2. Material and Methods

2.1 Animal Experiments

2.1.1 Zebrafish husbandry

Zebrafish were maintained and raised at 28.5°C under standard conditions (Westerfield, 1993), and staged according to Kimmel et al. (1995).
Embryos were kept in egg water (60 µg/ml Instant Ocean Sea Salts, Aquarium Systems Inc, USA), and 0.003% 1-phenyl-2-thiourea (PTU, Sigma) was used to suppress pigmentation. The construct for the *Tg*(*cldnB:GFP*) line was generated by fusing the 8 kb promoter sequence upstream of the ATG of zebrafish *claudinB* (Kollmar et al., 2001) to a fusion construct encoding membrane-tethered GFP (lynGFP) (Haas and Gilmour, 2006). Whole embryos were observed using a dissecting stereomicroscope (Leica MZ12, Leica, Germany) equipped with a digital camera (Spot Insight, Visitron, USA), and were staged by hours post fertilization (hpf) at 28.5°C or by counting somites.

2.1.2 Morpholino Injections

Morpholino antisense oligonucleotides were purchased from GENE TOOLS, LLC, USA. Sequences were chosen to target the ATG initiation site of translation region in the *lgl2* homologue gene, which resulted in a block of gene translation. The Morpholinos were injected at a concentration of 70, 80 and 100 µM in sterile ddH2O using glass micropipettes and a microinjector/micromanipulator setup (MPPI-2 Pressure Injector/ BP15 Back Pressure Unit, Applied Scientific Instrumentation, USA; MM33 Micromanipulator, Maerzhaeuser, Germany). One to two cell stage embryos were used for the injection of approximately 4 nl Morpholino-solution.
MO sequences (5'→3'):

*lgl2**UTRa***MO: 5'-TCCCTGGACGAGCCGGGACTCAAAC-3'
*lgl2**UTRb***MO: 5'-AGCCGGGACTCAAACTGCCCTCTCT-3'
*lgl2**ATG***MO: 5'-GCCCATGACGCCTGAACCTCTTCAT-3'
*has*MO: 5'-TGTCCCGCAGCGTGGGCATTATGGA-3'
*nok*MO: 5'-TGAGGTCAGCAGCGGCTCCAAACAC-3'
*myoVIa**ATG***MO: 5'-CCACACCGGCTTTCCATCGTCCATT-3'
*myoVIb**ATG***MO: 5'-CCACACAAGCTTCCCATCATCCATT-3'

2.1.3 Morpholino design

In order to design the Morpholinos, the genomic DNA from AB strain was extracted from 24 hpf embryos and was sequenced in the area flanking the ATG initiation site of translation or in the exon/intron boundary of the first WD repeats. The sequences were compared to the sequences published in the Sanger Zv4.0 genomic sequence zebrafish database (http://www.ensembl.org/Danio_rerio/index.html), and the Morpholinos were designed as antisense to the requested targeted area.

2.1.4 RNA and DNA Injections

Constructs were transcribed using the SP6 MessageMachine kit (Ambion). *In vitro* synthesized capped mRNA was dissolved in nuclease-free water. For rescue experiments, the mRNA was mixed with the MO prior to injection. Typically, 100ng/µl of RNA were injected. The pCS2+14xUAS *E1b lgl2:eGFP* construct was injected together with pB EF-1α Gal4-VP16 (Koester and Fraser, 2001) in a concentration of 30ng/µl each, with or without MO.

2.1.5 Mosaic Analysis

Genetic mosaics were generated by transplantation, described by Ho RK and Kane DA (1990) with some modulations. Donor embryos were injected at the one-cell stage with 1 mg/ml of the lineage tracer rhodamine dextran 10 kDa (Molecular Probes), with or without MO, under a dissecting stereomicroscope and were allowed to develop until the blastula stage. Approximately 20–30 cells were then transplanted into age-matched *Tg(cldnB:GFP)*-positive host embryos, wild type or injected with MO, at a region corresponding to the future pllp according to the zebrafish fate-map (Kimmel et al., 1990). The next day, recipient embryos were screened for the presence of red cells in the pllo and were imaged using a Leica TCS SP2 confocal microscope.

2.1.6 Time-Lapse Imaging

AB embryos were stained prior to imaging with 100 µM vital dye BODIPY-ceramide (Molecular Probes) in Danieau's for 1 hr and then transferred into egg water for 2 hrs. The embryos were mounted in 1.8% low melting Agarose and anesthetized with 0.2mg/ml tricaine (3-amino benzoic acid ethyl ester, Sigma, USA) prior to imaging with Zeiss LSM510 META confocal microscope using 40x magnification.

2.1.7 BrdU Injection

Embryos at the 20 somite stage were injected with 10 mM BrdU (5-bromo-2-deoxyuridine, Sigma) and were allowed to develop until 36 hpf prior to fixation in 4% PFA (paraformaldehyde).

2.1.8 Blebbistatin treatment

To block Myosin II activity, WT embryos were incubated in 10 µM Blebbistatin (Biomol) at 24 hpf stage, ON, and were washed several times with egg-water at the 48 hpf stage prior to analysis using a binocular microscope (LEICA MZFLIII).

2.2 Microbiological Methods

2.2.1 Transformation of bacteria with DNA

Competent *E. coli* DH5α cells were transformed with purified plasmid DNA or directly with DNA-ligation reactions. A 25 µl aliquot of competent *E. coli* DH5α cells was thawed on ice and was mixed with 10 ng of plasmid DNA. The mixture was incubated on ice for 30 min and then heat-shocked for 45 sec at 42°C
The cell suspension was mixed with 500 µl of LB medium (pre-warmed at 37°C) and incubated at 37°C for 1 hr. Cells were then plated on a LB agar plate (pre-warmed at 37°C) containing the appropriate selective agent.

2.2.2 Cryopreservation of bacteria

250 µl of an overnight culture of *E. coli* DH5α was mixed with 750 µl of 80% glycerol and immediately frozen at -80°C.

2.3 Molecular biology methods

2.3.1 Isolation of plasmid DNA from bacteria

5 ml of LB medium were inoculated with a single colony of *E. coli* DH5α picked from a LB agar plate containing the appropriate selection marker. The LB culture

was grown overnight at 37°C with vigorous shaking. The next day the cells were collected by centrifugation (14000xg; 5 min; RT). The pellet was resuspended in resuspension buffer (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNase A; pH 8.0) and subsequently lysed by adding an equal volume of lysis buffer (200 mM NaOH, 1% SDS (w/v)). The solution was mixed cautiously with an equal volume of neutralization buffer (3.0 M K-acetate; pH 5.5). Cellular debris and genomic DNA were removed by centrifugation of the solution (14000xg; 10 min; 4°C), followed by iso-propanol precipitation for 1hr. The solution was centrifuged (14000xg; 20 min; 4°C), and the alcoholic supernatant was removed. The DNA was resuspended in 70% alcohol and centrifuged (14000xg; 10 min; RT). The alcoholic supernatant was removed and the DNA was allowed to dry at RT. The DNA was then resuspend in 50 µl sterile ddH2O and stored at -20°C.

2.3.2 Isolation of genomic DNA from embryos

Genomic DNA was prepared from a single 3-dpf old AB or WIK embryo, for the designing of splice-variant MO. The embryos were soaked in Methanol and then dried at 70°C for 15 min. The embryos were digested with Proteinase K at a final concentration of 0.5 mg/ml in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 55°C for 4 hrs, followed by inactivation for 10 min at 75°C. The solution was diluted with sterile ddH2O 1:2.5 and stored at -20°C

2.3.3 Isolation of total RNA

Total RNA was isolated with the TRIZOL® reagent from Invitrogen, USA. Tissue samples were homogenized in 1 ml of TRIZOL® reagent per 50-100 mg of tissue and incubated for 5 min at RT. 0.2 ml of chloroform were added to the homogenate per 1 ml of TRIZOL® reagent. Samples were shaken vigorously by hand for 15 seconds and incubated at RT for 3 min. The phases were separated

by centrifugation (12000xg; 15 min; RT). Following centrifugation, the upper aqueous phase was transferred to a fresh tube and precipitated by adding 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL®. The RNA was collected by centrifugation (12000xg, 10 min, 4°C) and washed once with 1 ml of 70% ethanol per 1 ml TRIZOL®. The pellet was air-dried for 10 min redissolved in RNase-free water and stored at -80°C.

2.3.4 DNA and RNA concentration determination

The concentration of DNA and RNA samples was determined spectrophotometrically at a wavelength of 260 nm (OD260) since the concentration of DNA and RNA is a direct function of the optical density at this wavelength. For DNA, an OD260 of 1.0 equals a concentration of 50 µg/ml of double stranded DNA, for RNA an OD260 of 1.0 equals a concentration of 40 µg/ml of RNA.
DNA quality measurement was done by measuring the OD280 because proteins absorb UV-light maximally at this wavelength. Pure DNA solutions have an OD260:OD280 ratio of 1.8. A lower ratio indicates contamination of the sample with proteins.

2.3.5 Enzymatic digest of DNA

The appropriate amount of DNA was incubated with the corresponding restriction enzyme(s) and buffer at a ratio of 0.5 U emzyme/µg DNA. The digest was incubated at 37°C for 2 hrs to overnight. All restriction enzymes were obtained from Fermentas, Amersham or New England Biolabs, USA. After incubation, the digest was either column purified or subjected to gel electrophoresis to isolate the fragment of interest.

2.3.6 Agarose gel electrophoresis of DNA and RNA

DNA and RNA fragments were separated according to their molecular weight on 1.0% agarose gel. RNA was incubated for 3 min at 85°C in an equal volume of RNA-loading buffer (mMessage mMachine) prior to loading. Ethidium bromide was added to the gel at a final concentration of 0.5 µg/ml to facilitate visualization of the DNA or RNA.

2.3.7 Isolation of DNA from agarose gels

Polymerase chain reaction (PCR) products or DNA digests were resolved on 1.0% agarose gel containing ethidium bromide. By exposing the agarose gel to UV-light, the DNA was visualized and bands of interest were cut from the gel. The DNA was extracted using the QIAquick Kit® Gel Extraction Kit from Qiagen, Germany. The gel slice was incubated with binding buffer (3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5% ethanol (v/v); pH 6.6) at a ratio of 300 µl buffer/100 mg agarose at 50°C until the gel slice was completely dissolved. The sample was transferred to a filter column and subjected to centrifugation (14000xg, 1 min, RT). The filter column was washed ones with 750 µl washing buffer (2 mM Tris-HCl, 20 mM NaCl, 80% ethanol (v/v); pH 7.5) and the DNA was eluted with 50 µl elution buffer (10 mM Tris-HCl; pH 8.5) and stored at -20°C.

2.3.8 Reverse transcription

Generation of cDNA from RNA was done using the Sensiscript® Reverse Transcription Kit (Qiagen, Germany). 50 ng of total RNA were incubated with 2.0 µl 10x Buffer RT, 2.0 µl dNTP Mix (5 mM each dNTP), 1 µM Oligo-dT primer, 10 U

RNase inhibitor and 1.0 µl Sensiscript® Reverse Transcriptase. The volume of the reaction setup was adjusted to 20 µl with H2O and incubated at 37°C for 60 min. The reaction mix was then stored at -20°C.

2.3.9 Polymerase chain reaction

If not stated otherwise PCR was carried out according to the following parameters:
100 ng of template DNA were incubated in the presence of 200 µM dNTPs, 1 µM forward primer, 1 µM reverse primer, 2 units AmpliTaq® DNA-Polymerase (Roche, Switzerland), PCR Buffer (1.5 mM $MgCl_2$) in a thermocycler. During the first cycle the reaction mix was denatured at 95°C for 5 min. For the following 30 cycles the reaction mix was denatured at 95°C for 30 sec, primers were allowed to anneal at 60°C for 30 sec and DNA synthesis was carried out at 72°C for 45 sec. This was followed by a final elongation step at 72°C for 10 min. The reaction mix was then cooled down to 4°C and further processed. Alternatively, 2 units of Pfu DNA-Polymerase (Promega, USA) were used with 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10Mm $(NH_4)_2SO_4$, 2mM $MgSO_4$, nuclease-free BSA and 0.1% Triton X-100 (v/v).

2.3.10 PCR primers

Primer Identifier	Sequence (5'→3')
Lgl2ATG_for	GGCTCGAGCTTGCTCACCTTCACCG
Lgl2ATG_rev	CCGTAGAGTTTGATTGCACCTGATCTG
Lgl2WD40_for	GCATGGCTTCCCCCATCAACCC
Lgl2WD40_rev	GCTACGCATAGTTCAAATATAAGCC
Lgl2InSitu_for	CGGCTCGAGCTTGCTCACCTTCAC
Lgl2InSitu_rev	CCCATAACTGGCCCTCGGCATCCC
Lgl1InSitu_for	GGCCCTGCTGGGGAAGGAGATCC
Lgl1InSitu_rev	CGCAGAGACATCGTGAGGTCGCTC
Lgl2_ClaI_for	ATCGATGTCCCGGCTCGTCCAGGG
Lgl2_XhoI_rev	CTCGAGACTGAGAAGACTGTAGTGTG
Lgl2eGFP_XhoIBglII_rev	GGGCTCGAGAGATCTATCTCCATTGCTCAGCACAT
eGFP_BglII_for	GGCAGATCTGGAGGAGGAAGCGGGGGGAATGGTGA GCAAGGGCGAG
eGFP_XhoI_rev	GGCCCTCGAGTTACTTGTACAGCTCGTC
Lgl2Seq_for(2-21)	CTTCACTGATAATCCTTAAC
Lgl2Seq_for(397-416)	GCAATCAAACTCTACGGAGC
Lgl2Seq_for(808-827)	GAGACTCTTCATGAAAACCC
Lgl2Seq_for(1213-1232)	GACTTCACCTCTCGCATTAT
Lgl2Seq_for(1627-1646)	GGAGTCTTCCACACGGACGC
Lgl2Seq_for(2017-2035)	CACTCCGAGTGGAAACTAG
Lgl2Seq_for(2407-2426)	CACAGTACACCTTCACTGTG
Lgl2Seq_for(2818-2836)	GTGCTGACGAATCAGGGGG
Lgl2Seq_for(3220-3038)	GGAGATCAAACGACGTTCC
Lgl2Seq_for(3638-3657)	GTGTAAAATACTTTCGTTTG
Lgl2Seq_for(4021-4040)	CCGTACCTGTTTTTTTGGAC
Lgl2Seq_rev (784-801)	CTCCAGATTTCTGCGGCC
MetInSitu_for	CACTATTCTGAAGCTGCTTCCATCC

MetInSitu_rev	CGTGATGGAGATAAGGCAAACGGC

2.3.11 Site Directed Mutagenesis

For the mutation of the full-length *His-Myclgl2* (cloned inside pCS2+ vector) to *His-Myclgl2* ^{S5A} or *His-Myclgl2* ^{S3E}, a QuikChange® XL Site-Directed Mutagenesis kit (STRATAGENE USA) was used. 200 ng of *pCS2+/His-Myclgl2* were used as a template for 50 µl PCR reaction with 1x Reaction Buffer (10 mM KCL, 10mM $(NH_4)_2SO_4$, 20Mm Tris-HCL (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton® X-100 and 0.1 mg/ml nuclease-free bovine serum albumin (BSA), 125 ng forward and reverse primers each, 200 µM dNTPs, 3 µl QuickSolution and 2.5 units *pfuTurbo*® DNA polymerase in a thermocycler. During the first cycle, the reaction mix was denatured at 95°C for 1 min. For the following 18, cycles the reaction mix was denatured at 95°C for 50 sec, primers were allowed to anneal at 60°C for 50 sec and DNA synthesis was carried out at 68°C for 9 min. This was followed by a final elongation step at 68°C for 7 min. 10 units of *Dpn I* restriction enzyme were added in order to digest the non-mutated parental DNA template, for 1 hr at 37°C. 10 µl from the reaction mix were transformed into competent *E. coli* DH5α cells. To generate *pCS2+/ His-Myclgl2* ^{S5A} two PCR reactions were carried out, where in the first reaction the first 3 serines were mutated to alanines, and a new digest site for *StuI* was generated, and in second reaction the last 2 serines were mutated to alanines, and a new digest site for *XhoI* was generated. To generate *pCS2+/ His-Myclgl2* ^{S3E}*,* two PCR reactions were carried out, where in the first reaction 5 base pairs from the first 3 serines were mutated and in the second reaction another 4 base pairs from the first 3 serines were mutated, to generate 3 glutamates.

Primers for mutagenesis (5'→3')**:**

Mutation of first 3 Serines to Alanines forward

CACGAGTCAAG**GCC**ATCAAAAAG**GCT**CTGCGACAG**GCC**TTCCGCAG

Mutation of first 3 Serines to Alanines reverse

CTGCGGAAGGCCTGTCGCAGAGCCTTTTTGATGGCCTTGACTCGTG

Mutation of last 2 Serines to Alanines forward

GATTCGCCGC**GCT**CGAGTC**GCC**ATGCGCAAAC

Mutation of last 2 Serines to Alanines reverse

GTTTGCGCATGGCGACTCGAGCGCGGCGAATC

Mutation of first 3 Serines to Glutamates forward (5 base-pairs)

CACGAGTCAAG**G**C**G**ATCAAAAAGT**A**TCTGCGACAG**G**C**G**TTCCGCAG

Mutation of first 3 Serines to Glutamates reverse (5 base-pairs)

CTGCGGAACGCCTGTCGCAGATACTTTTTGATCGCCTTGACTCGTG

Mutation of first 3 Serines to Glutamates forward (4 base-pairs)

CACGAGTCAAGG**A**GATCAAAAAG**G**A**G**CTGCGACAGG**A**GTTCCGCAG

Mutation of first 3 Serines to Glutamates reverse (4 base-pairs)

CTGCGGAACTCCTGTCGCAGCTCCTTTTTGATCTCCTTGACTCGTG

2.3.12 Ligation of PCR-products

The ligation of a digested dephosphorylated (after 1 hr incubation with 10 units Alkaline Phosphatase, Calf Intestinal, New England BioLabs at 37°C) vector (containing selective agent) with PCR products or digested insert was performed after separation of the desired fragments electrophoretically on a 1% agarose gel containing ethidium bromide. The band of interest was cut from the gel and column purified. Ligation was performed using the Rapid DNA Ligation kit (Fermentas), with 50 ng dephosphorylated vector, insert in a molar ratio of 1:3 vector to insert, 1x Rapid Ligation Buffer, T4 DNA ligase and water were added as necessary, for 2 hrs. at 22°C. The reaction mix was transformed into competent *E. coli* DH5α cells.

2.3.13 Ligation of PCR-products in the pGEM-T® Easy Vector

The PCR reaction mix was separated electrophoretically on a 1% agarose-gel, and the band of interest was cut from the gel and column purified. Ligation of PCR products with the pGEM-T® Easy Vector (Promega, USA) was done according to the manufacturers instructions: 2 µl of the PCR product were incubated with 5 µl 2X Rapid Ligation Buffer®, 30 ng of pGEM-T Easy Vector and 3 U of T4 DNA Ligase. The volume of the reaction setup was adjusted to 10 µl with H2O and incubated at 16°C overnight. The next day 1/3 of the ligation reaction was used for transformation into competent *E. coli* DH5α cells.

2.3.14 *In-vitro* transcription of digoxigenin-labeled RNA

Digoxigenin (DIG)-labeled probes for in situ hybridization (ISH) were generated using the DIG labeling kit (Roche, Switzerland). 1 µg of linearized template plasmid-DNA was incubated with transcription buffer (40 mM Tris-HCl, 6 mM MgCl2, 10 mM dithiothreitol, 2 mM spermidine; pH 8.0), 1 mM ATP, 1 mM GTP, 1 mM CTP, 0.65 mM UTP, 0.35 mM DIG-11-UTP, 40 U RNA Polymerase and 20 U RNase Inhibitor. The volume of the reaction was adjusted to 20 µl and incubated for 2 hrs at 37°C. Template DNA was removed by incubating the reaction setup with 20 U *DNase I* at 37°C for 15 min. The RNA was precipitated by adding half of the reaction volume of ammonium acetate (7.8 M) and 3 times of the reaction volume of 100% ethanol at RT. The mixture was allowed to stand at RT for 50 min followed by centrifugation (14000xg; 40 min; RT). The pellet was washed with 80% ethanol followed by air drying and dilution with 20 µl RNase-free water and 80µl of Hyb buffer (5x SSC, 500 µg/ml Torula yeast RNA, 50 µg/ml Heparin, 0.1% Tween 9mM Citric Acid (Monohydrate) and 50% Formamide) and then stored at -20°C.

2.3.15 *In-situ* probes

Gene	Probe	Restr. site	Promoter
Lethal giant larve2	*lgl2*	NotI	T7
Lethal giant larve1	*lgl1*	NotI	T7
Eye absent1(Christine Petit, France)	*eya1*	EcoRI	T7
Receptor tyrosine kinase Met	*met*	SpeI	T7

2.3.16 ISH on whole-mount zebrafish embryos

Zebrafish embryos were dechorionated and fixed overnight in 4% PFA in PBS. After fixation the embryos were washed with PBS and dehydrated with 100% methanol, and stored at -20°C until use. For ISH, embryos were rehydrated through a series of graded methanol solutions in PBT: 75%, 50%, 25%, and 4 times PBT for 5 min each at RT. This was followed by a digest with proteinase K (10 µg/ml) in PBT (15 min for 24 hpf embryos 30 min for 32-72 hpf embryos) at RT. For embryos at younger stages, the digest with proteinase K was not necessary. The digest was stopped by washing 2 times with PBT for 1 and 5 min at RT. The embryos were refixed in 4% PFA in PBS for 20 min at RT and washed 5 times for 5 min with PBT at RT. Embryos were then incubated for at least 2 hrs in prehybridization buffer (5x SSC, 0.1% Tween 9mM Citric Acid (Monohydrate) and 50% Formamide). After prehybridization the embryos were incubated overnight at 67°C with the DIG-labeled RNA probe in fresh hybridization buffer (5x SSC, 500 µg/ml Torula yeast RNA, 50 µg/ml Heparin, 0.1% Tween 9mM Citric Acid (Monohydrate), at a final concentration of 1 µg/ml.
The next day, the embryos were washed extensively with a series of buffers: 1 time for 20 min at 67°C with prehybridization buffer, 3 times 20 min at 67°C with wash-solution I (50% SSCT 2 x / 50% Formamide), 1 time for 20 min at 67 °C

with wash-solution II (75% SSCT 2 x / 25% Formamide), 2 times 20 min at 67°C with wash-solution III (SSCT 2 x), 4 times 30 min at 67°C with wash-solution IV (SSCT 0.2 x) and 1 time for 5 min with PBT at RT. After washing the embryos were blocked in PBT containing 5% sheep serum and 10 mg/ml bovine serum albumin (BSA) for at least 2 hrs at RT and incubated with an anti-digoxigenin-alkaline-phosphatase (anti-DIG-AP) conjugate at a dilution of 1:2000 overnight in PBT containing 2 mg/ml BSA at 4°C. The anti-DIG-AP was preabsorbed with smashed zebrafish embryos in PBT containing 2 mg/ml BSA at a dilution of 1:400 for 3 hrs at 4°C before use. Unbound anti-DIG-AP was washed off the next day by incubating the embryos 8 times for 30 min at RT in PBT. The embryos were then incubated 3 times 5 min in NTMT staining buffer (100 mM NaCl, 100 mM Tris-HCl, 50 mM $MgCl_2$, 0,1% Tween 20; pH 9,5) and then in NTMT containing 1.88 mg/ml nitro blue tetrazolium chloride (NBT) and 0.94 mg/ml 5-Bromo-4-chloro-3-indolyl phosphate (BCIP). The staining reaction was stopped by washing the embryos 3 times 5 min in Stop-solution (50 mM NaH2PO4/Na2HPO4, 1 mM EDTA, 0.1% Tween; pH 5.8). Embryos were stored in Stop-solution at 4°C. For imaging with Axioplan 2 imaging microscope (Zeiss), embryos were transferred 3 times through methanol and then soaked in Benzylbenzoate/Benzylalcohol (2:1) and embedded in Permount (FisherChemicals, USA) prior to imaging.

2.3.17 BrdU Labeling

Embryos injected with BrdU were washed 3 times with PBS after fixation, followed by dehydration with methanol for at least 1 hr at -20°C. The embryos were rehydrated through a series of graded methanol solutions in PBS: 75%, 50%, 25%, and 100% PBS for 5 min each at RT. This was followed by a digest with proteinase K (10 µg/ml) in PBT for 15 min. The digest was stopped by washing 2 times with PBT for 1 and 5 min at RT. The embryos were refixed in 4% PFA in PBS for 20 min at RT and washed 5 times for 5 min each with PBT at RT. The embryos were blocked in PBDT (1xPBS, 1% DMSO, 0.1% Tween20)

containing 1% BSA and 2% NGS for 10 min at RT and incubated with mouse anti-BrdU antibody (Roche), 1:100 in blocking solution (PBDT, 1% BSA, 2% NGS), for 2 hrs at RT. The embryos were washed 4 times for 15 min each, with PBDT followed by 2 hrs incubation with anti-mouse antibody at RT. The embryos were washed 4 times for 15 min each, with PBDT and kept in -20°C until analysis by confocal microscopy.

2.3.18 Immunohistochemistry

Zebrafish embryos were dechorionated and fixed for 1 hr in 4% PFA in PBS at RT, followed by washing 4 times for 5 min each with PBT and once with PBDT (1xPBS, 1% DMSO, 0.1% Tween20). The embryos were blocked in PBDT containing 5% NGS for at least 100 min at RT and then incubated with primary antibody in PBDT containing 1% NGS, O.N. at 4°C. The next day the embryos were washed 2 times, 1 min each, and 6 times whole day in PBDT containing 0.1M NaCl with the last wash with PBDT containing 1% NGS and then incubated with secondary antibody in PBDT containing 5% NGS, O.N. at 4°C. The next day the embryos were washed 2 times, 1 min each, and 6 times whole day with PBDT containing 0.1 M NaCl then kept at -20°C until analysis by confocal microscopy.

2.3.18.1 TCA Fixation

For immunohistochemistry with anti Delta D and anti beta-catenin the embryos were fixed with 10% TCA (Trichloroacetic acid) on ice for 30 min, followed by 5 times washings with PBS. The embryos were permeabilized with 0.2% TritonX-100 in PBS for 30min and then incubated in blocking buffer at RT for 2 hrs. Embryos were incubated O.N. with primary antibody in PBDT containing 1% NGS, at 4°C and then washed 6 timed with PBS, followed by incubation with secondary antibody in PBDT containing 5% NGS, O.N. at 4°C. The next, day

embryos were washed 6 times with PBS and were transferred through a series of graded glycerol in PBS: 25%, 50% and 75% , at least 2 hrs for each, prior to mounting

2.3.19 antibodies

Primary antibodies:

Rabbit polyclonal anti-nPKCζ (C-20), 1:200 Santa Cruz Biotechnology, Inc.

Mouse monoclonal anti-ZO-1 clone 1A12, 1:200 Invitrogene.

Mouse monoclonal anti-E-Cadherin 1:200 BD Biosciences Pharmingen

Rhodamine Phalloidin Invitrogen 1:100

4',6-diamidino-2-phenylindole,dihydrochloride (Dapi), 1:1000 FluoroPure^{TM}grade

Mouse monoclonal anti-gamma Tubulin(GTU-88), 1:200 Biozol.

Mouse monoclonal anti-bromodeoxyuridine clone BMC 9318 Roche.

Rabbit anti Lgl2, 1:500, a gift from the Nuesslein-Volhard lab

Mouse anti Delta D, 1:400, a gift from Chitnis lab

Rabbit anti beta-catenin, 1:600, a gift from Birchmeier lab

Secondary antibodies:

Goat-anti-Rabbit Cy2, 1:200 Jackson ImmunoResearch Laboratories, Inc.

Goat-anti-Rabbit Cy5, 1:200 Jackson ImmunoResearch Laboratories, Inc.

Goat-anti-Mouse RRX, 1:200 Jackson ImmunoResearch Laboratories, Inc.

Goat-anti-Mouse Cy5, 1:200 Jackson ImmunoResearch Laboratories, Inc.

2.4 Histology

2.4.1 Vibratome Sectioning

Embryos were fixed in 4% PFA for 1 hr at RT and then incubated in 0.3 M sucrose/PBS O.N. at 4°C. The embryos were embedded in 4% low melting Agarose in 0.3 M succrose/PBS in Cryomolds and were oriented immediately vertically. The solid block was glued onto a round metal plate of the Vibratome (LeicaVT1000S) using special Cyanacrylat glue and positioned inside the Vibratome chamber. The Vibratome chamber was filled with cold 0.3 M sucrose/PBS and the block was sectioned using the following settings: Feed: 200μm, Speed: Freq: 2, Speed: 6. The slices were transferred immediately into a 6-well plate filled with cold 0.3 M sucrose/PBS. The embryos were washed with PBDT (1xPBS, 1% DMSO, 0.1% Tween20) and blocked in PBDT containing 5% NGS for at least 100 min, at RT, following by staining as described above in section 2.1.18, and kept at 4°C until analysis by confocal microscopy.

2.4.2 Zebrafish Embryos Embedding

Dissected embryos were embedded on a Microscope Glass slides (76x26 mm) using a binocular microscope (LEICA MZFLIII). The desired tissue was dissected out using fine needles and transferred onto the Microscope Glass slides and then covered with 1 drop of SlowFade Gold antifade reagent (Invitrogen). Cover glass (Roth, 18x18mm) was used to cover the samples, bordered with Glycerol. The samples were kept at 4°C until analysis by confocal microscopy.