hybridization images of 24 hpf embryos show a Pard3 transcript pattern in wild-type (a-d), which is reduced in the spinal cord of Mib^{ta52b} mutant (no. of embryos=5/5) embryos (e-h). Similarly, Pard6-gb transcript in brain, and spinal cord is shown in wild-type embryos (i-l). In Mib^{ta52b} Pard6-gb expression (m-p) is decreased in the brain and lost in the spinal cord (no. of embryos=5).

Our previous results have suggested that Notch signaling is important for the establishment of polarity between the 12 to 14 somites stage (14-16 hpf). We supposed that if Notch signaling is essential to regulate the transcription of apical polarity genes, then in wild-type embryos there should be an upregulation of the transcription of polarity genes, concomitant with the

establishment of polarity. Therefore, I checked if we see an upregulation of the crumbs and par complex genes at the 14 somites stage. Indeed I found that the transcriptional expression of *crb1* (Figure 40 a, b) and *crb2a* (Figure 40 e, f) is upregulated between the 6 and 14 somites stages (Figure 40 c, d, g, h). In contrast, pard6-gb is expressed at similar levels at 6 and 14 somites stages (Figure 40 i-l).

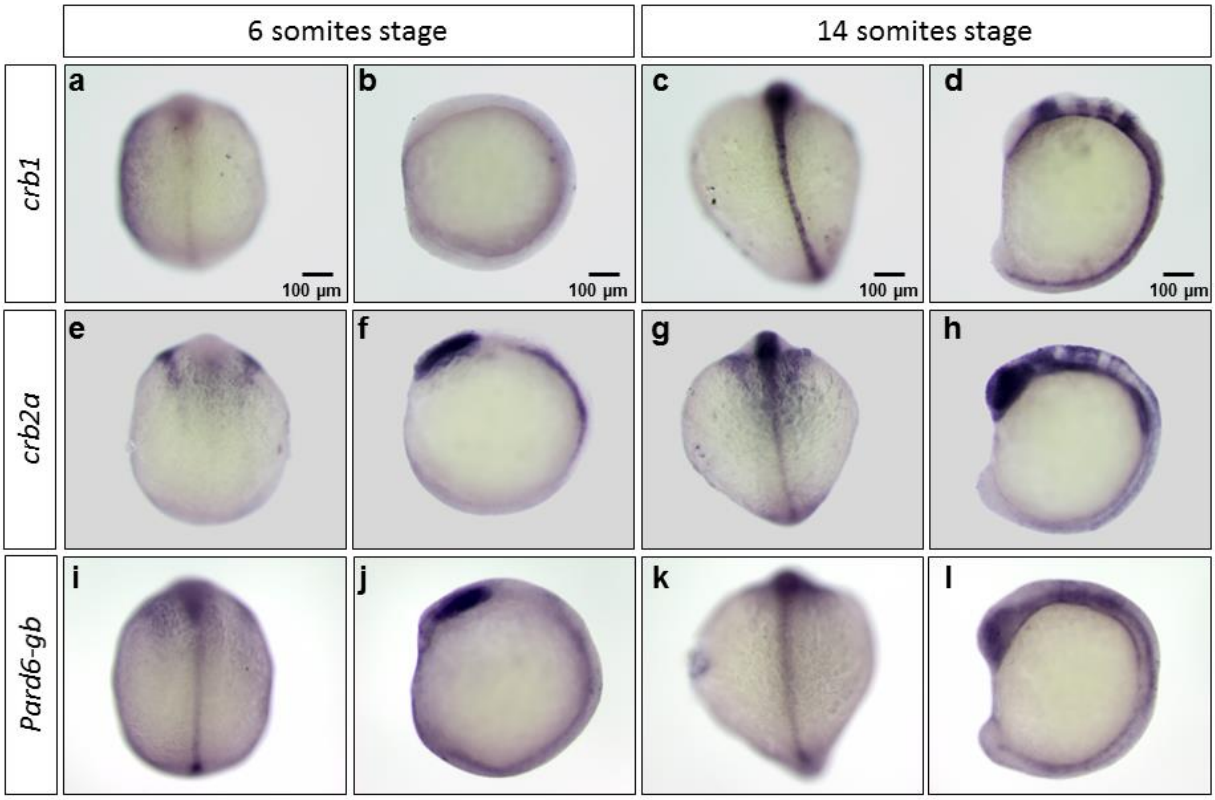


Figure 40: Transcriptional of polarity genes during early spinal cord morphogenesis
(a-b) In wild-type, there is almost no transcriptional expression of *crb1* at the level of the spinal cord in 6 somites stage embryos. Whole mount *in-situ* hybridization images showing dorsal (a) and lateral (b) views. No. of embryos (*crb1*) at 6 somite stage were analysed = 6.
(c-d) Transcriptional expression of *crb1* upregulates at the 14 somites stage. No. of embryos with upregulated *crb1*=6/6
(e-f) Similar to *crb1*, there is no transcriptional expression of *crb2a* at the level of the spinal cord at the 6 somites stage. No. of embryos (*crb2a*) were at 6 somites stage=7.
(g-h) Transcriptional expression of *crb2a* (g, h) also upregulates in the spinal cord at 14 somites stage in wild-type embryo. No. of embryos with upregulated *crb2a*=7/7.
(i-l) However, there is already trascrptional activity of *pard6-gb* at the 6 somites stage in the spinal cord.
(k-l) *pard6-gb* transcription expression do not differ significantly at the 14 somites stage. N(*pard6-gb*) at 6 somites stage=10 embryos and No. of embryos with upregulated *pard6-gb*=5/5

This was an interesting observation that genes of the different polarity complexes differ by their temporal expression dynamics. Therefore, we speculated that Notch signaling might regulate

specifically the transcription of the *crumbs* genes. For this, I checked the transcriptional expression of *crb1*, *crb2a* and *pard6-gb* at the 14-somites stage in Mib^{ta52b} mutant embryos. *In-situ* hybridization at the 14 somites stage shows Mib mutants display a severely reduced transcription of *crb1* and *crb2a*, whereas *pard6-gb* was not much affected (Figure 42). This suggests that Notch signaling regulates in particular the transcription of *crumbs* genes.

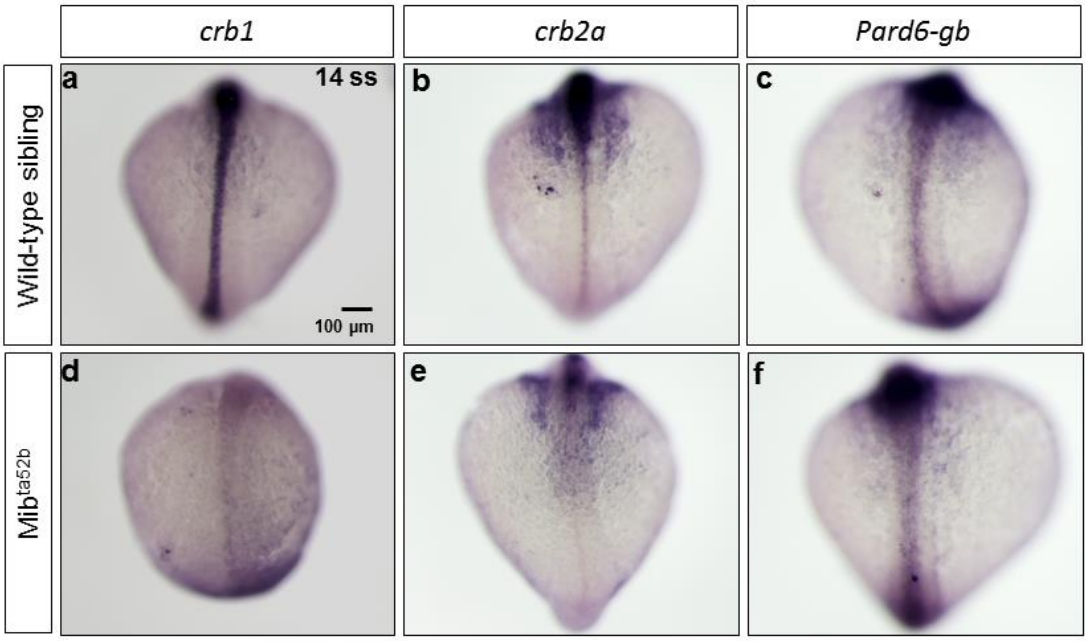


Figure 41: Transcription of polarity genes during early spinal cord morphogenesis in Mib mutant.

Whole mount in-situ hybridization dorsal view images showing (a) the transcript of *crb1* in wild-type sibling (no. of embryos=13) and (d) Mib mutant (no. of embryos=5/5).The transcription of *crb1* is failed to upregulate in a Mib^{ta52b} mutant embryo.
(b) *crb2a* in wild-type sibling (no. of embryos=10) and (e) mib mutant (no. of embryos=8/8). Similar to *crb1, crb2a* also do not upregulate at 14 somites stage in a Mib^{ta52b} mutant embryo.
(c) *pard6-gb* in wild-type sibling (no. of embryos=5) and in (f) Mib^{ta52b} mutant at the 14 somites stage (no. of embryos=5/5). In contrast to *crb1* and *crb2a*, *pard6-gb* transcription is upregulated at the 14 somites stage in a Mib^{ta52b} mutant embryo.

As we found that Mib loss-of-function Notch signaling caused a decrease in the transcription of *crumbs* genes, this suggests that Notch signaling might functions upstream of apical protein complexes. To make this observation strong, I examined the expression pattern of the *crumbs* genes when Notch signaling is hyper-activated. For this I injected wild-type embryos with a constitutively activated form of Notch i.e. Notch-intra-cellular-domain (NICD) RNA. Indeed, NICD-injected embryos showed an elevated expression levels of *crb1* (Figure 42 e-h) and *crb2a* (Figure 42 m-p) in comparison to wild-type embryos (Figure 42 a-d, i-l). It should however be noted that

the injection of activated Notch was sufficient to enhance the expression of *crumbs* genes in their endogenous expression domains, but not to induce their ectopic expression.

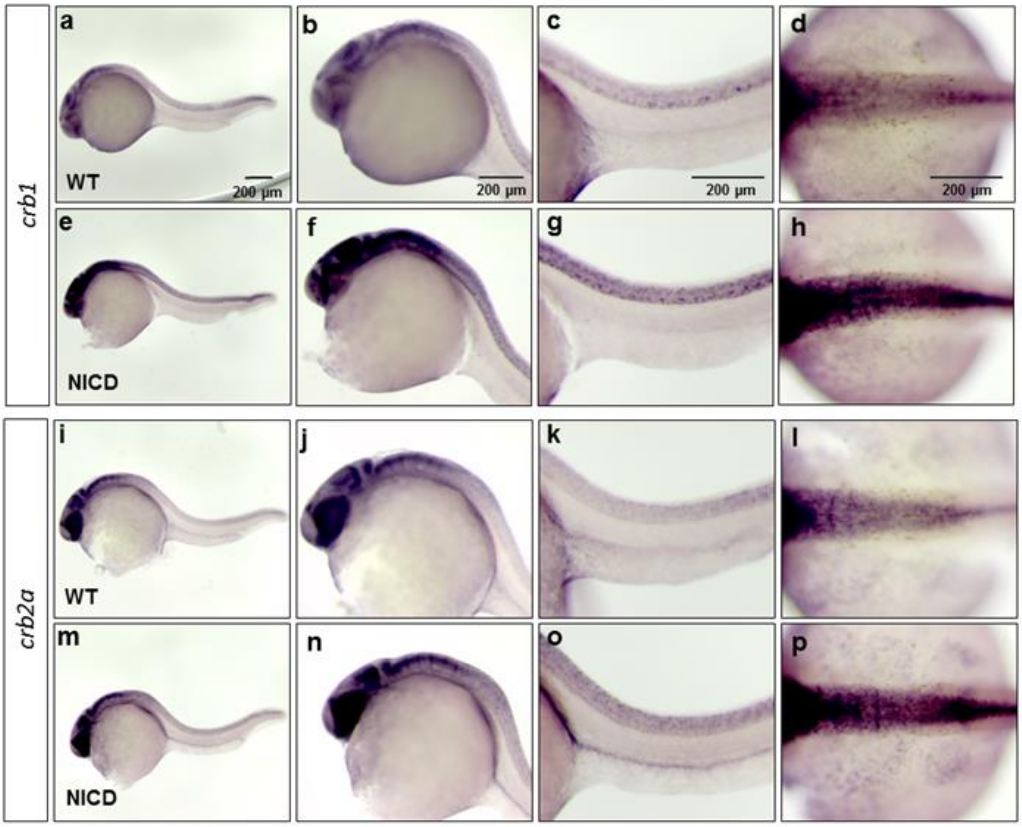


Figure 42: Activated Notch upregulates the transcription of *crumbs* genes
Whole mount *in situ hybridization* images at 24 hpf stage. Lateral view of the whole embryo (a, e, i, m); lateral view of the head (b, f, j, n); lateral view of the spinal cord (c, g, k, n); Dorsal view of the spinal cord (d, h, l, p).
*(*a-d) *crb1* transcript in wild-type embryo
(e-h) *crb1* expression is elevated upon Notch activation through NICD RNA injection.
(i-l) *crb2a* transcriptional expression in wild-type embryo
(m-p) When Notch signalling is overexpressed by introducing NICD RNA, *crb2a* expression is increased. No. of embryos analyzed=5 for each condition.

Our results shows that Mib depletion affect transcriptional expression of the crumbs and the par apical polarity complex genes. Mib is shown to be negatively regulated by the basolateral protein Par1, inducing neuronal differentiation (Ossipova et al., 2009). It would be interesting to see if Mib also regulate the expression of basolateral marker Mark2b (Par1 in mammals) through a feedback loop. Therefore, I further analysed the transcriptional expression of *mark2b* in Mib mutant embryos. I found that, both at 14 somites stage and 20 somites stage expression of *mark2b* does not change in Mib^{ta52b} (-/-) embryos (Figure 42 b, d), in comparison to their wild-type siblings (Figure 42 a, c). Altogether, our results suggest that Notch signalling modulate apico-basal polarity through apical polarity complex, particularly the crumbs complex.

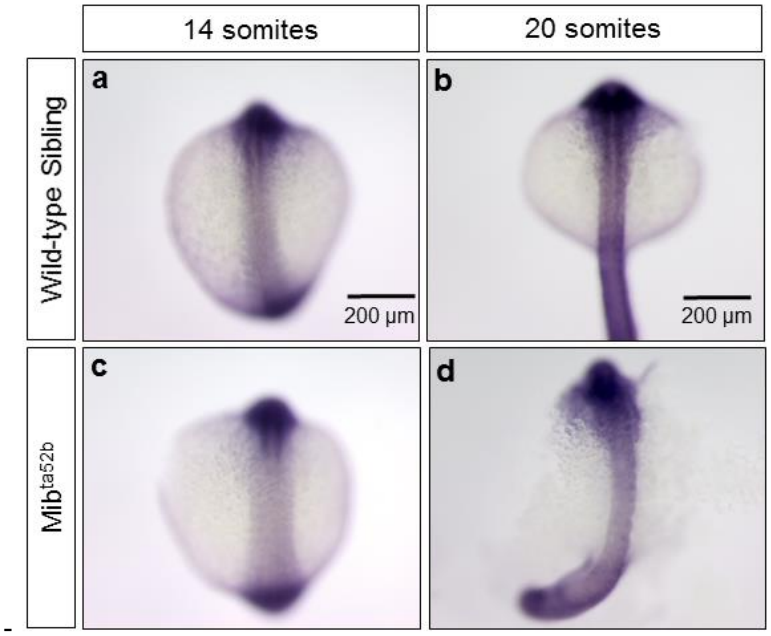


Figure 43: Transcription of the basolateral polarity gene *mark2b*
(a-b) *In situ hybridization* image showing dorsal view of the *mark2b* transcript in wild-type sibling embryos at 14 somites (a) and 20 somites (b). No. of embryos=5/5
(c-d) *mark2b* transcriptional expression in Mib^{ta52b} mutant embryos at 14 somites (c) and 20 somites (d). No. of embryos=5/5. At both 14 and 20 somites stage, transcription of *mark2b* does not change in Mib^{ta52b} mutant embryo.

3 Mindbomb is required for planar cell polarity

3.1 Mindbomb loss-of-function perturbs C-divisions

Establishment of apico-basal polarity is essential for the proper formation of the neural tube lumen. The zebrafish neural plate lacks typical apico-basal polarity until the late neural keel stages, when the neural tube midline has begun to form. The specialized C-division that takes place across the midline during this period, were considered to be a driving force for the establishment of polarity as Pard3-GFP accumulates at the abscission plane of C-dividing cells, which coincides with the forming midline (Tawk et al., 2007). However, more recently other studies have presented evidence against an obligatory role of C-divisions for apico-basal polarity establishment (Buckley et al., 2013; Žigman et al., 2011).

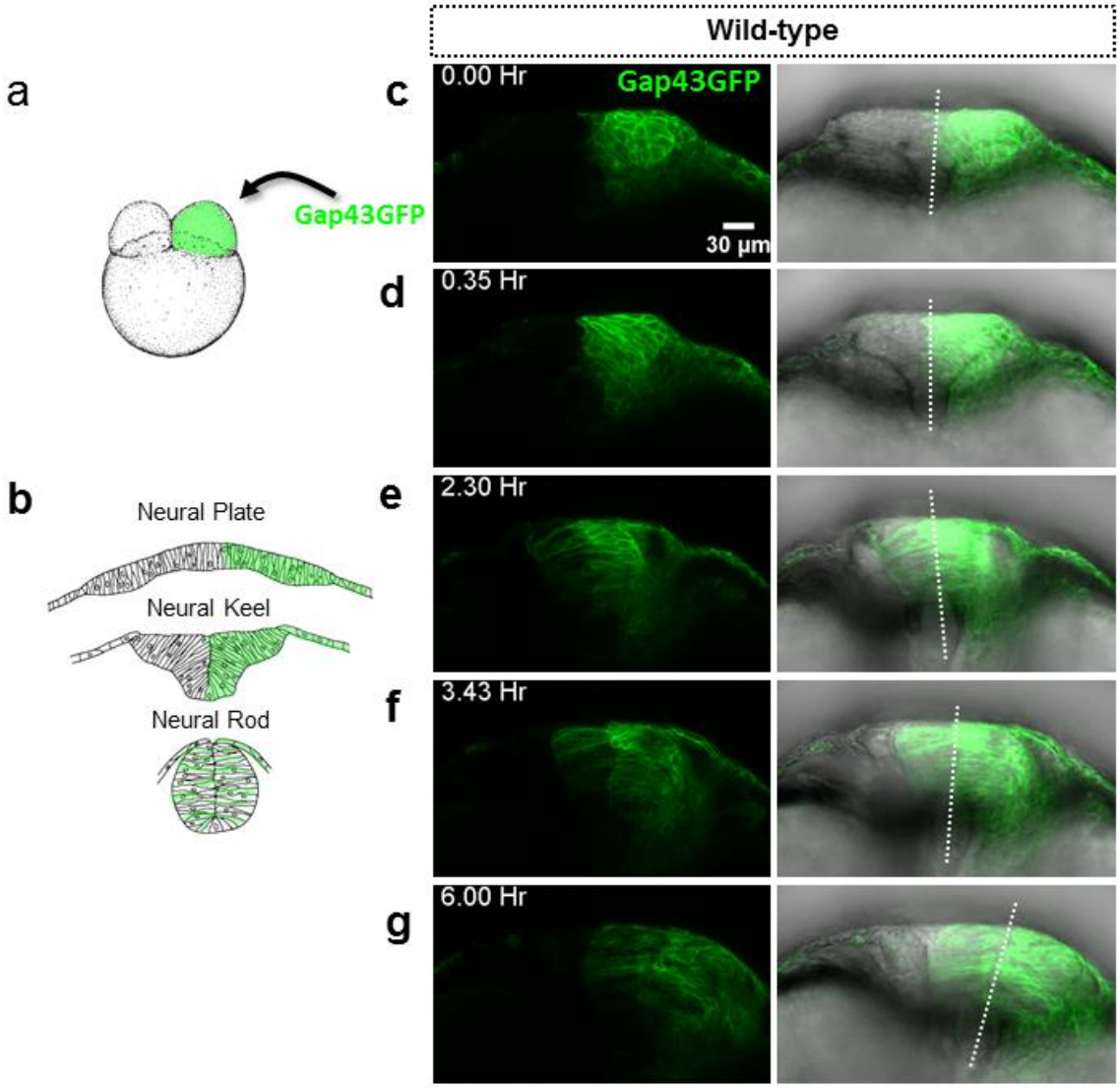


Figure 44: Midline crossing C-divisions in wild-type embryos

(a) Gap43GFP injections in one of the two cells, at the 2-cell stage embryo allows GFP labelling of the cells in half of the neural plate.

(b) A schematic representation of the spinal cord morphogenesis from the neural plate to the neural rod. Image adapted from (Kimmel et al., 1995).

(c-f) Transversal view of the neural tube morphogenesis. The images are taken at different time points from a time-lapse video. No. of time lapse of wild-type embryos= 5

(c) Transversal view of the neural plate with one side labelled with Gap43GFP. Right-hand side image shows the merge of Gap43GFP with the bright-field view.

(d) Transversal view of the early neural keel formation.

(e) During the neural keel stage, cells starts to form projections medially around the region where prospective midline would form.

(f) During the neural keel and the neural rod stages, in order to C-divide, cells shift their bodies to the tissue centre and round up as they enter mitosis.

(g) At the neural rod stage, due to the C-divisions, a homogenous distribution of cells from both sides occurs. Here, this is visible by Gap43GFP labelled green cells and unlabelled black cells that are equally distributed to the contra-lateral sides of the neural rod. A midline also appears at this time.

In my study, I found that the in the absence of Notch signalling, neural tube cells lose apico-basal polarity. Therefore I questioned, how Notch signalling affects C-divisions that orchestrate mirror symmetric apico-basal polarity in the neural tube? To answer this, I choose to follow the dynamic process of C-divisions during neural tube morphogenesis, using confocal time-lapse microscopy. A simple experimental setup was used, where a fluorescent membrane label (Gap43GFP RNA) was injected into one cell of a two cell stage embryo (Movie 3, Figure 44 a). If injected properly, Gap43GFP would label only one side of the CNS. Wild-type embryos showed that the half-labelled neural plate (Figure 44 c) starts to form mediolateral protrusions (Figure 44 d). Soon, the cells that are extending mediolateral protrusions shift their cell bodies medially, and start to round up at the start of the prophase. After the C-divisions, the more medial daughter cells move across the neural tube midline and elongate to cover the full apico-basal extent of the contra-lateral side of the neural tube epithelium (Figure 44 e-g).

To investigate the regulation of C-divisions by Notch signaling, I analyzed C-division in Mib loss-of-function embryo. Hereby, I injected Mib morpholino at the one cell stage and Gap43GFP in one of the two cells at the two cell stage embryo (Movie 4, Figure 45 a). Surprisingly, I found that in Mib-depleted embryos, cells do not form medial protrusions but stick together to stay on the ipsilateral side. Therefore, cells failed to have medially occurring C-divisions (Figure 45 c-e). Several studies have shown that in the zebrafish neural tube, C-divisions depends on the PCP pathway. PCP plays important roles, first in orienting the mitotic spindle of cells (Quesada-Hernández et al., 2010) and second, in the regulation of convergent extension movements of the neural plate (Ciruna et al., 2006).

Therefore, I next investigated, if the Mib ‘non-crossing’ phenotype resembles PCP loss-of-function conditions. Indeed, in embryos deficient for Vangl2, a component of the PCP pathway, neural plate cells do not cross to the contralateral side (Movie 5, Figure 46 a-e). This is very similar to what we observed in Mib morphants. In addition, in agreement with previous studies (Ciruna et al., 2006; Tawk et al., 2007), vangl2 morphants began to form a duplicated neural tube with a bifurcating midline (Figure 46 e). This later phenotype was however not observed in Mib morphants, as the loss of apico-basal polarity prevented us from visualizing the apical neural tube midline. Altogether, these results shows that the Notch signaling component Mib is important for the PCP-driven C-divisions. This further inspired us to investigate whether Mib plays part in other PCP-dependent processes as well such as convergent-extension during gastrulation and neural-tube morphogenesis.

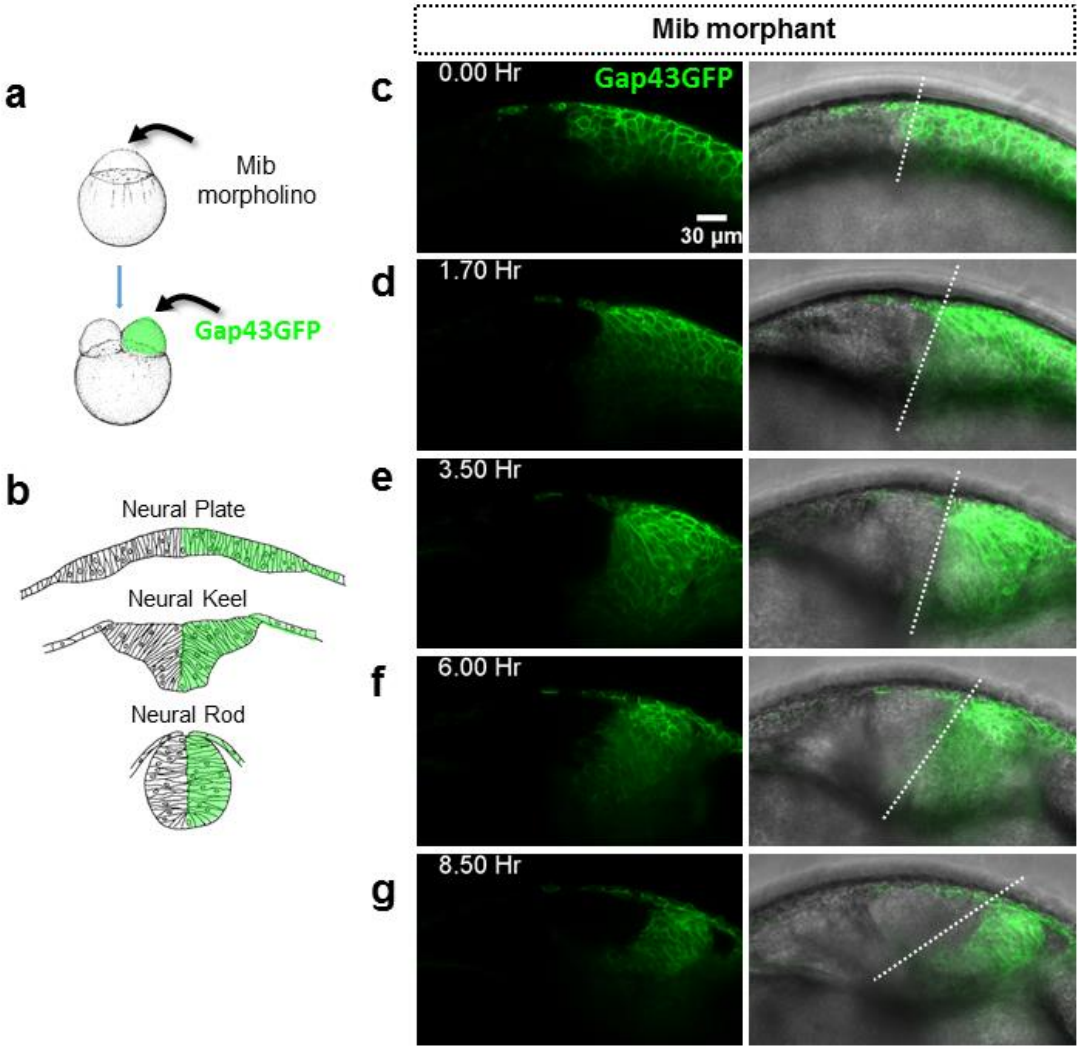


Figure 45: C-divisions do not occur in Mib loss-of-function embryo

(a) Experimentally, Mib morpholino is injected at the one cell stage embryo to get ubiquitous effect of morpholino and Gap43GFP is injected in one of the two cells, at the 2-cells stage embryo to allow GFP labelling of the cells in half of the neural plate.

(b). A schematic representation of the neural plate morphogenesis from the neural plate to the neural rod in the context of this experiment. Image is adapted from (Geldmacher-Voss et al., 2003; Kimmel et al., 1995)

(c-f) Transversal view of the neural tube morphogenesis. The images are taken at the different time points from a time-lapse video. No. of time lapse movies of Mib morphant embryos with the non crossing phenotype=4/4

(c) Transversal view of the neural plate with only one side labelled with Gap43GFP. Right-hand side image shows the merge of Gap43GFP with the bright-field view. The white dotted line shows the tissue centre.

(d) The neural plate starts to move inward in order to form the neural keel.

(e) During the neural keel stage, cells do not form any projections medially at the tissue centre.

(f) Transversal view the late neural keel, cells still do not form any projection to reach to the contralateral side of the tissue. Instead cells stays at the ipsilateral side.

(g) Transversal view of the neural rod stage of Mib morphant shows that cells do not cross to the contralateral side.

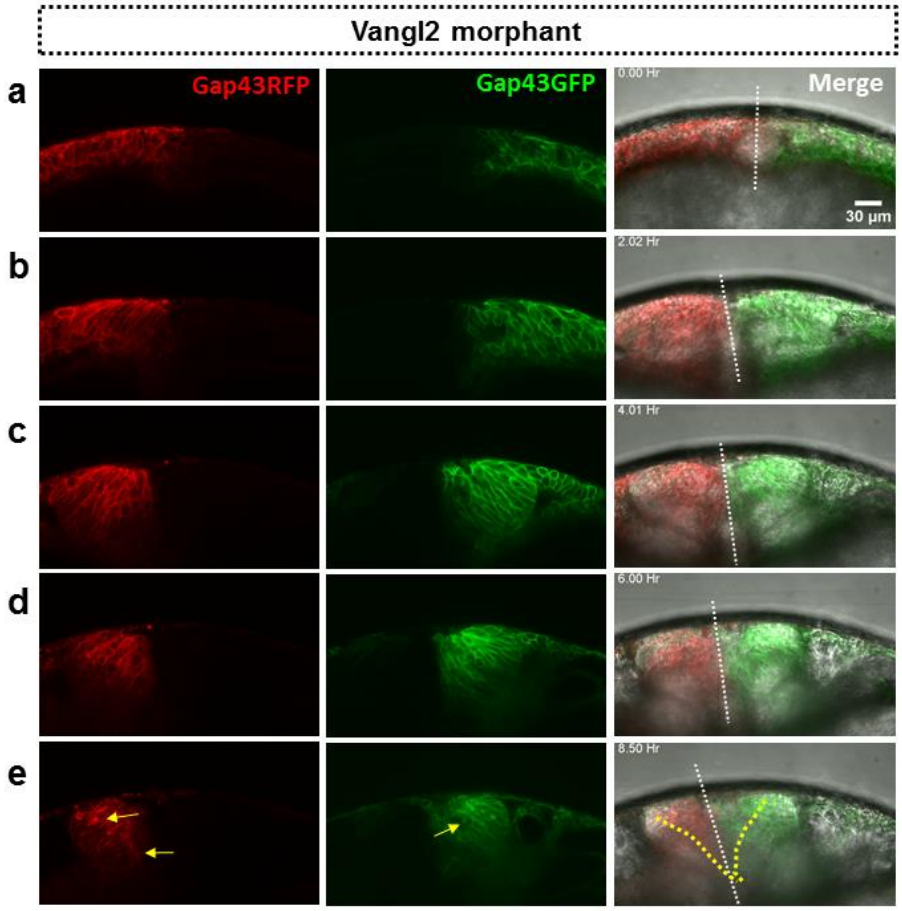


Figure 46: Vangl2 morphant embryos shows ectopic midline formation and lack of midline crossing.

(a) Transversal view of the neural plate of Vangl2 morphant embryo that is injected at the two cells simultaneously with Gap43GFP and Gap43RFP mRNA, in order to label half CNS with RFP and other half with GFP. The images are taken from a time-lapse at the level of anterior spinal cord.

(b-d) The neural plate transforms into the neural keel. The red and green cells do not interdigitate into each other's side.

(e) Ectopic midlines form at the lateral sides of the neural rod (yellow arrows), rather than a single midline at the tissue centre. In merged image the ectopic midlines are shown by dotted yellow lines and tissue centre by a white dotted line. No. of time lapse movies of vangl2 morphant embryos with the noncrossing phenotype=3/3

3.2 Mindbomb-depleted embryos exhibit convergent-extension defects

My previous results showed that Mib depleted embryo failed to have C-division that take place across the mediolateral axis. C-divisions during zebrafish neurulation are depended on the PCP pathway. The PCP pathway mainly contributes to the process of C-divisions by bringing close the two sides of the neuroepithelium and this is done by the convergent-extension movement. Therefore, there is a possibility that Mib depletion affects PCP-dependent convergent extension

and hence causes the failure of C-divisions. To explore this possibility, I looked for convergent-extension defects during gastrulation and neurulation.

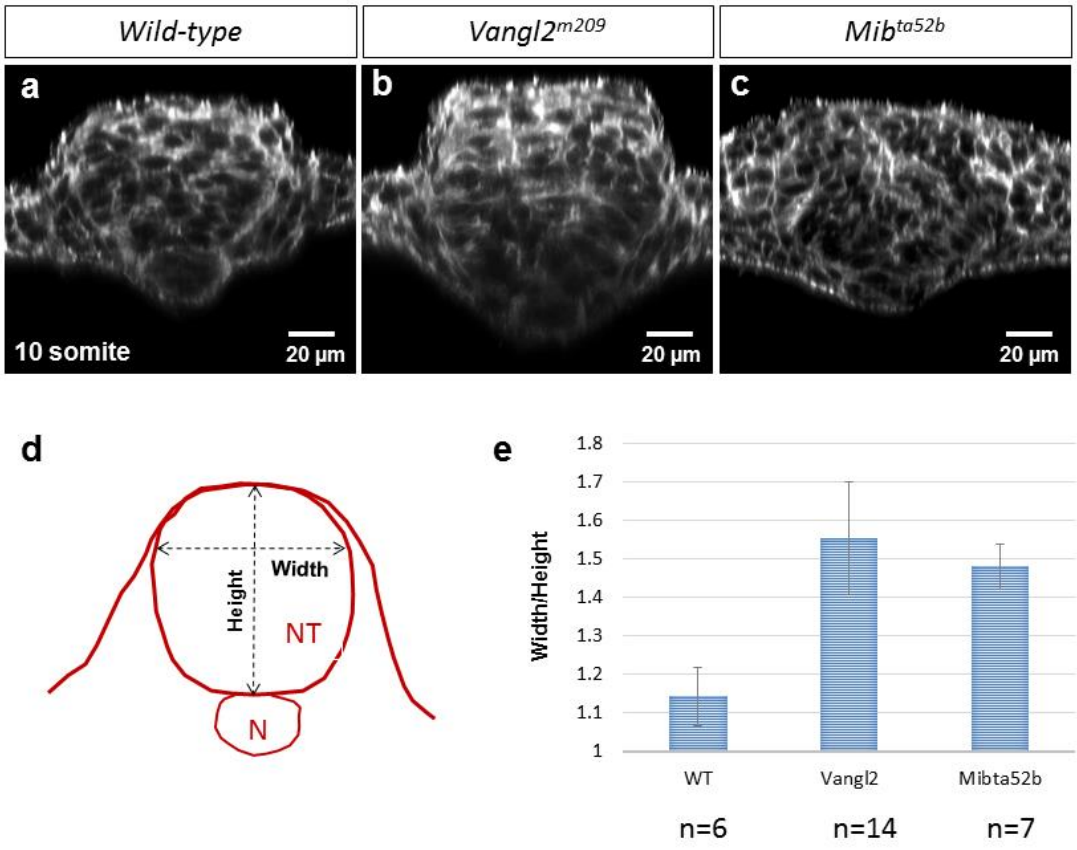


Figure 47: Mib loss-of-function embryos exhibit a broader neural tube.
(a) A confocal line scan at the level of first somite shows the transversal view of wild-type embryo at the10 somites stage.
(b-c) Line scan showing the transversal view of Vangl2 mutant (b) and Mib (c) mutant.
(d). A schamatic representation of width and height measurement in the neural rod.
(e) Quantification of the neural-rod broadening by measurement of width to height ratio shows that Mib morphants have the broader neural rod than wild-type. Error bars are standard error of the mean. No. of wild-type embryos=6, No. of vangl2 mutant embryos=14, and No. of Mib mutant embryos=7.

To determine convergent-extension defects, I measured the width-to-height ratio of the neural tube at the 10 somites stage in Vangl2 and Mib morphant embryos to observe convergent-extension defects. Due to the delay in convergent extension movements, PCP-defective embryos possess a shorter and less extended body axis and therefore, the neural tissue (Neural keel/rod/tube) size is wider than in wild-type embryos. I found that, indeed, Mib mutant embryos (Figure 47 c) show a broader neural tissue than wild-type embryos (Figure 47 a), just like vangl2 loss-of-function embryos (Figure 47 b).

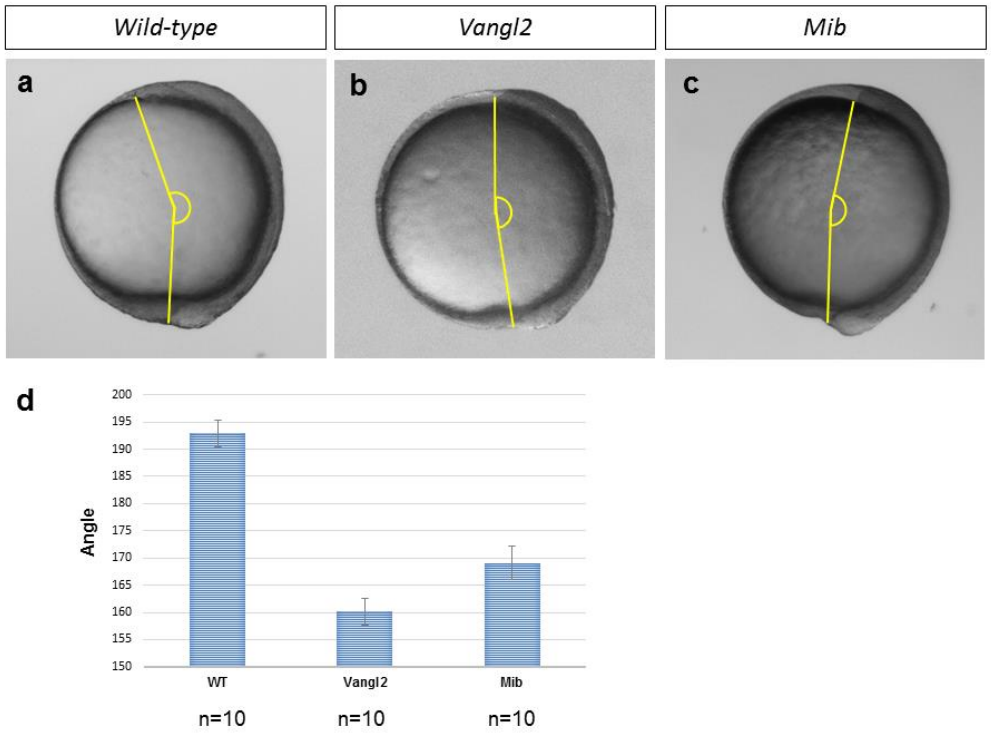


Figure 48: Mib depleted embryos display a shorter body axis
(a-c) Transmitted light lateral view images of wild-type (a), Vangl2 morphant (b), and Mib morphant (c) embryos at the tailbud stage. The angle between anterior and posterior ends are shown by yellow lines.
(d) Quantification of body axis elongation by measurement of angle between anterior and posterior extents, shows the reduction in body axis elongation in Mib morphants similar to Vangl2 morphants. Error bars are the standard error of the mean.

One classical way to examine convergent-extension defects during gastrulation is to measure the angle between the anterior and posterior extremities of the elongating body axis at the end of gastrulation (tail bud stage). PCP mutants are known to exhibit a shortened and broadened body axis. Lateral views of the bud stage Vangl2 morphant embryos shows a reduced angle between the head and the tail (Figure 48 b), in comparison to wild-type embryos (Figure 48 a). Similar to Vangl2, Mib-depleted embryos also displayed a shortened body axis i.e. a reduced axis extension angle (Figure 48 c, d). This suggests that Mib morphants also present convergent-extension defects. Convergent-extension defects at gastrulation were further confirmed in Mib^{ta52b} mutants (Figure 49).

Altogether, these results suggest that Mib is important for PCP-regulated convergent-extension movements. Another, important role of the PCP pathway is in the regulation of stereotypical division orientation during gastrulation and neurulation. I have earlier shown that Mib affects PCP-dependent C-divisions. There could be two ways to affect C-divisions, first by perturbing

convergent-extension movement, and second by affecting the division orientation of the neuroepithelial cells. I have already shown that Mib is important for convergent-extension. I next investigated if Mib is also required for stereotypical division orientation during gastrulation.

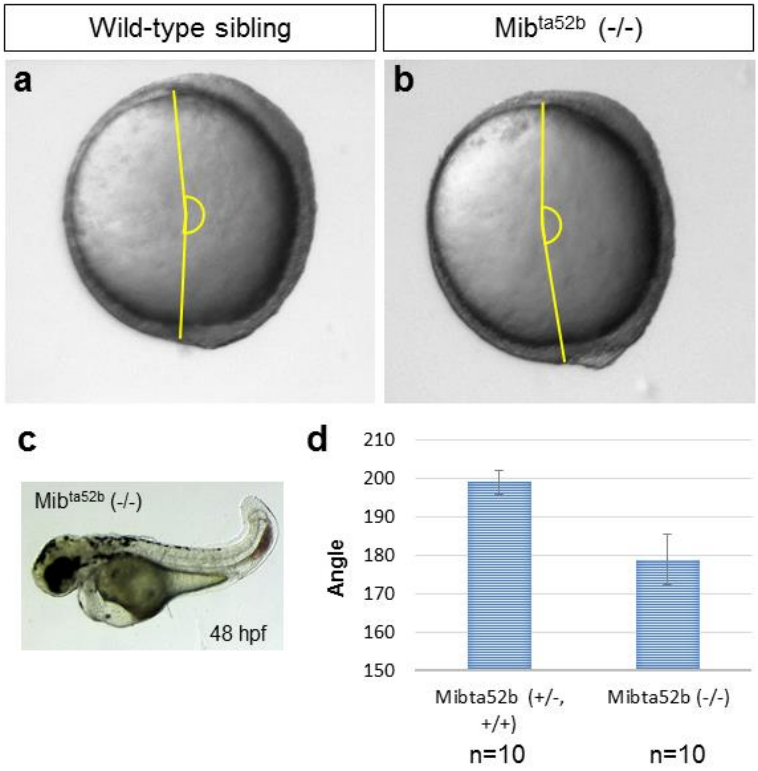


Figure 49: Mib mutant embryos show a shorter body axis.
(a-b) Transmitted light lateral view images of a wild-type sibling embryo (a) and a Mib homozygous mutant at the tailbud stage. The angle between anterior and posterior extent of the body axis are shown by yellow lines.
(c) Embryos were grown till 48 hpf and Mib^{ta52b} (-/-) embryos were selected based on the white-tail phenotype.
(d) Quantification of the body axis elongation by measuring the angle between anterior and posterior ends of the body. n= no. of embryos. Error bars are the standard error of the mean.

3.3 Mindbomb loss-of-function leads to disoriented divisions during zebrafish gastrulation

Stereotypical cell division orientation plays a key role in neural rod midline formation during zebrafish neurulation (Quesada-Hernández et al., 2010). My earlier results showed that Mib affects C-divisions during neurulation and convergent-extension during Zebrafish gastrulation and neurulation, two processes that are dependent on PCP. I further asked if Mib also affects stereotypical division orientation during zebrafish gastrulation, another PCP-dependent process.

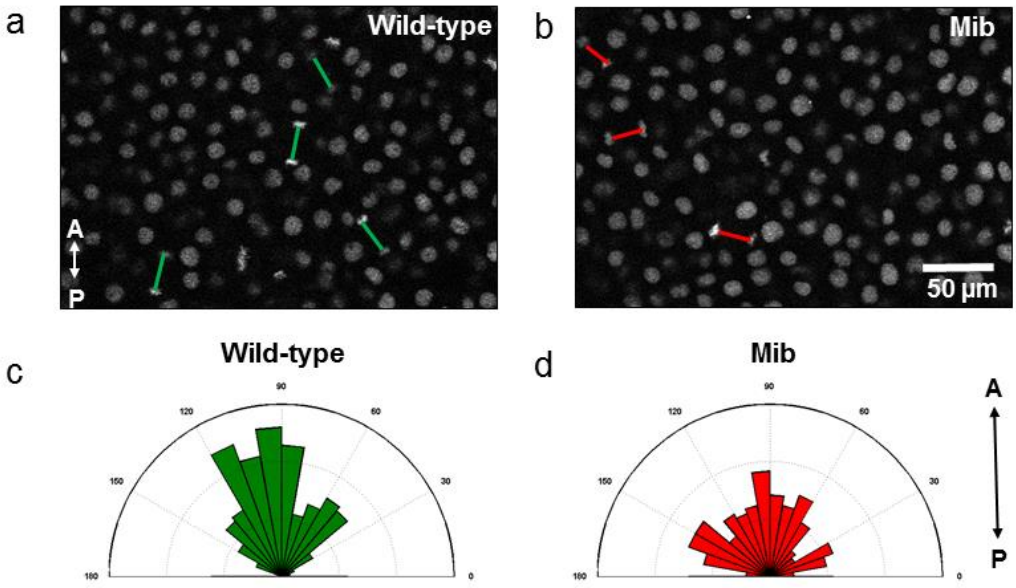


Figure 50: Mib loss-of-function embryo exhibit alterations in sterotypical division orientation during gastrulation.

(a) Orientation of the mitotic plane of epiblast cells with respect to the anterior-posterior axis in a Tg:H2A-GFP transgenic control embryo. The division planes of dividing cells are shown by green lines.
(b) Orientation of the mitotic plane of epiblast cells with respect to the anterior-posterior axis in a Tg:H2A-GFP transgenic embryo injected with Mib morpholino. The division planes of dividing cells are shown by red line.
(c) Polar graphs showing the frequency distribution of angles between the division axis and the anterior-posterior (A-P) axis in wild-type control embryos. No. of wild-type embryos=5 and cells=260
(d) Polar graphs showing the frequency distribution of angles between the division axis and the A-P axis in Mib morphant embryos. No. of Mib morphant embryos=4 and cells=220
The bidirectional arrow on the side shows the A-P axis.

During gastrulation, stereotypical cell division orientation contributes to body-axis elongation by positioning the daughter cells along the axis of elongation. In wild-type embryos the mitotic spindle is actively oriented along the anterior-posterior axis in the dorsal epiblast cells, a process that is disrupted in PCP mutants (Gong et al., 2004). Therefore, I carried out confocal imaging to analyse if Mib depletion also led to a disruption of division orientation in dorsal epiblast cells. Imaging was performed as described by Gong and colleagues. We found that, indeed, Mib morphant embryos displayed alterations in their division orientation i.e. epiblast cells no longer divide in an anterior-posterior oriented fashion (Figure 50 a). Quantifications of division orientation in wild-type and Mib morphant embryos are shown in Figure 50 (b, c). Altogether, these results shows that the Notch signalling component Mib is important for PCP-driven convergent-extension movements and oriented cell-divisions.

3.4 Mindbomb's role in PCP is independent of its function in Notch signaling

Several studies have shown that Notch pathway can contribute to several PCP driven processes e.g. photoreceptor cell fate determination during eye development in flies (Capilla et al., 2012; Strutt et al., 2002). We found that inhibition of the Notch signaling component Mindbomb lead to a disruption of C-divisions, convergent-extension defects and failures in stereotypical division orientation during gastrulation and neurulation. Therefore, next we asked ourselves whether the function of Mib in PCP is independent of its role in Notch signaling or whether overall Notch signaling contributes to PCP. First, I introduced a constitutively active-form of Notch (NICD) in Mib morphants, and measured the angle of body axis extension at the end of gastrulation. I found that introducing activated Notch could not rescue Mid-dependent convergent-extension defects (Figure 51 a-d).

In addition, I performed a similar rescue experiment to analyze the effect of Notch signaling on C-divisions. This experiment was based on the assumption that if Notch signaling were not required for C-divisions, introducing a constitutively active form of Notch would rescue the neural tube apico-basal polarity but not PCP-driven C-divisions. In this scenario, Mib loss-of-function embryos should produce a bifurcating double lumen just like other PCP mutants. The experimental scheme was to inject Mib morpholino and NICD RNA at the one cell stage along with a red membrane marker, (Gap43RFP), followed by the injection of a green membrane label (Gap43GFP) at the two cell stage (Figure 52 a). This complete (RFP) and half-labeling (GFP) of the neural tube allowed us to visualize the occurrence of midline-crossing C-divisions as well the whole structure of the neural tube (Figure 52 b).

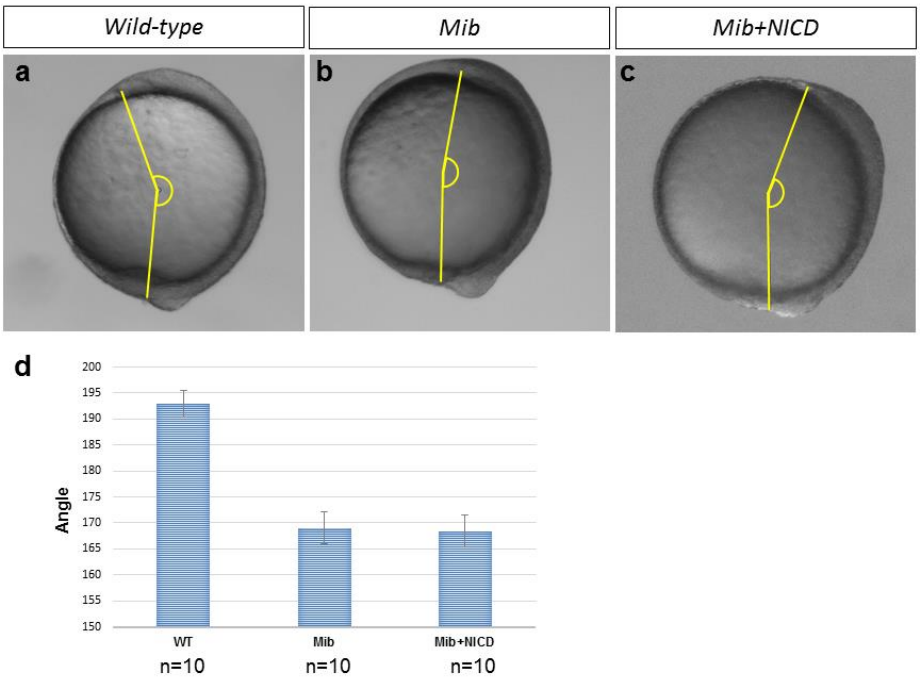


Figure 51: Activated Notch does not rescue the convergent-extension defects of Mib morphant embryos.

(a) Transmitted light lateral view image of wild-type embryo.
(b) Transmitted light lateral view image of Mib morphant embryo.
(c) Transmitted light lateral view image of MIb morphant embryo injected with NICD mRNA.
(a-d) The anglea between the anterior and the posterior ends are shown by yellow lines.
(e) Quantification of body axis elongation by measurement of angle between the anterior and the posterior ends shows that introducing an activated form of Notch in Mib morphant does not rescue convergent-extension defects. Error bars are standard error of the mean. n= no. of embryos.

Indeed, we found that introducing Notch in Mib morphants could not rescue the crossing phenotype, and cells (in green) lingered on the ipsilateral side (Movie 6, Figure 52 b-f). However, introducing activated Notch in Mib morphants could rescue apico-basal polarity, leading hence to the formation of a bifurcating double lumen in the two halves of the nervous system (Figure 52 f). Overall, these results show that Mib plays an additional role in the PCP pathway that is independent of its well-known function in Notch signaling.

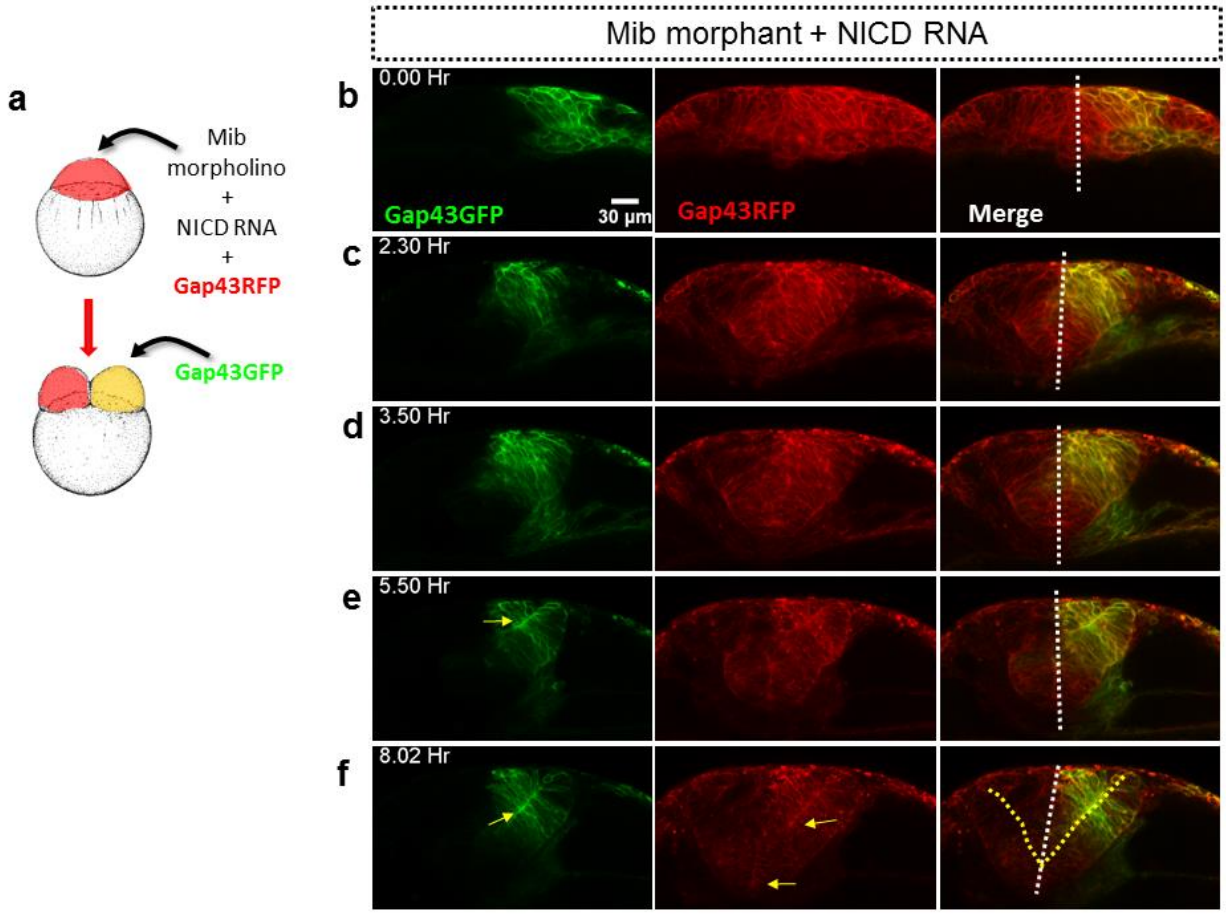


Figure 52: Constitutively activate Notch restores apico-basal polarity but not midline crossing of spinal cord cells

(a) A one cell stage embryo is injected with Mib morpholino and activated Notch (NICD RNA). In addition, Gap43RFP RNA in injected to label the cells of the whole embryo. At the two cell stage, one of the cells is injected with Gap43GFP to label only half of the neural tube.
(b) A transversal confocal image of the neural plate shows half GFP and full RFP labelling of the neural-plate.
(c-d) Morphogenesis of the neural plate into the neural keel. Green cells do not cross the tissue centre.
(e) A yellow arrow shows the initiation of midline formation at the lateral side. Green cells still do not cross to the contralateral side.
(f) Formation of the neural rod. Ectopic midlines (indicated by yellow dotted lines) form at the lateral sides. The tissue centre is represented by a white dotted line. No. of time lapse movies with=2/2

3.5 Mindbomb convergent-extension defects are rescued by a PCP downstream mediator.

Our results suggest that Mib plays a role in PCP signalling and is required for PCP-dependent convergent-extension movements. To further ensure the participation of Mib in PCP signalling, I wondered if the Mib loss-of-function phenotype can be rescued by a PCP protein. Thereby, I took

a candidate approach and performed experiments with several PCP proteins. In recent years, a role for Rho family GTPases downstream of Wnt/PCP has been demonstrated in a variety of morphogenetic processes associated with zebrafish development (Schlessinger et al., 2009). Thereby, I injected *rhoA* (a small GTPase) mRNA in Mib loss-of-function embryos. Interestingly, anterior-posterior body axis shortening is rescued by RhoA (Figure 53 a-e). Taken together, these results strongly indicate a role of Mib in PCP signalling.

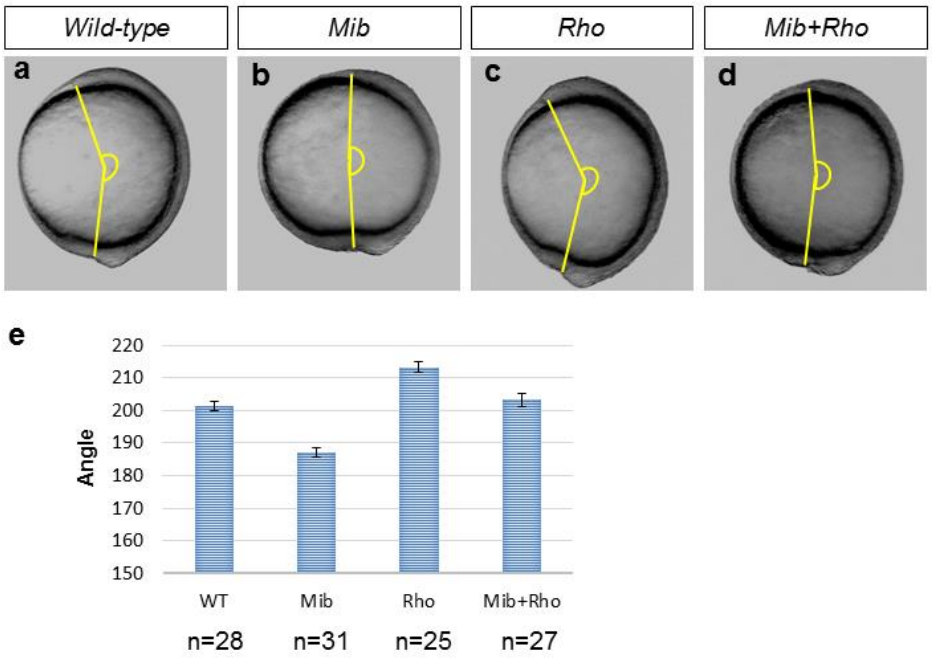


Figure 53: RhoA rescues the convergent-extension defects of Mib morphants.
(a) Transmitted light lateral view image of a wild-type embryo.
(b) Transmitted light lateral view image of a Mib morphant embryo.
(c) Transmitted light lateral view image of a wild-type embryo injected with RhoA mRNA.
(d) Transmitted light lateral view image of a Mib morphant embryo injected with RhoA mRNA.
(a-d) The angle between the anterior and the posterior ends is shown by yellow lines.
(e) Quantification of body axis elongation by measurement of the angle between the anterior and the posterior extent of the body axis shows that RhoA rescues the convergent-extension defects of Mib morphants. Error bars are standard error of the mean. n= no. of embryos

3.6 Mib might interact with Ryk to affect planar cell polarity

Our results shows that Mib loss-of-function affects PCP signaling. We next asked how Mib specifically contributes to the regulation of the PCP pathway. A recent study in *C. elegans* has shown the interaction of Mib with the Receptor related to tyrosine kinase (Ryk) to activate Wnt/β-catenin signaling (Berndt et al., 2011). In zebrafish, Ryk is shown to be a Wnt co-receptor. Ryk

deficiency led to several gastrulation defects (Lin et al., 2010). Hence, Ryk gives an interesting opportunity to determine how Mib contributes to the Wnt/PCP pathway.

To explore if Mib and Ryk interact with each other, I co-injected a hypomorphic dose of Mib and Ryk morpholinos. Under these conditions, single knockdown of Mib or Ryk has no effect on planar cell polarity and therefore, only single midline is formed these embryos (Figure 54 a-h). On the other hand, when Ryk and Mib morpholinos were coninjected, two midline starts to appear. This initial observation suggests that Ryk might interact with Mib and link Mib to the PCP pathway.

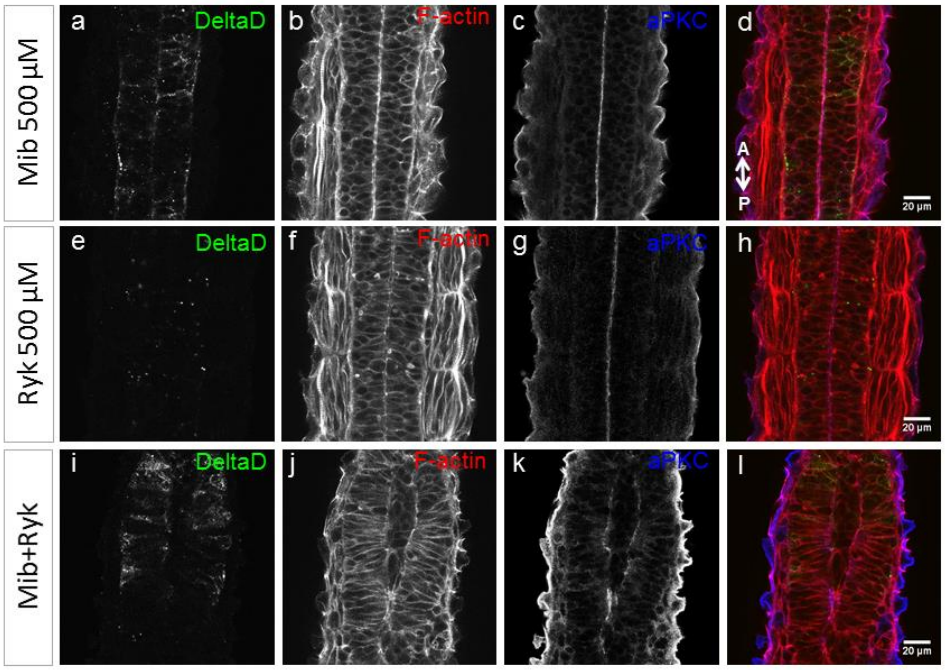


Figure 54: Mib interaction with Ryk

(a-d) Dorsal confocal image of an embryo injected with Mib morpholino with a hypomorphic dose of 500 µM. DeltaD (a), F-actin (b), aPKC (c) and Merge (d). No. of embryos=5

(e-h) Dorsal confocal image of an embryo injected with Ryk morpholino with a hypomorphic dose of 500 µM. DeltaD (e), F-actin (f), aPKC (g) and Merge (h). No. of embryos=5

(i-l) Dorsal confocal image of an embryo injected with Mib and Ryk morpholinos with a hypomorphic dose of 500 µM of each shows the ectopic midline formation. DeltaD (i), F-actin (j), aPKC (k) and Merge (l). Ryk+Mib moprphant embryos analysed with midline defects=5/8

4 Cell autonomy of Mib mutant phenotypes

C-division are an important process during the morphogenesis of the zebrafish neural tube. During C-divisions, Pard3-GFP localize at the apical end of the cells and generates two daughter cells with mirror symmetric apico-basal polarity, suggesting a role of C-divisions in establishing

apico-basal polarity (Tawk et al., 2007). However, further studies have shown, when C-division were inhibited, neuroepithelial cells are still able to polarized (Buckley et al., 2013; Quesada-Hernández et al., 2010). Hence, other mechanisms must contribute to the establishment of midline polarity.

Our observations that Notch signalling is important for apico-basal polarity and Mib is having a Notch-independent function in regulating C-divisions, led us to a hypothesis that C-dividing sister cells might interact with each other via Notch signalling to send signals across the midline to establish polarity. In order to determine whether extrinsic or intrinsic factors are important for certain developmental processes, cells can be transplanted into a different environment. Therefore, it is a suitable technique to study the importance of Notch signalling for C-dividing cells. To analyse if transplanted cells are able to C-divide normally and project all along the extent of the apico-basal axis, I used membrane-GFP (Gap43GFP) to label donor cells. Secondly, to determine if transplanted cells can polarize itself in the host environment, I injected donor embryos with Pard3-GFP mRNA. We observed that Gap43GFP wild-type cells transplanted into wild-type host behaved normally and elongated themselves along the apico-basal extent of the neuroepithelium (Figure 55 a-d).

Similarly, Pard3-GFP labelled donor were able to deposit Pard3-GFP at the apical end of the cells that lies at the midline. We observed two kinds of transplanted cells, around 69% of cells we analysed probably went through C-division, as the two sister cells connect to each other at the apical end, where Pard3-GFP accumulates (Figure 55 e-g). Secondly, 31% of cells were found to be single cells on either side of the neural tube that is having no sister cells on the contralateral side (Figure 55 h-j). These single cells are, however, able to deposit Pard3-GFP at the apical tips. This also suggests that not necessarily all cells undergo C-divisions in the zebrafish neural tube and moreover, this observation supports the argument that C-divisions alone are not essential for apico-basal polarity.

Next, I transplanted Gap43GFP or Pard3-GFP injected Mib mutant cells into the wild-type embryos. In this condition, transplanted cells cannot act as Delta ligand-presenting cells. However, surrounding wild-type cells can trigger Notch signalling in the transplanted cells. We found that Mib loss-of-function donor cells, injected with Gap43GFP could C-divide and elongate themselves (Figure 56 a-d). Similarly, Pard3-GFP injected Mib mutant cells accumulated Pard3-GFP at the apical midline (Figure 56 e-h). These results shows that Mib-deficient transplanted

cells behave like wild-type cells in a wild-type surrounding. Importantly, this experiment argues against a potentially relevant Delta/Notch signalling event between mitotic sister cells.

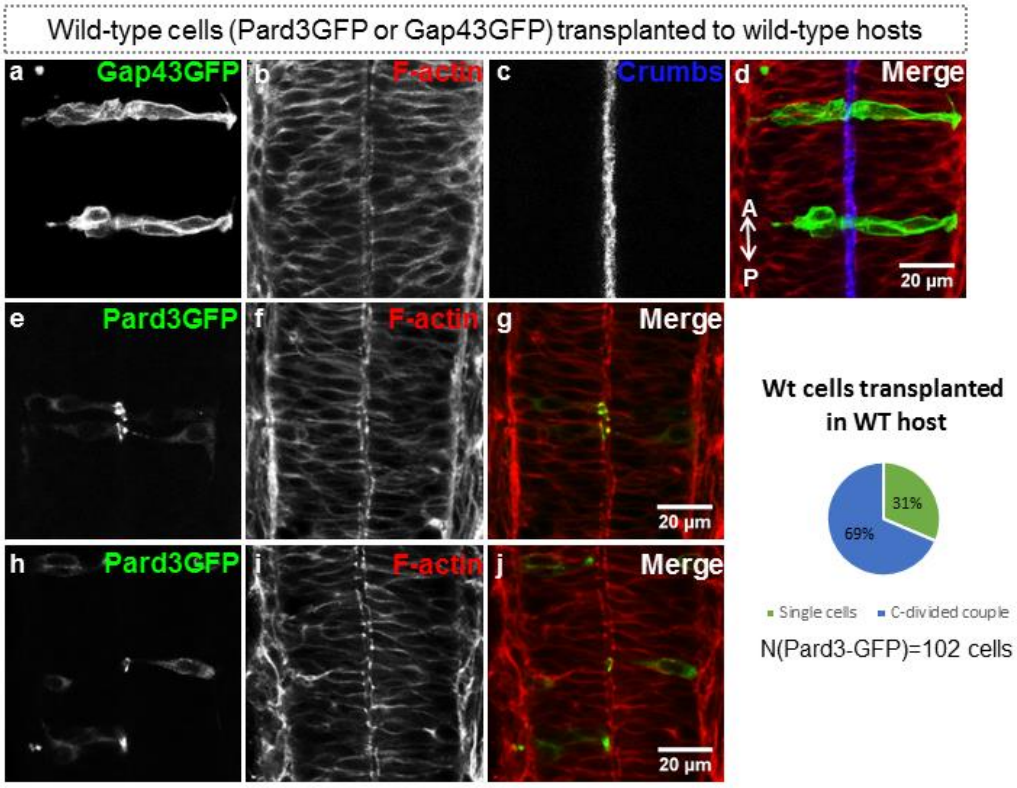


Figure 55: C-dividing cells in wild-type embryos

Dorsal views of the neural tube at the level of anterior spinal cord.

(a-d) Wild-type donor cells injected with Gap43GFP RNA (a), transplanted into a wild-type host that is stained for F-actin (b) and Zs-4(c) for the detection of Crumbs proteins. N= 2 embryos, 23 cells.

(e-g) Wild-type donor cells injected with Pard3-GFP RNA (e), transplanted into a wild-type host that is stained for F-actin (f). Transplanted cells that have undergone C-division, appeared in pairs, one cell to each side of the neural-tube. Pard3GFP accumulated at the apical tip of the cells.

(h-j) Wild-type donor cells injected with Pard3-GFP RNA (h), transplanted into a wild-type host that is stained for F-actin (i). Transplanted cells appeared as single cells that have not undergone C-division, but also accumulate Pard3-GFP at the apical tip of the cell.

N=13 embryos, 102 cells. A pie chart shows that out of 102 transplanted cells, 69% have C-divisions and 31% were single cells.

In a third set of transplantation experiments, Gap43GFP or Pard3-GFP mRNA was injected in wild-type donor cells that were transplanted into Mib mutant host embryos. In this case, Gap43GFP labelled transplanted cells were unable to adopt a typical columnar cell morphology (Figure 56 i-l). Donor cells with Pard3-GFP showed no sign of polarization, and Pard3-GFP is

seen as only as a diffuse signal. Additional Crumbs staining showed a complete absence of Crumbs in transplanted cells (Figure m-p).

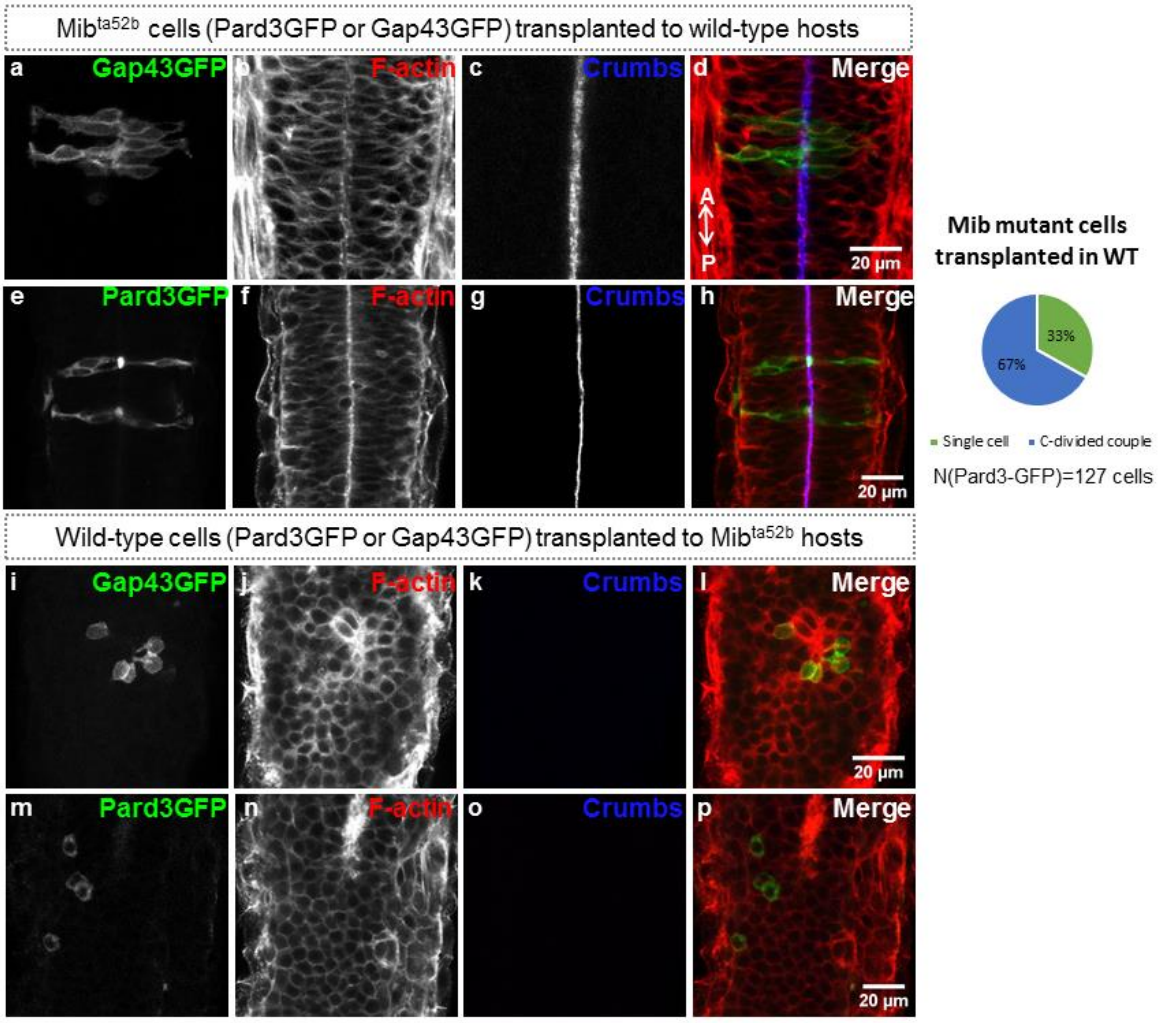


Figure 56: The spinal cord cells do not polarize in cell autonomous way

(a-d) Mib mutant donor cells injected with Gap43GFP RNA (a), transplanted into a wild-type host that is stained for F-actin (b) and crumbs(c). Mib mutant cell stretch itself to elongate along the apico-basal extent of the neuroepithelium. N=6 embryos, 27 cells

(e-h) Mib mutant donor cells injected with Pard3-GFP RNA (e), transplanted into a wild-type host that is stained for F-actin (f) and Crumbs (g). Mib mutant cell in Wild-type environment, are able to C-divide and accumulate Pard3-GFP at the apical end of the daughter cells. N=13 embryos, 127 cells. 67% of the cells could C-divide, and around 33% cells have not undergone C-division.

(i-l) Wild-type donor cells injected with Gap43GFP RNA (i), transplanted into a Mib mutant host that is stained for F-actin (j) and Crumbs (k). Wild-type transplanted cells in Notch-signalling depleted surrounding do not divide and do not exhibit a normal neuroepithelial cell morphology. N= 3 embryos, 8 cells

(m-p) Wild-type donor cells injected with Pard3-GFP RNA (m), transplanted into a Mib mutant host that is stained for F-actin (n) and Crumbs (o). Wild-type transplanted cells in Notch-signalling depleted surrounding do not polarise. N=6 embryos, 30 cells

In summary, these results suggest that neuroepithelial cells do not have cell autonomy to polarize themselves. Surrounding cells can induce apico-basal polarity in Mib-depleted transplanted cells. The question remains if the rescuing Delta/Notch signal originates from surrounding cells sitting next to the mutant cells on the ipsilateral side of the neural tube, or on the contralateral side, or on both sides. As wild-type cells do not exhibit cell autonomy in a Mib mutant host, this suggests that C-dividing cells do not interact with each other via Notch signalling.

5 Conclusion

In summary, my work produced several original findings. First, I found that DeltaD trafficking and links between Delta/Notch signaling and apico-basal polarity vary according to the developmental context: While Delta/Notch signaling is required for the establishment of apico-basal polartiy in the neural tube; it is dispensable for this same process in the inner ear.

In the course of studying the importance of apico-basal trafficking of Delta ligands for Notch signaling, I discovered that Notch signaling itself is important for apico-basal polarity in the zebrafish neural tube as Mib loss-of-function led to a complete loss of apico-basal polarity in the neuro-epithelium. I further showed that this phenotype is due to the failure of overall Notch signaling rather than a Notch-independent function of Mib: First, inhibition of Notch ligands and downstream transcriptional activators Rbpja and Rbpjb resulted in a loss of apico-basal polarity. Secondly, ectopic activation of Notch ensures a complete rescue of apico-basal polarity in Mib loss of function embryos. Through a temporal analysis, I further showed that the Notch signalling is required for the earliest steps of establishment of neuro-epithelial apico-basal polarity. Furthermore, I found that Notch signalling acts upstream of the Crumbs complex to regulate apico-basal polarity. This was evident by the downregulation of *crumbs* expression in Mib loss-of-function embryos.

Another striking result of this study is that the Notch signaling component Mib affects C-divisions through an effect on PCP and convergent extension movements. Remarkably, this effect of Mib on PCP is independent of its role in Notch signaling. I further showed that Mib-depleted embryos display alterations in cell-division orientation during gastrulation. Orientation of cell division is strictly dependent on the PCP pathway, thus indicating a novel role of Mib in the regulation of PCP signaling. In support of this, the convergent extension defects of Mib-depleted embryos can be rescued by the PCP downstream mediator RhoA.

Thus, Mib plays a dual role in the zebrafish neural tube development. On one hand, by participating in Notch signaling it regulates the apico-basal polarity of the neural tube. On the other hand, Mib takes part in PCP signaling to contribute to the regulation of morphogenetic movements and stereotypical division orientations. Altogether, this Ph.D. thesis presents a functional link between Delta/Notch signaling components and the cellular and tissular polarity of the neuroepithelium that is critical for the proper neural tube morphogenesis. A future study will aim to identify the precise mechanism through which Mib functions in PCP signaling.

General Discussion and Perspectives

In this thesis, I uncovered a dual role of the E3-ubiquitin ligase Mindbomb in zebrafish neural tube morphogenesis. I started this project with the initial aim to explore the dynamic process of apico-basal trafficking and transcytosis of Delta ligands *in vivo* and to characterize the significance of apico-basal transport of Delta ligands for the activation of Notch signaling. However, during the first year, I found that conversely, Notch signaling itself is crucial for apico-basal polarity of the neural tube. Moreover, I found an unexpected novel role of the Notch signaling component Mindbomb (Mib) in planar cell polarity. Thus, my study reveals a dual role of Mib in the regulation of apico-basal and planar cell polarity that is crucial for the proper neural tube morphogenesis. In the following, I will discuss the different findings that have emerged from my PhD work.

I. Trafficking of Delta ligands is differentially regulated in a tissue-context dependent manner

Epithelial cells present distinct functional apical and basolateral compartments. Although, it is well established that endocytosis of Delta ligands is essential for Delta-Notch signalling (Itoh et al., 2003), the actual reason for this requirement remains unclear. Using a trans-filter cell culture assay, Benhra and colleagues showed that Delta endocytosis ensures a basal-to-apical transcytosis of ligand molecules that is likely to be essential for productive ligand-receptor interactions (Benhra et al., 2010). My initial aim was to address the vectorial transcytosis model proposed by Benhra and colleagues, in the zebrafish neural tube. In my attempt to investigate the importance of endocytic trafficking for Delta-Notch signalling, I inhibited the function of the E3 ubiquitin ligase Mindbomb (Mib), using both antisense morpholinos and genetic mutants (Itoh et al., 2003). The analysis of Mib-depleted embryos led to the surprising observation that the apico-basal transport of DeltaD ligands is differentially regulated in different parts of the nervous system (Figure 57).

The transcytosis model of Benhra and colleagues postulates that Delta ligands are initially secreted to the baso-lateral cell surface, from where they are then relocalized towards the apical cell surface (Benhra et al., 2010). In accordance with this model, we observed that in the ear of Mib-depleted embryos DeltaD ligands accumulate at the basolateral membrane (Figure 24).

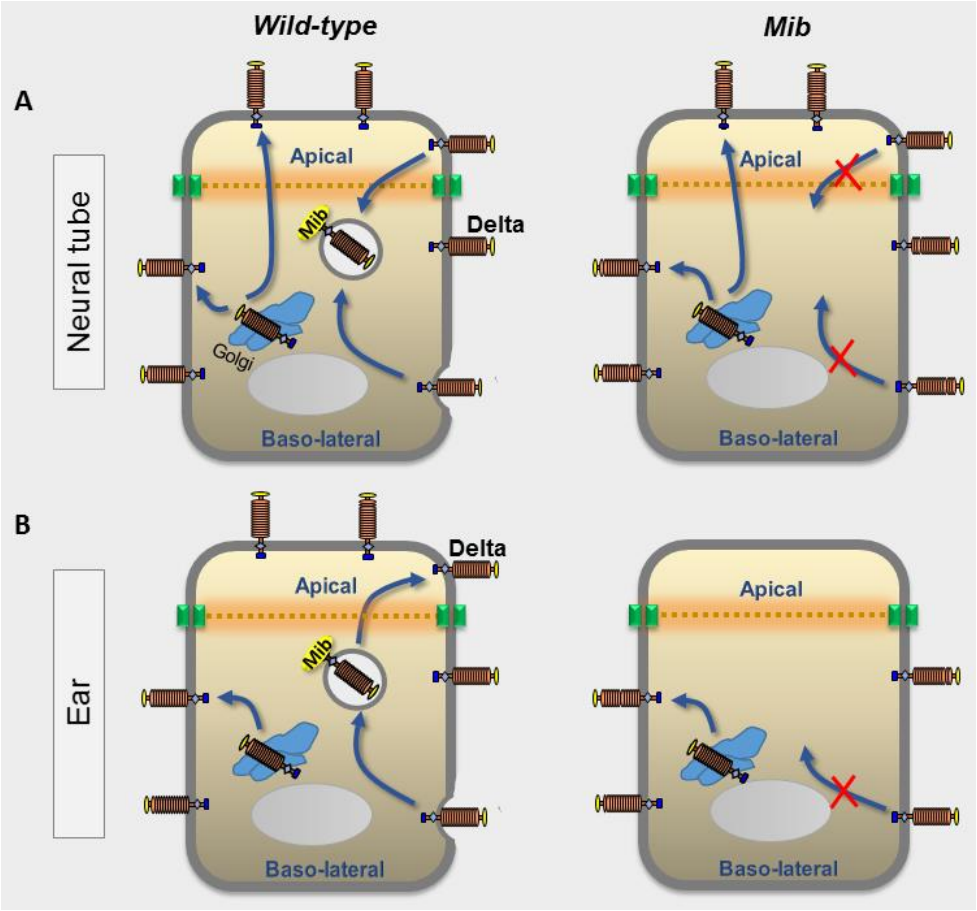


Figure 57: Model of differential regulation of DeltaD trafficking in the neural tube and the ear

(A) In the wild-type neural tube, DeltaD ligands are observed at both apical and basolateral domains in potential endocytic compartments. Upon inhibition of Mib, endocytosis of DeltaD ligands is blocked and therefore they remain at their initial site of secretion. In Mib loss-of-function embryo, DeltaD ligands are observed at both apical and basolateral domain.
(B) In the ear of Mib loss-of-function embryos, DeltaD ligands are found only at the basolateral domain, suggesting DeltaD ligands are secreted primarily at the basolateral membrane.

On the contrary, we found that in the spinal cord DeltaD accumulated on both apical and basolateral membranes upon partial inhibition of Mib. This suggests that the initial site of localization and secretion of delta ligands may not be restricted to the basolateral membrane (Figure 25). It would be very interesting to understand this differential regulation of deltaD trafficking in different part of the nervous system. A future study dedicated to the identification of the cellular regulators important for the internalization, the endocytic sorting and the intracellular transport of DeltaD ligand molecules in the ear versus the neural tube could provide important insights into the differential regulation of Delta trafficking in the two systems.

II. Notch signalling is important for the establishment of apico-basal polarity in the neural tube.

To study the importance of Delta ligand trafficking for Notch signaling, I inhibited the function of the E3 ubiquitin ligase Mindbomb (Mib). Using a high dose of Mib morpholino, I discovered that loss of function Mib led to the complete loss of apico-basal polarity in the spinal cord. This was evident by the loss of apical protein aPKC expression. In addition, the morphology of the neural tube was disrupted, and cells became round, and form rosette-like structure rather than adopting an elongated columnar cell shape (Figure 26). This was confirmed by observing a similar and even stronger phenotype in Mib^{ta52b} mutant (Figure 27). My observations therefore suggest a role of Mib in the regulation of neural tube apico-basal polarity. Recently a study in *Drosophila* has reported a crucial role of the E3-ubiquitin ligase Neuralized in the regulation of epithelial polarity that is independent of its function in Notch signaling (Chanet and Schweisguth, 2012). Neuralized has been previously shown to be essential for the endocytosis of Delta in flies (Pavlopoulos et al., 2001), similar to the function of Mib in zebrafish. Therefore, an obvious question was if overall Notch signaling is required to regulate apico-basal polarity, or like Neuralized, Mib also plays a Notch-independent role in apico-basal polarity.

In order to answer this I inhibited different components of the Notch pathway. First, inhibition of DeltaA ligands by morpholino in DeltaD mutant embryos caused a loss of apico-basal polarity that is similar (albeit somewhat weaker) to the one observed in Mib loss-of-function embryos (Figure 31). Secondly, the blocking of γ-secretase (using the pharmacological inhibitors DAPT and LY411575) that prevents the release of NICD and therefore activation of Notch signaling, also led to the loss of apico-basal polarity (Figure 32). Furthermore, ectopic activation of Notch by introducing a constitutive active form of Notch (NICD) (Iso et al., 2001), resulted in a complete rescue of apico-basal polarity in Mib loss-of-function embryos (Figure 33). These results indicate that overall Notch signaling is important for neural tube apico-basal polarity.

While a previous study suggested a role of noncanonical Notch signaling in the late maintenance of apico-basal polarity (Ohata et al., 2011), my subsequent results suggested that conversely the establishment of apico-basal polarity depends on canonical Notch signaling. I showed this by inhibiting the downstream transcriptional activators of Notch signaling RbpjA and RbpjB (Supressor of hairless Su(H) in *Drosophila* and CSL in mammals) that resulted in loss of

apico-basal polarity (Figure 34). These results altogether suggest that canonical Notch signaling is important for regulating apico-basal polarity in the neural tube of zebrafish.

How does Notch signaling contribute to the apico-basal polarity of the Zebrafish neural tube? In contrary to amniotes, the neuro-epithelial cells of zebrafish neural plate appear to lack typical apico-basal polarity, which only gradually appears at the late neural keel stage. During the neural plate/keel stages, deep and superficial cells from the two sides of the neural plate converge towards the tissue centre, form the neural keel and then the neural rod. At the neural rod stage, a morphologically recognizable midline of the neural tube (corresponding to the apical surface of the future neural tube lumen) becomes visible at the site of apposition of the cells from the two halves of the nervous system and becomes the site of localization of apical polarity proteins (Hong et al., 2010; Yang et al., 2009).

In order to understand the temporal regulation of polarity establishment, I performed a strict time course analysis of zebrafish neural tube development (Figure 36). I found that at 12-somites (14 hpf), polarity proteins start to accumulate at the midline of the neural tube in wild-type embryos. However, there was no accumulation of apical proteins in Mib mutants (Figure 37). This result suggests that Notch signaling regulates the establishment of apico-basal polarity. While previous studies have unraveled late roles of Notch signaling in the maintenance of apico-basal polarity in the zebrafish neuroepithelium (Ohata et al., 2011) and in mouse neural rosettes (Main et al., 2013), my work identifies a novel function of Notch signaling in polarity establishment.

In the murine developing neocortex, Par3 enhances Notch activity and inhibits the differentiation of neuroepithelial cells (Bultje et al., 2009). In *Drosophila*, Crumbs negatively regulates Notch signaling, and through a feedback loop, Notch induces *crumbs* (Herranz et al., 2006). In zebrafish, however, it has been shown that Crumbs binds to the extracellular domain of Notch and inhibits its activity (Ohata et al., 2011). These putative feedback loops may represent important linkage between the apico-basal polarity proteins and Notch signaling. In the context of this study, we found that depletion of Mib diminished the apical accumulation of Pard3 and Crumbs (Figure 28, and Figure 29).

Ohata and colleagues showed in their study that Crumbs binds the extracellular domain of Notch and inhibits Notch signaling. In association with another Crumbs complex protein, Mosaic eyes, noncanonical Notch signaling regulates the maintenance of apico-basal polarity (Ohata et

al., 2011). In my study, I found that transcription of polarity genes require Notch signaling (Figure 40). In addition, through *in-situ* hybridization experiments I showed that in loss-of-function Mib embryos, in particular *crumbs* expression is lost at the transcriptional level (Figure 41). This suggests that Notch might act upstream of the Crumbs complex. What is now needed is to show if Crumbs misexpression can rescue the Notch loss-of-function phenotype.

III. Mindbomb is required for planar cell polarity

Midline crossing C-divisions are one of the key features of zebrafish neurulation. The C-divisions that take place during neural keel and neural rod stages, generate two daughter cells with mirror symmetric apico-basal polarity. The apical polarity protein Pard3 accumulates at the cleavage furrow and is inherited by the two daughters equally at the abscission plane that coincides with the forming neural tube midline. After the division, the two daughter cells stretch themselves along the prospective apico-basal extent of the neuro-epithelium and form projections that reach the basal membrane, and remain connected at the apical tip, where Pard3 localizes (Tawk et al., 2007). Therefore, C-divisions were considered as the driving force for establishing apico-basal polarity in the zebrafish neuroepithelium. However, more recently other studies have presented evidence against the obligatory role of C-divisions for apico-basal polarity establishment (Buckley et al., 2013; Žigman et al., 2011), but still considered that C-division provide a morphogenetic advantage to the embryo (Buckley et al., 2013).

In my study, I found that Pard3 apical accumulation is lost when Mib is inhibited (Figure 28), therefore, it was interesting to know if Mib affected C-divisions. For this I carried out a time-lapse recording of neural tube morphogenesis, where membrane GFP was injected to mark only half of the nervous system. This resulted in a very surprising observation that in Mib loss-of-function embryos, C-divisions were severely perturbed (Figure 45). Several studies have shown that C-divisions are planar cell polarity (PCP) dependent (Ciruna et al., 2006; Quesada-Hernández et al., 2010; Tawk et al., 2007). In maternal zygotic mutants for a PCP core component, Vangl2/Trilobite, cells from the two sides of the neural rod never cross to the contralateral side. Instead C-divisions incorrectly take place at the lateral sides of the neural rod, leading to the ectopic generation of a bifurcating double neural tube midline (Ciruna et al., 2006). Thus Mib loss-of-function, gives a phenotype that is similar to the vangl2 mutant with respect to the loss of the midline crossing behaviour of neural keel/rod cells (Figure 46).

However, we did not observe a double neural midline in Mib loss-of-function embryos, since Mib depleted embryo also exhibit apico-basal polarity defects.

PCP signalling is required to direct intercalative behaviour of C-dividing cells along the medio-lateral axis by mediating convergent-extension movements (Ciruna et al., 2006). Therefore, there is a possibility that Mib depletion affects PCP, which then causes the failure of C-divisions. Accordingly, we found that Mib loss-of-function led to convergent-extension defects, resulting in embryos displaying a shorter body axis at the tail-bud stage (Figure 48) and a broadening of the central nervous system during neurulation (Figure 47). Furthermore, PCP signaling has been implicated in orienting the mitotic spindle along the medio-lateral axis of the dividing cells (C-divisions) during neurulation (Quesada-Hernández et al., 2010) and along the anterior-posterior axis during the gastrulation (Gong et al., 2004). My analysis of stereotypical cell division orientation during gastrulation led to the observation that Mib depletion indeed led to the alteration in cell-division orientation (Figure 50). The RhoA small GTPase protein has been identified as a downstream effectors of PCP signaling (Schlessinger et al., 2009). I further provided evidence in support of the role of Mib in PCP by rescuing the convergent-extension defects in Mib loss-of-function embryos through introducing RhoA mRNA (Figure 53).

The PCP and Notch pathways have been reported to be interrelated. In *Drosophila*, Notch signalling has been shown to contribute to PCP-dependent processes such as R3/R4 photoreceptor cell fate determination during eye development (Cooper and Bray, 1999; Strutt et al., 2002). Conversely, PCP signalling coordinates the spatial activity of the Notch pathway by regulating the endocytosis of Notch receptor during *Drosophila* leg development (Capilla et al., 2012). Thereby, I addressed the question if Mib loss-of-function affects PCP through Notch signalling. I found that introducing an activated form of Notch in Mib-depleted embryos did not rescue convergent-extension phenotypes (Figure 51). Moreover, time-lapse recording showed that introducing activated Notch in Mib morphants rescues the apico-basal polarity of the neural tube but fails to rescue the crossing of cells to the contralateral side. Interestingly, Notch signalling rescued Mib morphants present a bifurcating dual neural tube midline that is remindful of the phenotypes of the *vangl2* mutant (Figure 52). This clearly shows that 1) Notch signalling regulates apico-basal polarity, and 2) Mib has a Notch-independent role in PCP signalling (Figure 58).

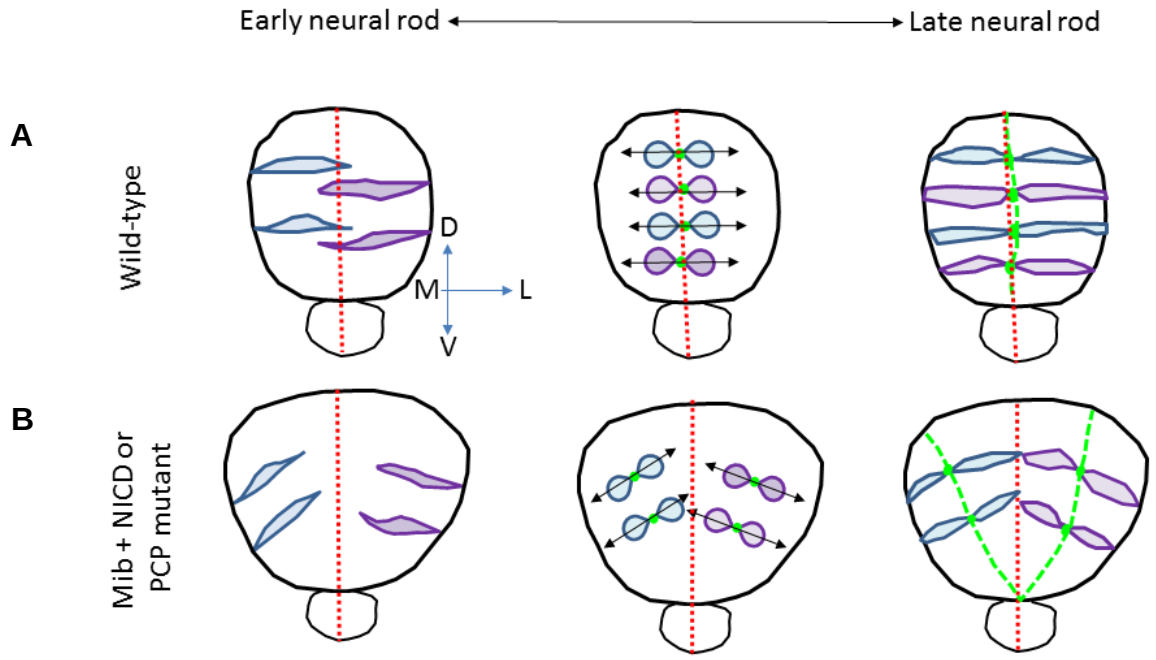


Figure 58: C-divisions in wild-type and PCP-affected conditions.
(A) In wild-type C-divisions occur along the medio-lateral (ML) axis (Black arrows) and deposit one daughter cell on the contralateral side. Pard3 accumulates at the abscission plane that coincides with the geometrical tissue center (Red dotted line) where the neural tube midline forms (Green dashed line).
(B) When activated Notch is introduced in Mib loss-of-function embryos, C-divisions do not occur along the ML axis at the tissue center. Divisions take place at the lateral sides of the neural rod, and as activated Notch restores apico-basal polarity, it gives rise to a dual neural tube midline (Green dashed line). Black arrows show that division orientation is changed and is perpendicular to the geometrical midline of each neural tube half.

IV. How does Mindbomb contribute to the PCP pathway?

We uncovered a novel role of Mib in the regulation of PCP-driven processes such convergent-extension movements and stereotypical division orientation. How Mib acts in PCP signaling is still unknown to us. Mib is an E3-ubiquitin ligase; therefore, it is possible that Mib takes part in the trafficking of one or more components of the PCP pathway. Several studies have reported that cellular levels of PCP components are modulated by the activity of E3-ubiquitin ligases. In *Drosophila*, the junctional pool of Flamingo and Dishvelled is regulated by Cullin-3/Diablo/ketch E3-ubiquitin ligase complex (Strutt et al., 2013) and Cullin1(Cul1)/SkpA/Supernumerary limbs(Slimb) regulates the stability of the peripheral membrane components Prickle (Pk) during wing development (Cho et al., 2015; Strutt et al., 2013). In mice the E3-ubiquitin ligases Smurf1/Smurf2 are important for neural tube closure and regulate PCP signalling by mediating

the degradation of Prickle (Narimatsu et al., 2009). Therefore, it would be interesting to explore if Mib also regulates the recycling and/or degradation of PCP components through its ubiquitin activity.

In *C. elegans*, MIb has shown to interact with a wnt co-receptor Ryk that promotes activation of Wnt/ β-catenin signaling (Berndt et al., 2011). Interestingly, Ryk has been shown to involve in Wnt/PCP pathway in vertebrates (Macheda et al., 2012). Similarly, another elegant study in zebrafish shows that Ryk acts downstream of Wnt5b (Lin et al., 2010) and make a complex with vangl2 in mammals (Andre et al., 2012). All these study suggest that Ryk is a promising candidate that could link Mib to the PCP signaling.

Our initial observation that partial inhibition of Mib and Ryk together results in synergistic PCP phenotypes suggests that Mib might interact with Ryk to affect PCP (Figure 54). To determine if Mib mediates endocytosis of Ryk, we will use a Ryk-eGFP fusion construct to analyze the subcellular localization of Ryk in Mib loss-of-function conditions.

V. Do C-dividing cells communicate with each other through Notch signaling?

During normal neural tube morphogenesis, Pard3-GFP accumulates at the abscission plane of cells undergoing mirror symmetric C-division (Tawk et al., 2007). However, cells are able to polarize roughly at the right place even without cell division, though the midline organization is not quite as precise (Buckley et al., 2013). Therefore, a second mechanism, other than cell division, must exist to target apical proteins to the right place within a cell at the right time. We hypothesized that cells on one side of the neural rod may contact cells on the opposite side to thus define a proper neural tube midline that lies at the tissue centre. Our observation that no C-divisions take place in Mib loss-of-function embryos, suggested that C-dividing cells might be interacting to each other through Notch signalling. To explore this possibility, I performed transplantation experiments to see the behaviour of a wild-type cell in a Notch-signalling depleted environment and the behaviour of Mib mutant cells in wild-type surroundings. We found that a mutant cell behaves normally in wild-type surrounding; however, wild-type cell do not polarize in the mutant environment (Figure 56). This suggest that cells do not function cell autonomously in the establishment of polarity and that surrounding cells contribute to the behaviour and polarity of a cell. As mutant cells are able to C-divide, it is more likely that Notch signaling acts between neighbouring cells. It would be interesting to see, how a Notch signaling

depleted cell would respond in a division blocked wild-type surrounding and *vice versa*. Therefore, further work needs to be done to find out the complete mechanism regarding the cross-talk of Notch signaling between the neighbouring and C-dividing cells.

VI. Conclusion

We found a novel role of Notch signalling in the establishment of apico-basal polarity. In addition, I showed that the Notch signalling component Mib participates in planar cell polarity. Thus, this study uncovers a dual role of Mib in the regulation of cellular and tissue polarity in the context of zebrafish neural tube morphogenesis. A future study to investigate the mechanism of action of Mib in planar cell polarity may further provide useful insights into the endocytic regulation of PCP components.

1 Fish lines and crossing

Wild-type and transgenic zebrafish were maintained under standard conditions (Westerfield, 2000) on a 14-hour photoperiod at 28-28.5 °C in the Institute of Biology fish facility. Embryos were obtained by crossing adult fish. Females and males can be identified based on their body shape and color. A healthy female has a big white belly and its color is bluish and silver grey. A male is usually thinner and yellowish golden in color. A female is generally able to lay eggs at morning in a 3 hours time window after the light is on at 8:30 AM. As the time of the production of fertilized eggs needs to be controlled according to the experiment, fish were also alternatively maintained in light-cycle cabinets where lights switch on at 11:30 AM. Eggs were obtained by separate pairing of a male with a female in a crossing cage. The crossing cages contain neatly fitting inlays with a mesh bottom that separates the adult zebrafish from the eggs. This is necessary, because the adult zebrafish would eat the eggs. Furthermore, the inlay itself can be subdivided into two compartments using a divider. If female and male are kept in separate compartments overnight, they can mate only if the divider is removed. This way the time of eggs production was controlled. This is very important for experiments such as microinjections, where early (zygote or one cell stage) embryos are needed with a synchronized development.
Wild-type and transgenic strains that were used in this study is listed below.

Fish Strain	Gene	References
AB	Wild-type	
Tubingen (TÜ)	Wild-type	
AB/ TÜ	Wild-type	
Tupfel long fin (TL)	Wild-type	
Aei^{AR33}	deltaD	(Latimer et al., 2002)
Mib^{ta52b}	mindbomb	(van Eeden et al., 1996)
Vangl2^{m209}	vangogh like-2	(Stemple et al., 1996)
Tg(Histone2A-GFP)		(Pauls et al., 2001)

Table 4: List of wild-type and transgenic zebrafish strains

2 Identification and genotyping of transgenic strains

2.1 Fin-clipping of adult fish

Fin-clipping is performed in order to isolate genetic material from individual fish for the purpose of genotyping. A small amount of tissue is clipped from the end of the tail in order to extract DNA that will be used for further analysis such as PCR. If done correctly, the caudal fin regenerates within two weeks.

Preparations:

All surfaces and materials used for tissue collection should be sterile. Therefore, surfaces and the tools used for the procedure were cleaned with ethanol prior to set up. PCR strips were labelled to transfer the clipped tissue into the PCR tubes. To anesthetize fish Tricaine (Ethyl 3 aminobenzoatec methylenesulfate; Sigma-Aldrich) 1.5 mM at pH 7.0 stock solution was prepared.

Procedure:

Fish were first anesthetized by immersion in 8-12 ml of tricaine stock solution in 200 ml water. The dose of tricaine can be different depending on the age of fish, so tricaine dose was tested gradually. A longer immersion in tricaine can be fatal for fish; therefore, it is important to take care of anesthesia time. Ideally, Fish should regain consciousness within a minute. Fish was taken with the plastic spoon with the tail protruding out of the spoon. A piece of tail was cut with the help of a surgical scissor. Very little tissue is sufficient to extract DNA, so no more than 50% of the fin area should be removed. This procedure should take less than one minute and should not result in bleeding. Fish are then immediately transferred to a container with fresh system water and monitored continuously until they have recovered. Upon immersion in fresh water, fish usually regain swimming ability within few minutes.

2.2 DNA preparation for the genotyping of individual embryo

- Dechorionated embryos were transferred to 0.5 or 1,5ml Eppendorf tubes using a glass Pasteur pipet
- Supernatant was taken off with Pasteur pipet and 50 µl of DNA prep buffer was added.
- Additionaly, embryos can be freezed in liquid nitrogen and stored at –80°C before proceeding further.
- Embryos were heated for 10min to 98°C, then cooled down to 55°C.

- Further, Proteinase K was added to a final concentration of 1 mg/ml and incubated for overnight at 55°C
- Next morning, PK was inactivated by heating for 10 min to 98°C
- The lysed DNA was then centrifuged for two mins at full speed and transferred to a clean Eppendorf tube.

DNA Prep Buffer

1.5 mM MgCl2
10 mM Tris-HCl pH 8.3
50 mM KCl
0.3% Tween 20
0.3% NP40

2.2.1 Mib^{ta52b} genotyping

To determine the presence of Mib^{ta52b} and WT alleles in a single PCR reaction, the following method was used:

This PCR is done with four primers.
Mib4P genP5 (generic forward primer for the Mib^{ta52b} amplicon): ACAGTAACTAAGGAGGGC
Mib4P WT rv (specific reverse primer for the Mib WT allele): AGATCGGGCACTCGCTCA
Mib4P MUT fw (specific forward primer for the Mib^{ta52b} allele): CAGCTGTGTGGAGACCGCAG
Mib4P genP3 (generic reverse primer for the Mib^{ta52b} amplicon): CTTCACCATGCTCTACAC

- Primers Mib4P genP5 and Mib4P genP3 amplify a 705 bp fragment for all genotypes (in theory, actually practically this band is most often barely visible).
- Primers Mib4P genP5 and Mib4P WT rv amplify a 303 bp fragment for fish carrying the WT allele.
- Primers Mib4P MUT fw and Mib4P genP3 amplify a 402 bp fragment for fish carrying the Mib^{ta52b} allele.

<u>To Assemble 20 µl PCR reactions:</u>
First in each tube, 1 µl undiluted DNA from the ProteinaseK digestion was added, followed by the addition of 19 µl of ice-cold PCR mix:

4 µl GoTaq Flexi Buffer (Promega)
1.2 µl $MgCl_2$ from GoTaq G2 kit (25 mM stock)
0.4 µl dNTP (from mix with each dNTP at 10 mM)
0.4 µl Primer **Mib4P genP5** (from 10 µM stock)
0.4 µl Primer **Mib4P WT rv** (from 10 µM stock)
0.4µl Primer **Mib4P MUT fw** (from 10 µM stock)
0.4µl Primer **Mib4P genP3** (from 10 µM stock)
0.1 µl GoTaq
Qsp 19 µl H_2O (11.7 µl)

PCR Program:
Initial denaturation 2min 95°
10 cycles
    30s Denaturation 95°
    30s Annealing 65°
    30s Annealing 55°
    60s Elongation 72°
25 cycles
    30s Denaturation 95°
    30s Annealing 55°
    60s Elongation 72°

Final extension 5 min 72°
Final hold 4°

15 µl of PCR product was run on a 2% agarose gel.

2.2.2 Vangl2^{m209} genotyping

Vangl2^{m209} genotyping was done as described before (Gao et al., 2011). The following primers were used:

WT	Fwd	GTGTGTCTGCCTGTGTCTTAC**T**
	Rev	GATAAACTCCTCCCCCAGGT
Mutant	Fwd	GTGTGTCTGCCTGTGTCTTAC**A**
	Rev	GATAAACTCCTCCCCCAGGT

To assemble 25 µl PCR reaction:

5.0 µl of 1:10 diluted fin-clipped DNA was used as a template for each reaction.

5.0 µl GoTaq Flexi Buffer (Promega)

0.5 µl Fwd WT/mutant primer

0.5 µl Rev WT/mutant primer

0.5 µl dNTPs (from mix with each dNTP at 10 mM)

0.125 µl Go Taq Pol

13.3 µl H_2O

PCR program

Initial denaturation 2 min 95°

35 cycles

30s Denaturation 95°

30s Annealing 55°

60s Elongation 72°

Final extension 5 min 72°

Final hold 4°

15 µl of PCR product was run on a 2% agarose gel.

3 Preparing RNA for microinjections

3.1 Plasmid digestion

In a 1.5ml Eppendorf tube 5 to 10 µg of plasmid DNA was digested with the appropriate restriction enzyme in a reaction volume of 250 µl and incubated for 2 hrs at the 37°C. Digested DNA was further cleaned up as explained below.

3.2 Plasmid clean-up

Digested DNA was further cleaned up and all the process was performed using gloves and RNAse free tips and reagents. DNA was purified using a phenol chloroform extraction that was followed by concentration and desalting on Amicon ultracentrifugation devices (Milipore).

- In 1.5 ml eppondorf tube 250 µl of Phenol/Chloroform/Isoamylalcohol was added and then vortexed and centrifuged for 1 min at full speed

- The upper aqueous phase was transferred to a new 1.5 ml Eppendorf tube and 250 µl Chloroform was added, vortexed and centrifuged 1 min at full speed.
- Again the upper aqueous phase was transferred to a new 1.5 ml Eppendorf tube and centrifuged for 1 min at full speed and further put in an Amicon Ultra 50K column (to avoid to carry over any chloroform onto the Amicon microfiltration device) and centrifuged for 7 min at 14000 g.
- Amicon column was then washed by adding 200 µl water, centrifuged 7 min at 14000 g.
- The microfiltration device was made upside down into a new collection tube, centrifuged 2 min at 1000 g.
- The collected purified DNA was checked for linearization and DNA quantity by running 1 µl of DNA on an agarose gel.

3.3 SP6 RNA synthesis

RNA synthesis was performed using the mMessage Machine SP6 kit from Ambion, followed by a phenol/chloroform purification and precipiation with Isopropanol.

To set up a 10 µl reaction the following composition was used:

3 µl linearized DNA
1 µl Reaction buffer (Ambion kit)
5 µl 2x dNTP-Mix (Ambion kit)
1 µl SP6 RNA polymerase (Ambion kit)
Incubated 2 to 4 hrs at 37°C

- After the incubation 0.5 µl DNAse I (Ambion kit) was added to digest template DNA for 20 min at 37°C.
- Then 25 µl Ammonium Acetate (Ambion kit) was added to stop the activity of DNAse I.
- 214 µl of sterile RNAse free water was added to make the volume 250 µl

Further, the following method was used to purify the prepared RNA.

- 250 µl Phenol/Chloroform/Isoamylalcohol was added, vortexed and centrifuged for 1 min at full speed
- The upper aqueous phase was shifted in a new 1.5 ml Eppendorf tube and 250 µl Chloroform added, vortexed and centrifuged for 1 min at full speed

- The upper aqueous phase was shifted in a new 15 ml Eppendorf tube, 250 µl Isopropanol added that was mixed shortly and left for precipitating for at least 20 min at – 20°C
- Then, it was centrifuged for 20 min at full speed at 4°C. Supernatant was discarded and RNA pellet was washed with 200 µl 70% Ethanol. Again centrifuged for 5 min at full speed and after discarding the supernatant, the RNA pellet was left for drying.
- The pellet was resuspended in 10 µl of sterile water from the Ambion kit and stored at – 20°C.

For RNA quantification, 0.5 µl RNA with 49.5 µl of water was mixed and the optical density (OD) was measured at the spectrophotometer to quantify.

To check the RNA quality, 0.5 µl was run on an agarose gel for 30 min maximum so that the RNA may not get degraded.

3.4 Microinjections

3.4.1 Dechorionation of embryos

At early stages of development zebrafish embryos can be easily dechorionated by pronase digestion of the chorion, which is composed of glycoproteins. Right after fertilization embryos were treated with 150 µl of 10 mg/ml pronase (Sigma) per petridish (Ø 35 cm) and washed with water as soon as the first embryo is observed that left the chorion. The petridish then was carefully submerged into a 250 ml beaker containing clean water. The water from the beaker was then decanted as much as possible and fresh water was added letting it run through the wall of the tilted beaker. The movement of the embryos by water stream helps embryos to dechorinate themselves. This washing step was repeated 2-3 times more and dechorinated embryos were transferred into agarose-coated petridishes using a glass pipettes. An alternative to enzymatic dechorionation with pronase is manual dechorionation with forceps

3.4.2 Preparation of injection solutions

Injection solutions for RNA were prepared by diluting the desired concentration of RNA in 0.1 M KCl 0.2% Phenol Red. The injection solutions for morpholinos were prepared diluting the desired concentration of morpholinos in Danieau (1x)-0.2% Phenol red.

Gene	Concentration	Sequence	Reference
mindbomb	1000 µM	GCAGCCTCACCTGTAGGCGCACTGT (splice Morpholinos exon/intron 1)	(Itoh et al., 2003)
deltaA	250 µM	CTTCTCTTTTCGCCGACTGATTCAT	(Matsuda and Chitnis, 2009)
rbpjA	125 µM	GCGCCATCTTCACCAACTCTCTCTA	This study
rbpjB	125 µM	GCGCCATCTTCCACAAACTCTCACC	This study
vangl2	100 µM	GTACTGCGACTCGTTATCCATGTC	(Park and Moon, 2002)
ryk	1000 µM	GGCAGAAACATCACAGCCCACCGTC	This study

Table 5: List of morpholinos

Gene	Concentration	Stage	Reference
pCS2-Gap43GFP	20 ng/µl	1 cell	(Concha et al., 2003)
	30 ng/µl	2-8 cell	
pCS2-Gap43RFP	20 ng/µl	1 cell	
	30 ng/µl	2-8 cell	
pCS2-Pard3-GFP	100 ng/ µl	1 cell	(Geldmacher-Voss et al., 2003)
	350 ng/ µl	16-32 cell	
Myc-Notch-Intra-pCS2	50 ng/ µl	1 cell	(Takke et al., 1999)
pCS2-RhoA	125 ng/ µl	1 cell	(Castanon et al., 2013)

Table 6: List of DNA constructs

3.4.3 Injections

Microinjections in zebrafish embryos were carried out manually, without micromanipulator. To ensure a homogenous distribution of the injected material, it is important to inject embryos as early as possible. For the ubiquitous expression, RNA and morpholinos were injected to the first blastomere. For mosaic labelling, RNA was injected into a single blastomere at the 16 to 32 cell stages. For the time-lapse imaging of the neural tube morphogenesis embryos were injected with Gap43GFP RNA at one cell stage. For the midline-crossing movies, Gap43GFP RNA was injected into one of the two cells at the two cells stage.

The embryos were injected with the injection solutions using a pressure microinjector (Femtojet Eppendorf). Special injection needles were prepared of glass capillaries (World Precision Instruments, outer diameter 1 mm) by using a needle puller (Sutter Instruments).

The injection solution were loaded in needle through microloaders (Eppendorf). The volume of the injection solution were estimated by a drop having diameter of 0.15 mm (approximately 1.76 nL in volume) on a 0.01mm x 1mm calibration slide (Meiji Techno). During and after the injection session, embryos were grown in 0.3x Danieau buffer.

30x Danieau buffer Stock

NaCl	1740 mM
KCl	21 mM
$MgSO_4 \cdot 7H_2O$	12 mM
$Ca(NO_3)_2$	18 mM
HEPES buffer	150 mM

Above mentioned reagents were dissolved in distilled water and pH was adjusted to 7.6.

4 Whole mount immunohistochemistry

- Embryos were fixed in 4% paraformaldehyde in PEM (PEM-PFA) for 1.5 hours, with gentle shaking at room temperature, or overnight at 4°C. Further, embryos were postfixed in PEM-PFA with 0.2% Triton for 30 minutes at room temparature (RT).
- After postfixation embryos were then washed 3x 5 mins in PEMT (PEM+ 0.2% Triton), 1x10 mins in PEMT+ 50mM NH4Cl and then for 3x5min in PEMT.
- Embryos were then preincubated in blocking solution (PEMT + 2% BSA) for three to four hours on RT.
- Embryos were incubated in primary antibody (Antibody + PEMT+ 2% BSA) 3 to 4 hours at RT, followed by multiple washes in PEMT.
- Embryos were then incubated in secondary preincubation in PEMT+2%BSA with 2% serum (such as Normal goat serum for the secondary antibodies that are from goat) at RT for 2 hours or at 4°C for overnight.
- Following this, embryos were incubated in secondary antibody in PEMT+ 2% BSA +2% Serum for 3-6 hours at RT.
- Finally embryos were washed 5 to 6 times in PEMT with gentle shaking and further stored in 50% Mowiol/ PEMT

Specific reagents:

PEM: The following reagents were dissolved in ddH_2O with the final concentration given below:

Na-Pipes 80 mM
EGTA 5 mM
$MgCl_2$ 1 mM
pH was adjusted to 7.4 with HCl

PEMT PEM + 0.2% TritonX100
NH4Cl 2.5 M stock solution

Name	Product	Concentration	Company
TRITC- Phalloidin		1:250	Sigma (D9542)
DAPI		1:5000	Sigma (P1951)
NGS (Normal goat serum)		1:50	Jackson Immunoresearch
Goat@Rabbit-Cy5	Secondary Antibody	1:250	Jackson Immunoresearch
Goat@Mouse-Alexa488	Secondary Antibody	1:500	Invitrogen
Mouse@zDeltaD	Primary Antibody IgG1	1:500	Abcam (ab73331) (Itoh et al., 2003)
Rabbit@aPKC	Primary Antibody	1:250	Santa Cruz (sc-216) (Cui et al., 2007)
Mouse@ZO-1	Primary Antibody IgG1kappa	1:500	Invitrogen (1A12) (Köppen et al., 2006)
Mouse@ZS-4 (Crumbs)	Primary Antibody	1:500	ZIRC (Vihtelic and Hyde, 2000)
Mouse@HuC/D	Primary Antibody	1:500	Invitrogen (16A11) (Fornaro et al., 2003)

Table 7: List of reagents for immunocytochemistry.

5 In situ hybridization on Zebrafish whole mount embryos

5.1 Making *in-situ* probes by PCR method

The following primers were used for preparing probes:

Gene		Primers sequence
Crumbs1	Forward	gcttatcgataccgtcgacTGTACCACCAGCCCATGTCATA
	Reverse	**TAATACGACTCACTATAGGG**cctcatcacagttttgacccac
Crumbs2a	Forward	gcttatcgataccgtcgacTGAGAGTGCCCCCTGCCTTAAT
	Reverse	**TAATACGACTCACTATAGGG**acagtcacagcggtagc
Pard3	Forward	GATCCAGGCAAAAACGCGAGAGATTCG
	Reverse	**TAATACGACTCACTATAGGG**gaagtagtcggcataacc
Pard6-gb	Forward	GACTACAGCAACTTTGGCACCAGCACTCT
	Reverse	**TAATACGACTCACTATAGGG**gtgatgactgtgccatcctcctc
Mark2b (Par1)	Forward	ATCTATGCTCAGCAGTGCAGAGAAGTCGGAGA
	Reverse	**TAATACGACTCACTATAGGG**cgtttcatgggagtcatgtggtgc

T7-promoter sequence (in bold letters) is added to the reverse primer to make antisense probe.

PCR

5 µl of 1:10 diluted Fin-clipped DNA was used as the template.

10 µl Herculase buffer (Agilant technologies)

2 µl dNTPs (from mix with each dNTP at 25 mM)

1.25 µl Forward primer

1.25 µl Reverse primer

1 µl Herculase II enzyme

29.5 µl H_2O

PCR program: For 30 cycles

10 cycles

30s Denaturation 95°

30s Annealing 65°

30s Annealing 55°

60s/kb Elongation 68°

25 cycles

30s Denaturation 95°

30s Annealing 55°

60s/kb Elongation 68°

Final extension 10 min at 68°

PCR products were sent to sequencing to confirm the probe sequence.

5.2 Synthesis of the probe

The method was developed by the Christine & Bernard Thisse lab.

Transcription of Probe antisense RNA:

1 µg PCR product

4 µl 5x transcription buffer (Stratagene)

2 µl NTP-DIG (Boehringer)

12.5 Units T3- or T7-RNA-Polymerase (Stratagene)

1 µl RNAse inhibitor (Boehringer)

H_20 qsp 20 µl.

2 hrs at 37°C

Digestion of DNA template

2 µl RNAse free DNAse I was added to digest the DNA template and incubated for 15-30 minutes at 37°C.

Reaction stop and precipitation:

- To stop the reaction following reagents were added one after the other and it is important to add them precisely in this order only.

EDTA 0,5M pH 8.0	1 µl
LiCl 4M	2.5 µl
Cold EtOH 100%	75 µl

- Stored for 30 mins at – 80°C (or overnight at -20°C)
- Centrifuged for 30 mins at maximum speed (in refrigerated centrifuge)
- Washed with 100 µl sterile 70% EtOH, and then centrifuged for 5 mins at maximum speed.
- Dry pellet (air or vacuum), and resuspend the pellet in 50 µl H20 or TE10/1
- RNA quality and quantity was checked by loading 1µl RNA on an agarose gel.

5.3 Hybridization on zebrafish embryos

- Embryos were fixed in 4%paraformaldehyde in PBS at 4 °C overnight with gentle shaking.
- Embryos were then dehydrated in 100% methanol and stored at –20 °C for minimum 2 hours.
- Embryos were further rehydrated through a methanol series :

  1 x 5 min 75% MeOH - 25% PBS

  1 x 5 min 50% MeOH - 50% PBS

  1 x 5 min 25% MeOH - 75% PBS

  4 x 5 min PBST

  (PBS1x / tween20 0.1%: 10ml of tween20 20% stock solution for 2 L of PBST)

- After rehydration, embryos were digested with 1 µg/ml Proteinase K. The duration of digestion depends on the the embryonic stage.

  Proteinase K digestion times:

  2-6 somites: 2 min

  14-20 somites: 4 min

  24h: 10 min
- Embryos were refixed in 4% PFA-PBST for 20 mins, followed by 5x 5mins washes with PBST.
- Next to this, embryos were incubated in hybridization buffer (Hyb) for 3-6 hours at 70 °C.
- Then probes diluted in prewarmed Hyb buffer added to the embryos and left overnight at 70 °C.
- The following day embryos were washed in the following solution:

  1 x fast washing with 100% Hyb at 70°C

  15 min 75% Hyb - 25% 2x SSC at 70°C

  15 min 50% Hyb - 50% 2x SSC at 70°C

  15 min 25% Hyb – 75% 2x SSC at 70 °C

  15 min 2x SSC at 70°C

  2 x 30 min with SSCx0.2 at 70°C

  10 min 75% 0.2x SSC- 25% PBST at RT

  10 min 50% 0.2x SSC- 50% PBST at RT

  10 min 25% 0.2x SSC- 75% PBST at RT

  10 min PBST at room temperature

- Embryos were put in blocking solution (PBST + 2% Sheep Serum) for 2 hours at RT then the anti-DIG antibody (1 :10000) diluted in PBST+2% BSA was added to the embryos and left overnight at 4 °C with gentle agitation.
- The next day embryos were washed with staining solution and then the staining was revealed using NBT/BCIP. Embryos were kept in the dark at RT until the staining is visible.
- The staining was stopped by washing in 3x 5 mins in PBST and then replacing PBST by PBS pH 5.5 with 1 mM EDTA. Embryos were incubated in Tris-Glycine pH 2.2 for few hours to overnight and stored in 80% Glycerol and 20% Tris-Glycine.
- Embryos were mounted on glass slide in 80% glycerol and images were taken on a dissecting microscope (Leica MZ205FA and Leica MZFLIII).

5.4 Preparations of reagents

SSC 20x stock solution

NaCl	175.3 g (3 M)
Cirtic acid trisodium salt	88.2 g (300 mM)

Dissolved in 1 L of distilled water pH was adjusted to 7.0.

Hybridization mix (HM)

- tRNA and Heparine were added for prehybridization and hybidization only.
- 1 ml of HM was prepared per in situ reaction (0.7 ml prehyb + 0.2 ml hyb)

Formamide	25 ml
SSC x20	12.5 ml
Heparine 5 mg/ml	0.5 ml
tRNA 50 mg/ml	0.5 ml
tween20 20%	0.25 ml
Citric acid 1M	0.46 ml
Sterile water	10.7 ml

staining buffer

100 mM Tris/HCL pH 9,5
50 mM MgCl2
100 mM NaCl

0,1% tween20

3 x 5 min Tris pH 9.5

Staining Solution

22.5 µl NBT

17.5 µl BCIP

5 ml Tris pH 9.5 solution

And to be kept in the dark.

NBT stock

50 mg of Nitro Blue Tetrazolium in 0.7 ml of Dimethylformamide anhydrous + 0.3 ml H_20

BCIP stock

50 mg of 5-Bromo4-Chloro3-IndoylPhosphate in 1 ml of Dimethylformamide anhydrous

6 Zebrafish cell transplantations

6.1 Preparations of the transplantations mould and needles

- A simple mould for casting dishes for transplantation was prepared by gluing together microscopic slide in order to get wells for individual embryos. This mould was put in the lid of a 90 mm petri dish and was covered with 2% agarose in 1x Danieau. For 15 mins this was left for solidifying and then the mould was removed. Excess of agarose was cut off using a subcutaneous needle. Usually two moulds were casted for a session of transplantation.
- Transplantation needles were made using glass capillaries, and by scratching a diamond pen a blunt mouth opening was achieved. The needle was filled with 1x Danieau with 5% Pen/Strep and screwed tight into the holder of the oil-filled transplantation apparatus.

6.2 Transplantation

- Transplantion dish were filled with 1x Danieau + 5% Pen/Strep.
- Donor embryos were placed in wells 1-3-5-7… and host embryos were placed in wells 2-4-6-8.
- After the cells were transplanted, donors were either discarded or processed for genotyping. In case, they needed to be genotyped like Mib^{ta52b} donor embryos, they were directly put into PCR tubes that contain DNA prep buffer.

- After the transplantation, host embryos were shifted from the transplantation dish to a new petri dish coated with 2% agarose in 0.3x Danieau and filled with 0.3% Danieau + 5% Pen/Strep.

6.3 Required consumables and equipment

- Transplantation needles that were pulled from thin wall capillaries without inner filament.
- 65 mm Petri dishes coated with 2% agarose in 0.3x Danieau
- 0.3 x Danieau media with 5% Pen/Strep
- 1x Danieau with 5% Pen/Strep
- Short Pasteur pipette
- Mineral oil (Trinity Biotec)
- 8.5 cm diameter petridishes
- Dissection scope with diascopic illumination
- Transplantation setup (Sutter instruments)
- Diamond pen
- 1 Pipette pump
- 200 µl pipette with 200 µl tips.

6.4 Cell transplantation experimental details

- Wild-type or Mib^{ta52b} embryos were used for transplants. Donor embryos were labelled by injection of GAP43GFP (50 ng/µl), a cell membrane marker, or Pard3-GFP (100 ng//µl) at the one-cell stage. Host embryos were collected, dechorinated and incubated at 28.5 °C in parallel with the donor embryo.
- All cell transplantations were carried out at blastula or early gastrula stages. Both donor and host embryos were of the same age (Figure 59).
- Approximately 20-30 cells were extracted from the donor embryo and drawn into the micropipette by turning the micrometer drive. Cells were then expelled into the host embryo by turning the micrometer device in the opposite direction.
- Transplanted embryos were grown till 24 hpf and then fixed. Further, embryos were stained with TRITC-phalloidin to label F-actin in order to label all cells and thus show the location of transplanted cells withinn the spinal cord.

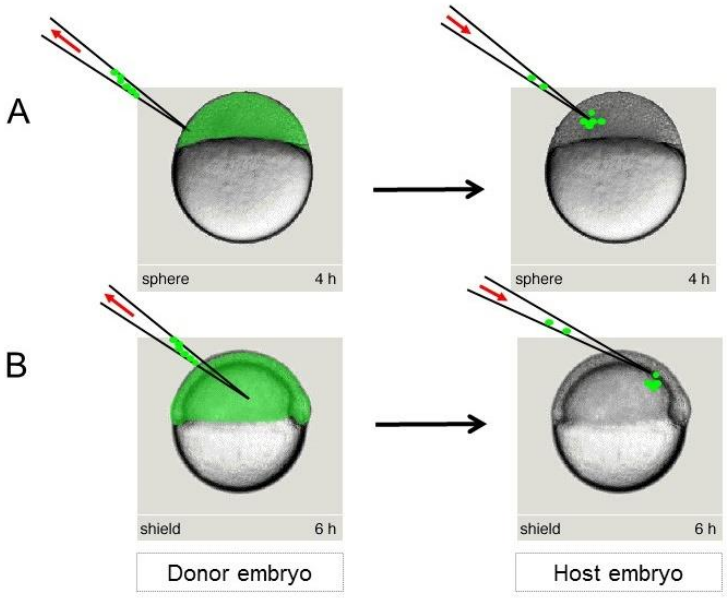


Figure 59: Cell transplantation
A. Transplantions of cells from Gap43GFP or Pard3GFP injected donor embryos to Wild-type or Mib^{ta52b} mutant embryos at the blastula stage. The green cells in host embryo show the approximate location of transplantation.
B. Transplanation of cells at the gastrulation stage.
(Images are adapted from(Kimmel et al., 1995).

7 Confocal Microscopy

7.1 Mounting embryos

Embryos were mounted in 0.75% low melting agarose (Sigma) in a glass bottom dish as shown in Figure 60 A (MatTek corporation). Live embryos older than 18 hpf were anesthetized with tricaine (Sigma Aldrich) using approximately a 1:20 dilution of the stock solution (1.5 mM). For dorsal view imaging of spinal cord embryos were oriented so that the spinal cord touches the glass (Figure 60 B). For transversal imaging embryos were oriented upside down so that their head touches the glass and tail stay upside (Figure 60 C)

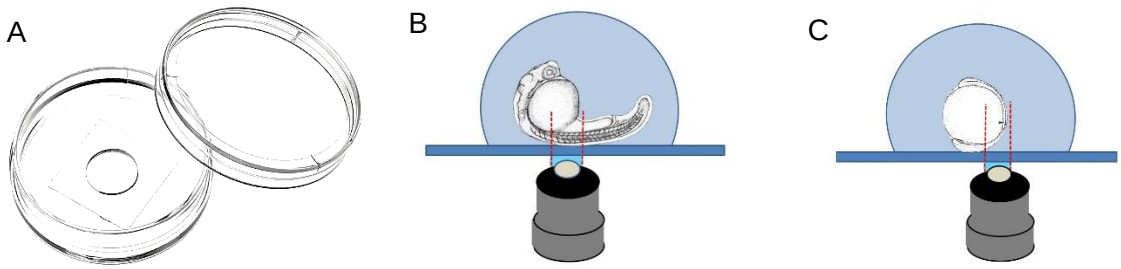


Figure 60: Mounting the embryos for confocal imaging
A. Glass bottom dish
B. Dorsal mounting of the embryo
C. Transversal mounting embryo

7.2 Imaging of fixed or live embryos

- Embryos were imaged on spinning disk (Olympus/Andor), Zeiss 510 NLO, Zeiss 510 Meta, Zeiss 710 and Zeiss 780 confocal microscopes using 40x (water) and 60x oil objectives.
- To image fixed embryos z-stacks of 10-30 sections covering a depth of around 50 µm (from EVL to Notochord) were taken using four channels.
- For making the long term time lapse movies of the neural tube morphogenesis, embryos were mounted transversally and imaged at the level of first to third somites. In every one minute interval, a single plane image was taken for 12-14 hours.
- For cell division orientation imaging in dorsal epiblast cells, shield stage transgenic Tg(H2A::GFP) embryos uninjected or injected with 1000 µM Mib morpholino were mounted. Z-stacks were collected at 2 min intervals. Embryos were imaged for approximately for 4 h (until bud stage). These time lapse movies were made on a Zeiss 780 inverted microscope with a 40x (NA 1.1) water immersion objectives.

7.3 Image analysis

All confocal microscopy images were analysed and quantified using the ImageJ (http://rbs.info.nih.gov/ij/) software. Images were enhanced for presentation by adjustments of levels, brightness and contrast and colour saturation. Projections of z-stack of images were maximum projections. Epiblast division orientation were quantified by rose plot analysis (Algorithem was provided by Irinka Castanon/Gonzalez-Gaitan lab) that were made in MATLAB software (Mathworks). Numerical analysis was carried out in Microsoft excel and figures were constructed in MS PowerPoint.

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