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 and Fernando Martín-Belmonte

**Corresponding author:** Fernando Martin-Belmonte, CBM-SO, Madrid

---

**Review timeline:**

- Submission date: 11 June 2013
- Editorial Decision: 18 July 2013
- Revision received: 17 October 2013
- Editorial Decision: 04 November 2013
- Revision received: 11 November 2013
- Accepted: 12 November 2013

---

**Transaction Report:**

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

---

**Editor:**

1st Editorial Decision 18 July 2013

Thank you for the submission of your manuscript entitled "The control of IQGAP membrane localization by EGFR regulates mitotic spindle orientation during epithelial morphogenesis" to The EMBO Journal and please accept my apologies for the delay in responding. We have just now received the full set of reports from the referees, which I copy below.

As you can see from their comments, referees #2 and #3 are generally positive and recommend the publication of your manuscript, provided some major issues that will need to be addressed. Referee #1 is less enthusiastic, but also believes in the suitability of your study if some improvements are implemented in a revised version.

In general, referee's concerns are rather explicit in their reports and thus I will not repeat them here, but I would like however to draw your attention to certain, particularly important points. Be aware that other, secondary points are also raised by the referees and, although your revised manuscript should address the referee concerns as completely as possible, they will not be determinant for acceptance. Both referee #1 (point 1) and referee #3 (point2), insist on the idea that the mechanism underlying IQGAP1-mediated spindle localization should be put into a wider context, taking into
account other known mechanisms involved. They explicitly mention the Galphai-LGN-NuMA system, and suggest additional experiments to address this issue. Referee #2, in turn, is concerned with the mechanisms of IQGAP1 localization. Finally, referee #3 is also concerned with the relationship between spindle orientation and lumen formation (point 1), and believes that a deeper analysis is warranted.

Please be aware that your revised manuscript must address the referees' concerns, as explained above, and their suggestions should be taken on board. It is 'The EMBO Journal' policy to allow a single round of revision only and, therefore, acceptance or rejection of your study will depend on the completeness of your responses included in the next, final version of the manuscript.

That being said do not hesitate to contact me by e-mail or on the phone in case you have any questions, need further input or anticipate any problems in fulfilling any of the referee requests.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). However, please contact me as soon as possible upon publication of any related work in order to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

When preparing your letter of response to the referees' comments, bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you very much for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS:

Referee #1:

In this paper, the authors analyze the mechanisms that regulate spindle orientation during epithelial morphogenesis in MDCK cysts, and established model for lumen formation. The authors analyzed the localization of IQGAP1 and EGFR in these cells, and find that they are restricted to the basolateral domains in the 3D-MDCK cysts. They also find that, in the absence of the proper function or localization of IQGAP1 or EGFR, spindle orientation and lumen formation is perturbed. In total, their work suggests that IQGAP1 is restricted to the basolateral domains in 3D-MDCK cysts by binding to EGFR using its IQ motif to promote proper oriented cell divisions. Overall, the data are high quality, and well documented. However, ultimately I do not believe that this paper represents a substantial advance in understanding the mechanisms for spindle orientation. In the absence of a clearer picture of the mechanisms by which IQGAP1 acts to promote spindle orientation (as detailed below), this paper is not suitable for publication in EMBOJ.

1. There is an extensive literature analyzing the mechanisms of spindle orientation. However, for this paper, it is almost as if EGFR and IQGAP1 function in a vacuum where these various other factors are not considered. Ultimately, this paper needs to define the contribution of IQGAP1 to controlling spindle orientation, and this means defining its relationship to the other key players in spindle orientation. For example, the relationship between EGFR-IQGAP and G i-LGN-NuMA complex is potentially very interesting. The authors claim that these don't affect each other, but this is only listed as data not shown. This needs to be thoroughly investigated. It is critical to show the localization dependency between these complexes and the effect of EGF addition on LGN localization to clarify whether EGFR-IQGAP pathway is sufficient to control spindle orientation. It seems very unlikely that there is a completely independent pathway for spindle orientation, and yet that both pathways make essential and non-overlapping contributions to spindle orientation. If this is the case, it would represent an important advance. However, to prove this the authors need to establish what is occurring downstream of IQGAP1.
2: The authors propose that IQGAP1 would polarize astral microtubule plus ends to the basolateral domain during "mitosis" (p15, L19). Although LGN/NuMA localizes to the cell cortex during mitosis, IQGAP1 localizes to the basolateral domain during "interphase" and previous work suggests that IQGAP1 interacts with +TIPs in "interphase" cells. It is critical to analyze whether IQGAP1 controls centrosome positioning/separation in interphase before mitosis similar to what is observed in Drosophila germ stem cells, or whether it is playing additional roles during mitosis that are distinct from its interphase localization.

Additional comments:
1: The authors show the positive regulation of IQGAP1 basolateral localization by EGFR, but the negative regulation of IQGAP localization at the apical cortex could also occur. It would strengthen their conclusions to test whether overexpressed IQGAP1 can localize to the apical domain when EGFR is saturated by IQGAP1.

2: In Supplemental Figure 4, the authors show that EGF treatment results in the depolarization of IQGAP1. This is an important result, but the authors also need to quantify spindle orientation under these conditions.

3. There are some persistent wording problems throughout the text. The authors need to carefully edit the manuscript.

4. It would be helpful to add a sentence saying that "this phenotype is rescued by expressing siRNA resistant IQGAP1 (described below)" after the Fig. 1D, E.

5. In Fig. 2C and D, the authors need to show single color images for -catenin. -catenin appears to localize to the basal domain at 15' after the treatment with PMT. Thus, other possibilities may exist for the spindle mis-orientation caused by PMT treatment. In addition, IQGAP1 accumulates at the apical domain in interphase, but less so in mitotic cells (Fig. 2D). Does this suggest that IQGAP1 controls centrosome positioning in interphase?

Referee #2:

The control of IQGAP membrane localization by EGFR regulates mitotic spindle orientation during epithelial morphogenesis.

This is an interesting study that examines mitotic spindle orientation during cystogenesis. There are, however, two concerns that need to be addressed.

First, the authors have an entire section dedicated to the IQ motifs and ultimately reach the conclusion that these motifs mediate the basolateral restriction of IQGAP1. In this section the conclusions are rather reaching and insufficiently supported by the data presented. In particular, is the use of the IQGAP1- IQm-GFP construct, which the authors state has both an apical and basolateral distribution. The authors do not sufficiently address how this would have any basolateral distribution if their conclusion is that the IQ motifs are responsible for basolateral sequestration. Overall, there are alternative explanations that should be examined and addressed based on the data provided.

Second, the authors need to show the level of EGFR-IQGAP1 association with EGF stimulation. The data presented shows very clearly the internalization of EGFR in response to stimulation, and the authors do show a shift of some IQGAP1 to the apical membrane. Given their conclusions, the absence of EGFR at the basolateral membrane should be accompanied by a concomitant loss of IQGAP1, however the images shown still have IQGAP present on the basolateral surfaces. This again relates back to the previous comment in that significant portions of the conclusions rest on the idea that EGFR is responsible for maintaining IQGAP localization however there is not a insignificant pool of IQGAP remaining even with the loss of EGFR, suggesting additional conclusions.
Referee #3:

In this article, the authors describe roles for IQGAP1 and EGFR in spindle orientation and in lumen formation in MDCK cell cysts. Although the data clearly show that IQGAP affects spindle orientation, which is a very interesting result, the link between spindle orientation and lumen formation has not been rigorously proven. In addition, several important experiments are missing and some of the data could be presented more clearly. I therefore think that this manuscript could be suitable for EMBOJ, but only after a major revision.

Major Points:

1) Most of the conclusions presented in this article are predicated on the assumption that spindle orientation is the only cause of abnormal lumen formation and this needs to be proven more rigorously.

At what angles does spindle misorientation result in abnormal lumen formation? The control shown in Figure 1G shows that only about 60% of spindles in the control condition have spindles perpendicular to the apicobasal axis. Many "tilted" spindles, about 35% of the total, appear in the control. Do tilted spindles cause abnormal lumen formation? What is the correlation between parallel spindles and abnormal lumens?

In Figure 1G it appears that there is a 4-5X increase in the number of parallel spindles between the control and the experimental siIGAP1 condition. This corresponds to an approximately 40% decrease in normal lumens. In Figure 4E there is a much greater increase (apparently about 10X) in the number of parallel spindles between the control and experimental IQm condition. However, the percent of normal lumens decreases by the same amount - roughly 40%.

In Figure 5J, spindle orientation in LLC-PK1 AP1B cells does not appear significantly different from MDCK cells, though this comparison is surprisingly not made directly. Despite the fact that spindle orientation is not different, normal lumen formation appears to be decreased by 20%.

These data show that the relationship between spindle misorientation and abnormal lumen formation is not linear, suggesting that it is also not entirely causal. The claim that spindle misorientation is the only cause of abnormal lumens is therefore weak. This conclusion needs to be strengthened with more evidence to show that IQGAP knockdown has no effect on polarity or apical secretion (supplementary figure 1 is not very convincing). For example, they could use the approach that Zheng et al used when they showed that the multiple lumen phenotype caused by the knock down LGN is suppressed by inhibitors of cell division.

2) In the discussion (page 16) the authors suggest that the IQGAP/EGFR complex acts independently of the Galphai/LGN/NuMA complex. This is based on the unpublished observations that 1) members of these complexes do not interact (via co-IP?) and 2) LGN localizes correctly even after silencing of IQGAP. As the observations are unpublished the reader cannot evaluate them, and neither observation is sufficient to justify the claim that the complexes do not interact functionally. In fact, at the end of this paragraph (Page 17), the authors note that "[f]urther studies will be required to demonstrate the link between these two complexes,” indicating that they believe such a link to exist.

As the Galphai/LGN/NuMA complex is implicated in mitotic spindle orientation in every cell type examined to date, I think it is reasonable to expect a more thorough investigation of functional interaction between the two complexes. Epistasis experiments should be possible given that siIQGAP1 does not randomize spindle orientation.

Minor Points

Abstract

The claim that experimental conditions randomize spindle angles is made multiple times in the manuscript, beginning in the abstract. This is usually not supported by the data. Randomization would produce a distribution whereby each class of spindle angle - parallel, perpendicular, and tilted
- accounts for one-third of the angles measured. This is not observed in any case except (perhaps) for the intraEGFR experiment (Figure 5L).

Introduction

Page 3, last sentence - Four papers, including the work of Elise Peyre in Xavier Morin's lab, are cited after the statement "LGN binds to Galphai exclusively at the basolateral membrane via the activity of aPKC...." In fact, the Peyre article demonstrates that lateral distribution of LGN is independent of aPKC in chick neuroepithelial cells.

Figure 1

In Figure 1D the percent of "Normal Lumens" is set to 100% based on the number of these lumens in the control condition. A decrease of about 40% is observed in siIQGAP1 #2. There is some data missing here. What is the percentage of abnormal lumens observed in the control condition? The same issue presents itself in several other figures, making the data difficult to evaluate. (If abnormal lumens appear in the control condition, the data as presented overemphasizes differences. For example, if 40% of lumens are abnormal in the control condition in Figure 1D, the difference between control and siIQAP1 #2 shrinks from 40% to about 20%.) I suspect that this isn't really a major problem. The graphs in Figure 5J and 6C presents the data differently, in that the control is not set to 100%, but rather presented against the total number of cysts. This reveals that about 10-15% of cysts have abnormal lumens under normal conditions. The graphs in the rest of the paper should be made consistent with these.

Given that both siIQGAP1#1 and #3 decrease normal lumen formation, they should also be expected to have an effect on spindle orientation. This effect should be shown as evidence that siIQGAP1#2 is not disrupting spindle orientation through an off-target effect.

Figure 2

The title of the relevant section in the manuscript is "The localization of IQGAP1 to the basolateral membrane is necessary for correct spindle orientation." The data presented in this figure offer only very weak evidence to support this claim. Both spindle misorientation and IQGAP1 mislocalization are observed after treatment with 4nM PMA. This shows correlation but not causation.

Figure 3

How do the IQGAP mutants affect spindle orientation?

Figure 4

Figure 4C - On page 10 the authors claim that the effect of IQGAP-deltaIQm-GFP on lumen formation is "even more significant in the absence of endogenous IQGAP1." Even more significant when compared to what? Does the p value shown in this figure indicate a significant difference between IQm (bar 2) and siIQGAP1+IQm (bar 3)? This is the relevant claim but doesn't seem to be likely based on the data shown. A comparison of IQm (bar 2) to siControl (bar 1) versus the comparison between siIQGAP + IQm (bar 4) and siControl (bar 1), which may have been intended, is irrelevant. A significant difference between siIQGAP1 and siIQGAP1+IQm also appears unlikely. These comparisons need to be clarified.

Figures 4C and 4D - These data indicate that spindle orientation is not randomized in the IQm but rather becomes worse than random, with only about 10% of spindles forming perpendicular to the apicobasal axis. How is this explained? It might be expected if IQm was more abundant at the apical axis than the lateral axes, but this is not the case (shown in Figure 4B).

Figure 5

Beta-catenin staining is shown in blue in Figures 5A-5E. It would be nice to see that its localization is unaffected by experimental treatments.
In Figure 5J, the authors show that expression of the intraEGFR fusion construct reduces the number of normal lumens by about 50%. Spindle orientation appears to be randomized. These results do not correspond very well with what appears to be a really very slight apical accumulation of IQGAP1 at the apical membrane (described as "partial" in the text) in Figure 5I. These data undermine the claim being made, which is that spindle misorientation and lumen abnormalities result from IQGAP1 mislocalization.

There is an important piece of data missing from this figure. What effect does EGF treatment have on lumen formation?

Figure 6

The authors claim that LLC-PK1 cells (which are inconsistently named LLC-PK1 in the figure) demonstrate more spindle misorientation and abnormal lumen formation than MDCK cells because EGFR and IQGAP1 are mislocalized at the apical cortex due to the lack of AP1B. In these cells EGFR and IQGAP1 remain at the apical cortex after addition of AP1B (Figures 6F and 6G) but are also localized laterally. In the case of IQGAP1 this would appear to be the same level of mislocalization observed in IQm mutants (Figure 4B), which have a much more severe spindle phenotype (Figure 4E).

Upon restoration of AP1B to this cell type, spindle angles are rescued to the level of MDCK cells (Figure 6J compared to Figure 6E). This figure therefore appears to show that spindle misorientation is not strictly due to EGFR / IQGAP1 mislocalization, since the spindles are oriented normally even though EGFR and IQGAP1 are mislocalized.

Figure 7

This figure is so speculative that it is not useful. The model depends on CLASP2, which has not been investigated in this report.

Discussion

Page 16, first paragraph, last sentence - "Interestingly, this protein complex is located exclusively at the basolateral membrane in the aPKC-mediated apical exclusion of LGN (Hao et al.)." This sentence should make note of the fact that the work of Hao et al applies to MDCK cells but is not true for all cell types.

Methods

Spindle angles were measured relative to the "apicobasal axis." How was this axis determined?

Typos

Page 9, middle of page - This sentence should read, "We analyzed how expressing these constructs might affect epithelial lumen formation and, as expected, neither of the mutants restored the normal phenotype..."

"Neither" should replace "none" since only two mutants are being discussed.

Page 10, top of page - Cdc42-binding rather than Cdc42 binding

Figure 1B and 1D - The nature of the siControl should be indicated (rather than just "control."). Error bars, as in Figure 4C, are missing from these control conditions.

Figure 2E - Perpendicular rather than Perpendicula
Referee #1:

In this paper, the authors analyze the mechanisms that regulate spindle orientation during epithelial morphogenesis in MDCK cysts, and established model for lumen formation. The authors analyzed the localization of IQGAP1 and EGFR in these cells, and find that they are restricted to the basolateral domains in the 3D-MDCK cysts. They also find that, in the absence of the proper function or localization of IQGAP1 or EGFR, spindle orientation and lumen formation is perturbed. In total, their work suggests that IQGAP1 is restricted to the basolateral domains in 3D-MDCK cysts by binding to EGFR using its IQ motif to promote proper oriented cell divisions. Overall, the data are high quality, and well documented. However, ultimately I do not believe that this paper represents a substantial advance in understanding the mechanisms for spindle orientation. In the absence of a clearer picture of the mechanisms by which IQGAP1 acts to promote spindle orientation (as detailed below), this paper is not suitable for publication in EMBOJ.

1. There is an extensive literature analyzing the mechanisms of spindle orientation. However, for this paper, it is almost as if EGFR and IQGAP1 function in a vacuum where these various other factors are not considered. Ultimately, this paper needs to define the contribution of IQGAP1 to controlling spindle orientation, and this means defining its relationship to the other key players in spindle orientation. For example, the relationship between EGFR-IQGAP and Gai-LGN-NuMA complex is potentially very interesting. The authors claim that these don’t affect each other, but this is only listed as data not shown. This needs to be thoroughly investigated. It is critical to show the localization dependency between these complexes and the effect of EGF addition on LGN localization to clarify whether EGFR-IQGAP pathway is sufficient to control spindle orientation. It seems very unlikely that there is a completely independent pathway for spindle orientation, and yet that both pathways make essential and non-overlapping contributions to spindle orientation. If this is the case, it would represent an important advance. However, to prove this the authors need to establish what is occurring downstream of IQGAP1.

We thank the reviewer for his/her constructive comments on the paper. To address these concerns we have characterized the potential functional interaction between the IQGAP1-EGFR and Gai-LGN-NuMA complex in the control of spindle orientation, which is included in the new figure 8. Indeed, we found that they are functionally related. We found that whereas LGN translocates to the basolateral membrane and spindle poles during mitosis independently of IQGAP1 (Fig. 8A), in the absence of IQGAP1, NuMA was depolarized and distributed all over the cell membrane (Fig. 8B). Furthermore IQGAP1 localization was not affected by the expression of a dominant negative form of LGN (Ct-LGN), even in mitotic cells with misorientated spindles (Fig. 8C). In summary, we identified that the specific localization of NuMA to the basolateral membrane of mitotic cells depends on the presence of IQGAP1 in this domain. We have hypothesized in the discussion that IQGAP1 could promote the binding of NuMA to LGN at the cell cortex. However, since NuMA is still associated with the plasma membrane in the absence of IQGAP1, and a pool of NuMA is at the apical cortex, with LGN only present at the BL membrane, this suggests that the interaction of NuMA with the plasma membrane must require an additional, different, still uncharacterized, binding partner. In fact a recent work has demonstrated that NuMA’s anaphase membrane localization is
independent of LGN and 4.1 interactions in mitotic keratinocytes (Seldin et al 2013).

2: The authors propose that IQGAP1 would polarize astral microtubule plus ends to the basolateral domain during "mitosis" (p15, L19). Although LGN/NuMA localizes to the cell cortex during mitosis, IQGAP1 localizes to the basolateral domain during "interphase" and previous work suggests that IQGAP1 interacts with +TIPs in "interphase" cells. It is critical to analyze whether IQGAP1 controls centrosome positioning/separation in interphase before mitosis similar to what is observed in Drosophila germ stem cells, or whether it is playing additional roles during mitosis that are distinct from its interphase localization.

We have characterized centrosome positioning/separation in control cells and cells silenced for IQGAP1 (new figure Supplemental 3). Immunofluorescence staining in cysts with control or IQGAP1 silenced cells showed that IQGAP1 absence does not affect centrosome in interphase cells (Suppl. Fig. 3A). Quantification indicates that in all conditions tested, centrosomes localize next to the apical membrane (Suppl. Fig. 3B) and with the same centrosomal separation (Suppl. Fig. 3C).

Additional comments:
1: The authors show the positive regulation of IQGAP1 basolateral localization by EGFR, but the negative regulation of IQGAP localization at the apical cortex could also occur. It would strengthen their conclusions to test whether overexpressed IQGAP1 can localize to the apical domain when EGFR is saturated by IQGAP1.

We have generated cell clones with a moderated expression of IQGAP1 GFP that mimic the basolateral membrane localization of the endogenous protein (Fig. 3B). We have also obtained clones with a higher expression of IQGAP1 GFP, which appears non-polarized all over the plasma membrane and also partially diffused in the cytoplasm (below). This result suggests that IQGAP1 can localize to the apical domain when EGFR is saturated by IQGAP1. However, IQGAP1 overexpression also seems to drastically affect cell viability in our hands, and thus, we have not included this information in the article.

IQGAP1-GFP (green)
Tubulin (red)
DNA (blue)
2: In Supplemental Figure 4, the authors show that EGF treatment results in the depolarization of IQGAP1. This is an important result, but the authors also need to quantify spindle orientation under these conditions.

This is an interesting question that has been addressed before in MDCK cysts treated with HGF (Yu et al., 2003). Induction of MDCK cells forming cysts with HGF induces cells to remodel into tubes, which is associated with a 90° rotation of the mitotic spindle. We observed the same tubulogenesis induction in our experiments with EGF. We do not consider that the analysis of spindle orientation in cells treated with EGF, which is a very laborious task, would add important information to our conclusions.

3. There are some persistent wording problems throughout the text. The authors need to carefully edit the manuscript.

We have carefully revised the text for these wording problems and modified it accordingly.

4. It would be helpful to add a sentence saying that "this phenotype is rescued by expressing siRNA resistant IQGAP1 (described below)" after the Fig. 1D, E.

This sentence is included in the version of the manuscript (P6 L12)

5. In Fig. 2C and D, the authors need to show single color images for β-catenin. β-catenin appears to localize to the basal domain at 15’ after the treatment with PMT. Thus, other possibilities may exist for the spindle mis-orientation caused by PMT treatment. In addition, IQGAP1 accumulates at the apical domain in interphase, but less so in mitotic cells (Fig. 2D). Does this suggest that IQGAP1 controls centrosome positioning in interphase?

We now show single images of β-catenin. Indeed β-catenin remains normally associated with the lateral membrane during the whole PMA treatment. IQGAP1 localized in the apical domain also in dividing cells. This was just a visual effect caused by the confocal plane selected for the image. As discussed before, IQGAP1 does not control centrosome positioning/separation in MDCK cysts (comment 2).

Referee #2:

Bañón-Rodríguez et al: The control of IQGAP membrane localization by EGFR regulates mitotic spindle orientation during epithelial morphogenesis.

This is an interesting study that examines mitotic spindle orientation during cystogenesis. There are, however, two concerns that need to be addressed.

We thank the reviewer for her/his overall constructive and positive comments on the paper. As requested, we have performed new experiments to support our model.

First, the authors have an entire section dedicated to the IQ motifs and ultimately reach the conclusion that these motifs mediate the basolateral restriction of IQGAP1.
In this section the conclusions are rather reaching and insufficiently supported by the data presented. In particular, is the use of the IQGAP1-ΔIQm-GFP construct, which the authors state has both an apical and basolateral distribution. The authors do not sufficiently address how this would have any basolateral distribution if their conclusion is that the IQ motifs are responsible for basolateral sequestration. Overall, there are alternative explanations that should be examined and addressed based on the data provided.

We agree the IQ motifs are not the only domain that could link IQGAP1 to the plasma membrane. Indeed, IQGAP1 binds numerous membrane/cortical proteins. IQGAP1 has been described to interact with actin (Erickson et al., 1997), which is highly enriched in the apical cortex, through its calponin homology domain (CHD) present at the N-terminus. In addition, the RasGAP C-terminus (RGCT) domain of IQGAP1 interacts with other basolateral proteins, such as E-cadherin or β-catenin (Kuroda et al., 1998). Therefore, these interactions could mediate IQGAP1 basolateral membrane localization even in the absence of IQ motifs. However, our results have characterized a role for IQ motifs in the IQGAP1 exclusive association with the basolateral membrane. We have now better clarified this issue in the article (P11 L7-9).

Second, the authors need to show the level of EGFR-IQGAP1 association with EGF stimulation. The data presented shows very clearly the internalization of EGFR in response to stimulation, and the authors do show a shift of some IQGAP1 to the apical membrane. Given their conclusions, the absence of EGFR at the basolateral membrane should be accompanied by a concomitant loss of IQGAP1, however the images shown still have IQGAP present on the basolateral surfaces. This again relates back to the previous comment in that significant portions of the conclusions rest on the idea that EGFR is responsible for maintaining IQGAP localization however there is not a insignificant pool of IQGAP remaining even with the loss of EGFR, suggesting additional conclusions.

This is a very interesting question and we have performed new immunoprecipitation assays to analyze EGFR-IQGAP1 association in control vs. EGF treated cells (new figure Supplemental 5B). This new experiment clearly shows that the EGFR-IQGAP1 interaction is drastically decreased in the presence of EGF, which confirms our previous hypothesis (P13 L9).

As previously described in comment 1, IQGAP1 can bind to basolateral proteins such as E-cadherin or β-catenin through its C terminal domain and to actin through its CHD domain present at the N-terminus, which might explain the pool of IQGAP1 that remains at the BL membrane when the interaction with EGFR is lost.

Referee #3:

In this article, the authors describe roles for IQGAP1 and EGFR in spindle orientation and in lumen formation in MDCK cell cysts. Although the data clearly show that IQGAP affects spindle orientation, which is a very interesting result, the link between spindle orientation and lumen formation has not been rigorously proven. In addition, several important experiments are missing and some of the data could be presented
more clearly. I therefore think that this manuscript could be suitable for EMBOJ, but only after a major revision.

We thank the reviewer’s constructive comments on the paper.

Major Points:

1) Most of the conclusions presented in this article are predicated on the assumption that spindle orientation is the only cause of abnormal lumen formation and this needs to be proven more rigorously.

At what angles does spindle misorientation result in abnormal lumen formation? The control shown in Figure 1G shows that only about 60% of spindles in the control condition have spindles perpendicular to the apicobasal axis. Many “tilted” spindles, about 35% of the total, appear in the control. Do tilted spindles cause abnormal lumen formation? What is the correlation between parallel spindles and abnormal lumens?

We agree that previous classification of mitotic spindle angles in the quantifications made difficult to accurately associate spindle orientation and single lumen formation. To improve the analysis we have now represented each angle individually, and the mean angle of all mitotic-spindles analyzed. This new analysis shows that in the absence of IQGAP1 the mean angle of mitotic spindles appear randomized (≈ 45°). The detailed description of how the angle of mitotic spindles were measured is included in the material and methods section.

In Figure 1G it appears that there is a 4-5X increase in the number of parallel spindles between the control and the experimental siGAP1 condition. This corresponds to an approximately 40% decrease in normal lumens. In Figure 4E there is a much greater increase (apparently about 10X) in the number of parallel spindles between the control and experimental IQm condition. However, the percent of normal lumens decreases by the same amount - roughly 40%.

Our new quantifications, that we believe are more accurate clearly show that silencing of IQGAP1 randomize spindle orientation, which induces a decrease of 40% in single lumen formation. We found this association of random spindle orientations and a decrease of 40-50% in single lumen formation to be consistent throughout the paper.

In Figure 5J, spindle orientation in LLC-PK1 AP1B cells does not appear significantly different from MDCK cells, though this comparison is surprisingly not made directly. Despite the fact that spindle orientation is not different, normal lumen formation appears to be decreased by 20%.

Our new quantifications show that indeed the spindle orientation in LLC-PK1 AP1B cells is quite different from MDCK cells (61.80° ± 5.30 vs. 74.73° ± 3.52), which fairly correlate with the difference in single lumen formation LLC-PK1-AP1B: 65.57 ± 5.23% vs. MCDK, 82.96 ± 3.17%)
These data show that the relationship between spindle misorientation and abnormal lumen formation is not linear, suggesting that it is also not entirely causal. The claim that spindle misorientation is the only cause of abnormal lumens is therefore weak. This conclusion needs to be strengthened with more evidence to show that IQGAP knockdown has no effect on polarity or apical secretion (supplementary figure 1 is not very convincing). For example, they could use the approach that Zheng et al used when they showed that the multiple lumen phenotype caused by the knock down LGN is suppressed by inhibitors of cell division.

Following the reviewer’s comments, we have now characterized the role of IQGAP1 in apical secretion using surface biotinylation assays (new supplemental figure 2B), and junctional integrity and apical compartment exocytosis using calcium switch experiment (Suppl figure 2C). We found that IQGAP1 does not play any role in these processes. In addition, we performed the approach described in Zhen et al 2010, to characterize whether inhibitors of cell division could suppress IQGAP1 silencing effect (new figure 1G and 1H). Indeed, we observed that thymidine addition to cells silenced for IQGAP1 for two days completely recover normal lumen formation (Fig. 1G), although as expected reducing the size of the cyst, which confirm our proposed model of IQGAP1 functioning specifically in spindle orientation.

2) In the discussion (page 16) the authors suggest that the IQGAP/EGFR complex acts independently of the Galphai/LGN/NuMA complex. This is based on the unpublished observations that 1) members of these complexes do not interact (via co-IP?) and 2) LGN localizes correctly even after silencing of IQGAP. As the observations are unpublished the reader cannot evaluate them, and neither observation is sufficient to justify the claim that the complexes do not interact functionally. In fact, at the end of this paragraph (Page 17), the authors note that “[further studies will be required to demonstrate the link between these two complexes,” indicating that they believe such a link to exist. As the Galphai/LGN/NuMA complex is implicated in mitotic spindle orientation in every cell type examined to date, I think it is reasonable to expect a more thorough investigation of functional interaction between the two complexes. Epistasis experiments should be possible given that silIQGAP1 does not randomize spindle orientation.

We thank the reviewer’s 1 and 3 for their questions regarding the potential functional interaction of IQGAP1 with the Gαi/LGN/NuMA complex. Indeed we found that they are functionally related (figure 8). We found that whereas LGN translocates to the basolateral membrane and spindle poles during mitosis independently of IQGAP1 (Fig. 8A), in the absence of IQGAP1, NuMA was depolarized and distributed all over the cell membrane (Fig. 8B). Furthermore IQGAP1 localization was not affected by the expression of a dominant negative form of LGN (Ct-LGN), even in mitotic cells with misoriented spindles (Fig. 8C). In summary, we identified that the specific localization of NuMA to the basolateral membrane of mitotic cells depends on the presence of IQGAP1 in this domain. We have hypothesized in the discussion that IQGAP1 could promote the binding of
NuMA to LGN at the cell cortex. However, since NuMA is still associated with the plasma membrane in the absence of IQGAP1, and a pool of NuMA is at the apical cortex, with LGN present only at the BL membrane, this suggests that the interaction of NuMA with the plasma membrane must require a different, still uncharacterized, binding partner. In fact a recent work has demonstrated that NuMA’s anaphase membrane localization is independent of LGN and 4.1 interactions in mitotic keratinocytes (Seldin et al 2013).

Minor Points

Abstract

The claim that experimental conditions randomize spindle angles is made multiple times in the manuscript, beginning in the abstract. This is usually not supported by the data. Randomization would produce a distribution whereby each class of spindle angle - parallel, perpendicular, and tilted - accounts for one-third of the angles measured. This is not observed in any case except (perhaps) for the intraEGFR experiment (Figure 5L).

In the revised version of the manuscript, we have improved the analysis of mitotic spindle angles by representing each angle individually, and the mean angle of all spindles analyzed per condition. This new analysis shows that the mean angle of the mitotic spindles is randomized (≈ 45°) in the absence of IQGAP1.

Introduction

Page 3, last sentence - Four papers, including the work of Elise Peyre in Xavier Morin’s lab, are cited after the statement “LGN binds to Galphai exclusively at the basolateral membrane via the activity of aPKC...” In fact, the Peyre article demonstrates that lateral distribution of LGN is independent of aPKC in chick neuroepithelial cells.

We have eliminated this reference following the reviewer’s suggestion

Figure 1

In Figure 1D the percent of “Normal Lumens” is set to 100% based on the number of these lumens in the control condition. A decrease of about 40% is observed in siIQGAP1 #2. There is some data missing here. What is the percentage of abnormal lumens observed in the control condition? The same issue presents itself in several other figures, making the data difficult to evaluate. (If abnormal lumens appear in the control condition, the data as presented overemphasizes differences. For example, if 40% of lumens are abnormal in the control condition in Figure 1D, the difference between control and siIQAP1 #2 shrinks from 40% to about 20%.) I suspect that this isn’t really a major problem. The graphs in Figure 5J and 6C presents the data differently, in that the control is not set to 100%, but rather presented against the total number of cysts. This reveals that about 10-15% of cysts have abnormal lumens under normal conditions. The graphs in the rest of the paper should be made consistent with these.
We thank the reviewer for this comment. We have already changed the graphs to make the quantifications more consistent, and control data is now set to total numbers of cyst in all the figures of the paper.

Given that both siIQGAP1#1 and #3 decrease normal lumen formation, they should also be expected to have an effect on spindle orientation. This effect should be shown as evidence that siIQGAP1#2 is not disrupting spindle orientation through an off-target effect.

We have characterized spindle orientation using siIQGAP1#1 and #3 (new figure Supplemental 1). Quantification of mitotic spindle angles indicates that in all cases mitotic spindles were randomized. This data corroborates the results obtained for siIQGAP1#2.

Figure 2

The title of the relevant section in the manuscript is "The localization of IQGAP1 to the basolateral membrane is necessary for correct spindle orientation." The data presented in this figure offer only very weak evidence to support this claim. Both spindle misorientation and IQGAP1 mislocalization are observed after treatment with 4nM PMA. This shows correlation but not causation.

Following reviewer's comment we have now changed the title to "Depolarization of IQGAP1 induces randomized mitotic spindle orientation.

Figure 3

How do the IQGAP mutants affect spindle orientation?

We have performed this analysis following reviewer’s comment, and mitotic spindle angles were measured and represented in new figure 3F.

Figure 4

Figure 4C - On page 10 the authors claim that the effect of IQGAP-deltaIQm-GFP on lumen formation is "even more significant in the absence of endogenous IQGAP1."

Even more significant when compared to what? Does the p value shown in this figure indicate a significant difference between IQm (bar 2) and siIQGAP1+IQm (bar 3)? This is the relevant claim but doesn’t seem to be likely based on the data shown. A comparison of IQm (bar 2) to siControl (bar 1) versus the comparison between siIQGAP + IQm (bar 4) and siControl (bar 1), which may have been intended, is irrelevant. A significant difference between siIQGAP1 and siIQGAP1+IQm also appears unlikely. These comparisons need to be clarified.

Following reviewer’s comment, we have introduce the statistic in the figure to better clarify the comparison between cells expressing IQGAP1-dIQm GFP vs cells expressing this construct and depleted of endogenous IQGAP1.
Figures 4C and 4D - These data indicate that spindle orientation is not randomized in the IQm but rather becomes worse than random, with only about 10% of spindles forming perpendicular to the apicobasal axis. How is this explained? It might be expected if IQm was more abundant at the apical axis than the lateral axes, but this is not the case (shown in Figure 4B).

We have performed new quantifications to improve this analysis, as described before, and we show that cells expressing IQGAP1-ΔIQm GFP present randomized mitotic spindle angles (Mean angle: IQGAP1-ΔIQm = 43.09° ± 4.40).

Figure 5

Beta-catenin staining is shown in blue in Figures 5A-5E. It would be nice to see that its localization is unaffected by experimental treatments.

We have now changed figures 5A, 5C and 5D to show that neither PMA nor EGF treatments modifies β-catenin basolateral localization.

In Figure 5J, the authors show that expression of the intraEGFR fusion construct reduces the number of normal lumens by about 50%. Spindle orientation appears to be randomized. These results do not correspond very well with what appears to be a really very slight apical accumulation of IQGAP1 at the apical membrane (described as "partial" in the text) in Figure 5I. These data undermine the claim being made, which is that spindle misorientation and lumen abnormalities result from IQGAP1 mislocalization.

We agree with the reviewer that the levels of IQGAP1 at the apical membrane are not very high. However, this partial translocation of IQGAP1 seems to be sufficient for mitotic spindles to orient randomly. Similar effects are observed after 5 min treatment of PMA where most IQGAP1 maintains its basolateral localization and there is just a partial translocation of the protein to the apical membrane. Also in this case mitotic spindles orientate randomly. These results consistently suggest that just a slight translocation of IQGAP1 to the apical membrane is sufficient to misorientate mitotic spindles.

There is an important piece of data missing from this figure. What effect does EGF treatment have on lumen formation?

We agree with reviewer’s comment and we have performed new experiments in this direction represented in new figure Supplemental 5C. Treatment of MDCK cysts with EGF for 24 hours disrupts cyst morphology inducing the development of multiple small lumens surrounding the preformed central lumen and the accumulation of luminal cell clusters (Single lumens: Control=75.09% ± 1.41 vs EGF treated=55.44% ± 5.21).

Figure 6

The authors claim that LLC-PK1 cells (which are inconsistently named LLCPK1 in the figure) demonstrate more spindle misorientation and abnormal lumen formation.
than MDCK cells because EGFR and IQGAP1 are mislocalized at the apical cortex due to the lack of AP1B. In these cells EGFR and IQGAP1 remain at the apical cortex after addition of AP1B (Figures 6F and 6G) but are also localized laterally. In the case of IQGAP1 this would appear to be the same level of mislocalization observed in IQm mutants (Figure 4B), which have a much more severe spindle phenotype (Figure 4E).

We regret our mistake and we have corrected in the revised version of the manuscript the nomenclature of LLC-PK1 cells. New graphs representing mitotic spindle angles show that in both cases spindles are randomly oriented (Mean angles: LLC-PK1 = 45.37° ± 5.07 vs IQGAP1-IQm = 43.09° ± 4.40).

Upon restoration of AP1B to this cell type, spindle angles are rescued to the level of MDCK cells (Figure 6I compared to Figure 6E). This figure therefore appears to show that spindle misorientation is not strictly due to EGFR/IQGAP1 mislocalization, since the spindles are oriented normally even though EGFR and IQGAP1 are mislocalized.

Although AP1B expression in LLC-PK1 partially restores the phenotype, the efficiency of LLC-PK1 AP1B cells forming single lumens is not as high as MDCK cells. This is now clearer represented with the measure of individualize mitotic spindle angles. Mean angle: LLC-PK1 AP1B = 61.80° ± 5.30 vs. MDCK = 74.73° ± 3.52. These angles fairly correlate with the difference in single lumen formation LLC-PK1-AP1B: 65.57 ± 5.23% vs. MDCK, 82.96 ± 3.17%.

Figure 7

This figure is so speculative that it is not useful. The model depends on CLASP2, which has not been investigated in this report.

We agree with the reviewer and we have changed the model following the reviewer´s comment.

Discussion

Page 16, first paragraph, last sentence - "Interestingly, this protein complex is located exclusively at the basolateral membrane in the aPKC-mediated apical exclusion of LGN (Hao et al)." This sentence should make note of the fact that the work of Hao et al applies to MDCK cells but is not true for all cell types.

We agree and have modified this sentence following reviewer’s suggestion

Methods

Spindle angles were measured relative to the "apicobasal axis." How was this axis determined?

We have included a section in methods titled “measurement of spindle angles” explaining the procedure for measuring the angle of mitotic spindles.

Typos
Page 9, middle of page - This sentence should read, "We analyzed how expressing these constructs might affect epithelial lumen formation and, as expected, neither of the mutants restored the normal phenotype... "Neither" should replace "none" since only two mutants are being discussed.

We regret this mistake, which is now corrected in the new version of the manuscript.

Page 10, top of page - Cdc42-binding rather than Cdc42 bindin

We regret this typo, which is now corrected in the new version of the manuscript.

Figure 1B and 1D - The nature of the siControl should be indicated (rather than just "control.") Error bars, as in Figure 4C, are missing from these control conditions.

This mistake is now corrected in the new version of the manuscript. We have included the error bars in figure 4C.

Figure 2E - Perpendicular rather than Perpendicula

Again, this typo is now corrected in the new version of the manuscript.
Thank you for the submission of your revised manuscript to The EMBO Journal and please accept my apologies for the delay in our response. Your study was sent back to former referees #1 and #3, who after careful consideration now believe that all major concerns have been properly addressed and your manuscript is almost ready for publication. That being said, a few minor issues that still require your attention, mostly related to the presentation of your data and the discussion of recently published reports, have been however pointed out by referee #3. Once these minor concerns have been addressed, I will be glad to accept your manuscript for publication in The EMBO Journal.

Every paper now includes a 'Synopsis' to further enhance their discoverability. Synopses are displayed on the html and they are freely accessible to all readers. The synopsis includes an image, normally cropped by us from one of the final figures of the manuscript, as well as 2-5 one-sentence bullet points that summarize the article and should be complementary to the abstract - i.e. not repeat the same text. Could I ask you to provide the bullet points as a separate word file as part of your final manuscript?

I would also like to mention that we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels presented? The PDF files should be labeled with the appropriate figure/panel number and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files.

Do not hesitate to contact me in case you have any questions.

Thank you very much again for your patience. I am looking forward to seeing the final version of your manuscript.

REFEREE REPORTS:

Referee #1 (Report):

In this revised paper, the authors have made several experimental additions and have edited the text and figures. The combination of these changes has addressed the majority of comments from my initial review. As such, I find this paper suitable for publication.

Referee #3 (Report):

The authors have put a great deal of work into revising the manuscript. It is now appropriate for publication in EMBOJ provided that the following three concerns, which are small but important, are addressed.

1) The first major point made during the earlier evaluation, namely that spindle misorientation does not correlate well with abnormal lumen formation, has not been fully addressed. The authors state in their rebuttal that "To improve the analysis we have now represented each angle individually, and the mean of all the mitotic spindles analyzed. This new analysis shows that in the absence of IQGAP the mean angle of mitotic spindles appears randomized (45\degree)". This is somewhat misleading. A mean angle of 45 degrees is not sufficient to demonstrate spindle angle randomization. The relevant statistic is not the average of the distribution but rather its slope. A mean of 45 degrees would occur if every spindle angle measured were at 45 degrees, which would hardly be random. A mean of 45 degrees would also occur if the distribution of spindle angles was simply biphasic, as it appears in Figure 1F and was apparent in the first submission. A claim about the effect of spindle randomization on lumen formation is thus not supported by the data as it is presented.

Other labs now present spindle angles in cumulative data plots. These are easier to evaluate and
perhaps more informative.

2) The authors show that PMA treatment affects both spindle orientation and IQGAP localization. They do not show that the spindle-misorienting effect of PMA treatment relies on IQGAP mislocalization. Thus the title of their second section, "Depolarization of IQGAP randomizes spindle orientation," is still incorrect.

3) "Gi localizes to the plasma membrane and recruits LGN to this region. Although Gi is distributed throughout the cell membrane in polarized tissues, LGN binds to Gi 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. In turn, Gi-LGN binds to NuMA, thereby anchoring the astral microtubules to the basolateral membrane (Blumer et al., 2006; Morin et al., 2007; Peyre et al., 2011; Zheng et al., 2010)."

In the first evaluation, I noted that the work of Elise Peyre is incorrectly cited here. As I wrote, her work indicates that aPKC is dispensable for spindle orientation in chick neuroepithelium. In their rebuttal letter the authors claim to have addressed this discrepancy in their revised manuscript by removing the citation. It appears that they have not. In the meantime, another article has been published (Bergstralh et al, 2013) which demonstrates that aPKC is dispensable for spindle orientation in at least one type of Drosophila epithelia. Rather than simply removing the citation, the work of Peyre (and now Bergstralh) should be addressed.

2nd Revision - authors' response 11 November 2013

Referee #1 (Report):

In this revised paper, the authors have made several experimental additions and have edited the text and figures. The combination of these changes has addressed the majority of comments from my initial review. As such, I find this paper suitable for publication.

We thank the reviewer for his positive evaluation of the manuscript.

Referee #3 (Report):

The authors have put a great deal of work into revising the manuscript. It is now appropriate for publication in EMBOJ provided that the following three concerns, which are small but important, are addressed.

1) The first major point made during the earlier evaluation, namely that spindle misorientation does not correlate well with abnormal lumen formation, has not been fully addressed. The authors state in their rebuttal that "To improve the analysis we have now represented each angle individually, and the mean of all the mitotic spindles analysed. This new analysis shows that in the absence of IQGAP the mean angle of mitotic spindles appears randomized (≅ 45[degree sign])." This is somewhat misleading. A mean angle of 45 degrees is not sufficient to demonstrate spindle angle randomization. The relevant statistic is not the average of the distribution but rather its slope. A mean of 45 degrees would occur if every spindle angle measured were at 45 degrees, which would hardly be random. A mean of 45 degrees would also occur if the distribution of spindle angles was simply biphasic, as it appears in Figure 1F and was apparent in the first submission. A claim about the effect of spindle randomization on lumen formation is thus not supported by the data as it is presented.

Other labs now present spindle angles in cumulative data plots. These are easier to evaluate and perhaps more informative.

With all due respect we do not agree with the reviewer in this point. We have demonstrated that spindle misorientation correlate with abnormal lumen formation. There are different possibilities to represent spindle angles and we believe that show each angle individually is easier to evaluate and
more informative than cumulative data plots. Furthermore different recent studies have used this methodology to show defects in spindle orientation and lumen formation. I agree that a mean angle of 45 degrees is not sufficient to demonstrate spindle angle randomization, but since this is not essential to the main message of the article, and that it would take further new experiments to make these statistical evaluations, we have just eliminated that our results demonstrate “that in the absence of IQGAP the mean angle of mitotic spindles appears randomized”, with that “in the absence of IQGAP the mean angle of mitotic spindles are misoriented and they seem to be randomized (which is what suggests the distribution of every single angle)

2) The authors show that PMA treatment affects both spindle orientation and IQGAP localization. They do not show that the spindle-misorienting effect of PMA treatment relies on IQGAP mislocalization. Thus the title of their second section, "Depolarization of IQGAP randomizes spindle orientation," is still incorrect.

We agree and we have modified this title.

3) "Gai localizes to the plasma membrane and recruits LGN to this region. Although Gai is distributed throughout the cell membrane in polarized tissues, LGN binds to Gai 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. In turn, Gai-LGN binds to NuMA, thereby anchoring the astral microtubules to the basolateral membrane (Blumer et al., 2006; Morin et al., 2007; Peyre et al., 2011; Zheng et al., 2010)."

In the first evaluation, I noted that the work of Elise Peyre is incorrectly cited here. As I wrote, her work indicates that aPKC is dispensable for spindle orientation in chick neuroepithelium. In their rebuttal letter the authors claim to have addressed this discrepancy in their revised manuscript by removing the citation. It appears that they have not. In the meantime, another article has been published (Bergstralh et al, 2013) which demonstrates that aPKC is dispensable for spindle orientation in at least one type of Drosophila epithelia. Rather than simply removing the citation, the work of Peyre (and now Bergstralh) should be addressed.

In the new version, we properly address the work of Peyre and Bergstralh.