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ICK is essential for cell type-specific ciliogenesis and the regulation of ciliary transport

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Editor: David del Alamo

1st Editorial Decision

26 November 2012

Thank you for the submission of your research manuscript to our editorial office and please accept my apologies for the delay in responding. I have read and considered your study on the background of the related literature and evaluated its suitability from the scope of The EMBO Journal.

Your study provides a detailed analysis of the phenotype of ICK with particular attention to neuronal development. I certainly appreciate your conclusive demonstration that ICK loss-of-function causes impaired proliferation of neuronal progenitors and that this correlates with defective ciliogenesis and Shh signaling. However, some concerns arise regarding the depth of analysis of your study. First, whether the phenotypes observed in knockout mice are actually caused by defects in ciliogenesis and not other cellular defects, remains largely unclear. Furthermore, the molecular role of ICK in the ciliogenesis process and how this would explain the phenotypes observed in terms of intraflagellar transport or Shh signaling, are not sufficiently explored. With these caveats in mind, while I consider that the involvement of ICK in ciliary morphogenesis is both a novel and potentially interesting idea, I believe that your manuscript is at this stage at a rather descriptive level. Without significant development in the directions suggested above, particularly regarding the molecular mechanism of action in the cilium, I am afraid that your manuscript does not provide the

kind of advance of high functional and mechanistic insight that would justify its publication in The EMBO Journal. I have therefore decided not to proceed with the review process.

Please note that we subject to external review only a small percentage of the manuscripts that we receive. I am sorry to disappoint you on this occasion and I hope for the rapid publication of your study somewhere else.

Resubmission

03 October 2013

We would like to submit our manuscript entitled "ICK is essential for ciliogenesis and the regulation of protein transport at ciliary tips" by Chaya et al., which we wish to have considered for publication in The EMBO Journal.

One of the important features of cilia is that IFT particles switch from anterograde to retrograde transport at the tip of the cilia. A recent study reported the involvement of BBSome and IFT144 molecules in transport switching in *C. elegans* (Wei et al., *Nat. Cell Biol.* 2012;14:950-957.), however, the regulatory mechanisms underlying protein transport switch at the ciliary tip are still poorly understood. We found that ICK localizes to the tips of the cilia. We generated and analyzed both conventional and central nervous system-specific ICK knock-out mice. Loss of ICK caused aberrant localization of Shh signaling molecules in the cilia and ciliary defects in neural progenitor cells, but not in mature neurons, indicating that ICK is essential for ciliogenesis in neuronal progenitors but dispensable in mature neurons.

Interestingly, we found that both IFT-A and IFT-B components were concentrated at the ciliary tips in ICK-deficient cells. Overexpression of ICK also induced a strong accumulation of IFT-B but not IFT-A components at the ciliary tip, indicating that loss or overexpression of ICK affects the localization of IFT components at the ciliary tip differently. Our results show that ICK regulates IFT turnaround at the tips of the cilia. We also found that Kif3a is directly phosphorylated by ICK and that inhibition of Kif3a phosphorylation impairs Kif3a function. These results suggest that ICK functions at ciliary tips through phosphorylating Kif3a.

It should be noted that a missense mutation in human ICK is associated with a disease with multiple organ defects, ECO (Lahiry et al., *Am. J. Hum. Genet.* 2009;84:134-147.). We found that the ICK protein with the human mutation has a reduced ability to localize to the ciliary tip and to rescue cilia formation in ICK-deficient MEFs, suggesting that normal localization of ICK at the ciliary tip is important for ICK function.

Taken together, this study sheds light on regulatory mechanisms for the protein transport switch from anterograde to retrograde at cilia tips that previously have been almost totally unknown.

We appreciate your understanding of the significance of our study. All of the authors in this paper reviewed the paper and agreed to submit. We would be grateful to have this manuscript considered for publication in The EMBO Journal. We look forward to hearing from you.

2nd Editorial Decision

05 November 2013

Thank you for the submission of your manuscript entitled "ICK is essential for ciliogenesis and the regulation of protein transport at ciliary tips" to The EMBO Journal. I have just received the reports from the two referees that were asked to evaluate your manuscript and I regret to say that their assessment is not a positive one.

As you will see from their reports, besides a number of important technical concerns, both referees believe that, while the connection of ICK to the Hedgehog pathway as the cause of the phenotypes observed is well established, the molecular details regarding the role of ICK in the cilium are rather unclear. Unfortunately, the referees believe that the experimental evidence in support of your main hypothesis for the role of ICK as a regulator of IFT complexes during the anterograde/retrograde transport switch is not sufficiently convincing. Considering these criticisms and the fact that despite their absolute interest in your work none of the referees considers your manuscript suitable for publication, after discussing your case within the editorial team, we do not believe it would be

productive to call for a revised version of your manuscript at this stage, and therefore we cannot offer to publish it.

That being said, however, if you feel that you can obtain data that would significantly address the referees' concerns and strengthen the study along the lines pointed out in their reports, we would be glad to consider a new manuscript in the near future. To be completely clear, however, I have to inform you that a new manuscript would need to be treated as a new submission rather than a revision and, while we will try to contact the same referees, it would be reviewed afresh, also with respect to the literature and the novelty of your findings at the time of resubmission. Please do not hesitate to contact me should you need any further input or have any related questions.

I am sorry that I have to disappoint you at this stage. I hope, however, that the referee comments will help you improve your study and I thank you once more for the opportunity to consider your manuscript.

REFEREE REPORTS:

Referee #1 (Report):

Technical Quality: Adequate
Novelty: Medium
General Interest: Medium

In this manuscript, the authors report the characterization of mice lacking a cilia-associated kinase, ICK. ICK appears to be a very interesting protein - a kinase related to other kinases known to regulate cilia length that is enriched at cilia tips. This is extremely provocative, as many interesting but illusive regulatory events are believed to take place at cilia tips.

The authors show that ICK mutants die at the end of gestation with phenotypes associated with a mild disruption of cilia-dependent Hedgehog signaling (e.g. polydactyly and a small cerebellum). They attribute these organismal phenotypes to structural defects in cilia, which is plausible given their data. They propose that ICK plays a role in the reorganization of IFT complexes that must happen at cilia tips to reorganize the IFT complexes as they switch from anterograde to retrograde trafficking; this would be an important finding. To define the ICK mechanism of action, they identify Kif3a, an essential subunit of the kinesin that directs anterograde trafficking, as a candidate substrate; these experiments are unfortunately not convincing.

The defects in the ICK mutants appear to be surprisingly cell-type specific. For example, cilia in the E10.5 neural tube of the midgestation embryo, although not examined directly (e. g. by SEM) are normal enough to promote normal Shh-dependent neural patterning. But at E15.5, there are few cilia in a similar neural progenitor population in the cerebral cortex. These are unexpected results, and need to be supported by analysis of ICK expression in these tissues and stages.

The cilia phenotypes are interesting, but they are analyzed at rather low resolution. The authors should compare the ICK cilia phenotype to the phenotype of Bromi mutants (Ko et al., 2010), as Bromi binds to CCRK, an ICK substrate.

The weakest part of the paper is the attempt to demonstrate that Kif3a is a relevant ICK substrate. They show that ICK can phosphorylate Kif3a *in vitro*. They generate an antibody that recognizes phosphorylated Kif3a, which shows a lovely localization to cilia tips in NIH 3T3 cells (although there is no staining a marker at the base of the cilium, so it might localize to the base rather than the tip). However they fail to do the obvious control: they need to test whether the cilia tip signal for phospho-Kif3a is lost in ICK mutant MEF cilia. The zebrafish experiments they do carry out to test the importance of phosphorylation actually tests whether the C-terminal 50 amino acids of the protein is important for function, not whether phosphorylation is important.

Additional points:

1. In Page 8 the authors claim that Smo is localized to "shortened" cilia in ICK^{-/-}. Measurements of cilia length are needed to support this conclusion.
2. It doesn't make sense that the ICK Nes CKO does not exhibit hydrocephaly while ICK^{-/-} does.
3. In Fig 1N, Fig S3 W and X the authors show that only about 20% of ICK^{-/-} MEFs form cilia, comparing to 80% in the wild type. The markers used to label cilia are acetylated α -tub. ACIII and Arl13b. However, in page 13, it reads " Smo signal displayed on cilia-like structure in the vicinity of centriole in more than 80% ICK^{-/-} MEFs. This result shows that ciliary structure is retained in ICK^{-/-} MEFs." This appears to be internally contradictory.
4. For ICK^{-/-} and ICK CKO experiments, Western blot analysis is needed to confirm the absence of ICK protein at indicated time points.
5. It appears that in ICK CKO mutants, cilia in both cortex and photoreceptors are longer than wild type. Given that longer cilia are reported in Mak mutants, measurements of cilia length in those experiments should be included.
6. The authors claim that the developmental defects in ICK^{-/-} or ICK CKO could be attributed to loss of Shh signaling. Analysis of Gli1 or Patched expression in ICK^{-/-} embryos would be required to determine whether those defects are directly related to Shh.
7. ICK^{-/-} MEFs appear to have strong defects in retrograde trafficking and accumulate most, if not all, IFT proteins at cilia tip. Shh proteins, including Smo and Gli accumulate in the cilia regardless of pathway activity. Those phenotypes are similar to mutants that lack retrograde motor dynein, which the authors should consider. Are those defects present in cilia of mesenchymal or neural epithelial cells?
8. In Fig1O, the authors show that cilia in ICK^{-/-} MEFs are shorter than wild type. Does expression of ICK wt construct rescue cilia length? Please include the measurement in Fig 5. Can the author compare the endogenous level of ICK to that of rescue or over-expression of ICK? Does over-expression of ICK induce longer cilia? In fact the ICK expression vector is under a CAG promoter, which almost guarantees over-expression of the protein.
9. Can the authors speculate how ICK gets to the cilia tip? If ICK were trafficked by Kinesin-II, would ICK phosphorylate Kif3a at cilia base instead of cilia tip? Figure 1J shows there is ICK signal at cilia base.
10. In the model, the author propose that lack of ICK leads to excessive assembly of IFT complex at cilia tip because of accumulation of IFT particles seen in ICK^{-/-} cilia tips. In fact, accumulations of IFT proteins and Smo in the cilia have been reported in mutants that lack cytoplasmic dynein heavy chain 2, the retrograde IFT motor. Therefore, a more plausible explanation for ICK phenotype would be that ICK^{-/-} mutant cilia have defects in IFT retrograde trafficking. Analysis of cilia ultrastructure would make it possible to determine whether the ICK mutant cilia resemble dynein mutants or bromi mutants, or have a distinct effect on cilia structure.

Referee #2 (Report):

Technical Quality: Adequate
Novelty: Medium
General Interest: Medium

Review of manuscript by Chaya et al.

In the manuscript titled "ICK is essential for ciliogenesis and the regulation of protein transport at

ciliary tips" Chaya et al. describe the function of the vertebrate protein kinase ICK. The authors describe the phenotypes associated with ICK (conditional) knockout mice, which are highly suggestive of ciliary pathology and abrogated Hedgehog signalling. This is consistent with known ciliary roles of related MAP kinase proteins from various organisms, including mammalian MAK and CCRK, *Chlamydomonas* LF4, *C. elegans* DYF-5, etc., in intraflagellar transport (IFT) and cilium length control. Complementary experiments in MEFs, showing for example how a human patient mutation does not rescue the function of ICK, and studies in zebrafish, round out what is a fairly detailed study on this ICK kinase.

The manuscript is on the whole well written, and aside from issues noted below, presents a series of experiments that are generally well conducted and presented. However, there is one particular major point (#5) below that is particularly problematic. Other major points are noted below, together with some minor points, that would help to improve the manuscript.

MAJOR POINTS

1. The authors claim that although cilia are almost entirely lacking in the ICK^{-/-} brain, they are present and 'normal' in the nasal pit. Fig. 2GH suggests the presence of cilia in the nasal pit because of AC3 staining, but the resolution of the image shown does not clearly indicate that the cilia are present (as opposed to AC3 being found outside of a missing cilium, for example) and certainly do not indicate that the cilia are 'normal'. The authors should provide superior images revealing the presence of cilia in this tissue. Furthermore, it is of interest that some cilia are present in some tissues, but not in the brain. To address this interesting observation better and complement the studies carried out in mature photoreceptors (where they show some or all? cilia are present), the authors can easily show the presence or absence of cilia in at least two more tissues, e.g., kidney cells.
2. The absence of neural tube defects in the ICK^{-/-} mice is of interest. The authors claim localization to 'shortened' cilia (Fig. S3Q, R) in this tissue, they do not quantitate these results, and state that there are "some ciliary abnormality in the ICK^{-/-} neural tube". Such a statement is vague and insufficient to understand the nature/scope of the ciliary defect; what percentage of cells are ciliated (as they quantitate for MEFs in Fig. S3)? Can the authors provide information about the length distribution of these cilia?
3. In the various experiments carried out with MEFs, it is not clear what proportion of cells have cilia, or do not, and if they have cilia, whether they have 'short' cilia or not. These data are essential for the reader to understand the results. For example, in Fig. S6, the percent cilia with Gli2 is noted, but what is the percent ciliation? Is it, as suggested in Fig. 5E, approximately 10%? It would also be useful to know the distribution of lengths for the cilia (as mentioned above), and both of these data should be mentioned in the text, to ease the interpretation of the results for the author; for example, on page 13 of the manuscript, in the first paragraph the authors state: "This result shows that ciliary structure is retained in ICK^{-/-} MEFs." However, in the third paragraph, a new section, the authors describe the rescuing of ciliogenesis in MEFs with wild type ICK (and not by the ECO-associated or kinase-dead variants).
4. Because ICK and MAK or CCRK may have overlapping targets, and thus, potentially overlapping functions, one hypothesis for the tissue specific effects of ICK loss may be upregulation of MAK or CCRK. The authors should test for upregulation of MAK and CCRK in ICK^{-/-} cells and tissues.
5. Hypothesizing that ICK phosphorylates Kif3a directly, which may explain some of the IFT defects observed, the authors show that a form of Kif3a mutated at the T674 residue (in a consensus phosphorylation site) is not a substrate of ICK *in vitro*. This result is very interesting, but it is then perplexing that when the authors attempt to confirm the findings *in vivo*, they do not use this mutant for rescue experiments in zebrafish (they find that overexpression of Kif3a abrogates cilium formation in cultured cells). Rather, they use a plasmid in which the Ser/Thr residues of the last 50 amino acids of Kif3a are changed to alanine. Why? Furthermore, they find that this extensively modified Kif3a ('Kif3a-CA') is unable to rescue Kif3a functionality, and mention that "This result suggests that the C-terminal portion of Kif3a including an ICK phosphorylation site is essential for

normal Kif3a function". It is probably not surprising that Kif3a's function is abrogated, and one can easily argue that the authors are not specifically testing the potential effect of ICK on Kif3a. As such, the mechanistic understanding of ICK function provided by this study is highly limited. In particular, examining only body curvature in zebrafish rescue experiments will not be directly indicative of the function of ICK in cilium formation; authors should examine effects on the cilium itself (including ciliogenesis and distribution of selected IFT proteins).

MINOR POINTS

1. The authors should include brief introductory material on CCRK/broad minded proteins from mouse and *C. elegans*, as they are relevant to the overall roles of MAP kinases in ciliogenesis/cilium length control.
2. The title indicates that ICK is required for ciliogenesis, but this does not appear to be consistent across cell types, thereby overstating its importance.
3. Regarding the Shh pathway protein mislocalization in the knockout, these results do not appear in the abstract, and there is no mention of the Shh pathway in the introduction.
4. The authors do not indicate that their observation is supportive of the data by Burghoorn et al., 2007, which indicates that the worm ortholog of ICK (DYF-5) has been shown to regulate the kinesin II complex. This is supportive of their data indicating that ICK phosphorylates Kif3a (a component of kinesin II). Please incorporate into the discussion.

Resubmission

11 February 2014

Referee #1:

In this manuscript, the authors report the characterization of mice lacking a cilia-associated kinase, ICK. ICK appears to be a very interesting protein - a kinase related to other kinases known to regulate cilia length that is enriched at cilia tips. This is extremely provocative, as many interesting but illusive regulatory events are believed to take place at cilia tips.

The authors show that ICK mutants die at the end of gestation with phenotypes associated with a mild disruption of cilia-dependent Hedgehog signalling (e.g. polydactyly and a small cerebellum). They attribute these organismal phenotypes to structural defects in cilia, which is plausible given their data. They propose that ICK plays a role in the reorganization of IFT complexes that must happen at cilia tips to reorganize the IFT complexes as they switch from anterograde to retrograde trafficking; this would be an important finding. To define the ICK mechanism of action, they identify Kif3a, an essential subunit of the kinesin that directs anterograde trafficking, as a candidate substrate; these experiments are unfortunately not convincing.

Thank you for reviewing our manuscript. We appreciate your understanding of our paper. We sincerely considered your comments. We performed additional experiments and prepared a new version of the manuscript accordingly.

The defects in the ICK mutants appear to be surprisingly cell-type specific. For example, cilia in the E10.5 neural tube of the midgestation embryo, although not examined directly (e. g. by SEM) are normal enough to promote normal Shh-dependent neural patterning. But at E15.5, there are few cilia in a similar neural progenitor population in the cerebral cortex. These are unexpected results, and need to be supported by analysis of ICK expression in these tissues and stages.

Following the referee's comment, we examined *ICK* mRNA expression in the E10.5 neural tube and E15.5 brain by *in situ* hybridization analysis. We found that *ICK* is expressed in both the E10.5 neural tube and E15.5 brain, including the cerebral cortex (Supplementary Figure S1A and B). We analysed cilia in the E10.5 *ICK*^{+/+} and *ICK*^{-/-} neural tube in more detail. We first immunostained these cilia using an anti-Arl13b antibody and found that ciliary numbers and length decrease in the *ICK*^{-/-} neural tube compared to those in the *ICK*^{+/+} neural tube (Supplementary Figure S3Q–T). We also observed E10.5 *ICK*^{-/-} neural tube cilia using scanning electron microscopy. We found that *ICK*^{-/-} neural tube cilia are shorter than those of *ICK*^{+/+} mice (Figure 1J and K). To examine ciliary localization of Hedgehog signalling components in the E10.5 *ICK*^{+/+} and *ICK*^{-/-} neural tube, we immunostained the cilia using antibodies against polyglutamylated tubulin and Smo. We found an accumulation of Smo in cilia in the *ICK*^{-/-} neural tube (Figure 4K and L). Previously, *IFT88*^{ko} and *IFT88*^{tm1Rpw} were identified as hypomorphic and null mutants of *IFT88*, respectively (Murcia *et al.*, *Development*, 2000; 127:2347-2355; Huangfu *et al.*, *Nature*, 2003; 426:83-87). Neural tube patterning defects observed in *IFT88*^{ko} embryos are milder than those in *IFT88*^{tm1Rpw} mice (Huangfu *et al.*, *Nature*, 2003; 426:83-87). While *IFT88*^{ko} mice develop shortened cilia, *IFT88*^{tm1Rpw} mutants lack cilia (Huangfu *et al.*, *Nature*, 2003; 426:83-87; Liu *et al.*, *Development*, 2005; 132:3103-3111). These observations suggest that the severity of neural tube patterning phenotypes is related to that of ciliary abnormalities. Ciliary defects in the neural tube caused by loss of *ICK* may not be severe enough to disrupt neural tube patterning. We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 6, line 4 – page 6, line 6)

“We observed that *ICK* mRNA is expressed in the embryonic day 10.5 (E10.5) neural tube and E15.5 brain including the cerebral cortex (Supplementary Figure S1A and B).”

(Page 8, line 10 – page 8, line 14)

“To investigate the ciliary integrity of neural progenitors, we examined cilia in the neural tube by immunohistochemistry. We found that cilia numbers and lengths decrease in the *ICK*^{-/-} neural tube (Supplementary Figure S3Q–T). To analyse the ultrastructure of neural tube cilia, we performed scanning electron microscopic analysis. We found that ciliary length decreases in the *ICK*^{-/-} neural tube (Figure 1J and K).”

(Page 8, line 7 from the bottom – page 8, line 6 from the bottom)

“Although we observed ciliary defects in the *ICK*^{-/-} neural tube, it may not be severe enough to disrupt neural tube patterning.”

(Page 14, line 9 from the bottom – page 14, line 8 from the bottom)

“Smo signals in the cilia increased in the E10.5 *ICK*^{-/-} neural tube and paraxial mesoderm (Figure 4K–N).”

The cilia phenotypes are interesting, but they are analysed at rather low resolution. The authors should compare the ICK cilia phenotype to the phenotype of Bromi mutants (Ko et al., 2010), as Bromi binds to CCRK, an ICK substrate.

To compare the cilia phenotype of *ICK*^{-/-} mice with that of *bromi* mutants at high resolution, we observed E10.5 *ICK*^{-/-} neural tube cilia using scanning electron microscopy. We observed that *ICK*^{-/-} neural tube cilia are shorter than that of *ICK*^{+/+} mice (Figure 1J and K). While *bromi* mutant cilia display a swollen or bulbous morphology (Ko *et al.*, *Dev. Cell*, 2010; 18:237-247), cilia in the *ICK*^{-/-} neural tube did not show that morphology. We added this data to the new manuscript, modified the text, and added descriptions in the Introduction, Results, and Discussion as follows:

(Page 4, line 8 from the bottom – page 4, line 5 from the bottom)

“It was reported that ICK is a substrate of Cell cycle-related kinase (Ccrk) (Fu *et al.*, 2006). A mutant of *Dyf-18*, the *C. elegans* ortholog of *Ccrk*, occasionally forms long curved cilia (Phirke *et al.*, 2011). Broad-minded (*Bromi*), which interacts with *Ccrk*, is required for the formation of proper structure in cilia (Ko *et al.*, 2010).”

(Page 8, line 13 from the bottom – page 8, line 7 from the bottom)

“To analyse the ultrastructure of neural tube cilia, we performed scanning electron microscopic analysis. We found that ciliary length decreases in the *ICK*^{-/-} neural tube (Figure 1J and K). Mutations in *Dync2h1*, which encodes the heavy chain of the cytoplasmic dynein-2 motor, or *Bromi* cause morphological changes in the cilia (Ko *et al.*, 2010; Ocbina *et al.*, 2011). Unlike *Dync2h1* or *bromi* mutants, cilia in the *ICK*^{-/-} neural tube did not show a swollen morphology.”

(Page 23, line 12 – page 23, line 15)

“A mutation in *Bromi*, which encodes an interaction partner of *Ccrk*, leads to a swollen or bulbous morphology in cilia (Ko *et al.*, 2010), whereas *ICK*^{-/-} neural tube cilia do not display that morphology. Although ICK is a substrate of *Ccrk* (Fu *et al.*, 2006), ICK may have a distinct function from that of *Bromi*.”

The weakest part of the paper is the attempt to demonstrate the Kif3a is a relevant ICK substrate. They show that ICK can phosphorylate Kif3a in vitro. They generate an antibody that recognizes phosphorylated Kif3a, which shows a lovely localization to cilia tips in NIH 3T3 cells (although there is no staining a marker at the base of the cilium, so it might localize to the base rather than the tip). However they fail to do the obvious control: they need to test whether the cilia tip signal for phospho-Kif3a is lost in ICK mutant MEF cilia.

Following the referee’s suggestion, instead of NIH3T3 cells, we immunostained *ICK*^{+/+} and *ICK*^{-/-} MEFs using antibodies against phosphorylated Kif3a Thr-674 (p-Kif3a) and acetylated α -tubulin. We observed that p-Kif3a localizes to the cilia in *ICK*^{+/+} MEFs, and p-Kif3a signals were often enriched at ciliary bases and tips (Figure 7D). On the other hand, the proportion of cilia with p-Kif3a at ciliary tips decreased in *ICK*^{-/-} MEFs (Figure 7E). This result suggests that Kif3a is phosphorylated by ICK at the tips of cilia. We added this data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 17, the last line – page 18, line 3)

“We found that p-Kif3a localizes to the cilia in *ICK*^{+/+} MEFs, and that p-Kif3a signals were often enriched at ciliary bases and tips (Figure 7D). In *ICK*^{-/-} MEFs, the proportion of cilia with p-Kif3a at ciliary tips decreased (Figure 7E).”

The zebra fish experiments they do carry out to test the importance of phosphorylation actually tests whether the C-terminal 50 amino acids of the protein is important for function, not whether phosphorylation is important.

To examine the role of phosphorylation of Kif3a in ciliary formation, we generated a construct expressing the Kif3a mutant in which 8 serine or threonine residues clustered in the C-terminal region, including residue 674, are replaced with alanines (Kif3a-8xA) in the previous version of the manuscript (Figure 7F). To clarify the numbers of the substituted serine or threonine residues in the C-terminal region of Kif3a, we replaced the name Kif3a-CA with Kif3a-8xA throughout the manuscript. In the previous version of the manuscript, we observed that Kif3a-8xA has a decreased ability to rescue the *Kif3a* antisense morpholino (*Kif3a* MO)-induced curly tail phenotype compared to that of Kif3a-WT (Figure 7M and N) (Figure 7D and E in the previous version of the manuscript).

In the new version of the manuscript, we investigated the role of phosphorylation of Kif3a in ciliary formation in more detail.

We first performed rescue experiments using NIH3T3 cells (Figure 7F–L). We previously tried to perform rescue experiments using RPE-1 cells, however, in our experimental conditions, overexpression of even Kif3a-WT inhibited ciliary formation in these cells, making us unable to observe the effect of the mutant form of Kif3a in ciliary formation. We, therefore, used NIH3T3 cells instead of RPE-1 cells to perform rescue experiments. FLAG-tagged constructs expressing GFP or shRNA-resistant Kif3a (WT or T674A) were transfected with control shRNA or shKif3a into NIH3T3 cells. Cells were immunostained with anti-FLAG and anti-acetylated α -tubulin antibodies. We found that this shKif3a inhibits ciliary formation (Figure 7H and L). Expression of shRNA-resistant Kif3a-WT rescued shKif3a-mediated inhibition of ciliation (Figure 7I and L). Unexpectedly, shRNA-resistant Kif3a-T674A had a slightly increased ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT (Figure 7J and L). Since ciliary numbers decreased in *ICK*^{-/-} cells, we first expected that Kif3a-T674A would show a reduced ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT. The reason why the effect of Kif3a-T674A on ciliary formation in knockdown and rescue experiments using cultured cells is the opposite of the results obtained from *ICK* knockout experiments is unclear, however, a recent study reported that knockdown of *ICK* and *Mak* promotes ciliary formation in cultured cells (Yang *et al.*, *EMBO Rep.*, 2013; 14:741-747). Suppression of the expression of *ICK* may promote ciliary formation through reducing the level of Kif3a phosphorylation at residue 674 in cultured cells. On the other hand, since Kif3a-C-T674A was still phosphorylated by *ICK* (Figure 7C), we thought that phosphorylation of other serine or threonine residues in the C-terminal region of Kif3a may also be important for Kif3a function in ciliary formation. To further analyse the role of phosphorylation of the Kif3a C-terminal region in ciliary formation, we constructed a plasmid encoding the Kif3a mutant, Kif3a-8xA, as described above (Figure 7F). Expression of shRNA-resistant Kif3a-8xA failed to rescue shKif3a-mediated inhibition of ciliary formation (Figure 7K and L). These results suggest that phosphorylation of the C-terminal region of Kif3a affects ciliary formation in cultured cells.

Furthermore, to investigate the role of phosphorylation of Kif3a in ciliary formation *in vivo*, we performed rescue experiments using zebra fish embryos and observed the nasal cilia. At 3 dpf, cilia have developed in nasal pit epithelia in control larvae (Figure 7O and S). In contrast, larvae injected with Kif3a MO and GFP lost cilia at this stage (Figure 7P and S). Loss of cilia by Kif3a MO injection was partially rescued by co-injection with Kif3a-WT mRNA (Figure 7Q and S). However, co-injection with Kif3a-8xA mRNA failed to rescue the ciliary loss by knockdown of *Kif3a* (Figure 7R and S). Taken together, these results suggest that phosphorylation of the C-terminal portion of Kif3a is essential for normal cilia formation.

Interestingly, a majority of shortened cilia lost p-Kif3a signals at the tips of cilia (left and middle panels in Figure 7E), whereas cilia of normal length often had p-Kif3a signals at ciliary tips in *ICK*^{-/-} MEFs (right panels in Figure 7E). This result suggests that phosphorylation of Kif3a at residue 674 is linked to ciliary formation. We added these data to the new manuscript, modified the text, and added descriptions in the Abstract, Introduction, Results, and Discussion as follows:

(Page 2, line 5 from the bottom – page 2, line 3 from the bottom)

“In addition, *ICK* directly phosphorylated Kif3a, while inhibition of this Kif3a phosphorylation affected ciliary formation”

(Page 5, line 4 from the bottom – page 5, line 2 from the bottom)

“We also found that *ICK* directly phosphorylates Kif3a, a subunit of Kinesin-2, and that Kif3a phosphorylation is required for normal cilia formation *in vivo*.”

(Page 17, line 9 – page 17, line 10)

“Kif3a is phosphorylated by *ICK* and Kif3a phosphorylation is required for ciliary formation”

(Page 18, line 3 – page 19, line 3)

“Interestingly, a majority of shortened cilia lost p-Kif3a signals at the tips of cilia (left and middle panels in Figure 7E), whereas cilia of normal length often had p-Kif3a signals at ciliary tips in *ICK*^{-/-} MEFs (right panels in Figure 7E). This result suggests that Kif3a is phosphorylated by ICK at ciliary tips, and that phosphorylation of Kif3a at residue 674 is linked to ciliary formation.

To investigate the role of phosphorylation of Kif3a in ciliary formation, we performed rescue experiments using NIH3T3 cells (Figure 7F–L). We constructed a short hairpin RNA (shRNA) to knockdown *Kif3a* (Supplementary Figure S8D), and found that shKif3a inhibits ciliary formation (Figure 7H and L). Expression of shRNA-resistant Kif3a-WT rescued shKif3a-mediated inhibition of ciliation (Figure 7I and L). Unexpectedly, shRNA-resistant Kif3a-T674A had a slightly increased ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT (Figure 7J and L). Since Kif3a-C-T674A was still phosphorylated by ICK (Figure 7C), we thought that phosphorylation of other serine or threonine residues in the C-terminal region of Kif3a may also be important for Kif3a function in ciliary formation. To further analyse the role of phosphorylation of the Kif3a C-terminal region in ciliary formation, we constructed a plasmid encoding the Kif3a mutant in which 8 Ser or Thr residues clustered in the C-terminal region, including residue 674, are replaced with Ala (Kif3a-8xA) (Figure 7F). Expression of shRNA-resistant Kif3a-8xA failed to rescue shKif3a-mediated inhibition of ciliary formation (Figure 7K and L). These results suggest that phosphorylation of the C-terminal region of Kif3a affects ciliary formation in cultured cells.

To investigate the role of phosphorylation of Kif3a in ciliary formation *in vivo*, we performed rescue experiments using zebra fish embryos.”

(Page 19, line 9 from the bottom – page 19, the last line)

“We also investigated ciliogenesis in the nasal pits of the larvae injected with the morpholino and mRNA (Figure 7O–S). At 3 dpf, cilia have developed in nasal pit epithelia in control larvae (Figure 7O and S). In contrast, larvae injected with Kif3a MO and GFP lost cilia at this stage (Figure 7P and S). Loss of cilia by Kif3a MO injection was partially rescued by co-injection with Kif3a-WT mRNA (Figure 7Q and S). However, co-injection with Kif3a-8xA mRNA failed to rescue the ciliary defect by knockdown of *Kif3a* (Figure 7R and S). Taken together, these results suggest that phosphorylation of the C-terminal portion of Kif3a is essential for normal ciliary formation.”

(Page 22, line 7 from the bottom – page 23, line 4)

“We found that shRNA-resistant Kif3a-T674A has a slightly increased ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT. Since ciliary numbers decreased in *ICK*^{-/-} cells, we first expected Kif3a-T674A would show reduced ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT. The reason why the effect of Kif3a-T674A on ciliary formation in knockdown and rescue experiments using cultured cells is the opposite of the results obtained from *ICK* knockout experiments is unclear, however, the study by Yang *et al.* reported that knockdown of *ICK* and *Mak* promote ciliary formation in cultured cells (Yang *et al.*, 2013). Suppression of the expression of *ICK* may promote ciliary formation through reducing the level of Kif3a phosphorylation at residue 674 in cultured cells.”

(Page 24, line 9 from the bottom – page 24, line 8 from the bottom)

“The inhibition of phosphorylation of Kif3a affected its function in ciliary formation.”

Additional points:

1. In Page 8 the authors claim that *Smo* is localized to "shortened" cilia in *ICK*^{-/-}. Measurements of cilia length are needed to support this conclusion.

We immunostained cilia in the E10.5 $ICK^{+/+}$ and $ICK^{-/-}$ neural tube using an anti-Arl13b antibody and measured ciliary length. We found that ciliary length decreases in the $ICK^{-/-}$ neural tube (Supplementary Figure S3Q–R' and T). We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 8, line 10 – page 8, line 12)

“To investigate the ciliary integrity of neural progenitors, we examined cilia in the neural tube by immunohistochemistry. We found that cilia numbers and lengths decrease in the $ICK^{-/-}$ neural tube (Supplementary Figure S3Q–T).”

2. *It doesn't make sense that the ICK Nes CKO does not exhibit hydrocephaly while ICK-/- does.*

Thank you for pointing this out. We simply forgot to present this data in the previous version of the manuscript. We performed Nissl staining of sagittal sections from the P21 control and $ICK Nes$ CKO brain. We observed no obvious difference in the size of lateral ventricles between the control and $ICK Nes$ CKO brain (Supplementary Figure S5H and I). It was recently reported that ciliary defects cause increased apoptosis and impaired proliferation of $NG2^+PDGFR-a^+$ neural progenitors, resulting in neonatal hydrocephalus (Carter *et al.*, *Nat. Med.*, 2012; 18:1797-1804), suggesting that hydrocephalus observed in $ICK^{-/-}$ mice is due to ciliary abnormalities of those progenitors. On the other hand, defects of ependymal cilia cause progressive hydrocephalus at postnatal stages (Tissir *et al.*, *Nat. Neurosci.*, 2010; 13:700-707). It is known that cilia in ependymal cells are formed after birth (Spassky *et al.*, *J. Neurosci.*, 2005; 25:10-18). To investigate the effect of loss of ICK on ependymal cilia, we immunostained P4 control and $ICK Nes$ CKO brains using an anti-acetylated α -tubulin antibody. We did not observe any obvious differences in the ciliary formation of ependymal cells between P4 control and $ICK Nes$ CKO brains (Supplementary Figure S5V and W). Perinatal hydrocephalus caused by the absence of ICK may be compensated for in postnatal development. We added these data to the new manuscript, modified the text, and added descriptions in the Results and Discussion as follows:

(Page 11, line 4 – page 11, line 6)

“In contrast to the $ICK^{-/-}$ mice, no expanded ventricle was observed in the $ICK Nes$ CKO brain at P21 (Supplementary Figure S5H and I).”

(Page 12, line 9 from the bottom – page 12, line 7 from the bottom)

“We did not observe any obvious differences in ependymal cell ciliary formation between the P4 control and $ICK Nes$ CKO brain (Supplementary Figure S5V and W).”

(Page 21, line 3 – page 21, line 12)

“We observed that $ICK^{-/-}$ mice display hydrocephalus, whereas $ICK Nes$ CKO mice did not. It was recently reported that ciliary defects cause increased apoptosis and impaired proliferation of $NG2^+PDGFR-a^+$ neural progenitors, resulting in neonatal hydrocephalus (Carter *et al.*, 2012), suggesting that the hydrocephalus observed in $ICK^{-/-}$ mice is due to ciliary abnormalities in those progenitors. On the other hand, defects of ependymal cilia cause progressive hydrocephalus at postnatal stages (Tissir *et al.*, 2010). It is known that cilia in ependymal cells are formed after birth (Spassky *et al.*, 2005). We did not observe any obvious differences in ciliary formation of ependymal cells between the P4 control and $ICK Nes$ CKO brain. Perinatal hydrocephalus caused by the absence of ICK may be compensated for in postnatal development.”

3. *In Fig 1N, Fig S3 W and X the authors show that only about 20% of ICK-/- MEFs form cilia, comparing to 80% in the wild type. The markers used to label cilia are acetylated α -tub. ACIII and Arl13b. However, in page 13, it reads " Smo signal displayed on cilia-like structure in the vicinity of*

centriole in more than 80% ICK^{-/-} MEFs. This result shows that ciliary structure is retained in ICK^{-/-} MEFs." This appears to be internally contradictory.

About 10% of ICK^{-/-} MEFs formed acetylated α -tubulin-positive cilia (Figure 1N–P), whereas more than 80% of ICK^{-/-} MEFs had Smo signals in the vicinity of centrioles with cilia-like structures (Supplementary Figure S6A–D). Since acetylated α -tubulin is a marker for the ciliary axoneme and Smo is a membrane protein, these data suggest that ICK^{-/-} MEF cilia have ciliary membrane structures without acetylated α -tubulin-positive microtubules. Although AC3 and Arl13b are ciliary membrane proteins, we observed that about 10% of ICK^{-/-} MEFs form AC3-positive cilia and that about 30% of ICK^{-/-} MEFs have Arl13b-positive cilia (Supplementary Figure S3U–Z). The transport system for AC3 and Arl13b in cilia may be different from that for Smo. We added this comment to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 14, line 2 – page 14, line 9)

“We found that about 10% of ICK^{-/-} MEFs formed acetylated α -tubulin-positive cilia (Figure 1N–P). Since acetylated α -tubulin is a marker for the ciliary axoneme and Smo is a membrane protein, these data suggest that ICK^{-/-} MEF cilia have ciliary membrane structures without acetylated α -tubulin-positive microtubules. Although AC3 and Arl13b are ciliary membrane proteins, we observed that about 10% of ICK^{-/-} MEFs form AC3-positive cilia and that about 30% of ICK^{-/-} MEFs have Arl13b-positive cilia (Supplementary Figure S3U–Z). The transport system for AC3 and Arl13b in cilia may be different from that for Smo.”

4. For ICK^{-/-} and ICK CKO experiments, Western blot analysis is needed to confirm the absence of ICK protein at indicated time points.

Following the referee’s comment, we examined ICK protein expression in ICK^{-/-} MEFs, the P0 ICK *Dkk3* CKO retina, and the P4 ICK *Nes* CKO brain by Western blot analysis. We confirmed that ICK protein expression is lost in all of these cells or tissues (Supplementary Figure S2B, 4B and 5E). We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 6, line 10 from the bottom – page 6, line 8 from the bottom)

“Absence of ICK mRNA and protein expression in the ICK^{-/-} mice was confirmed (Supplementary Figure S2A and B).”

(Page 9, line 11 from the bottom – page 9, line 10 from the bottom)

“ICK protein expression was lost in the ICK *Dkk3* CKO retina (Supplementary Figure S4B).”

(Page 10, line 7 from the bottom – page 10, line 6 from the bottom)

“ICK protein expression was lost in the ICK *Nes* CKO brain (Supplementary Figure S5E).”

5. It appears that in ICK CKO mutants, cilia in both cortex and photoreceptors are longer than wild type. Given that longer cilia are reported in Mak mutants, measurements of cilia length in those experiments should be included.

We presented data about the lengths of neuronal cilia in the control and ICK *Nes* CKO cerebral cortex at one month of age in the previous version of the manuscript (Figure 3I). There was no significant difference in ciliary length between the control and ICK *Nes* CKO cerebral cortex. According to the referee’s comment, we measured the ciliary length of photoreceptors in the adult

control and *ICK Dkk3* CKO retina. We immunostained ciliary axoneme and connecting cilia using antibodies against acetylated- α -tubulin and RPGR, respectively. We observed no significant difference in length of ciliary axoneme and connecting cilia between control and *ICK Dkk3* CKO photoreceptors (Supplementary Figure S4N and O). We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 10, line 3 – page 10, line 5)

“We observed no obvious difference in the number and length of the ciliary axoneme and connecting cilia between control and *ICK Dkk3* CKO retinas (Supplementary Figure S4J–O).”

6. The authors claim that the developmental defects in ICK^{-/-} or ICK CKO could be attributed to loss of Shh signalling. Analysis of Gli1 or Patched expression in ICK^{-/-} embryos would be required to determine whether those defects are directly related to Shh.

According to the referee’s suggestion, we examined *Gli1* mRNA expression in the E15.5 *ICK^{+/-}* and *ICK^{-/-}* brain by Q-PCR. We found that *Gli1* mRNA expression is significantly decreased in the *ICK^{-/-}* brain compared to that in *ICK^{+/-}* brain. We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 7, line 6 – page 7, line 7)

“The expression of *Gli1*, a downstream gene of the Shh signalling cascade, decreased in the *ICK^{-/-}* brain (Supplementary Figure S2I).”

7. ICK^{-/-} MEFs appear to have strong defects in retrograde trafficking and accumulate most, if not all, IFT proteins at cilia tip. Shh proteins, including Smo and Gli accumulate in the cilia regardless of pathway activity. Those phenotypes are similar to mutants that lack retrograde motor dynein, which the authors should consider. Are those defects present in cilia of mesenchymal or neural epithelial cells?

According to the referee’s comment, we examined ciliary localization of Smo in the E10.5 *ICK^{+/+}* and *ICK^{-/-}* neural tube and paraxial mesoderm. We immunostained these tissues using antibodies against polyglutamylated tubulin and Smo. We found an accumulation of Smo in cilia in the *ICK^{-/-}* neural tube and paraxial mesoderm (Figure 4K–N). We next investigated ciliary localization of IFT88 in the E10.5 *ICK^{+/+}* and *ICK^{-/-}* neural tube. We immunostained the neural tube using antibodies against Arl13b and IFT88. We observed no obvious difference in ciliary localization of IFT88 between *ICK^{+/+}* and *ICK^{-/-}* embryos (Supplementary Figure S6O–P’). Unlike *Ift122^{sopb}* mutant mice, which show defects in retrograde ciliary transport (Qin *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2011; 108:1456-1461), *ICK^{-/-}* neural tube cilia did not show an accumulation of IFT88 at the ciliary tips. Although *Ift122^{sopb}* mutant mice show defects in the neural tube patterning, we did not observe any obvious differences in patterning between *ICK^{+/+}* and *ICK^{-/-}* embryos (Supplementary Figure S3A–P). Abnormalities of IFT localization caused by loss of *ICK* may be weaker than those due to defects in retrograde ciliary transport. We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 14, line 9 from the bottom – page 14, line 8 from the bottom)

“Smo signals in the cilia increased in the E10.5 *ICK^{-/-}* neural tube and paraxial mesoderm (Figure 4K–N).”

(Page 16, line 9 from the bottom – page 16, line 6 from the bottom)

“Unlike *Ift122^{sopb}* mutant mice, which show defects in retrograde IFT (Qin *et al.*, 2011), we did not observe an accumulation of IFT88 at the tips of cilia in the *ICK^{-/-}* neural tube at E10.5 (Supplementary Figure S6O–P).”

8. In Fig1O, the authors show that cilia in *ICK^{-/-}* MEFs are shorter than wild type. Does expression of *ICK* wt construct rescue cilia length? Please include the measurement in Fig 5. Can the author compare the endogenous level of *ICK* to that of rescue or over-expression of *ICK*? Does over-expression of *ICK* induce longer cilia? In fact the *ICK* expression vector is under a CAG promoter, which almost guarantees over-expression of the protein.

We transfected constructs expressing FLAG-tagged GFP or *ICK*-WT into *ICK^{-/-}* MEFs and immunostained cilia using antibodies against acetylated- α -tubulin and FLAG. We found that expression of *ICK*-WT rescues ciliary length in *ICK^{-/-}* MEFs (Figure 5F). We compared the *ICK* protein expression level in non-transfected cells to that in *ICK*-overexpressing cells. We transfected constructs expressing FLAG-tagged *ICK*-WT into NIH3T3 cells and immunostained cilia using antibodies against acetylated- α -tubulin, FLAG, and *ICK*. We observed that *ICK* signals at ciliary tips increased in *ICK*-overexpressing cells compared to those in control (FLAG-negative) cells (Supplementary Figure S7A and B). To examine whether overexpression of *ICK* induces ciliary elongation, we transfected constructs expressing FLAG-tagged GFP or *ICK*-WT into NIH3T3 cells and immunostained cilia using antibodies against acetylated- α -tubulin and FLAG. We found that overexpression of *ICK* slightly elongates cilia in NIH3T3 cells (Supplementary Figure S7C–E). We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 15, line 6 – page 15, line 7)

“*ICK*-WT rescued ciliary length in *ICK^{-/-}* MEFs (Figure 5F).”

(Page 16, line 3 from the bottom – page 16, the last line)

“We found that *ICK* signals at ciliary tips increase in *ICK*-overexpressing cells (Supplementary Figure S7A and B). Overexpression of *ICK* induced slight ciliary elongation (Supplementary Figure S7C–E).”

9. Can the authors speculate how *ICK* gets to the cilia tip? If *ICK* were trafficked by Kinesin-II, would *ICK* phosphorylate *Kif3a* at cilia base instead of cilia tip? Figure 1J shows there is *ICK* signal at cilia base.

We have no experimental evidence to explain how *ICK* gets to the ciliary tips. If *ICK* is transported to ciliary tips by anterograde IFT, which is driven by Kinesin-2, the activity of *ICK* may be suppressed by unknown mechanisms while this protein is being transported as a cargo. This is because proteins that should function at a distinct cellular region must be properly trafficked to that region by Kinesin and play their roles only after arriving. In Figure 1L (Figure 1J in the previous version), a red signal near the ciliary base is thought to be background for the following reasons: First, when we immunostained cells using an anti-*ICK* antibody, we observed signals in the cytoplasm of both *ICK^{+/+}* and *ICK^{-/-}* MEFs. Although we found that signals at ciliary tips disappear in *ICK^{-/-}* MEFs, those in the cytoplasm did not (Figure 1L and M) (Figure 1J and K in the previous version). Second, we rarely observed signals like this at or near ciliary bases. Thus, we concluded that *ICK* is localized at ciliary tips. We added this comment to the new manuscript, modified the text, and added descriptions in the Results and Discussion as follows:

(Page 8, the last line – page 9, line 1)

“We observed background signals in the cytoplasm of both $ICK^{+/+}$ and $ICK^{-/-}$ MEFs (Figure 1L and M).”

(Page 23, line 9 from the bottom – page 23, line 5 from the bottom)

“How does ICK get to ciliary tips? If ICK is transported to ciliary tips by anterograde IFT, the activity of ICK may be suppressed by unknown mechanisms while this protein is being transported as a cargo. This is because proteins that should function at a distinct cellular region must be properly trafficked to that region by Kinesin and play their roles only after arriving.”

10. In the model, the author propose that lack of ICK leads to excessive assembly of IFT complex at cilia tip because of accumulation of IFT particles seen in $ICK^{-/-}$ cilia tips. In fact, accumulations of IFT proteins and Smo in the cilia have been reported in mutants that lack cytoplasmic dynein heavy chain 2, the retrograde IFT motor. Therefore, a more plausible explanation for ICK phenotype would be that $ICK^{-/-}$ mutant cilia have defects in IFT retrograde trafficking. Analysis of cilia ultrastructure would make it possible to determine whether the ICK mutant cilia resemble dynein mutants or bromi mutants, or have a distinct effect on cilia structure.

Thank you for this comment. To compare the ultrastructure of cilia in $ICK^{-/-}$ mice with that in *Dync2h1* or *bromi* mutants, we observed E10.5 $ICK^{-/-}$ neural tube cilia using scanning electron microscopy. We observed that $ICK^{-/-}$ neural tube cilia are shorter than that of $ICK^{+/+}$ mice (Figure 1J and K). It was reported that *Dync2h1* mutants have a swollen cilia (Ocbina *et al.*, *Nat. Genet.*, 2011; 43:547-553). *bromi* mutant cilia also show a swollen or bulbous morphology (Ko *et al.*, *Dev. Cell*, 2010; 18:237-247). Unlike *Dync2h1* or *bromi* mutants, cilia in the $ICK^{-/-}$ neural tube did not show a swollen or bulbous morphology. Loss of ICK has a distinct effect on ciliary structure. We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 8, line 13 from the bottom – page 8, line 7 from the bottom)

“To analyse the ultrastructure of neural tube cilia, we performed scanning electron microscopic analysis. We found that ciliary length decreases in the $ICK^{-/-}$ neural tube (Figure 1J and K). Mutations in *Dync2h1*, which encodes the heavy chain of the cytoplasmic dynein-2 motor, or *Bromi* cause morphological changes in the cilia (Ko *et al.*, 2010; Ocbina *et al.*, 2011). Unlike *Dync2h1* or *bromi* mutants, cilia in the $ICK^{-/-}$ neural tube did not show a swollen morphology.”

Referee #2:

Review of manuscript by Chaya et al.

In the manuscript titled "ICK is essential for ciliogenesis and the regulation of protein transport at ciliary tips" Chaya et al. describe the function of the vertebrate protein kinase ICK. The authors describe the phenotypes associated with ICK (conditional) knockout mice, which are highly suggestive of ciliary pathology and abrogated Hedgehog signalling. This is consistent with known ciliary roles of related MAP kinase proteins from various organisms, including mammalian MAK and CCRK, Chlamydomonas LF4, C. elegans DYF-5, etc., in intraflagellar transport (IFT) and cilium length control. Complementary experiments in MEFs, showing for example how a human patient mutation does not rescue the function of ICK, and studies in zebra fish, round out what is a fairly detailed study on this ICK kinase.

The manuscript is on the whole well written, and aside from issues noted below, presents a series of experiments that are generally well conducted and presented. However, there is one particular major point (#5) below that is particularly problematic. Other major points are noted below, together with some minor points, that would help to improve the manuscript.

Thank you for reviewing our manuscript. We appreciate your understanding of our paper. We sincerely considered your comments. We performed additional experiments and prepared a new version of the manuscript accordingly.

MAJOR POINTS

1. The authors claim that although cilia are almost entirely lacking in the ICK^{-/-} brain, they are present and 'normal' in the nasal pit. Fig. 2GH suggests the presence of cilia in the nasal pit because of AC3 staining, but the resolution of the image shown does not clearly indicate that the cilia are present (as opposed to AC3 being found outside of a missing cilium, for example) and certainly do not indicate that the cilia are 'normal'. The authors should provide superior images revealing the presence of cilia in this tissue. Furthermore, it is of interest that some cilia are present in some tissues, but not in the brain. To address this interesting observation better and complement the studies carried out in mature photoreceptors (where they show some or all? cilia are present), the authors can easily show the presence or absence of cilia in at least two more tissues, e.g., kidney cells.

According to the referee's comment, we presented higher magnification images of Supplementary Figure S2J and K (Supplementary Figure S2G and H in the previous version) (Supplementary Figure S2J' and K'). To further investigate ciliary formation in ICK^{-/-} mice, we observed cilia in the kidney, skin, and intestine at E15.5 (Supplementary Figure S2L–Q). Cilia in the nephric duct exhibited no obvious change between ICK^{+/+} and ICK^{-/-} embryos (Supplementary Figure S2L and M). We found fewer cilia in the epidermis and dermis of the skin of ICK^{-/-} embryos (Supplementary Figure S2N and O). In addition, there were markedly fewer cilia in the intestinal muscular layers and submucosa of ICK^{-/-} mice (Supplementary Figure S2P and Q). These results suggest that ICK is essential for ciliogenesis in a tissue-specific manner. We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 7, line 6 from the bottom – page 8, line 4)

“In contrast, epithelial cilia in the nasal pit were normal in the ICK^{-/-} mice (Supplementary Figure S2J–K'). To further investigate the effect of loss of ICK on ciliary formation, we observed cilia in the kidney, skin, and intestine at E15.5 (Supplementary Figure S2L–Q). Cilia in the nephric duct exhibited no obvious change between ICK^{+/+} and ICK^{-/-} embryos (Supplementary Figure S2L and M). We found fewer cilia in the epidermis and dermis of the skin of ICK^{-/-} embryos (Supplementary Figure S2N and O). In addition, there were markedly fewer cilia in the intestinal muscular layers and submucosa of ICK^{-/-} mice (Supplementary Figure S2P and Q). These results suggest that ICK is essential for ciliogenesis in a tissue-specific manner.”

2. The absence of neural tube defects in the ICK^{-/-} mice is of interest. The authors claim localization to 'shortened' cilia (Fig. S3Q, R) in this tissue, they do not quantitate these results, and state that there are "some ciliary abnormality in the ICK^{-/-} neural tube". Such a statement is vague and insufficient to understand the nature/scope of the ciliary defect; what percentage of cells are ciliated (as they quantitate for MEFs in Fig. S3)? Can the authors provide information about the length distribution of these cilia?

According to the referee's suggestion, we analysed cilia in the E10.5 ICK^{+/+} and ICK^{-/-} neural tube in more detail. We immunostained these cilia using an anti-Arl13b antibody and found that ciliary

numbers and length decrease in the $ICK^{-/-}$ neural tube compared to those in the $ICK^{+/+}$ neural tube (Supplementary Figure S3Q and R). We also quantified these results in Supplementary Figure S3S and T. We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 8, line 10 – page 8, line 12)

“To investigate the ciliary integrity of neural progenitors, we examined cilia in the neural tube by immunohistochemistry. We found that cilia numbers and lengths decrease in the $ICK^{-/-}$ neural tube (Supplementary Figure S3Q–T).”

(Page 8, line 7 from the bottom – page 8, line 6 from the bottom)

“Although we observed ciliary defects in the $ICK^{-/-}$ neural tube, it may not be severe enough to disrupt neural tube patterning.”

3. In the various experiments carried out with MEFs, it is not clear what proportion of cells have cilia, or do not, and if they have cilia, whether they have 'short' cilia or not. These data are essential for the reader to understand the results. For example, in Fig. S6, the percent cilia with Gli2 is noted, but what is the percent ciliation? Is it, as suggested in Fig. 5E, approximately 10%? It would also be useful to know the distribution of lengths for the cilia (as mentioned above), and both of these data should be mentioned in the text, to ease the interpretation of the results for the author; for example, on page 13 of the manuscript, in the first paragraph the authors state: "This result shows that ciliary structure is retained in $ICK^{-/-}$ MEFs." However, in the third paragraph, a new section, the authors describe the rescuing of ciliogenesis in MEFs with wild type ICK (and not by the ECO -associated or kinase-dead variants).

We presented the data about ciliary numbers and length in $ICK^{+/+}$ and $ICK^{-/-}$ MEFs in the previous version of the manuscript (Figure 1N–Q; Supplementary Figure S3U–Z (Figure 1L–O; Supplementary Figure S3S–X in the previous version)). We immunostained these cilia using antibodies against acetylated α -tubulin, AC3, and Arl13b and found that ciliary numbers and length decrease in $ICK^{-/-}$ MEFs. While we observed that about 10% of $ICK^{-/-}$ MEFs formed acetylated α -tubulin-positive cilia (Figure 1N–P), more than 80% of $ICK^{-/-}$ MEFs had Smo signals in the vicinity of centrioles with cilia-like structures (Supplementary Figure S6A–D). Since acetylated α -tubulin is a marker for the ciliary axoneme and Smo is a membrane protein, these data suggest that $ICK^{-/-}$ MEF cilia have ciliary membrane structures without acetylated α -tubulin-positive microtubules. Although AC3 and Arl13b are ciliary membrane proteins, we observed that about 10% of $ICK^{-/-}$ MEFs form AC3-positive cilia and that about 30% of $ICK^{-/-}$ MEFs have Arl13b-positive cilia (Supplementary Figure S3U–Z). The transport system for AC3 and Arl13b in cilia may be different from that for Smo. For the rescue experiments in Figure 5, we observed acetylated α -tubulin-positive cilia in $ICK^{-/-}$ MEFs. We added this comment to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 14, line 2 – page 14, line 9)

“We found that about 10% of $ICK^{-/-}$ MEFs formed acetylated α -tubulin-positive cilia (Figure 1N–P). Since acetylated α -tubulin is a marker for the ciliary axoneme and Smo is a membrane protein, these data suggest that $ICK^{-/-}$ MEF cilia have ciliary membrane structures without acetylated α -tubulin-positive microtubules. Although AC3 and Arl13b are ciliary membrane proteins, we observed that about 10% of $ICK^{-/-}$ MEFs form AC3-positive cilia and that about 30% of $ICK^{-/-}$ MEFs have Arl13b-positive cilia (Supplementary Figure S3U–Z). The transport system for AC3 and Arl13b in cilia may be different from that for Smo.”

(Page 15, line 3 – page 15, line 5)

“We transfected these constructs into $ICK^{-/-}$ MEFs and observed the acetylated- α -tubulin-positive cilia (Figure 5B–E).”

(Page 15, line 3 from the bottom – page 15, the last line)

“We next examined the ability of the WT hICK and the human ECO mutant to rescue the formation of acetylated- α -tubulin-positive cilia in $ICK^{-/-}$ MEFs (Figure 5L–O).”

4. Because ICK and MAK or CCRK may have overlapping targets, and thus, potentially overlapping functions, one hypothesis for the tissue specific effects of ICK loss may be upregulation of MAK or CCRK. The authors should test for upregulation of MAK and CCRK in ICK -/- cells and tissues.

According to the referee's comment, we first examined by Q-PCR whether *Ccrk* and *Mak* mRNA expression are upregulated in $ICK^{-/-}$ MEFs. We found that there is no significant difference in *Ccrk* mRNA expression between $ICK^{+/+}$ and $ICK^{-/-}$ MEFs (Supplementary Figure S2C). We did not detect *Mak* mRNA expression in either $ICK^{+/+}$ or $ICK^{-/-}$ MEFs. We next analysed the mRNA expression level of *Ccrk* and *Mak* in the *ICK Nes* CKO brain by Q-PCR. There was no significant difference in *Ccrk* mRNA expression between the control and *ICK Nes* CKO brain (Supplementary Figure S5F). We did not detect *Mak* mRNA expression in either the control or *ICK Nes* CKO brain. We added these data to the new manuscript, modified the text, and added descriptions in the Results as follows:

(Page 6, line 8 from the bottom – page 6, line 7 from the bottom)

“*Ccrk* mRNA expression was not upregulated in $ICK^{-/-}$ MEFs (Supplementary Figure S2C). We did not detect *Mak* mRNA expression in either $ICK^{+/+}$ or $ICK^{-/-}$ MEFs.”

(Page 10, line 6 from the bottom – page 10, line 4 from the bottom)

“*Ccrk* mRNA expression was not upregulated in the *ICK Nes* CKO brain (Supplementary Figure S5F). We did not detect *Mak* mRNA expression in either control or *ICK Nes* CKO brains.”

5. Hypothesizing that ICK phosphorylates Kif3a directly, which may explain some of the IFT defects observed, the authors show that a form of Kif3a mutated at the T674 residue (in a consensus phosphorylation site) is not a substrate of ICK in vitro. This result is very interesting, but it is then perplexing that when the authors attempt to confirm the findings in vivo, they do not use this mutant for rescue experiments in zebra fish (they find that overexpression of Kif3a abrogates cilium formation in cultured cells). Rather, they use a plasmid in which the Ser/Thr residues of the last 50 amino acids of Kif3a are changed to alanine. Why? Furthermore, they find that this extensively modified Kif3a ('Kif3a-CA') is unable to rescue Kif3a functionality, and mention that "This result suggests that the C-terminal portion of Kif3a including an ICK phosphorylation site is essential for normal Kif3a function". It is probably not surprising that Kif3a's function is abrogated, and one can easily argue that the authors are not specifically testing the potential effect of ICK on Kif3a. As such, the mechanistic understanding of ICK function provided by this study is highly limited. In particular, examining only body curvature in zebra fish rescue experiments will not be directly indicative of the function of ICK in cilium formation; authors should examine effects on the cilium itself (including ciliogenesis and distribution of selected IFT proteins).

To analyse the role of phosphorylation of Kif3a in ciliary formation, we performed rescue experiments using NIH3T3 cells (Figure 7F–L). We previously tried to perform rescue experiments using RPE-1 cells, however, in our experimental conditions, overexpression of even Kif3a-WT inhibited ciliary formation in these cells, making us unable to observe the effect of the mutant form of Kif3a in ciliary formation. We, therefore, used NIH3T3 cells instead of RPE-1 cells to perform rescue experiments. We constructed three types of short hairpin RNAs (shRNAs) to knockdown

Kif3a. We co-transfected control shRNA or each of three types of shKif3a (shKif3a-1, -2, and -3) expression plasmids with GFP-expressing plasmids and FLAG-tagged Kif3a-WT-expressing constructs into HEK293T cells. We observed the FLAG-tagged protein expression by Western blot analysis using an antibody against FLAG. All the shRNAs against *Kif3a* showed an inhibitory effect on *Kif3a* (Supplementary Figure S8D). We used shKif3a-1 for rescue experiments. FLAG-tagged constructs expressing GFP or shRNA-resistant Kif3a (WT or T674A) were transfected with control shRNA or shKif3a into NIH3T3 cells. Cells were immunostained with anti-FLAG and anti-acetylated α -tubulin antibodies. We found that this shKif3a inhibits ciliary formation (Figure 7H and L). Expression of shRNA-resistant Kif3a-WT rescued shKif3a-mediated inhibition of ciliation (Figure 7I and L). Unexpectedly, shRNA-resistant Kif3a-T674A had a slightly increased ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT (Figure 7J and L). Since ciliary numbers decreased in *ICK*^{-/-} cells, we first expected that Kif3a-T674A would show a reduced ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT. The reason that the effect of Kif3a-T674A on ciliary formation in knockdown and rescue experiments using cultured cells is the opposite of the results obtained from *ICK* knockout experiments is unclear, however, a recent study reported that knockdown of *ICK* and *Mak* promote ciliary formation in cultured cells (Yang *et al.*, *EMBO Rep.*, 2013; 14:741-747). Suppression of the expression of *ICK* may promote ciliary formation by reducing the level of Kif3a phosphorylation at residue 674 in cultured cells. On the other hand, since Kif3a-C-T674A was still phosphorylated by *ICK* (Figure 7C), we thought that phosphorylation of other serine or threonine residues in the C-terminal region of Kif3a may also be important for Kif3a function in ciliary formation. To further analyse the role of phosphorylation of Kif3a C-terminal region in ciliary formation, we constructed a plasmid encoding a Kif3a mutant in which 8 Ser or Thr residues clustered in the C-terminal region, including residue 674, are replaced by Ala (Kif3a-8xA) (Figure 7F). To clarify the numbers of the substituted serine or threonine residues in the C-terminal region of Kif3a, we replaced the name Kif3a-CA with Kif3a-8xA throughout the manuscript. Expression of shRNA-resistant Kif3a-8xA failed to rescue shKif3a-mediated inhibition of ciliary formation (Figure 7K and L). These results suggest that phosphorylation of the C-terminal region of Kif3a affects ciliary formation in cultured cells.

Furthermore, to investigate the role of phosphorylation of Kif3a in ciliary formation *in vivo*, we performed rescue experiments using zebra fish embryos and observed the nasal cilia. At 3 dpf, cilia are developed in nasal pit epithelia in control larvae (Figure 7O and S). In contrast, larvae injected with Kif3a MO and GFP lost cilia at this stage (Figure 7P and S). Loss of cilia by Kif3a MO injection was partially rescued by co-injection with Kif3a-WT mRNA (Figure 7Q and S). However, co-injection with Kif3a-8xA mRNA failed to rescue the ciliary loss by knockdown of *Kif3a* (Figure 7R and S). Taken together, these results suggest that phosphorylation of the C-terminal portion of Kif3a is essential for normal ciliary formation.

To further investigate whether Kif3a is phosphorylated by *ICK*, we immunostained *ICK*^{+/+} and *ICK*^{-/-} MEFs using antibodies against p-Kif3a and acetylated α -tubulin. We observed that p-Kif3a localizes to the cilia in *ICK*^{+/+} MEFs, and that p-Kif3a signals were often enriched at ciliary bases and tips (Figure 7D). On the other hand, the proportion of cilia with p-Kif3a at ciliary tips decreased in *ICK*^{-/-} MEFs (Figure 7E). This result suggests that Kif3a is phosphorylated by *ICK* at the tips of cilia. Interestingly, a majority of shortened cilia lost p-Kif3a signals at the tips of cilia (left and middle panels in Figure 7E), whereas cilia with normal length often had p-Kif3a signals at ciliary tips in *ICK*^{-/-} MEFs (right panels in Figure 7E). This result suggests that phosphorylation of Kif3a at residue 674 is linked to ciliary formation. We added these data to the new manuscript, modified the text, and added descriptions in the Abstract, Introduction, Results, and Discussion as follows:

(Page 2, line 5 from the bottom – page 2, line 3 from the bottom)

“In addition, *ICK* directly phosphorylated Kif3a, while inhibition of this Kif3a phosphorylation affected ciliary formation”

(Page 5, line 4 from the bottom – page 5, line 2 from the bottom)

“We also found that *ICK* directly phosphorylates Kif3a, a subunit of Kinesin-2, and that Kif3a phosphorylation is required for normal cilia formation *in vivo*.”

(Page 17, line 9 – page 17, line 10)

“Kif3a is phosphorylated by ICK and Kif3a phosphorylation is required for ciliary formation”

(Page 17, the last line – page 19, line 3)

“We found that p-Kif3a localizes to the cilia in *ICK*^{+/+} MEFs, and that p-Kif3a signals were often enriched at ciliary bases and tips (Figure 7D). In *ICK*^{-/-} MEFs, the proportion of cilia with p-Kif3a at ciliary tips decreased (Figure 7E). Interestingly, a majority of shortened cilia lost p-Kif3a signals at the tips of cilia (left and middle panels in Figure 7E), whereas cilia of normal length often had p-Kif3a signals at ciliary tips in *ICK*^{-/-} MEFs (right panels in Figure 7E). This result suggests that Kif3a is phosphorylated by ICK at ciliary tips, and that phosphorylation of Kif3a at residue 674 is linked to ciliary formation.

To investigate the role of phosphorylation of Kif3a in ciliary formation, we performed rescue experiments using NIH3T3 cells (Figure 7F–L). We constructed a short hairpin RNA (shRNA) to knockdown *Kif3a* (Supplementary Figure S8D), and found that shKif3a inhibits ciliary formation (Figure 7H and L). Expression of shRNA-resistant Kif3a-WT rescued shKif3a-mediated inhibition of ciliation (Figure 7I and L). Unexpectedly, shRNA-resistant Kif3a-T674A had a slightly increased ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT (Figure 7J and L). Since Kif3a-C-T674A was still phosphorylated by ICK (Figure 7C), we thought that phosphorylation of other serine or threonine residues in the C-terminal region of Kif3a may also be important for Kif3a function in ciliary formation. To further analyse the role of phosphorylation of the Kif3a C-terminal region in ciliary formation, we constructed a plasmid encoding the Kif3a mutant in which 8 Ser or Thr residues clustered in the C-terminal region, including residue 674, are replaced with Ala (Kif3a-8xA) (Figure 7F). Expression of shRNA-resistant Kif3a-8xA failed to rescue shKif3a-mediated inhibition of ciliary formation (Figure 7K and L). These results suggest that phosphorylation of the C-terminal region of Kif3a affects ciliary formation in cultured cells.

To investigate the role of phosphorylation of Kif3a in ciliary formation *in vivo*, we performed rescue experiments using zebra fish embryos.”

(Page 19, line 9 from the bottom – page 19, the last line)

“We also investigated ciliogenesis in the nasal pits of the larvae injected with the morpholino and mRNA (Figure 7O–S). At 3 dpf, cilia have developed in nasal pit epithelia in control larvae (Figure 7O and S). In contrast, larvae injected with Kif3a MO and GFP lost cilia at this stage (Figure 7P and S). Loss of cilia by Kif3a MO injection was partially rescued by co-injection with Kif3a-WT mRNA (Figure 7Q and S). However, co-injection with Kif3a-8xA mRNA failed to rescue the ciliary defect by knockdown of *Kif3a* (Figure 7R and S). Taken together, these results suggest that phosphorylation of the C-terminal portion of Kif3a is essential for normal ciliary formation.”

(Page 22, line 7 from the bottom – page 23, line 4)

“We found that shRNA-resistant Kif3a-T674A has a slightly increased ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT. Since ciliary numbers decreased in *ICK*^{-/-} cells, we first expected Kif3a-T674A would show reduced ability to rescue shKif3a-induced inhibition of ciliary formation compared to that of Kif3a-WT. The reason why the effect of Kif3a-T674A on ciliary formation in knockdown and rescue experiments using cultured cells is the opposite of the results obtained from *ICK* knockout experiments is unclear, however, the study by Yang *et al.* reported that knockdown of *ICK* and *Mak* promote ciliary formation in cultured cells (Yang *et al.*, 2013). Suppression of the expression of *ICK* may promote ciliary formation through reducing the level of Kif3a phosphorylation at residue 674 in cultured cells.”

(Page 24, line 9 from the bottom – page 24, line 8 from the bottom)

“The inhibition of phosphorylation of Kif3a affected its function in ciliary formation.”

MINOR POINTS

1. The authors should include brief introductory material on CCRK/broad minded proteins from mouse and *C. elegans*, as they are relevant to the overall roles of MAP kinases in ciliogenesis/cilium length control.

According to the referee's comment, we added the description about previous studies on the function of Ccrk and Bromi in ciliary formation in *C. elegans* and mouse in the Introduction as follows:

(Page 4, line 8 from the bottom – page 4, line 5 from the bottom)

“It was reported that ICK is a substrate of Cell cycle-related kinase (Ccrk) (Fu *et al.*, 2006). A mutant of *Dyf-18*, the *C. elegans* ortholog of *Ccrk*, occasionally forms long curved cilia (Phirke *et al.*, 2011). Broad-minded (Bromi), which interacts with Ccrk, is required for the formation of proper structure in cilia (Ko *et al.*, 2010).”

2. The title indicates that ICK is required for ciliogenesis, but this does not appear to be consistent across cell types, thereby overstating its importance.

According to the referee's advice, we changed the title of this manuscript as follows:

ICK is essential for cell type-specific ciliogenesis and the regulation of ciliary transport

3. Regarding the Shh pathway protein mislocalization in the knockout, these results do not appear in the abstract, and there is no mention of the Shh pathway in the introduction.

We added the description about mislocalization of Shh pathway proteins in *ICK*^{-/-} cells in the Abstract. We also added the description of the Shh pathway in the Introduction.

(Page 2, line 8 – page 2, line 9)

“*ICK*-deficient cells formed cilia with mislocalized Hedgehog signalling components.”

(Page 3, line 7 – page 3, line 12)

“Cilia are known to be important for Shh signal transduction (Huangfu and Anderson, 2005). In the absence of Shh pathway stimulation, Smo rarely localizes in the cilia. In this condition, low levels of Gli transcription factors localize to the tip of cilia. However, Shh pathway stimulation induces the accumulation of Smo and Gli in the cilia, and then triggers the transcription of target genes (Corbit *et al.*, 2005; Haycraft *et al.*, 2005; Kim *et al.*, 2009).”

4. The authors do not indicate that their observation is supportive of the data by Burghoorn *et al.*, 2007, which indicates that the worm ortholog of ICK (*DYF-5*) has been shown to regulate the kinesin II complex. This is supportive of their data indicating that ICK phosphorylates Kif3a (a component of kinesin II). Please incorporate into the discussion.

According to the referee's suggestion, we added a description of a previous study showing that loss of function of *Dyf-5*, the *C. elegans* orthologue of *ICK*, affects the function of Kinesin-2 motors in the Discussion as follows:

(Page 24, line 6 from the bottom – page 24, line 3 from the bottom)

“Our data on *ICK* phosphorylation of Kif3a is supported by a previous study showing that loss of function of *Dyf-5*, the *C. elegans* orthologue of *ICK*, affects the function of Kinesin-2 motors in cilia (Burghoorn *et al.*, 2007).”

3rd Editorial Decision

14 March 2014

Thank you for your patience while your manuscript has been re-reviewed and please accept my apologies for the delay in responding. Your study has been sent to former referees #1 and #2, who now consider that the manuscript has been significantly improved although some concerns remain, particularly from referee #1.

While referee #2 is reasonably convinced of the suitability of your manuscript for The EMBO Journal without further modification, I would like to draw your attention to comments from referee #1 (pasted below at the end of this letter), who is not yet convinced by the experimental evidence of your conclusions regarding Kif3 modulation by *ICK*. After further consultation, s/he not only agrees on the comprehensive nature of your study and its interest, but also suggests very specific experiments that need to be performed for your manuscript to be accepted:

"The authors have done an enormous amount of work and *Ick* is clearly an interesting kinase. However I would insist on two things:

(1) Is *ICK* really at cilia tips? Fig. 1L appears to show two dots of *ICK* localization, which could be at the tip and base of the cilium. They need to do triple labels for *ICK*, axoneme and a basal body marker like gamma-tubulin to show that the arrowheads actually mark cilia tips.

(2) The authors need to do quantitative analysis of their images to show whether the intensity of Kif3a staining/cilia tip is decreased in the *Ick* mutant (Fig. 7D, E). They say the "proportion of cilia" with tip staining is decreased, but present no numbers. In particular, the third example of p-Kif3a staining in wt looks weaker than the third example in mutant, suggesting that there is considerable variation and that there may be no significant difference in tip p-Kif3a between wt and mutant. This certainly means that *Ick* is not the only Kif3a kinase and may mean that it is not a Kif3a kinase at all. This must be clarified, in my view."

Note that point 2 may need further discussion in the manuscript or even toning down the conclusions as evidence suggests, particularly the results obtained by the 8xA mutant vs. the single T to A mutant, that *ICK* is probably not the only kinase of Kif3a.

Although it is our policy to allow a single round of revision, in this case, given the very positive consideration of your manuscript by the referees in terms of novelty and interest, I would like to give you the opportunity to address these remaining issues.

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

Thank you again for your patience and the opportunity to consider your work for publication. I look forward to the final version of your manuscript.

REFeree REPORTS:

Referee #1:

The authors have responded conscientiously to the comments of the reviewers and the revised manuscript is significantly improved. The single most important point of the manuscript, that Ick modulates cilia trafficking by phosphorylation of Kif3a, remains unconvincing.

Figure 7D, E compares the staining of p-Kif3a in wild-type and mutant MEFs. The authors state that there is less staining of p-Kif3a at the tips of mutant cilia, compared to wt. In these images, p-Kif3a appears to be distributed along the wild-type cilium, with some enrichment at both the base and the tip, as often seen for IFT proteins. The mutant cilia are short and appear to have less p-Kif3a staining. However staining is still observed, it is not clear whether the staining is at the base or the tip because there is no marker for the base of the cilium, and no quantitation of the data is presented. Thus the new data show that Ick cannot be the only Kif3a kinase present in the MEFs and do not provide a compelling argument that Ick is actually a Kif3a kinase.

Referee #2:

Chaya and colleagues have adequately addressed all of the comments I suggested in my review. In addition, it is clear that they have addressed other relevant referee comments, resolving issues in most cases by performing additional experiments. In particular, the experiments involving the Kif3a-8xA mutant construct, previously my main criticism, now help to strengthen the manuscript considerably. I believe that this study contributes significantly to our understanding of vertebrate ICK, and its role in cilium function. The findings are novel and of broad interest to those interested in cilia, ciliary trafficking, and cellular signalling.

1st Revision - authors' response

29 March 2014

Referee #1:

The authors have done an enormous amount of work and Ick is clearly an interesting kinase. However I would insist on two things:

(1) Is ICK really at cilia tips? Fig. 1L appears to show two dots of ICK localization, which could be at the tip and base of the cilium. They need to do triple labels for ICK, axoneme and a basal body marker like gamma-tubulin to show that the arrowheads actually mark cilia tips.

Thank you for reviewing our manuscript. We performed additional experiments and prepared a revised manuscript.

Because of background signals with ICK immunostaining (Figure 1L, left), we displayed a high magnification cilia image containing background signalling (old Figure 1L, right bottom) to avoid misrepresentation by showing only the best cilia images with no background signals.

In order to address the referee's concern, we investigated ciliary localization of ICK by triple immunolabeling. We immunostained $ICK^{+/+}$ and $ICK^{-/-}$ MEFs using antibodies against ICK (red), acetylated α -tubulin (green), and Pericentrin (a marker for centrosomes, magenta), and found that ICK localizes to the ciliary tips but not to the ciliary bases in $ICK^{+/+}$ MEFs (Figure 1L). No ICK signals were detected in $ICK^{-/-}$ MEF cilia (Figure 1M). We also showed additional images of ICK ciliary localization in $ICK^{+/+}$ MEFs as below (Figure L1). Thus, we confirmed ICK localization at ciliary tips. In our current manuscript, we presented the new triple immunostaining images as Figure 1L and M. We also modified the figure legend for Figure 1L and M as follows:

(Page 34, line 10 from the bottom – page 34, line 8 from the bottom)

" $ICK^{+/+}$ and $ICK^{-/-}$ MEFs were immunostained with antibodies against ICK (red), acetylated α -tubulin (a marker for the ciliary axoneme, green), and Pericentrin (a marker for centrosomes, magenta)."

WT MEF AcTub ICK Peri

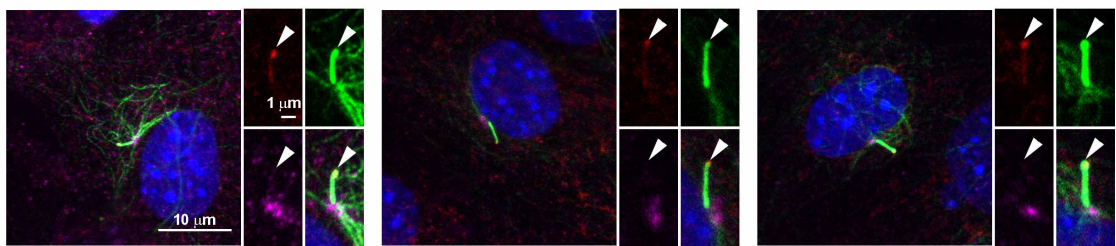


Figure L1 ICK is localized at ciliary tips. $ICK^{+/+}$ MEFs were immunostained with antibodies against ICK (red), acetylated α -tubulin (green), and Pericentrin (magenta). Nuclei were stained with DAPI (blue). Arrowheads indicate ciliary tips.

(2) The authors need to do quantitative analysis of their images to show whether the intensity of Kif3a staining/cilia tip is decreased in the *Ick* mutant (Fig. 7D, E). They say the "proportion of cilia" with tip staining is decreased, but present no numbers. In particular, the third example of *p*-Kif3a staining in wt looks weaker than the third example in mutant, suggesting that there is considerable variation and that there may be no significant difference in tip *p*-Kif3a between wt and mutant. This certainly means that *Ick* is not the only Kif3a kinase and may mean that it is not a Kif3a kinase at all. This must be clarified, in my view.

According to the referee's comment, we quantified the results shown in Figure 7D and E. We found that the percentage of cilia with *p*-Kif3a at the ciliary tips significantly decreased in $ICK^{-/-}$ MEFs (new Figure 7F), supporting the idea that ICK phosphorylates Kif3a. Since *p*-Kif3a signals at ciliary tips did not completely disappear in $ICK^{-/-}$ MEFs, ICK may not be the only Kif3a kinase. We observed that Kif3a-8xA more strongly affects ciliary formation than single point mutant Kif3a-T674A. This observation and the result that Kif3a-C-T674A is still phosphorylated by ICK suggest that ICK regulates Kif3a function through phosphorylating residue 674 as well as other residues in the Kif3a C-terminal region. However, we cannot exclude the possibility that other kinases contribute to the phosphorylation of that region of Kif3a. We further suppose that there may be other ICK phosphorylation target proteins playing roles in the regulation of ciliary transport. We added the quantification data to the new manuscript as Figure 7F, and modified the text in the Results, Discussion, and Figure legends as follows:

(Page 18, line 2 – page 18, line 3)

"In $ICK^{-/-}$ MEFs, the percentage of cilia with *p*-Kif3a at the ciliary tips markedly decreased (Figure 7E and F)."

(Page 24, line 5 from the bottom – page 25, line 4)

“Since p-Kif3a signals at the ciliary tips did not completely disappear in *ICK*^{-/-} MEFs, ICK may not be the only Kif3a kinase. We observed that Kif3a-8xA more strongly affects ciliary formation than Kif3a-T674A. This observation and the result that Kif3a-C-T674A is still phosphorylated by ICK suggest that ICK regulates Kif3a function through phosphorylation of residue 674 as well as other residues in the Kif3a C-terminal region. However, we cannot exclude the possibility that other kinases contribute to the phosphorylation of that region of Kif3a. We further suppose that there may be other ICK phosphorylation target proteins playing roles in the regulation of ciliary transport.”

(Page 39, line 6 from the bottom)

“(F) The percentage of cilia with p-Kif3a signals at the ciliary tips was quantified.”

4th Editorial Decision

01 April 2014

Thank you for the submission of your revised manuscript to The EMBO Journal. As you have properly addressed the concerns raised by the referees, I am writing with an 'accept in principle' decision, which means that I will be glad to accept your manuscript for publication once just a very minor detail has been addressed, as follows.

Browsing through the manuscript, I have noticed that the Materials and Methods section in the main text is rather limited. While we fully understand that a full description of the methodology employed might be out of line in the main text and can be much further developed as supplementary information, we also believe that a description of at least key techniques with details specific to this study, must accompany the main body of the manuscript to ensure proper understanding of the results by the reader. With this in mind, I would suggest moving the subsections "Generation of ICK knock-out (KO) mice and ICK conditional knock-out (CKO) mice", "Antibodies", "Plasmid constructs" and "Statistical analysis" from the supplementary data into the Materials and Methods section of the main text.

I would also like to mention that 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?

If you have any questions or comments, 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 study. Congratulations in advance for a successful publication.