Par3 in chick lens placode development

Maraysa de Oliveira Melo | Ricardo Moraes Borges | Chao Yun Irene Yan

Department of Cell and Developmental Biology, Institute for Biomedical Sciences, Universidade de São Paulo, Av. Prof. Lineu Prestes, São Paulo, SP 05508-900, Brazil

Correspondence
Chao Yun Irene Yan, Department of Cell and Developmental Biology, Institute for Biomedical Sciences, Universidade de São Paulo, Av. Prof. Lineu Prestes, São Paulo, SP 05508-900, Brazil.
Email: ireneyan@usp.br

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Abstract
The lens originates from a simple cuboidal epithelium, which, on its basal side, contacts the optic vesicle, whilst facing the extraembryonic environment on its apical side. As this epithelium changes into the pseudostratified lens placode, its cells elongate and become narrower at their apical ends. This is due to the formation of an apical actin network, whose appearance is restricted to cells of the placodal region, as a result of region-specific signaling mechanisms that remain largely unknown. Here, we investigated the role of the polarity protein PAR3 and the phosphorylation state of its Threonine 833 (T833) aPKC-binding site in the recruitment of aPKC and in the establishment of actin network in the chick lens placode. Overexpression of wild type PAR3 recruited aPKC and punctate actin clusters to the basolateral membranes of the placodal cells. Recruitment of aPKC depended on the charge of the residue that replaced the T833 residue. In contrast, induction of the ectopic actin spots was independent on the charge of this residue.

1 | INTRODUCTION

The lens of the eye forms early in embryonic development from a non-neural pre-lens ectoderm that overlies the optic vesicle. In response to signals delivered through the extracellular matrix from the apposed optic vesicle, the pre-lens ectoderm grows along the apical-basal axis to four times its original length and forms the pseudostratified tissue called the lens placode (Hendrix and Zwaan, 1975; Rajagopal et al., 2008; Schook, 1980, Supporting Information Figure 1). During this period, actomyosin filaments accumulate in the apical domain culminating in an extensive network change in the lens placode (Borges, Lamers, Forti, Santos, & Yan, 2011; Plageman et al., 2011). This actin network is required for the acto-myosin contraction that reduces the apical surface and drives the placode inward. In the mouse embryo, the placodal invagination also depends on the presence of filopodia that emerge from the base of the placode, tethering it to the underlying optic tissue. Loss of basal filopodia reduces the depth of the lens pit. Likewise, this tethering process depends on the contractile activity of the acto-myosin cytoskeleton present in the filopodia (Chauhan et al., 2009). Together, these data emphasize the importance of asymmetric distribution of the actin skeleton along the placodal apical-basal axis for the ensuing apical contraction and invagination that will shape the lens. Although the morphological aspects of these changes have been described in detail, the molecular mechanisms of cell polarity that drive cytoskeletal rearrangement in this scenario are still unknown. We are particularly interested in the formation of the apical actin network. The establishment and maintenance of this network is a necessary step for the next morphogenesis event of placodal invagination (Borges et al., 2011).

Clearly, remodeling and reallocation of cytoskeletal elements during conversion of the pre-lens ectoderm to placode occurs in accordance to cellular apical-basal polarity. Establishment and maintenance of cell polarity is determined by a conserved set of proteins, the PAR (partition-defective) proteins. PAR proteins determine the asymmetric distribution of subcellular components according to the apical-basal domains, through the formation of complexes with signaling proteins.

The apical domain is defined by PAR3/PAR6/aPKC and CRB/PALS1/PATJ protein complexes and the basolateral domains by PAR1, Lgl, and PAR4 (reviewed in Macara, 2004; Suzuki and Ohno, 2006). Several studies implicate PAR3, in particular, as the upstream determinant of apical identity. PAR3 alone is sufficient to target the PAR3/PAR6/aPKC complex to the apical membrane domain (Harris and Peifer, 2005). Further, cell polarity can be dynamically regulated by modulation of PAR3 levels in the apical domain or by its association with distinct isoforms of aPKC (Eom, Amarnath, Fogel, & Agarwala, 2011; Parker et al., 2013). PAR3 is also important in the asymmetric distribution of the cytoskeleton. For instance, knockdown of PAR3 disorganizes the actin cytoskeleton during tight junction reassembly in MDCK
cells and abolishes formation of F-actin-rich structures in endodermal cells of gastrulating nematode embryos (Chen and Macara, 2005; Roh-Johnson and Goldstein, 2009).

Here, we explore the possibility that PAR3 can determine the formation of actin networks in the lens placode. We first identified the subcellular localization of PAR3 during the establishment of the placode. Then, to investigate if PAR3 was sufficient to recruit actin we overexpressed wild type PAR3 in placodal and periplacodal tissue. Finally, we tested if modulation of PAR3-aPKC would affect accumulation of actin. Our findings suggest that: (1) PAR3 is sufficient to recruit actin in the placodal and periplacodal cells; (2) The quality of PAR3-induced actin clusters differs according to the subcellular domain: in the basolateral domain the ectopic clusters are punctate while in the apical domain it appears like a line at the apical membrane; (3) changes in the aPKC-binding site of PAR3 affect recruitment of aPKC but not of actin.

2 | RESULTS

2.1 | Characterization of the apical compartment in pre-lens ectoderm and lens placode

During lens placode morphogenesis, actin filaments accumulate in the apical domain of the cells (Borges et al., 2011). To characterize in spatial detail the evolution of apical actin accumulation in the early stage of lens placode elongation, relative to the apical membrane, we compared the location of the apical actin network with the focal plane that contained ZO-1, a tight junction protein. As shown in Figure 1a–b”, ZO-1 expression is restricted to the apical domain at all stages of the placode, while, in the pre-lens ectoderm, actin is homogeneously present under all of the plasma membrane (Figure 1c–c”). When the pre-lens ectoderm elongates to form the pseudostratified lens placode, actin concentrates in the same compartment as ZO-1 (Figure 1d–d”).

To verify if reallocation of actin cytoskeleton during lens development is modulated by dynamic reallocation of PAR3 we first detected PAR3 localization during placodal elongation and prior to apical actin accumulation. We followed PAR3 localization during conversion from pre-lens ectoderm to placode. PAR3 is apically located in the pre-lens ectoderm and it remains in the cell apex throughout lens placode formation (Figure 2a–b”). As the apical domain is defined by the PAR3/ PAR6/aPKC complex, we also analyzed the localization of aPKC and PAR6 in pre-lens ectoderm and lens placode. Because of the lack of suitable antibodies that recognize chick PAR6, we analyzed its subcellular localization indirectly, through immunofluorescence for GFP after the electroporation of non-phenotypic concentrations of PAR6 fused to GFP (0.1 μg μL⁻¹ of GFP::PAR6). GFP::PAR6 and aPKC were detected at the apical domain (Figure 2c–f”), but in a subdomain slightly more apical to PAR3, from pre-lens ectoderm through lens placode stages (Figure 3a–f). This relative subcompartmentalization, with aPKC/PAR6 slightly more apical than PAR3, also occurs in the neural tube, in polarized epithelia of invertebrate embryos and adult mammals (Afonso and Henrique, 2006; Harris and Peifer, 2005). These data suggest that apical actin accumulation is not driven by reallocation of PAR3. We speculated that formation of apical actin in this context results from modulation of PAR3 affinity for downstream partners.

2.2 | Par3 induces basolateral ectopic actin aggregates

In the chick neural tube, overexpression of PAR3 is sufficient to promote the formation of ectopic PAR3/PAR6/aPKC complexes and to recruit adhesive junction components (Afonso and Henrique, 2006). Because the relationship between PAR3 and f-actin has not been previously addressed in the placode, we first needed to determine if PAR3 alone was sufficient to recruit f-actin ectopically.

Thus, we overexpressed the full length sequence of chicken PAR3 fused to GFP (cPAR3::GFP) (2.5 μg μL⁻¹) in the context of lens placodal cells. Because apical f-actin is already intensely labeled in control placodes, which hinders detection of changes, we focused our attention on ectopic cPAR3::GFP punctate clusters in the basolateral membrane domains of placodal cells (Figure 4a,b, arrows). There, both beta-catenin and actin co-localized to the ectopic basolateral clusters (Figure 4a’,b’).

PAR3 has a conserved amino-terminal domain involved in self-oligomerization (NTD/CR1) (Feng, Wu, Chan, & Zhang, 2007; Zhang et al., 2013). To verify if oligomerization is necessary for the establishment of the ectopic actin clusters, we overexpressed a truncated form of cPAR3 that lacks this domain (cPAR3-N::GFP; Afonso & Henrique, 2006). The truncated form was found throughout the cytoplasm, did not form clusters nor elicited ectopic basolateral actin accumulation in the placode (Figure 4c–c’). These data suggest that PAR3 is sufficient to initiate accumulation of actin and adhesive proteins and that oligomerization is important for this event.

2.3 | Par3 recruits apical actin in the periplacodal ectoderm

Because the apical domain of placodal cells contain high amounts of actin, we could not observe if overexpression of PAR3 enhanced the accumulation of apical actin. Thus, to confirm that PAR3 is sufficient to recruit f-actin to the apical domain in this embryonic region, we analyzed the effect of PAR3 in the apical domain of the cells that surround the placode (periplacodal ectoderm), where actin filaments are homogeneously distributed along the apical-basal domain (Figure 5a’–a”) and an apical f-actin network is normally not evident (Borges et al., 2011). Here, PAR3 recruited ectopic f-actin apically. Interestingly, the pattern of the PAR3-generated ectopic apical actin differed from the ectopic basolateral clusters seen in the placodal cells. Whereas the basolateral actin clusters were all punctate, in the apical domain of periplacodal ectoderm ectopic actin appeared as a line in the subapical domain (Figure 5b’–d”). The similarity of the phalloidin fluorescence intensity profiles in the basal-apical axis compared to the immunostaining for cPAR3::GFP or MYC::PAR3 mutants suggests that this actin network was induced by the presence of ectopic PAR3 in the apical domain (Figure 5b”–d”)
FIGURE 1  Actin polarizes to the apical domain during placode elongation. Pre-lens ectoderm (a, c, e) and lens placode (b, d, f) were immu-
nostained for the occlusive junction protein ZO-1 (a–f; a’–f’) and adhesive junction protein beta-catenin (e’, e”, f’, f”) and counterstained for
cell membrane with Dio (a’–a”, b’–b”) or actin (c’–c”; d’–d”). ZO-1 is consistently in the apical domain at all stages analyzed (a–f). Actin is
distributed throughout the cell periphery in pre-lens ectoderm (c’, c”) but is polarized to the apical domain after placode elongation (d’, d”).
Beta-catenin is localized along the apico-basal sides (e’, e”) in pre-lens ectoderm and enriched in the apical domain in the elongated placode
cells (f’, f”). The apical membrane faces the right edge of the images. The dotted line delimits the interface between the lens precursor tissue
(right) and optic vesicle (left). The boxes in the diagrams to the right represent the region of the image relative to the developing lens.
P = placode; E = Ectoderm; OV= Optic Vesicle Bar = 5μm
FIGURE 2 The PAR complex is apical in pre-lens ectoderm and lens placode. Pre-lens ectoderm (a–a’; c–c”; e–e”) and lens placode (b–b”; d–d”; f–f”) were immunostained for ZO-1, PAR3, aPKC, and GFP, in embryos electroporated with GFP::cPAR6. PAR3, aPKC, and PAR6 are in the apical domain in the pre-lens ectoderm (a’–a”, c’–c”, and e’–e”, respectively) and in elongated lens placode (b’–b”, d’–d”, and f’–f”). Apical membrane faces the right edge of the images. The placode and pre-lens ectoderm lie to the right of the dotted line. Bar (a–f and a’–f’): 5 μm. (a”–f”) show magnified view of the boxed areas. Bar: 1μm. The boxes in the diagrams to the right represent the region of the image relative to the developing lens. P = placode; E = Ectoderm; OV = Optic Vesicle
2.4 Phosphorylation of threonine 833 does not affect par3-mediated actin recruitment

To test if PAR3-driven accumulation of ectopic actin requires recruitment of other known components of the PAR complex we decreased PAR3 affinity for other members of the PAR complex. One of the key sites that modulate affinity of PAR3 for other members of the PAR complex is threonine 833 (T833). T833 charge state changes with its phosphorylation status. Substitution of PAR3 threonine 833 by the acidic residue aspartic acid (PAR3T833D), diminishes its affinity for aPKC and, as a consequence, to the rest of the apical PAR complex (Nakayama et al., 2008). Conversely, substitution of PAR3 T833 by an alanine residue (PAR3T833A) increases the stability of the complex. Therefore, we compared the effect of PAR3T833A or PAR3T833D in recruiting actin ectopically.

Both mutants formed aggregates in the basolateral membrane of the cells that colocalize actin (Supporting Information Figure 2). When we quantified actin recruitment, all three forms (PAR3FL and both mutants) colocalized with f-actin at a similar frequency; around 10% of PAR3 clusters recruited actin, suggesting that the ability to recruit actin ectopically did not depend on the charge of residue T833 (Figure 6e).

No significant differences were detected between native PAR3 (PAR3FL) and PAR3T833D in their ability to recruit aPKC (Figure 6, arrows). However, as expected, the PAR3T833A mutant colocalized with aPKC more often than native PAR3 (Figure 6e). These data suggest that, in the placode, the phosphorylation state of PAR3T833 also modulates PAR3 affinity for aPKC. Furthermore, it indicates that actin recruitment was not proportional to the affinity of PAR3 for aPKC.

3 DISCUSSION

In the present study, we show that in the lens placode the PAR3/PAR6/aPKC complex is apically localized, albeit at different subcellular levels. This finding is similar to what was reported in the chick neural tube, where PAR3, PAR6, and aPKC are all localized in the apical compartment, but do not overlap. Instead, similar to what we observe here, PAR6 and aPKC are localized slightly above PAR3. Whereas PAR3 co-localizes with adhesive junctions (Afonso and Henrique, 2006).

Our functional data supports the hierarchical importance of PAR3 shown in other systems. For instance, during wound-healing of epithelial sheets, junctional complexes are re-established and PAR3 and aPKC are recruited—in this order—to form punctate aggregates (Suzuki et al., 2002). We show here that the PAR3 ectopic clusters do contain aPKC, suggesting that PAR3 recruited aPKC. In the neural tube, overexpression of PAR3 alone is sufficient to induce punctate clusters of beta-catenin (Afonso and Henrique, 2006). In the lens placode, PAR3 was also sufficient to promote formation of ectopic clusters of beta-catenin. Clustering of PAR3 itself in the basolateral membrane and its ability to recruit beta-catenin was mediated by the CR1 domain in our experimental paradigm. Likewise, ectopic clustering of PAR3 in the chick neural tube was also dependent on the presence of the CR1 domain (Afonso and Henrique, 2006). Taken together, these data suggest that the role of PAR3 and its mechanism of action as an initiator of polarity cue changes seem to be preserved in the lens placode.

While the CR1/NTD domain is responsible for membrane targeting of the PAR complex, another region mediates the direct interaction between PAR3 and aPKC (Chen and Zhang, 2013). Single residues in
PAR3 aPKC-interacting domain are directly relevant to the stabilization of the apical polarity complex (Khazaei and Puschel, 2009; Nagai-Tamai, Mizuno, Hirose, Suzuki, & Ohno, 2002; Nakayama et al., 2008). Amongst these, T833 in rat PAR3 has been reported to be a key residue involved in the regulation of PAR3-aPKC interaction (Nakayama et al., 2008). Phosphorylation of T833 by Rho-ROCK or its substitution by an aspartate residue inhibits interaction of PAR3 with aPKC and PAR6 in cell extracts. Here, we confirm the importance of T833 in regulating affinity to aPKC. Wild type (PAR3FL) and the phosphomimetic mutant (PAR3T833D) were similar in their ability to recruit aPKC. In contrast, substitution of T833 for a nonphosphorylatable residue (PAR3T833A) enhanced significantly the appearance of aPKC in the ectopic basolateral clusters. Together, these data suggest that in the basolateral domain, the ectopically expressed wild type PAR3 most
likely is phosphorylated at T833 and thus behaves similarly to PAR3T833D. An additional point is that increased effectiveness in recruiting aPKC was not translated into enhanced actin recruitment. There was no difference in the number of actin-positive ectopic clusters generated by either forms of PAR3.

A possible explanation is that the aPKC recruited to ectopic basolateral clusters is not active. The basolaterally located ectopic actin clusters never evolved beyond punctuate clusters. Punctate actin clusters are characteristic of initial establishment of spot-like adhesive junctions and their establishment do not require aPKC kinase activity (Vasioukhin, Bauer, Yin, & Fuchs, 2000). aPKC activity is essential for the transition of spot-like junctions to belt-like adhesive junctions (Kishikawa, Suzuki, & Ohno, 2008; Suzuki et al., 2002). In the absence of aPKC activity the primordial punctate aggregates remain in their immature state (Suzuki et al., 2002). Further, apical adhesive junctions require the presence of active aPKC for their maturation (Kishikawa et al., 2008). In this scenario, the variation that we observe in the quality of ectopic actin clusters in the basolateral (punctate) and apical membranes (lines) might be due to differences in the availability of activated aPKC between these two domains. In other words, although the PAR3 T833 mutants do differ in their ability to recruit aPKC, both mutants and wild-type ectopic PAR3 would bind to inactive aPKC in the basolateral clusters and thus the adhesive junctions remain arrested at a nascent state. This would explain why PAR3FL, PAR3T833A, and PAR3T833D do not differ in their ability to form actin-positive ectopic clusters. In contrast, the availability of activated aPKC in the apical domain would allow the
maturation of ectopic apical actin into more complex formations in the periplacodal cell. An alternative possibility is that distinct isoforms of aPKC are distributed along the polarized cell. For instance, in embryonic hippocampal neurons, the different isoforms of aPKC: aPKC-lambda and PKM-zeta are spatially segregated according to neuronal polarity. Both isoforms interact with PAR3 but have opposite effects in axon outgrowth. Whereas aPKC-lambda/PAR3 is associated with axonal outgrowth, PKM-zeta/PAR3 represses formation of axons (Parker

**FIGURE 6** PAR3FL, PAR3T833A or PAR3T833D recruited actin and aPKC ectopically. PAR3FL (a), PAR3T833A (b) and PAR3T833D (c) overexpression (green channel) formed aPKC clusters at the basolateral plasma membrane (arrows in a', b' and c', red). (a', b' and c') are magnifications of the boxes in (a–c) respectively. The overexpressed protein is detected by the presence of GFP for PAR3FL or Myc for the mutants (green channel). The nuclear counterstain is DAPI (blue channel). PAR3T833A ectopic clusters recruited aPKC more frequently than native PAR3 (PAR3FL) (e). The difference between Par3FL x T833A in their ability to recruit aPKC was considered significant under Dunn's test pairwise comparison (0.05 < p < 0.08; asterisk) but not compared to PAR3T833D. In contrast, PAR3FL, PAR3T833A and PAR3T833D do not differ significantly in their ability to recruit actin. Bar: 10 μm (a–c), 2 μm (a', b', c'). The images were captured from the lens placode as shown in the drawing d). P = placode, OV = optic vesicle.
et al., 2013). In this scenario, we would have an isoform of aPKC in the basolateral domain that inhibits formation of potentially contractile complex networks of actin, while in the apical region another isoform of aPKC that allows their formation is present. Unfortunately, the reagents available for our experimental paradigm are not sufficiently sensitive to distinguish between active and inactive or different isoforms of aPKC in the ectopic clusters.

An alternative, but not exclusive explanation, is that further evolution of the ectopic junctions requires additional downstream elements. A possible missing element in basolateral regions is ARP/WASP. ARP2/3 alone is a low-efficiency nucleator and requires binding to a nucleation promoting factor such as WASP (Campellone and Welch, 2010). When ARP2/3 is inhibited, the apical localization of actin is disrupted and the continuity of junctional belts is affected (Baum and Georgiou, 2011; Georgiou, Marinari, Burden, & Baum, 2008). PAR3 can interact with WASP/ARP2 via the scaffolding membrane 14-3-3 (Hurd et al., 2003; Jin et al., 2004). Thus, it is possible that the arrest of the ectopic basolateral actin clusters in punctate conformation is due to lack of both aPKC activity and availability of ARP2/3.

Finally, we would like to end by presenting the chick lens placode as a model system to explore modulation of polarity signals in vertebrate epithelia during embryonic morphogenesis. Most studies in cell polarization in embryos revolve around establishment of apical-basal identity in immature invertebrate epithelia/zygotes. Likewise, the invertebrate ovary follicle is the most popular site for investigation of polarity changes in established embryonic epithelia (St Johnston and Ahringer, 2010). On the other hand, calcium-depletion-induced repolarization of mammalian tissue cultured cells, while contributing significantly to our understanding of cell polarity, do not cover the complexity of embryonic morphogenesis (Roignon, Peng, & Mostov, 2013; Wang and Margolis, 2007). Given the accessibility of the lens placode for imaging and the amount of genetic tools available for the chick embryo, this is a tissue that could contribute with general understanding of the control mechanisms for cell polarity in embryogenesis.

Abbreviations: PAR, partition-defective proteins; aPKC, atypical protein kinase C; ZO-1, zona occludens protein – 1; GFP, green fluorescent protein; ARP1/2, actin-related protein 1/2; WASP, Wiskott-Aldrich syndrome protein.

4 | MATERIALS AND METHODS

4.1 | Chick embryo harvest and cryosection

Fertilized eggs from Leghorn hens (Yamaguishi Farm, São Paulo, Brazil) were incubated at 37°C and 50% of humidity to obtain embryos at the stages of pre-lens ectoderm (HH11, 40-45 h) and lens placode (HH13, 18-21 somites, 48–52 h) (Hamburger and Hamilton, 1951). The embryos were collected by the filter paper method (Chapman, Colignon, Schoenwolf, Lumsden, & Communication, 2001); the head was sectioned at the level of heart, fixed in 2% paraformaldehyde for 30 min, washed three times with PBS and cryoprotected for 1 h on 20% sucrose at 4°C. Thereafter, they were embedded in 1:1 of 20% of sucrose and OCT (TissueTek) and stored at -80°C. Cryosections (10 μm in thickness) were collected on gelatinized slides for immunofluorescence analysis. All the experimental procedures have been approved by the Ethical Committee for Animal Research of the Biomedical Institute at our University.

4.2 | Immunofluorescence

The slides were air-dried at 37°C for 30 min, fixed in 4% paraformaldehyde for 30 min, washed three times with PBS and blocked for 1 h with 3% NGS (Jackson ImmunoResearch), 1% BSA (Jackson Immunoresearch) diluted in PBST (PBS containing 0.1% Triton X-100 (Sigma) at room temperature in a humid chamber. The primary antibodies were diluted in blocking solution and applied overnight. The primary antibodies used were mouse anti-PKCα (Biosciences, no. 610175) and rabbit anti-PKCζ (Cell Signaling no. C24E6), mouse anti-beta-catenin (BD Biosciences, no. 610153), mouse anti-GFP (Molecular Probes, no. A11120), rabbit anti-GFP (Molecular Probes, no. A6455), mouse anti-myC (Invitrogen, no. R950-25), rabbit anti-myC (Cell Signaling Technology, 71D10), rabbit anti-PAR3 (Millipore, no. 07-330), mouse anti-ZO-1 (Molecular Probes, no. 339100), and rabbit anti-ZO-1 (Invitrogen, no. 617300). After incubation with the primary antibodies, the slides were washed with PBS and incubated with secondary antibodies. The lipophylic stain DIO was used as a counterstain to delineate cell membranes. No commercially available antibodies could detect chicken PAR6 (cPAR6). Thus, we resorted to indirect detection through immunofluorescence for GFP following electroporation with low concentration of GFP::cPAR6 (0.1 μg μL⁻¹).

Secondary antibodies were Alexa 488-conjugated goat anti-rabbit (Molecular Probes, no. A11008), Alexa 488-conjugated donkey anti-mouse (Molecular Probes, no. A21202), TRITC conjugated goat anti-mouse (Zymed, no.B1-6541) and TRITC conjugated goat anti-rabbit (Sigma, no.T6778). The cryosections were incubated with rhodamine-phalloidin (Molecular Probes, no.R415) for actin filaments detection, Vybrant DIO (Invitrogen, no.V22886) for membrane staining and DAPI (Sigma) for nuclei staining.

4.3 | In ovo electroporation

The embryos were manipulated in ovo and visualized with 10% Indian ink diluted in Ringers solution injected under the embryo. The platinum electrodes (0.5-mm diameter) were placed parallel to the head, at the level of the optic vesicles, with the negative electrode next to the right pre-lens ectoderm. Electroporation was performed at earlier stages to allow time for the exogenous gene expression to develop in the placode. An aperture was made over the vitelline membrane next to the exogenous gene expression to develop in the placode. The slides were air-dried at 37°C for 30 min, fixed in 4% paraformaldehyde for 30 min, washed three times with PBS and blocked for 1 h with 3% NGS (Jackson ImmunoResearch), 1% BSA (Jackson Immunoresearch) diluted in PBST (PBS containing 0.1% Triton X-100 (Sigma) at room temperature in a humid chamber. The primary antibodies were diluted in blocking solution and applied overnight. The primary antibodies used were mouse anti-PKCα (Biosciences, no. 610175) and rabbit anti-PKCζ (Cell Signaling no. C24E6), mouse anti-beta-catenin (BD Biosciences, no. 610153), mouse anti-GFP (Molecular Probes, no. A11120), rabbit anti-GFP (Molecular Probes, no. A6455), mouse anti-myC (Invitrogen, no. R950-25), rabbit anti-myC (Cell Signaling Technology, 71D10), rabbit anti-PAR3 (Millipore, no. 07-330), mouse anti-ZO-1 (Molecular Probes, no. 339100), and rabbit anti-ZO-1 (Invitrogen, no. 617300). After incubation with the primary antibodies, the slides were washed with PBS and incubated with secondary antibodies. The lipophylic stain DIO was used as a counterstain to delineate cell membranes. No commercially available antibodies could detect chicken PAR6 (cPAR6). Thus, we resorted to indirect detection through immunofluorescence for GFP following electroporation with low concentration of GFP::cPAR6 (0.1 μg μL⁻¹).

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or containing the above clones in the pre-lens ectoderm (HH49, 33 h of incubation). Plasmids [diluted in 0.1% Fast Green (Sigma)] were injected by the aperture next to the pre-lens ectoderm of the right eye, and five square pulses of 9 V and 50 ms of duration and 100 ms of intervals were administered. Embryos were reincubated and analyzed ~16 h later at lens placode stage and processed for immunofluorescence. Special care was taken so that the placode was properly aligned for the cryosections. To guarantee that the sections were aligned parallel to the apical-basal axis, we only used cryosections where the placodal cells were at their maximal height. Slides were mounted in Vecta Shield (Vector Laboratories, USA) and analyzed with a LSM 510 Zeiss microscope and Zeiss LSM-780 NLO (CEFAP, ICB-USP, FAPESP 2009/53994-8). The images are representative of at least three independent experimental sets.

4.4 Quantification of fluorescent images

The intensity of labeling for actin was obtained using the free software Image J v1.43 (NIH) as previously described (Borges et al., 2011). Briefly, the apico-basal length was delimited with a box on unprocessed confocal-captured images and the intensity of the pixels was tabulated relative to the apico-basal distance. We attributed to each image the value of 100 for the highest intensity and all other values were expressed as a fraction of this value. The quantification profiles shown are representative of at least three independent experimental sets. In total, we analyzed confocal slices from six embryos electroporated with PAR3FL (full length-wild type) and four embryos with T833A or T833D.

To quantify the number of ectopic clusters that contained aPKC and actin, confocal images were analyzed in Image J v1.43 (NIH). PAR3::GFP clusters were identified and labelled by arrows. The arrows were added in a way that they appear in all channels (PAR3 and aPKC or phalloidin channels) and counted in each confocal frame of the appropriate channel. Then, phalloidin or aPKC labels that were identified in the same location as the PAR3::GFP clusters (arrows) were counted. Next, we calculated how many clusters of PAR3::GFP colabeled for aPKC or phalloidin. We analyzed all the confocal frames captured from different embryos. In PAR3FL embryos 135 images (11 embryos) were analyzed for both actin and aPKC clusters; on PAR3T833A embryos 91 images (8 embryos) and 34 images (3 embryos) were analyzed for actin and aPKC, respectively; and on PAR3T833D embryos 49 images (5 embryos) for actin and 49 images (4 embryos) for aPKC were analyzed. The colocalization index of wild-type (PAR3FL) and mutant PAR3 forms and actin or aPKC was analyzed for variance distribution with Kruskal-Wallis test followed by Dunn’s pairwise comparison.

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AUTHOR CONTRIBUTION

MOM and CYIY designed the experiments, MOM performed the experiments. MOM, RB and CYIY analyzed and discussed the results and CYIY wrote the manuscript.

REFERENCES


**SUPPORTING INFORMATION**

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