

# aPKC, Crumbs3 and Lgl2 control apicobasal polarity in early vertebrate development

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## Summary

In early vertebrate development, apicobasally polarised blastomeres divide to produce inner non-polarised cells and outer polarised cells that follow different fates. How the polarity of these early blastomeres is established is not known. We have examined the role of Crumbs3, Lgl2 and the apical aPKC in the polarisation of frog blastomeres. Lgl2 localises to the basolateral membrane of blastomeres, while Crumbs3 localises to the apical and basolateral membranes. Overexpression aPKC and Crumbs3 expands the apical domain at the expense of the basolateral and repositions tight junctions in the new apical-basolateral interface. Loss of aPKC function causes loss of apical markers and redirects basolateral markers ectopically to

the apical membrane. Cell polarity and tight junctions, but not cell adhesion, are lost and outer polarised cells become inner-like apolar cells. Overexpression of *Xenopus* Lgl2 phenocopies the aPKC knockout, suggesting that Lgl2 and aPKC act antagonistically. This was confirmed by showing that aPKC and Lgl2 can inhibit the localisation of each other and that Lgl2 rescues the apicalisation caused by aPKC. We conclude that an instrumental antagonistic interaction between aPKC and Lgl2 defines apicobasal polarity in early vertebrate development.

Key words: aPKC, Crumbs, Lgl, Epithelial polarity, *Xenopus*

## Introduction

A conserved aspect of early vertebrate development is the polarisation of early blastomeres along an apicobasal axis and the generation of inner and outer cells by division of these polarised blastomeres. For example, in the mouse, *Xenopus* and zebrafish morula and blastula stage embryos, divisions perpendicular to the apicobasal axis of polarity generate both outer polar and inner apolar cells, while divisions parallel to this axis generate only outer polar cells (Chalmers et al., 2003; Johnson and Ziomek, 1981; Kimmel et al., 1995) (reviewed by Johnson and McConnell, 2004; Müller and Bossinger, 2003). The outer cells establish an epithelial layer, which is sometimes called a proto-epithelium (Johnson and McConnell, 2004). The production of outer epithelial cells enveloping an inner non-epithelial apolar cells is the first sign of morphological diversification of cells in development. In *Xenopus*, the orientation of division is controlled by the shape of the cells (Chalmers et al., 2003); in the mouse this is also the case (reviewed by Johnson and McConnell, 2004). In the mouse, outer cells give rise to trophectoderm, while inner cells give rise to the inner cell mass (ICM) (Tarkowski and Wroblewska, 1967), the precursor of the embryo proper. In *Xenopus*, in the animal pole, outer cells are fated to become secondary neuron precursors, while inner cells become primary neurons (Chalmers et al., 2002; Hartenstein, 1989). In zebrafish, outer cells give rise to the enveloping layer (EVL), while inner cells

give rise to the embryo (Kimmel et al., 1995). Thus, although the details differ, a common theme is that these two populations of cells remain segregated and follow different fates. The conserved aspect of early vertebrate development, the polarisation of the blastomeres, is a prerequisite for generating these two distinct populations of cells: outer polar epithelial cells and inner apolar cells. Thus, cell polarisation underpins the generation of cell fate diversity. Apicobasal polarisation starts at the two-cell stage in *Xenopus* embryos and slightly later, at the eight-cell stage, in the mouse (Fesenko et al., 2000) (reviewed by Johnson and McConnell, 2004). How the apicobasal polarity of these early blastomeres is established and maintained in the vertebrate embryo is not known.

Work in *Drosophila* embryonic epithelia and neuroblasts, has shown that apical and basolateral membrane domains are defined by the antagonistic action of protein complexes, which are localised to the apical and basal lateral membrane domains (reviewed by Muller and Bossinger, 2003; Tepass et al., 2001). The same network of interactions is conserved in the polarity of the *C. elegans* zygote (reviewed by Pellettieri and Seydoux, 2002). Apical complexes include the Par3/Par6/aPKC/Cdc42 complex and the Crumbs/stardust/discs-large complex. Basolateral proteins include Lethal Giant Larvae (LGL) and Par1. Homologues of these proteins have been identified in vertebrates and shown to form evolutionarily conserved complexes (Hurd et al., 2003; Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000; Plant et al., 2003; Roh et al., 2003;

Yamanaka et al., 2003). In mammalian cultured epithelial cells, members of these complexes play a role in polarity, primarily by regulating the formation of tight junctions (TJs) rather than defining apical or basolateral membrane identity (Hirose et al., 2002; Roh et al., 2003; Suzuki et al., 2002; Yamanaka et al., 2003). For example, inhibiting aPKC or Par6 blocks TJ formation during the re-polarisation of MDCK cells but does not have an effect on already polarised cells (Suzuki et al., 2002; Yamanaka et al., 2001). Similarly, mammalian Lgl overexpression does not affect the polarity of polarised cells but does block the formation of TJs (Yamanaka et al., 2003). Therefore, how apical and basolateral membrane identify is regulated in vertebrates, is unknown.

Here, we investigate the roles of three molecules – aPKC, Crumbs3 and Lgl2 (each representing one of the three major protein complexes involved in cell polarity in invertebrates) – in the polarisation of frog blastomeres. We have shown previously that aPKC is apically localised in frog blastomeres (Chalmers et al., 2003). We show here that Lgl2 localised specifically to the basolateral membrane, while Crumbs3 localised to the apical and basolateral membrane domains. Overexpression of *aPKC* expands the apical membrane, correspondingly reduces the basal side and repositions the TJs in the new apicobasal border. *Crumbs3* also expands the apical side but is less effective than *aPKC*. Loss of aPKC function with a dominant-negative construct, causes loss of apical identity and expansion of basolateral identity into the apical side. Cells lose their polarity and tight junctions, and become similar to inner apolar cells. Overexpressing *Lgl2* phenocopies the aPKC loss of function. Finally, aPKC and Lgl2 can inhibit the localisation of each other and *Lgl2* can rescue the over-apicalisation caused by overexpression of *aPKC*. These findings suggest that aPKC/Crumbs3 and Lgl2 are involved in polarisation of vertebrate embryonic epithelial cells by defining apical and basolateral membrane identity. Furthermore, aPKC and Lgl2 show an antagonistic interaction, which appears to have been evolutionarily conserved in embryogenesis between vertebrates and invertebrates.

## Materials and methods

### DNA constructs

Xt aPKC  $\lambda$  was isolated using the *Xenopus tropicalis* EST database (Gilchrist et al., 2004), clone Tgas015a22 was picked, sequenced (GenBank AY884235) and the coding sequence subcloned into pCS2. Xt PKC  $\lambda$  N-terminal construct was made by cloning the sequence coding for the first 126 amino acids of XtPKC $\zeta$  into CS2. A Xt Crumbs3 clone (Tegg038L10) was identified using ESTs and the coding sequence (GenBank AY884237) cloned into pCS2. An Xt Lgl2 clone was identified (Tegg006o20) from the ESTs, sequenced (GenBank AY884236) and the coding sequence cloned into pCS2. A pCS2 GFP construct was made for RNA overexpression and producing fusion proteins by cloning the coding sequence of GFP3 into pCS2. GFP-Lgl2 and Crumbs3-GFP were made by cloning the coding sequence of each protein into pCS2 GFP. The following constructs were also used: His-tagged mouse aPKC  $\lambda$  pSP64T (Nakaya et al., 2000) and *lacZ* pCS2 (Chalmers et al., 2002).

### RNA overexpression

The constructs described above were used to make RNA for overexpression using the message machine kit (Ambion) and the RNA injected into embryos at the two-cell stage. The embryos were then cultured until the required stage and fixed in PBSF (phosphate-

buffered saline+4% formaldehyde), photographed and if required embedded in gelatin albumen and sectioned on a vibratome (Chalmers et al., 2002). Alternatively the embryos were fixed in Dent's (80% methanol + 20% DMSO) at  $-20^{\circ}\text{C}$  for antibody staining. Embryos injected with GFP fusion constructs (or GFP fusion constructs and RLDX) were fixed in 4% paraformaldehyde in PBS for 1 hour and stored in Dent's.

### Antibody staining and GFP localisation studies

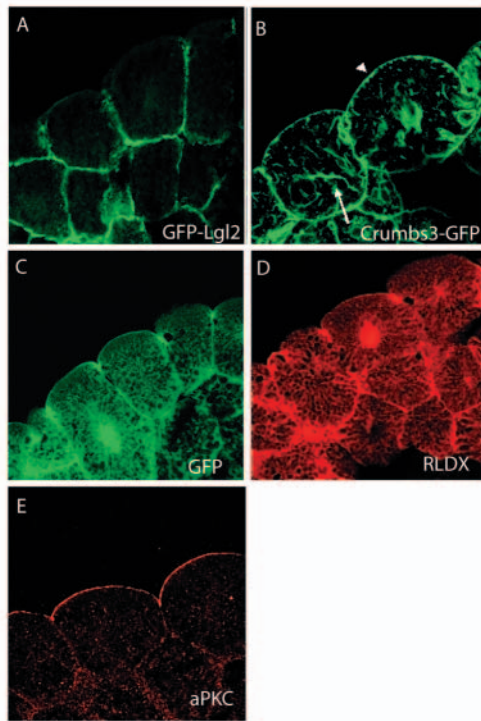
Embryos injected with GFP fusion proteins were cryosectioned using the fish gelatin protocol (Fagotto and Gumbiner, 1994), mounted in Vectashield (Vector Laboratories) and imaged directly on the confocal (BioRad Radiance confocal). GFP and the cytoplasmic lineage label RLDX (Rhodamine-labelled lysinated dextran, Molecular Probes) were injected as a control.

Antibody staining was carried out on fish gelatin cryosections (Fagotto and Gumbiner, 1994) as described (Chalmers et al., 2003). The following antibodies were used. Anti-pan cytokeratin clone C-11 (Sigma, C2931), anti-occludin (Cordenonsi et al., 1997), anti  $\beta$ 1 integrin 8C8 (Gawantka et al., 1992), Developmental Hybridoma Bank), anti-cingulin (Cardellini et al., 1996), anti-aPKC (Santa Cruz, nPKC $\zeta$  C-20 SC-216; unfortunately there seems to be a big variation in quality between batches of this antibody), anti-GFP (Molecular Probes, A11122). The following secondary antibodies were used anti rabbit Alexa 568 (Molecular Probes, A11011), anti-rabbit Alexa 488 (Molecular Probes, A11008) and anti-mouse Alexa 568 (Molecular Probes, A11004). When Cytex Green (Molecular Probes) was used as a nuclear stain it was added with the secondary antibody at final concentration of 1/5000.

## Results

### aPKC, Crumbs3 and Lgl2 show specific subcellular localisation in embryonic epithelial cells

A full-length clone for the *Xenopus* homologue of *Lgl* was identified in a *Xenopus tropicalis* EST database (Gilchrist et al., 2004) and was isolated from the corresponding arrayed library. The encoded protein is more similar to mouse Lgl2 than mouse Lgl1 and was named *Lgl2*. Similarly, we also identified a homologue of *Crumbs* from *Xenopus tropicalis* which was most similar to human *CRB3* (Crumbs 3) and is therefore referred to as *Crumbs3*. A GFP-Lgl2 fusion protein showed basolateral localisation in the early epithelial cells of blastula stage (stage 8) embryos (Fig. 1A), similar to the localisation of  $\beta$ -integrin and occludin (Chalmers et al., 2003; Cordenonsi et al., 1997; Gawantka et al., 1992). At the same stage, Crumbs3-GFP showed localisation to the inner side of apical membrane. Interestingly, the Crumbs3-GFP apical localisation was punctate as if in vesicles (Fig. 1B, arrowhead). Crumbs3-GFP was also localised to the basolateral membranes, which was surprising for a protein that is apical in other systems. However, we note that such has been reported for other apical proteins in *Xenopus* and is thought to be due to the increased demand for basolateral membrane during the rapid embryonic cleavages, which overwhelms the sorting ability of the cells (Roberts et al., 1992). Crumbs3-GFP was also observed in internal 'filamentous' or 'loop'-like structures, the identity of which is not known at present (Fig. 1B, arrow). The localisation of both these proteins was clearly distinct from the control GFP or the cytoplasmic lineage label RLDX (Fig. 1C+D). aPKC was apically localised (Fig. 1E), as previously reported (Chalmers et al., 2003). Therefore, in these early epithelial cells aPKC localises to the apical membrane, Lgl2



**Fig. 1.** aPKC, Crumbs3 and Lgl2 show specific localisation in early epithelial cells. (A) GFP-Lgl2 localised exclusively to the basolateral membrane at stage 8. (B) Crumbs3-GFP localised to the apical (arrowhead) and basolateral membrane at stage 8, and to unknown internal structures (arrow). (C) GFP control. The examples shown are after injecting 1 ng of RNA. (D) RLDX control. GFP localised nonspecifically in the cytoplasm, nucleus and points of cell contact, as did the lineage label RLDX. Because of the high yolk content of early *Xenopus* cells, cytoplasmic fluorescence of the controls has a latticed appearance. This is very distinct from the localisation of the fusion proteins shown. (E) Antibody staining showing that aPKC localises to the apical membrane.

to the basolateral membrane and Crumbs3 to both apical and basolateral membranes.

### aPKC and Crumbs3 overexpression expands the apical membrane

To test the function of aPKC in the polarisation of the blastomeres, first, we overexpressed the mouse and *Xenopus* aPKC in the two-cell stage *Xenopus* embryo. The mouse gene is the His-tagged mouse PKC lambda ( $\lambda$ ) construct used previously (Nakaya et al., 2000). The *Xenopus* gene was identified in the *Xenopus tropicalis* EST database and is more similar to mouse PKC $\lambda$  than PKC $\zeta$ .

The early cleavages of injected embryos were normal but at the blastula stage, the epithelium of the surface of the embryo had lost its normal appearance (Fig. 2A,B; see also whole mount in Fig. 7A), which can be seen with GFP or *lacZ*-injected embryos (Fig. 2C,D). The epithelial cells in aPKC-injected embryos were rounded and protruded from the surface of the embryo (Fig. 2A,B). In the animal hemisphere of wild-type *Xenopus* embryos only the apical surface is pigmented. In aPKC-overexpressing embryos, the pigmented surfaces of the protruding cells were expanded and the non-pigmented reduced. These protruding superficial cells were

not extruded from the embryo, as they did not fall off after the removal of the vitelline membrane; manual 'teasing' confirmed that they were firmly attached to their neighbours. Both mouse and *Xenopus* constructs showed a dose dependence in the percentage of affected embryos (Fig. 2, right panels) although, for reasons that are not clear, the mouse gene was more effective than the *Xenopus* one, at all concentrations tested.

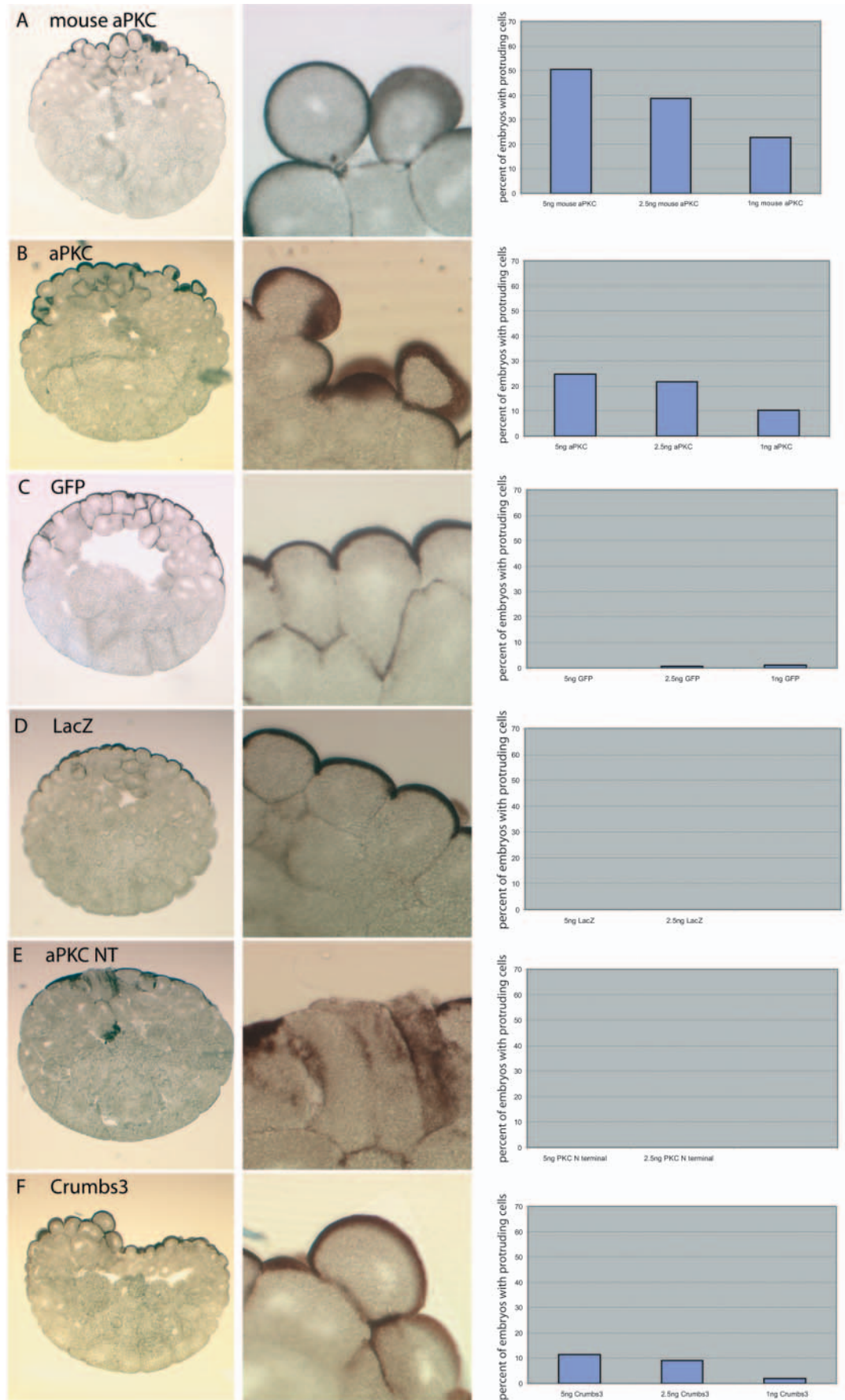
A truncated version of the *Xenopus tropicalis* protein, aPKC NT, which lacks the entire kinase domain was injected. This did not produce any apicalisation (Fig. 2E), showing that the kinase domain is required for apicalisation. Although the aPKC NT construct did not cause apicalisation, it did have another effect on the cells (see below).

This is the first time that aPKC has been shown to have the ability to expand the apical membrane. To our knowledge, the only other molecule which has been shown to have a similar effect in *Drosophila* and mammalian epithelia is Crumbs (Roh and Margolis, 2003; Wodarz et al., 1995). Therefore, we tested if Crumbs3 would also cause apicalisation in *Xenopus* embryonic epithelia. Over expression of *Crumbs3* did cause apicalisation similar to that observed with aPKC, although the phenotype was weaker (Fig. 2F).

### aPKC overexpression skews the ratio of apical to basolateral membrane domain and repositions tight junctions

To characterise the effect further, we looked at alterations of apicobasal polarity with antibodies for apical and basolateral markers in albino embryos. As an apical marker, we used keratin, which is localised all around the cortex of these early epithelial cells but is particularly enriched on the apical side (Jamrich et al., 1987; Klymkowsky et al., 1987). As basolateral markers we used occludin, a component of TJs, initially targeted to the basolateral surface (Fesenko et al., 2000) and  $\beta$ 1-integrin, a basolateral transmembrane protein (Gawantka et al., 1992). In aPKC-injected embryos, the keratin-enriched membrane was expanded (Fig. 3B compare with 3A), consistent with the pigmented surface expansion observed in pigmented embryos (Fig. 2). By contrast, the cell membrane that was positive for occludin and  $\beta$ 1-integrin was greatly reduced (Fig. 3D compare with 3C, Fig. 3F compare with 3E) suggesting that the apical domain is expanded at the expense of the basolateral one. We also looked at TJs, as aPKC has been implicated in TJ formation in mammalian epithelial cells. As a marker, we used cingulin, a protein found in the cytoplasmic plaque of TJs (Cordenonsi et al., 1999) (reviewed by D'Atri and Citi, 2002). Interestingly, cingulin staining was maintained but the position was shifted to the basal side, marking the new interface of the extended apical and reduced basolateral membrane domains (Fig. 3H compare with 3G). Immunostaining of aPKC-injected embryos with an aPKC antibody confirmed that the affected area was positive for overexpressed aPKC (Fig. 3J). This analysis showed that overexpression of aPKC causes the formation of super-apical epithelial cells, while tight junctions are maintained but shifted in their position (Fig. 3K). Apicobasal polarity is maintained as the cells still have a distinct apical and basolateral membrane. However, polarity is distorted, as the allocation of cell membrane to the apical and basolateral sides becomes heavily biased in favour of the apical side.



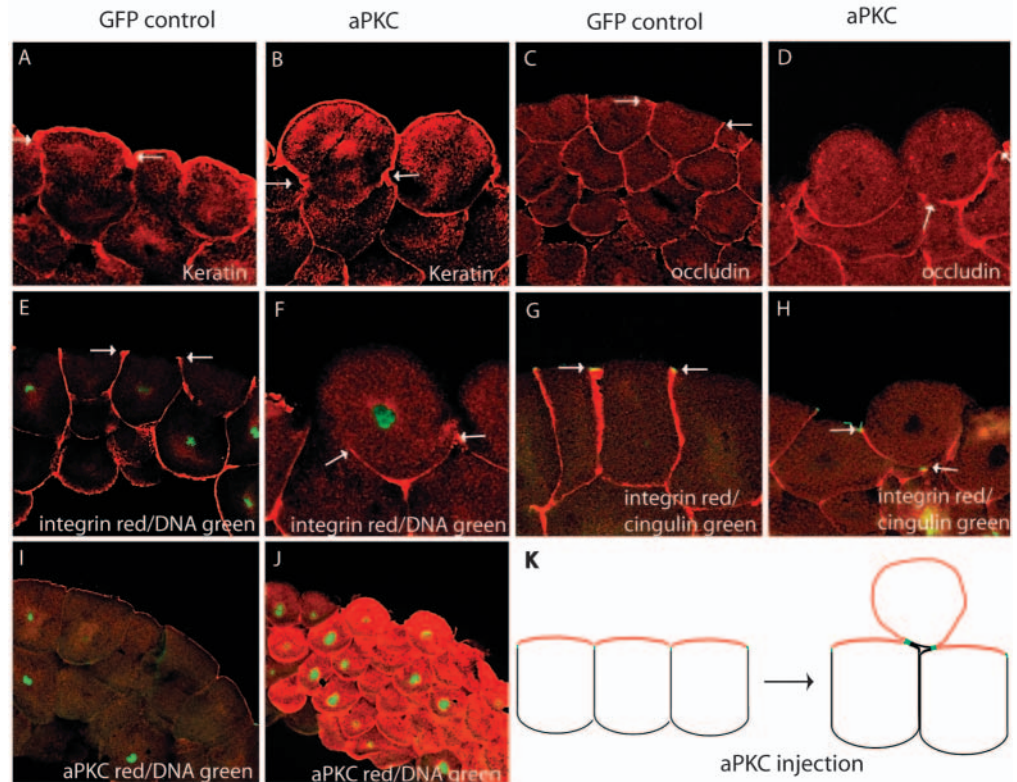


**Fig. 2.** aPKC overexpression produces rounded, protruding, hyper-pigmented cells. (A,B) Mouse and *Xenopus* aPKC overexpression produced embryos with protruding superficial cells and extended pigmented (apical) surface when compared with controls (C,D).

(E) Overexpression of a truncated version of the *Xenopus tropicalis* protein, PKC NT, which lacks the entire kinase domain, failed to produce this phenotype. (F) *Crumbs3* overexpression caused cell protrusion and over-apicalisation, similar to that of aPKC, but was less effective in that the percentage of affected embryos was lower.

Quantification was carried out blind, by counting the number of embryos with protruding cells. Right hand panels show the percentage of affected embryos at each concentration of injected RNA. Each experiment was carried out at least three times and the average is shown.

**Fig. 3.** aPKC is sufficient to promote apical and inhibit basal lateral membrane identity without disrupting tight junctions. (A,B) aPKC overexpression (B) caused expansion of the apical marker keratin compared with GFP control (A). (C-F) aPKC caused reduction in the basolateral markers, occludin (D) and  $\beta$ 1-integrin (F) compared with controls (C,E). (G,H) aPKC caused tight junctions (as marked by cingulin) to be maintained but relocated to the new apicobasolateral border. The borders of the markers used in each panel are delineated with arrows. (I,J) aPKC staining in GFP-injected controls (I) and aPKC staining in aPKC-injected (J) embryos. The apicalised cells have inherited overexpressed aPKC. (K) Diagrammatic representation of the result; aPKC causes protruding hyper-apical cells, which still have tight junction markers. Apical, red; basolateral, black; tight junctions, green. Albino embryos were injected with aPKC RNA and stained for antibody markers of cell polarity. Each experiment was carried out three times.



### Loss of aPKC function causes expansion of the basolateral membrane domain

The *Xenopus* egg contains a supply of maternal aPKC RNA and protein (Chalmers et al., 2003; Nakaya et al., 2000). To knockout aPKC, first, we targeted the maternal RNA by injecting antisense oligos into oocytes and recovering them with the host-transfer method (Heasman et al., 1994). Although we were able to identify several antisense oligos that were efficient in knocking down the RNA level in the egg, the protein level was not reduced up to the blastula stages, presumably indicating the persistence of stable maternal protein (data not shown). Such antisense oligos are usually not effective at later stages of embryogenesis. Second, we injected morpholino oligos (MOs) for aPKC $\lambda$  that were effective in reducing the protein level at post-mid-blastula transition (MBT) stages (see Fig. S1A in the supplementary material), presumably reflecting an effect on the zygotic protein. These also did not have an effect at early stages. After MBT, these embryos developed severe ectodermal defects (see Fig. S1B-F in the supplementary material). TUNEL assay in aPKC $\lambda$  morpholino embryos showed increased cell death at the gastrula stage (see Fig. S1G-I in the supplementary material), which is consistent with the reported role of aPKC in cell survival (reviewed by Moscat and Diaz-Meco, 2000). However, it is not clear that cell death is the primary cause of the ectodermal defects, because cell death seemed confined to the deep cells of the embryos (see Fig. S1I in the supplementary material). In any case, the severity of this phenotype hindered the analysis of any effects on cell polarity.

Third, we injected RNA at the two-cell stage for the truncated form of aPKC, aPKC NT, which lacks the kinase domain but retains the Par6-interacting domain (Fig. 4A).

Therefore, this fragment of aPKC can bind its normal partner Par6 but has no kinase activity and so acts as a dominant-negative for endogenous aPKC function (Gao et al., 2002). The advantage of this approach is that aPKC is inhibited before the apoptotic pathway becomes activated at MBT, so any pre-MBT effects cannot be due to apoptosis. As expected, injection of this RNA did not cause apicalisation (see above, Fig. 2). Instead, cells in the injected region lost their pigmentation, indicating loss of apical identity (Fig. 4C,D). The effect was small in terms of percentage of embryos (Fig. 4B) and the number of cells effected, but reproducible. Co-expressing the wild-type aPKC with the dominant-negative fragment reduced the number of affected embryos to almost background levels (Fig. 4B). This rescue confirms that the effect of the dominant-negative fragment is caused by inhibiting aPKC and not a non-specific effect.

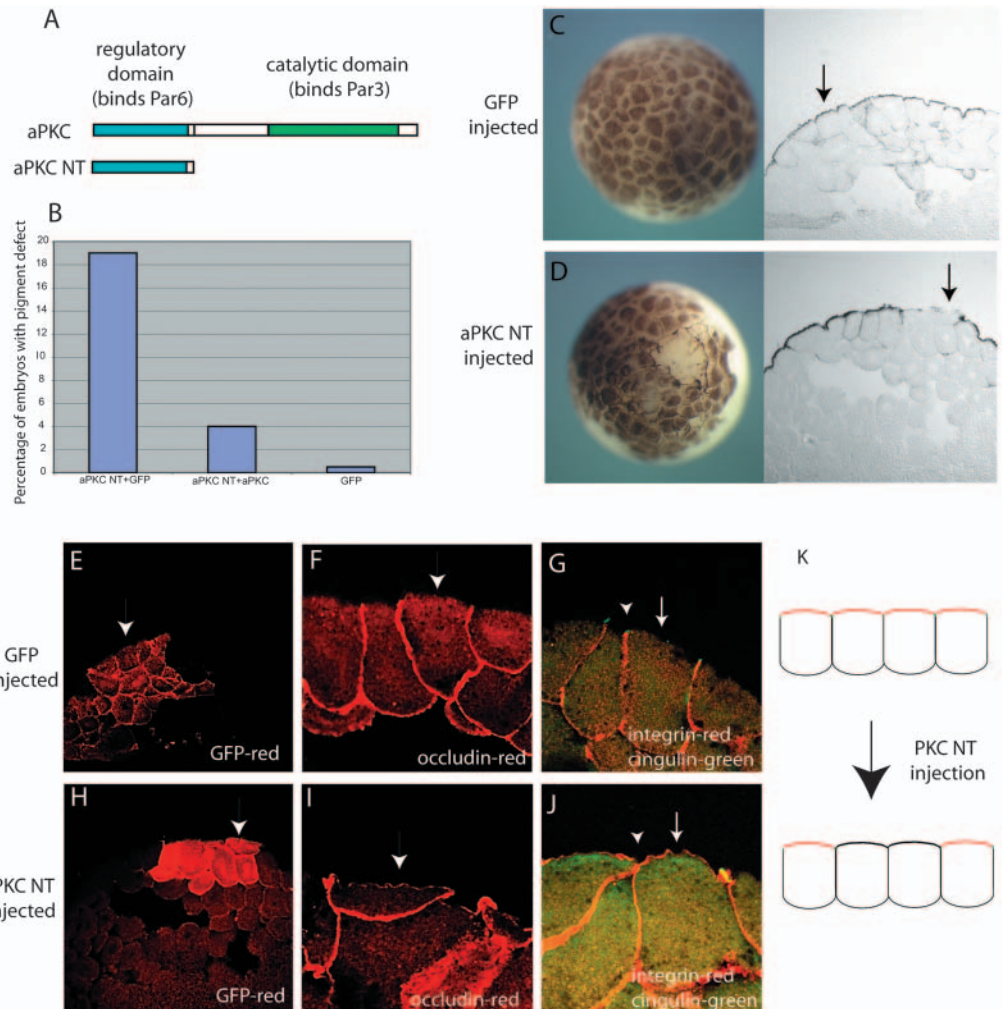
The loss of apical identity was confirmed and extended by staining for membrane protein markers. The basolateral markers  $\beta$ 1-integrin and occludin now appeared ectopically on the apical side of the injected cells (Fig. 4F-J). The effect was stronger for  $\beta$ 1-integrin than for occludin. TJs, marked by cingulin, were lost in the affected region (Fig. 4J). However, cell adhesion was maintained, as one would expect by the fact that cadherin-mediated adhesion is restricted to the basolateral membrane (Angres et al., 1991; Schneider et al., 1993).

Thus, although overexpression of aPKC drives the expansion of the apical domain of the cell membrane, loss of aPKC function has the opposite effect of expanding the basolateral domain (Fig. 4K). Localisation of basolateral markers all around the cell membrane is a distinctive feature of inner apolar cells. Therefore, we conclude that the epithelial



**Fig. 4.** Loss of aPKC function expands basolateral membrane domain into the apical side and disrupts the apical domain.

(A) The aPKC NT construct has the Par6-binding site but no kinase domain and so acts as a dominant-negative fragment. (B) The effect of this construct can be rescued by overexpressing full-length aPKC. Injections of 4.5 ng aPKC NT + 0.5 ng GFP, 4.5 ng aPKC NT + 0.5 ng aPKC, or 5 ng GFP were carried out. The average of four experiments scored blind is shown. (C,D) aPKC NT dominant-negative fragment caused pigment defects (D) compared with control (C) (5 ng of each). (E,H) *aPKC NT* was co-injected with *GFP* showing that the pigment defects occurred in the injected region. The arrows in C,E and D,H highlight the same cells. (F-J) *aPKC NT* (I,J) caused ectopic localisation of the basolateral markers  $\beta$ 1-integrin and occludin to the apical side (arrow) and tight junctions were also lost (J, arrowhead) when compared with *GFP* control (F,G). (K) Diagram of the observed phenotype. Colours are as above. Pigmented embryos were injected as this allowed the affected area to be easily identified, they were then fixed and stained for markers of cell polarity.



polarity of the cells is lost and that they now resemble, in their membrane characteristics, the non-polarised deep cells.

### Lgl2 promotes basal lateral identity and inhibits apical identity

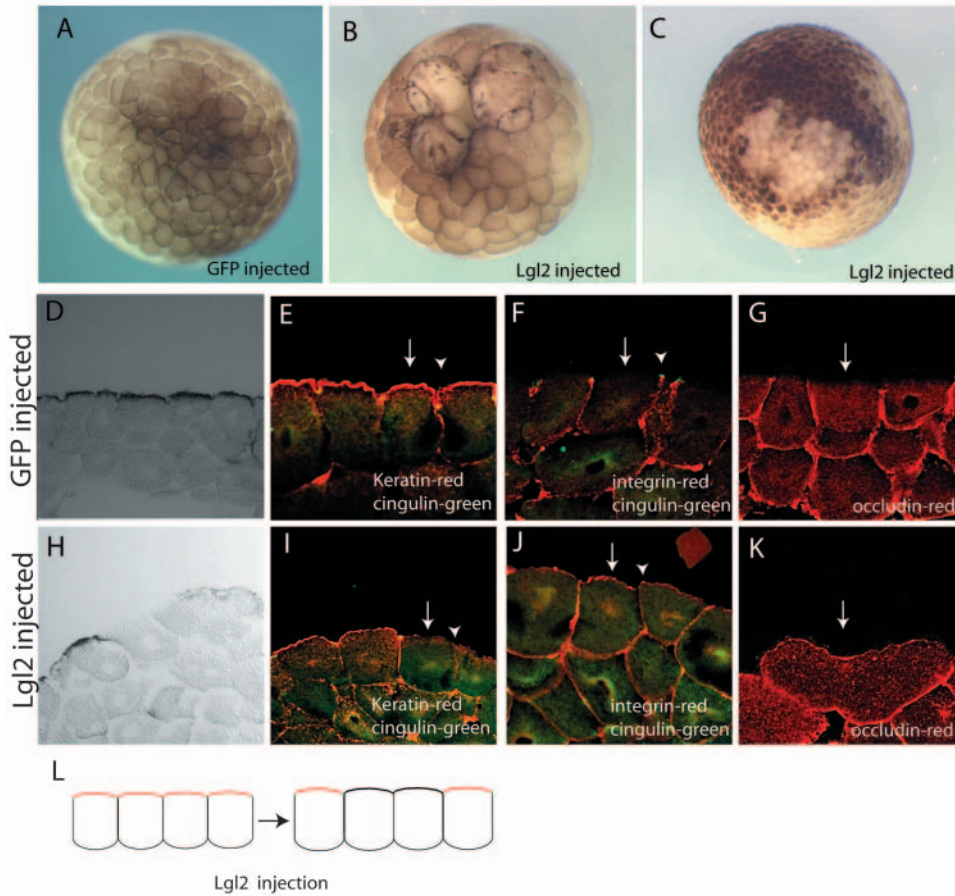
These experiments showed that aPKC is necessary and sufficient to define apical domain identity. To find out whether basolateral proteins have similar instructive roles for the basolateral side, we tested the activity of Lgl2. Overexpression of *Lgl2* caused loss of pigmentation in the outer cells (Fig. 5). At high doses, we also observed a defect in cytokinesis, such that it started normally but was abandoned before completion, resulting in large non-pigmented cells. (Fig. 5B). At lower doses, cytokinesis proceeded normally, but pigmentation was again lost from the apical side of the cells (Fig. 5C). Immunostaining showed that Lgl2 inhibited keratin stain (Fig. 5I, arrow) and expanded  $\beta$ 1-integrin and occludin ectopically on the apical side (Fig. 5J,K, arrow). As in the aPKC knockout, the effect was stronger for  $\beta$ 1-integrin than occludin, TJ were lost (cingulin; Fig. 5J) but cell adhesion was maintained.

To verify that this phenotype did not reflect the shedding of outer pigmented cells from the embryo and their replacement by inner cells, we filmed *Lgl2*-injected embryos. The time lapse clearly showed that disruption and eventual loss of pigmentation occurs gradually in outer cells and that these

were not extruded from the embryo (Fig. 6). We conclude that Lgl2 inhibits apical and promotes basolateral membrane identity (Fig. 5L). Upon *Lgl2* overexpression, outer epithelial cells lose apicobasal polarity and tight junctions, and assume the membrane phenotype of inner non-epithelial cells, without losing their position in the embryo.

### aPKC and Lgl2 act by a process of mutual inhibition

The phenotype of overexpressing *Lgl2* is remarkably similar to the phenotype of the dominant-negative aPKC and opposite to the phenotype of the wild-type aPKC. These findings suggest that aPKC may be working by inhibiting Lgl2 and Lgl2 may function by inhibiting aPKC, consistent with work in *Drosophila* (Betschinger et al., 2003; Hutterer et al., 2004). To test this idea, we looked to see if aPKC and Lgl2 would inhibit the localisation of each other. This was indeed the case; overexpressing aPKC caused delocalisation of Lgl2 from the basolateral membrane to the cytoplasm (Fig. 7A,B), whereas overexpression of Lgl2 caused delocalisation of aPKC from the apical membrane (Fig. 7C,D). Finally, to test the model of mutual inhibition, we asked whether *Lgl2* would rescue the aPKC overexpression phenotype. aPKC (2.5 ng) was co-injected with *Lgl2* or *GFP* (Fig. 7E-G). *Lgl2* but not *GFP* co-injection rescued the aPKC-induced rounding and protrusion of cells, which is due to the expansion of the apical membrane.



**Fig. 5.** Lgl2 promotes basolateral and inhibits apical identity. (A) Injection of 5 ng GFP did not affect the cells. (B,C) Injection of *Xenopus Lgl2* caused loss of pigment and also a block in cytokinesis at high doses (B, 5 ng; C, 0.5 ng). (D-K) Injection of GFP (D-G) or *Lgl2* (H-K) and immunostaining with the markers shown in each panel. GFP-injected embryos were entirely normal. (I) Injection of *Lgl2* caused a reduction in keratin to the levels normally seen in the basolateral region (arrow) and loss of tight junctions (cingulin, arrowhead). (J,K) Injection of *Lgl2* caused ectopic localisation of  $\beta$ 1-integrin (J) and occludin (K) to the apical side (arrow) and loss of tight junctions (arrowhead). (L) diagrammatic representation of phenotype, colours as above. Experiments were carried out three times in both albino and pigmented embryos (except for the keratin where the staining is obscured by the pigment and therefore was carried out only in albinos), and the same result was obtained in both.

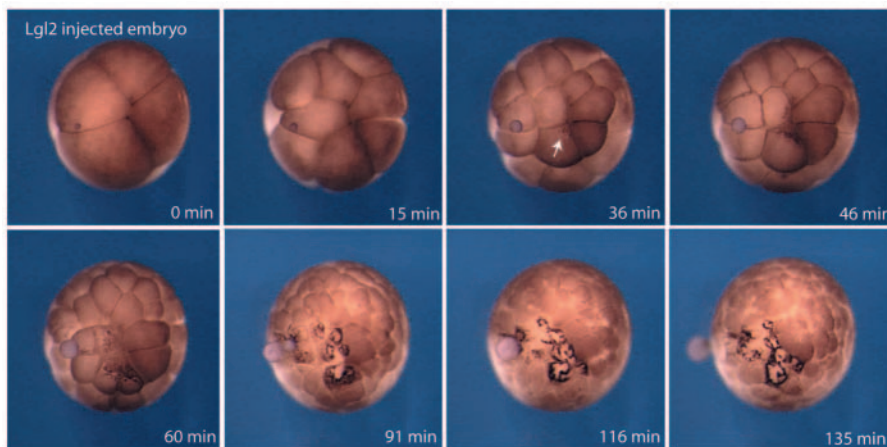
These results support the idea of an antagonistic interaction between aPKC and Lgl2, which define the apical and basolateral sides of the membrane of an epithelial cell, respectively (Fig. 8).

## Discussion

We have shown that aPKC and Crumbs3 are functionally sufficient to define apical membrane identity in epithelial cells of the frog blastula. Lgl2 is localised basolaterally and is similarly sufficient to define the basolateral domain. aPKC is not only sufficient but also necessary for apical membrane

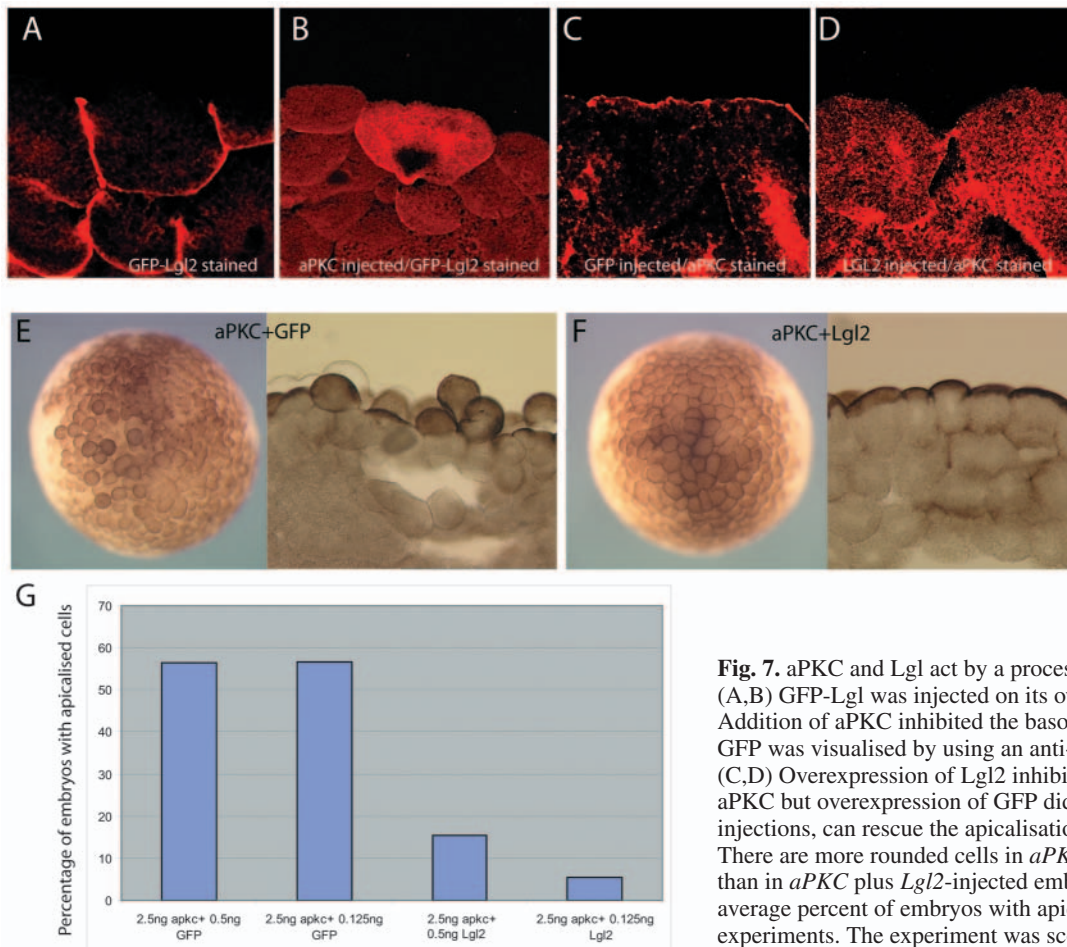
identity in these cells. aPKC and Lgl2 act antagonistically to establish and maintain distinct membrane domains, a mode of action that seems to have been highly conserved in evolution (Betschinger et al., 2003; Hutterer et al., 2004) and this work (summarised in Fig. 8).

These findings are important for two reasons. First, they have uncovered a function for these vertebrate proteins in polarisation, beyond a role in tight junction formation, that had not been appreciated from work in mammalian epithelial cells. Second, they have shown that these proteins have a role in the polarisation of cells in early vertebrate development, a prerequisite in generating cell fate diversity in the early embryo.



**Fig. 6.** Time-lapse images showing the gradual but direct depigmentation of the apical side by *Lgl2*. A pigmented embryo was injected anally with *Lgl2* RNA at the two-cell stage and filmed. A small site of cytoplasmic leakage helps to verify the site of injection. Evidence of apical membrane disruption starts as a concentration of pigment spots (arrow) that appear quite suddenly and spread quickly. The even distribution of pigmentation is lost and the pigment is gradually cleared from the apical side. Interestingly, pigment becomes concentrated to the periphery of the apical domain. There is no evidence of inner cells coming to the surface of the embryo or outer cells falling in.





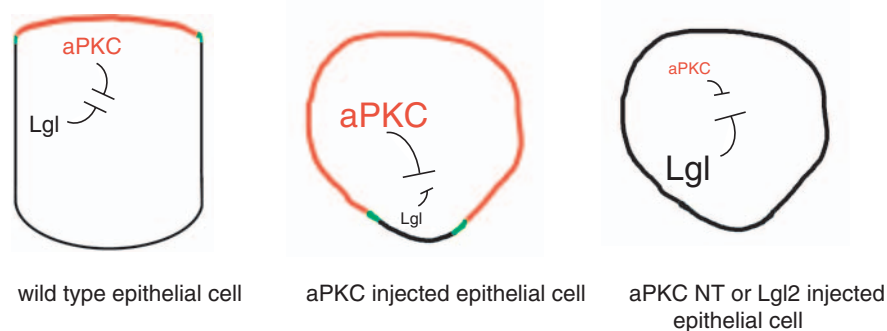
**Fig. 7.** aPKC and Lgl act by a process of mutual inhibition. (A,B) GFP-Lgl was injected on its own (A) or with aPKC (B). Addition of aPKC inhibited the basolateral localisation of GFP-Lgl2. GFP was visualised by using an anti-GFP antibody. (C,D) Overexpression of Lgl2 inhibited the apical localisation of aPKC but overexpression of GFP did not. (E-G) *Lgl2*, but not *GFP* injections, can rescue the apicalisation caused by injecting *aPKC*. There are more rounded cells in *aPKC* plus *GFP*-injected embryos than in *aPKC* plus *Lgl2*-injected embryos. The graph shows the average percent of embryos with apicalised cells from three experiments. The experiment was scored blind as for Fig. 2.

### A role for aPKC, Crumbs3 and Lgl2 in the establishment and maintenance of polarity

In the *Xenopus* proto-epithelium, the apical membrane is inherited from the egg ('old' membrane), while the basolateral membrane is newly synthesised ('new' membrane) (Müller and Hausen, 1995; Roberts et al., 1992). Therefore, effects on the apical and basolateral membrane are indicative of effects in the maintenance and establishment of membrane identity, respectively. The apical defects associated with aPKC downregulation and Lgl2 overexpression suggest that the

presence of aPKC and/or the absence of Lgl2 is necessary for the maintenance of apical membrane identity. However, the reduction of the basolateral side by aPKC and Crumbs3, and the expansion by Lgl2, suggest that misregulation of these proteins also affects the establishment of polarity.

In mammalian epithelial cell lines, inhibition of aPKC and overexpression of Lgl do not have an effect on the polarity of cells that are already polarised, but do have an effect on newly polarising cells, suggesting that these proteins act on the establishment, rather than the maintenance of polarity (Suzuki et al., 2002; Yamanaka et al., 2003). Our findings show for the first time that, in early vertebrate embryogenesis, these proteins are involved in both aspects of polarisation. This difference between mature and developing epithelia is also reflected in the localisation of aPKC protein. In confluent epithelial cells, aPKC is restricted to the tight junctions but absent from the apical membrane. By contrast, in *Xenopus* embryonic epithelia, aPKC is localised to the apical membrane (Chalmers et al., 2003), consistent with the suggested role in maintaining the identity of the apical membrane, a role additional to tight junction formation in these cells. Interestingly, in early mouse and zebrafish, aPKC is also



**Fig. 8.** A model showing the antagonistic action of aPKC and Lgl2 in maintaining the apical and basolateral domain. Increased aPKC causes expansion of the apical domain (red), while reduced aPKC or increased Lgl2 causes expansion of the basolateral domain (black). Tight junctions are shown in green.



localised to the apical membrane and not just the tight junctions (Horne-Badovinac et al., 2001; Pauken and Capco, 2000), suggesting that the mechanism described here may be evolutionarily conserved.

### A model for the mechanism of action for aPKC, Crumbs3 and Lgl2 in embryonic epithelial polarity – the potential role of tight junctions and vesicle transport

Previous work in mammalian epithelial cell lines has mainly focused on the role of polarity proteins in the formation of TJs (Hirose et al., 2002; Hurd et al., 2003; Suzuki et al., 2002; Suzuki et al., 2001; Yamanaka et al., 2001). TJs, apart from acting as permeability barriers, are thought to form physical fences that prevent intermixing of apical and basolateral membrane components. Could an effect on tight junctions explain the phenotypes that we report? When aPKC is overexpressed, TJs are re-positioned, but not abolished. These misplaced TJs are positioned as they normally are, at the interface of the apical and basolateral membrane domains. As in the wild-type situation, in experimental embryos these two domains appear cleanly segregated, but the apical domain is expanded and the basolateral diminished. Therefore, it seems likely that the primary effect of aPKC and Crumbs3 overexpression is on partitioning of the membrane into apical and basolateral domains, rather than on the TJs themselves. How aPKC and Crumbs3 overexpression cause the expansion of the apical domain is not clear at present. Perhaps some of the newly synthesised membrane acquires apical character instead of basal, or perhaps the apical domain stretches mechanically, or both. The apical domain is capable of constriction, brought about, for example, by the overexpression of the actin-binding protein Shroom (Haigo et al., 2003), so it is conceivable that it would also be capable of stretching.

In the aPKC knockout and Lgl2 overexpression, TJs are lost and basolateral membrane markers spread to the apical side of the cells. In this case, it is possible that the basolateral expansion is a consequence of the loss of a physical barrier. Alternatively, basolateral markers could appear on the apical side by an active mode of transport, independent on the presence or absence the TJs. Although we cannot formally distinguish between these two possibilities, we favour the second one. The basolateral membrane is newly synthesised during division and it is known that  $\beta$ 1-integrin is inserted into this membrane by fusion of stored vesicles (Gawantka et al., 1992). It seems that misdirection of such vesicles to the apical side during division, would be a straightforward and rapid way for the insertion of  $\beta$ 1-integrin to the entire apical membrane. This scenario is consistent with the observation that mammalian Lgl biochemically interacts with syntaxin 4, a component of basolateral exocytic machinery (Musch et al., 2002). It is also important to note that *Xenopus* blastula cells develop and maintain their epithelial polarity autonomously, in the complete absence of cell-cell contacts (Chalmers et al., 2003; Fesenko et al., 2000; Müller and Hausen, 1995) (reviewed by Müller and Bossinger, 2003). Therefore, in this system, functional TJs seem to play a secondary role in the establishment and/or maintenance of polarity and are unlikely to be the primary targets of the polarity complex proteins. We favour a model whereby aPKC/Lgl2 maintain distinct membrane domains not only by playing a role in TJ formation

but by more direct mechanisms, such as directing vesicle trafficking.

### aPKC-, Crumbs3- and Lgl2-driven epithelial polarity underlies a conserved event of cell fate diversification in vertebrates

A conserved aspect of vertebrate embryogenesis is the polarisation of the blastomeres and the generation of two phenotypically different cell populations via their division (reviewed by Müller and Bossinger, 2003). Cell polarisation is a prerequisite in generating two phenotypically distinct populations of cells in the early embryo.

Based on the localisation of membrane markers and pigment, we have shown that in aPKC knockout and Lgl2-overexpressing embryos, the outer epithelial cells lose their polarity and become phenotypically similar to inner cells. There are several possible ways in which cells could lose their polarity, such as regionalised membrane proteins failing to localise to the membrane altogether, or mixing of apical and basal markers. Instead, what we have observed is loss of polarity by specific transformation of the apical membrane to basolateral. The transformation of outer cells to inner-like reduces cell diversity in the affected area of the embryo. Furthermore, because these outer cells have lost their polarity, they would no longer be able to generate two phenotypically distinct cell types by division, at least as far as their membrane protein localisation is concerned.

In conclusion, aPKC and Crumbs3 act to promote apical membrane and inhibit basolateral, while Lgl2 acts to promote basolateral and inhibit apical membrane identity. The balance between these two antagonistic activities acts to establish and maintain apical and basal lateral membrane domains during early vertebrate development. Similar interactions have been reported in the establishment of embryonic epithelial polarity in *Drosophila* (Hutterer et al., 2004). These findings highlight an evolutionary conservation in the mechanisms that generate polarity and hence phenotypic cell diversity in the early vertebrate embryo.

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### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/5/977/DC1>

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