The aPKC/Par3/Par6 polarity complex and membrane order are functionally inter-dependent in epithelia during vertebrate organogenesis

Ahmed Abu-Siniyeh\textsuperscript{1,5}, Dylan M. Owen\textsuperscript{2}, Carola Benzing\textsuperscript{1}, Silke Rinkwitz\textsuperscript{3}, Thomas S. Becker\textsuperscript{3}, Arindam Majumdar\textsuperscript{4*}, Katharina Gaus\textsuperscript{1*}

\textsuperscript{1}School of Medical Sciences, ARC Centre for Advanced Molecular Imaging and Australian Centre for NanoMedicine, The University of New South Wales, Australia
\textsuperscript{2}Department of Physics and Randall Division of Cell and Molecular Biophysics, King’s College London, UK
\textsuperscript{3}Brain and Mind Research Institute, Sydney Medical School and Department of Health Sciences, University of Sydney, Australia
\textsuperscript{4}Department of Immunology, Genetics, and Pathology, Uppsala University, Sweden
\textsuperscript{5}Present address: Department of Chemistry and Medical Analysis, Faculty of Science, Al-Balqa’ Applied University, Al-Salt 19117, Jordan

* Equal contribution

Running title: Membrane order in zebrafish epithelia
Keywords: membrane organisation, lipid phases, polarity proteins, apical and basolateral membranes, epithelial cell, zebrafish larvae

Correspondence to:
Katharina Gaus (k.gaus@unsw.edu.au)
Level 3, Lowy Cancer Research Centre
The University of New South Wales
Sydney, 2052, NSW, Australia

Arindam Majumdar (arindam.majumdar@igp.uu.se)
Department of Immunology Genetics and Pathology
Rudbeck Laboratory, Uppsala University
Dag Hammarskjölds väg 20
751 85 Uppsala, Sweden
Abstract
The differential distribution of lipids between apical and basolateral membranes is necessary for many epithelial cell functions, but how this characteristic membrane organization is integrated within the polarity network during ductal organ development is poorly understood. Here we quantified membrane order in the gut, kidney, and liver ductal epithelia in zebrafish larvae at 3-11 days post fertilization (dpf) with Laurdan 2-photon microscopy. We then applied a combination of laurdan imaging, antisense knockdown, and analysis of polarity markers to understand the relationship between membrane order and apical basal polarity. We found a reciprocal relationship between membrane order and the cell polarity network. Reducing membrane condensation by exogenously added oxysterol or depletion of cholesterol reduced apical targeting of the polarity protein, aPKC. Conversely, using morpholino knockdown in zebrafish, we found that membrane order was dependent upon the Crb3 and Par3 polarity protein expression in ductal epithelia. Hence our data suggest that the biophysical property of membrane lipid packing is a regulatory element in apical basal polarity.

Synopsis (66 words)
The differential distribution of lipids between apical and basolateral membranes is necessary for many epithelial cell functions, but how membrane organization integrated within the polarity network poorly understood. Here we quantified and manipulated membrane order in the gut, kidney, and liver ductal epithelia in developing zebrafish larvae with Laurdan 2-photon microscopy and found a reciprocal relationship between membrane order and the apical-basal distribution of polarity proteins.
Introduction

Apical basal epithelial polarity is the basis for the secretory and absorptive physiological properties of ductal organs. Epithelial cell organization and function are dependent upon the polarized distribution of lipids and proteins to their respective apical and basolateral membrane domains and the segregation of these membrane domains by tight junctions. During epithelial differentiation, membrane biogenesis, junction formation, and polarized trafficking are all intimately interconnected. How asymmetry is established and maintained within epithelial cells is a fundamental question in cell and developmental biology.

The differential distribution of lipids within cell membranes reflects apical basal polarity and also provides a mechanism for membrane specific protein sorting. For example, apical membranes are enriched in glycosphingolipids and cholesterol and the physical association of sphingolipids with proteins in the trans-Golgi network directs these protein cargos to the apical membrane thereby maintaining cell polarity. Phosphoinositides are likewise distributed within polarized epithelial cells. PIP₃ is predominant over other phosphoinositide species in basolateral membranes and confers basolateral identity by recruitment of basolateral proteins. In contrast, PIP₂ is concentrated in apical membranes and endows membranes with apical identity.

Though the global organization of epithelial plasma membranes are divided into relatively stable apical and basolateral domains, at the nanoscale, membranes appear to be much more molecularly heterogeneous and temporally dynamic. Lipid rafts are transient aggregates of sphingolipids, cholesterol, and proteins, smaller than 200 nm in size, with functions as signaling platforms and in lipid/protein trafficking. Lipid rafts behave biophysically as a liquid ordered phase, in contrast to neighboring non-raft membranes, which correspond to a liquid disordered phase and are relatively fluid.

The functional requirement for cholesterol rich rafts in protein trafficking and polarity has been investigated using pharmacological agents, like methyl-β-cyclodextrin (mßCD) and 7-ketocholesterol (7KC), which deplete plasma membrane cholesterol content and increase membrane fluidity, respectively. Using cyclodextrins and 7KC, many examples of raft dependent protein trafficking have been found, however, within epithelial cells, the clearest example is found in the apical marker Hemeagglutinin viral protein (HA). Pharmacologically induced cholesterol
depletion with mβCD specifically reduces trafficking of the HA to apical membranes in MDCK cells. In addition, genetic screens in C. elegans suggest a potential link between lipid rafts, sphingolipids, and apical protein sorting during epithelial polarization. Mutations in biosynthetic enzymes resulting in reduced glycosphingolipid synthesis cause apical basal polarity and lumen morphogenesis defects in vivo.

The formation of apical and basolateral membrane domains and their proper segregation is governed by competitive interactions between the Crb/Pals1/Patj and the Lgl/Scribble/Dlg cell polarity protein complexes. Furthermore, a third protein complex, Par3/Pard6/aPKC mediates interactions between the Crb and Scribble activities, which dictates the balance of apical to basolateral membrane. In vertebrates and invertebrates, atypical protein kinase C, aPKC, activity is enriched in apical membranes in differentiated epithelia, and it’s absence results in loss of apical membranes and the expansion of basolateral membranes. Stabilization of apical aPKC activity is necessary for lumen formation and apical aPKC localization has been used as a molecular readout of apical basal polarity in epithelia.

In vertebrates, the Crb family is represented by the Crb1, Crb2, and Crb3 homologs. Of these, Crb3 is widely expressed in mammalian ductal epithelial organs such as the lungs, kidneys, gut, and liver. In Drosophila and MDCK cells, apical aPKC localization depends on the integrity of the Crb complex. Crb proteins localize to apical membranes and their interaction with Par6/aPKC is required for apical aPKC enrichment and associated with apical membrane formation. Overexpression of Drosophila Crb protein induces apical membrane expansion at the expense of basolateral domain in embryonic epidermal cells, while genetic loss of function mutations in crb result in the opposite phenotype. Recently, a Crb3 knock out mouse has been reported with phenotypes in several organ systems. In zebrafish, antisense knockdown using morpholinos shows that crb3 is required for the differentiation of retinal photoreceptor, renal, and otic cilia. Par3 localizes to tight junctions and its interactions with Pard6/aPKC are required for apical membrane definition and cell polarity. Knockdown of Par3 in MDCK cells delays apical membrane formation with possible defects in vacuolar apical compartment delivery to target plasma membranes. In zebrafish, the par3 functions in multiple developmental contexts, but has been intensively studied.
during polarity driven symmetric neuroblast division and cell fate determination events in the neural tube 23, 24.

While both the Crb3 and Par3 complexes are needed for establishing apical basal polarity, the functional relationship of these polarity complexes to epithelial membrane organization is unknown. Could cell polarity complexes have a functional role in the epithelial membrane organization and lipid raft formation? Conversely, might lipid rafts regulate polarity complex localization and function?

One of the technical challenges in studying lipid rafts and membrane organization in vivo has been the ability to visualize them. Zebrafish embryos are optically transparent, develop rapidly ex utero, and allow excellent bioimaging opportunities. In previous work, we innovated a method using the environmentally sensitive dye Laurdan to visualize membrane order in living vertebrate zebrafish embryos 25. By simultaneously recording the emission in two spectral channels, a normalized intensity ratio, termed Generalized Polarization (GP), can be calculated that reflects membrane order with +1 the most ordered and -1 the most fluid membranes 26. In this report, we attempted to test a link between polarity complex function and membrane organization using the zebrafish experimental system by combining Laurdan imaging and functional gene knockdown. We chose zebrafish crb3 and par3 as intervention points for disabling polarity complex function where antisense morpholinos are used to knockdown gene function. In combination with mβCD- and 7KC-mediated perturbation of lipid raft assembly, we found that polarity complex function and epithelial membrane order are mutually interdependent.

Results
As a first step towards visualizing the emergence of membrane order during tubular organ morphogenesis in zebrafish, we labeled live or fixed 3-11 days post fertilization (dpf) embryos with 50 µM of the membrane dye Laurdan for 30 min. Laurdan is non-fluorescent in water and incorporates spontaneously into membranes, where it is strongly fluorescent. Importantly, the emission profile depends on the degree of membrane order since Laurdan is an environmentally sensitive dye, reporting the water content of lipid bilayers 5, 27. We have previously shown that Laurdan microscopy can be applied to zebrafish embryos 25. Laurdan was excited with a 2-photon laser and the intensity images in the two emission channels converted to pseudo-colored GP images (Fig. 1). We
confirmed that the polarized light of the 2-photon laser did not result in a photo-selection effect, resulting in similar GP values independently of the orientation of the sample relative to the excitation direction (Supplementary Fig. 1). Further, the dye does not appear to be to harmful to the embryos since embryos labeled with Laurdan at 3 dpf developed normally to adulthood (Supplementary Fig. 2A).

We focused our investigation on the polarized epithelial cells of the gut, kidney and liver and examined their membrane order over 3-11 dpf. Even without additional staining, individual cells were clearly visible and apical membranes (AM) and basolateral membranes (BM) identified (Figure 1). Fixation caused minor changes in the GP values in some tissues but preserved differences in membrane order between apical membranes and basolateral membranes (Supplementary Fig. 2B). Despite the differences in tissue morphology between individual samples at the same developmental stage, GP values were remarkably consistent (Supplementary Fig. 3), suggesting that the apical and basolateral membrane composition, and not the gross tissue morphology, regulated membrane order throughout development.

In all three tissues and at all the examined developmental stages the membrane order was higher in the apical compared to the basolateral membrane as indicated by the red and green hues in Figure 1. Similarly, there appears to be a change in membrane order during development with particularly high membrane orders recorded at 6 dpf for the three tissues. To quantify these changes, we measured the GP value in regions corresponding to the apical and basolateral membranes. As expected from studies with cultured epithelial cells 28, we found the apical membrane to be significantly more ordered than the basolateral membranes in gut, kidney and liver epithelial cells at all five developmental stages (Supplementary Fig. 4). In addition, membrane order in these cells was significantly remodeled during development and peaked at 6 dpf in both apical and basolateral membrane (Fig. 2A-C).

In order to investigate whether membrane order affects the distribution of polarity networks, we aimed to manipulate the membrane order without disturbing the overall tissue architecture. We thus treated embryos 6 dpf with either 100 µM 7-ketocholesterol for 30 min (7KC) or 2.5 mM methyl-β-cyclodextrin (mβCD) for 40 min. The additional ketone group in 7KC acts as a spacer to preferentially increasing the fluidity of highly ordered membranes while having little effect on already fluid membranes 5, 6. We expected that treatment
with mβCD would remove membrane cholesterol and thus also decrease membrane order. Indeed, both treatments resulted in more fluid membranes in the gut, kidney and liver at 6 dpf (Fig. 3). The quantification confirmed that 7KC and mβCD significantly decreased membrane order in both the apical and basolateral membranes in these tissues (Fig. 3B-D) indicating that the treatments were not specific to certain membranes and tissues. Importantly, epithelial cells retained their architecture (Fig. 3A) and the tissue morphology was unaltered by 7KC enrichment and cholesterol depletion (Supplementary Fig. 5A-C); epithelial cells remained polarized, and tight junctions were intact as evidenced by α-ZO-1 staining so that apical and basolateral membranes were visible after the treatments. This was confirmed with phalloidin staining of epithelial junctional actin networks which were unaltered in sterol treated embryos (Supplementary Figure 6). Hence these treatment conditions were found suitable to manipulate membrane order.

Next, we treated 4 dpf embryos with 7KC or mβCD and either immediately measured their membrane order or let them develop to 6 dpf and recorded the membrane order 2 days after treatment (Fig. 4). As with the membrane manipulations at 6 dpf, both 7KC enrichment and short exposure to mβCD significantly and acutely reduced membrane order of apical and basolateral membranes in the gut, liver and kidney (Fig. 4A) and the effect was still detectable after the additional 2-day incubation period after treatment (Fig. 4B). However, both apical and basolateral membranes in all three examined tissue recovered and significantly increased their membrane order so that the difference to the GP values in untreated Control tissue was reduced by 6 dpf (Fig. 4B and Supplementary Figure 7). The recovery was most noticeable in the kidney where the membrane order of apical membranes returned to GP values that were not significantly different to Control GP values (Supplementary Fig. 7B). We concluded that membrane order is dynamic.

To assess the effect of membrane sterol manipulations and recovery on the polarity network, we immuno-stained 4 dpf and 6 dpf embryos, that were treated with mβCD and 7KC at 4 pdf, with antibodies against atypical protein kinase C (aPKC), a readout of apical basal polarity. The staining was specific for the apical membrane in gut, kidney and liver, even after the acute manipulation of the membrane order (Fig. 5A). In order to quantify the degree of polarization, we selected regions of the apical membrane...
and the cytoplasm and calculated the ratio of the area-normalized fluorescence intensity of the aPKC staining in these two regions (Fig. 5B). This revealed that 7KC enrichment and cholesterol depletion significantly impaired the apical accumulation of aPKC immediately after the treatment at 4 dpf and to a lesser extent, after the recovery period (6 dpf after treatment at 4 dpf) in the liver and gut (Fig. 5B). The apical targeting of aPKC had fully recovered in the kidney after either treatment and in the gut after 7KC enrichment. Hence there is a correlation between the membrane order and apical localization of aPKC under conditions where membrane order is acutely decreased immediately after the manipulation and post recovery (Fig. 4B, Fig. 5B). In fact, the greatest recovery in membrane order observed in the kidney also resulted in the greatest recovery of aPKC apical targeting in this tissue, strongly suggesting a mechanistic link between the two processes. We concluded that polarity complex function was dynamic and dependent upon membrane order.

We then asked whether the loss of polarity proteins would result in a loss of membrane order. We used morpholinos directed against the polarity proteins Par3 (par3 MO) and crumbs 3a (crb3a MO) to reduce the Par3 and Crb3a protein levels, respectively, at 4 dpf (Supplementary Fig. 8) 21; 23; 29. We again quantified apical targeting of aPKC by measuring the apical to cytoplasmic ratio of confocal images (Fig. 6). We found that in zebrafish embryo, the loss of expression of Par3 or Crb3a resulted in a significant decrease in apical localization of aPKC in gut, kidney and liver at 4 dpf.

Interestingly, knock-down of either polarity protein resulted in a significant loss of membrane order in both the apical and basolateral membranes at 4 dpf (Fig. 7). This was again observed in all three examined tissue types of in gut, kidney and liver. Importantly, functional α-ZO-1 staining was preserved in morphants indicating that the reduced membrane order is not due to mixing of apical and basolateral membrane domains (Figure 6A). Taken together, similar to the lipid manipulations, impaired apical localization of aPKC in MO-treated larvae was also accompanied by loss of membrane order, strongly suggesting a reciprocal relationship between the establishment of a polarity network and membrane order in apical and basolateral membranes.

Discussion
While the requirement for membrane trafficking in generating an apical-basolateral axis of polarity is known
how the biophysical properties of the apical versus basolateral contributes to apical basal polarization has not been experimentally addressed. Here, we used zebrafish embryos and intravital imaging of membrane order to address this question. Our results suggest that lipid organisation and polarity complexes are functionally inter-dependent during epithelial polarization in the zebrafish gut, liver, and kidney. These results are consistent with a role for lipid rafts in regulating overall apical basal polarity.

The zebrafish embryo has an endogenous source of cholesterol and other lipids in the yolk. After fertilization, lipids are released from the yolk into the blood stream. We observed an increase in membrane order occurs over several days that paralleled epithelial differentiation. This may indicate the continued trafficking of sphingolipids and cholesterol to the forming membranes. We can only speculate that the observed decrease in membrane order post 6 pdf is due to food intake or changes in lipid species during organogenesis (Sampaio and Gerl, 2001).

During epithelial polarization, the interactions between the Crb, Par, and Scribble complexes result in the stabilization of aPKC in, and the exclusion of the Scribble complex from, apical membranes. The apical enrichment of active aPKC is an important event in the establishment of apical basal polarity. Both Crb3 and aPKC are early apical membrane markers and their apical localizations presage lumen formation during tubulogenesis. It is thought that aPKC phosphorylates basolateral proteins, like Lgl, thereby excluding them from the apical domain. The integrity of the Crb complex is required for apical aPKC localization since aPKC apical targeting is disrupted in Drosophila crumbs or stardust/pals1 mutants or in Crb3 knockdown MDCK cells. However, there may be other routes for aPKC to become enriched in apical membranes.

Our results suggest that membrane order is required for apical aPKC stabilization. Conversely, polarity complex function regulates epithelial membrane order. Using morpholino knockdown in zebrafish, we found that membrane order in gut, kidney, and liver ductal epithelia was dependent upon the Crb and Par3 polarity protein function. Furthermore, in either crb3a MO or par3 MO embryos, reduced apical aPKC was seen in all organs tested, consistent with observations in Drosophila epithelia. These results lead us to conclude that biophysical properties of the lipid bilayer must play some role in the cascade of events resulting in apical basal polarity.
Our results are consistent with earlier observations pointing to an instructive role for lipids in polarity and a molecular link between lipid modifying enzymes and polarity complexes. The phosphoinositide phosphatase PTEN associates with the Par3/Par6/aPKC complex in MDCK cells and PTEN activity endows membranes with apical character. In Drosophila retinal photoreceptors, Pten is recruited to a Par3/Par6/aPKC complex where it locally produces PtdIns(3,4,5)P3 required for the differentiation of photoreceptor apical membranes. This suggests that polarity complexes and lipid modification work together and could be a way to locally modify membrane lipid composition.

How can lipid organization direct polarity networks? It is possible that aPKC, or other polarity complex proteins, need to associate with lipid rafts in order for apical aPKC accumulation and proper cell polarity. In Drosophila, the phosphoinositide binding domain of Bazooka/Par3 is required for apical membrane localization and apical basal polarity. In our experiments, cholesterol depletion and 7KC enrichment may disrupt the assembly of the Crb complex in lipid rafts and therefore interfere with apical aPKC accumulation. Reduced apical aPKC may in turn disturb membrane trafficking defects involving both apical and basolateral membranes, which could explain the higher membrane fluidity we observed. However, the reduction in membrane order in knockdown embryos is unlikely to be due to loss of tight junctions as we observed that tight junctions were intact, matching observations with the Crb3 knock-out mouse. Hence, a more localized influence of polarity complexes on the lipid environment is more likely. For example, it is possible that the Crb/Pals1/Patj complex is able to recruit proteins that locally increase membrane packing, perhaps through synthesis of cholesterol, sphingolipids or lipids and proteins that contribute to vesicle biogenesis.

An alternative possibility is that vesicular trafficking within the TGN or from the TGN to the apical and basolateral plasma membrane is itself affected by the degree of lipid condensation of these membranes. We noticed that the sterol manipulations used here were not specific to a subcellular membrane compartment and affected intracellular membranes to a similar extent than the plasma membrane (data not shown). Hence cholesterol depletion and 7KC enrichment could directly interfere with vesicular trafficking and this notion is consistent with a previous report that found vesicles destined for apical delivery to be enriched...
in sphingolipids and cholesterol \(^{38}\). However, it is currently not known how cell polarity complexes control vesicular trafficking.

In summary, we reported the co-dependence of polarity complex and membrane order in developing zebrafish that suggest that not only physical properties like cell shape and tension but also localized lipid organization is integrated with the polarity network in ductal epithelia.
Materials and Methods

Fish strain and handling
We used the zebrafish mutant strain, Casper, throughout the study because of its transparency in development stages [39]. Adult fish were grown and mated according to standard protocols. Briefly, adult zebrafish were maintained on regular light-dark cycle with 14 h of light and 10 h dark at pH 7.4 and 28°C in salinity controlled conditions (Westerfield 1995). To mate the fish, three females and two males were placed in a breeding tank but physically separated from each other. On the next day, the barrier was removed and females begun to lay eggs shortly after the start of the light cycle. Eggs were kept in E3 medium at 28°C (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4). Larvae were culled by immersion in 4°C ice water.

Laurdan staining and mounting
Zebrafish larvae were treated with 50 µM Laurdan (Invitrogen, D250) for 30 min in E3 medium. Larvae were washed with Phosphate Buffer Saline (PBS), and allowed to recover for 30 min in E3 medium. Larvae were anesthetized by adding 50 µl of 0.04% Tricaine (Sigma, E10521) to 1 ml of E3 medium and embedded in 1 ml of 1% low melting agarose (Sigma, A9414) in a glass-bottom microscope chamber.

Laurdan Imaging and images analysis
Imaging was conducted on a laser-scanning confocal microscope (SP5; Leica Microsystems, Wetzlar, Germany). Laurdan was excited at 800 nm using a femtosecond-pulsed Ti: Sapphire laser (Mai-Tai; Spectra-Physics, Mountain View, CA). Fluorescence was detected in the two channels simultaneously ranging from 400–460 nm and 470–530 nm using internal photomultiplier tubes (PMT) with the confocal pinhole open and recorded as 8-bit, 512 X 512 pixels digital images. Live and fixed zebrafish larvae were imaged with a 63× glycerol-immersion objective with a numerical aperture (NA) of 1.3. GP images were constructed by using specific plugin [Owen, 2012 #75] in ImageJ (National Institutes of Health, Bethesda, MD). For each pixel, the GP was calculated from the two channel intensity values as:

$$GP = \frac{I_{400-460} - GI_{470-530}}{I_{400-460} + GI_{470-530}}$$

Here, G is the G-factor, used to calibrate the GP values for differences in detector sensitivities. To calculate G, a solution of 0.5 µM Laurdan in DMSO was imaged under the same imaging conditions as the zebrafish larvae and its GP value measured (GPmes). This standard sample has a
literature reference GP (GP_{ref}) value of 0.207. The G factor is then calculated using:

\[ G = \frac{GP_{ref} + GP_{ref}GP_{mes} - GP_{mes} - 1}{GP_{mes} + GP_{ref}GP_{mes} - GP_{ref} - 1} \]

**Sterol manipulations**

Larvae were divided in three groups as follow: Control group were not treated with any compound and were kept in E3 medium. Methyl-β-cyclodextrin (mβCD) group: larvae were treated by 2.5 mM (mβCD) for 40 min (0.0033 g of mβCD dissolved in 1 ml E3 medium). 7-ketocholesterol (7KC) group: larvae were treated with 100 µM (mβCD/7KC) for 30 min (10.066 µl of 4.967 µM 7-ketocholesterol in 1ml E3 medium). Then treated larvae were washed with PBS for three times. These concentrations of mβCD and 7KC were used since higher concentrations result in embryonic lethality making analysis impossible. 7KC/mβCD complex preparation: ~1 ml of 15 mg/ml 7KC in ethanol were prepared (7KC = 5-cholesten-3β-OL-7-ONE, Steraloids, C6970-000). 5% mβCD stock were prepared by dissolving 500 mg of mβCD in 10 ml distilled H2O (mβCD = methyl-b-cyclodextrin, Sigma, C4555). 400 µl of 5% mβCD stock were added to HPLC vial on a hotplate at 80 °C with stirring. 4 × 10 µl aliquots of 15 mg/ml 7KC were added to vial; leaving 5-10 minutes between adding each 10 µl aliquot. After the fourth aliquot has been added, the preparations were left on the hotplate at 80 °C for 1 hour – without covering the vials. After 1 hour, all aliquots of the prepared 7KC/ MβCD complexes were collected into 15 ml falcon tube.

**Immunofluorescence - Zebrafish whole mount staining**

Larvae were fixed in 4% Paraformaldehyde (PFA) for overnight at 4°C. Then larvae were washed in PBST (1XPBS pH7.4, 0.1% Tween 20) for 5 min followed by washing in distilled H2O for 5 min. After that larvae were frozen in pre-chilled acetone at -20°C for 7 min. Then washed in distilled H2O for 5 min followed by washing in PBST buffer for 5 min. Larvae were Incubate in 2% normal goat serum (NGS) (Sigma, G9023) in PBDT (1XPBS pH7.4, 1% bovine serum albumin (BSA) (Sigma, A9056), 1% Dimethyl sulphoxide (DMSO) (Sigma, D2650), 0.1% Tween-20 (Sigma, P1379) for 1 hour at room temperature. Next, they were soaked in primary mouse antibody ZO-1 (Invitrogen, 339100) diluted at 1:300, wheat germ agglutinin, Alexa Fluor 594 conjugate (WGA) (Invitrogen, W11262) diluted at 1:50 and overnight at
4 °C with gentle rocking. Then larvae were washed eight times 15 min each in PBST at room temperature. Then, larvae were treated with Alexa Fluor 488 Goat anti-mouse IgG secondary antibody (Invitrogen, A11001) diluted 1:500 in 2% NGS-PBDT overnight at 4°C. Finally, larvae were washed eight times 15 min each in PBST then mounted with Dako (Dako, S302380) for imaging. Actin staining experiments were performed with Phalloidin rhodamine (Invitrogen, R415) diluted 1:500 in PBST.

**aPKC immunostaining**

Larvae were fixed with 4% PFA overnight at 4°C. Next day, larvae were washed in PBS three times for 5 min each. Larvae were placed in molten 4% low melting agarose into plastic sectioning mold. Larvae were sectioned at 50 μM by Leica vibratome (Vibratome, Inc.). Sections were incubated with blocking solution PBDT for 3 hours at room temperature then sections were incubated with primary mouse anti-ZO-1 antibody (Invitrogen, 339100) diluted at 1:300 and rabbit anti-PKC ζ antibody (Santa Cruz Biotechnology, (C-20): sc-216) diluted at 1:500 in the blocking solution for overnight at 4°C. Next day, sections were washed four times (10 min, 15 min, 30 min, and 60 min, each) in PBT (1% BSA, 1X PBS, 0. 1% Tween-20). Then sections were treated with Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Invitrogen, A-11001) and Alexa Fluor 594 goat anti-rabbit IgG secondary antibody (Invitrogen, A-11012) diluted at 1:500 in blocking solution for overnight at 4°C. Sections were washed four times (10 min, 15 min, 30 min, and 60 min, each) in PBT. Finally, sections were treated with DAPI (0.1% DAPI in 50 ml 1X PBS; Invitrogen, D1306) for 30 min to stain the nuclei. Then they were washed three times in PBS for 5 min each then mounted on a fluoro dish by using Dako (Jayachandran et al. 2010; Shim 2011).

**Morpholino knockdown**

Two antisense morpholino oligos were synthesized by (GeneTools LLC, Corvalis, OR, USA) and designed based on the 5’ UTR sequence to reduce protein expression of Par3 and Crb3a in zebrafish. Morpholino sequences for par3 MO 5’ TCAAAGGCTCCCCGTCCTGTGGGTTC 3’ and crb3a MO 5’ AGCCCAACCTGCTGGATCATTTCGG 3’ have been described and validated in previous work 21; 23; 29. The par3 MO and crb3a MO were re-suspended in water (1-2 μg/μl) containing 0.1% phenol. Par3 MO was injected at 125 μM, crb3a MO at 250 μM. 1 nl of MO (0.1-0.25 mM) was injected into the yolk of 1 to 4 cell stage.
Statistics
Statistical comparisons between two populations were made using Student’s t-test assuming unequal variances. Comparison between multiple groups was performed by one-way ANOVA with Bonferroni post-test, and results were considered statistically significant at $P < 0.05$. Unless stated otherwise, data were presented as mean ± standard deviation (SD) from at least three replicates of a single experiment. All statistical analyses were done with Prism software (Graphpad Software Inc., USA).

Acknowledgements
This work was supported by fellowships and grants from the National Health and Medical Research Council of Australia (to K.G., grant numbers APP1059278 and APP1037320) and the Australian Research Council (to K.G., grant number CE14010011). We thank Swedish Cancerfonden for supporting this work with an international research travel award. We thank Lena Claesson-Welsh (IGP/Uppsala University) and Lars Holmgren (CCK/Karolinska Institute) for their encouragement and support. We are grateful to the technical staff and the Mind and Brain Institute zebrafish facility for taking care of fish and the Biomedical Imaging Facility (BMIF) at UNSW.
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