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SHORT COMMUNICATION

aPKCζ cortical loading is associated with Lgl cytoplasmic release and tumor growth in *Drosophila* and human epithelia

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Atypical protein kinase C (aPKC) and Lethal giant larvae (Lgl) regulate apical–basal polarity in *Drosophila* and mammalian epithelia. At the apical domain, aPKC phosphorylates and displaces Lgl that, in turn, maintains aPKC inactive at the basolateral region. The mutual exclusion of these two proteins seems to be crucial for the correct epithelial structure and function. Here we show that a cortical aPKC loading induces Lgl cytoplasmic release and massive overgrowth in *Drosophila* imaginal epithelia, whereas a cytoplasmic expression does not alter proliferation and epithelial overall structure. As two aPKC isoforms (ι and ζ) exist in humans and we previously showed that *Drosophila* Lgl is the functional homologue of the Human giant larvae-1 (Hugl-1) protein, we argued if the same mechanism of mutual exclusion could be impaired in human epithelial disorders and investigated aPKCι, aPKCζ and Hugl-1 localization in cancers deriving from ovarian surface epithelium. Both in mucinous and serous histotypes, aPKCζ showed an apical-to-cortical redistribution and Hugl-1 showed a membrane-to-cytoplasm release, perfectly recapitulating the *Drosophila* model. Although several recent works support a causative role for aPKCι overexpression in human carcinomas, our results suggest a key role for aPKCζ in apical–basal polarity loosening, a mechanism that seems to be driven by changes in protein localization rather than in protein abundance.
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A correct apical–basal cell polarity is the main feature of a healthy epithelial tissue (Nelson, 2003). From invertebrates to mammals, cells composing the epithelial sheets possess complex junctional structures that define an apical and a basolateral domain, favor cell–cell communication and cytoskeleton architecture, restrict motility and maintain tissue integrity making the cell layer work as a functional unit (Pilot and Lecuit, 2005; Christiansen and Rajasekaran, 2006; Shin *et al*., 2006). Loosening of these complexes is often associated to epithelial cancer progression, especially during the epithelial-to-mesenchymal transition (reviewed in Thiery, 2002), also if tumors exist in which cells exhibit diverse differentiated phenotypes as it is emerging from cancer stem cells theory (Polyak and Hahn, 2006; Filip *et al*., 2006). Ovarian carcinoma is a highly lethal form of cancer (Scott and McCluggage, 2006); the lack of appropriate animal models has strongly delayed either morphological or genetic characterization (Vanderhyden *et al*., 2003) and the mesothelial origin of the ovarian surface epithelium (OSE) has long kept researchers away from considering this tissue as a potential source of ovarian carcinomas (Auersperg *et al*., 2001). Only in the late 1980s methods to culture OSE have become available (Siemens and Auersperg, 1988) and genetic approaches led to conclusive evidence that mucinous and serous histotypes derive from OSE cells, whereas endometrioid and clear-cells subtypes are rather believed to arise from ectopic endometrium (Auersperg *et al*., 2001; Katabuchi and Okamura, 2003; Bell, 2005). OSE is a monolayer of a flat-to-cuboidal epithelium coating human ovaries and cortical inclusion cysts in which cell–cell adhesiveness is not so strong (Sundfeldt, 2003): the cyclic rupture and proliferative repair at the sites of ovulation are probably a reason for this junctional plasticity (Katabuchi and Okamura, 2003; Okamura *et al*., 2006). Curiously, opposite to the majority of carcinomas, a columnar, more cohesive morphology is for OSE the first step towards malignancy (Ong *et al*., 2000; Sarrío *et al*., 2006). In the most advanced stages, however, both mucinous and serous carcinomas are composed of dedifferentiated, round-shaped cells that grow in 3D and exfoliate giving rise to local metastases vehiculated by peritoneal fluids (Auersperg *et al*., 2001; Naora and Montell, 2005). A genetic link between cell shape and

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proliferation is given by molecules involved in the establishment and maintenance of apical–basal cell polarity in the epithelial tissues. The atypical protein kinase C (aPKC) proteins are a subgroup of the PKC family of serine-threonine protein kinases that comprise the ι/λ and ζ isoforms in mammals, whereas only the aPKCζ isoform exists in *Drosophila* (Suzuki *et al*., 2001; Rolls *et al*., 2003; Kovac *et al*., 2007). These proteins belong to the evolutionarily conserved PAR apical complex (Harris and Peifer, 2004; Suzuki and Ohno, 2006) and are involved in the determination of the apical identity both in the invertebrate and vertebrate epithelial cells. Studies in invertebrate models and in mammalian *in vitro* systems have partly elucidated the role of aPKC in the maintenance of the epithelial cell polarity and its relationship with the main determinant of the basolateral region, Lethal giant larvae (Lgl) (Rolls *et al*., 2003; Yamanaka *et al*., 2003; Chalmers *et al*., 2004). Lgl is an evolutionarily conserved molecule that functions linking cell polarity regulation to cell proliferation control in the epithelial tissues (Manfruelli *et al*., 1996; Bilder *et al*., 2000; Humbert *et al*., 2003; Bilder, 2004). Both in invertebrate and mammals it has been demonstrated that, when aPKC is found in an active apical PAR complex, it is able to phosphorylate Lgl inducing its detachment from the plasma membrane owing to a conformational change (Plant *et al*., 2003; Betschinger *et al*., 2005). This displacement results in an Lgl inhibition at the apical domain. Lgl, in turn, inhibits aPKC function at the basolateral domain, probably recruiting a still unknown PAR antagonist or sequestering aPKC in an inactive complex at that region (Hutterer *et al*., 2004; Yamanaka *et al*., 2006). This mechanism of mutual exclusion plays a fundamental role in the epithelial structure; when disrupted, cells indeed fail to maintain the correct shape and overproliferate, as can be observed upon Lgl depletion or aPKC ectopic expression (Rolls *et al*., 2003). We have previously demonstrated that *Drosophila* Lgl is the functional homologue of the Human giant larvae-1 (Hugl-1) protein, which is able to rescue lethality and cell polarity defects when expressed in *lgl* zygotic mutants (Grifoni *et al*., 2004). Moreover, Hugl-1 shows the same basolateral distribution as the endogenous Lgl and its ectopic expression in a wild-type (wt) background does not perturb epithelial morphogenesis (unpublished data); protein localization might thus be epistatic to protein abundance for these membrane-anchored shape determinants, *c'est à dire*: no matter how many molecules are available, but they have to stay at the right site to work properly. Hugl-1 deregulation has been associated with several types of epithelial cancers (Grifoni *et al*., 2004; Schimanski *et al*., 2005; Kuphal *et al*., 2006) and several recent papers support a causative role for aPKCι overexpression in carcinomas (Eder *et al*., 2005; Regala *et al*., 2005); in the same works, from 'low' to 'almost undetectable' levels of aPKCζ have been found in normal and cancer tissues, but in the light of our hypothesis it could play a still secret role in cell polarity and proliferation. An *in situ* analysis performed on mouse embryo sections also revealed substantial differences in expression between ι and ζ isoforms; the level of aPKCζ transcript was on average substantially lower compared to that of aPKCι but often restricted to the epithelial layer of organs (Kovac *et al*., 2007). To better characterize the molecular interplay between aPKC and Lgl we used *Drosophila* epithelia as a model in which to investigate the effect on Lgl redistribution and tissue morphology of several aPKC forms targeted to different cell

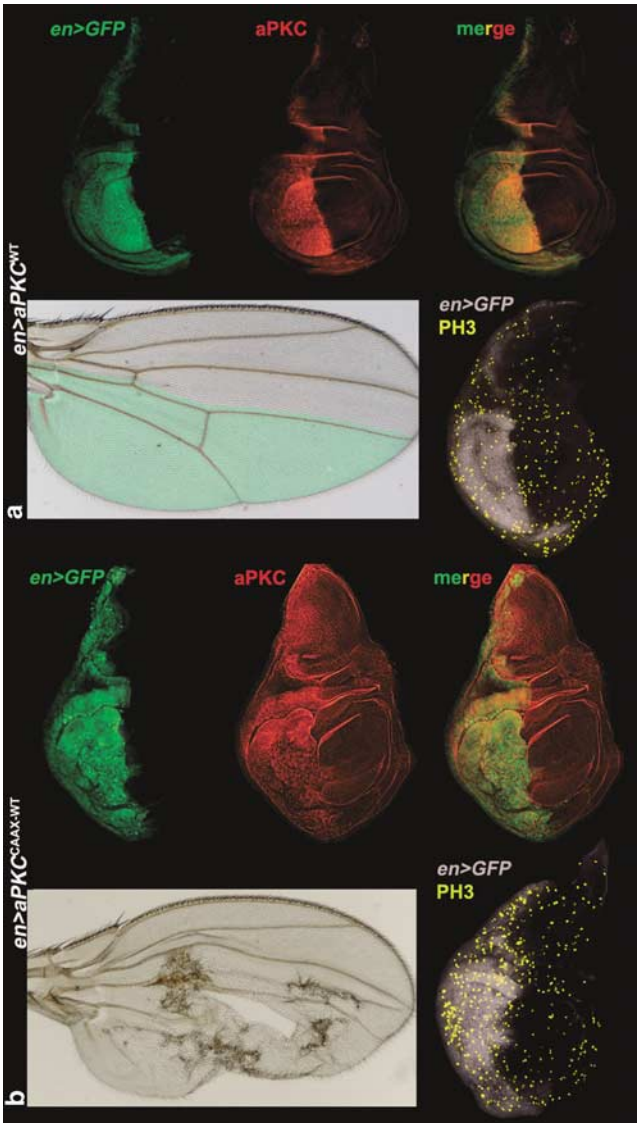

Figure 1 aPKC cortical localization alters proliferation and morphogenesis in imaginal discs. *en > aPKC*^{WT} (**a**) and *en > aPKC*-$^{CAAX-WT}$ (**b**) imaginal wing discs and adult wings; *en* promoter drives also the expression of a cytoplasmic GFP. In the adult wing, the posterior compartment is false-colored in light green. Immunofluorescence staining of third instar imaginal discs was performed according to standard protocols. Rabbit α-aPKCζ (1:200) and TRITC goat α-rabbit (1:50) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit α-phospho-histone H3 (mitosis marker, 1:250) was from Upstate (Charlottesville, VA, USA). Transgenic lines are described in Lee *et al*. (2006). Images were captured with a Nikon Eclipse 90i wide-field microscope. Magnification is × 200 for imaginal discs and × 40 for wings.

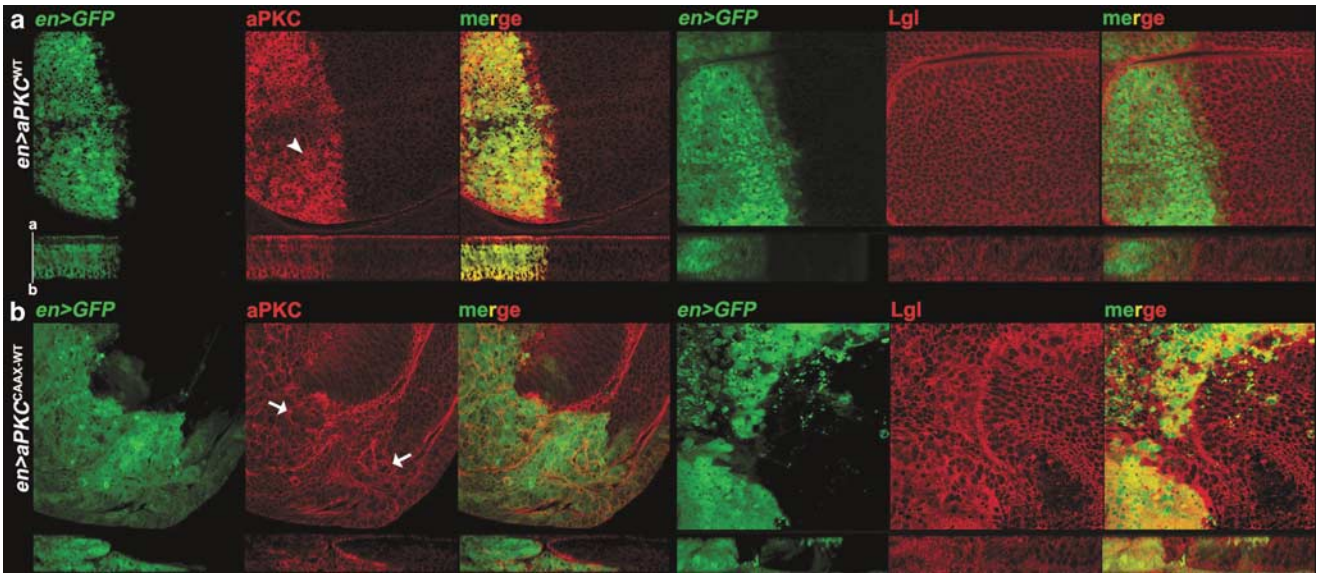

Figure 2 aPKC cortical loading induces Lgl cytoplasmic release in imaginal discs. Confocal zeta stacks and zeta axis projections of *en > aPKC*^{WT} (**a**) and *en > aPKC*$^{CAAX\text{-}WT}$ (**b**) imaginal wing discs. *en* promoter drives also the expression of a cytoplasmic GFP. Rabbit α-Lgl (1:200) was described in Grifoni *et al*. (2004). Images were captured with a Leica TSC SP2 AOBS confocal microscope at ×400 magnification.

compartments. *Drosophila* imaginal discs are larval structures that give rise to adult organs such as wings, eyes and legs. They are composed of a monolayered epithelium that perfectly recapitulates many morphological, structural and molecular features of human epithelia coating internal organs. For this reason, *Drosophila* imaginal discs are broadly utilized as a model system in which to investigate *in vivo* genetic mechanisms integrating cell proliferation and tissue morphogenesis (Bryant and Schmidt, 1990; Weinkove and Leevers, 2000; Bilder, 2004). We thus expressed a wt cytoplasmic form of the *Drosophila* aPKC under the control of the *engrailed* (*en*) promoter, using the UAS-Gal4 binary system (Brand and Perrimon, 1993). *en* promoter activity is restricted to the posterior compartment of the wing disc throughout the organ development, so it is feasible to observe the transgene effects bounded to that district (marked with a cytoplasmic green fluorescent protein (GFP)) and use the anterior compartment, where the transgene is not expressed, as an internal control. As shown in Figure 1a, the expression of the wt cytoplasmic aPKC (*UAS-aPKC*^{WT}) has no effects on the overall morphology of the posterior compartment of the wing disc; phospho–histone H3 mitosis marker shows a homogeneous distribution and the adult wings everting from these larval structures are comparable to those from wt animals.

Conversely, when we induce an aPKC prenylated form, which targets the expression to the plasma membrane (*UAS-aPKC*$^{CAAX\text{-}WT}$), hyperproliferation, massive overgrowth and deep morphogenetic alterations are observed both in discs and adult wings (Figure 1b). Hence, a cortical aPKC distribution is strictly required to perturb tissue architecture. Moreover, the ectopic expression of a kinase-dead membrane-targeted form (*UAS-aPKC*$^{CAAX\text{-}KD}$) is not able to alter morphogenesis, indicating that, besides cortical loading, aPKC kinase activity is essential for its oncogenic potential to arise (data not shown). It is hence possible that a membrane ectopic localization of this kinase promote Lgl phosphorylation also at the basolateral domain, displacing it from its working site and compromising apical–basal polarity. Indeed, at the cellular level, confocal images in Figure 2 show that in *en > aPKC*^{WT} discs Lgl is retained at the membrane such as in the anterior, wt compartment whereas in *en > aPKC*$^{CAAX\text{-}WT}$ discs Lgl is released in the cytoplasm in the regions where *en* promoter is active the most.

Focusing on cell shape, *en > aPKC*$^{CAAX\text{-}WT}$ cells exhibit a circular outline and appear larger (arrows) than *en > aPKC*^{WT} cells, showing instead the typical polygonal shape (arrowhead) of all epithelial surfaces (Gibson *et al*., 2006). It is however possible that changes in cell shape are favored in the highly proliferating tissues of the imaginal discs. To test the role of apical–basal polarity perturbation in a non-proliferating context we analyzed the follicular epithelium, an adult tissue of somatic origin that lines the egg chamber, in which cells stop dividing at stage 7 of oogenesis. Using a CY2 promoter, active in all the follicular cells covering the oocyte from stage 8 onward (Queenan *et al*., 1997), we induced the expression of the same transgenes as above in adult females. As shown in Figure 3a, where the apical–basal axis is indicated by a double-headed arrow, the wt expression of aPKC and Lgl in the follicular epithelium is apical and basolateral, respectively.

Following *UAS-aPKC*^{WT} transgene induction, aPKC is abundantly expressed in the cytoplasm (3b) and Lgl is retained at its natural membrane domain (3c), whereas the induction of the *UAS-aPKC*$^{CAAX\text{-}WT}$ transgene

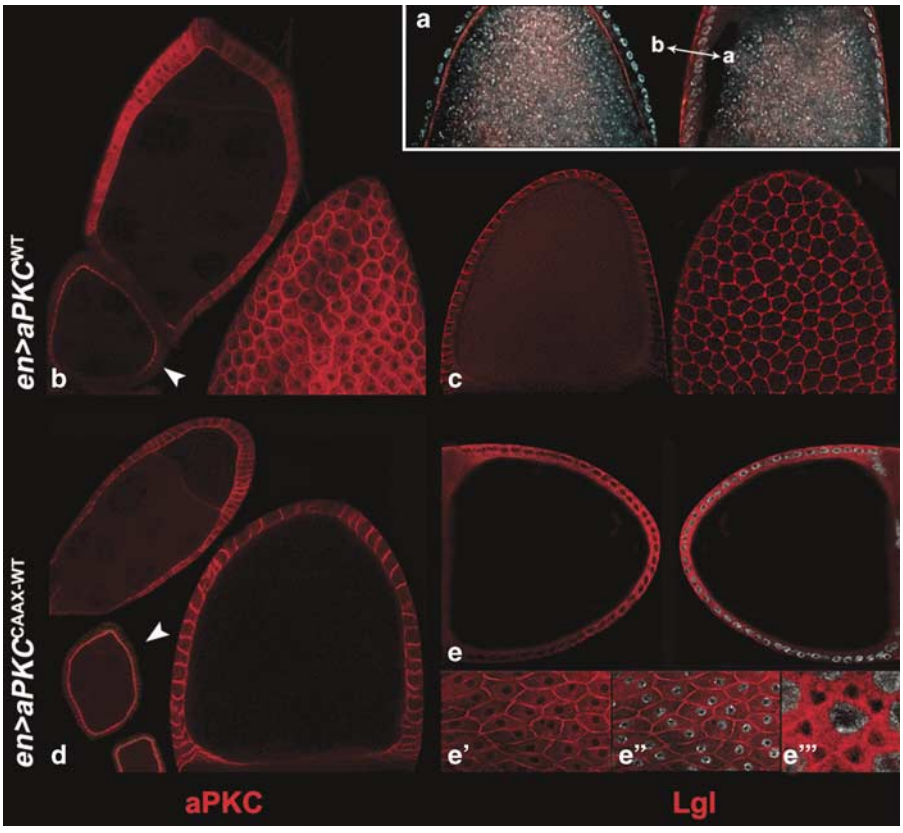

Figure 3 aPKC cortical loading induces changes in cell shape and Lgl cytoplasmic release in follicular epithelium. (**a**) Wide-field aPKC (left) and Lgl (right) localization in wt egg chambers; the apical–basal axis polarity of the follicular epithelium is indicated by the double-headed arrow. (**b** and **c**) *CY2 > aPKC*^{WT} egg chambers confocal cross and surface sections stained for aPKC and Lgl; (**d** and **e**) *CY2 > aPKC*$^{CAAX\text{-}WT}$ egg chambers confocal cross sections stained for aPKC and Lgl, a surface section is shown in (**e′**, **e″**) and a $\times 630$ magnification of a star-like, delaminating follicular cell is shown in (**e‴**). In (**b**) and (**d**), arrowheads indicate chambers before stage 8 in which CY2 promoter is still inactive. Immunofluorescence staining of egg chambers was performed according to standard protocols. Visible nuclei are counterstained with Hoechst 33258 (Sigma Aldrich, Milano, Italy).

enriches aPKC expression at the lateral domain (3d) and this event triggers a massive Lgl cytoplasmic release (3e). In Figure 3, a surface section (e′, e″) and a high magnification of a star like, misshaped follicular cell that is losing contact with the adjacent cells (e‴) are also shown. *CY2 > aPKC*$^{CAAX\text{-}WT}$ chambers are indeed distorted, with the follicular epithelium partially or completely delaminated (data not shown); star-like structures are very frequent and these females produce very few mature eggs with respect to the *CY2 > aPKC*^{WT} females (about 1/50; $n = 50/2500$); in the figure the least impaired chambers are presented to make aPKC and Lgl subcellular localization visible. In summary, our results indicate that, also in a tissue where cell proliferation has ceased, changes in subcellular localization of molecules involved in cell polarity are able to cause deep morphogenetic alterations, among which the most relevant is cell contour deformation with a consequent detachment from the adjacent units. Following our hypothesis concerning the importance of protein localization for membrane-anchored shape determinants, strongly supported by our *in vivo* analysis in *Drosophila* epithelia, we investigated aPKC*ι*, aPKCζ and Hugl-1 distribution in human ovarian cancers. A total of 19 mucinous and serous ovarian cancers were analyzed besides normal OSE and the results, summarized in Figure 4, show that aPKC*ι* and aPKCζ localization is mainly apical in normal samples (Figure 4a and b); intensity is instead different, with aPKCζ showing low levels of expression with respect to the other form (see Figure 4 legend). Hugl-1 localization is mainly basolateral such as in *Drosophila* epithelia, as can be seen in Figure 4c–c′.

In all the mucinous carcinomas, aPKC*ι* positivity is cytoplasmic (d, d′) whereas aPKCζ lines the whole-cell contour (e–e″) and Hugl-1 shows a cytoplasmic reactivity (f–f″). The same trend is observed in all the serous histotypes analyzed: aPKC*ι* is cytoplasmic (g), aPKCζ is still apically enriched in cells showing a columnar shape (arrows) but it is cortical at the membrane in the tumor regions where cells are round-shaped (arrowheads) and grow in multiple layers (h). Hugl-1 localization is cytoplasmic such as in mucinous carcinomas (i).

Apical–basal polarity is a conserved feature of epithelial sheets, whose cells build up a series of junctions in order to adhere to each other and work as a functional unit (Pilot and Lecuit, 2005; Shin *et al*., 2006). Cell polarity molecules are often associated to

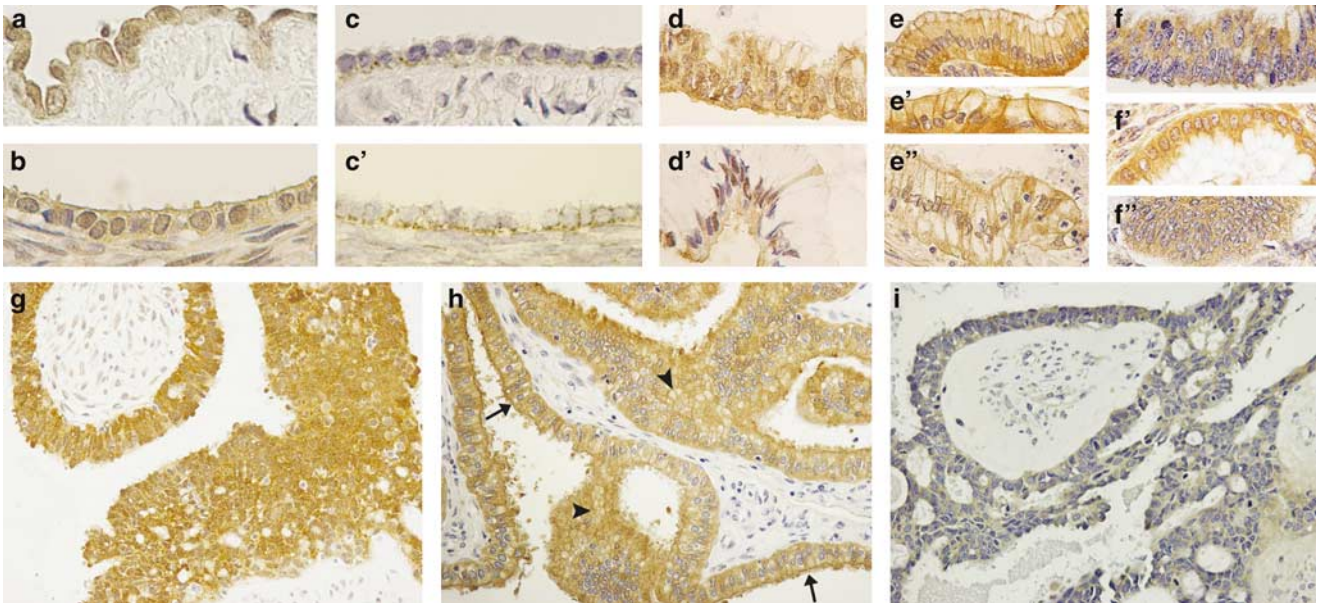

Figure 4 aPKCζ and Hugl-1 localization in ovarian cancers shows the same impairments observed in the *Drosophila* model. (**a**–**c**′) Healthy OSE, aPKC*ι* (**a**), aPKCζ (**b**) and Hugl-1 (**c** and **c**′) localization; (**d**–**f**″) mucinous cancers, aPKC*ι* (**d** and **d**′), aPKCζ (**e**–**e**″) and Hugl-1 (**f**–**f**″) localization; (**g**–**i**) serous cancers, aPKC*ι* (**g**), aPKCζ (**h**) and Hugl-1 (**i**) localization. (**a**–**c**′) pictures are at ×400 magnification, (**d**–**f**″) are at ×300 and (**g**–**i**) are at ×200. Immunohistochemistry was performed according to standard ab–biotin–streptavidin–peroxidase protocols, except for aPKCζ for which a signal amplification was carried out using the Primary Antibody Enhancer/HRP Polymer system, Lab Vision. Rabbit α-aPKC*ι* (1:50) was from Santa Cruz Biotechnology and rabbit α-Hugl-1 (1:200) was described in Grifoni *et al*. (2004). Following to peroxidase reaction, samples were hematoxylin–eosin counterstained for histopathological observation.

cancer because of their role in cell shape and adhesiveness. aPKC and Lgl are molecules that link cell polarity and proliferation control in *Drosophila* epithelia (Rolls *et al*., 2003) and their human homologues aPKC*ι* and Hugl-1 have been shown to be associated to several forms of epithelial cancers. In particular, transcript and protein abundance has been investigated and aPKC*ι* increasing (Eder *et al*., 2005; Regala *et al*., 2005) or Hugl-1 reduction (Grifoni *et al*., 2004; Schimanski *et al*., 2005; Kuphal *et al*., 2006) was observed in cancers. Our results show that protein mislocalization is also an important tumoral marker for this class of molecules; the *in vivo* model highlights how an aPKC cortical distribution can induce changes in cell polarity and growth, whereas a strong overexpression of the same molecule in the cytoplasmic compartment does not interfere with morphogenesis. The same results emerged from human ovarian cancers, where aPKC*ι* is abundantly cytoplasmic whereas aPKCζ is mainly cortical both in the mucinous and serous histotypes. Moreover, it seems to colonize ectopic cell domains in a stepwise manner, especially in the serous samples. We thus propose a model in which, during tumor formation, aPKCζ spreads from its apical sites along the lateral domain where it phosphorylates Hugl-1 that is consequently released in the cytoplasm in an inactive form, so mimicking its loss of function phenotype. This aPKCζ spreading is probably due to compromising of a still unknown PAR apical complex inhibitor/antagonist at the basolateral domain (Hutterer *et al*., 2004). Our work points out an interesting conservation both in molecules and mechanisms underlying changes in cell polarity during tumor formation and the perfect recapitulation of the *Drosophila* system in human samples highlights the relevance of this organism in cancer investigation, such a complex genetic disease meets the model in which genetic analysis is the most advanced.

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