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Cell cycle-dependent ubiquitylation and destruction of NDE1 by CDK5-FBW7 regulates ciliary length

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Transaction Report:

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

1st Editorial Decision

22 December 2014

Thank you for submitting your manuscript to our editorial office. I have now carefully assessed it and discussed it with the other editors. In addition, I also chose to send your study to an expert Advisory Editorial Board member of the journal for further consultations. I am afraid our conclusion from these considerations is that we cannot offer publication of your study at this point. We appreciate that the identification a mechanisms linking CDK5 via the important FBW7 E3 component to NDE1-mediated regulation of ciliogenesis represents an interesting and potentially important finding. Nevertheless, given that your previous report already established NDE1 regulation of ciliary length as well as cell cycle-related NDE1 oscillations, we feel that more conclusive and definitive insight into the pathway would be required to make this manuscript a sufficiently major overall advance warranting publication as a full EMBO Journal article. In particular, our expert advisor (whose detailed concerns you will find copied below for your information) retained important reservations regarding the differential effects on cilia biogenesis and cilium length regulation, and the importance of NDE1 overall protein levels as compared to localization. Given these concerns, I am afraid we cannot consider the manuscript at present a sufficiently strong candidate for publication in The EMBO Journal. It may however be suited for our sister journal EMBO reports, which focusses on concise, significant new findings that don't need to be mechanistically fully fleshed out; should you be interested in having the study considered further at EMBO reports, please briefly get back to me, and I would be happy to discuss the study with my EMBO reports colleagues following the holiday break. At this stage, however, I am very sorry to have to disappoint you.

Editorial Advisor's comments:

- I found it puzzling that the effect of NDE1 is on cilium length (rather than formation/biogenesis, as the title and Fig. 9 both wrongly imply), whereas that of FBW7 is on cilium formation (the authors discuss this a bit, arguing that FBW7 must have other targets); given this, why should the depletion of NDE1 (which has not impact on the fraction of ciliated cells (see Fig. 8C) partially rescue the lower ciliary number observed upon FBW7 depletion (see Fig. 4B)?
 - it was not clear to me whether the story was really about overall NDE1 protein levels (e.g. Fig. 1A, 24h or 36h, shows that there is still plenty of protein left in control conditions, certainly enough for it to be present at the primary cilium it seems); maybe regulation of centrosomal localization is key?
 - it was also not clear to me why the authors did not use the FBW7 mutant cells in further experiments given the contradictory results with the two siRNAs
-

Additional correspondence (author)

02 June 2015

Thank you for the consideration of our ms at The EMBO Journal and your offer to discuss it with The EMBO Reports editors. We would love to have our work eventually published by EMBO Press, so please go ahead. However, I would also like to briefly rebut the Editorial Advisor's comments just for clarity:

1) When we say that FBW7 depleted cells show a reduction in the number of ciliated cells at 24 h following serum starvation, it means that cilia were not visible to be counted. As we have shown in our Nature Cell Biology paper in 2011, at earlier points, cells depleted of NDE1 do show reduced numbers of ciliated cells. Therefore, we could easily count the number of ciliated cells in wild type, FBW7-, NDE1-, or FBW7-/NDE- depleted cells at 12, 24 (already done), and 48 h following serum starvation to test whether FBW7 caused a definitive block or a delay in ciliogenesis and this can be rescued by the depletion of NDE1. We picked up the 24 h time point to be consistent throughout the paper. We didn't realize that that point was not clear, but now I understand the reviewer's point. This can be easily rectified in revision.

2) In Figure 1A, it is shown that NDE1 is reduced by 80-90% when cells are in G1 in RPE1 cells and almost 100% in BALB/C 3T3 cells (Fig. S2). Therefore, there is a massive overall reduction. As the reviewer commented, the relevant pool here is the centrosomal pool, which is also massively reduced. We will be happy to present the data-they are available. In fact, this is why we investigated the presence of FBW7 and p35 at the centrosome. By the way, neither NDE1 nor FBW7 is present at the cilium. They are both present at the centrosome/basal body.

3) FBW7-deleted cells (DLD-1-FBXW7-/-) cannot be used for ciliation studies, because they are cancer cells and are not ciliated. We have already tested. There is nothing wrong with the second siRNA-simply, it was less efficient than the first one.

Overall, I strongly believe that our study is highly mechanistic and still suitable for The EMBO Journal, but it is also perfectly fine for us to be considered at The EMBO Reports. My concern is that after successful revisions, it will get too big for EMBO Reports. Thank you again and best wishes for a Happy Holiday Season

Additional correspondence (author)

14 January 2015

In the meantime, we did some experiments and here is a brief update:

1) Cells depleted of Fbw7 show an increase in ciliated cells from 24 to 48 h following serum starvation, indicating that Fbw7 depletion did not cause a permanent block in ciliation, but rather an inhibition. This could mean that cilia are there, but too small to be seen and counted.

2) Cells depleted of Fbw7 show a marked suppression of full length GLi2 levels and Gli3 cleavage (both cilia-dependent events), that were rescued in double mutant cells (Fbw7 and NDE1). This finding which is completely novel indicates that FBW7 and NDE1 do function antagonistically in the same pathway to regulate the Hedgehog pathway and support the point that they regulate ciliary length/ciliogenesis. If FBW7 depletion would cause a permanent block in ciliogenesis that could not be rescued by NDE1 depletion, we would have seen no modulation of the Hedgehog pathway, which is not the case. We are excited about these data, because no connection between FBW7 or NDE1 and the Hedgehog pathway has been reported before.

In light of these data, I would like to suggest that we add them in the existing paper formatted for EMBO Journal (it'd be much easier) and rectify the text to indicate that FBW7/NDE1 regulate ciliary length rather cilium biogenesis, as was recommended by the advisor, and re-submit to EMBO Journal, perhaps as a new manuscript.

Additional correspondence (editor)

14 June 2015

Thanks for the update on your further data, as well as your earlier clarifications which I have now been able to consider. In this light, I would agree to now having a modified manuscript reviewed for The EMBO Journal at first - so please go ahead and resubmit a revised manuscript incorporating the changes and additional data. The simplest way would be for you to email me the modified files (if allowed by the file size) and we'd upload them into our submission system, sparing you from having to go through the submission procedures once more.

Editorial Decision

10 March 2015

We have now received reports from three expert referees, who have evaluated your study on NDE1 regulation by CDK5/FBW7. I am pleased to inform you that all referees in principle acknowledge the potential interest and overall quality of this work, although there are nevertheless a number of specific concerns raised by referees 1 and 3 that will require close attention. Should you be able to adequately address these points (with the exception of Ref 3 point 8, which appears somewhat beyond the scope of this revision), we would be happy to consider a revised manuscript further for publication in The EMBO Journal.

I would therefore like to invite you to prepare a revised manuscripts along the lines suggested by the referees, keeping in mind that we allow only a single round of major revision, making it important to carefully respond to all points raised at this stage. We generally grant three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study; should you foresee a problem in meeting this three-month deadline, please let me know in advance and we could discuss the possibility of an extension. Finally, I should point out that we now require a completed 'author checklist' to be submitted with all revised manuscripts - see below for more detail.

Thank you again for the opportunity to consider this work for The EMBO Journal, and please do not hesitate to contact me should you have any feedback or questions regarding the referee reports or this decision. I look forward to your revision.

Referee #1:

Review of Cell cycle-dependent ubiquitylation and destruction of NDE1 by CDK5-FBW7 regulates cilium biogenesis.

The same group has previously demonstrated that the conserved microtubule-binding protein, Nde1, controls cilia length via a dynein-dependent mechanism. In particular, its depletion in cultured cells and zebrafish leads to the formation of overly long cilia. In this manuscript the authors identify the molecular pathway responsible for regulating Nde1 levels in G1/G0 cells. They report that the ubiquitin ligase FBW7 is essential for Nde1 degradation and that the kinase CDK5 primes this interaction. The findings here also highlight a broader role for FBW7 in ciliogenesis; while FBW7 depletion causes a reduction in the number of cilia formed, Nde1 depletion does not have such an effect. Likewise, co-depletion of FBW7 with Nde1 only partially rescues the ciliogenesis defect. The authors propose a linear pathway for the role of these proteins in cilia length control: Nde1 is phosphorylated by CDK5, then phosphorylated Nde1 is targeted for degradation through ubiquitylation by FBW7.

The data in the manuscript is novel, good quality and experiments seem to be performed to a high standard. The paper reads well and in my view the conclusions are well supported. Nevertheless, the manuscript would benefit from a few additional experiments and changes before publications, which I outline below.

1. The authors clearly demonstrate that total Nde1 levels are sensitive to CDK5 and FBW7 activities. However, currently there is no direct proof in the manuscript to show that it is the centrosomal/basal body pool of Nde1 that is affected. Therefore, it would be important to analyse the centrosomal/basal body pool of Nde1 in cells depleted of FBW7 or CDK5 by microscopy and quantitate these results. I would suggest doing these experiments both in presence and absence of serum. Surprisingly, there does not seem to be a change in Nde1 levels at the centrosome in Fig. 3C between control and FBW7 deleted DLD-1 cells.
2. While the results with Nde1T191I largely support the authors' hypothesis, the effects seen are not massive (i.e. small reduction in binding FBW7 and in ubiquitylation). It would be important to include some functional evidence as to whether Nde1T191I prevents/supports formation of long cilia. Does Nde1T191I localise to centrosomes/basal bodies?
3. Basal body localisation of FBW7 is not entirely convincing due to poor staining of cilia in Fig. 3B.
4. It would be useful to accompany Fig. 7 with representative images demonstrating correct localisation/accumulation of overexpressed products.
5. Specificity of centrosomal staining should be demonstrated by siRNA depletion of p35 in Fig. 6E.
6. Western blot in Fig 1D is quite dirty. Blot in Fig. 2A shows a different Nde1 pattern between FBW7 siRNA 1 and 2; considering that lanes have been loaded equally, and the siRNA efficiencies are comparable, this is difficult to explain. Can the authors show lesser exposed blots?
7. In Fig. E2A CDK5 signal seems prominent after release from nocodazole, and the inverse correlation between Nde1 and CDK5 levels is very clear. It is surprising therefore that in serum-starved cells CDK5 levels are much weaker. Are these blots comparable? If not, it would be informative to show CDK5, Nde1 and FBW7 levels in cycling vs quiescent cells on the same blot. Since levels of CDK5 may not be indicative of its activity, including p25 and p35 would be useful.
8. In the legend of Fig. 6D, I assume the authors meant to say p39 does NOT interact with p39.
9. The authors should use the same terminology (either FBXW7 or FBW7) through the manuscript.

Referee #2:

Review of Maskey et al (Tsiokas Lab).

The paper nicely describes the role of the Fbw7 E3 ligase and tumor suppressor as the trigger for destruction of Nde1, a critical regulator of centrosomal dynein traffic and a negative regulator of the formation of primary cilia in Go. By triggering destruction of Nde1 in Go, Fbw7 plays a key role in establishing ciliation. Concurrent inactivation of Nde1 partially rescues the effect of Fbw7 knockdown on ciliation, arguing that Nde1 is important but possibly not the only Fbw7 target that shows negative regulation of ciliation in Go. Completing the story, the authors show that the known upregulation of Cdk5 kinase in Go is important for phosphorylating and triggering destruction of Nde1. The authors define and mutate the Cdk5 phosphorylation site on Nde1, which stabilizes the protein and blocks Go. A TCGA-defined cancer mutation in an independent site also blocks degradation of Nde1.

The mechanism is supported by showing in vitro ubiquitination of Nde1 by Fbw7 dependent on Cdk5 phosphorylation of Nde1 substrate. The authors also show colocalization of Nde1 substrate and Fbw7 at centrosomes.

This is a straightforward and nicely executed study that shows how a well-established tumor suppressor, may direct loss of cilia by a known mechanism (via Cdk5) and potentially by additional pathway mutations. The paper suggests a potential role for Nde1 as a marker for cancer progression. I appreciate that an earlier Nature Cell Biology paper described a role for Nde1 in ciliary control, but that paper did not present the more complete and detailed mechanism here. I find the overall story easier to understand with knowledge of the key switches. The present story also suggests a search for parallel pathways using similar regulators, such as other Fbw7 targets.

The data are very simply presented and convincing, figure by figure.

I think the paper is appropriate for EMBO Journal. It may be worth having a commentary on this paper, esp. if there are other papers published on related topics. The cell cycle control of cilia is still murky and this is a clear story.

Referee #3:

In this study by Maskey and colleagues, the authors focus on the role of NDE1 as a regulator of ciliary length. Most of the paper focuses on defining a novel relationship between CDK5 and its phosphorylation of NDE1 as source of a signal that allows degradation by FBW7, a ubiquitin ligase. In general, these experiments are well performed, and the results are novel, and help clarify the regulation of ciliary dynamics. There are some questions related to the data and the model where clarification could help improve the certainty of the proposed relationships, and further address mechanism.

1. What is the evidence for the motif in Fig 1C as a phospho-degron completely specific for FBW7, as opposed to other E3 ligases? It would increase confidence if a negative control (alternative E3 ligase) was assessed.

2. For Figure 2A versus 2B, a better explanation of how siRNA knockdown of FBW7 can be effective in both contexts, but only result in removal of NDE1 with emetine, would aid in understanding the argument that FBW7 is essential.

3. Fig 3: The data demonstrate colocalization of FBW7 and NDE1. This is appropriate to support an interaction but also a bit peculiar, as if FBW7 is causing degradation of NDE1, one might expect a pattern of mutual exclusion, dependent on timing of ciliary synthesis. How do the authors explain this?

4. One point the authors do not consider is that AURKA is a negative regulator of cilia, and is a well-defined partner and substrate of FBXW7, based on multiple papers. AURKA has also been shown to interact with NDE2/NDEL1 in post-mitotic neurons. It is very possible that AURKA is a partner of NDE1 or FBXW7 contributing to the observed phenotypes. The authors should determine whether inhibition or depletion of AURKA influences the NDE1-associated phenotypes, and determine whether expression of AURKA at the basal body is affected by FBW7 (Figure 4 analyses) - hypothetically, if loss of FBW7 elevates AURKA, this could poise cells for disassembly or shortening of cilia. It is also a bit surprising that NDE1 knockdown by itself does not affect ciliation (Fig 4D).

5. On what basis do the authors say CDK5 causes phosphorylation of NDE1 on a single residue, or two residues (Fig 5A)? It is not possible to extrapolate phosphorylation events from migration changes. It would also be helpful to have the actual phosphorylation data presented for Fig 5B, at least in a supplemental figure.

6. Please provide quantification for Figure 8A, from multiple experiments. The claim of later elevation of NDE1 elevation in the absence of CDK5 is not convincing.

7. What happens to expression of FBW7 when CDK5 is depleted, and vice versa? This is important to know, to provide additional clarity to the model. It would also be useful to do knockdown experiments with p35, for completeness.

8. The authors emphasize in the introduction that CP110, trichoplein, and OFD1 as important defined regulators of ciliary length. Some basic experiments addressing the functional relationship between these proteins and NDE1, FBW7, and CDK5 would enhance the manuscript.

Minor points.

1. FBW7 is more commonly known as FBXW7, the official gene symbol. The authors should either use the official name, or make clear alternate nomenclature in the abstract, to aid the reader. Similarly, please incorporate use of the official gene symbol, CDK5R1, for p35/p25/etc, to help disambiguate.

2. Fig 1A. MG132 does not seem to lead to an increase in NDE1 levels; rather, it minimizes the decrease induced by starvation.

3. The introduction to the phospho-degron that is subject to mutation could be more clearly laid out in discussion of Fig 1C - for instance, a reference, and indications in the results text as to what typically phosphorylates this motif.

1st Revision - authors' response

07 June 2015

Referee #1:

The data in the manuscript is novel, good quality and experiments seem to be performed to a high standard. The paper reads well and in my view the conclusions are well supported. Nevertheless, the manuscript would benefit from a few additional experiments and changes before publications, which I outline below.

1. The authors clearly demonstrate that total Nde1 levels are sensitive to CDK5 and FBW7 activities. However, currently there is no direct proof in the manuscript to show that it is the centrosomal/basal body pool of Nde1 that is affected. Therefore, it would be important to analyse the centrosomal/basal body pool of Nde1 in cells depleted of FBW7 or CDK5 by microscopy and quantitate these results. I would suggest doing these experiments both in presence and absence of serum. Surprisingly, there does not seem to be a change in Nde1 levels at the centrosome in Fig. 3C between control and FBXW7 deleted DLD-1 cells.

Please see new Figures 2B and E6. We have followed the reviewer's suggestion and quantified NDE1 levels in the basal body/centrosome in wild type and FBW7-or CDK5-depleted cells in the presence (0 h time point) or absence of serum (12 and 24 h time points). Quantification of the amount of NDE1 in the centrosome of DLD-1 and knockout cells shows about ~2-fold increase in signal intensity. Please use high magnification to visualize the image in Figure 3C.

2. While the results with Nde1T191I largely support the authors' hypothesis, the effects seen are not massive (i.e. small reduction in binding FBW7 and in ubiquitylation). It would be important to include some functional evidence as to whether Nde1T191I prevents/supports formation of long cilia. Does Nde1T191I localise to centrosomes/basal bodies?

Please see new Figure E2. We now provide evidence that overexpressed Flag-tagged NDE1^{T191I} shows an accumulation at the centrosome resulting in almost invisible cilia. This effect seems more severe than the overexpression of wild type NDE1 which caused stumpy but still visible cilia (Kim et al, 2011). This observation is consistent with the stabilization of NDE1^{T191I} at the centrosome/basal body compared to wild type NDE1. In regard to the small reduction in FBW7 binding and ubiquitylation of NDE1^{T191I} compared to wild type NDE1 (Figure 1F and G), please note that only a small fraction of massively expressed, transfected wild type NDE1 is likely to be primed by endogenous CDK5 for recognition by transfected FBW7 (Figure 1F) or ubiquitylation by endogenous FBW7 (Figure 1G). Thus, a small difference in these parameters is not unreasonable.

3. *Basal body localisation of FBW7 is not entirely convincing due to poor staining of cilia in Fig. 3B.*

We replaced the image with an image that shows clear labeling of the primary cilium. In our hands, staining of endogenous ARL13B in RPE1-hTERT shows clear and robust expression along the primary cilium, exclusion from the transition zone, and some weak staining- not always consistent, at the area of the centrosome. Thus, using ARL13B as a ciliary marker in this set of experiments, we concluded that FBW7 was not present along the cilium, but instead, it was present in the centrosome/basal body (based on co-localization with a variety of centrosomal proteins). To provide further evidence that FBW7 was present in the basal body, we co-stained FBW7 with CEP164, a distal appendage marker of the mature mother centriole, in serum-starved cells. Please see Figure E3.

4. *It would be useful to accompany Fig. 7 with representative images demonstrating correct localisation/accumulation of overexpressed products.*

We would be happy to provide the results from this experiment, but they are difficult to interpret. In Figure 7, CDK5 and p25 are both tagged with GFP, while NDE1 is Flag-tagged. Transient expression in HEK293T cells results in massive overexpression everywhere in the cell without specific expression at the basal body/centrosome. Thus, although we do see extensive co-localization of p25 (labeled with rabbit α -p35/25), NDE1 (labeled with mouse α -Flag), and GFP, that labels both transfected CDK5 and p25, we cannot be certain that there is specific co-localization at the centrosome. However, recognition of NDE1 by FBW7 and subsequent degradation by the SCF^{FBW7} complex could occur in sites other than the centrosome, since endogenous FBW7 is expressed in multiple subcellular sites, in addition to the centrosome, such as nucleus, nucleolus, and in the cytoplasm.

5. *Specificity of centrosomal staining should be demonstrated by siRNA depletion of p35 in Fig. 6E.*

Please see new Figure 6E (right image). We have depleted p35 and now show that signal in the centrosome was undetectable. We also show that levels of NDE1 are increased (Figure 6F).

6. *Western blot in Fig 1D is quite dirty. Blot in Fig. 2A shows a different Nde1 pattern between FBW7 siRNA 1 and 2; considering that lanes have been loaded equally, and the siRNA efficiencies are comparable, this is difficult to explain. Can the authors show lesser exposed blots?*

In regard to old Figure 1D, we think that reviewer is referring to the lower panel. This was due to the fact that we had re-probed the blot initially probed with α -NDE1 with α -FBW7 without stripping the α -NDE1 from the blot. We have now replaced the bottom panel showing efficient immunoprecipitation of FBW7 (Figure 1D, lower panel) and expanded the upper panel to show almost the entire blot. In regard to old Figure 2A, we have repeated this experiment multiple times using two FBW7 siRNAs and the results are now shown side-by-side in new Figure 2A. We believe that the confusion in the previous blot (old Figure 2A) was due to non-accurate determination of FBW7 knockdown efficiency, although knockdown efficiencies looked similar. This issue has now been resolved.

7. *In Fig. E2A CDK5 signal seems prominent after release from nocodazole, and the inverse correlation between Nde1 and CDK5 levels is very clear. It is surprising therefore that in serum-starved cells CDK5 levels are much weaker. Are these blots comparable? If not, it would be informative to show CDK5, Nde1 and FBW7 levels in cycling vs quiescent cells on the same blot. Since levels of CDK5 may not be indicative of its activity, including p25 and p35 would be useful.*

Please see new Figure E4. We have repeated these experiments and new samples are loaded in the same blot, as the reviewer recommended. We have included p25/p35 levels.

8. *In the legend of Fig. 6D, I assume the authors meant to say p39 does NOT interact with p39.*

We made the correction.

9. *The authors should use the same terminology (either FBXW7 or FBW7) through the manuscript.*

We are aware of the confusion, but try to be consistent with current nomenclature. FBW7 is referring to the protein and *FBXW7* is referring to the gene. We made this distinction in the text.

Referee #2:

Review of Maskey et al (Tsiokas Lab).

The paper nicely describes the role of the Fbw7 E3 ligase and tumor suppressor as the trigger for destruction of Nde1, a critical regulator of centrosomal dynein traffic and a negative regulator of the formation of primary cilia in Go. By triggering destruction of Nde1 in Go, Fbw7 plays a key role in establishing ciliation. Concurrent inactivation of Nde1 partially rescues the effect of Fbw7 knockdown on ciliation, arguing that Nde1 is important but possibly not the only Fbw7 target that shows negative regulation of ciliation in Go. Completing the story, the authors show that the known upregulation of Cdk5 kinase in Go is important for phosphorylating and triggering destruction of Nde1. The authors define and mutate the Cdk5 phosphorylation site on Nde1, which stabilizes the protein and blocks Go. A TCGA-defined cancer mutation in an independent site also blocks degradation of Nde1.

The mechanism is supported by showing in vitro ubiquitination of Nde1 by Fbw7 dependent on Cdk5 phosphorylation of Nde1 substrate. The authors also show colocalization of Nde1 substrate and Fbw7 at centrosomes.

This is a straightforward and nicely executed study that shows how a well-established tumor suppressor, may direct loss of cilia by a known mechanism (via Cdk5) and potentially by additional pathway mutations. The paper suggests a potential role for Nde1 as a marker for cancer progression. I appreciate that an earlier Nature Cell Biology paper described a role for Nde1 in ciliary control, but that paper did not present the more complete and detailed mechanism here. I find the overall story easier to understand with knowledge of the key switches. The present story also suggests a search for parallel pathways using similar regulators, such as other Fbw7 targets.

The data are very simply presented and convincing, figure by figure.

I think the paper is appropriate for EMBO Journal. It may be worth having a commentary on this paper, esp. if there are other papers published on related topics. The cell cycle control of cilia is still murky and this is a clear story.

We thank the reviewer for the positive evaluation.

Referee #3:

In this study by Maskey and colleagues, the authors focus on the role of NDE1 as a regulator of ciliary length. Most of the paper focuses on defining a novel relationship between CDK5 and its phosphorylation of NDE1 as source of a signal that allows degradation by FBW7, a ubiquitin ligase. In general, these experiments are well performed, and the results are novel, and help clarify the regulation of ciliary dynamics. There are some questions related to the data and the model where clarification could help improve the certainty of the proposed relationships, and further address mechanism.

1. What is the evidence for the motif in Fig 1C as a phospho-degron completely specific for FBW7, as opposed to other E3 ligases? It would increase confidence if a negative control (alternative E3 ligase) was assessed.

Please see Fig. E1. APC/C and β -TrCP1 were tested as alternative E3 ligases for NDE1. There were no detectable effects on degradation of NDE1 (APC/C) or binding to NDE1 (β -TrCP1).

2. For Figure 2A versus 2B, a better explanation of how siRNA knockdown of FBW7 can be effective in both contexts, but only result in removal of NDE1 with emetine, would aid in understanding the argument that FBW7 is essential.

Please see new Figure 2A and also our response to point 6 of reviewer 1. For clarification, steady-state levels of NDE1 were also affected in cells transfected with siRNA#2 (please compare lanes 2 and 6 of old Figure 2B), but steady-state levels did not seem to be affected significantly in old Fig. 2A (please compare lanes 2 and 3 of old Figure 2A), although efficiency of FBW7 knockdown seemed to be similar (lanes 2 and 3, old Figure 2A). We believe that assessment FBW7 silencing efficiency was problematic in old Figure 2A. Therefore, we repeated these experiments multiple times and re-organized this figure

(now Figure 2A), so that there is no confusion about the efficiency of FBW7 silencing and upregulation of NDE1 using both siRNAs.

3. Fig 3: The data demonstrate colocalization of FBW7 and NDE1. This is appropriate to support an interaction but also a bit peculiar, as if FBW7 is causing degradation of NDE1, one might expect a pattern of mutual exclusion, dependent on timing of ciliary synthesis. How do the authors explain this?

We show co-localization of NDE1 and FBW7 in cells that are not serum-starved (Figure 3A), in which condition NDE1 is not primed by CDK5 for degradation by SCF^{FBW7}. The reason that we did not show expression of NDE1 in serum-starved cells (Figure 3B) was that it was barely detectable. As per reviewer 1's suggestion, we now quantify this reduction at the centrosome/basal body region (new Figure 2B). Thus, under conditions of serum starvation, there is a marked reduction of NDE1 in the centrosome/basal body, showing a pattern of mutual exclusion, dependent on timing of ciliary formation.

4. One point the authors do not consider is that AURKA is a negative regulator of cilia, and is a well-defined partner and substrate of FBXW7, based on multiple papers. AURKA has also been shown to interact with NDE2/NDEL1 in post-mitotic neurons. It is very possible that AURKA is a partner of NDE1 or FBXW7 contributing to the observed phenotypes. The authors should determine whether inhibition or depletion of AURKA influences the NDE1-associated phenotypes, and determine whether expression of AURKA at the basal body is affected by FBW7 (Figure 4 analyses) - hypothetically, if loss of FBW7 elevates AURKA, this could poise cells for disassembly or shortening of cilia. It is also a bit surprising that NDE1 knockdown by itself does not affect ciliation (Fig 4D).

The reviewer is correct that Aurora A kinase is a known partner and target of FBW7 and has an established role in ciliary disassembly. It also phosphorylates NDEL1 upon mitotic entry and in postmitotic neurons. However, there is no direct evidence that it phosphorylates NDE1. As per the reviewer's suggestion, we tested whether depletion of FBW7 could result in the upregulation of Aurora A Kinase in serum-starved cells (G1/G0). Our data show that this was not case in RPE1-hTERT cells (Figure 2A). This is not entirely unexpected, since well-established FBW7 targets such as Cyclin E, Myc, etc are not subject to degradation via FBW7 in every tissue and/or cell line examined. We do show, however, a significant downregulation of Aurora A Kinase in G1 (please compare lane 1 with lanes 2-4, Figure 2A), which is consistent with its degradation by the APC/C. These data make us believe that the major pathway that degrades Aurora A kinase in G1 is through APC/C and not SCF^{FBW7}, and that seems to be also consistent in RPE1-hTERT cells. An additional important point arguing against a role of Aurora A kinase in the NDE1-associated phenotype depletion is that knockdown or chemical inhibition of Aurora A kinase does not affect cilia formation and/or ciliary length in RPE1-hTERT cells. This has been specifically examined in (Pugacheva et al, 2007). Aurora A Kinase has a specific effect on ciliary disassembly induced by serum-re-addition. In this manuscript, we do not examine the effect of NDE1 on ciliary disassembly. Because of these reasons, we did not investigate further a role of Aurora A Kinase in modulating ciliary phenotypes induced by the depletion of NDE1. In regard to the effect of NDE1 knockdown on ciliation (% of ciliated cells), NDE1 does have an effect on ciliation, but at earlier time points. After ciliation is completed (varies from 24h to 48h), the effect of NDE1 depletion is more pronounced on ciliary length.

5. On what basis do the authors say CDK5 causes phosphorylation of NDE1 on a single residue, or two residues (Fig 5A)? It is not possible to extrapolate phosphorylation events from migration changes. It would also be helpful to have the actual phosphorylation data presented for Fig 5B, at least in a supplemental figure.

We agree with the reviewer, but please note that gels in Figures 5A and C and E5 contained 50 μ M Phos-tagTM reagent, which allows resolution of protein phosphorylation in a step-wise manner, depending on the degree of phosphorylation. We have added the actual phosphorylation data in Figure E5, as the reviewer requested.

6. Please provide quantification for Figure 8A, from multiple experiments. The claim of later elevation of NDE1 elevation in the absence of CDK5 is not convincing.

Please see new Figure 8A.

7. What happens to expression of FBW7 when CDK5 is depleted, and vice versa? This is important to

know, to provide additional clarity to the model. It would also be useful to do knockdown experiments with p35, for completeness.

Please see new Figures 2A and 8A. We have also knocked down p35 and presented the data in new Figure 6F.

8. The authors emphasize in the introduction that CP110, trichoplein, and OFD1 as important defined regulators of ciliary length. Some basic experiments addressing the functional relationship between these proteins and NDE1, FBW7, and CDK5 would enhance the manuscript.

We are afraid that addressing these important questions could diffuse the focus of the manuscript. Perhaps, they could form the core of a new manuscript, where combinatorial effects of more than one negative regulator of ciliary formation and length, such as NDE1, CP110, trichoplein, etc could be examined in wild type and FBW7-depleted cells more thoroughly. Preliminary data looking at overall levels of CP110 in the cells depleted of FBW7, though, did not show significant differences.

Minor points.

1. FBW7 is more commonly known as FBXW7, the official gene symbol. The authors should either use the official name, or make clear alternate nomenclature in the abstract, to aid the reader. Similarly, please incorporate use of the official gene symbol, CDK5R1, for p35/p25/etc, to help disambiguate.

We made appropriate changes, as we saw fit, to avoid confusion. In the case of p25/p35, however, we kept the p25/p35 terminology, because we need to discriminate between p25 and p35 in several places in the manuscript.

2. Fig 1A. MG132 does not seem to lead to an increase in NDE1 levels; rather, it minimizes the decrease induced by starvation.

We made the change.

3. The introduction to the phospho-degron that is subject to mutation could be more clearly laid out in discussion of Fig 1C - for instance, a reference, and indications in the results text as to what typically phosphorylates this motif.

We followed the reviewer's suggestion.

References

Kim S, Zaghoul NA, Bubenshchikova E, Oh EC, Rankin S, Katsanis N, Obara T, Tsiokas L (2011) Nde1-mediated suppression of ciliogenesis affects cell cycle re-entry. *Nat Cell Biol* **13**: 351-360

Pugacheva EN, Jablonski SA, Hartman TR, Henske EP, Golemis EA (2007) HEF1-dependent Aurora A activation induces disassembly of the primary cilium. *Cell* **129**: 1351-1363

Acceptance letter

29 June 2015

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Referee #1

The authors have satisfactorily addressed my comments and I now recommend the manuscript for publication.

Referee #3

In this revision, the authors have done an excellent job of addressing the critiques made by all reviewers. The current story is interesting, and bears on the long-standing issue of ciliary length control. This is a very complete and valuable study.