

Optogenetic Frizzled 7 reveals a permissive function of Wnt/PCP signaling in directed mesenchymal cell migration

by

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Abstract

The Wnt/planar cell polarity (Wnt/PCP) pathway determines planar polarity of epithelial cells in both vertebrates and invertebrates. The role that Wnt/PCP signaling plays in mesenchymal contexts, however, is only poorly understood. While previous studies have demonstrated the capacity of Wnt/PCP signaling to polarize and guide directed migration of mesenchymal cells, it remains unclear whether endogenous Wnt/PCP signaling performs these functions instructively, as it does in epithelial cells. Here we developed a light-switchable version of the Wnt/PCP receptor Frizzled 7 (Fz7) to unambiguously distinguish between an instructive and a permissive role of Wnt/PCP signaling for the directional collective migration of mesendoderm progenitor cells during zebrafish gastrulation. We show that prechordal plate (ppl) cell migration is defective in maternal-zygotic *fz7a* and *fz7b* (MZ *fz7a,b*) double mutant embryos, and that Fz7 functions cell-autonomously in this process by promoting ppl cell protrusion formation and directed migration. We further show that local activation of Fz7 can direct ppl cell migration both *in vitro* and *in vivo*. Surprisingly, however, uniform Fz7 activation is sufficient to fully rescue the ppl cell migration defect in MZ *fz7a,b* mutant embryos, indicating that Wnt/PCP signaling functions permissively rather than instructively in directed mesendoderm cell migration during zebrafish gastrulation.

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About the Author

Daniel Čapek completed an MA in Celtic Studies at the University of Vienna in 2008 writing a diploma thesis about translation and syntactic analysis of a Middle Welsh manuscript under the supervision of Prof. Stefan Schumacher. During this time, he also began a Bachelor's degree in biology, with a focus on Zoology and Evolutionary Biology, which culminated in a project on the 3D imaging of avian embryonic digits, supervised by Dr. Brian Metscher. He continued this work in a subsequent MSc in Genetics and Developmental Biology at the University of Vienna, which he completed with a thesis on avian digit homology under the supervision of both Dr. Brian Metscher and Prof. Gerd Müller. Said thesis later led to the publication of the following article:

Čapek D, Metscher BD, and Müller GB. 2014. Thumbs down: A molecular-morphogenetic approach to avian digit homology. *J. Exp. Zool. (Mol. Dev. Evol.)* 322B: 1–12.

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List of Publications Appearing in Thesis

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2. Smutny M, Ákos Z, Grigolon S, Shamipour S, Ruprecht V, Čapek D, Behrndt M, Papusheva E, Tada M, Hof B, Vicsek T, Salbreux G, and Heisenberg CP, 2017. Friction forces position the neural anlage, Nature Cell Biology 19 (4), 306–317.
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List of Symbols/Abbreviations

SI units and chemical elements and formulas are not listed here.

Aa	amino acids
AP	alkaline phosphatase
A-V	animal-vegetal
BCIP	5-Brom-4-chlor-3-indoxylphosphat
BFP	blue fluorescent protein
BMP	bone morphogenetic protein
Bp	base pairs
Cas	casanova
Cas9	CRISPR associated 9
C&E	convergence and extension
CE	convergent extension
Cf.	confer
CIL	contact inhibition of locomotion
CRD	cysteine-rich domain
CRE	causes recombination
CRISPR	clustered regularly interspaced palindromic repeats
crRNA	CRISPR RNA
C-term	carboxy-terminus
Cy5	cyanine 5
Daam	dvl associated activator of morphogenesis
DEP	dvl, Egl-10, pleckstrin
Dgo	diego
DIX	dishevelled-axon
DMEM	Dulbecco's modified eagle's medium

DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	deoxynucleotide
DREADD	designer receptor exclusively activated by designer drugs
Dsh	dishevelled
Dvl	dishevelled
Ecad	e-cadherin
ECL	extracellular loop
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia
EtOH	ethanol
EVL	enveloping layer
Fab	fragment antigen-binding
FFFF	frizzled- frizzled- frizzled- frizzled
Fmi	flamingo
For	forward
Fz	frizzled
GFP	green fluorescent protein
GPCR	g-protein coupled receptor
Gsc	goosecoid
Gsk3β	Glycogen synthase kinase 3 β
GW	gateway
H2A	histone 2a
H2B	histone 2b
Hgg	hatching gland gene
HH	Hamburger-Hamilton
HM	hybridization mix
Hp_f	hours post fertilization

HR	homologous recombination
ICL	intracellular loop
i.e.	id est
IgG	immunoglobulin G
JNK	c-jun N-terminal kinase
Kb	kilobases
Kny	knypek
LED	light emitting diod
LoxP	locus of Crossover in P1
LWR	length to width ratio
MBT	mid-blastula transition
MeOH	methanol
Mmp	matrix metalloproteinase
mRNA	messenger RNA
Mypt1	Myosin phosphatase target subunit 1
MZ	maternal zygotic
NA	numerical aperture
NBT	nitro-blue tetrazolium chloride
Nc	notochord
NCr	neural crest
Ndr	nodal related
Ne	neuroectoderm
NHEJ	non-homologous end joining
Ntl	notail
ORF	open reading frame
PAM	protospacer adjacent motif
Papc	paraxial protocadherin
PBS	phosphate buffered saline
PCP	planar cell polarity

PCR	polymerase chain reaction
P-D	proximo-distal
PDGF	platelet-derived growth factor
PDZ	postsynaptic density 95, discs large, zonula occludens-1
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
Pk	prickle
Ppl	prechordal plate
Ppt	pipetail
Ptk	protein tyrosine kinase
Rab5c	ras-related protein 5c
Rac	ras-related C3 botulinum toxin substrate
RASSL	receptor activated solely by a synthetic ligand
Refs	references
Rev	reverse
RFP	red fluorescent protein
Rho	ras homolog gene family
RNA	ribonucleic acid
RNase	ribonuclease
ROCK	rho-associated protein kinase
ROI	region of interest
Rok2	rho kinase 2
Ror2	receptor tyrosine kinase like orphan receptor 2
RRFF	rhodopsin-rhodopsin-frizzled-frizzled
RRFR	rhodopsin-rhodopsin-frizzled-rhodopsin
RT	room temperature
sgRNA	single guide RNA
Slb	silberblick
Sox	sry-related HMG box

SSC	saline sodium citrate buffer
Stbm	strabismus
TGF	transforming growth factor
TL	tüpfel longfin
TM	transmembrane domain
tracRNA	trans-activating RNA
Tri	trilobite
UTR	untranslated region
Vang	van gogh
Vangl	van gogh like
Vs.	versus
Wg	wingless
Wnt	wingless-type MMTV integration site
Wt	wildtype
YSL	yolk syncytial layer

1 Introduction

1.1 *Zebrafish as a model system*

The zebrafish (*Danio rerio*) was been discovered as an organism with potential for genetic studies in the 1980s¹⁻³ and has since progressed to a widely used vertebrate model system^{4,5} due to its multitude of advantages over other model organisms. Indeed, not only is the embryo large, and the embryonic development external, its clutch size is very high, a single couple yielding hundreds of eggs. Altogether, this offers the possibility of many diverse manipulation techniques, such as high throughput injections of mRNA or morpholinos. These features are major advantages compared to other vertebrate models (e.g. mouse (*Mus musculus*) or chicken (*Gallus gallus*)) and make the zebrafish a powerful model organism for genetic screens (reviewed in ⁶). Another important feature of the zebrafish embryo is its transparency, which allows for live fluorescent imaging of cells and proteins using confocal and light sheet microscopy^{7,8}. For the same reason it also is an excellent model for optogenetic studies⁹. Finally it has a reasonable generation time of 3 to 4 months, and thus a wealth of mutant lines and transgenic lines exist, which makes zebrafish a well suited model for the study of Wnt/PCP signaling¹⁰.

1.2 *Zebrafish development*

The early development of zebrafish can be categorized into 7 periods: the zygotic, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods¹¹. Given a stable temperature of 28.5°C, the first 5 of these periods are finished within the first day of development, and the larva hatches after three days. The egg consists of a mix of cytoplasm and yolk and is tightly surrounded by a membrane called the chorion. Fertilization occurs externally immediately after laying, through a pore in the chorion, termed micropyle¹². The zygotic period starts directly after fertilization and only includes the 1-cell stage, with the zygote itself measuring about 0.7mm in diameter. The chorion starts swelling after fertilization, increasing the size of the egg, which comes equipped with maternal proteins and mRNAs that regulate the early stages of development before the onset of zygotic transcription.

30 minutes after fertilization the first stream of yolk-free cytoplasm towards the animal pole arises, segregating the first cell from the yolk. Actin-dependent pulsations have been suggested as drivers for this process¹³. Due to this separation, the first polarity in the embryo is established: the cytoplasm-containing cell side is termed animal pole and the yolk side vegetal pole (Fig. 1.2.1). The animal pole marks the later anterior part of the embryo, while posterior tissues arise at the vegetal pole¹¹. After 45 minutes the cells start to divide, heralding the cleavage period. The divisions occur synchronously roughly every 15 minutes and are meroblastic, leaving cytoplasmic bridges that connect the blastomeres with each other and the yolk early on¹⁴.



Figure 1.2.1: Zebrafish embryo at 1-cell stage. Brightfield microscopy, modified from Kimmel¹¹. Lateral view, the animal pole with the cell is on top, the vegetal pole down. The scale bar is 250μm.

During early cleavage stages, cytoplasmic streaming towards the animal pole continues. The first cleavage furrow is vertically oriented, and the subsequent cleavages are oriented relative to the first one. Whether the dorso-ventral axis can be predicted from the orientation of these cleavages is highly debated¹⁵⁻¹⁸, although the dorsal side appears to be determined already during early development due to asymmetric localization of Wnt8a mRNA and the nodal ligand squint^{19,20}. During the 16-cell stage the four central blastomeres separate completely from the yolk and the other cells, while the marginal cells retain the incomplete separation. During the cleavage leading to the 64-cell stage, the vegetal of the two daughter cells of each of these central blastomeres will be covered entirely by other

cells. These cells are referred to as deep cells, the remaining superficial cells give rise to the enveloping layer (EVL)¹¹.

The blastula period features several important events: the mid-blastula transition (MBT), the formation of the yolk syncytial layer (YSL), as well as doming and the onset of epiboly. At the 512-cell stage the cell cycles lengthen and lose their synchrony. This process is called the mid blastula transition and marks the onset of zygotic transcription²¹. Shortly afterwards the marginal EVL cells collapse into the yolk and thereby form a multi-nucleated layer between the yolk and the margin of the blastoderm called the yolk syncytial layer¹¹. The remaining EVL cells differentiate into a multicellular epithelium that contributes to the embryonic epidermis²². At 4 hours post fertilization (hpf) the embryo has a roughly spherical shape, due to continuous shortening along the animal-vegetal axis (Fig 1.2.2 A), and about 20 minutes later a process called doming starts, in which the blastoderm and EVL spread over the yolk sac (epiboly) and the yolk bulges up towards the animal pole (Fig. 1.2.2 B). The blastoderm is thinning along the animal-vegetal axis due to radial intercalations²³, triggered by epithelial surface expansion²⁴. The moment, when epiboly movements reach the equator of the embryo (50% epiboly), marks the end of the blastula period and the onset of gastrulation (Fig. 1.2.2 C).

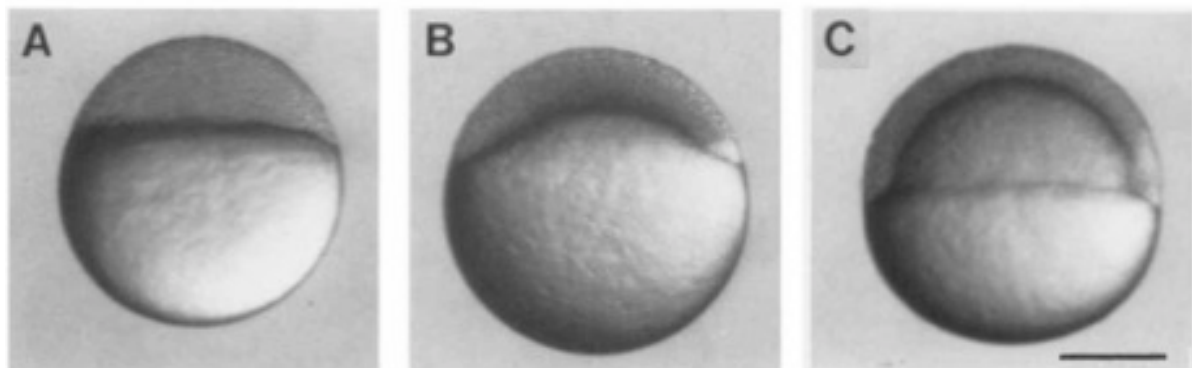


Figure 1.2.2: Blastula stages. Brightfield microscopy images, modified from Kimmel¹¹. Lateral view, the animal pole with the blastoderm is on top, the vegetal pole down. The scale bar is 250µm. A shows sphere stage, B dome stage, and C 50% epiboly.

Shortly after 50% epiboly, marginal cells start folding back and internalizing, leading to a thickening called the germ ring. Cells that stay on the outside form the ectodermal germ

layer, while ingressing cells are precursors of the mesendoderm that later splits into meso- and endoderm^{11,25}. Epiboly is temporarily arrested during shield formation but continues shortly afterwards¹¹. Convergence movements to the dorsal side lead to the formation of the shield, the zebrafish embryonic organizer²⁶⁻²⁸. In this region cells ingress as individuals^{25,29,30}, while cells in the ventral and lateral region undergo synchronous ingression that is reminiscent of involution in other organisms^{25,31,32}.

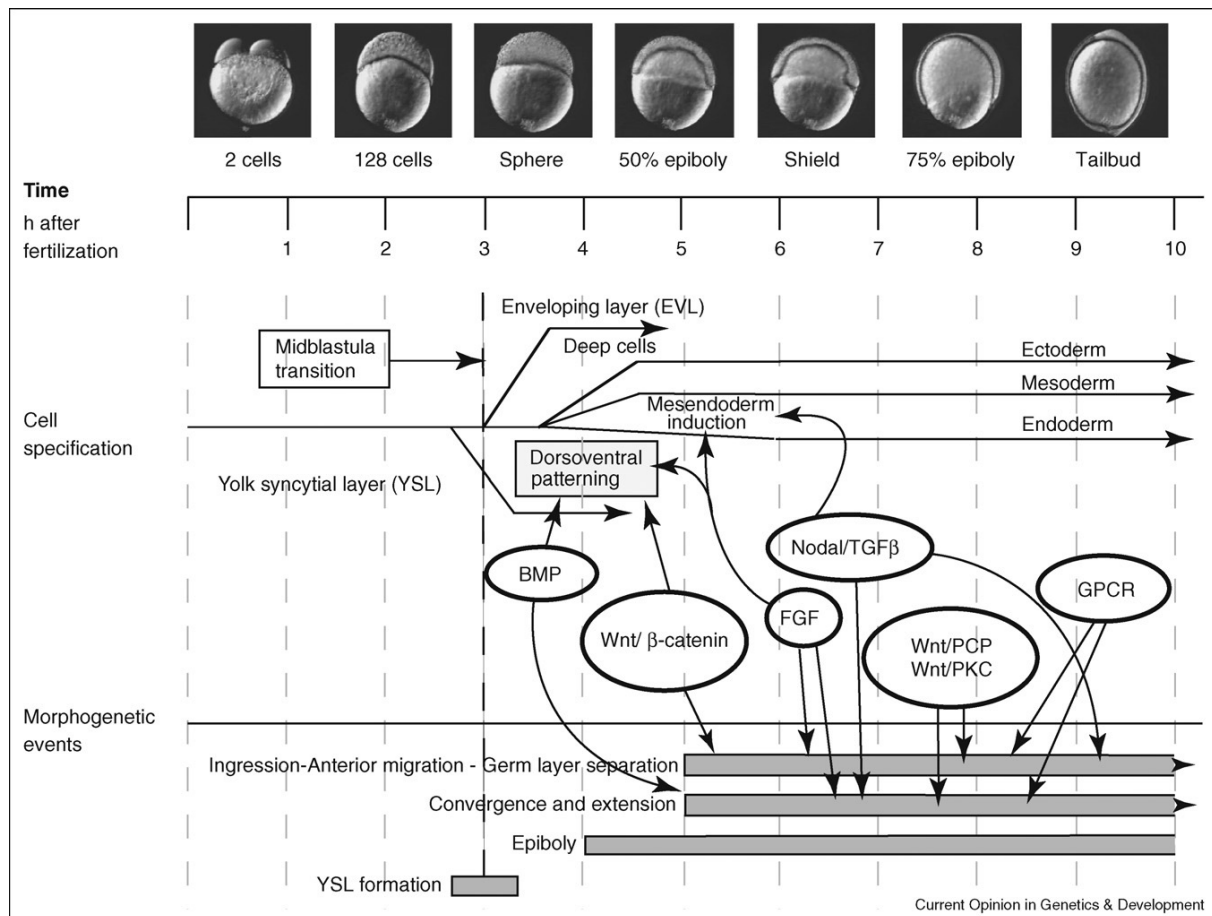


Figure 1.2.3: Morphogenetic events and cell fate specification. The roles of involved pathways are indicated. The relative timing in hpf and stage is shown on top. Taken from Heisenberg & Solnica-Krezel³³.

Gastrulation is a major event in morphogenesis, during which organisms develop from a ball of early blastomeres to an embryo with distinct germ layers and polarities³³ (Fig. 1.2.3). It is a complex network of cell fate specification and cell and tissue migration processes that influence each other³³. Besides the already mentioned epiboly and ingression/involution movements, a third morphogenetic tissue rearrangement takes place: convergent extension (CE). During convergent extension (also referred to as convergence & extension, C&E)

movements, the anterior-posterior body axis is elongated and the mediolateral one is narrowed (see chapter 1.3)^{34,35}. Ingression of deep cells to form the inner layers of the gastrula, epiboly movements towards the vegetal pole, and CE movements continue concomitantly until the end of the gastrula period.

Cell fate specification events during gastrulation include mesendoderm induction and dorsoventral patterning³³. Mesendoderm induction is controlled by Nodal signaling, which is also required for normal gastrulation movements³⁶⁻³⁸. Dorso-ventral patterning is regulated by canonical Wnt/ β -catenin³⁹ and BMP signaling^{40,41}, the latter of which appears to be also involved in regulating convergence and extension movements⁴².

Gastrulation concludes through blastopore closure at the vegetal pole and formation of the tailbud at 10hpf. During the following segmentation period the somites and primary organs form and the anterior-posterior body axis further elongates. Neurulation, the formation of the primitive nervous system, also falls into this period. Embryonic development then continues for two more days through the phylotypic pharyngula period⁴³, in which axis lengthening and organogenesis continues, the pharyngeal arches form, and pigmentation begins. The embryos hatch at some point during the third day (48-72 hpf), though the exact time point is variable, and during the hatching period the embryo continues to grow, while the morphogenesis of most organs is completed. After hatching they are referred to as larvae instead of embryos¹¹.

1.3 *Convergence and extension*

Convergent extension is a widely occurring process throughout the animal kingdom, in which one axis narrows while another elongates^{34,35}. On a cellular level this is primarily achieved by collective migration, during which cells remain physically interconnected and the cell-cell junctions stay intact⁴⁴, and cell intercalation, where they do not³⁵. A similar outcome can be achieved by applying different strategies; while zebrafish paraxial mesoderm cells primarily migrate to the dorsal side and only then intercalate, *Xenopus* C&E uses cell intercalation mediated by mediolaterally oriented protrusions from the start⁴⁵. Independent of the strategy, both organisms look alike by the end of gastrulation³⁵.

1.3.1 Tissue movements during convergence and extension

C&E movements in the inner layers of the zebrafish gastrula differ between the axial and paraxial mesoderm (Fig. 1.3). Gastrulation starts when cells internalize at the margin and subsequently migrate away towards the animal pole^{25,35,46}. Cells that internalize first in the shield region give rise to the most anterior part of the axial mesoderm, the prechordal plate (ppl). While the remaining mesendoderm precursor cells migrate only loosely associated and individually⁴⁶, the ppl has a very cohesive mode of migration^{35,47}. The leading edge cells of the ppl are highly polarized and display protrusions oriented into the direction of movement^{48,49}. It furthermore has been shown that all ppl cells share these features and are actively involved in migration⁴⁷.

Directly after ingressión axial, paraxial, and lateral cells migrate towards the animal pole. At mid gastrulation (around 70% epiboly) however, paraxial and lateral mesoderm cells change direction and start migrating towards the dorsal midline, while axial tissues continue moving towards the animal pole (Fig. 1.3)^{46,50}. Despite several differences, the migration of lateral mesoderm and ppl cells both seem to depend on a tight regulation of protrusive behavior and cell cohesion. Interfering with the ratio between lamellipodia and bleb formation disturbs the directionality of migration^{49,51}. Likewise interfering with the cadherin-dependent stability of cell contacts reduces the ability of either cell population to migrate efficiently and directionally⁵²⁻⁵⁵. Once lateral cells approach the dorsal area of the future notochord, they initiate mediolateral and radial intercalation behavior that contributes to the extension of the body axis^{25,35,50,56}. Unlike the ppl and lateral mesoderm, the notochord appears to be exclusively shaped by mediolateral intercalation⁵⁷.

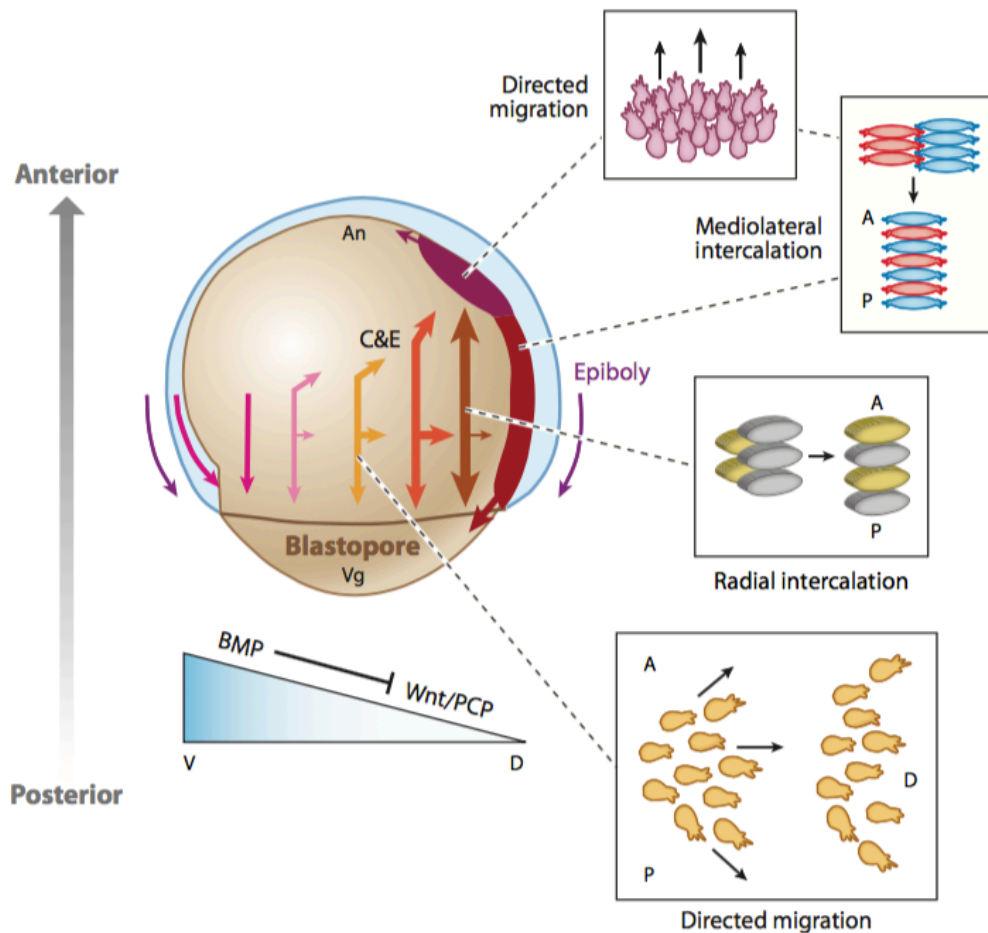


Figure 1.3: Schematic of movement patterns of cell populations during gastrulation. Boxes show specific modes of cell behavior. The wedge-shaped box shows the ventral to dorsal BMP gradient that inhibits expression of Wnt/PCP components. Modified from Solnica-Krezel & Sepich²⁵.

1.3.2 Molecular regulation of convergence and extension

The precise nature of the molecular mechanisms that regulate C&E is not entirely understood yet, but several involved molecules and pathways have been identified. Most prominently the Wnt/PCP pathway is required for proper C&E, since numerous PCP mutants display C&E defects (see chapter 1.4 below). It has also been shown that protrusion formation of the prechordal plate depends on PDGF/PI3K signaling and the small GTPases RhoA and Rac1^{47,48}. The polarity of ppl protrusions, however, depends on E-cadherin mediated cell cohesion, since isolated ppl cells keep their ppl identity but randomly direct their protrusions⁴⁷, and Wnt/PCP signaling⁵². The Wnt/PCP effect on ppl cell polarity appears to be mediated again by RhoA and Rac1, as loss of function of either protein results

in misorientation⁴⁷. Wnt/PCP signaling is also involved in the convergence of lateral mesoderm and the intercalation of notochord progenitor cells by controlling cell polarity and cell motility, since many Wnt/PCP mutants display shortened and widened paraxial and notochord domains as well as aberrant cell shapes⁵⁸⁻⁶⁵. It has been suggested that the direction of the convergence movements is regulated by BMP signaling (Fig. 1.3), through ventral inhibition of Wnt/PCP component expression⁴². It has also been suggested that a ventral to dorsal BMP gradient negatively regulates cadherin-dependent cell cohesion and thus creates a dorsal to ventral adhesion gradient, which directs converging cells⁵³.

1.4 The Wnt/PCP pathway

All epithelia by definition have an apical-basal polarity that is necessary for the epithelium to maintain its barrier function. Besides that, however, most epithelia have a second polarity within said plane. This planar cell polarity (PCP) was discovered in insects more than 30 years ago⁶⁶⁻⁶⁸ and has been thoroughly studied and very well reviewed in *Drosophila*, where cuticular structures display obvious defects in planar cell polarity⁶⁹⁻⁷⁵. Although different mechanisms appear to be at place in different tissues, e.g. cell fate regulation in the eye, spindle orientation in the thorax epithelium, and asymmetric actin localization in the wing^{74,76-78}, all these processes are controlled by two sets of core proteins. The Fz/PCP and the Ft/Ds (which will not be discussed in this project) systems appear to work in parallel and partially redundantly⁷⁹⁻⁸¹. In *Drosophila*, the Fz/PCP pathway consists of 6 core proteins (see table 1): Frizzled (Fz), a seven-pass transmembrane receptor, Van Gogh (Vang), a four-pass transmembrane protein, the atypical cadherin Flamingo (Fmi), and three cytosolic proteins, Dishevelled (Dsh), Prickle (Pk), and Diego (Dgo)^{73,74}. Originally the role of Wnts in insect Fz/PCP signaling was unclear due to a lack of PCP-like phenotypes in *wnt* mutants⁷³. However, experiments showing that the PCP axes in the wing and eye are oriented towards Wnt sources^{82,83} and gain-of-function experiments showing the capacity of Wnt and Wingless (Wg) expressing clones to re-orient the polarity of nearby cells⁸⁴ have changed that view and support a role for Wnts in *Drosophila* PCP signaling.

One of the most studied and best understood model systems for Wnt/PCP signaling is the *Drosophila* wing, in which PCP is activated during late third instar larval and early pupal

stages^{82,85} and sets up the proximodistal polarity of the epithelial cells from the primitive radial one^{82,86}. This becomes apparent when the proteins of the Fz/PCP core group change from an original uniform subapical ring localization, which partially overlaps with the adherens junctions, to an asymmetric localization along the proximodistal axis (Fig. 1.4 A)⁸⁷.

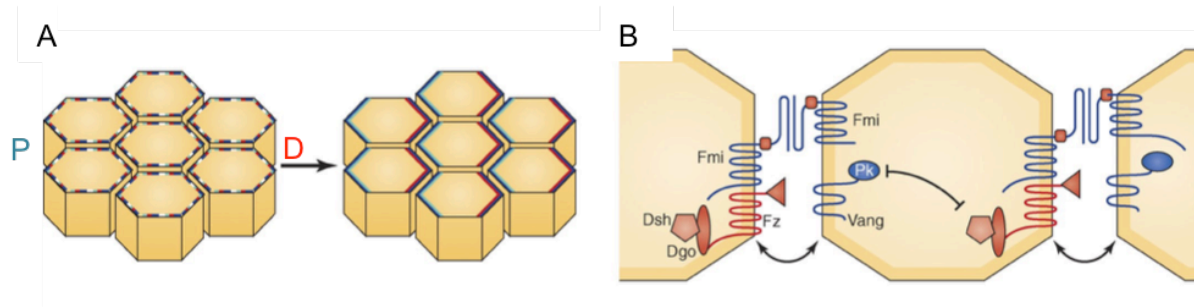


Figure 1.4: PCP establishment in *Drosophila* epithelia, modified from Devenport⁷². A: Schematic of *Drosophila* wing epithelial cells, before planar polarization (left) and after (right), proximal is left, distal is right, P marks the proximal side of the cells, D the distal. From originally unbiased distribution two clusters form, Pk and Vang (turquoise) localize at the proximal membrane, Fz, Dsh, and Dgo (red) at the distal side; Fmi co-localizes with both clusters. B: Feedback interactions stabilize the asymmetric localization. Fmi-Fz complexes interact with Fmi-Vang complexes in the neighboring cells, while the complexes antagonize each other within a cell.

During this relocation, two complexes are formed based on negative interactions between Prickle and Dishevelled, which are regulated by Diego^{88,89}, and clustering of Dsh with Fz and Pk with Vang respectively^{88,90}. Flamingo localizes on both sides and helps stabilize the asymmetric distribution by forming complexes with Fz and Vang that interact intercellularly (Fig 1.4 B)⁹¹⁻⁹⁵. The asymmetric localization is facilitated by microtubules directed along the P-D-axis, which direct the movement of the Fz-Fmi complexes^{74,96-98}. This process describes specifically how planar polarity is set up in the wing epithelium, but it is thought to be the general mechanism in *Drosophila* tissues and possibly also in other epithelial systems⁷⁴.

1.4.1 Vertebrate homologues and functions

Vertebrates have homologues for all Fz/PCP core components, but also contain novel proteins that do not have a *Drosophila* orthologue, or orthologues with unknown function in the pathway. Most prominent among those are the secreted glycoproteins Wnt5 and Wnt11⁷⁴. Hence the pathway is usually referred to as Wnt/PCP pathway in vertebrates. In *Drosophila* PCP signaling appears to mostly function in epithelial systems. Since the core complex is conserved in vertebrates (see table 1), it is not surprising that planar polarity in many vertebrate epithelia is regulated similarly. Examples include the orientation of hair cells in the cochlea^{99,100}, the orientation of skin hair in mammals¹⁰¹, the ciliated cells of the larval *Xenopus* skin¹⁰², the orientation of the lateral line cells in fish¹⁰³, left-right symmetry breaking in the node or Kupfer's vesicle¹⁰⁴⁻¹¹⁰, or neurulation^{63,111,112}. Besides these epithelial functions, vertebrate PCP signaling is involved in mesenchymal contexts like limb elongation¹¹³, neural crest cell (NCR) migration¹¹⁴⁻¹¹⁶, muscle fiber orientation¹¹⁷, and convergent extension^{58,59,61,63,118-121}. In fact, convergence and extension phenotypes without apparent cell fate defects are considered a hallmark of vertebrate PCP mutations¹⁰. Since mutants for the chordate additions to the pathway such as *wnt11* (*silberblick*, *slb*^{58,122,123}), *wnt5* (*pipetail*, *ppt*⁶¹), *knypek* (*kny*⁵⁹), or *protein tyrosine kinase 7* (*ptk7*¹¹⁹), all display C&E defects (see table 1), it is an intriguing thought that this new task made the recruitment of these genes necessary.

During the convergence and extension movements in gastrulation and neurulation, Wnt/PCP signaling seems to regulate both cell migration and intercalation^{45,51,75,124-126}. Another system in which Wnt/PCP components are required for cell intercalation events is the formation of the primitive streak in chick embryos¹²⁷. The necessity of Wnt/PCP signaling for neural crest migration provides another example for its role in cell motility. In a process termed contact inhibition of locomotion (CIL), Wnt and Fz downregulate actin polymerization at the sites of cell contacts such that the NCR cell polarizes and migrates away from its neighbors^{114,115}. There is also a non-vertebrate example where PCP signaling is involved in cell migration, namely *Drosophila* border cell migration during oogenesis¹²⁸. Cells have to continuously form and release contacts among themselves and with the extracellular matrix (ECM) when they migrate, which might be regulated by Wnt/PCP³⁵. It

has been shown that the persistence of cell contacts is increased by accumulation of Fz7 at the contact sites upon binding of Wnt11¹²⁹. Furthermore Wnt/PCP signaling can modulate the Rab5-mediated endocytosis of E-cadherin and thus modulate cell cohesion in mesendoderm cells⁵². Wnt/PCP has been also shown to regulate ECM reorganization^{130,131}, which is partially done via the metalloproteinase *mmp14*¹³².

Vertebrates contain multiple copies of the Wnt/PCP core proteins, and in zebrafish there exist additional paralogues due to the teleost genome duplication (reviewed in ¹³³; see table 1). For instance, there are five copies of *prickle* (*pk1a*, *b*, *2a*, *b*, and *3*), as well as four *flamingo* (*celsr1a*, *b*, *2*, and *3*), PCP *frizzled* (*fz2*, *7a*, *7b*, and *10*), and *dishevelled* (*dvl1a*, *b*, *2*, *3a*, *b*) homologues^{74,75,134}. They often act redundantly, since knockdowns of single paralogues only cause mild phenotypes, but enhance the mutant phenotypes of other genes¹³⁵. Additionally many of these genes are maternally contributed, leading to mild or no phenotypes in zygotic single mutants, e.g. *celsr1a* mutants show no phenotypes, while morphants do¹³⁶. Furthermore, the zygotic mutants for either paralogue of *fz7* do not show mutant gastrulation phenotypes. Only the maternal and zygotic double mutant (MZ *fz7a,b*) displays classical C&E PCP phenotypes⁶³. Another interesting feature of Wnt/PCP signaling is the similarity of overexpression and knock-out phenotypes, which is likely due to a delicate balance of protein amounts^{128,137}.

Table 1.4.1: PCP components in *Drosophila* and vertebrates (adapted from Singh & Mlodzik⁷⁴, Yang & Mlodzik⁷³).

<i>Drosophila</i> Genes	Vertebrate* Genes	Molecular Features	<i>Drosophila</i> defects	Vertebrate defects	Refs
<i>frizzled (fz)</i>	<i>fz2, fz3, fz6, fz 7a&b, fz10</i>	Seven-pass transmembrane receptor; binds Wnt ligands, interacts with Dsh/Dvl	All adult tissues	Cochlea, epidermis, neurulation, C&E, NCr migration	68,101,114,134,137-143
<i>dishevelled (dsh)</i>	<i>dishevelled (dvl) 1a & b, 2, 3 a& 3b</i>	Cytoplasmic protein with DIX, PDZ, and DEP domains; binds Fz, Pk, Stbm, and Dgo	All adult tissues	C&E, cochlea	109,118,126,144-151
<i>strabismus (stbm)/van gogh (vang)</i>	<i>vangl1, vangl2/trilobite (tri)</i>	Four-pass transmembrane protein; binds Pk, Dsh, and Dgo	All adult tissues	C&E, cochlea, limb elongation, growth cone guidance	60,99,108,113,152-155
<i>flamingo (fmi) / starry night (stan)</i>	<i>celsr1a, b, 2, 3</i>	Atypical cadherin with seven-pass transmembrane receptor features; homophilic cell adhesion	All adult tissues	C&E, cochlea, epidermis, growth cone guidance	62,155-160
<i>prickle (pk)</i>	<i>pk1a, b, pk2a, b, pk3</i>	Cytoplasmic protein with LIM and PET domains, interacts with Dsh, Stbm, and Dgo	All adult tissues	C&E	89,135,161-163
<i>diego (dgo)</i>	<i>diversin, inversin</i>	Cytoplasmic ankyrin repeat protein; binds Dsh, Stbm, and Pk	All adult tissues	C&E	88,164-168
Not determined	<i>glypican 4/6 /knypek (kny)</i>	Heparan sulfate proteoglycan; binds Wnt11, Wnt5, and Fz7; enhances Wnt/PCP signaling	Not determined	C&E	59,169
Not determined	<i>wnt5/ppt</i>	Secreted glycoprotein; acts as ligand for Fz receptors	Not determined	C&E, limb elongation, growth cone guidance	61,113,155,170-173
Not determined	<i>wnt11/slb</i>	Secreted glycoprotein; acts as ligand for Fz receptors	Not determined	C&E, NCr migration	58,114,118,137,170,172
Not determined	<i>Ptk7</i>		Not determined	C&E, Cochlea development, NCr migration	174,175

* I list here mostly fish genes, in Singh & Mlodzik⁷⁴, Yang & Mlodzik⁷³ mainly mouse and *Xenopus* orthologues are listed.

1.4.2 Dishevelled pathway selection and downstream targets

Dishevelled (Dsh or Dvl) was originally discovered in the *Drosophila* PCP pathway, but is also a key element in the transcriptionally active canonical Wnt/ β -catenin pathway^{144,146,176}. The key to pathway selection lies in differential binding of Dvl and thus differential downstream activation. Dvl features three conserved functional domains: the N-terminal 80 amino-acid DIX (Dishevelled-Axin) domain, the central PDZ (Postsynaptic density 95, Discs Large, Zonula occludens-1) of 90aa, and the C-terminal DEP (Dvl, Egl-10, Pleckstrin) domain, which consists of 80aa¹⁴⁴. The DIX domain promotes Dvl polymerization in the cytosol¹⁷⁶ and acts in canonical Wnt signaling¹⁴⁴. The PDZ domain is required in both pathways, as its protein-binding abilities are able to transduce signals from Frizzleds to downstream targets^{177,178}. The DEP domain is Wnt/PCP specific and binds downstream targets of Dvl, like the formin homology adaptor protein Daam1 (Dvl associated activator of morphogenesis 1; Fig 1.4.2).

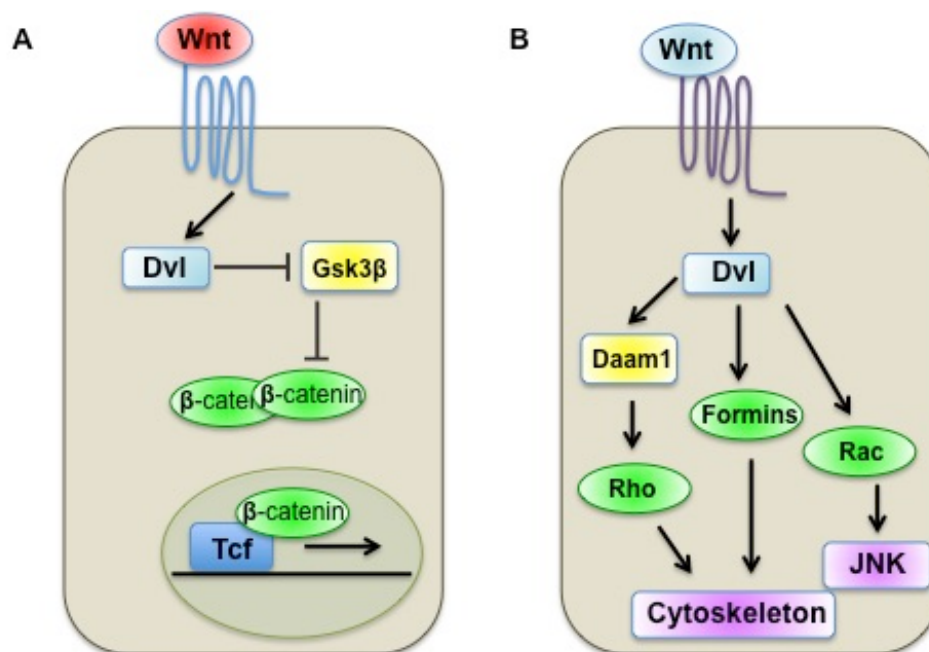


Figure 1.4.2: Schematic of the canonical and the Wnt/PCP pathways, after Gao & Chen¹⁴⁴. A: In the canonical Wnt pathway Dvl inhibits Gsk3 β upon activation by Wnt and Fz. This stabilizes β -catenin, which can thus promote transcription in the nucleus. B: In the non-canonical Wnt/PCP pathway different Wnts and Fzs activate the same Dvls as in the canonical pathway, but the downstream cascade involves other molecules and directly regulates cell behavior.

As mentioned above vertebrates possess several *dishevelled* paralogues (3 in mammals, 5 in zebrafish). This could lead to the assumption that different Wnt pathways use different

dishevelleds, especially since this is the case for *wnts* (where *wnt5* and *wnt11* are dedicated Wnt/PCP ligands^{58,61}) and *fzs* (*fz2*, *fz7a,b*, and *fz10* seem to be Wnt/PCP receptors in zebrafish^{134,138}). However, all *dishevelled* paralogues appear to have the same capacities and to act redundantly, likely owing to their overlapping expression patterns and conserved structure¹⁴⁴. While single knockout mice display only very mild defects, *dvl1*^{-/-}, *dvl2*^{-/-} double mutants show classical PCP phenotypes, like neural tube closure and cochlea defects, but can be rescued by exogenous *dvl3* expression^{151,179-181}.

The downstream targets of Dvl in the PCP pathway are not entirely known, but involve multiple small GTPases¹⁴⁴. In one branch of the pathway Dvl interacts via Daam1 with a Rho guanine exchange factor, to form RhoGTP, which in turn activates the Rho-dependent kinase (ROCK, Rok2^{124,182,183}, Fig. 1.4.2 B). ROCK phosphorylates the regulatory light chain of Myosin and thus increases contractility of the cell cortex^{124,184,185}, while at the same time inhibiting Mypt1, the regulatory subunit of the Myosin phosphatase complex, which – when active – dephosphorylates Myosin⁵¹. Dvl can also interact with other small GTPases, such as Rac1 or RhoB. In the presence of a Wnt5a gradient RhoB signaling can polarize melanoma cells and lead to directed migration¹⁸⁶. Signaling via Rac1 is not entirely understood, but likely involves Jun-N-terminal kinase (JNK)^{144,187,188}. Additionally Wnt/PCP signaling can act via the actin-binding protein Profilin in *Xenopus* blastopore closure and axon guidance^{189,190}.

The common theme of these branches of Wnt/PCP signaling is remodeling of the cytoskeleton and control of migration^{124,144}. Wnt11 and Fz7 have also been shown to act via another Dishevelled independent pathway. In this case the signal transduction happens via trimeric G-proteins and protein kinase C and it is required for tissue separation in *Xenopus*¹⁹¹.

1.4.3 Instructive or permissive function

Despite the tremendous advances in our understanding of planar cell polarity over the past decades, many questions remain. A major point of interest is the mode in which Wnts function as input into the pathway. To this day it remains heavily debated, if Wnt ligands form a gradient that acts as a guidance cue, and if receiving cells translate this into polarity

information^{73-75,192,193}. This modus operandi is usually referred to as *instructive* in the Wnt/PCP field^{75,84,192-194}. The alternative model suggests that although Wnts and Frizzleds are required for PCP, their direction does not matter; as long as they are present within a cell, they can fulfill their purpose, no matter from which direction they came. This type of function is called *permissive*^{58,74,75}. There is evidence supporting both hypotheses, partly context-dependent, which shall be discussed in the following section.

1.4.3.1 Instructive Evidence

Originally, the requirement for Wnts in *Drosophila* PCP was doubtful, because no loss-of-function phenotypes were known. However, the PCP axes are oriented towards a Wnt source^{82,83} and more recently it has been shown that exogenous clonal expression of Wingless (Wg) or *Drosophila* Wnt4 can reorient the PCP axis of nearby cells (Fig 1.4.3.1)⁸⁴. This does not only suggest that Wnts have a function in *Drosophila* but also shows clearly that they have instructive capacity.

In vertebrates many different systems have been investigated leading to similar results. Fz deficient mouse embryos have defects in axon guidance of serotonergic and dopaminergic neurons, and Wnt coated beads could redirect polarity in explant culture¹⁶⁰. Similarly, clonal expression of *wnt5* and *wnt11* caused asymmetric localization of Pk3 in the ventral *Xenopus* epidermis (Fig. 1.5.3.1)¹⁹⁴. The instructive capacity of Wnt/PCP in mesenchymal cell populations was demonstrated in chick embryos. When *wnt11* expressing cells were transplanted between two somites or into the myotome, the surrounding myofibers of the host embryo changed their orientation to face the Wnt source¹¹⁷. In the developing mouse limb bud phosphorylated Vangl2 forms a complex with Ror2 that is asymmetrically localized within the cells. In Wnt5a mutant mice, Vangl2 phosphorylation, Ror2-Vangl2 complex formation, as well as asymmetric Vangl2 localization was significantly reduced¹¹³. More evidence for the instructive function of Wnt ligands in PCP signaling was found in chick limb development: when acrylic beads soaked with Wnt5a protein were implanted into the lateral plate mesoderm of HH stage 16 embryos, chemotactic cell movement towards the bead could be frequently observed¹⁹⁵.

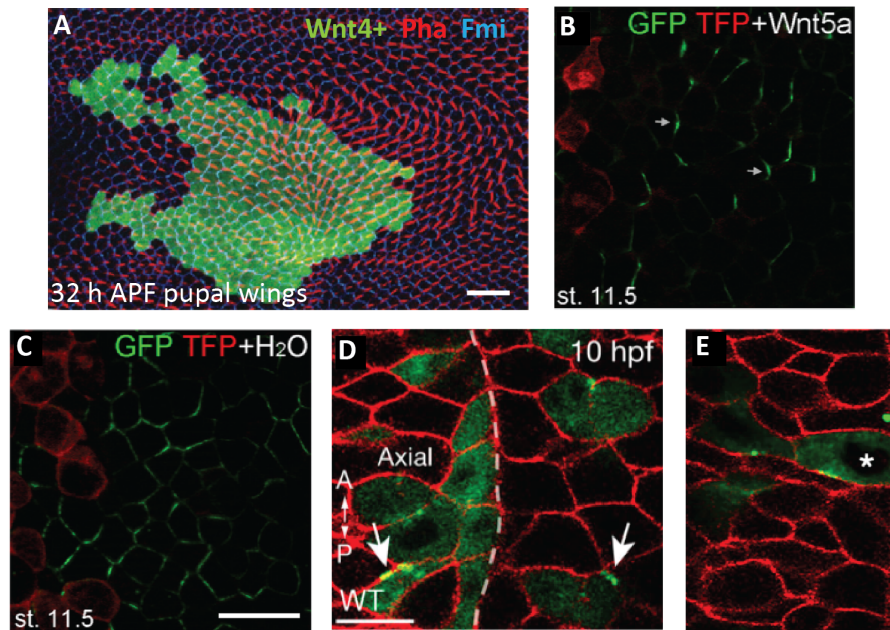


Figure 1.4.3.1: Evidence for instructive function. A: Clonal expression of Wnt4 (green) reorients the polarity of neighboring cells, marked by actin (Phalloidin, red) and Fmi (blue). The scale bar is 10 μ m. Taken from Wu et al.⁸⁴. B & C: Pk3 in *Xenopus* larval ectoderm is polarized by ectopic Wnt5a expression (B, red), but not by water (C, red), the scale bar is 50 μ m. Taken from Chu & Sokol¹⁹⁴. D&E: Localization of Pk1 (green, D) to the anterior cell membrane and of Dvl (green, E) to the posterior one. The scale bar is 20 μ m. Taken from Yin et al.⁵⁶.

In zebrafish it was also shown that a GFP-tagged *Drosophila* Pk construct localizes to the anterior edge of notochord and neural tube cells during the segmentation period¹¹¹. Another study found the same Pk-GFP localization together with posterior enrichment of a *Xenopus* Dsh-GFP construct in mediolaterally intercalating cells of the presomitic mesoderm at the very end of gastrulation (95% epiboly to bud stage, 9-10hpf, Fig. 1.4.3.1)⁵⁶. Similarly, cells expressing an N-terminally tagged Vangl2 construct showed anterior Vangl2 enrichment during the segmentation period, when transplanted to unlabeled WT hosts¹⁹⁶. This is reminiscent of the opposing localization of the Vang-Pk and Fz-Dsh-Dgo clusters in *Drosophila* epithelia. Taken together these data clearly show the instructive capacity of Wnt/PCP signaling in various epithelial and mesenchymal contexts.

1.4.3.2 Permissive evidence

The idea that Wnt/PCP signaling could have permissive function goes back to the discovery that the gene locus causing the *silberblick* (*slb*) phenotype encodes *wnt11*⁵⁸. *Silberblick* fish embryos display defects in anterior axial mesoderm migration, more specifically a shortened

and widened anterior notochord and an elongated prechordal plate, that leads to a squint-eyed phenotype, reminiscent of strabismus, or even fusion of the eyes later in development (Fig. 1.4.3.2)^{58,122,123}. These defects can be rescued by injection of *wnt11* mRNA into the 1-cell stage of the embryo, leading to uniform expression. The same rescue effect could be achieved with mRNA encoding an amino-terminally truncated version of *Xenopus dsh*, which can signal through the PCP pathway, but not through the canonical Wnt pathway, or with *wnt5b* mRNA^{58,61}. The ubiquitous expression of exogenous mRNA is unlikely to recapitulate positional information, and thus this experiment provides very strong evidence against an instructive function of Wnt/PCP signaling in vertebrate gastrulation¹⁰. This would, however, still leave the possibility that the downstream receptor is expressed in a gradient, which would lead to gradient-like signaling transmission despite a ubiquitously present ligand.

A lot of the data that support an instructive function of Wnt/PCP signaling come from overexpression experiments (e.g. ^{117,160,195}) and do not necessarily recapitulate the endogenous function. The same is true for data showing biased localization of core PCP components. Besides being overexpression studies, they often use proteins from other species^{10,56}. In the case of Vangl2, the asymmetry of its localization during zebrafish segmentation was only visible using exogenous fluorescently tagged protein, but not in immunofluorescent detection of the endogenous Vangl2¹⁹⁶. In the case of *Xenopus*, it has been shown that the proposed asymmetric Dsh localization actually reflects yolk-free cytoplasm and not cell polarity¹⁹⁷. As for the parallels between the asymmetric localization of PCP components in *Drosophila* epithelia and vertebrate mesenchyme, it has been pointed out that in the fly the PCP axis of cells aligns with the proximodistal tissue axis; the asymmetry in vertebrates is oriented in anterior-posterior direction, while the cells undergo mediolateral intercalation⁵⁶. These results show important differences between epithelial and mesenchymal functions of Wnt/PCP signaling and challenge the view of Wnt ligands as instructive guidance cues.

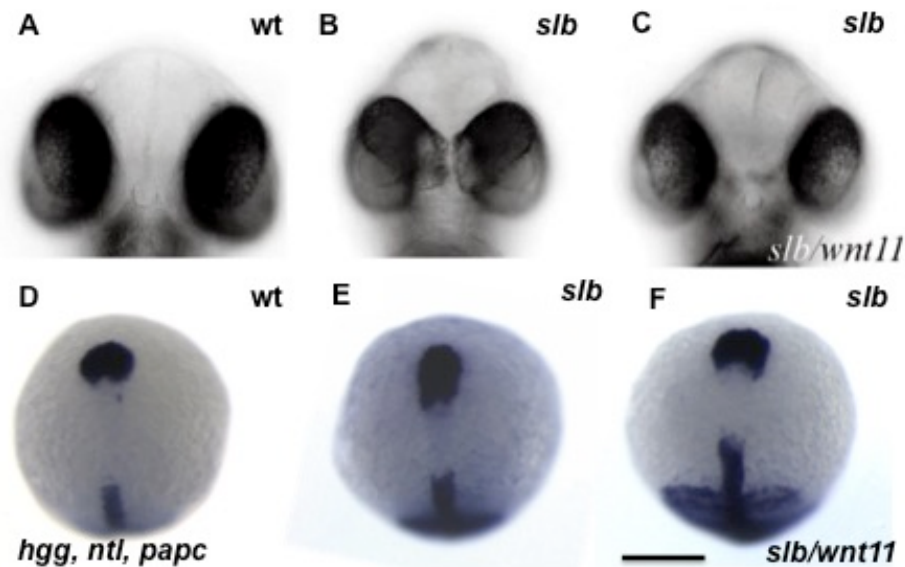


Figure 1.4.3.2 Permissive evidence. A-C: *Slb* embryos (B) display reduced eye spacing at pharyngula stages compared to wt (A). This phenotype can be rescued by ubiquitous expression of *wnt11* (C). Taken from Heisenberg et al.⁵⁸. D-F: In situ hybridizations with *hgg*, *ntl*, and *papc* probes. *Slb* embryos (E) have a narrow and elongated ppl at bud stage (10hpf) compared to wt (D). This phenotype can also be rescued by ubiquitous ectopic *wnt11* (F). The scale bar is 250µm.

1.5 Tools for the study of Wnt/PCP signaling

Two obstacles have hindered the study of PCP signaling. Firstly, the necessity to mimic endogenous signaling, which requires a high spatial and temporal control over the pathway activity, beyond the abilities of exogenous overexpression cell clones. The other problem is the required balance of Wnt/PCP proteins, which makes visualizing protein localization faithfully very difficult; indeed, on the one hand a potential asymmetric localization of a PCP component could be lost due to disturbed balance of Wnt/PCP proteins in overexpression experiments. On the other hand, asymmetry found in overexpressed tagged fusion proteins could also be an artifact of imbalanced dosage.

To overcome these problems we decided to extend the experimental toolkit in this project beyond established techniques (see methods section). In order to gain a high spatial and temporal control over PCP signaling activity, we designed a chimeric light-activated version of the Wnt/PCP receptor Fz7. In addition, to tackle the overexpression problem when visualizing PCP core components, we planned to knock-in a single copy of a fluorescent

protein into the endogenous Fz7 locus, which would allow to faithfully monitor Fz7 localization.

1.5.1 Optogenetics

Since the possibility of a receptor gradient, operating downstream of a gradient or ubiquitous ligand, could not be excluded by previous studies, we needed a Fz7 receptor that is non-responsive to endogenous Wnt signaling, but can be activated in spatially and temporally controlled fashion. These reasons suggested a chimeric receptor approach that separates signaling input from downstream signaling. To avoid the necessity of washout steps and get faster reaction time, we decided to use an optogenetic approach rather than activation by synthetic ligands or designer drugs (RASSLs and DREADDs)¹⁹⁸⁻²⁰⁰. The zebrafish system also offered an ideal match for an optogenetic approach due to its external development and transparent embryos as has been shown recently²⁰¹.

1.5.2 CRISPR mediated knock-ins

The CRISPR/Cas9 (clustered regularly interspaced palindromic repeats/CRISPR-associated 9) system is a bacterial defense mechanism against foreign DNA, i.e. plasmids and bacteriophages²⁰²⁻²⁰⁵. The system consists of the Cas9 endonuclease, small CRISPR RNAs (crRNA), and trans-activating crRNAs (tracRNA). After infection, crRNAs recognize intruder DNA via base pairing and recruit tracRNAs, which then foster binding of Cas9 to the complex. Cas9 cleaves the infecting DNA and thus inactivates the pathogen²⁰⁶. For its application as a genome-editing tool, the crRNA and the tracRNA have been fused to a single guide RNA (sgRNA) that contains both the target and the Cas9 recruiting sequence. Therefore the system can be customized to target any desired locus by including a gene-specific sequence in the sgRNA. The only restriction is the requirement of a recognition sequence, called the protospacer adjacent motif (PAM), which typically has the sequence NGG (N can be any nucleotide). The nuclease cleaves upstream of this sequence²⁰⁷.

This technique has been used frequently for site-specific mutagenesis in zebrafish (e.g. ^{208,209}). Combined approaches of CRISPR and DNA templates for integration, homologous recombination (HR), and non-homologous end joining (NHEJ) have previously succeeded in knocking-in sequences of varying length at specific sites in the genome^{210,211}. Here, we want

to integrate a *loxP* site via HR and subsequently introduce the fluorescent protein in a second step using CRE/lox recombination.

1.6 Aims of this project

Given the amount of contradicting evidence concerning an instructive or permissive function of Wnt/PCP signaling, it is reasonable to assume different functions in different contexts¹⁰. It is conceivable that the same pathway polarizes cells within the plane of an epithelium with respect to a signaling molecule gradient, but acts permissively in the three dimensional environment of mesenchymal mesendoderm, where it might encounter a uniform ligand distribution. A wealth of experiments applying exogenous Wnt signals^{84,117,160,194,195} have impressively demonstrated that the pathway indeed has instructive capacity, if an artificial gradient is applied. However, to what extent this reflects the endogenous situation remains unclear^{194,212}. In this project, we want to address this question in the very system that has provided the clearest evidence for permissive function to date: zebrafish gastrulation⁵⁸.

- Aim 1: Analyze the phenotype of MZ *fz7a,b* embryos, since Fz7 is a likely candidate to be the receptor for Wnt11^{129,137}.
- Aim 2: Create a light-activated Frizzled receptor (OptoFz7) to unambiguously distinguish between instructive and permissive function.
- Aim 3: Investigate instructive capacity of OptoFz7 by localized activation.
- Aim 4: Try to rescue the phenotypes on embryo, tissue, and cell level by uniform activation of OptoFz7 to test for permissive function.
- Aim 5: Knock a fluorescent protein into the *fz7b* locus to create a single copy transgenic line that faithfully displays the localization of endogenous *fz7b*.

2 Material and Methods

2.1 *Reagents, media, and buffers*

E3 medium	5 mM NaCl, 0.17 mM KCl, 0.33 mM $\text{CaCl}_2 \times 2 \text{ H}_2\text{O}$, 0.33 mM $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$, pH 6.5
Danieus medium	58 mM, 0.7 mM KCl, 0.4 mM MgSO_4 , 0.6 mM $\text{Ca}(\text{NO}_3)_2$, 5 mM HEPES, pH 7.6
Agarose	0.7% (w/v) low melting point agarose (Invitrogen) in E3 medium for mounting live embryos 2% (w/v) agarose in E3 medium for injection and transplantation molds
PBS	1.7 mM KH_2PO_4 ; 5.2 mM Na_2HPO_4 ; 150 mM NaCl
PBST	1.7 mM KH_2PO_4 ; 5.2 mM Na_2HPO_4 ; 150 mM NaCl, 0.1% Tween-20
9-cis-retinal	Sigma (R5754), diluted to 10mM in MeOH, stored in 50 μ l aliquots at -20°C
DMEM	Dulbecco's modified eagle medium/Nutrient Mixture F-12 HAM, (Sigma D6434)
LB Medium	10 g/L Tryptone, 5g/L Yeast Extract, 5 g/L NaCl

2.1.1 Reagents for in situ hybridization

20x SSC	3.0 M NaCl and 0.34M Trisodium citrate in dH_2O
2X SSC	1:10 dilution of 20X SSC
0.2X SSC	1:10 dilution of 2X SSC
Hybridization mix plus (HM+)	50% Deionized formamide (Merck, 109684), 5X SSC, 0.1% Tween-20, pH6 (1M citric acid), 0.5mg/mL RNase-free tRNA (Sigma, R6875), 50mg/ml Heparin (Sigma, H3393) in dH_2O
Hybridization mix (HM)	50% Deionized formamide (Merck, 109684), 5X SSC, 0.1% Tween-20, pH 6 (1M citric acid), 0.5mg/mL in dH_2O
Solution A	75% (v/v) HM, filled up with 2x SSC

Solution B	50% (v/v) HM, filled up with 2x SSC
Solution C	25% (v/v) HM, filled up with 2x SSC
Solution D	75% (v/v) 0.2x SSC, filled up with PBST
Solution E	50% (v/v) 0.2x SSC, filled up with PBST
Solution F	25% (v/v) 0.2x SSC, filled up with PBST
Blocking buffer	2% (v/v) sheep serum, 2 mg/mL BSA in PBST
	Store at -20°C
Alkaline Tris buffer	100mM Tris/HCl pH 9.5, 50mM MgCl ₂ , 100mM NaCl, 0.1% Tween-20 in dH ₂ O
NBT/BCIP Staining solution	prepare fresh and keep in the dark
	Use 4.4ul of NBT and 3.3ul of BCIP per ml of solution
	Put in NBT (Sigma, 11383213001 Roche) first in Alkaline Tris buffer and mix
	Put in BCIP (Sigma, 11383221001 Roche) and mix again

2.1.2 Reagents for Immunohistochemistry

Washing buffer 1	0.5% Tween-20 in PBS
Washing buffer 2	0.5% Tween-20, 0.5% Triton X-100, 0.1 M glycine
IF buffer	0.5% Tween-20, 0.5% Triton X-100, 1% DMSO, 1% BSA

2.2 Antibodies

Digoxigenin	Roche, AP-labeled FAB fragments (11093274910)
E-cadherin	Rabbit Anti fish E-cadherin (MPI-CBG)
Laminin	Rabbit Anti Laminin (Sigma, L9393)
Secondary	Cy5-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson 111-175-003)

2.3 *Plasmids, Primers & Morpholinos*

Morpholinos

sox32 Morpholino GCATCCGGTCGAGATACATGCTGTT

Genotyping primers

fz7a-forward	CAAACCCGACGCCCTAC
fz7a-reverse	ATGAGGTACCGATGAAGAGG
fzd7b_hu3495_DpnI/fwd	ATCGTCATTGCGTGTTACTTCTACGAT
fzd7b_hu3495_rev	TGGTTGTTGCCTAGCCGTTTG

Gateway primers

Rhodopsin GW for	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCAATGAA AACGATCATCGCC
Rhodopsin GW rev	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGAAAACGAT
OptoFz7 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATACCGTCGTCTCGCC
Frizzled GW rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATACCGTCGTCTCGCCCTG
Wnt11 GW for	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGATGACAG AATACAGGAAC
Wnt11 GW rev	GGGGACCACTTTGTACAAGAAAGCTGGGTACTCGAGACGTATCTCTCT
3xcherry GW for	GGGGACAAGTTTGTATAGAAAAGTTGTAATGGTGAGCAAGGGCGAG
3xcherry GW rev	GGGGACCACTTTGTACAAGAAAGCTGGGTATCACTCGAGCTTGTACAG

CRISPR primers

gRNA Primer 1 (T7)	GCGTAATACGACTCACTATAG
gRNA primer 2	AAAGCACCGACTCGGTGCCAC
sgDNA rev	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTA GCCTTATTTAACTTGCTATTTCTAGCTCTAAAAC
genotyping primer for	TCATGGTGCTGTATTTCTTTGG
genotyping primer rev	AAGGCATGTTCTTTGGAGTTGT
loxP for	ATAACTTCGTATAATGTATGCTATACGAAGTTAT
loxP rev	ATAACTTCGTATAGCATACATTATACGAAGTTAT

Table 2.3.1: In situ probes

Number	Gene	Backbone	Polymerase	Reference
#68	<i>ntl</i>	pCS2+	T7	⁵⁸
#41	<i>hgg</i>	pBluescript	T3	¹³⁵
#73	<i>papc</i>	pBluescript	T3	¹³⁵
#110	<i>fz7a</i>	psport	SP6	
#172	<i>fz7b</i>	pBluescript	T3	

Table 2.3.2: plasmids for mRNA injection

Number	Gene	Backbone	Polymerase	Source
#427	<i>h2A-mcherry</i>	PCS2	SP6	²¹³
#554	<i>h2B-tagBFP</i>	PCS2	SP6	Gateway, Heisenberg group
#249	<i>h2B-GFP</i>		SP6	Tanaka lab
#675	<i>mkate2-CAAX</i>	PCS2Dest	SP6	MPI-CBG, gateway
#106	<i>wnt11</i>	pBSII-SK(-)	T7	⁵⁸
#740	<i>RRFR</i>	pCS2	SP6	Golden Gate
#741	<i>RRFR-mcherry</i>	pCS2	SP6	Gateway
#742	<i>FFFF</i>	pCS2	SP6	Golden Gate
#743	<i>FFFF-mcherry</i>	pCS2	SP6	Gateway
#738	<i>RRFF</i>	pcDNA3.1	T7	Golden Gate
#739	<i>RRFF-mcherry</i>	PCS2Dest	SP6	Gateway
#744	<i>rhodopsin</i>	pCS2	SP6	Golden Gate
#678	<i>dvl2-GFP</i>	pCS2	SP6	Tada lab
#714	<i>wnt11-3x-mcherry</i>	PCS2Dest	SP6	Gateway
#632	<i>cas9</i>		T7	Addgene ^{#42251,} ²¹⁴
#698	<i>cre.zf1</i>	pCS2	SP6	²¹⁵

Table 2.3.3: Gateway constructs

Number & Name	Type	Source
pDONR221, #208	pDONR(P1-P2)	Lawson lab
pDONR 3', #211	pDONR(P2r-P3)	Lawson lab
pCS2, #444	pDEST(R1-R3)	Lawson lab
mcherry, #441	p3ENTR(R2-L3)	Lawson lab
mkate2, #542	p3ENTR(R2-L3)	MPI-CBG
wnt11, #569	pENTR(L1-L2)	⁵⁸
3xcherry, #570	pENTR(R2-L3)	Siekhaus lab, cloned

2.4 Technical Equipment

Confocal microscopes	Leica SP5 upright Leica SP5 inverted Zeiss LSM 700 upright Zeiss LSM 880 upright LaVision Biotec TrimScope
Stereomicroscopes	Leica MZ 125 Leica M165 FC
Camera for stereoscope	Leica DFC 425C with Leica software
Epifluorescence microscopes	Visitron TIRF/FRAP
Glass capillaries (injection)	Harvard GC100F-15
Transplantation needles	Biomedical instruments ES-blastocyst Injection pipettes with spike, straight - VESp-ID20 micrometers, PL 55mm
Microinjectors	PV820 and Pico-Pump with foot pedal (WPI)
Micromanipulators	Narishige MN-15 (injection set up) MO-155 (transplantation set up)
Needle puller	Flaming/Brown P87 (Sutter instruments)
Pipette holders	MN-151 (injection)

	MPH3 (transplantation)
Nanospectrometer	Nanodrop 2000 spectrophotometer (Thermo scientific)
Heating block	Dri-block DB-2D (Techne)
Temperature chamber (upright microscopes)	IST Austria imaging facility
Stageheater (inverted microscopes)	IST Austria workshop
Incubator for optogenetics	Herp Nursery II, 69802, Lucky Reptile
LEDs for optogenetics	Bivar SMD Hochleistungs-LED 3,3 V, 505nm, IST Austria machine shop
Glass-bottom dishes	CellView Cell Culture Dishes, Glass Bottom. 4 compartment (Greiner 627975); P35G-0-10-C case (Matek)
Plasmacleaner	Harrick Plasma, with Edwards XDS 5 vacuum pump
Fluorescence lamp	CoolLED pE-300
PCR cycler	Bio-Rad C1000, Applied Biosystems Veriti 96 Well Thermal Cycler
Gel imaging	Bio-Rad, Gel Doc XR+

2.5 *Molecular Biology methods*

Most constructs used in this project are available in the Heisenberg lab. New constructs were obtained using either Golden Gate cloning or Gateway cloning. The kits used are mentioned at the procedure they were used for and were applied according to the supplier's protocol. If additional steps were performed they are stated separately.

2.5.1 Gateway cloning

Gateway cloning was performed as described before²¹⁶. Briefly a fragment was amplified by PCR using primers that are specific to the target sequence while adding attB sites to the amplicon. A recombination reaction was then performed using the appropriate donor vector

and BP Clonase (Thermo Fisher, 11789020) according to the supplier's protocol with all volumes halved. The reaction was stopped by adding Proteinase K, and 2µl of the reaction was used for transformation of Top 10 chemically competent cells (Thermo Fisher C404010). 50µl and 200µl were spread on Kanamycin (50µg/ml) containing agarose plates (IST Austria mediakitchen). Clones were amplified in liquid culture of LB medium containing Kanamycin and the recombination confirmed by sequencing. To combine two or three Donor vectors, a LR reaction was performed using LR clonase (Thermo Fisher 11791100) over night. The remaining steps were performed as mentioned above except that Ampicillin (100µg/ml) was used as an antibiotic.

2.5.2 Golden Gate cloning

The golden gate cloning approach was used by Maurizio Morri (MM) to clone the first two chimeras (RRFR, FFFF, see results 3.2 below), and by myself to add the third chimera (RRFF). It was based on a mother construct designed by MM and Harald Janovjak that consisted mostly of bovine Rhodopsin: the intracellular loops (ICLs) and C-terminus were substituted by artificial loops, and in the N-terminus a VSV-G-epitope²¹⁷ (amino acid sequence YTDIEMNRLGK, from the vesicular stomatitis viral glycoprotein) for antibody detection was included. The artificial loops were designed to enable the cloning procedure based on Type II restriction enzymes²¹⁸ and Golden Gate cloning^{219,220}. Golden Gate cloning was chosen because it does not leave restriction sites behind in the cloned sequence ("seamless cloning"), digestion and ligation are combined into one step, and hence no gel purification steps are required, and more than one loop could be replaced at the same time. The artificial sequences were placed in the mother construct based on previously published cutting sites²²¹. These sequences were designed such that each one contained a unique set of Type II restriction sites and a specific start and end sequence, to insert a target sequence in a specific direction and prevent re-ligation of the artificial loops. Also a third analytical restriction site was included, in order to screen for unsuccessful cloning (see table 2.5.2, for the full sequence of the mother construct see Appendix A).

Table 2.5.2 Golden Gate construct

Loop	Start	End	Restriction sites	Analytical site
ICL1	CGTC	CTCA	GAGACC, GGTCTC - BsaI	ACCGGT - AgeI
ICL2	GGTG	GCCA	GAGACG, CGTCTC, BsmBI	GCTAAGC – BlnI
ICL3	CGGA	CGCA	GTCTTC, GAAGAC – BbsI	GATATC - EcoRV
C-term	CGC/CGT	ACA	GAAGACG, GCTCTTC - SapI	GCGGCCGC - NotI

The reaction was assembled as follows:

100ng of mother construct in pcDNA 3.1 vector

7.8ng of each target loop (aiming at a 5:1 ratio between mother construct and loops)

1µl T4 DNA ligase (NEB M0202)

2µl ligation buffer

5 units of the first restriction enzyme of the respective loop

water to 20µl

The reaction was incubated in a BioRad PCR cycler for 45 minutes at 37°C. Then 5 units of the second loop-specific enzyme were added and incubated for another 35 minutes. Next, the enzymes were heat inactivated for 5 minutes at 50°C and 10 minutes at 80°C. The product was then digested with the analytical enzyme of the respective loops. Chemically competent One Shot Top 10 (Thermo Fisher C404010) or E. coli XL10 Gold (Janovjak lab) were subsequently transformed with the products and spread on Ampicillin containing agarose plates (IST Austria mediakitchen) and incubated at 37°C for up to 12hours. Two to five clones per condition were picked, amplified over night at 37°C in LB medium on a shaker, minprepped (Pure Yield Plasmid MiniPrep system, Promega A1223), and sequenced (Eurofins). The constructs were subcloned to the pCS2+ vector or transcribed directly from the pcDNA3.1 to generate mRNA (see chapter 2.5.3). Alternatively they were used as a template for gateway cloning.

2.5.3 mRNA and antisense in situ probe synthesis

mRNA was synthesized *in vitro* using the mMessage mMachine T7 or Sp6 kit respectively (Thermofisher AM1344, 1340). To this end the plasmid containing the respective promoter was cut just after the ORF, usually using NotI-HF enzyme (NEB R3189) and purified afterwards using the Wizard Gel and PCR clean-up kit (Promega A9281). In vitro transcription was then performed according to the kit using 1-1.5µg of digested template for 2-3 hours. Afterwards, the template DNA was digested using RNase free Turbo DNase, and 0.5µl were analyzed on an agarose Gel. The mRNA was purified using LiCl precipitation and EtOH washes. The mRNA was eventually eluted in 20 µl of RNase free water, and the concentration was measured using a Nanodrop photospectrometer. The stock was stored at -20°C.

In case of in situ hybridization probe synthesis the same kits were used, but the supplied NTP mix (2x NTP/CAP) was replaced by a digoxigenin RNA labeling mix (Sigma 11277073910). Like for mRNA, the DNA template was digested and the quality of the probe synthesis analyzed by gel electrophoresis. After LiCl precipitation and EtOH washes, the probes were eluted in 20µl of RNase free water, 180µl HM+ buffer (see buffer section) was added and the stock stored at -20°C.

2.6 Fish husbandry

Fish were raised and maintained in the fish facility at IST Austria as described previously²²², either in groups of up to 10 in 3L plastic tanks or in larger groups in 10L glass tanks (Aqua Schwarz). The fish were set up in the evening prior to the start of an experiment in setup boxes (Aqua Schwarz or Tecniplast), with male and female fish separated by a transparent wall or a sieve. On the next day, they were put together and eggs were collected. They were incubated at 28°C or 31°C and staged according to Kimmel et al.¹¹. Wildtype (wt) fish were a cross between AB and TL lines.

2.6.1 Transgenic and mutant lines

The following transgenic lines were used in this project: Tg(*gsc:GFP-CAAX*)²²³, in which membranes of ppl cells are labeled in green; Tg(*gsc:turbo-RFP*)^{201,224} in which the cytosol of

ppl cells is labeled red, and Tg(*mezzo:GFP*), in which the cytosol of all mesendoderm progenitor cells is labeled in green.

A mutant line was also used: maternal zygotic *frizzled 7a and b* double mutant (MZ *fz7a,b*⁶³). This line can be maintained as homozygous double mutant for 2-3 generations. Afterwards, it was outcrossed to wt, resulting in double heterozygotes. Incrosses mostly resulted in single mutants, double heterozygotes, and some hetero/homozygotes (i.e. *fz7a* +/-, *fz7b* -/-; or *fz7a* -/-, *fz7b* +/-), but no homozygous double mutants. The fish were genotyped when adult (see below) and the hetero/homozygotes were backcrossed. The next generation usually contained a few homozygous double mutants, again assessed by genotyping. To generate transgenic mutant lines Tg(*gsc:GFP-CAAX*), or Tg(*gsc:turbo-RFP*) males were crossed to MZ *fz7a,b* females and the offspring was screened for fluorescence in its ppl cells and raised. These heterozygous transgenic fish were backcrossed to MZ *fz7a,b* fish as described above until heterozygous transgenic and homozygous mutant fish were obtained. The resulting lines were called MZ *fz7a,b*; Tg(*gsc:GFP-CAAX*), and MZ *fz7a,b*; Tg(*gsc:turbo-RFP*) respectively.

Besides the Frizzled mutant lines, *wnt* mutants were also used. *Wnt5* is maternally contributed⁶¹, however the phenotype still also occurs in homozygous *wnt5* mutants, called *pipetail* (*ppt*^{61,120,225}), from a heterozygous incross. For experiments only homozygote incrosses were used. The fish were maintained as homozygous mutants for 2 generations, after which a new homozygous generation was made from incrosses of heterozygotes. Homozygotes were identified by tail morphology after 24 hours^{61,120,225}. The *wnt11* mutant is called *silberblick* (*slb*⁵⁸). For *wnt11* mutants also only homozygote incrosses were used in experiments. Incrosses from heterozygous parents can still be identified by phenotype during pharyngula stages, at which mutants look squint-eyed to the degree of eye fusion (see 1.4.3.2).

2.6.2 Genotyping of *fz7a,b* double mutants

Since only maternal-zygotic homozygous double mutants display the C&E phenotype it was necessary to frequently genotype for the correct fish. The tips of caudal fins of anesthetized (0.024% Ethyl 3-aminobenzoate methanesulfonate, Sigma 886-86-2) fish was cut and DNA extracted using the Kappa Express Extract (Sigma KK7100) DNA extraction kit. For *fz7a* a PCR with the following protocol was performed:

<u>94°C</u>	<u>1 min</u>	
94°C	20s	
57°C	30s	
<u>72°C</u>	<u>1 min</u>	<u>35x</u>

72°C	3 min
4°C	∞

Protocol for *fz7b*:

<u>94°C</u>	<u>3 min</u>	
94°C	20s	
56.5°C	30s	
<u>72°C</u>	<u>30s</u>	<u>20x</u>

94°C	20s	
58.5°C	30s	
<u>72°C</u>	<u>30s</u>	<u>20x</u>
72°C	3 min	
4°C	∞	

Following the PCR a diagnostic digest was performed at 37°C over night. The restriction enzyme for *fz7a* was MseI (NEB R0525), for which a restriction site was gained due to the mutation, and the one for *fz7b* was DpnII (NEB R0543), for which a restriction site is lost in the mutant. The results were then compared to wt and mutant control.

2.7 *Inject*ions

Inject

ions into 1-cell stage embryos were performed as described previously²²². Needles were pulled from capillaries with filament (see 2.4) allowing loading of mRNA. The needle was placed on a needle holder on a micromanipulator connected to a pneumatic pump and the length of the pulse was adjusted such that a droplet of about 0.5nl was ejected with one shot. The 1-cell embryos were put into grooves in an agarose chamber containing E3 buffer and oriented using a brush. The needle was then inserted in a way that it pierced the chorion and a part of the yolk. One or two droplets were then placed into the yolk in the vicinity of the cell.

In order to achieve laterally biased expression, 60pg of *wnt11-3x-mcherry* mRNA was injected into a single blastomere at the 32-cell stage (1.75 hpf). The embryos were then screened by fluorescence for samples that had a left-right asymmetry.

2.7.1 *Inducing ppl cell fate*

It has been shown that activation of Nodal signaling and simultaneous inhibition of *casanova* causes all cells of an embryo to adopt prechordal plate cell fate²²⁶⁻²²⁹. To achieve all ppl embryos, we injected 100pg *cyclops* (*ndr2*) mRNA, together with 4ng of *casanova* (*sox32*) morpholino. Whenever possible Tg(*gsc:GFP-CAAX*) embryos were used and the success of the induction was confirmed by GFP expression.

2.8 *Transplantations*

To achieve mosaic labeling of ppl cells or to address the question of whether Fz7 functions cell-autonomously, transplantations were used. To this end, donor embryos were ppl cell

fate induced as described above, while at the same time injected with additional agents according to the experiment. Host embryos were of the MZ *fz7a,b*; Tg(*gsc:GFP-CAAX*) or Tg(*gsc:GFP-CAAX*) line. At 5hpf host and donor embryos were selected based on the fluorescent signal and dechorionated in E3 buffer in glass dishes. At 6hpf both were moved to a prepared agarose chamber containing E3 buffer and aligned in grooves. The transplantation setup consisted of a transplantation needle (inner diameter = 20µm, with spike, Biomedical instruments) that was mounted on a needle holder, which was connected to a 1ml syringe. The needle holder was mounted on a micromanipulator. By inserting the needle into the blastoderm of donor embryos and cautiously moving the lever of the syringe, groups of 5-20 cells were aspirated into the tip of the needle. The cells were then transplanted to the leading edge of the ppl of host embryos by the same means. The efficiency was examined according to the fluorescent signal.

2.9 *In vitro explant assays*

The *in vitro* assays were performed based on previously described experiments²³⁰. Embryos were injected as described above to induce ppl cell fate. Glass bottom dishes were plasma-cleaned (2 minutes, maximum level) and coated with fibronectin (Sigma F1141). The coating of the dishes was performed by diluting the fibronectin stock 1:4 in distilled water, applying a 50µl drop to the dish and letting the water evaporate. When uninjected control embryos were at dome stage, the injected and induced embryos were transferred to glass dishes containing Danieus medium. After dechorionation, explants were made by cutting the blastoderm just above the yolk, leaving behind about 2 rows of cells. These explants were transferred to glass dishes with pre-warmed DMEM cell culture medium (stock diluted with 10% distilled water) and cut to smaller explants of roughly 50 cells. The smaller explants were then transferred to the fibronectin coated glass bottom dishes in cell culture medium as described above. The samples were incubated (28°C) for two hours and then imaged.

2.10 *Optogenetic experimental procedures*

For the embryo scale rescue experiments, samples were injected at the 1-cell stage and then scored for fertilization at 4-cell stage, as MZ *fz7a,b* fish frequently have a high number

of unfertilized eggs. The batch was split into two groups of equal size and kept in E3 buffer. One batch was put into a cryobox and wrapped in aluminum foil to avoid light exposure. Both batches were incubated together at 28 or 31°C in an incubator equipped with 505nm light emitting diodes (LEDs) with an intensity of $3\mu\text{W}/\text{mm}^2$. At bud stage, the samples were fixed in 4% PFA. Since the morphological phenotype was analyzed, the short light exposure before fixing the samples was no problem in this assay.

For the localization experiments the procedure was the same aside from the fact that the embryos were fixed at 30% epiboly and that the dark group was fixed in a dark room, with only red light sources, to affect the localization of Wnt/PCP components as little as possible.

For *in vitro* and *in vivo* experiments that involved localized activation, the embryos were incubated until the desired stage similar to the above mentioned experiments, but already dechorionated at 4-cell stage before incubation in a regular incubator without LEDs in a cryo box and wrapped in aluminum foil. Further procedures (agarose mounting, explantations, imaging) were carried out in the dark with only red light sources and using a custom made red filter for the stereoscopes. Localized activation experiments were performed on Leica SP5 microscopes using the Live Data Mode. General imaging was done with the 561nm laser, since this wave length is out of the activation range for Rhodopsin-based chimeras²³¹. OptoFz7 was activated in a part of the sample using the 488nm laser. The incubation chambers or sample holding sections were covered with light-tight blankets. *In vivo* experiments were done on an upright scope using a 25x/0.95 NA water-dipping lens, with the Argon laser set to 25%, the 488nm laser to 15%, 400Hz bidirectional scanning speed, yielding a radiant exposure of $0.013\text{nj}/\mu\text{m}^2$. For *in vitro* an inverted scope with a 20x/0.7 NA air objective was used, the Argon laser was set to 25%, the 488nm laser to 12%, the speed was 400Hz with bidirectional scanning, resulting in a radiant exposure of $0.009\text{nj}/\mu\text{m}^2$.

For ubiquitous activation experiments *in vitro* and *in vivo*, the samples were incubated as described previously, but not light protected during further procedures like agarose mounting, explantation or imaging. Ubiquitous *in vitro* experiments were imaged using a Visitron TIRF/FRAP epifluorescence microscope with brightfield and 475nm illumination at

12% light intensity and 20ms exposure time, resulting in a radiant exposure of $0.018\text{nj}/\mu\text{m}^2$. The ubiquitous *in vivo* experiments were imaged with a LaVision Trim two-photon microscope with 800nm (12% laserpower), 830nm (12% laserpower), and 1100nm (20% laserpower) illumination, with a radiant exposure of $0.044\text{nj}/\mu\text{m}^2$.

2.11 *In situ* hybridizations

The *in situ* hybridizations in this project were carried out based on a protocol modified after Thisse & Thisse²³² (updated 2010 Zfin.org). Until hybridization, the steps were done using 1.5 or 2ml Eppendorf tubes. From the stringency washes onwards, little baskets in 24 well plates were used. The baskets were made from vessels cut from 15ml falcon tubes with 120 μm nylon mesh (Scientific Laboratory Supplies; Flystuff 57-102) fused to the bottom.

Protocol:

- 1) Fix embryos at desired stage in 4% PFA overnight at 4°C.
- 2) Wash 4 times 15 minutes in PBST at RT.
- 3) Dechorionate in PBST.
- 4) Wash 15 minutes in 100% MeOH at RT.
- 5) Store overnight in 500 μl 100% MeOH at -20°C.

Note: embryos can be stored at this point for several months.

- 6) Rehydrate embryos at RT.
 - 75 % (v/v) MeOH/PBS, 5 min
 - 50 % (v/v) MeOH/PBS, 5 min
 - 25 % (v/v) MeOH/PBS, 5 min

- 7) Wash 4x with PBST, 5 min each.

- 8) Change to 500 μl HM+ buffer.

Note: at this point embryos can be stored at -20°C for several days.

- 9) Prehybridize at 66.6°C in a waterbath for 2-5hrs.
- 10) Replace HM+ with 200 μl of HM+ containing antisense RNA probe.

Probe dilutions: 1:200 for *hgg* and *ntl*; 1:100 for *papc*

- 11) Hybridize at 66.6°C over night or up to 48 hours.
- 12) Preheat solutions A-C, 2X, and 0.2X SSC to 66.6°C.

- 13) Stringency washes: take off probe and store for future usage at -20°C and add 1ml of to a basket in a 24 well plate. Incubate at 66.6°C in a waterbath, not entirely submerged for 10 minutes. Fill following wells with solutions B and C, 2X SSC, and twice 0.2X SSC.
wash 10 minutes in solution B at 66.6°C
wash 10 minutes in solution C at 66.6°C
wash 10 minutes in 2X SSC at 66.6°C
wash twice 30 minutes in 0.2X SSC at 66.6°C
- 14) RT washes: 10 minutes each in solutions D, E, and F, and PBST on a rocker.
- 15) Block samples in Blocking buffer (ISH) for 2 hrs at RT on a rocker.
- 16) Incubate with anti-DIG antibody (diluted 1:2000-1:5000) for 2 hours at RT.
Over night at 4°C also works
- 17) Discard antibody solution and wash the embryos briefly in PBST.
- 18) Wash 6x 15 min in PBST on rocker at RT (up to 10 hours or even overnight at 4°C).
- 19) Wash 3x 5 min in Alkaline Tris buffer on a rocker at RT.
- 20) Remove alkaline Tris buffer and replace with 700 µL freshly-prepared-and-kept-in-the-dark Staining solution. From here keep in the dark as much as possible.
- 21) Incubate at RT until the desired staining is reached. For the *hgg-ntl-papc* mix this is typically 60-90 minutes.
- 22) Wash twice briefly in PBST at RT.
- 23) Wash 10 minutes in PBST at RT.
- 24) Optional: clear in 100% MeOH for 30 mins at RT.
- 25) Wash twice 10 minutes in PBST at RT.
- 26) Add some drops of EDTA before storage.
- 27) Optional: clear in 70% glycerol up to several days at 4°C.
- 28) Image in glycerol or PBS.

2.12 Immunohistochemistry

- 1) Fix embryos in 2% PFA in PBS over night at 4°C.
- 2) Wash embryos in Washing buffer 1 at 4°C overnight.
- 3) Dechorionate.

- 4) Wash dechorionated embryos in Washing buffer 2 for 1 hour.
- 5) Wash in Washing buffer 1 for 10 min.
- 6) Pre-block embryos in IF buffer.
- 7) Incubate with 1st antibody (rabbit anti-cdh1, #94) diluted 1:200 in IF at 4°C overnight.
- 8) Wash 4x in IF buffer, each wash at least 30 min, at RT
- 9) Incubate with 2nd antibody (anti-rabbit) 1: 2000 overnight at 4°C
- 10) Wash 4x in Washing buffer 1, at least 30 min per wash.
- 11) Image

2.13 CRISPR/Cas9 mediated genome editing

2.13.1 Site selection & construct creation

We chose a target sequence for cleavage that is situated in the 3rd extracellular loop of Fz7b using the ChopChop tool (<http://chopchop.cbu.uib.no/>). Then a DNA template was designed such that a site-specific guide RNA can be transcribed using T7 polymerase, containing a **short clamp**, a **RNA polymerase promoter (T7)**, the **fz7b target site**, and the **Cas9 binding scaffold**:

GCGTAATACGACTCACTATA
GGAGCAGTGGGAAAAAACC
GTTTTAGAGCTAGAAATAGCAAGTTAA
AATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTT

It was assembled by PCR using an ordered specific Oligonucleotide (Eurofins) and 3 standard primers (see primers section 2.3). The oligonucleotide for insertion of the *loxP* cassette was also ordered (Eurofins). It contains the *loxP* site itself, the **homology arms** and two extra nucleotides to avoid frameshifts:

TTCGTGAGCAGTGGGAAAAA
ATAACTTCGTATAATGTATGCTATACGAAGTTAT
ctACCTGGCATATG
CAAACGTG

PCR-amplification of guide RNA template:

64.5µL H₂O
20µL 5x GoTaq Green PCR buffer (Promega M7911)
2µL dNTPs, 10mM each
2.5µL gRNA Primer 1, 10µM
2.5µL gRNA Primer 2, 10µM
2µL sgDNA_rv, 1µM
2µL site-specific Oligo1, 1µM
3µL DMSO
1.5µL GoTaq (Promega M3001)

The reaction was split in two 50µL reactions, because this is the maximum volume of the PCR cycler.

PCR Protocol:

95 °C 1 min

95 °C 15s

60 °C 30s x 40 cycles

72 °C 20s

72 °C 5 min

4 °C ∞

The product was run on a 3% agarose/TAE gel, the 120bp fragments were cut from the gel, and cleaned up with a Qiagen Gel Extraction kit (Qiagen 28704). From this template, the sgRNA was transcribed (Ambion MAXIScript T7, Thermo Fisher, AM1312) for 3 hours at 37°C and the DNA templates removed by Turbo DNase. 2µL of 8M LiCl and 75µL absolute EtOH were added and the RNA was precipitated over night at -80°C. Afterwards it was pelleted by centrifugation at 16000g at 4°C, washed in 80% EtOH, and centrifuged again. After 20

minutes of air-drying, the pellet was eluted in 30µL RNase-free water. *Cas9* mRNA was transcribed from a plasmid by *in vitro* transcription as described above (see chapter 2.5.3).

2.13.2 Injection and Genotyping for generating CRISPR/Cas9 knock-in lines

A mix of *cas9* mRNA, the *fz7b* targeting sgRNA, and the *loxP* oligonucleotide was injected into 1-cell stage embryos. The mixture contained 40ng/µL sgRNA, 80ng/µL *cas9* mRNA, 1µM, 0.025% phenol red, and 0.2M KCl. About 200 embryos were injected, incubated at 28.5°, and monitored throughout day 1 to check for phenotypes. At 30 hpf the embryos were collected in pools of three and DNA was extracted using the HotSHOT protocol²³³. In brief: the E3 buffer was replaced with 30µL of 50mM NaOH, and the samples were heated to 95°C for 20 minutes. Afterwards, the reaction was cooled to 4°C and 3µL of Tris-HCl (pH 8) was added. The samples were then centrifuged for one minute at maximum speed to pellet the debris. 1µL of the supernatant was used as template for amplification by PCR. For genotyping 3 PCR reaction were set up: one with a *loxP* specific forward and a *fz7b* specific reverse primer located in the 3'UTR, one with a *loxP* specific reverse primer and a *fz7b* specific forward primer within the open reading frame, and one using the *fz7b* primers as positive control.

PCR for genotyping:

1µL template DNA
4µL GoTaq Green PCR buffer
0.4µL forward primer
0.4µL reverse primer
0.4µL dNTPS
13.7µL water

PCR protocol:

94°C	1 min	
94°C	20 sek	
52°C	30 sek	30X

72°C 1 min

72°C 7 min

4°C ∞

All three PCR products were analyzed by gel electrophoresis. Once the efficiency of the CRISPR process was determined, the experiment was repeated, the embryos raised to adulthood and genotyped by finclipping and PCR analysis as described above. Samples that had bands in the *loxP* PCRs were sent for sequencing (Eurofins). Fish that had the *loxP* cassette inserted in the proper frame were backcrossed to wt fish, and the efficiency of germ-line transmission was determined as described above. Offspring from such founder fish were raised, maintained as a line, and used for the CRE/lox recombination experiment.

2.13.3 CRE/lox recombination

The CRE/lox recombination experiment was performed according to published work^{215,234}. Briefly, *CRE* mRNA was injected together with a plasmid containing a *loxP* site, the *fz7b* sequence downstream of the knocked-in *loxP* site, followed by a short 27bp linker and the *GFP* sequence, but no eukaryotic promoters. First, 20pg of plasmid and 10pg of *CRE* mRNA, then the double or triple amount of both was injected. The embryos were then screened for GFP fluorescence and with PCR using the *fz7b* forward and a *GFP* reverse primer (Eurofins).

PCR protocol:

94°C 1 min

94°C 20s

55°C 30s 30x

72°C 1 min

72°C 7 min

4°C ∞

2.14 Data analysis

2.14.1 Statistics

Statistical analysis was done using GraphPad Prism 6. To test for normality of a sample, a D'Agostino & Pearson omnibus normality test was used. If two samples were compared and normal distribution could be assumed an unpaired t-test was performed. If two samples were not normally distributed, a Mann-Whitney test was performed instead. In case more than two normally distributed samples were compared an ANOVA was performed followed by Sidak's multiple comparison test. If no normal distribution could be assumed, A Kruskal-Wallis test followed by Dunn's multiple comparison test was used.

2.14.2 Image Processing

Images acquired by multiphoton or confocal microscopy were imported to Imaris (Bitplane) to 3D visualize the recorded channels and further analysis. For correlation analysis, all embryos were rotated and oriented along the animal-vegetal axis at the dorsal side of the embryo based on the *gsc*:GFP-CAAX signal in Imaris 9 in such a way that the animal-vegetal axis corresponded to the Z-axis of the image. Ppl nuclei were separated from other nuclei by surface masking of the *gsc*:GFP channel, neuroectoderm cells were defined by negative surface masking and restriction of the area of analysis in X, Y, and Z. Nuclei positions of ppl progenitors and neuroectoderm cells in xyz-dimensions were exported for each time point and used for the correlation analysis. In case of the *in vivo* analysis of protrusive behavior, images were processed using Imaris 8. Transplanted cells were segmented based on the membrane signal and converted to surfaces. Those were exported using the *Surface to Obj file export* extension (Bitplane) and used for further analysis.

2.14.3 Analysis of local correlation

The local correlation was analyzed as described previously²²³. In brief, 3D velocity vectors were averaged in sectors of 50 by 50 μm^2 in xy planes and the full z direction for every time point. Thus, a grid covered the whole imaged area, centered on the leading edge. To quantify the correlation between the ppl and the overlying ectoderm, the directional correlation of the two was calculated for every sector of the grid. The correlation could

adopt values from 1 (in case the ppl and the neuroectoderm move in the same direction) to -1 (if the two tissues move in opposite directions).

2.14.4 Analysis of protrusion formation

It has previously been shown that the difference between maximum and minimum curvature can be used to quantify shape change²³⁵. To perform this analysis in a semi-automated fashion, we segmented the transplanted cells in 3D using Imaris 8 and exported the surface as mentioned above (2.14.2). We then calculated the Gaussian curvature of the surface using a Matlab script and normalized it to the cell radius. This resulted in a number of change-of-curvature events, which we plotted as histogram of the relative frequency of the analyzed images.

2.14.5 Fiji analysis of in situ hybridized samples and cell culture experiments

To quantify the expression domains of marker genes in *in situ* hybridized samples in a semi-automated fashion, we used two custom-made Fiji scripts (Gabriel Krens, IST Austria Imaging Facility). In the script used for measuring the distance between notochord and prechordal plate, a region of interest (ROI) was selected in lateral images that contained the posterior edge of the ppl and the anterior border of the nc (as determined from *hgg* and *ntl* expression respectively), with the pixel size normalized to a scale bar that was added during image acquisition. The script then automatically measured the distance between the two expression domains. The other script was used for measuring the length to width ratio (LWR) of nc and ppl, as well as the width of the *papc* domain. Again an ROI was selected and after thresholding, the script automatically measured the longest axis of the expression domain as well as the perpendicular one, and returned both lengths as well as their ratio.

For measuring the perimeter ratio of explants in cell culture, we used a previously published Fiji script (Gabriel Krens, IST Austria Imaging Facility, ²³⁶). In brief the cells were segmented using the software Illastik and the segmented file and the original file were imported back to Fiji, where the script compared the perimeter length of the segmented area to the perimeter of an ellipsoid that had the same area and longest axis.

3 Results

3.1 *Fz7a,b* expression patterns

In zebrafish gastrulae the PCP *wnts* are expressed laterally, with *wnt5* being expressed in the posterior paraxial and lateral mesoderm, and *wnt11* in the anterior paraxial and lateral mesoderm⁶¹. However, the expression patterns of the *fz7* paralogues at gastrulation stages have not been well documented yet. *Fz7b* is reported to be weakly ubiquitously expressed, but posterior lateral expression is visible²³⁷. *Fz7a* has been described as not spatially restricted²³⁸ or expressed in the dorsal part of the gastrula^{239,240}. During the segmentation period, however, *fz7b* is documented quite well and is expressed in presomitic and somitic mesoderm, and anterior axial mesoderm²³⁷. Since this is reminiscent of the expression of *wnt* ligands, we decided to stain for expression patterns at the end of gastrulation. The two *fz7* paralogues appear to partially overlap and partially complement each other in their expression domains. Anteriorly *fz7b* is expressed in the ppl, not in the tissue directly surrounding it, and again more laterally. In the posterior part it is expressed in the paraxial mesoderm, with the adaxial cells staining strongest, reminiscent of the *wnt5* expression pattern. It is absent from the notochord and tailbud mesenchyme (Fig. 3.1).

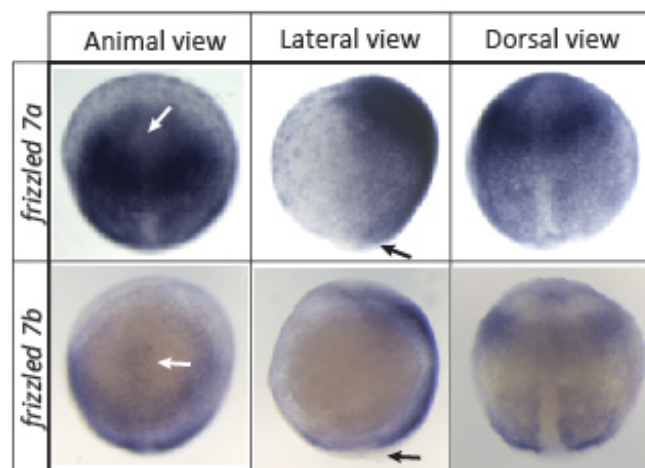


Figure 3.1: Expression patterns of *fz7a* & *b* at the end of gastrulation (10hpf). White arrows show the ppl, black arrows the tailbud.

fz7a is expressed at the leading edge of the ppl and the anterior lateral mesoderm, but is absent from the main body of the ppl (Fig. 3.1 animal view). Posterior to that, it has some level of expression in the entire dorsal half of the embryo, with the exception of the

notochord (Fig. 3.1 lateral view). Together they cover the entire anterior axial and paraxial mesoderm; posteriorly the only tissue of the dorsal hemisphere without expression of either paralogue is the notochord. The combined *fz7* expression pattern, therefore, overlaps with the expression patterns of *wnt5* and *wnt11*, and additionally covers the ppl.

3.2 Analysis of the Mz *fz7a,b* mutant phenotype

The MZ *fz7a,b* mutant was published in 2010 and its phenotype analyzed with respect to body axis elongation⁶³. It was shown that the body axis is significantly shortened and that injection of *fz7* mRNA could rescue this effect to some extent. Following up on this, we first analyzed the ppl and paraxial phenotype of this mutant.

3.2.1 Whole mount analysis

We fixed MZ *fz7a,b* mutant and wt embryos at 10hpf (bud stage¹¹) and *in situ* hybridized them with RNA antisense probes against *notail* (*ntl*), *hatching gland gene* (*hgg*), and *paraxial protocadherin* (*papc*). The expression patterns of these genes correspond to the notochord (*ntl*), the prechordal plate (*hgg*), and the paraxial mesoderm (*papc*) respectively. We measured the areas expressing the marker genes using Fiji and found that multiple tissues are affected and contribute to the overall morphological defects of MZ *fz7a,b* embryos at the end of gastrulation (Fig 3.2.1).

The notochord was shortened and widened as described for C&E phenotypes of other Wnt/PCP mutants (Fig. 3.2.1 A, B, & C)^{58,61}, and the paraxial mesoderm also displayed convergence defects and was therefore widened (Fig. 3.2.1 A & D). The prechordal plate was elongated, compared to the widened kidney shaped ppl of wt embryos, reminiscent of the *slb* phenotype (Fig. 3.2.1 A & E)^{58,122,123}. In addition, it also showed a reduced distance to the notochord (Fig. 3.2.1 A & F), suggesting a ppl migration defect. The ppl defect supports the notion that Fz7 might be the receptor for Wnt11, which is known for anterior mesendoderm phenotypes⁶¹. This is in agreement with experiments that showed co-localization between fluorescently tagged versions of Wnt11 and Fz7, as well as with co-immunoprecipitation experiments in *Xenopus*¹³⁷.

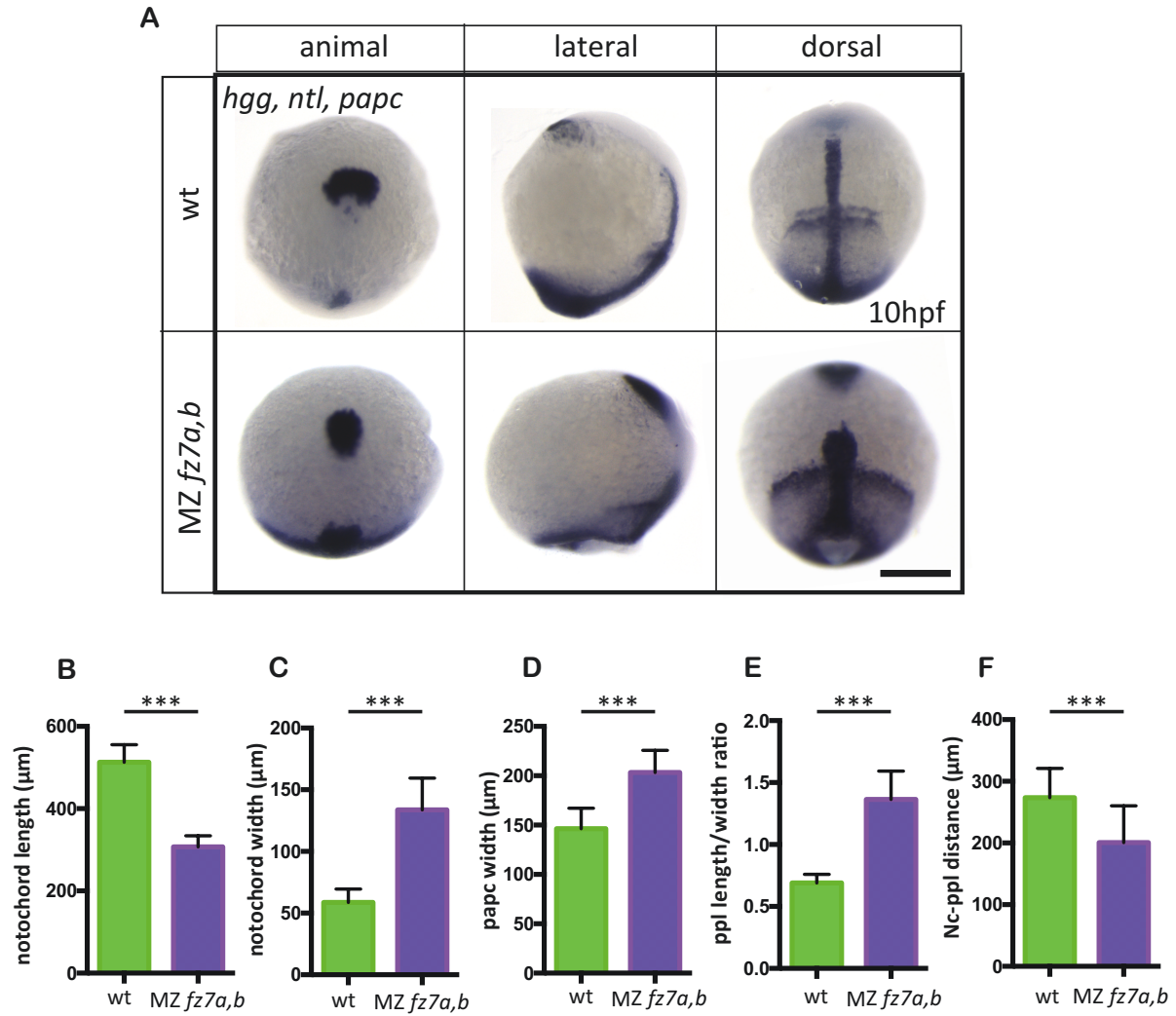


Figure 3.2.1: Whole mount analysis of MZ *fz7a,b* mutants. A: *In situ* hybridization with RNA antisense probes against *hgg*, *ntl*, and *papc*. The left column is an animal pole view displaying the prechordal plate, the middle column shows a lateral view highlighting the length of the notochord and the distance between nc and ppl, the right column is viewed from dorsal to show convergence phenotypes of the paraxial mesoderm and the notochord. The scale bar is 250µm. B-F: Quantification of C&E phenotypes, green columns are wt, purple columns are mutant embryos. The measured parameter including unit is shown left of the graph. Error bars are standard deviation. For statistical analysis Student's t-tests were used with *** $p < 0.001$. N = 78 for wt and 63 MZ *fz7a,b* (B-D), N=38 each (E), N=22 for wt and N=17 for MZ *fz7a,b* (F).

3.2.2 Prechordal plate migration analysis

Intrigued by the phenotype of the ppl in MZ *fz7a,b* mutant embryos, we decided to study ppl migration and polarization behavior in wt and mutant embryos. To this end, the Tg(*gsc:GFP-CAAX*) line, in which the prechordal plate cells express membrane-anchored GFP, was crossed to the MZ *fz7a,b* line, thus resulting in a transgenic mutant MZ *fz7a,b*; Tg(*gsc:GFP-CAAX*) line. To use this line to analyze prechordal plate migration the nuclei of the embryos were labeled red via mRNA injection. The embryos were then mounted in

agarose at shield stage and imaged by confocal microscopy. We found that the prechordal plate of both mutant and wt embryos moved at about the same velocity towards the animal pole during onset and early gastrulation. From mid gastrulation, however, the ppl of MZ *fz7a,b* embryos slowed down dramatically, stopped their animal directed movements, and were subsequently moving in the opposite direction towards the vegetal pole together with the overlying ectoderm undergoing epiboly (Fig. 3.2.2 B & C).

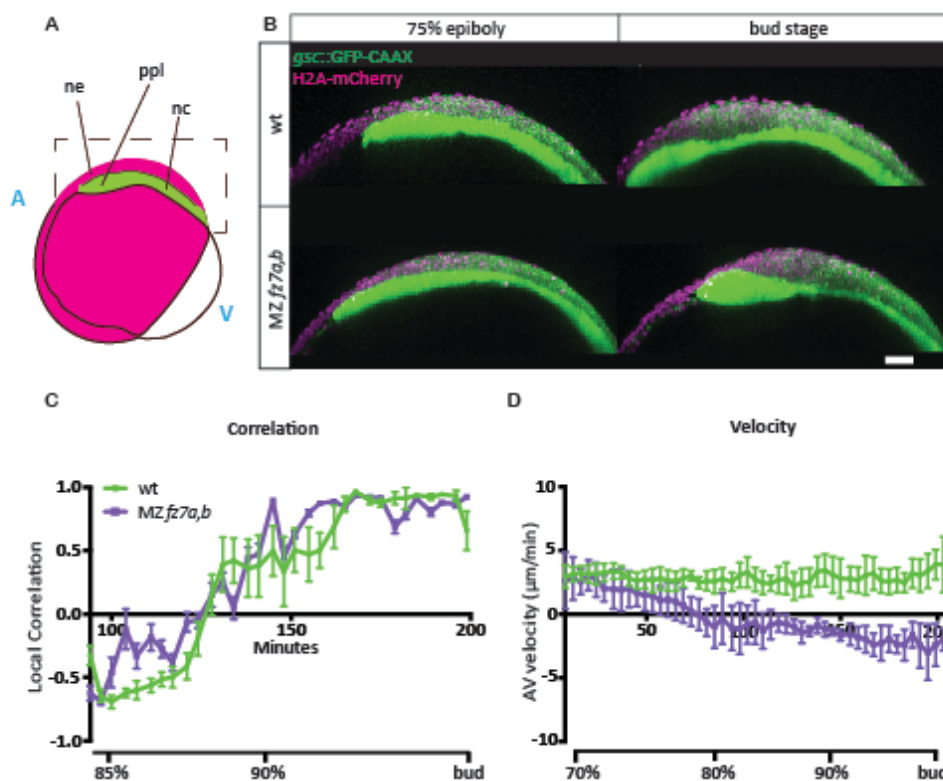


Figure 3.2.2: Analysis of prechordal plate migration in MZ *fz7a,b* and wt embryos. A: Schematic of the field of view in B. The embryo is shown laterally, with the animal pole marked by A and the vegetal pole by V, green shows the prechordal plate (ppl) and the notochord (nc), magenta the overlying prospective neuroectoderm (ne), the dashed line the field of view in B. B: Confocal microscopy images of wt (upper row) and mutant (lower row) embryos at mid (left, 75%) and late (right, bud stage) gastrulation; the scale bar is 50μm. All cells are labeled by H2A-mCherry (magenta). C: Quantification of the local correlation between leading edge ppl cells and overlying ectodermal cells in wt (green) and mutant (purple) embryos. The X-axis is time (minutes, stage), the Y-axis local correlation (order parameter). N=3 per genotype. D: Quantification of velocities of ppl leading edge cells in wt (green) and mutant (purple) embryos. The X-axis is time (minutes, stage), the Y-axis velocity in vegetal to animal pole direction (μm/min). N=3 per genotype.

Since defects in Wnt/PCP signaling were reported to cause impaired germ layer separation¹⁹¹, we were wondering, if the deceleration of MZ *fz7a,b* mutant ppl cells might be caused by increased adhesion between the prechordal plate and the overlying

prospective neuroectoderm. To investigate this possibility we analyzed the local movement correlation between cells of the leading edge of the prechordal plate and the overlying ectoderm cells as described previously²²³. We found that the correlation between meso- and ectoderm in mutants does not differ from the one in wt embryos (Fig. 3.2.2 C). The two tissues have a high correlation from mid gastrulation on, but in the wt the ectoderm follows the direction of mesoderm movements towards the animal pole, while in the mutant the mesoderm and ectoderm move together towards the vegetal pole, which is reflected in the different vegetal to animal velocities of the genotypes (Fig. 3.2.2 D). This argues against a tissue separation defect and rather points to impaired migratory abilities of the mesendoderm progenitor cells. This is also in agreement with the finding that the position of the neural anlage along the animal-vegetal axis is correlated with the position of the prechordal plate²²³.

3.2.3 Analysis of Cell-autonomy

Next, we sought to find out if the defect in prechordal plate migration was intrinsic to the prechordal plate cells themselves or caused by other neighboring tissues, such as the overlying ectoderm or the surrounding paraxial mesoderm, in which PCP components, e.g. *wnt11*, are expressed⁶¹. Signaling between neighboring cells of the same or a different germ layers has been shown to be of importance for functional Wnt/PCP signaling in various contexts²¹². Another possible scenario was that the phenotype is caused by a lack of convergence movements of posterior, lateral, and paraxial tissues, especially since the onset of the prechordal plate migration defect (Fig. 3.2.2) coincides with the onset of convergence movements⁴⁶. We also investigated, if Fz7 signals autonomously on a cellular level, since it was shown that it can also act non-autonomously on neighboring cells^{84,140}.

To address these questions we decided to perform heterotypic transplantation experiments, in which mutant ppl cells were transplanted to wt hosts and vice versa, and compare them to homotypic controls, in which donor and host embryo were of the same genetic background. To this end we induced ppl cell fate in donor embryos (see methods section 2.7.1) and co-injected them with *mkate2-CAAX* mRNA in order to label the cell membranes red.

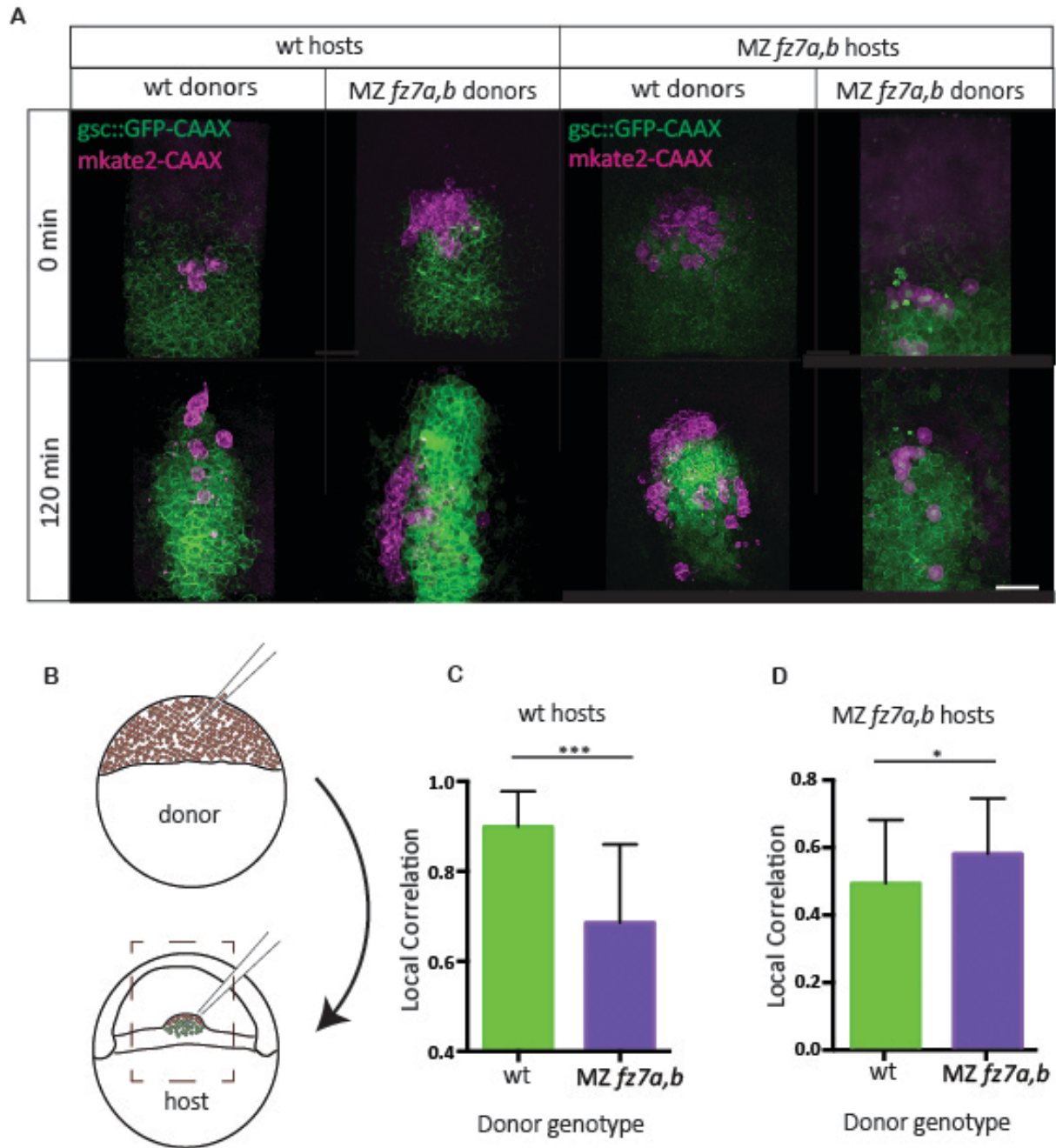


Figure 3.2.3: Transplantation experiments to investigate a potential cell-autonomous or non-autonomous role of Fz7 in the ppl. **A:** Images from confocal microscopy at early (upper row) and late (lower row) gastrulation, view from dorsal, animal pole is to the top. Donor cells are labeled in magenta, host cells in green. Donor and host genotypes are labeled above the images. The scale bar is 50 μ m. **B:** Schematic of the transplantation procedure, the colors used are the same as in **A**, the dashed line shows the field of view in **A**. For a detailed description see Method section (2.8). **C & D:** Quantification of the local correlation in **A**, for statistical analysis Student's t-tests were used with *** $p < 0.001$, and * $p < 0.05$.

We then transplanted cells from the induced donor embryos to the leading edge of the prechordal plate of MZ *fz7a,b*; Tg(*gsc:GFP-CAAX*) and normal Tg(*gsc:GFP-CAAX*) embryos at shield stage. We found that wt cells transplanted to wt embryos stay mostly at the leading

edge (Fig. 3.2.3 A), with little positional change relative to each other, similar to what was described previously⁴⁷. However, when MZ *fz7a,b* cells were transplanted to wt hosts, they separated from host ppl cells and moved towards the vegetal pole (Fig. 3.2.3 A), reminiscent of the vegetal-ward movement of the prechordal plate in MZ *fz7a,b* mutants (Fig. 3.2.2 B & C).

We quantified this cellular behavior by analyzing the coordination between transplanted cells and their neighboring host cells. We found that the local correlation was significantly reduced in the case of mutant donors (Fig. 3.2.3 C). When transplanted to MZ *fz7a,b* hosts, mutant cells mostly stayed at the relative position they were transplanted to with some mixing between host and donor cells (Fig. 3.2.3 A & D). Wt cells in mutant ppl moved to the leading edge of prechordal plate and stayed there throughout gastrulation, resulting in a mild but significant reduction of local correlation compared to the mutant-to-mutant homotypic transplantations (Fig. 3.2.3 A & D).

Since the transplantation of mutant cells to wt hosts could not rescue their migratory behavior, it could be ruled out that the environment of the ppl is responsible for the defects in MZ *fz7a,b* embryos, or that the phenotype was due to a lack of pushing force from posterior converging tissues, because both parameters were wt. Rather, the defects seem to arise from a lack of migratory force, exerted by the prechordal plate cells themselves, and thus their inability to overcome the vegetal-ward movement of the ectoderm.

3.2.4 MZ *fz7a,b* have protrusion formation defects *in vitro* and *in vivo*

Since various Wnt/PCP components have been implicated in the formation, mode, and orientation of cellular protrusions^{47,51,65,241}, we asked whether impaired protrusive behavior could be the reason for the defective migratory behavior in MZ *fz7a,b* mutants. To reduce the complexity of this experiment, we turned towards an *in vitro* approach. Explants of roughly 50 cells from ppl fate induced embryos of either the MZ *fz7a,b*; Tg(*gsc:GFP-CAAX*) or the normal Tg(*gsc:GFP-CAAX*) line were cultured on fibronectin-coated glass bottom dishes as described previously^{230,236}, and imaged by brightfield and epifluorescence microscopy.

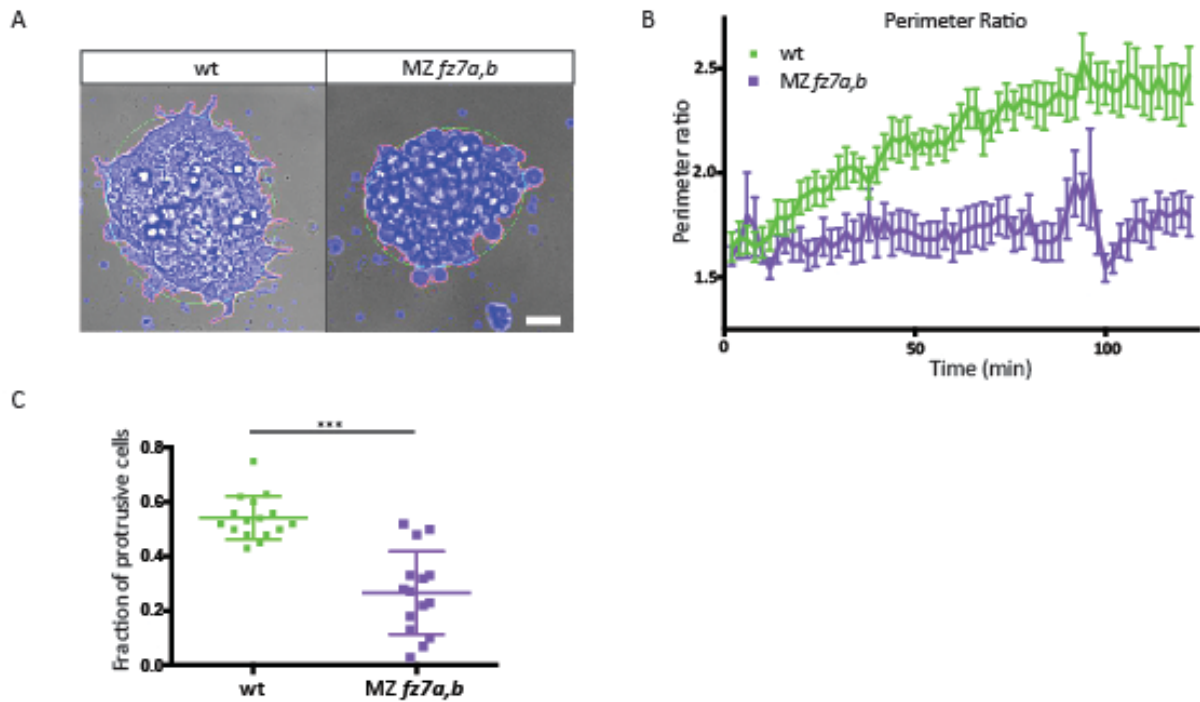


Figure 3.2.4.1: Analysis of protrusive activity of MZ *fz7a,b* mutants *in vitro*. A: Explants of wt and mutant embryos. Brightfield image, the outline of the cluster is segmented (red line), and an ellipsoid (green line) fitted to the same area (purple). Debris or single cells are not included in the analysis. The scale bar is 40 μ m. B: Quantification of the ratio between the segmented perimeter (red) and the fitted ellipsoid (green) in A (Y-axis) over time (minutes, X-axis), N=12 explants per genotype. C: Fraction of cells at the margin of a cluster that display protrusions after 4 hours in culture (last time point in B). N= 12 explants per genotype, for statistical analysis a Mann-Whitney test was used with ***p<0.001.

The clusters were segmented and the circumference, normalized to the outline of an ellipsoid of the same area, plotted over time. This ratio reflects the roughness of the outline of the cluster and therefore serves as an efficient and unbiased proxy for protrusive activity²³⁶. Additionally we counted the fraction of cells at the margin of a cluster that displayed protrusions at the end of the experiment. Clusters of both genotypes started at about the same level of protrusiveness. However, wt cells increased their protrusive activity over time, while mutant cells retained roughly the same level (Fig. 3.2.4.1 A). This was also reflected in the perimeter ratio, which went up for wt cells but stayed relatively unchanged in mutants (Fig. 3.2.4.1 B), and the fraction of protrusive marginal cells (Fig. 3.2.4.1 C).

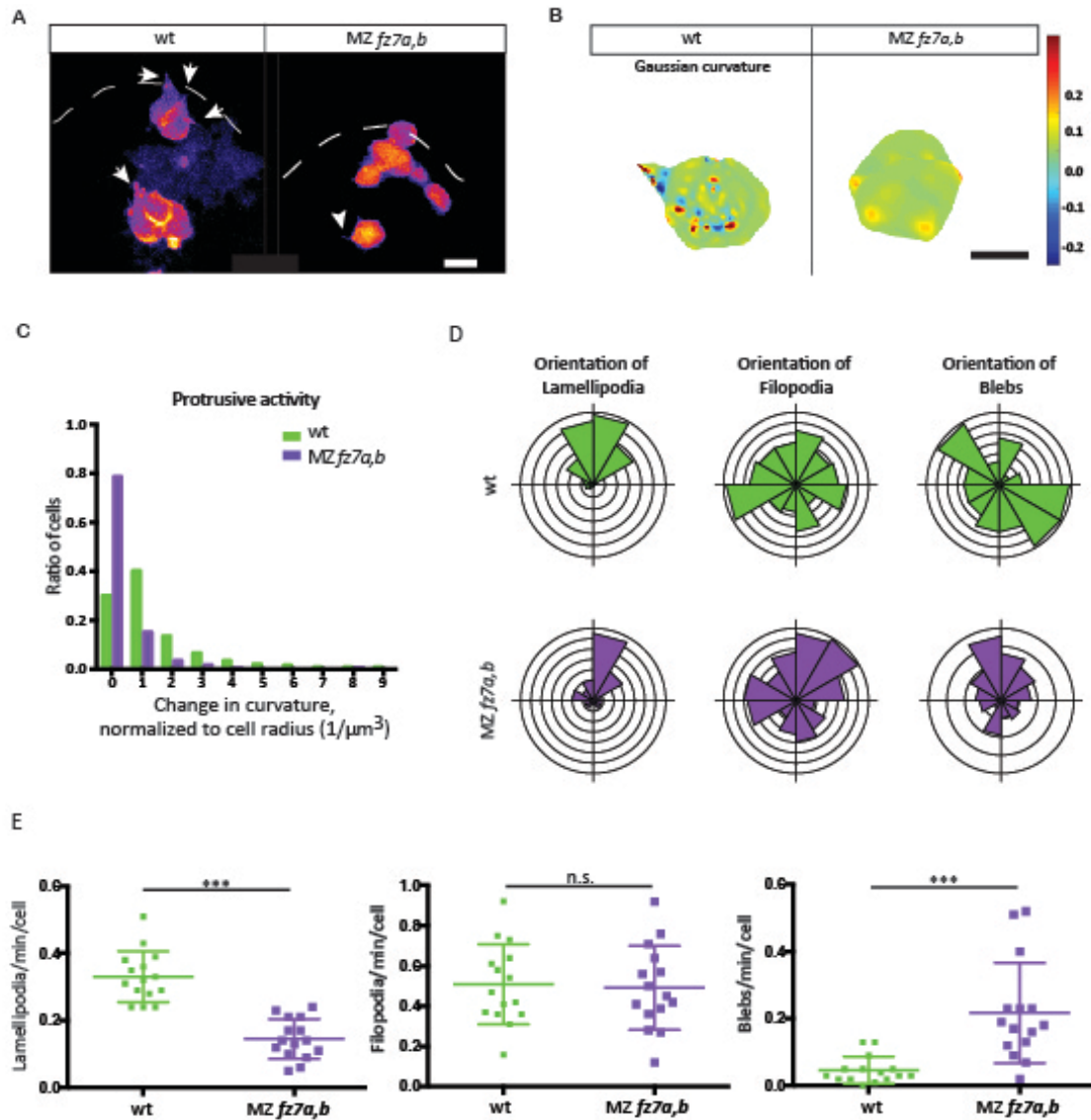


Figure 3.2.4.2: Analysis of protrusive activity of MZ *fz7a,b* mutants *in vivo*. **A:** Homotypically transplanted cells from ppl-fate induced donor embryos labeled with mkate2-CAAX, the host ppl is only outlined (dashed white line). The donor genotype is shown above the images, the scale bar is 50 μm . Arrows indicate lamellipodia-like protrusions, arrowheads filopodia. **B:** Homotypically transplanted cells from ppl fate induced donor embryos labeled with mkate2-CAAX. The samples were segmented in Imaris and the Gaussian curvature of the surface calculated in Matlab, and displayed as a heatmap. The scale bar is 10 μm . **C:** Histogram showing the relative frequency of curvature change events per μm^3 in analyzed frames. N=10 cells per genotype. **D:** Orientation of different types of protrusions in wt and MZ *fz7a,b* mutant embryos, with animal pole on the top. **E:** Frequency of different types of protrusions in wt and MZ *fz7a,b* mutant embryos. N=15 cells per genotype. For statistical analysis Student's t-tests were used with *** $p < 0.001$.

Following up on the *in vitro* results we wanted to confirm our findings *in vivo*. To this end we turned towards the transplantation assay again. Cells from prechordal plate fate induced embryos of either MZ *fz7a,b* mutant or wt background with the cell membranes labeled in red (mkate2-CAAX) were transplanted to the leading edge of the prechordal plate of

Tg(*gsc:GFP-CAAX*) host embryos of the same respective background (i.e. wt to wt, mutant to mutant). Due to the mosaic labeling nature of the transplantation assay it was possible to visualize protrusions. The embryos were imaged by 2-photon confocal microscopy. We found that wt embryos indeed display frequent lamellipodia-like protrusions, whereas cells from mutant embryos are mostly roundish with occasional filopodia (Fig. 3.2.4.2 A). We counted the different types of protrusions and could confirm that MZ *fz7a,b* mutant cells display significantly less lamellipodia (0.14 ± 0.059 protrusions/cell/minute) compared to wt embryos (0.33 ± 0.075 protrusions/cell/minute), while the number of protrusions resembling filopodia remained unchanged (Fig. 3.2.4.2 E). Blebs were slightly but significantly more frequent in mutant than in wt cells (Fig. 3.2.4.2 E). Interestingly, the preferential orientation of these different protrusion types along the direction of ppl progenitor cell migration towards the animal pole was indistinguishable between MZ *fz7a,b* and wt embryos (Fig. 3.2.4.2 D).

Since highly protrusive cells undergo significant shape deformations, while rounded cells do not, we used the difference between maximal and minimal curvature to quantify shape deformations, as described previously²³⁵. This curvature change index was used as a measure for protrusive activity to compare wt and mutant cells *in vivo*. As expected from the counting of protrusions, we found that high curvature changes are absent in mutant cells in the majority of cases, while wt cells display mostly higher changes in curvature, indicating more protrusive activities (Fig. 3.2.4.2 B & C).

Taken together, our *in vitro* and *in vivo* analysis shows that MZ *fz7a,b* mutant cells have defects in lamellipodia formation, providing an explanation for the overall defective prechordal plate migration in MZ *fz7a,b* mutant embryos. However Fz7 appears to be dispensable for protrusion orientation.

3.2.5 Cell-cell adhesion

Since it has been shown that Wnt/PCP signaling regulates the endocytosis of E-cadherin through Rab5c mediated endocytosis⁵², we wanted to investigate if this is at least partially

also responsible for the ppl migration defect in MZ *fz7a,b* mutants. This was especially interesting, since cell cohesion is required for collective migration in many systems^{53,114}, including ppl migration itself⁴⁷. We therefore fixed MZ *fz7a,b*; Tg(*gsc::GFP-CAAX*) and the normal Tg(*gsc::GFP-CAAX*) embryos at bud stage and performed antibody stainings for E-cadherin. The samples were imaged and the membrane to cytosol ratio of E-cadherin localization was analyzed. However, we could not find a significant difference between wt and mutant embryos (Fig. 3.2.5). This indicates that defects in the regulation of E-cadherin mediated cell cohesion do not contribute to the prechordal plate phenotype in MZ *fz7a,b* mutant embryos.

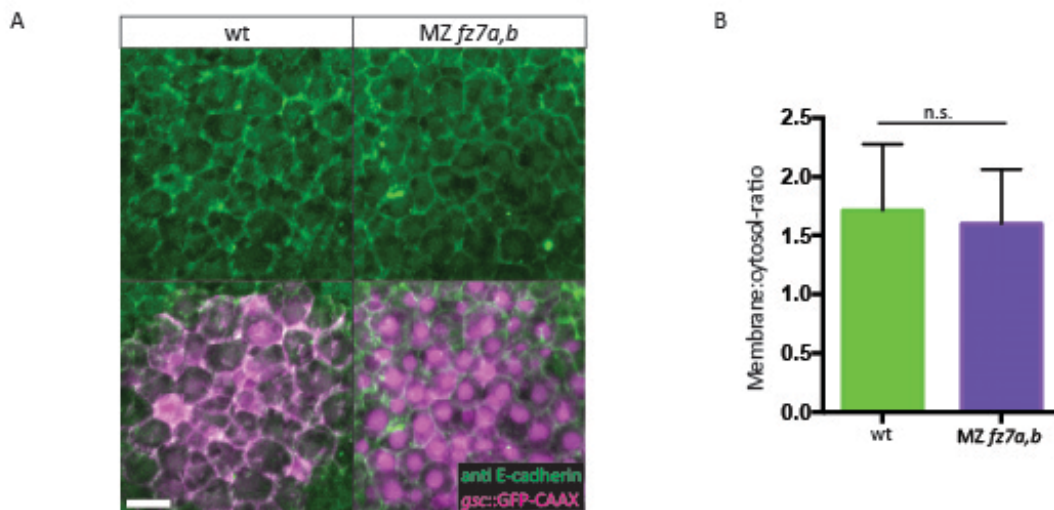


Figure 3.2.5: E-cadherin localization in wt and mutant embryos. A: Antibody staining against E-cadherin (green) in the ppl of 10hpf embryos. Magenta is *gsc::GFP-CAAX* to show that the analyzed cells are ppl. The scale bar is 20 μ m. B: Quantification of the membrane to cytosol fluorescence intensity ratio. For statistical analysis Student's t-test was performed. N= 94 (wt) and 86 (MZ *fz7a,b*) cells from 15 (each genotype) embryos.

3.3 Construction of OptoFz7

Frizzled receptors belong to the superfamily of G-protein coupled receptors (GPCRs), in which they form their own family of “Frizzled Class” receptors²⁴². Frizzleds have a 120 amino acids long extracellular domain, which contains the Frizzled- or cysteine rich domain (CRD, Fig. 3.3 B), binding site for Wnt ligands²⁴³. Like other GPCRs, it has seven transmembrane domains (TM), and therefore 3 extracellular (ECL) and intracellular (ICL) loops, while the C-terminus faces the cytosol (Fig. 3.3 B). TM5 contains a leucine zipper domain²⁴⁴, ICL3 two

Dishevelled binding motifs²⁴⁵, and the C-terminus a PDZ domain binding motif and the C-terminal amino acid sequence ETTV, which both can bind Dvl as well^{178,242,245}. Among the functional domains the CRD domain appears to be at least partially dispensable for PCP signaling^{87,246,247}, but complete removal of the extracellular amino terminus affects the non-autonomous PCP function in *Drosophila* epithelia, because Fz binds Vang with its extracellular domain⁹⁴. The extracellular loops do not have functional domains and there are only two *Drosophila* mutations in the extracellular domains known that affect PCP signaling²⁴⁶. Among the TM domains TM4-7 appear to be the most important ones, as mutations there affect canonical Wnt and PCP signaling²⁴³. Vertebrate and *Drosophila* data suggest that all 3 internal loops are required for the signal transduction²⁴⁶⁻²⁴⁸.

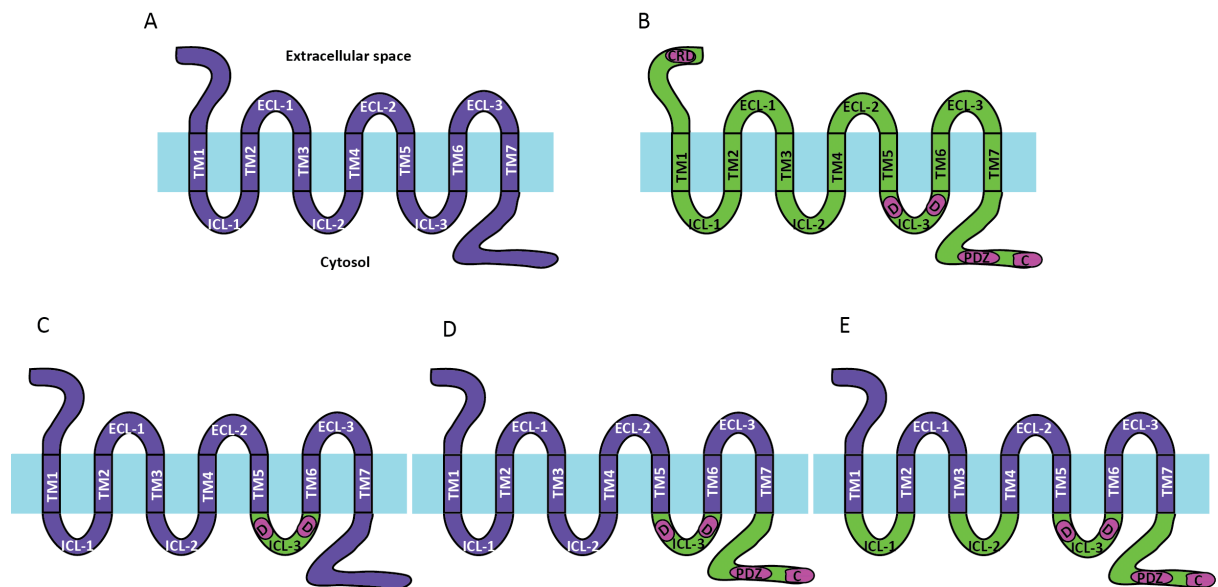


Figure 3.3: Architecture of OptoFz7 chimeras. A: Schematic of the Rhodopsin structure, light blue shows the cell membrane, up is extracellular, down intracellular space, as indicated in A. TM1-7 labels the transmembrane domains, ECL 1-3 the extracellular loops, ICL 1-3 the intracellular loops. B: Schematic of the Fz7 protein structure, beside the before mentioned domains, there is a cysteine-rich frizzled domain in the N-terminus (CRD), two Dishevelled binding sites in ICL3 (M1, M2), a PDZ domain binding site (PDZ) near the C-terminus, and the last 4 amino acids (ETTV), which also have Dvl binding function. C: Minimal construct (RRFR) with only ICL3 replaced with the Fz7 (green) sequence, the rest is still Rhodopsin (purple). D: Intermediate construct (RRFF), ICL3 and everything after TM7 are replaced with Fz7 sequence. E: Maximal construct, all intracellular domains are Fz7 (FFFF).

Like Frizzled, Rhodopsin also belongs to the GPCR superfamily²⁴⁹ and shares most structural features with Frizzleds (Fig. 3.3), although there is almost no sequence homology between

GPCRs of different classes²⁵⁰. Based on previously published chimeric proteins^{251,252}, we designed 3 different versions of OptoFz7, as it is difficult to predict which configurations of intra-class GPCR chimeras work best²⁵⁰. The minimal construct (dubbed RRFR) was based on reports that ICL3 is the most important part for signal transduction of a GPCR^{251,253}, and had the aim to keep the structure of the Rhodopsin backbone as intact as possible (Fig. 3.3 C). Other sources state that all ICLs are required for signaling²⁴⁶⁻²⁴⁸, we thus also created a maximal construct (FFFF, Fig. 3.3 E), and eventually an intermediate one (RRFF), that contained all elements with known Dvl binding domains, but otherwise had the Rhodopsin sequence (Fig. 3.3 D).

Normal Rhodopsin is composed of the 40KD opsin and the carotenoid 11-cis-retinal, which is covalently linked to it^{249,254,255}. In cell culture experiments that applied optogenetic chimeric GPCRs, cells are usually incubated in cis-retinal containing media before activation^{231,251}. Since this project focuses mostly on the whole embryo, we decided to co-inject cis-retinal with the mRNA to provide all cells with roughly equal amounts. Moreover, instead of 11-cis-retinal we apply the isomere 9-cis-retinal, which has similar properties²⁵⁶, but is supposed to be more stable and was also already successfully used in Rhodopsin-based chimeric proteins²⁰⁰.

3.4 Testing OptoFz7 by rescue of the MZ *fz7a,b* *ppl* phenotype

Based on the results of the *silberblick* rescue⁵⁸ and MZ *fz7a,b* mutant rescue⁶³ by injection of the respective mRNA to the 1-cell stage, we were curious if the ubiquitous expression and activation of OptoFz7 could also rescue the phenotypes of MZ *fz7a,b* embryos. To this end, we injected mRNA encoding for the three OptoFz7 versions together with the Rhodopsin co-factor 9-cis-retinal to 1-cell stage embryos, split the batch in two groups and kept one in the dark, while the other group was exposed to 505nm light. The samples were fixed at bud stage (10hpf) and *in situ* hybridized for *hgg*, *ntl*, and *papc*.

We first assessed the *ppl* phenotype and found the shape of the *ppl* could be rescued to various extents, depending on the construct used (Fig. 3.4.1). We therefore used this assay to distinguish between the different constructs and found that the RRFF one had the best rescue effect as well as the best light vs. dark ratio (Fig. 3.4.1).

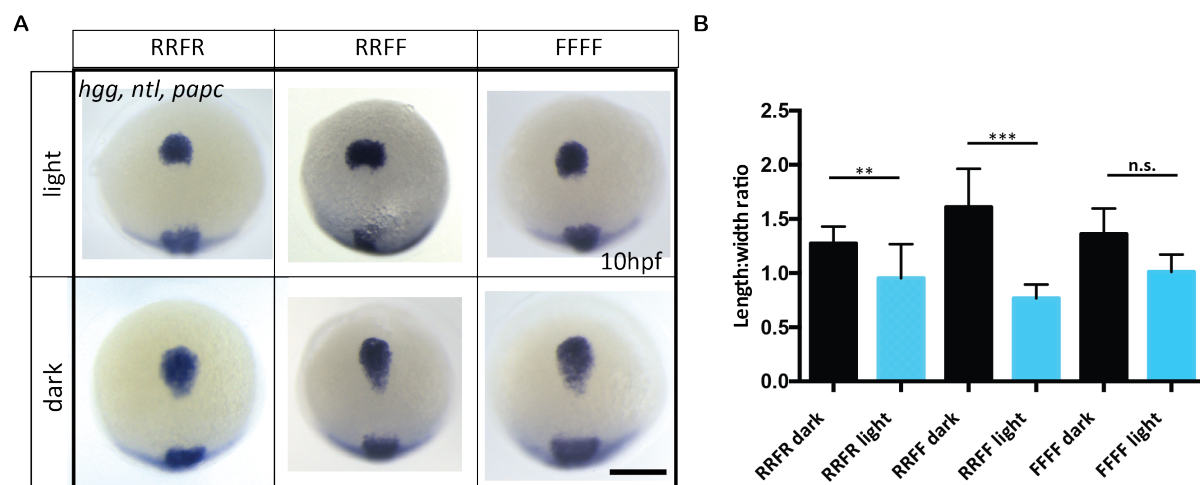


Figure 3.4.1: Rescue of the *ppl* shape by ubiquitous activation of OptoFz7. A: *In situ* hybridization with RNA antisense probes against *hgg*, *ntl*, and *papc*. The columns display the different constructs, the upper row the light condition, the lower row the dark condition. 20pg of mRNA were injected for RRFF, and FFFF, 40pg for RRFR, based on previous experiments. The scale bar is 250μm. **B:** Quantification of length to width ratio of the *ppl* in A. Black columns are dark conditions, blue ones light. Error bars are standard deviation. For statistical analysis a Kruskal-Wallis, followed by Dunn's multiple comparison test was used with ***p<0.001, **p<0.01. N (from left)= 18, 30, 29, 40, 27, 11.

Based on these results, we decided to use exclusively the RRFF receptor from now on. In addition to the ppl shape, we next analyzed the distance from the notochord and compared both parameters to wt and MZ *fz7a,b* conditions (Fig. 3.4.2).

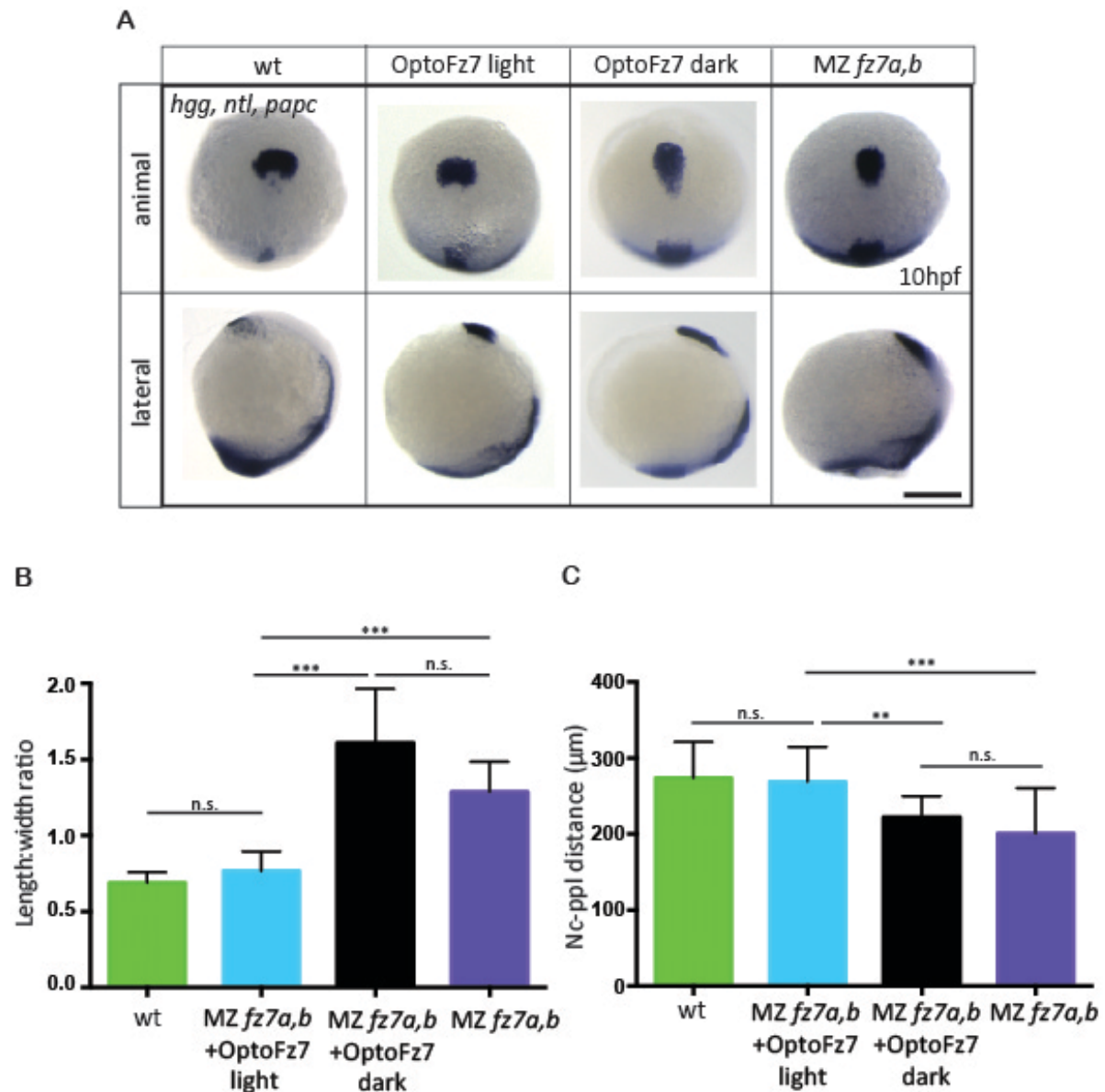


Figure 3.4.2: Rescue of the ppl phenotypes by OptoFz7 compared to wt and uninjected MZ *fz7a,b* mutant embryos. **A:** *In situ* hybridization with RNA antisense probes against *hgg*, *ntl*, and *papc*. The upper row is animal pole view to visualize the prechordal plate shape, the lower one is lateral to assess the distance between ppl and nc. The first column is wt, the others are mutants. 20pg of mRNA was injected. The scale bar is 250μm. **B & C:** Quantification of the ppl phenotypes in A, B shows the ppl shape; C shows the distance between nc and ppl. The columns correspond to the ones in A. Error bars are standard deviation. For statistical analysis a Kruskal-Wallis, followed by Dunn's multiple comparison test was used with *** $p < 0.001$, ** $p < 0.01$. N in B (from left)= 22, 30, 29, 17; in N in C (from left)= 37, 40, 29, 17.

Ubiquitous OptoFz7 could rescue both phenotypes (Fig. 3.4.2). The light groups were indistinguishable from the wt and significantly different from both dark and uninjected MZ *fz7a,b* mutant samples. The dark groups mostly behaved like the untreated mutants. In regards to the ppl shape, there was a slight but insignificant lengthening of the ppl compared to mutants (Fig. 3.4.2 A & B).

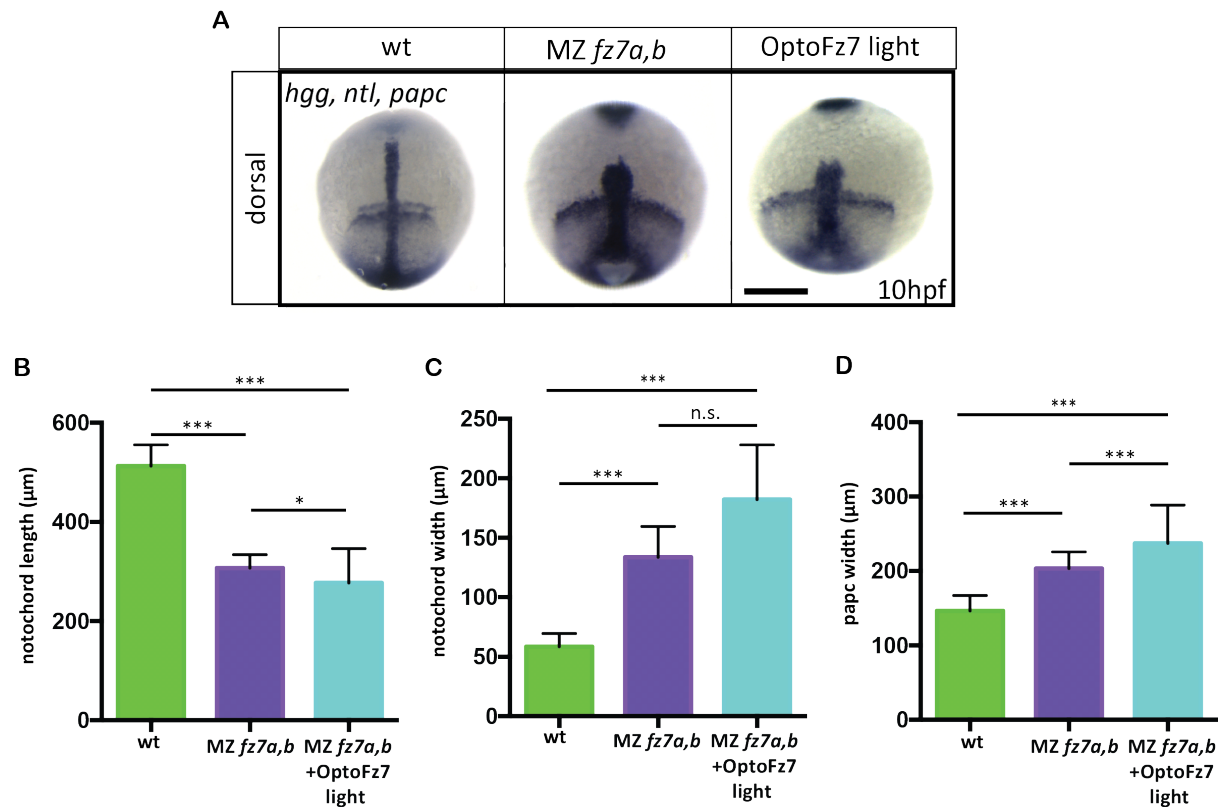


Figure 3.4.3: Rescue of dorsal phenotypes with OptoFz7 compared to wt, and uninjected MZ *fz7a,b* mutant embryos. A: *In situ* hybridization with RNA antisense probes against *hgg*, *ntl*, and *papc*. The view is from dorsal to visualize the length and width of the notochord and the width of the paraxial mesoderm. 20pg of mRNA was injected. The scale bar is 250μm. B-D: Quantification of measurements in A. The columns correspond to the ones in A. B shows the nc length, C the nc width, and D the width of the paraxial mesoderm. Error bars are standard deviation. For statistical analysis a Kruskal-Wallis, followed by Dunn's multiple comparison test was used for C, ANOVA with Tukey's multiple comparison test in B & D, with *** $p < 0.001$, * $p < 0.05$. N (from left)= 78, 63, 22.

Next, we analyzed the posterior phenotypes of the MZ *fz7a,b* mutant by the same method. We were not able to rescue any of them (Fig. 3.4.3). The notochord length defect was mildly but significantly exacerbated (Fig. 3.4.3 B). The width of the notochord showed a high variability and was not significantly different from uninjected mutants (Fig. 3.4.3 C). The convergence of the paraxial mesoderm, as marked by *papc* expression, was more strongly

impaired than in mutant embryos without treatment (Fig. 3.4.3 D). Taken together, we can use ubiquitous activation of the OptoFz7 by 505nm light illumination to rescue the typical anterior PCP C&E phenotypes of the MZ *fz7a,b* mutant embryos (ppl shape and distance to the nc); however, defects of posterior C&E cannot be rescued.

3.5 Gain of function in wildtype

Since overexpressing OptoFz7 in wt explants led to reduced protrusion formation *in vitro*, we wondered what effect it would have at the embryo level. The most obvious phenotype and the one that was rescued most efficiently by OptoFz7 activation in MZ *fz7a,b* mutants was the shape of the prechordal plate. Therefore, we injected *optoFz7* mRNA with cis-retinal to wt embryos, incubated them in light or dark conditions, and fixed them for *in situ* hybridization. The OptoFz7 injected samples displayed the elongated ppl shape characteristic for impaired Wnt/PCP signaling. Surprisingly however, the phenotype also occurred in the dark and even was a bit stronger (Fig. 3.5 A & B). This demonstrates that a specific level of Wnt/PCP signaling is required to achieve a proper ppl shape.

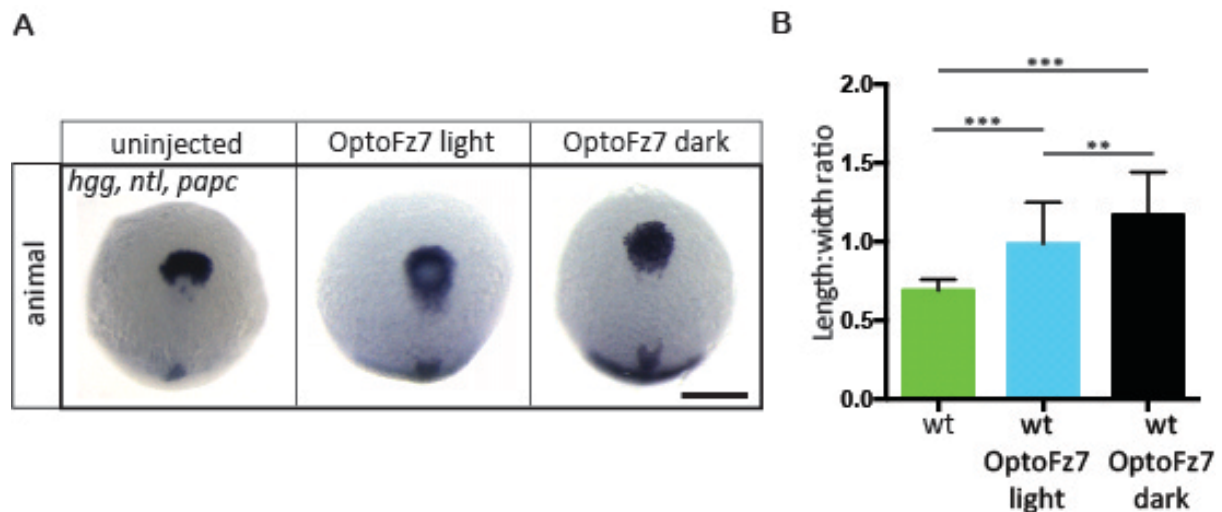


Figure 3.5: Gain of function experiment in wt. A: *In situ* hybridization with RNA antisense probes against *hgg*, *ntl*, and *papc*. The view is from animal pole displaying the prechordal plate. The scale bar is 250µm. B: Quantification of ppl length to width ratio. The green column shows untreated wt embryos, the blue column wt with OptoFz7 and light, and the black one wt with OptoFz7, but without light. For statistical analysis ANOVA followed by Tukey's multiple comparison test was used with *** $p < 0.001$ and ** $p < 0.01$. N = 37 for wt, 23 for the light group, and 32 for the dark group.

3.6 Rescue of Wnt mutants

The *wnt11* mutant *silberblick* has a ppl shape defect that is equally or even more pronounced than the one in MZ *fz7a,b*. Since the Wnt11 protein is thought to be the ligand for Fz7^{58,122,123,129,137}, OptoFz7 should be able to rescue the ppl phenotype of *slb* embryos. To investigate this possibility we repeated the original whole embryo rescue assay, but used *slb* embryos instead of Mz *fz7a,b*. OptoFz7 rescued the shape of the ppl frequently and significantly (Fig. 3.6 A & B), if the samples were exposed to light. In the dark group there was no significant effect (Fig. 3.6 A & B).

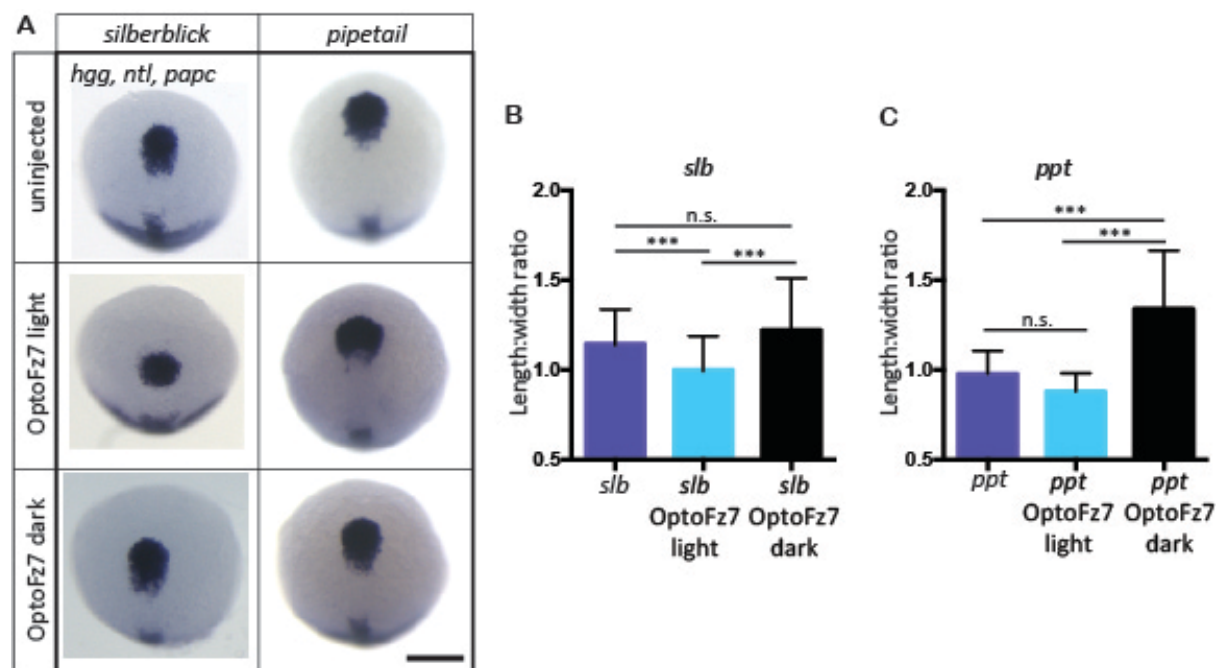


Figure 3.6: Attempted rescue of ppl shape of *wnt* mutants. A: *In situ* hybridization with RNA antisense probes against *hgg*, *ntl*, and *papc*. The view is from the animal pole displaying the prechordal plate. The scale bar is 250µm. B: Quantification of ppl length to width ratio. Purple columns are mutants, blue ones mutants with OptoFz7 under light conditions, black columns are mutants with OptoFz7, but without light. B shows the rescue of *silberblick*, C of *pipetail*. For statistical analysis a Kruskal-Wallis test followed by Dunn's multiple comparison test was used, ***p<0.001.

The *wnt5* mutant *pipetail* has mostly posterior phenotypes, but when we stained the ppl shape with the *hgg* antisense probe, we found the ppl also to be rounder than in wt embryos (Fig. 3.6 A, and ⁶¹). We thus tried to rescue the phenotype with OptoFz7 in the same fashion as with the *slb* and MZ *fz7a,b* mutants. Interestingly, some rescued embryos displayed a ppl shape similar to wt (Fig. 3.6 A), but overall there was no significant

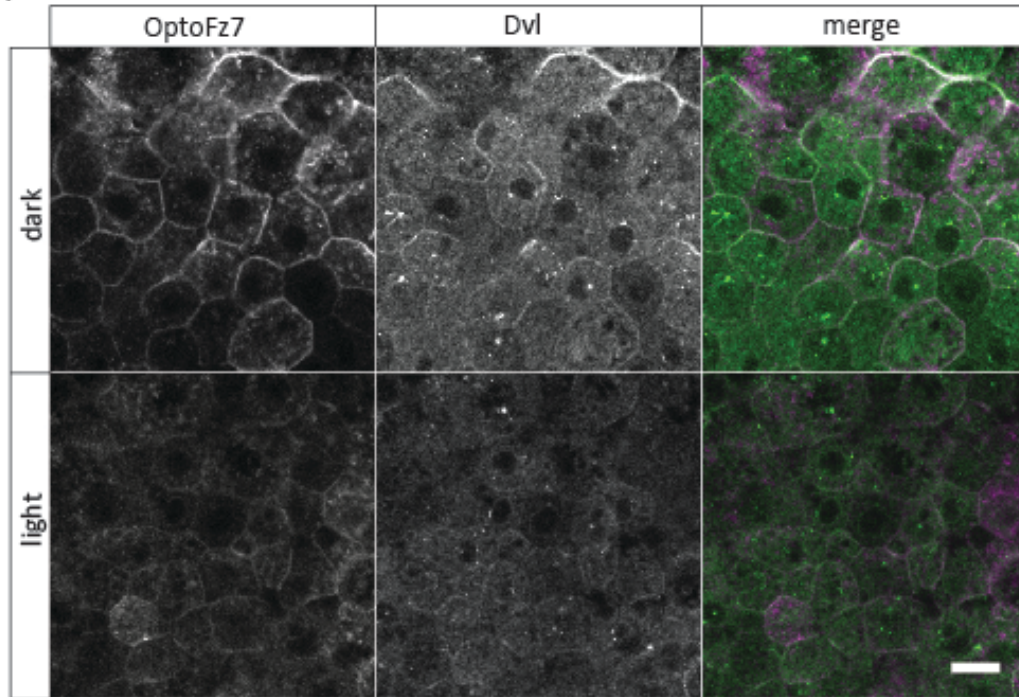
improvement (Fig. 3.6 C). On the other hand, in the dark condition OptoFz7 appeared to exacerbate the phenotype towards the level of *slb* and MZ *fz7a,b* mutants (Fig. 3.6 A), similar to what occurred in the gain of function experiment (Fig. 3.5 A & B).

3.7 PCP signaling of OptoFz7 depends on light activation and cis-retinal

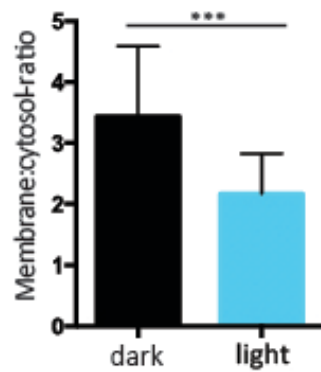
After finding that OptoFz7 can rescue the ppl phenotypes of MZ *fz7a,b* mutants in the presence of light, but not without light, we wanted to further investigate the light-dependent behavior of the chimera. A hallmark of functional Fz7 is the recruitment of Dvl to the plasma membrane if co-expressed^{129,257}. We therefore co-injected GFP tagged zebrafish *dishevelled 2* (*dvl2-GFP*; courtesy of Masazumi Tada) mRNA together with *optoFz7* tagged C-terminally with *mCherry* mRNA and 9-cis retinal. Half of the injected embryos were exposed to light, while the rest was kept in the dark. We fixed all embryos at 30% epiboly and imaged them from the animal pole as described previously¹²⁹. The idea of this animal pole assay is, to image wt embryos before endogenous Wnt/PCP components are expressed; this way the imaged localization is exclusively due to the injected constructs¹²⁹. The amount of mRNA injected was double the molar equivalent of the amount needed for rescuing ppl phenotypes, in order to make the signal bright enough. We found that Dvl2-GFP protein is recruited to the membrane and co-localizes there with OptoFz7-mCherry (Fig. 3.7.1 A).

Frizzled like other GPCRs undergoes endocytosis upon activation by a ligand^{231,258}. We therefore looked for the same property in OptoFz7. To this end, embryos injected with *optoFz7-mCherry* mRNA and 9-cis-retinal were incubated in either the absence or presence of 505nm light, fixed and imaged by confocal microscopy, using the same animal pole assay described above. Measuring the cell membrane to cytosol ratio of the fluorescence signal, we found significantly reduced OptoFz7 surface levels in the light group compared to the dark control group (Fig. 3.7.1 A & B). We therefore could trust that our receptor is light-responsive and transmitting Wnt/PCP signaling. Dvl-GFP displayed similar endocytosis behavior as OptoFz7-mCherry (Fig. 3.7.1 A&C).

A



B OptoFz7 localization



C Dishevelled localization

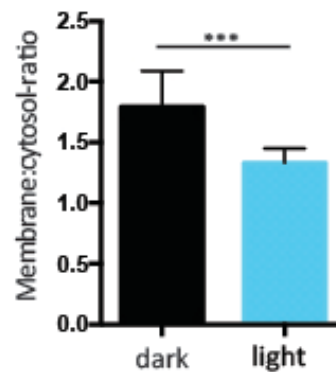


Figure 3.7.3.7.1: Localization of OptoFz7. A: Confocal images of embryos injected with 85pg *optoFz7-mcherry*, and 100pg *dvl2-GFP* mRNA, and 140pg 9-cis-retinal, fixed at 30% epiboly. The first column shows OptoFz7 localization, the second Dvl2-GFP. The third column shows both signals, with Dvl2-GFP being green, and OptoFz7-mcherry magenta. The upper row is in dark condition, the lower one light. The scale bar is 20µm. B & C: Quantification of the cytosol to membrane signal ratios in A. For statistical analysis Student's t-tests were used, with *** $p < 0.001$. N in B is 67 cells in the dark group, and 108 in the light group, N in C is 31 (dark) and 36 (light).

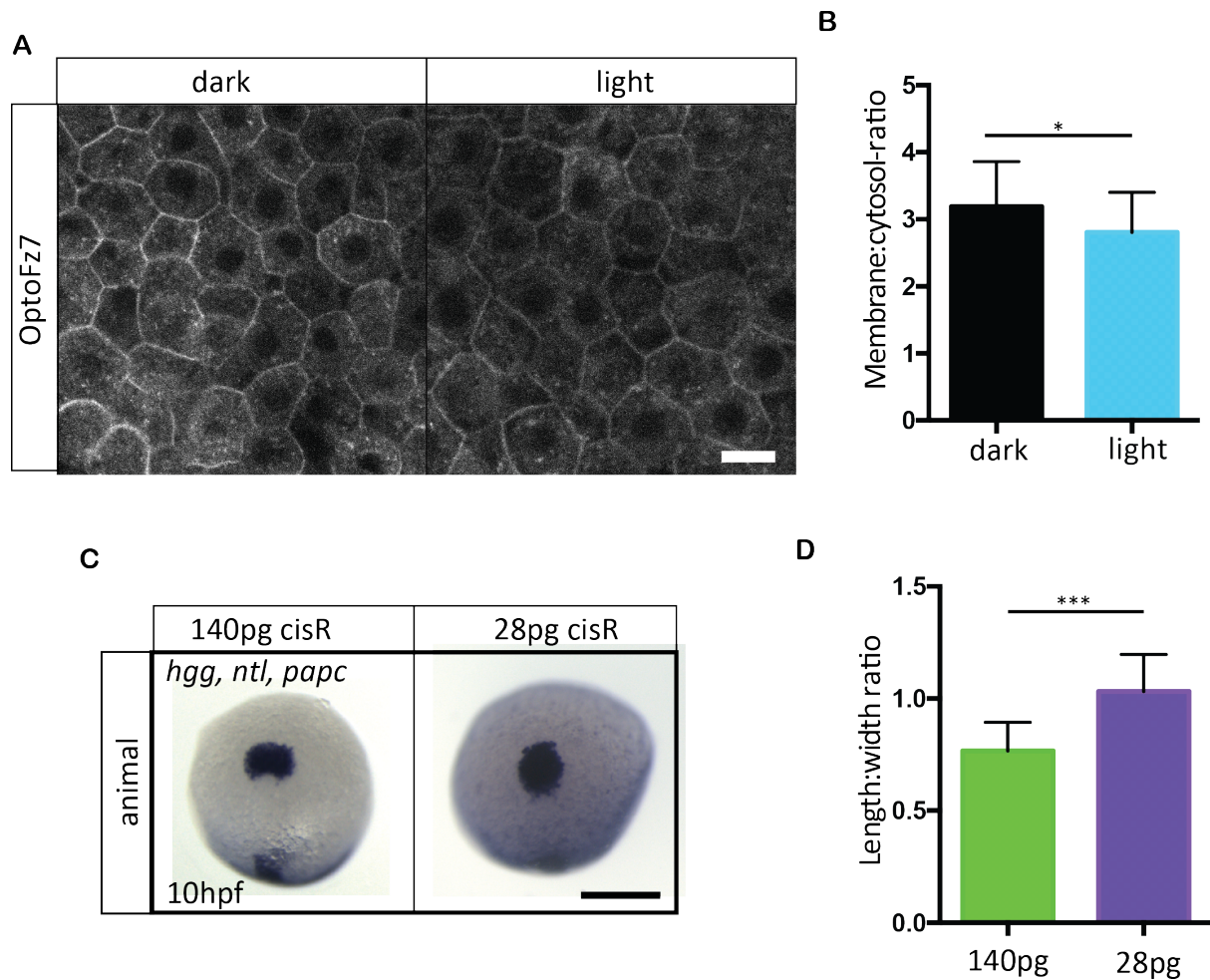


Figure 3.7.3.7.2: Dependence of OptoFz7 on exogenous cis-retinal. **A:** Confocal images of embryos injected with 85pg *optoFz7-mCherry* mRNA, fixed at 30% epiboly. The scale bar is 20µm. **B:** Quantification of the cytosol to membrane signal ratios in **A**. **C:** *In situ* hybridization with RNA antisense probes against *hgg*, *ntl*, and *papc*. The view is from animal pole to visualize ppl shape. 20pg of mRNA were injected, but either normal (left panel) or five fold reduced level of 9-cis-retinal. The scale bar is 250µm. **D:** Quantification of the ppl shape in **C**. Error bars show the standard deviation. For statistical analysis Student's t-tests were used with *** $p < 0.001$, * $p = 0.433$. N in **B** is 24 cells (dark) and 23 (light). N in **D** is 40 (140pg), and 13 (28pg).

It has been reported that the addition of retinal is unnecessary for investigating vertebrate neural systems, because cells contain a sufficient amount in form of vitamin A²⁵⁹. However, we did not know if this is also true for gastrulation stage embryos. To test the dependence of OptoFz7 on cis-retinal for signaling we repeated the rescue experiment and the localization assay with reduced levels of cis-retinal or entirely without (Fig. 3.7.2). The difference in subcellular protein localization was reduced, if no cis-retinal was co-injected (Fig. 3.7.2 A & B; $p = 0.433$). The value for the dark group stayed at roughly the same level, but the ratio of the light group went up (cf. 3.7.1), suggesting that endocytosis upon

activation was reduced. In the rescue experiment, the same amount of *optoFz7* mRNA as usual was injected, but the level of co-injected 9-cis-retinal was reduced 5-fold. In those conditions, the rescue effect was severely reduced compared to the efficiency with normal levels (Fig. 3.7.2 C & D). Both results suggest that at least certain levels of exogenous cis-retinal are required for *in vivo* optogenetic experiments in zebrafish gastrulae and further support the light specificity of OptoFz7.

3.8 Localized activation of OptoFz7

Ectopic expression of Wnt ligands in PCP regulated systems can result in instructive cell behavior, as has been shown in chicken myofibres, the *Drosophila* wing epithelium, or the *Xenopus* larval ventral epidermis^{84,117,194}. Therefore, we wanted to investigate if this was also the case in zebrafish prechordal plate migration. As a first experiment we injected mRNA encoding *wnt11* tagged with *3x-mCherry* into a single blastomere of Tg(*mezzo:GFP*) at the 32-cell stage. Since the cells are not connected by cytoplasmic bridges anymore at this stage, this results in mosaic labeling. The later localization of the cells arising from a single blastomere in the embryo cannot be predicted. Therefore, samples with a left/right bias in *wnt11-3x-mCherry* expression were selected based on the fluorescence signal at shield stage. We frequently found mild and, in rare cases, strong tilting of the body axis towards the Wnt11 source (Fig. 3.8.1). In both severity levels the *wnt11* expressing half of the body also displayed epiboly delay.

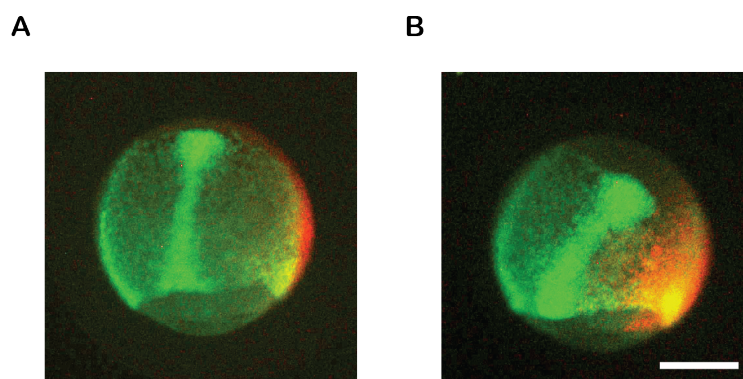


Figure 3.8.3.8.1: Asymmetric expression of *wnt11-3x-mCherry*. Epifluorescence images of Tg(*mezzo:GFP*) embryos, the images are overlays of both channels. Red shows Wnt11-3x-mCherry, green mezzo:GFP. The scale bar is 250µm. A shows the mild phenotype, B the strongest example.

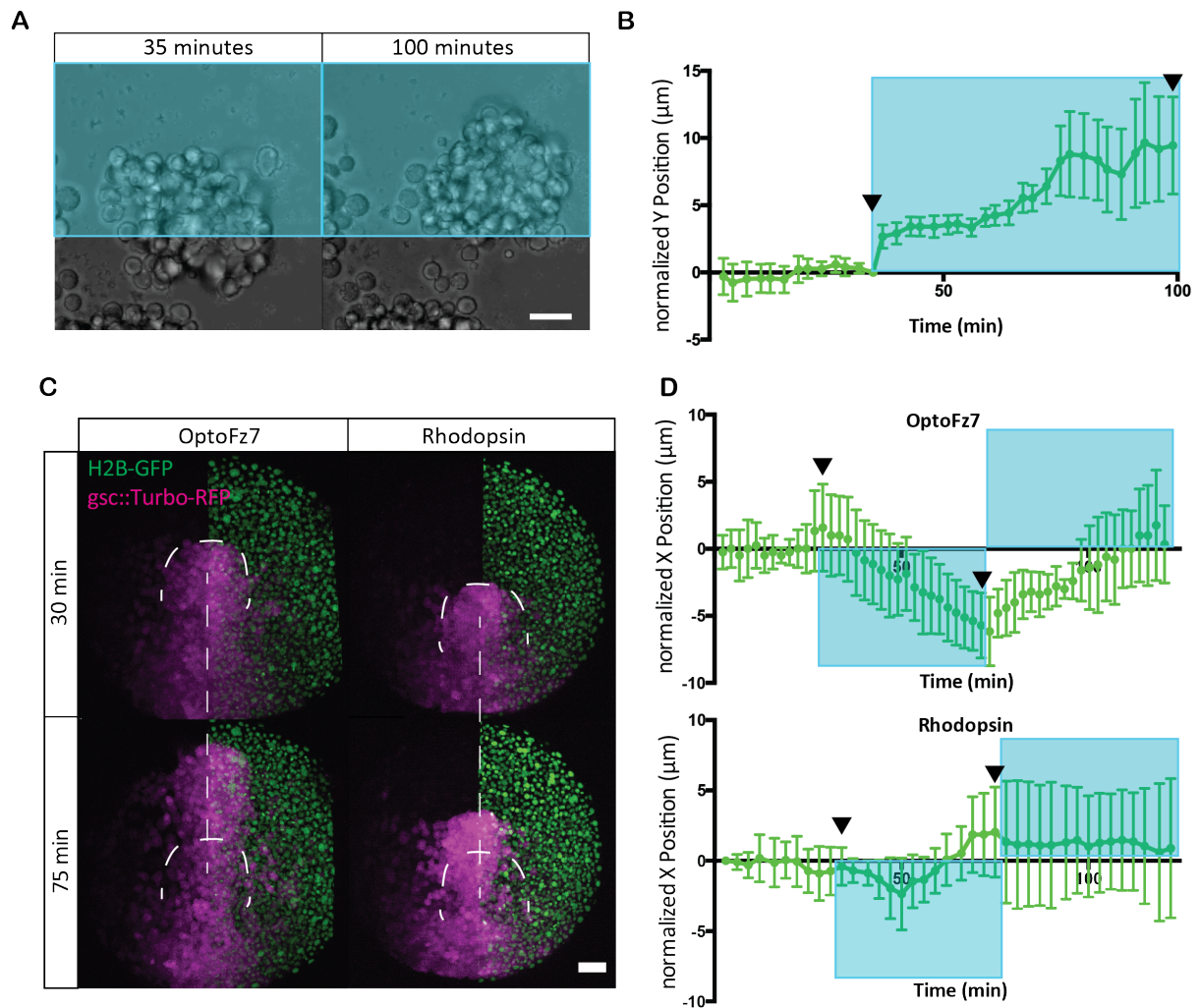


Figure 3.8.3.8.2: Local activation of OptoFz7. **A:** Explants of ppl cell fate induced MZ *fz7a,b* embryos, injected with *optoFz7* mRNA and 9-cis-retinal, in cell culture. Left panel is at the start of activation, 35 minutes into the experiment, the right panel at the end of illumination. The blue box shows the area of 488nm light activation. The scale bar is 40 μm . **B:** Quantification of the Y-position of the explants normalized to the time step before activation. The blue box symbolizes the area of 488nm light illumination in **A**, the arrows mark the time points of the images in **A**. **C:** Confocal images of MZ *fz7a,b*; Tg(*gsc::TurboRFP*) embryos during gastrulation. Embryos have been injected with 9-cis-retinal and *h2b-GFP* mRNA to highlight the area of 488nm light activation, and either with *optoFz7* (left column) or *rhodopsin* (right column) mRNA. The upper panels are imaged at the start of activation, 30 minutes into the experiment, the lower ones at the end of first activation. Dashed rounded lines outline the ppl in the early images in both rows. Dashed show that they are at the same X-position in both rows. The scale bar is 50 μm . **D:** Quantification of the X-positions of ppl's normalized to the start of the experiment. The blue box symbolizes the area of 488nm light illumination in **C**, the arrows mark the time points of the images in **G**.

As a next step we were interested if OptoFz7 could have the same instructive capacity, if it was expressed ubiquitously but activated locally. We first applied the OptoFz7 receptor in the *in vitro* assay. Explants of ppl fate induced MZ *fz7a,b*; Tg(*gsc::GFP-CAAX*) embryos, co-injected with *optoFz7* mRNA and 9-cis retinal were cultured in the dark on fibronectin

coated glass bottom dishes and imaged with an inverted confocal microscope using a 561nm laser. We then applied pulses of 488nm light to half of the explant. As expected from literature and from the asymmetric Wnt11 experiments the activation of a part of the explant caused a directional response, as the cell clusters moved towards the area that was exposed to light (Fig. 3.8.2 A). We quantified these movements by tracking the positions of cell clusters and plotted them over time (Fig. 3.8.2 B).

Next we wanted to investigate, if this instructive potential of OptoFz7 would also be preserved *in vivo*. To test this we crossed the MZ *fz7a,b* line to the *Tg(gsc:TurboRFP)* line, in which prechordal plate cells are labeled in red^{201,224}. This was necessary because imaging of GFP of the *Tg(gsc:GFP-CAAX)* line would at the same time activate OptoFz7. We injected MZ *fz7a,b; Tg(gsc:Turbo-RFP)* embryos with *optoFz7* mRNA, 9-cis-retinal, and *h2b-GFP* mRNA as a marker for light activation, and incubated them in the dark until shield stage. Then, they were mounted dorsal side up under red light and imaged using a confocal microscope with 561nm laser. We exposed half of the sample to light from the 488nm laser, which activates OptoFz7. Indeed, we found that the prechordal plate moved towards the area of light exposure, which was marked by fluorescence from the co-injected H2B-GFP (Fig 3.8.2 C). We quantified this by segmenting the prechordal plate and plotting the change of position along the X-axis (Fig. 3.8.2 D). To ensure that this was Fz7 specific and not due to phototoxicity, we repeated the same experiment using normal Rhodopsin instead of OptoFz7 (Fig. 3.8.2 C). Changes in the X-position of the ppl occurred only weakly and randomly and did not correlate with the light exposure (Fig. 3.8.2 D). To conclude, these experiments demonstrate that OptoFz7 can cause a directional response, when activated in parts of a cell population, and corroborate the instructive capacity of Wnt/PCP signaling.

3.9 Ubiquitous activation of OptoFz7

The results of the local activation experiments *in vitro* and *in vivo* clearly demonstrated the instructive capacity of OptoFz7. On the other hand the ability of OptoFz7 to rescue ppl shape and the distance to the nc by uniform activation suggested a permissive function. Therefore, we were interested in the effect uniform OptoFz7 activity would have on the sorting behavior (Fig. 3.2.3) or the protrusive activity (chapter 3.2.4). The first aim was to

rescue the sorting out and falling back phenotype of MZ *fz7a,b* cells in heterotypic transplantations (Fig. 3.2.3 A & B). We transplanted cells from induced MZ *fz7a,b* donors injected with *optoFz7* mRNA, 9-cis-retinal, and *mkate2-CAAX* mRNA to *Tg(gsc::GFP-CAAX)* wt embryos and imaged them using two-photon microscopy. Since we could not image them without the GFP channel, we did not protect the samples from light, thus causing ubiquitous activation of OptoFz7 in transplanted cells. We found that the transplanted mutant cells did not sort out and fall back, but stubbornly stuck to the leading edge (Fig. 3.9.1 A). This was also obvious from the local correlation between the transplanted cells and their neighbors (Fig. 3.9.1 B).

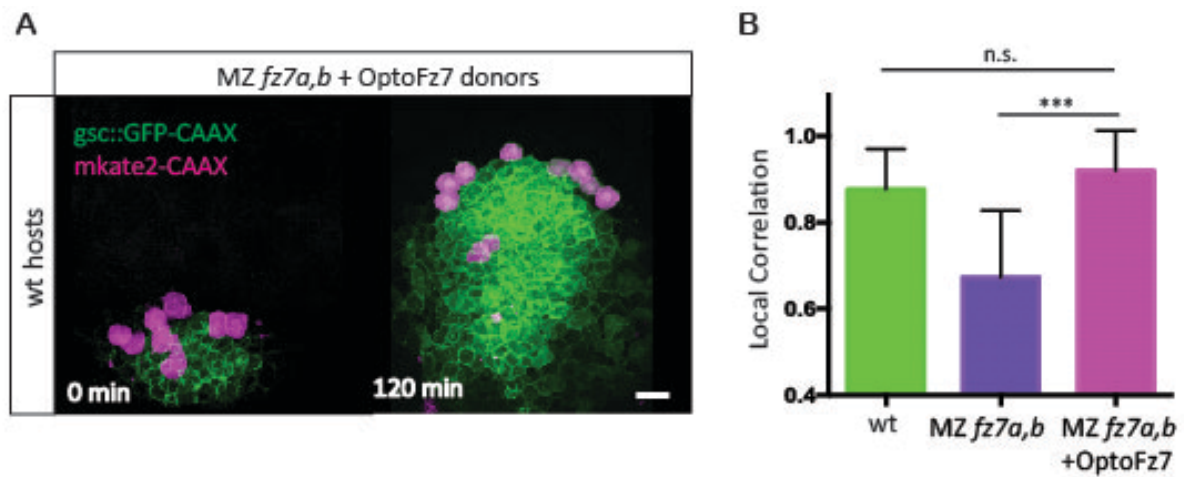


Figure 3.9.1: Optogenetic rescue of sorting behavior in heterotypic transplantation experiment with wt hosts and mutant donors injected with *optoFz7*, *mkate2-CAAX*, and 9-cis-retinal. A: Images from confocal microscopy at early (left) and late (right) gastrulation, view from dorsal, animal pole is to the top. Donor cells are labeled in magenta, host cells in green. The scale bar is 40 μ m. B: Quantification of the local correlation in A. For statistical analysis a Kruskal-Wallis test followed by Dunn's multiple comparison test was used, ***p<0.001.

We then used the same approach to investigate, whether transplanted mutant cells would recover their protrusive activity. Indeed, cells uniformly activated by OptoFz7 resembled the protrusive activity of wt cells (Fig. 3.9.2 A). Analysis of the Gaussian curvature of 3D-segmented cells and plotting the change in curvature normalized to cell size (cf. 3.1.4 D & E) confirmed the rescue efficiency (Fig. 3.9.2 B & C). We additionally counted the types of protrusions and found that the number of lamellipodia-like protrusions and blebs was restored to wt level (0.32 ± 0.083 lamellipodia/cell/minute, 0.06 ± 0.041 blebs/cell/minute Fig. 3.9.2 D & F).

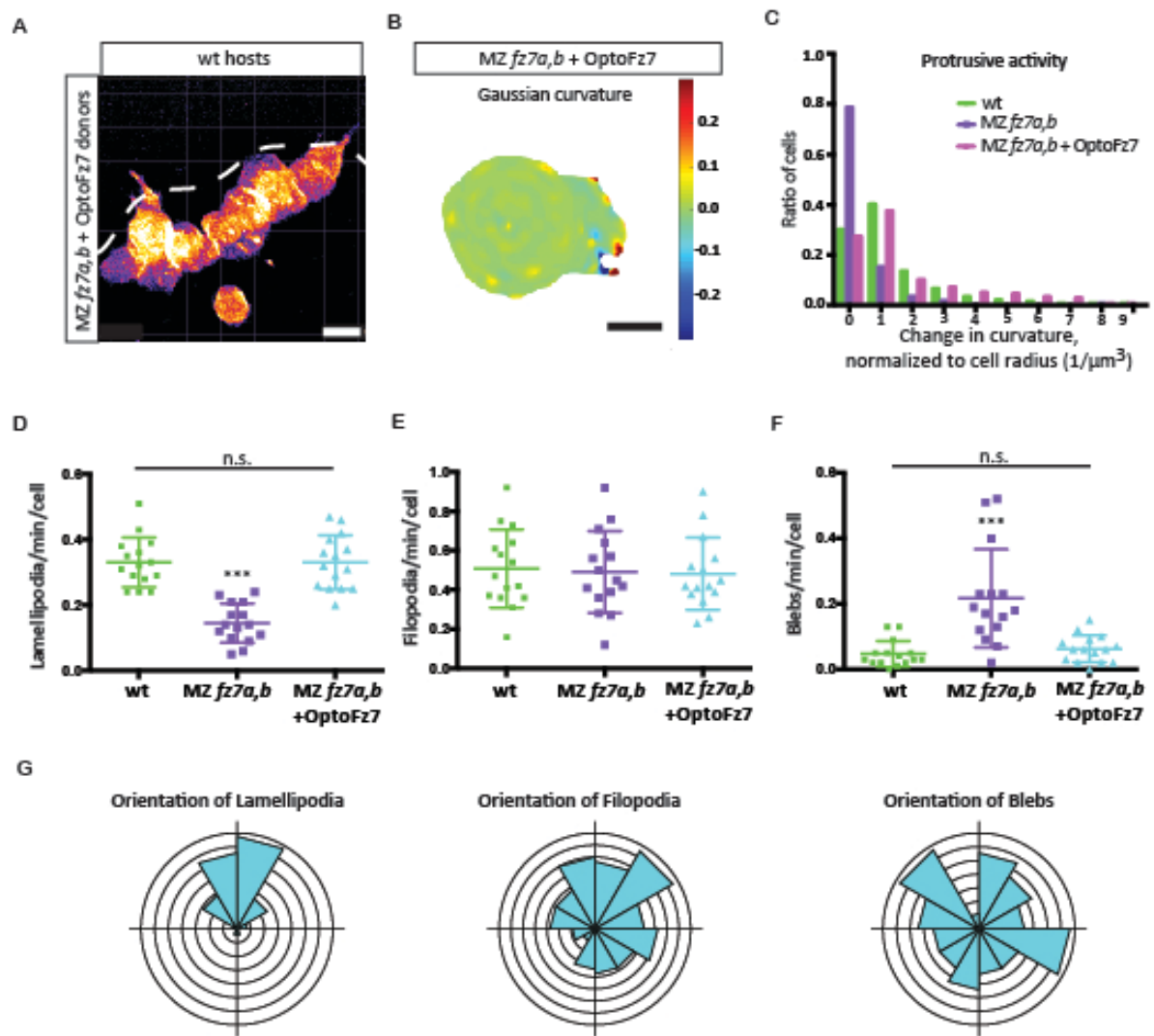


Figure 3.9.2: Rescue of protrusive behavior *in vivo*. A: MZ *fz7a,b* cells from ppl-fate induced donor embryos injected with *mkate2-CAAX* (green), 9-cis-retinal, and *optofz7* mRNA. The ppl of the host wt embryo is only outlined (dashed white line). The scale bar is 30 μm . B: Transplanted cell from same experimental setup as in A. The cells were 3D-segmented in Imaris, the Gaussian curvature of the surface calculated in Matlab, and displayed as a heatmap. The scale bar is 10 μm . C: Histogram showing the relative frequency of curvature change events per μm^3 in analyzed images for wt, mutants and mutants with active OptoFz7. N=10 cells per genotype or treatment. D – F: Frequency of lamellipodia (D), filopodia (E), and blebs (F) in transplanted MZ *fz7a,b* mutant cells, injected with mRNA encoding for OptoFz7. For statistical analysis ANOVA followed by Tukey's multiple comparison test was used, *** $p < 0.001$, N=15 cells per genotype. G: Orientation of different types of protrusions in transplanted MZ *fz7a,b* mutant cells, injected with mRNA encoding for OptoFz7.

The number of filopodia (Fig. 3.9.2 E) and the orientation of protrusions (Fig.3.9.2 G) remained unchanged (0.48 ± 0.184 filopodia/cell/minute).

We were curious if this rescue would also work in the simplified *in vitro* setup and therefore made explants from ppl cell fate induced MZ *fz7a,b*; *Tg(gsc:eGFP-CAAX)* embryos, injected with *optoFz7* mRNA and 9-cis-retinal. The explants were incubated on fibronectin coated glass bottom dishes and imaged with brightfield microscopy. During imaging the samples were also exposed to 475nm LED light to activate OptoFz7. As described above, we segmented the outline of the clusters, normalized it to the area, and plotted the circumference over time.

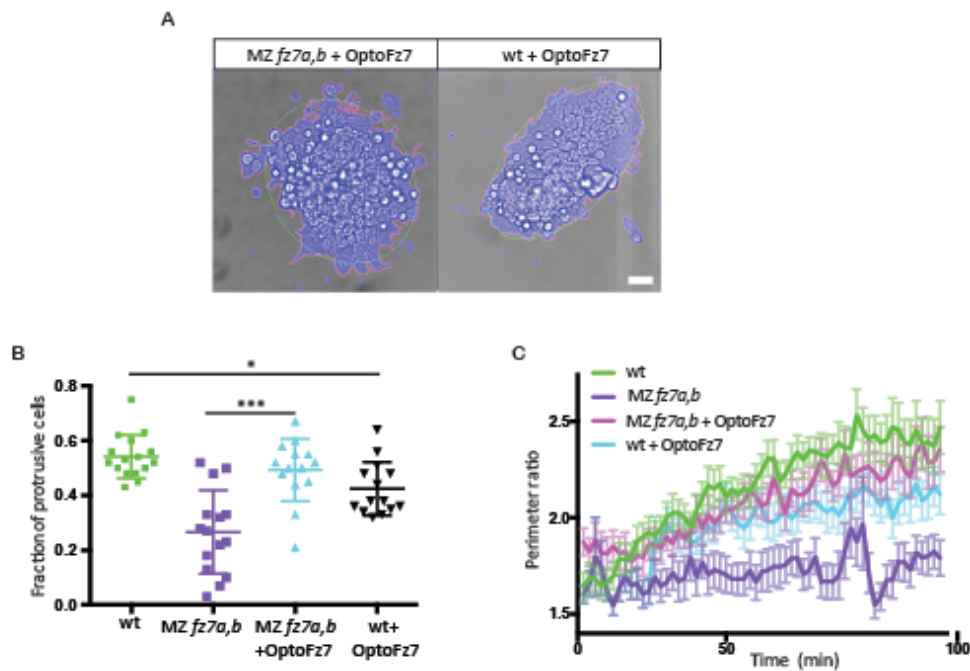


Figure 3.9.3: Effect of OptoFz7 on protrusive behavior of wt and mutant explants *in vitro*. A: Brightfield microscopy images of explants from ppl cell fate induced *Tg(gsc:eGFP-CAAX)* embryos, the genotypes were either mutant (left) or wt (right). The scale bar is 30 μ m. B: Quantification of the fraction of marginal cells that display protrusions after 4 hours in culture. For statistical analysis a Kruskal-Wallis test followed by Dunn's multiple comparison test was used, *** $p<0.001$, * $p<0.05$, N=12 samples per genotype. C: Quantification of the ratio between segmented perimeter (red line in A) and fitted ellipsoid (green line in A) in A (Y-axis) over time (minutes, X-axis). N= 12 samples per genotype.

Additionally we counted the number of marginal cells that displayed protrusions at the end of the experiment (49% after 4 hours in culture). Mutant embryos rescued by OptoFz7 behaved much more like wt explants than like mutant explants (Fig. 3.9.3 A-C).

We also expressed OptoFz7 in explants made from wt embryos and found that continuous and ubiquitous activation in culture led to early plateauing of protrusive activity in these samples, leaving the explants on a level halfway between untreated wt and mutant clusters

(Fig. 3.9.3 A & C). Also the fraction of marginal cells with protrusions was significantly reduced (42%, Fig. 3.9.3 B). Wnt/PCP signaling has been shown to be very sensitive to dosage, with overexpression of Wnt/PCP components often resembling mutant or morphant phenotypes¹³⁷. Therefore the over-activation of the pathway likely disturbs the PCP balance causing the observed gain of function phenotype.

Taken together, these results show that uniform activation of Wnt/PCP signaling is sufficient to rescue the ppl phenotypes of MZ *fz7a,b* mutant embryos and restore the proper balance of Wnt/PCP activity required for protrusion formation.

3.10 CRISPR/Cas9 mediated knock-in

In order to overcome the limitations that ectopic overexpression of tagged constructs pose to the visualization of Wnt/PCP components, we wanted to knock-in a fluorescent protein into the endogenous *fz7b* locus. We targeted a sequence situated in the 3rd extra-cellular loop of the *fz7b* ORF for *loxP* insertion, since the ECLs do not contain any reported functional domains (see Chapter 3.2) and a promising site was predicted by the ChopChop online tool (<http://chopchop.cbu.uib.no/>).

We injected wt embryos with a mixture of *cas9* mRNA, sgRNA, and an oligonucleotide containing the *loxP* site flanked by 20bp homology arms (see Methods chapter 2.13 for a more detailed description). We screened for insertion by PCR and found *loxP* positive bands in 5 out of 16 samples (Fig. 3.10). Since pools of 3 embryos were genotyped this results in efficiency between 10.42% and 31.25%. Fish with this CRISPR treatment were raised, screened by finclip and PCR, and positive ones were sent for sequencing.

We identified three fish, in which the *loxP* site was inserted at the right position and in the right frame. These founders were backcrossed to wt to determine the frequency of germ line transmission. One fish did not appear to transmit the insertion through the germ line; the other two had a transmission rate of 1/8 and 1/16 respectively. The offspring was raised and genotyped.

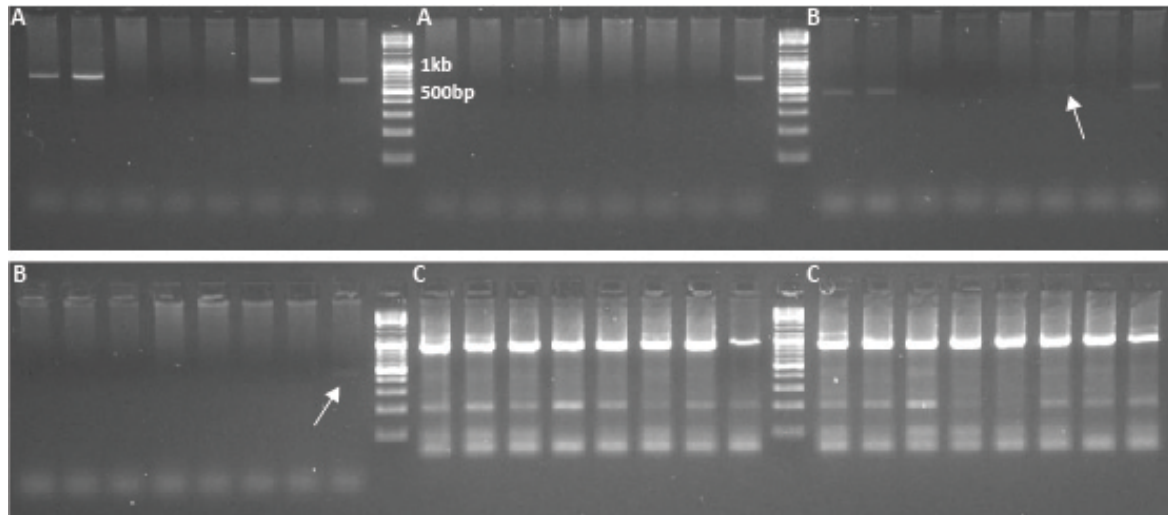


Figure 3.10: Screening for *loxP* insertion after CRISPR/Cas9 mutagenesis. Shown are images from gel electrophoresis of 3 different PCR products. **A:** PCR using *fz7b*-specific forward and *loxP*-specific reverse primers. **B:** PCR using *loxP*-specific forward and *fz7b*-specific reverse primers. **C:** PCR using *fz7b*-specific forward and reverse primers, as a positive control. 16 pools of 3 embryos were analyzed. White arrows mark weak bands.

We used this F1 generation to inject *CRE* mRNA and the recombination plasmid, containing GFP (see methods for details). We started with the amount used in published studies²³⁴, and went up to double and triple amounts, but could not identify any positive embryos by fluorescence signal or PCR.

4 Conclusion

In this project we investigated the mode of function of the Wnt/PCP pathway in a mesenchymal context, namely the directed collective migration of mesendodermal progenitor cells in zebrafish gastrulation. These different cell populations with diverse properties, in contrast to epithelia where the apical-basal polarity determines the principal tissue architecture, have to move into different, sometimes opposite or changing directions during convergence and extension movements. This does not mean that the epithelial situation is trivial; questions about the upstream input, the role of Wnt ligands, or the interaction between the Fat/Ds and Fz/PCP pathways have only recently been resolved or are still under debate. Overall the epithelial function of PCP signaling is still far better understood than the mesenchymal one^{73,74}.

Here, we especially wanted to address the question of whether the Wnt/PCP pathway acts as an instructive guidance cue that polarizes cells towards the animal pole or dorsal midline by forming a signaling gradient, or whether it is a permissive regulator of cell behavior. We addressed this problem directly at the receptor level and chose an optogenetic approach to be able to activate the pathway at specific time points and in specific cell populations. Our results demonstrate that the uniform activation of Fz7 in the whole embryo is sufficient to rescue the ppl phenotype in MZ *fz7a,b* mutant embryos. This result is not compatible with the idea of a required endogenous signaling gradient, be it a ligand or a receptor gradient, and therefore rule out an indispensable instructive function in the ppl.

We show that MZ *fz7a,b* mutants have a ppl migration defect that effectively stops the movement towards the animal pole at mid gastrulation. Since the correlation of mesendodermal and ectodermal cells remains unchanged compared to wt, a tissue separation defect like in *Xenopus*¹⁹¹ cannot be responsible for this phenotype. Heterotypic transplantation experiments reveal that the defect is tissue and cell autonomous. Furthermore through homotypic transplantations and *in vitro* cell culture experiments we demonstrate that protrusion formation of mutant ppl cells is impaired. Uniform activation of OptoFz7 can rescue all these phenotypes and restore cellular behavior that is

indistinguishable from wt cells. Taken together, these results clearly demonstrate that the permissive aspect of Wnt/PCP signaling is sufficient for collective ppl migration.

By activating OptoFz7 either uniformly or in a spatially restricted manner, we show that the very same mechanism can act instructively to guide the direction of cell movement or permissively to regulate migratory behavior without providing a spatial cue. This supports previous findings that show the instructive capacity of PCP signaling if ectopic sources of Wnt are supplied, but also suggests that this is not the way endogenous Wnt/PCP works^{10,58,194}. We furthermore analyzed a PCP gain of function in wt explants and embryos. Over-activation of Wnt/PCP signaling disturbs protrusion formation and consequently also causes aberrant ppl shapes. This finding is in agreement with published data showing that the dosage of Wnt/PCP needs to be tightly regulated in order to function properly¹³⁷.

Importantly, while our data clearly show that a uniform activity of Wnt/Fz7 signaling is sufficient for ppl migration, it is still possible that other parts of the PCP pathway work instructively in the ppl. Ror2, Ryk and Ptk7 for instance have previously been shown to functionally interact with Wnt11 and/or Fz7 in regulating non-canonical Wnt signaling^{113,119,175,260,261}. They might thus also be involved in mediating Wnt11 signaling within the ppl.

4.1 Why not convergence?

Given how well the rescue of various ppl phenotypes worked, from cellular to tissue to embryo level, it was surprising that the convergence of the paraxial and lateral mesoderm and consequently the notochord phenotype of MZ *fz7a,b* mutants could not be rescued at all. On the contrary, the phenotype actually became aggravated. This suggests that the mechanism underlying convergence might be somewhat different from the one in the ppl animal pole migration, although both involve collective migration^{25,35}.

This could be explained by a model in which the Wnt/PCP pathway acts permissively in one tissue but instructively in the other, and therefore one phenotype can be rescued by ubiquitous activity and the other one cannot. This, however, would require a localized

source of PCP signaling. Yet the expression patterns of the two *fz7s* (Fig. 3.9), and both *wnts*, dedicated to PCP signaling⁶¹, show co-expression rather than a sender and a receiver population. The sole exception is the ppl that appears to express *frizzleds* but no *wnts*. The absence of *wnt* expression from the notochord argues against a role as a secreted attractor.

This leaves the possibility of another pathway that interacts with the same downstream targets as Wnt/PCP and functions in a gradient-like fashion. A candidate for this role is the BMP pathway, which forms a ventral to dorsal gradient and has been suggested to instruct the directions of convergence movements by interfering with cell-cell adhesion⁵³, which is in turn required for collective migration⁴⁴. Still, it is unclear, why this would cause the rescue with OptoFz7 to fail or even exacerbate the convergence defect. A possible explanation is that the BMP dependent adhesion cue is very subtle and by activating the Wnt/PCP pathway everywhere it is simply overwritten. The co-operation of uniform Wnt/PCP signaling with another pathway that acts in a gradient-like way actually resembles instructive function.

The idea of subtle gradients opens other options for guidance cues. For example a gradient of tissue stiffness could guide the cells towards the dorsal region, as has been suggested for neural crest cell migration²⁶². This possibility is especially intriguing since the extracellular matrix (ECM) starts to be deposited at the time convergence movements start and ECM organization has been reported to be Wnt/PCP dependent^{131,263-265}. The concept of a non-Wnt/PCP guidance cue is also supported by the finding that Vangl2 localization in the lateral mesoderm is stage-specific, but independent of Wnt/PCP signaling¹⁹⁶.

It is also conceivable that the converging lateral mesoderm is more sensitive to PCP dosage than the prechordal plate, since it has to change direction at mid gastrulation⁶¹. Over-activation of the Wnt/PCP pathway can change cell behavior from stable protrusions to random protrusive activity⁶⁵, and stable mediolaterally oriented protrusions are required, for example, for convergence in *Xenopus*^{45,126}. Although the mechanism of convergence is different in frogs and fish^{35,45}, randomizing protrusion directions generally interferes with collective migration⁴⁷. Finally, it is possible that the rescue actually works but at the same

time a gain of function effect is achieved due to ectopic activation of the pathway, for example in the normally Wnt/PCP-free ventral or notochord region. This gain of function effect might conceal a rescue that is actually happening.

Irrespective of the actual nature of the guidance cue, the convergence of lateral mesoderm cells is controlled by some sort of instructive mechanism that can be disturbed by over-activation of the Wnt/PCP pathway. This can be interpreted as Wnt/PCP instructive function that is not based on a gradient of either receptor or ligand, but a co-factor or interaction with another pathway.

4.2 What is the directionality cue, if not PCP?

The results from this project clearly demonstrate that Wnt/PCP signaling is required for collective migration of the prechordal plate but not to guide the direction of migration. Consequently, the question arises what actually does serve as the guidance cue. Transplantation experiments suggest that the cue is intrinsic to the moving group of ppl cells⁴⁷. In the lateral line primordium, for example, directed migration based on a self-deposited chemokine gradient and polarization based on differential canonical Wnt and FGF expression have been shown^{266,267}. FGF signaling is also essential for ppl migration in *Medaka*, but so far no patterned expression of signaling molecules has been reported in the ppl^{47,268}.

Due to the lack of a known chemical signal, the idea of a physical cue becomes increasingly attractive. The above mentioned hypothesis that cells follow a tissue stiffness gradient regulated by the extracellular matrix would not work for the ppl, since ECM deposition starts only during gastrulation, when the ppl is already on its way²⁶⁹. The vegetally directed movements of the overlying ectoderm could, however, govern the direction of ppl migration, as shear force has the ability to polarize cells²²³. Another possibility is that the ppl polarizes along the highest tension, a process called plithotaxis²⁷⁰. Membrane surface tension can influence cell shape and motility²⁷¹ and the trailing axial mesoderm cells that are pulled by the ppl, could provide such tension. Single *Xenopus* cells, on which pulling force is applied by magnetic beads, polarize along the direction of this pulling force away from the

bead²⁷². If cells in the region between the nc and the ppl, which are sometimes referred to as notochord tip cells are removed by laser ablation, the gap between the two tissues opens, supporting the idea of pulling force between the anterior and posterior axial mesoderm²⁷³. Taken together, these data suggest that the ppl is polarized by a tension gradient that originates from the fact that there are no hypoblast cells in front of the ppl, but the following notochord cells are pulling from behind⁴⁷.

4.3 *Wnt mutants*

Previous studies have suggested that Fz7 is the receptor for Wnt11^{129,137} and the similarity of the gastrulation phenotypes support this notion. However, the phenotypes are not exact copies of each other. MZ *fz7a,b* embryos have a stronger posterior and slightly weaker anterior phenotype than *slb* mutants^{58,63}. *Ppt* embryos, on the other hand, have a mild anterior and severe posterior phenotype. Combined *slb/ppt* double mutants resemble MZ *fz7a,b* embryos with respect to the posterior phenotype, but the combined anterior phenotype is stronger, suggesting redundancy between the *wnts*⁶¹ and also the presence of an additional receptor in the ppl. The combined evidence of expression and phenotype analysis thus suggests a model in which *wnt11* is the prime anterior ligand (with some *wnt5* redundancy) and *wnt5* the posterior one. On the receptor level, *fz2* is a posterior receptor, the two *fz7s* are required along the whole A-V axis, and act partially redundantly with *fz2*¹³⁸, and there is one additional receptor in the ppl. A possible candidate is *fz10*¹³⁴. This idea is also supported by the attempts to rescue the *wnt* mutants with OptoFz7 that resulted only in partial or insignificant rescues (Fig. 3.8).

It is still surprising that the activation of the pathway via the receptor – in this case OptoFz7 - results in different phenotypes than the ubiquitous expression of the ligand^{58,61}. This suggests that Fz7 and the additional receptor have different downstream signaling targets. Since Wnt11 controls cell cohesion in the ppl through E-cadherin surface levels⁵², and we could not detect any E-cadherin localization defects in MZ *fz7a,b* embryos, E-cadherin is a good candidate. Taken together, these data suggest a model, in which Wnt proteins regulate collective migration via protrusive behavior and cell cohesion by signaling through different Frizzled receptors in the anterior and posterior paraxial mesoderm and the ppl.

Furthermore permissive function appears to be sufficient for migration that is aligned with the animal-vegetal axis, but not for medio-lateral convergence movements.

4.4 Outlook

Although we showed in this project that the mode of operation of Wnt/PCP signaling in collective ppl migration is permissive, a number of unanswered questions remain. It is still unclear where the PCP core components localize within the cells of the ppl and the lateral mesoderm and what significance the reported asymmetries of Dvl and Pk have^{56,111}. To address this, live imaging of the endogenous protein would be required, as we planned with the *fz7b* knock-in line. Recent success in CRISPR/Cas-9 mediated knock-ins of fluorescent reporter proteins²⁷⁴ suggest that this might soon be possible. It also remains uncertain why the rescue for the ppl works so well, while the rescue for the converging paraxial mesoderm does not. It would be interesting to test whether this was due to dosage sensitivity or if Wnt/PCP really has a different function in lateral tissues, and thus can act instructive or permissive depending on the context as has been suggested¹⁰. Finally, the question of the guidance cue for the ppl remains. The idea that a tension gradient polarizes the ppl cells away from their followers is intriguing and should be addressed *in vitro* and *in vivo*.

5 References

1. Driever, W. *et al.* A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123**, 37–46 (1996).
2. Haffter, P. *et al.* The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* **123**, 1–36 (1996).
3. Streisinger, G., Walker, C., Dower, N., Knauber, D. & Singer, F. Production of Clones of Homozygous Diploid Zebra Fish (*Brachydanio-Rerio*). *Nature* **291**, 293–296 (1981).
4. Dooley, K. & Zon, L. I. Zebrafish: a model system for the study of human disease. *Current Opinion in Genetics & Development* **10**, 252–256 (2000).
5. Howe, K. *et al.* The zebrafish reference genome sequence and its relationship to the human genome. *Nature Publishing Group* 1–7 (2013). doi:10.1038/nature12111
6. Patton, E. E. & Zon, L. I. The art and design of genetic screens: Zebrafish. *Nat Rev Genet* **2**, 956–966 (2001).
7. Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J. & Stelzer, E. Optical sectioning deep inside live embryos by selective plane illumination microscopy. *Science* **305**, 1007–1009 (2004).
8. PETERS, K. G., RAO, P. S., BELL, B. S. & KINDMAN, L. A. Green Fluorescent Fusion Proteins - Powerful Tools for Monitoring Protein Expression in Live Zebrafish Embryos. *Developmental Biology* **171**, 252–257 (1995).
9. Portugues, R., Severi, K. E., Wyart, C. & Ahrens, M. B. Optogenetics in a transparent animal: circuit function in the larval zebrafish. *Current Opinion in Neurobiology* 1–8 (2012). doi:10.1016/j.conb.2012.11.001
10. Jussila, M. & Ciruna, B. Zebrafish models of non-canonical Wnt/planar cell polarity signalling: fishing for valuable insight into vertebrate polarized cell behavior. *WIREs Dev Biol* **1**, e267 (2017).
11. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of Embryonic Development of the Zebrafish. *Dev. Dyn.* **203**, 1–58 (2004).
12. Yanagimachi, R. *et al.* Sperm Attractant in the Micropyle Region of Fish and Insect Eggs1. *Biology of Reproduction* **88**, 41 (2013).
13. Fuentes, R. & Fernández, J. Ooplasmic segregation in the zebrafish zygote and early embryo: Pattern of ooplasmic movements and transport pathways. *Dev. Dyn.* **239**, 2172–2189 (2010).
14. Kimmel, C. B. & Law, R. D. Cell lineage of zebrafish blastomeres. I. Cleavage pattern and cytoplasmic bridges between cells. *Developmental Biology* **108**, 78–85 (1985).
15. Kimmel, C. B. & Warga, R. M. Indeterminate Cell Lineage of the Zebrafish Embryo. *Developmental Biology* **124**, 269–280 (1987).
16. Abdelilah, S., SolnicaKrezel, L., Stainier, D. & Driever, W. Implications for Dorsoventral Axis Determination From the Zebrafish Mutation Janus. *Nature* **370**, 468–471 (1994).
17. STREHLOW, D. & GILBERT, W. A Fate Map for the 1st Cleavages of the Zebrafish. *Nature* **361**, 451–453 (1993).
18. STREHLOW, D., HEINRICH, G. & GILBERT, W. The Fates of the Blastomeres of the 16-Cell Zebrafish Embryo. *Development* **120**, 1791–1798 (1994).
19. Lu, F.-I., Thisse, C. & Thisse, B. Identification and mechanism of regulation of the zebrafish dorsal determinant. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 15876–15880 (2011).
20. Gore, A. V. *et al.* The zebrafish dorsal axis is apparent at the four-cell stage. *Nature*

- Publishing Group* **438**, 1030–1035 (2005).
21. Kane, D. A. & Kimmel, C. B. The Zebrafish Midblastula Transition. *Development* **119**, 447–456 (1993).
 22. Fukazawa, C. *et al.* Developmental Biology. *Developmental Biology* **346**, 272–283 (2010).
 23. Warga, R. M. & Kimmel, C. B. Cell Movements During Epiboly and Gastrulation in Zebrafish. *Development* **108**, 569–580 (1990).
 24. Morita, H. *et al.* The Physical Basis of Coordinated Tissue Spreading in Zebrafish Gastrulation. *Developmental Cell* **40**, 354–366.e4 (2017).
 25. Solnica-Krezel, L. & Sepich, D. S. Gastrulation: Making and Shaping Germ Layers. *Annu. Rev. Cell Dev. Biol.* **28**, 687–717 (2012).
 26. Thisse, B. & Thisse, C. Seminars in Cell & Developmental Biology. *Seminars in Cell & Developmental Biology* **42**, 94–102 (2015).
 27. Saude, L., Woolley, K., Martin, P., Driever, W. & Stemple, D. L. Axis-inducing activities and cell fates of the zebrafish organizer. *Development* **127**, 3407–3417 (2000).
 28. Shih, J. & Fraser, S. E. Characterizing the zebrafish organizer: microsurgical analysis at the early-shield stage. *Development* **122**, 1313–1322 (1996).
 29. Montero, J. A. Shield formation at the onset of zebrafish gastrulation. *Development* **132**, 1187–1198 (2005).
 30. Shih, J. & Fraser, S. E. Distribution of tissue progenitors within the shield region of the zebrafish gastrula. *Development* **121**, 2755–2765 (1995).
 31. Kane, D. A. & Adams, R. in *Pattern Formation in Zebrafish. Results and Problems in Cell Differentiation* (ed. Solnica-Krezel, L.) **40**, (Springer, 2002).
 32. Keller, P. J., Schmidt, A. D., Wittbrodt, J. & Stelzer, E. H. K. Reconstruction of Zebrafish Early Embryonic Development by Scanned Light Sheet Microscopy. *Science* **322**, 1065–1069 (2008).
 33. Heisenberg, C.-P. & Solnica-Krezel, L. Back and forth between cell fate specification and movement during vertebrate gastrulation. *Current Opinion in Genetics & Development* **18**, 311–316 (2008).
 34. Keller, R. *et al.* Mechanisms of convergence and extension by cell intercalation. *Philosophical Transactions of the Royal Society B: Biological Sciences* **355**, 897–922 (2000).
 35. Tada, M. & Heisenberg, C. P. Convergent extension: using collective cell migration and cell intercalation to shape embryos. *Development* **139**, 3897–3904 (2012).
 36. Carmany-Rampey, A. & Schier, A. F. Single-cell internalization during zebrafish gastrulation. *Curr. Biol.* **11**, 1261–1265 (2001).
 37. Feldman, B. *et al.* Lefty antagonism of squint is essential for normal gastrulation. *Curr. Biol.* **12**, 2129–2135 (2002).
 38. Branford, W. W. & Yost, H. J. Lefty-dependent inhibition of nodal- and Wnt-responsive organizer is essential for normal gene expression gastrulation. *Curr. Biol.* **12**, 2136–2141 (2002).
 39. Schier, A. F. & Talbot, W. S. Molecular genetics of axis formation in zebrafish. *Annual Review of Genetics* **39**, 561–613 (2005).
 40. Neave, B., Holder, N. & Patient, R. A graded response to BMP-4 spatially coordinates patterning of the mesoderm and ectoderm in the zebrafish. *Mechanisms of Development* **62**, 183–195 (1997).

41. Tucker, J. A., Mintzer, K. A. & Mullins, M. C. The BMP Signaling Gradient Patterns Dorsoventral Tissues in a Temporally Progressive Manner along the Anteroposterior Axis. *Developmental Cell* **14**, 108–119 (2008).
42. Myers, D. C., Sepich, D. S. & Solnica-Krezel, L. Bmp Activity Gradient Regulates Convergent Extension during Zebrafish Gastrulation. *Developmental Biology* **243**, 81–98 (2002).
43. Ballard, W. W. Morphogenetic Movements and fate maps. *American Zoologist* **21**, 301–399 (1981).
44. Friedl, P. & Gilmour, D. Collective cell migration in morphogenesis, regeneration and cancer. 1–13 (2009). doi:10.1038/nrm2720
45. Wallingford, J. B., Fraser, S. E. & Harland, R. M. Convergent extension: the molecular control of polarized cell movement during embryonic development. *Developmental Cell* **2**, 695–706 (2002).
46. Sepich, D. S., Calmelet, C., Kiskowski, M. & Solnica-Krezel, L. Initiation of convergence and extension movements of lateral mesoderm during zebrafish gastrulation. *Dev. Dyn.* **234**, 279–292 (2005).
47. Dumortier, J. G., Martin, S., Meyer, D., Rosa, F. M. & David, N. B. Collective mesendoderm migration relies on an intrinsic directionality signal transmitted through cell contacts. *Proceedings of the National Academy of Sciences* **109**, 16945–16950 (2012).
48. Montero, J.-A., Kilian, B., Chan, J., Bayliss, P. E. & Heisenberg, C.-P. Phosphoinositide 3-Kinase Is Required for Process Outgrowth and Cell Polarization of Gastrulating Mesendodermal Cells. *Current Biology* **13**, 1279–1289 (2003).
49. Diz-Muñoz, A. *et al.* Control of Directed Cell Migration In Vivo by Membrane-to-Cortex Attachment. *PLoS Biol* **8**, e1000544 (2010).
50. Sepich, D. S. *et al.* Role of the zebrafish trilobite locus in gastrulation movements of convergence and extension. *genesis* **27**, 159–173 (2000).
51. Weiser, D. C., Row, R. H. & Kimelman, D. Rho-regulated Myosin phosphatase establishes the level of protrusive activity required for cell movements during zebrafish gastrulation. *Development* **136**, 2375–2384 (2009).
52. Ulrich, F. *et al.* Wnt11 Functions in Gastrulation by Controlling Cell Cohesion through Rab5c and E-Cadherin. *Developmental Cell* **9**, 555–564 (2005).
53. Hardt, von der, S. *et al.* The Bmp Gradient of the Zebrafish Gastrula Guides Migrating Lateral Cells by Regulating Cell-Cell Adhesion. *Current Biology* **17**, 475–487 (2007).
54. Warga, R. M. & Kane, D. A. A role for N-cadherin in mesodermal morphogenesis during gastrulation. *Developmental Biology* **310**, 211–225 (2007).
55. Arboleda-Estudillo, Y. *et al.* Movement Directionality in Collective Migration of Germ Layer Progenitors. *Current Biology* **20**, 161–169 (2010).
56. Yin, C., Kiskowski, M., Pouille, P. A., Farge, E. & Solnica-Krezel, L. Cooperation of polarized cell intercalations drives convergence and extension of presomitic mesoderm during zebrafish gastrulation. *The Journal of Cell Biology* **180**, 221–232 (2008).
57. Glickman, N. S., Kimmel, C. B., Jones, M. A. & Adams, R. J. Shaping the zebrafish notochord. *Development* **130**, 873–887 (2003).
58. Heisenberg, C.-P., Tada, M. & Rauch, G.-J. Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature* **405**, 76–81 (2000).

59. Topczewski, J. *et al.* The zebrafish glypican knypek controls cell polarity during gastrulation movements of convergent extension. *Developmental Cell* **1**, 251–264 (2001).
60. Jessen, J. R. *et al.* Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. *Nat Cell Biol* (2002). doi:10.1038/ncb828
61. Kilian, B. *et al.* The role of Ppt/Wnt5 in regulating cell shape and movement during zebrafish gastrulation. *Mechanisms of Development* **120**, 467–476 (2003).
62. Carreira-Barbosa, F. *et al.* Flamingo regulates epiboly and convergence/extension movements through cell cohesive and signalling functions during zebrafish gastrulation. *Development* **136**, 877–877 (2009).
63. Quesada-Hernández, E. *et al.* Stereotypical Cell Division Orientation Controls Neural Rod Midline Formation in Zebrafish. *Current Biology* **20**, 1966–1972 (2010).
64. Li, X. *et al.* Gpr125 modulates Dishevelled distribution and planar cell polarity signaling. *Development* **140**, 3028–3039 (2013).
65. Cheng, X.-N. *et al.* Leucine repeat adaptor protein 1 interacts with Dishevelled to regulate gastrulation cell movements in zebrafish. *Nature Communications* 1–14 (2017). doi:10.1038/s41467-017-01552-x
66. Gubb, D. & García-Bellido, A. A genetic analysis of the determination of cuticular polarity during development in *Drosophila melanogaster*. *J Embryol Exp Morphol* **68**, 37–57 (1982).
67. Lawrence, P. A. & Shelton, P. M. The determination of polarity in the developing insect retina. *J Embryol Exp Morphol* **33**, 471–486 (1975).
68. Vinson, C. R., Conover, S. & Adler, P. N. A *Drosophila* tissue polarity locus encodes a protein containing seven potential transmembrane domains. *Nature* **338**, 263–264 (1989).
69. Adler, P. N. Planar signaling and morphogenesis in *Drosophila*. *Developmental Cell* **2**, 525–535 (2002).
70. Mlodzik, M. Planar cell polarization: do the same mechanisms regulate *Drosophila* tissue polarity and vertebrate gastrulation? *Trends Genet.* **18**, 564–571 (2002).
71. Goodrich, L. V. & Strutt, D. Principles of planar polarity in animal development. *Development* **138**, 1877–1892 (2011).
72. Devenport, D. Cell biology in development: The cell biology of planar cell polarity. *The Journal of Cell Biology* **207**, 171–179 (2014).
73. Yang, Y. & Mlodzik, M. Wnt-Frizzled/Planar Cell Polarity Signaling: Cellular Orientation by Facing the Wind (Wnt). *Annu. Rev. Cell Dev. Biol.* **31**, 623–646 (2015).
74. Singh, J. & Mlodzik, M. Planar cell polarity signaling: coordination of cellular orientation across tissues. *WIREs Dev Biol* **1**, 479–499 (2012).
75. Gray, R. S., Roszko, I. & Solnica-Krezel, L. Planar Cell Polarity: Coordinating Morphogenetic Cell Behaviors with Embryonic Polarity. *Developmental Cell* **21**, 120–133 (2011).
76. Bellaiche, Y., Gho, M., Kaltschmidt, J. A., Brand, A. H. & Schweisguth, F. Frizzled regulates localization of cell-fate determinants and mitotic spindle rotation during asymmetric cell division. *Nat Cell Biol* **3**, 50–57 (2001).
77. Bellaiche, Y. The planar cell polarity protein Strabismus promotes Pins anterior localization during asymmetric division of sensory organ precursor cells in *Drosophila*. *Development* **131**, 469–478 (2003).
78. Ségalen, M. *et al.* The Fz-Dsh Planar Cell Polarity Pathway Induces Oriented Cell

- Division via Mud/NuMA in *Drosophila* and Zebrafish. *Developmental Cell* **19**, 740–752 (2010).
79. Donoughe, S. & DiNardo, S. dachsous and frizzled contribute separately to planar polarity in the *Drosophila* ventral epidermis. *Development* **138**, 2751–2759 (2011).
 80. Yang, C.-H., Axelrod, J. D. & Simon, M. A. Regulation of Frizzled by Fat-like Cadherins during Planar Polarity Signaling in the *Drosophila* Compound Eye. *Cell* **108**, 675–688 (2002).
 81. Lawrence, P. A., Struhl, G. & Casal, J. Planar cell polarity: one or two pathways? *Nat Rev Genet* **8**, 555–563 (2007).
 82. Aigouy, B. *et al.* Cell Flow Reorients the Axis of Planar Polarity in the Wing Epithelium of *Drosophila*. *Cell* **142**, 773–786 (2010).
 83. Sagner, A. *et al.* Establishment of Global Patterns of Planar Polarity during Growth of the *Drosophila* Wing Epithelium. *Curr. Biol.* **22**, 1296–1301 (2012).
 84. Wu, J., Roman, A.-C., Carvajal-Gonzalez, J. M. & Mlodzik, M. Wg and Wnt4 provide long-range directional input to planar cell polarity orientation in *Drosophila*. *Nat Cell Biol* **15**, 1045–1055 (2013).
 85. Strutt, H., Warrington, S. J. & Strutt, D. Dynamics of Core Planar Polarity Protein Turnover and Stable Assembly into Discrete Membrane Subdomains. *Developmental Cell* **20**, 511–525 (2011).
 86. Olguin, P. & Mlodzik, M. A New Spin on Planar Cell Polarity. *Cell* **142**, 674–676 (2010).
 87. Wu, J., Klein, T. J. & Mlodzik, M. Subcellular Localization of Frizzled Receptors, Mediated by Their Cytoplasmic Tails, Regulates Signaling Pathway Specificity. *PLoS Biol* **2**, e158 (2004).
 88. Jenny, A., Reynolds-Kenneally, J., Das, G., Burnett, M. & Mlodzik, M. Diego and Prickle regulate Frizzled planar cell polarity signalling by competing for Dishevelled binding. *Nat Cell Biol* **7**, 691–697 (2005).
 89. Tree, D. R. *et al.* Prickle mediates feedback amplification to generate asymmetric planar cell polarity signaling. *Cell* **109**, 371–381 (2002).
 90. Jenny, A., Darken, R. S., Wilson, P. A. & Mlodzik, M. Prickle and Strabismus form a functional complex to generate a correct axis during planar cell polarity signaling. *EMBO J.* **22**, 4409–4420 (2003).
 91. Amonlirdviman, K. Mathematical Modeling of Planar Cell Polarity to Understand Domineering Nonautonomy. *Science* **307**, 423–426 (2005).
 92. Chen, W.-S. *et al.* Asymmetric Homotypic Interactions of the Atypical Cadherin Flamingo Mediate Intercellular Polarity Signaling. *Cell* **133**, 1093–1105 (2008).
 93. Strutt, H. & Strutt, D. Differential Stability of Flamingo Protein Complexes Underlies the Establishment of Planar Polarity. *Current Biology* **18**, 1555–1564 (2008).
 94. Wu, J., Jenny, A., Mirkovic, I. & Mlodzik, M. Frizzled–Dishevelled signaling specificity outcome can be modulated by Diego in *Drosophila*. *Mechanisms of Development* **125**, 30–42 (2008).
 95. Wu, J. & Mlodzik, M. A quest for the mechanism regulating global planar cell polarity of tissues. *Trends in Cell Biology* **19**, 295–305 (2009).
 96. Shimada, Y., Yonemura, S., Ohkura, H., Strutt, D. & Uemura, T. Polarized Transport of Frizzled along the Planar Microtubule Arrays in *Drosophila* Wing Epithelium. *Developmental Cell* **10**, 209–222 (2006).
 97. Eaton, S., Wepf, R. & Simons, K. Roles for Rac1 and Cdc42 in planar polarization and

- hair outgrowth in the wing of *Drosophila*. *The Journal of Cell Biology* **135**, 1277–1289 (1996).
98. Turner, C. M. & Adler, P. N. Distinct roles for the actin and microtubule cytoskeletons in the morphogenesis of epidermal hairs during wing development in *Drosophila*. *Mechanisms of Development* **70**, 181–192 (1998).
 99. Dabdoub, A. & Kelley, M. W. Planar cell polarity and a potential role for a Wnt morphogen gradient in stereociliary bundle orientation in the mammalian inner ear. *J. Neurobiol.* **64**, 446–457 (2005).
 100. Montcouquiol, M. *et al.* Identification of *Vangl2* and *Scrb1* as planar polarity genes in mammals. *Nature* **423**, 173–177 (2003).
 101. Guo, N., Hawkins, C. & Nathans, J. Frizzled6 controls hair patterning in mice. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 9277–9281 (2004).
 102. Mitchell, B. *et al.* The PCP Pathway Instructs the Planar Orientation of Ciliated Cells in the *Xenopus* Larval Skin. *Current Biology* **19**, 924–929 (2009).
 103. López-Schier, H. & Hudspeth, A. J. A two-step mechanism underlies the planar polarization of regenerating sensory hair cells. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 18615–18620 (2006).
 104. Borovina, A., Superina, S., Voskas, D. & Ciruna, B. *Vangl2* directs the posterior tilting and asymmetric localization of motile primary cilia. *Nat Cell Biol* **12**, 407–412 (2010).
 105. Guirao, B. *et al.* Coupling between hydrodynamic forces and planar cell polarity orients mammalian motile cilia. *Nat Cell Biol* **12**, 341–350 (2010).
 106. Nonaka, S. *et al.* De Novo Formation of Left–Right Asymmetry by Posterior Tilt of Nodal Cilia. *PLoS Biol* **3**, e268 (2005).
 107. Okada, Y., Takeda, S., Tanaka, Y., Belmonte, J.-C. I. & Hirokawa, N. Mechanism of Nodal Flow: A Conserved Symmetry Breaking Event in Left-Right Axis Determination. *Cell* **121**, 633–644 (2005).
 108. Song, H. *et al.* Planar cell polarity breaks bilateral symmetry by controlling ciliary positioning. *Nature* **466**, 378–382 (2011).
 109. Hashimoto, M. *et al.* Planar polarization of node cells determines the rotational axis of node cilia. *Nat Cell Biol* **12**, 170–176 (2010).
 110. Oteiza, P., Koppen, M., Concha, M. L. & Heisenberg, C. P. Origin and shaping of the laterality organ in zebrafish. *Development* **135**, 2807–2813 (2008).
 111. Ciruna, B., Jenny, A., Lee, D., Mlodzik, M. & Schier, A. F. Planar cell polarity signalling couples cell division and morphogenesis during neurulation. *Nat Cell Biol* **439**, 220–224 (2006).
 112. Tissir, F. & Goffinet, A. M. Shaping the nervous system: role of the core planar cell polarity genes. *Nature Publishing Group* **14**, 525–535 (2013).
 113. Gao, B. *et al.* Wnt Signaling Gradients Establish Planar Cell Polarity by Inducing *Vangl2* Phosphorylation through *Ror2*. *Developmental Cell* **20**, 163–176 (2011).
 114. Theveneau, E. *et al.* Chase-and-run between adjacent cell populations promotes directional collective migration. *Nat Cell Biol* **15**, 763–772 (2013).
 115. Mayor, R. & Theveneau, E. The role of the non-canonical Wnt–planar cell polarity pathway in neural crest migration. *Biochem. J.* **457**, 19–26 (2013).
 116. De Calisto, J. Essential role of non-canonical Wnt signalling in neural crest migration. *Development* **132**, 2587–2597 (2005).
 117. Gros, J., Serralbo, O. & Marcelle, C. WNT11 acts as a directional cue to organize the elongation of early muscle fibres. *Nature* **457**, 589–593 (2009).

118. Tada, M. & Smith, J. C. Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through. *Development* **127**, 2227–2238 (2000).
119. Hayes, M., Naito, M., Daulat, A., Angers, S. & Ciruna, B. Ptk7 promotes non-canonical Wnt/PCP-mediated morphogenesis and inhibits Wnt/ -catenin-dependent cell fate decisions during vertebrate development. *Development* **140**, 2245–2245 (2013).
120. Hammerschmidt, M. *et al.* Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, *Danio rerio*. *Development* **123**, 143–151 (1996).
121. Tada, M., Concha, M. L. & Heisenberg, C.-P. Non-canonical Wnt signalling and regulation of gastrulation movements. **13**, 251–260 (2002).
122. Heisenberg, C. P. *et al.* Genes involved in forebrain development in the zebrafish, *Danio rerio*. *Development* **123**, 191–203 (1996).
123. Heisenberg, C. P. & Nüsslein-Volhard, C. The function of *silberblick* in the positioning of the eye anlage in the zebrafish embryo. *Developmental Biology* **184**, 85–94 (1997).
124. Marlow, F., Topczewski, J., Sepich, D. & Solnica-Krezel, L. Zebrafish Rho kinase 2 acts downstream of Wnt11 to mediate cell polarity and effective convergence and extension movements. *Curr. Biol.* **12**, 876–884 (2002).
125. Kim, S. K. *et al.* Planar Cell Polarity Acts Through Septins to Control Collective Cell Movement and Ciliogenesis. *Science* **329**, 1337–1340 (2010).
126. Wallingford, J. B. *et al.* Dishevelled controls cell polarity during Xenopus gastrulation. *Nature* **405**, 81–85 (2000).
127. Voiculescu, O., Bertocchi, F., Wolpert, L., Keller, R. E. & Stern, C. D. The amniote primitive streak is defined by epithelial cell intercalation before gastrulation. *Nature* **449**, 1049–1052 (2007).
128. Bastock, R. & Strutt, D. The planar polarity pathway promotes coordinated cell migration during Drosophila oogenesis. *Development* **134**, 3055–3064 (2007).
129. Witzel, S., Zimyanin, V., Carreira-Barbosa, F., Tada, M. & Heisenberg, C.-P. Wnt11 controls cell contact persistence by local accumulation of Frizzled 7 at the plasma membrane. *The Journal of Cell Biology* **175**, 791–802 (2006).
130. Skoglund, P. & Keller, R. Integration of planar cell polarity and ECM signaling in elongation of the vertebrate body plan. *Current Opinion in Cell Biology* **22**, 589–596 (2010).
131. Jessen, J. R. Recent advances in the study of zebrafish extracellular matrix proteins. *Developmental Biology* **401**, 110–121 (2015).
132. Coyle, R. C., Latimer, A. & Jessen, J. R. Membrane-type 1 matrix metalloproteinase regulates cell migration during zebrafish gastrulation: Evidence for an interaction with non-canonical Wnt signaling. *Experimental Cell Research* **314**, 2150–2162 (2008).
133. Glasauer, S. M. K. & Neuhauss, S. C. F. Whole-genome duplication in teleost fishes and its evolutionary consequences. *Mol Genet Genomics* **289**, 1045–1060 (2014).
134. Nikaido, M., Law, E. W. P. & Kelsh, R. N. A systematic survey of expression and function of zebrafish frizzled genes. *PLoS ONE* **8**, e54833 (2012).
135. Carreira-Barbosa, F. Prickle 1 regulates cell movements during gastrulation and neuronal migration in zebrafish. *Development* **130**, 4037–4046 (2003).
136. Formstone, C. J. & Mason, I. Combinatorial activity of Flamingo proteins directs

- convergence and extension within the early zebrafish embryo via the planar cell polarity pathway. *Developmental Biology* **282**, 320–335 (2005).
137. Djiane, A., Riou, J., Umbhauer, M., Boucaut, J. & Shi, D. Role of frizzled 7 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*. *Development* **127**, 3091–3100 (2000).
 138. Yu, H., Ye, X., Guo, N. & Nathans, J. Frizzled 2 and frizzled 7 function redundantly in convergent extension and closure of the ventricular septum and palate: evidence for a network of interacting genes. *Development* **139**, 4383–4394 (2012).
 139. Vinson, C. R. & Adler, P. N. Directional non-cell autonomy and the transmission of polarity information by the frizzled gene of *Drosophila*. (1987).
 140. Zheng, L., Zhang, J. & Carthew, R. W. frizzled regulates mirror-symmetric pattern formation in the *Drosophila* eye. *Development* **121**, 3045–3055 (1995).
 141. Wang, Y., Thekdi, N., Smallwood, P. M., Macke, J. P. & Nathans, J. Frizzled-3 is required for the development of major fiber tracts in the rostral CNS. *Journal of Neuroscience* **22**, 8563–8573 (2002).
 142. Yu, H. *et al.* Frizzled 1 and frizzled 2 genes function in palate, ventricular septum and neural tube closure: general implications for tissue fusion processes. *Development* **137**, 3707–3717 (2010).
 143. Quesada-Hernández, E. *et al.* Stereotypical cell division orientation controls neural rod midline formation in zebrafish. *Curr. Biol.* **20**, 1966–1972 (2010).
 144. Gao, C. & Chen, Y.-G. Dishevelled: The hub of Wnt signaling. *Cellular Signalling* **22**, 717–727 (2010).
 145. Axelrod, J. D., Miller, J. R., Shulman, J. M., Moon, R. T. & Perrimon, N. Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. *Genes & Development* **12**, 2610–2622 (1998).
 146. Boutros, M., Paricio, N., Strutt, D. I. & Mlodzik, M. Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell* **94**, 109–118 (1998).
 147. Park, T. J., Mitchell, B. J., Abitua, P. B., Kintner, C. & Wallingford, J. B. Dishevelled controls apical docking and planar polarization of basal bodies in ciliated epithelial cells. *Nat. Genet.* **40**, 871–879 (2008).
 148. Klingensmith, J., Nusse, R. & Perrimon, N. The *Drosophila* segment polarity gene dishevelled encodes a novel protein required for response to the wingless signal. *Genes & Development* **8**, 118–130 (1994).
 149. Sokol, S. Y., Klingensmith, J., Perrimon, N. & Itoh, K. Dorsalizing and neuralizing properties of Xdsh, a maternally expressed *Xenopus* homolog of dishevelled. *Development* **121**, 3487 (1995).
 150. Sussman, D. J. *et al.* Isolation and characterization of a mouse homolog of the *Drosophila* segment polarity gene dishevelled. *Developmental Biology* **166**, 73–86 (1994).
 151. Etheridge, S. L. *et al.* Murine Dishevelled 3 Functions in Redundant Pathways with Dishevelled 1 and 2 in Normal Cardiac Outflow Tract, Cochlea, and Neural Tube Development. *PLoS Genet* **4**, e1000259 (2008).
 152. Goto, T. & Keller, R. The Planar Cell Polarity Gene Strabismus Regulates Convergence and Extension and Neural Fold Closure in *Xenopus*. *Developmental Biology* **247**, 165–181 (2002).
 153. Taylor, J., Abramova, N., Charlton, J. & Adler, P. N. Van Gogh: a new *Drosophila*

- tissue polarity gene. *Genetics* **150**, 199–210 (1998).
154. Wolff, T. & Rubin, G. M. Strabismus, a novel gene that regulates tissue polarity and cell fate decisions in *Drosophila*. *Development* **125**, 1149–1159 (1998).
 155. Shafer, B., Onishi, K., Lo, C., Colakoglu, G. & Zou, Y. Vangl2 Promotes Wnt/Planar Cell Polarity-like Signaling by Antagonizing Dvl1-Mediated Feedback Inhibition in Growth Cone Guidance. *Developmental Cell* **20**, 177–191 (2011).
 156. Usui, T. *et al.* Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. *Cell* **98**, 585–595 (1999).
 157. Curtin, J. A. *et al.* Mutation of Celsr1 Disrupts Planar Polarity of Inner Ear Hair Cells and Causes Severe Neural Tube Defects in the Mouse. *Current Biology* **13**, 1129–1133 (2003).
 158. Das, G., Reynolds-Kenneally, J. & Mlodzik, M. The atypical cadherin Flamingo links Frizzled and Notch signaling in planar polarity establishment in the *Drosophila* eye. *Developmental Cell* **2**, 655–666 (2002).
 159. Qu, Y. *et al.* Atypical Cadherins Celsr1-3 Differentially Regulate Migration of Facial Branchiomotor Neurons in Mice. *Journal of Neuroscience* **30**, 9392–9401 (2010).
 160. Fenstermaker, A. G. *et al.* Wnt/Planar Cell Polarity Signaling Controls the Anterior-Posterior Organization of Monoaminergic Axons in the Brainstem. *Journal of Neuroscience* **30**, 16053–16064 (2010).
 161. Takeuchi, M. *et al.* The prickle-Related Gene in Vertebrates Is Essential for Gastrulation Cell Movements. *Current Biology* **13**, 674–679 (2003).
 162. Veeman, M. T., Slusarski, D. C., Kaykas, A., Louie, S. H. & Moon, R. T. Zebrafish Prickle, a Modulator of Noncanonical Wnt/Fz Signaling, Regulates Gastrulation Movements. *Current Biology* **13**, 680–685 (2003).
 163. Antic, D. *et al.* Planar Cell Polarity Enables Posterior Localization of Nodal Cilia and Left-Right Axis Determination during Mouse and *Xenopus* Embryogenesis. *PLoS ONE* **5**, e8999 (2010).
 164. Das, G. Diego interacts with Prickle and Strabismus/Van Gogh to localize planar cell polarity complexes. *Development* **131**, 4467–4476 (2004).
 165. Simons, M. *et al.* Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways. *Nat. Genet.* **37**, 537–543 (2005).
 166. Feiguin, F., Hannus, M., Mlodzik, M. & Eaton, S. The ankyrin repeat protein Diego mediates Frizzled-dependent planar polarization. *Developmental Cell* **1**, 93–101 (2001).
 167. Schwarz-Romond, T. *et al.* The ankyrin repeat protein Diversin recruits Casein kinase I ϵ to the beta-catenin degradation complex and acts in both canonical Wnt and Wnt/JNK signaling. *Genes & Development* **16**, 2073–2084 (2002).
 168. Itoh, K., Jenny, A., Mlodzik, M. & Sokol, S. Y. Centrosomal localization of Diversin and its relevance to Wnt signaling. *Journal of Cell Science* **122**, 3791–3798 (2009).
 169. Ohkawara, B. Role of glypican 4 in the regulation of convergent extension movements during gastrulation in *Xenopus laevis*. *Development* **130**, 2129–2138 (2003).
 170. Dabdoub, A. Wnt signaling mediates reorientation of outer hair cell stereociliary bundles in the mammalian cochlea. *Development* **130**, 2375–2384 (2003).
 171. Qian, D. *et al.* Wnt5a functions in planar cell polarity regulation in mice. *Developmental Biology* **306**, 121–133 (2007).

172. Andre, P., Song, H., Kim, W., Kispert, A. & Yang, Y. Wnt5a and Wnt11 regulate mammalian anterior-posterior axis elongation. *Development* **142**, 1516–1527 (2015).
173. Ye, Z. *et al.* Wnt5a uses CD146 as a receptor to regulate cell motility and convergent extension. *Nature Communications* **4**, 1–11 (2013).
174. Shnitsar, I. & Borchers, A. PTK7 recruits dsh to regulate neural crest migration. *Development* **135**, 4015–4024 (2008).
175. Yen, W. W. *et al.* PTK7 is essential for polarized cell motility and convergent extension during mouse gastrulation. *Development* **136**, 2039–2048 (2009).
176. Wallingford, J. B. & Habas, R. The developmental biology of Dishevelled: an enigmatic protein governing cell fate and cell polarity. *Development* **132**, 4421–4436 (2005).
177. Punchihewa, C., Ferreira, A. M., Cassell, R., Rodrigues, P. & Fujii, N. Sequence requirement and subtype specificity in the high-affinity interaction between human frizzled and dishevelled proteins. *Protein Science* **18**, 994–1002 (2009).
178. Wong, H.-C. *et al.* Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled. *Molecular Cell* **12**, 1251–1260 (2003).
179. Hamblet, N. S. Dishevelled 2 is essential for cardiac outflow tract development, somite segmentation and neural tube closure. *Development* **129**, 5827–5838 (2002).
180. Wang, J. *et al.* Regulation of polarized extension and planar cell polarity in the cochlea by the vertebrate PCP pathway. *Nat. Genet.* **37**, 980–985 (2005).
181. Wang, Y. The Role of Frizzled3 and Frizzled6 in Neural Tube Closure and in the Planar Polarity of Inner-Ear Sensory Hair Cells. *Journal of Neuroscience* **26**, 2147–2156 (2006).
182. Habas, R., Kato, Y. & He, X. Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1. *Cell* **107**, 843–854 (2001).
183. Tanegashima, K., Zhao, H. & Dawid, I. B. WGEF activates Rho in the Wnt-PCP pathway and controls convergent extension in *Xenopus* gastrulation. *EMBO J.* **27**, 606–617 (2008).
184. Zhu, S., Liu, L., Korzh, V., Gong, Z. & Low, B. C. RhoA acts downstream of Wnt5 and Wnt11 to regulate convergence and extension movements by involving effectors Rho Kinase and Diaphanous: Use of zebrafish as an in vivo model for GTPase signaling. *Cellular Signalling* **18**, 359–372 (2006).
185. Etienne-Manneville, S. & Hall, A. Rho GTPases in cell biology. *Nature* **420**, 629–635 (2002).
186. Witze, E. S., Litman, E. S., Argast, G. M., Moon, R. T. & Ahn, N. G. Wnt5a control of cell polarity and directional movement by polarized redistribution of adhesion receptors. *Science* **320**, 365–369 (2008).
187. Habas, R., Dawid, I. B. & He, X. Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. *Genes & Development* **17**, 295–309 (2003).
188. Rosso, S. B., Sussman, D., Wynshaw-Boris, A. & Salinas, P. C. Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development. *Nat Neurosci* **8**, 34–42 (2004).
189. Sato, A. *et al.* Profilin is an effector for Daam1 in non-canonical Wnt signaling and is required for vertebrate gastrulation. *Development* **133**, 4219–4231 (2006).

190. Matussek, T. *et al.* Formin Proteins of the DAAM Subfamily Play a Role during Axon Growth. *Journal of Neuroscience* **28**, 13310–13319 (2008).
191. Winklbauer, R., Medina, A., Swain, R. K. & Steinbeisser, H. Frizzled-7 signalling controls tissue separation during *Xenopus* gastrulation. *Nature* **413**, 856–860 (2001).
192. Collu, G. M. & Mlodzik, M. Planar Polarity: Converting a Morphogen Gradient into Cellular Polarity. *Curr. Biol.* **25**, R372–R374 (2015).
193. Humphries, A. C. & Mlodzik, M. ScienceDirectFrom instruction to output: Wnt/PCP signaling in development and cancer. *Current Opinion in Cell Biology* **51**, 110–116 (2017).
194. Chu, C.-W. & Sokol, S. Y. Wnt proteins can direct planar cell polarity in vertebrate ectoderm. *eLife Sciences* **5**, (2016).
195. Wyngaarden, L. A. *et al.* Oriented cell motility and division underlie early limb bud morphogenesis. *Development* **137**, 2551–2558 (2010).
196. Roszko, I., S Sepich, D., Jessen, J. R., Chandrasekhar, A. & Solnica-Krezel, L. A dynamic intracellular distribution of Vangl2 accompanies cell polarization during zebrafish gastrulation. *Development* **142**, 2508–2520 (2015).
197. Panousopoulou, E., Tyson, R. A., Bretschneider, T. & Green, J. B. A. The distribution of Dishevelled in convergently extending mesoderm. *Developmental Biology* 1–8 (2013). doi:10.1016/j.ydbio.2013.07.012
198. Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S. & Roth, B. L. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 5163–5168 (2007).
199. Coward, P. *et al.* Controlling signaling with a specifically designed Gi-coupled receptor. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 352–357 (1998).
200. Oh, E., Maejima, T., Liu, C., Deneris, E. & Herlitze, S. Substitution of 5-HT_{1A} Receptor Signaling by a Light-activated G Protein-coupled Receptor. *Journal of Biological Chemistry* **285**, 30825–30836 (2010).
201. Sako, K. *et al.* Optogenetic Control of Nodal Signaling Reveals a Temporal Pattern of Nodal Signaling Regulating Cell Fate Specification during Gastrulation. *CellReports* 1–13 (2016). doi:10.1016/j.celrep.2016.06.036
202. Barrangou, R. *et al.* CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**, 1709–1712 (2007).
203. Marraffini, L. A. & Sontheimer, E. J. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* **322**, 1843–1845 (2008).
204. Garneau, J. E. *et al.* The CRISPR/Cas bacterial immunesystem cleaves bacteriophage and plasmid DNA. *Nature Publishing Group* **468**, 67–71 (2010).
205. Hale, C. R. *et al.* RNA-Guided RNA Cleavageby a CRISPR RNA-Cas Protein Complex. *Cell* **139**, 945–956 (2009).
206. Wiedenheft, B., Sternberg, S. H. & Doudna, J. A. RNA-guided genetic silencing systems in bacteria and archaea. *Nature Publishing Group* **482**, 331–338 (2012).
207. Auer, T. O. & Del Bene, F. CRISPR/Cas9 and TALEN-mediated knock-in approaches in zebrafish. *METHODS* 1–9 (2014). doi:10.1016/j.ymeth.2014.03.027
208. Hwang, W. Y. *et al.* Heritable and Precise Zebrafish Genome Editing Using a CRISPR-Cas System. *PLoS ONE* **8**, e68708 (2013).
209. Jao, L.-E., Wente, S. R. & Chen, W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proceedings of the National Academy of Sciences* **110**, 13904–13909 (2013).

210. Chang, N. *et al.* Genome editing with RNA-guided Cas9 nuclease in Zebra sh embryos. *Cell Res* **23**, 465–472 (2013).
211. Auer, T. O., Durore, K., De Cian, A., Concordet, J. P. & Del Bene, F. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Research* **24**, 142–153 (2014).
212. Williams, M. L. & Solnica-Krezel, L. ScienceDirectRegulation of gastrulation movements by emergent cell and tissue interactions. *Current Opinion in Cell Biology* **48**, 33–39 (2017).
213. Campinho, P. *et al.* Tension-oriented cell divisions limit anisotropic tissue tension in epithelial spreading during zebrafish epiboly. *Nat Cell Biol* **5**, 1–13 (2013).
214. Hwang, W. Y. *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnology* 1–3 (2013). doi:10.1038/nbt.2501
215. Pan, X., Wan, H., Chia, W., Tong, Y. & Gong, Z. Demonstration of site-directed recombination in transgenic zebrafish using the Cre/loxP system. *Transgenic Res* **14**, 217–223 (2005).
216. Kwan, K. M. *et al.* The Tol2kit: A multisite gateway-based construction kit forTol2 transposon transgenesis constructs. *Dev. Dyn.* **236**, 3088–3099 (2007).
217. Yun, C. O. *et al.* dl-VSVG-LacZ, a vesicular stomatitis virus glycoprotein epitope-incorporated adenovirus, exhibits marked enhancement in gene transduction efficiency. *Human Gene Therapy* **14**, 1643–1652 (2003).
218. Pingoud, A., Fuxreiter, M., Pingoud, V. & Wende, W. Type II restriction endonucleases: structure and mechanism. *Cellular and Molecular Life Sciences* **62**, 685–707 (2005).
219. Engler, C. & Marillonnet, S. in *DNA Cloning and Assembly Methods* (eds. Valla, S. & Lale, R.) 119–131 (Humana Press, 2014).
220. Engler, C., Gruetzner, R., Kandzia, R. & Marillonnet, S. Golden Gate Shuffling: A One-Pot DNA Shuffling Method Based on Type IIs Restriction Enzymes. *PLoS ONE* **4**, e5553 (2009).
221. Airan, R. D., Thompson, K. R., Fenno, L. E., Bernstein, H. & Deisseroth, K. Temporally precise in vivo control of intracellular signalling. *Nature* **458**, 1025–1029 (2009).
222. Westerfield, M. *The zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio)*. (Univ. of Oregon Press, 2000).
223. Smutny, M. *et al.* Friction forces position the neural anlage. *Nat Cell Biol* **19**, 306–317 (2017).
224. Barone, V. *et al.* An Effective Feedback Loop between Cell-Cell Contact Duration and Morphogen Signaling Determines Cell Fate. *Developmental Cell* **43**, 198–211.e12 (2017).
225. Rauch, G. J. *et al.* Wnt5 is required for tail formation in the zebrafish embryo. *Cold Spring Harbor Symposia in Quantitative Biology* **62**, 227–234 (1997).
226. Alexander, J., Rothenberg, M., Henry, G. L. & Stainier, D. Y. casanova plays an early and essential role in endoderm formation in zebrafish. *Developmental Biology* **215**, 343–357 (1999).
227. David, N. B. & Rosa, F. M. Cell autonomous commitment to an endodermal fate and behaviour by activation of Nodal signalling. *Development* **128**, 3937–3947 (2001).
228. Aoki, T. O. *et al.* Molecular integration of casanova in the Nodal signalling pathway controlling endoderm formation. *Development* **129**, 275–286 (2002).
229. Krieg, M. *et al.* Tensile forces govern germ-layer organization in zebrafish. *Nat Cell*

- Biol* **10**, 429–436 (2008).
230. Ruprecht, V. *et al.* Cortical Contractility Triggers a Stochastic Switch to Fast Amoeboid Cell Motility. *Cell* **160**, 673–685 (2015).
 231. Xu, Y. *et al.* Optogenetic control of chemokine receptor signal and T-cell migration. *Proceedings of the National Academy of Sciences* (2014). doi:10.1073/pnas.1319296111
 232. Thisse, C. & Thisse, B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nature Protocols* **3**, 59–69 (2008).
 233. Meeker, N., Hutchinson, S., Ho, L. & Trede, N. Method for isolation of PCR-ready genomic DNA from zebrafish tissues. *Biotech.* **43**, 610–614 (2007).
 234. Horstick, E. J. *et al.* Increased functional protein expression using nucleotide sequence features enriched in highly expressed genes in zebrafish. *Nucleic Acids Research* **43**, e48–e48 (2015).
 235. Campàs, O. *et al.* Quantifying cell-generated mechanical forces within living embryonic tissues. *Nat Meth* **11**, 183–189 (2014).
 236. Krens, S. F. G. *et al.* Interstitial fluid osmolarity modulates the action of differential tissue surface tension in progenitor cell segregation during gastrulation. *Development* **144**, 1798–1806 (2017).
 237. Rauch, G. J. *et al.* Submission and Curation of Gene Expression Data. *ZFIN Direct Data Submission* (<http://zfin.org>). (2003). Available at: (Accessed: 23rd February 2018)
 238. Thisse, C. & Thisse, B. High Throughput Expression Analysis of ZF-Models Consortium Clones. *ZFIN Direct Data Submission* (<http://zfin.org>). (2005). Available at: (Accessed: 23rd February 2018)
 239. Thisse, B. & Thisse, C. Fast Release Clones: A High Throughput Expression Analysis. *ZFIN Direct Data Submission* (<http://zfin.org>). (2004). Available at: (Accessed: 20 February 2004)
 240. El-Messaoudi, S. & Renucci, A. Expression pattern of the frizzled 7 gene during zebrafish embryonic development. *Mechanisms of Development* **102**, 231–234 (2001).
 241. Ulrich, F. Slb/Wnt11 controls hypoblast cell migration and morphogenesis at the onset of zebrafish gastrulation. *Development* **130**, 5375–5384 (2003).
 242. Schulte, G. & Bryja, V. The Frizzled family of unconventional G-protein-coupled receptors. *Trends in Pharmacological Sciences* **28**, 518–525 (2007).
 243. Strutt, D., Madder, D., Chaudhary, V. & Artymiuk, P. J. Structure-Function Dissection of the Frizzled Receptor in *Drosophila melanogaster* Suggests Different Mechanisms of Action in Planar Polarity and Canonical Wnt Signaling. *Genetics* **192**, 1295–1313 (2012).
 244. Katoh, M. & Katoh, M. Comparative Genomics on Fzd7 orthologs. *International Journal of Molecular Medicine* 1–5 (2005).
 245. Tauriello, D. V. *et al.* Wnt/ β -catenin signaling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E812–E820 (2012).
 246. Povelones, M. Genetic Evidence That *Drosophila* frizzled Controls Planar Cell Polarity and Armadillo Signaling by a Common Mechanism. *Genetics* **171**, 1643–1654 (2005).
 247. Jones, K. H., Liu, J. & Adler, P. N. Molecular analysis of EMS-induced frizzled

- mutations in *Drosophila melanogaster*. *Genetics* **142**, 205–215 (1996).
248. Cong, F. Wnt signals across the plasma membrane to activate the β -catenin pathway by forming oligomers containing its receptors, Frizzled and LRP. *Development* **131**, 5103–5115 (2004).
 249. Palczewski, K. Crystal Structure of Rhodopsin: A G Protein-Coupled Receptor. *Science* **289**, 739–745 (2000).
 250. Kleinlogel, S. Optogenetic user's guide to Opto-GPCRs. *Frontiers in bioscience (Landmark edition)* (2015).
 251. Kim, J.-M. *et al.* Light-Driven Activation of β 2-Adrenergic Receptor Signaling by a Chimeric Rhodopsin Containing the β 2-Adrenergic Receptor Cytoplasmic Loops $\dagger$. *Biochemistry* **44**, 2284–2292 (2005).
 252. Liu, T. *et al.* G protein signaling from activated rat frizzled-1 to the beta-catenin-Lef-Tcf pathway. *Science* **292**, 1718–1722 (2001).
 253. Rasmussen, S. G. F. *et al.* Crystal structure of the β 2 adrenergic receptor–Gs protein complex. *Nature* **477**, 549–555 (2011).
 254. OVCHINNIKOV, Y. A. Rhodopsin and Bacteriorhodopsin - Structure-Function-Relationships. *FEBS Lett.* **148**, 179–191 (1982).
 255. Hargrave, P. A. *et al.* Rhodopsins Protein and Carbohydrate Structure - Selected Aspects. *Vision Res.* **24**, 1487–& (1984).
 256. Sekharan, S. & Morokuma, K. Why 11- cis-Retinal? Why Not 7- cis-, 9- cis-, or 13- cis-Retinal in the Eye? *J. Am. Chem. Soc.* **133**, 19052–19055 (2011).
 257. Park, T. J., Gray, R. S., Sato, A., Habas, R. & Wallingford, J. B. Subcellular Localization and Signaling Properties of Dishevelled in Developing Vertebrate Embryos. *Current Biology* **15**, 1039–1044 (2005).
 258. Onishi, K. *et al.* Antagonistic Functions of Dishevelleds Regulate Frizzled3 Endocytosis via Filopodia Tips in Wnt-Mediated Growth Cone Guidance. *Journal of Neuroscience* **33**, 19071–19085 (2013).
 259. Carter, M. E. & de Lecea, L. Optogenetic investigation of neural circuits in vivo. *Trends in Molecular Medicine* **17**, 197–206 (2011).
 260. Lin, S., Baye, L. M., Westfall, T. A. & Slusarski, D. C. Wnt5b-Ryk pathway provides directional signals to regulate gastrulation movement. *The Journal of Cell Biology* **190**, 263–278 (2010).
 261. Yu, A. *et al.* Ryk cooperates with Frizzled 7 to promote Wnt11-mediated endocytosis and is essential for *Xenopus laevis* convergent extension movements. *The Journal of Cell Biology* **182**, 1073–1082 (2008).
 262. Barriga, E. H., Franze, K., Charras, G. & Mayor, R. Tissue stiffening coordinates morphogenesis by triggering collective cell migration in vivo. *Nature Publishing Group* 1–19 (2018). doi:10.1038/nature25742
 263. Dohn, M. R., Mundell, N. A., Sawyer, L. M., Dunlap, J. A. & Jessen, J. R. Planar cell polarity proteins differentially regulate extracellular matrix organization and assembly during zebrafish gastrulation. *Developmental Biology* **383**, 39–51 (2013).
 264. Dzamba, B. J., Jakab, K. R., Marsden, M., Schwartz, M. A. & DeSimone, D. W. Cadherin Adhesion, Tissue Tension, and Noncanonical Wnt Signaling Regulate Fibronectin Matrix Organization. *Developmental Cell* **16**, 421–432 (2009).
 265. Goto, T., Davidson, L., Asashima, M. & Keller, R. Planar Cell Polarity Genes Regulate Polarized Extracellular Matrix Deposition during Frog Gastrulation. *Current Biology* **15**, 787–793 (2005).

- 266. Donà, E. *et al.* Directional tissue migration through a self-generated chemokine gradient. *Nature Publishing Group* **503**, 285–289 (2013).
- 267. Aman, A. & Piotrowski, T. Wnt/b-Catenin and Fgf Signaling Control Collective Cell Migration by Restricting Chemokine Receptor Expression. *Developmental Cell* **15**, 749–761 (2008).
- 268. Shimada, A. *et al.* Maternal-zygotic medaka mutants for fgfr1 reveal its essential role in the migration of the axial mesoderm but not the lateral mesoderm. *Development* **135**, 281–290 (2007).
- 269. Latimer, A. & Jessen, J. R. Matrix Biology. *Matrix Biology* **29**, 89–96 (2010).
- 270. Trepap, X. & Fredberg, J. J. Plithotaxis and emergent dynamics in collective cellular migration. *Trends in Cell Biology* **21**, 638–646 (2011).
- 271. Diz-Muñoz, A., Fletcher, D. A. & Weiner, O. D. Use the force: membrane tension as an organizer of cell shape and motility. *Trends in Cell Biology* **23**, 47–53 (2013).
- 272. Weber, G. F., Bjerke, M. A. & DeSimone, D. W. A Mechanoresponsive Cadherin-Keratin Complex Directs Polarized Protrusive Behavior and Collective Cell Migration. *Developmental Cell* **22**, 104–115 (2012).
- 273. Bosze, B. *et al.* Pcdh18a-positive tip cells instruct notochord formation in zebrafish. doi:10.1101/257717
- 274. Kesavan, G., Chekuru, A., Machate, A. & Brand, M. CRISPR/Cas9-Mediated Zebrafish Knock-in as a Novel Strategy to Study Midbrain-Hindbrain Boundary Development. *Front. Neuroanat.* **11**, 278 (2017).

Appendix A – Construct sequences

Golden Gate Mother construct

ACCATGAAAACGATCATCGCCCTGAGCTACATCTTCTGCCTGGTATTGCCATGTACACCGATATAGA
GATGAACAGGCTGGGAAAGGATAGCCTCATGAACGGGACCGAGGGCCCAAACCTTCTACGTGCCTTT
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Rhodopsin

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RRFR

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TAA

RRFF

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FFFF

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