EGFR controls IQGAP basolateral membrane localization and mitotic spindle orientation during epithelial morphogenesis

Inmaculada Bañón-Rodríguez, Manuel Gálvez-Santisteban, Silvia Vergarajauregui[†],
Minerva Bosch, Arantxa Borreguero-Pascual & Fernando Martín-Belmonte^{*}

Abstract

Establishing the correct orientation of the mitotic spindle is an essential step in epithelial cell division in order to ensure that epithelial tubules form correctly during organ development and regeneration. While recent findings have identified some of the molecular mechanisms that underlie spindle orientation, many aspects of this process remain poorly understood. Here, we have used the 3D-MDCK model system to demonstrate a key role for a newly identified protein complex formed by IQGAP1 and the epithelial growth factor receptor (EGFR) in controlling the orientation of the mitotic spindle. IQGAP1 is a scaffolding protein that regulates many cellular pathways, from cell-cell adhesion to microtubule organization, and its localization in the basolateral membrane ensures correct spindle orientation. Through its IQ motifs, IQGAP1 binds to EGFR, which is responsible for maintaining IQGAP1 in the basolateral membrane domain. Silencing IQGAP1, or disrupting the basolateral localization of either IQGAP1 or EGFR, results in a non-polarized distribution of NuMA, mitotic spindle misorientation and defects in single lumen formation.

Keywords cell polarity; cytoskeleton; epithelial morphogenesis; lumenogenesis; spindle orientation

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Cell Cycle

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Introduction

One of the most fundamental processes during development is the formation of epithelial sheets and tubes. These structures constitute the main elements in most of our internal organs (e.g. the kidney, breast or liver) and the surfaces across which an organism interacts with its environment (e.g. the skin, mucosa). In internal organs, epithelial cells usually become organized into tubules, which play

essential roles in normal body functions (e.g. nutrient transport and waste disposal). During tubule formation cells first become polarized and they are then organized three-dimensionally around a central lumen, a process that is wholly dependent on the correct orientation of the mitotic spindle during cell division (Fischer *et al*, 2006; Rodríguez-Fraticelli *et al*, 2011).

During cell division, astral microtubules are guided to specific sites in the cell cortex where they anchor the mitotic spindle in the correct orientation. Epithelial cells divide along a plane of division perpendicular to the apicobasal axis, which requires the spindle to be anchored to the junctional plasma membrane. Misorientation of the spindle results in tissue and organ misshaping, and it is implicated in diseases like cancer and polycystic kidney disease (Castanon & Gonzalez-Gaitan, 2011). The G α i-LGN-NuMA protein complex controls spindle orientation by anchoring astral microtubules to the cell cortex (Siller & Doe, 2009; Hao *et al*, 2010). G α i localizes to the plasma membrane and recruits LGN to this region. Although G α i is distributed throughout the cell membrane in polarized tissues, LGN binds to G α i exclusively at the basolateral membrane via the activity of aPKC, which is restricted to the apical membrane where it phosphorylates LGN, thereby excluding it from this domain. However, in some epithelial cells such as chick neuroepithelial cells and *Drosophila* follicular epithelium, lateral distribution of LGN is independent of aPKC (Peyre *et al*, 2011; Bergstrahl *et al*, 2013). In turn, G α i-LGN binds to NuMA, thereby anchoring the astral microtubules to the basolateral membrane (Blumer *et al*, 2006; Morin *et al*, 2007; Zheng *et al*, 2010; Peyre *et al*, 2011). Despite this knowledge, the precise nature of the signals that drive mitotic spindle orientation in epithelial cells is poorly understood.

The IQGAP family is an evolutionarily conserved family of multi-domain proteins that are expressed in organisms from yeast to mammals. IQGAP1 is ubiquitously expressed and it is the best-characterized member of the family. IQGAP2 is mainly expressed in the liver, while IQGAP3 is restricted to the brain, lung, testis, small intestine and colon (White *et al*, 2012). IQGAP1 is a 190 kDa protein with an F-actin-binding calponin-homology domain (CHD), a coiled-coil homodimerization domain, a polyproline binding region (WW), an IQ

Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain

^{*}Corresponding author. Tel: 34 911964615; Fax: 34 911964420; E-mail: fmartin@cblm.uam.es

[†]Present address: National Heart, Lung and Blood Institute (NHLBI), National Institutes of Health (NIH), Bethesda, Maryland, USA

domain with four IQ motifs, a Ras GTPase-activating protein related domain (GRD), and a RasGAP C-terminus (RGCT) that mediates binding to proteins such as E-cadherin, β -catenin, APC and CLIP-170 (Fukata *et al*, 2002; Johnson *et al*, 2009; White *et al*, 2009). Over 90 proteins have been described as IQGAP1 binding partners, linking IQGAP1 to several cellular functions, including cell-cell adhesion, the reorganization of actin filaments or microtubules, cell migration and the regulation of signal transduction (White *et al*, 2012). Indeed, the angiogenesis and metastasis seen in different types of tumors is frequently correlated with altered IQGAP1 distribution and protein levels (Johnson *et al*, 2009). Furthermore, IQGAP1 appears to be involved in the microtubule dynamics associated with cell polarization and silencing IQGAP1 expression inhibits the reorientation of the microtubule-organizing centre (MTOC) in several cell types (Watanabe *et al*, 2004; Kanwar & Wilkins, 2011). In addition, IQGAP1 is known to mediate the capture and stabilization of microtubules at the cell cortex through its binding partner CLIP170, which is essential for protein polarization and directional cell migration (Noritake *et al*, 2005). However, while IQGAP1 anchors microtubules to the cell cortex in a variety of cell models, its role in anchoring astral microtubules to the plasma membrane during epithelial cell division has not yet been evaluated.

Previous studies of epithelial cells revealed a positive role for IQGAP1 in E-cadherin-mediated cell-cell adhesion (Noritake *et al*, 2004). However, the role of IQGAP1 in other aspects of epithelial morphogenesis, such as lumen formation, remains unclear. Interestingly, IQGAP1 binds to the EGFR via its IQ motifs (McNulty *et al*, 2011) and this receptor has recently been shown to localize to the basolateral membrane in MDCK cysts in 3D cultures. Furthermore, this distribution of EGFR appears to be essential for single lumen formation, as mutations that drive the EGFR to the apical membrane severely affect cyst formation (Cotton *et al*, 2013).

Using the organotypic 3D-MDCK cell system, we describe here a new mechanism whereby the EGFR mediates the distribution of IQGAP1 in the membrane and thereby regulates mitotic spindle orientation during epithelial morphogenesis. We demonstrate that IQGAP1 localizes to the basolateral membrane to correctly orient the mitotic spindle and that the IQ motifs in IQGAP1, which are essential for its efficient binding to the EGFR, determine its specific basolateral distribution. Disrupting the localization of EGFR to the basolateral membrane or IQGAP1 binding to EGFR interferes with the restricted distribution of IQGAP1 in this domain, provoking misorientation of the mitotic spindle and defects in single lumen formation. Furthermore, IQGAP1 is required for the basolaterally restricted distribution of NuMA but not that of LGN, the localization of which is independent of IQGAP1.

Results

IQGAP1 orientates the mitotic spindle to control single lumen formation

IQGAP1 is an effector of Rac1 and Cdc42 (Briggs & Sacks, 2003) that is expressed ubiquitously in different polarized tissues of vertebrates, playing a central role in cell-cell adhesion, cell polarization, directional cell migration and the regulation of protein trafficking (Noritake *et al*, 2005; Osman, 2010). To define how IQGAP1 participates in epithelial lumen formation during morphogenesis, we silenced endogenous IQGAP1 and investigated its role in 3D

epithelial morphogenesis in the 3D-MDCK model system (Fig 1A). A siRNA heteroduplex (#2) was seen to decrease endogenous IQGAP1 levels by 90% and therefore, it was used in the subsequent experiments (Fig 1B). When IQGAP1 expression was silenced in this way, a significant number of MDCK cysts displayed defects in single lumen formation when compared to the control cysts that formed (Fig 1C,D). Since this effect was rescued by expressing a siRNA-resistant mouse IQGAP1 isoform (described below in Fig 3), it would appear that IQGAP1 is required for correct lumen formation during 3D epithelial morphogenesis.

It was recently demonstrated, both *in vivo* and in 3D cultures, that the formation of single lumens is dependent on the correct orientation of the mitotic spindle during cell division (Jaffe *et al*, 2008; Hao *et al*, 2010; Rodriguez-Fraticelli *et al*, 2010). Indeed, disrupting any component of the machinery involved in spindle orientation affects single lumen formation. For lumen formation, the mitotic spindle is correctly orientated perpendicular to the apicobasal cell axis when the astral microtubules of the spindle are captured and attached to the lateral membrane of polarized epithelial cells. Since IQGAP1 captures and stabilizes microtubules at the cell cortex (Noritake *et al*, 2005) we investigated the specific role of IQGAP1 in spindle orientation in the 3D-MDCK model system. In the absence of IQGAP1, misorientation of the mitotic spindle could be detected by immunofluorescence (Fig 1E), and whereas the spindle was mainly oriented perpendicular to the apicobasal axis in control cysts, in IQGAP1-silenced cysts the mitotic spindles appeared to be randomly arranged (Fig 1F). Moreover, silencing IQGAP1 using different siRNAs produced similar effects on mitotic spindle orientation (supplementary Fig S1).

In addition to controlling spindle orientation, vectorial transport to a nascent apical domain is one of the molecular processes required for the formation of biological tubes (Lubarsky & Krasnow, 2003). Each cell in the cyst must coordinate its vectorial membrane transport in order to form a single lumen (Bryant *et al*, 2010; Galvez-Santisteban *et al*, 2012). As IQGAP1 is also involved in exocytosis and vesicle trafficking (Osman, 2010; White *et al*, 2012), and single lumen formation is defective in its absence, we investigated whether defects in protein trafficking contribute to the phenotype observed following IQGAP1 silencing. To investigate the possible role of IQGAP1 in vesicle trafficking, we silenced IQGAP1 and as a positive control, we also silenced the synaptotagmin-like protein 2a (Slp2a) that is known to play an essential role in membrane trafficking during lumen formation (Galvez-Santisteban *et al*, 2012). Similar defects in single lumen formation were observed in both Slp2a- and IQGAP1-silenced cells (supplementary Fig S2A), although most Slp2-silenced cells displayed defects in vesicle trafficking (the accumulation of internal vesicles) that were not observed in IQGAP1-silenced cells (supplementary Fig S2A). The failure of IQGAP1 to affect vesicle trafficking was also evident through the apical recovery of podocalyxin, which was unaffected in biotinylation assays in the absence of IQGAP1 (supplementary Fig S2B). Finally, we performed a calcium switch experiment to analyze the recovery of transepithelial resistance (TER) *in vivo* using an electrical cell-substrate impedance system (ECIS; Lo *et al*, 1995). In this way we demonstrated that IQGAP1 does not regulate the formation of apical junctions (supplementary Fig S2C) and moreover, we found that IQGAP1 does not control the position of centrosomes (supplementary Fig S3).

Since it was previously demonstrated that spindle misorientation induces multilumen formation, then defects in cystogenesis would be blocked by inhibiting cell division (Zheng *et al*, 2010). To further

Figure 1. IQGAP1 is necessary for correct spindle orientation and single lumen formation.

- A** MDCK cells were transfected with three different siRNA heteroduplexes targeting canine IQGAP1. Transfected cells were grown in 3D cultures for 72 h and the efficiency of the siRNAs was analyzed in Western blots (WB).
- B** Quantification of IQGAP1 depletion. The siRNA#2 was most effective in silencing IQGAP1, resulting in 90% depletion, and it was therefore used in all the subsequent experiments ($n = 3$; values represent mean $\pm$ s.d.).
- C** MDCK cells were transfected with control or IQGAP1 siRNA and grown in 3D cultures. After 72 h the cysts were stained for Podxl (red), actin (green) and ToPro3 (blue). Scale bars, 5 μ m.
- D** Single lumen formation was quantified with respect to that observed in control cysts. Only 59.5 $\pm$ 5.75% of siRNA#2 cysts polarized and formed a single lumen ($n = 3$; values represent mean $\pm$ s.d.).
- E** Control and IQGAP1 siRNA cysts were stained for actin (red) and acetylated tubulin (green). Scale bars, 5 μ m.
- F** The angle formed between the mitotic spindle and the apicobasal axis was measured and the mean angles were: siControl = 75.53 $\pm$ 2.69°; siIQGAP1 = 45.47 $\pm$ 4.57° ($n = 3$, >40 cysts per experiment, values represent mean $\pm$ s.e.m.).
- G** Scheme of the experimental design (left panel). Control cells and IQGAP1 KD cells were grown in 3D cultures immediately after nucleofection. After 4 days, control cells organized in epithelial organoids surrounding a central lumen (group G1), while IQGAP1-KD cysts displayed defects in single lumen formation when compare to the controls (group G3). Control cells treated with thymidine (2 mM), formed smaller cysts with single lumens in the same rate as those not treated with the mitotic inhibitor (group G2). After 48 h, IQGAP1 interference starts to be effective, although the addition of thymidine at this point completely recovered normal lumen formation (group G4: Right panel) Representative images of cysts grown for 4 days under the conditions described previously. Scale bars, 5 μ m.
- H** Single lumen quantification under each condition described in (G). Cysts forming single lumen: group G1 = 73.66 $\pm$ 1.23%; group G2 = 71.36 $\pm$ 7.84%; group G3 = 52.16 $\pm$ 5.13%; group G4 = 69.98 $\pm$ 6.19% ($n = 3$, >100 cysts per experiment; values represent mean $\pm$ s.d.). Error bars represent the s.e.m. or s.d.: * $P < 0.05$; ** $P < 0.01$.

Source data are available online for this figure.

confirm the role of IQGAP1-silencing in single lumen formation through the control of spindle orientation, we analyzed how IQGAP1 silencing affected cells maintained in the presence or absence of the mitotic inhibitor, thymidine. Importantly, we observed that when cells with silenced IQGAP1 expression were exposed to thymidine for 2 days they completely recovered normal lumen formation (Fig 1G, H), despite reducing the size of the cyst, as would be expected.

Taken together, these results indicate that IQGAP1 controls the orientation of the mitotic spindle but that it is not required for vesicle trafficking when lumens are formed during epithelial morphogenesis.

PMA treatment induces IQGAP1 depolarization and mitotic spindle misorientation

We next characterized the distribution of IQGAP1 to determine if its localization in 3D cysts is essential for spindle orientation. In mitotic cells, IQGAP1 was distributed throughout the basolateral membrane and it was completely absent from the apical surface (Fig 2A), while in non-mitotic cells, IQGAP1 was localized at cell-cell junctions, as observed previously in epithelial monolayers (Fig 2B; Katata *et al*, 2003). We assessed how altering the distribution of IQGAP1 affected mitotic spindle orientation. Stimulating gastric glands with PMA, a potent PKC activator, promotes the redistribution of IQGAP1 to the apical pole of cells lining the gland lumen (Chew *et al*, 2005). In MDCK cysts treated with PMA (4 nM), we observed a rapid translocation (15 min) of endogenous IQGAP1 from the basolateral to the apical surface, while the distribution of the cell-cell junction protein β -catenin remained unaffected (Fig 2C). Relocalization of IQGAP1 to the apical domain upon exposure to PMA was accompanied by a significant decrease in the mean spindle angle in MDCK cells (Fig 2D,E). These data suggest that the spatial restriction of IQGAP1 to the basolateral membrane is essential for proper spindle orientation, and that translocation of IQGAP1 to the apical membrane underlies the spindle misorientation.

PMA induces robust phosphorylation of human IQGAP1, which in turn promotes neurite outgrowth (Li *et al*, 2005), with Ser1443 and Ser1441 identified as the major sites of IQGAP1 phosphorylation following PMA treatment (Li *et al*, 2005). However, the alignment of human IQGAP1 with homologues from other species (note that MDCK cells are canine kidney cells) revealed that Ser1441 but not Ser1443 is conserved (supplementary Fig S4A). To study the role of PMA-mediated IQGAP1 phosphorylation in the apical translocation of IQGAP1, we expressed a phospho-incompetent S1441A mutant that localizes to the junctions of 3D-MDCK cells. Upon exposure to PMA, the IQGAP1-S1441A mutant translocated to the apical membrane at the same rate as the wild-type protein (supplementary Fig S4B), indicating that the apical translocation of IQGAP1 is not driven by PMA-mediated phosphorylation of IQGAP1 at Ser-1441. Hence, an alternative mechanism must be responsible for this effect.

IQ motifs mediate the basolateral restriction of IQGAP1

The IQGAP1 protein contains multiple protein-protein interacting domains that have been implicated in a various functions in the cell (White *et al*, 2012). To better characterize the role of IQGAP1 in epithelial morphogenesis and to analyze the role of these domains in IQGAP1 localization, we generated a GFP-tagged full-length IQGAP1

and a set of GFP-IQGAP1 constructs containing different regions of this protein (Fig 3A). We analyzed the distribution of these fusion proteins by immunofluorescence (IF) in 3D-MDCK cells and like the endogenous protein, IQGAP1-GFP localized to cell junctions (Fig 3B). Interestingly, neither IQGAP1:N nor IQGAP1:C were polarized to the apical and basolateral membranes, these constructs containing the N-terminal and C-terminal domains of IQGAP1, and sharing some of the IQ motifs (Fig 3B). We analyzed how expressing these constructs affected epithelial lumen formation and as expected, neither of the mutants restored the normal phenotype when endogenous IQGAP1 was silenced. Indeed, not only did cells expressing these constructs form multiple lumens with an abnormal morphology in most cysts, similar to IQGAP1 silenced cells (Fig 3C, D), but they also significantly attenuated normal lumen formation in cells expressing normal levels of endogenous IQGAP1 (Fig 3E). Moreover, in the absence of endogenous IQGAP1, neither of these constructs could correctly orientate the spindle, which appeared to be randomized in all cases (Fig 3F).

Together, these results suggest that the N-terminal and C-terminal domains of IQGAP1 drive the specific apical localization of this protein, and that this signal is overcome by a stronger signal directing IQGAP1 to the basolateral membrane. Indeed, the actin-binding N-terminal CHD domain and the Cdc42-binding GRD domain may account for the apical localization of IQGAP1:N and IQGAP1:C, respectively. However, in cells expressing both the IQGAP1:N and IQGAP1:C constructs, which only share part of the IQ motifs, a fraction of IQGAP1 was still retained at the basolateral membrane (Fig 3A,B), suggesting that the IQ motifs of IQGAP1 drive its polarized localization. Furthermore, an IQGAP1 construct lacking the IQ motifs, IQGAP1- Δ IQm-GFP, partially redistributed to the apical membrane domain (Fig 4A,B). The fraction of IQGAP1- Δ IQm-GFP maintained in the basolateral membrane is most likely due to the RGCT-domain of IQGAP1 which interacts with other basolateral proteins such as E-cadherin or β -catenin (Kuroda *et al*, 1998). Importantly, IQGAP1- Δ IQm-GFP expression in MDCK cells affected single lumen formation, an effect that was even more pronounced in the absence of endogenous IQGAP1 (Fig 4C) and that resulted in a dramatic misorientation of the mitotic spindle (Fig 4D, quantification in 4E). Finally, exposing MDCK cells expressing IQGAP1- Δ IQm-GFP to PMA did not affect the distribution of the protein (Fig 4F). Taken together, these results suggest that IQ motifs specifically restrict the distribution of IQGAP1 to the basolateral membrane. In the absence of these IQ motifs, or upon exposure to PMA, IQGAP1 becomes depolarized at the cell membrane, resulting in aberrant mitotic spindle orientation and disrupting single lumen formation.

EGFR stimulation induces IQGAP1 depolarization and mitotic spindle misorientation

Several proteins have been shown to bind IQGAP1 via its IQ motifs, including calmodulin, MEK1/2, B-Raf and EGFR (Takahashi & Suzuki, 2006; Johnson *et al*, 2009; McNulty *et al*, 2011). The EGF receptor (EGFR) is known to play several essential roles in kidney homeostasis and renal tubule repair (Zeng *et al*, 2009), and it is specifically distributed in the basolateral membranes of the kidney collecting ducts (Hobert & Carlin, 1995; He *et al*, 2002) and of MDCK cells (Hobert & Carlin, 1995; Dempsey *et al*, 1997). Membrane polarization of EGFR is necessary to control the proliferation

Figure 2. IQGAP1 localizes to the basolateral membrane in interphase and in mitotic cells, and its depolarization results in defective spindle positioning.

- A Cysts stained for tubulin (green), actin (red) and IQGAP1 (blue). Scale bars, 5 μ m.
- B Cysts stained for IQGAP1 (green), actin (red) and β -catenin (blue). Scale bars, 5 μ m.
- C Cysts were grown for 72 h, treated with 4 μ M PMA for 5, 10 or 15 min, and stained for IQGAP1 (green), actin (red) and β -catenin (blue). The basolateral (arrows) and apical (arrowheads) localization of endogenous IQGAP1 is indicated. Scale bars, 5 μ m.
- D Cysts stained with IQGAP1 (green), actin (red) and ToPro3 (blue). The yellow line represents the angle of the mitotic spindle. Scale bars, 5 μ m.
- E The angle formed between the mitotic spindle and the apicobasal axis was measured and the mean angles were: Control = $72.19 \pm 3.59^\circ$; PMA 5 min = $44.45 \pm 5.33^\circ$; PMA 10 min = $39.30 \pm 5.41^\circ$; PMA 15 min = $39.46 \pm 5.29^\circ$ ($n = 3$, >30 cells per experiment; values represent the mean $\pm$ s.e.m.). Error bars represent the s.e.m.: * $P < 0.05$; ** $P < 0.01$.

of epithelial tissues and interestingly, IQGAP1 was recently shown to modulate EGFR activation (McNulty *et al*, 2011). Thus, EGFR has emerged as a candidate to associate IQGAP1 to the basolateral membrane and therefore, we hypothesized that the phenotype resulting from IQGAP1- Δ IQm expression could be explained by defective IQGAP1-EGFR binding.

To investigate this hypothesis, we studied the interaction between IQGAP1 and EGFR in 3D-MDCK cyst cultures. First, we

confirmed that EGFR localizes to the basolateral membrane (Fig 5A, left panels), as described previously in MDCK monolayers (Hobert & Carlin, 1995), and we showed that the distribution of EGFR in this membrane domain was totally independent of IQGAP1 (Fig 5A, right panels). During cell division, EGFR was distributed throughout the basolateral membrane and it was totally excluded from the apical membrane domain, displaying the same distribution pattern as IQGAP1 in mitotic cells (Fig 5B). We confirmed that IQGAP1

Figure 3. IQ motifs are required to localize IQGAP1 to the basolateral membrane.

- A Scheme showing the IQGAP1 constructs used, each of which was designed as a GFP fusion protein.
- B MDCK cells stably expressing each construct were grown in 3D cultures to form cysts, which were fixed and stained for actin (red). The basolateral (arrows) and apical (arrowheads) localization of the mutant constructs is indicated.
- C MDCK clones for each construct transfected together with the IQGAP1 siRNA were grown in 3D cultures, and the cysts were fixed and stained for Pdxl (red) and actin (blue). The basolateral (arrows) and apical (arrowheads) localization of the mutant constructs is indicated.
- D The ability of each construct to allow single lumen cysts to form in the absence of endogenous IQGAP1 was quantified and represented as a percentage of the total number of cells ($n = 3$, >50 cysts per experiment; values represent the mean $\pm$ s.d.).
- E The ability of each clone to allow single lumen cysts to form was quantified and represented as a percentage of the total number of cells ($n = 3$, >50 cysts per experiment; values represent the mean $\pm$ s.d.).
- F The angle formed between the mitotic spindle and the apicobasal axis was measured and the mean angles were: IQGAP1 GFP = $71.56 \pm 2.91^\circ$; IQGAP1:N = $48.45 \pm 3.67^\circ$; IQGAP1:C = $48.01 \pm 3.41^\circ$ ($n = 3$, >35 cells per experiment; values represent the mean $\pm$ s.e.m.).

Data information: Error bars represent the s.e.m. or s.d.: * $P < 0.05$; ** $P < 0.01$. Scale bars, 5 μ m.

vesicles independently of IQGAP1 expression (Fig 5C). As PMA activation of PKC is thought to mediate signaling through several pathways, we stimulated EGFR with EGF to specifically study the response of this receptor. The internalization of the EGFR in MDCK cysts treated with EGF (2 ng/ml) was similar to that observed following PMA treatment (Fig 5D).

Accordingly, we characterized the role of EGFR in the localization and function of IQGAP1. Importantly, EGF treatment clearly dampened the interaction of IQGAP1 with EGFR (supplementary Fig S5B) and the exclusive basolateral distribution of IQGAP1 was lost in EGF-treated cells (Fig 5E, quantification in 5F). Moreover, EGF stimulation induced significant defects in mitotic spindle orientation, similar to those observed in IQGAP1-silenced cells (Fig 5G, quantification in 5H) and when prolonged (24 h), it induced the development of multiple small lumens surrounding the preformed central lumen and the accumulation of luminal cell clusters (supplementary Fig S5C). To further demonstrate the importance of the basolateral localization of the EGFR in restricting the distribution of IQGAP1, we generated a construct in which the intracellular region of EGFR was fused to the C2A/B domains of Slp2a (IntraEGFR) that localize exclusively to the apical membrane in 3D-MDCK cells (Galvez-Santisteban *et al*, 2012). Expressing the C2A/B-IntraEGFR fusion protein in MDCK cells induced the partial translocation of IQGAP1 to the apical membrane (Fig 6A), disrupting single lumen formation (Fig 6B) and mitotic spindle orientation (Fig 6C, quantification in 6D). Together, these results suggest that EGFR, an IQGAP1 binding partner, is essential to restrict the distribution of IQGAP1 to the basolateral membrane, and that exposure to EGF modifies the basolateral localization of IQGAP1, the normal orientation of the mitotic spindle and consequently, the formation of single lumens. We assessed whether this mechanism might be involved in crucial physiological processes that require reorientation of the mitotic spindle, such as epithelial tubulogenesis and branching (Yu *et al*,

interacts with EGFR by co-immunoprecipitation, an interaction that was strongly attenuated in the absence of IQGAP1 IQ motifs (supplementary Fig S5A). It was previously demonstrated that the phosphorylation of EGFR controls its subcellular distribution in 3D-MDCK cultures, allowing it to modulate epithelial morphogenesis (Cotton *et al*, 2013). Moreover, PMA phosphorylates and transactivates EGFR in glioblastoma cells and in dermal fibroblasts (Amos *et al*, 2005; Fukaya *et al*, 2013), and we found that exposing 3D-MDCK cyst cultures to PMA induced the internalization of EGFR in

2003). Indeed, EGFR activation has been shown to be crucial for embryonic kidney tubulogenesis in a model *in vitro* system (Sakurai *et al*, 1997). Exposing mature 3D-MDCK organoids to EGF (2 ng/ml) for 24 h induced epithelial tubulogenesis and the depolarization of IQGAP1 (supplementary Fig S6), suggesting a physiological role of IQGAP1 depolarization in mitotic spindle reorientation.

These findings indicate that the localization of IQGAP1 depends on EGFR, and that both proteins exhibit a polarized basolateral distribution in resting and mitotic cells. Stimulation of MDCK cysts with EGF induces the endocytosis of EGFR and IQGAP1 depolarization, which in turn provokes mitotic spindle reorientation.

Figure 4. IQ motifs restrict IQGAP1 to the basolateral membrane.

- A Scheme showing the constructs used, each designed as a GFP fusion protein.
- B MDCK cells stably expressing IQGAP1-ΔIQm eGFP were transfected with the control or IQGAP1 siRNA and grown in 3D cultures to form cysts, which were fixed and co-stained for actin (red) and β-catenin (blue). The basolateral (arrows) and apical (arrowheads) localization of the mutant construct is indicated.
- C The ability of the IQGAP1-ΔIQm stable clone to form single lumen cysts was quantified and represented as the percentage of the total number of cysts: Control, $98 \pm 1.4\%$; IQGAP1-ΔIQm, $61.5 \pm 7.4\%$; siIQGAP1, $59.5 \pm 7.05\%$; IQGAP1-ΔIQm + siIQGAP1, $46.2 \pm 4.18\%$ ($n = 3$, >50 cysts per experiment; values represent the mean $\pm$ s.d.).
- D MDCK IQGAP1-ΔIQm eGFP cysts were stained for acetylated tubulin (red) and the yellow line represents the mitotic spindle angle.
- E The angle formed between the mitotic spindle and the apicobasal axis was measured and the mean angles were: control = $76.10 \pm 2.29^\circ$, IQGAP1-ΔIQm = $43.09 \pm 4.40^\circ$ ($n = 3$, 40 cells per experiment; values represent the mean $\pm$ s.e.m.).
- F MDCK IQGAP1-ΔIQm eGFP cysts were treated with PMA for 15 min, fixed and co-stained for actin (red) and β-catenin (blue). The basolateral (arrows) and apical (arrowheads) localization of the mutant construct is indicated.

Data information: Error bars represent the s.d. or s.e.m.: * $P < 0.05$; ** $P < 0.01$. Scale bars, 5 μ m.

LLC-PK1 proximal tubule cells exhibit a non-polarized distribution of IQGAP1 and EGFR, spindle misorientation and defective lumen formation

IQGAP1 is a multidomain protein that binds to several partners distributed differentially in the cell. While it appears that the basolateral localization of the EGFR is fundamental to restrict IQGAP1 to this membrane domain, we used LLC-PK1 cells to confirm this hypothesis. LLC-PK1 cells are proximal tubule cells derived from the pig kidney in which the absence of the AP1B clathrin adaptor leads to the accumulation of the EGFR in the apical membrane domain (Folsch *et al*, 1999; Cotton *et al*, 2013). We initially confirmed that endogenous EGFR is mainly found in the apical membrane domain of these cells (Fig 7A), where IQGAP1 was also highly enriched (Fig 7B), clearly reproducing the distribution in MDCK cells previously observed for IQGAP1-ΔIQm and IQGAP1 following EGF stimulation. The formation of a single lumen by these cells in 3D organotypic cultures was significantly less efficient than that observed for MDCK cells (only $51.86 \pm 5.94\%$ of cysts formed a single lumen; Fig 7C). Hence, we measured the spindle angle in these cells to determine whether this deficiency in single lumen formation was due to defective mitotic spindle positioning and in marked contrast to MDCK cells, spindle orientation appeared to be randomized in LLC-PK1 cells (Fig 7D,E).

Finally, we attempted to restore a normal basolateral distribution of EGFR and normal lumen formation in LLC-PK1 cells by stably expressing the μ 1B isoform of the AP1 μ -subunit (Gan *et al*, 2002). In LLC-PK1-AP1B cells, we observed a partial redistribution of EGFR to the basolateral membrane, together with that of IQGAP1, although a small amount of both proteins persisted in the apical domain (Fig 7F, G). Importantly, LLC-PK1-AP1B cells were significantly more efficient than LLC-PK1 cells in forming single lumens (Fig 7H), although not as efficient as MDCK cells. Moreover, there was significantly more mitotic spindles orientated perpendicular to the apicobasal axis in LLC-PK1-AP1B cells than in the parental cells (Fig 7I,J).

In summary, these data demonstrate the importance of the basolateral membrane localization of EGFR to restrict IQGAP1 to this domain, and consequently, for correct mitotic spindle orientation and single lumen formation.

The absence of IQGAP1 disrupts the basolateral localization NuMA

It is known that LGN and NuMA localize to the basolateral membrane and control correct mitotic spindle orientation (Du *et al*,

2001; Gordon *et al*, 2001; Zheng *et al*, 2010). Hence, we investigated the potential role of IQGAP1 in the localization and/or activity of LGN/NuMA in spindle orientation. As described previously, LGN-GFP translocates to the basolateral membrane and orientates spindle poles during mitosis (Du *et al*, 2001), and its distribution was completely independent of IQGAP1 (Fig 8A). By contrast, while NuMA localize to the basolateral membrane in control mitotic cells, it was distributed all over the cell membrane in the absence of IQGAP1 (Fig 8B). We addressed whether the distribution of IQGAP1 depends on LGN using the C-terminal domain of LGN (Ct-LGN), which acts as a dominant negative form of LGN, disrupting both the endogenous basolateral distribution of LGN and mitotic spindle orientation in MDCK cells (Rodríguez-Fraticelli *et al*, 2010). Interestingly, IQGAP1 localization was not affected by the expression of Ct-LGN (under the control of the Tet-off inducible promoter), even in mitotic cells with misorientated spindles (Fig 8C).

Together these results indicate that while the activity and basolateral localization of LGN and IQGAP1 do not appear to be related, the specific localization of NuMA to the basolateral membrane of mitotic cells depends on the presence of IQGAP1 in this domain.

Discussion

Although mitotic spindle orientation is of fundamental importance in epithelial morphogenesis and in the generation of polarized cells, it is a process that remains poorly understood (Castanon & Gonzalez-Gaitan, 2011). The findings presented here describe a new molecular mechanism involved in mitotic spindle orientation and that drives the formation of the central lumen during epithelial morphogenesis. We demonstrate that the IQGAP1 scaffolding protein localizes to the basolateral membrane where it serves to orientate the mitotic spindle perpendicular to the apicobasal axis of the cell. Indeed, when this specific localization of IQGAP1 is compromised in the 3D-MDCK organotypic model, the mitotic spindle is misoriented and single lumen formation is disrupted. The localization of IQGAP1 to the basolateral membrane depends on its IQ motifs, which mediate IQGAP1 binding to EGFR. Indeed, EGFR appears to act as a linker to restrain IQGAP1 in the basolateral membrane, and it is distributed in the same pattern as IQGAP1 in interphase and mitotic cells. Moreover, EGFR appears to orientate the mitotic spindle, since the activation of EGFR by EGF induces its internalization, provoking a redistribution of IQGAP1 throughout the cell membrane and ultimately disrupting the orientation of the mitotic spindle (see model Fig 9).

Figure 5. EGFR mediates IQGAP1 localization to the basolateral membrane and controls spindle orientation.

- A** Control cysts and those transfected with IQGAP1 siRNA were stained for EGFR (green), actin (red) and β -catenin (blue). Arrows indicate the basolateral localization of EGFR in the absence of IQGAP1.
- B** Control cysts were stained for tubulin (green), actin (red) and EGFR (blue).
- C** Control and IQGAP1 siRNA-transfected cysts were grown for 72 h, exposed to 4 μ M PMA and then stained for EGFR (green), actin (red) and β -catenin (blue).
- D** Control cysts were grown for 72 h, treated with EGF (2 ng/ml) and then stained for EGFR (green), actin (red) and β -catenin (blue).
- E** Control cysts were grown for 72 h, treated with EGF (2 ng/ml) and then stained for IQGAP1 (green), actin (red) and β -catenin (blue). The basolateral (arrows) and apical (arrowheads) localization of the endogenous IQGAP1 is indicated.
- F** After EGF treatment IQGAP1 was evenly distributed between the apical and basolateral membranes ($45.66 \pm 2.21\%$ IQGAP1 at the apical membrane). Fluorescence intensity was quantified with ImageJ software and represented as a percentage ($n = 3$, >50 cysts per experiment; values represent the mean $\pm$ s.d.).
- G** Control and EGF-treated cysts were stained for tubulin (green), actin (red) and IQGAP1 (blue). The yellow line represents the mitotic spindle angle.
- H** The mean angles formed between the mitotic spindle and the apicobasal axis were: control = $74.33 \pm 2.70^{\circ}$, EGF-treated = $43.04 \pm 4.14^{\circ}$ ($n = 3$, >40 cells per experiment; values represent mean $\pm$ s.e.m.).

Data information: Error bars represent the s.d. or s.e.m.: * $P < 0.05$; ** $P < 0.01$. Scale bars, 5 μ m.

Figure 6. EGFR mediates the localization of IQGAP1 to the basolateral membrane and it controls spindle orientation.

A Scheme showing the constructs used, each designed as a GFP fusion protein. MDCK cells stably expressing C2A/B intraEGFR were grown for 72 h to form cysts, and then stained for actin (red) and IQGAP1 (blue). The apical localization of endogenous IQGAP1 is indicated (arrowheads).
 B The ability of the C2A/B intraEGFR stable clone to form single lumen cysts was quantified and represented as a percentage of the total number of cysts: control, 88.5 ± 0.7%; C2A/B intraEGFR, 45.96 ± 1.23% (n = 3, >50 cysts per experiment; values represent the mean ± s.d.).
 C C2A/B intraEGFR cysts were stained for tubulin (red) and the yellow line represents mitotic spindle angle.
 D The mean angle formed between the mitotic spindle and the apicobasal axis was: control = 75.14 ± 2.24°, intraEGFR = 44.90 ± 4.61° (n = 3, 40 cells per experiment; values represent mean ± s.e.m.).

Data information: Error bars represent the s.d. or s.e.m.: *P < 0.05; **P < 0.01. Scale bars, 5 µm.

IQGAP1 binds to and stabilizes plus-end microtubules at the cell cortex by interacting with plus-end-associated proteins, such as CLIP170 (Fukata *et al*, 2002; Noritake *et al*, 2004). IQGAP1 has also been shown to interact directly with CLASP2 (Watanabe *et al*, 2009), a family of proteins that bind to the plus-tip protein EB1 and that mediate the interaction between microtubules and the cell cortex (Mimori-Kiyosue *et al*, 2005). Taken together with the results presented here, the interaction of IQGAP1 with different microtubule binding proteins (e.g. CLIP170 and CLASP2) would appear to polarize plus-end astral microtubules to the basolateral membrane during mitosis, correctly orientating the spindle in dividing epithelial cells as required for lumen formation. Indeed, MDCK cells stably expressing the GFP-tagged C-terminus of IQGAP1, which acts as a dominant negative form of IQGAP1 as it prevents the binding of endogenous IQGAP1 to CLIP170 microtubules (Fukata *et al*, 2002), displayed significant defects in single lumen formation (data not shown). Hence, IQGAP1 binding to microtubules appears to be essential for correct spindle orientation.

It was previously demonstrated that the NuMA/LGN/Gα protein complex is capable of generating pulling forces on astral MTs during mitosis (Du & Macara, 2004), acting as a specific controller of mitotic

spindle orientation in epithelia (Hao *et al*, 2010; Zheng *et al*, 2010; Peyre *et al*, 2011). LGN (Leu-Gly-Asn repeats)/GPSM2 (G-protein signaling modulator 2) belong to a family of proteins that modulate G-protein activation and that transduce extracellular signals received by cell surface receptors into integrated cellular responses. LGN is distributed in the cytoplasm in interphase cells but it translocates to the cell cortex during mitosis by interacting with the Gαi membrane protein. There, LGN interacts with NuMA, a microtubule-binding protein that binds to cytoplasmic dynein (Merdes *et al*, 1996; Du *et al*, 2001) and that tethers the mitotic spindle to the membrane (Fig 9). Indeed, disrupting the interactions between endogenous LGN and Gα, or LGN and NuMA, results in spindle misorientation and defective cystogenesis (Zheng *et al*, 2010). Interestingly, this protein complex is localized exclusively at the basolateral membrane in MDCK cells via the aPKC-mediated apical exclusion of LGN (Hao *et al*, 2010).

We found that EGFR-IQGAP1 forms another complex that also seems to be required for the polarized anchoring of astral microtubules during epithelial cell division and to control of spindle orientation. Our data indicate that a functional connection exists

Figure 7. LLC-PK1 cells have a non-polarized distribution of EGFR and IQGAP1, and they consequently suffer spindle misorientation.

A, B LLC-PK1 cysts were grown for 72 h, fixed and then stained for (A) EGFR (green), actin (red) and β-catenin (blue), or (B) IQGAP1 (green), actin (red) and β-catenin (blue). The apical localization of endogenous EGFR or IQGAP1 is indicated (arrowheads).

C Percentage of cysts forming a single lumen: MCDK, $82.96 \pm 3.17\%$; LLC-PK1, $51.86 \pm 5.94\%$ ($n = 3$, >100 cysts per experiment; values represent the mean $\pm$ s.d.).

D LLC-PK1 cysts were stained for acetylated tubulin (green), actin (red) and β-catenin (blue). The yellow line represents mitotic spindle angle.

E Mean spindle angles: MCDK = $74.73 \pm 3.52^\circ$, LLC-PK1 = $45.37 \pm 5.07^\circ$ ($n = 3$, 30 cysts per experiment; values represent the mean $\pm$ s.e.m.).

F, G LLC-PK1 AP1B cysts were grown for 72 h, fixed and then stained for (F)EGFR (green), actin (red) and β-catenin (blue), or (G) IQGAP1 (green), actin (red) and β-catenin (blue). The basolateral localization of endogenous EGFR or IQGAP1 is indicated (arrows).

H The percentage of LLC-PK1-AP1B cysts forming a single lumen was quantified: LLC-PK1-AP1B, $65.57 \pm 5.23\%$ ($n = 3$, >80 cysts per experiment; values represent the mean $\pm$ s.d.).

I LLC-PK1-AP1B cysts were stained for acetylated tubulin (green), actin (red) and β-catenin (blue). The yellow line represents mitotic spindle angle.

J Mean spindle angles: LLC-PK1 = $45.29 \pm 5.09^\circ$, LLC-PK1 AP1B = $61.80 \pm 5.30^\circ$ ($n = 3$, 30 cysts per experiment; values represent the mean $\pm$ s.e.m.).

Data information: Error bars represent the s.d. or s.e.m.: * $P < 0.05$; ** $P < 0.01$. Scale bars, 5 μ m.

Figure 8. The distribution of NuMA but not LGN is altered in the absence of IQGAP1.

- A Stable Tet-Off T23 cells expressing LGN-GFP were transfected with control or IQGAP1 siRNA. Doxycycline was removed from medium of stably expressing cells and they were immediately plated and grown in 3D cultures for 48 h. The cells were then fixed and stained for tubulin (red) and IQGAP1 (blue). The basolateral localization of the construct is indicated (arrows). Scale bars, 5 μ m.
- B MDCK cells were transfected with control or IQGAP1 siRNA and grown in 3D cultures. Cysts were fixed and stained for NuMA (green), tubulin (red) and IQGAP1 (blue). The basolateral (arrows) and apical (arrowheads) localization of NuMA is indicated. Scale bars, 5 μ m.
- C Stable Tet-Off T23 cells expressing Ct-LGN-myc were grown in 3D cultures for 72 h (left panel) or 48 h before doxycycline was removed from the medium for a further 24 h (right panel). The cells were then fixed, and stained for IQGAP1 (red) and ZO1 (blue). Scale bars, 5 μ m.

between both complexes in terms of their influence on mitotic spindle orientation. We found that LGN binding to G α and its specific exclusion from the apical domain is independent of IQGAP1, since IQGAP1 silencing had no effect on the basolateral localization of LGN. However, IQGAP1 is necessary for NuMA polarization to the basolateral membrane. Thus, the question arises as to how IQGAP1 modulates the distribution of NuMA? One hypothesis is that IQGAP1 could promote the binding of NuMA to LGN at the cell cortex. Thus, in the absence of IQGAP1, NuMA still maintains the ability to bind plus end

MTs in mitotic cells and it could be dragged by the astral MTs through the cell cortex, interacting in the apical membrane domain with binding partners other than LGN. Indeed, it was recently shown that spindle positioning in symmetrically dividing human anaphase cells also depends on the interaction of NuMA with the cortical adaptor 4.1G/R (Kiyomitsu & Cheeseman, 2013). However, other studies recently demonstrated that NuMA's anaphase distribution is independent of interactions with LGN and 4.1 in mitotic keratinocytes (Seldin *et al*, 2013). In addition, 4.1R localized to the basolateral

Figure 9. Model illustrating role of EGFR-IQGAP1 in epithelial polarization.

Top panel. EGFR binds to the IQ motifs of IQGAP1 and restricts its localization to the basolateral membrane. *Middle panel.* In the absence of IQGAP1, EGFR is restricted to the basolateral membrane. However, NuMA and the +TIPs of astral microtubules attach arbitrarily to the membrane, resulting in mitotic spindle misorientation. *Bottom panel.* EGF treatment activates the EGFR, resulting in receptor internalization via vesicles. The restriction of IQGAP1 to the basolateral membrane is subsequently lost and mitotic spindles appeared to be randomly orientated, favoring tubulogenesis.

membrane in gastric epithelial cells (Yang *et al*, 2009) and MDCK cells (our unpublished results), and its cortical interaction is independent of IQGAP1 (Ruiz-Saenz *et al*, 2011). Therefore, further studies will be required to better characterize the interaction of NuMA with the cell cortex and how IQGAP1 regulates the specific localization of NuMA to the basolateral membrane. Moreover, it remains to be determined whether other proteins might also be involved in controlling the orientation of the epithelial mitotic spindle.

We demonstrate here that the polarization of IQGAP1 to the basolateral membrane is dependent on its interaction with the EGFR. The EGFR is a receptor for members of the epidermal growth factor (EGF) family, and EGF binding to its extracellular domain causes receptor dimerization and tyrosine autophosphorylation, promoting cell proliferation, growth and migration (Yarden, 2001). The localization of EGFR to the basolateral membrane is controlled at multiple levels. First, PKC-dependent phosphorylation of EGFR at

Thr654 mediates its basolateral membrane sorting (Cotton *et al*, 2013). Two atypical PKC (aPKC) isoforms (λ/ι and ζ) are localized to the apical membrane in 3D-cultured MDCK cells, from where they can phosphorylate and exclude LGN, thereby influencing mitotic spindle orientation (Hao *et al*, 2010). Although information regarding the substrate specificity of each PKC protein is limited, some peptides are thought to be specific to individual PKC isozymes. Indeed, EGFR Thr654 may be phosphorylated by aPKC as well as PKC (Nishikawa *et al*, 1997). We also demonstrate that fusion of an apical-targeting domain (C2A/B) to the intracellular region of EGFR localizes the construct to the apical region. However, this construct is unable to stably maintain its apical localization and it is rapidly redistributed to sub-apical vesicles. These observations suggest a possible role for aPKC in EGFR phosphorylation and apical exclusion, preventing spindle misorientation via a mechanism similar to that which mediates the apical exclusion of LGN. Alternatively, the C2A/B domain could induce the dimerization and phosphorylation of the intracellular region of EGFR, causing its internalization. AP1B directs EGFR to the basolateral membrane via the biosynthetic route (Cotton *et al*, 2013) and the EGFR accumulates apically in AP1B-deficient LLC-PK1 cells, as does IQGAP1 as a consequence. AP1B expression in LLC-PK1 induced a partial but not complete redistribution of EGFR to the basolateral membrane, reducing the apical accumulation of IQGAP1, and favoring perpendicular mitotic spindle orientation and single lumen formation. These findings indicate that AP1B expression is not sufficient to restrict EGFR to the basolateral membrane in LLC-PK1 cells, and that a small accumulation of EGFR at the apical membrane is enough to induce apical IQGAP1 localization, resulting in spindle misorientation.

During the morphogenesis of an epithelial tissue, cells often organize into biological tubes, structures required for the function of many organs. Thus, tubule formation is a fundamental event in the generation of diverse tissue types during metazoan development and it depends on the activity of soluble growth factors, like EGF, that modulate essential cellular processes associated with cell plasticity and transcription (Bryant & Mostov, 2008). We found that EGF induced tubulogenesis *in vitro*, suggesting a physiological role for EGFR activation in renal tubule development. Indeed, EGFR has been attributed an important morphogenetic role in early nephron development (Sakurai *et al*, 1997) and it also appears to be essential for tubule regeneration: high concentrations of EGF accumulate in urine (Tsukumo *et al*, 1987) and mammalian kidneys produce high levels of the EGF precursor, preproEGF (Gesualdo *et al*, 1996). As EGF is in contact with the apical surface of the lumen in kidney tubules, and EGFR is exclusively located in the basolateral membrane domain, activation of EGFR is prevented by its spatial separation from its agonist (Salido *et al*, 1986; Zeng *et al*, 2009). However, when epithelial integrity is compromised due to tubule damage and the disruption of the cell junctions, the basolateral membrane becomes exposed to EGF, which activates EGFR, triggering cell proliferation and re-establishing the epithelial barrier. Thus, extrapolating our results to a physiological context suggests that IQGAP1 would be restricted to the basolateral membrane in normal kidney due to its binding to EGFR. By contrast, EGFR activation, following epithelial injury in the mature kidney or due to stimulation of tubule branching during morphogenesis, would depolarize the distribution of IQGAP1, promoting altered cell division, proliferation and restoring epithelial integrity.

Epithelial growth factor receptor is one of the growth factor receptors most commonly affected in cancer and altered EGFR has

been described in several tumor types, including breast, colon and lung tumors (Sainsbury *et al*, 1987; Bauknecht *et al*, 1989; Spano *et al*, 2005; Ding *et al*, 2008). Most drugs designed to treat such tumors target the signaling activity of this receptor. However, our data demonstrate a role for EGFR in IQGAP1 localization and spindle orientation, and since the latter has been shown to be disrupted in cancer (Ellenbroek *et al*, 2012; Marongiu *et al*, 2012; Martin-Belmonte & Perez-Moreno, 2012), it may be this activity of EGFR that is compromised in cancer. There is currently increasing evidence that cancer stem cells are responsible for the long-term maintenance of tumor growth (Clarke *et al*, 2006; Dick, 2008; Blanpain, 2013). Asymmetric division of stem/progenitor cells allows the generation of the differentiated cells necessary for tissue development and the maintenance of a stem cell pool, both essential processes during development, adult tissue homeostasis and regeneration. Failure to carry out asymmetric stem cell division promotes symmetric cell division, increasing the population of stem cells with a highly proliferative potential. During asymmetric cell division cell polarization is tightly coupled to spindle orientation (Betschinger & Knoblich, 2004; Roegiers & Jan, 2004; Siller & Doe, 2009). The complex formed by $G\alpha$ /LGN/NuMA, together with other proteins like the Par complex and the Rho GTPase Cdc42, has been implicated in both symmetric and asymmetric cell division (Siller & Doe, 2009; Williams *et al*, 2011). Accordingly, determining the role of IQGAP in asymmetric cell division will be an important challenge for the future.

In summary, our data describe a new molecular mechanism involving EGFR and IQGAP1 that controls spindle orientation in epithelial cells. We show that EGFR acts as a basolateral linker for IQGAP1, which in turn anchors astral microtubules of the mitotic spindle to the junctional/basolateral membrane, and drives the correct orientation of cell division, which is essential for single lumen formation during tubulogenesis.

Materials and Methods

Cell culture

MDCKII cells were cultured in complete MEM supplemented with 5% fetal bovine serum, 50 U/ml penicillin, 50 μ g/ml streptomycin and 2 mM L-Gln (Gibco, Life Technologies, Carlsbad, CA, USA). MDCK cells stably expressing IQGAP1-GFP and the different constructs were generated by co-transfection with the blasticidin-resistant gene, and they were selected for 10 days with 0.5 mg/ml blasticidin. LLC-PK1, LLC-PK1 AP1B and 293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μ g/ml streptomycin and 2 mM L-Gln. Cyst cultures were performed as described previously (Rodríguez-Fraticelli *et al*, 2010).

Antibodies and reagents

The primary antibodies used were: IQGAP1 (1:1000; 610612; BD Transduction, San Jose, CA, USA), EGFR (1:100; GR01; Calbiochem, Darmstadt, Germany), tubulin [YL1/2] (1:500; ab6160; Abcam, Cambridge, UK), acetylated tubulin (1:1000; T7451; Sigma-Aldrich, Seelze, Germany), GFP (1:500; A5455; Invitrogen, Carlsbad, CA, USA), β -catenin (1:1000; sc7199; Santa Cruz, Dallas, TX, USA), NuMA (1:250; ab36999; Abcam), γ -tubulin (1:500; T6557; Sigma),

ZO1 (1:500; R40.76; Millipore, Darmstadt, Germany) and myc (1:1000; 9E10; Roche, Basel, Switzerland). The Podxl/gp135 antibody was a gift from the Ojakian laboratory (State University of New York Downstate Medical Center, USA). Peroxidase-conjugated donkey anti-mouse IgG and anti-rabbit IgG were used as secondary antibodies in western blots (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Alexa Fluor-conjugated secondary antibodies (Alexa Fluor 488, 555 or 647; Invitrogen) and TOPRO3 (nuclear/DNA staining; Molecular Probes [Carlsbad, CA, USA], Invitrogen) were used for microscopy. PMA (P1585; Sigma), EGF (01-107; Millipore) and thymidine (T1895; Sigma) were used to treat the cells.

Vectors

IQGAP1, as well as, IQGAP1:C, IQGAP1:N, IQGAP1:CT and IQGAP1 Δ IQm, and C2A/B intraEGFR were cloned into a pEGFP-N1 vector (Takara Bio Inc, Otsu, Shiga, Japan). LGN and DN-LGN were cloned into pTRE2hyg vector (Adgene, Cambridge, MA, USA). The pcDNA6A EGFR-myc construct was a kind gift from Antonio Villalobo (IIB-CSIC, Madrid, Spain).

RNAi

Stealth siRNA duplexed targeting messenger RNA sequences of 25 nucleotides from canine IQGAP1 were purchased from Invitrogen. The sequences were submitted to the BLAST search engine to ensure targeting specificity. For siRNA transfection, MDCK cells were trypsinized and nucleofected (Lonza, Basel, Switzerland) with the siRNAs. After 24 h, the cells were resuspended and plated in 12-well plates and in coverglass chambers coated with Matrigel to grow cysts. Total cell lysates from 3D cultures were analyzed in western blots to confirm siRNA efficiency.

Immunofluorescence, microscopy and quantification

MDCK cells were fixed at different time points and stained by immunofluorescence using the primary antibodies indicated. Alexa-fluor488/555/647 conjugated anti-rabbit and anti-mouse were used as the secondary antibodies (Life technologies). Images were acquired on an LSM510 inverted confocal microscopes (Zeiss, Jena, Germany) using the ZEN software and 63X/NA1.4 oil Plan-Apochromat Objectives (Zeiss). Finally, the images were treated using ImageJ software (NIH, Bethesda, MD, USA; <http://rsb.info.nih.gov/ij/>).

To analyze spindle orientation, cysts were stained for acetylated tubulin, and the angle formed between the spindle and the apicobasal axis was measured. At least three experiments per condition were quantified, analyzing >30 cysts/experiment. Significance was calculated using a paired, two-tailed Student's *t*-test, and the *P*-values are indicated in each experiment.

Measurement of spindle angle

Confocal images of mitotic cells in the middle region of the cysts were collected. Using ImageJ software, a line connecting the two spindle poles was drawn, and another line was drawn from the center of the apical membrane of the cell to the midpoint of the basal membrane, defining the apicobasal axis. The angle between the two lines was measured.

Calcium switch

Cells were incubated overnight in calcium-free MEM medium supplemented with dialyzed FBS, subsequently restoring calcium conditions with complete MEM. Monolayer impedance was measured in real time to determine transepithelial permeability.

Apical biotinylation assay

Control and IQGAP1-silenced cells were grown to confluency on six well plates. The cells were treated twice for 30 min on ice with trypsin (100 µg/ml), they were then washed with ice-cold PBS and new medium was added. After 0, 30, 60 or 90 min of incubation at 37°C, the cells were extensively washed with ice-cold PBS before they were biotinylated for 20 min on ice (1 mg/ml sulfo-NHS-LC-biotin in PBS, supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂ [pH 7.8]). The excess biotin was removed and quenching buffer (Tris-HCl 50 mM pH 6.8) was added for 10 min on ice. Finally, to carry out the pull-down assay the cells were lysed with RIPA buffer containing 0.2% Triton Tx-100 (50 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS) and protease inhibitors (Boehringer, Ingelheim, Germany).

Immunoprecipitation

The 293T cells were grown on p100 dishes until they reached 60–70% confluence and they were then co-transfected with EGFR myc-IQGAP1 eGFP or EGFR myc-IQGAP1 ΔIQm eGFP. Transfection with a GFP construct alone or with IQGAP1 eGFP served as the controls. In supplementary Fig S5A, EGFR was immunoprecipitated with an anti-myc antibody and IQGAP1 binding was assessed in Western blots. In supplementary Fig S5B, IQGAP1 was immunoprecipitated with an anti-GFP antibody and EGFR binding was assessed in Western blots probed with anti-myc. In both cases the input material was 1/20th of the immunoprecipitate volume. Binding was analyzed by quantifying the pixels using ImageJ software.

Supplementary information for this article is available online: <http://emboj.emboexpress.org>

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Author contributions

I.B.-R., M.G.-S., S.V. and F.M.-B. designed the experiments. I.B.-R., M.G.-S., S.V., M.B. and A.B.-P. did the experimental work. I.B.-R., M.G.-S. and S.V. analyzed the experiments. I.B.-R. and F.M.-B. wrote the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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