THE EMBO JOURNAL

Manuscript EMBO-2011-79984

Identification of a novel Wnt5a-CK1 -Dvl2-Plk1-mediated primary cilia disassembly pathway

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Review timeline:

Submission date:	30 October 2011
Editorial Decision:	14 December 2011
Revision received:	29 March 2012
Editorial Decision:	17 April 2012
Accepted:	18 April 2012

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.)

1st Editorial Decision 14 December 2011

Thank you very much for submitting your research paper reporting distinct signaling events within cilia disassembly for consideration to The EMBO Journal editorial office.

I do apologize for the slight delay in getting back to you with a decision on the study, caused by relatively late incoming reports. In fact, one of the scientists that initially agreed to assess your work did until now not return any comments. Having received however two relatively consistent assessments, I am in a position to reach a final decision to not further delay the proceedings of your study.

As you will recognize, both scientists find the proposal in principle interesting. However, they also bring up significant critiques that would have to be thoroughly addressed to establish confidence in the conclusions of your study. As the comments are rather explicit and seem very reasonable, there is no reason to repeat them here in full. They range from confirming major results indeed in ciliated cells (a point crucial to the overall proposal), essential localization of the components at the right cellular compartment, revealing in-vitro binding and interrogating Dvl2 phosphomimic mutants for effects on both HEF1-activity respective ciliogenesis defects. In sum, both argue that the paper would need much stronger molecular and functional support to overcome the current relatively preliminary state.

On balance we would be willing to offer the chance to appropriately address the current concerns. As this entails however time-consuming additional experimentation with partially uncertain outcome(s), we would fully understand if you might find it easier to seek more rapid publication elsewhere.

In case you would like to embark on a potential revision, I urge you to take the demands into careful consideration to not waste your time and avoid unpleasant disappointments much later in the process. Please do not hesitate to contact me with in case of further questions or indeed outlining possible experiments and timeline in case you plan to revise the study (preferably via E-mail).

I am sorry that I cannot be more encouraging at this stage of analysis but I hope that clearly communicating our demands and expectations might facilitate efficient proceedings in the interest of you and your study.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this manuscript by Lee and colleagues, the authors show a new role for Plk1 and Dvl2 in the signaling pathway, relevant for cilia disassembly. They demonstrate that Dvl2 is phosphorylated by CK1 in response to Wnt5a stimulation, then forms a complex with Plk1 in the early stages of the cell cycle. Together as a complex, Plk1 and Dvl2 contribute to cilia disassembly by stabilizing HEF1 and activating the associated Aurora A kinase, known to be crucial for cilia disassembly. The association of Plk1 and Dvl2 during mitosis has been characterized before (Kikuchi, Niikura et al. 2010, EMBO 29:3470 - not referenced in this study, and should be added), as has the interaction between CK1 and Dvl (Corbit et al, Nat Cell Biol 10:70-6, 2008 - not referenced, and should be added), and Dvl2 has been reported recently to be important for apical docking and planar polarization in ciliated epithelial cells (Park, Mitchell et al. 2008). However, in this study the authors more extensively characterize the phospho-dependant binding of Plk1 and Dvl2, place all the components in sequence, and demonstrate regulation of HEF1/Aurora A dependant cilia disassembly, creating a novel and potentially significant finding. Most of the experiments are well conducted and the data clearly presented and described. However, some important issues need to be addressed.

1) Page 6 and elsewhere in the study: the authors should clarify whether work is performed in ciliated or non-ciliated cells. Corbit et al have demonstrated that CK1 phosphorylation of Dvl is inhibited by Kif3A in cells with intact cilia, but this is lost in unciliated cells. Clarifying this point is extremely important: if the relationship only pertains in unciliated cells, it is hard to see how it is relevant to ciliary disassembly.
2) Continuing this point, localization of CK1 and Dvl2 to ciliary basal bodies, rather than just centrosomes, is important. Although the authors use acetylated alpha-tubulin as a marker in some experiments, the only localization marker used for these two proteins is to gamma-tubulin, which will react equally with both. They need to demonstrate some localization of the proteins to the correct compartment in ciliated cells.
3) In regard to analyzing the association between CK1 and Dvl2, it would be important to determine whether recombinant proteins interact in vitro, to exclude contributions from other proteins in complex with them.
4) Figure 2. It is surprising there is no additive effect between the knockdown of CK1 and CK1 ; instead, loss of either one completely eliminates phosphorylation. How do the authors explain this.
5) Figure 3C and other Figures: To be sure that the effect of Plk1 knockdown, or expression of Dvl2 mutants, on cilia is not due to a difference in cell cycle compartmentalization, it is essential to perform a FACS analysis. This is particularly the case because the authors are only tracking the second wave of ciliary disassembly, which occurs at G2/M transition (as reviewed in the Plotnikova et al paper cited) and could certainly be affected by cell cycle issues when mutating proteins associated with Plk1. Tellingly, Figure S4C for Plk1 and Aurora A inhibition FACS has been performed18h and 24h after serum inhibition and indeed shows a drug-induced accumulation in G2.

Thus the slowed down ciliary resorption might be due to a drug-induced difference in cell cycle compartmentalization. Cyclin A increases less in Plk1 depleted cells and it is not possible to exclude that cyclin A might be activated due to serum stimulation without proper progression through the cell cycle. Therefore it would be important to see the matching results in FACS analysis also for PLk1 depletion. In addition, for a complete analysis a 2-6 h time point after serum stimulation should be included in this study to state whether Plk1 only has an effect on the 2nd wave or on both waves of cilia resorption. Further, in figure 3C t is not obvious whether at the 6h time point the reduction in Plk1 depleted cells is significantly less compared to control cells, as before serum stimulation less control cells where ciliated. It would be more descriptive to show in addition the reduction of ciliated cells in % at the various time points in Plk1 depleted cells versus control cells. In the later time points the difference is more obvious. The omission of early time points is really notable because these time points are included for studies of Wnt5a in the latter part of the paper.

6) On page 9, the paragraph describing results has several confusing statements. "in contrary to the role of Dvl2 in multiciliated cells" is not supported by a reference. If the authors refer to Park et al. that Dvld2 to indicated plays a role in ciliogenesis in multiciliated cells, it only has been shown that Dvld2 Is located at the base of the cilium.

7) Figure 4C/D: As the percentage of ciliated cells and cilia length is very different after serum starvation, it would be descriptive to show an additional graph with the reduction in percent of the 0h time point for each time point of serum stimulation and calculate under which condition the reduction is significant.

8) Figure 5: Although an effect of S143A and T224A mutaions has been shown in the context of Wnt5 induced ciliary disassembly, to complete this chain of evidence, the effect of downregulation or inhibition of CK1 / on the Wnt5-induced ciliary disassembly would be interesting. It would be extremely valuable to add a small molecule inhibitor of CK1 to the analysis, to support the results with knockdowns, for this and other experiments.

9) To further support the hypothesis of a physiological role of Wnt5 in cilia disassembly, it would be important to see the expression level of Wnt5 in a time course while ciliary disassembly or it should be shown Wnt5 in IF before and after serum stimulation in ciliated cells.

10) General technical points: For data in Figure 1 and throughout the study, relative densities in western blots have been calculated but normalization would be helpful in some cases. As some western blots are overexposed, a shorter exposure would be necessary for a proper normalization of the data. Further, information about the number of times results were repeated in independent experiments is generally lacking, and should be supplied. p values indicating significance of results should be also included in some figures. Was the cilia counting performed blinded? It is difficult to assess the solidity of the results without this information.

11) On page 13, in association with Figure 5, the authors state that centrosomal CK1 signals increase gradually following addition of serum, and become abundant at mitotic centrosomes, as would be expected when cilia are totally reabsorbed. This is troubling on several levels. First, the increase shown in 5E/F is minor; certainly not enough to demonstrate a change in activity. It is much more likely that activity would spike at the time of ciliary disassembly, if activity were relevant to the process. Is there any antibody that allows analysis of activated CK1 kinase at the basal body, rather than total kinase? Further, by the logic the authors use, CK1 activity is NOT relevant to disassembly at the early time point, as the expression has not changed. This makes it likely the observed effects are mediated indirectly, through inhibiting G2/M entry, and again makes it urgent to back up the results with detailed FACS.

Some minor comments:

- Figure 1A: The asterisk in this figure is not explained
- In some figures the bands shown in CBB are not clear and should be labeled
- Figure 3D: Why is the 6h time point not included in this experiment like in the previous ones? Figure 3D is not showing a delayed ciliary disassembly, it is showing failure to disassemble cilia.
- Figure 6C: CK1 is overexpressed and difficult to compare and both CK1 and are not mentioned

in the description of the results.

Referee #2:

In this manuscript, Lee and colleagues study the process of primary cilia disassembly prior to mitosis, a phenomenon described several decades ago but which has only recently started to be addressed. Through the use of biochemical and cell biology approaches the authors uncovered a new part of the pathway(s) involved in this process and describe new specific roles for Wnt5a, CK1 , Dvl2 and Plk1. The major findings of this work are the following: Wnt5a promotes the phosphorylation of Dvl2 at the critical residues S143 and T224 which is dependent on CK1 but not on the closely related CK1 . Phosphorylated Dvl2 binds Plk1 an event that ultimately regulates HEF1-AurkA-HDAC6-dependent primary cilia disassembly through an indirect regulation of HEF1's degradation by the Smad3-APC10 complex. These findings are very relevant to the cilia and cell cycle fields.
In general the experiments were well conducted and interpreted, and the results are of very good quality. Below are some concerns about several aspects of the manuscript but if addressed satisfactorily I would enthusiastically support publication in EMBO J.
Major concerns:
- The model proposed by the author would be considerably strengthened by showing that the expression of a Dvl2 S143 and T224 phosphomimetic mutant that would constitutively bind Plk1 would hyperactivate HEF1-mediated cilia disassembly leading to either shorter cilia or defects in ciliogenesis altogether.
- The results presented in FigS3 need clarification. In this experiment the authors massively over-express Dvl2 (from a shRNA resistant transcript) in cells expressing either control Luciferase shRNAs or Dvl2 shRNAs. In cells expressing Luciferase shRNAs the over-expression of Dvl2 prevented primary cilia assembly after 48h of serum starvation. However, in cells supposedly depleted of endogenous Dvl2 but over-expressing the wt protein, primary cilia assembly is somehow restored. I am unable to understand this result given the levels of over-expressed Dvl2 present compared with the much lower levels of endogenous Dvl2 present in control Luciferase + Vector cells. Indeed even if the endogenous levels are depleted the over-expressed protein in Dvl2i + Dvl2 cells should prevent cilia assembly as in Gli+Dvl2, unless the over-expressed protein is not functional.
- In Fig3C the authors show that after starvation the population of PLK1 depleted cells presents more ciliated cells than the control population but this is never mentioned or discussed. Is this difference significant? Could PLK1 have a role regulating cilia assembly?
- Regarding Fig4A, the authors say on page in page 11 "Depletion of each of the components did not significantly alter the levels of other proteins (Figure 4A). Induction of a slow migrating Dvl2 (b form) in cells silenced for AurA and Plk1 (AurAi and Plk1i, respectively) could be due to the presence of a small fraction of mitotically arrested cells accumulated during depletion of these proteins.". However there are several alterations in protein levels that can be observed in these western blots:
- AurkA silencing significantly increases the total level of Dvl2 and not only the appearance of a slow migrating band. Furthermore, the appearance of this band is suggested to be due to the presence of a small percentage of mitotic cells but before the authors suggested, when referring to Fig1B, that Dvl2 is expressed at lower levels in mitosis. The same applies for PLK1 depletion in which the total levels of Dvl2 seem to be higher than in control cells.
- The silencing of Dvl2 causes an increase in -catenin levels.
- Dvl2 silencing in this figure seems to decrease the levels of AurkA which is not observed later on.
- CK1 levels are increased in the RNAi of Dvl2, -cat, AurkA, PLK1 and CK1 .
- In Fig4C the authors show that the populations depleted of Dvl2 and AurkA have more ciliated cells than control cells. However this was not mentioned or discussed. This observation is curious since for example the RNAi of AurkA apparently did not have the same effect in the study of Pugacheva et al 2007. Are differences observed here statistically significant? What could be the reasons for them and their biological meaning?
- In different experiments there are differences in the observed percentages of ciliated cells for control cells (e.g. Fig3D and 4C). The authors point out that, lower percentages were observed for infected cells, but do not try to explain this. Is this due to the virus infection, due to the constant production of shRNAs? Did the authors try a different shRNA delivery method to clarify this

matter? Although the results presented are clear and support the conclusions taken from them regarding cilia disassembly, this issue is relevant to analyse the experiment as a whole since the conditions regarding cilia assembly might not be ideal.
- In Fig4C the authors show that the populations depleted of -catenin or CK1 present a significantly reduced number of ciliated cells than controls after serum starvation. However this is not discussed. Are these proteins involved in cilia assembly or is this a new finding?
- The authors never mention the fate of Dvl2 depleted cells that fail to disassemble their cilia. The recent data regarding this process indicates that indeed the presence of the primary cilium prevents the progression to mitosis supporting a ciliary role in the regulation of cell cycle progression. Therefore one wonders if this was observed in this study. As the authors mention, Dvl2 was shown to have roles in mitosis being for example required for proper spindle orientation. However Dvl2 silencing in HeLa cells did not prevent mitotic entry and spindle formation. Was this the case for Dvl2 silencing in starved ciliated RPE-1 cells or were these blocked due to the presence of the primary cilium? And if these cells were indeed arrested, would cycling (non ciliated) RPE-1 cells also arrest or would they behave like HeLa when silenced for Dvl2?
Minor concerns:
- For all the experiments the authors should indicate the number of times the assay was performed not only the number of cells counted.
- The manuscript needs to be proofread extensively to remove grammatical errors and typos.
- I recommend that the nomenclature chosen to indicate silencing by RNAi in all the figures, i.e. adding an (i) in front of the name of the gene (e.g. Dvl2i) is changed. I believe it to be confusing particularly when rescue experiments are being shown.
- The authors often refer to cells expressing control Luciferase shRNAs as cells depleted of control GLi (e.g. page 11). This is incorrect and should be changed in the main text and figure legends. As human cells do not express luciferase they cannot be depleted of it.
- In page 6 the authors say "The level of Dvl2 bound to GSTPBD was reduced in nocodazole (Noc)-treated (M phase) cells, apparently due to a low level of Dvl2 expression at this stage (Figure 1B)". Have the authors or previous studies analysed Dvl2's expression during the cell cycle? Have they tried another method for blocking cells in mitosis to discard the hypothesis that this observation is due to microtubule depolymerization by nocodazole?
- In Fig1A left panel there is a band (third lane) marked with an asterisk that is not explained.
- In FigS2C the authors present the results of a Luciferase reporter assay based on the SuperTopFlash and FopFlash reporter plasmids that is never properly explained. A brief explanation of the assay should be included either in the figure legend or the Materials and Methods section. Still regarding this figure, the authors say in pages 8 and 9 "However, Plk1 activity neither altered the degree of -catenin ubiquitination nor influenced the level of -catenin-dependent transcription activity (Supplementary FigureS2), thereby diminishing the likelihood that Dvl2-bound Plk1 contributes to the canonical pathway.". However, there is a difference in expression of the reporter between the DMSO and PLK1 inhibitor treated cells blocked in mitosis and in the presence of Wnt3a that is not commented. The meaning of this difference should be explained.
- In the legend of FigS4C the cell cycle analysis should be explained a bit more saying for example that it was the DNA content of the cells that was analysed. The figure itself could be improved by saying that the first peak corresponds to 2N DNA content and the second to 4N and maybe by including the percentages of cells in each population.
- In Fig4E the band marked with the asterisk is not explained.
- In Fig 6A the left panel is not explained. One assumes that those cells were used in order to obtain medium containing Wnt5a but this should be explained.
- In FigS8D Smad2/3 seems to be absent from the inputs.

1st Revision - Authors' Response 29 March 2012

We thank the anonymous reviewers for the constructive and positive comments on our manuscript, " Identification of a novel Wnt5a-CK1ε-Dvl2-Plk1-mediated primary cilia disassembly pathway." We have performed several additional experiments and made several other modifications to the manuscript to address the reviewers' comments/suggestions. To assist in evaluating changes made to our original manuscript, portions that are revised have been highlighted in red color. Below are our point-by-point responses.

Reviewer #1

In this manuscript by Lee and colleagues, the authors show a new role for Plk1 and Dvl2 in the signaling pathway, relevant for cilia disassembly. They demonstrate that Dvl2 is phosphorylated by CK1ε in response to Wnt5a stimulation, then forms a complex with Plk1 in the early stages of the cell cycle. Together as a complex, Plk1 and Dvl2 contribute to cilia disassembly by stabilizing HEF1 and activating the associated Aurora A kinase, known to be crucial for cilia disassembly. The association of Plk1 and Dvl2 during mitosis has been characterized before (Kikuchi, Niikura et al. 2010, EMBO 29:3470 - not referenced in this study, and should be added), as has the interaction between CK1ε and Dvl (Corbit et al, Nat Cell Biol 10:70-6, 2008 - not referenced, and should be added), and Dvl2 has been reported recently to be important for apical docking and planar polarization in ciliated epithelial cells (Park, Mitchell et al. 2008). However, in this study the authors more extensively characterize the phospho-dependant binding of Plk1 and Dvl2, place all the components in sequence, and demonstrate regulation of HEF1/Aurora A dependant cilia disassembly, creating a novel and potentially significant finding. Most of the experiments are well conducted and the data clearly presented and described. However, some important issues need to be addressed.

The Kikuchi et al, EMBO J paper was indeed discussed and cited in the original manuscript. As suggested, we added Corbit et al, Nat Cell Biol paper in page 20 line 8.

1) Page 6 and elsewhere in the study: the authors should clarify whether work is performed in ciliated or non-ciliated cells. Corbit et al have demonstrated that CK1ε phosphorylation of Dvl is inhibited by Kif3A in cells with intact cilia, but this is lost in unciliated cells. Clarifying this point is extremely important: if the relationship only pertains in unciliated cells, it is hard to see how it is relevant to ciliary disassembly.

This is a very important comment that we further clarified. The initial Dvl2-Plk1 PBD interaction was observed in asynchronously growing HeLa cells (as indicated in page 6 line 5). In this revised version, we showed that the PBD-dependent Plk1-Dvl2 interaction also occurs efficiently under serum-starved and 3 h serum-stimulated hTERT-RPE cells, where the majority of cells are ciliated. Consistent with the Dvl2-Plk1-dependent primary cilia disassembly, the interaction between Dvl2 and Plk1 was increased ~5 times 3 h after serum stimulation, in comparison to the basal level of binding under serum starved (0 h) condition. Similar to the Plk1-Dvl2 interaction in asynchronously growing HeLa cells, both S143, and to a lesser extent, T224 residues were also required for the interaction under serum-starved conditions. These results are provided in Supplementary Figure S8A and S8B, and are described in page 13 line 9.

2) Continuing this point, localization of CK1ε and Dvl2 to ciliary basal bodies, rather than just centrosomes, is important. Although the authors use acetylated alpha-tubulin as a marker in some experiments, the only localization marker used for these two proteins is to gamma-tubulin, which will react equally with both. They need to demonstrate some localization of the proteins to the correct compartment in ciliated cells.

We carried out co-immunostaining analyses using serum-starved hTERT-RPE cells. In this version, we showed that both Dvl2 and CK1ε are localized to basal bodies. In order to avoid cross-recognition of the same source primary antibodies by secondary antibodies, we used either anti-acetylated tubulin or anti-detyrosinated tubulin (Glu-tub) antibody to visualize primary cilia. The results are provided in Supplementary Figure S14A, and are described in page 20 line 5.

3) In regard to analyzing the association between CK1ε and Dvl2, it would be important to determine whether recombinant proteins interact in vitro, to exclude contributions from other proteins in complex with them.

As suggested, we performed in vitro binding analyses and showed that Dvl2 directly interact with CK1ε. This result is provided in Supplementary Figure S14B, and is described in page 20 line 8.

4) Figure 2. It is surprising there is no additive effect between the knockdown of CK1ε and CK1δ; instead, loss of either one completely eliminates phosphorylation. How do the authors explain this.

This result was also somewhat surprising to us. However, since it was difficult to efficiently deplete both CK1ε and CK1δ at the same time (especially a fraction of CK1ε remains after shCK1δ/ε double knockdown, as indicated in Figure 2C and 2D, arrows), one possibility would be that this remaining fraction contributes to the generation of the p-S143 and p-T224 epitopes. Another possibility would be

the presence of other unidentified kinase(s) phosphorylating the S143 and T224 sites. These possibilities are mentioned in page 8 line 6.

5) Figure 3C and other Figures: To be sure that the effect of Plk1 knockdown, or expression of Dvl2 mutants, on cilia is not due to a difference in cell cycle compartmentalization, it is essential to perform a FACS analysis. This is particularly the case because the authors are only tracking the second wave of ciliary disassembly, which occurs at G2/M transition (as reviewed in the Plotnikova et al paper cited) and could certainly be affected by cell cycle issues when mutating proteins associated with Plk1. Tellingly, Figure S4C for Plk1 and Aurora A inhibition FACS has been performed18h and 24h after serum inhibition and indeed shows a drug-induced accumulation in G2. Thus the slowed down ciliary resorption might be due to a drug-induced difference in cell cycle compartmentalization. Cyclin A increases less in Plk1 depleted cells and it is not possible to exclude that cyclin A might be activated due to serum stimulation without proper progression through the cell cycle. Therefore it would be important to see the matching results in FACS analysis also for PLk1 depletion. In addition, for a complete analysis a 2-6 h time point after serum stimulation should be included in this study to state whether Plk1 only has an effect on the 2nd wave or on both waves of cilia resorption. Further, in figure 3C t is not obvious whether at the 6h time point the reduction in Plk1 depleted cells is significantly less compared to control cells, as before serum stimulation less control cells where ciliated. It would be more descriptive to show in addition the reduction of ciliated cells in % at the various time points in Plk1 depleted cells versus control cells. In the later time points the difference is more obvious. The omission of early time points is really notable because these time points are included for studies of Wnt5a in the latter part of the paper.

The reviewer concerns whether the observed delay in primary cilia disassembly in Plk1 RNAi cells could be attributable to a delay in the cell cycle in these cells. As requested, we closely examined with much narrower time intervals the effect of Plk1 depletion on primary cilia disassembly following serum stimulation. The results showed that depletion of Plk1 did not appear to significantly alter the cell cycle until the cells reach the late stages (12 h – 18 h) of the cell cycle. Under these conditions, the percentage of cells with primary cilia in Plk1 RNAi cells was noticeably diminished as early as several hours (4–6 h) after serum stimulation and was steadily declined over time. These findings suggest that Plk1 functions early in the cell cycle to promote primary cilia disassembly in a sustained manner. These results are provided in Supplementary Figure S5, and are described in page 10 line 11 (see also page 13 line 9 for related results). These results are consistent with the findings that the Dvl2-Plk1 interaction is greatly (~5 fold) enhanced 3 h after serum stimulation (Supplementary Figure S8) and that the elevated levels of p-S143 and p-T224 epitopes were steadily maintained following serum stimulation {Figure 6A and B (*Figure 5C and D in the original version*)}. As requested, the data in Supplementary Figure S5A were replotted to show relative % of cells with primary cilia.

In a separate experiment, our data showed that the S143A mutant exhibited a sizable defect in primary cilia disassembly as early as 3-6 h after serum stimulation (4-7 h after Wnt5a treatment) {Figure 7E (*Figure 6E in the original version*)}. These findings strongly diminish the likelihood that the defect in primary cilia disassembly in the S143A mutant is contributed by an altered cell cycle in the late stages. This finding is described in page 16 line 7.

6) On page 9, the paragraph describing results has several confusing statements. "in contrary to the role of Dvl2 in multiciliated cells" is not supported by a reference. If the authors refer to Park et al. that Dvld2 to indicated plays a role in ciliogenesis in multiciliated cells, it only has been shown that Dvld2 Is located at the base of the cilium.

We agree with the reviewer on this helpful comment. As suggested, we took out the phrase “in contrary to -----.“ Thanks for the clarification on the role of Dvl2 in multiciliated cells!

7) Figure 4C/D: As the percentage of ciliated cells and cilia length is very different after serum starvation, it would be descriptive to show an additional graph with the reduction in percent of the 0h time point for each time point of serum stimulation and calculate under which condition the reduction is significant.

As suggested, the results in Figure 4C and 4D were replotted and provided in Supplementary Figure S7. It is clear that shDvl2, shPlk1, shAurA, and shCK1ε cells are significantly defective in primary cilia disassembly in comparison to the shGL control cells.

8) Figure 5: Although an effect of S143A and T224A mutaions has been shown in the context of Wnt5 induced ciliary disassembly, to complete this chain of evidence, the effect of downregulation or inhibition of CK1δ/ε on the Wnt5-induced ciliary disassembly would be interesting. It would be

extremely valuable to add a small molecule inhibitor of CK1 to the analysis, to support the results with knockdowns, for this and other experiments.

We examined the effect of downregulation of CK1δ or CK1ε on the Wnt5-induced ciliary disassembly and the results are provided in Supplementary Figure S11A, and are described in page 15 line 20. The results confirm our observation that CK1ε, but not CK1δ, is critical for mediating Wnt5a-induced primary cilia disassembly. We have employed RNAi-based CK1δ or CK1ε-specific depletion, rather than IC261-based inhibition, because IC261 has been shown to inhibit both CK1δ and CK1ε equally well with an IC_{50} value of ~1 μM (Mashhoon N, et al., 2000 J. Biol. Chem. 275:20052). In addition, a delay in primary cilia disassembly after Dvl2 depletion was confirmed by a second shDvl2 targeting a distinct sequence, as shown in Supplementary Figure S4A-B. These findings suggest that the observed delay in primary cilia disassembly in shDvl2 cells is not the consequence of an off-target effect associated with any given shRNA targeting Dvl2.

9) To further support the hypothesis of a physiological role of Wnt5 in cilia disassembly, it would be important to see the expression level of Wnt5 in a time course while ciliary disassembly or it should be shown Wnt5 in IF before and after serum stimulation in ciliated cells.

As suggested, we examined the expression level of Wnt5a after serum stimulation. The results are provided in Supplementary Figure S11B, and are described in page 16 line 11. Our results show that Wnt5a is detectably expressed following serum stimulation and its expression is maintained in a sustained manner. These findings are consistent with Supplementary Figure S5, S8, and Figure 6A-D (*Figure 5C-F in the original version*).

10) General technical points: For data in Figure 1 and throughout the study, relative densities in western blots have been calculated but normalization would be helpful in some cases. As some western blots are overexposed, a shorter exposure would be necessary for a proper normalization of the data. Further, information about the number of times results were repeated in independent experiments is generally lacking, and should be supplied. p values indicating significance of results should be also included in some figures. Was the cilia counting performed blinded? It is difficult to assess the solidity of the results without this information.

Where appropriate, normalized Western blot intensities have been calculated (See the blots with numbers in the figures). In Figure 2C, relative intensities for both slow-migrating (hyperphosphorylated) and fast-migrating (underphosphorylated) forms under CK1δ and/or CK1ε RNAi conditions are provided. Shorter exposures are provided for the entire Figure 4A and Figure 7C (*Figure 6C in the original version*) bottom panel. The relative signal intensities in Figure 4A are normalized based on the levels of total proteins in the α-tubulin and Coomassie-stained gel. P values have been provided for Figure 4C and 7B (*Figure 4C and 6B in the original version*). Primary cilia quantifications for all the graphs were carried out 'blind'. Additional information on the number of cells counted and/or the number of experiments repeated was provided.

11) On page 13, in association with Figure 5, the authors state that centrosomal CK1ε signals increase gradually following addition of serum, and become abundant at mitotic centrosomes, as would be expected when cilia are totally reabsorbed. This is troubling on several levels. First, the increase shown in 5E/F is minor; certainly not enough to demonstrate a change in activity. It is much more likely that activity would spike at the time of ciliary disassembly, if activity were relevant to the process. Is there any antibody that allows analysis of activated CK1ε kinase at the basal body, rather than total kinase? Further, by the logic the authors use, CK1ε activity is NOT relevant to disassembly at the early time point, as the expression has not changed. This makes it likely the observed effects are mediated indirectly, through inhibiting G2/M entry, and again makes it urgent to back up the results with detailed FACS.

This turned out to be a very helpful suggestion! In the absence of anti-phospho-CK1ε antibody that can be used to assess the status of CK1ε activation either at basal bodies or in total lysates, we carried out in vitro kinase assays to determine the total kinase activity of CK1ε following serum stimulation. The result showed that the CK1ε activity was not detectable in 48 h serum-starved hTERT-RPE cells. However, it was detectably increased following serum stimulation. This result is now provided in Figure 6E (*Figure 5G in the original version*), and is described in page 14 line 21. This finding helps explain the drastic elevation of the p-S143 and p-T224 epitopes 3 h after serum stimulation {Figure 6B; *Figure 5D in the original version*)} in the absence of proportionally increased total Dvl2.

Minor comments:

• Figure 1A: The asterisk in this figure is not explained

Now, it is explained as a degradation product. Thanks!

• In some figures the bands shown in CBB are not clear and should be labeled

Where appropriate, missing labels for Coomassie-stained gel (CBB) are provided. Except the CBB panels for GST-PBD ligand, all other CBB panels were provided as loading controls.

• Figure 3D: Why is the 6h time point not included in this experiment like in the previous ones? Figure 3D is not showing a delayed ciliary disassembly, it is showing failure to disassemble cilia.

As suggested, now the 6 h data are provided. These results together with other data in the manuscript support the notion that the kinase activity of Plk1 is required for proper primary cilia disassembly.

• Figure 6C (KSL wrote: Figure 7C in the revised version): CK1ε is overexpressed and difficult to compare and both CK1ε and δ are not mentioned in the description of the results.

I suspect the reviewer have meant that CK1ε is "overexposured" not "overexpressed", since the cellular lysates are from untransfected hTERT-RPE cells. Thus, we provided a blot with shorter exposure.

Reviewer #2

In this manuscript, Lee and colleagues study the process of primary cilia disassembly prior to mitosis, a phenomenon described several decades ago but which has only recently started to be addressed. Through the use of biochemical and cell biology approaches the authors uncovered a new part of the pathway(s) involved in this process and describe new specific roles for Wnt5a, CK1ε, Dvl2 and Plk1. The major findings of this work are the following: Wnt5a promotes the phosphorylation of Dvl2 at the critical residues S143 and T224 which is dependent on CK1ε but not on the closely related CK1δ. Phosphorylated Dvl2 binds Plk1 an event that ultimately regulates HEF1-AurkA-HDAC6-dependent primary cilia disassembly through an indirect regulation of HEF1's degradation by the Smad3-APC10 complex. These findings are very relevant to the cilia and cell cycle fields. In general the experiments were well conducted and interpreted, and the results are of very good quality. Below are some concerns about several aspects of the manuscript but if addressed satisfactorily I would enthusiastically support publication in EMBO J.

- The model proposed by the author would be considerably strengthened by showing that the expression of a Dvl2 S143 and T224 phosphomimetic mutant that would constitutively bind Plk1 would hyperactivate HEF1-mediated cilia disassembly leading to either shorter cilia or defects in ciliogenesis altogether.

We examined whether mutation of S143 or T224 to a negatively charged residue (Asp or Glu) increases the interaction between Dvl2 and Plk1. Unfortunately, none of the four mutants that we generated exhibited an enhanced interaction, thus preventing us from further characterizing these mutants. This result is provided as Supplementary Figure S9C, and is discussed in page 13 line 18. The above results are not unexpected because, unlike mutational activation of activating phospho-Ser or phospho-Thr residues on kinases, literature shows that analogous mutations at polo-box binding phospho-Ser/Thr residues rather diminish binding affinity (Kang YH et al, 2006 Mol Cell 24:409; Soung et al, 2009 Dev Cell 16:539; Johmura et al, 2011 Proc Natl Acad Sci USA 108:11446). It is now well appreciated that phosphorylated Ser/Thr residues are strictly required for polo-box binding.

- The results presented in FigS3 need clarification. In this experiment the authors massively over-express Dvl2 (from a shRNA resistant transcript) in cells expressing either control Luciferase shRNAs or Dvl2 shRNAs. In cells expressing Luciferase shRNAs the over-expression of Dvl2 prevented primary cilia assembly after 48h of serum starvation. However, in cells supposedly depleted of endogenous Dvl2 but over-expressing the wt protein, primary cilia assembly is somehow restored. I am unable to understand this result given the levels of over-expressed Dvl2 present compared with the much lower levels of endogenous Dvl2 present in control Luciferase + Vector cells. Indeed even if the endogenous levels are depleted the over-expressed protein in Dvl2i + Dvl2 cells should prevent cilia assembly as in Gli+Dvl2, unless the over-expressed protein is not functional.

This is a very good comment for further clarification of our data. Indeed, we found that overexpressed Dvl2 generates a lot of aggregated intracellular assemblies that may not be functional in the Dvl2-mediated primary cilia disassembly pathway. This result is provided in Supplementary S4E and described in the Online Supplementary Information, page 18 line 4. Thanks for the helpful suggestion!

- In Fig3C the authors show that after starvation the population of PLK1 depleted cells presents more ciliated cells than the control population but this is never mentioned or discussed. Is this difference significant? Could PLK1 have a role regulating cilia assembly?

We believe that longer cilia are the result of the loss of Plk1-dependent primary cilia disassembly process. This is described in page 10 line 9 and page 12 line 7. Also, a new result, demonstrating a significantly increased ciliary population in shPlk1 cells, is provided in Supplementary Figure S5 to further support our view.

- Regarding Fig4A, the authors say on page in page 11 "Depletion of each of the components did not significantly alter the levels of other proteins (Figure 4A). Induction of a slow migrating Dvl2 (b form) in cells silenced for AurA and Plk1 (AurAi and Plk1i, respectively) could be due to the presence of a small fraction of mitotically arrested cells accumulated during depletion of these proteins.". However there are several alterations in protein levels that can be observed in these western blots: AurkA silencing significantly increases the total level of Dvl2 and not only the appearance of a slow migrating band. Furthermore, the appearance of this band is suggested to be due to the presence of a small percentage of mitotic cells but before the authors suggested, when referring to Fig1B, that Dvl2 is expressed at lower levels in mitosis. The same applies for PLK1 depletion in which the total levels of Dvl2 seem to be higher than in control cells. The silencing of Dvl2 causes an increase in β-catenin levels. Dvl2 silencing in this figure seems to decrease the levels of AurkA which is not observed later on. CK1δ levels are increased in the RNAi of Dvl2, β-cat, AurkA, PLK1 and CK1ε.

To fully address this comment, we carried out immunoblotting analyses with a new set of samples prepared. The new data provided in Figure 4A showed that depletion of each of the components in the pathway does not significantly alter the levels of other proteins, as we originally described. In a separate experiment, we observed that treatment of nocodazole-treated lysates with λ phosphatase significantly increased the amount of Dvl2 detected by immunoblotting analyses (Supplementary Figure S1). This finding suggests that the apparent low level of Western-detected Dvl2 in the nocodazole-treated sample in Figure 1B was likely due to a failure to detect hyperphosphorylated Dvl2 under our experimental conditions. To incorporate this finding, we eliminated "Induction of a slow migrating Dvl2 -----" from the original version. Thanks for this helpful comment!

- In Fig4C the authors show that the populations depleted of Dvl2 and AurkA have more ciliated cells than control cells. However this was not mentioned or discussed. This observation is curious since for example the RNAi of AurkA apparently did not have the same effect in the study of Pugacheva et al 2007. Are differences observed here statistically significant? What could be the reasons for them and their biological meaning?

Similar to the Plk1-depleted cells explained above, our data strongly suggest that Dvl2 and AurA play a critical role in primary cilia disassembly. Consistent with this opinion, depletion of Dvl2 or AurA leads to more (and also longer as shown in Figure 4D) ciliated cells than depletion of luciferase control. These observations are described in page 12 line 7. We observed that the maximum ciliary population for lentivirus-based shRNA-treated cells after 48 h starvation was significantly less (~50%) than that for siRNA-treated cells (~80%) (See the result provided in Supplementary Figure S4I and related text in page 10 line 4). Therefore, we speculate that this much diminished ciliogenesis condition may have allowed us to better observe enhanced ciliary formation in shDvl2, shPlk1, or shAurA cells.

The differences in the formation of primary cilia between control shGL and shDvl2 or shAurA cells have been statistically analyzed and the p-values are provided in Figure 4C.

- In different experiments there are differences in the observed percentages of ciliated cells for control cells (e.g. Fig3D and 4C). The authors point out that, lower percentages were observed for infected cells, but do not try to explain this. Is this due to the virus infection, due to the constant production of shRNAs? Did the authors try a different shRNA delivery method to clarify this matter? Although the results presented are clear and support the conclusions taken from them regarding cilia disassembly, this issue is relevant to analyse the experiment as a whole since the conditions regarding cilia assembly might not be ideal.

To address this question, we comparatively examined the efficiency of primary cilia formation in hTERT-RPE cells transfected with siLuciferase (siGL), or infected with lentivirus expressing

shLuciferase (shGL) or empty vector itself. The results provided in Supplementary Figure S4I confirmed that lentivirus infection itself significantly diminishes the level of primary cilia formation in these cells. However, we believe that the shRNA-based method is more adequate than the siRNA method to stably knockdown target proteins during the lengthy period of primary cilia disassembly assays.

In Fig4C the authors show that the populations depleted of β-catenin or CK1δ present a significantly reduced number of ciliated cells than controls after serum starvation. However this is not discussed. Are these proteins involved in cilia assembly or is this a new finding?

Our results hint that β-catenin or CK1δ are rather involved in primary cilia assembly process. As suggested, this view is now mentioned in page 12 line 15.

The authors never mention the fate of Dvl2 depleted cells that fail to disassemble their cilia. The recent data regarding this process indicates that indeed the presence of the primary cilium prevents the progression to mitosis supporting a ciliary role in the regulation of cell cycle progression. Therefore one wonders if this was observed in this study. As the authors mention, Dvl2 was shown to have roles in mitosis being for example required for proper spindle orientation. However Dvl2 silencing in HeLa cells did not prevent mitotic entry and spindle formation. Was this the case for Dvl2 silencing in starved ciliated RPE-1 cells or were these blocked due to the presence of the primary cilium? And if these cells were indeed arrested, would cycling (non ciliated) RPE-1 cells also arrest or would they behave like HeLa when silenced for Dvl2?

To investigate whether primary cilia disassembly is required prior to mitotic entry in hTERT-RPE cells, we quantified mitotic cells with primary cilia. The result showed that not a single mitotic hTERT-RPE cell possessed primary cilia. This finding hints the presence of a primary cilia-imposed cell cycle delay prior to G2/M transition, and suggests that disassembly of primary cilia is likely a prerequisite for mitotic entry in these cells. The results are provided in Supplementary Figure S15A and are described in page 20 line 14.

Interestingly, unlike HeLa cells, which rarely have primary cilia, depletion of Dvl2 in hTERT-RPE cells did not induce multipolar spindle morphologies, even though a significant fraction of the shDvl2 population exhibited no detectable primary cilia. hTERT-RPE cells may have a tighter regulation in the establishment of spindle bipolarity than HeLa cells. The results are shown in Supplementary Figure S15B and these findings are discussed in page 22 line 17.

Minor comments:

- For all the experiments the authors should indicate the number of times the assay was performed not only the number of cells counted.

This information is now provided in the figure legends.

- The manuscript needs to be proofread extensively to remove typos.

This has been done!

- I recommend that the nomenclature chosen to indicate silencing by RNAi in all the figures, i.e. adding an (i) in front of the name of the gene (e.g. Dvl2i) is changed. I believe it to be confusing particularly when rescue experiments are being shown.

Now, we changed the nomenclature, as suggested.

The authors often refer to cells expressing control Luciferase shRNAs as cells depleted of control GLi (e.g. page 11). This is incorrect and should be changed in the main text and figure legends. As human cells do not express luciferase they cannot be depleted of it.

This has been taken care of. Thanks for the correction!

- In page 6 the authors say "The level of Dvl2 bound to GSTPBD was reduced in nocodazole (Noc)-treated (M phase) cells, apparently due to a low level of Dvl2 expression at this stage (Figure 1B)". Have the authors or previous studies analysed Dvl2's expression during the cell cycle? Have they tried another method for blocking cells in mitosis to discard the hypothesis that this observation is due to microtubule depolymerization by nocodazole?

We closely examined the level of Dvl2 expression during the cell cycle. We carried out λ treatment and found that the apparent low level of mitotic Dvl2 was mainly due to the inefficiency of detecting hyperphosphorylated Dvl2 forms under our experimental conditions. This result is provided in Supplementary Figure S1, and is described in page 6 line 14.

- In Fig1A left panel there is a band (third lane) marked with an asterisk that is not explained.

We now indicated it as a degradation product. Thanks!

In FigS2C the authors present the results of a Luciferase reporter assay based on the SuperTopFlash and FopFlash reporter plasmids that is never properly explained. A brief explanation of the assay should be included either in the figure legend or the Materials and Methods section. Still regarding this figure, the authors say in pages 8 and 9 "However, Plk1 activity neither altered the degree of β-catenin ubiquitination nor influenced the level of β-catenin-dependent transcription activity (Supplementary FigureS2), thereby diminishing the likelihood that Dvl2-bound Plk1 contributes to the canonical pathway.". However, there is a difference in expression of the reporter between the DMSO and PLK1 inhibitor treated cells blocked in mitosis and in the presence of Wnt3a that is not commented. The meaning of this difference should be explained.

We now provided brief explanations for SuperTopFlash and FopFlash reporter plasmids in Online Supplementary Information, page 6 line 20. We agree with the reviewer that there is a moderate level of difference in the reporter expressions between Plk1 inhibitor (BI2536)-treated samples and shPlk1-treated samples. However, both data are consistent with the argument that Plk1 is not involved in the canonical Wnt signaling pathway. Generally speaking, although pharmacological inhibitors can effectively inhibit their target enzyme activities, they may exhibit non-specific cross-reactivities against unintended targets. Thus, the moderately elevated reporter expression in BI2536-treated samples in comparison to the reporter expression in shPlk1 cells could be due to the differences in the level of target inhibition combined with off-target effects. To avoid any potential confusion and to effectively deliver the main point of the results, we decided to omit the Supplementary Figure S2C (BI2536-treated samples), while keeping the Supplementary Figure 2D (shPlk1-treated samples) as Figure S2C in the current version.

- In the legend of FigS4C the cell cycle analysis should be explained a bit more saying for example that it was the DNA content of the cells that was analysed. The figure itself could be improved by saying that the first peak corresponds to 2N DNA content and the second to 4N and maybe by including the percentages of cells in each population.

This suggestion has been incorporated into the Supplementary Figure S5 and S6 legends.

- In Fig4E the band marked with the asterisk is not explained.

This is a degradation product. Now, this is done!

- In Fig 6A (KSL wrote: Figure 7A in the revised version) the left panel is not explained. One assumes that those cells were used in order to obtain medium containing Wnt5a but this should be explained.

This is explained in the legend.

- In FigS8D Smad2/3 seems to be absent from the inputs.

As suggested, we provided a longer exposure in the Supplementary Figure S13D to show Smad2/3 in the input.

We hope and believe that these comments and the changes to the manuscript should satisfy all the concerns about the original submission. We look forward to your response.

2nd Editorial Decision 17 April 2012

Thank you very much for the revised study.

One of the original referees assessed the rather significant revisions and seems, as visible from the enclosed comment, fully satisfied with the improvements provided.

Please allow me to congratulate to the study! The editorial office will soon be in touch with necessary paperwork related to official acceptance.

Please also notice that we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We thus publication of a single PDF file per figure comprising the original, uncropped and unprocessed scans at least for the key data of your paper. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. This PDF will be published online with the article as a supplementary "Source Data" file. Please let me know if you have any questions about this policy or check this link for a recent example (http://www.nature.com/emboj/journal/v30/n20/suppinfo/emboj2011298as1.html).

I am very much looking forward to your response.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT:

This manuscript by Lee et al has improved significantly following the performance of a large number of suggested experiments. Of particular importance, the authors now convincingly demonstrate the interactions of Dvl2 and Plk1, and the activation of CK1epsilon during the process of ciliary resorption. The resulting data are of high quality, and the study is worthy of publication.