THE EMBO JOURNAL

Manuscript EMBO-2015-93003

Phosphorylation-dependent Akt-Inversin interaction at the basal body of primary cilia.

Kohki Kimura, Tatsuma Edamura, Tsutomu Tanaka, Satoko Ishigaki, Thoria Donia, Hiroko Noguchi, Toshihiko Iwanaga and Masayuki Noguchi

Corresponding author: Masayuki Noguchi, Hokkaido University

Review timeline:

Submission date:	04 September 2015
Editorial Decision:	22 October 2015
Revision received:	20 January 2016
Editorial Decision:	23 February 2016
Revision received:	04 May 2016
Accepted:	06 April 2016

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: Daniel Klimmeck

1st Editorial Decision 22 October 2015

Thank you for the submission of your manuscript entitled "Akt and Inversin interaction is important for cystic formation in nephronophthisis type II in humans" (EMBOJ-2015-93003) to The EMBO Journal and please accept my apologies for the delay in responding, which was caused by the difficulties in finding referees during the busy weeks after the summer break, affecting our usually much shorter editorial handling time. Your study has been sent to three referees, and we have so far received reports from two of them, which I copy below. As both referees are convinced about the high interest, novelty and quality of your study, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this decision is made in the interest of time, and I will forward you the third report very likely including further requests, as soon as I receive it.

Without going into the details that you will find below, both referees are very positive as I already mentioned. They express, however, besides a number of other important issues, rather fundamental concerns regarding the physiological relevance of your study that would need your attention during the review process. In particular, both referees point out, that it would be important to address the relevance of Akt-INVS signaling for cilia integrity in an in vivo mutant setting. Also, the referees ask you to re-consider the claims being made on the human context, and accordingly the study title. These views were well supported by the comments of a third expert in the field, from whom we

sought advice before sending out for review. I judge the comments of the referees to be generally reasonable and agree that adding insights into the one of the established in vivo model systems would be an essential point for consideration.

In any case, please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems.

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.

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). Nevertheless, 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://emboj.msubmit.net/html/emboj_author_instructions.html#a2.12

As you have probably seen already, every paper now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Finally, in order to ensure good reporting standards and to improve the reproducibility of published results, our guidelines to authors are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Accordingly, we now require the submission of a completed author checklist, which covers in a systematic manner your practices regarding animal welfare, human subjects, data deposition, statistics and research ethics. It needs to be filled (most of the fields will not apply to your study in particular) and returned to the editorial office at revision, either via the online submission system as a supplementary file or by email (contact@embojournal.org). Please, click on the link below and follow the instructions to download the checklist file:

http://emboj.embopress.org/authorguide

Again, please contact me at any time during revision if you need any help or have further questions.

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

REFEREE REPORTS:

Referee #1:

The study by Suizu et al convincingly shows that Akt interacts with Inversin, that the two proteins co-localize in the inversin compartment of cilia and that Akt can phosphorylate Inversin.

Furthermore, they show that this complex is likely important for mitotic spindle alignment in 2D and presumably 3D cultures in vitro, important for lumen formation. In general, the study is interesting, well controlled and novel.

This reviewer has a couple of conceptual issues that might need to be discussed and/or tested experimentally and a few technical issues.

What would be the contribution of ciliary Akt to the entire pool of cellular Akt? Would the PI3k/Akt pathway activated by receptors other that PDFR (InsR for instance) affect the pool of Akt at cilia? Presumably not. It would be interesting to test which proportion of the entire Akt pool sits in cilia and which of the many pathways known to activate it fail to act on the ciliary one.

The second issue deals with the physiological relevance of the discovery. Akt mutant mice were previously reported. Do they have any type of ciliary defect and more specifically renal tubular or cystic malformations?

Specific Major concerns:

1. The title is not appropriate. The study does not investigate any tissue from humans affected by NPHP nor it makes any point related to cysts. The implications are strong, but the title misrepresents the content of the study.

2. In figure 1 A,B and E: IP is performed with either HA or Flag and the corresponding pull-downs are only shown for aHA. The corresponding aFlag blots (from the same IPs) should be included in all cases.

3. In figure 2E the authors nicely use the anti pAkt substrate for their immunoblots. The same antibody should be used to show that in physiological conditions endogenous inversin is phosphorylated at pAKT consensus sites upon PDFG treatment.

4. The pAkt stainings are convincing, but the corresponding negative controls upon treatment with Pi3kinhibitors should also be included in figure 3 to show the specificity of the staining.

5. The conclusions drawn by the authors from the 3D cultures experiments are excessive. It is not appropriate to conclude that the studies on lumen formation in 3D cultures of MDCK cells (figure 7) can be indicative of "correct development of the tubular lumen" in vivo. 3D cultures of MDCK cells are a good system to study lumen formation in terms of apico-basal polarity. The defective tubulogenesis which is thought to lead to cyst formation in the renal tubule in vivo is instead based on planar polarity which cannot be recapitulated by this simple system in vitro. Thus, the conclusions from this part should be more moderate both in the results and in the discussion sections.

6. On page 9 there are 2 consecuitive paragraphs that are repeated twice.

7. Figure 6D, the blot appears very weired. Was somehow manipulated and/or bands cut out?

Referee #2:

The manuscript by Suizu et al. reports that Akt and inversin (INVS) interact in a phosphorylation-dependent fashion. The authors identified inversin as a novel interactor of Akt in a yeast two hybrid screen. This interaction depends on PDGF-AA mediated signaling. Interestingly, application of PDGF-AA in ciliated cells appears to result in a translocation of INVS from the cilium to the basal body, where INVS co-localizes with Akt. The authors show that Akt phosphorylates INVS at residues 864-866. In subsequent experiments Suizu and colleagues study the cellular effects of INVS lacking the Akt phosphorylation sites (3A INVS). They report that 3A INVS inhibits proliferation in MDCK cells and that spindle axis orientation and 3D lumen formation is impaired in cells expressing this construct. Based on these results the authors conclude that the interaction of INVS and Akt is important for cyst formation in humans.

The finding that INVS and Akt interact in a phosphorylation-dependent fashion is novel and potentially very interesting. The biochemical analysis to support this claim is thorough and the data appear to be very solid. The interpretation that this interaction is "important for cyst formation in nephronophtisis type II in humans", as the authors state in the title, is less convincing in my view. The cellular models used in this study are not sufficient to make this claim. Unfortunately, there are no data in a model organism to support the in vivo relevance of the proposed model which dampens my enthusiasm for this otherwise very thorough biochemical study.

Please find my detailed comments below:

1. In the title, the authors make a rather bold statement about the relevance of this interaction in human disease. In the absence of any in vivo data it is certainly not appropriate to state that the interaction between INVS and Akt is important for cyst formation in humans (or any other organism). The cell culture models used here are not sufficient to make statements about cyst formation in vivo (see later comments). This study would be much stronger if the authors presented data in an animal model to support a role of Akt in INVS-dependent cyst formation: e.g. genetic interaction between Akt and INVS in an animal model (zebrafish, mouse), or effect of pharmacological inhibition of Akt in cyst formation. The different isoforms of Akt have been extensively studied in knockout mice. To my knowledge there is no report about involvement of Akt genes in cyst formation. In a recent publication Akt 1 and 2 have been studied in the kidney using knockout mice (Canaud G et al. Nature Medicine 2013). This study focused on podocytes rather than on tubular cells (which give rise to cysts). However, there is no evidence on the kidney sections of Akt-deficient mice in this paper to support a role in cyst formation. At the very least the authors have to discuss the literature on the role of Akt in cyst formation in the kidney. Without additional data in an animal model (or humans) the claims about the significance of the Akt-INVS interaction in cyst formation need to be deleted or phrased more carefully (the last sentence of the discussion is one example).

2. As the authors state, there is some evidence to suggest that misalignment of the spindle axis during mitosis might contribute to cyst formation. If this is relevant, it occurs in 3 dimensions, because the spindle alignment has to occur relative to the axis of the kidney tubule. Here the authors analyze the spindle axis in cells grown on glass cover slips. I don't understand the biological significance of this experiment because the cells are forced to stay within the plane of the cover slip since they form an epithelial monolayer. The significance of these results for cyst formation in a three-dimensional organ is questionable. The fact that a disease-causing patient mutation (R899X) does not show a misalignment in these experiments although patients harboring this mutation have cysts underscores this issue. In addition, the role of misalignment of the spindle axis in cyst formation is controversial. The authors should cite an important study by Stefan Somlo and

colleagues (Nishio S et al. JASN 2010) showing that loss of oriented cell division does not initiate cyst formation in PKD mouse models.

3. The authors study the role of the INVS and Akt in cilia growth and propose that Akt controls ciliary development through phosphorylation of INVS. Yet, the role of INVS in ciliogenesis is another controversial topic as the authors correctly discuss. Experiments in vivo have shown that loss of INVS does not affect ciliogenesis. This raises the question about the relevance of findings in cell culture models in the pathogenesis of cystic kidney disease and supports the need to study the role of the the Akt-INVS interaction in animal models if claims were to made about cyst formation.

4. The authors use 3D cultures of MDCK cells and relate lumen formation of epithelial MDCK cysts to "proper formation of the renal tubule". Although this model has been widely used to study epithelial morphogenesis the interpretation of results of these types of experiments in the context of cyst formation in polycystic kidney disease has been difficult (wild-type cells form cysts, tubules can grow in the presence of HGF or after transfection of PKD genes, etc.). The lumen formation in MDCK cyst has no obvious mechanistic relevance for proper renal tubulogenesis as opposed to cyst formation in vivo. Therefore, sentences like the last sentence on page 20 are not a valid conclusion of the data.

5. Nephronophtisis type 2 is a recessive disease. In the cell culture models used in this studies, the authors observe "normal" phenotypes in non-transfected cells. How can the transfection of recessive loss of function mutations of INVS cause cellular phenotypes if a wild-type background. Are these dominant-negative effects? I would find it more logical if deletion of INVS in MDCK cells caused a phenotype. This phenotype could then be rescued with WT or mutant forms of INVS to assess the function of INVS variants. Please discuss this issue.

6. I could not find a satisfying description of the statistical analysis of many of the cell biological experiments. What was number of independent experiments that was used for the statistical analysis. The numbers (n) depicted in the figures probably refer to cells rather than independent experiments. The statistical tests should be performed with independent experiments (e.g. independent transfections). Please indicate this for experiments depicted in Figures 4B, 4E, 5E, 5G, 6A, 6C, 7A-D.

7. The translocation of INVS from the cilium to the basal body is very interesting. The analysis of the data, however, appears somewhat superficial. Please include statistics of several independent experiments to substantiate this observation (e.g. by analyzing the ratio of eGFP-INVS fluorescence intensity relative to acetylated tubulin and gamma-tubulin before and after PDGF stimulation. It is somewhat surprising that the 3A mutation does not affect the translocation to the basal body. Maybe the authors could speculate about a mechanism of translocation.

8. There is no reference to Fig. 5B in the text. Please include this. It looks like the labels of the subsequent panels of Figure 5 were mixed up. Please change 5C to 5B, 5D to 5E etc.

1st Revision - authors' response 20 January 2016

Phosphorylation dependent Akt-Inversin interaction at the ciliary pocket of primary cilia.

Referee #1

First, as the referee suggested we have changed the title as "**Phosphorylation dependent Akt-Inversin interaction at the ciliary pocket of primary cilia"** in order to tone down the clarification of the mechanistic insight of human nephronophthisis in our manuscript.

The referee #1 asked that what would be the contribution of ciliary Akt to the entire pool of cellular Akt?

In order to efficiently grow the cilia, in addition to the confluency of the cell on the culture dish, over 48 hours of serum starvation is required. Therefore, the levels of overall Akt phosphorylation within the cell/cytosol would be inhibited and became quite low when the cilia are present at the surface of the plasma membrane of the cells.

It is of note that even after the 48 hours of serum starvation condition to facilitate the primary cilia to grow, the levels of Akt phosphorylation and its substrates (detected by CST #9611) at the ciliary pocket remain high in the absence of growth factor stimulation (i.e. PDGF-AA) or serum starvation condition. Inversin is known to (and we did confirmed) mainly be present at the "Inversin compartment" of primary cilia which is located proximal portion of the primary cilia, but not at the ciliary pocket of the basal portion of primary cilia under normal culture condition allow cilia to grow. After PDGF-AA stimulation, Inversin moved from the Inversin compartment of the primary cilia to the ciliary pocket (at the ciliary base) and co-localized with Akt which is already high levels of phosphorylation (even after 48 hours of serum starvation which allow cilia to grow), and presumably get phosphorylated by the active Akt present at the ciliary pocket.

The referee asked whether any Akt mutant mice (or equivalent) have any types of cystic formation. Polycystic kidneys are known to be caused by an amazingly broad array of genetic mutations and manipulations. The PI3K-Akt-mTOR pathways are suggested to be involved in clinical manifestation of polycystic kidney. Indeed, renal cysts form in ~30% of patients with TSC (Tubero sclerosis complexes), in which *TSC* gene, a major downstream effector of Akt, is mutated (Huang & Manning, 2009, Inoki, Corradetti et al., 2005) (Winyard & Jenkins, 2011). Moreover, knock out mice of Bcl-2, which is activated through phosphorylation of BAD at S136 by Akt (del Peso, Gonzalez-Garcia et al., 1997) (Datta, Dudek et al., 1997), demonstrate the manifestation of severe polycystic kidneys (Veis, Sorenson et al., 1993). Akt signal is a major important intracellular mediator for anti-apoptotic responses. In this regard, disruption of Akt signal coincides with the histological observation of the increased levels of apoptosis which is associated with the progressive deterioration of renal function that occurs in human polycystic kidney disease patients (Woo, 1995). It is noteworthy, however, since three isoforms of Akt are present in human genome, all of which physically interact with INVS, single or double knock out of Akt (1/2) mice (triple knock out mice is lethal) may not result in the failure of INVS phosphorylation, which might affect the manifestation of the cystic formation of the kidney.

1. As the referee#1 suggested, we have changed the title as "**Phosphorylation dependent Akt-Inversin interaction at the ciliary pocket of primary cilia"**

2. As the referee suggested we have performed the Flag blot to confirm the co-immunoprecipitation of Akt with Inversin in Fig.1A-E.

3. The referee asked to show the endogenous INVS is phosphorylated upon PDGF stimulation. Using anti-phospho-Akt substrate antibody (CST #9611) in combination with anti-INVS antibody to detect phosphorylation of INVS, we have conducted indicated experiment and the results were presented in Fig S2C-D.

4. As the referee suggested we treated the cells with PI3K inhibitor LY294002, which

inhibited the phosphorylation of Akt at the ciliary pocket supporting the specificity of the immunostaining. The results were presented in the supplemental data S2B-C.

5. The ciliary hypothesis has evolved as the unifying concept of cystogenesis: cilia bend by fluid flow, initiate a calcium influx that prevents cyst formation. It is noteworthy that series evidences supported that cell motility is regulated by Akt (Xue & Hemmings, 2013). Dysfunction of cilia is suggested to play a role in cystic formation (Oh & Katsanis, 2012). In zebra fish, INVS knockdown by antisense morpholino oligonucleotides causes pronephric cysts (Simons, Gloy et al., 2005). The cystic epithelia displayed increased proliferation and apoptosis rates(Simons & Walz, 2006 12722). We have added one sentence with the above reference on page 18 of the revised manuscript. We admit the referee's comment so that we have toned down the statement in the revised manuscript (last sentence of page 20 in the discussion).

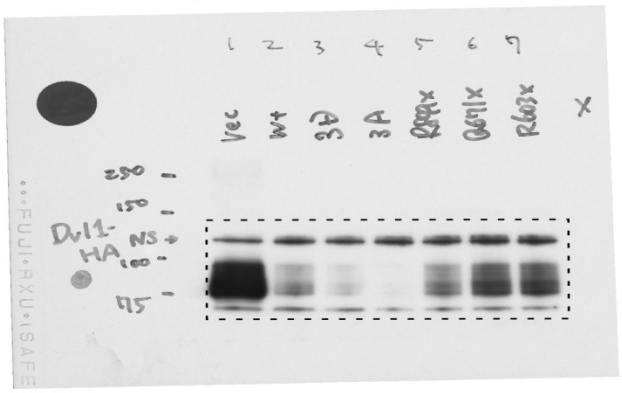
   There are several reports in which 3D culture system of MDCK cells was utilized for evaluating cystic formation of the kidney in vivo (Takiar, Mistry et al., 2012, Veikkolainen, Naillat et al., 2012). However, we agree that as the referee suggested we have restated and carefully choose the wording about the results of the 3D culture and its interpretation for the cystic formation in NPHP2.

6. Repeated sentences were deleted on page 9.

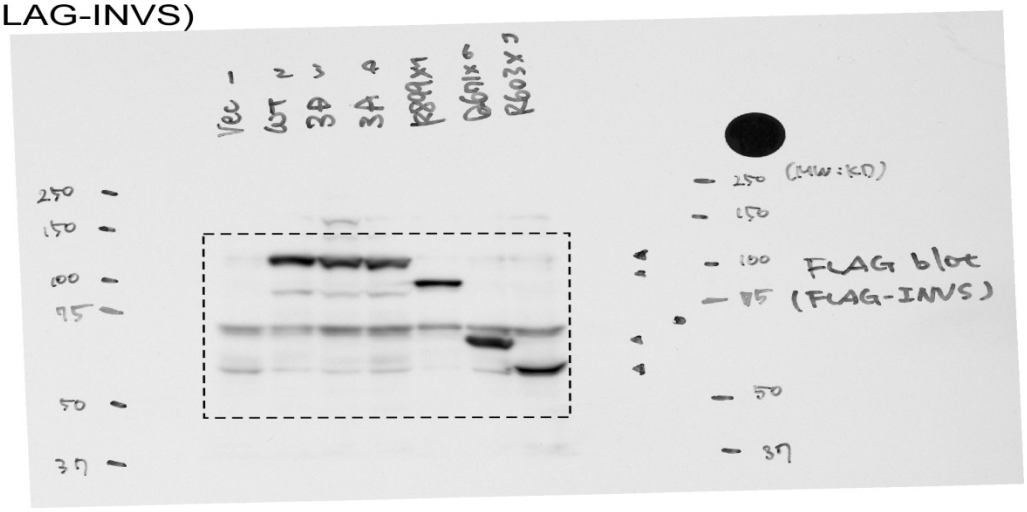
7. The referee raised a concern of the artificial manipulation of Fig 6D. The panels presented were original and no artificial manipulations were conducted on this or any other panels of this manuscript. We have provided the original blot of Fig 6D (please see below).

Fig. 6D. Original data of immunoblot.

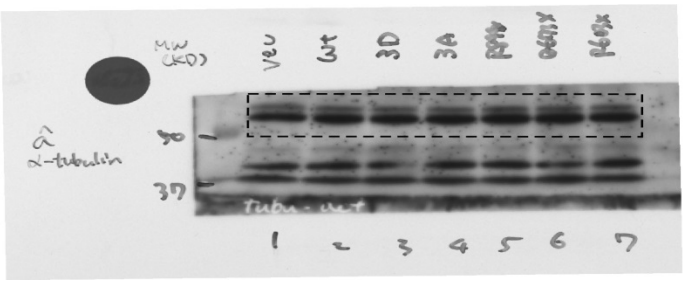
HA blot (Dvl1-HA)



FLAG blot (FLAG-INVS)



anti-α-tubulin blot



References

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91: 231-41

del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 278: 687-9

Huang J, Manning BD (2009) A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans 37: 217-22

Inoki K, Corradetti MN, Guan KL (2005) Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet 37: 19-24

Oh EC, Katsanis N (2012) Cilia in vertebrate development and disease. Development 139: 443-8

Simons M, Gloy J, Ganner A, Bullerkotte A, Bashkurov M, Kronig C, Schermer B, Benzing T, Cabello OA, Jenny A, Mlodzik M, Polok B, Driever W, Obara T, Walz G (2005) Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways. Nat Genet 37: 537-43

Simons M, Walz G (2006) Polycystic kidney disease: cell division without a c(l)ue? Kidney Int 70: 854-64

Takiar V, Mistry K, Carmosino M, Schaeren-Wiemers N, Caplan MJ (2012) VIP17/MAL expression modulates epithelial cyst formation and ciliogenesis. American journal of physiology Cell physiology 303: C862-71

Veikkolainen V, Naillat F, Railo A, Chi L, Manninen A, Hohenstein P, Hastie N, Vainio S, Elenius K (2012) ErbB4 modulates tubular cell polarity and lumen diameter during kidney development. J Am Soc Nephrol 23: 112-22

Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75: 229-40

Winyard P, Jenkins D (2011) Putative roles of cilia in polycystic kidney disease. Biochim Biophys Acta 1812: 1256-62

Woo D (1995) Apoptosis and loss of renal tissue in polycystic kidney diseases. N Engl J Med 333: 18-25

Xue G, Hemmings BA (2013) PKB/Akt-dependent regulation of cell motility. J Natl Cancer Inst 105: 393-404

Referee #2.

We have changed the title as "**Phosphorylation dependent Akt-Inversin interaction at the ciliary pocket of primary cilia."** as the referee suggested in our revised manuscript.

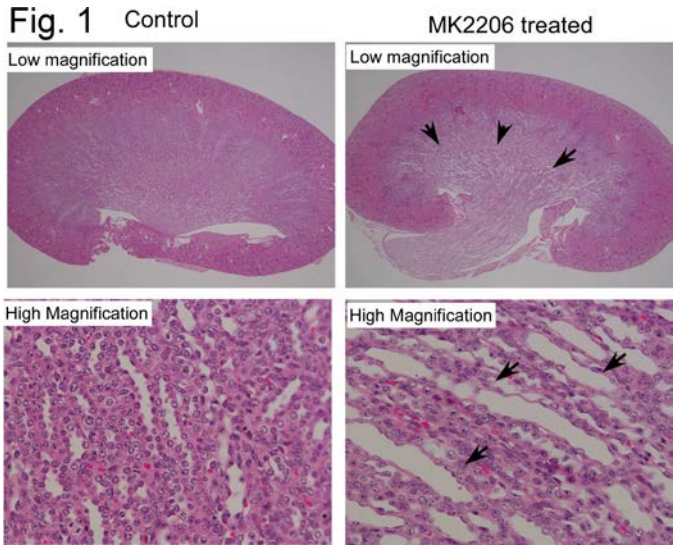
1. The referee asked whether any Akt mutant mice or equivalent (i.e. Zebrafish) have any types of ciliary defect. In Zebrafish, INVS knockdown by antisense morpholino oligonucleotides causes pronephric cysts(Simons, Gloy et al., 2005). However, consecutive phosphorylation sites at 864 to 866 are not conserved in the Zebrafish, so we cannot use Zebrafish as an *in vivo* model. We certainly admit the limitation of our study in vivo, hence, we have carefully restate the issues in the revised manuscript (title as well as the statement on page 20 and the discussion).

   We would like to emphasize, however, that the PI3K-Akt-mTOR pathway is suggested to be involved in clinical manifestation of polycystic kidney in vivo both mice and humans. It is known that renal cysts form in ~30% of patients with TSC (Tubero sclerosis complexes), a major downstream effector of Akt (Huang & Manning, 2009, Inoki, Corradetti et al., 2005) (Winyard & Jenkins, 2011). Moreover, knock out mice of anti-apoptotic protein Bcl-2, which is activated through phosphorylation of BAD via Akt mediated phosphorylation (Datta, Dudek et al., 1997, del Peso, Gonzalez-Garcia et al., 1997), is also known to result in severe manifestation of polycystic kidneys (Veis, Sorenson et al., 1993).

   Akt signal is a major important intracellular mediator for anti-apoptotic responses. In this regard, disruption of Akt signal coincides with the histological observation of the increased levels of apoptosis which is associated with the progressive deterioration of renal function that occurs in human polycystic kidney disease patients (Woo, 1995). It is of note, however, three isoforms of Akt with redundant biological function are present in human genome, all of which physically interact with INVS. Double knock out mice of Akt1/3 is lethal around embryonic day 12, (Dummler, Tschopp et al., 2006), thus no triple knock out mouse of Akt has been created. Single or double knock out of Akt1/2 mice may not result in the failure of INVS

phosphorylation, which might affect the absence of the manifestation of cystic formation of the kidney in these animals.

In order to address the in vivo effect of inhibition of Akt kinase activity in vivo, we have examined the effect of Akt inhibitor MK 2206 on C57BL/6 mice.



Methods: Two microgram per gram (body weight) of MK2206 (#11593, Cayman Chemical) was orally administered to the mouse after 5 days of pregnancy every other days. Seventeen days after DOB, the kidneys were resected, formalin-fixed, and paraffin-embedded sections were subjected for H&E staining.

Results: Macroscopically, the size of the kidneys were almost normal. Microscopically, over all architecture of the kidney was almost normal without remarkable cystic change. However, it is noted that mild to moderate dilatation of the renal tubules and medullary collecting ducts appeared to be more obvious in MK 2206 treated mouse (arrow heads, **Fig. 1**, upper panel, low power view, lower panel, high power view, right side panels). The numbers of the tubular and ductal epithelial cells appeared to be modestly increased, which exhibited enlarged vesicular nuclei with prominent nucleoli in MK2206 treated mouse (**Fig. 1,** lower panels, arrow heads). The observation, which is consistent with the histological findings reported in the autosomal recessive polycystic kidney (Lonergan, Rice et al., 2000, Lubarsky & Krasnow, 2003, Paul & Vanden Heuvel, 2014), at least supported that Akt kinase activity possibly be playing a role for the normal development of kidney in vivo.

As the referee suggested that we will include the article published by Canaud and

co-workers about the involvement of Akt2 as an underlying mechanisms for chronic renal diseases (Canaud, Bienaime et al., 2013). We also added the observation reported in these literatures listed above in the discussion section of the revised manuscript (Page 24~25 in the discussion section of the revised manuscript).

2~3.The referee raised a concern of the misalignment of the spindle axis and the cystic formation. Regarding the cell culture system as a possible model for the cystic formation, we can provide the following statements in the literatures:

Tubular epithelial cells are endowed with a pre-determined cellular program that controls the orientation of cell division relative to the neighboring cells. Therefore, spatially controlled division of regenerating tubular cells is suggested to be required to ensure nephron integrity (Simons & Walz, 2006). It has been reported that normal tubules in the kidney undergo oriented cell divisions, as a consequence, resulted in an elongation of the tubule after division (Lubarsky & Krasnow, 2003). When oriented cell division is lost, either through defective PCP (Planar cell polarity) or through mutations that give rise to PKD (polycystic kidney diseases), tubules become broader and remain short (McNeill, 2009). Precisely defined structure and architecture not only at the single cell level, but also at the tissue level appeared to be required for proper nephron function. It is suggested that oriented cell division is result of a correctly positioned spindle axis. Therefore, Inversin is suggested to control the orientation of cell division relative to the neighboring cells. When tubules elongate during renal development, tubular cells undergo massive proliferation (Simons & Walz, 2006).

Further, Hildebrandt reported that defects of cystoproteins lead to disruption of planar cell polarity and thereby to renal cysts through to malorientation of the centrosome or mitotic spindle complex (Hildebrandt, Attanasio et al., 2009, Hildebrandt, Benzing et al., 2011). Consistently, Happe reported that renal cyst is characterized by progressive development of fluid-filled cyst derived from renal tubular epithelial cells. Altered ciliary signaling caused disoriented cell division in renal tubules, which resulted in renal cyst formation (Happe, de Heer et al., 2011). The article suggested by referee#2 also stated that “loss of oriented cell division is a feature of pkhd1 mutation and cyst formation” (Nishio, Tian et al., 2010).

We believe that these published reports supported the mechanistic connections

between in vitro experimental models and in vivo cystic formation of kidney, in the revised manuscript (we have added couple of statement from the literatures in the discussion section of the revised manuscript (page 25~26 of the revised manuscript in the discussion section). We certainly admit the referee's concern so that we also have toned down the statement of the 3D culture and the cystic formation, as the referee suggested.

4. Regarding the cell culture system as a possible model for the cystic formation, we have listed the above (please see the responses for #2-3). We certainly admit the points the referee raised so that we have restate the sentence on Page 20 accordingly as the referee suggested.

5. The referee raised a question how recessive disease like nephronophthisis type II can be evident in overexpression experiment, perhaps as dominant-negative effect of the mutation. We admit that nephronophthisis type II is caused by the mutation of *NPHP2* is considered as an autosomal recessive disease. Thus, the biological effect presented in MDCK cells were considered as primarily overexpression of the dominant negative effects which are overshadowing on top of endogenous expression derived from the wild type allele. However, in the literature, two cases of human NPHP2 are reported to be caused by the heterozygous mutation of the single chromosome (A10-1: heterozygous mutation of deletion of 2908, F75-1: heterozygous variant mutation of pA650P) (Tory, Rousset-Rouviere et al., 2009). Although pathogenetic details were not described in details, it is also reported that three cases of NPHP3, another autosomal recessive type of human nephronophthisis, carried only one heterozygous mutation in humans (Tory et al., 2009). We also would like to notify that we have created series of mIMCD3 cell lines (mouse inner medullary collecting duct cell line**,** ATCC) in which wild type and/or mutant forms of INVs were overexpressed. However, when we introduced siRNA for INVS to inhibit endogenous expression of INVS in these cell lines, the cells could not tolerate the elimination of endogenous INVS. As this is the situation, we cannot

eliminate the endogenous INVS to examine the function of INVS mutants, but just overexpressed the INVS in the presence of endogenous wild type INVS to examine the function.

6. The numbers of the experiments with statistical analysis were indicated in all the experiments as below. We have added the sentences in the figure legends.

Fig. 4B

The results presented are means ±SE of the pixels of yellow color which indicates colocalization of INVS (red) and phospho-Akt (green) in hTERT-RPE1 cells (n=31 for time 0, 35 for 1 min., and 33 for 3 min, respectively). Three independent experiments have conducted with similar results.

Fig. 4E

The results presented are means ±SE of the pixels of yellow color which indicates colocalization of EGFP-INVS (green) with γ-tubulin (red) in hTERT-RPE1 cells (n=28). Three independent experiments have done and they showed similar results. Statistical analysis was verified by student's *t* test.

Fig. 5E

The results presented are means ±SE of the longitudinal length of acetylated-tubulin (red), a marker of primary cilia, in Akt siRNA transfected (green) hTERT-RPE1 cells (n=175 for control siRNA, 175 for Akt siRNA, and 195 for Akt siRNA plus siRNA resistant Akt, respectively). Three independent experiments have conducted with similar results. Statistical analysis was verified by student's *t* test.

Fig. 5G

The results presented are means ±SE of longitudinal length of acetylated-tubulin (blue), a marker of primary cilia, in EGFP-INVS (green) transfected in hTERT-RPE1 cells (n=58 for EGFP vector, 58 for EGFP-INVS WT, and 52 for EGFP-INVS 3A mutant, respectively). Three independent experiments have conducted with similar results. Statistical analysis was verified by student's *t* test.

Fig. 6A

The results presented are scatter plot of the angle (θ) of mitotic spindle (n=28 for all

samples of vector, HA-WT, -3D, -3A, -R899X, -Q671X, and -R603X INVS stably transfected MDCK cells). Three independent experiments have conducted with similar results. Statistical analysis was verified by Mann-Whitney's non-parametric median test.

Fig. 6C

The results presented are means ±SE of the ratio of triplicates between sequentially measured TCF/LEF fire fly signal and Renilla signal in indicated HA-INVS transfected 293T cells by using Dual-Luciferase reporter® Assay System (Promega) with triplicated experiments. Two independent experiments have conducted with similar results. Statistical analysis was verified by student's *t* test.

Fig. 7A and C

The results presented are means ±SE of percentage of the normal acini structure as uniform as Vector- or WT INVS-transduced MDCK cells form (A) (n=166 for Vector, 204 for HA-WT, 233 for -3D, 150 for -3A, 164 for -R899X, 128 for -Q671X, and 137 for -R603X INVS stably transfected MDCK cells, respectively). Two independent experiments have conducted with similar results. Statistical analysis was verified by student's *t* test.

Fig. 7B and D

The results presented are scattered plots of the size of acini (B) (n=205 for Vector, 143 for HA-WT, 131 for -3D, 167 for -3A, 144 for -R899X, 128 for -Q671X, and 144 for -R603X INVS stably transfected MDCK cells, respectively). Two independent experiments have conducted with similar results. Statistical analysis was verified by Mann-Whitney's non-parametric median test.

7. We have added the statistics analysis of the experiments of the translocation of INVS as the referee suggested in the revised manuscript.

8. Citations of Fig 5B were fixed (page 15 of the revised manuscript).

References

Canaud G, Bienaime F, Viau A, Treins C, Baron W, Nguyen C, Burtin M, Berissi S, Giannakakis K, Muda AO, Zschiedrich S, Huber TB, Friedlander G, Legendre C, Pontoglio M, Pende M, Terzi F (2013) AKT2 is essential to maintain podocyte viability and function during chronic kidney disease. Nat Med 19: 1288-96

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91: 231-41

del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science 278: 687-9

Dummler B, Tschopp O, Hynx D, Yang ZZ, Dirnhofer S, Hemmings BA (2006) Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. Mol Cell Biol 26: 8042-51

Happe H, de Heer E, Peters DJ (2011) Polycystic kidney disease: the complexity of planar cell polarity and signaling during tissue regeneration and cyst formation. Biochim Biophys Acta 1812: 1249-55

Hildebrandt F, Attanasio M, Otto E (2009) Nephronophthisis: disease mechanisms of a ciliopathy. J Am Soc Nephrol 20: 23-35

Hildebrandt F, Benzing T, Katsanis N (2011) Ciliopathies. N Engl J Med 364: 1533-43

Huang J, Manning BD (2009) A complex interplay between Akt, TSC2 and the two mTOR complexes. Biochem Soc Trans 37: 217-22

Inoki K, Corradetti MN, Guan KL (2005) Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet 37: 19-24

Lonergan GJ, Rice RR, Suarez ES (2000) Autosomal recessive polycystic kidney disease: radiologic-pathologic correlation. Radiographics 20: 837-55

Lubarsky B, Krasnow MA (2003) Tube morphogenesis: making and shaping biological tubes. Cell 112: 19-28

McNeill H (2009) Planar cell polarity and the kidney. J Am Soc Nephrol 20: 2104-11

Nishio S, Tian X, Gallagher AR, Yu Z, Patel V, Igarashi P, Somlo S (2010) Loss of oriented cell division does not initiate cyst formation. J Am Soc Nephrol 21: 295-302

Paul BM, Vanden Heuvel GB (2014) Kidney: polycystic kidney disease. Wiley Interdiscip Rev Dev Biol 3: 465-87

Simons M, Gloy J, Ganner A, Bullerkotte A, Bashkurov M, Kronig C, Schermer B, Benzing T, Cabello OA, Jenny A, Mlodzik M, Polok B, Driever W, Obara T, Walz G

(2005) Inversin, the gene product mutated in nephronophthisis type II, functions as a molecular switch between Wnt signaling pathways. Nat Genet 37: 537-43

Simons M, Walz G (2006) Polycystic kidney disease: cell division without a c(l)ue? Kidney Int 70: 854-64

Tory K, Rousset-Rouviere C, Gubler MC, Moriniere V, Pawtowski A, Becker C, Guyot C, Gie S, Frishberg Y, Nivet H, Deschenes G, Cochat P, Gagnadoux MF, Saunier S, Antignac C, Salomon R (2009) Mutations of NPHP2 and NPHP3 in infantile nephronophthisis. Kidney Int 75: 839-47

Veis DJ, Sorenson CM, Shutter JR, Korsmeyer SJ (1993) Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75: 229-40

Winyard P, Jenkins D (2011) Putative roles of cilia in polycystic kidney disease. Biochim Biophys Acta 1812: 1256-62

Woo D (1995) Apoptosis and loss of renal tissue in polycystic kidney diseases. N Engl J Med 333: 18-25

2nd Editorial Decision 23 February 2016

Thank you for submitting the revised version of your manuscript. It has now been seen by the two original referees, whose comments are enclosed below.

As you will see both referees find that their concerns have been sufficiently addressed and are broadly in favour of publication, pending satisfactory minor revision, and a few editorial issues concerning text and figures that I need you to address.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript using the link enclosed below, addressing the comments of all reviewers.

In more detail, referee 2 asks you to include the in vivo Akt inhibitor experiment added during revision, into the manuscript. Also, both referees point out, that it would be important to re-consider the claims being made, and accordingly the study title as well as several points in the discussion. In addition, it would be necessary to add relevant literature and have the manuscript language edited by a native speaker, or equivalent service.

Further, we have realized that there are some formal issues with figure quality, which we ask you to adjust at re-submission (Figures 2A and 2E). Regarding your current" supplementary information": Please consider that we now have "Expanded View and Appendix "format instead of the former "supplementary information". This is detailed at
http://emboj.embopress.org/authorguide#expandedview
but in short, this means for you now:

Please combine /convert all your "Supplementary information" into ONE Appendix-PDF, i.e. kindly provide all supplementary text, figures and related legends within one Appendix-PDF, call it Appendix, and call the figures inside "Appendix figure S1".....

Please insert a "Table of Contents" as the first page of this Appendix-PDF and ensure that within the article file all references/call-outs to the Appendix-items are adjusted accordingly (also referring to "Appendix figure S1"....).

** EMBO Press encourages all authors and reviewers to associate an Open Researcher and Contributor Identifier (ORCID) to their account. ORCID is a community-based initiative that provides an open, non-proprietary and transparent registry of unique identifiers to help disambiguate research contributions.

Thank you very much in advance!

Again, please contact me at any time if you need any help or have further questions.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

REFEREE REPORTS

Referee #1:

The authors have made a good effort to respond to all issues raised by this reviewer. The study is much improved. The results on the interaction between Akt and INV and the phosphorylation of the

last by the first are totally convincing. The potential role of this phosphorylation in the regulation of inversin localization and in ciliogenesis are convincing. The data on the role of the triple mutant (and other mutants) in the mitotic spindle orientation with respect to the apico-basal polarity axis in the MDCK cells and on lumen formation are also convincing.

The authors have providied a convincing discussion on as why the individual Akt mutants were not reported to manifest any ciliary dysfunction suggesting that triple (Akt1,2,3) mutants would need to be generated to appreciate this phenotype.

This reviewer has only two minor issues remaining to be addressed:

1. The cystic phenotype in nephronophtisis patients cannot be really considered as polycystic kidney disease. ESRD is rarely reached due to cysts in this disease. So, the manifestation should be called "cystic kidney disease" and not "polycystic kidney disease" throughout the text. PKD is a peculiar and different type of manifestation which might not be explained by the results of this study.

2. The discussion on the mitotic spindle orientation is improved, but still it is a bit confusing on the role of planar cell polarity. Spindles in epithelia can orient along two different axis: apico-basal and planar. The defective orientation observed in this study is NOT planar (impossible to mimick in vitro) but it is instead along the apico-basal axis. Thus, the discussion on this aspect should be further smoothened. However, because the distorted lumen formation in the MDCK 3D cultures in vitro has been previously reported as a readout of function for other NPHP genes (Delous et al, HMG 2009), the authors can refer to those studies and avoid (or limit) trying to link this to the PCP alteration proposed to regulate cystogenesis. This is per se questioned in the field. But irrespective of this, the process described in this study is an entirely different biological process.

Referee #2:

In the revised manuscript Suizu and colleagues have addressed many of the issues I had raised. However, in my opinion there are still a few issues that need clarification.

1. Both referees raised the question about the physiological significance of the findings in vivo. I appreciate that it is not trivial to address this issue experimentally. The authors have attempted to address this with an experiment using the Akt inhibitor MK 2206 in mice, which are presented in the response to referee 2 (Fig. 1, comment #1.). These observations are potentially interesting. However, if these observations are reproducible and result in statistically significant differences, they should be included in the manuscript. If not, these data cannot be used to support the relevance of the findings in vivo.

The authors have toned down the title. But in my view, there are still sentences in the manuscript that need to be rephrased: last sentence of abstract: "These observation underscore the significance of the Akt-INVS interaction for abnormal cyst formation in NPHP2" and last sentence of the Discussion: "Therefore, clarification of the Akt-INVS functional interaction will pivotal to achieve a therapeutic breakthrough for NPHP2 patients." In my opinion, sentences like this would need to be supported by data in an animal model of NPHP2. I would suggest to replace these sentences with statements that highlight the biochemical and cell biological aspects in the paper. Closing sentences that highlight the need for future experiments evaluating the physiological significance of the reported findings in animal models would be more appropriate than referring to issues related to NPHP2 patients.

2. In my review I asked how PCP can be studied in 2 D cell culture. This question has not been answered: "As the authors state, there is some evidence to suggest that misalignment of the spindle axis during mitosis might contribute to cyst formation. If this is relevant, it occurs in 3 dimensions, because the spindle alignment has to occur relative to the axis of the kidney tubule. Here the authors analyze the spindle axis in cells grown on glass cover slips. I don't understand the biological significance of this experiment because the cells are forced to stay within the plane of the cover slip since they form an epithelial monolayer. The significance of these results for cyst formation in a three-dimensional organ is questionable. The fact that a disease-causing patient mutation (R899X) does not show a misalignment in these experiments although patients harboring this mutation have cysts underscores this issue." Please explain this to me.

3. In question #5 of the initial review I asked how a recessive disease, which is thought to be caused by INVS loss of function, can be modeled by overexpression of mutant forms of INVS in the presence of endogenous INVS: "Nephronophtisis type 2 is a recessive disease. In the cell culture models used in this studies, the authors observe "normal" phenotypes in non-transfected cells. How can the transfection of recessive loss of function mutations of INVS cause cellular phenotypes in a wild-type background. Are these dominant-negative effects? I would find it more logical if deletion of INVS in MDCK cells caused a phenotype. This phenotype could then be rescued with WT or mutant forms of INVS to assess the function of INVS variants. Please discuss this issue."

I am not entirely convinced that the response addresses this conceptual issue. The authors explain that they attempted to suppress INVS expression using siRNA in mIMCD3 cells to study the cellular phenotype. They state that these "cells could not tolerate the elimination of endogenous INVS". The effects in overexpressed mutants were performed in MDCK, not in IMCD cells. Suppression of INVS in MDCK cells by shRNA has been published and the authors refer to this paper in their manuscript (Mergen et al.). Moreover, Mergen et al. found differences in cilia formation/disassembly that could easily be studied using the mutants. Why haven't the authors tried to suppress INVS in MDCK cells, using this published procedure (or asked for the cells from Mergen et al.). I still think that a loss of function cell culture model and rescue with INVS mutants would strengthen the claims made by the authors. At the very least this issue should be discussed more thoroughly in the manuscript.

4. Point 6 of the previous review: The authors have now added a more thorough description of the statistics. In all the panels where this applies they have stated that following referring to multiple experimental series: "Three (or two) independent experiments have conducted with similar results." I assume that the data from all three (in some panels two) experiments were included in the statistical analysis. If this is not the case, all experiments should be included in the analysis.

5. The manuscript might benefit from language editing by a native speaker prior to publication.

2nd Revision - authors' response 04 May 2016

Referee #1.

1. We have changed all instances of “polycystic kidney disease” to “cystic kidney disease” in the revised manuscript.

2. We agree that spindles orient along the apico-basal and planar axes, and we have modified our statements in the revised manuscript to more appropriately reflect our observations within this context. Based on the referee’s suggested reference (Delous, Hellman et al., 2009) as well as existing literature (Takiar, Mistry et al., 2012, Veikkolainen, Naillat et al., 2012), we carefully restate the link between distorted lumen formation in the 3D culture system with cystogenesis.

Referee #2.

1. We note that five pups born from MK-2206-treated pregnant dams showed similar histological findings. However, we agree with the reviewer that it is difficult to quantitatively evaluate the small dilatation of renal tubules and medullary collecting ducts, given that such measurements can vary with changes in the section-cutting plane of the histological samples. We also appreciate the complexities involved in precisely counting cells and comparing differences in cell numbers in histological sections of renal tubules and medullary collecting ducts. As pointed out by the referee, we agree that our observations of MK-2206-treated mice can only point to a possible involvement of Akt signaling in kidney development at this time and that we will need further evidence to conclusively attribute a role for Akt signaling in cystogenesis.

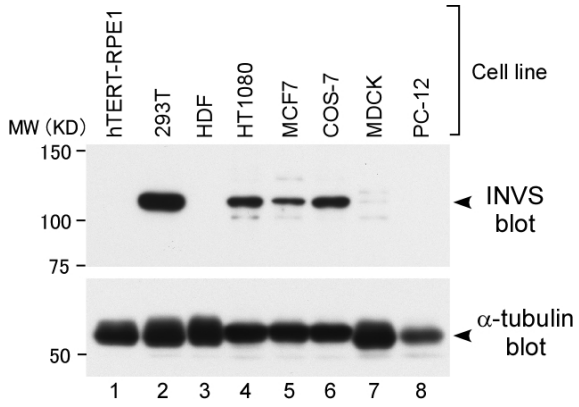
   As suggested, we have changed the following sentences in the revised manuscript: 1. "These observations underscore the significance of the Akt-*INVS* interaction for abnormal cyst formation in NPHP2" 2. Discussion: "Therefore, clarification of the Akt-INVS functional interaction is pivotal to achieve a therapeutic breakthrough for NPHP2 patients."

2. A 2D culture system of PCP (planar cell polarity) has been previously described (Kikuchi, Niikura et al., 2010, Toyoshima & Nishida, 2007). However, since spindles can orient along both, apico-basal and planar axes, we agree that there are technical limitations in the measurement of the spindle axes in 2D systems. Both methodological and technical limitations may contribute to a part of the readout of

the misalignment of the spindle axis in the disease-causing mutation, R899X, which exhibits an apparently normal spindle axis alignment in 2D systems. There are several reports that describe a 3D culture system of MDCK cells for evaluating cystic formation of the kidney *in vivo* (Takiar et al., 2012, Veikkolainen et al., 2012). We have also attempted to analyze the observed misalignment of spindle axis orientation in 3D culture systems. However, since cells can divide in any direction, particularly during distorted lumen formation stage, it is not technically facile to precisely measure small differences in the angle of orientation of the spindle axis in 3D culture systems (Banon-Rodriguez, Galvez-Santisteban et al., 2014, Mao, Streets et al., 2011). Of note, determination of the standard axis, which is required for the measurement of angles to demonstrate spindle misalignment, is not easy because cells can divide in multiple directions during each cell division over the course of the lumen formation. It is also difficult to accurately determine the direction of the standard axis of cell division for evaluating the angles of “misalignment” of spindle axes, specifically during the distorted lumen formation stages.

3. We admit that the results of the mutation study of MDCK cells presented in our revised manuscript are overexpression of mutant INVS in the presence of wild type INVS so that the observations are essentially dominant negative effect over the endogenous wild type expression. Notably, however, the expression levels of endogenous INVS in MDCK cell appeared to be very low (almost undetectable) compared to the other cell lines (293T, HT1080, MCF7, Cos-7 cells) we have examined (see below). Therefore, the biological effect of the mutant INVS can be relatively clearly observed by the overexpression experiments in MDCK cells. We

certainly admit, however, the biological effects of mutant forms of INVS observed in the MDCK overexpression experiments were possibly due to the dominant negative effect of mutant INVS in the presence of minimally expressing endogenous INVS.



Method: Cells were maintained in DMEM with 10 % FBS (or 10 % Horse Serum and 5 % FBS for PC-12 cells) and lysed with ice-cold Brij97 cell lysis buffer (see below). Twenty μg of proteins was resolved onto SDS-PAGE and immunoblotted by anti-INVS antibody (10585-1-AP, Proteintech) or anti-α-tubulin antibody (DM1A, #9026, Sigma) and detected by ECL.

Brij97 cell lysis buffer:
0.875% Brij97 (Sigma), 0.125% NP40, 150mM NaCl, 10mM Tris HCl pH 7.5, and 2.5 mM EDTA containing proteinase inhibitor mix (Leupeptin and AEBSF), phosphatase inhibitors (1 mM Na3VO4 and 10 mM NaF).

We have added the observation (MDCK cells expressing undetectable levels of INVs) in the revised manuscript in the main text as well as supplemental information (Appendix figure S4B).

I would also like to note that NPHP2 is considered as an autosomal recessive disease in the literature. Thus, genetic mutation in most of the NPHP2 cases are associated with either homozygous or combination of two different heterozygous mutation of each allele. However, it has been reported that at least two cases of human NPHP2 are caused by the heterozygous mutation of the single chromosome (A10-1: heterozygous mutation of deletion of 2908, F75-1: heterozygous variant mutation of

pA650P) (Tory, Rousset-Rouviere et al., 2009). Although pathogenetic details were unclear, it has also reported that three cases of NPHP3, another autosomal recessive type of human nephronophthisis, carried only one heterozygous mutation in humans(Tory et al., 2009).

4. The referee asked whether all the independent experiments are included into the single set of statistical analysis. Since in most of the data for statistical analysis were conducted several different combination of immunostaining by different antibodies and analyzed using confocal microscopy. The intensities of the immunostaining as well as the uptake conditions of the confocal images (High Voltage, Gain, and Offset) may not necessarily identical or occasionally variable depending on the condition of the experiments. Thus, we independently analyzed the statistics in separate experiments. I would like to emphasize, however, that the results were reproducible and consistent in indicated numbers of independent experiments.

References

Banon-Rodriguez I, Galvez-Santisteban M, Vergarajauregui S, Bosch M, Borreguero-Pascual A, Martin-Belmonte F (2014) EGFR controls IQGAP basolateral membrane localization and mitotic spindle orientation during epithelial morphogenesis. EMBO J 33: 129-45

Delous M, Hellman NE, Gaude HM, Silbermann F, Le Bivic A, Salomon R, Antignac C, Saunier S (2009) Nephrocystin-1 and nephrocystin-4 are required for epithelial morphogenesis and associate with PALS1/PATJ and Par6. Hum Mol Genet 18: 4711-23

Kikuchi K, Niikura Y, Kitagawa K, Kikuchi A (2010) Dishevelled, a Wnt signalling component, is involved in mitotic progression in cooperation with Plk1. EMBO J 29: 3470-83

Mao Z, Streets AJ, Ong AC (2011) Thiazolidinediones inhibit MDCK cyst growth through disrupting oriented cell division and apicobasal polarity. American journal of physiology Renal physiology 300: F1375-84

Takiar V, Mistry K, Carmosino M, Schaeren-Wiemers N, Caplan MJ (2012) VIP17/MAL expression modulates epithelial cyst formation and ciliogenesis. American journal of physiology Cell physiology 303: C862-71

Tory K, Rousset-Rouviere C, Gubler MC, Moriniere V, Pawtowski A, Becker C, Guyot C, Gie S, Frishberg Y, Nivet H, Deschenes G, Cochat P, Gagnadoux MF, Saunier S, Antignac C, Salomon R (2009) Mutations of NPHP2 and NPHP3 in infantile nephronophthisis. Kidney Int 75: 839-47

Toyoshima F, Nishida E (2007) Integrin-mediated adhesion orients the spindle parallel to the substratum in an EB1- and myosin X-dependent manner. EMBO J 26: 1487-98

Veikkolainen V, Naillat F, Railo A, Chi L, Manninen A, Hohenstein P, Hastie N, Vainio S, Elenius K (2012) ErbB4 modulates tubular cell polarity and lumen diameter during kidney development. J Am Soc Nephrol 23: 112-22

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: Masayuki Noguchi
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2015-93003R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Fig 4B, Fig 4E, Fig 5E, Fig 5G, Fig.6B, Fig. 6C, Fig. 7C, and Fig. 7D.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA