

Manuscript EMBO-2012-82995

NDR2-mediated Rabin8 phosphorylation is crucial for ciliogenesis by switching binding specificity from phosphatidylserine to Sec15

Shuhei Chiba, Yuta Amagai, Yuta Homma, Mitsunori Fukuda and Kensaku Mizuno

Corresponding author: Kensaku Mizuno, Tohoku University

Review timeline:

Submission date:	17 August 2012
Editorial Decision:	04 September 2012
Additional Correspondence:	06 September 2012
Revision received:	04 January 2013
Editorial Decision:	24 January 2013
Revision received:	30 January 2013
Accepted:	31 January 2013

Transaction Report:

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

Editor: David Del Alamo

1st Editorial Decision

04 September 2012

Thank you for the submission of your manuscript to The EMBO Journal. It has been sent to three referees, and so far we have received reports from two of them, which I copy below. As both referees are convinced about the interest and quality of your study, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and I will forward you the third report, probably including further requests, as soon as I receive it.

Without going into details that you will find below, both referees are very positive and ask mainly for minor clarifications. Two concerns, however, have been pointed out that must be addressed experimentally before your manuscript can be accepted for publication. Referee #1 suggests two precise experiments that will in principle settle the question on the relative contributions of NDR1 and NDR2, while referee #2 in turn, proposes an alternative experiment to further confirm the lipid-binding capabilities of Rabin8.

Please be aware that acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is 'The EMBO Journal' policy to allow a single round of revision only. Acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For

more details on our Transparent Editorial Process initiative, please visit our website:
<http://www.nature.com/emboj/about/process.html>

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Do not hesitate to contact me by e-mail or on the phone in case you have any questions or need further input.

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

REFEREE REPORTS

Referee #1

In this paper Chiba et al. analyze the molecular events that are involved in the formation of ciliary membranes at the onset of ciliogenesis. They show that NDR1 and 2 can phosphorylate Rabin8 at residue serin-272 by in vitro kinase assays. Importantly, this reaction is necessary for efficient cilium formation as shown in RPE tissue culture cells after serum starvation. They further show that Rabin8 transiently associates with pericentrosomal vesicles after serum starvation and phosphorylation of Rabin8 is necessary to release the protein into the cytosol, because the S272A mutant form of Rabin8 was defective in detaching from the pericentrosomal vesicles. Interestingly, phosphorylation of Rabin8 switches its binding affinities from binding to phosphatidylserin and Rab11-GTP to binding to the exocyst component Sec15 presumably allowing for vesicle tethering and subsequent release of Rabin8 from the membranes. Because, this paper bridges cellular signaling to cilium outgrowth it should be of broad interest to EMBO J readers.

Major comments:

- 1) Although it seems that the number of ciliated cells is not reduced upon depleting NDR1, from the images chosen it looks like that cilia that were formed in the cells with depleted NDR1 are shorter in comparison to the control cells (figure 2B). This should be further investigated, as it would strengthen the author's argument that NDR1 and NDR2 both can phosphorylate Rabin8. If indeed only NDR2 would phosphorylate Rabin8 in vivo, how would this be accomplished? Does only NDR2 but not NDR1 localize to pericentrosomal vesicles? This should be tested.
- 2) Upon treatment of RPE cells with siRNA to knock down NDR1 or NDR2, it looks like NDR2 is completely depleted whereas NDR1 is not. Could it be that NDR1 depletion has no obvious effect on ciliogenesis because the depletion was not 100%? Is there an additive effect if NDR1 and NDR2 are depleted at the same time? This could easily be tested.

Minor comments:

- 1) From the data shown in figure 4B, I don't think the authors can claim with confidence that "Rabin8 binds more tightly to membrane-associated Rab11-GTP than to membrane-free Rab11-GTP", because there is no way to know how Triton X-100 effects the binding interface between Rab11 and Rabin8. Please rephrase.
- 2) The discussion is lengthy and should be shortened. Indeed, right now the discussion contains many repetitions of arguments and descriptions that were already made in the result section.

Referee #3

Summary:

In this manuscript the authors describe the regulation of ciliogenesis by the NDR2-mediated phosphorylation of the Rab8 guanine nucleotide exchange factor (GEF) Rabin8. Mutations in NDR2

have been identified in canine retinal ciliopathies, supporting its crucial role in ciliogenesis. The authors now show that Rabin8 is a substrate for the serine/threonine kinase NDR2 and that the phosphorylation of Rabin8 switches its affinity from the Rab11/phosphatidyl serine (PS) to Sec 15 binding. The authors define the similarities and differences between the regulation of Rabin8 and its yeast counterpart Sec2 that point to the evolutionary conservation of the regulation of vesicular trafficking by NDR-family kinases. However, they provide evidence that different phospholipids are responsible for the recruitment of Rabin8 and Sec 2 to Rab11-positive membranes. This is an important and carefully executed study, and the data generally support the conclusions. My comments/suggestions are listed below.

Major comments:

The lipid binding assays often provide information that is contradictory to the protein-phospholipid-binding affinities *in vivo* (as was the case with *evt2* used in this study). To strengthen their evidence for the Rabin8 binding to PS, the authors may consider generating a lipid-binding domain of Rabin8 fused to a fluorescent reporter to further examine the lipid binding properties of Rabin8. The binding data shown in Figure 5 should be quantified. Preferably, they should be directly compared to the Sec2 lipid binding data as lipid-binding assays vary.

Minor comments:

1. Labeling of different panels in Figure 1 (and similarly in other figures) is confusing. For example the top panel in Fig 1B represents an autoradiogram but the label "Autoradiography" is actually closer to the lower panel that presumably represents Amido black staining. The authors should clearly group the images with the corresponding labels in all figures.
2. The authors do not provide the information about the RPE cell line used in this study, which is essential for comparison to other data generated in immortalized RPE cells.
3. Why is NDR2 almost undetectable in siRNA control and NDR1 siRNA treated cells in Figure 2A?
4. On page 5 the authors state "NDR2 siRNAs significantly decreased the number of ciliated cells". What is the P value?
5. Although the text on page 5 refers to "ciliated" cells Figure 2C and E state "ciliary cells". This should be corrected.
6. On page 7 the authors state that "Quantitative analysis showed that the number of cells with pericentrosomal localization of YFP-Rabin8(WT) in NDR2-depleted cells was higher than that observed in non-NDR2-depleted cells". How many experiments were performed and how many cells were counted? There are no error bars in Figure 3B and D and no P-value is given.
7. On page 7 (and elsewhere) the authors describe detergent-assisted membrane "abstraction". This term is confusing as abstraction mostly relates to ideas and concepts. The authors probably meant "membrane removal" or "membrane extraction". This should be corrected throughout the manuscript.
8. On page 8 the authors state: "Since the S272E mutation weakens the ability of Rabin8 to PS...". Did they mean the ability of Rabin8 to bind to PS?
9. On page 9 the authors mention the "Rabin8 deleted mutant". This should be replaced with "Rabin8 deletion mutant".
10. The Discussion is too long and repetitive. Many statements from the results are repeated in the first part of the Discussion. This repetition should be eliminated and the authors should focus more on discussing their data in the context of current literature.

Thank you again for the submission of your research manuscript to The EMBO Journal. As I mentioned in my previous letter, your manuscript was sent to three referees and we have just received the third report, which I copy below.

As you can see below, referee #2 is more negative than referees #1 and #3. S/he is essentially concerned with your description of the dynamics of Rabin8, Rab11 and Sec15 and how it relates to subcellular localizations and vesicle association. Two major points, 5 and 6 in his/her list, relate to these concerns and will need to be addressed experimentally in the revised version of your manuscript. Other points raised, particularly related to control experiments, will also need your attention, although point 10 is out of the scope of this manuscript and will not be taken into consideration for acceptance of your study. Naturally, if you feel that you can address this concern, addition of extra experimental data would only be in the best interest of your manuscript.

Do not hesitate to contact me by e-mail or on the phone in case you have any questions or need further input.

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

REFEREE REPORT

Referee #2

This paper analyzes the effect of phosphorylation of the Rab8 GEF, Rabin8, on its role in ciliogenesis. They propose that phosphorylation by the NDR2 kinase regulates a switch in Rabin8 binding affinity from phosphatidyl serine to Sec15 and that this switch is necessary for the tethering and fusion of precursor vesicles needed for ciliogenesis. In general, I found many of the experimental aspects of the paper to be too preliminary, furthermore there is considerable vagueness regarding the overall mechanism. The overriding problem I have is understanding the series of events that they are proposing. Presumably, Rabin8 is recruited from the cytosol to precursor vesicles by the combination of Rab11-GTP and PS. The vesicles concentrate at the centriole and at some point Rabin8 is phosphorylated by NDR2. This causes a loss of affinity for PS and an increase in affinity for Sec15. So does this cause dissociation of Rabin8 from vesicles? If so, how does that facilitate tethering? "Tethering" implies the linkage of vesicles to some structure. What structure are the vesicles tethered to? What is Sec15 bound to? Does a block in phosphorylation block fusion or recycling? I find this all rather vague and confusing.

Specific comments:

1. Although they demonstrate that S272 is important for phosphorylation, they don't actually show that this residue is phosphorylated. In principle, this site could be required for recognition by NDR2 and some other site is phosphorylated. If it is phosphorylated, what is the stoichiometry?
2. Where is NDR2? Is it concentrated at the centriole?
3. Is there any increased effect of knocking down both NDR1 and NDR2 together?
4. They need to show complementation of NDR2 knockdown with a resistant NDR2 construct (but not kinase dead).
5. The prolonged association of Rabin8 with the centrosome when phosphorylation is blocked can be interpreted in several ways and needs to be explored in greater depth. A time course of association after starvation would help determine if the onset of Rabin8 localization is normal or if the entire process is just slowed down. FRAP analysis could establish if the rate of Rabin8 turnover has been altered. Is Rabin8 actually associated with vesicles when phosphorylation is blocked? This could be established by immuno-EM. It is essential that the authors define the exact step in the ciliogenesis reaction that is blocked in this situation. Are vesicles concentrated but not fused? In Fig 3A, lower panels, there appears to be 3 Ac-tubulin spots, only one of which is labeled with Rabin8(SA).

6. The experiment shown in Figure 4B is very weak. They are comparing the binding of Rabin8 to soluble, monomeric Rab11 in detergent versus Rab11 embedded in a complex biological membrane with lots of other proteins present. It is impossible to make any definitive conclusion from this comparison. A far better experiment would be to compare binding of pure non-prenylated Rab11 to pure prenylated Rab11 that has been reconstituted into artificial liposomes of varying composition. Even in that more defined situation they would need to keep the Rab11 concentration below 1 molecule per liposome to make sure that any apparent increase in affinity is not due to increased copy number/particle.
7. The effect of the S to E mutation on PS binding (Figure 5D) is not very dramatic. This would be more convincing if done as binding curves over a range of PS concentrations. Actual kD's would be best.
8. Figure 5 F is not convincing. The evt(PH) does not appear to be concentrated at the centriole at all.
9. Is the carboxy terminus of Rabin8 (350-460) sufficient for PS binding or is it just necessary? To definitively assess the role of PS binding it is important to define the PS binding site and then construct mutants that are specifically defective in PS binding. In addition they could determine the effect of reducing PS synthesis, but I imagine that would have many consequences in vivo.
10. The authors propose a conformational switch in Rabin8 from a Rab11- and PS-binding conformation to a Sec15-binding conformation. A direct, physical assay for conformational change upon phosphorylation would make this argument far more convincing.

1st Revision - authors' response

04 January 2013

Replies to Referee #1:

Thank you for reviewing our manuscript. According to the reviewer's comments, we carried out additional experiments and revised the manuscript carefully.

In this paper Chiba et al. analyze the molecular events that are involved in the formation of ciliary membranes at the onset of ciliogenesis. They show that NDR1 and 2 can phosphorylate Rabin8 at residue serin-272 by in vitro kinase assays. Importantly, this reaction is necessary for efficient cilium formation as shown in RPE tissue culture cells after serum starvation. They further show that Rabin8 transiently associates with pericentrosomal vesicles after serum starvation and phosphorylation of Rabin8 is necessary to release the protein into the cytosol, because the S272A mutant form of Rabin8 was defective in detaching from the pericentrosomal vesicles. Interestingly, phosphorylation of Rabin8 switches its binding affinities from binding to phosphatidylserin and Rab11-GTP to binding to the exocyst component Sec15 presumably allowing for vesicle tethering and subsequent release of Rabin8 from the membranes. Because, this paper bridges cellular signaling to cilium outgrowth, it should be of broad interest to EMBO J readers.

Major comments:

1) Although it seems that the number of ciliated cells is not reduced upon depleting NDR1, from the images chosen it looks like that cilia that were formed in the cells with depleted NDR1 are shorter in comparison to the control cells (figure 2B). This should be further investigated, as it would strengthen the author's argument that NDR1 and NDR2 both can phosphorylate Rabin8. If indeed only NDR2 would phosphorylate Rabin8 in vivo, how would this be accomplished? Does only NDR2 but not NDR1 localize to pericentrosomal vesicles? This should be tested.

According to the reviewer's comment, we measured the length of cilia in NDR1-depleted cells, in comparison to the control cells. As shown in [Fig. 2D](#) in the revised manuscript, the average length of cilia that were formed in NDR1-depleted cells was ~15% shorter than that in control cells. Additionally, the length of cilia in NDR2-depleted cells was also shorter than that in control cells, and the length of cilia in NDR1/NDR2 double knockdown cells was shorter than those in NDR1 or NDR2 single knockdown cells. These results indicate that both NDR1 and NDR2 are involved in lengthening cilia.

To examine how NDR2 has a more active role in ciliogenesis than NDR1, we analyzed the localization of NDR1 and NDR2 in RPE1 cells. As shown in [Fig. 2F, 2G, and Supplementary Fig. S2D](#), NDR1 was diffusely distributed in the cytoplasm and the nucleus, but NDR2 localized in the vesicular structures, as previously reported in other cell types (Devroe et al., 2004). Time-lapse fluorescence analysis of YFP-NDR2 revealed that NDR2 vesicles were often associated with the centrosome ([Fig. 2H and Supplementary Movie S2](#)). These results suggest that the preferential role of NDR2 over NDR1 in ciliogenesis may be due to the difference in their subcellular localization.

2) Upon treatment of RPE cells with siRNA to knock down NDR1 or NDR2, it looks like NDR2 is completely depleted whereas NDR1 is not. Could it be that NDR1 depletion has no obvious effect on ciliogenesis because the depletion was not 100%? Is there an additive effect if NDR1 and NDR2 are depleted at the same time? This could easily be tested.

According to this comment, we analyzed the effect of various concentrations (12.5 to 100 nM) of NDR1 siRNA on ciliogenesis (we usually used 25 nM siRNA). As shown in [Supplementary Fig. S2B](#), the higher dose of NDR1 siRNA weakly suppressed ciliogenesis, although the effect was still weaker than that of NDR2 siRNA. We also analyzed the effect of double knockdown of NDR1 and NDR2 on ciliogenesis. Double knockdown slightly reduced the number of ciliated cells, compared with the effect of NDR2 single knockdown ([Supplementary Fig. S2C](#)). These results indicate that NDR1 siRNA has the weak potential to suppress ciliogenesis, but the effect of NDR1 siRNA is weaker than that of NDR2 siRNA. We added these data in the revised manuscript.

Minor comments:

1) From the data shown in figure 4B, I don't think the authors can claim with confidence that "Rabin8 binds more tightly to membrane-associated Rab11-GTP than to membrane-free Rab11-GTP", because there is no way to know how Triton X-100 effects the binding interface between Rab11 and Rabin8. Please rephrase.

We agree the reviewer's comment. We rephrased to "Exposure to Triton X-100 drastically decreased the amount of Rab11(QL) bound to Rabin8, compared to those not exposed to Triton X-100 (Figure 4C in revised manuscript), which raises the possibility that some of the membrane components are involved in promoting Rabin8 binding to Rab11-containing vesicles." (in page 9).

2) The discussion is lengthy and should be shortened. Indeed, right now the discussion contains many repetitions of arguments and descriptions that were already made in the result section.

According to the reviewer's comment, we deleted repetitive descriptions and shortened 'Discussion' in the revised manuscript.

Replies to Referee #2:

Thank you for reviewing our manuscript. According to the reviewer's comments, we carried out additional experiments and revised the manuscript carefully.

This paper analyzes the effect of phosphorylation of the Rab8 GEF, Rabin8, on its role in ciliogenesis. They propose that phosphorylation by the NDR2 kinase regulates a switch in Rabin8 binding affinity from phosphatidyl serine to Sec15 and that this switch is necessary for the tethering and fusion of precursor vesicles needed for ciliogenesis. In general, I found many of the experimental aspects of the paper to be too preliminary, furthermore there is considerable vagueness regarding the overall mechanism. The overriding problem I have is understanding the series of events that they are proposing. Presumably, Rabin8 is recruited from the cytosol to precursor vesicles by the combination of Rab11-GTP and PS. The vesicles concentrate at the centriole and at some point Rabin8 is phosphorylated by NDR2. This causes a loss of affinity for PS and an increase in affinity for Sec15. So does this cause dissociation of Rabin8 from vesicles? If so, how does that facilitate tethering? "Tethering" implies the linkage of vesicles to some structure. What structure are the vesicles tethered to? What is Sec15 bound to? Does a block in phosphorylation block fusion or recycling? I find this all rather vague and confusing.

To answer the reviewer's queries, we depicted our proposed model for the role of Rabin8 phosphorylation in the trafficking, tethering and fusion of vesicles for ciliary membrane formation

([Supplementary Fig. S8](#)). According to our model, phosphorylation of Rabin8 causes the transfer of Rabin8 from the Rab11/PS vesicles to Sec15 (exocyst) on the distal end of the mother centriole. Because Rabin8 activates Rab8, and Sec15 is an effector of Rab8 (and Rab11), Rab11/Rab8-containing vesicles can tether to the centriole by the interactions between Sec15 and Rab8 (or Rab11). We speculate that Rabin8 on the distal end of mother centriole stimulates local activation of Rab8, which further induces the tethering of vesicles to the centriole and the tethering and fusion between vesicles for 'ciliary vesicle' formation and expansion. Since a block in phosphorylation caused the inhibition of both ciliogenesis (Fig. 2B) and recycling (Fig. 3A, right), we assume that Rabin8 phosphorylation is involved in both processes. We added descriptions in 'Discussion' in the revised manuscript (page 13), as follows:

"Rabin8 is initially recruited to the Rab11/PS-bearing vesicles, which become transported to the pericentrosome. Rabin8 converts Rab8-GDP to active Rab8-GTP. Since the exocyst localizes on the distal end of the mother centriole (Hehly *et al*, 2012), these vesicles are anchored to the distal end of the mother centriole via the interaction between Rab8-GTP (or Rab11-GTP) and Sec15. Rabin8 is then phosphorylated by NDR2 and transferred from the Rab11/PS-vesicles to Sec15 (exocyst) on the mother centriole. The interaction between Rabin8 and Cep164 (a centriolar protein that was recently identified as a Rabin8 interactor) (Schmidt *et al*, 2012) may also contribute to anchor Rabin8 on the centriole. Since Sec15 is an effector of Rab8, the interaction between Rabin8 (Rab8 GEF) and Sec15 (Rab8 effector) can induce a positive feedback loop, by which local activation of Rab8 is attainable. Given the critical role of Rab8 in ciliary membrane formation (Nachury *et al*, 2007; Westlake *et al*, 2011), Rabin8-mediated local activation of Rab8 on the distal end of the mother centriole probably promotes the tethering of precursor vesicles to the mother centriole as well as the tethering and fusion between precursor vesicles, which leads to ciliary vesicle formation and expansion. Defects in Rabin8 phosphorylation probably block the transfer of Rabin8 to the centriole and local activation of Rab8, resulting in the inhibition of tethering and fusion of vesicles for ciliogenesis. It could be that the phosphorylation event is also required as the initial step for the release of Rabin8 from the centrosome and its recycling, although the mechanism of Rabin8 release from the centrosome has yet to be elucidated."

Specific comments:

1. *Although they demonstrate that S272 is important for phosphorylation, they don't actually show that this residue is phosphorylated. In principle, this site could be required for recognition by NDR2 and some other site is phosphorylated. If it is phosphorylated, what is the stoichiometry?*

To demonstrate that Ser-272 is actually phosphorylated by NDR2, we raised the antibody against the Ser-272-phosphorylated peptide, corresponding to amino acid residues 266-279 of Rabin8. The antibody recognized Rabin8(WT), but not its S272A or S272E mutant ([Supplementary Fig. S1A](#)), and the immunoreactivity of Rabin8(WT) was diminished after the treatment with 1-phosphatase ([Supplementary Fig. S1B](#)), indicating that the anti-pRabin8 antibody specifically recognizes the Ser-272-phosphorylated Rabin8. In vitro kinase assays showed that NDR2(WT), but not kinase-dead NDR2, yielded the anti-pRabin8-immunoreactive GST-Rabin8 ([Supplementary Fig. S1C](#)), which indicates that NDR2 actually phosphorylates Rabin8 at Ser-272. We do not show the stoichiometry, but NDR1 effectively phosphorylated Rabin8, to the extent similar to histone H1, a substrate generally used for measuring the kinase activity of NDR ([Supplementary Fig. S1D](#)). We added these data in [Supplementary Fig. S1](#).

2. *Where is NDR2? Is it concentrated at the centriole?*

We analyzed the subcellular localization of NDR1 and NDR2. YFP- or Myc-tagged NDR1 and NDR2 were expressed in hTERT-RPE cells and analyzed by YFP fluorescence or anti-Myc immunostaining. As previously reported (Devroe *et al*, 2004), NDR1 was diffusely distributed in the cytoplasm and the nucleus, but NDR2 was localized on the vesicular structures in the cytoplasm ([Fig. 2F and 2G](#); [Supplementary Fig. S2D](#)). To examine the possibility that NDR2-containing vesicles associate with the centrosome, YFP-NDR2 was expressed in RPE1 cells and analyzed by time-lapse fluorescence microscopy. This analysis showed that NDR2-containing vesicles were often associated with the centrosome ([Fig. 2H](#); [Supplemental Movie S2](#)).

3. *Is there any increased effect of knocking down both NDR1 and NDR2 together?*

We analyzed the effect of double knockdown of NDR1 and NDR2, in comparison to the effect of single knockdown of NDR1 or NDR2. Double knockdown of NDR1 and NDR2 slightly decreased the number of ciliated cells, compared with the effect of single knockdown of NDR2 ([Supplementary Fig. S2C](#)). We also showed that double knockdown of NDR1 and NDR2 decreased the length of cilia, compared with the single knockdown of NDR1 or NDR2 ([Fig. 2D](#)). These results suggest that NDR1 siRNA has a weak effect on ciliogenesis.

4. *They need to show complementation of NDR2 knockdown with a resistant NDR2 construct (but not kinase dead).*

According to the reviewer's comment, we examined whether the effect of NDR2 siRNA is recovered by expression of siRNA-resistant (sr) mouse (m) NDR2. We generated RPE1 cell lines stably coexpressing GFP and mNDR2(WT) or its kinase-dead (KD) mutant using an IRES-AcGFP vector, and exposed to NDR2 siRNA. Exposure to NDR2 siRNA significantly reduced the number of ciliated cells in control cells, but NDR2 siRNA-induced inhibition of ciliation was significantly blocked in cells expressing mNDR2(WT), but not in cells expressing mNDR2(KD) ([Fig. 2E](#)), indicating that NDR2 siRNA inhibits ciliogenesis by suppressing endogenous NDR2 expression and that the kinase activity of NDR2 is required for ciliogenesis.

5. *The prolonged association of Rabin8 with the centrosome when phosphorylation is blocked can be interpreted in several ways and needs to be explored in greater depth. A time course of association after starvation would help determine if the onset of Rabin8 localization is normal or if the entire process is just slowed down. FRAP analysis could establish if the rate of Rabin8 turnover has been altered. Is Rabin8 actually associated with vesicles when phosphorylation is blocked? This could be established by immuno-EM. It is essential that the authors define the exact step in the ciliogenesis reaction that is blocked in this situation. Are vesicles concentrated but not fused? In Fig 3A, lower panels, there appears to be 3 Ac- tubulin spots, only one of which is labeled with Rabin8(SA).*

According to the reviewer's comment, we analyzed time-dependent changes in the localization of YFP-Rabin8(S272A) after serum starvation. In contrast to Rabin8(WT), Rabin8(S272A) almost constantly accumulated on the pericentrosome, regardless of time after serum starvation ([Fig. 3A, right panel](#); [Supplementary Fig. S3B](#)). Accumulation of Rabin8(S272A) in serum-fed cells suggests the possibility that Rabin8-containing vesicles are usually transported to the centrosome and recycled into the cytoplasm and that phosphorylation of Rabin8 is required for recycling of Rabin8 and Rabin8-containing vesicles from the centrosome, in addition to the role in ciliary membrane formation (see also [Supplementary Fig. S8](#) and [Discussion](#) (page 13)).

The reviewer suggests the FRAP analysis to examine whether the turnover rate of Rabin8 is altered by a block of phosphorylation. However, it is difficult to compare the turnover rates of Rabin8(WT) and Rabin8(S272A), because Rabin8(WT) does not accumulate on the pericentrosome when it rapidly turns over. Rabin8(WT) accumulates on the pericentrosome only transiently after serum starvation, and at this time point, the rate of turnover of Rabin8(WT) is probably slowed down to the level similar to that of Rabin8(S272A).

The reviewer asked whether Rabin8(S272A) is actually associated with vesicles. In place of immuno-EM, we measured the fluorescence images of YFP-Rabin8(S272A), together with CFP-Rab11(QL) and mCherry-evt2(PH), which are described to localize in endosomal vesicles and trans-Golgi network (Fairn *et al*, 2011; Uchida *et al*, 2011). Rabin8(S272A) was partially colocalized with Rab11(QL) and evt2(PH) in the vesicular structures around the centrosome ([Fig. 4B](#), [5F](#) and [Supplementary Fig. S6](#)), indicating that Rabin8(S272A) is actually associated with Rab11/PS-containing vesicles.

The reviewer also asks about the step in ciliogenesis that is blocked when Rabin8 phosphorylation is inhibited. As described above, we depicted our proposed model in [Supplementary Fig. S8](#). If phosphorylation is blocked, transfer of Rabin8 from the vesicle to the centriole is blocked. In our model, Rabin8 localization on the distal end of the centriole promotes local activation of Rab8, which in turn stimulates the tethering of vesicles to the centrosome and the tethering and fusion between vesicles for 'ciliary vesicle' formation and ciliogenesis. Therefore, we assume that a block in phosphorylation inhibits local activation of Rab8 on the distal end of centrosome and thereby inhibits Rab8-mediated tethering of vesicles to the centrosome and the tethering and fusion between vesicles.

As for the lower panels of Fig. 3A ([Fig. 3B](#) in the revised manuscript), we made an error in coloring. We corrected to Ac-tubulin (blue) and pericentrin (red). Two extra pericentrin (red) spots may be the non-specific background.

6. The experiment shown in Figure 4B is very weak. They are comparing the binding of Rabin8 to soluble, monomeric Rab11 in detergent versus Rab11 embedded in a complex biological membrane with lots of other proteins present. It is impossible to make any definitive conclusion from this comparison. A far better experiment would be to compare binding of pure non-prenylated Rab11 to pure prenylated Rab11 that has been reconstituted into artificial liposomes of varying composition. Even in that more defined situation they would need to keep the Rab11 concentration below 1 molecule per liposome to make sure that any apparent increase in affinity is not due to increased copy number/particle.

We agree the reviewer's comment. As the reviewer pointed out, the data in [Figure 4C](#) ([Fig. 4B](#) in the previous manuscript) is weak to make a conclusion that Rabin8 binds more tightly to membrane-associated Rab11-GTP than to membrane-free Rab11-GTP. However, this figure still raises the possibility that some of the membrane components (lipids or membrane proteins) may be involved in the promotion of Rabin8 binding to Rab11-containing vesicles.

To examine the possibility that membrane association of Rab11 affects the interaction between Rab11 and Rabin8, the reviewer suggests the reconstitution experiments using pure prenylated or non-prenylated Rab11 inserted into artificial liposomes, but insertion of pure Rab11 into artificial liposomes was technically difficult. Rab11 is anchored to the lipid membrane via C-terminal geranylgeranyl modifications. As an alternative experiment, we tested the binding of Rabin8 to Rab11(QL) and Rab11(QL, 2CA), a Rab11(QL) mutant defective in C-terminal geranylgeranyl modifications (Cys-212 and Cys-213 near the C-terminal region is replaced by Ala). Rabin8 bound to Rab11(QL) and Rab11(QL, 2CA) to a similar extent ([Supplementary Figure S4D](#)), indicating that the C-terminal lipid modification of Rab11 (therefore the membrane association of Rab11) does not directly affect the interaction between Rab11 and Rabin8, at least in in vitro binding assays. Based on these results and the previous observations that Rab GTPases and phospholipids cooperatively control the specificity of protein recruitment, we postulated that some of the membrane lipid components may be involved in the promotion of Rabin8 binding to Rab11-containing vesicles in cells and tested the binding ability of Rabin8 to lipid components.

7. The effect of the S to E mutation on PS binding (Figure 5D) is not very dramatic. This would be more convincing if done as binding curves over a range of PS concentrations. Actual kD's would be best.

According to the reviewer's comment, we analyzed the binding of S272A and S272E mutants over a range of PS liposomes. The apparent K_d values of the S272A and S272E mutant to the total lipid in the liposome containing 15% PS were 45.3 mM and 102.1 mM, respectively ([Fig. 5D](#)), indicating the S272A mutant has a higher affinity to PS than the S272E mutant.

8. Figure 5 F is not convincing. The evt(PH) does not appear to be concentrated at the centriole at all.

PS is known to be located in endosomes and trans-Golgi network (Uchida et al, 2011; Fairn et al, 2011). Since evt2(PH) was used to measure the localization of PS, it does not concentrate at the centriole. To show the colocalization of Rab11 and evt2(PH) more clearly, [Fig. 5E](#) (corresponding Fig. 5F in the previous manuscript) was replaced by the enlarged one. The data in [Fig. 5E](#), [5F](#) and [Supplemental Fig. S6](#) suggest that Rab11-GTP and Rabin8(SA) partially colocalize with PS on the pericentrosomal vesicles, while PS localizes more widely on vesicular structure throughout the cytoplasm.

9. Is the carboxy terminus of Rabin8 (350-460) sufficient for PS binding or is it just necessary? To definitively assess the role of PS binding it is important to define the PS binding site and then construct mutants that are specifically defective in PS binding. In addition they could determine the effect of reducing PS synthesis, but I imagine that would have many consequences in vivo.

According to this comment, we constructed additional Rabin8 deletion mutants and analyzed their PS-binding activity by liposome-binding assays. DN1 and DN2 bound to PS, but the

binding of DN3 to PS was barely detectable (Fig. 5G; Supplemental Fig. S5C, D), indicating that DN2 (amino acids 251-460) is the minimum PS-binding region of Rabin8 at this moment and that the C-terminal DN3 region (amino acids 351-460) is required but not sufficient for PS binding. As shown in Fig. 6E and 6G, Rabin8(I329S) mutant was defective in PS binding and was not accumulated in the pericentrosomal region. However, this mutant and Rabin8(D300-305) (Fig. 6F and Feng et al, 2012) were defective in binding to either PS or Rab11. As the reviewer pointed out, it is important to construct mutants that are specifically defective in PS binding, but the trial to separate the PS and Rab11 binding activity has not been successful. We did not examine the effect of reducing PS synthesis, because this would not provide convincing evidence for the function of PS in Rabin8 localization and functions.

10. The authors propose a conformational switch in Rabin8 from a Rab11- and PS-binding conformation to a Sec15-binding conformation. A direct, physical assay for conformational change upon phosphorylation would make this argument far more convincing.

It is an important issue to determine the phosphorylation-dependent conformational change in Rabin8. Although we are planning to do the three-dimensional structural analysis as the collaborative research, it is difficult to answer this comment immediately.

Replies to Referee #3:

Thank you for reviewing our manuscript. According to the reviewer's comments, we carried out additional experiments and revised the manuscript carefully.

Summary:

In this manuscript the authors describe the regulation of ciliogenesis by the NDR2-mediated phosphorylation of the Rab8 guanine nucleotide exchange factor (GEF) Rabin8. Mutations in NDR2 have been identified in canine retinal ciliopathies, supporting its crucial role in ciliogenesis. The authors now show that Rabin8 is a substrate for the serine/threonine kinase NDR2 and that the phosphorylation of Rabin8 switches its affinity from the Rab11/phosphatidyl serine (PS) to Sec 15 binding. The authors define the similarities and differences between the regulation of Rabin8 and its yeast counterpart Sec2 that point to the evolutionary conservation of the regulation of vesicular trafficking by NDR-family kinases. However, they provide evidence that different phospholipids are responsible for the recruitment of Rabin8 and Sec 2 to Rab11-positive membranes. This is an important and carefully executed study, and the data generally support the conclusions. My comments/suggestions are listed below.

Major comments:

The lipid binding assays often provide information that is contradictory to the protein-phospholipid-binding affinities in vivo (as was the case with evt2 used in this study). To strengthen their evidence for the Rabin8 binding to PS, the authors may consider generating a lipid-binding domain of Rabin8 fused to a fluorescent reporter to further examine the lipid binding properties of Rabin8. The binding data shown in Figure 5 should be quantified. Preferably, they should be directly compared to the Sec2 lipid binding data as lipid-binding assays vary.

According to the reviewer's comment, we constructed additional Rabin8 deletion mutants and analyzed their PS-binding activity by liposome-binding assays. DN1 and DN2 bound to PS, but the binding of DN3 to PS was barely detectable (Fig. 5G; Supplemental Fig. S5C, D), indicating that DN2 (amino acids 251-460) is the minimum PS-binding region of Rabin8 at this moment and that the C-terminal DN3 region (amino acids 351-460) is required but not sufficient for PS binding.

To examine the lipid binding properties of Rabin8 in cells, we expressed YFP-tagged Rabin8 and its mutants together with CFP-Rab11(QL) and mCherry-evt2(PH) (as a marker of PS) in RPE1 cells and analyzed their subcellular localization. Full-length Rabin8(WT or SA) was colocalized with Rab11(QL) and evt2(PH) on the pericentrosomal vesicles; in contrast, colocalization of DN2 with Rab11(QL) and evt2(PH) was scarcely detectable (Supplemental Figure S6), indicating that the C-terminal DN2 region is not sufficient for effective localization of Rabin8 on the PS/Rab11-containing vesicles in cells and that the N-terminal region appears to be involved in its vesicular localization.

According to the reviewer's comment, we added quantitative data of Rabin8 binding to PS in Fig. 5D and Supplemental Fig. 5A. We also analyzed the PS binding activity of yeast Sec2p by liposome-binding assays. GST-Sec2p did not bind to PS liposomes, under the conditions where GST-Rabin8 bound (Supplemental Fig. S5B).

Minor comments:

1. Labeling of different panels in Figure 1 (and similarly in other figures) is confusing. For example the top panel in Fig 1B represents an autoradiogram but the label "Autoradiography" is actually closer to the lower panel that presumably represents Amido black staining. The authors should clearly group the images with the corresponding labels in all figures.

According to the reviewer's comment, we clarified the grouping of the images with the corresponding labels by adjusting the space (Fig. 1A, 1B, 2A, 2I, 4C, 6B and 6D).

2. The authors do not provide the information about the RPE cell line used in this study, which is essential for comparison to other data generated in immortalized RPE cells.

We used human telomerase reverse transcriptase (hTERT)-immortalized RPE1 cell lines in all experiments in this study. We described it in page 5 and replaced the descriptor of "RPE cells" by "RPE1 cells" in the revised manuscript.

3. Why is NDR2 almost undetectable in siRNA control and NDR1 siRNA treated cells in Figure 2A?

NDR2 was weak but detectable in control siRNA and NDR1 siRNA cells (Fig. 2A). The signal of NDR2 was weaker than that of NDR1, probably because the low content of endogenous NDR2 protein in cells or the low titer of the anti-NDR2 antibody.

4. On page 5 the authors state "NDR2 siRNAs significantly decreased the number of ciliated cells". What is the P value?

We analyzed the data in Fig. 2C by one-way ANOVA followed by Turkey test. We added the P value (***, $p < 0.001$, compared with control siRNA) in the legend of Fig. 2C.

5. Although the text on page 5 refers to "ciliated" cells Figure 2C and E state "ciliary cells". This should be corrected.

We corrected the description of "ciliary cells" to "ciliated cells".

6. On page 7 the authors state that "Quantitative analysis showed that the number of cells with pericentrosomal localization of YFP-Rabin8(WT) in NDR2-depleted cells was higher than that observed in non-NDR2-depleted cells". How many experiments were performed and how many cells were counted? There are no error bars in Figure 3B and D and no P-value is given.

We performed three independent experiments, at least 100 cells in each experiment. We added error bars in Fig. 3C and 3E (corresponding Fig. 3B and 3D in the previous manuscript), and the P-values were described in the legend of Fig. 3.

7. On page 7 (and elsewhere) the authors describe detergent-assisted membrane "abstraction". This term is confusing as abstraction mostly relates to ideas and concepts. The authors probably meant "membrane removal" or "membrane extraction". This should be corrected throughout the manuscript.

We corrected the description of "membrane abstraction" to "membrane removal" or "membrane extraction".

8. On page 8 the authors state: "Since the S272E mutation weakens the ability of Rabin8 to PS...". Did they mean the ability of Rabin8 to bind to PS?

We corrected and added "to bind" in this sentence.

9. On page 9 the authors mention the "Rabin8 deleted mutant". This should be replaced with "Rabin8 deletion mutant".

We corrected to "Rabin8 deletion mutant".

10. The Discussion is too long and repetitive. Many statements from the results are repeated in the first part of the Discussion. This repetition should be eliminated and the authors should focus more on discussing their data in the context of current literature.

We shortened "Discussion" by eliminating the repetitive descriptions, and discussed more in the context of current literatures, including Hehnly et al. (2012) and Schmidt et al., (2012).

2nd Editorial Decision

24 January 2013

Thank you for the submission of the revised version of your manuscript entitled "NDR2-mediated Rabin8 phosphorylation is crucial for ciliogenesis by switching binding specificity". It has been sent to two of the original reviewers, who now consider that your study is basically ready for publication, pending some very minor revisions (see comments pasted below). I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once these few details have been addressed.

Browsing through the manuscript myself I have noticed that, despite the outstanding work you have done describing in detail your statistical analysis, a few tiny additions need to be made, as follows. In the figure legends to panels 3C, 3E, 4B, 5D and 6A, you present a P value suggesting that some kind of statistical analysis has been performed, but the nature of this analysis is not specified. In particular, in figure 5D, you use an asterisk to indicate (probably) statistical significance, but neither the asterisk nor the data analysis method are defined. Similarly, the error bars used in the graph depicted in panel A of supplementary figure 5 are not defined either.

I would also like to mention that, as a novel initiative in The EMBO Journal, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this initiative do not hesitate to contact me.

Thank you very much for your patience and congratulations in advance on a successful publication. Once these minor changes suggested by the referees and myself are incorporated into the manuscript, you will receive an official acceptance letter with further instructions on how to proceed with the publication process.

REFEREE REPORTS

Referee #1

The authors have largely responded satisfactorily to my and the other reviewers' comments, and they describe an interesting model for the regulation of Rabin8 through phosphorylation by NDR2.

I now support publication, given that the additional 2 minor comments I have are addressed.

Minor comment 1: I have an issue with the liposome-binding assays. The term 'liposome' normally implies small or larger vesicles that would not sediment by a 10 min centrifugation at 12,000g. Instead, normally larger liposomes and vesicles pellet only after 1 h centrifugation at 100,000g, whereas small liposomes are typically analyzed using floatation assays where membranes are floated in sucrose gradient at high speeds. Because the authors use low-speed spins to sediment

'liposomes', the binding assays probably reflect binding to large uni- or multi-lamellar vesicles as opposed to vesicle-like liposomes. This should be clarified for the reader in the main text.

Minor comment 2: In Figure S1C: What is NDR2 DA? Please explain.

Referee #2

The revised paper has addressed many of my prior criticisms. There is one issue that I feel must still be addressed. This concerns Fig 4C. Comparing the binding of Rabin 8 to Rab11 solubilized in detergent vs Rab11 embedded in a complex biological membrane is pointless. The membrane fragments may have many copies of Rab11 in each fragment, therefore a much higher signal would result from each interaction. This does not necessarily imply that any other interaction is involved. The authors use this result as justification to pursue possible interactions between Rabin8 and lipids. In fact, an interaction between the yeast homologue, Sec2, and lipids has already been published, so no additional justification is needed. I strongly urge the authors to remove this panel from the figure since it could be very misleading and is not necessary for the rest of the paper.

minor points:

In Fig2 J, the axis is labeled "srDA". I assume they meant srSA since that is what the legend says.

In Fig6C, the sequence is labelled "Rabib8" instead of Rabin8.

2nd Revision - authors' response

30 January 2013

Point-By-Point Response

Replies to Referee #1:

Thank you for reviewing our revised manuscript. According to the reviewer's comments, we revised the manuscript, as follows:

The authors have largely responded satisfactorily to my and the other reviewers' comments, and they describe an interesting model for the regulation of Rabin8 through phosphorylation by NDR2. I now support publication, given that the additional 2 minor comments I have are addressed. Minor comment 1: I have an issue with the liposome-binding assays. The term 'liposome' normally implies small or larger vesicles that would not sediment by a 10 min centrifugation at 12,000g. Instead, normally larger liposomes and vesicles pellet only after 1 h centrifugation at 100,000g, whereas small liposomes are typically analyzed using floatation assays where membranes are floated in sucrose gradient at high speeds. Because the authors use low-speed spins to sediment 'liposomes', the binding assays probably reflect binding to large uni- or multi-lamellar vesicles as opposed to vesicle-like liposomes. This should be clarified for the reader in the main text.

As the reviewer pointed out, we performed lipid-binding assays using "multilamellar vesicles". We therefore replaced the term "liposomes" by "multilamellar vesicles".

Minor comment 2: In Figure S1C: What is NDR2 DA? Please explain.

Thank you for your comment. We corrected "NDR2(DA)" to "kinase-dead (KD)" in Supplementary Figure S1(C) and Figure legend.

Replies to Referee #2:

Thank you for reviewing our revised manuscript. According to the reviewer's comments, we revised the manuscript, as follows:

The revised paper has addressed many of my prior criticisms. There is one issue that I feel must still be addressed. This concerns Fig 4C. Comparing the binding of Rabin 8 to Rab11 solubilized in

detergent vs Rab11 embedded in a complex biological membrane is pointless. The membrane fragments may have many copies of Rab11 in each fragment, therefore a much higher signal would result from each interaction. This does not necessarily imply that any other interaction is involved. The authors use this result as justification to pursue possible interactions between Rabin8 and lipids. In fact, an interaction between the yeast homologue, Sec2, and lipids has already been published, so no additional justification is needed. I strongly urge the authors to remove this panel from the figure since it could be very misleading and is not necessary for the rest of the paper.

According to the reviewer's comment, we removed Figure 4C and deleted the sentences related to this Figure.

Minor points:

In Fig2 J, the axis is labeled "srDA". I assume they meant srSA since that is what the legend says.

In Fig6C, the sequence is labelled "Rabib8" instead of Rabin8.

Thank you for your comment. We corrected "srDA" to "srSA" in Fig. 2J and "Rabib8" to "Rabin8" in Fig. 6C.