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## **A combination of Wnt and growth factor signaling induces Arl4c expression to form epithelial tubular structures**

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### **Review timeline:**

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### **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: David Del Alamo*

1st Editorial Decision

16 October 2013

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Thank you for the submission of your manuscript entitled "Wnt and growth factor signaling expresses Arl4c co-operatively to form epithelial tubular structures" to The EMBO Journal. We have just now received the full set of reports from the referees, which I copy below. As both referees agree on the high interest of your manuscript and their comments are in general positive, I would like to invite you to revise it.

Without going into details that you will find below rather explicitly, all referees consider that your manuscript should be published in The EMBO Journal provided that a number of points -technical concerns for the most part- are addressed in order to improve your message. I would like to draw your attention in particular to the referees' major points. Be aware that other, secondary points are also raised and, although your revised manuscript should address the referee concerns as completely as possible, they will not be determinant for acceptance.

On a side note, and also mentioned by referee #1, we believe that your manuscript could substantially benefit from some editing and polishing of your writing, as your message becomes confusing at times. Just as an example, the title of the manuscript itself is not entirely clear.

Please be aware that it is 'The EMBO Journal' policy to allow a single round of revision only and

that, therefore, acceptance of the manuscript will essentially depend on the completeness of your responses included in the next version of the manuscript. Do not hesitate to contact me by e-mail or on the phone in case you have any questions, you need further input or you anticipate any problem during the revision process.

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.

#### REFEFREE REPORTS:

##### Referee #1:

The paper presented by Matsumoto and coworkers shows that co-stimulation with Wnt3a and epidermal growth factor (Wnt3a/EGF) induced the formation of tubular structures in a three-dimensional culture of intestinal epithelial cells through the expression of an Arf-like small GTP-binding protein (Arl4c). Then, they characterized the transcriptional regulation of Arl4c, which depend on the cooperation of the growth factor-MAP kinase (MAPK) pathway with, Wnt3a- -catenin pathway. The authors also show the signaling pathways downstream of the co-stimulation with Wnt3a/EGF, which resulted in the rearrangement of the cytoskeleton through the activation of Rac and inactivation of Rho; nuclear translocation of YAP/TAZ in leading cells of the tubular structures that promoted cell growth, and the branching morphogenesis of embryonic kidney ureteric buds.

Altogether the proposed mechanistic pathways seem to be correct, the experimental design is accurate and clear, and most of the data satisfactorily validated. I believe that this paper represents a substantial advance in understanding the mechanisms for epithelial tubulogenesis downstream of Wnt3a/EGF signaling both *in vitro* and *in vivo*. However, there are some experiments that will contribute to better elucidate this mechanism.

##### Major Concerns:

1- Fig. 7C: the authors describe that treatment of ureteric bud with IWP2, U0126, IWP2/U0126, and FIIN-1 inhibits branching tubulogenesis. However, the cells at the tip of these UBs look very unhealthy. It is critical to analyze whether this treatments are affecting ureteric bud cells viability for example by performing immunofluorescence analysis of these cells with apoptotic markers like Caspase3. Similarly, treatments with SecinH3 or NSC23766 (figure 3G and 5B) seem to induce apoptosis (apoptotic nucleus), are they affecting cell viability? Are these drugs affecting also cell proliferation and cell polarity?

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

There are also many mistakes that should be addressed. For instance, in page 6 the authors state, "Wnt3a/EGF-dependent expression of Arl4c mRNA was suppressed by knockdown of -catenin (Figure. 2A; Supplementary Figure S3A)". However Arl4c mRNA levels were reduced, not suppressed in this experiment.

In page 9, first paragraph "Knockdown of Arl4c rescued Wnt3a/EGF-dependent suppression of RhoA activity (Figure 3D)". This sentence is just not correct. The authors should revise this sentence.

Additional comments:

- 1- In order to get more insights in the molecular mechanism regulated by Arl4c, it would be also interesting to see the localization of Arl4c in these cells at subcellular level. They could use the Anti-Arl4c antibody to detect the endogenous protein, or alternatively, since they are using a Arl4c-GFP cell line, they could show the GFP staining together with other markers such as polarity markers, junctions, etc.
- 2-The authors use a protocol for induction of tubulogenesis, both in IEC6 and MDCK cells, with Wnt3a and EGF added to the solid matrigel at the same time that the cells are plated to form 3D cultures, which is different from the protocol used before by other authors working in 3D organoid tubulogenesis (Wei et al, 2005). Do these cells respond to Wnt3a/EGF once the cells have already formed 3D organoids in matrigel, which represent a more physiological mechanism for tubulogenesis?
- 3-Fig. 1F: The authors should include a separated panel with Arl4c-GFP expression in the picture to see the percentage of cells that are positive for Arl4c-GFP
- 4-Fig. 3B (left blot): The levels of activated Rac1 in this western blot does not seem to correspond with the results showed in the graph, is this experiment representative? In general the levels of total Rac1 does not seem to be well adjusted in figure 3, panels A and B
- 5-Fig. 5A: Arl4c siRNA also seem to affect cell viability since nucleus staining shows abnormal nucleus. Is the absence of Arl4c affecting cell viability?
- 6-Fig. 5B: In this panel the authors correlate the overexpression of Arl4c with an activation of YAP/TAZ. To further validate this correlation, they should also co-stain YAP/TAZ with Arl4c.
- 7-In figure 7D the authors should include magnifications of the kidney tubules of all different treatments, not just the control. It is difficult to get any conclusions from the images as they are currently being presented.

Referee #2:

In this manuscript the authors report that extracellular ligands such as Wnt 3a, EGF or HGF, work in concert to induce the expression of Arl4c to promote epithelial tubule formation.

Specific points.

1. Figure S1 indicates that there are significant morphological changes with Wnt5a treatment (lack of a lumen) and Wnt5a/EGF treatment (overgrowth, potential lumen abnormalities). Is this a conical/non-canonical Wnt phenomenon?
2. Based on reported studies it is possible that the cysts are too immature to respond to singular signals such as HGF. This needs to be addressed.
3. The selection of Arl4c needs to be better explained. If the author's contention is that the individual treatments do not cause phenotypic changes then the comparison of microarray data should have been changes between the individual treatments and the dual treatment at all time points rather than just the control. At a minimum this data should be appended to Table S1.
4. Does Arl4c siRNA suppress the morphological changes in 3D culture? The cyst image shows indications of branching and bears little to no resemblance to the control.
5. Does beta-catenin interact with Ets/Tcf4 complex when the cells are solely treated with Wnt3a? Is this bound to a TCF binding region?
6. What is the level of Arf6 activation with Wnt3a, Wnt5a or EGF treatment in IEC6 cysts?

8. There are several reports documenting the effect of the Wnts, HGF and EGF on Arf6 and Rac1-mediated signaling during epithelial morphogenesis, matrix invasion and even in epithelial tubule formation. These findings should be discussed and put into context on the current study.

9. The data is documented with one cell line only.

1st Revision - authors' response

22 November 2013

Referee #1:

Major Concerns:

*1- Fig. 7C: the authors describe that treatment of ureteric bud with IWP2, U0126, IWP2/U0126, and FIIN-1 inhibits branching tubulogenesis. However, the cells at the tip of these UBs look very unhealthy. It is critical to analyse whether this treatments are affecting ureteric bud cells viability for example by performing immunofluorescence analysis of these cells with apoptotic markers like Caspase3. Similarly, treatments with SecinH3 or NSC23766 (figure 3G and 5B) seem to induce apoptosis (apoptotic nucleus), are they affecting cell viability? Are these drugs affecting also cell proliferation and cell polarity?*

Apoptosis has been implicated in luminal formation of salivary or mammary glands and MCF-10A mammary epithelial cells. According to the referee's comment, kidney rudiments were stained with anti-active (cleaved) caspase-3 antibody. Apoptotic cells were observed in the interior of ureteric ducts and the mesenchyme region, but only a few cells died at ureteric bud (UB) tips. Treatment with IWP2, U0126, IWP2/U0126, and FIIN-1 did not increase apoptotic cells at UB tips. Therefore, these inhibitors do not affect cell viability of UB epithelial cells. The results are shown in Supplementary Figure S10D and described in the text (page 14, the bottom through page 15, line 3)

We also stained cysts and tubes consisting of IEC6 cells with anti-active caspase-3 antibody. Apoptotic cells were observed in the luminal space of IEC6 cysts and in the interior of developing tubes induced by Wnt3a/EGF. These nuclei were abnormally shaped. However, IEC6 cells forming cysts and tubes were active caspase-3 negative, with intact nuclei. Active caspase-3-positive cells with abnormal nuclei were also observed in the luminal space of tubes treated with SecinH3 and NSC23766, but apoptotic cells did not increase by the treatment, suggesting that the inhibitors do not affect viability of IEC6 cells forming cysts and tubes. The results are shown in Supplementary Figure S4E and G and described in the text (page 7, lines 2 through 7; page 10, lines 5 through 9 from the bottom).

*2-There are some persistent wording problems throughout the text. The authors need to carefully edit the manuscript. There are also many mistakes that should be addressed. For instance, in page 6 the authors state, "Wnt3a/EGF-dependent expression of Arl4c mRNA was suppressed by knockdown of  $\beta$ -catenin (Figure. 2A; Supplementary Figure S3A)". However Arl4c mRNA levels were reduced, not suppressed in this experiment.*

*In page 9, first paragraph "Knockdown of Arl4c rescued Wnt3a/EGF-dependent suppression of RhoA activity (Figure 3D)". This sentence is just not correct. The authors should revise this sentence.*

We appreciate the referee's suggestion and asked native English-speaking experts of "BioMed Proofreading" to edit our manuscript. We also revised the sentences that the referee pointed out.

Additional comments:

*1- In order to get more insights in the molecular mechanism regulated by Arl4c, it would be also interesting to see the localization of Arl4c in these cells at subcellular level. They could use the Anti-Arl4c antibody to detect the endogenous protein, or alternatively, since they are using a Arl4c-GFP cell line, they could show the GFP staining together with other markers such as polarity markers, junctions, etc.*

As already shown in Supplementary Figure S3C and S6A, endogenous and ectopically expressed Arl4c were clearly localized to the leading edge of elongated IEC6 cells on 2D Matrigel. Arl4c was colocalized with F-actin, suggesting that Arl4c is involved in the regulation of the actin dynamics. The statements are described in the text (page 6, lines 12 through 14; page 9, lines 8 and 9).

However, in 3D Matrigel culture endogenous Arl4c was not detected in IEC6 cells, and ectopically expressed Arl4c-GFP was diffusely distributed throughout the cytosol and the plasma membrane as shown in Supplementary Figure S4C, probably due to antibody sensitivity and resolution of our microscopy. Since Arl4c is modified with myristic acid and located to the plasma membrane, its intracellular localization in 3D culture would be the same as 2D culture.

*2-The authors use a protocol for induction of tubulogenesis, both in IEC6 and MDCK cells, with Wnt3a and EGF added to the solid matrigel at the same time that the cells are plated to form 3D cultures, which is different from the protocol used before by other authors working in 3D organoid tubulogenesis (Wei et al, 2005). Do these cells respond to Wnt3a/EGF once the cells have already formed 3D organoids in matrigel, which represent a more physiological mechanism for tubulogenesis?*

We appreciate the referee's suggestion. According to the referee's comment, once IEC6 cells formed cysts in 3D Matrigel culture, they were treated with Wnt3a/EGF. Under the condition, IEC6 cells also developed tubes, however, the efficiency was lower compared with our original protocol. We speculated the reason as follows. Wnt3a is an insoluble protein and readily attached to extracellular matrix components, including proteins and glycans, through lipid and glycan modifications. Therefore, Wnt3a may not be easily accessible to IEC6 cysts embedded in 3D Matrigel. In order to induce tube formation efficiently we added Wnt3a and EGF at the same time that the cells were plated on the Matrigel. The results are shown in Supplementary Figure S2 and described in the text (page 5, lines 6 through 14).

*3-Fig. 1F: The authors should include a separated panel with Arl4c-GFP expression in the picture the to see the percentage of cells that are positive for Arl4c-GFP.*

We generated IEC6 cells expressing Arl4c-GFP using lentiviruses. The percentage of cells that express Arl4c was approximately 80%. The results are shown in Supplementary Figure S4C and described in its legend.

*4-Fig. 3B (left blot): The levels of activated Rac1 in this western blot does not seem to correspond with the results showed in the graph, is this experiment representative? In general the levels of total Rac1 does not seem to be well adjusted in figure 3, panels A and B*

According to the referee's comment, we repeated the Rac assay in Figure 3A and B. As shown in new Figure 3B, Arl4c-GFP clearly activated Rac. In addition, the levels of total Rac1 were normalized.

*5-Fig. 5A: Arl4c siRNA also seem to affect cell viability since nucleus staining shows abnormal nucleus. Is the absence of Arl4c affecting cell viability?*

As responded to major concerns 1, active caspase-3-positive cells were observed in the luminal space of IEC6 cysts and in the interior of developing tubes induced by Wnt3a/EGF, and these nuclei were abnormally shaped. However, IEC6 cells forming cysts and tubes were active caspase-3 negative, with intact nuclei. Knockdown of Arl4c did not increase apoptotic cells in the luminal space. Therefore, depletion of Arl4c did not affect cell viability. These results were shown in Supplementary Figure S4E and F and described in the text (page 7, lines 8 through 10).

*6-Fig. 5B: In this panel the authors correlate the overexpression of Arl4c with an activation of YAP/TAZ. To further validate this correlation, they should also co-stain YAP/TAZ with Arl4c.*

As responded to additional comments 3, approximately 80% of cells in this cell line expressed Arl4c-GFP although expression levels were variable in different cells. YAP/TAZ was observed in the nucleus of morphologically extended IEC6 cells expressing Arl4c-GFP in the presence of EGF. We assume that EGF could act on IEC6 cells in which Arl4c is expressed properly, thereby causing

cells to form tubes. The results are shown in Supplementary Figure S8E and described in the text (page 12, lines 11 through 13 from the bottom).

*7-In figure 7D the authors should include magnifications of the kidney tubules of all different treatments, not just the control. It is difficult to get any conclusions from the images as they are currently being presented.*

According to the referee's comments, magnifications were added to each panels treated with different reagents. In addition, we repeated the experiments and presented more clear images so that effects of various reagents on bud formation from kidney organoids are readily understood. The results are shown in Figure 7D.

Referee #2:

Specific points.

*1. Figure S1 indicates that there are significant morphological changes with Wnt5a treatment (lack of a lumen) and Wnt5a/EGF treatment (overgrowth, potential lumen abnormalities). Is this a conical/non-canonical Wnt phenomenon?*

Although the referee commented that Wnt5a or Wnt5a/EGF induce significant morphological changes, the treatment did not affect morphology of IEC6 cysts with clear apical lumen except that Wnt5a/EGF enlarged cysts. Recently we showed that IEC6 cells express high levels of Wnt5a and that knockdown of Wnt5a leads to loss of lumen integrity with fragmented apical and disrupted basolateral structures (Gon, H., Mol. Biol. Cell, in press). Given that endogenous Wnt5a is sufficient to regulate the functions of IEC6 cells, the cells may not respond to exogenous Wnt5a. The statements are described in the text (page 4, line 11 from the bottom and lines 3 through 7 from the bottom).

*2. Based on reported studies it is possible that the cysts are too immature to respond to singular signals such as HGF. This needs to be addressed.*

This comment is related with the referee #1's additional comment 2. After IEC6 cells formed cysts in 3D Matrigel culture, they were treated with EGF or HGF. Under the condition, IEC6 cells did not respond to the single stimulation, either. However, the cells induced tube formation in response to Wnt3a/EGF, although efficiency was lower compared with simultaneous stimulation with these growth factors at the same time cells were plated on Matrigel. Therefore, the reason why IEC6 cysts do not respond to single stimulation is not due to the maturation state of IEC6 cysts. The results were shown in Supplementary Figures S2 and described in the text (page 5, lines 6 through 14).

*3. The selection of Arl4c needs to be better explained. If the author's contention is that the individual treatments do not cause phenotypic changes then the comparison of microarray data should have been changes between the individual treatments and the dual treatment at all time points rather than just the control. At a minimum this data should be appended to Table S1.*

DNA microarray analyses were performed at 4 and 24 h after stimulation with Wnt3a, EGF, or Wnt3a/EGF in 3D culture. Arl4c was selected based on the criterion that expression levels were higher in cells treated by Wnt3a/EGF than in cells treated by Wnt3a or EGF at both time points. The detailed results were shown in Supplementary Table S1 and described in the text (page 6, lines 7 through 9).

*4. Does Arl4c siRNA suppress the morphological changes in 3D culture? The cyst image shows indications of branching and bears little to no resemblance to the control.*

Knockdown of Arl4c did not affect the morphology of IEC6 cysts. However, it inhibited the elongation and branching of tubes induced by Wnt3a/EGF. Among the four processes of tube formation, "extension" was significantly inhibited. This could reflect the ability of Arl4c to regulate

the actin cytoskeleton at initiation of tubulogenesis. The results are shown in Figure 1F and Supplementary Figure S4A and described in the text (page 6, lines 5 through 8 from the bottom).

*5. Does beta-catenin interact with Ets/Tcf4 complex when the cells are solely treated with Wnt3a? Is this bound to a TCF binding region?*

When the cells were treated with CHIR99021 alone to activate the b-catenin pathway, the association of Tcf4 with Ets1 was enhanced, and b-catenin was also observed in the complex. When DN-Tcf4, which lacks the b-catenin-binding site, was expressed instead of wild-type Tcf4, DN-Tcf4 and b-catenin were not associated with Ets1, suggesting that the b-catenin/Tcf4 complex is able to interact with Ets1 and that Tcf4 binds to b-catenin and Ets1 through different sites. These results suggest that the EGF-MAPK pathway induces the interaction of the b-catenin/Tcf4 complex with Ets1 and the Wnt3a-b-catenin pathway enhances it. The results are shown in Figure 2G and Supplementary Figure S5G and described in the text (page 8, lines 6 through 13 from the bottom).

*6. What is the level of Arf6 activation with Wnt3a, Wnt5a or EGF treatment in IEC6 cysts?*

Using a GST-GGA3 PBD pull-down assay, Wnt3a, EGF, or Wnt3a/EGF-dependent activation Arf6 was examined. Wnt3a/EGF activated Arf6 compared with either Wnt3a or EGF. The results are shown in Supplementary Figure S6B and described in the text (page 9, lines 7 and 8 from the bottom).

*8. There are several reports documenting the effect of the Wnts, HGF and EGF on Arf6 and Rac1-mediated signalling during epithelial morphogenesis, matrix invasion and even in epithelial tubule formation. These findings should be discussed and put into context on the current study.*

As pointed out by the referee, HGF regulates Arf6 and Rac1 signalling during tube formation of MDCK cells, and treatment with HGF and Y27632 increases the length and number of extensions in type I collagen gel. However, in 3D Matrigel HGF did not affect cyst morphology of MDCK cells but developed tube formation when Arl4c was expressed. These results suggest that Matrigel renders cells immotile and less-proliferative compared with type I collagen and that Arl4c expression relieves prevention of tube formation by Matrigel. We showed that the combination of HGF and Y27632 or blebbistatin induces wild-type MDCK cell tube formation and that SecinH3 inhibits HGF-induced tube development of MDCK/Arl4c cells, indicating that the combination of cytoskeletal rearrangement and HGF signalling is necessary for tubulogenesis of MDCK cells in the presence of Matrigel. These results support a common mechanism of tube formation in IEC6 and MDCK cells.

Although it has been shown that cell division is required for tube formation, how epithelial morphological changes influence cell growth is not known. We showed here that Wnt3a/EGF-induced morphological changes leads to cellular proliferation through the nuclear localization of YAP/TAZ during tube formation.

In addition, Wnt signalling is known to control tubulogenesis in several organs including the kidney. The b-catenin-dependent pathway regulates branching and maintains ureteric bud (UB) cells in an undifferentiated state. However, the Wnt ligand that mediate the b-catenin-dependent pathway remains to be elucidated. We developed a new *in vitro* culture system of UBs in the presence of R-spondin1, GDNF, and FGF1 without conditioned medium. Because R-spondin1 enhances Wnt-dependent b-catenin signalling, an Wnt ligand expressed by UB could act in an autocrine manner. Furthermore, we suggested that the b-catenin-dependent pathway induces Arl4c expression, which is involved in branching morphogenesis of UBs. These statements are described in the text (page 16, line 9 from the bottom through page 17, line 2; page 17, lines 9 through 13 from the bottom and lines 4 through 6 from the bottom; page18, lines 4 through 9).

*9. The data is documented with one cell line only.*

HGF did not induce tube formation of MDCK cells in 3D Matrigel culture. By stably expressing Arl4c, MDCK cells could respond to HGF and developed tubes. A low concentrations of Y27632 or blebbistatin induced tube formation of MDCK cells in the presence of HGF, and SecinH3 inhibited HGF-induced tube formation of MDCK cells stably expressing Arl4c. These results suggest that changes in cell morphology through Arf6 and Rho contribute to induce development of tubes by MDCK cells as well as IEC6 cells. In addition, Arl4c expression was important for the

branching morphogenesis of UBs prepared from the embryonic kidney. Therefore, our data is not restricted to one cell line only and suggest that the importance of Arl4c expression in tubulogenesis would be common among several tube organs, if not all. The results are shown in Figure 6 and Supplementary Figure S9 and described in the text (page 13, lines 3 and 4 from the bottom; page 15, line 4 through 6 from the bottom; page 16, lines 3 from the bottom through page 17, line 2; page 17 lines 7 through 9 from the bottom).

2nd Editorial Decision

07 January 2014

Thank you for the submission of your revised manuscript to The EMBO Journal and please accept my apologies again for the unexpected delay in responding due to the holiday break.

Your study was sent back to referees #1 and #2, who now believe that all major concerns have been properly addressed and your manuscript is almost ready for publication (see below). That being said, a few minor issues raised by referee #2 that still require your attention, mostly related to the presentation of your data, will have to be addressed. Once these minor problems have been solved, I will be glad to accept your manuscript for publication in The EMBO Journal. Along the same lines, I would like to suggest for you to move the "Statistical Analyses" section from the supplementary information into the main text, for further clarity.

Every paper now includes a 'Synopsis' to further enhance their discoverability. Synopses are displayed on the html version of the article 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-short-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 now 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 used in the figures? 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.

If you have any questions regarding this initiative or any other part of the publication process, please do not hesitate to contact me.

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

#### REFEREE REPORTS:

Referee #1:

In this article Matsumoto and coworkers characterize the formation of tubular structures in intestinal epithelial cells through the Wnt/EGF-mediated expression of Arl4c. In this revised version of the manuscript, the authors have performed new experiments that, in my opinion, address most of the major issues raised by me, and the other reviewer, on the previous version, and the new data provides more in-depth evidence supporting the proposed model. In addition, they have also edited the manuscript and it is now more accurate and easier to read. Thus, I consider that this new version of the manuscript is ready for publication in EMBO J.



Referee #2:

1. It is unclear what the authors mean 'by the efficiency was lower when cysts were treated with Wnt? Less tubules form or less cysts form tubules? In any event, it represents the more physiological setting. Given that Wnts can affect cyst morphogenesis, this point becomes especially important to support the conclusions drawn for the effects on Wnts in tubule formation.
2. The authors state that 80% of the cells in 3D cultures express Arl4C, but from the data shown it is hard to tell which cells do not. A phase image should be shown alongside or replace with a figure with better resolution and magnification.
3. Arl4si expression could promote caspase-independent mechanisms of decreased cell viability. The cells appear as though viability is compromised. This should be put into context in the discussion.
4. The authors have not responded to whether Wnt5A/EGF promote changes via canonical or non-canonical signaling.
5. The authors need to better speculate on the link between Arl4, ARF6 and Rac1 in tubule formation.

2nd Revision - authors' response

13 January 2014

Referee #1:

*In this article Matsumoto and co-workers characterize the formation of tubular structures in intestinal epithelial cells through the Wnt/EGF-mediated expression of Arl4c. In this revised version of the manuscript, the authors have performed new experiments that, in my opinion, address most of the major issues raised by me, and the other reviewer, on the previous version, and the new data provides more in-depth evidence supporting the proposed model. In addition, they have also edited the manuscript and it is now more accurate and easier to read. Thus, I consider that this new version of the manuscript is ready for publication in EMBO J.*

I am very happy to hear that our revised manuscript is worth publishing in EMBO J.

Referee #2:

*1. It is unclear what the authors mean 'by the efficiency was lower when cysts were treated with Wnt? Less tubules form or less cysts form tubules? In any event, it represents the more physiological setting. Given that Wnts can affect cyst morphogenesis, this point becomes especially important to support the conclusions drawn for the effects on Wnts in tubule formation.*

In the method that IEC6 cells are treated with growth factors after they formed cysts, the efficiency of tube formation from cysts by Wnt3a/EGF was low. As already shown, addition of Wnt3a or Wnt5a alone did not affect cyst morphogenesis. These statements were described in the text (page 4, lines 9 and 10 from the bottom; page 5, lines 11 and 12). Wnt5a signalling might be required for cytogenesis of IEC6 cells because knockdown of Wnt5a affected cyst morphology (Gon, H., Mol. Biol. Cell, 2013;24:3764–3774). Given that endogenous Wnt5a is sufficient to regulate the functions of IEC6 cells, the cells may not respond to exogenous Wnt5a. We described the statements (page 4, lines 3 from the bottom through page 5, line 1) in response to the reviewer#2's original comment(1).

*2. The authors state that 80% of the cells in 3D cultures express Arl4C, but from the data shown it is hard to tell which cells do not. A phase image should be shown alongside or replace with a figure with better resolution and magnification.*

We replaced with a figure with better resolution and magnification in Supplementary Figure 4C and indicated cells which do not express Arl4c-GFP with white arrowheads.

*3. Arl4si expression could promote caspase-independent mechanisms of decreased cell viability. The cells appear as though viability is compromised. This should be put into context in the discussion.*

We showed the morphology of cysts and tubes treated with control and Arl4c siRNAs in Figures 1F and 5A and Supplementary Figure S4A and F. Although the referee said that the cells appear as though viability is compromised, it seems to us that depletion of Arl4c did not affect cell morphology. Aberrant formed nuclei, which were stained with anti-active caspase-3 antibody, were observed in the lumen of cysts and tubes, but treatment of the cells with Arl4c siRNA did not affect the numbers of abnormal shaped nuclei. IEC6 cells forming cysts and tubes were active caspase-3 negative, with intact nuclei. Knockdown of Arl4c did not increase apoptotic cells in the luminal space. Therefore, we consider that Arl4c siRNA does not affect cell viability.

The referee suggested that we should discuss that depletion of Arl4c promotes caspase-independent mechanisms of decreased cell viability. I am afraid that the statement may make readers misunderstand that our results with Arl4c siRNA were artifacts. Therefore, it is hard to describe this point in "Discussion". I would really appreciate it if the referee could accept our response.

*4. The authors have not responded to whether Wnt5A/EGF promote changes via canonical or non-canonical signalling.*

As shown in Figure 1A and Supplementary Figure 1A, Wnt5a did not affect EGF-dependent enlargement of cysts. It is generally believed that Wnt5a activates the b-catenin-independent pathway (non-canonical signalling). In our recent paper (Gon, H., Mol. Biol. Cell, 2013;24:3764–3774) we showed that IEC6 cells express high levels of Wnt5a and that knockdown of Wnt5a suppressed Rac1 activation, supporting that Wnt5a is involved in the b-catenin-independent pathway in IEC6 cells. The statements were described in the text (page 4, lines 3, line 5, and 10 and 11 from the bottom). We believe that Wnt5a activates the non-canonical signalling but that further activation of the signalling is not involved in tube formation. Because we used Wnt5a as a control for Wnt3a and the results using Wnt5a were negative, we did not emphasize effects of Wnt5a signalling on tube formation.

*5. The authors need to better speculate on the link between Arl4, ARF6 and Rac1 in tubule formation.*

In this manuscript we showed that Arl4c expression by Wnt3a/EGF in IEC6 cells activates Rac1 through ARNO and Arf6, resulting in proper inhibition of RhoA, during tube formation of IEC6 cells in Matrigel. In addition, as shown in Figure 6, we found that HGF does not affect cyst morphology of MDCK type II cells in Matrigel but develops tube formation when Arl4c is expressed. The combination of HGF and Y27632 or blebbistatin induced wild-type MDCK II cell tube formation and SecinH3 inhibited HGF-induced tube development of MDCK/Arl4c-GFP cells. It was reported that unlike MDCK type II cells, HGF induces tubes from MDCK type I cells in 3D Matrigel. In this model, HGF-induced Arf6 activation promotes the recruitment of Rac1 to the cell surface at the initiation of tube formation. Further, Arf6 activation also upregulates MAPK activity and the expression of the urokinase-type plasminogen activator receptor, which induces Rac1 activation probably through the DOCK180/Elmo complex, a Rac1 activator. Therefore, a cellular apparatus downstream of Arf6 activation, including the Rac and Rho axis, might be common in tube formation of IEC6 and MDCK I and II cells. The link between Arl4c, Arf6, and Rac1 was discussed in the text (page 16, line 7 from the bottom through page 17, line 5).